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January 28, 2016

Declaration by MARTIN BLANK, PhD

1. My name is Martin Blank PhD. Given my expertise as a cellular biologist and experience as a professor and researcher having authored over 90 scientific papers and a recent book “Overpowered” regarding electromagnetic fields effects on cells, I strongly recommend that the City of Los Angeles, the Mayor, the City Council and the Office of the City Attorney **NOT** proceed with the Citywide WiFi/Citylink LA program due to the damage to public health. The research attached and cited shows that, at the power levels required for WiFi to operate reliably over the project’s large areas, the Radio Frequency (RF) Radiation will have significant biological health effects, especially on electrosensitive individuals and children. Many research studies have documented the damage that will result from the ongoing exposure to electromagnetic fields and corresponding Radio Frequency Radiation emitted by the wireless transmitters used by Citywide WiFi. The radiation may not cause thermal or heating effects but will certainly cause non-thermal biological effects that are not being accounted for, and are not protected by our current FCC safety standards.

From 1962 to 2011, I was a professor in the Department of Physiology and Cellular Biophysics at Columbia University, New York, NY where I both taught and conducted research. Currently, I am a Special Lecturer in that department.

My formal education included a Bachelor of Science degree (Magna Cum Laude) in Chemistry from City College of New York, a PhD in Physical Chemistry from Columbia University, and a PhD in Colloid Science from Cambridge University, England. The focus in the Colloid Science department, under the direction of Professor F.J. W. Roughton was on the electrical properties of biological surfaces and membranes. This provided unique training for research on electric and magnetic field effects in biological cells.

My research has focused on living cells, their components (e.g. DNA, proteins, ions, electrons) and their interactions with the environment. The research (detailed in my Curriculum Vitae in Attachment 1) has concentrated on electric and magnetic field effects on electron transfer reactions, enzymes, DNA and fluxes in the ion channels of excitable membranes. This entailed determining how electrically charged components (ions and electrons) of cells are affected by external fields. Studies of electric field effects on proteins, lipids and ions provide insight into the effects of electric and magnetic fields (EMF) on cells in living organisms.

2. To render a professional opinion regarding the health risks associated with exposure to EMF (Electromagnetic Fields) from many sources, including ELF (extremely low frequency) from power lines, and RF (radio frequency) from cell phones, WiFi, smart meters, etc. I have reviewed the relevant information and commented on the reported harmful effects, as well as protective biological reactions of cells to this unnatural (i.e., man-made) radiation in the environment.

3. In addition to teaching and research, I have been involved in EMF related activities for many years. I served as President of the Bioelectromagnetics Society 1989-1990, and was selected to open the First Congress of the European Bioelectromagnetics Association in Belgium in 1992. I was Editor-in-Chief of the First World Congress on "Electricity and Magnetism in Biology and Medicine" proceedings, and Plenary Lecturer on Bioelectromagnetics for international conferences in Brazil, Canada, India, Israel, Italy and Japan. I also served on the "Bioelectrochemistry and Bioenergetics" Editorial Board and as Biology Divisional Editor of the Journal of the Electrochemical Society for thirteen years. In 2015, I was a consultant for the Canadian Parliament regarding EMF safety standards, and was spokesperson for the over 200 published EMF scientists who petitioned the UN and the World Health Organization (W.H.O.) regarding the strong scientific evidence showing the need for stricter control of EMF exposure to protect the public. (See Attachment 2)

I was one of the organizers of the Bioinitiative Report (BIR) and wrote online reviews on the Cellular Stress Response in both 2007 and 2012 editions (See

Attachments 3 and 4). The most recent report, <http://www.bioinitiative.org/freeaccess/report/docs/report.pdf>, summarizes over 1800 recent epidemiology studies, as well as cell and molecular biology research (See Attachment 5). A key summary from the report states:

‘Bioeffects are clearly established and occur at very low levels of exposure to electromagnetic fields and radiofrequency radiation. Bioeffects can occur in the first few minutes of exposure to power lines as well as at levels associated with cell and cordless phone use. Bioeffects can also occur from just minutes of exposure to mobile phone masts (cell towers), WiFi, and wireless utility ‘smart’ meters. Chronic base station level exposures can result in illness.’

I recently published **Overpowered (2014)**, a book to introduce the public to the potentially harmful effects of EMF in the environment and how to protect oneself.

4. Based on a wide range of research studies, I conclude that:

- Electricity and magnetism are fundamental forces that interact with charged particles, i.e., primarily with electrons in our cells. The organism, in reaction to these conditions, produces **the cellular stress response, a DNA mechanism that is activated by many potentially harmful stimuli** (e.g., high and low temperature, changes in pH, toxic metals). In other words, **cells react to EMF as potentially harmful.**
- Stress protein synthesis starts with activation of DNA. **Higher RF-EMF levels can cause chemical changes in DNA that lead to mutations and cancer** and other abnormal biological processes (e.g., development and growth of tumors).
- **Biological systems are affected by a wide range of EMF frequencies, including ELF, RF and MW (microwave) ranges.** Because of the many sources in the environment (cell phone towers, WiFi, smart meters) the effects are additive. Unfortunately, the divisions of the EM (electromagnetic) spectrum were created by engineers and physicists who assumed arbitrary frequency boundaries that do not relate to the biology. **Human cells do not recognize EM spectrum divisions. They react to electromagnetic fields across the spectrum.**

- Furthermore, the same engineers and physicists assumed that the biological response was caused by the energy of the EMF stimulus, and could be measured by an increase in temperature. ***The biological response is stimulation of stress protein synthesis in DNA, and the stress response occurs across the EM spectrum.*** When stress protein synthesis is stimulated by EMF, the body is essentially telling us that exposure is harmful to living cells.
- ***The stress protein synthesis occurs*** at field strength and duration thresholds that are very low and ***below the temperature-based thresholds set by safety standards.*** (This is especially true in the ELF range where epidemiology studies indicate increased risk of leukemia at 3-4mG and the U.S. Standards are at 1000mG) This means that cells in the body respond at very low exposure levels.

Because cells activate the stress response to a wide range of EMF frequencies, this reaction would appear to be highly relevant to the setting of safety standards.

However, the stress response has been ignored in the setting of safety standards.

Safety standards have been set based on the ability of EMF to heat tissue! Both non-thermal and thermal EMF signals activate the stress response, (See Attachment 6 - Blank, Goodman. 2004) but thresholds triggering stress on biological systems occur at levels on the order of 0.5 to 1.0 μ T (5-10 mG) for ELF, **thousands of times lower energy than the ‘safe levels’ in the RF range.** However, this information has not been included in prior scientific reviews because **insufficient attention was paid to the relevant cell biology.**

5. The stress response has provided vital evidence about cellular defense mechanisms – it shows that the reaction starts when EMF interacts with DNA.

Protein synthesis begins when the two chains of DNA come apart and make an mRNA copy of the amino acid code (that is in the DNA composition) for a particular protein. This normally is initiated when a particular chemical stimulus (transcription factor) binds to a specific DNA, and in forming a bond changes the electron distribution. Research has shown electron conduction in DNA (See Attachment 7 - Wan et al, 1999)

enables communication along the molecule, and so EMF affects electron distribution and movement in DNA and enables the two chains of the double helix to come apart to initiate protein synthesis. During this coming apart, along with normal functioning, abnormal processes can also occur (See Attachment 8 - Blank, Goodman. 2001).

Several studies have reported both single and double strand breaks in the DNA ‘double helix’, and other chromosome damage after exposure to extremely low frequency (ELF) fields (See Attachment 9 - Lai, Singh. 1997). Similar malfunction has also been reported after exposure to higher frequency, radiofrequency (RF) fields. The REFLEX Project, a collaboration of twelve laboratories in the European Union (Attachment 10 - REFLEX. 2004), found that **both ELF and RF exposures modified the expression of many genes and proteins well below the safety limits.**

For a long time, agencies such as ICNIRP (International Commission on non-Ionizing Radiation Protection) and WHO maintained that an EMF must increase the temperature in order to cause changes in cells. Many lines of research now point to changes in DNA without elevation of temperature. The thresholds for a number of biological systems are shown in Table 1 (below), and many are in the range of 0.5 to 1.0 μ T (5-10mG), not very much higher than the usual environmental backgrounds of \sim 0.1 μ T (1mG). The effects occur in basic cellular systems at relatively low field strengths, similar to those in the environment. **Non-thermal ELF and RF fields can cause DNA damage, and therefore represent health and safety concerns.**

Table 1. Cells React to Very Low EMF (well below safety limit)

<u>Biological System</u>	<u>Threshold</u>	<u>Reference</u>
Stress proteins in cells		
HL60, Sciara, yeast	<8mG	Goodman, Blank, 1998
breast (HTB124, MCF7)	<8mG	Lin et al, 1999
chick embryo (anoxia)	\sim 20mG	DiCarlo et al, 1999
Accelerate electron transfer		
Na,K-ATPase	2-3mG	Blank & Soo, 2001
cytochrome oxidase	5-6mG	Blank & Soo, 2001
ornithine decarboxylase	\sim 20mG	Litovitz et al, 1991

Belousov-Zhabotinsky	<5mG	Blank & Soo, 2003
Disease related		
leukemia epidemiology	3-4mG	Ahlbom et al, 2000
		Greenland et al, 2000
ELF Safety Limit	1000mG	ICNIRP, 1997

All of the reported thresholds are well below the safety limit!!!

In the RF range, research by Lai and Singh (1997) and Litovitz et al. (1991) have shown similar effects (See Attachments 9 and 11). As articulated by the Bioinitiative Report citing over 130 sources showing Radio Frequency radiation emitted by wireless transmitters has biological effects at levels millions of times lower than current FCC Safety Standards (see Attachment 22 Reported Biological Effects from RFR at Low-Intensity Exposure). In addition to very low thresholds, exposure durations in the RF range do not have to be very long to be effective.

Table 2. Cells React to Very Low RF (well below safety limit). Excerpted from the Bioinitiative Report 2012 (see Attachment 23 for the full chart)

Power Density (microWatts/cm ²)	Observed Effects	Reference
As low as (10 ⁻¹³) or 100 femtowatts/cm2	Super-low intensity RFR effects at MW resonant frequencies resulted in changes in genes; problems with chromatin conformation (DNA)	Belyaev, 1997
0.00034 uW/cm2	Chronic exposure to mobile phone pulsed RF significantly reduced sperm count,	Behari, 2006
0.0005 uW/cm2	RFR decreased cell proliferation at 960 MHz GSM 217 Hz for 30-min exposure	Velizarov, 1999
0.0006 - 0.0128 uW/cm2	Fatigue, depressive tendency, sleeping disorders, concentration difficulties, cardiovascular problems reported with exposure to GSM 900/1800 MHz cell phone signal at base station level exposures.	Oberfeld, 2004
0.003 - 0.02 uW/cm2	In children and adolescents (8-17 yrs) short-term exposure caused headache, irritation, concentration difficulties in school.	Heinrich, 2010

0.003 to 0.05 uW/cm ²	In children and adolescents (8-17 yrs) short-term exposure caused conduct problems in school (behavioral problems)	Thomas, 2010
0.005 uW/cm ²	In adults (30-60 yrs) chronic exposure caused sleep disturbances, (but not significantly increased across the entire population)	Mohler, 2010
0.005 - 0.04 uW/cm ²	Adults exposed to short-term cell phone radiation reported headaches, concentration difficulties (differences not significant, but elevated)	Thomas, 2008
0.006 - 0.01 uW/cm ²	Chronic exposure to base station RF (whole-body) in humans showed increased stress hormones; dopamine levels substantially decreased; higher levels of adrenaline and nor-adrenaline; dose-response seen; produced chronic physiological stress in cells even after 1.5 years.	Buchner, 2012
1000 uW/cm ²	FCC RF Safety Limit	

Litovitz et al (1991), working with the enzyme ornithine decarboxylase, have shown **a full response to EMF when cells were exposed for only 10 sec** (See Attachment 11). This occurred with ELF sine waves or ELF modulated 915 MHz sine waves. (915MHz is RF but the ELF modulation was effective!) Kultz (2005) summarized the evidence that specific groups of genes are activated along with stress genes and are involved in sensing and repairing damage to DNA and proteins.

The stress response is a natural defense mechanism activated by molecular damage caused by environmental forces. The response involves reaction with DNA, i.e., stimulating stress genes as well as genes that sense and repair damage to DNA and proteins. At high EMF intensities, the interaction with DNA can lead to DNA strand breaks that can result in mutations, an initiating step in the development of cancer. (See Attachment 12 - Blank, Electromagnetic Biology and Medicine, 2008).

EMF have been shown to cause other potentially harmful biological effects, such as leakage of the blood brain barrier that can lead to damage of neurons in the brain,

increased micronuclei (DNA fragments) in human blood lymphocytes, all at exposures well below the limits in the current FCC guidelines in the US.

In summary, the human health consequences of long-term exposure to high EMF levels lead to molecular damage, including DNA. If the molecular damage is not fully repaired and the damaged cells are not eliminated by apoptosis (cell suicide), the diseases that are most likely to develop are: (a) cancer, primarily leukemia in children and breast cancer in women; (b) neurodegenerative diseases such as Alzheimer's disease and ALS; (c) immunological disorders, including electrohypersensitivity (EHS).

6. Epidemiology studies

Epidemiology research, that is, research on large populations over time, has served as a key guide for EMF policy on health risks associated with ELF (power lines) and RF (cell phones). These studies, which show the effects of long term exposures demonstrate quantitative dose-response relations (i.e., the health effects are proportional to the EMF dose).

The paper published in 1979 by Wertheimer and Leeper (1979) showed a dose-response link between EMF and leukemia (See Attachment 13). Since then, there have been many studies on the relation between EMF and human disease. Among the key studies are two pooled analyses by Greenland et al (2000) and Ahlbom et al (2000) which confirmed a statistically significant doubling of the risk of leukemia in children when exposures exceed 3-4mG (See Attachments 14 and 15). The link between DNA damage and development of cancer is further supported by Yang et al (2009) who correlated a significantly increased risk of leukemia in children with a deficiency in DNA repair genes (See Attachment 16). (i.e., when repair genes were present, they appear to be able to repair some of the damage and prevent disease.)

Dr. Neil Cherry found strong corroborating evidence for these effects in the archives of public health statistics of all childhood cancers around the Sutro Broadcasting Tower in San Francisco between the years 1937 and 1988. The 50 years of data from the archives involved a total of 123 cases of childhood cancer from a population of 50,686 children, and included 51 cases of leukaemia, 35 cases of brain cancer and 37 cases of lymphatic cancer. The risk ratio (RR) for all childhood cancers was elevated in

the area studied. **The risk declined with radial distance from the antennas, but it was still above a risk ratio of 5 even at a distance of 3km where the field was measured to be $1\mu\text{W}/\text{cm}^2$, comparable to what has been measured near cellphone towers (See Attachment 17). (Similar results have been reported around RF broadcasting antennas in Sydney, Australia and Rome, Italy, and there are now studies of effects of cellphones on brain cancer and cancer of the salivary glands.)**

There is also evidence that EMF plays a role in breast cancer in women by inhibiting the ability of normally secreted melatonin to slow the growth of breast cancer cells. Liburdy et al. (1993) showed that the threshold for inhibiting melatonin lies between 2-12mG (See Attachment 18).

Inhibition of melatonin secretion by the pineal gland is also associated with sleep disorders and disturbances of the immune system through various allergic and inflammatory responses and effects on tissue repair processes. The pineal gland also secretes serotonin, and a deficiency in serotonin due to EMF is also associated with insomnia, as well as memory and mood disorders.

In addition to the risk of cancer and effects on the immune system, Huss et al (2009) found an increased risk of Alzheimer's disease and death from neurodegenerative diseases for people who live within 50 meters of 220-380 kV power lines compared with people who live 600 meters or more, where the fields were about 1mG. **The estimated fields at 50 meters for the 220kV line are about 5mG and for the 380kV line are about 8mG. The fields would be very much higher at 20 meters.** (See Attachment 19)

After reviewing the full range of studies, the International Agency for Research on Cancer (IARC) in 2002 found that there is reliable scientific evidence that EMFs in the ELF range are a possible human carcinogen (In 2011, IARC made a similar evaluation regarding the RF range.) (See Attachments 20 and 21). Since 2002, additional evidence has supported the IARC statement. Hence, like the cell biology studies, epidemiological studies show adverse biological changes on exposure to EMF. The EMF interactions with DNA and the low levels at which these reactions occur offer a plausible mechanism connecting environmental exposure and human carcinogenesis (See Attachment 12 - Blank, Electromagnetic Biology and Medicine, 2008).

7. Mechanism of EMF Interaction with DNA as a Fractal Antenna

The responses of deoxyribonucleic acid (DNA) to electromagnetic fields (EMF) in different frequency ranges can be understood in terms of the double helical structure of the DNA and the electronic conduction within the DNA molecule and its compact structure in the nucleus. Human DNA is 2 meters long and it is coiled many fold in order to fit into a nucleus that is only microns in size. The need to fit into this cramped space results in the DNA being coiled many times, and a molecule having electron conduction paths of many different lengths. The many different lengths mean that the DNA can act as an antenna that is sensitive to many non-ionizing frequencies in the extremely low frequency (ELF) and radio frequency (RF) ranges.

The wide frequency range of interaction with EMF is the functional characteristic of a fractal antenna, and **DNA appears to possess the two structural characteristics of fractal antennas, electronic conduction and self symmetry. These properties contribute to greater reactivity of DNA with EMF in the environment, and the DNA damage could account for increases in cancer epidemiology,** as well as variations in the rate of chemical evolution in early geologic history.

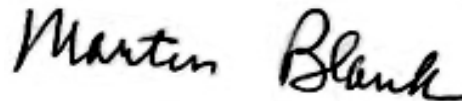
8. The Growing Presence of EMF in the Environment

All of the studies cited above occurred when EMF levels were lower than they are today. Increasingly, people are exposed to a much wider range of EMF as a result of advancing technological developments, such as cell phones, WiFi, smart meters, radiation from installation of cell towers, etc. Also, the scientists (primarily engineers and physicists) who set the divisions of the EM spectrum, selected frequency boundaries that do not relate to the biology. For example, they incorrectly assumed that the only dangerous range was EMF that caused the body temperature to increase. Human cells do not recognize EM spectrum divisions. The same biological reactions (including the cellular stress response), can be stimulated in more than one subdivision of the EM spectrum and in subdivisions that do not cause temperature increases.

There are now sufficient scientific data about the biological effects of EMF to limit human exposure. We can state unequivocally that EMF can cause damage (single and double strand breaks) to DNA at exposure levels that are considered safe under the FCC guidelines in the USA (See Attachment 9 - Lai and Singh, 1997). Further, these

guidelines do not take into account the accumulation of changes or mutations in DNA that occur with prolonged exposure—and the actual use of the various devices involves prolonged exposure, indeed increasingly prolonged exposure.

In conclusion, given my expertise as a cellular biologist and experience as a professor and researcher authoring over 90 scientific papers and a recent book “Overpowered” regarding EMF effects on the cells, I strongly recommend that the city of Los Angeles, the Mayor, the City Council and the office of the City Attorney **NOT** proceed with the Citywide WiFi/Citylink LA program due to the damage to public health, that will result from the ongoing exposure to electromagnetic fields and corresponding Radio Frequency Radiation emitted by the wireless transmitters used by citywide WiFi. The proposed system may not cause thermal or heating effects but will certainly cause non-thermal biological effects that are not being accounted for or protected by our current FCC safety standards. It is clear that the safety standards must be revised to take into account the potentially harmful non-thermal biological processes that occur. I’m available for further consultation or questions.



Martin Blank, PhD.

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Attachment 1

MARTIN BLANK - CURRICULUM VITAE

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Education

Cambridge University, England, PhD (Colloid Science)
Columbia University, PhD (Physical Chemistry)
City College of New York, BS Magna Cum Laude (Chemistry)

Academic Appointments

Research Fellow (Chemistry), Columbia University
Postdoctoral Research Fellow, Cambridge University, England
Instructor in Physiology, Columbia University
Assistant Professor of Physiology, Columbia University
Associate Professor of Physiology and Cellular Biophysics, Columbia University
Special Lecturer, Department of Physiology and Cellular Biophysics, Columbia University,
630 West 168th Street, New York, NY 10032

Other Appointments

Chemist, California Research Corp, Richmond, CA.
Chemist, Esso Research and Engineering Co, Linden, NJ.
Chemist, Unilever Research Lab, Cheshire, England
Visiting Scientist, Polymer Dept, Weizmann Institute, Israel
Chemist, Unilever Research Lab, Hertfordshire, England
Visiting Scholar, Bioengineering Dept, University of California, Berkeley
Research Chemist, Unilever Research Lab, Vlaardingen, Netherlands
Visiting Professor, Pharmacology Dept, Hebrew University, Israel
Physiologist, Office of Naval Research, London, England
Visiting Lecturer, Biochemistry Dept, Monash University, Australia
Biologist, Office of Naval Research, Arlington, VA
Visiting Professor, Acad Sci USSR, Inst Electrochemistry, Moscow, and
Dept of Biophysics, Univ of Warsaw, Poland
Visiting Professor, Tata Institute, Bombay, India
Visiting Professor, Dept of Chemistry, University of the Negev, Beersheba, Israel
Visiting Scientist, Dept of Biology, University of Victoria, BC, Canada
Visiting Professor, Dept of Theoretical Physics, Kyoto University, Japan

Honors

Consumers Union Research Fellowship, Columbia University
Research Career Development Award (USPHS), Columbia University
Distinguished Visiting Professor, Univ Western Australia
Distinguished Lecturer in Physiology, Wayne State University
Certificate of Commendation, Office Naval Research, Arlington
Certificate of Appreciation, The Electrochemical Society
Yasuda Award, Bioelectrical Repair and Growth Society
Visiting Professor, Tata Institute, Bombay, India
Invited expert, House of Commons Committee on Health (HESA), Canada Parliament, Ottawa
Invited expert, House Committee on Natural Resources and Energy (HNRE), Vermont
Invited expert, on Biological Effects of EM Fields, for 'Open Court' in Brazil
Invited expert, on Biological Effects of EM Fields before BC Utilities Commission

Areas of Research

General Experimental and Theoretical Areas:

Electromagnetic field effects on cells (cellular stress response, enzyme reactions, DNA reactions)

Membrane biophysics and transport mechanisms (active, passive, excitation mechanisms)

Biopolymers (surface and electrical properties of proteins, DNA)

Theoretical Models of Processes in Membranes and Biopolymers:

Electric and magnetic field effects on electron transfer reactions, enzymes, DNA

Ion fluxes in excitable membranes and ion gating in channels

Cooperative reactions in membranes, hemoglobin

Specific Biological Systems:

Electron transfer reactions: Belousov-Zhabotinski (oxidation of malonic acid), cytochrome oxidase

Enzymes: Na,K-ATPase, cytochrome oxidase, F₀F₁ATPase (effects of ions and EM fields)

Proteins: hemoglobin, red cell membrane, lung surfactant, Sciara salivary gland proteomics

Cells: red blood cells, sperm cells, HL60, Sciara salivary gland, E. coli

Membranes: red blood cells, sperm cells, membrane enzymes

Interfaces, Monolayers (proteins, lipids, ions), Bilayers: Permeability, Rheology, Electrical effects

Teaching

Faculty of Medicine - College of Physicians and Surgeons, Columbia University

Faculty of Pure Science - Graduate School of Arts and Sciences, Columbia University

Ettore Majorana Center, Erice, Italy-International School of Biophysics

City University of New York (Graduate School) - Lectures on Surface Chemistry in Biology

Tata Institute, Bombay, India

University of the Negev, Beersheba, Israel

Society Memberships

American Association for the Advancement of Science

Bioelectromagnetics Society

Bioelectrochemical Society

American Chemical Society (Colloid and Surface Chemistry Division)

Biophysical Society

Electrochemical Society (Organic and Biological Division)

Professional Activities

Editorial Boards

Bioelectrochemistry and Bioenergetics - Co-Editor, 1981 - 1987; North American Editor, 1993 - 1998

Journal of Electrochemical Society - Divisional Editor (Biology), 1978 -1991

Journal of Colloid and Interface Science - Advisory Board, 1978 -1981

Colloids and Surfaces (founded 1979) - Editorial Board, 1979 -1986

Pathophysiology, Guest Editor, special issue on Electromagnetic Fields, 2007-2009

Electromagnetic Biology and Medicine - Editorial Board, 2012 -

Bioelectrochemical (BES) Society

Founding Member, March 1979; Vice President, 1979 - 1988; President, 1988 - 1992.

Co-organizer, 4th International Symposium on Bioelectrochemistry, Woods Hole, MA, 1977.

Plenary Lecturer, Weimar, 1979; Jerusalem, 1981; Stuttgart, 1983; Bologna, 1985; Szeged, 1987;

Pont-a-Mousson, France, 1989; Bielefeld, 1992; Seville, 1994; Israel, 1996; Denmark, 1998;

Bratislava, 2001; Florence, Italy, 2003.

Bioelectromagnetics (BEMS) Society

Invited Lecturer: San Francisco, 1985; Madison, 1986; Stamford, 1988; Quebec, 2002; Bethesda, 1988,;

Los Angeles, 1993
Board of Directors, 1989-1992; President Elect, 1996; President, 1997-1998; Past President, 1998-1999
Plenary Lecturer, Quebec, Canada, 2002
Symposium Organizer, Speaker, Washington, 2004; Cancun, 2006

BioInitiative Working Group (2005 - present)

- An international group of scientists focused on EMF issues (including science, public policy and public health). **The BioInitiative Report**, entitled 'A Scientific Perspective on Health Risk of Electromagnetic Fields' was published on line on August 31, 2007.
<http://www.bioinitiative.org/report/index.htm>
- Author of Section 7, pp. 1-40. Evidence for Stress Response (Stress Proteins)
- Peer- reviewed update of Report published in **Pathophysiology** 16 (2,3): 67-249, 2009.
- **BioInitiative Report 2012** update, released online at www.bioinitiative.org

World Congress on Electricity and Magnetism in Biology and Medicine

1992-3 Executive Committee, Site Selection Committee, Program Committee.
1992-3 Editor-in-Chief of Proceedings Volume, First World Congress
1994-7 Vice President, Executive Committee for Second World Congress
Chairman, Technical Program Committee, Second World Congress

International School of Biophysics, Erice, Italy; Co-Director and Lecturer in following:

Bioelectrochemistry I: Biological Redox Reactions and Energetics, 1981.
Bioelectrochemistry II: Membrane Phenomena, 1984.
Bioelectrochemistry III: Charge Separation Across Biomembranes, 1988.
Bioelectrochemistry IV: Nerve-Muscle Function, 1991.

Division of Colloid and Surface Chemistry, American Chemical Society

Symposium Chairman, "Surface Chemistry of Biological Systems".
Symposium Chairman, "Bioelectrochemistry", Miami, 1978; Cleveland, 1981; Washington, 1983;
Denver, 1987.
Program Committee, Biology and Medicine, Chairman, 1979-1983.
Lecturer, Ann Arbor, 1987; Biological Interfacial Reactions, Atlanta, 1991.

Division of Organic and Biological Electrochemistry (Electrochemical Society)

Symposium Chairman, "Electrochemical Processes at Biological Membranes", Seattle, 1978
Secy-Treas; Vice Chair; Chair 1983-1985; Board of Directors, Electrochemical Society, 1983-1985.
Symposium Chairman, "Electrical Double Layers in Biology", Toronto, 1985.
Speaker, Boston, 1986; Washington, 1991.

Gordon Research Conferences

Day Chairman and speaker, "Chemistry at Interfaces", 1974.
Speaker, "Sensory Transduction in Microorganisms", 1978.
Organizing Chairman, First Conference "Bioelectrochemistry", 1980.
Day Chairman and speaker, "Bioelectrochemistry", 1982. Speaker, 1984, 1986, 1988, 2004.
Discussion Leader, 1990, 1992, 1994, 1996, 1998, 2000, 2002.
Speaker, "Protons and Membrane Reactions", 1985.
Speaker, "Physicochemical Aspects, Transport in Microvasculature", 1985.

Grant Review Consultant

Office of Naval Research, Department of Defense
IPA Biologist, Manager of Membrane Electrochemistry ARI, 1986-1988

Chairman, Panel on Biological Sciences Div, August 1986
 Member, Panel on Interdisciplinary Research, April 1979
 Electric Power Research Institute, Palo Alto, CA
 Member, Basic Sciences Advisory Committee, 1987-1991
 National Institutes of Health
 Radiation Study Section, 1991 and several ad hoc Study Sections and site visit committees
 National Science Foundation
 US Army Research Office
 US-Israel Binational Science Foundation
 Petroleum Research Fund
 Medical Research Council - Canada
 Australian Research Grants Committee
 Research Corporation (Providence, Rhode Island)
 University and Polytechnic Grants Committee, Hong Kong
 International Science Foundation (for Former Soviet Union), Washington, DC
 Breast Cancer Research Program, University of California
 US Army Medical Research and Materiel Command, Neurotoxin Exposure Program, AIBS
 US Army Radiofrequency Radiation Research Program, AIBS

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Consultant to Private Corporations

California Research Corp.
Esso Research and Engineering Co.
Unilever Research Labs
Procter and Gamble Co.
Electro-Biology Inc.
Lever Brothers Co.
Electric Power Research Institute (EPRI)
Pfizer, Hospital Products Group
SENMED Medical Ventures, Sentron Medical, Inc.
Leigh, Day & Co., Solicitors, London, England

Attachment 2



**To: His Excellency Ban Ki-moon, Secretary-General of the United Nations
Honorable Dr. Margaret Chan, Director-General of the World Health Organization
Honorable Achim Steiner, Executive Director of the U.N. Environmental Programme
U.N. Member Nations**

International Appeal: Scientists call for Protection from Non-ionizing Electromagnetic Field Exposure

We are scientists engaged in the study of biological and health effects of non-ionizing electromagnetic fields (EMF). Based upon peer-reviewed, published research, we have serious concerns regarding the ubiquitous and increasing exposure to EMF generated by electric and wireless devices. These include—but are not limited to—radiofrequency radiation (RFR) emitting devices, such as cellular and cordless phones and their base stations, Wi-Fi, broadcast antennas, smart meters, and baby monitors as well as electric devices and infra-structures used in the delivery of electricity that generate extremely-low frequency electromagnetic field (ELF EMF).

Scientific basis for our common concerns

Numerous recent scientific publications have shown that EMF affects living organisms at levels well below most international and national guidelines. Effects include increased cancer risk, cellular stress, increase in harmful free radicals, genetic damages, structural and functional changes of the reproductive system, learning and memory deficits, neurological disorders, and negative impacts on general well-being in humans. Damage goes well beyond the human race, as there is growing evidence of harmful effects to both plant and animal life.

These findings justify our appeal to the United Nations (UN) and, all member States in the world, to encourage the World Health Organization (WHO) to exert strong leadership in fostering the development of more protective EMF guidelines, encouraging precautionary measures, and educating the public about health risks, particularly risk to children and fetal development. By not taking action, the WHO is failing to fulfill its role as the preeminent international public health agency.

Inadequate non-ionizing EMF international guidelines

The various agencies setting safety standards have failed to impose sufficient guidelines to protect the general public, particularly children who are more vulnerable to the effects of EMF.

The International Commission on Non-Ionizing Radiation Protection (ICNIRP) established in 1998 the “Guidelines For Limiting Exposure To Time-Varying Electric, Magnetic, and Electromagnetic Fields (up to 300 GHz)”¹. These guidelines are accepted by the WHO and numerous countries around the world. The WHO is calling for all nations to adopt the ICNIRP guidelines to encourage international harmonization of standards. In 2009, the ICNIRP released a statement saying that it was reaffirming its 1998 guidelines, as in their opinion, the scientific literature published since that time “has provided no evidence of any adverse effects below the basic restrictions and does not necessitate an immediate revision of its guidance on limiting exposure to high frequency electromagnetic fields”². ICNIRP continues to the present day to make these assertions, in spite of growing scientific evidence to the contrary. It is our opinion that, because the ICNIRP guidelines do not cover long-term exposure and low-intensity effects, they are insufficient to protect public health.

The WHO adopted the International Agency for Research on Cancer (IARC) classification of extremely low frequency electromagnetic field (ELF EMF) in 2002³ and radiofrequency radiation (RFR) in 2011⁴. This classification states that EMF is a *possible human carcinogen (Group 2B)*. Despite both IARC findings, the WHO continues to maintain that there is insufficient evidence to justify lowering these quantitative exposure limits.

Since there is controversy about a rationale for setting standards to avoid adverse health effects, we recommend that the United Nations Environmental Programme (UNEP) convene and fund an independent multidisciplinary committee to explore the pros and cons of alternatives to current practices that could substantially lower human exposures to RF and ELF fields. The deliberations of this group should be conducted in a transparent and impartial way. Although it is essential that industry be involved and cooperate in this process, industry should not be allowed to bias its processes or conclusions. This group should provide their analysis to the UN and the WHO to guide precautionary action.

Collectively we also request that:

1. children and pregnant women be protected;
2. guidelines and regulatory standards be strengthened;
3. manufacturers be encouraged to develop safer technology;
4. utilities responsible for the generation, transmission, distribution, and monitoring of electricity maintain adequate power quality and ensure proper electrical wiring to minimize harmful ground current;
5. the public be fully informed about the potential health risks from electromagnetic energy and taught harm reduction strategies;
6. medical professionals be educated about the biological effects of electromagnetic energy and be provided training on treatment of patients with electromagnetic sensitivity;
7. governments fund training and research on electromagnetic fields and health that is independent of industry and mandate industry cooperation with researchers;
8. media disclose experts’ financial relationships with industry when citing their opinions regarding health and safety aspects of EMF-emitting technologies; and
9. white-zones (radiation-free areas) be established.

¹ <http://www.icnirp.org/cms/upload/publications/ICNIRPemfgdl.pdf>

² <http://www.icnirp.org/cms/upload/publications/ICNIRPStatementEMF.pdf>

³ <http://monographs.iarc.fr/ENG/Monographs/vol80>

⁴ <http://monographs.iarc.fr/ENG/Monographs/vol102/>

Initial release date: May 11, 2015

This version's date: October 15, 2015

Inquiries, including those from qualified scientists who request that their name be added to the Appeal, may be made by contacting Elizabeth Kelley, M.A., Director, EMFscientist.org, at info@EMFscientist.org.

Note: the signatories to this appeal have signed as individuals, giving their professional affiliations, but this does not necessarily mean that this represents the views of their employers or the professional organizations they are affiliated with.

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Dr. Don Maisch, Ph.D., Australia

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Attachment 3



SECTION 7

Evidence for Stress Response (Stress Proteins)

Health Risk of Electromagnetic Fields: Research on the Stress Response

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Prepared for the BioInitiative Working Group

July 2007

A Scientific Perspective on Health Risk of Electromagnetic Fields: Research on the Stress Response

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I. Abstract

The stress response is a protective cellular mechanism that is characterized by stress protein synthesis. The stress response, by its very nature, shows that *cells react to EMFs as potentially harmful*. The stress response is an important protective mechanism that enables cells from animals, plants and bacteria to survive environmental stressors with the aid of heat shock proteins (hsp). It is stimulated by both non-thermal power (ELF), and non-thermal radiofrequency (RF) as well as thermal radio (RF) frequency EMFs, so the greatly differing energies are not critical in activating the DNA to synthesize proteins. Direct interaction of both ELF and RF EMFs with DNA is likely, since specific DNA sequences are sensitive to EMFs and retain their sensitivity when transferred to artificial molecular constructs. Basic science research is essential for determining the biological parameters needed to assess health risks of electromagnetic fields (EMFs) and the molecular mechanisms that explain them. However, the adversarial nature of the debate about risk has clouded the evaluation of the science. To clarify the results of research on EMF stimulation of the stress response, it is necessary to consider the scientific context as well as the research. There is ample evidence that ELF and RF fields activate DNA in cells and cause damage at exposure levels that are considered 'safe' (i.e., below current exposure limits that are based on tissue heating as measured in Specific Absorption Rate or SAR). Because non-thermal EMFs are biologically active and potentially harmful, new safety standards must be developed to protect against possible damage at non-thermal levels, and the standards must be defined in terms of a non-thermal biological dose. Fewer than one quarter of the relevant references listed in Table 1 appear in the IEEE list leading to the newly revised IEEE C95.1 recommendations (April, 2006).

II. Stress Proteins - Conclusions (Heat Shock Proteins)

Conclusion: *Scientific research has shown that the public is not being protected from potential damage that can be caused by exposure to EMF, both power frequency (ELF) and radio frequency (RF).*

Conclusion: *DNA damage (e.g., strand breaks), a cause of cancer, occurs at levels of ELF and RF that are below the safety limits. Also, there is no protection against cumulative effects stimulated by different parts of the EM spectrum.*

Conclusion: *The scientific basis for EMF safety limits is flawed when the same biological mechanisms are activated in ELF and RF ranges at vastly different levels of the Specific Absorption Rate (SAR). Activation of DNA to synthesize stress proteins (the stress response), is stimulated in the ELF at a non-thermal SAR level that is over a billion times lower than the same process activated in the RF at the thermal level.*

Conclusion: *There is a need for a biological standard to replace the thermal standard and to also protect against cumulative effects across the EM spectrum.*

III. ELF and RF activation of the stress response

Much detailed information about the stress response will be presented in the following sections and in the tables, but the most important finding to keep in mind is that *both ELF and RF fields activate the synthesis of stress proteins*. All cells do not respond to EMF, but activation of the same cellular mechanism by both thermal and non-thermal stimuli in a variety of cells shows that both ELF and RF are biologically active and that a biological ‘dose’ of EMF cannot be described in terms of SAR (Blank and Goodman, 2004a). SAR is irrelevant for non-thermal ELF responses, where energy thresholds are many orders of magnitude lower than in RF. A new definition of EMF dose is necessary for describing a safety limit, and SAR must be replaced by a measure of exposure that can be defined in biological terms.

The stress response, by its very nature, shows that *cells react to EMFs as potentially harmful*. The stress response is an important protective mechanism that enables cells from animals, plants and bacteria to survive environmental stressors, such as sharp increases in temperature (originally called ‘heat shock’), hypoxia, and dissolved toxic heavy metals like Cd^{+2} and oxidative species that can damage proteins and DNA (‘oxidative stress’). The stress response is evolutionarily conserved in essentially all eukaryotic and prokaryotic organisms, but not all stressors are effective in all cells, and different stress proteins are activated under different conditions. Stress proteins are a family of about 20 different proteins, ranging in size from a few kilodaltons to over 100kD. The 27kD and 70kD protein families are the most common and most frequently studied.

Kültz (2005) has called the stress response a ‘... defense reaction of cells to damage that environmental forces inflict on macromolecules.’, based on evidence from gene analysis showing that the stress response is a reaction to molecular damage. The genes activated as a group along with stress genes, which Kültz calls the ‘universally conserved proteome’, are those associated with sensing and repairing damage to DNA and proteins. Stress proteins help damaged proteins refold to regain their conformations, and also act as “chaperones” for transporting cellular proteins to their destinations in cells. The molecular damage stimulated by non-thermal ELF fields occurs in the absence of an increase in temperature. ELF energy thresholds are estimated to be about 10^{-12} W/kg, over a billion times lower than the thermal stimuli that cause damage in the RF range (Blank and Goodman, 2004a).

The classic stress response to a sharp increase in temperature (i.e., ‘heat shock’) is associated with a biochemical pathway where transcription factors known as heat shock factors, HSFs, translocate from the cytoplasm to the nucleus, trimerize and bind to DNA at the heat shock elements (HSEs) in the promoters of the genes. The promoter is the DNA segment where protein synthesis is initiated and it is not part of the coding region.

The HSEs contain specific nucleotide sequences, nGAAn, that are the consensus sequences for thermal stimuli. The binding of HSFs to HSEs, etc is similar for heat shock in plant, animal and bacterial cells. ELF range EMFs have been shown to follow the same sequence of events in inducing stress response proteins in human cells, including breast (MCF7, HTB124), leukemia (HL60), epithelial cells, as well as *E. coli* and yeast cells.

Studies done with chick embryos and cells from *Drosophila* and *Sciara* salivary gland chromosomes have produced graphic evidence of the effects of EMF. In *Drosophila* and *Sciara* salivary gland chromosomes, EMF causes the formation of ‘puff’s, enlarged regions along the chromosome, at loci associated with activation of heat shock genes. This is followed by elevated concentrations of transcripts at the sites and eventually stress protein synthesis (Goodman and Blank, 1998). The changes in chromosome morphology are characteristic of the stress response to both EMF and elevated temperature. Chick embryos develop hearts that stop beating when the oxygen concentration is lowered, but that can be protected and kept beating if stress proteins have been induced by ELF fields (DiCarlo et al, 1998) and in the RF range (Shallom et al, 2002).

The cellular response pathways to EMF have been characterized in the ELF range (Goodman and Blank, 2002), and have been found to share some of the characteristics of heat shock stress, such as the movement of heat shock factor monomers from the cytoplasm to the nucleus. The biochemical mechanism that is activated, the MAPK signaling pathway, differs from the thermal pathway (Goodman and Blank, 2002), but is the same as the non-thermal pathway in the RF range (Leszczynski et al, 2002).

The HSP70 gene is activated within minutes in cells exposed to ELF fields (Lin et al, 1997), and is accompanied by the binding of HSFs to the specific nucleotide sites in the promoter of the gene. However, different segments of the DNA promoter function as HSEs. Research in the ELF range has shown that the promoter of the major stress protein, hsp70, has two domains that respond to two different physical stimuli, EMF and an increase in temperature (Lin et al, 1999). The stimulus-specific domains have different DNA sequences that cannot be interchanged. The ***DNA consensus sequences that respond to EMF are nCTCTn*** (Lin et al, 1997; 1999). These differ from the nGAAn consensus sequences for thermal stimuli. The existence of two different consensus sequences that respond to EMF and temperature increase, respectively, are molecular evidence of different pathways that respond to non-thermal and thermal stimuli.

In another series of experiments, a DNA sequence from the promoter of an EMF sensitive gene was included in a construct containing a reporter gene, either chloramphenicol amino transferase (CAT) or luciferase. In each case, the construct proved to be EMF sensitive and reacted when an ELF field was applied (Lin et al, 2001). The ability to transfer EMF sensitive DNA sequences that subsequently respond to an EMF is further evidence linking the cellular response to a DNA structure.

In heat shock, the stress response is activated when extracellular signals affect receptors in the plasma membrane. This probably does not happen with an EMF, which can easily penetrate throughout the cell and whose actions are therefore not limited to the

membrane. One can transfer the EMF response by transferring the DNA consensus sequences (Lin et al, 2001), so it is likely that the activation mechanism involves direct EMF interaction with the DNA consensus sequences. The cell based signal transduction pathways of the heat shock response are involved in regulation of the EMF stimulated process, probably through the feedback control mechanisms that respond to the stress proteins synthesized or the mRNA concentrations that code for them (Lin et al, 1998).

Repeated induction of the stress response in a cell has been shown to induce cytoprotection, a reduced response associated with restimulation (Blank and Goodman, 1998). This is analogous to thermotolerance, the reduced response to an increase in temperature after an initial heat shock response. Experiments with developing chick embryos show similar habituation to repeated stimulation in the ELF range (DiCarlo et al, 2002). There are different effects of continuous and intermittent EMF exposures that show feedback control features in the EMF stimulated stress response (Lin et al, 1997). This autoregulatory reaction is an indication that the thermotolerance mechanism is inherent in the response to a single stimulus as well.

It has now been shown in many laboratories that RF also stimulates the cellular stress response and cells start to synthesize stress proteins in many different kinds of cells (e.g., Kwee et al, 2001; Shallom et al, 2002; Leszczynski et al, 2002; Weisbrot et al, 2004). Cotgreave (2005) included many cells that did not synthesize stress proteins in response to RF stimulation in his summary of data. The listings in Table 1 contain additional positive and negative results. It is quite clear that certain cell lines do not respond to EMF by synthesizing stress proteins. The reasons are not known, but the changes in cells in tissue culture and in cancer cells may render some of them unable to respond to EMF. In addition to mutations in cell lines, pre-exposure to ambient ELF and RF fields in the laboratory can also affect an ability to respond. What we can say in summary at this stage is that:

- the stress response has been demonstrated in many cells and linked to changes in the DNA and chromosomes.
- there are similarities in stress protein synthesis stimulated in the non-thermal ELF and thermal RF frequency ranges.
- the biochemical mechanism that is activated is the same non-thermal pathway in both ELF and RF, and is not associated with the thermal response.

IV. DNA activation mechanisms: EMFs and electrons

We think of DNA as a very stable polymer that stores and transmits genetic information from generation to generation. However, DNA must also come apart relatively easily to enable the continuous protein synthesis that is needed to sustain living cells. Usually, this process is started when specialized proteins called transcription factors bind to DNA.

However, both ELF and RF fields also stimulate DNA to start protein synthesis. EMF stimulation of stress protein synthesis indicates activation of DNA, even by relatively weak non-thermal ELF. This raises the possibility that EMF can cause other changes in DNA that interfere with the copying and repair processes in DNA, and that can lead to mutations and cancer.

Protein synthesis starts when the two chains of DNA come apart to make an mRNA copy of the amino acid code for a particular protein. This occurs at the specific DNA segment where the transcription factor binds, and in forming a bond changes the electron distribution. Since recent research has shown electron conduction in DNA (Wan et al, 1999; 2000; Ratner, 1999; Porath et al, 2000; Giese and Spichty, 2000), it is possible that EMF affects electron distribution and movement in DNA, and helps it to come apart to initiate protein synthesis, not unlike the action of a transcription factor. Charge transport through DNA depends on the DNA sequence (Shao et al, 2005), and there are reasons to believe that EMFs would cause the DNA to come apart at the EMF consensus sequence, nCTCTn (Blank and Goodman, 2002).

The ability of relatively small perturbations to stimulate DNA to initiate biosynthesis is consistent with larger perturbations that lead to DNA strand breaks. Several experimental studies have reported both single and double strand breaks in DNA and other chromosome damage after exposure to ELF fields (Lai and Singh, 1997a; Ivancsits et al, 2005, Diem et al, 2005; Winker et al, 2005). Ivancsits et al (2005) found DNA damage in fibroblasts, melanocytes and rat granulosa cells, but not in lymphocytes, monocytes and skeletal muscle cells. Single and double strand breaks and other DNA damage after exposure to RF fields have also been reported (Phillips et al, 1998; Sarimov et al, 2004; Lai and Singh, 2005).

The Ivancsits, Diem and Winker studies cited above are part of the REFLEX Project, a collaboration of twelve laboratories in seven countries of the European Union (REFLEX, 2004). The group found that both ELF and RF exposures, below the current safety limits, modified the expression of many genes and proteins. They also reported DNA damage (e.g., strand breaks, micronuclei, chromosomal damage) due to ELF fields at exposures of 35 μ T. Similar genotoxic effects were produced in fibroblasts, granulosa cells and HL60 cells by RF fields at SARs between 0.3 and 2W/kg. The expression and phosphorylation of the stress protein hsp27 was one of the many proteins affected.

The REFLEX Project Report (2004) is available on the internet and well worth consulting as a source of much information about the effects on cells *in vitro* due to the ELF and RF exposures we encounter in our environment. The Report has an introduction by Ross Adey, one of the last things he wrote, telling us about the importance of establishing "...essential exposure metrics ... based on mechanisms of field interactions in tissues". One needs a biological metric in order to characterize EMF exposure.

The possibility that EMFs could cause greater damage to DNA in the RF range and at longer exposures was demonstrated by Phillips et al (1998) who reported more DNA breaks when cells were exposed at higher SARs. They suggested that the rate at which

DNA damage can be repaired (or eliminated by apoptosis) is limited, and when the rate of damage at the higher SARs exceeds the repair rate, there is the possibility of retaining mutations and initiating carcinogenesis. Chow and Tung (2000) reported that exposure to a 50Hz magnetic field enhances DNA repair through the induction of DnaK/J synthesis. The eternal struggle in cells and organisms between the forces tending to break things down (catabolism) and those tending to build up and repair (anabolism) probably accounts for much of the variability one finds in experiments with cells as well as with people.

The changes in DNA initiated by ELF fields cannot be explained by thermal effects. Electric and magnetic fields interact with charges and magnetic dipoles, and fundamental mechanisms must ultimately be based on these interactions. From the data in Table 2, it is clear that relatively little energy is needed for effects on electron transfer (Blank and Goodman, 2002; 2004b; Blank, 2005). The low energies needed to perturb DNA in the ELF range suggest that the mechanism involves electrons, e.g., probably in the H-bonds that hold the two chains of DNA together. Electrons have very high charge to mass ratio and are most likely to be affected even by weak electric and magnetic fields.

There are many indications that electrons are involved in EMF reactions with DNA. In experiments that stimulate the stress response, the estimated force of $\sim 10^{-21}$ newtons that activates DNA can move a free electron about the length of a H-bond ($\sim 0.3\text{nm}$) in 1ns. The calculated electron velocity is comparable to electron velocities measured in DNA (Wan et al, 1999; 2000), and is also expected if electrons move at the \sim nanometer/picosecond flickering rate of protons in H-bonded networks (Fecko et al, 2003) that would be present at normally hydrated DNA sites. Electrons can tunnel nanometer distances in proteins (Gray and Winkler, 2003), and experiments have shown comparable electron movement in DNA (Wan et al, 1999; 2000). Electrons might be expected to move more readily from the CTCT bases in the consensus sequence, because of their low electron affinities. Finally, ELF fields have been shown to accelerate electron transfer in oxidation-reduction reactions (Blank and Soo, 1998; 2003).

The fact that the same non-thermal mechanism is activated in ELF and RF ranges emphasizes that it is not the total energy associated with the EMF that is critical, but rather the regular oscillations of the stimulating force. As already mentioned earlier, the energy associated with each wave (i.e., energy/cycle) is more or less independent of the frequency. If the same energy is needed to reach threshold in both ELF and RF, the many repetitions at the higher frequency cause the non-thermal threshold to be reached in a shorter time and the total energy absorbed over time to increase with frequency. Even in the ELF range, where SAR levels are very low, the stress response is activated by short exposures to fields of less than $1\mu\text{T}$, while single and double strand breaks in DNA have been reported at longer exposures to higher field strengths $\sim 0.1\text{mT}$ (Lai and Singh, 2005). The two mechanisms appear to be related in that breaks in DNA appear to result from free radical mechanisms that also involve electron transfer reactions (Lai and Singh, 1997b).

The reaction of EMFs with DNA differs from those listed in Table 2 in that they appear

to occur with equal ease at the widely differing frequencies in ELF and RF ranges. The frequency dependence of a reaction provides information about how time constants of charge transfer processes are affected by fields, and the frequency responses of the few EMF sensitive biological systems that have been studied suggest that fields are most effective at frequencies that are close to the natural rhythms of the processes affected (Blank and Soo, 2001a; Blank and Goodman, 2004b; Blank, 2005). Frequency optima for the enzymes, Na,K-ATPase and cytochrome oxidase, differ by an order of magnitude with maximums at about 60Hz and 800Hz, respectively (Blank and Soo, 2001a), in both cases close to the observed frequency maximum of the enzyme reaction. The rate constant of the BZ reaction is about 250Hz, the frequency of the rate limiting step in a multi-step process with at least 10 sub-reactions (Blank and Soo, 2003).

The electrons in DNA that are affected by EMFs are probably not engaged in electron transfer reactions. They respond to frequencies that range from ELF to RF and are more likely to be tied to the wide frequency range of fluctuations than to the frequency of a particular reaction. The displacement of electrons in DNA would charge small groups of base pairs and lead to disaggregation forces overcoming H-bonds, separating the two chains and enabling transcription. Studies have shown that biopolymers can be made to disaggregate when the molecular charge is increased (Blank, 1994; Blank and Soo, 1987). This explanation would also apply to the effect of applied electric fields that also activate DNA. Electric fields exert a force on electrons, and have been shown to stimulate protein synthesis in HL60 cells (Blank et al, 1992), E coli (Laubitz et al, 2006) and muscle *in vivo* (Blank, 1995). The genes for the hsp70 stress protein are more likely to be activated since they have been shown to be 'bookmarked' on the DNA chain, that is, more exposed to externally applied forces (Xing et al, 2005).

The outline of a plausible mechanism to account for EMF activation of DNA through interaction with electrons has relied on evidence from many lines of research. This mechanism may or may not hold up under further testing, but the experimental facts it is based on have been verified. It has been clearly demonstrated that exposure of cells to non-thermal power and thermal radio frequency EMFs, at levels deemed to be safe for human exposure, activate DNA production of stress proteins and could increase the number of DNA breaks. There is ample experimental evidence to support the possibility of DNA damage at non-thermal levels of exposure, and the need for greater protection.

V. The critical role of scientific research

The connection between the results of scientific research and assessing EMF risk does not appear to be working well. We all agree that EMFs are unsafe at the level where they cause electrocution, and that we must protect against that possibility. We also agree that if other risks are associated with EMFs, we must identify them and determine the exposure levels at which they occur. This task requires that we define a biological dose of EMF, and that we obtain information about cellular mechanisms activated at different doses. As we have seen, the currently accepted measure of EMF dose, the specific absorption rate (SAR), is definitely not a measure of the effective biological dose when

stress protein synthesis can be stimulated by SAR levels that differ by many orders of magnitude in the ELF and RF ranges (Blank and Goodman, 2004a). Yet, there is strong opposition to accepting the consequences of these experimental facts.

Regarding EMF mechanisms, we still have much to learn, but we know that the energy and field strength thresholds of many biological reactions are very low (Table 2). These findings indicate that safe exposure levels for the public should be substantially lowered, if only as a precautionary measure. Even when stated in vague terms, so as to require little more than lip service, a precautionary policy has not yet been recommended by the WHO. Thus, the two main problems of research on EMF risk, defining a biological dose and the desired level of exposure protection, remain to be solved.

Scientific research can contribute to defining a biological dose, but the desired level of exposure protection is a more complicated issue. Guidance for EMF policy on exposure protection has come primarily from epidemiology studies of health risks associated with power lines in the case of ELF, and cell phones in the case of RF. Basic research studies do not provide insight into the effects of long term exposures that are so important in determining risk, and they appear to have been used almost entirely to probe biochemical mechanisms that might underlie health risks identified in epidemiology studies. However, the research does overcome a basic weakness of epidemiology studies, an inability to determine a causal relation and to rule out effects of possible confounders. Epidemiology studies can correlate EMF exposure and health effects in human populations, and show quantitative dose-response relations, but it is only when coupled with basic research on molecular mechanisms that one can test and establish the scientific plausibility of effects of exposure. This scientific capability has become more important with recent advances in research on DNA, where mutations associated with initiation and promotion of cancer can be identified. EMF laboratory research has also played an indirect role in the practical aspects of risk by showing that:

- many biological systems are affected by EMFs,
- EMFs compete with intrinsic forces in a system, so effects can be variable,
- many frequencies are active,
- field strength and exposure duration thresholds are very low,
- molecular mechanisms at very low energies are plausible links to disease (e.g., effect on electron transfer rates linked to oxidative damage, DNA activation linked to abnormal biosynthesis and mutation).

Research on the stress response, a protective mechanism that involves activation of DNA and protein synthesis, was not included in previous scientific reviews prior to evaluating safety standards, and thus provides additional insights into EMF interactions (Blank and Goodman, 2004a). Activation of this protective mechanism by non-thermal as well as thermal EMF frequencies has demonstrated:

- the reality and importance of non-thermal effects of EMFs,

- that cells react to an EMF as potentially harmful,
- the same biological reaction to an EMF can be activated in more than one division of the EM spectrum,
- direct interaction of ELF and RF with DNA has been documented and both activate the synthesis of stress proteins,
- the biochemical pathway that is activated is the same pathway in both ELF and RF and it is non-thermal,
- thresholds triggering stress on biological systems occur at environment levels on the order of 0.5 to 1.0 μT for ELF,
- many lines of research now point to changes in DNA electron transfer as a plausible mechanism of action as a result of non-thermal ELF and RF.

Given these findings, the *specific absorption rate (SAR)* is not the appropriate measure of biological threshold or dose, and should not be used as a basis for a safety standard since it regulates against thermal effects only.

Cellular processes are unusually sensitive to non-thermal ELF frequency fields. The thresholds for a number of biological systems are shown in Table 2, and many are in the range of 0.5 to 1.0 μT , not very much higher than the usual environmental backgrounds of $\sim 0.1 \mu\text{T}$. The low biological thresholds in the non-thermal ELF range undermine claims that an EMF must increase the temperature in order to cause changes in cells. They also show that many biochemical reactions can be affected by relatively low field strengths, similar to those in the environment. Non-thermal ELF fields can also cause DNA damage, and therefore add to health and safety concerns.

In addition to very low thresholds, exposure durations do not have to be very long to be effective. Litovitz et al (1991, 1993), working with the enzyme ornithine decarboxylase, have shown a full response to an EMF when cells were exposed for only 10sec. This occurred with ELF sine waves or ELF modulated 915MHz sine waves. The exposure had to be continuous, since gaps in the sine wave resulted in a reduced response. Interference with the sine wave in the form of superimposed ELF noise also reduced the response (Mullins et al, 1998). The interfering effect of noise has been shown in the RF range by Lai and Singh (2005), who reported that noise interferes with the ability of an RF signal to cause breaks in DNA strands. The decreased effect when noise is added to a signal is yet another indication that EMF energy is not the critical factor in causing a response.

The finding that the stress response threshold can be stimulated in both ELF and RF frequency ranges appears to suggest that the threshold is independent of EMF energy. Energy increases with the frequency, so compared to an ELF energy of ~ 1 a.u. (arbitrary unit of energy), the energy at RF is $\sim 10^{11}$ a.u. Actually, it is the energy/cycle that is independent of frequency. A typical ELF cycle at 10^2 Hz lasts 10^{-2} sec and a typical RF

cycle at 10^{11} Hz lasts 10^{-11} sec. Because the energy is spread over a different number of cycles each second in the two ranges, the same value of $\sim 10^{-2}$ a.u./cycle applies to both ELF and RF ranges.

An early review of the stress response in the ELF range (Goodman and Blank, 1998) summarized basic findings, and a more recent review by Cotgreave (2005) has provided much additional information, primarily on the RF range. Table 1 summarizes both ELF and RF studies (mainly frequencies 50Hz, 60Hz, 900MHz, 1.8GHz) relevant to stimulation of DNA and stress protein synthesis in many different cells. The list is not exhaustive, but the citations represent the different frequencies and biological systems, as well as the diversity of results in the literature. As already noted by Cotgreave (2005), the stress response does not occur in reaction to EMFs in all cells. A paper by Jin et al (2000), to be discussed later, shows that even the same cell line can give opposite results in the same laboratory. The stress response is an important topic in its own right, but its importance for EMF research is that it offers insights into EMF interaction mechanisms in the stimulation of DNA. On the practical level, the stress response has shown the need to replace the SAR standard to take into account non-thermal biological effects.

Differences in experimental results shown in Table 1 are not uncommon when studying phenomena that are not as yet well understood, and this frequently gives rise to controversy. In EMF research, however, other factors have contributed to a controversial scientific atmosphere. The following sections on the scientific context, as well as a critique of the review by Cotgreave, will show how discussion of the stress response and the absence of discussion on related topics have compromised the evaluation of the science. The discussion of stress response stimulation in ELF and RF ranges together with ideas on DNA mechanisms, has important implications regarding EMF risk and safety.

VI. The troubling context of today's science

The need to include basic research findings in assessment of health risks is clear, but it is equally important to make sure that these findings are properly evaluated. No less an authority on science than Donald Kennedy (2006), the current Editor of *Science*, wrote "...how competitive the scientific enterprise has become, and the consequential incentive to push (or shred) the ethical envelope". He was referring primarily to the controversial religious/ political atmosphere over such issues as evolution, stem cell research, etc, but he could just as easily have included economic factors. In the following quote, editors of the *Journal of the American Medical Association* (JAMA 284:2203-2208, 2000) pointed out distortions in the proof of effectiveness of drugs in studies supported by the drug industry:

"There is a growing body of literature showing that faculty who have industry ties are more likely to report results that are favorable to a corporate sponsor, are more likely to conduct research that is of lower quality, and are less likely to

disseminate their results to the scientific community”.

Even *The Wall Street Journal* (Jan 9, 2007), which generally presents favorable views of business, had a front page article on the controversy over whether mycotoxins produced by molds are harmful, that was critical of scientist-business community connections. They pointed out that some scientific experts in the professional societies, who had issued statements minimizing harmful effects, had not disclosed their links to companies defending lawsuits in this area.

The connection between scientific expertise, the research that is done, and the source of support, has always been an ethical gray area, but the above examples and recent instances of experimental fraud have reinforced the impression that the ethical standards of scientists have deteriorated considerably. In our area of interest, insufficient attention has been paid to the influence the power and communication industries may be having on the research of those assessing EMF safety. At the Third International Standard Setting Seminar (October 2003) in Guilin, China, Prof. Henry Lai of the University of Washington summarized 179 cell phone studies showing that independent researchers were twice as likely to report biological effects due to RF in comparison to those funded by industry. This was very much in line with the earlier JAMA comment on the drug industry. Published reports have started to appear (Hardell et al, 2006; Huss et al, 2007) documenting the correlation of EMF research outcome with the source of support. Recognition of the phenomenon is a first step toward minimizing abuses, and one hopes that this information will eventually be factored into evaluation of the experimental results. I am not overly optimistic, since those who wish their influence to remain hidden can channel support through unaffiliated committees with non-committal names.

Science is a cooperative enterprise in the long run, but in day-to-day practice, there has always been competition among scientists for recognition and support. In EMF research, the atmosphere has become especially adversarial in the selection of participants and subjects to be covered in recent evaluations. Two important examples are the International Committee on Electromagnetic Safety (ICES) and IEEE sponsored symposium on "Reviews of Effects of RF Energy on Human Health" (BEMS Supplement 6, 2003), and the more recent WHO sponsored symposium "Sensitivity of Children to EMF Exposure" (BEMS Supplement 7, 2005). Both collections of papers appeared in *Bioelectromagnetics*, the journal of the primary research society in this scientific specialty, where publication carries a certain aura of authority in the field. Of course, one expects the highest of ethical standards, and the editor assured everyone that normal reviewing procedures, etc, had been followed. However, all that had come after the scope of the papers had been narrowly defined so that there was no coverage of recent research on the EMF stimulated stress response or stimulation of DNA to initiate protein synthesis. An older mind set pervaded the choice of the topics and the papers. That mind set appeared to be stuck in the belief that non-thermal EMF was biologically inert, that the nucleus was an impregnable structure that unlocked the genetic information in its DNA only at the time of cell division, etc. These two meetings took place only a few years ago, in a world of science where it had already been known for some time that biochemical signals are continuously changing DNA in cell nuclei and mitochondria,

turning on protein synthesis, checking and repairing DNA itself, etc. Research on the stress response had even shown that DNA was unusually sensitive to EMF by finding responses in the non-thermal ELF range. One expects to find such papers in symposia organized by the Mobile Manufacturers Forum, but not in *Bioelectromagnetics*.

A science based evaluation process cannot limit its scope of interest so as to ignore a research area that is so central in biology today, and that is obviously affected by EMF. Information on the EMF stimulated stress response and stimulation of DNA to initiate protein synthesis must be an integral part of the evaluation process, and its omission in earlier evaluations compromised the scientific basis of those reviews and distorted their conclusions.

It is ironic that the review in *Bioelectromagnetics* Supplement 6 listed as its first guiding principle that “The RF safety standard should be based on science”, essentially a reaffirmation of the IEEE guideline for the revision of C95.1-1991 safety standards. Scientific research is designed to answer questions, and answers do not come from deciding *a priori* that certain types of studies are not relevant or can be ignored because they have not been adequately proven in the eyes of the organizers. Scientific method is not democratic. The word ‘proof’ in ‘scientific proof’ is best understood in terms of its older meaning of ‘test’. It does not rely on an adversarial ‘weight of the evidence’, where opposing results and arguments are presented and compared. Answers do not come from keeping a scoreboard of positive versus negative results and merely tallying the numbers to get a score. In scientific proof, number and weight do not count. It is hard to see how the review in *Bioelectromagnetics* Supplement 6 could reconcile its advocacy of science as a guiding principle with its subsequent endorsement of “the weight of evidence approach” to be used in their assessment.

We should be reminded that ‘scientific proof’ is not symmetric (Popper, 1959). One cannot prove that EMF is harmless no matter how many negative results one presents. One single reproducible (significant) harmful effect would outweigh all the negative results.

The above characteristics of science are generally acknowledged to be valid as abstract principles, but in EMF research, it has been quite common to list positive and negative findings and thereby imply equal weights. Table 1 is an alphabetical listing by first author of positive and negative findings, with the negative studies indicated as **NO** in bold. There is no scoreboard, since the studies are on many different systems, etc, and not of the same quality. The listing is not meant to be complete or to be scored, but rather to present the variety of biological systems studied in the different EMF ranges. Negative studies play an important role in science, and there is good reason to publish them when they are failures to replicate earlier positive results. This can often lead to important clarifications of the effect, the technique, etc. However, negative studies are being used in another way. Although they cannot prove there is no positive effect, they do have an influence in the unscientific ‘weight of evidence approach’. In epidemiology, where it is difficult to compare studies done under different conditions, it is common to make a table of the positive and negative results. The simple listing has the effect of a

tally, and the overall score substitutes for an evaluation. In any case, one can write that the evidence is ‘not consistent’, ‘not convincing’ or claims are ‘unsubstantiated’ and therefore ‘unproven’. The same is true in experimental studies. Funds are generally not available for an independent study to track down the causes of the differences in results, so the contradictory results are juxtaposed and a draw is implied. This is a relatively cheap but effective way to neutralize or negate a positive study.

VII. Replication and failures to replicate experimental results

Independent replication of experiments is an essential criterion for acceptance of a result and one of the pillars of scientific proof. However, as we shall see below, it is very difficult to actually replicate a biological experiment. We need only remember the experience with the ‘Henhouse’ project run by the Office of Naval Research many years ago, when chicken eggs from different suppliers led to different effects of EMFs on chick embryo development.

While scientists generally shun replications, some failures to replicate have been analyzed and explained. The two discussed below had the earmarks of replications, but neither was. In one case, it was clearly shown by Jin et al (2000) that the investigators failed to use the precise cell type population of the original experiment. Jin et al obtained HL60 cells from the two different sources used in the papers with the contradictory results, and showed that the cells had very different growth characteristics, significantly different reactivities and reactions to EMFs. It appears that even different samples of the same cell line in the same laboratory can have different responses to EMFs. The changes that occur in tissue culture over time can result in very different responses to EMFs.

In another example, Utteridge et al (2002) published a paper in *Radiation Research* meant to test the positive results of an earlier study (Repacholi et al, 1997) that had shown a twofold increase in lymphoma in mice exposed to cell phones. They failed to replicate the findings, but even a cursory reading of the paper showed that the study was poorly designed and executed, and was definitely not a replication. They had used a different exposure regimen and had manually handled the animals, an added stress on the mice. The cancer rate in the control group was three times the rate of the earlier study, possibly due to the handling, making it almost impossible to find any effect of cell phone exposure. There were also unusual inconsistencies in the published data, such as listing the weights of animals that had died months earlier. It is hard to see how the paper passed peer review. The Utteridge study self-destructed, and the results of the Repacholi study are still looked upon as showing a relation between RF and cancer in an animal model. However, there were scientific casualties, the peer review process of the journal and the credibility of its editors.

It may be appropriate to mention that *Radiation Research*, a journal devoted to research with ionizing radiation frequencies, has published studies that almost exclusively show no EMF effects. A quick glance at Table 1 will show that many of the ‘**NO** effect’ listings are published in that journal. It has even gone beyond the frequency range

defined in its title and published 'negative' studies in the non-ionizing frequency range. The internet edition of *Microwave News* has an explanation for why this journal repeatedly publishes negative research and appears to have become so politicized on the EMF issue.

It is not unusual for scientists to deviate from an original experimental protocol when repeating an experiment. They generally view the deviations as improvements in technique. Readers who have not worked on that particular system are unlikely to focus on a small difference that does not appear to be significant. Yet, even a small difference may lead to a failed replication. Blank and Soo (2003) showed that EMF accelerated the Belousov-Zhabotinsky (BZ) reaction, which is the catalyzed oxidation of malonic acid. A subsequent study reported no effect of EMF on the BZ reaction (Sontag, 2006), in essence a failed replication. In the second study, the authors did not apply the field at the time the reactants were mixed, as in the original, but only after the reaction was well under way for about seven minutes. This time difference was critical for a reaction that responds to EMF. Other reactions had responded to EMF (Blank and Soo, 2001b; Blank, 2005) only when the field was applied at time zero, when the intrinsic chemical forces were relatively weak. The effect of EMF was even shown to vary inversely with the opposing chemical forces of an enzyme (Blank, 2005). After seven minutes, the BZ reaction was running at full speed and the applied ELF fields were not strong enough to overcome the built up chemical forces.

The above paragraph points up a critical factor often overlooked in EMF experiments. EMF is only one of the factors that can affect the rate of a biochemical reaction, and a relatively weak one in the ELF range. It appears that when an EMF accelerates charge movements associated with a reaction, the applied field competes with intrinsic forces, and the ability to see an effect of the applied EMF depends on minimizing the other forces in the system. It is obvious that an important strategy to minimize unwanted biological effects due to EMF is to maintain intrinsic forces at optimal (healthy) levels.

In the above mentioned experiments with the Na,K-ATPase (Blank, 2005), it was found that the effect of an applied electric or magnetic field varied inversely with the activity of the enzyme, which could be changed by changing ion concentrations, temperature, inhibitors, or by the normal aging of the preparation. The effect of intrinsic activity was also observed in other systems, electron transfer from cytochrome C to cytochrome oxidase (Blank and Soo, 1998), and in the effect of temperature on the oxidation of malonic acid (Blank and Soo, 2003). Since the effect of EMF in an experiment can vary depending on the other forces acting in the system, it is important to make sure that all relevant parameters are identified and controlled. Replication of biological experiments must ensure a comparable level of intrinsic biological activity before a perturbing EMF is applied. This is especially difficult with enzyme preparations as they age.

In studies of stress protein synthesis, many factors must be considered, but the choice of cells is particularly important. Not all cells respond to EMF, and the results of many experiments have suggested ideas about critical properties that are apt to determine the

response and also affect the ability to replicate an experimental result.

A quick look at Table 1 shows that tissue culture cells are more likely to show ‘**NO** effect’. That is not really surprising. Cells in tissue culture have changed significantly to enable them to live indefinitely in the unnatural conditions of a flask in a laboratory, and the changes could have made them unresponsive to EMF. The same is true of the changes in cancer cells, although some (e.g., MCF7) have responded to EMF (e.g., Liburdy et al, 1993), and in one cell line, HL60, some samples respond to EMF and others do not (Jin et al, 2000). On the other hand, the study by Czyz et al (2004) found that p53-deficient embryonic stem cells showed an increased EMF response, but the wild type did not. It is obviously difficult to make generalizations about the necessary conditions for a response to EMF when there are so many variations, and cells can undergo changes in tissue culture.

Some insight into differences between cells has been obtained from a broad study of genotoxic effects in different kinds of cells (Ivancsits et al, 2005). They found no effects with lymphocytes, monocytes and skeletal muscle cells, but did find effects with fibroblasts, melanocytes and rat granulosa cells. Other studies (e.g., Lantow et al, 2006b; Simko et al, 2006) have also found that the blood elements, such as lymphocytes and monocytes are natural cells that have not responded. From an evolutionary point of view, it may be that mobile cells can easily move away from a stress and there is little selective advantage to develop the stress response. The lack of response by skeletal muscle cells is easier to explain (Blank, 1995). It is known that cells containing fast muscle fibers do not synthesize hsp70, while those with slow fibers do. This evolutionary development protects cells from over-reacting to the high temperatures reached in fast muscles during activity.

Other natural cells listed in Table 1, such as epithelial, endothelial and epidermal cells, fibroblasts, yeast, E coli, developing chick eggs, the cells of *Drosophila*, *Sciara* and *C elegans*, have all been shown to respond. While experiments with non-responding cells have provided little information, studies of the differences between responding and non-responding cells may be the best experimental strategy for studying the stress response mechanism. Proteomics appears to be an excellent tool for answering many of the questions about the molecular mechanisms that are activated (Leszczynski et al, 2004).

In studies of stress protein synthesis, the time course of a response must be determined. There is generally a rapid induction and a slower falloff of response, but the kinetics can be affected by many other conditions of the experiment. It is, therefore, important to look for stress proteins when they are apt to be present, and not before they have been synthesized or after the response has decayed. This may be the explanation for the inability of Cleary et al, (1997) to observe stress proteins twenty-four hours after exposure. Some additional cautions to be aware of in contemplating or evaluating a study. For example, different stresses elicit different responses, so it is important to determine which of the ~20 different stress proteins are synthesized. The most frequently studied stress proteins are hsp70 and hsp27, but others may be involved and undetected. The exposure history of a cell population must be known, since there are differences in

the responses to an initial stimulus and subsequent ones. The need to provide shielding for cells becomes far more complicated when they respond to RF as well as ELF fields and one must insure no pre-exposure.

Obviously, many experiments must be done to determine the optimal conditions for the study of a particular system. This does not shift the burden of proof to those unable to find an effect, but it adds weight to the cautions generally voiced in papers that state their failure to observe stress proteins ‘under our experimental conditions’. Those words mean just that, and not that stress proteins were absent.

An experiment on EMF stimulation of cell growth that has almost disappeared from the EMF literature is the work of Robert Liburdy (Liburdy et al, 1993). He reported that weak 60Hz fields can interfere with the ability to inhibit growth in MCF7 breast cancer cells. This finding has been replicated six times, but the original experiment and its replications have been ignored by many health oriented scientists (Liburdy, 2003), including the recent WHO review (BEMS Supplement 7, 2005). Even breast cancer researchers (e.g., Loberg et al, 1999), who have not been directly involved in the EMF debate, appear to be totally unaware of results showing the ability of weak 60Hz fields to affect cancer cell growth. It is shocking when an EMF research review by a presumably scientifically neutral WHO fails to even mention any of the papers that offers insight into the mechanism of a devastating disease that is so prevalent in the population (Blank and Goodman, 2006). Let us not forget the asymmetry in scientific proof (Popper, 1959), where a single reproducible harmful effect would outweigh all the negative results. The many replications of the Liburdy experiment have given us a crucial finding regarding the question of EMF risk, and they cannot be ignored.

VIII. A critical look at a recent review of the stress response

The earlier discussion of non-scientific influences in the design and presentation of the results of EMF research serves as an introduction to a critical look at the recent review on RF and the stress response by Cotgreave (2005) ‘with contributions of the Forschungsgemeinschaft Funk’. I agree with the major conclusion-of the review, the need for more research on the stress response with better controls. However, Cotgreave was highly selective in his omission of papers on ELF and stress proteins. Given that there are many relevant ELF papers reporting effects on stress proteins at non-thermal levels, this omission results in significant under-reporting of what is scientifically established. These obvious and scientifically questionable omissions were used to cast doubt on the ability of RF to have a significant biological effect, at a time when much evidence pointed in the opposite direction.

Cotgreave stated correctly that RF is pleiotropic (produces more than one gene effect) for many regulatory events, in addition to the stress response. That observation comes as no surprise to biologists who know that cellular systems are interconnected and that the complexity of the signaling pathways resembles that of the old interlinked intermediary metabolism charts. It is also no surprise to those familiar with early papers on EMFs,

which showed activation of genes such as *c-myc* (Goodman and Shirley-Henderson, 1991; Lin et al, 1994;1996) and *c-fos* (Rao and Henderson, 1996) at about the same time the EMF stress response was first described (Blank et al, 1994; Goodman et al, 1994). The EMF stimulated synthesis of many proteins (Goodman and Henderson, 1988) and the binding of specific transcription factors AP-1, AP-2 and SP-1 were also previously described (Lin et al, 1998).

By highlighting the previously known pleiotropic nature of the EMF response, Cotgreave played down the role of the stress response as a protective mechanism. Had he analyzed the biological implications of the many genes activated, he could have pointed to evidence from proteomics and gene analysis that there is a relevant pattern to the pleiotropism. Kültz (2005) recently summarized the evidence that specific groups of genes are activated along with stress genes across the biological spectrum. It is of particular interest to the EMF discussion that this ‘universally conserved proteome’ consists largely of genes involved in sensing and repairing damage to DNA and proteins, evidence that the stress response is a reaction to molecular damage across the biological spectrum. The stress response is one of many stimulated by RF, but other parts of the response also show evidence of damage control in reaction to an EMF.

By limiting the scope of his review to effects of RF, Cotgreave overlooked much that is relevant to understanding the effects of EMFs. That was a bit like writing a review on the physiological effects of alcohol and limiting the discussion to scotch whiskey. The EM spectrum is continuous and its divisions arbitrary, so there is no good reason to limit the discussion to RF when living cells are activated and synthesize stress proteins in both RF and ELF ranges (Blank and Goodman, 2004a). Furthermore, emissions from cell phones include both RF and ELF frequencies (Linde and Mild, 1997; Jokela, 2004; Sage et al, 2007). The bulk of the original research on EMFs and the stress response was done using ELF (see review by Goodman and Blank, 1998). ELF studies also led to information about the DNA consensus sequence sensitive to EMFs that differs from the ‘heat shock’ consensus sequence (Lin et al, 1999). This is a critical piece of molecular evidence showing the difference between thermal and non-thermal responses. Cotgreave described the heat shock consensus sequence, but not the EMF consensus sequence or the experiments in which such sequences were transferred and retained sensitivity to an EMF (Lin et al, 2001). For any insight into EMF-DNA interaction, it was absolutely essential to describe the molecularly based biological sensitivity to EMFs, inherent in DNA structure, that differs from thermal sensitivity and that can be manipulated.

More importantly, by considering both ELF and RF responses, it becomes obvious that the practice of describing EMF ‘dose’ in terms of SAR is meaningless for the stress response (Blank and Goodman, 2004a). The research on ELF stimulated stress response has shown unequivocally that SAR at the threshold is many orders of magnitude lower than in the RF range. The separation of thermal and non-thermal mechanisms had already been shown by Mashevich et al (2002), where chromosomal damage observed under RF in lymphocytes was not seen when the cells were exposed to elevated temperatures. The importance of non-thermal mechanisms was also made clear in the experiments of Bohr and Bohr (2000) in a much simpler biochemical system, showing

that both denaturation and renaturation of β -lactoglobulin are accelerated by microwave EMF, and by de Pomerai et al (2003), who showed that microwave radiation causes protein aggregation without bulk heating. These as well as the ELF enzyme kinetics studies listed in Table 2 should have indicated that EMFs can cause changes in molecular structure without requiring heating.

Cotgreave overlooked a similarity between electric and magnetic ELF stimulation of DNA and endogenous electric stimulation of protein synthesis. Blank (1995) had reviewed this effect in striated muscle, and recently Laubitz et al (2006) showed that myoelectrical activity in the gut can trigger heat shock response in *E coli* and Caco-2 cells. The mechanism in striated muscle is well known. Body builders stimulate muscle activity to increase muscle mass, and biologists have known that the electric fields associated with muscle action potentials stimulate the synthesis of muscle proteins. The particular proteins synthesized appear to be related to the frequency of the action potentials, and one can even change the protein composition of a muscle by changing the frequency of the action potentials (Pette and Vrbova, 1992). Under normal physiological conditions, the action potentials along the muscle membrane drive currents across the DNA in nuclei adjacent to the membrane. The estimated magnitude of electric field, $\sim 10\text{V/m}$, provides a large safety margin in muscle, since fields as low as 3mV/m stimulate biosynthesis in HL60 cells (Blank et al, 1992). The fact that a physiological mechanism links electric stimulation to protein synthesis suggests that EMF can cause stress protein synthesis by a similar mechanism.

As a matter of proper scholarly attribution ‘heat shock’ was first described in *Drosophila* by Ritossa (1962), and the first description of stress response due to EMF was in back-to-back papers showing similar protein distributions stimulated by temperature and ELF (Blank et al, 1994), and that both stimuli resulted in proteins that reacted with the same specific antibody for the stress protein hsp70 (Goodman et al, 1994). The ability of power frequency fields to alter RNA transcription patterns had been reported even earlier by Goodman et al (1983).

The above discussion acknowledges that Cotgreave’s review was a positive contribution that summarized much useful information, but one that failed to properly assess the state of knowledge in EMF stress protein research. He gave the impression that much of the information was tenuous and that the thermal mechanism was the only one to consider. This may be his point of view and that of co-contributor, Forschungsgemeinschaft Funk. However, at the very least, he should have incorporated relevant research on stimulation of the stress response by non-thermal EMFs. The ELF data have convinced many to reject the paradigm of thermal effects only. A reader would have learned more about the stress response had the author devoted more space to the ELF papers than to papers on something called ‘athermal heating’.

IX. Rethinking EMF safety in a biology context

Studies of the stress response in different cells under various conditions have enabled us to characterize the molecular mechanisms by which cells respond to EMF and their effects on health risk. That information can now correct assumptions about biological effects of EMF, and establish a scientific basis for new safety standards.

In setting standards, it is essential that basic findings in all relevant research areas are taken into account. Relevance is not subjective. It is determined by whether a study adds to our knowledge of how cells react to EMF, and this criterion determined inclusion of the references in Table 1. The criteria for the references in the IEEE list were not focused on the molecular biology of cellular responses that illuminate disease mechanisms, but were based on such assumptions as arbitrarily defined divisions of the spectrum, on thermal responses only, etc. It is therefore not surprising that many relevant studies were omitted in the IEEE literature review. Fewer than one quarter of the references listed in Table 1 appear in the IEEE list. The result of having omitted many EMF studies, including those on the stress response, is that many research results have not been utilized in setting EMF safety standards. A careful examination of basic assumptions will show that the omissions are crucial and that they indicate an urgent need to reconsider the entire basis for EMF safety standards. Here in bold are the assumptions, followed by the re-evaluations:

- **Safety standards are set by division of the EM spectrum.** It may come as a surprise to the engineers and physicists who set up the divisions of the EM spectrum, but biology does not recognize EM spectrum divisions. The same biological reaction can be stimulated in more than one subdivision of the EM spectrum. The arbitrarily defined divisions of the spectrum do not in any way confine the reactions of cells to EMF, and ELF studies do indeed contribute to an understanding of how cells respond to RF. This was discussed in the critique of Cotgreave's (2005) review. This area clearly demands immediate attention. People are getting ELF and RF simultaneously from the same device, and they are being protected from thermal effects only. This ignores the potentially harmful effects from non-thermal ELF and RF discussed next.

- **EMF standards are based on the assumption that only ionizing radiation causes chemical change.** The stress response in both ELF and RF ranges has shown that non-ionizing radiation also causes chemical change. Several additional examples of EMF stimulated chemical change in the ELF range are listed in Table 2.

- **EMF standards are based on the assumption that non-ionizing EMF only causes damage by heating (i.e., damage by thermal effects only).** Research on the stress response in the ELF range has shown that a thermal response to a rise in temperature and the non-thermal response to EMF are associated with different DNA segments of the same gene. Both the thermal and the non-thermal mechanisms are natural responses to potential damage.

Furthermore, the non-thermal stress response can occur in both the ELF and RF ranges. Other non-thermal effects of EMF have been demonstrated, e.g., acceleration of electron transfer reactions and DNA strand breaks.

- **Safety limits in the non-ionizing range are in terms of rate of heating (SAR).** The above described effects occur below the thermal safety limits in the non-ionizing range, so the safety limits provide no protection against non-thermal damage. Safety limits must include non-thermal effects.

X. Summary

It is generally agreed that EMF safety standards should be based on science, yet recent EMF research has shown that a basic assumption used to determine EMF safety is not valid. The safety standard assumes that EMF causes biological damage only by heating, but cell damage occurs in the absence of heating and well below the safety limits. This has been shown in the many studies, including the cellular stress response where cells synthesize stress proteins in reaction to potentially harmful stimuli in the environment, including EMF. The stress response to both the power (ELF) and radio (RF) frequency ranges shows the inadequacy of the thermal (SAR) standard.

The same mechanism is stimulated in both ranges, but in the ELF range, where no heating occurs, the energy input rate is over a billion times lower than in the RF range.

The stress response is a natural defense mechanism activated by molecular damage caused by environmental forces. The response involves activation of DNA, i.e., stimulating stress genes as well as genes that sense and repair damage to DNA and proteins. Scientific research has identified specific segments of DNA that respond to EMF and it has been possible to move these specific segments of DNA and transfer the sensitivity to EMF. At high EMF intensities, the interaction with DNA can lead to DNA strand breaks that could result in mutation, an initiating step in the development of cancer.

Scientific research has shown that ELF/RF interact with DNA to stimulate protein synthesis, and at higher intensities to cause DNA damage. The biological thresholds (field strength, duration) are well below current safety limits. To be in line with EMF research, a biological standard must replace the thermal (SAR) standard, which is fundamentally flawed. EMF research also indicates a need for protection against the cumulative biological effects stimulated by EMF across the EM spectrum.

XI. References

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**Table 1. Studies of EMF Stimulation of DNA and Protein Synthesis
(page 1)**

Table 1 summarizes both ELF and RF studies (mainly frequencies 50Hz, 60Hz, 900MHz, 1.8GHz) relevant to stimulation of DNA and stress protein synthesis in many different cells.

Study/Journal	Frequency	Cells/effect on hsps
Balcer-Kubicek et al, 1996 Radiation Res	60Hz	HL60 NO synthesis of myc
Blank et al, 1994 Bioelectrochem Bioenerg	60Hz	<i>Sciara</i> salivary glands [temperature, EMF, cause same new proteins]
Capri et al, 2004 Int J Radiat Biol	1800MHz	monocytes NO effect on apoptosis, hsp70
Caraglia et al, 2005 J Cell Physiol	1.95GHz	epidermoid cancer cells Induces apoptosis, hsp70
Chauhan et al, 2006 Radiation Res	1.9GHz	human lymphoblastoma (TK6) NO hsp response
Chauhan et al, 2006 Int J Radiat Biol	1.9GHz	two human immune cell-lines HL60,MM6 NO hsp response
Cleary et al, 1997 Bioelectromagnetics	27MHz	HeLa, CHO (also at 2450MHz mammalian cells NO hsp after 2 hr exposure, 24 hr to measurement
Chow and Tung, 2000 FEBS Letters	50Hz	<i>E. coli</i> strain XL-1 BLUE + plasmid pUCB DNA repair improved
Czyz et al, 2004 Bioelectromagnetics	modulated 1.71GHz	p53-deficient embryonic stem cells hsp70 expression, but not in wild type

Table 1. Studies of EMF Stimulation of DNA and Protein Synthesis
(page 2)

Daniells et al, 1998 Mutat Res	750MHz	C elegans induced hsp16
Dawe et al, 2005 Bioelectromagnetics	750MHz	C elegans (same lab as above paper) hsp 16 may be due to temperature rise
Di Carlo et al, 2002 J Cell Biochem	60Hz	chick embryo repeated EMF causes lower hsp response
Diem et al, 2005. Mutation Res	1800MHz	fibroblasts, GFSH-R-17 granulosa cells non-thermal DNA breakage
Fritze et al, 1997 Neuroscience	900MHz	rat brain blood brain barrier leakage at high SAR
Goodman et al, 1983 Science	pulsed 60Hz	<i>Sciara</i> larvae induce cellular transcription
Goodman et al, 1994 Bioelectrochem Bioenerg	60Hz	<i>Sciara</i> larvae increased hsp70 transcripts
Harvey et al, 2000 Cell Biol Int	864.3MHz	human mast cell line, HMC-1 effects on protein kinase C , stress genes
Hirose et al, 2006a Bioelectromagnetics	2.1425GHz	Human IMR-90 fibroblasts NO effect on gene expression of p53
Hirose et al, 2006b Bioelectromagnetics	2.1425GHz	human glioblastoma A172, IMR-90 fibroblasts NO effect on apoptosis, phosphorylation of hsp27
Ivancsits et al, 2005 Mutation Res	intermittent 50Hz	NO effect lymphocyte, monocyte, muscle: DNA damage: fibroblast, melanocyte, rat granulose
Jin et al, 1997 Bioelectrochem Bioenerg	60Hz	HL60 cells from two sources <i>myc</i> expression in one population, not in other
Kwee et al, 2001 Electro- and Magnetobiology	960MHz	human epithelial amnion (AMA) cells hsp70 increased

Table 1. Studies of EMF Stimulation of DNA and Protein Synthesis
(page 3)

Lacy-Hulbert et al, 1995 Radiation Res	50Hz	HL60 NO synthesis of myc or β -actin
Lai & Singh, 1997a Bioelectromagnetics	60Hz	rat brain cells melatonin blocks DNA strand breaks
Lai & Singh, 2005 Electromag Biol Med	1800MHz	rat brain cells noise blocks DNA strand breaks
Lantow et al, 2006a Radiation Res	1800MHz	human Mono Mac 6 and K562 cells NO hsp response
Lantow et al, 2006b Radiat Environ Biophys	1800MHz	primary human monocytes, lymphocytes NO hsp response
Lantow et al, 2006c Radiation Res	1800MHz	human Mono Mac 6 and K562 cells NO effect on apoptosis or necrosis
Laszlo et al, 2005 Radiation Res	835MHz	cultured mammalian cells NO 'effect within sensitivity of assay'
Laubitz et al, 2006 Experimental Physiol	muscle generated ELF	E coli, Caco-2 cells induce hsp70, protect vs apoptosis
Lee JS et al, 2005 Int J Radiat Biol	849, 1763 MHz	hsp70.1-deficient mice NO hsp induction
Lee S et al, 2005 FEBS Lett	2.45GHz	cultured human cells gene regulation: apoptosis 88, cell cycle99
Leszczynski et al, 2002 Differentiation	900MHz	human endothelial cells activate hsp27/p38MAPK stress pathway
Liburdy et al, 1993 J Pineal Res	60Hz	ER ⁺ MCF7 breast cancer cells block melatonin's oncostatic action
Lim et al, 2005 Radiation Res	900MHz	human leukocytes. NO effect on hsp
Lin et al, 1994 J Cell Biochem	60Hz	human HL60 cells EMF region of the c-myc promoter

**Table 1. Studies of EMF Stimulation of DNA and Protein Synthesis
(page 4)**

Lin et al, 1996 Bioelectrochem Bioenerg	60Hz	human HL60 cells changes in c-myc transcript levels
Lin et al, 1999 J Cell Biochem	60Hz	human HL60 cells EMF consensus sequence in HSP70 promoter
Lin et al, 2001 J Cell Biochem	60Hz	human HL60 cells EMF consensus sequence response elements
Lixia et al, 2006 Mutat Res	1.8GHz	human lens epithelial cells increased hsp70 protein
Maes et al, 2006 [Epub] Mutagenesis	900MHz	peripheral blood lymphocytes NO effect on DNA damage
Malagoli et al, 2004 Comp Biochem Physiol	50Hz	mussel immunocyte activate p38 MAP kinase, induce hsp70, hsp90
Mashevich et al, 2003 Bioelectromagnetics	830MHz	human peripheral blood lymphocytes chromosomal instability
McNamee et al, 2002 Radiat Res	1.9Ghz	human leukocytes NO effect on DNA damage, micronuclei
Miyakawa et al, 2001 Bioelectromagnetics	60Hz	C elegans induction of hsp16
Nylund & Leszczynski,2004 Proteomics	900MHZ	human endothelial cell line EA.hy926 effects on cytoskeletal proteins
Nylund & Leszczynski,2006 Proteomics	900MHZ	human endothelial cell line EA.hy926 response genome- and proteome-dependent
Oktem et al, 2005. Arch Med Res	900MHz	rats (oxidative kidney damage) oxidative damage protected by melatonin
Ozguner et al, 2005 Toxicol Ind Health	900MHz	rats (oxidative myocardial damage) protection by caffeic acid phenethyl ester

Table 1. Studies of EMF Stimulation of DNA and Protein Synthesis

(page 5)

Penafiel et al, 1997 Bioelectromagnetics	840MHz (AM, FM)	mouse L929 cells (ornithine decarboxylase activity) frequency dependent AM effect, no FM effect
Phillips et al, 1998 Bioelectrochem Bioenerg	813, 836MHz	Molt-4 T-lymphoblastoid cells DNA damage (and ability to repair) varied with SAR
Saffer & Thurston, 1995 Radiation Res	60Hz	HL60, Daudi cells NO synthesis of myc
Sanchez et al, 2006 FEBS J	900MHz	human skin cells slight but significant increase in hsp70
Sarimov et al, 2004 IEEE Trans Plasma Sci	895, 915MHz	transformed human lymphocytes affect chromatin conformation
Shallom et al, 2002 J Cell Biochem	915MHz	chick embryos induces hsp70, protects against hypoxia
Shi et al, 2003. Environ health Perspect	60Hz	human keratinocytes NO phosphorylation, expression of hsp27
Simko et al, 2006 Toxicol Lett	900MHz	human Mono Mac 6 cells NO hsp reponse
Vanderwaal et al, 2006 Int J Hyperthermia	900MHz	cultured HeLa, S3 and EA Hy296 cells NO hsp27 phosphorylation increases
Velizarov et al, 1999 Bioelectrochem Bioenerg	960MHz	human epithelial cells cell proliferation
Wang et al, 2006 Bioelectromagnetics	2450MHz	human glioma A172 cells NO hsp70, hsp27
Weisbrot et al, 2003 J Cell Biochem	900MHz	<i>Drosophila</i> hsp708, affects development, reproduction
Winker et al, 2005 Mutation Res	intermittent 50Hz	human diploid fibroblasts micronuclei, chromosomal damage

Table 2 Biological Thresholds in the ELF Range

Biological System	Threshold*	Reference
<i>Enzyme reaction rates</i>		
Na,K-ATPase	.2-.3 μ T	Blank & Soo, 1996
cytochrome oxidase	.5-.6 μ T	Blank & Soo, 1998
ornithine decarboxylase	\sim 2 μ T	Mullins et al, 1999
<i>Oxidation-reduction rate</i>		
Belousov-Zhabotinsky	<.5 μ T	Blank & Soo, 2001b
<i>Biosynthesis of stress proteins</i>		
HL60, Sciara, yeast,	<.8 μ T	Goodman et al, 1994
breast (HTB124, MCF7)	<.8 μ T	Lin et al, 1998
chick embryo (anoxia)	\sim 2 μ T	DiCarlo et al, 2000
<i>Disease related block melatonin inhibition</i>		
of breast carcinoma	.2<1.2 μ T	Liburdy et al, 1993
leukemia epidemiology	.3-.4 μ T	Ahlbom et al, 2000 Greenland et al, 2000

*The estimated values are for departures from the baseline, although Mullins et al (1999) and DiCarlo et al (2000) generally give inflection points in the dose-response curves. The leukemia epidemiology values are not experimental and are listed for comparison.

Attachment 4



SECTION 7

The Cellular Stress Response: EMF-DNA Interaction

2012 Supplement

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Prepared for the BioInitiative Working Group

September 2012

ABSTRACT

The research on stress proteins stimulated by EMF was reviewed by the author in the BioInitiative Report (2007) as well as in the special issue of Pathophysiology (2009) devoted to EMF. This review emphasizes the more recent research on the mechanism of interaction of EMF with DNA. It appears that the DNA molecule is particularly vulnerable to damage by EMF because of the coiled-coil configuration of the compacted molecule in the nucleus. The unusual structure endows it with the self similarity of a fractal antenna and the resulting sensitivity to a wide range of frequencies. The greater reactivity of DNA with EMF, along with a vulnerability to damage, underscores the urgent need to revise EMF exposure standards in order to protect the public. Recent studies have also exploited the properties of stress proteins to devise therapies for limiting oxidative damage and reducing loss of muscle strength associated with aging.

I. INTRODUCTION

The cellular stress response is a protective reaction of individual cells to potentially harmful stimuli in the environment. It is characterized by the synthesis of a class of proteins referred to as stress proteins. The cellular stress response differs from the more familiar responses of entire organisms to stresses that lead to secretion of cortisol and adrenalin and that result in the activation of various systems throughout the body. The cellular stress response, as the name indicates, is a specific response of individual cells, and stress proteins are the chemical agents that also serve as markers.

The cellular stress response was first described as a reaction to elevated temperature (Ritossa, 1962), which accounts for the proteins initially being called heat shock proteins. Several physical and chemical environmental influences have since been found to evoke the response, and in 1994, Goodman and Blank (1994) were the first to show that the response was stimulated by EMF. In fact, the cells were far more sensitive to EMF than to thermal stimuli, the threshold energy of the EMF stimulus being more than one billion times weaker than an effective thermal stimulus (Blank , Goodman, 1994).

The ‘heat shock’ response, i.e., hsp synthesis, is activated by a variety of potentially harmful stresses, including physical stimuli like pH and osmotic pressure changes, as well as chemicals such as ethanol and toxic metal ions like Cd^{2+} . The ability of EMF in the power frequency (extremely low frequency, ELF) range (Goodman, Blank, 1998) to evoke this response was followed by reports of similar effects due to radio frequency (RF) fields (de Pomeraï et al. 2003) and amplitude modulated RF fields (Czyz et al, 2004).

The finding that EMF evoked the cellular stress response had obvious and important biological implications:

- Because the cellular stress response is a reaction to potentially harmful stimuli in the environment, the cells were asserting that *EMF is potentially harmful* to cells.
- Because EMF stimulated protein synthesis, it meant that *EMF causes the two strands of DNA to come apart* for the protein code to be read and for synthesis to proceed.
- Since *EMF can interact with DNA*, it can cause *errors during replication*, as well as during protein synthesis, and higher energy EMF could be expected to cause *DNA strand breaks*, as has been observed (Lai and Singh, 1995).
- The incremental increase of DNA strand breaks with increases in field strength indicates a *dose-response*, evidence in support of EMF as the responsible agent.

II. CELLULAR STRESS PROTEINS ARE A NEW CLASS OF PROTEINS

Proteins are important components of cells and make up about 50% of the dry weight of most cells. The many different proteins are classified according to their functions, and stress proteins are now recognized as a new class of proteins with functions related to cell protection. Stress proteins join such well-known categories as contractile proteins (e.g. actin, myosin), catalytic proteins or enzymes (e.g. pepsin, amylase), transport proteins

(e.g. ATPases for ions across membranes, hemoglobins for blood gases, cytochromes for electrons), etc. Stress proteins were originally described as being synthesized in response to external stimuli and that is currently the area of greatest interest. However, they are also present constitutively.

Cellular stress proteins are synthesized when cells come in contact with stimuli that cause damage to macromolecules (Kultz, 2005), and the stress proteins aid in the repair and transport of these molecules. Because the first stimulus identified was an increase in temperature, the proteins were called 'heat shock' proteins and designated using the original terminology that starts with 'hsp' (for 'heat shock' protein) and a number equal to the molecular weight in kilodaltons.

The transition from heat shock protein to stress protein should alert (perhaps even alarm) the government agencies responsible for setting EMF safety standards. The thermal stimuli that evoked synthesis of protective proteins were believed to be dangerous for cells, but now we see that non-thermal EMF stimuli cause the same protective reactions in cells. The heat shock response and the EMF stress response both relate to the threshold for biological damage, and we should realize that EMF damage is caused by non-thermal stimuli. Compared to the energy needed to stimulate heat shock, EMF requires but a small fraction of the thermal energy needed to produce the same response (Blank et al., 1992).

The government agencies that assess safety of EMF exposure assume that danger is associated with an increase in temperature, i.e., a thermal criterion. It is clear from the responses of cells that the safety of EMF exposure, as indicated by the synthesis of protective stress proteins, is unrelated to the temperature increase. The cells are very sensitive to EMF, and the protective biological response to EMF occurs long before there is a significant change in temperature. It should be obvious that EMF safety standards are based on false assumptions and must be revised to reflect the scientific evidence. Non-thermal EMF stimuli are potentially harmful.

III. PROTEIN SYNTHESIS

The stress response, like all protein synthesis, indicates that all of the different physical and chemical stimuli that can initiate this response cause the two strands of DNA to come apart for the amino acid code for protein synthesis code to be read. Therefore, the observed stress protein synthesis is evidence that EMF has interacted with the DNA to start this process. The research showing that EMF in both the ELF and RF frequency ranges can also cause DNA strand breaks (Lai, Singh, 1995; 1996; Reflex Report 1994), suggests that the two phenomena are due to the same interaction mechanism, and that there is greater molecular damage with greater EMF energy.

Many research papers and some reviews have been published since the cellular stress response was reported to be stimulated by EMF. In addition to earlier reviews on EMF stimulation of the cellular stress response in the ELF (Goodman, Blank, 1998) and RF (Cotgreave, 2005) ranges, the subject was reviewed in Pathophysiology (Blank, 2009). Also, Calderwood (2007) has edited the volume on cell stress proteins in volume 7 of the series Protein Reviews. A recent (ICEMS, 2010) review on EMF and Bio-Effects includes many papers focused on a variety of possible EMF interaction mechanisms, but does not review the stress response, the stimulation of DNA or biosynthesis.

Section 7 of the Bioinitiative Report summarized both ELF and RF studies, mainly at frequencies 50 Hz, 60 Hz, 900MHz and 1.8 GHz. The citations in that review were not exhaustive, but the different frequencies and many different cells indicated the diversity of results on stimulation of DNA and stress protein synthesis. The many different types of cells that respond to EMF, both *in vivo* and *in vitro*, include epithelial, endothelial and epidermal cells, cardiac muscle cells, fibroblasts, yeast, *E. coli*, developing chick eggs, and dipteran cells.

It is clear that the stress response does not occur in reaction to EMF in all types of cells, and that tissue cultured cells (as opposed to natural cells) are less likely to show an effect of EMF, probably because immortalized cells have been changed significantly to enable them to live indefinitely in unnatural laboratory conditions. Even the same cell line from

two different suppliers can respond differently. Jin et al. (1997) showed that HL60 cells from one supplier reacted to EMF while identically labeled cells from another supplier did not respond. Some cancer cells (e.g., MCF7 breast cancer cells) have responded to EMF (Liburdy et al., 1993; Lin et al., 1998), and Czyz et al. (2004) found that p53-deficient embryonic stem cells showed an increased EMF response, but the wild type did not. Ivancits et al., (2005) found no genotoxic effects (i.e., DNA damage) in lymphocytes, monocytes and skeletal muscle cells, but did find effects with fibroblasts, melanocytes and rat granulosa cells. Lantow et al. (2006) and Simko et al. (2006) found that blood elements, such as lymphocytes and monocytes did not respond. Obviously, the cellular stress response is widespread but not universal.

IV. MECHANISM OF PROTEIN SYNTHESIS BY EMF

The stress response has provided an opportunity to investigate EMF interaction with DNA, and in particular, how this results in stimulating DNA to start the synthesis of proteins. Because the DNA sequence is known for hsp70, it was possible to study the effects of changes in the DNA sequence on protein synthesis. As a result of these experiments, it was possible to identify two distinct regions in the promoter region of the HSP 70 gene - an EMF sensitive region that was not sensitive to increased temperature, as well as a region sensitive only to temperature. The EMF sensitive domain contains number of nCTCTn myc-binding sites relative to the transcription initiation site and upstream of the temperature sensitive binding sites (Lin et al. 1999; 2001). These electromagnetic response elements (EMREs) are also found on the *c-myc* promoter which also reacts to EMF.

The EMF sensitivity of the DNA sequences, nCTCTn, was demonstrated by transfecting these sequences into CAT and Luciferase reporter genes and stimulating those genes (with EMF) to synthesize CAT and luciferase, respectively (Lin et al., 1999; 2001). Thus, the HSP70 promoter contains different DNA regions that are specifically sensitive to thermal and non-thermal stressors. This biological mechanism is obviously based on direct interaction with specific segments of DNA, and there is reason to believe that EMF can interact similarly with other segments of DNA. In our experiments, induction of

increased levels of hsp70 by EMF is rapid and occurs at extremely low levels of energy input, 14 orders of magnitude lower than with a thermal stimulus (Blank et al. 1994).

V. EMF INTERACTION WITH SIGNALING PATHWAYS

EMF penetrate cells unattenuated and so can interact directly with the DNA in the cell nucleus, as well as with other cell constituents. The above-cited experiments demonstrating the ability of electromagnetic response elements (EMREs) to interact with EMF, after being transferred to another DNA chain, is further support for direct EMF-DNA interaction as the most likely mechanism for EMF initiation of the cellular stress response.

In contrast to EMF, most biological agents are impeded by membranes and require special mechanisms to gain access to the cell interior. Friedman et al, (2007) have demonstrated that, in those situations, the initial step in transmitting extracellular information from the plasma membrane to the nucleus of the cell occurs when NADH oxidase rapidly generates reactive oxygen species (ROS). These ROS stimulate matrix metalloproteinases that allow them to cleave and release heparin binding epidermal growth factor. This secreted factor activates the epidermal growth receptor, which in turn activates the extracellular signal regulated kinase 1/2 (ERK) cascade. The ERK cascade is one of the four mitogen-activated protein kinase (MAPK) signaling cascades that regulate transcriptional activity in response to extracellular stimuli.

Stress protein synthesis can occur by direct interaction of EMF with DNA, as well as by membrane mediated stimulation via chemical signaling. While both mechanisms are possible, it is of interest to note that the body responds directly to physical inputs when there is a need for a rapid response. The body cannot rely upon slowly responding pathways for the synthesis of a relatively large amount of urgently needed protein molecules. The signal pathways function primarily as a mechanism for maintaining homeostasis by minimizing change and responding slowly to stimuli.

VI. INSIGHTS FROM MUSCLE PROTEIN SYNTHESIS

EMF stimulated protein synthesis may appear to be an unnatural mechanism, but it is essentially the same as the natural process in striated muscle. The only difference is that the electrons in DNA are driven by EMF, while in striated muscle, they are driven by the changes in electric (membrane) potential that cause contraction. Striated muscle is a tissue that requires steady protein synthesis to ensure proper function. Protein synthesis is initiated by the same electric currents that stimulate the muscle contractions. Body builders know that one must stimulate muscle contraction in order to increase muscle mass, and biologists have shown that the electric currents that flow across the muscle membranes during contraction pass through the DNA in the muscle nuclei and stimulate protein synthesis.

Muscle nuclei are not spread evenly throughout a muscle fiber, but are located near the muscle membranes that carry the currents. This means that the DNA in the nuclei can be stimulated every time the muscle is stimulated. The estimated magnitude of electric field along the muscle nuclei, $\sim 10\text{V/m}$, provides a large safety margin in muscle, since fields as low as 3mV/m were found to stimulate biosynthesis in HL60 cells (Blank et al, 1992).

Studies showing effects of EMF on electron transfer reactions in solution suggest that ionic (electric) currents affect electron movements within DNA in much the same way (Blank, 1995). Both electric and EMF (AC magnetic fields) stimulate protein synthesis in HL60 cells and have similar effects on electron transfer in the Na,K-ATPase (Blank and Soo, 2001a; 2001b). This suggests that interaction with DNA, of both electric fields and EMF, initiate stress protein synthesis by a similar mechanism.

Studies on muscle protein synthesis also suggest the possibility of a

frequency code that controls the particular segment of DNA that is activated. Studies have shown that different proteins can be synthesized by changing the frequency of the action potentials that stimulate the process. These experiments were possible because ‘fast’ and ‘slow’ muscles contract at different rates because they are composed of different proteins. For this reason it was possible to stimulate muscles at different rates and to study changes in the proteins as a result of changing the frequency of the action potentials (Pette, Vrbova, 1992). The review by Blank (1995) includes many additional experiments that show the importance of the frequency in controlling the segment of the muscle DNA that is affected by the current and translated into protein.

Studies of effects of EMF on well characterized electron transfer reactions, involving cytochrome oxidase, ATP hydrolysis by Na,K-ATPase, and the Belousov–Zhabotinski (BZ) redox reaction, have shown that:

- EMF can accelerate electron transfer rates
- EMF acts as a force that competes with the chemical forces driving a reaction. This means that the effect of EMF varies inversely with the intrinsic reaction rate, and that EMF effects are only seen when intrinsic rates are low. (*N.B. EMF has a greater effect when the system is in a rundown state.*)
- Experimentally determined thresholds are low ($\sim 0.5 \mu\text{T}$).
- Effects vary with frequency, with different optima for the reactions studied: The two enzymes showed broad frequency optima close to the reaction turnover numbers for Na,K-ATPase (60 Hz) and cytochrome oxidase (800 Hz), suggesting that EMF interacted optimally when in synchrony with the molecular kinetics. EMF interactions with DNA in both ELF and RF ranges and do not appear to involve electron transfer reactions with well-defined kinetics.

The effects of EMF on electron transfer reactions were studied in the ELF frequency range, and one would expect differences in the RF range. However, the situation is more

complicated. The effects of EMF on electrons in chemical reactions were detected in the Na,K-ATPase when electric or magnetic fields, each accelerated the reaction only when the enzyme was relatively inactive, i.e., the chemical driving forces were weak. These experiments enabled an estimate of the electron velocity as approximately 10^3 m/s (Blank and Soo, 2001a; 2001b), a velocity similar to that of electrons in DNA. An electron moving at a velocity of 10^3 m/s crosses the enzyme ($\sim 10^{-8}$ m) before the ELF field has had a chance to change. This means that a low frequency effect on fast moving electrons in DNA or in enzymes should be viewed as effectively due to a repeated DC pulse. In the RF range, the pulse train is longer.

VII. DNA IS A FRACTAL ANTENNA

Human DNA is about 2 m long, and the molecule is greatly compacted so that it fits into the nuclei of cells that are microns in diameter.

DNA has a unique double helical structure where two strands of DNA are bound together by hydrogen bonds between pairs of nucleotide bases (one on each strand) and they form a long twisted ribbon with delocalized π electrons that form continuous planar clouds on both surfaces of the ribbon. The result is a structure with two continuous paths that can conduct an electron current along the DNA.

Many studies, initially from the laboratory of Barton at Cal Tech (Hall et al, 1996), have shown that DNA does indeed conduct electrons. As would be expected, the rate of conduction can be influenced by the detailed structure of DNA. Changes, such as hairpin turns and mismatched bases, can lead to the disruption of the ordered double helical structure and anomalies in the rate of electron flow (Arkin et al, 1996; Hall et al, 1997; Lewis et al, 1997; Kelley et al, 1999; Giese, 2002). Electron flow can lead to local charging as well as oxidative damage.

Variations in the rate of electron flow can lead to the accumulation of charge at bottlenecks. The temporary buildup of charge at a site results in strong repulsive forces that can cause a disruption of H-bonds. A net charge can even disrupt the structure of a complex molecule, such as occurs when the four protein chains of hemoglobin

disaggregate in response to a gradual buildup of charge in the hemoglobin tetramer (Blank, 1984; Blank and Soo, 1998). For similar reasons, one would expect disaggregating forces at the DNA site where charge builds up. This would be expected to occur more easily in a compact structure such as DNA in the nucleus.

The tightly coiled DNA in the nucleus uses fractal patterns in order to occupy space efficiently. A fractal is a shape that displays *self-similarity*, where each part of the shape resembles the entire shape. Thus, the double helix is wound into a coil and that coil is wound into a larger coil, and so on. DNA in a cell nucleus is a coiled-coil many times over.

Since the DNA molecule in the nucleus conducts electricity and is organized in a self-similar pattern, it has the two key characteristics of *fractal antennas* when interacting with EMF (Blank, Goodman 2011). Fractal design is desirable for an antenna because it minimizes the overall size, while reacting to a wide range of electromagnetic frequencies. However, these characteristics are not desirable in DNA, because of the many frequencies in the environment that can and do react with DNA. The almost continuous cloud of delocalized electrons along both faces of the ‘ribbon’ formed by the base pairs provides a conducting path for responding to EMF and makes it more vulnerable to damage. The chemical changes that result from electron transfer reactions, are associated with molecular damage in DNA.

VIII. DNA DAMAGE AND CANCER

Stress proteins are essential for cell protection. They help defend cells against damaging forces like increases in temperature and reductions in oxygen supply that could be life-threatening. Similarly, the body generates stress proteins to strengthen cellular resistance to the effects of EM radiation. However, stress protein synthesis is really only an emergency measure that is designed to be effective in the short term. The response to repeated stimuli diminishes with repeated exposure and this could be dangerous.

Thermotolerance, the ability to tolerate higher temperatures as a result of repeated exposures to high temperature, was originally demonstrated at the molecular level in connection with heat shock. Repeated exposure to increased temperature resulted in a decreased heat shock response. A similar mechanism applies when the cellular stress response is stimulated by EMF, since repeated EMF stimuli result in lower production of stress proteins. This could very well be a mechanism by which repeated exposure to EMF can result in less protection and more damage to molecules like DNA. The lower protection predisposes exposed individuals to an increased risk of mutation and initiation of cancer.

DiCarlo and Litovitz (2008) at Catholic University in Washington, D.C. demonstrated the development of EMF tolerance in an experiment performed on chicken embryos. In those eggs exposed to ELF-radiation of 8 μT for 30 or 60 minutes at a time, twice a day for four days, production of hsp70 in response to oxygen deprivation declined. The same response was noted in those eggs exposed to RF radiation of 3.5 $\mu\text{W}/\text{cm}^2$ for 30 or 60 minutes, once a day, for four days. The researchers noted that these eggs produced 27% less hsp70 following these exposures, and had correspondingly reduced ability to fend off cell damage (reduced *cytoprotection*). Similar experiments have been carried out with short, repeated exposures (in contrast to extended exposures). There too, the rate of stress protein synthesis is reduced with each repetition. The reduction in stress protein synthesis as a result of continuous exposure to EMF would predispose an individual to the accumulation of DNA damage and the development of cancer.

Cancers are believed to be the long term result of the errors in DNA that occur during the normal functioning of cells. Living cells are continuously growing (making protein) and dividing (making DNA), and errors in synthesis occur. The error rate is a very small but finite, so the vast majority of errors is repaired, but not all. When the error rate is too high, the cell activates apoptosis and destroys itself. However, the small number of errors that is retained accumulates over time as mutations, some of which can affect function. It is particularly bad when mutation inactivates a tumor suppressor gene or a

DNA repair gene and enables creation of an oncogene, since this accelerates the development of a cancer.

Although damage can occur during protein synthesis and cell division, as well as upon exposure to oxidizing chemicals, the probability of developing cancer is increased as a result of damage to DNA structure caused by exposure to EMF (Verschaeve, 2008). EMF induced oxidative damage to DNA has even been reported on exposure to high ELF fields (Yokus et al, 2008).

IX. STRESS RESPONSE: BIOLOGICAL GUIDE TO SAFETY

The cellular stress response is the way the body tells us that it has come in contact with a potentially harmful stimulus. Since cells react to relatively low levels of EMF, both ELF and RF, one would think that the low biological thresholds for a protective reaction to harmful stimuli would provide critical guidance for the authorities seeking to establish meaningful safety standards. By ignoring the information from the cellular stress response, the authorities appear to be saying that they are better judges of what is harmful to cells than the cells themselves.

Research on the cellular stress response has drawn attention to the inadequacy of EMF safety standards. The synthesis of stress proteins at EMF levels that are currently considered safe indicates that ambient exposure levels can influence the molecular processes involved in protein synthesis needed to provide new molecules and replace damaged molecules. The ability of EMF to interfere with normal function and damage the protein and DNA molecules that are being synthesized is definitely a reason to consider this effect for guidance regarding its health implications. The system of safety standards is not at all protective because processes stimulated at non-thermal levels have been overlooked. The standards must be revised.

The authorities have been misguided in assuming that only thermal stimuli could affect chemical bonds and that non-thermal stimuli cannot cause chemical changes. Non-thermal biological mechanisms activated by EMF have been known for some time, and

some experiments have even been aimed specifically at demonstrating unusual changes in biological systems due to non-thermal EMF stimuli. Bohr and Bohr (2000) showed that both a reaction and its reverse, the denaturation and renaturation of β -lactoglobulin, are accelerated by microwave EMF, and de Pomerai et al (2003) showed that microwave radiation causes protein aggregation in the absence of bulk heating. A clear separation of thermal and non-thermal mechanisms in biology was shown by Mashevich et al (2002) in experiments where chromosomal damage in lymphocytes that had been observed under RF was not seen when the cells were exposed to elevated temperatures. The neglect of non-thermal mechanisms by regulators is based on their ignorance of reactions in biological systems. By greatly underestimating the risk of EMF exposure, they continue to endanger the public.

The cellular stress response is activated by a mechanism that involves interaction of EMF with the DNA molecule. This reaction of DNA, and/or the stress proteins that are synthesized, could be used to develop new EMF safety standards (Blank and Goodman, 2012). A biologically-based measure of EMF radiation could replace the misguided energy-based “specific absorption rate” (SAR). (It should be noted that SAR is the safety standard in the radiofrequency (RF) range, but it fails as a standard for predicting cancer risk in the ELF range.) A standard based on stress proteins would have several advantages compared to SAR:

- it is based on a protective cellular mechanism that is stimulated by a variety of potentially harmful environmental agents
- it is stimulated by a wide range of frequencies in the EM spectrum so there would be no need for different standards in different frequency ranges.

Cancers are believed to arise from mutations in DNA, and changes in DNA induced by interaction with EMF could be a better measure of the biologically effective dose. It may be possible to measure the changes by transcriptional alterations and/or translational changes in specific proteins. A biologically-based standard related to stimulation of DNA

could apply over a much wider range of the electromagnetic spectrum and include ionizing radiation.

X. STRESS RESPONSE: GUIDE TO NEW THERAPIES

Since activation of the cellular stress response by EMF was shown to be a protective mechanism, it was only a matter of time before the response would be studied as a potential therapeutic agent. Thermal activation of the stress response has already been shown to be effective in cardiac bypass surgery (Currie et al., 1993; Udelsman et al., 1993; Nitta et al., 1994). Stress protein activation can apparently minimize the oxidative damage of ischemia (low oxygen level in a tissue) reperfusion that occurs when the blood supply is reconnected to the heart after surgery. However, the temperature control required for thermal activation is cumbersome and the technique is not easily applied compared to EMF. A study of non-invasive EMF induction of hsp70, prior to cardiac bypass surgery, has shown that myocardial function can be preserved, and at the same time decrease ischemic injury (George et al, 2008).

EMF activation of stress protein synthesis has a clear advantage over thermal activation. The biological response is not related to the EMF energy, so protective biological responses should occur far below thermal levels. 60 Hz fields were shown to induce elevated levels of hsp70 protein in the absence of elevated temperature (Goodman et al., 1994; Goodman and Blank, 1998; Han et al., 1998; Lin et al., 1998, 1999, 2001; Carmody et al., 2000) in cells including cultured rodent cardiomyocytes (Goodman and Blank, 2002). Also, Di Carlo et al. (1999) and Shallom et al. (2002) confirmed that cardiomyocytes were protected from anoxic damage in EMF exposed chick embryos.

Another potential therapeutic application has come from a study of the stress protein hsp10 in relation to striated muscle function. Kayani et al (2010) at the University of Liverpool found that this stress protein can prevent the age-related deterioration of muscle strength in skeletal muscle of transgenic mice. Hsp10 is often linked with hsp60 in supporting mitochondrial function. In cardiac myocytes this combination protects mitochondrial function as well as preventing cell deaths induced by ischemia-reperfusion.

These results suggest that mitochondrial hsp10 and hsp60 in combination or individually play an important role in maintaining mitochondrial integrity and ability to generate ATP, which are crucial for survival of cardiac myocytes during ischemia/reperfusion.

Research on therapeutic effects using stress proteins is obviously just beginning and we can expect other applications where EMF is used to generate this group of therapeutic agents essentially instantaneously and in situ.

XI. THE ENVIRONMENTAL EMF ISSUE AND CONCLUSIONS

Research has shown that the EMF-activated cellular stress response:

- is an effective protective mechanism for cells exposed to a wide range of EMF frequencies
- thresholds are very low (safety standards must be reduced to limit biological responses)
- mechanism involves direct interaction of EMF with the DNA molecule (claims that there are no known mechanisms of interaction are patently false)
- the coiled-coil structure of DNA in the nucleus makes the molecule react like a fractal antenna to a wide range of frequencies (there is a need for stricter EMF safety standards)
- biologically-based EMF safety standards could be developed from the research on the stress response.

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Attachment 5

BIOINITIATIVE 2012 - CONCLUSIONS Table 1-1

Overall, these 1800 or so new studies report abnormal gene transcription (Section 5); genotoxicity and single-and double-strand DNA damage (Section 6); stress proteins because of the fractal RF-antenna like nature of DNA (Section 7); chromatin condensation and loss of DNA repair capacity in human stem cells (Sections 6 and 15); reduction in free-radical scavengers - particularly melatonin (Sections 5, 9, 13, 14, 15, 16 and 17); neurotoxicity in humans and animals (Section 9), carcinogenicity in humans (Sections 11, 12, 13, 14, 15, 16 and 17); serious impacts on human and animal sperm morphology and function (Section 18); effects on offspring behavior (Section 18, 19 and 20); and effects on brain and cranial bone development in the offspring of animals that are exposed to cell phone radiation during pregnancy (Sections 5 and 18). This is only a snapshot of the evidence presented in the BioInitiative 2012 updated report.

BIOEFFECTS ARE CLEARLY ESTABLISHED

Bioeffects are clearly established and occur at very low levels of exposure to electromagnetic fields and radiofrequency radiation. Bioeffects can occur in the first few minutes at levels associated with cell and cordless phone use. Bioeffects can also occur from just minutes of exposure to mobile phone masts (cell towers), WI-FI, and wireless utility 'smart' meters that produce whole-body exposure. Chronic base station level exposures can result in illness.

BIOEFFECTS WITH CHRONIC EXPOSURES CAN REASONABLY BE PRESUMED TO RESULT IN ADVERSE HEALTH EFFECTS

Many of these bioeffects can reasonably be presumed to result in adverse health effects if the exposures are prolonged or chronic. This is because they interfere with normal body processes (disrupt homeostasis), prevent the body from healing damaged DNA, produce immune system imbalances, metabolic disruption and lower resilience to disease across multiple pathways. Essential body processes can eventually be disabled by incessant external stresses (from system-wide electrophysiological interference) and lead to pervasive impairment of metabolic and reproductive functions.

LOW EXPOSURE LEVELS ARE ASSOCIATED WITH BIOEFFECTS AND ADVERSE HEALTH EFFECTS AT CELL TOWER RFR EXPOSURE LEVELS

At least five new cell tower studies are reporting bioeffects in the range of 0.003 to 0.05 $\mu\text{W}/\text{cm}^2$ at lower levels than reported in 2007 (0.05 to 0.1 uW/cm^2 was the range below which, in 2007, effects were not observed). Researchers report headaches, concentration difficulties and behavioral problems in children and adolescents; and sleep disturbances, headaches and concentration problems in adults. Public safety standards are 1,000 – 10,000 or more times higher than levels now commonly reported in mobile phone base station studies to cause bioeffects.

EVIDENCE FOR FERTILITY AND REPRODUCTION EFFECTS: HUMAN SPERM AND THEIR DNA ARE DAMAGED

Human sperm are damaged by cell phone radiation at very low intensities in the low microwatt and nanowatt/cm² range (0.00034 – 0.07 uW/cm²). There is a veritable flood of new studies reporting sperm damage in humans and animals, leading to substantial concerns for fertility, reproduction and health of the offspring (unrepaired de novo mutations in sperm). Exposure levels are similar to those resulting from wearing a cell phone on the belt, or in the pants pocket, or using a wireless laptop computer on the lap. Sperm lack the ability to repair DNA damage.

Studies of human sperm show genetic (DNA) damage from cell phones on standby mode and wireless laptop use. Impaired sperm quality, motility and viability occur at exposures of 0.00034 uW/cm² to 0.07 uW/cm² with a resultant reduction in human male fertility. Sperm cannot repair DNA damage.

Several international laboratories have replicated studies showing adverse effects on sperm quality, motility and pathology in men who use and particularly those who wear a cell phone, PDA or pager on their belt or in a pocket (Agarwal et al, 2008; Agarwal et al, 2009; Wdowiak et al, 2007; De Iuliis et al, 2009; Fejes et al, 2005; Aitken et al, 2005; Kumar, 2012). Other studies conclude that usage of cell phones, exposure to cell phone radiation, or storage of a mobile phone close to the testes of human males affect sperm counts, motility, viability and structure (Aitken et al, 2004; Agarwal et al, 2007; Eroglu et al., 2006). Animal studies have demonstrated oxidative and DNA damage, pathological changes in the testes of animals, decreased sperm mobility and viability, and other measures of deleterious damage to the male germ line (Dasdag et al, 1999; Yan et al, 2007; Otitoloju et al, 2010; Salama et al, 2008; Behari et al, 2006; Kumar et al, 2012). There are fewer animal studies that have studied effects of cell phone radiation on female fertility parameters. Panagopoulous et al. 2012 report decreased ovarian development and size of ovaries, and premature cell death of ovarian follicles and nurse cells in *Drosophila melanogaster*. Gul et al (2009) report rats exposed to stand-by level RFR (phones on but not transmitting calls) caused decrease in the number of ovarian follicles in pups born to these exposed dams. Magras and Xenos (1997) reported irreversible infertility in mice after five (5) generations of exposure to RFR at cell phone tower exposure levels of less than one microwatt per centimeter squared (μ W/cm²).

EVIDENCE THAT CHILDREN ARE MORE VULNERABLE

There is good evidence to suggest that many toxic exposures to the fetus and very young child have especially detrimental consequences depending on when they occur during critical phases of growth and development (time windows of critical development), where such exposures may lay the seeds of health harm that develops even decades later. Existing FCC and ICNIRP public safety limits seem to be not sufficiently protective of public health, in particular for the young (embryo, fetus, neonate, very young child).

The Presidential Cancer Panel (2010) found that children ‘are at special risk due to their smaller body mass and rapid physical development, both of which magnify their vulnerability to known carcinogens, including radiation.’

The American Academy of Pediatrics, in a letter to Congressman Dennis Kucinich dated 12 December 2012 states “*Children are disproportionately affected by environmental exposures, including cell phone radiation. The differences in bone density and the amount of fluid in a child’s brain compared to an adult’s brain could allow children to absorb greater quantities of RF energy deeper into their brains than adults. It is essential that any new standards for cell phones or other wireless devices be based on protecting the youngest and most vulnerable populations to ensure they are safeguarded through their lifetimes.*”

FETAL AND NEONATAL EFFECTS OF EMF

Fetal (*in-utero*) and early childhood exposures to cell phone radiation and wireless technologies in general may be a risk factor for hyperactivity, learning disorders and behavioral problems in school.

Fetal Development Studies: Effects on the developing fetus from *in-utero* exposure to cell phone radiation have been observed in both human and animal studies since 2006. Divan et al (2008) found that children born of mothers who used cell phones during pregnancy develop more behavioral problems by the time they have reached school age than children whose mothers did not use cell phones during pregnancy. Children whose mothers used cell phones during pregnancy had 25% more emotional problems, 35% more hyperactivity, 49% more conduct problems and 34% more peer problems
(Divan et al., 2008).

Common sense measures to limit both ELF-EMF and RF EMF in these populations is needed, especially with respect to avoidable exposures like incubators that can be modified; and where education of the pregnant mother with respect to laptop computers, mobile phones and other sources of ELF-EMF and RF EMF are easily instituted.

Sources of fetal and neonatal exposures of concern include cell phone radiation (both paternal use of wireless devices worn on the body and maternal use of wireless phones during pregnancy).

Exposure to whole-body RFR from base stations and WI-FI, use of wireless laptops, use of incubators for newborns with excessively high ELF-EMF levels resulting in altered heart rate variability and reduced melatonin levels in newborns, fetal exposures to MRI of the pregnant mother, and greater susceptibility to leukemia and asthma in the child where there have been maternal exposures to ELF-EMF.

A precautionary approach may provide the frame for decision-making where remediation actions have to be realized to prevent high exposures of children and pregnant woman.

(Bellieni and Pinto, 2012 – Section 19)

EMF/RFR AS A PLAUSIBLE BIOLOGICAL MECHANISM FOR AUTISM (ASD)

- Children with existing neurological problems that include cognitive, learning, attention, memory, or behavioral problems should as much as possible be provided with wired (not wireless) learning, living and sleeping environments,
 - Special education classrooms should observe 'no wireless' conditions to reduce avoidable stressors that may impede social, academic and behavioral progress.
 - All children should reasonably be protected from the physiological stressor of significantly elevated EMF/RFR (wireless in classrooms, or home environments).
 - School districts that are now considering all-wireless learning environments should be strongly cautioned that wired environments are likely to provide better learning and teaching environments, and prevent possible adverse health consequences for both students and faculty in the long-term.
 - Monitoring of the impacts of wireless technology in learning and care environments should be performed with sophisticated measurement and data analysis techniques that are cognizant of the non-linear impacts of EMF/RFR and of data techniques most appropriate for discerning these impacts.
 - There is sufficient scientific evidence to warrant the selection of wired internet, wired classrooms and wired learning devices, rather than making an expensive and potentially health-harming commitment to wireless devices that may have to be substituted out later, and
 - Wired classrooms should reasonably be provided to all students who opt-out of wireless environments.
- (Herbert and Sage, 2012 – Section 20)

Many disrupted physiological processes and impaired behaviors in people with ASDs closely resemble those related to biological and health effects of EMF/RFR exposure. Biomarkers and indicators of disease and their clinical symptoms have striking similarities. Broadly speaking, these types of phenomena can fall into one or more of several classes: a) alteration of genes or gene expression, b) induction of change in brain or organismic development, c) alteration of phenomena modulating systemic and brain function on an ongoing basis throughout the life course (which can include systemic pathophysiology as well as brain-based changes), and d) evidence of functional alteration in domains such as behavior, social interaction and attention known to be challenged in ASD.

Several thousand scientific studies over four decades point to serious biological effects and health harm from EMF and RFR. These studies report genotoxicity, single-and double-strand DNA damage, chromatin condensation, loss of DNA repair capacity in human stem cells, reduction in free-radical scavengers (particularly melatonin), abnormal gene transcription, neurotoxicity, carcinogenicity, damage to sperm morphology and function, effects on behavior, and effects on brain development in the fetus of human mothers that use cell phones during pregnancy. Cell phone exposure has been linked to altered fetal brain development and ADHD-like behavior in the offspring of pregnant mice.

Reducing life-long health risks begins in the earliest stages of embryonic and fetal development, is accelerated for the infant and very young child compared to adults, and is not complete in young people (as far as brain and nervous system maturation) until the early 20's. Windows of critical development mean that risk factors once laid down in the cells, or in epigenetic changes in the genome may have grave and life-long consequences for health or illness for every individual.

All relevant environmental conditions, including EMF and RFR, which can degrade the human genome, and impair normal health and development of species including homo sapiens, should be given weight in defining and implementing prudent, precautionary actions to protect public health.

Allostatic load in autism and autistic decompensation - we may be at a tipping point that can be pushed back by removing unnecessary stressors like EMF/RFR and building resilience.

The consequence of ignoring clear evidence of large-scale health risks to global populations, when the risk factors are largely avoidable or preventable is too high a risk to take. With the epidemic of autism (ASD) putting the welfare of children, and their families in peril at a rate of one family in 88, the rate still increasing annually, we cannot afford to ignore this body of evidence. The public needs to know that these risks exist, that transition to wireless should not be presumed safe, and that it is very much worth the effort to minimize exposures that still provide the benefits of technology in learning, but without the threat of health risk and development impairments to learning and behavior in the classroom.

(Herbert and Sage, 2010 – Section 20)

THE BLOOD-BRAIN BARRIER IS AT RISK

The BBB is a protective barrier that prevents the flow of toxins into sensitive brain tissue. Increased permeability of the BBB caused by cell phone RFR may result in neuronal damage. Many research studies show that very low intensity exposures to RFR can affect the blood-brain barrier (BBB) (mostly animal studies). Summing up the research, it is more probable than unlikely that non-thermal EMF from cell phones and base stations do have effects upon biology. A single 2-hr exposure to cell phone radiation can result in increased leakage of the BBB, and 50 days after exposure, neuronal damage can be seen, and at the later time point also albumin leakage is demonstrated. The levels of RFR needed to affect the BBB have been shown to be as low as 0.001 W/kg, or less than holding a mobile phone at arm's length. The US FCC standard is 1.6 W/kg; the ICNIRP standard is 2 W/kg of energy (SAR) into brain tissue from cell/cordless phone use. Thus, BBB effects occur at about 1000 times lower RFR exposure levels than the US and ICNIRP limits allow.

(Salford, 2012 - Section 10)

If the blood-brain barrier is vulnerable to serious and on-going damage from wireless exposures, then we should perhaps also be looking at the blood-ocular barrier (that protects the eyes), the blood-placenta barrier (that protects the developing fetus) and the blood-gut barrier (that protects proper digestion and nutrition), and the blood-testes barrier (that protects developing sperm) to see if they too can be damaged by RFR.

EPIDEMIOLOGICAL STUDIES CONSISTENTLY SHOW ELEVATIONS IN RISK OF BRAIN CANCERS

Brain Tumors: There is a consistent pattern of increased risk of glioma and acoustic neuroma associated with use of mobile phones and cordless phones.

“Based on epidemiological studies there is a consistent pattern of increased risk for glioma and acoustic neuroma associated with use of mobile phones and cordless phones. The evidence comes mainly from two study centres, the Hardell group in Sweden and the Interphone Study Group. No consistent pattern of an increased risk is seen for meningioma. A systematic bias in the studies that explains the results would also have been the case for meningioma. The different risk pattern for tumor type strengthens the findings regarding glioma and acoustic neuroma. Meta-analyses of the Hardell group and Interphone studies show an increased risk for glioma and acoustic neuroma. Supportive evidence comes also from anatomical localisation of the tumor to the most exposed area of the brain, cumulative exposure in hours and latency time that all add to the biological relevance of an increased risk. In addition risk calculations based on estimated absorbed dose give strength to the findings. (Hardell, 2012 – Section 11)

“There is reasonable basis to conclude that RF-EMFs are bioactive and have a potential to cause health impacts. There is a consistent pattern of increased risk for glioma and acoustic neuroma associated with use of wireless phones (mobile phones and cordless phones) mainly based on results from case-control studies from the Hardell group and Interphone Final Study results. Epidemiological evidence gives that RF-EMF should be classified as a human carcinogen.

Based on our own research and review of other evidence the existing FCC/IEE and ICNIRP public safety limits and reference levels are not adequate to protect public health. New public health standards and limits are needed.

EVIDENCE FOR GENETIC EFFECTS

Eighty six (86) new papers on genotoxic effects of RFR published between 2007 and mid-2012 are profiled. Of these, 54 (63%) showed effects and 32 (37%) showed no effects.

Forty three (43) new ELF-EMF papers and two static magnetic field papers that report on genotoxic effects of ELF-EMF published between 2007 and mid-2012 are profiled. Of these, 35 (81%) show effects and 8 (19%) show no effect.

EVIDENCE FOR NEUROLOGICAL EFFECTS

One hundred fifty five (155) new papers that report on neurological effects of RFR published between 2007 and mid-2012 are profiled. Of these, 98 (63%) showed effects and 57 (37%) showed no effects.

Sixty nine (69) new ELF-EMF papers (including two static field papers) that report on genotoxic effects of ELF-EMF published between 2007 and mid-2012 are profiled. Of these, 64 (93%) show effects and 5 (7%) show no effect.

EVIDENCE FOR CHILDHOOD CANCERS (LEUKEMIA)

With overall 42 epidemiological studies published to date power frequency EMFs are among the most comprehensively studied environmental factors. Except ionizing radiation no other environmental factor has been as firmly established to increase the risk of childhood leukemia.

Sufficient evidence from epidemiological studies of an increased risk from exposure to EMF (power frequency magnetic fields) that cannot be attributed to chance, bias or confounding. Therefore, according to the rules of IARC such exposures can be classified as a **Group 1 carcinogen (Known Carcinogen)**.

There is no other risk factor identified so far for which such unlikely conditions have been put forward to postpone or deny the necessity to take steps towards exposure reduction. As one step in the direction of precaution, measures should be implemented to guarantee that exposure due to transmission and distribution lines is below an average of about 1 mG. This value is arbitrary at present and only supported by the fact that in many studies this level has been chosen as a reference.

Base-station level RFR at levels ranging from less than 0.001 uW/cm² to 0.05 uW/cm². In 5 new studies since 2007, researchers report headaches, concentration difficulties and behavioral problems in children and adolescents; and sleep disturbances, headaches and concentration problems in adults.

MELATONIN, BREAST CANCER AND ALZHEIMER'S DISEASE

MELATONIN AND BREAST CANCER

Conclusion: Eleven (11) of the 13 published epidemiologic residential and occupational studies are considered to provide (positive) evidence that high ELF MF exposure can result in decreased melatonin production. The two negative studies had important deficiencies that may certainly have biased the results. There is sufficient evidence to conclude that long-term relatively high ELF MF exposure can result in a decrease in melatonin production. It has not been determined to what extent personal characteristics, e.g., medications, interact with ELF MF exposure in decreasing melatonin production

Conclusion: New research indicates that ELF MF exposure, in vitro, can significantly decrease melatonin activity through effects on MT1, an important melatonin receptor.

ALZHEIMER'S DISEASE

There is strong epidemiologic evidence that exposure to ELF MF is a risk factor for AD. There are now twelve (12) studies of ELF MF exposure and AD or dementia which . Nine (9) of these studies are considered positive and three (3) are considered negative. The three negative studies have serious deficiencies in ELF MF exposure classification that results in subjects with rather low exposure being considered as having significant exposure. There are insufficient studies to formulate an opinion as to whether radiofrequency MF exposure is a risk or protective factor for AD.

There is now evidence that (i) high levels of peripheral amyloid beta are a risk factor for AD and (ii) medium to high ELF MF exposure can increase peripheral amyloid beta. High brain levels of amyloid beta are also a risk factor for AD and medium to high ELF MF exposure to brain cells likely also increases these cells' production of amyloid beta.

There is considerable in vitro and animal evidence that melatonin protects against AD. Therefore it is certainly possible that low levels of melatonin production are associated with an increase in the risk of AD.

(Davanipour and Sobel, 2012 – Section 13)

STRESS PROTEINS AND DNA AS A FRACTAL ANTENNA FOR RFR

DNA acts as a 'fractal antenna' for EMF and RFR.

The coiled-coil structure of DNA in the nucleus makes the molecule react like a fractal antenna to a wide range of frequencies.

The structure makes DNA particularly vulnerable to EMF damage.

The mechanism involves direct interaction of EMF with the DNA molecule (claims that there are no known mechanisms of interaction are patently false)

Many EMF frequencies in the environment can and do cause DNA changes.

The EMF-activated cellular stress response is an effective protective mechanism for cells exposed to a wide range of EMF frequencies.

EMF stimulates stress proteins (indicating an assault on the cell).

EMF efficiently harms cells at a billion times lower levels than conventional heating.

Safety standards based on heating are irrelevant to protect against EMF-levels of exposure. There is an urgent need to revise EMF exposure standards. Research has shown thresholds are very low (safety standards must be reduced to limit biological responses). Biologically-based EMF safety standards could be developed from the research on the stress response.

EVIDENCE FOR DISRUPTION OF THE MODULATING SIGNAL HUMAN STEM CELL DNA DOES NOT ADAPT OR REPAIR

Human stem cells do not adapt to chronic exposures to non-thermal microwave (cannot repair damaged DNA), and damage to DNA in other cells generally do not repair as efficiently.

Non-thermal effects of microwaves depend on variety of biological and physical parameters that should be taken into account in setting the safety standards. Emerging evidence suggests that the SAR concept, which has been widely adopted for safety standards, is not useful alone for the evaluation of health risks from non-thermal microwave of mobile communication. Other parameters of exposure, such as frequency, modulation, duration, dose should be taken into account.

Lower intensities are not always less harmful; they may be more harmful.

Intensity windows exist, where bioeffects are much more powerful.

A linear, dose-response relationship test is probably invalid for testing of RFR and EMF (as is done in chemicals testing for toxicity).

Resonant frequencies may result in biological effects at very low intensities comparable to base station (cell tower) and other microwave sources used in mobile communications.

These exposures can cause health risk. The current safety standards are insufficient to protect from non-thermal microwave effects.

The data about the effects of microwave at super-low intensities and significant role of duration of exposure in these effects along with the data showing that adverse effects of non-thermal microwave from GSM/UMTS mobile phones depend on carrier frequency and type of the microwave signal suggest that microwave from base-stations/masts, wireless routers, WI-FI and other wireless devices and exposures in common use today can also produce adverse effects at prolonged durations of exposure.

Most of the real signals that are in use in mobile communication have not been tested so far. Very little research has been done with real signals and for durations and intermittences of exposure that are relevant to chronic exposures from mobile communication. In some studies, so-called “mobile communication-like” signals were investigated that in fact were different from the real exposures in such important aspects as intensity, carrier frequency, modulation, polarization, duration and intermittence.

New standards should be developed based on knowledge of mechanisms of non-thermal effects. Importantly, because the signals of mobile communication are completely replaced by other signals faster than once per 10 years, duration comparable with latent period, epidemiologic studies cannot provide basement for cancer risk assessment from upcoming new signals.

In many cases, because of ELF modulation and additional ELF fields created by the microwave sources, for example by mobile phones, it is difficult to distinguish the effects of exposures to ELF and microwave. Therefore, these combined exposures and their possible cancer risks should be considered in combination.

As far as different types of microwave signals (carrier frequency, modulation, polarization, far and near field, intermittence, coherence, *etc.*) may produce different effects, cancer risks should ideally be estimated for each microwave signal separately.

The Precautionary Principle should be implemented while new standards are in progress.

It should be anticipated that some part of the human population, such as children, pregnant women and groups of hypersensitive persons could be especially sensitive to the non-thermal microwave exposures.

N. EFFECTS OF WEAK-FIELD INTERACTIONS ON NON-LINEAR BIOLOGICAL OSCILLATORS AND SYNCHRONIZED NEURAL ACTIVITY

A unifying hypothesis for a plausible biological mechanism to account for very weak field EMF bioeffects other than cancer may lie with weak field interactions of pulsed RFR and ELF-modulated RFR as disrupters of synchronized neural activity. Electrical rhythms in our brains can be influenced by external signals. This is consistent with established weak field effects on coupled biological oscillators in living tissues. Biological systems of the heart, brain and gut are dependent on the cooperative actions of cells that function according to principles of non-linear, coupled biological oscillations for their synchrony, and are dependent on exquisitely timed cues from the environment at vanishingly small levels (Buzsaki, 2006; Strogatz, 2003). The key to synchronization is the joint actions of cells that co-operate electrically - linking populations of biological oscillators that couple together in large arrays and synchronize spontaneously. Synchronous biological oscillations in cells (pacemaker cells) can be disrupted by artificial, exogenous environmental signals, resulting in desynchronization of neural activity that regulates critical functions (including metabolism) in the brain, gut and heart and circadian rhythms governing sleep and hormone cycles (Strogatz, 1987). The brain contains a population of oscillators with distributed natural frequencies, which pull one another into synchrony (the circadian pacemaker cells). Strogatz has addressed the unifying mathematics of biological cycles and external factors disrupt these cycles (Strogatz, 2001, 2003). *“Rhythms can be altered by a wide variety of agents and that these perturbations must seriously alter brain performance”* (Buzsaki, 2006).

“Organisms are biochemically dynamic. They are continuously subjected to time-varying conditions in the form of both extrinsic driving from the environment and intrinsic rhythms generated by specialized cellular clocks within the organism itself. Relevant examples of the latter are the cardiac pacemaker located at the sinoatrial node in mammalian hearts (1) and the circadian clock residing at the suprachiasmatic nuclei in mammalian brains (2). These rhythm generators are composed of thousands of clock cells that are intrinsically diverse but nevertheless manage to function in a coherent oscillatory state. This is the case, for instance, of the circadian oscillations exhibited by the suprachiasmatic nuclei, the period of which is known to be determined by the mean period of the individual neurons making up the circadian clock (3–7). The mechanisms by which this collective behavior arises remain to be understood.” (Strogatz, 2001; Strogatz, 2003)

Synchronous biological oscillations in cells (pacemaker cells) can be disrupted by artificial, exogenous environmental signals, resulting in desynchronization of neural activity that regulates critical functions (including metabolism) in the brain, gut and heart and circadian rhythms governing sleep and hormone cycles. The brain contains a population of oscillators with distributed natural frequencies, which pull one another into synchrony (the circadian pacemaker cells). Strogatz has addressed the unifying mathematics of biological cycles and external factors disrupt these cycles.

EMF AND RFR MAKE CHEMICAL TOXINS MORE HARMFUL

EMF acts on the body like other environmental toxicants do (heavy metals, organic chemicals and pesticides). Both toxic chemicals and EMF may generate free radicals, produce stress proteins and cause indirect damage to DNA. Where there is combined exposure the damages may add or even synergistically interact, and result in worse damage to genes.

EMF IS SUCCESSFULLY USED IN HEALING AND DISEASE TREATMENTS

“The potential application of the up-regulation of the HSP70 gene by both ELF-EMF and nanosecond PEMF in clinical practice would include trauma, surgery, peripheral nerve damage, orthopedic fracture, and vascular graft support, among others. Regardless of pulse design, EMF technology has been shown to be effective in bone healing [5], wound repair [11] and neural regeneration [31,36,48,49,51,63,64,65,66]. In terms of clinical application, EMF-induction of elevated levels of hsp70 protein also confers protection against hypoxia [61] and aid myocardial function and survival [20,22]. Given these results, we are particularly interested in the translational significance of effect vs. efficacy which is not usually reported by designers or investigators of EMF devices. More precise description of EM pulse and sine wave parameters, including the specific EM output sector, will provide consistency and “scientific basis” in reporting findings.”

“The degree of electromagnetic field-effects on biological systems is known to be dependent on a number of criteria in the waveform pattern of the exposure system used; these include frequency, duration, wave shape, and relative orientation of the fields [6,29,32,33,39,40]. In some cases pulsed fields have demonstrated increased efficacy over static designs [19,21] in both medical and experimental settings.”

(Madkan et al, 2009)

ELF-EMF AND RFR ARE CLASSIFIED AS POSSIBLE CANCER-CAUSING AGENTS – WHY ARE GOVERNMENTS NOT ACTING?

The World Health Organization International Agency for Research on Cancer has classified wireless radiofrequency as a Possible Human Carcinogen (May, 2011)*. The designation applies to low-intensity RFR in general, covering all RFR-emitting devices and exposure sources (cell and cordless phones, WI-FI, wireless laptops, wireless hotspots, electronic baby monitors, wireless classroom access points, wireless antenna facilities, etc). The IARC Panel could have chosen to classify RFR as a Group 4 – Not A Carcinogen if the evidence was clear that RFR is not a cancer-causing agent. It could also have found a Group 3 designation was a good interim choice (Insufficient Evidence). IARC did neither.

NEW SAFETY LIMITS MUST BE ESTABLISHED - HEALTH AGENCIES SHOULD ACT NOW

Existing public safety limits (FCC and ICNIRP public safety limits) do not sufficiently protect public health against chronic exposure from very low-intensity exposures. If no mid-course corrections are made to existing and outdated safety limits, such delay will magnify the public health impacts with even more applications of wireless-enabled technologies exposing even greater populations around the world in daily life.

SCIENTIFIC BENCHMARKS FOR HARM PLUS SAFETY MARGIN = NEW SAFETY LIMITS THAT ARE VALID

Health agencies and regulatory agencies that set public safety standards for ELF-EMF and RFR should act now to adopt new, biologically-relevant safety limits that key to the lowest scientific benchmarks for harm coming from the recent studies, plus a lower safety margin. Existing public safety limits are too high by several orders of magnitude, if prevention of bioeffects and minimization or elimination of resulting adverse human health effects. Most safety standards are a thousand times or more too high to protect healthy populations, and even less effective in protecting sensitive subpopulations.

SENSITIVE POPULATIONS MUST BE PROTECTED

Safety standards for sensitive populations will more likely need to be set at lower levels than for healthy adult populations. Sensitive populations include the developing fetus, the infant, children, the elderly, those with pre-existing chronic diseases, and those with developed electrical sensitivity (EHS).

PROTECTING NEW LIFE - INFANTS AND CHILDREN

Strong precautionary action and clear public health warnings are warranted immediately to help prevent a global epidemic of brain tumors resulting from the use of wireless devices (mobile phones and cordless phones). Common sense measures to limit both ELF-EMF and RFR in the fetus and newborn infant (sensitive populations) are needed, especially with respect to avoidable exposures like baby monitors in the crib and baby isolettes (incubators) in hospitals that can be modified; and where education of the pregnant mother with respect to laptop computers, mobile phones and other sources of ELF-EMF and RFR are easily instituted.

Wireless laptops and other wireless devices should be strongly discouraged in schools for children of all ages.

STANDARD OF EVIDENCE FOR JUDGING THE SCIENCE

The standard of evidence for judging the scientific evidence should be based on good public health principles rather than demanding scientific certainty before actions are taken.

WIRELESS WARNINGS FOR ALL

The continued rollout of wireless technologies and devices puts global public health at risk from unrestricted wireless commerce unless new, and far lower exposure limits and strong precautionary warnings for their use are implemented.

EMF AND RFR ARE PREVENTABLE TOXIC EXPOSURES

We have the knowledge and means to save global populations from multi-generational adverse health consequences by reducing both ELF and RFR exposures. Proactive and immediate measures to reduce unnecessary EMF exposures will lower disease burden and rates of premature death.

DEFINING A NEW 'EFFECT LEVEL' FOR RFR

On a precautionary public health basis, a reduction from the BioInitiative 2007 recommendation of 0.1 $\mu\text{W}/\text{cm}^2$ (or one-tenth of a microwatt per square centimeter) for cumulative outdoor RFR down to something three orders of magnitude lower (in the low nanowatt per square centimeter range) is justified.

A scientific benchmark of 0.003 $\mu\text{W}/\text{cm}^2$ or three nanowatts per centimeter squared for 'lowest observed effect level' for RFR is based on mobile phone base station-level studies. Applying a ten-fold reduction to compensate for the lack of long-term exposure (to provide a safety buffer for chronic exposure, if needed) or for children as a sensitive subpopulation yields a 300 to 600 picowatts per square centimeter precautionary action level. This equates to a 0.3 nanowatts to 0.6 nanowatts per square centimeter as a reasonable, precautionary action level for chronic exposure to pulsed RFR.

These levels may need to change in the future, as new and better studies are completed. We leave room for future studies that may lower or raise today's observed 'effects levels' and should be prepared to accept new information as a guide for new precautionary actions.

Attachment 6

Initial Interactions in Electromagnetic Field-Induced Biosynthesis

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Low frequency electromagnetic (EM) fields induce gene expression, and recent insights into physical interactions of EM fields with model systems suggest a mechanism that could initiate this process. The consistently low thresholds at which EM fields stimulate biological processes indicate that they require little energy. Since it has been shown that such weak fields accelerate electron transfer reactions, they could stimulate transcription by interacting with electrons in DNA to destabilize the H-bonds holding the two DNA strands together. Such a mechanism is consistent with the low electron affinity of the bases in previously identified electromagnetic response elements (EMREs) needed for EM field interaction with DNA. It is also in line with both endogenous and in vitro stimulation of biosynthesis by electric fields. The frequency response of several EM sensitive biological systems suggests that EM fields require repetition and are most effective at frequencies that coincide with natural rhythms of the processes affected. *J. Cell. Physiol.* 199: 359–363, 2004. © 2004 Wiley-Liss, Inc.

STIMULATION OF BIOSYNTHESIS BY ELECTROMAGNETIC FIELDS

Cells are unusually sensitive to electromagnetic (EM) fields. Transcription is stimulated by both low frequency (~ 10 – 10^2 Hz) electric fields (Blank et al., 1992; Blank, 1995) and magnetic fields (Goodman and Blank, 1998), as well as by high frequency radio/microwave fields ($\sim 10^{12}$ Hz) (dePomerai et al., 2000; Leszczynski et al., 2002; Weisbrot et al., 2003). The high frequency fields are truly electromagnetic in that the electric and magnetic fields propagate together, whereas at low frequencies the fields can be effectively separated as alternating electric or magnetic fields. The low frequency alternating magnetic fields are usually referred to as EM fields to distinguish them from DC, or fixed, magnetic fields.

Although, many physical stimuli induce transcription, the biosynthetic response to EM fields occurs after exposures of only a few minutes and at 14 orders of magnitude lower energy density than the stimulus of elevated temperature (Blank and Goodman, 2000). Very low thresholds for EM field interaction, given in Table 1, have been found in a variety of biological systems that include changes in rates of enzyme and redox reactions, the biosynthesis of stress proteins, as well as disease-related studies. The consistency of the findings may indicate that these biological EM field effects are due to a similar mechanism.

Since the weak fields that initiate transcription of DNA (Goodman and Blank, 1998) also accelerate electron transfer reactions (Blank and Soo, 2001a,b, 2003), interaction with electrons in DNA could be the basis for initiating transcription. Electrons are most likely to be affected by weak fields because of their low

mass, and interaction with electrons could affect the primary biological functions of DNA, information conservation and retrieval. These two processes appear to require contradictory specifications, that DNA be stable to preserve the integrity of the information while retaining the ability to come apart readily for retrieval of the information. In cells, this is accomplished by the large number of H-bonds between the complementary base pairs on the two DNA strands. In H-bonds (indicated as :), electrons from an O or N that is part of a stable bond are shared with a proton in another bond, as in NH:O or NH:N. Since electrons in these bonds are less strongly held than in covalent bonds, control of H-bonds through the electrons would enable DNA to come apart easily for the code to be read, and then 'zipped' up again for storage. Some of this is usually accomplished by enzymes such as topoisomerase and polymerase. We suggest that electric and magnetic fields generate forces on electrons that weaken the H-bonds holding the two DNA strands together. Oscillating fields set up vibrations that eventually destabilize H-bonds, and vibration frequency is an important factor to consider.

Our earlier focus on the specific signaling pathways in the interaction of EM fields with cells to induce stress

Abbreviations: Hz, hertz; mG, milligauss; bp, base pairs.

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Received 3 August 2003; Accepted 6 November 2003

DOI: 10.1002/jcp.20004

TABLE 1. Estimated biological thresholds

Biological system	EM field (mG)	Reference
Enzyme reaction rates		
Na,K-ATPase	2–3	Blank and Soo (1996)
Cytochrome oxidase	5–6	Blank and Soo (1998)
Ornithine decarboxylase	~20	Mullins et al. (1999)
Oxidation–reduction reaction rate		
Belousov–Zhabotinski	<5	Blank and Soo (2001a)
Biosynthesis of stress proteins		
HL60, Sciara, yeast cells	<8	Goodman et al. (1994)
Breast (HTB124, MCF7) cells	<8	Lin et al. (1998b)
Chick embryo (anoxia protection)	~20	DiCarlo et al. (2000)
Disease related		
Block inhibition of (MCF7) breast		
Carcinoma cells by melatonin	2 < 12	Liburdy (2003)
Leukemia epidemiology	3–4	Ahlbom et al. (2000); Greenland et al. (2000)

protein synthesis showed that they stimulate distinctly different pathways from those implicated in response to elevated temperature (Goodman and Blank, 2002). However, those studies did not provide information about the physical interactions that activated these pathways. At the time, we thought in terms of electron currents in DNA (Ratner, 1999; Porath et al., 2000) that could interact with the EM fields and lead to DNA chain separation. It is now questionable if these currents occur with any frequency, and whether they occur over a large enough number of bases to activate DNA during short exposures (Tran et al., 2000; Boon and Barton, 2002; Zhang et al., 2002).

We have learned, however, that specific DNA sequences (nCTCTn) are involved in the response to EM fields (Lin et al., 1998a, 1999, 2001). Inserting these electromagnetic response elements (EMREs) into a promoter of a reporter gene that is unresponsive to EM fields makes that gene EM field-responsive. Removing or mutating these EMREs eliminates the EM field response. There is also evidence that the EM field response is proportional to the number of EMREs (Lin et al., 1998b). When we assumed that EM fields interacted with electron currents in DNA, our estimate of the relative balance of forces holding DNA together showed that larger forces can be generated at C and T bases (Blank and Goodman, 2002). A simpler, more straightforward rationale for the nCTCTn composition of EMREs can be seen immediately from the electron affinities of the four bases in DNA ($A = 0.97$, $G = 1.51$, $T = 0.81$, $C = 0.57$). The C and T bases have the lowest electron affinities and are most likely to give up their electrons when external forces are applied. Therefore, the physical properties of EMREs provide a plausible basis for interaction with electrons in DNA as an initiating mechanism.

ELECTRON TRANSFER ACCELERATION BY EM FIELDS

The responses of simpler biological systems to EM fields also support interaction with electrons as an initiating mechanism. The systems studied include two well-characterized enzymes, Na,K-ATPase (Blank and Soo, 1992, 1996) and cytochrome oxidase (Blank and Soo, 1998, 2001b), as well as the Belousov–Zhabotinski (BZ) reaction, the catalyzed oxidation of malonic acid

(Blank and Soo, 2001a, 2003). The cytochrome oxidase reaction involves electron transfer between cytochrome C and the enzyme complex; the Na,K-ATPase reaction is the splitting of ATP that precedes the ionic currents of the ‘ion pump.’ Electron transfer in the cytochrome oxidase reaction is accelerated, as is the ATP-splitting rate of the Na,K-ATPase, where the calculated speed of the charges affected by the field suggests that they are electrons (Blank and Goodman, 2000). An earlier study of the Na,K-ATPase (Britten and Blank, 1973) correlated the non-specific inhibition of cations with their redox potentials, suggesting that electron transfer may be a critical step in enzyme function.

The rapid charge movement in the Na,K-ATPase, calculated to be about 10^3 m/sec, means that it takes about 10^{-11} sec to cross the membrane-spanning distance of about 10 nm. Since a 60 Hz sine wave lasts 1/60 sec, the charge ‘sees’ a constant DC magnetic field while crossing, and the interactions are effectively with repeated DC fields. Fixed DC fields of this magnitude do not affect the Na,K-ATPase (Blank and Soo, 1997), so the effect on the enzyme must be due to the regular repetitions that occur in ‘tune’ with the normal molecular motions.

Recent studies on the BZ reaction (Blank and Soo, 2003), a chemical system with no tissue extracts, have confirmed previous observations with the two enzyme reactions. The three reactions show common characteristics:

- EM fields accelerate the reactions;
- the effect of the EM field *varies inversely with the intrinsic rate of the reaction*, that is, the magnetic driving force competes with the chemical driving forces;
- there are frequency optima in the three systems; in the enzyme studies, the frequencies are close to the turnover numbers of the enzyme reactions.

These characteristics are consistent with interaction of EM fields with electrons.

ELECTRONS IN H-BONDS AS TARGETS FOR EM FIELDS

The H-bonds between base pairs that stabilize DNA are much weaker than covalent bonds. They have

energies in the range of 5 kcal/mol, but they vary considerably. The varying bond lengths and bond angles suggest a relatively wide range of bond energies and abilities to withstand perturbations. In some H-bonds, the bonding electrons are shared between more than two nuclei (Suehnel, 2002). Electrons in such non-intra-base-pair H-bonds are more easily displaced. EM fields probably interact with electrons associated with bases having low electron affinity and in non-intra-base-pair H-bonds. In any case, EM fields generate sufficient force to displace them, since the force [in newtons (N)] on an electron,

$$F = qvB,$$

where $q = 1.6 \times 10^{-19}$ coulombs, v = velocity (in m/sec), and the magnetic flux density, B , is approximately $10 \mu\text{T}$ (100 mG) in our experiments stimulating stress protein synthesis. The electron velocity, $v = 10^3$ m/sec, estimated from electric and magnetic field thresholds in experiments with the Na,K-ATPase (Blank and Soo, 1992, 1996), is comparable to electron velocities measured in DNA (Wan et al., 1999). This magnitude of electron velocity is also expected if electrons move at the \sim nanometer/picosecond flickering rate of protons in H-bonded networks (Fecko et al., 2003). The assumed value for ' v ' leads to $F \sim 10^{-21}$ N, and an acceleration of $\sim 10^9$ m/sec² for an electron of mass 9.1×10^{-31} kg. With this magnitude of acceleration, an electron can move 1 nm in 1 nsec, a displacement greater than the ~ 0.3 nm overall length of an average H-bond. Repeated pulses could set up vibrations that destabilize an H-bond.

The displacement of electrons in an H-bonded network may also occur by the unusual charge movements characteristic of the "Grotthuss mechanism." In aqueous systems, during the conduction of protons (H^+) in electric fields, an approaching proton 'bonds' with the oxygen of a water molecule and releases the hydrogen on the other end of the molecule as a free proton. The flipping of the bond and its electrons results in a proton moving forward at a much faster rate, even if it is not the same proton. If this type of process occurred in adjacent H-bonds in DNA subjected to an external force, there could be some unusual movements, especially when water molecules are present (Fecko et al., 2003). The more complex H-bonds in DNA with lower energies and more constrained angles would be more vulnerable.

STIMULATION OF TRANSCRIPTION BY ELECTRIC FIELDS

Interaction with electrons should occur with electric fields as with EM fields, and that has been shown. Increases in transcripts of *c-myc* and histone H2B in human cells occur in 60 Hz electric fields of 3 mV/m (Blank et al., 1992), where the force on an electron, $\sim 5 \times 10^{-19}$ N, is almost three orders of magnitude greater than the EM field force that causes the same effect. An even more dramatic example is the pronounced effect of *endogenous* electric stimulation on protein synthesis in mammalian striated muscle (Pette and Vrbova, 1992; Blank, 1995). Different muscle proteins are synthesized at 150 and 20 Hz frequencies, with action potentials delivered by either the nerve or external electrodes. It is even possible to change the

protein composition of a 'fast' muscle to that of a 'slow' muscle over a period of a few weeks by changing the frequency of stimulation. The change in protein composition is probably due to activation of different DNA coding regions. In electric stimulation of muscle, the waxing and waning of forces generated by continuously oscillating fields would set up oscillations that could destabilize H-bonds.

The currents of action potentials that penetrate muscle membranes and flow near nuclei can be shown to be large enough to stimulate DNA. The current magnitudes can be estimated from muscle action potentials, which rise from resting level to a peak of ~ 100 mV in about 1 msec, and propagate at ~ 10 m/sec. In the 1 msec that it takes the potential to peak, the front of an action potential advances 10 mm, so peak and resting potentials are separated by 10 mm, for a gradient of 10 V/m. This electric field gradient is three orders of magnitude larger than the 3 mV/m that stimulates transcription in human cells, and suggests a large margin of safety in muscle. The gradient is even larger than the 0.5 mV/m electric field threshold to change Na,K-ATPase activity (Blank and Soo, 1992).

Recent use of nanosecond electric pulses in electroporation has resulted in stimulation of the cell interior (Joshi et al., 2002). Normally, electroporation is used to permeabilize cell membranes with high voltage (\sim kV/m) microsecond pulses that do not readily penetrate cell membranes. The high frequency electric fields that penetrate activate apoptosis through caspase release and cause DNA fragmentation (Beebe et al., 2003). It appears that stimulation of cell interiors is possible with high frequency (nanosecond pulses) electric fields, as well as with low frequency EM fields, but the lower energy EM fields do not damage cellular structures and are to be preferred.

FIELD FREQUENCY AND REACTION SPECIFICITY

Studies of the frequency dependence of biochemical reaction rates in electric or magnetic fields indicate optimal frequencies (Table 2). The increased Na,K-ATPase activity at 60 Hz is very close to the natural rate of the enzyme (Blank and Soo, 2001a). In cytochrome oxidase (Blank and Soo, 1998), the optimal frequency of about 800 Hz is close to the range of its function in mitochondria. These data suggest that the field is most effective when it coordinates with the natural rhythm of a reaction. Since the effects on both enzymes vary inversely with intrinsic enzyme rates, it is clear that the fields compete with biochemical driving forces.

Studies of *E. coli* F_0F_1 -ATPase activity in electric fields (Martirosov and Blank, 1995) also show that inhibition is a function of frequency, and that the optimal frequency is close to F_0F_1 -ATPase turnover numbers (10–80 Hz). Frequency dependence requires the native enzyme structure, since mutant strains show lower frequency dependence, and there is a loss of frequency dependence in precursor F_0F_1 when activity decreases in cold storage. The F_0F_1 -ATPase optimal frequency increases with field strength, with minima around 1,000 Hz at 2.3 V/cm, and about 30 Hz at 3.6 V/cm. The existence of two characteristic frequencies for an active center could occur if higher fields cause

TABLE 2. Optimal stimulation frequency

Biological system	Frequency (Hz)	Reference
Enzyme reaction rates		
Na,K-ATPase (EM field)	60	Blank and Soo (2001a)
Cytochrome oxidase (EM field)	800	Blank and Soo (2001a)
F ₀ F ₁ ATPase (low E field)	1,000	Martirosov and Blank (1995)
(High E field)	30	Martirosov and Blank (1995)
Oxidation–reduction reaction rate		
Belousov–Zhabotinski (EM field)	250	Blank and Soo (2001b)
In vitro biosynthesis		
Human HL60 cells (EM field)	45	Wei et al. (1990)
In vivo biosynthesis (mammalian)		
‘Fast’ muscle (E field)	150	Pette and Vrbova (1992)
‘Slow’ muscle (E field)	20	Pette and Vrbova (1992)

significant structural changes or changes in the rate limiting steps of the reaction. It is possible that this energy-converting enzyme, optimized for its function through evolution, might work at a slower rate when there is a greater driving force.

The stimulation of biosynthesis in EM fields also shows a dependence on frequency. Increases in transcripts of *c-myc* and histone H2B in HL60 cells, reported in 60 Hz electric fields (Blank et al., 1992), are optimal at 45 Hz (Wei et al., 1990). The optimal frequency is in the range of RNA synthesis rates (bases/sec), and may be related to an effect on charge transfer in the RNA polymerase reaction.

Endogenous electric field stimulation of biosynthesis by the currents of action potentials also shows a relation between rate of stimulation and the proteins synthesized. Neither the speed nor the magnitude of an action potential is affected by frequency, but electrons at a particular site on the DNA are subjected to more perturbations at higher frequency, and summation is possible. Both effects would lead to threshold events more easily at the higher frequency, and could be related to different effects of high or low frequency action potentials in muscle. The repeated electric stimuli of cardiac action potentials or central nervous system rhythms probably have similar effects on the nuclei of adjacent cells, and may be involved in regulation of natural biosynthetic mechanisms, as well as the onset and pace of development. Endogenous electric fields may also interact with non-coding DNA to accomplish some of the critical timing during development.

CONCLUSION

We have proposed that interaction of EM and electric fields with electrons in DNA is a plausible basis for activation of DNA. Because of the low energy required, interaction with electrons in H-bonds may be the initial perturbation that leads double stranded DNA to come apart and begin the complex process of transcription to messenger RNA. The response to EM fields takes advantage of the natural mechanism that responds to internal electric forces.

Stimulation and modulation of DNA function by magnetic and electric fields have shown that physical forces can lead to specific effects as with biochemical reactions. In addition to the frequency, stimulus duration and different patterns of pulsing of magnetic and electric fields can further alter responses and specificity.

The correlation between magnetic field frequency and the rate of charge transfer reactions offers promise for clinical applications.

Finally, when considering environmental influences, EM field activation of DNA reinforces concerns about human exposure to the exogenous fields due to power lines, communication devices, etc. The Liburdy study, listed in Table 1 (Liburdy, 2003) and replicated in four other laboratories, shows that low level EM fields affect the growth of human estrogen receptor positive breast cancer cells. The melatonin-induced growth inhibition, overcome by 12 mG fields but not by 2 mG fields, places the threshold between these two values, which are on either side of the epidemiology threshold for childhood leukemia (Ahlbom et al., 2000; Greenland et al., 2000). Health concerns are often expressed in terms of the need to prevent oxidative damage. Acceleration of electron transfer in EM fields is acceleration of oxidation.

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Attachment 7

Femtosecond dynamics of DNA-mediated electron transfer

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Contributed by Ahmed H. Zewail, March 26, 1999

ABSTRACT Diverse biophysical and biochemical studies have sought to understand electron transfer (ET) in DNA in part because of its importance to DNA damage and its repair. However, the dynamics and mechanisms of the elementary processes of ET in this medium are not fully understood and have been heavily debated. Two fundamental issues are the distance over which charge is transported and the time-scale on which the transport through the π -stack of the DNA base pairs may occur. With femtosecond resolution, we report direct observation in DNA of ultrafast ET, initiated by excitation of tethered ethidium (E), the intercalated electron acceptor (A); the electron donor (D) is 7-deazaguanine (Z), a modified base, placed at different, fixed distances from A. The ultrafast ET between these reactants in DNA has been observed with time constants of 5 ps and 75 ps and was found to be essentially independent of the D–A separation (10–17 Å). However, the ET efficiency does depend on the D–A distance. The 5-ps decay corresponds to direct ET observed from 7-deazaguanine but not guanine to E. From measurements of orientation anisotropies, we conclude that the slower 75-ps process requires the reorientation of E before ET, similar to E/nucleotide complexes in water. These results reveal the nature of ultrafast ET and its mechanism: in DNA, ET cannot be described as in proteins simply by a phenomenological parameter, β . Instead, the involvement of the base pairs controls the time scale and the degree of coherent transport.

The striking resemblance of the base-pair stack of DNA to conductive one-dimensional aromatic crystals prompted, over 30 years ago, the proposal that long-range charge transport might proceed through DNA (1). In the three decades since, biochemical, biophysical, and theoretical studies have sought to address the possibility and efficiency of the transport (2–29). Such charge migration through DNA is significant, because radical migration is a critical issue to our understanding of carcinogenesis and mutagenesis (5, 6).

Photoinduced electron transfer (ET) reactions have provided a useful tool in elucidating parameters governing ET through DNA. In the 1980s and early 90s, a class of experiments on noncovalently bound electron donors (D) and electron acceptors (A) in DNA was reported (7–12). A major debate focused on whether or not ET through DNA may proceed rapidly and differently from that found in σ -bonded systems. These experiments provided valuable information and raised many questions, but a key issue was the distance between D and A, which was not well defined.

With D and A covalently bonded to DNA, studies of ET on more well defined assemblies were made possible, and the effect of distance could be addressed (13–20). Values of the parameter β , which reflects the distance scale of ET through a given medium (30), remarkably, ranged from $\leq 0.1 \text{ Å}^{-1}$ to $> 1.4 \text{ Å}^{-1}$; β was estimated by using one system of a fixed

distance (13, 14) or by varying the distance (15–20). The fact that values of β could vary so enormously created substantial controversy. Recently, studies of ET between modified bases in DNA (20) were reported with β spanning this full range depending on stacking of reactants (16). Long-range oxidative damage and repair to DNA have been reported for covalently bound oxidants (17, 27–29). Moreover, recent conductivity measurements have shown the existence of both dark and photoinduced one-dimensional currents along DNA that were aligned in flexible-cast DNA films (3). Theoretical efforts (21–26) have discussed the different modes of ET, direct tunneling vs. hopping, and the possible range of β for DNA; a prediction of β for DNA of $1.2\text{--}1.6 \text{ Å}^{-1}$ was made (21). The importance of the β -value is in relating rates to the mechanism, an area that has received considerable attention in the studies of ET in proteins [refs. 31 (and references therein), 32, and 33].

The direct observation of the ultrafast dynamics of DNA-mediated ET is critical to our understanding of the mechanism. With femtosecond resolution, it is now possible to obtain the actual time scale of ET and to relate the rates to the distance between D and A. The time scale of orientational coherence and solvation can also be examined, allowing us to elucidate the role of molecular motions.

The DNA Assemblies

ET between ethidium (E) and 7-deazaguanine (Z) has been characterized previously in aqueous solution (34). By using femtosecond spectroscopy, ET was found to proceed between photoexcited E and associated Z triphosphate (ZTP) but not between E and the natural analogue GTP. The measured peak potential of the cyclovoltammetry, which is taken as an estimate of the standard potential, is 1.0 V and 1.3 V (vs. normal hydrogen electrode) for $\text{ZTP}^{+}/\text{ZTP}$ and $\text{GTP}^{+}/\text{GTP}$, respectively; the peak potential for $\text{E}^{+}/\text{E}^{\bullet}$ is 1.2 V (after subtracting the 0–0 excitation energy from the ground-state peak potential for $\text{E}^{+}/\text{E}^{\bullet}$) (18). The ET dynamics of the E/ZTP system were ultrafast but reflected the time required for the reorientation of the complex into an active conformation for charge transfer (34). The quenching of the E^{\bullet} fluorescence was studied in a DNA assembly as a function of distance and sequence (18). In Z-containing duplexes covalently modified with E, steady-state fluorescence measurements revealed a shallow dependence of the efficiency of the quenching on distance, but the reaction dynamics could not be resolved by picosecond single-photon counting.

Fig. 1a illustrates models of three DNA assemblies used in the studies reported here. In these 14-mer duplexes containing tethered E, the position of Z has been varied so as to give a range of D–A separations; the sequence representing the binding site for E and the sequence flanking Z have been kept constant. Fluorescence-decay profiles, steady-state fluores-

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Abbreviations: ET, electron transfer; E, ethidium; A, electron acceptor; D, electron donor; Z, 7-deazaguanine; ZTP, Z triphosphate; G, guanine; B, bridge.

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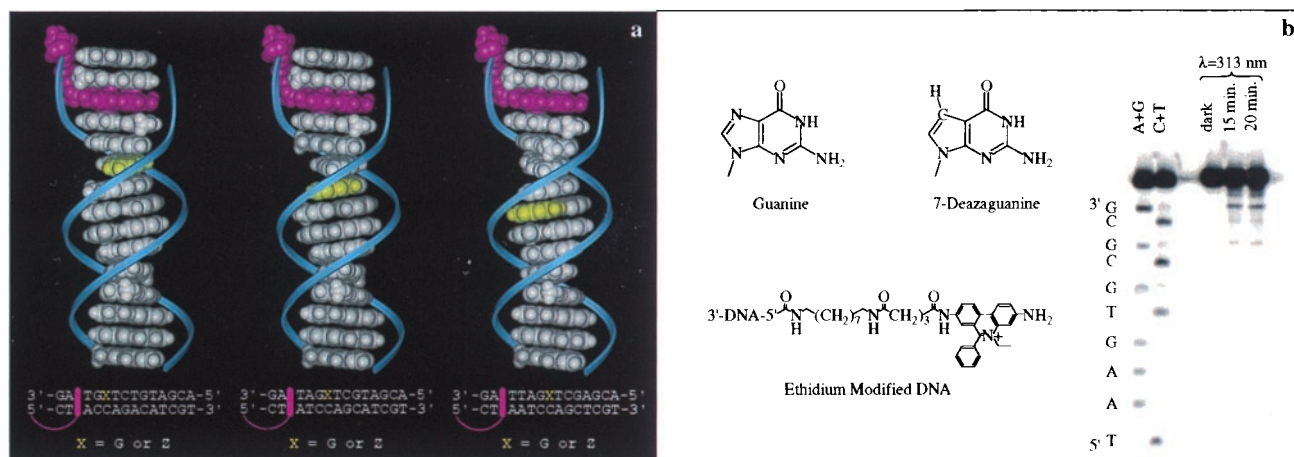


FIG. 1. The DNA assemblies. (a) Molecular models (Insight II) illustrating the E-tethered (red) DNA assemblies (left to right) 5Z, 6Z, and 7Z. The Z base is shown in yellow. Sequences are given below. (b) Structures of guanine (G), Z, and the E-modified tether, as well as an autoradiogram (right) after denaturing 18% PAGE, showing photoinduced damage of an E-modified duplex generated by irradiation at 313 nm of the duplex (10 μ M) in 5 mM phosphate/50 mM NaCl, pH 7, 5' 32 P-labeled on the strand complementary to that containing the tether. Crosslinking occurs at the first two base steps on the 3' side (near E); see *The DNA Assemblies* and ref. 35. The sequence 3'-CGCGCACTTA-5' also was examined and gave consistent results.

cence polarization measurements, thermal denaturation profiles, ionic-strength effects, and studies using various linker lengths all indicate that the tethered E moiety is intercalated within the DNA duplex. These data are also consistent with a homogeneous assembly in the steady-state regime (16, 18). Fig. 1a depicts E intercalated at the second base step of the assembly; molecular modeling indicates this second base step to be the farthest possible site from the end of the hybridized duplex into which the tethered E can intercalate, although the first base step is preferred.

To examine experimentally the distribution of binding sites for E within the tethered duplex, a series of photocrosslinking experiments were undertaken. Irradiation of E-modified duplexes at a short wavelength (313 nm) leads to covalent crosslinking at the site of intercalation and strand breakage; the resultant damage can be visualized by denaturing PAGE (35). Fig. 1b shows the damage on the strand complementary to that modified with E. Quantitation of gel bands indicates a narrow distribution of binding sites, with $\geq 70\%$ of tethered E intercalated at the first base step and the remaining E bound predominantly within the second base step from the terminus. Here, then we consider, conservatively, tethered E as intercalated within the second base step, such that the intrahelix distance separating E and Z in the 5Z assembly is 10 Å (assuming 3.4-Å stacking); for 6Z and 7Z the distance is 14 Å and 17 Å, respectively.

ET Dynamics

To study the dynamics of ET in real time, we performed femtosecond transient absorption measurements on the molecular assemblies shown in Fig. 1. The detailed experimental setup has been described elsewhere (34). Briefly, a femtosecond pulse at 500 nm was employed to excite E into its excited state (S_1), and a second pulse at ≈ 400 nm, after a variable time delay, was used to probe the dynamics of this state. The dramatic differences in the transient absorption decays of 5G and 5Z can be seen in Fig. 2a and d. In the short time range, 5G shows the expected lifetime of tethered E in DNA: ≈ 2 -ns decay background with a small (14%) 1.5-ps decay component; the lifetime of E^* functionalized on the exocyclic amine is ≈ 2 ns when tethered to DNA (16), compared with ≈ 20 ns for unfunctionalized E^* in DNA (36, 37). In contrast, 5Z decays with a much stronger 5-ps component (34%); at longer times,

the decay of 5Z shows a 75-ps (23%) component, which is absent in 5G, with the ≈ 2 -ns decay (43%) present (Fig. 2d).

To ensure that the decay is that of the initial excited E population, we also performed femtosecond fluorescence up-conversion measurements. The fluorescence transients were detected in the wavelength range between 570 nm and 670 nm. Fig. 3 shows the transients of 5G and 5Z at 600 nm and 670 nm. As in the transient absorption, significant differences in the fluorescence transients of 5G and 5Z were observed. In the short time range (at 600 nm), the lifetime of E^* (Fig. 3a) is shortened in 5Z ($\tau \approx 5$ ps); As with transient absorption, we can fit the early time decay to the same 1.5-ps component, but the signal-to-noise ratio for the fluorescence up-conversion is not as good as for transient absorption. At longer times and at 600 nm (Fig. 3b), the fluorescence decay of 5G consists predominantly of the ≈ 2 -ns component (with a possible small 20-ps component), whereas 5Z shows a stronger decay with the expected time constant of ≈ 75 ps and the ≈ 2 -ns decay. After the 5-ps and 75-ps decays, the fluorescence intensity of 5Z decreases significantly when compared with 5G; i.e., the ultrashort fluorescence lifetime component in 5Z reflects the onset of a major nonradiative process brought about by the D-B-A (B = bridge) of the DNA assembly.

The unambiguous presence of two distinguishable time scales for such processes in the assemblies containing Z raises the following question: Are the 5-ps and 75-ps decays caused by ET? As determined by electrochemical studies (18), ΔG for ET is ≈ -0.2 eV (E^*/Z) and $+0.1$ eV (E^*/G), respectively (note that 1 eV = 1.602×10^{-19} J). Hence, there is a favorable driving force for ET with Z but not with G. Energy transfer is not significant because of the lack of spectral overlap between D and A. Because Z and G differ by only one atom, other nonradiative processes can be excluded. We conclude that ET leads to the dynamics observed in the Z assemblies. It is important to note that both time constants observed (5 ps and 75 ps) reflect the *forward* ET dynamics, because the fluorescence intensity of the product radical state is supposed to be much weaker than that of the initial state, monitored in both the transient absorption and fluorescence up-conversion experiments.

For the same D-A system, without DNA mediation, it was found that solvation of E occurs on the time scale of femtoseconds and up to 1 ps in water; furthermore, the orientation time of E was measured to be ≈ 70 ps (34). To examine solvation and internal rotation when DNA is mediating the

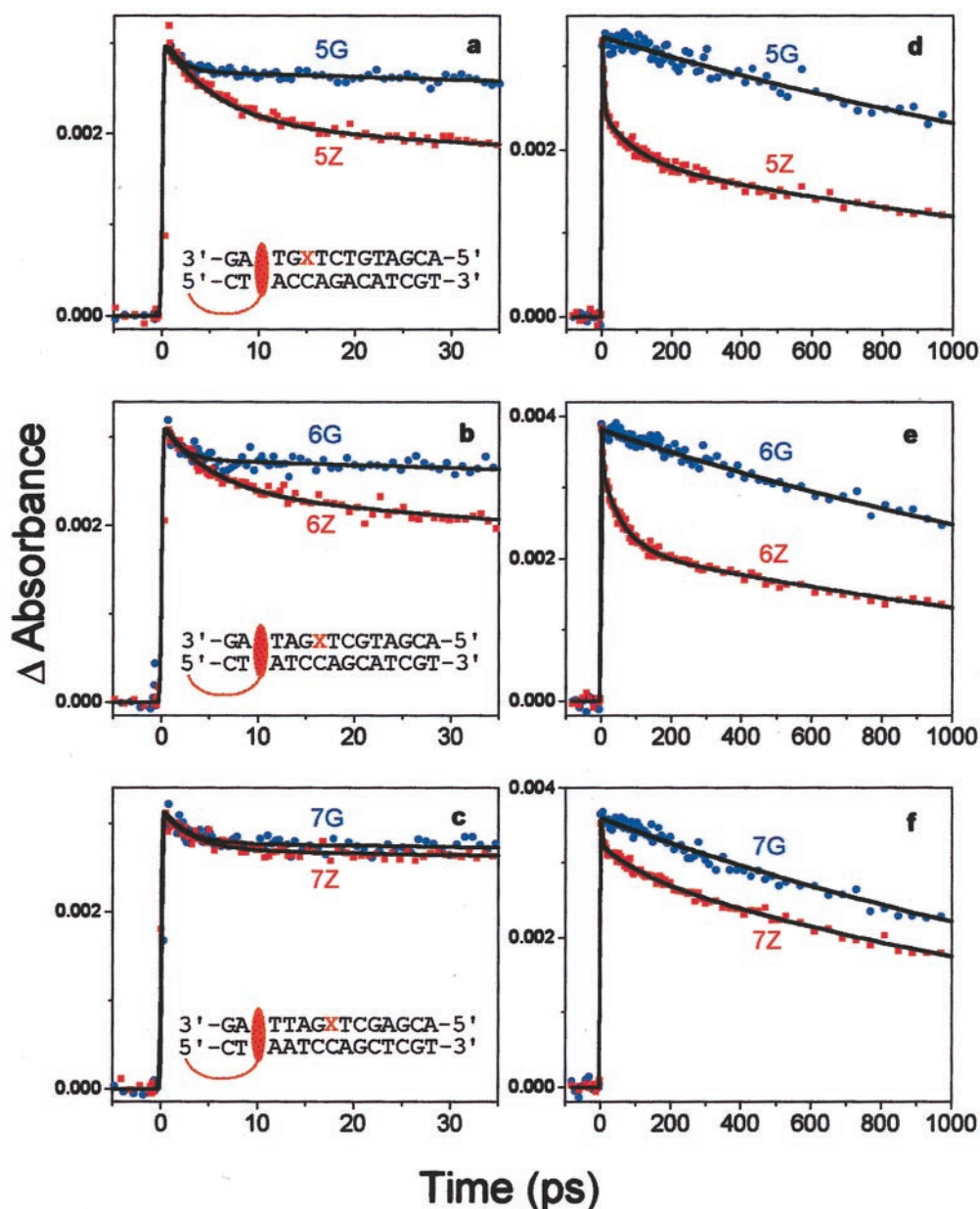


FIG. 2. Femtosecond transient absorption of DNA assemblies with 5Z/5G, 6Z/6G, and 7Z/7G after excitation at 500 nm (probed at 400 nm); short (*Left*) and long (*Right*) time scales are shown. All samples contained 10 μ M duplex DNA in 5 mM phosphate/50 mM NaCl, pH 7. The sequences are displayed with closest position of E indicated.

transfer, we studied the wavelength dependence of the fluorescence transients and the time dependence of the fluorescence anisotropy. At the longer wavelength (670 nm; Fig. 3 *c* and *d*) the fluorescence signal of 5G rises, and we fitted this picosecond rise to 1.5-ps and 20-ps components (10–20%); the signal-to-noise is not sufficient to provide accurate values but the picosecond rise is definite. As shown in Fig. 3*b*, 5Z exhibits the same 75-ps decay component observed at 600 nm. The fact that the transient appears as a decay at the short wavelength and as a rise at the longer wavelength suggests that the ultrafast dynamics in 5G reflect a temporal spectral shift. Such a shift can be caused by solvation dynamics and/or structural relaxation processes; a 1.5-ps decay is consistent with the solvation process (34). The longer picosecond component may be attributed to structural relaxation, such as the phenyl-ring rotation (38), probed in fluorescence (not transient absorption) because of the sensitivity of fluorescence detection to a subset of molecular configurations. Naturally, the same processes are expected to be present in 5G and 5Z; however, the dominant

5-ps decay in 5Z almost cancels the picosecond rise components, which would be expected at 670 nm because of the spectral shift, hence the observed flattening in Fig. 3*c*.

To answer the question as to why there are two apparent time scales for ET (5 ps and 75 ps), we performed femtosecond fluorescence anisotropy measurements. The anisotropies $r(t)$ are derived from the intensity of the parallel $I_{\parallel}(t)$ and perpendicular $I_{\perp}(t)$ up-conversion signals by using the following formula: $r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)]$. Fig. 4 *a* and *c* shows the anisotropy of the 5G and 5Z assemblies. It is interesting that 5Z shows a single slow decay (≈ 5 ns); 5G shows a similar decay but with an additional decay component ($\approx 12\%$) of ≈ 100 ps. The ≈ 100 -ps and ≈ 5 -ns anisotropy decays of 5G are ascribed to the restricted rotation of E in DNA and other slower rotations, including that of the whole DNA duplex (37, 39, 40). As discussed elsewhere (34), the fact that the ≈ 100 -ps anisotropy decay exists in 5G but not in 5Z indicates that there is a correlation of the slow ET process (75 ps) with the rotation of E in DNA. Note that the ≈ 100 -ps anisotropy

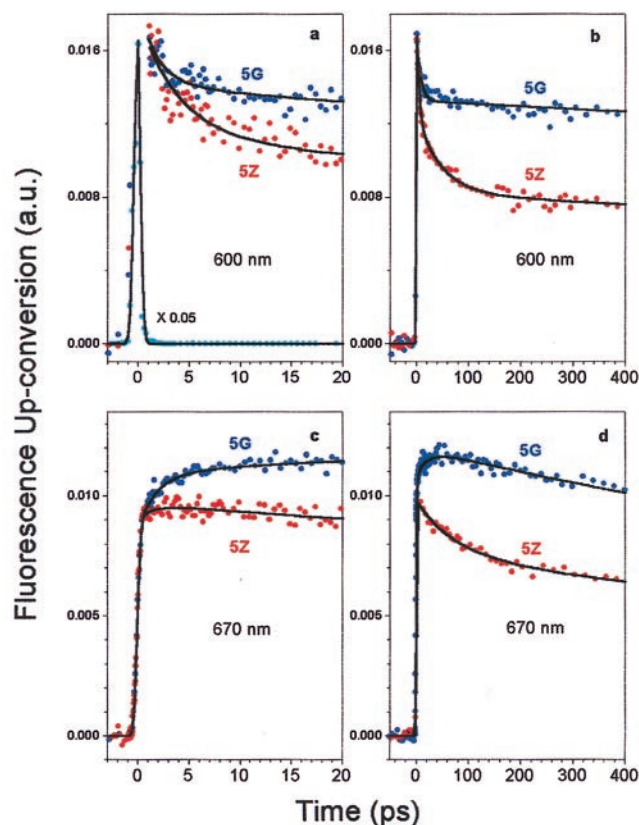


FIG. 3. Femtosecond fluorescence up-conversion of DNA assemblies with 5Z and 5G, detected at 600 nm (Upper) and 670 nm (Lower) in the short (Left) and the long (Right) time range. At the short wavelength, there is a solvent-alone spike around $t = 0$ (see *a*), which is absent at the longer wavelength (670 nm). The sample conditions are given in Fig. 2.

decay component appears also in the parallel fluorescence signal $I_{\parallel}(t)$ shown in Fig. 4*b*. $I(t)$ is calculated in the following way: $I(t) = I_{\parallel}(t) + 2I_{\perp}(t)$, normalized to $I_{\parallel}(t)$ at longer times. It is equivalent to the signal at the magic angle (54.7°). Comparing $I(t)$ and $I_{\parallel}(t)$, it becomes evident that the latter contains an additional decay component of ≈ 100 ps caused by an orientational motion.

There are two types of trajectories with E having different geometries (orientations), one being initially *favorable* and the other initially *unfavorable* for ET. In reality, there is a whole distribution of orientations, but the ET emphasizes two families; those poised and those requiring time to dynamically switch on. Hence, the orientational motion within the DNA duplex is important for ET. We conclude that the 5-ps decay reflects the ET dynamics in those structures where E is “perfectly stacked,” whereas the slow quenching process (75 ps) is caused by the fraction of molecules where E has to reorient/rotate first before ET can occur. The reason for the absence of the ≈ 100 -ps anisotropy decay in 5Z is that the faster rotating molecules are immediately drained away by the ET process once they convert into the favorable geometry of ET. The 2-ns component represents those molecules that do not align favorably for ET.

Distance Dependence

An important question is how ET depends on the D–A distance. This dependence was examined by varying the position of Z within the 14-mer duplex while keeping the tethered E position constant. Both fluorescence up-conversion and transient absorption experiments reveal similar behavior, and

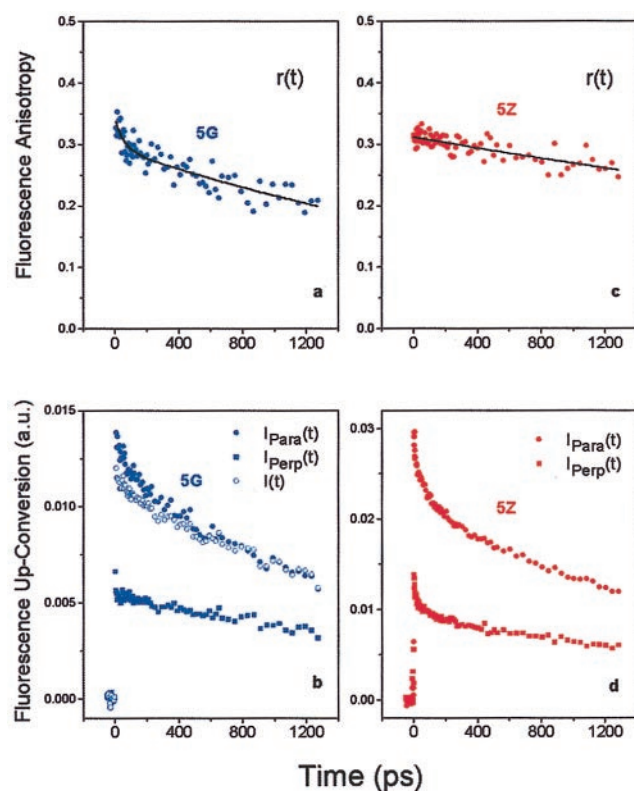


FIG. 4. Polarization dependence of the time-resolved fluorescence. The decays of the fluorescence anisotropy for 5G (*a*) and 5Z (*c*) are shown. Fluorescence decays I_{\parallel} and I_{\perp} (polarization of the detection with respect to the excitation) for 5G and 5Z are also given (*b* and *d*). The magic angle (54.7°) $I(t)$, deduced from I_{\parallel} and I_{\perp} for 5G (see *ET Dynamics*), is shown for comparison with I_{\parallel} . The sample conditions are given in Fig. 2.

we show only the transient absorption of 5Z, 6Z, and 7Z with a D–A separation of at least 10 Å, 14 Å, and 17 Å, respectively, and in reference to 5G, 6G, and 7G (Fig. 2). Clearly, the Z assemblies show unusual distance dependencies in their dynamical times and amplitudes. The G assemblies show essentially no variations within our experimental error; all transient absorption measurements of 5G, 6G, and 7G are similar in both the short and the long time scans and show ≈ 1.5 -ps and ≈ 2 -ns decays (Fig. 2, curve G).

In contrast, the transient absorption data of 5Z, 6Z, and 7Z indicate that ET occurs essentially with the same characteristic time constants for all distances studied but with a decreasing amplitude as the distance increases (Fig. 2, curve Z). In the order of 5Z, 6Z, and 7Z, the fast component (5 ± 1 ps) has the amplitude of 34%, 24%, and 14%. The slower component (75 ± 20 ps for 5Z and 6Z; 103 ± 34 ps for 7Z) has the amplitude of 23%, 22%, and 9%. Consequently, the total ET efficiency (5-ps and 75-ps decays) of the 5Z, 6Z, and 7Z are 57%, 46%, and 23%, respectively; here the efficiency is defined as the intensity drop in the ET decay normalized to the initial intensity. These efficiencies should be compared with the steady-state measurements: 70%, 56%, 28% for 5Z, 6Z, and 7Z, respectively, calibrated to the steady-state intensities of 5G, 6G, and 7G (18).

Mechanism: Transport and Molecular Motions

The striking observations reported here elucidate the elementary mechanism of DNA-mediated ET: independent of the D–B–A distance (10–17 Å), the rates are similar, whereas the ET efficiency decreases with increasing distance over the same

range. We consider first the determinants of the ultrafast time scale for ET and then the basis for variations in ET efficiency.

There are two fundamental concepts governing the ET process, the energetics of the D–B–A system and the time scale of the molecular motions during the transfer. The energetics play an important role in defining two extreme limits, that of *transport* through the B by multiple-step hopping and the other that involves a one-step *transfer* between D and A. The literature is rich in the application of these two extreme cases for one-dimensional excitation transfer in solids (ref. 41 and references therein), in molecular assemblies (42) and electronics (43), for ET in biological systems (44), and for many D–A systems (45). These issues of debating the role of chemical and physical transfer in bridged systems and the relative importance of thermodynamic and kinetic effects have roots in many studies of ET reactions in solutions (46, 47).

If the transfer is a one-step process, then the distance dependence of the rates is dramatic and can be described by the parameter β in a superexchange mechanism (48). In this case, there is an effective coupling between D and A that depends exponentially on the length of B. On the other hand, a transport process involves a real population residence in B, resulting in a weak distance dependence. Here the electronic coupling (V) is critical between D and B, within B, and between B and A. This picture is incomplete, however, as one must consider also the time scale for the orientational motion relative to that of ET in order for the transfer or transport to be effective. Achieving orientational coherence could in fact become the rate-determining step for ET (34).

In the system described here, with the initial femtosecond excitation of E, which is a cation in the ground state, an electron is promoted from the highest occupied molecular orbital to the lowest unoccupied molecular orbital (HOMO and LUMO, respectively) of E creating an electron-hole localized on E, the electron acceptor (hole donor). The interaction of E^{+*} with the DNA B leads to a hole injection from E^{+*} to B or more accurately from E^{+*} –B–Z to E^+ –B $^{+*}$ –Z. Because of the net driving force ($\Delta G \approx -0.2$ eV), there is a final “trapping” step which produces E^+ –B–Z $^{+*}$. The energies of the three configurations are determining factors for the transport. Significantly, the overall transport is controlled by the rate of electron injection, governed by the electronic coupling V_{E-B} ; the transport in the B, determined by V_{B-B} ; and the trapping rate, governed by V_{B-Z} . Implicit in these rates is their dependence on the energetics of the base pairs and on the stacking of E, B, and Z.

We may estimate the time scale of ET between the E^{+*} –B–Z and E^+ –B $^{+*}$ –Z configurations by using the semiclassical Marcus expression (49). We assume that pulse radiolysis (50) and electrochemical (51) studies on the oxidation of the nucleotides and reduction of E (18) provide reasonable estimates of their values within DNA so that $\Delta G_{E,B} = 0.1$ eV and $\Delta G_{B,Z} = -0.3$ eV. Then if λ , the reorganization energy, is 0.1 eV and the electronic coupling matrix elements, V_{E-B} and V_{B-Z} , are 200 cm^{-1} at room temperature, we estimate k^{-1} for the first and the final steps to be ≈ 2 ps; changing λ from 0.1 eV to 0.2 eV changes the value of k^{-1} for the first step to ≈ 4 ps. The similarity of this value to the observed time scale for ET may suggest the adequacy of the parameters estimated for the transport, with the indication that the transfer in B is faster than that of the initial and/or final step. In fact, we observe no significant change in the rates with distance, and thus ET involves a rate-determining step that is independent of the steps in B.

We have carried out preliminary quantum calculations by using the ZINDO method (52, 53) and found that the coupling matrix elements range from 100 cm^{-1} to 400 cm^{-1} for adjacent base pairs and that the energy difference between E, Z, and the intervening B states, or the energy spread, is on the order of 0.3 eV. Thus we estimate ≈ 300 fs per step in the B for near

resonance transport. There is no one defined energy difference to consider, because the energy spread is comparable to that of ΔG . It is important to realize that the effective coupling to B is sequence dependent, and we believe that the entire sequence should be considered collectively in calculating ΔG . Thus, ΔG for hole injection from E^+ to adenine will be smaller in EAAAG than in EATTG. The λ value given above is lower than those reported for ET in polar solvents (54) and in some proteins [refs. 31 (and references therein), 32, and 33], but it is comparable to those found for ET in less polar solvents (54) and in the photosynthetic reaction center (55); in DNA, the proximity of D and A to the B and the intercalation provides a unique environment that influences the value of λ . It should be noted that, in our systems, the transfer is not accompanied by (+–) charge separation; only carrier (+) transport is involved.

What about the efficiency of the transport? It is apparent that the structural dynamics of the DNA double helix must come into play. The ultimate yield of the transport depends on the spatial orientation of D, A, and the B base pairs—stacking—which dictates the magnitude of the effective coupling. As the dynamical nature of the DNA base stack gives rise to a distribution of conformations, only a fraction of the population is active for ultrafast ET. This distribution of local conformations will vary with DNA sequence (56). Moreover, all these conformations vary with time as a result of the dynamical motions within DNA, which occur on picosecond to millisecond time scales (57). In the DNA assemblies, the favorable and nonfavorable conformations give rise to the observed rates of ET, as discussed above. For DNA duplexes that contain certain defects (e.g., transiently destacked base pairs or reactants), coupling is reduced to such an extent that ET becomes impossible during the lifetime of the excited E. These inactive structures will contribute to the long (≈ 2 -ns) decay that we observe in both fluorescence and transient absorption experiments. As we do not observe a significant change in the three decay rates (5 ps, 75 ps, and ≈ 2 ns) with distance, the dynamical defect motions important here occur primarily on a time scale slower than the lifetime of E.

Accordingly, the observed dependence of the efficiency (yield) on distance is controlled by the “static” (on the time scale of the lifetime of E) distribution of ground-state conformations. Statistically, over longer distances, there will be a higher probability of defects in stacking, which explains our observed reduction in the amplitudes of both the 5-ps and the 75-ps components as we increase the distance from 5Z to 7Z. This decrease in yield is expected to depend exponentially on distance, but in duplexes with finite length, the dependence could deviate somewhat; the total ET efficiency changes in the order of 5Z, 6Z, and 7Z from 57% to 46% and to 23%. As such, these yield results will give a β value that does not reflect the actual behavior of the rates vs. distance; the yield reflects only the degree of disorder in the stacking. Finally, we expect that the coherence length of the B transport is on the order of the next-neighbor base-pair distances, because a coupling matrix element, V_{B-B} , of ≈ 200 cm^{-1} is comparable to kT at room temperature (T).

In conclusion, these results elucidate the elementary mechanism for carrier transport in DNA. The dynamics of this biological ET in D–B–A assemblies are governed by the local effective interactions of D (A) with the intervening base pairs and by the time scale of molecular motions. In the systems reported, where D and A are at a fixed distance apart, the ultrafast efficient ET becomes inefficient by 17 Å of separation, indicating the increased role of stacking disorder among members of the B. The use of the parameter β must be handled with care, as it is valid only if the energetics allow for a virtual coupling of D and A to the B. β may be used as a “figure of merit,” but the physics must be clear regarding the mechanism of the transfer or transport.

This work would have not been possible without the support of the Laboratory for Molecular Sciences. T.F. is grateful for financial support from the Deutsche Forschungsgemeinschaft.

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Attachment 8

PROSPECT

Electromagnetic Initiation of Transcription at Specific DNA Sites

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Abstract Initiation of transcription by electromagnetic (EM) fields offers an insight into mechanism. EM field stimulated transcription appears to require specific DNA sequences, and these bases may be sites where EM fields generate large repulsive forces between chains by accelerating electrons that move within DNA. We can estimate the repulsion between chains by assuming that electron affinity is a measure of electron density at each base, and inversely related to the velocity of electrons (and the force). The repulsive force can be compared to the attraction between chains due to H-bonds. From the difference between repulsion and attraction, we show that sites rich in C and T, as in the specific sequences, would be more likely to come apart in EM fields. These calculations suggest a plausible mechanism for initiation of transcription by EM fields, and provide a rationale for specific sequences to function as EM field response elements. Electron flow could also be a factor in DNA chain melting due to Joule heating. *J. Cell. Biochem.* 81:689–692, 2001. © 2001 Wiley-Liss, Inc.

Key words: EM fields; transcription; specific DNA sequences; EM response elements

INTRODUCTION

Transcription is initiated via many different signaling pathways and by transcription factors that bind at different DNA sites. What appears to be common to all is that the reactions cause DNA chains to separate so RNA polymerase can function. The involvement of many chemical reactions makes it difficult to think in terms of a general mechanism, but the ability of relatively weak electromagnetic (EM) fields to initiate transcription [Goodman and Blank, 1998] offers an alternative approach to mechanism. As a first approximation, we can think of EM fields as generating repulsive forces that aid chain separation. Studies of acceleration of reaction rates by electromagnetic (EM) fields indicate that the fields affect electron transfer reactions [Blank and Soo, 1998, 2000], and recent observations that DNA can conduct electrons within its stacked base pairs [Wan et al., 1999; Porath et al., 2000], suggest the possibility that EM

fields initiate transcription by interacting directly with moving electrons in DNA [Blank and Goodman, 1999, 2000]. The EM field forces (proportional to electron velocities) that initiate transcription in DNA are comparable to those that accelerate enzyme reactions. EM fields penetrate tissues without attenuation, so they must penetrate to the nuclear DNA and interact with the conducting electrons. Furthermore, since different DNA sequences have different conductivities [Meggers et al., 1998], EM fields could theoretically interact more strongly with specific DNA sequences. Studies of EM stimulated transcription have identified special sequences in the *c-myc* and HSP70 promoters that are required for the response to EM fields. Eight nCTCTn sequences in a 900bp region of the *c-myc* promoter [Lin et al., 1994], and three nCTCTn sequences in a 70bp region of the HSP70 promoter [Lin et al., 1998], appear to be needed [Lin et al., 2000]. We have called these nCTCTn sequences electromagnetic field response elements (EMRE). Removing EMREs eliminates the EM field response, and introducing them imparts the ability to respond to applied EM fields. (The 900bp segment of the *c-myc* promoter containing eight EMREs, when placed upstream of CAT or luciferase reporter

Grant sponsor: SOL Foundation.

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Received 26 December 2000; Accepted 5 January 2001

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This article published online in Wiley InterScience, March 26, 2001.

constructs that are otherwise unresponsive to EM fields, induces an EM field response.) It would appear likely that the nCTCTn sequences generate especially large repulsive forces between DNA chains. In this paper, we develop the assumption that EM field interaction with electron currents in DNA can generate a force that contributes to chain repulsion, and we show how the force would be expected to vary with the base composition of DNA.

Electron Movement in DNA

In studies of DNA, electron flow has been generated by applied electric fields, light-induced electron release, etc. [Arkin et al., 1996; Dandliker et al., 1997; Meggers et al., 1998; Wan et al., 1999; Porath et al., 2000]. We do not know if electron flow occurs as a result of processes within the DNA that are part of its normal function, and if so, the nature of that flow. Electron flow could be a mechanism for testing the integrity of the DNA, or restricted segments of it, to see if breaks have occurred. Also, transcription factors may isolate segments of DNA for local electron flow as part of the mechanism for initiation of transcription by generating a force or heat, as discussed below.

To proceed, we shall assume that rapid, tonic flows of electrons within DNA are needed to coordinate reactions in different parts of the linear DNA polymer, and that electron currents flow intermittently along each DNA chain separately. Experiments show that double stranded DNA is needed for electronic conduction, but other studies show that bases from one chain can be flipped out of the double helix while the second chain is virtually unaffected, suggesting independent function of the two chains [Klimasauskas et al., 1994]. In any case, if we assume independent conduction in each chain, it is possible to have a circuit of the two DNA chains that includes the entire molecule. It is also possible to conceive of repulsive forces generated by the EM fields when currents flow in opposite directions in a circuit made up of the two chains.

Evaluating the effects of electronic conduction in DNA along the lines of classical physics, the forces generated by EM fields will be proportional to the velocity, v , of the electrons, as given by the Lorentz equation, $F = qvB$, where q is a unit charge and B is the strength of the magnetic field. Assuming independent conduction in each chain, we estimate the

relative repulsive force between chains generated by an EM field and compare it to the strength of H-bonding holding the chains together at that base. From the difference we can determine how that base would be affected by an EM field. We use the electron affinity of the different bases to estimate the local electron density, and estimate the velocity at each base in terms of the distribution of electrons. The forces should depend on the properties of the constituent bases, and hence on the detailed DNA composition.

DNA Chain Repulsion Model

We calculate the forces of repulsion between chains due to EM fields, assuming that the electron affinity is a measure of the electron density at each base. That is, in a population of delocalized electrons, there will be a higher relative concentration (density) at the base with the greater electron affinity. The values we use for electron affinity at each base [Chen and Chen, 1998] are as follows: A = 0.97, G = 1.51, T = 0.81, C = 0.57, and we assume that electron affinities are directly proportional to the local charge density. When a current flows through the DNA, the charge carriers must move faster when there are fewer of them, so we estimate that the relative electron velocity at each point is inversely related to electron density. It is the electron velocity, v , that determines the force, F , for a particular value of EM field.

To estimate the forces of attraction between chains, we assign known H-bond interaction energies for A-T and C-G bonds. A-T bonds have two H-bonds (N-H-N, N-H-O) for a total of ~10 kcal/mol, and C-G bonds have three H-bonds (N-H-N, N-H-O, O-H-N) for a total of ~15 kcal/mol, so C-rich chains have greater attraction. The importance of C-G bonds in raising the DNA chain melting point is well known.

In these calculations, we consider individual bases, but electrons are delocalized and a better approximation would average adjacent electron affinities over n bases ($3 < n < 10$). This more realistic electron distribution over a length of DNA would provide a better estimate of the probability of opening a turn of the DNA helix. Obviously, the average would consider attractive as well as repulsive forces.

Estimating Force Balance

Table I provides an estimate of the relative repulsive force and relative attraction energy at

TABLE I. Steps in Calculating Force Balance

	Bases	C	T	A	G
1	Electron affinity (EA)	0.57	0.81	0.97	1.51
2	Repulsive force~ Velocity~ (EA) ⁻¹	1.75	1.25	1.03	0.66
3	Attraction~H-bonds	3	2	2	3
4	Attraction~H-bonds	1.5	1	1	1.5
5	Repulsive force-attraction	0.25	0.25	0.03	-0.84
6	Net effect	repulsion	repulsion	—	attraction

each of the four bases, as outlined in the above section. Since the values are relative, no units are given. The first line lists values of the electron affinity for each of the four bases. The second line takes the inverse of the electron affinity to estimate the relative electron velocity at each base, since the velocity should be inversely related to the concentration. The electron velocity is directly proportional to the force generated by an EM field at each base. Obviously, the magnitude of the repulsive force would increase as the EM field strength increased. The third line lists the number of H-bonds, which is apportioned between the two bases in a pair and listed in the fourth line. The fifth line calculates the difference between the estimated repulsive forces and attraction energy at each base. The net effect, repulsion or attraction, is listed on the sixth line.

Our calculations show that sites rich in C and T would be more likely to come apart when repulsive forces are generated by EM fields. This provides a rationale for our identification of electromagnetic response elements (EMREs) associated with the response of the *c-myc* and HSP70 promoters to EM fields. In those studies, we identified eight nCTCTn elements in *c-myc* and three in HSP70 that were needed for the response. In later studies, we incorporated the 900bp segment of the *c-myc* promoter, with its eight EMREs, upstream of CAT or luciferase reporter constructs that were otherwise unresponsive to EM fields, and induced an EM field response.

DNA Chain Melting

The above EM field mechanism may provide insight into other transcription mechanisms, since it focuses on the structurally based attractive and repulsive forces that are involved in any process of chain separation. The attractive forces are those due to H-bonds, and if the

processes generally initiated by chemical reactions weaken attractive forces by processes that depend on electronic conduction, then the EM field mechanism may be relevant. DNA chain melting (i.e., disruption of intermolecular bonds due to greater molecular motion) may be such a process.

Let us assume that DNA chain melting results from Joule heating due to electronic currents that are generated by reactions involving transcription factors. Using the same approach as with EM fields, we assume electron affinity is a measure of the electron density at each base, and that electron affinities are proportional to the local charge density. We estimate that the electrical resistance (R) at each base is inversely related to electron density. That is, the resistance is greater when there are fewer electrons to function as charge carriers. Since the current (I) would be the same at all points along the DNA, the heating associated with local electron flow (I^2R) will vary with the value of R. Obviously, the greater the heating, the greater the possibility of causing local DNA melting. Here too, as a first approximation, C-rich chains should have higher resistances and higher attractive forces, but the detailed code will be needed to give the effect on different regions of the DNA.

It would appear that calculations of the velocity, v , and the resistance, R , are closely related, since they are similarly derived from the electron affinities in our model. Therefore, the reasoning based on chain repulsion (an EM field mechanism) or chain melting (a more general mechanism) would lead to similar conclusions.

CONCLUSION

The calculations in this paper suggest a plausible mechanism for initiation of transcrip-

tion by the generation of repulsive forces between DNA chains when EM fields interact with flowing electrons. They also provide a rationale for the ability of the EMREs, in the two promoters we have studied, to function as EMRE's.

In addition to the response to EM fields, electron flow may be involved in other processes in DNA. For this reason, the approach of this paper may be useful in relating the details of DNA sequence to function. It would be interesting to see if a map of estimated electronic velocities (or resistances) correlates with a DNA function map, i.e., if regions that favor chain separation correlate with regions associated with control of DNA transcription as opposed to coding regions.

ACKNOWLEDGMENTS

We thank Hana Lin and Lily Soo for their excellent technical assistance.

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Attachment 9

Acute Exposure to a 60 Hz Magnetic Field Increases DNA Strand Breaks in Rat Brain Cells

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Acute (2 h) exposure of rats to a 60 Hz magnetic field (flux densities 0.1, 0.25, and 0.5 mT) caused a dose-dependent increase in DNA strand breaks in brain cells of the animals (assayed by a microgel electrophoresis method at 4 h postexposure). An increase in single-strand DNA breaks was observed after exposure to magnetic fields of 0.1, 0.25, and 0.5 mT, whereas an increase in double-strand DNA breaks was observed at 0.25 and 0.5 mT. Because DNA strand breaks may affect cellular functions, lead to carcinogenesis and cell death, and be related to onset of neurodegenerative diseases, our data may have important implications for the possible health effects of exposure to 60 Hz magnetic fields. *Bioelectromagnetics* 18:156–165, 1997. © 1997 Wiley-Liss, Inc.

Key words: 60 Hz magnetic fields; DNA single-strand and double-strand breaks; brain cells; microgel electrophoresis

INTRODUCTION

In recent years, both the popular media and the science media have raised concerns about possible health hazards of environmental exposure to extremely-low-frequency (ELF) electromagnetic fields, especially to 50 Hz and 60 Hz [Abelson, 1989; Bridges and Preache, 1981; Brodeur, 1990; Florig, 1992; Morgan et al., 1990; Pool, 1990a–c]. With increased use of electricity and equipment emitting electromagnetic fields, many people are subjected to intermittent and chronic exposure to ELF fields of various intensities and forms. There are speculations that ELF magnetic fields can act as copromoter or promoter of cancer [see reviews in Holmberg, 1995; Loscher and Mevissen, 1994; Wrensch et al., 1993]. Epidemiological studies have suggested that ELF electromagnetic fields may increase the risk of various types of cancer, including leukemia and brain and breast tumors [e.g., Juutilainen et al., 1990; Loomis et al., 1994; Savitz and Loomis, 1995; see also review in Wrensch et al., 1993].

In the present study, we investigated the effect of acute exposure to a 60 Hz magnetic field on DNA strand breaks, a common form of DNA damage, in brain cells of the rat. DNA damage that accumulates in cells over a period of time could be the cause of slow-onset diseases, such as cancer. Indeed, DNA strand breaks have been correlated with carcinogenicity

[Ames, 1989a,b; Cerutti, 1985; Tice, 1978], cell death [Onishi et al., 1993; Prigent et al., 1993; Walker et al., 1991; Ward, 1990], aging [Hart and Setlow, 1974; Tice, 1978], and neurodegenerative diseases [Mullaart et al., 1990b; Robins et al., 1983].

The method of Singh et al. [1995] was used in this research to measure DNA single-strand and double-strand breaks in individual brain cells of the rat. The method is one the most sensitive for assaying DNA strand breaks and can be used to evaluate breaks in a single cell. It has been used in numerous studies on DNA damage and genotoxicity [see reviews in Fairbairn et al., 1995; McKelvey-Martin et al., 1993]. The technique involves making a microgel on microscope slides, consisting of a cell suspension imbedded in low-melting-temperature agarose and phosphate-buffered saline (PBS). The cells are then lysed in the microgel in high salt and detergents, treated with proteinase K, and electrophoresed in a highly alkaline condition for single-strand break and in a neutral condition for dou-

Contract Grant Sponsor: National Institute of Environment Health Sciences; Contract Grant number: ES-06290.

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Received for review 10 January 1996; Final revision received 1 July 1996.

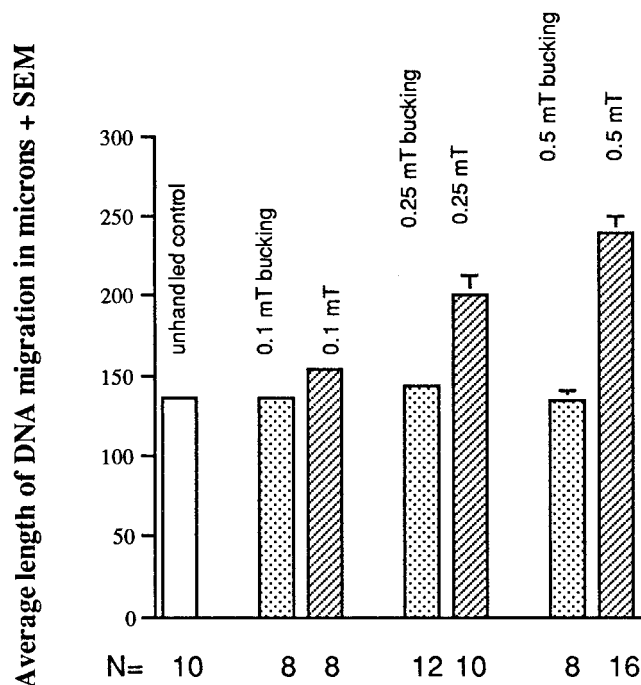


Fig. 1. Levels of DNA single-strand breaks (expressed in length of DNA migration) in the different treatment groups. The average of 0.1 mT magnetic field-exposed group is significantly different from its "bucking" control group at $P < .05$ (two-tailed Newman-Keuls test) and averages of 0.25 and 0.5 mT magnetic field-exposed groups are significantly different from their respective bucking controls at $P < .01$. N is the number of animals studied in each condition. Fifty cells from each animal were measured, and the mean migration length of the 50 cells was a data point used in data analysis.

ble-strand break measurements. The DNA is then stained with a fluorescent dye to allow visual measurement of the extent of DNA migration, an index of DNA damage. This technique is more sensitive than other available methods in detecting DNA strand breaks. It can detect one break per 2×10^{10} daltons of DNA in lymphocytes induced by X-rays [Singh et al., 1995].

METHODS AND PROCEDURES

Animals and General Experimental Conditions

Male Sprague-Dawley rats (2–3 months old, 250–300 g) purchased from B & K Laboratory were used in this research. They were housed in a vivarium on a 12 h light-dark cycle (lights on 7:00–19:00 h) and were given food and water ad libitum. The experimental environment was kept at 22 °C with a relative humidity of 65%. Animal care was provided by the University of Washington Department of Comparative Medicine, which conforms to the NIH guidelines for the care and use of laboratory animals.

Rats were exposed to a 60 Hz magnetic field in a Helmholtz coil pair. The design and characteristics of this exposure system have been described previously [Lai et al., 1993]. Briefly, each Helmholtz coil is made of 80 turns of No. 6 wire wound in rectangular loops, with minimum internal dimensions of 0.86×0.543 m. During construction, epoxy was layered between loops to glue them together. This minimizes vibration noise when the coils are activated. The coils are completely shielded against emission of electric fields; they are wound on frames fabricated from wood and aluminum. The coils were designed with split windings terminated on multiterminal blocks so that they can be wired in various series or parallel combinations for impedance matching and connecting to multichannel or multifrequency sources. A switch can be used to put the coils "in phase" to generate magnetic fields or in the "bucking mode." Because there are two sets of coils in each Helmholtz coil, in the bucking mode they are activated in an antiparallel direction to cancel mutually the fields generated by each of the coils. This condition controls for possible effects of heat and vibration generated when the coils are activated.

By varying the input current to the coils, exposure fields could be set anywhere from the ambient level to the maximum coil-designed magnetic field strength

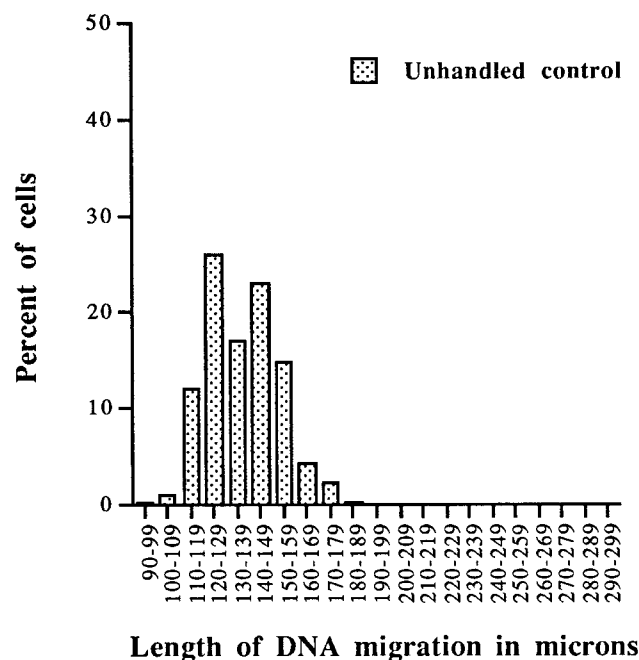
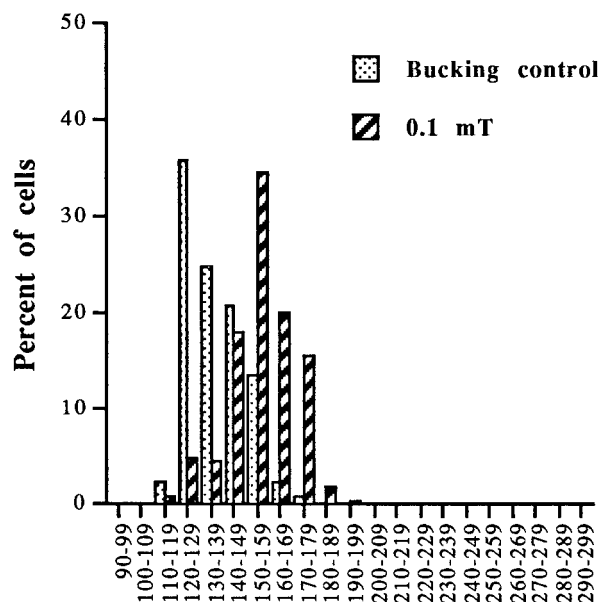


Fig. 2. Percentage distribution of brain cells vs. DNA migration length (single-strand breaks) of unhandled animals (N = 10). Fifty cells were scored from each animal. Therefore, data from the unhandled controls represent the distribution of 500 cells from 10 animals.



Length of DNA migration in microns

Fig. 3. Percentage distribution of brain cells vs. DNA migration length (single-strand breaks) of rats exposed to a 60 Hz magnetic field of 0.1 mT ($N = 8$; 400 cells) and rats exposed in the bucking mode ($N = 8$; 400 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 311.6$, $df = 7$, $P < .001$).

(5.6 mT). With an exposure level set at 1 mT, the heat dissipation for each coil is less than 8 W. The heat generated is efficiently dissipated due to the large surface area of the coil and good ventilation in the exposure room. During exposure, animals were housed in a plastic cage enclosure (length 45 cm, width 21 cm, height 22 cm) placed in the center of the space between the coils. Three animals could be exposed in the cage at the same time. The ambient magnetic field (i.e., when the power supply to the coils is turned off) was 0.14 μ T.

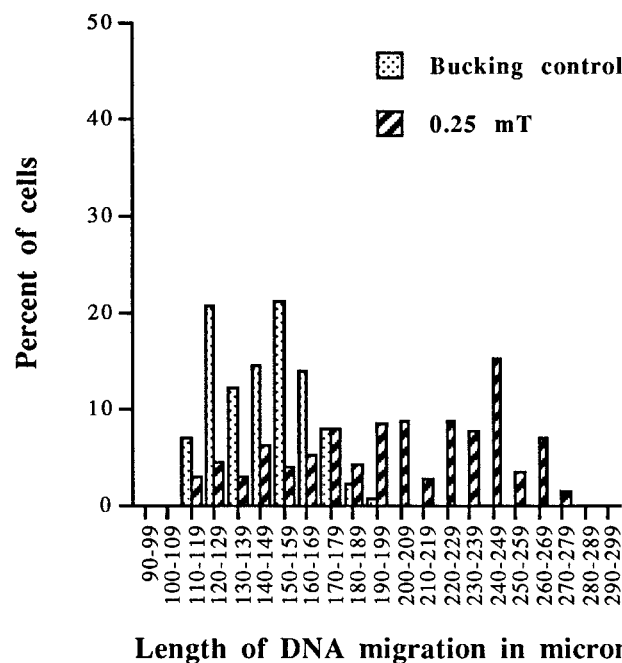
Exposure was between 8:00 and 11:00 AM, to control for possible variation in responses due to circadian rhythm. All experiments were run blind; i.e., the experimenters performing the DNA strand break assay did not know the treatment (exposure) conditions of the animals. Controls for these experiments were animals placed for the same period of time in the Helmholtz coil pair activated in the bucking mode with the same electric current. Thus, a group of bucking mode controls was used for each flux density of the magnetic field-exposed experimental group. In addition, DNA strand breaks were also analyzed from brain cells of a group of unhandled rats to control for the possible effect of exposure procedures.

In the experiment, rats were exposed in the Helmholtz coil system to different intensities (flux densities of 0.1, 0.25, and 0.5 mT) of a 60 Hz magnetic field for 2 h. Four hours after exposure, rats were sacrificed and DNA single-strand and double-strand breaks in brain cells were assayed. This experimental schedule was used because in other research we observed an increase in brain cell DNA breaks in rats after 2 h of exposure to microwaves and assayed at 4 h postexposure [Lai and Singh, 1995].

An animal was sacrificed by placing it in a closed foam box containing dry ice (CO_2) for 65 s. (Cardboard was placed on top of the dry ice to prevent its direct contact with the animal.) This method of euthanasia minimizes red blood cell contamination of tissue samples. Rats were then decapitated with a small animal guillotine, and their brains were dissected out immediately. Cells from the brains were isolated for DNA single-strand and double-strand break measurements.

DNA Single-Strand and Double-Strand Break Assays

The microgel electrophoresis assay described by Lai and Singh [1996] was used to study DNA single-strand and double-strand breaks in brain cells. All



Length of DNA migration in microns

Fig. 4. Percentage distribution of brain cells vs. DNA migration length (single-strand breaks) of rats exposed a 60 Hz magnetic field of 0.25 mT ($N = 10$; 500 cells) and rats exposed in the bucking mode ($N = 12$; 600 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 569.9$, $df = 16$, $P < .001$).

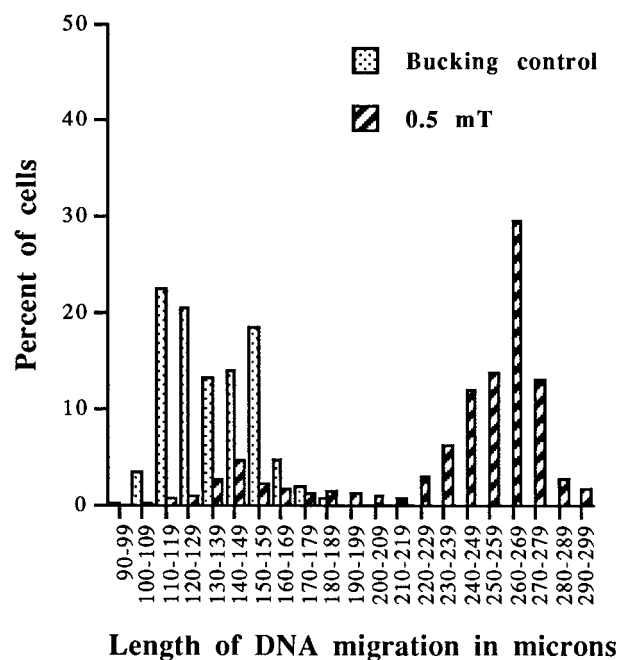


Fig. 5. Percentage distribution of brain cells vs. DNA migration length (single-strand breaks) of rats exposed a 60 Hz magnetic field of 0.5 mT ($N = 16$; 800 cells) and rats exposed in the bucking mode ($N = 8$; 400 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 851.3$, $df = 19$, $P < .001$).

chemicals used in the assay were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. All procedures were carried out in minimum indirect light. Immediately after removal from the skull, the brain was immersed in ice-cold PBS (NaCl 8.01 g, KCl 0.20 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.20 g, per liter; pH 7.4) containing 200 mM of N-t-butyl- α -phenylnitron, a spin-trap compound, and quickly washed four times with the PBS to remove most of the red blood cells. A pair of sharp scissors was used to mince (approximately 200 cuts) the tissue in a 50 ml polypropylene centrifuge tube containing 5 ml of ice-cold PBS to obtain pieces of approximately 1 mm^3 . Four more washings with cold PBS removed most of the remaining red blood cells. Finally, tissue pieces were dispersed into single-cell suspensions in 5 ml of PBS by using a 5 ml Pipetman. This cell suspension consisted of different types of brain cells.

Ten microliters of this cell suspension was mixed with 200 μl of 0.5% agarose (high-resolution 3:1 agarose; Amresco, Solon, OH) maintained at 37°C , and 30 μl of this mixture was pipetted onto a fully frosted slide (Erie Scientific Co., Portsmouth, NH) and immediately covered with a $24 \times 50 \text{ mm}$ square No. 1 coverglass (Corning Glass Works, Corning, NY) to

make a microgel on the slide. Slides were put in an ice-cold steel tray on ice for 1 min to allow the agarose to gel. The coverglass was removed from the slide and 200 μl of agarose solution was layered as before. Slides were then immersed in an ice-cold lysing solution (2.5 M NaCl, 1% sodium N-lauroyl sacosinate, 200 μM disodium EDTA, 10 mM Tris base; pH 10) containing 1% Triton X-100. After overnight lysing at 4°C , slides were treated with DNAase-free proteinase K (Boehringer Mannheim Corp., Indianapolis, IN) in the lysing solution (pH 7.4, without Triton X-100) for 2 h at 37°C .

For single-strand DNA breaks, slides were put on the horizontal slab of an electrophoretic assembly (Hoefer Scientific, San Francisco, CA), modified so that both ends of each electrode were connected to the power supply. One liter of an electrophoretic buffer (300 mM NaOH, 0.1% 8-hydroxyquinoline, 2% dimethyl sulfoxide, 100 mM Tris, and 10 mM tetrasodium EDTA; pH > 13) was gently poured into the assembly to cover the slides to a height of 6.5 mm above their surface. After 20 min, to allow for DNA unwinding, electrophoresis was started (0.4 V/cm, approximately 250 mA, for 60 min); the buffer was recirculated during electrophoresis. At the end of the electrophoresis, excess electrophoretic buffer was removed. The slides were gently removed from the electrophoretic apparatus and immersed for neutralization in 35 ml of 0.4 M Tris (pH 7.4) in a Coplin jar (two slides per jar) for 15 min. After two more similar steps of neutralization, the slides were dehydrated in absolute ethanol in a Coplin jar for 30 min and then dried.

For double-strand DNA breaks, microgel preparation and cell lysis were performed as described above. Slides were then treated with ribonuclease A (Boehringer Mannheim Corp.; 10 $\mu\text{g}/\text{ml}$ in the lysing solution without Triton X-100; pH 7.4) at 37°C for 2 h and then with proteinase K (1 mg/ml in the lysing solution without Triton X-100; pH 7.4) for 2 h at 37°C . They were then placed for 20 min in an electrophoretic buffer (100 mM Tris, 300 mM sodium acetate and acetic acid; pH 9) and electrophoresed for 1 h at 0.4 V/cm (approximately 100 mA). The slides were treated with 300 mM NaOH for 15 min and neutralized as before with 0.4 M Tris (pH 7.4). Slides were dehydrated as described above for the single-strand break assay.

Staining and DNA migration measurement procedures were similar for both single-strand and double-strand breaks. One slide at a time was stained with 50 μl of 1 mM solution of YOYO-1 (stock, 1 mM in DMSO from Molecular Probes, Eugene, OR) and then covered with a $24 \times 50 \text{ mm}$ coverglass. Two slides were prepared from each brain sample. One slide was

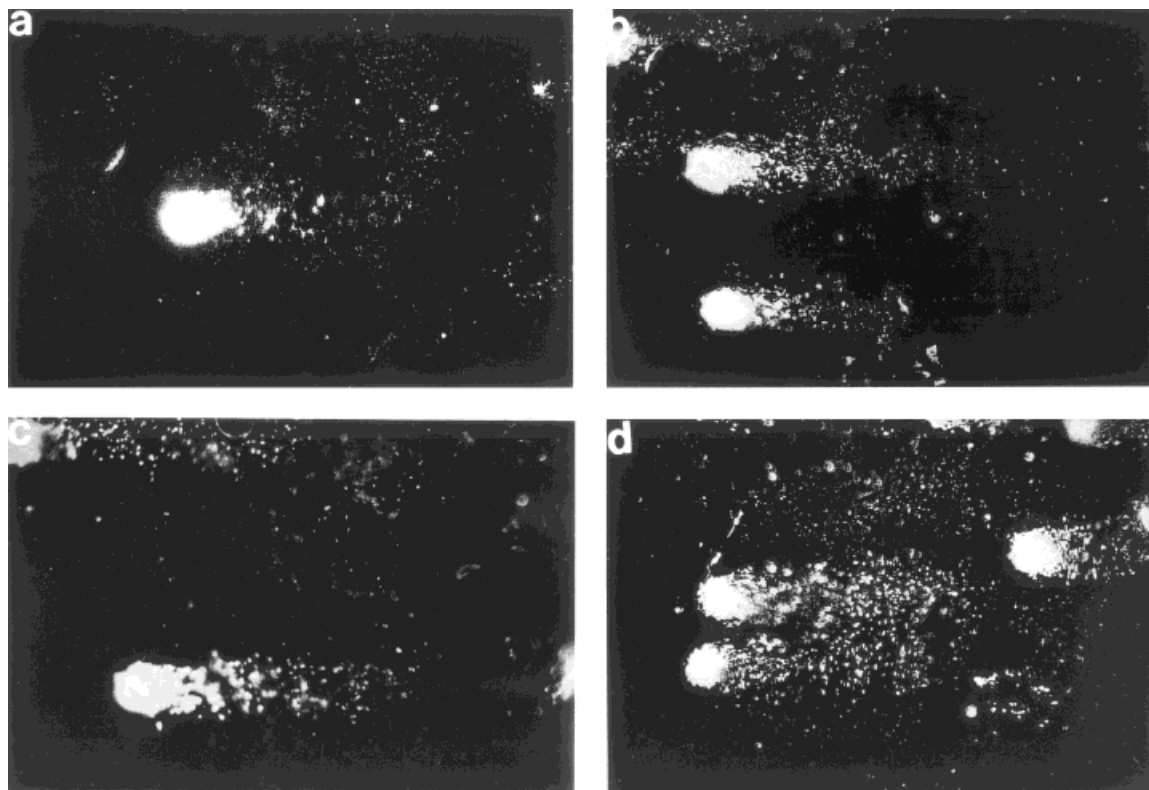


Fig. 6. Photographs of single-strand break DNA migration pattern of individual brain cells from rats exposed to bucking condition (0.1 mT) (a) or magnetic fields of 0.1 mT (b), 0.25 mT (c), and 0.5 mT (d). $\times 400$.

assayed for DNA single-strand breaks and the other for double-strand breaks. The length of DNA migration (including the nucleus diameter to the last three particles of DNA visible perpendicular to the direction of migration) of each cell was measured. Fifty representative cells were scored from each slide. Therefore, from each brain sample, 50 cells each were scored for DNA single-strand and double-strand breaks.

Data Analysis

The averaged migration length (in micrometers) of the 50 cells scored from a sample was used as a data point in data analysis. Analysis of variance (ANOVA) followed by the Newman-Keuls test to determine significance of difference between two treatment groups was used in statistical analysis of averaged data. In addition, distribution of cells (in percentage of total) with respect to DNA migration lengths was also plotted. The χ^2 test was used to determine significant difference in patterns of migration between treatment groups. A difference at $P < .05$ was considered statistically significant.

RESULTS

Effects of acute magnetic field exposure on DNA single-strand breaks in brain cells of the rat are presented in Figures 1–6. Figure 1 shows the average length of DNA migration from the various treatment groups. One-way ANOVA of the data showed a significant treatment effect ($F[6,65] = 29.13$, $P < .005$). Data from magnetic field-exposed rats were compared with their respective “bucking control” by using the Newman-Keuls test. Data showed a significant increase in DNA single-strand breaks in brain cells of rats after exposure to a 60 Hz magnetic field at 0.1 mT ($P < .05$), 0.25 mT ($P < .01$), and 0.5 mT ($P < .01$). A dose-dependent effect, with longer average migration length at higher flux density of exposure, was observed. Plots of percentage of cells vs. length of DNA migration (in intervals of 10 μm) for each group of animals are presented in Figures 2–5. These data show a shift of the distribution to longer migration lengths as the flux density of the magnetic field increases. Figure 6 shows photographs of the DNA migration pattern (single-strand breaks) of individual brain cells from rats of the different treatment groups.

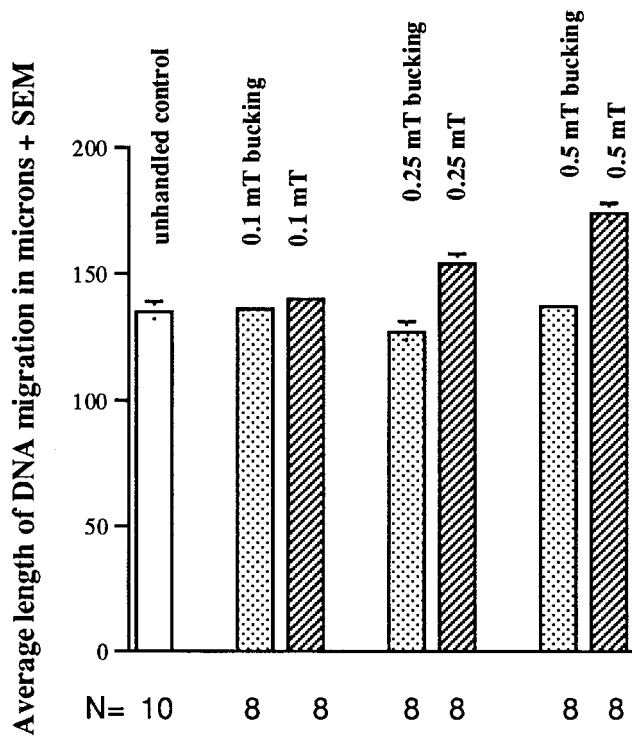


Fig. 7. Levels of DNA double-strand breaks (expressed in length of DNA migration) in the different treatment groups. A two-tailed Newman-Keuls test showed a significant difference at $P < .01$ between the groups exposed to 0.25 and 0.5 mT magnetic field and the respective bucking control groups. No significant difference was found between the 0.1 mT-exposed group and its bucking control.

The effects of magnetic field exposure on DNA double-strand breaks in brain cells of the rat are shown in Figures 7–12. Figure 6 shows the average DNA migration lengths of the various treatment groups. One-way ANOVA of the data showed a significant treatment effect ($F[6,51] = 27.57, P < .005$). No significant effect on double-strand breaks was observed after exposure at a magnetic field flux density of 0.1 mT (compared to that of the respective bucking controls), whereas a significant increase was observed at flux densities of 0.25 mT ($P < .01$) and 0.5 mT ($P < .01$). Distributions of length of DNA migration in brain cells are shown in Figures 7–10. An increased shift of the distribution to longer migration lengths at higher flux densities of exposure was observed. Figure 12 shows photographs of DNA migration pattern (double-strand breaks) of individual brain cells from rats of the different treatment groups.

DISCUSSION

Our results show that acute exposure to a 60 Hz magnetic field causes an increase in both single-strand

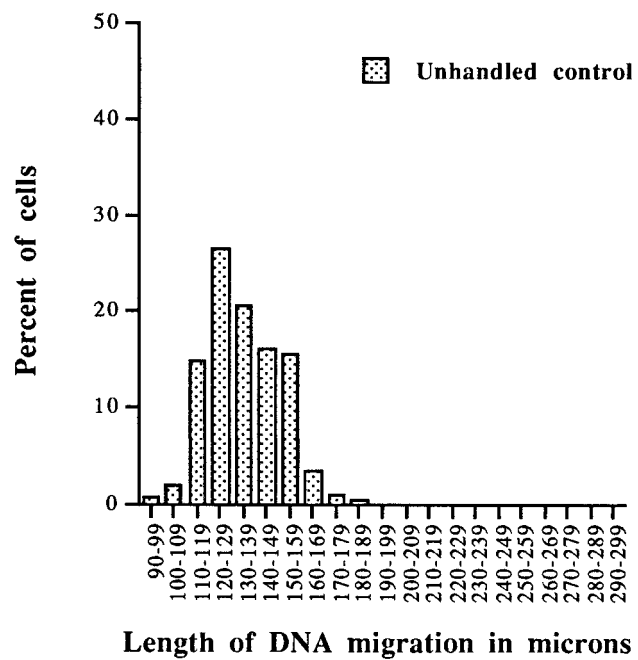


Fig. 8. Distribution of DNA migration length (double-strand breaks) of brain cells of unhandled rats ($N = 10$; 500 cells).

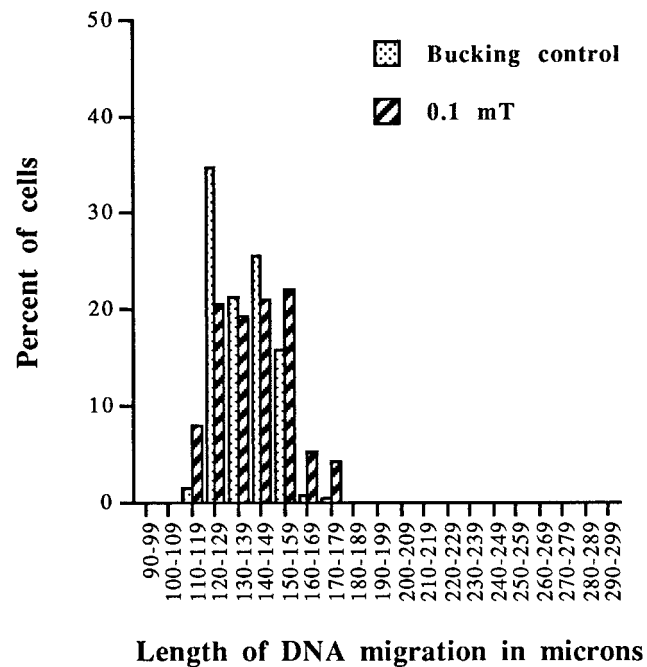


Fig. 9. Percentage distribution of brain cells vs. DNA migration length (double-strand breaks) of rats exposed to a 60 Hz magnetic field of 0.1 mT ($N = 8$; 400 cells) and rats exposed in the bucking mode ($N = 8$; 400 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 66, df = 6, P < .001$).

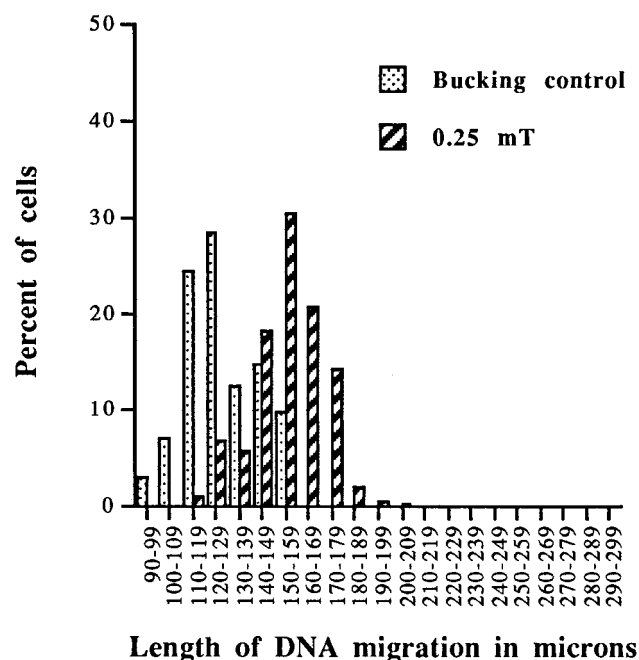


Fig. 10. Percentage distribution of brain cells vs. DNA migration length (double-strand breaks) of rats exposed a 60 Hz magnetic field of 0.25 mT (N = 8; 400 cells) and rats exposed in the bucking mode (N = 8; 400 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 390.8$, df = 9, $P < .001$).

and double-strand DNA breaks in brain cells of the rat. ELF magnetic fields do not have enough energy to break chemical bonds directly in DNA molecules. A possible explanation of the present observations is that 60 Hz magnetic fields affect enzymatic processes involved in DNA repair, leading to an accumulation of DNA strand breaks. This hypothesis is supported by a recent report by Phillips et al. [1995] that acute exposure to a 60 Hz magnetic field significantly affected the activity of poly-ADP-ribose polymerization, an enzymatic activity involved in DNA repair. A similar effect on poly-ADP-ribose polymerization has also been observed by Behari et al. (personal communication) in brain cells of rats after chronic exposure to a 50 Hz magnetic field.

In two previous studies [Fairbairn and O'Neill, 1994; Reese et al., 1988], no significant effect of ELF magnetic fields on DNA strand breaks in cells was reported. In the study by Reese et al. [1988], Chinese hamster ovary cells were exposed to a 60 Hz magnetic field at 0.1 and 2 mT for 1 h; no significant effect on DNA single-strand breaks was observed in these cells immediately after exposure as measured by the alkaline elution technique. In this study, cell samples were kept throughout the experiment under "iced" condition,

which would preclude any effect due to change in enzymatic activity in the cells. In the Fairbairn and O'Neill [1994] study, human cells were first suspended in agarose on a slide before being exposed for 1 or 24 h to a 50 Hz pulsed magnetic field (peak flux density 5 mT, pulse duration 3 ms). Cells suspended in agarose are not in a good physiological environment. Thus, any possible effect of magnetic fields on enzymatic activity might not be revealed under such an experimental condition.

The ELF magnetic flux density in the environment varies over a wide range. For example, household and office levels can vary from 0.01 to 1 μ T. Intermittently, levels can reach more than 10 μ T. Levels near a power transmission line can be 10–30 μ T, whereas the magnetic flux density can vary between 0.1 and 1 mT near some electrical appliances (e.g., electric blankets, hair dryers). Much higher levels are expected in occupational exposures [Bernhardt, 1985; Gauger, 1984; Krause, 1986; Tenforde and Kaune, 1987]. Recommended maximum levels of magnetic field exposure also vary. For example, the interim guidelines of the International Nonionizing Radiation Committee of the International Radiation Protection Association [IRPA/INIRC, 1990] for occupational situations are 0.5 mT

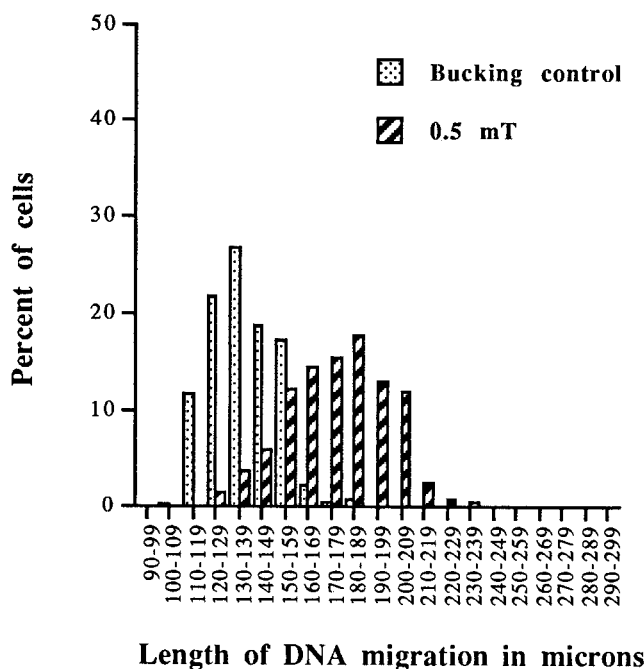


Fig. 11. Percentage distribution of brain cells vs. DNA migration length (double-strand breaks) of rats exposed a 60 Hz magnetic field of 0.5 mT (N = 8; 400 cells) and rats exposed in the bucking mode (N = 8; 400 cells). Distribution patterns of the two treatments were significantly different from each other ($\chi^2 = 492.5$, df = 10, $P < .001$).

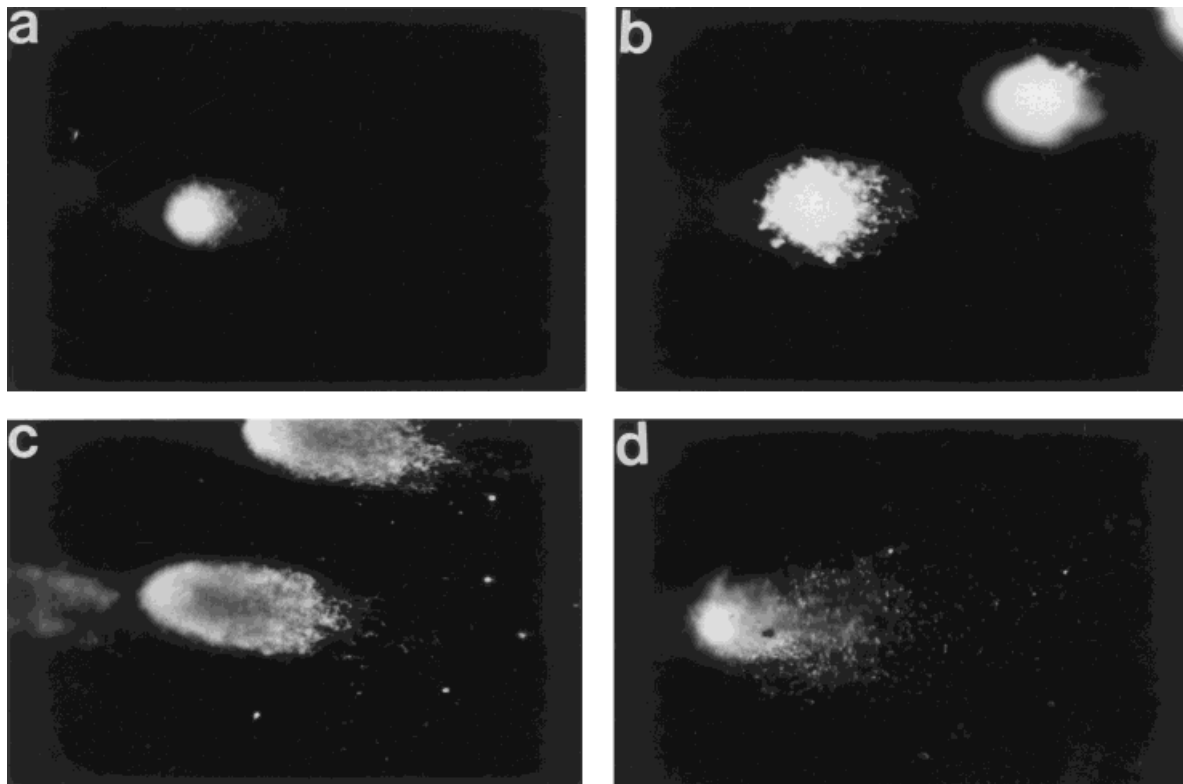


Fig. 12. Photographs of double-strand break DNA migration pattern of individual brain cells from rats exposed to bucking condition (0.1 mT) (a) or magnetic fields of 0.1 mT (b), 0.25 mT (c), and 0.5 mT (d). $\times 400$.

for workday exposure and 5 mT for short-term (2 h) exposure, whereas for the general public they are 0.1 mT for 24 h/day exposure and 1.0 mT for exposure of a few hours per day. The National Radiological Protection Board (NRPB) of England recommends a limit of 2.0 mT for both occupational and general public exposure to ELF magnetic fields [NRPB, 1989]. In our study, we found that a 60 Hz magnetic field causes DNA single-strand breaks in rat brain cells at a flux density ≥ 0.1 mT and double-strand breaks at 0.5 and 1.0 mT. Magnetic fields of these intensities are within the limits of the IRPA/INIRC and NRPB guideline standards and exist in both the public and the occupational environments. Furthermore, the intensity of the magnetic field studied and found to have a significant effect on DNA is well below the level for producing the classical induced electric current effects [Bernhardt, 1985] and within the IRPA/INIRC and NRPB recommended magnetic field-induced current density threshold of $1 \mu\text{A}/\text{cm}^2$. However, the effects we observed after magnetic field exposure are probably not caused by induced electric currents in the tissue by the oscillating magnetic field. In our research, we found that direct application of convulsive electric currents to the head

(40 mA, 60 Hz, 2 s) did not significantly affect the amount of DNA single-strand breaks in brain cells of the rat [Khan et al., 1995].

An increase in DNA strand breaks in body cells could have an important implication for the possible health effect of exposure to ELF magnetic fields in the environment. According to the multistep and clonal model of the origin of cancer [reviewed in Goldberg et al., 1991; Stein, 1991], tumorigenesis is a multistage process, mainly initiation followed by promotion, beginning with only one cell escaping from immune surveillance. Damage to cellular DNA or lack of its repair could be an initial event in carcinogenesis (clonal origin).

Cumulative damage in DNA in cells has been shown also for aging [Ames et al., 1993; Chetsanga et al., 1977; Mullaart et al., 1990a; Targovnik et al., 1985; Wheeler and Lett, 1974]. Based on the suggestion of Alexander [1967], several investigators contend that accumulated DNA damage in neurons and other post-mitotic cells is the primary factor in aging and death of an organism. Indeed, Wheeler and Lett [1974] have shown that DNA repair is age related in cerebellar neurons in beagle dogs, and Chetsanga et al. [1977]

showed that the rate of neuronal DNA single-strand breakage is higher in old than in young mice. Cumulative DNA damage in cells, particularly in neurons, has also been associated with Alzheimer's disease [Jones et al., 1989; Mullaart et al., 1990b; Robbins et al., 1983], Huntington's disease [Bridges, 1981; Scudiero et al., 1981], and Parkinson's disease [Robbins et al., 1983]. Aside from neurodegenerative conditions, such as Alzheimer's and Parkinson's diseases, increases in DNA strand breaks are seen also in disorders of premature senility, such as xeroderma pigmentosum, Werner's syndrome, Cockayne syndrome, ataxia telangiectasia, and retinal dystrophies [Robbins et al., 1983]. This may be relevant to recent epidemiological studies reporting that occupational exposure to electromagnetic fields could increase the risks of development of Alzheimer's disease [Sobel et al., 1995] and amyotrophic lateral sclerosis [Davanipour et al., 1995].

Thus, DNA strand breaks could lead to disruption of cell functions and carcinogenesis, and a relationship between an increase in DNA strand breaks and aging and neurodegenerative disorders has been suggested. It is imperative that further studies be carried out to characterize and understand the effect of ELF magnetic field exposure on DNA strand breaks in cells. In particular, interaction effects of exposure parameters, such as intensity and duration of exposure, intermittent vs. continuous exposure, etc., and the effect of magnetic fields on DNA metabolic enzyme activities should be investigated. It would also be interesting to investigate whether in vivo magnetic field exposure affects DNA in cells of other organs in the body.

ACKNOWLEDGMENTS

We thank Mrs. Monserrat Carino for technical assistance in performing the experiment. This research was supported by a grant from the National Institute of Environmental Health Sciences (ES-06290).

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Melatonin and a Spin-Trap Compound Block Radiofrequency Electromagnetic Radiation-Induced DNA Strand Breaks in Rat Brain Cells

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Effects of in vivo microwave exposure on DNA strand breaks, a form of DNA damage, were investigated in rat brain cells. In previous research, we have found that acute (2 hours) exposure to pulsed (2 μ sec pulses, 500 pps) 2450-MHz radiofrequency electromagnetic radiation (RFR) (power density 2 mW/cm², average whole body specific absorption rate 1.2 W/kg) caused an increase in DNA single- and double-strand breaks in brain cells of the rat when assayed 4 hours post exposure using a microgel electrophoresis assay. In the present study, we found that treatment of rats immediately before and after RFR exposure with either melatonin (1 mg/kg/injection, SC) or the spin-trap compound N-tert-butyl- α -phenylnitron (PBN) (100 mg/kg/injection, IP) blocks this effect of RFR. Since both melatonin and PBN are efficient free radical scavengers, it is hypothesized that free radicals are involved in RFR-induced DNA damage in the brain cells of rats. Since cumulated DNA strand breaks in brain cells can lead to neurodegenerative diseases and cancer and an excess of free radicals in cells has been suggested to be the cause of various human diseases, data from this study could have important implications for the health effects of RFR exposure. *Bioelectromagnetics* 18:446–454, 1997. © 1997 Wiley-Liss, Inc.

Key words: radiofrequency electromagnetic radiation (RFR); brain cells; DNA single- and double-strand breaks; melatonin; N-tert-butyl- α -phenylnitron (PBN); free radicals

INTRODUCTION

Recently, we reported an increase in DNA single- and double-strand breaks in the brain cells of rats exposed for 2 hours to pulsed 2450-MHz radiofrequency electromagnetic radiation (RFR) at averaged whole body specific absorption rates (SAR) of 0.6 and 1.2 W/kg [Lai and Singh, 1995, 1996]. In these experiments, DNA strand breaks were assayed 4 hours post exposure.

The mechanism by which RFR causes this effect is not known. In the present study, we investigated whether free radicals play a role. Rats were treated with the free radical scavengers melatonin and N-tert-butyl- α -phenylnitron (PBN) to investigate whether they can block RFR-induced DNA single- and double-strand breaks in brain cells. Melatonin has been reported to be a free radical scavenger [Reiter et al., 1995]. It has been shown to inhibit DNA-adduct forma-

tion induced by the carcinogen safrole in vivo [Tan et al., 1993] and to protect lymphocytes from radiation-induced chromosome damage in vitro [Vijayalaxmi, 1995]. In addition, an advantage of using melatonin in this study is that it can readily pass through the blood-brain barrier and cell and nuclear membranes [Costa et al., 1995; Menendez-Pelaez and Reiter, 1993; Menendez-Pelaez et al., 1993]. PBN has been shown to protect cells from free radical-induced apoptosis [Slater et al., 1995]. In particular, various studies have reported that PBN can reverse free radical-related damage to the nervous system. For example, it has been shown to

Contract Grant sponsor: National Institute of Environmental Health Sciences; Contract Grant number; ES-03712.

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Received for review 5 December 1996; revision received 28 January 1997

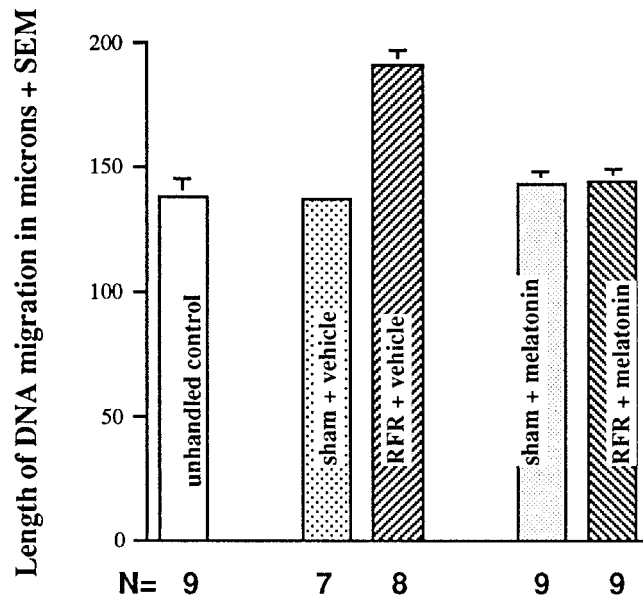


Fig. 1. Effect of treatment with melatonin on RFR-induced increase in DNA single-strand breaks in rat brain cells. Data was analyzed using the one-way ANOVA, which showed a significant treatment effect ($F[4,37] = 16.59, P < .001$).

reverse age-related changes in protein chemistry in the brain and deterioration in spatial memory functions in the rat [Carney and Floyd, 1991; Carney et al., 1991]. It can reverse ischemia-induced free radical injury in the brain [Oliver et al., 1990], inhibit free radical release after experimental brain concussion [Sen et al., 1994], and reduce infarct size in the brain following transient middle cerebral artery occlusion [Zhao et al., 1994].

METHODS AND PROCEDURES

Animals

Male Sprague-Dawley rats (250-300 g) purchased from B & K Laboratory, Bellevue, WA, were used in this research. They were housed three to a cage in a room adjacent to the RFR exposure room for 48 hours before an experiment. The laboratory was maintained on a 12-hour light-dark cycle (light on 6:00–18:00 h) and at an ambient temperature of 22 °C and a relative humidity of 65%. Animals were given food and water ad libitum.

RFR Exposure System and Exposure Conditions

The cylindrical waveguide system developed by Guy et al. [1979] was used for RFR exposure. The system consists of individual cylindrical waveguide

tubes connected through a power divider network to a single RFR power source. Each tube consists of a section of circular waveguide constructed of galvanized wire screen in which a circularly polarized TE_{11} mode field configuration is excited. The tube contains a plastic chamber that houses a rat with enough space to allow free motion. The floor of the chamber is formed of glass rods, allowing waste to fall through plastic funnels into a collection container outside the waveguide. Waveguides were calibrated and checked from time to time.

This waveguide system, using circularly polarized radiation, enables efficient coupling of radiation energy to the animal exposed. For example, a spatially averaged power density of 1 mW/cm² in the circular waveguide produces a whole-body SAR of 0.6 W/kg in the rat [Chou et al., 1984]. The range of power densities for exposure to a linearly polarized plane-wave associated with an SAR of 0.6 W/kg is approximately 3–6 mW/cm². By connecting this system to a pulsed signal source (Applied Microwave, model PG5KB), rats were irradiated with pulsed (2 μ sec pulse width, 500 pulses per second) 2450-MHz radiation at a spatially averaged power density of 2 mW/cm², which gave an averaged whole-body SAR of 1.2 W/kg. Since each waveguide can be activated individually, an animal can be subjected to either RFR- or sham-exposure in a waveguide. Both RFR- and sham-exposed animals were included in each exposure session.

In the experiment, animals were injected with melatonin (Sigma Chemical Co., St. Louis, MO; 1 mg/kg/injection, SC, dissolved in a concentration of 1 mg/ml in 1% ethanol-saline solution) or an equal volume of its vehicle, or with N-tert-butyl- α -phenylnitron (PBN) (Sigma Chemical Co., St. Louis, MO; 100 mg/kg/injection, IP, dissolved at 25 mg/ml in physiological saline) or an equal volume of its vehicle. Injections were given immediately before and after exposure. The drug dosages used were based on previous studies showing efficient free radical scavenging effects, especially in the brain [Carney et al., 1991; Chen et al., 1994; Kothari et al., 1995; Lafon-Cazal et al., 1993a,b; Melchiorri et al., 1995; Tan et al., 1993; Zhao et al., 1994]. Melatonin and PBN solutions were prepared immediately before injection, and exposure to light and air were kept at a minimum. Since the drugs have a short half-life (0.5–2 hours) in the blood, the experimental schedule involved two hours of exposure and four hours of post-exposure waiting, and the exact time when DNA strand breaks occurred was not known, we decided to inject the animals twice: before and after exposure.

Therefore, there were four treatment groups for each drug (melatonin and PBN)-treatment experiment: RFR/drug; RFR/vehicle; sham/drug; and sham/vehicle.

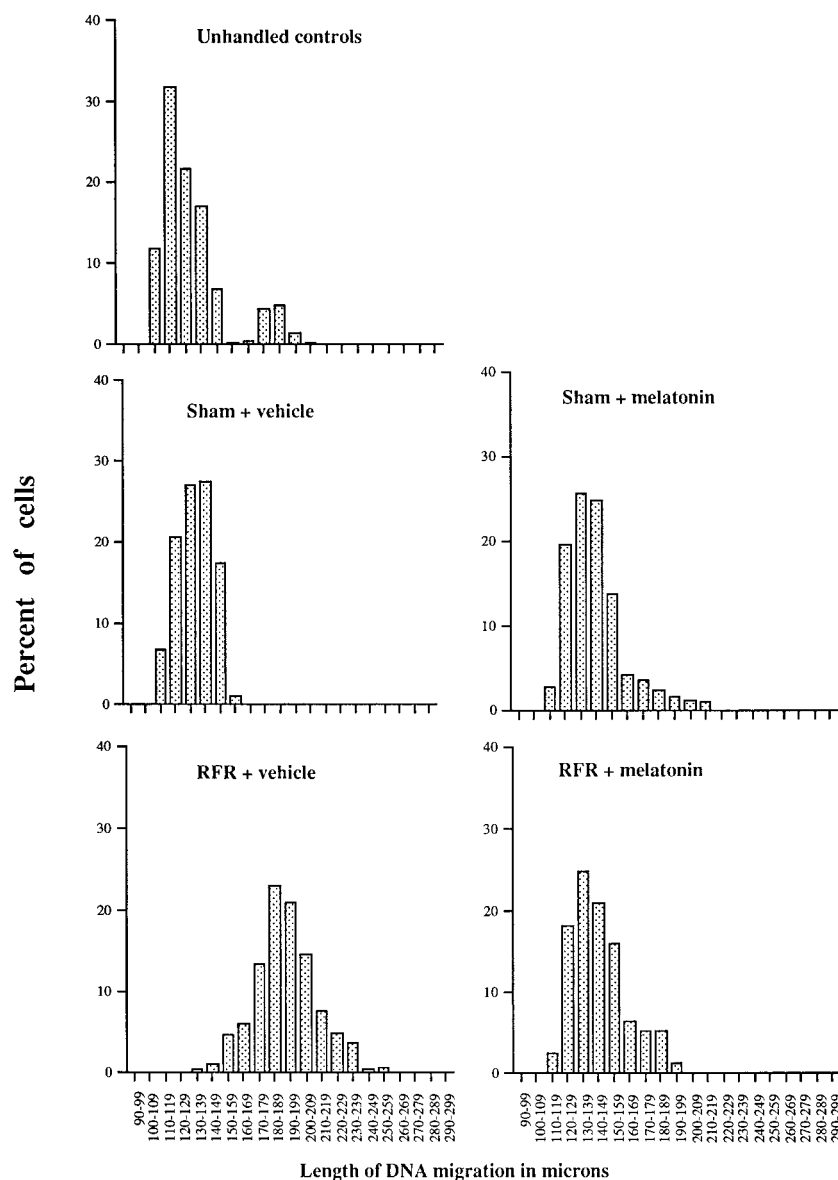


Fig. 2. Percent distribution of cells as a function of DNA migration length of the five groups of animals shown in Fig. 1.

In addition, a group of unhandled animals was included in each experiment. These animals were housed in their home cage for the entire period of the experiment, and DNA strand breaks were assayed in their brains without experimental treatment and handling. These animals controlled for the possible effect of experimental procedures on DNA strand breaks in brain cells.

The animals were returned to their home cages after exposure. Four hours later, each rat was placed for 60 seconds in a closed foam box containing dry ice (a cardboard was put on top of the dry ice to prevent its direct contact with the animal) and then decapitated

with a small animal guillotine. Dry ice was used in the euthanasia because its use minimizes red blood cell contamination of tissue samples which could affect DNA strand break measurements. All procedures from this step onward were done in minimum indirect light. Brains were immediately dissected out from the skull for assay of DNA strand breaks. Dissection of a brain took approximately 30 seconds.

All experiments were run blind. The on/off conditions of the waveguides were determined by an experimenter before an experiment. Two other experimenters, who did the animal exposure/brain dissection and

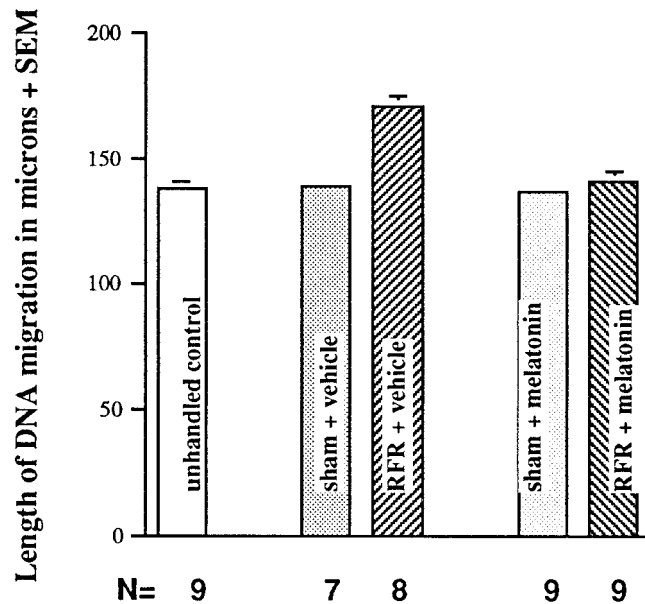


Fig. 3. Effect of treatment with melatonin on RFR-induced increase in DNA double-strand breaks in rat brain cells. One-way ANOVA of the data showed a significant treatment effect ($F[4,37] = 19.02$, $P < .001$).

DNA strand-break assay, respectively, did not know the treatment conditions (RFR or sham exposure) of the rats.

Assay Methods for DNA Strand Breaks

The microgel electrophoresis assay for DNA single- and double-strand breaks in rat brain cells was carried out as described previously in Lai and Singh [1996]. All chemicals used in the assay were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Immediately after dissection, a brain was immersed in ice-cold phosphate-buffered saline (PBS) (NaCl , 8.01 g; KCl , 0.20 g; Na_2HPO_4 , 1.15 g; KH_2PO_4 , 0.20 g, per liter, pH 7.4) containing 200 μM of *N*-t-butyl- α -phenylnitron. The tissue was quickly washed four times with the PBS to remove most of the red blood cells. A pair of sharp scissors was used to mince (approximately 200 cuts) the tissue in a 50-ml polypropylene centrifuge tube containing 5 ml of ice-cold PBS to obtain pieces of approximately 1 mm^3 . Four more washings with cold PBS removed most of the remaining red blood cells. Finally, in 5 ml of PBS, tissue pieces were dispersed into single-cell suspensions using a P-5000 Pipetman. This cell suspension consisted of different types of brain cells. Ten microliters of this cell suspension were mixed with 0.2 ml of 0.5% agarose (high-resolution 3:1 agarose; Amresco, Solon, OH) maintained at 37 $^{\circ}\text{C}$, and 30 μl of this mixture was pipetted onto a fully frosted slide (Erie

Scientific Co., Portsmouth, NH) and immediately covered with a 24 \times 50 mm square #1 coverglass (Corning Glass Works, Corning, NY) to make a microgel on the slide. Slides were put in an ice-cold steel tray on ice for 1 min to allow the agarose to gel. The coverglass was removed and 200 μl of agarose solution was layered as before. Slides were then immersed in an ice-cold lysing solution (2.5 M NaCl , 1% sodium *N*-lauroyl sarcosinate, 100 mM disodium EDTA, 10 mM Tris base, pH 10) containing 1% Triton X-100.

To measure single strand DNA breaks, after lysing overnight at 4 $^{\circ}\text{C}$, slides were treated with DNAase-free proteinase K (Boehringer Mannheim Corp., Indianapolis, IN) in the lysing solution for 2 hours at 37 $^{\circ}\text{C}$. They were then put on the horizontal slab of an electrophoretic assembly (Hoefer Scientific, San Francisco, CA) modified so that both ends of each electrode were connected to the power supply. One liter of an electrophoresis buffer (300 mM NaOH , 0.1% of 8-hydroxyquinoline, 2% dimethyl sulfoxide, and 10 mM tetra-sodium EDTA, pH 13) was gently poured into the assembly to cover the slides to a height of 6.5 mm above their surface. After allowing 20 min for DNA unwinding, electrophoresis was started (0.4 volt/cm, approximately 250 mA, for 60 min) and the buffer was recirculated.

At the end of the electrophoresis, electrophoretic buffer above the slides was gently removed. Slides were then removed from the electrophoresis apparatus and immersed in neutralization buffer (0.4 M Tris at pH 7.4) in a Coplin jar (two slides per jar) for 10 min. After two more similar steps of neutralization, the slides were dehydrated in absolute ethanol in a Coplin jar for 30 min and then dried.

For double-strand breaks, microgel preparation and cell lysis were done as mentioned above. Slides were then treated with ribonuclease A (Boehringer Mannheim Corp., Indianapolis, IN) (10 $\mu\text{g}/\text{ml}$ in the lysing solution) for 2 hours and then with proteinase K (1 mg/ml in the lysing solution) for 2 hours at 37 $^{\circ}\text{C}$. They were then placed for 20 min in an electrophoretic buffer (100 mM Tris, 300 mM sodium acetate and acetic acid at pH 9.0), and then electrophoresed for 1 hour at 0.4 volt/cm (approximately 100 mA). The slides were treated with 300 mM NaOH for 10 min and neutralized as before with 0.4 M Tris (pH 7.4). Slides were then dehydrated in absolute ethanol for 30 min and dried.

Staining and DNA migration measurement procedures were similar for both single- and double-strand breaks. One slide at a time was taken out and stained with 50 μl of 1 μM solution of YOYO-1 (stock, 1 mM in DMSO from Molecular Probes, Eugene, OR) and then covered with a 24 \times 50-mm coverglass. Slides were examined and analyzed with a Reichert vertical fluores-

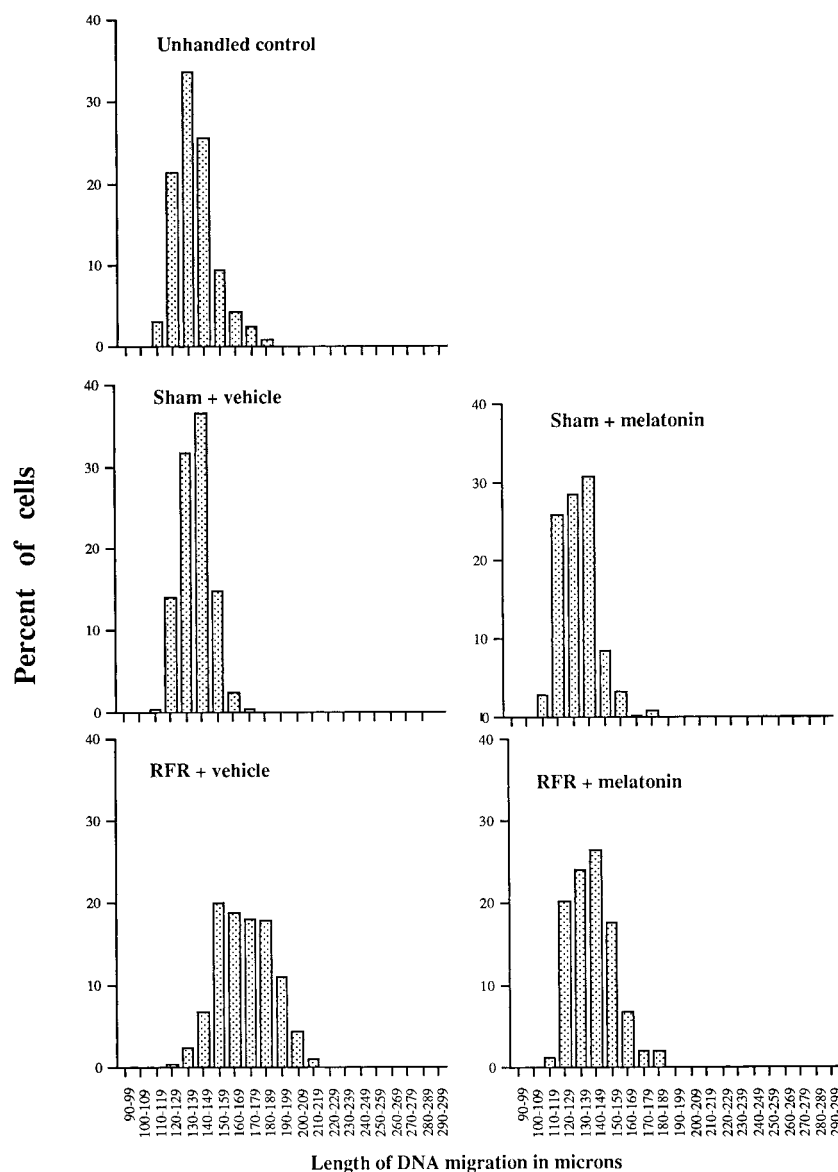


Fig. 4. Percent distribution of cells as a function of DNA migration length of the five groups of animals shown in Fig. 3.

cent microscope (model 2071) equipped with a filter combination for fluorescence isothiocyanate (excitation at 490 nm, emission filter at 515 nm, and dichromic filter at 500 nm). We measured the length of DNA migration (in microns) from the beginning of the nuclear area to the last 3 pixels of DNA perpendicular to the direction of migration at the leading edge. The migration length is used as the index of DNA strand breaks. In the present assay procedure, precipitation with ethanol enabled detection of smaller DNA fragments and increased the sensitivity and resolution of the assay. With this treatment, a significantly higher DNA migration length was detected.

Without ethanol precipitation, the migration lengths of DNA from brain cells of a sham-exposed animal would be 40–50 microns.

Two slides were prepared from the brain sample of each animal: one for assay of single-strand DNA breaks, and the other for double-strand breaks. Fifty cells were randomly chosen and scored from each slide. However, cells that showed extensive damage, with DNA totally migrated out from the nuclear region, were not included in the measurement. These highly damaged cells probably resulted from the tissue and cell processing procedures. They occurred equally in RFR-

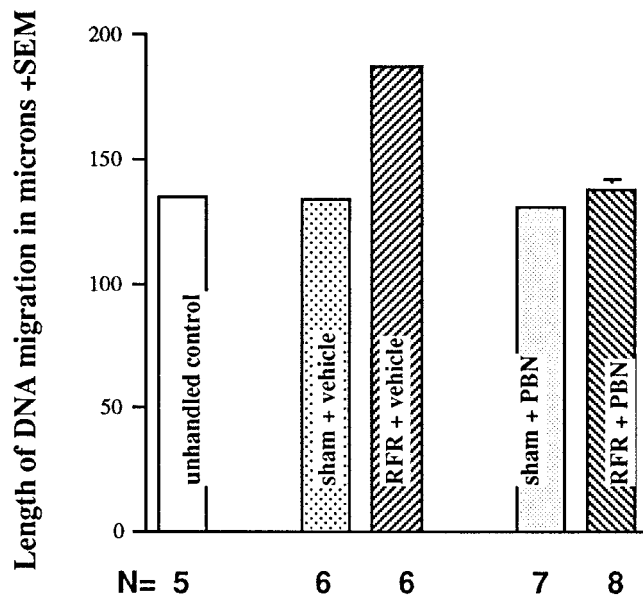


Fig. 5. Effect of treatment with PBN on RFR-induced increase in DNA single-strand breaks in rat brain cells. Data was analyzed using the one-way ANOVA, which showed a significant treatment effect ($F[4,27] = 75.5$, $P < .001$).

exposed, sham-exposed, and unhandled samples. Therefore, from each animal, 50 cells each were scored for single- and double- strand DNA breaks.

Data Analysis

The average length of DNA migration from the 50 cells, measured for single- and double-strand breaks in each rat, was used in data analysis using the one-way ANOVA. The difference between the two treatment groups was compared by the Newman-Keuls Test with a difference of $P < .05$ considered statistically significant. Percentages of cells with respect to DNA migration length (in intervals of 10 microns) were also plotted.

RESULTS

Figure 1 shows the data of melatonin treatment on RFR-induced DNA single-strand breaks in brain cells of rats. RFR significantly increased DNA single-strand breaks in brain cells ('RFR + vehicle' vs 'sham + vehicle', $P < .01$, Newman-Keuls test), whereas treatment with melatonin completely blocked the effect of RFR (i.e., no significant effect was found between 'RFR + melatonin' and 'sham + melatonin'). It should be pointed out that melatonin by itself has no significant effect on DNA single-strand breaks, i.e., no significant difference was found between the 'sham + melatonin' and 'sham + vehicle' groups. Experimental procedures also had no significant effect on DNA sin-

gle-strand breaks in brain cells of the rat (i.e., there is no significant difference between the unhandled control and 'sham + vehicle' groups). Percentage distributions of cells as a function of DNA migration length for the five treatment groups in this experiment are shown in Figure 2. Exposure to RFR caused a shift of distribution to longer lengths (i.e., to the right), and treatment with melatonin restored the distribution to a pattern similar to that of the 'sham + vehicle' animals.

A similar conclusion can be drawn from data of the study on treatment with melatonin on DNA double-strand breaks in brain cells. Melatonin treatment blocked RFR-induced increase in DNA double-strand breaks in rats brain cells. Figures 3 and 4 plot the mean migration length and percent cell versus migration length distribution, respectively.

The results of treatment with PBN on RFR-induced increase in DNA single- and double-strand breaks in rat brain cells are presented in Figures 5–8. Similar to the effect of melatonin treatment, PBN blocked the RFR-induced increases in DNA single- and double-strand breaks in rat brain cells.

DISCUSSION

Data from the present experiment confirm our previous finding [Lai and Singh, 1995, 1996] that acute RFR exposure causes an increase in DNA single- and double-strand breaks in brain cells of the rat. In addition, we have found that the effect can be blocked by treating the animals with melatonin or PBN. Since a common property of melatonin and spin-trap compounds is that they are efficient free radical scavengers [Carney and Floyd, 1991; Carney et al., 1991; Floyd, 1991; Lafon-Cazal et al., 1993 a,b; Lai et al., 1986; Oliver et al., 1990; Reiter et al., 1995; Sen et al., 1994; Zhao et al., 1994], these data suggest that free radicals may play a role in the RFR-induced DNA single- and double-strand breaks observed in brain cells of the rat. Consistent with this hypothesis is the fact that free radicals can cause damage to DNA and other macromolecules in cells. Particularly, oxygen free radicals have been shown to cause DNA strand breaks [McCord and Fridovich, 1978]. In addition, a study has implicated free radicals as the cause of some of the biological effects observed after exposure to RFR. Phelan et al. [1992] reported that RFR can interact with melanin-containing cells and lead to changes in membrane fluidity consistent with a free radical effect.

If free radicals are involved in the RFR-induced DNA strand breaks in brain cells, results from the present study could have an important implication on the health effects of RFR exposure. Involvement of free radicals in human diseases, such as cancer and atherosclerosis, have been suggested. Free radicals also play

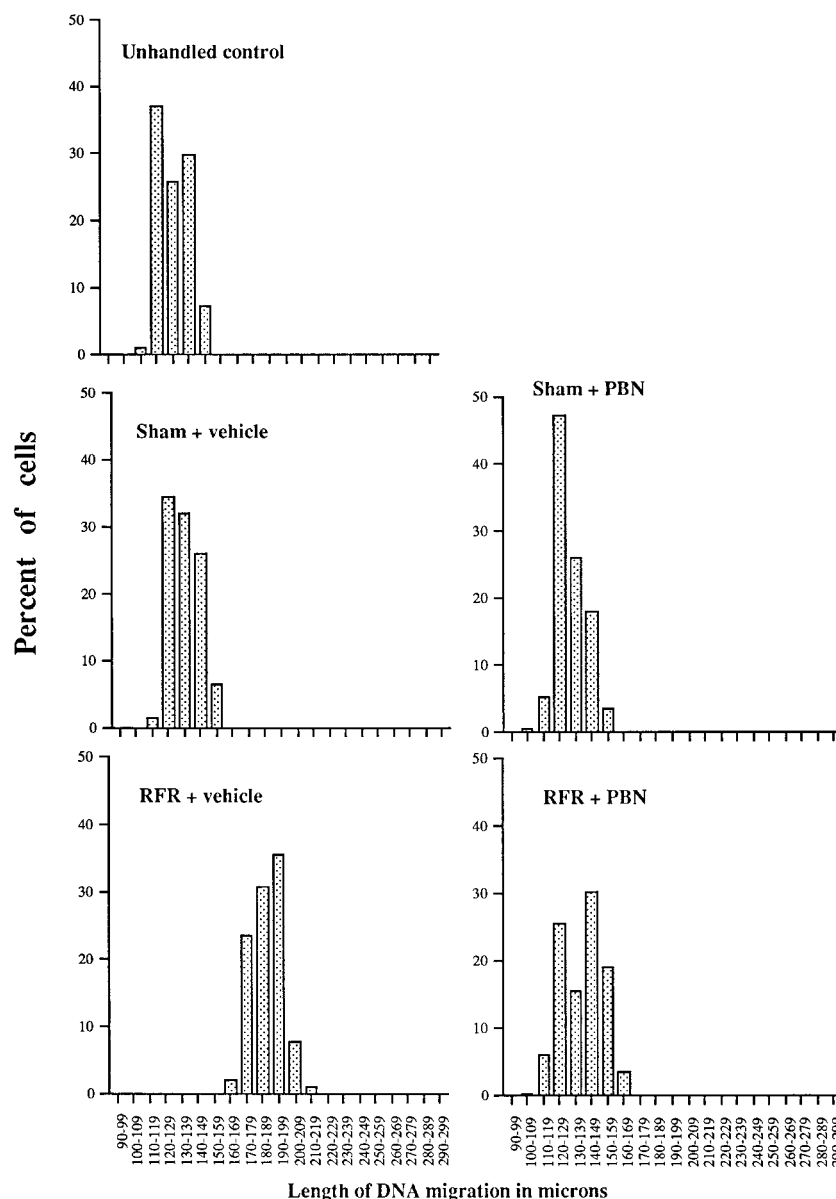


Fig. 6. Percent distribution of cells as a function of DNA migration length of the five groups of animals shown in Fig. 5.

an important role in aging processes [Reiter, 1995]. Aging has been ascribed to accumulated oxidative damage to body tissues [Forster et al., 1996; Sohal and Weindruch, 1996], and involvement of free radicals in neurodegenerative diseases, such as Alzheimer's, Huntington's, and Parkinson's, has also been suggested [Borlongan et al., 1996; Owen et al., 1996]. Furthermore, the effect of free radicals can depend on the nutritional status of an individual, e.g., availability of dietary antioxidants [Aruoma, 1994], consumption of ethanol [Kurose et al., 1996], and dietary restriction [Wachsman, 1996]. Various life conditions, such as psychological stress [Haque et al., 1994] and strenuous

physical exercise [Clarkson, 1995], have been shown to increase oxidative stress and enhance the effect of free radicals in the body. Thus, one can speculate that some individuals may be more susceptible to the effects of RFR exposure.

However, it must be pointed out that both melatonin and PBN can have other actions on cells in the brain that can decrease DNA damage. Further support for our hypothesis can be obtained by studying whether other compounds with free radical scavenging properties can similarly block the effect of RFR, and by measurement of other free radical-related cellular effects, such as oxidative molecular damages in lipids, protein, and DNA.

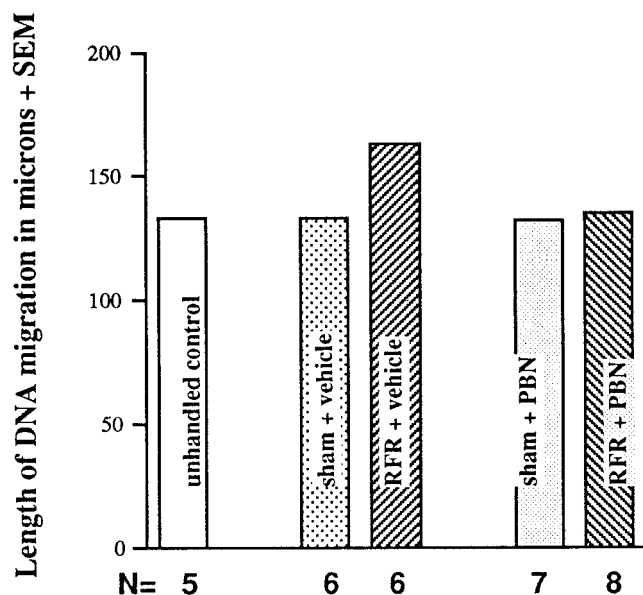


Fig. 7. Effect of treatment with PBN on RFR-induced increase in DNA double-strand breaks in rat brain cells. One-way ANOVA of the data showed a significant treatment effect ($F[4,27] = 47.83, P < .001$).

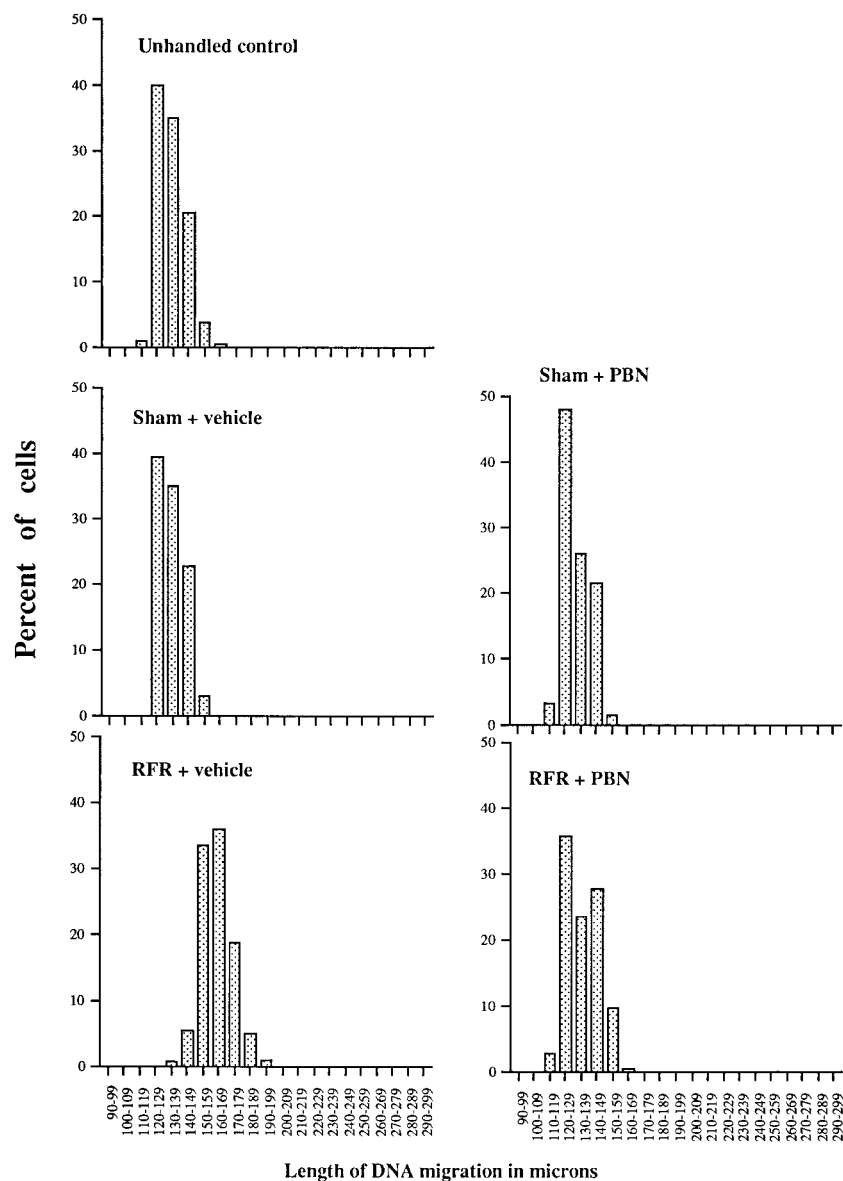


Fig. 8. Percent distribution of cells as a function of DNA migration length of the five groups of animals shown in Fig. 7.

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Attachment 10



FINAL REPORT

Risk Evaluation of Potential Environmental Hazards From Low Frequency Electromagnetic Field Exposure Using Sensitive *in vitro* Methods

**A project funded by the European Union
under the programme**

Quality of Life and Management of Living Resources

Key Action 4 "Environment and Health"

Contract: QLK4-CT-1999-01574

Start date: 01 February 2000

End date: 31 May 2004

Acronym: REFLEX



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ANNEX I

ANNEX II

FOREWORD

Prof. William Ross Adey, who made fundamental contributions to the emerging science of the biological effects of electromagnetic fields (EMFs), died on May 20, 2004, in Redlands, California, USA. He was scheduled to deliver his personal views of EMF research at a REFLEX workshop held in Bologna in October 2002. But by then he was already too frail to travel. Dr. Adey who was an informal advisor of the REFLEX consortium sent us his talk in written form. In memory of his achievements as a scientist and in recognition of his support of the REFLEX work, the consortium decided that his message would be an inspiration to all those scientists who are willing to accept the challenges posed by EMF research, and in addition, make a fitting introduction to the final report.

THE FUTURE OF FUNDAMENTAL RESEARCH IN A SOCIETY SEEKING CATEGORIC ANSWERS TO HEALTH RISKS OF NEW TECHNOLOGIES

The Challenge to Conventional Wisdom

The history of bioelectromagnetics epitomizes a range of problems that arise whenever a community of sciences is confronted with a frontier that delves deeply into the established orthodoxies of biology, the physical sciences and engineering. These conflicts have become even more sharply defined when emerging new knowledge in bioelectromagnetics research has challenged the conventional wisdom in each part of this trinity.

Thirty-five years ago, we, who first voiced our observations of physiological responses to a spectrum of environmental EMFs at levels below thresholds for significant tissue heating, were promptly challenged by acolytes of orthodoxies in the biological and physical sciences. At best, we were euphemistically described as “controversial,” a designation that persists to this day. A Yale physicist recently added the charming term “crackpot” to describe a highly qualified biophysicist investigator.

What is the basis of this deep thorn of discontent? Historically, excitation in biological systems has been modeled and tested in terms of equilibrium thermodynamics. In this classic tradition, it was assumed that the potential effectiveness of an exciting agent could be assessed by its ability to transfer energy to the receptor in excess of its random thermal atomic and molecular collisions. Thus, the physical expression kT , the union of the Boltzmann constant and temperature, has been regarded as an expression of an immutable threshold below which an exciting agent would not be physiologically effective. In like fashion from the quantum realm of the physicist, photon energies of low-frequency magnetic fields, now known to act as effective physiological stimuli, would also fall below this thermal barrier.

Here is one example: The human auditory threshold involves a hair cell vibration of 10-11 meters, or about the diameter of a single hydrogen atom. But, by an as-yet-unknown mechanism, the ear suppresses the vastly larger noise of its thermal atomic and molecular collisions, functioning as an almost “perfect” amplifier close to 0°K.

Clearly, we face a profound paradox, with answers to be sought in cooperative states and nonequilibrium thermodynamics, as first suggested in a biological context almost 60 years ago by Herbert Fröhlich.

The lesson is clear. The awesome complexity of biological organization demands our most careful consideration.

The Recent History of Technology Applications

We also find the heat of controversy in the recent history of technological applications in western societies. At no point in the last 20 years has public school education ensured that a majority of citizens has even a basic understanding of sophisticated communication devices and systems, such as telephones, radio and television. Similarly, automotive engineering remains a sea of vast ignorance for most users. Nor is such knowledge considered appropriate or necessary.

In summary, we have become superstitious users of an ever-growing range of technologies, but we are now unable to escape the web that they have woven around us.

Media reporters in general are no better informed. Lacking either responsibility or accountability, they have created feeding frenzies from the tiniest snippets of information gleaned from scientific meetings or from their own inaccurate interpretation of published research. In consequence, the public has turned with pleading voices to government legislatures and bureaucracies for guidance.

Public Concerns and the Evolving Pattern of Research Funding

We face the problem brought on by the blind leading the blind. Because of public pressure for rapid answers to very complex biological and physical issues, short-term research programs have been funded to answer specific questions about certain health risks.

Participating scientists have all too often accepted unrealistic expectations that, in a matter of a few years, they will provide answers to pivotal questions in cell and molecular biology that can only be achieved slowly, painstakingly and collaboratively over a decade or more.

Using EMFs as tools, we have launched our ship on a vast, uncharted ocean, seeking a new understanding of the very essence of living matter in physical processes at the atomic level. This is an awesome and humbling prospect, surely not to be ignored or forgotten in the pragmatic philosophies of most risk research.

In many countries, and particularly in the USA, the effects of such harassing and troublesome tactics on independent, careful fundamental research have been near tragic. Beguiled by health hazard research as the only source of funding, accomplished basic scientists have diverted from a completely new frontier in physical regulation of biological mechanisms at the atomic level. Not only have governments permitted corporate interests in the communications industry to fund this research, they have even permitted them to determine the research questions to be addressed and to select the institutions performing the research.

These policies overlook the immutable needs of the march of science. In their hasty rush to judgment, they have sought a scientific consensus where none can yet exist. Such a consensus will occur only after experimental convergence emerges from a spectrum of related but certainly not identical experiments.

Defining the Role of Epidemiology in Current Controversies

Much in the fashion of ancient Romans, standing four-square and reading the auguries of future events by noting flight patterns of passing birds, the modern-day epidemiologist has become the high priest in the search for correlates of disease processes with a constellation of environmental observables. It is rare for them to be competent in delving into questions of causality, particularly where no exposure metric has been established for a suspected environmental factor. Nevertheless, in courts of law, in legislatures, and among a concerned public, epidemiological opinions have become a gold standard, typically outranking evidence based on a balanced and often cautionary review of current medical science.

We should remind ourselves that their professional tool is biostatistics —they build endless Byzantine edifices of levels of statistical risk, with little or no commitment to the underlying science or medicine. Their mutual discussions have produced the technique of meta-analysis, the pooling of statistical analyses from a series of epidemiological studies. The method ignores the nuances of both experimental design and epidemiological findings in the separate studies, and blinds us to options for further research based on the possible uniqueness of these separate observations.

It appears reasonable that there should be no more large epidemiological studies on human EMF exposures until essential exposure metrics are established, based on mechanisms of field interactions in tissues.

Repairing the Body Politic of Science: Some Personal Reflections

The passage of time across the years has not diminished in any way the importance, even the urgency, that one feels towards the growing edifice of science. We must not fail to engender in younger minds a passionate curiosity and an imagination sufficient to kindle their commitment to all that is great and good in the scientific method.

As I reflect on major changes wrought in the U.S. national research scene over the past 40 years, I sense a deep and growing concern that research training and the culture of research accomplishment have stifled

the burning thorn of personal discontent that should be the creative option of all young minds entering on a research career.

Graduate students are assigned a project that is typically a segment of their advisor's grand vista. They may not deviate to ask creative "what if?" questions. They emerge from the chrysalis of their training, bearing a parchment for the professional market place, affirming proficiency in certain techniques, but in no way proclaiming the arrival of that precious citadel of a creative mind.

Please allow me to conclude with an urgent proposal that comes from my own research experience. Formal instruction in physics, theoretical and applied, has become the weakest link for those entering on a career in medical research. Bioelectromagnetics research has opened the door to a new understanding of the very essence of living matter in physical regulation at the atomic level, beyond the realm of chemical reactions in the exquisite fabric of biomolecules. Without versatility in biophysics that matches their typical knowledge in molecular biology and biochemistry, none of these students may cross this threshold to the cutting edge of in future medical research. Let us not see this opportunity lost prematurely through prostitution of mechanistic research in the market place of possible health risks.

Thank you for the great privilege of offering these personal reflections.

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1.0 INTRODUCTION

Based on the state of knowledge acquired during the last 50 years of research on possible biological effects of electromagnetic fields (EMF), the majority of the scientific community is convinced that exposure to EMF below the existing security limits does not cause a risk to the health of the general public. However, this position is questioned by others, who are of the opinion, that the available research data are contradictory or inconsistent and therefore, unreliable. As a consequence, it is necessary that the methodology applied in EMF research to be considerably improved and complemented by the most recent molecular biological techniques. In the REFLEX project, biological effects of extremely low frequency electromagnetic fields (ELF-EMF) and radio frequency electromagnetic fields (RF-EMF) are studied using sophisticated and diverse research methodologies separately since it is assumed that the generation of effects, if verifiable at all, may be based on different mechanisms.

Many laboratory investigations have been performed to test the hypothesis that ELF-EMF exposure may constitute a risk to the health of people. This hypothesis is almost entirely based on epidemiological studies, some of which indicate that ELF-EMF may contribute to the development of leukaemia in children, and other cancers in adults chronically exposed in residential environments or occupational settings (NRPB 2001; California EMF Program 2002; IARC Monographs 2002). The existing uncertainty is a source of increasing concern for the public, the health authorities and also the industry. *In vitro* studies have shown that ELF-EMF induces significant biological alterations in a variety of cells and tissues. These changes concern the up-regulation of several early response genes, including c-myc (Jin et al. 2000), c-fos (Rao and Henderson 1996) and hsp70 mRNA (Goodman and Blank 1998), thus increasing the production of stress inducible heat shock proteins (Goodman and Henderson 1988; Tokalov and Gutzeit 2003). In spite of this, it is still an unsolved issue whether or not exposure to ELF-EMF may promote pathological processes such as carcinogenesis and if so, whether or not the field effects are exerted through mechanisms influencing the genome of cells, cell proliferation, differentiation or programmed cell death (apoptosis). Results from several studies have indicated that ELF-EMF does not exert any direct genotoxic effect, but may promote carcinogenesis indirectly by interfering with the signal transduction pathways of cells (Blackman et al. 1985; Liburdy et al. 1993). Of course, the present uncertainty could considerably be diminished by increasing our knowledge on the parameters of the electromagnetic field which are critical for the generation of biological effects and of the biological systems which are crucial for the occurrence of pathological cellular events.

As with ELF-EMF, several epidemiological and animal studies also cast suspicion on RF signals to promote cancer and other diseases in chronically exposed individuals (Stewart Report 2000; Hardell et al. 2003). Because of its overwhelming presence in our society, the potential influence of RF-EMF exposure on the development of adverse health effects has become a major topic of interest for all concerned, including the government, the general public, and the industry. Putatively non-thermal, immediate and reversible responses have been described in the literature for several years (Roschke and Mann 1997; Wagner et al. 1998; Borbely et al. 1999; Preece et al. 1999; Koivisto et al. 2000; Huber et al. 2000; Krause et al. 2000). However, these effects, because of their unspecific nature have been regarded as indications of potential biological responses to electrical excitation, rather than harmful effects able to produce permanent damage to health. To date, several *in vitro* studies have been carried out to investigate the disease causing potential of RF radiation. While most of these studies using different cell systems, exposure set-ups and molecular-biological and toxicological methodologies did not show any biological effect, increasing numbers of studies have come up with contradicting results (Moulder et al. 1999; Vescovic et al. 2002).

As stated above, although investigations of possible biological effects of EMF have been conducted for decades, reliable answers are still missing. Extensive epidemiological and animal studies commonly expected to provide the answer as to whether or not EMF might be hazardous are in progress. However, this approach alone might not be able to provide certain evidence whether EMF can or cannot contribute to the pathogenesis of diseases such as cancer or neurodegenerative disorders. The low sensitivity of the epidemiological methodology in detecting low risk associations is probably insufficient to reliably identify any risk to health caused by EMF. Therefore, although epidemiological studies will be needed to ultimately validate the extent of any potential health hazard of EMF, such research must be supplemented and supported by data from animal and *in vitro* studies. Therefore, *in vitro* studies using the most modern molecular biological techniques such as genomics and proteomics are urgently needed in order to create at least a hypothetical basis for the understanding of disease development through EMF-exposure. If it can be determined that such a basis exists, it becomes even more important, to search for marker

substances which are specific for EMF exposure. Such marker substances could considerably increase the accuracy of epidemiological studies, so that even a low health risk due to EMF exposure would not escape epidemiological detection.

The main goal of the REFLEX project is to investigate the effects of EMF on single cells *in vitro* at the molecular level below the energy density reflected by the present safety levels. Most, if not all chronic diseases, including cancer and neurodegenerative disorders, are of diverse and heterogeneous origins. This variability is to a great extent generated by a relatively small number of critical events, such as gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis). Gene mutations, cell proliferation and apoptosis are caused by or result in an altered gene and protein expression profiles. The convergence of these critical events is required for the development of all chronic diseases. The REFLEX project is, therefore, designed to answer the question whether or not any of these disease-causing critical events could occur in living cells after EMF exposure. Failure to observe the key critical events in living cells *in vitro* after EMF exposure would suggest that further research efforts in this field could be suspended and financial resources should be reallocated for the investigation of more important issues.

2.0 MATERIAL AND METHODS

2.1 Exposure setups (Participant 10)

In order to compare the results of investigations carried out in the different laboratories and to ensure the conclusiveness of the data obtained in the studies, it is of the utmost importance that the conditions of exposure to EMF be strictly controlled. It was the task of Participant 10 to evaluate and modify already existing setups, to develop new optimised exposure systems and to provide technical quality control during the entire period of exposure. The latter was realised by (1) the conduct of a thorough dosimetry including an analysis of possible artefacts, (2) the continuous monitoring of exposure and environmental parameters and (3) blinded exposure protocols. Details about this work can be found in the appendix.

2.1.1 ELF-EMF exposure setup

A novel ELF setup was developed, and four copies were installed in the laboratories of Participants 3, 4, 7 and 11. The setup consists of two four-coil systems, each of which is placed inside a μ -metal shielding box. The coils produce a linearly polarised B-field over the area of the Petri dishes with a B-field vector perpendicular to the dish plane. The shielded design of the chamber guarantees non-interference between the two units, such that they can be kept close to each other inside the same incubator in order to guarantee identical ambient conditions for the cell dishes. Two fans per coil system ensure fast atmospheric exchange between the chambers and incubator. The airflow temperature is monitored with accurate Pt100 probes fixed inside the exposure chamber.

The signal is generated by a computer-controlled arbitrary function generator. A custom-designed current source allows arbitrary field variations in the range from MHz to 1.5 kHz. The maximum achievable magnetic flux density for a sinusoidal with a frequency of less than 80 Hz is 3.6 mT RMS. Sinusoidal signals with a frequency range from 3 Hz up to 1000 Hz can be applied, controlled and monitored. A powerline signal was defined which represents a worst-case scenario with respect to spectral content and corresponds to the maximum accepted distortions for power systems by the International Electrotechnical Commission (IEC 1995) (Figure 1). In addition to these waveforms, arbitrary field on/off intermittently in the range from seconds to hours can be applied.

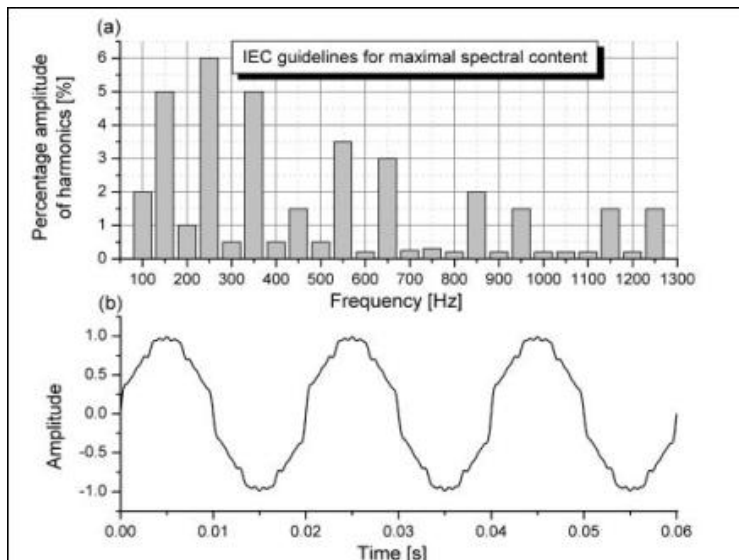


Figure 1. (a) Frequency composition of the powerline signal, corresponding to the maximum allowed spectral content according the IEC guidelines for low and medium voltage power networks (IEC 1995). Shown are the spectral amplitudes of the harmonics in relation to the main 50 Hz component. (b) The resulting powerline signal in the time domain.

The coil current and consequently the magnetic field is quasi-continuously (10s intervals) recorded and regulated using resistors providing low-temperature sensitivity. The currents in the bifilar coils can be randomly switched parallel for field exposure or nonparallel for sham control by the computer. This procedure is used to apply blind protocols and additionally to avoid temperature artefacts between exposed and control coils, since they are heated by the same current.

The evaluation and optimisation of the coil configuration was performed using numerical techniques (Mathematica V4.1) and experimental methods (3-axis Hall meter, FH49 Magnet Physik, Germany). A non-uniformity of less than 1% for the magnetic field over the exposure area of 16 cm x 16 cm x 23 cm is achieved. An uncertainty of 4.3% for the B-field assessment and a B-field variability of 1.6% were found. The average deviation of 2.9% between simulation and measurement is integrated in the uncertainty assessment. Parasitic electric fields generated by the coil system are reduced to less than 1 V/m by a grounded, metallic shielding box between the coil and Petri dishes. The temperatures inside the cell media have been analysed and no temperature differences due to field or sham exposures could be detected (i.e., temperature differences were below 0.1°C). The induced electric fields resulting from a sinusoidal exposure can be expressed as $E = 3.2 \cdot f \cdot B \cdot r$ [V/m] and for the powerline exposure as $E = 664 \cdot B \cdot r$ [V/m], whereby f is the frequency of the sinusoidal [Hz], B is the average B-field [T] and r is the radial distance from the dish centre [m]. The estimated vibration of the exposed cells is less than 1 m/s^2 ($= 0.1 \text{ g}$), which is a factor of 20 above the minimal background level for sham. If an elastically damped dish holder is used (as provided for Participant 4) the vibration load can be further reduced by a factor 12 to 0.1 m/s^2 .

In addition to the newly developed exposure systems, it was decided to use two existing setups (Participants 5, 8).

The ELF setup of Participant 5 is based on a pair of Helmholtz coils placed inside a μ -metal shield; exposure and sham are kept in different incubators (no blinded protocols); and sinusoidal B-fields (50 Hz) up to 0.1 mT can be applied.

The ELF setup of Participant 8 is based on two unshielded 4-coil systems arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied under non-blinded exposure conditions.

2.1.2 RF-EMF exposure setup

A novel RF setup (GSM) was developed, and four copies were installed in the laboratories of Participants 2, 5, 6, and 8. The system enables EMF exposure of cells under defined conditions with respect to field strengths, polarisation, modulation and temperature and is operated within the GSM DCS mobile frequency band. The setup consists of two single-mode resonator cavities for 1.8 GHz that are placed within an CO₂ incubator. Up to six 35 mm diameter Petri dishes can be exposed in one waveguide resonator. A dish holder guarantees that the dishes are placed exactly in the H-field maximum of the standing wave inside the waveguide.

Each waveguide is equipped with a fan for rapid environmental atmospheric exchange. In order to ensure stable exposure independent of the loading and drifts, monopole antennas are integrated to monitor and control the incident field. The system enables the exposure of monolayers of cells with a non-uniformity of SAR of less than 30% and an efficiency of better than 20 W/kg per W input power.

Much care has been taken to avoid artefacts due to temperature differences between exposed and sham exposed cells. The temperature response of the medium has been assessed by measurements in terms of the incident field strength, cell medium volume and air flow. A temperature load of less than 0.03°C per W/kg SAR was found. The air flow temperature is monitored with accurate Pt100 probes, resulting in differences of less than 0.1°C between the air flow temperature of the exposed and sham waveguides. A numerical heat flow analysis has shown that the possibility of temperature hot spots inside the medium can be excluded.

Field strengths, temperatures and fan currents as well as all commands are continuously logged to encrypted files which are evaluated after the experiments in order to ensure studies under 'blind' conditions (exposure and sham conditions are blindly assigned to the two waveguides by the computer-controlled signal unit).

Field, SAR and temperature characterisations were performed with numerical methods (FDTD simulation platform SEMCAD, SPEAG, Switzerland) and were experimentally verified using the near field scanner DASY3 (SPEAG, Switzerland) equipped with dosimetric field and temperature probes. An uncertainty and variability analysis resulted in an absolute uncertainty for the SAR assessment of 20% and a variability of 5%. The average deviation of 15% between SAR measurement and simulation is within the range of the uncertainty and therefore verifies the reliability of the numerical dosimetry.

The signal unit allows the application of the following five different exposure signals (Figure 2):

- Continuous Wave (CW): An unmodulated CW signal can be applied as a reference (same thermal load, but no ELF modulation components).
- GSM-217Hz: GSM signals are amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms), corresponding to the dominant modulation component of GSM. The ratio between slot average SAR and time average SAR is 8.
- GSM-Basic: In addition to this basic GSM-217Hz TDMA frame, every 26th frame is idle, which adds an 8 Hz modulation component to the signal. The ratio between slot average SAR and time average SAR is 8.3.
- GSM-DTX: The discontinuous transmission mode (DTX) is active during periods without speaking into the phone. To save battery power, the transmission is reduced to 12 frames per intermediate multiframe of 104 frames (compared to 100 frames for GSM Basic). The frame structure of the DTX signal results in 2, 8 and 217 Hz components. The ratio between slot average SAR and time average SAR is 69.3.
- GSM-Talk: GSM-Talk generates temporal changes between GSM-Basic and GSM-DTX and simulates a conversation with an average duration of 97s and 50s for Basic and DTX, respectively. The ratio between slot average SAR and time average SAR is 11.9. Furthermore, arbitrary field on/off intermittence in the range from seconds to hours can be applied.

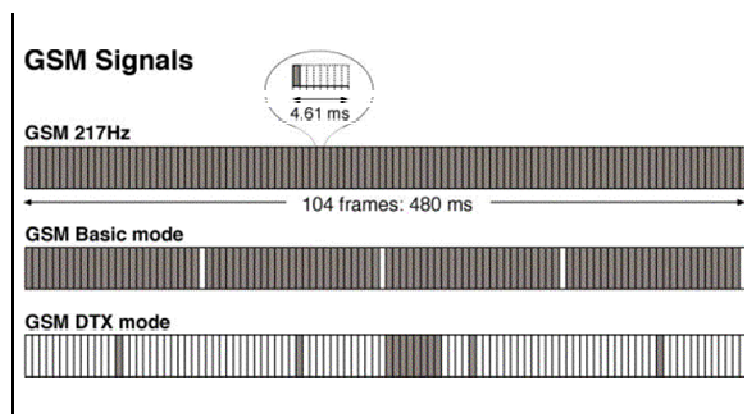


Figure 2. Pulse structure of the applied GSM signals. The basic frame has a period of 4.61 ms and contains a 576 μ s burst including 15 μ s rising and falling edges. 26 frames make up a GSM multiframe (MF) and 104 frames a GSM intermediate multiframe (IMF). GSM-217Hz is composed of a repetition of basic frames (104 bursts per IMF), whereby every 26th frame is blanked for the GSM-Basic signal (100 burst per IMF). The GSM DTX mode is active during periods of silence and transmission is reduced to 12 bursts per IMF.

In addition to the newly developed exposure systems, it was decided to use three existing setups (Participants 4, 6, 9) and to modify and improve the two setups of Participants 4 and 9.

The RF setup of Participant 4 is based on two R14 waveguides operated at 1710 MHz (Schönborn et al. 2000). Eight 60 mm diameter Petri dishes are exposed with the same concept as for the RF setup (GSM). An identical signal unit is applied, and similar performance is achieved. However, different settings for GSM-Talk were used: Average duration of Basic and DTX was 50s and 97s (instead of 97s and 50s). Therefore the ratio between slot average SAR and time average SAR is 19.8 (instead of 11.9).

The existing RF setup of Participant 6 is based on a R9 resonator cavity operated at 900 MHz. Active water cooling of the Petri dishes is integrated; for details see Toivo et al. (2001). The signal unit can apply GSM-217Hz. Blinded exposure protocols are not possible. In the course of the project, Participant 6 was additionally equipped with the standard RF-setup (GSM).

The RF setup of Participant 9 is based on the Wire-Patch cell and is operated at 900 MHz (Laval et al. 2000). The system was equipped with a new signal unit, allowing the full spectrum of GSM signals. However, also this setup does not allow for computer-controlled blinded exposure conditions. On the other hand, Participant 9 assures that exposure using this system was done blinded (see 2.6.2 below)

2.2 Experiments with human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and with granulosa cells of rats (Participant 3)

2.2.1 ELF and RF-EMF exposure setups

See 2.1

2.2.2 Cell culture and exposure conditions

Human diploid fibroblasts (obtained from healthy donors) and SV40 transformed GFSH-R17 rat granulosa cells (Keren-Tal 1993) (provided by Participant 7) were cultivated in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS), 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Human melanocytes (male, 3 years old) and skeletal muscle cells (male, 63 years old) were received from Promocell (Heidelberg, Germany) and cultured according to the supplied protocol. Cells were incubated at 37°C in an atmosphere of 5% CO₂ and at 90-100% relative humidity and supplied with fresh culture medium every 48h.

Leukocytes from a healthy donor (female, 31 years old) were isolated from venous blood using Ficoll Paque gradient centrifugation. Cells were washed twice with PBS, resuspended in DME medium with additives and seeded into 35 mm Petri dishes. After 2 hours monocytes had become completely attached on the bottom surface and were separated from lymphocytes by decantation. Monocytes were washed twice with PBS and taken up in 3 ml DME medium.

Lymphocytes from a healthy donor (female, 27 years old) were isolated from venous blood with Ficoll Paque gradient centrifugation. Cells were resuspended in fresh culture medium (DME, 25% FCS, 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) with or without stimulation with phytohemagglutinine (1%). The cells were seeded into 35 mm Petri dishes at a density of 2×10^5 cells/3 ml, 24 hours prior to ELF-EMF exposure.

The cells mentioned above were exposed or sham-exposed in suitable waveguides connected with an ELF-EMF generation system provided by Participant 10 within a Heraeus incubator (model Kendro BBD 6220). After exposure cells were detached with trypsin and suspended in fresh culture medium for Comet assay analysis or maintained in culture for cytogenetic testing. Each exposure level was tested in duplicate. In another series of experiments human fibroblasts (cell strain ES-1) and SV40 transformed GFSH-R17 rat granulosa cells (Keren-Tal 1993) (provided by Participant 7) were exposed or sham-exposed in suitable waveguides connected with a RF-EMF generation system (RF 1800 MHz) provided by Participant 10 within a Heraeus incubator (model Hera cell 1501). These experiments were first performed by E. Diem in the laboratories of Participant 2 and later on continued in our own laboratories with a comparable RF-EMF exposure setup, but a RF of 1950 MHz. After exposure cells were detached with trypsin and suspended in fresh culture medium for Comet assay analysis or maintained in culture for cytogenetic testing. Each exposure level was tested in duplicate.

Combined exposures to UVC and ELF-EMF were performed on ice using a germicidal lamp (60 W, Desaga, Heidelberg, Germany), the output of which predominantly contains UVC (253.7 nm). Exposure to UVC prior to ELF-EMF was carried out at an intensity of 2 W/m² (measured with a radiometer, Blak-

ray®, Ultra-violet products. Inc., model J225, San Gabriel, USA) for 10 minutes, which equals 1.2 kJ/m². Exposure to UVC post to ELF-EMF exposure was performed at an intensity of 2.5 W/m² for 30 minutes, which equals 4.5 kJ/m².

For thermal exposure cells were incubated at 38.5°C for 4 hours in a commercial incubator (BBD 6220, Kendro, Vienna, Austria). To study repair kinetics, cells were further incubated at 37°C for up to 24 hours.

2.2.3 Comet assay

We followed the technique described by Östling and Johanson (1984) with minor modifications by Singh et al. (1988, 1991). EMF-exposed and sham-exposed cells (10,000 – 30,000) were mixed with 100 µl low melting agarose (0.5%, 37°C) to form a cell suspension, pipetted onto 1.5% normal melting agarose pre-coated slides, spread using a cover slip, and maintained on a cold flat tray for about 10 minutes to solidify. After removal of the cover slip the third layer of 0.5% low melting agarose was added and solidified. The slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, pH 10) and lysed for 90 minutes at 4°C. Subsequently, the slides were drained and placed in a horizontal gel electrophoresis tank side by side, nearest the anode. The tank was filled with fresh electrophoresis buffer (1mM Na₂EDTA, 300 mM NaOH, pH>13 or pH=12.1 in case of alkaline Comet Assay and 100 mM Tris, 300 mM sodium acetate, 500 mM sodium chloride, pH 8.5 in case of neutral Comet Assay) to a level approximately 0.4 cm above the slides. For both, alkaline and neutral Comet assay, slides were left in the solution for 40 minutes for equilibration and unwinding of the DNA before electrophoresis. Electrophoresis conditions (25 V, 300 mA, 4°C, 20 min, field strength: 0.8 V/cm) were the same for neutral and alkaline Comet assay. All steps were performed under dimmed light to prevent the occurrence of additional DNA damage. After electrophoresis the slides were washed 3 times with Tris buffer (0.4 M Tris, pH 7.5) to neutralise, then air-dried and stored until analysis. Comets were visualised by ethidium bromide staining (20 µg/ml, 30 seconds) and examined at 400 X magnification using a fluorescence microscope (Axiophot, Zeiss, Germany). One thousand DNA spots from each sample were classified into 5 categories corresponding to the amount of DNA in the tail according to Anderson et al. (1994) with modifications. The proposed classification system provides a fast and inexpensive method for genotoxic monitoring. Due to the classification to different groups by eye, no special imaging software is required. The different classification groups are not weighted equally, due to the fact that they do not represent equal grades of damage. Moreover, the technique becomes more sensitive, because many cells can be scored in a short time (1000 cells instead of 50-100 cells with image analysing). The subsequent calculation of a “Comet tailfactor” allows quantifying DNA damage as a single figure, which makes it easier to compare results. Due to the scoring of 1000 cells in one experiment, which are tenfold the cells processed with image analysing, standard deviations are very low. Reproducibility has been thoroughly checked. Results expressed as “Comet tailfactors” were calculated according to Diem et al. (2002). All experiments were performed in duplicate by the same investigator.

2.2.4 Micronucleus assay

Micronucleus (MN) assay was performed according to Fenech and Morley (1985) and Fenech (1993). Fifty thousand cells were seeded into slide flasks (Nunc, Roskilde, Denmark) and exposed to ELF-EMF. In order to block cytokinesis, cytochalasin B (3 µg/ml, Sigma, St. Louis, USA) was added four hours before the first round of replication. After termination of the culture, fibroblasts were treated with hypotonic KCl solution (0.075 M, 30 min.) and fixed with a mixture of methanol : aqua bidest. (7:3) for 10 min. Slides were air-dried and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, USA) for 3 minutes. MNs were visualised under a fluorescence microscope and 2000 binucleated cells were scored according to criteria published by Lasne et al. (1984). The results are expressed as MN events/500 binucleated cells.

2.2.5 Chromosomal aberrations

For evaluation of chromosomal aberrations 2 x 10⁵ cells were seeded into 35 mm petri dishes (Nunc, Roskilde, Denmark) and exposed to EMF at conditions producing maximum effects in the Comet assay.

After EMF exposure, fibroblasts were trapped at metaphase by incubation with colcemid (0.2 µg/ml, Invitrogen Corporation, Paisely, Scotland) for the last 4h prior to harvesting. Subsequently, the cells were detached with trypsin (Invitrogen Corporation, Paisely, Scotland) and subjected to a hypotonic treatment (0.075 M KCl, 37°C, 30 min.). Thereafter, cold fixative (methanol : acetic acid 3 : 1) was slowly added and cells were collected by centrifugation. Fixation procedure was repeated twice. Finally, the cells were resuspended in 0.5 ml of fixative, dropped on clean slides, air dried, stained for 12 minutes with 4% GIEMSA, prepared in Sorensen's buffer (38 mM KH₂PO₄, 60 mM Na₂HPO₄ x 12 H₂O, pH=7) and rinsed with aqua bidest. Chromosomal aberrations were evaluated in 10,000 well-spread and complete (46 chromosomes) metaphases (5,000 ELF-exposed, 5,000 sham-exposed). The identification of chromosome aberrations was carried out following the criteria recommended by the WHO (1985). Different types of aberrations (chromosome gaps, chromosome breaks, ring chromosomes, dicentric chromosomes and acentric fragments) were scored separately. Five independent experiments were performed. Results are expressed as percent chromosomal aberrations per cell.

2.2.6 Fluorescence *in situ* hybridisation (FISH)

For evaluation of stable translocations, cells were seeded into 35 mm petri dishes at cell density of 2×10^5 /3ml. After ELF-EMF exposure and an additional repair time of one replication round, fibroblasts were trapped at metaphase by incubation with colcemid (0.2 µg/ml, Invitrogen Corporation, Paisely, Scotland) for the last 4h prior to harvesting. Subsequently, the cells were detached with trypsin (Invitrogen Corporation, Paisely, Scotland) and subjected to a hypotonic treatment (0.075 M KCl, 37°C, 30 min.). Thereafter, cold fixative (methanol : acetic acid 3 : 1) was slowly added and cells were collected by centrifugation. Fixation procedure was repeated twice. Finally, the cells were resuspended in 0.5 ml of fixative, dropped on clean slides and air-dried. Subsequently, slides were denatured (70% formamide/2×SSC pH 7, 72°C, 2 min) and immediately dehydrated through a cold (-20°C) ethanol series (70%, 80%, 90%). The rhodamine-labelled whole chromosome probes (chromosome 1-22, X, Y) were prepared according to the recommendation of the manufacturer (Appligene Oncor Q-biogene, Illkirch, France). Aliquots of 30 µl were applied to the metaphase preparations, the slides were covered with a cover slip and incubated overnight in a moist chamber at 37°C. The following day, slides were immersed in 0.5×SSC at 72°C for 5 min, transferred to 1×PBD at room temperature for 2 min and counterstained with DAPI (4,6-diamidino-2-phenylindole, 0.02 µg/ml, Sigma, St. Louis, USA). Fluorescence signals were evaluated using a fluorescence microscope equipped with filters capable of simultaneously passing DAPI/Rhodamine. 1,000 well spread and complete (46 chromosomes) metaphases were scored for translocations for each labelled chromosome.

2.2.7 Changes in mitochondrial membrane potential (JC-1 staining)

Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) upon ELF-EMF exposure were assessed by staining mitochondria with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbo-cyanine iodide (JC-1, Molecular Probes, Leiden, The Netherlands), a fluorescent dye with high sensitivity to $\Delta\Psi_m$ in intact cells (Cossarizza et al. 1993b; Salvioli et al. 1997). This lipophilic cation forms J-aggregates in the matrix of intact mitochondria (emitting at 590 nm) or is released in a monomeric form (527 nm) from depolarised mitochondria. A good correlation between the J-aggregate fluorescence of JC-1 and $\Delta\Psi_m$ has been reported previously (Smiley et al. 1991). Immediately after exposure to ELF-EMF (50 Hz, 1 mT, 15h, intermittent 5 min on/10 min off) or RF-EMF (GSM basic 1950 MHz, SAR=1 W/kg, 15h, intermittent 5 min on/10 min off), cells were trypsinated, centrifuged (700 g, 5 min) and resuspended in medium (RPMI 1640 with 10% fetal calf serum). Subsequently, the cells were incubated in triplicates at a density of 0.45×10^6 cells/ml in complete medium for 15 min at 37°C in the dark with 10 µg/ml JC-1. After incubation with JC-1 the fibroblasts were washed twice with phosphate buffered saline (Gibco, Vienna, Austria) and adjusted to 2.15×10^5 cells/ml. From each of the three stained samples per ELF-EMF exposure and sham exposure, respectively, 8 x 250 µl cell suspension have been transferred to a 96 well sample plate. Measurement of red fluorescence (excitation 540 nm, emission 590 nm) and green fluorescence (excitation 485 nm, emission 535 nm) was done on a Wallac Victor 2 fluorescence plate reader (EG&G Wallac, Turku, Finland). Results were expressed as ratios of red/green fluorescence. For positive controls cell cultures were treated for 18h with 20 µM camptothecin (Sigma-Aldrich, Vienna, Austria), a well known inducer of apoptosis.

2.2.8 Statistical analysis

Statistical analysis was performed with STATISTICA V. 5.0 package (Statsoft, Inc., Tulsa, USA) and SPSS 10.0 package (SPSS Inc., Illinois, USA). All data are presented as mean \pm standard deviation (SD). The differences between exposed and sham-exposed, as well as between different exposure conditions were tested for significance using independent Student's t-test or one-factorial ANOVA with post hoc Student's t-test Bonferroni-correction. A difference at $p < 0.05$ was considered statistically significant. Correlation was assessed by multiple regression analysis using linear regression.

2.3 Experiments with human HL-60 cells (Participant 2)

2.3.1 RF-EMF exposure setup

See 2.1.2

2.3.2 Cell culture and exposure conditions

Human HL-60 cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Promocell, Heidelberg, Germany), 1% L-glutamine, 1% HEPES buffer and 2% penicillin/ streptomycin (Gibco BRL Life Technologies, Rockville, MD, USA) under temperature- and pH-control conditions. The cell line was maintained in logarithmic growth phase at 37°C in a 5% CO₂ atmosphere. For radio-frequency (RF) exposure experiments the initial seeding density per 35 mm petri dish was 7.5×10^5 cells. In addition to sham-exposed cells, cells incubated under these normal cell culture conditions without the waveguides being connected to the generator system (see below) were examined as incubator controls. In positive control cells DNA breakage was induced either by incubation of cells for 60 min in cell culture medium containing hydrogen peroxide at a final concentration of 100 $\mu\text{mol/l}$ or by 6 MeV γ -irradiation (0.5 Gy, exposure time: 5.2 s). In case of assessing indirectly the generation of reactive oxygen species and directly the modulation of detoxifying capacities of HL-60 cells, culture medium was supplemented with ascorbic acid (10 $\mu\text{mol/l}$) prior to RF-exposure.

The following exposure conditions were examined with respect to direct and indirect genotoxic effects in HL-60 cells using the alkaline Comet assay, the cytokinesis-block micronucleus assay, the flow cytometric measurement of micronuclei induction and DNA alterations, cytotoxicity testing, assessment of cell viability and cell growth:

- 1800 MHz, continuous wave (C.W.) exposure, 24 hours, SAR=0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg and 3.0 W/kg, compared with the corresponding sham exposed cells, incubator control cells and positive controls.
- At 1800 MHz, SAR 1.3 W/kg, C.W., different periods of exposure, ranging from 2h up to 72h exposure.
- 1800 MHz, SAR of 1.3 W/kg different RF-signals: C.W., 5 min on/10 min off; GSM-217Hz; GSM-Talk were examined.
- 1800 MHz, GSM-DTX, 24 hours, 5 min on/5 min off, SAR 1.0 W/kg, compared with the corresponding sham exposed cells, for gene expression profiling studies.
- 1800 MHz, C.W., 24 hours, SAR 1.3 W/kg, compared with the corresponding sham exposed cells and incubator controls for gene expression profiling studies.

Cells were exposed or were sham-exposed in waveguides connected with a RF-generator system in a Heraeus incubator (Model Hera Cell). After each run the cells were immediately taken out of the incubator for subsequent analyses unless otherwise stated. Experiments were performed under blinded conditions in the following way: after the cells were placed in the two waveguides, and the incubator chamber was closed, the selection which of the two waveguides was connected to the RF generator and which remained disconnected, i.e. served as a sham control, was controlled by the computer system provided by Participant 10 and remained concealed to the experimentalists until analyses by the Comet assay and the micronucleus assay were completed and results documented. For decoding which of the two waveguides was connected to the RF-generator or remained disconnected, respectively, and for control of

the experimental conditions of each run, technical data were mailed independently to Participant 10 and returned electronically with electronic documentation of the time points of dispatching and return. Temperature within the waveguides was monitored during each run for as well sham-exposed and RF-field exposed cells and documented electronically. Additionally, at the end of the experiment pH values were controlled within the cell culture medium of sham-exposed, RF-field exposed and control cells.

An independent experiment consisted of 6 exposed and 6 sham-exposed petri dishes (35 mm diameter) with an initial seeding density of 7.5×10^5 cells per petri dish. In order to rule out potential differences of the six positions within the waveguide, the cells from the six exposed petri dishes and of the six sham-exposed dishes, respectively, were pooled prior to further analysis. Differences between exposed cells and corresponding controls were tested for significance, employing the Student's t-test at a level of $p \leq 0.05$.

2.3.3 *in vitro* genotoxicity tests

The cytokinesis-block in vitro micronucleus (MN) assay

The MN assay was carried out as described by Natarajan and Darroudi (1991) according to the guidelines developed by Fenech (1993, 2000), Fenech and Morley (1985, 1986), Fenech and Rinaldi (1995), Fenech et al. (1994) and Garriott et al. (2002). In order to evaluate the frequency of MN in binucleated (BN) human HL-60 cells, cytochalasin B (final concentration 3.0 µg/ml) was added to the growth medium after exposure and washing. Cytochalasin B prevents the cells from completing cytokinesis resulting in the formation of multinucleated cells (Fenech and Morley 1985). The cells were fixed after 24 hours. For fixation the cells were washed and treated with cold hypertonic KCl solution (5.6 g/l). Then the cells were fixed 3 times with a solution of acetic acid/methanol (1:3) and subsequently air dried preparations were made. For the detection of MN in binucleated cells (BNC) the slides were stained with 2.0% Giemsa solution. To determine the frequency of MN of RF-exposed, sham-exposed or control cultures the number of MN in 1000 BNC cells were scored microscopically at 400 fold magnification by one person in 2 slides for each experimental point. All particles about the size smaller than one-third that of the main nuclei, round-shaped with similar staining characteristics as the main nuclei were counted as micronuclei. In particular after high doses of γ -irradiation (2 and 3 Gy, respectively), it was sometimes difficult to distinguish between "true" micronuclei and apoptotic bodies that occurred also in BN cells. Experiments were repeated at least three times independently.

To study the effects of RF-EMF on cell division, the number of BNC relative to the number of mono-, bi-, tri- and tetranucleated cells (% BNC) was determined according to Fenech (2000). Furthermore, apoptotic cells can be recognised by a characteristic pattern of morphological changes, which may be broadly defined as cell shrinkage, cell shape change, condensation of cytoplasm, nuclear envelope changes, nuclear fragmentation and loss of cell surface structures.

Alkaline single cell gel electrophoresis assay (SCGE, Comet assay)

The alkaline SCGE assay was carried out as described by Singh et al. (1988) according to the guidelines developed by Tice et al. (1990, 2000), Fairbairn et al. (1995) and Klaude et al. (1996). After exposure and washing, a single cell suspension of 1×10^4 cells was mixed with 100 µl of 0.7% LMP agarose in PBS and transferred to the fully frosted slides precoated first with 1% and then with 0.5% NMP agarose in PBS. Finally, a covering layer (0.7% LMP agarose) was transferred to the slide. All procedures were performed under dimmed light. Subsequently, the slides were covered with a coverslip and allowed to solidify in the refrigerator. Then the coverslips were removed and the slides were immersed for 1 hour at 4°C in lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10) containing 1% Triton-X 100 and 10% DMSO added just before use. Thereafter, the slides were exposed to 0.3 M NaOH for 20 min to allow the DNA to unwind. After this, the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na₂EDTA and 300 mM NaOH, pH 13.5) following electrophoresis at 0.8 V/cm (25 V, 300 mA) for 20 min. After electrophoresis, the slides were rinsed 2x with 400 mM Tris, pH 7.5 and were stained with 80 µl ethidium bromide (0.02% in water), covered with a coverslip. To prevent additional DNA damage all steps described above were conducted under dimmed light or in the dark. 50 randomly chosen cells per slide (two slides per culture) were analysed using a 400-fold magnification with a Zeiss fluorescence microscope (Zeiss Axioplan). A computerized image analysis system (Kinetic Imaging 4.0, Optilas, München, Germany) was employed to measure different Comet parameters. As a measure of DNA damage tail length (in µm), Tail Extent Moment, Olive Tail Moment (OTM) and % of DNA in tail were automatically calculated. To determine

DNA migration of exposed, sham-exposed or control cultures, 100 cells were scored microscopically for Comet formation on 2 slides for each experimental point. As a positive control hydrogen peroxide at a final concentration of 100 $\mu\text{mol/l}$ for 1h was used. Experiments were repeated at least three times independently. The results reported are the mean values \pm standard deviation (SD).

Positive control through Gamma-Irradiation

Irradiation was administered with 6 MeV X-rays on an linear accelerator to the HL-60 cells (0.75×10^6) in dishes at doses of 0.5, 1.0, 2.0 and 3.0 Gy (dose rate: 5.8 Gy/min). A control dish received no irradiation. Then, both irradiated and non-irradiated samples were returned to the incubator and cultured until analyses at each relevant point were performed.

Viability test

Viability of the cell samples was assessed using the trypan blue exclusion test. The percentages of viable cells were then determined by placing aliquots of the treated cells in a Neubauer chamber and scoring cells for either the absence (viable cells) or the presence (dead cells) of blue staining. Only cultures with a viability more than 90% were analysed.

2.3.4 in vitro cytotoxicity testing

In order to exclude in vitro cytotoxic effects different approaches were used to verify cell viability, including trypan blue staining, flow cytometry tests, by which cells with reduced viability are marked by nuclear propidium iodide and the MTT assay, a colorimetric assay, that is based on the ability of viable, i.e. metabolically active cells to cleave tetrazolium salts to form formazan dye.

Cell viability and cell cytotoxicity were assessed by using the MTT assay. MTT is a sensitive first indicator of mitochondrial damage induced by oxidative stressors (Wasserman and Twentyman 1988). To analyse HL-60 cell proliferation, the MTT assay (Cell Proliferation Kit I, Roche, Mannheim, Germany) was used according to the manufacturer's protocol, and the data reported as OD units. This assay is very sensitive for the measurement of cell proliferation based upon the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

Briefly, around 2×10^3 cells per well were plated in 96-well microtiter plates with 100 μl of medium. 10 μl of a MTT (5 mg/ml) solution (Roche, Mannheim) was added. Incubation occurred for 4h at 37°C. 100 μl of solubilisation solution was added to each well. The plate was allowed to stand overnight in the incubator (37°C, humidified atmosphere). Absorbance was measured at 570 nm. Performing analysis on micronuclei induction, the ratio of BNC against mono-, bi-, tri- and tetranucleated cells is determined, giving a measure of cell division and cell cycle progression. Performing the flow cytometric analysis of micronuclei induction, an assessment of cell viability and also of DNA distribution and therefore of cell cycle alterations become feasible.

2.3.5 Preparation of nuclei suspensions from cells for flow cytometry analysis

The method was performed according to Nüsse and Kramer (1984), Nüsse and Marx (1997) and Wessel and Nüsse (1995). After exposure of cells and subsequent incubation for 24h (recovery time) the medium was removed and cells were washed twice with PBS and counted. Cells were then spun at 100 x g for 5 min at room temperature. Supernatants were removed carefully, the remaining cell pellet was re-suspended by gently shaking. FACS solution I (10 mM NaCl, 3 mM sodium citrate, 10 mg/l RNase A, 3 ml/l of 10% Igepal solution in water, 25 $\mu\text{g/l}$ ethidium bromide freshly prepared before use) was added to the cell pellet and cells were suspended to a density of approximately 1×10^6 cells per ml. The suspension was stirred for 2 sec and was kept for 30-60 min at room temperature in the dark. After adding FACS solution II (70 mM citric acid, 250 mM sucrose, 40 mg/l of ethidium bromide) the suspension was stirred for 2 sec and subjected to FACS analysis.

2.3.6 Flow cytometric exclusion of apoptosis via Annexin V assay and TUNEL assay

Annexin V assay

After exposure to RF-EMF 10^7 cells were centrifuged, washed several times with PBS and the pellet was resuspended with Annexin V binding buffer (Becton Dickinson Biosciences, Heidelberg, Germany), 1 ml of this suspension was incubated for 20 min in the dark in the presence of 100 μ l Annexin V binding buffer and 3 μ l Annexin V FITC (Becton Dickinson Biosciences, Heidelberg, Germany). After washing with Annexin V binding buffer, cells were resuspended in 300 μ l Annexin V binding buffer + 5 μ l Propidium Iodide (PI) solution (50 μ g/ml) and were analysed by flow cytometry using a FACSCalibur Analytic Flow Cytometer (Becton Dickinson Biosciences, Heidelberg, Germany).

TUNEL assay

After exposure to RF-EMF 10^7 cells were centrifuged, washed several times with PBS and then fixed for 1h by PBS/formaldehyde (4%). Then the cells were washed again, resuspended with PBS and permeabilised with 100 μ l Triton X solution (0.01% Triton in 0.1% sodium citrate solution) for 2 minutes, followed by labelling with 50 μ l TUNEL reaction mixture (Roche, Mannheim) for 1 h at 37°C. After this incubation the cells were washed and resuspended with 500 μ l PBS. The cells then underwent flow cytometric analysis in order to determine the number of green stains (representing apoptotic DNA fragmentation). DNA content analysis was performed on a Becton Dickinson FACScan according to the manufacturer's protocol.

2.3.7 Reactive oxygen species (ROS) and antioxidant enzyme activity

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH^\cdot) and singlet oxygen (1O_2) have powerful oxidative potential. ROS are capable of attacking lipids, nuclear acids and proteins, resulting in certain degree of oxidative damage. The cell possesses an efficient antioxidant defence system, mainly composed of antioxidative enzymes such as superoxide dismutase, and glutathione peroxidase. These enzymes are able to scavenge excessive ROS to cellular metabolism, and thereby lead to a relative stabilisation of the ROS level under physiological conditions. To evaluate the ROS level differences in RF-exposed and sham-exposed HL-60 cells the Nitric Oxide Assay, the oxyDNA assay, the direct detection by flow cytometry using the fluorescent dye Dihydrorhodamine 123 and the Lipid Peroxidation Assay were used. For measuring the antioxidative enzyme capacity the activities of superoxide dismutase and glutathione peroxidase were evaluated. The tests mentioned above were chosen within a first screening approach in order to assess qualitatively gross changes in ROS levels and antioxidative enzyme activities. All analyses were performed at room temperature unless otherwise stated.

Nitric oxide (NOx)

Nitric oxide (NOx) was measured using the colorimetric Nitric Oxide Assay Kit from Calbiochem (Cat. No. 482650, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, nitrate in aqueous solutions (supernatant after centrifugation of 7×10^5 cells/ml) was reduced to nitrite by enzymatic conversion by nitrate reductase and was estimated spectrophotometrically at 540 nm using the Griess reaction. The absorbance obtained is compared against a standard curve of known concentrations of NOx (1-25 μ mol/l) and the results were expressed as μ mol/l NOx. Detection limit is <1 μ mol/l for NOx (Miles et al. 1996).

oxy DNA

Oxidative DNA damage, with 8-oxoguanine as the major oxidative DNA product, was measured using the fluorogenic OxyDNA Assay Kit from Calbiochem (Cat. No. 500095, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). The assay utilizes a direct fluorescent probe directly binding to the DNA adduct of 8-oxoguanine (de Zwart et al. 1999, Kasai 1997, Cooke 1996). Briefly, cells (1×10^6) were washed first in $1 \times$ PBS, then in wash solution, and then by the addition of 100 μ l blocking solution with a 1-hour incubation at 37°C. After 2 washes in working solution, cells were incubated with 100 μ l FITC conjugate for 1 hour in the dark at room temperature before they were washed twice in washing solution and once in PBS. The FITC labelled protein conjugate binds to the 8-oxoguanine moiety present in the 8-oxoguanosine of oxidized DNA. Finally, cells were resuspended in FACS buffer and were analysed by flow cytometry (FACScan, Becton Dickinson). The presence of oxidized DNA is indicated by a

green/yellow fluorescence. A partial augmentation (shoulder at the right side of the signal) of FL-1 fluorescence intensity indicates an increase in level of oxidative DNA damage, i.e. 8-oxoguanine. In the present study, assays for the screening of oxidative DNA damage were performed after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) or sham-exposed cells. Oxidatively damaged DNA was quantified by determination of the area under the curve (AUC) of the shoulder at the right side of the signal fluorescence intensity in RF-field exposed cells.

Detection of ROS level with Dihydrorhodamine 123

7.5×10^5 cells were incubated with 5 $\mu\text{mol/l}$ dihydrorhodamine123 (DHR123, Sigma, Germany), as a ROS capture (Lopez-Ongil et al. 1998), during sham- or RF-exposure for 24h. Additionally, positive controls were run, in which 100 $\mu\text{mol/l}$ of hydrogen peroxide (H_2O_2) was added for 1h prior to the end of the experiment. Intracellularly, DHR123 is oxidized by ROS to form the fluorescent compound rhodamine123 (Rh123), which is pumped into mitochondria and remains there. After the experiment, cells were harvested, washed with PBS and immediately analysed for Rh123 fluorescence intensity by flow cytometry (FACScan, Becton Dickinson). The percentage of oxidative damage was defined as the percentage of gated HL-60 cells with Rh123 fluorescence. The results presented represent the means of three independent experiments.

Lipid Peroxidation Assay

Lipid Peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit from Calbiochem (Cat. No. 437634, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, can be estimated spectrophotometrically at 586 nm after reaction with a chromogenic reagent at 45°C to obtain an index for lipid peroxidation (Melchiorri et al. 1995, Öllinger and Brunmark 1994, Sewerynek et al. 1995).

Briefly, 3×10^6 cells were used per assay. The cells were lysed by repetitive freezing/thawing in 1000 μl distilled water. The cellular membranes were not removed until after the incubation with reagent R1 and R2. The samples were centrifuged at $15.000 \times g$ for 10 minutes to clarify the homogenate supernatant. Immediately prior to reading the absorbance at 586 nm, 200 μl of sample solution, 650 μl of diluted reagent R1 and 150 μl of diluted reagent R2 were mixed to a volume of 1000 μl . A least-square linear regression demonstrates that the standard curve (concentration range 0-20 $\mu\text{mol/l}$) is a linear function of the concentration of either MDA or 4-HNE. The absorbance values obtained were compared against a standard curve of known concentrations of MDA/4-HNE (1-20 μM). The results were reported as μmol (MDA + 4-HNE)/l. Detection limit is 0.1 $\mu\text{mol/l}$ for (MDA + 4-HNE).

To screen the possible effect of RF-EMF on endogenous antioxidant enzyme activity, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined in HL-60 cells that exposed to RF-EMF (1800 MHz, continuous wave at SAR 1.3 W/kg for 24h) or sham-exposed

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem (Cat. No. 574600, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, 4×10^6 cells were washed with PBS buffer, diluted in 250 μl of PBS buffer and extracted with 400 μl of a chloroform/ethanol mixture (62.5/35.5 v/v). 40 μl of the aqueous layer of this sample extract was mixed with 30 μl of diluted chromogenic reagent (R1) and 30 μl reagent R2 in 900 μl of an aqueous alkaline solution buffer. The SOD-mediated increase in the rate of auto-oxidation of this reaction mixture was utilised to yield a chromophore with maximum absorbance at 525 nm (Wang et al. 1991, Vilim and Wilhelm 1989). Results were expressed as SOD units. Detection limit for SOD activity is 0.2 U/ml.

Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity of cell homogenates was determined using a cellular glutathione peroxidase assay Kit from Calbiochem (Cat. No. 354104, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, to assay cellular glutathione peroxidase, 70 μl of cell homogenisate of 1×10^6 cells is added to a 1050 μl of a solution containing glutathione (GSH, 1 mmol/l), as a source of reducing equivalents, GSH reductase (0.4 U/ml) and NADPH. The reaction is initiated by the addition of 350 μl of the diluted organic peroxide t-butyl hydroperoxide and the absorbance at 340 nm was recorded over a period of 5 minutes. The rate of decrease in the absorbance (NADPH is converted to NADP) is directly proportional to the GPx activity in the cell homogenisate. Therefore, the difference in absorbance per min

was used to calculate the enzyme activity and results were expressed as GPx units/ mg protein. As a positive control cellular glutathione peroxidase at an activity of 288 mU/ml was assayed at 23°C.

2.3.8 Analysis of cellular growth behaviour

Cellular doubling time

Cellular growth behaviour of HL-60 cells following RF-exposure for 24h (1800 MHz, continuous wave, SAR 1.3 W/kg) with respect to growth velocity as compared to sham and incubator controls was assessed by determination of the cellular doubling time t_d :

$$t_d = \frac{\log 2 \cdot dt}{\log N - \log N_0}$$

with dt = time of exposure with RF field or sham-exposure, N_0 = number of cells at the beginning of the experiment and N = number of cells at the end of experiment.

Thymidine kinase (TK) assay

Thymidine kinase activities were determined by radioenzyme assay Prolifigen® TK-REA (AB Sangtec Medical, Bromma, Sweden) with ^{125}I -deoxyuridinemonophosphate as substrate. Briefly, assay buffer containing ^{125}I -labelled substrate was added to the HL-60 cell lysate (lysate diluted 1:100) and incubated for 4h. Lysis was performed with 5×10^4 cells and a NP40/Tween 20 containing lysis buffer. The reaction was stopped by addition of a separator tablet which binds the phosphorylated product. After washing, radioactivity was measured. The level of radioactivity is directly proportional to the enzyme activity in the original sample. The TK value was calculated from the standard curve and expressed as U/l.

2.3.9 Statistics

To compare the results of the different groups listed above, the Student's t-test (two-sided test) was used.

2.3.10 Proteomics

Different optimisation strategies for enhancement of 2-dimensional resolution of highly complex cellular protein mixtures were performed during the project. First of all we started with the IPG-strip approach/Pharmacia. Alternatively we also tested the tube gel approach and found, that for our system this proved to be the most suitable one with the best resolution. This is essential for the following identification steps. Therefore we here focus on the description of the tube gel technology. In general, the methodology for two-dimensional electrophoresis of protein mixtures described by Klose and Kobalz (1995), was performed. Alternatively, a protocol with slight modifications was applied, described below.

IEF-sample preparation

For separation of cellular proteins by 2-dimensional gel electrophoresis cells were lysed after thorough washing with PBS by repeated freezing and thawing. Proteins were solubilised in: 56 mg urea lyophilised with 16.8 µl 0.6 M DTE, 41 µl protein (corresponds to 250 µg of protein)/water, 5 µl Ampholyte 9-11, 8 µl 25% CHAPS, mixed for 30 minutes at room temperature, centrifuged 6 minutes at 14000 g, then the supernatant was taken off as sample.

IEF/2-D PAGE

High resolution 2-dimensional SDS-polyacrylamide gel electrophoresis (2-D PAGE, 23 x 30 cm gels, pI 2-9.5; 14 x 16 cm gels alternatively, pI 2-11) was performed as follows. For the isoelectric focussing of the small gels a pH-gradient extending from pH 2 to pH 11 was generated by means of carrier ampholytes. For the 14x16 cm 2D-gels six 13 cm long and 2.2 mm thick isoelectric focusing gels were prepared with 3.3 g urea, 780 µl acrylamide / N,N'-methylene-bis-acrylamide (30% T, 5.4% C), 480 µl 25% CHAPS, 1.92 ml water and 300 µl ampholyte mix. The ampholyte mix, which can be stored at -20°C, consisted of 340 µl servalytes 5-7, 340 µl ampholines, 113 µl servalytes 3-10, 113 µl servalytes 2-11 and 113 µl servalytes 3.5-10. The IEF-samples were loaded on the tube gel, overlaid with 10 µl sample overlay (12 M urea, 8% w/v CHAPS, 4% ampholyte 9-11, 0.16 M DTE) and with the upper chamber

buffer consisting of a sodium hydroxide solution (20 ml 1N NaOH in 980 ml water). The lower chamber buffer contained 0.6 ml 85% phosphoric acid in 900 ml water. Gels were run in a tube gel apparatus at room temperature. The samples are focused for 9000 Vh. After extrusion gels were equilibrated for 5 minutes in SDS equilibration buffer (10% v/v 0.5 M Tris HCl pH 6.8, 5% v/v 20 mM EDTA 2Na, 20% of a 10% SDS solution, before usage 1.54% w/v DTE was added) and then loaded onto the 14 x 16 cm second dimension vertical slab gel. This gel consisted of a 12.5% SDS separation gel (7.5 ml 2M Tris HCl pH 8.8, 0.4 ml 10% SDS, 1.9 ml 20 mM EDTA 2Na, 12.7 ml water, 15.1 ml of an acrylamide/bisacrylamide solution (30% T, 2.67% C), 12.7 ml water; polymerisation with 20 µl TEMED and 200 µl of a 10% ammonium persulfate solution) and a stacking gel (2 ml Tris HCl pH 6.8, 80 µl 10% SDS, 400 µl 20 mM EDTA 2 Na, 1.1 ml of an acrylamide/bisacrylamide solution (30% T, 2.67% C), polymerised with 8 µl TEMED and 80 µl of a 10% ammonium persulfate solution). The lower chamber buffer for the second dimension consisted of 400 ml Tris/glycine 10x stock solution, 40 ml 10% SDS, diluted to 4000 ml with water, the upper chamber buffer consists of 50 ml Tris/glycine 10x stock solution, 5 ml 10% SDS, diluted to 500 ml with water, 230 mg DTE and 50 µl 0.5% BPB. The isoelectric focussing gel was layered on top of the SDS stacking gel and overlaid with top chamber buffer. Each gel was run for approximately 4 h beginning with 20 mA, 30 mA within the stacking gel and finally 50 mA in the separation gel. After finishing electrophoresis the gels were fixed in 40% ethanol/10% glacial acetic acid overnight, then silver stained according to the method applied by Klose and Kobalz (1995) and then dried.

Two-dimensional polyacrylamide gels were first qualitatively analysed on a light box visually. Clear changes in the protein secretion pattern like newly expressed or disappearing proteins can be detected by this means. The human eye is capable of registering even very small variations like an increasing or decreasing secretion of a characteristic protein, whereas a quantitative analysis of changes protein secretion is not possible. Protein spots of the 2-D gels were displayed by standard staining procedures with silver for image analysis. The corresponding gels were digitised. The spots were detected and the master gel image was calibrated. Image analysis was performed with Proteom Weaver (Definiens, München, Germany). Known proteins serve as "landmark proteins". With the help of their isoelectric points and molecular weights an internal two-dimensional co-ordinate system can be generated. In this system an evaluation of the isoelectric point and molecular weight of any protein of interest together with the determination of its amount expressed in the sample is possible. Qualitative and quantitative analysis of single gels and a direct gel-to-gel comparison was performed by this method.

2.3.11 Gene expression profiling

In an approach to examine effects of RF-EMF on gene expression on the transcriptional level (transcriptome), changes of cellular RNA profiles were analysed by use of the array technology in collaboration with the Resource Center of the German Human Genome Project (Participant 12). Methodological details and detailed presentations of the results obtained together with Participant 12 are provided under 2.10.3 and 2.10.4.

2.4. Experiments with the human neuroblastoma cell line NB69 (Participant 5)

2.4.1 ELF-EMF exposure setup

50 Hz, sine wave magnetic fields (MF) at 10 µT, 100 µT or 2000 µT_{rms} were generated by a pair of coils in a Helmholtz configuration energised by a wave generator Newtronic Model 200MSTPC, (Madrid, Spain). The exposure setup used in these experiments was reproduced from that described by Blackman et al., (1993). Each exposure system consisted of two 1000-turn, 20-cm-diameter coils of enamelled wire, aligned coaxially 10 cm apart and oriented to produce vertically polarised magnetic fields. Cell culture dishes were placed in the uniform MF space within the coils for exposure or sham-exposure. Currents in the coils were adjusted and monitored using a multimeter (Hewlett Packard, model 974A, Loveland, CO) after the flux density was established with fluxgate magnetometers (Bartington, model MAG-3, GMW Assoc and Wandel and Goltermann S.A, EFA-3, Model BN 2245/90.20. Two pairs of coils were mounted

in the centre of magnetically shielded (co-netic alloy) boxes (Amuneal Corp., Philadelphia, PA) housed in incubators (Forma, Models 3121 and 3194) with a 5% CO₂, 37°C. The magnetic shielding allowed for reduced environmental fields at the samples' locations. With DC MF = 0.02-0.08 µT and AC MF = 0.07 - 0.1 µT. Each incubator contained a coil system and shielding box, but only one set was energised for each experiment.

Two sets of coils, shielding rooms and incubators were used. In each experimental run, one set of coils was energised at random. The samples in the unenergised set were considered sham-exposed control. See also 2.1.1

2.4.2 RF-EMF exposure setup

See 2.1.2

2.4.3 Cell culture and EMF-Exposure

The human neuroblastoma cell line NB69 was obtained from Dr. M.A. Mena, (Hospital Ramón y Cajal, Madrid) and cultured in Dulbecco's Minimum Essential Medium (D-MEM) supplemented with 15% (ELF-EMF) or 10% (RF-EMF) heat inactivated foetal calf serum (FCS, Gibco), 2 mM L-Glutamine and 100 U/ml penicillin / 100 U/ml streptomycin. The cells were grown at 37°C in a CO₂ incubator. In each experiment, cells were seeded at a density of 4.5×10^4 cells/ml in Ø 60 mm plastic dishes. NB69 cells cultured in D-MEM were exposed and/or incubated in the presence or absence of retinoic acid (RA). In the experiments with RA, 40 dishes were supplemented with 0.0 µM (20 dishes) or 2.0 µM all trans RA (20 dishes) in absolute ethanol dissolved (1:1000) in culture medium. This vehicle was proven not to affect significantly cell growth when compared to cultures treated with the same volume of medium alone. In the RF-EMF experiment, cells were seeded at a density of 4.5×10^4 cells / ml in Ø 35 mm dish (NUNC) in 12 dishes, supplemented with 0.0 ng/ml (6 dishes) or 20 ng/ml of bFGF (human recombinant, Boehringer Mannheim GmbH, 6 dishes). Immunocytochemical and *in situ* hybridisation studies were carried out 4 days post-plating.

Isolated embryonic neural stem cells

Striata from E15 Sprague-Dawley rat embryos were dissected and mechanically dissociated. Cell suspensions were grown in a defined medium (DF12), composed of Dulbecco's modified Eagle's medium and Ham's F-12 (1:1), 2 mM L-glutamine, 1 mM sodium pyruvate (all from Gibco BRL, Life Technologies Inc, Grand Island, New York), 0.6% glucose, 25 mg/ml insulin, 20 nM progesterone, 60 µM putrescine, and 30 nM sodium selenite (all from Sigma Chemical Co, St Louis, MO), 100 mg/ml human transferrin and 50 ng/ml human recombinant EGF (both from Boehringer Mannheim GmbH, Germany). After a minimum of five passages, cells were plated at a density of 500.000 cells/dish (Ø 35 mm) on 12 mm glass coverslips coated with 15 µg/ml poly-L-ornithine (immunocytochemistry) or 50 µg/ml poly-L-lysine (in situ hybridisation). The cultures were maintained in DF12 and EGF for 3 days and then switched to DF12 without EGF for longer culture periods. Immunocytochemical and in situ hybridisation studies were carried out at day 3 post-plating. For additional information see Reimers et al., (2001). Neural stem cells (NSCs) are self-renewable, multipotential cells capable of differentiating into the three major neural cell types. The mechanisms, involved in the regulation of NSC's differentiation are not fully understood.

Test for cellular response to retinol (ROL) or retinoic acid (RA)

Different concentrations of ROL and RA were selected within the physiological range in mammals: 0.1 µM to 5 µM. Cells were seeded and supplemented with ROL concentrations of 0.0, 0.1, 0.5, 1.0, 2.0, or 5.0 µM or with RA concentrations of 0.0, 0.5, 1.0, 2.0, or 5.0 µM. Seventy-two hours after plating, the medium was removed and replaced with fresh medium supplemented with the corresponding ROL or RA concentrations. Each of the retinoid concentrations was triple tested (a total of 15 petri dishes per experimental replicate), and a total of 3 experimental replicates were carried out. At the end of five days of incubation in the absence or presence of ROL or RA the cells were scrape-collected in 1 ml of culture medium. Aliquots of the cell suspensions received 1 ml of 0.4% Trypan Blue, and the number of total cells and of viable cells were calculated using a Neubauer chamber. Each sample was double counted.

ELF-EMF exposure conditions

In each experimental replicate 20 dishes with cells (10 with 0.0 µg/ml RA and 10 with 2.0 µg/ml RA) were incubated for three days inside pairs of unenergised Helmholtz coils placed in a shielded chamber, inside a 5% CO₂ incubator, at a 37°C and 100% RH atmosphere. At the end of day 3 the media were renewed and 10 dishes (5 with 0.0 µg/ml RA and 5 with 2 µg/ml RA) were placed in one incubator; the remaining ten dishes (5 with 0.0 µg/ml RA and 5 with 2 µg/ml RA) were placed in an identical incubator. Both incubators were used, in a random sequence, alternatively for MF exposure and sham-exposure. The exposed group was treated intermittently, 3h on/3h off, to 50 Hz ELF-EMF at 10 or 100 µT magnetic flux densities (MFD) for 42h or 63 hours. At the end of this period the cells were checked for appropriate viability and proper immunocytochemical characteristics before being processed for analysis of their responses to the physical and chemical treatments. Spectrophotometric analysis of total protein and DNA contents were done following the methods described in Mena et al., (1995). As we described below, cell counting by Trypan Blue exclusion, BrdU incorporation in DNA, PCNA labelling and flow cytometry were assayed in cells exposed to 10 or 100 µT as an estimation of the proliferative activity of NB69 cell line. The percent of apoptotic cells was estimated with TUNEL-labelling (TdT-mediated dUTP nick labelling) and the expression of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB) was analysed using phosphorylation site-specific antibodies. Assays and analysis were performed blinded to treatment condition. The statistical test used was ANOVA followed by Student's T-test for unpaired data.

RF-EMF exposure condition

Neural stem cells (NSC) and NB69 cells grown on coverslips were exposed for 21 or 24 hours, respectively to 1800 GSM signals (Talk, Basic, CW and DTX signals), at 1-2 W/kg SAR), in 5 min On/10 min Off cycles. In the experiments with neural stem cells a total of 10 replicates were carried out. In each replicate a total of 12 dishes (with three coverslips per dish) were RF-EMF-exposed or sham-exposed, in groups of 6 samples. In the experiments with human neuroblastoma cells a total of 27 replicates were carried out. In each replicate a total of 12 dishes were exposed, in groups of 3 samples, to one of the following treatment combinations: untreated controls, bFGF alone, EMF alone, bFGF + EMF. The sham-exposure and the RF-EMF exposure were carried out inside shielded chambers (IT'IS Setup, Schuderer et al., 2001), in a 5% CO₂, 37°C and 100% humidity atmosphere. At the end of the 21-h (NSC) or 24-h (NB69) period of RE-EMF exposure or sham-exposure the cell responses were checked for appropriate viability and proper immunocytochemical characteristics before being processed for analysis of their responses to the physical and chemical treatments through Trypan Blue exclusion, immunocytochemical and in situ hybridisation studies.

Cell counting and cell viability

After treatment the cells were detached from culture dishes and resuspended in 1 ml of media. The cell number was determined in aliquots of 50 µl using a haemocytometer and each sample was double-counted by Trypan Blue exclusion. Doubling time (DT) of proliferating cells was calculated according to Falasca et al., (1999) using the formula $DT = T2 - T1 / 3.32 \log (X2 / X1)$, where T1 and T2 are the culture times in hours, and X1 and X2 are number of cells at the corresponding time. The ANOVA test followed by Student's T-test for unpaired data was used for statistical significance ($p < 0.05$).

2.4.4 Immunocytochemical characterisation of NB69 Cells

Cells were grown in Ø 60 mm dishes onto 12 mm-diameter round coverslips for immunocytochemical detection. After fixation with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, the cells were incubated for 30 min in a blocking solution containing 2.5% (wt/vol) BSA in phosphate-buffered saline (PBS) to prevent non-specific antibody binding. The same solution was used to dilute the different antibodies. Cells were successively incubated with a mouse monoclonal antibody raised against the neuron-specific intermediate filament BIII-tubulin, and for the astrocyte-specific glial fibrillary acidic protein, GFAP), (1:1000, Promega; 1:300, Sigma, respectively). Nestin immunostaining was carried out by 1:5000 dilution of a rabbit antiserum, followed by an anti-rabbit IGL labelled with FITC (1:200; Jackson ImmunoResearch, West Grove, PA, USA).

2.4.5 Immunocytochemical characterisation of neural stem cells (NSC)

EGF-expanded neurospheres were seeded onto adherent substrate and treated with EGF during their first 3 days in culture, in order to enhance expansion of precursor cells. After this period the mitogen was withdrawn, and cells grew in a defined medium, which promoted cell differentiation to neurons, astrocytes and oligodendrocytes. Between 2h and 3 days, the cultures, mainly contained nestin-positive, undifferentiated precursors. At later stages, the total number of cells dropped, as a gradual loss of nestin content occurred, together with an enhancement in the differentiation processes of neurons, oligodendrocytes and astrocytes.

2.4.6 Immunocytochemical staining for the Cell Nuclear Antigen (PCNA).

PCNA, the auxiliary component of DNA polymerase delta, is a proliferation-induced, 36 kD nuclear protein. The expression of PCNA in tissues has been found to be correlated with proliferative activity. In fact, it has been suggested (Kawasaki et al. 1995) that PCNA levels may reflect differences in the proliferative activity of neuroblastomas, as they evolve through different stages of the disease. However, PCNA is also necessary for nucleotide-excision repair of DNA. In the present work, we estimate PCNA positive cells in eight experiments with cells exposed to the MF at 10 μ T on day 3 after plating. At the end of the 42-h exposure period, PCNA positive cells were determined by immunostaining, using PCNA-labelling and Hoechst for quantification of total number of cells. Cells were stained with 2 μ g/ml Hoechst dye 33342 for 10 min at room temperature, studied and photographed with fluorescence microscope. In a total of 13 experiments the cells were exposed to the MF at 100 μ T on day 3 after plating, and processed for PCNA labelling at the end of the 42 and 63-h exposure (day 5 and 6 postplating). The proportion of PCNA+ cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips (two control and two exposed) per experimental replicate. All determinations were carried out following blind protocols.

2.4.7 5-bromo-2'-deoxyuridine (BrdU) labelling for identification of cells synthesising DNA.

Samples exposed to 100 μ T were labelled with BrdU at different times during the exposure period: Time 0 of exposure, (day 3 after plating); time 21h (day 4) or time 42h (day 5). The cells were always analysed 21 hours after the BrdU application, i.e. at 21, 42 or 63 hours of exposure. The results were compared to those in the respective controls (BrdU-treated, MF-unexposed). The analysis was performed through total cell number counting (Trypan Blue exclusion protocol) and by quantification of anti-BrdU antibody positive cells. The proportion of BrdU+ cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips per experimental replicate. All determinations were carried out following blind protocols.

2.4.8 Flow cytometry assay

DNA content and cell cycle phase distribution were analysed by flow cytometry with propidium iodide DNA staining, in cells exposed to 100 μ T for 42 or 63 hours (day 5 or 6 post-plating, N= 3 or 6 experiments, respectively, with two replicates per experimental condition). Cells were harvested, fixed with 70% ethanol and incubated with RNase A (100 μ g/ml) and the DNA intercalating dye propidium iodide (20 μ g/l) in citrate buffer (3.4 mM). The cell cycle phase analysis was performed by flow cytometry using a Becton Dickinson FAC flow cytometer and Becton Dickinson CellQuest software. All determinations were carried out following blind protocols.

2.4.9 Apoptosis assay

In order to investigate the potential influence of 50 Hz MF on apoptosis the percent of apoptotic cells was estimated with TUNEL-labelling (TdT-mediated dUTP nick labelling) after 63 hours of exposure. Additional assays for apoptosis were carried out through flow cytometry in propidium iodide-stained neuroblastoma cells, exposed to the MF for 42 or 63 h (N= 7 experimental replicates). The distribution pattern of apoptotic nuclear DNA was determined using a Becton Dickinson FAC flow cytometer and Becton Dickinson CellQuest software. Three additional experiments were conducted and the cellular

response was analysed through TUNEL procedure. After fixation in 4% paraformaldehyde, cells were washed with PBS, permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate, washed again with PBS, and incubated for 60 min at 37°C with biotin-conjugated dUTP in a TdT (terminal deoxynucleotidyl transferase, 25 U/ml) catalysed reaction (Roche Molecular Biochemicals) in a humidified atmosphere in the dark. The labelled nuclei (dUTP) were revealed with 3,3'-diaminobenzidine. The cells were counterstained with methyl green. *In situ* labelled nuclei were quantified by image analysis and photographed under light microscope. All determinations were carried out following blind protocols.

2.4.10 Immunocytochemical staining for the expression p-CREB

CREB is a nuclear transcription factor that regulates expression of genes controlling cell proliferation, differentiation, and survival. In fact, this protein is known to play an important role in neuronal survival and plasticity. Besides, different alterations of the CREB family of transcription factors have been observed in tumours. The cells were grown on coverslips. The MF-exposed samples (50 Hz; 100 μ T, 30 or 60 minutes exposure) and their respective controls were labelled at the end of the exposure period for 30 min, 60 min, or 2 hours using phosphorylation site-specific antibodies. As positive controls, samples were treated with bFGF at a concentration known to activate p-CREB immunoreactivity in neuronal cells. The proportion of p-CREB cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips per experimental replicate. All determinations were carried out following blind protocols. For analysis of Ser¹³³-phosphorylated CREB and total CREB, Western blotting was performed on 2×10^6 cells per experimental point. Cell pellets were added at 4°C with a lysis buffer containing 1% deoxycholate, 1 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1mM sodium orthovanadate for 10 min. Cell lysates were sonicated and either immediately processed by Western or kept frozen until assayed. Protein concentration in the samples was estimated by the method of Lowry et al., (1951). Equivalent (50 μ g) amounts of proteins per sample were subjected to electrophoresis on a 10% sodium dodecyl sulphate-acrilamide gel. The gel was then blotted onto a nitrocellulose membrane. Blotted membranes were blocked for 1h in a 4% suspension of dried skimmed milk in PBS and incubated overnight at 4°C with a rabbit polyclonal anti-P-CREB serum; against the phosphorylated Ser133 form of CREB (1:1000 dilution). The membranes were washed and incubated for 1h at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G (dilution 1:1000). Specific reactions were revealed by the ECL Western blotting detection reagent (Amersan Biosciences).

2.4.11 Indirect immunocytochemistry

Rabbit polyclonal antibodies and mouse monoclonal antibodies against neural antigens and receptors for FGF were used as primary antibodies for indirect immunocytochemistry. Polyclonal anti-FGFR1, anti-FGFR2 and anti-FGFR3 were purchased to Santa Cruz Biotechnology Inc. Burlingame, CA. Anti-GFAP was obtained from Dakopatts a/s, Glostrup, Denmark. Monoclonal anti β -tubulin isotype III and anti-GFAP were obtained from Developmental Studies Hybridoma Bank (University of Iowa), Sigma Chemical Co, and Boehringer Mannheim GmbH, respectively. Secondary antibodies raised in goat against rabbit, and in sheep against mouse immunoglobulins, conjugated to alexafluor were purchased to Jackson Boehringer Mannheim GmbH. After appropriate culture time, cells grown on poly-L-ornithine coated coverslips were fixed with 4% paraformaldehyde for 10 min, rinsed 3 times in phosphate-buffered saline, then blocked with 10% foetal calf serum, and subsequently incubated in primary antibodies for 1 hour at room temperature (for growth factor receptors). Permeabilisation for intracellular antigens was achieved by incubation with ethanol acetic solution at -20°C for 20 min. To assess non-specific binding for each antibody, adjacent cultures were incubated in buffer, without primary antibody. For dual labelling, primary antibodies generated by different species were added together. Secondary antibodies were administered for 45 min in the dark at room temperature. The coverslips with cells were mounted in a medium containing p-phenylenediamine and bisBenzimide (Hoechst 33342, Sigma Chemical Co). In selected experiments, FGFR1 was immunoperoxidase detected, using a biotin-linked (Vector Laboratories Inc, Burlingame, CA), instead of a fluorescence-linked secondary antibody, followed by incubation with an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories Inc). Finally, peroxidase was developed with 0.05% DAB, 0.005% hydrogen peroxide. The samples were counterstained with haematoxylin.

2.4.12 Hybridisation histochemistry

The hybridisation protocol has been adapted for all cell cultures from that reported by Simmons et al. (1989). The cells were fixed with 4% paraformaldehyde for 30 min at room temperature, dehydrated, air dried for 2 hours, and stored at -80°C . Before hybridisation, the cultures were treated with proteinase K at a doses ranging from 0.1 to 1 $\mu\text{g/ml}$ for 10 min at 37°C , acetylated with acetic anhydride, dehydrated and air dried. Each coverslip was overlaid with 50 μl of hybridisation solution (50% formamide, 0.3M CNa, 10mM Tris pH 8.0, 1mM EDTA, 0.5 mg/ml transfer RNA, 0.5 mg/ml total yeast RNA, 1x Denhardt's solution, 10% dextran sulphate, 10 mM DTT) containing $1 \times 10^7 \text{cpm/ml}$ [^{35}S]-radio-labelled probe (FGFR1, FGFR2, FGFR3, FGFR4 cRNAs). In each experiment parallel cultures were hybridised with the complete probes and with the hydrolysis product of each type of probe. Alkaline hydrolysis was performed by incubating probes at 60°C with bicarbonate buffer, pH 10.2, during appropriate periods of time, in order to obtain fragments of 250 bp. Hybridisation was carried out at 55°C overnight, and thereafter coverslips were treated with 20 $\mu\text{g/ml}$ ribonuclease A for 30 min at 37°C . Coverslips were washed at increasing stringency with the final wash in 0.1x SSPE (10mM NaH_2PO_4 , pH 7.4, 0.15M NaCl, 1mM EDTA), containing 1mM DTT, for 1 hour at 45°C . After dehydration, cultures were air dried for 2 hours. In order to facilitate subsequent manipulation of coverslips, they were fixed to slides with DPX, so that 2 similar cultures, hybridised with the antisense and sense probes respectively, were attached to the same slide. Cultures were exposed to X-ray film for 5 days, and then dipped in Kodak NTB-2 liquid autoradiographic emulsion, and exposed for 4 weeks at 4°C prior to development in Kodak D-19. Finally, the cultures were counterstained with haematoxylin and eosin and analysed.

2.4.13 Nucleic acid probes

[^{35}S]-UTP (ICN Pharmaceuticals Inc, Irvine, CA) labelled probes were synthesised in a run-off transcription reaction, using T3 or T7 and SP6 or T7 polymerases to generate antisense or sense RNAs, respectively.

2.4.14 Analysis of immunocytochemical data

The results were expressed as mean \pm SEM from 4 to 6 independent experiments (per treatment) done in duplicates (two coverslips). Where indicated, data are normalised in relation to their own controls, and represent the mean \pm SEM of two coverslips from 4 independent experiments. In each coverslip, 30 predetermined visual fields (400X amplification) were counted under fluorescence microscopy through a program of Image Analysis. Statistical analyses were performed using Student's T test, and differences were considered significant when $p \leq 0.05$.

2.5 Experiments with human lymphocytes and thymocytes and embryonic stem cells of mice during cardiac differentiation (Participant 8)

2.5.1 ELF-EMF exposure setup

The ELF-EMF exposure setup used by Participant 8 is based on two unshielded 4-coil systems arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied (see 2.1.1). It was composed by two systems, one used for the active exposure and one used as sham. Each system was composed by four circular coils; each coil being double-wrapped, in order to obtain a wound (active) or counter-wound (sham) configuration. The characteristics of the coils were the following: internal radius of the top and bottom coils 9.2 cm, numbers of turns 40 (20+20); internal radius of the two central coils 6.6 cm, numbers of turns 40 (20+20); distances between the coil centres 7.9 cm. The coil configuration was calculated in order to have a large zone of high uniformity (1%). The two systems were powered in series by an home-made DC amplifier connected to a function generator (Beckman FG3A). The ELF set up was kept inside a commercial CO_2 incubator (HeraCell) and the temperature was monitored by means of an high precise thermoresistor. At variance with the ELF set up used by Participant 4 the automated blind protocol was not implemented in the system by means of a suitable switcher and the experiments were

done in blind by the experimental protocol.

2.5.2 RF-EMF exposure setup

See 2.1.2

2.5.3 Cell proliferation by ³H-TdR incorporation test

Peripheral blood mononuclear cells (PBMCs) from 20 young donors, were separated by centrifugation on Histopaque-1077 (Ficoll Histopaque, Sigma Chemical, St. Louis, MO, USA) discontinuous density gradient (Böyum 1968). One hundred µl of cell suspension containing 10⁵ PBMC in complete medium (RPMI 1640 with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated AB serum from a pool of 10 human donors) was distributed in microplate wells (Costar, Cambridge, Ma, USA) and added with 0.1 ml of medium with or without mitogen. The following mitogens were used to promote lymphocyte proliferation: phytohemagglutinin (PHA-P, Difco, Detroit, MI, USA) at the final concentrations of 0.1, 1, 5 and 10 µl/ml; anti-CD3 monoclonal antibody (mAb) (OKT3, an IgG2a mAb, from ATCC, Rockville, MD, USA) at the final concentration of 10 ng/ml. Each point was performed in quadruplicate. Cultures were incubated and sham-exposed or exposed to 50 Hz magnetic field (50 µT) for 2 up to 6 days (5% CO₂ in a humidified atmosphere). ³H-methyl-thymidine (³H-TdR, Amersham Int., UK, specific activity 5 Ci/mM) was added for the last 6 hours of culture (0.5 µCi/well). At the end of the incubation period, PBMC were harvested and washed on fibre filters by a multiple cell culture harvester (Skatron, Norway); ³H-TdR incorporation was measured by liquid scintillation counting (b-counter, Beckman).

2.5.4 Cell proliferation by flow cytometry

PBMCs from young donors were marked by using the fluorescent cell tracer Carboxyfluorescein diacetate, succinimidyl ester (CFDAse, Serotec, UK). It couples irreversibly intracellular proteins by reaction with lysine side-chains and other available amine groups. Cells become fluorescent and after mitogenic stimulation (with 1 or 0.1 µl/mL PHA, phytohaemoagglutinin; in some experiments anti-CD3 10 ng/mL was also used), the dye is divided between the daughter cells. Each division results in generation of a population of cells that is marked by half of the cellular fluorescence intensity. PBMCs were cultured in petri dishes (35mm) at the concentration of 1x10⁶/mL of medium and exposed to RF. Two intermittent types of exposure were applied using Talk modulated RF signal (SAR 2 W/kg): (1) 10 min on/20 min off for 44 h; (2) 2h on/22h off for 72 hours. We performed experiments with cells from 6 donors using the former, from 11 donors using the latter. All cells were acquired and analysed after 72 h and 120 h of culture. At the end of time culture, cells were harvested and labelled by anti-CD4, anti-CD8 and anti-CD28 monoclonal antibodies (Serotec, UK), in order to discriminate helper and cytotoxic T cells with or without the co-stimulating molecule CD28, fundamental for the activation of lymphocytes.

2.5.5 Cell cycle analysis by flow cytometry

Cell cycle was analysed in PBMCs exposed either to ELF and RF-EMF. In ELF experiments, PBMCs from 9 young donors were stimulated by PHA (optimal dose, i.e. 1 µl/ml) or anti-CD3 (10 ng/mL) and exposed or sham-exposed for 24, 48, 72 and 96 hours. In RF experiments, PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR= 1.3 W/Kg). The exposure time was 10 minute on and 20 minute off for 44 hours.

Cell cycle analysis was performed by the method of 5-bromo-2'-deoxyuridine (BrdU) incorporation and propidium iodide (PI) staining. Briefly, at the end of the incubation period, cells were labelled with 20 µM BrdU for 30 min., centrifuged for 1 min., washed twice with 1 ml of PBS solution containing 0.5% Tween 20, and resuspended in 1 ml HCl 1N. After a 30 min. incubation at room temperature, cells were centrifuged at 300g for 1 min. washed once in 0.1 M Na₂B₄O₇, and added with 5 µl of anti-BrdU mAb (Becton Dickinson, San José, CA, USA). Cells were incubated for 60 min. at 4°C, washed twice and

resuspended in 200 µl of diluted secondary antibody (goat-anti-mouse IgG conjugated with fluorescein isothiocyanate, FITC). After a 30 min. incubation at 4°C, cells were washed twice and resuspended in 200 µl of PBS solution with 0.5% Tween 20 and 200 µl of PI working solution (50 mg/ml in 3.4 mM trisodium citrate, 9.65 mM NaCl plus 0.03% Nonidet P40). After 15 min. at 4°C in the dark, cells were acquired and analysed by flow cytometer.

2.5.6 Expression of membrane receptors on T lymphocytes by flow cytometry

Phenotypical analysis of T lymphocytes was performed in PBMCs exposed either to ELF and RF. In ELF experiments, HLA-DR and CD25 membrane expression were analysed on CD3+ T and CD4+ T helper lymphocytes respectively. The analysis was performed on cells from 9 young donors, before and after stimulation with PHA (1 µl/ml) or anti-CD3 mAb (10 ng/ml). Briefly, cells were stimulated and exposed from 24h to 72 h, collected, washed twice with cold PBS and stained with different mAbs directly conjugated with FITC or phycoerythrin (PE). The following mAbs from Becton Dickinson and from Serotec (Oxford, UK), were used: anti-CD3, recognising all T cells; anti-HLA-DR, recognising B cells and activated T cells; anti-CD4, reactive with helper/inducer T cell subset; anti-CD25, reactive with the p55 chain of IL-2 receptor. The expression of HLA-DR and CD25 molecules was studied on CD3+ and CD4+ lymphocytes at 24, 48, 72h after mitogen stimulation. In RF experiments, PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors were exposed to TALK signal (SAR=2 W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). CD25, CD95 and CD28 in CD4+ helper and CD8+ cytotoxic T lymphocytes, respectively, were analysed by flow cytometry technique. (In specific: CD95 is the receptor activating the pathway of programmed cell death). Lymphocytes before and after exposure to RF were phenotypically analysed. Cells, unstimulated or stimulated with anti-CD3 mAb (10 ng/ml), immediately after exposure or sham-exposure, were collected, washed twice with cold PBS and stained with three different mAbs (CD25, CD95, CD28, CD4 and CD8, Serotec, Oxford, UK) directly conjugated with FITC or phycoerythrin (PE), or tricolor fluorocromes. The analysis was performed on 10,000 lymphocytes for each sample and the three fluorescences were analysed using "paint-a-gate" software (Becton Dickinson).

We performed also from 5 up to 8 replications, using PBMCs from the same young donor. Cells were unstimulated or anti-CD3-stimulated (10 ng/mL) and exposed or sham-exposed to TALK modulated RF (SAR 2 W/kg) for 44h (10 min on/20 min off). After exposure, cells were stained using the same protocol describe before. The following membrane molecules were analysed: CD25, CD95, CD28, CD45RO, HLA-DR on CD4+ and CD8+ T lymphocytes, respectively. Moreover, a more sophisticated analysis was performed on fluorescence distribution of CD4+ helper T lymphocytes from 10 young and 8 elderly donors

2.5.7 Spontaneous and induced apoptosis by flow cytometry

PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). PBMCs were induced to undergo apoptosis by 2-deoxy-D-ribose (dRib) (Barbieri et al, 1994), which acts through an oxidative pathway (Monti et al. 2000) and the early stage of apoptosis was assessed by Annexin-V and propidium iodide kit (ANX-V, Bender, Vienna, Austria) using flow cytometry technique. Briefly, cells were collected, washed in PBS and resuspended in 200 µL of ANX-V binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), stained with 5 µL of ANX-V and incubated for 10 minutes at room temperature. Then, cells were washed with binding buffer to remove the excess of ANX-V, resuspended in PBS, counterstained with 5 µg/ml PI and analysed by flow cytometry. ANX-V, the probe used for the detection of early stage apoptotic cells, is able to recognise phosphatidylserine (PS) when present on the outer leaflet of the plasma membrane. It is well known that PS is normally found only on the inner leaflet of the cell membrane double layer, but it is actively transported to the outer layer as an early event in apoptosis and becomes available for annexin binding (Green and Steinmetz 2002).

2.5.8 MMP modifications in induced and spontaneous apoptosis

PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young

and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). Changes in MMP have been evaluated by using the lipophilic cationic probe JC-1, which changes reversibly its colour from green to orange as MMP increases (over values of about 80-100 mV). This property is due to the reversible formation of JC-1 aggregates upon membrane polarisation that causes a shift in the emitted light from 530 nm (*i.e.*, emission of JC-1 monomeric form) to 590 nm (*i.e.*, emission of J-aggregates) when excited at 490 nm; the colour of the dye changes reversibly from green to orange/red as the mitochondrial membrane becomes more polarised. Both colours can be detected using the filters commonly mounted on flow cytometers, so that green emission can be analysed in one fluorescence channel, and orange/red emission in the other. Briefly, cells were stained with 2.5 µg/mL JC-1 and kept at room temperature for 10 minutes, washed twice with PBS, resuspended in a total volume of 400 µL PBS and analysed (Cossarizza et al. 1993b; Salvioli et al. 1997).

2.5.9 Cytokine production by ELISA

The production of interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) in unstimulated and stimulated PBMCs from young donors was determined in the supernatant of cultures. Two types of stimuli were used: a) 10 ng/mL anti-CD3, b) 10 ng/mL TPA (12-O-tetradecanoylphorbol-13-acetate) plus 1 µ/mL PHA (phytohaemoagglutinin). PBMCs from 26 donors were stimulated with the former and 24 were stimulated with the latter. The second stimulus is stronger than the first, since it is directed on monocytes. PBMCs were exposed both to GSM talk (2 W/kg SAR) and DTX only (SAR 1.4 W/kg) RF for 44 hours (10 min on/20 min off). At the end of time culture, supernatants were harvested and frozen. After the collection of all the samples we analysed the presence of cytokines by ELISA (Enzyme linked Immuno-adsorbant assay), a well known immuno-enzymatic method, by which cytokine is revealed by a coloured end-product. By using an ELISA plate reader final data are produced as pg/mL units.

2.5.10 Hsp70 levels in induced and spontaneous apoptosis by flow cytometry

PBMCs, obtained from 7 healthy young donors, were treated by 2-deoxy-d-Ribosio (dRib, 10 mM) for 44 h and in the meantime cells were exposed to RF (GSM Talk signal, SAR 2W/kg; 10 min ON and 20 min OFF). Detection of intracellular Hsp70 was performed by flow cytometry techniques (Bachelet et al., 1998). At the end of culture, cells were collected, fixed and permeabilised by ethanol 95% and acetic acid 5% for 15-30 minutes, at -20°C. Then, cells were washed with PBS with 1% of BSA and labelled with Hsp70 primary antibody (Mouse IgG, 70 KDa for human target, Pharmingen, BD, San Josè, CA, USA) for 1h at 4°C in the dark. Then, cells were incubated with a secondary fluorescent antibody (GOT anti-mouse, Becton Dickinson), FITC conjugated (isothiocyanate of fluorescein) for 30 min at 4°C in the dark. At the end of the incubation cells were washed and analysed by flow cytometer (FCSalibur, BD). Fluorescence intensity was evaluated by CellQuest® programme.

2.5.11 Thymocyte development and apoptosis by HTOC and flow cytometry

Pieces of human thymus were obtained by cardio-surgery from S.Orsola-Malpighi Hospital (Bologna, Italy) from 6 human newborn. (5 days-8 months). Small fragments of tissue (2-3 mm³) were cut and cultured above a sterilised filter on a small piece of gelfoam which was embedded of medium (20% of FCS, 1% of penicillin-streptomycin, 79% of DMEM). Each gelfoam was placed inside the Petri dishes containing the DMEM medium. This is a standard technique used to analyse in vitro thymocyte differentiation (Anderson and Jenkinson 2000). Human thymus organ cultures (HTOC) were incubated for 48 h, but the exposure (or sham-exposure) was performed only during the first 24h (DTX only at SAR 1.4 W/kg; 10 min on / 20 min off). At the end of culture (48 h), thymocytes were separated by gentle pressing through a fine stainless steel screen submerged in PBS. Single cell suspensions were obtained by passing the cells through a steel filter, and washing twice in PBS. Thymocytes were directly labelled with FITC or PE conjugated mAbs; such as, CD4, CD8, $\alpha\beta$ TCR (T cell receptor), $\gamma\delta$ TCR, CD71 (transferrin receptor on proliferating cells) and CD16 (receptor for IgG) in order to discriminate different phenotypical phases of differentiation. Moreover, apoptosis and viability were assessed by annex-V (see the methods described above) and PI staining, respectively. 10,000 cells from each sample were acquired by flow cytometer and analysed by paint-a gate-software.

2.5.12 T lymphocyte gene expression by microarray technology

Quiescent T lymphocytes were separated by MACS® (Magnetic Cell Sorting, Miltenyi Biotec, Germany). Three samples were obtained from control cultures, sham-exposure and exposure to DTX only RF for 44h (10 min on/20 min off). These samples were sent to Participant 12 for gene expression analysis by microarray technology. Data analysis were performed in Bologna by Participant 8.

2.5.13 Cell culture of embryonic stem cells and EMF-Exposure

GTR1 ES cells, a derivative of R1 ES mouse cells (Nagy 1993) bearing the puromycin-resistance gene driven by the cardiomyocyte-specific MHC promoter (GTR1 cells were kindly provided by Dr. William L Stanford (University of Toronto and Centre for Modelling Human Disease, Canada). ES cells were maintained in the undifferentiated state by culturing in DMEM containing 15% FBS, supplemented with a final concentration of 1000 U/ml ESGRO-LIF (LIF). To induce cardiac differentiation, cells were plated onto bacterial Petri dishes, containing DMEM lacking supplemental LIF. After 2 days of culture, the resulting embryoid bodies (EBs) were plated onto tissue culture dishes. When spontaneous contractile activity was noticed, puromycin (2 µg/ml) was added to eliminate non-cardiomyocytes. After 2 days, puromycin-selected myocytes were transferred to new tissue culture dishes. As indicated in the legend of each figure, EBs, collected at several stages after plating, as well as puromycin-selected cells, were processed for gene expression analyses. Following LIF removal and throughout puromycin selection, GTR1 cells were exposed to ELF-EMF (50 Hz, 0.8 mT_{rms}).

2.5.14 Analysis of mRNA expression

Expression of GATA-4, Nkx-2.5, prodynorphin, alpha-myosin heavy chain and myosin light chain-2V mRNA was assessed by RT-PCR as previously described (Ventura 2000), using GAPDH mRNA as a measure of equal loading and mRNA stability. GATA-4 mRNA levels were also quantitated by RNase protection assay, as described elsewhere (Ventura 1997, Ventura 2003a, Ventura 2003b). Briefly, fragments of the main exon of the mouse GATA-4 (292 bp) gene was inserted into pCRII-TOPO (Invitrogen, CA). Transcription of the plasmid linearised with *Bam*HI generated a sense strand of GATA-4 mRNA, which was used to construct a standard mRNA curve. Transcription in the presence of [³²P]CTP (800 Ci/mmol) (Amersham International) of plasmids linearised with *Xba*I produced an antisense strand of GATA-4 mRNA (radio-labelled cRNA probe). Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was performed for 48h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and cpm values were translated to pg values on a correlated standard curve. Data were expressed as pg of mRNA/µg of total RNA.

2.5.15 Transcriptional analysis in isolated nuclei

Nuclear run-off was performed. 90 µl of nuclear suspension were added with 100 µl of 2 x reaction buffer (10 mmol/L Tris/HCl, pH 7.5, 5 mmol/L MgCl₂, 0.3 mol/L KCl, 5 mmol/L dithiothreitol, 1 mmol/L each of ATP, GTP, and CTP), and 5 µl of [α-³²P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 µl of 20,000 units/ml RNase-free DNase. Equal counts of ³²P-labeled nuclear RNA (about 5 x 10⁶ cpm) were then subjected to a solution hybridisation RNase protection assay and were hybridised for 12h at 55°C in the presence of unlabelled antisense GATA-4 mRNA. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was for 48h. ³²P-labeled nuclear RNA was also hybridised with unlabeled antisense cyclophilin mRNA synthesised from a *Nco*I-linearised pBS vector containing a 270-base pair fragment of pIB15, a cDNA clone encoding for rat cyclophilin (6). Cyclophilin mRNA was utilised as a constant mRNA for control.

2.6 Experiments with brain cells of different origin and human monocytes and endothelial cells (Participant 9)

2.6.1 Exposure setup and exposure conditions

The wire-patch cell (WPC) is the setup that was used for exposure to frame-scheme (FS) GSM-900 signals. This exposure system accommodates 8 Petri dishes to be built as a double Petri dish, i.e. a 3.5-cm diameter Petri dish (where cells are cultured) is positioned inside a 5-cm diameter dish (outer dish with distilled water inside). Two double Petri dishes are piled-up and placed in the wire-patch antenna (Figure 3) so that a total of 8 dishes can be exposed at a time. Two successive WPC models were used. The second one shown in Figure 1, built by Participant 10, was fitted with electric field probes that allowed monitoring of the SAR in real time.

Cells were cultured in 3.2 ml of culture medium and the outer Petri dishes are filled with 5 ml of distilled water. Cultured cells in Petri dishes were placed in a standard CO₂ air-flow incubator inside a WPC for a minimum of 3 hours to allow for temperature stabilisation. Sham exposed samples were treated in the same way in a non-activated WPC placed in a second, identical incubator. Each WPC was fitted with a square annular ring made of absorbing foam (Figure 3) to block emission sideways towards the metallic walls of the incubators and allow for a good adaptation at 900 MHz. The foam is sealed with waterproof lining.

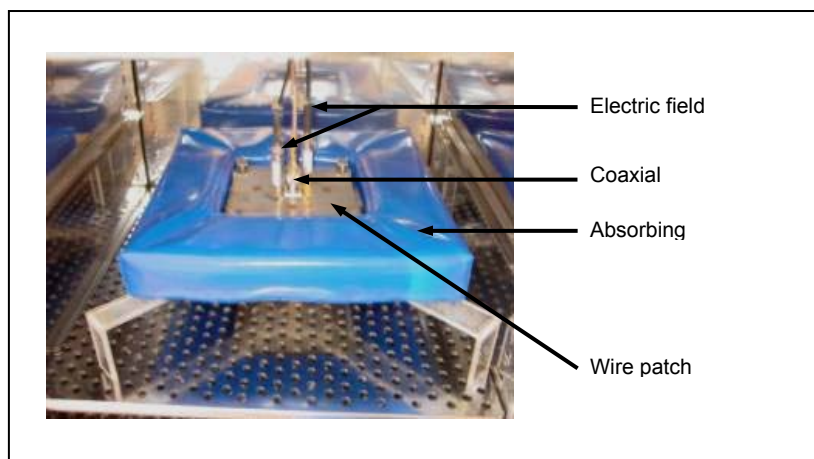


Figure 3. Image of a wire-patch antenna surrounded by the absorbing foam in a dedicated incubator

Dosimetry

The WPC's dosimetry (temperature measurement and modelling) was performed in collaboration with French experts (P. Lévêque, IRCOM, France and J. Wiart, France Télécom R&D). A good efficiency was found (around 0.6 W/kg per incident watt) and the uniformity for cell exposure was found to be very good (ca. 15%) (Figure 4). Experimental Specific Absorption Rate (SAR) evaluation was undertaken in order to validate the data obtained by numerical dosimetry (see Participant 10). Based on the measurement of temperature increase after the RFR generator was turned on, this technique gives also information on the thermal consequences at the level of the whole setup. Temperature was recorded in the inner part of a double Petri dish using optical fibres (Luxtron probes, that are immune to the microwaves), as well as in the incubator throughout the first hours of exposure (until temperature stabilisation was reached). Temperature measurements also showed that it took at least 2 hours for temperature to equilibrate, after the cell cultures were introduced, in the absence of microwaves. This needed to be taken in account in the exposure protocol. Experimental dosimetry showed that the mean SAR was 0.77 W/kg at the level of the cell monolayer, which is in good agreement (within 15%) with numerical data. The corresponding temperature rise was 0.2°C (i.e. temperature difference between the Petri dish inner part and the incubator).

Since heating is produced by absorption of the microwaves by the samples, care was taken to keep the temperature of the exposed and sham-exposed samples identical during the experimental trial. For that purpose, the “exposed” incubator temperature was first set at a lower value than that of the “sham” side, depending on the SAR level chosen for a given experiment. For a SAR of 2 W/kg for instance, the temperature difference was 0.5°C for an input power of 3.4 W, in very good agreement with numerical FDTD modelling (Figure 4) Those data have been confirmed for quality control purposes by Participant 10.

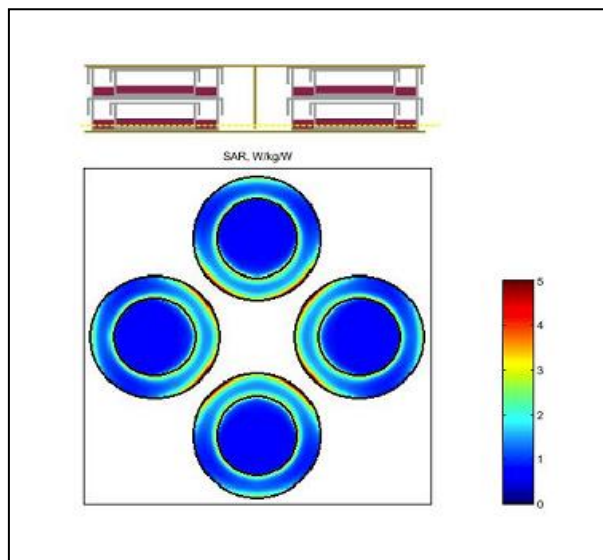


Figure 4. FDTD simulation of SAR in the Petri dishes (outer and inner parts) using an input power of 1 W. Upper panel shows the Petri dishes disposition in the wire-patch antenna.

2.6.2 Cell culture and RF-EMF exposure

For each exposure condition, at least three independent experiments were performed in a blind manner. For that purpose, all samples are coded prior to exposure, one researcher is in charge of exposure and codes are broken after completion of the analysis of all parameters investigated in the experiment by researchers unaware of the exposure conditions.

Culture of nerve cells

Rat primary neurons (granule cells) - a very critical cell type in the central nervous system-, rat primary astrocytes and rat C6 astrocytic cell line were used. Both primary cell types were prepared from newborn rat cerebella. Human nerve cell lines were also used to look at possible species specificity as well as at differences between transformed and normal cells. Human U87 astrocytic- and SH-SY5Y neuronal- cell lines were chosen as models.

i) Rat brain primary cultures: Primary cultures were prepared from postnatal day 4-9 (P4-P9) rat cerebella. Two types of cultures were prepared: neuronal-astroglial and glial (astrocytes) cultures.

Neuronal-astroglial cultures, with approximately 80% neurons and 20% glia in the cell population, were obtained from P4 rat cerebella. The cells were plated on polylysine-coated dishes at 10^6 cells/dish in Hanks Minimum Essential Medium supplemented with 10% horse serum. They were maintained in 3% CO₂ in air at 37°C for 4-6 days. Astrocyte cultures, where very few neurons survived, were obtained from P8 rat cerebella and plated at 10^6 cells/dish in Dulbecco Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (AB, 100 U/ml penicillin and 100 µg/ml streptomycin) and maintained in 9% CO₂ in air at 37°C for 13-15 days. For the experiments, all primary cells were plated in 35 mm diameter Petri dishes and used as it, without any further handling. The endpoint investigated in these cells was apoptosis. Rat primary neurons were sham-exposed or exposed to GSM-900 for one or 24 hours and apoptosis was evaluated following a time-kinetics (4, 8 and 24 hours

after exposure began) or immediately after exposure, respectively. Rat primary astrocytes were sham-exposed or exposed to GSM-900 for 1 hour and apoptosis was evaluated as described previously. Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

ii) *Human neuroblastoma cells*: Human SH-SY5Y neuroblastoma cells (ECACC N° 94030304) were cultured in Ham's F12 medium supplemented with 15% FCS, 1% non-essential amino-acids and AB. The endpoint investigated in these cells was apoptosis. Human SH-SY5Y neuronal cells – as rat primary neurons – were sham-exposed or exposed to GSM-900 for one or 24 hours and apoptosis was evaluated following a time-kinetics (4, 8 and 24h after exposure began) or immediately after exposure, respectively. Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

iii) *Human glioblastoma cells*: Human U87 glioblastoma cells (ECACC N° 89081402) were grown in Eagle Minimum Essential Medium supplemented with 10% FCS, 1% non-essential amino-acids and AB. The endpoint investigated in these cells was apoptosis. Human U87 astrocytic cells -as rat primary astrocytes - were sham-exposed or exposed to GSM-900 for 1 hour and apoptosis was evaluated following a time-kinetics (4, 8 and 24h after exposure began). Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

iv) *Rat glioma cells* : Rat C6 glioma cells were obtained from the European Collection of Cell Cultures (ECACC N° 85040101, UK) and maintained in DMEM-F12 medium (Biomedica, France) containing 10% fetal bovine serum (Gibco), 2 mM sodium pyruvate (Biomedica, France) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Biomedica, France) at 37°C in a standard culture incubator. The endpoint investigated in these cells was expression and activity of the inducible isoform of the Nitric Oxide Synthase (iNOS or NOS₂). C6 cells were sham-exposed or exposed to GSM-900 alone (0.2 W/kg or 2.0 W/kg, 48h) or in the presence of a cocktail of lipopolysaccharide and cytokines CK (see below). Following RFR or sham-exposure the cells were harvested for western blot analysis and culture medium collected for the determination of nitrite accumulation, to test iNOS expression and activity, respectively. Randomised sham/sham exposures were included in the schedule of exposure, so that the engineer responsible for the analysis never was aware of any exposure condition. A total of four sham/sham exposures, six sham/exposed experiments at 0.2 W/kg and three sham/exposed experiments at 2 W/kg were conducted. The Student t test was used for statistical analyses.

Culture of immune cells

i) *Human monocytes*: Human U937 monocytic cells (ECACC N° 85011440) are grown as a cell suspension in RPMI 1640 medium complemented with 10% FCS plus AB. Two endpoints were investigated in these cells:

- Apoptosis in cells submitted either to a 48 hour-exposure to GSM-900 at a SAR of 0.7 W/kg or to a 1 hour-exposure at 0.7 W/kg and 2.0 W/kg followed by a treatment with camptothecin (CPT). Three to six independent experiments were performed for each exposure condition. Results were expressed as the ratio of apoptotic cells in GSM-900-exposed versus sham-exposed samples with or without CPT treatment. The Student t test was used for statistical analyses.
- Gene expression in cells submitted to a 1 hour-exposure to GSM-900 at a SAR of 2.0 W/kg.

ii) *Human microglial cells*: The human cloned microglial cells (CHME 5) were plated at a density of 10⁶ cells/35mm diameter dishes in 2 ml of complete Dulbecco's MEM medium. Cultures were carried out for 3 days in water-saturated 5% CO₂ in air at 37°C before GSM exposure. The endpoint investigated in these cells was gene expression in cells after a 1 hour-exposure to GSM-900 at a SAR of 2.0 W/kg.

Culture of endothelial cells

Two EA.hy926 cell lines were tested: one was a generous gift from Participant 6, the other one from Dr. Cora-Jean S. Edgell who first developed the cell line and gave permission to use these cells in Bordeaux. The purpose for using both cell lines was to look at potential different behaviour of cells cultured in slightly different conditions that may have led to possible genotypic drift. Human EA.hy926 endothelial cells were cultured according to the provider's instructions.

i) *EA.hy926* (a gift from Participant 6): Cells were grown in DMEM supplemented with 1% penicillin-streptomycin, 2% L-glutamine (200 mM), HAT-supplement and 10% FCS.

ii) *EA.hy926* (a gift from Dr. Cora-Jean S. Edgell, North Carolina University at Chapel Hill, NC, USA): Cells were grown in DMEM supplemented with 1% penicillin-streptomycin, 2% L-glutamine (200 mM) and 10% FCS. For the RF-EMF experiments, cells were removed from culture flasks with trypsin, washed and seeded at a density of 0.26×10^6 cells/12 mm-diameter glass coverslips corresponding to 1.2×10^6 cells/55 mm-diameter dishes (as mentioned in Leszczynski et al. 2002). After an overnight culture, coverslips were transferred to 35-mm diameter Petri dishes and *EA.hy926* cells were sham-exposed or exposed to RF-EMF for one hour at 2.0 W/kg. The endpoint to be studied in these cells is the expression of the heat-shock protein 27 (hsp27). *EA.hy926* cells were sham-exposed or exposed to RFR for one hour at 2.0 W/kg.

2.6.3 Chemicals and other treatments

Positive controls used chemicals or other treatments. In order to look at possible interactions between RFR and chemicals, different protocols used RFR exposure combined to or prior chemical treatment.

Lipopolysaccharide plus cytokine treatment

Lipopolysaccharide plus cytokine treatment was used as a positive control for iNOS expression in C6 glial cells (Hewett et al., 1993; Nomura 1998). Two days before RFR exposure, C6 cells are plated in custom-made Petri dishes at a density of 5×10^4 cells/dish. At the day of experiment, Petri dishes are filled with culture medium containing 4% FCS for cell deprivation. Half of the samples are treated with a cocktail of *e.coli* lipopolysaccharide (LPS 10 µg/ml) and cytokines IFN γ (50 U/ml) plus TNF α (50 ng/ml) before Petri dishes are placed in the wire-patch antenna. Cells were then put in the exposure-dedicated incubators during 3 hours for temperature stabilisation before exposure to RFR started.

Camptothecin treatment

The apoptosis-inducer camptothecin (4 µg/ml, 4 hours) as a positive control in U937 monocytic cells. Camptothecin is a topoisomerase I inhibitor. As such, it inhibits the topoisomerase molecule from religating DNA strands after cleavage. This leaves a cell with DNA breaks, which if not repaired, become lethal (Holden 2001). When cells of the immune system are exposed the topoisomerase I inhibitor camptothecin, they rapidly undergo cell death via apoptosis, irrespective of what phase of the cell cycle a cell is in (Cotter 1992).

Heat shock

Positive controls for heat shock proteins (Hsp27 and Hsp70) induction were performed by exposing the different cell lines (U87, C6 and SH-SY5Y cell lines) to a heat shock at 43°C for 20 min.

2.6.4 Detection of apoptosis

The occurrence of apoptosis was assessed using two markers and flow cytometry.

Double staining with Annexin-V/FITC and propidium iodide

During apoptosis, phosphatidyl-serine is exposed on the outer leaflet of the plasma membrane that causes a loss of membrane asymmetry. Annexin V preferentially binds to phosphatidylserine (Van Engeland et al. 1998) and can be detected by flow cytometry using the APOTESTTM-FITC kit (Dako, France) according to manufacturer's instructions. Immediately after the complete treatment, cells were harvested so that all cells, including floating cells, were taken in account for the apoptotic test. Where needed, cells were scrapped (nerve cells) before being washed with PBS, and centrifuged at 200 g for 5 minutes. Cell pellet was resuspended and 10^6 cells were incubated for 15 minutes in 100 µl of cold labelling solution (1 µl of Annexin-V/FITC and 2.5 µl of propidium iodide (PI) 250 µg/ml) in 96 µl of the kit's labelling buffer. Then 250 µl of labelling buffer were added and samples are analysed on a flow cytometer.

Double staining with DiOC₆(3) and propidium iodide

Mitochondrial physiology is disrupted in cells undergoing apoptosis via intrinsic pathways. Mitochondrial membrane potential ($\Delta\Psi_m$) decrease has been largely described which can be measured using the

carbocyanine dye (DiOC₆(3), Zamzami et al., 1995). Briefly, immediately after exposure, cells were washed and centrifuged as indicated above. Then 10⁶ cells were incubated for 10-15 minutes in 500 µl of PBS containing 40 nM of DiOC₆(3). Propidium iodide (2.5 µl of PI; 50 µg/ml) was added before analysis on a flow cytometer.

Data acquisition was performed using a FacScan[®] flow cytometer (Becton Dickinson) with the following parameters: 488 nm excitation, 515 nm bandpass filter for the Annexin V and DiOC₆(3) dyes and filter > 560 nm for PI detection. Analysis was performed on 10000 events using the Cell-Quest[®] software. Analysis was performed blindly.

2.6.5 Western Blot analysis

Western Blotting was used for the detection of iNOS expression in C6 cells. C6 cells were lysed using RIPA buffer [0.5 mM Tris (pH 8.0), 0.5% Sodium Deoxycholate, 10% SDS, 150 mM NaCl, 1% Triton X100 and protease inhibitors (16 mg/ml Benzamidin, 10 mg/ml Aprotinin, 10 mg/ml Pepstatin, 10 mg/ml Leupeptin, 10 mg/ml Phenanthroline and 1 mM Phenylmethylsulfonyl Fluoride)] using methods adapted from Schreiber et al. (1989).

Proteins were extracted from cell lysates and the concentration was determined by Bradford reaction (Biorad Protein Assay[®]). Protein samples (10-20 µg) were electrophoretically separated through a 7.5% polyacrylamide SDS-page gel, electroblotted to polyvinylidene difluoride membranes and probed with mouse anti-iNOS (Transduction Laboratories N-39120, 1/5000[°]). In addition, we used β-actin as an internal control for protein loading (all blots were de-hybridised and reprobed for β-actin detection). Immunoreactive bands were visualised using ECL Western Blotting System[®] (Amersham-Pharmacia Biotech, RPN 2108) followed by exposure to autoradiography film (Biomax, Kodak). The NIH Image 1.54 software was used for blot quantitative analysis (based on OD measurements).

2.6.6 Griess reaction

iNOS activity in C6 cells was quantified as nitrites accumulation in culture media by the colorimetric assay based on the Griess reaction. 50 µl of culture medium collected in triplicate from the samples were added to 60 µl of Griess A solution (sulfanilamide 1% in 1.2N HCl) and 60 µl of Griess B solution (Naphthylene Diamine Dichlorhydrate 0.3% in distilled water). The mixture was incubated 10 min at room temperature and read at 540 nm with a spectrophotometer. Fresh corresponding culture medium served as blank for NO₂⁻ content determination. Results were expressed as µg of nitrite per million cells.

2.6.7 Hsp immunolabelling and image analysis

Hsp70 expression was evaluated in human neuronal (SH-SY5Y) and rat (C6) or human (U87) astrocytic cell lines. Three days before the experiment, cells were plated on glass coverslips in 24-well plates at a density of 0.5 x 10⁵ cells/well. The day before the experiment, coverslips were transferred to 35-mm diameter Petri dishes before being placed in the sham- and RFR-dedicated incubators.

U87, C6 and SH-SY5Y cell lines were sham-exposed or exposed to GSM-900 for 24 hours and the expression of Hsp70 was evaluated at the end of exposure. Hsp27 expression was evaluated in human EA.hy926 cells at the end of a one hour sham- or RFR exposure at 2.0 W/kg. Following RFR or sham exposure, the cells were fixed in PBS-paraformaldehyde (4%) for immunocytochemistry. Anti-hsp70 and anti-hsp27 antibodies were obtained from Stressgen[®]. The first antibody was revealed using a FITC-labelled antibody. Coverslips were mounted on slides with Mowiol[®] before microscopy observation. For each exposure condition, three (hsp70) to five (hsp27) independent experiments have been performed in a blind manner. After immunocytochemistry labelling, fluorescence analysis was performed using the Aphilion[®] image software. Results are expressed as arbitrary units of fluorescence intensity.

2.6.8 RNA extraction and cDNA array hybridisation

Based on the data available within the REFLEX consortium on the effect of RF-EMF exposure on gene expression (see Participant 12), we chose two human cell lines involved in inflammatory processes (brain

human microglial and monocytes cells). Indeed, one of the gene families that were shown to be sensitive to exposure to electromagnetic fields is the gene family involved in inflammation. The human cloned microglial CHME-5 cells and the monocytic U937 cells were sham-exposed or exposed for one hour at 2 W/kg. Immediately after exposure, they were harvested for RNA extraction using Nucleospin® RNA purification kit (BD Biosciences Clontech, Palo Alto, USA). Total RNA purification was performed following user manual instructions. The amount of total RNA was measured by spectrophotometry. RNA samples were then frozen at -80°C before being sent to Participant 12 who performed cDNA array hybridisation.

2.7 Experiments with embryonic stem cells of mice (Participant 4)

2.7.1 Exposure setups

See 2.1.1 and 2.1.2.

2.7.2 Cell culture and EMF exposure

Pluripotent R1 ES cells (Nagy et al. 1993), wild type (wt) D3 (Doetschman et al. 1985) and p53-deficient ES cells (p53-/-; a gift of Dr. T. Jacks, Howard Hughes Medical Institute, Cambridge, MA, see Jacks 1994) derived from D3 cells were cultured as described (Wobus et al. 2002) except that p53-/- ES cells were maintained on neomycin-resistant SNL feeder cells (a gift of Dr. A. Bradley, Baylor College of Medicine, Houston, TX) in presence of 300 µg/ml G418. EC cells of line P19 (Edwards and McBurney 1983) were cultured without feeder cells (Wobus et al. 1994). For differentiation, P19, R1, and wt or p53-/- D3 cells were cultivated as EB in hanging drops in Dulbecco's modified minimal essential medium (DMEM, Gibco) supplemented with 20% FCS, L-glutamine, non-essential amino acids (NEAA) and β-mercapthoethanol (β-ME) as described (Wobus et al. 2002). Briefly, cells (n=400) in 20 µl of differentiation medium were incubated in hanging drops as embryo-like aggregates ("embryoid bodies", EBs) for 2 days and in suspension for 3 days. EBs were plated separately onto gelatin-coated 24-well microwell or tissue culture plates (Ø 6cm) at day 5 for morphological and reverse transcriptase polymerase chain reaction (RT-PCR) analyses, respectively. For the induction of P19 cell differentiation, EBs were cultivated in the presence of 1% DMSO (Sigma) during the first 2 days of EB development (Wobus 1994).

EBs derived from p53-/- and wt D3 ES cells were RF- or ELF-EMF- exposed in hanging drops for 6 or 48 hours (Figure 5). For 6h experiments, samples were collected immediately after exposure and used for RT-PCR analysis (Figure 5). After 48h exposure, EBs were further cultivated and samples were sequentially collected during differentiation for RT-PCR analyses. EBs derived from R1 cells were EMF (GSM-217)- or sham-exposed in hanging drops (SAR: 1.5 W/kg) for 2 days and in suspension (SAR: 2.0 W/kg) for 3 days. P19 cells (n=200,000) were seeded into 0.1% gelatin-coated tissue culture dishes, cultured in DMEM (see above) and after 2h pre-incubation at 37°C placed into the exposure setup for EMF and sham-exposition. As control, non-exposed cells were cultured in a separate humidified 5% CO2 incubator at 37°C. P19 cells were exposed to EMF at SAR value of 2.0 W/kg for 22 or 40h. After exposure, cells were immediately processed for flow cytometric analysis (P19), and in parallel, R1 and P19-derived EBs were prepared for differentiation and RT-PCR analysis

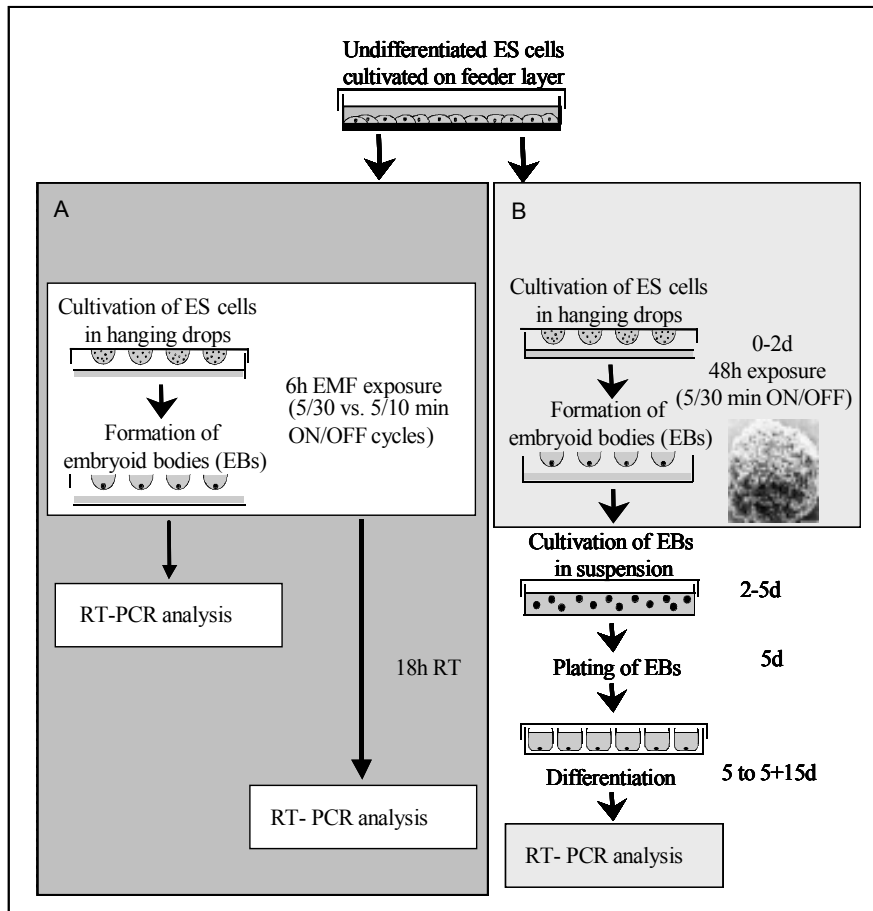


Figure 5. Experimental protocol for the exposure of undifferentiated and differentiating ES cells to EMF. Wild type (wt) and p53-deficient (p53^{-/-}) D3 ES cells were grown on feeder layer. (A) ES cells were RF-EMF or PL-MF exposed for 6 h at the initial stage of hanging drop formation, when the differentiation processes are initiated. mRNA levels of genes encoding *egr-1*, *p21*, *c-jun*, *c-myc*, *hsp70* and *bcl-2* were analysed immediately after exposure (or after 18 h recovery time for PL-MF). (B) ES cells were exposed to EMF in hanging drops for 48 h and were monitored at different stages of the differentiation process.

For differentiation of neural phenotypes, R1 ESs were cultivated in 'hanging drops' ($n = 200$ cells/drop) for 2 days. EBs were transferred to bacteriological petri dishes (Greiner, Germany) and cultivated for two days in Iscove's modification of DMEM (IMDM, GIBCO) containing 20% FCS and supplements as described (Wobus et al., 2002), with the exception that β -mercaptoethanol was replaced by $450 \mu\text{M}$ α -monothioglycerol (Sigma, Steinheim, Germany). EBs ($n=20-30$) were plated onto tissue culture dishes (\varnothing 6cm) at day 4 and cultivated in IMDM +20% FCS. The selection of neural precursor cells was carried out according to (Rolletschek 2001). After attachment of EBs, one day later, the medium was exchanged by DMEM/F12 medium supplemented with $5 \mu\text{g/ml}$ insulin, 30 nM sodium selenite (all from Sigma), $50 \mu\text{g/ml}$ transferrin and $5 \mu\text{g/ml}$ fibronectin (all from GIBCO) referred as "nestin-selection media". The culture medium was replenished every 2 days. RF-EMF or ELF-EMF exposure was performed for 48h between 4+4d and 4+6d. Nestin-positive neural precursor cells were selected after cultivation for 7 days (= 4+7d). At day 4+8, EBs were dissociated by 0.1% trypsin (GIBCO)/ 0.08% EDTA (Sigma) in PBS (1:1) for 1 min, collected by centrifugation, and replated onto poly-L-ornithine/laminin-coated tissue culture dishes into DMEM/F12 containing 20 nM progesterone, $100 \mu\text{M}$ putrescine, $1 \mu\text{g/ml}$ laminin (all from Sigma), $25 \mu\text{g/ml}$ insulin, $50 \mu\text{g/ml}$ transferrin and 30 nM sodium selenite, referred to as "nestin-expansion media", for six days until day 4+13. The medium was changed every 2 days. 10 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF; Strathmann Biotech, Hannover, Germany) were added daily. At day 4+14, the differentiation of neurons was induced by 'Neurobasal' medium plus 2% B27 (GIBCO), 10% FCS and maintained by the addition of survival

promoting factors such as interleukin-1 β (IL-1 β , 200 pg/ml daily; PeproTech, London, UK) and db-cAMP (700 μ mol every four days; Sigma). Glial cell line-derived neurotrophic factor (GDNF, 2 ng/ml; R&D Systems) and transforming growth factor- β 3 (TGF- β 3, 2 ng/ml) were applied at day 4+18 and at day 4+21, respectively. Neurturin (NTN, 10 ng/ml; all from PeproTech) was applied at day 4+21. The application of survival promoting factors during terminal stages was combined with medium changes at three-day intervals. The total time of cultivation was 4+23d (Figure 6).

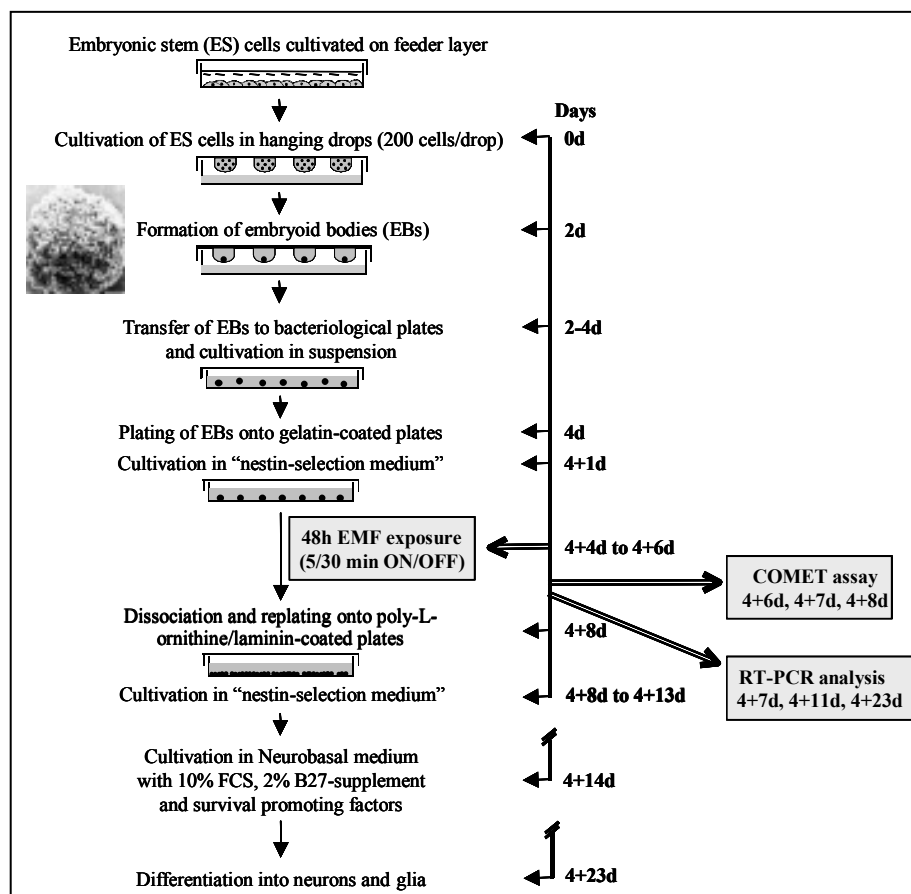


Figure 6. Experimental protocol for EMF exposure of R1 ES cells differentiating into the neural lineage. For differentiation of neural phenotypes, ES were cultivated in 'hanging drops' as EBs (embryoid bodies) for two days, then transferred to bacteriological petri dishes and cultivated two more days. EBs (n = 20-30) were plated onto tissue culture dishes (\varnothing 6cm) at day 4. After attachment of EBs, one day later, the medium was exchanged with medium supporting the development of neural precursor cells (replenished every 2 days). Nestin-positive neural precursor cells were selected after cultivation for 7 days (= 4+7d). Cell samples were analysed for primary DNA damage (24 and 48h after exposure) measured by the Comet assay, and mRNA levels of various regulatory, tissue-specific and neuronal genes at different stages of differentiation.

2.7.3 Detection of mRNA levels by semi-quantitative RT-PCR analysis

The expression of early response and growth regulatory genes as well as genes involved in neural and cardiac differentiation was analyzed in ES and EC cells after differentiation by semi-quantitative RT-PCR as described (Wobus et al. 2002). EBs or cells differentiating after the dissociation of EBs collected at days 4+4, 4+7, 4+11 and 4+23 were suspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 1% β -mercaptoethanol). Total RNA was isolated by the single step extraction method according to (Chomczynski, 1987). RNA was reverse transcribed using Oligo d(T)₁₆ primers (Perkin-Elmer, Überlingen, Germany) for the genes encoding c-fos, c-jun, c-myc, early growth response-1 (egr-1), hsp70, p21, p53, bcl-2, bax, growth arrest and DNA-damage inducible -45

(GADD45), engrailed-1 (en-1), nurr-1, nestin, tyrosine hydroxylase (TH), GFAP (glial fibrillary acidic protein), α -myosin heavy chain (α -MHC) (primer sequences and the number of PCR cycles are available on request).

Reverse transcription was performed with MuLV reverse transcriptase (Perkin-Elmer) for 1h at 42°C, followed by denaturation for 5 min at 99°C and cooling to 4°C according to the protocol supplied by the manufacturer. For semi-quantitative determination of mRNA levels, PCR analyses were carried out with Ampli Taq DNA polymerase (Perkin-Elmer). For determination of relative mRNA levels, two separate PCR reactions, either using primers of the analyzed gene or primers specific for β -tubulin were performed with 3 μ l from each RT reaction.

One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.35 μ g/ml ethidium bromide. Gels were illuminated with UV light and the ethidium bromide fluorescence signals were stored by the E.A.S.Y. system (Herolab, Wiesloch, Germany) and analysed by the TINA2.08e software (Raytest Isotopenmeßgeräte, Straubenhardt, Germany). Data of the target genes were plotted as percentage changes in relation to the expression of the housekeeping gene β -tubulin. Gels of four independent experiments were analysed.

2.7.4 Detection of mRNA levels by quantitative RT-PCR (Q-RT-PCR)

Quantitative RT-PCR was performed with specific primers and TaqMan probes designed with Primer Express 2.0 (Applied Biosystems). All oligonucleotides were obtained by Metabion (Germany). The TaqMan probes for Q-RT-PCR were 5'-labelled with FAM (6-carboxyfluorescein) and with 3' prime quencher, TAMRA. The primer and TaqMan probe sequences are available on request. The mouse GAPDH gene was used as endogeneous reference. Reactions were carried out in 96-well plates using iCycler, Version 3.0.6070 (BioRad). The threshold cycle (Ct), which is the cycle number, at which the amount of the amplified product of the analysed genes reached a fixed threshold, was determined subsequently. The relative quantitation of bcl-2 and GADD45 mRNA levels was calculated using the comparative Ct method. In order to avoid amplification of contaminating DNA, all primers were designed with an intron sequence inside the amplicon, template-free controls were used as negative controls, the melting temperature of the TaqMan probe was adjusted to be at least 10° higher than the melting temperatures of the sense and anti-sense primers. After Real-time RT-PCR, gel electrophoresis was performed to confirm the correct size of the amplified product.

2.7.5 Single cell gel electrophoresis (Comet assay)

The alkaline version of the Comet assay was applied for detection of single-strand break and alkali-labile site induction, and neutral Comet assay for detection of double-strand breaks. In order to obtain single-cell suspensions, control, sham-exposed or exposed EB outgrowths were trypsinised by addition of 2 ml prewarmed at 37°C mixture of 0.1% Trypsin : 0.01% EDTA (1:1, v/v) per 6 cm tissue culture dish, incubated for 60 sec at room temperature, then the mixture was carefully aspirated as previously described (Wobus et al. 2002). The procedure was repeated. Thereafter, the cells were resuspended into cold Dulbecco's modified Eagle's medium (Gibco BRL, cat. No. 52100-039) with 15% FCS, the cell density was adjusted at $\sim 5 \times 10^5$ cells/ml and the test-tubes were placed on ice.

For the Comet assay procedure, the protocol of the original technique described by Östling and Johanson (1984) was followed with minor modifications by (Singh et al. 1991, Morris et al. 1999, Speit et al. 2000, Ivancsits et al. 2002b). Briefly, 20 μ l of cell suspension ($\sim 10^5$ cells) was mixed with 200 μ l prewarmed (37°C) 0.5% low melting point agarose in PBS. The cell suspension was rapidly pipetted onto slides with frosted ends precoated with 1.5% normal melting point agarose in PBS and evenly spread using a coverslip. The slides were incubated at 4°C for 15 min. to allow the microgel to solidify. Then the coverslips were removed by pulling them carefully aside. The slides were immersed in precooled (4°C) lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% sodium sarcosinate, 10% DMSO, pH 10 for the alkaline Comet assay and pH 7.5 for the neutral COMET assay) and lysed for 60 min at 4°C in the dark. After lysis, the slides were removed from the lysis buffer, drained, placed into a horizontal electrophoresis tank and covered with the precooled (4°C) electrophoresis buffer (1 mM Na₂EDTA, 300 mM NaOH, pH 13 for the alkaline Comet assay and 100 mM Tris, 300 mM sodium acetate, 500 mM sodium chloride, pH 8.5 for the neutral Comet assay, respectively). After 20 min incubation for DNA

alkaline denaturation and expression of the various types of alkaline labile sites (alkaline Comet assay) and for washing away the lysis buffer and equilibration (neutral Comet assay), the electrophoresis was performed in the dark at 4°C for 10 min. The electrophoretic conditions (25 V, 300 mA, field strength 0.7 V/cm) were identical for both versions (alkaline and neutral) of the single cell gel electrophoresis assay. After removal from the electrophoresis tank, the slide surface was carefully covered 3x with a neutralization buffer (400 mM Tris, pH 7.5, 5 min. incubation), then the slides were rinsed briefly in distilled water and fixed in 100% ethanol for 5 min. The slides were air-dried at a slanted angle for at least 2 hours and stained with 50 µl ethidium bromide solution (20 µg/ml in bidistilled water). The analysis was done using the fluorescence microscope ECLIPSE E600 (Nikon, Germany), appropriate excitation and barrier filters (excitation 540-580 nm, barrier 600-660 nm) at 200 fold magnification and the imaging software Lucia (Version 4.71 for Windows). A total of 1000 nuclei were scored (500 per slide) for exposed, sham exposed and control cells and classified into 5 groups according to tail length and intensity using the classification proposed by Anderson et al. (1994): Group A corresponding to <5% DNA damage; B (5-20%); C (20-40%); D (40-95%) and E (>95%). All analyses were performed by the same investigator blind. Results were expressed as 'tail factors', calculated according to Ivancsits et al. (Ivancsits et al. 2002a; Ivancsits et al. 2002b) by the following formula: Tailfactor (%) = $\frac{AF_A + BF_B + CF_C + DF_D + EF_E}{n}$, where A is the number of nuclei classified to group A, F_A is the average DNA damage of group A, which is 2.5% in Anderson's classification; B is the number of nuclei classified to group B, F_B is the average DNA damage of group B (12.5%); C is the number of nuclei classified to group C, F_C is the average DNA damage of group C (30%); D is the number of nuclei classified to group D, F_D is the average DNA damage of group D (67.5%); E is the number of nuclei classified to group E, F_E is the average DNA damage of group E (97.5%); n – the number of scored nuclei (n= 1000).

The results on DNA damage in the alkaline and neutral Comet assay were obtained from 6 separate experiments. The statistical analysis was performed with the SigmaPlot for Windows Version 3.06 package (Jandel Corp.). All data are presented as mean values ± standard error of the mean (SEM). The differences between exposed, sham exposed and control cells were checked for statistical significance using the independent Student's *t* test.

2.7.6 Analysis of cardiac differentiation

Cardiac differentiation of EC or ES cells was used as a parameter of differentiation according to the embryonic stem cell test, EST (Spielmann et al. 1997), established for in vitro analysis of embryotoxic agents. Spontaneously beating cardiomyocytes were estimated at various stages after EB plating. The percentage of EB containing beating cardiomyocytes and mRNA levels of α -MHC were used for the estimation of the degree of cardiac differentiation.

2.7.7 Flow cytometric analysis of cell cycle phases

RF-EMF- and sham-exposed P19 cells were processed according to the two-step procedure of DNA staining (Sehlmeyer et al. 1996). Cells were analysed with a FACStar^{PLUS} flow cytometer (Becton Dickinson, Heidelberg, Germany). Data from 3 (22h) and 4 (40h) independent experiments with 3 to 6 parallels were subjected to statistical analysis performed with the 'ModFitLT' software (Verity Software House, Inc.).

2.8 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

2.8.1 ELF-EMF exposure setup

See 2.1.1

2.8.2 Cell culture and exposure conditions

The human neuroblastoma cell line was grown in RPMI, 10% foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C and 5% CO₂. Cells were plated one day prior to exposure at densities varying with the exposure protocol: 2.6x10⁶ cells per 100 mm dish for 16 hours exposure protocol; 2 x 10⁶ cells per 100 mm dish for 48 hours exposure protocol followed by recovery of the cells immediately after the end of the exposure; and 10⁶ cells per 100 mm dish when cells were exposed to the electromagnetic field for 48 hours and harvested 48 hours after the end of the exposure. This was to ensure the collection of the same amount of cells at the end of the different exposure protocols.

Human neuroblastoma cells (SY5Y) were exposed to ELF-EMF (50 Hz, powerline) in a “blind trial” system that allows direct comparison to control unexposed cells. Different exposure protocols, varying in the density of ELF-EMF and in the time of exposure, were applied: a) 2 mT magnetic flux density, intermittent exposure of 5 min on/5 min off, duration 16h; b) 1 mT magnetic flux density, intermittent exposure 5 min on/5 min off, duration 16h; c) 2 mT magnetic flux density, continuous exposure, duration 16h; d) 1 mT flux density, continuous exposure, duration 16hs; e) 1 mT magnetic flux density, continuous exposure, duration 48h. Harvesting of the cells was performed immediately after the end of exposure, unless indicated otherwise.

2.8.3 RNA preparation and Northern blot analysis

Total RNA from exposed and sham exposed SY5Y cells was extracted using RNA Fast II (Molecular Systems, San Diego, CA, USA) according to manufacturer's instructions and size fractionated on 1% agarose gel containing 2.2 M formaldehyde as described in Sambrook et al. (1989). RNA was subsequently transferred and cross-linked to a nylon membrane (Biodyne A, Pall Europe Ltd., UK). After two hours of pre-hybridisation at 65°C in 0.125 M Na₂HPO₄, 1 mM EDTA, 0.25 M NaCl, 7% SDS, 10% polyethylene glycole and 1% BSA, RNA was hybridised with 10⁶ cpm/ml of ³²P labelled cDNA probe corresponding to the cytoplasmic domain of the human α3 (nucleotides +975/+1404; Fornasari et al. 1997) and α5 (nucleotides +1005/+1263, Chini et al. 1992) nAChR subunits and to the full length coding region of the human α7 nAChR subunit (Groot Kormelink and Luyten 1997). Following hybridisation, membrane was washed at a final stringency depending on the probe used: α3, 0.1x SSC/0.1% SDS at 50 °C; α5, 0.2x SSC/0.1% SDS at 55 °C; α7, 0.1x SSC/0.1 % SDS at 65°C.

The human Phox2a probe corresponds to the 5'UTR region (nucleotides +26/+219) obtained by digestion of the construct SacI-NcoI (Flora, 2001) with Eag I and NcoI. The human Phox2b probe corresponds to the 5'UTR specifying region (nucleotides -299/-90 with respect to the ATG; GenBank accession number NM_003924) and was obtained by RT-PCR. The primers used were: upper primer 5'-GTG CCA GCC CAA TAG ACG GAT G-3'; lower primer 5'-CTC AAC GCC TGC CTC CAA ACT G-3'. The human DβH probe (nucleotides +728/+1337; GenBank accession number NM_000787) was obtained by RT-PCR using the following primers: upper primer 5'-GCT TCT CTC GGC ACC ACA TTA TC-3'; lower primer 5'-TGA GGG CTG TAG TGA TTG TCC TG-3'. The final stringency washings were Phox2a, 0.2x SSC/0.1% SDS at 55°C; Phox2b, 0.2x SSC/0.1% SDS at 50°C; DβH, 0.1x SSC/0.1% SDS at 55°C, respectively.

After stripping the probe, blots were re-hybridised to a human 18S cDNA probe (nucleotides 715-794; Ambion, Austin, TX, U.S.A.) to check the quality of the RNAs and normalise the amount of RNA loaded.

2.8.4 Radioligand assay with ¹²⁵I-α Bungarotoxin and ³H-Epipatidine

After the end of the exposure, the cells were detached with buffer A (50 mM Tris-HCl pH 7, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 2mM PMSF (buffer A) and centrifuged at 10000g for 60 min. The pellets were washed and then homogenised using an Ultra Turrax homogeniser in an excess of buffer A containing 10 µg/ml of a mixture of the protease inhibitors leupeptin, bestatin, pepstatin A and aprotinin in order to block possible proteolysis during the incubation time of the assays.

¹²⁵I-αBungarotoxin (αBgtx) was from Amersham, England, and had a specific activity of 200 Ci/mmol; ³H-Epipatidine (Epi; NEN, Boston, USA) had a specific activity of 56 Ci/mmol.

In preliminary experiments we determined the affinity of ^{125}I - αBgtx by performing saturation binding experiments on the cell homogenate. The ^{125}I - αBgtx concentrations ranged from 0.1 to 20 nM, and aspecific binding was determined, after overnight incubation at room temperature, using 1 μM unlabeled αBgtx . The affinity of ^3H -Epi was determined by performing saturation binding experiments on the cell homogenate using ^3H -Epi concentrations between 0.005 and 10 nM, diluted in buffer A, and incubated overnight at 4°C.

After having determined the affinity of nicotinic ligands, the determination of the number of nicotinic receptors was performed using ^3H -Epi binding and ^{125}I - αBgtx -binding to membrane homogenates using saturating concentrations of nicotinic ligands (2 nM ^3H -Epi or 10 nM ^{125}I - αBgtx) and subtracted for the aspecific binding performed in parallel using 2 nM ^3H -Epi or 10 nM ^{125}I - αBgtx and 100 nM cold Epi or 1 μM cold αBgtx . For total and aspecific ^3H -Epi binding membranes were always preincubated with 2 mM cold αBgtx . ^{125}I - αBgtx binding was performed overnight at room temperature and the ^3H -Epi binding overnight at 4°C. At the end of the incubation, the samples were filtered on GF/C filters and radioactivity counted in a α - or β - counter, respectively. The number of receptor present was expressed as fmol of ^3H -Epi or ^{125}I - αBgtx bound/mg of protein. Protein measurement was done using the BCA protein assay (Pierce) with bovine serum albumin as the standard.

2.8.5 Protein preparation and Western blot analysis

Total protein extract was prepared from sham and exposed cells by the freezing and thaw method. Briefly, cells were detached by scraping in PBS 1x and collected by centrifugation at 1000 rpm for 15 min at 4°C. The pellet was resuspended in PBS 1x containing protease and phosphatase inhibitors (purchased by SIGMA) and 20 mM Phenyl-Methyl-Sulphonyl-Fluoride (PMSF). Cells were lysed by four repeated passages between liquid nitrogen to freeze and 37°C to thaw. NaCl at a final concentration of 400 mM was subsequently added, to allow extraction of nuclear protein. Samples were incubated 10 min on ice and extract clarified by centrifugation at 14000 rpm for 30 min at 4°C in a table-top centrifuge (Eppendorf). Twenty micrograms of total extract were then separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were pre-incubated with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) for one hour, after which the primary antibodies were added at appropriate dilutions and incubated for two hours; the secondary antibodies conjugated with horseradish peroxidase were then added and incubated for one hour. The bands were revealed using Super Signal West Dura (Pierce). Standard molecular weights (New England Biolabs) were loaded in parallel.

2.9 Experiments with *Xenopus laevis* oocytes, granulosa cells of rats, HeLa cells, Chinese Hamster Ovary (CHO) cells and human fibroblasts (Participant 7)

2.9.1 ELF-EMF-exposure setup

See 2.1.1

2.9.2 ELF-EMF exposure, expression in *Xenopus* oocytes and RNA preparation of rCx46

The cDNA for rCx46 were subcloned in the SP64T vector for RNA transcription. SP64T contains 250 bp of the non-coding sequence from *Xenopus laevis* β -globin including a poly-A tract that increases translational efficiency. RNA was prepared by using a synthesis kit containing SP6 RNA polymerase and CAP analogue purchased from Ambion (Austin, USA). The *Xenopus* expressions construct was linearised with XbaI for RNA transcription. The transcript concentration was estimated spectrophotometrically and analysed on agarose gels. The oocytes were isolated from *Xenopus laevis* ovaries and stage V-VI oocytes were collected and defolliculated by collagenase treatment (5 mg/ml, 355 U/mg, 1.5 h; Worthington, Type 2) in Ca^{2+} -free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , Na-HEPES at pH 7.4 and adjusted with sorbitol to 240 mosmol/l). An injection apparatus

(Nanoliter Injector, World Precision Instruments) was used to inject 23 nl of 25 ng/μl of Cx46 cRNA and 23 nl of DNA antisense to the endogenous XenCx38 oligo 5'-gCT gTG AAA CAT ggC Agg Atg (500 ng/μl) (Tib Molbiol) to eliminate endogenous hemi-channel currents. Oocytes were incubated in ND96 supplemented with antibiotics (100 U/ml penicillin/streptomycin) at 17°C. During the expression period of 14h, 17h and 20h the oocytes were exposed to ELF-EMF of 50 Hz powerline or sham exposed. Field strength of 2.3 mT was either continuously or intermittently (5 min on/10 min off) applied. In a further series of experiments 1.0 mT was applied with the intermittent application protocol. For the cell-to-cell coupling assay of paired oocytes, each cell of a pair of oocytes were injected with 23 nl of DNA antisense to the endogenous XenCx38 in order to suppress endogenous coupling in addition 23 nl of rCx46 cRNA (25 ng/μl) were injected. The expressing oocytes were incubated for 16h in ND96 medium, followed by manual removal of the vitelline layers. Two oocytes were paired at their vegetal poles and incubated with ND96 at 17°C for further 8h in the ELF-EMF setup (Participant 10). During this incubation the oocytes pairs were exposed to ELF-EMF (50 Hz, powerline, 1.0 mT, intermittently (5 min on/10 min off)).

2.9.3 Electrophysiological recordings of single and paired oocytes

The two electrode voltage clamp technique was applied to measure the expressed and conducting rCx46 gap-junctional hemi-channels in single *Xenopus laevis* oocytes using a voltage-clamp amplifier Turbo TEC-10 CD (npi electronic, Tamm, Germany). Voltage protocols were applied by a Pentium 100 MHz Computer linked to an ITC-16 interface (Instrutech. Corp., NY). The following pulse protocol was used throughout the experiments: From a constant holding potential of -90 mV or -80 mV variable test potentials were applied for 15 s in the range from -110 mV to +70 mV in steps of 10 mV after repolarisation of the oocyte at a holding potential of -90 mV or -80 mV. The latter was constantly applied for at least 70s. The current signals were filtered at 1 kHz and were sampled at 0.5 or 0.25 kHz. Data acquisition and analysis were performed by using Pulse/PulseFit (HEKA, Germany), Igor Pro (Wave Metrics, USA), Origin (Microsoft), PatchMaschine (V. Avdonin, University of Iowa, USA). n denotes the numbers of individual oocytes. The data are given as mean ± s.e.m. For electrophysiological recordings on paired oocytes the setup was extended by a second amplifier and a further pair of micromanipulators /electrodes. For the pulse protocol of paired oocytes a holding potential of -40 mV, close to the resting potential, was used for both oocytes. The depolarising test pulses were applied for 5s (10s) in the range from -120 mV to +120 mV and the corresponding holding potential was held for 15s (30s). For the electrophysiological recordings the micropipettes were filled with 3 M KCl resulting in input resistances of 1-1.5 M. During the current recordings the oocytes were continuously superfused with the corresponding solution at a rate of 0.5 ml/min and all recordings were performed at room temperature (20-22°C). The standard bath was a nominal Ca²⁺- free ND96 solution at pH 7.4. The different Ca²⁺ concentrations of ND96 were obtained by addition of suitable concentrations of CaCl₂ to the standard solution. For experiments on single oocytes Ca²⁺-concentrations of 0.0, 0.25 and 0.5 mM and on paired oocytes 1.8 mM and 5 mM were used.

2.9.4 Voltage-jump current-relaxation and membrane conductance of hemi-channels

The steady-state current amplitudes were leak-subtracted and denoted as I_{ss}. The I_{ss} values are presented as function of driving voltage (V-V_{rev}). The leak current at the applied test potential V was determined by extrapolation of the corresponding current values in the range of -100 mV to -70 mV, at this voltage the voltage dependent hemi-channels are closed. The reversal potential (V_{rev}) of the rCx46-mediated current was calculated by a 4-point interpolation polynomial of third order. The corresponding membrane conductance G(V) was calculated from the steady-state current amplitude divided by the driving voltage (V-V_{rev}) and plotted as function of test potential V. In the absence of a significant time- and voltage-dependent current inactivation the corresponding G(V) values in the range of -110 mV < V < +40 mV could be fitted by a simple Boltzmann distribution according to: $G(V) = (A / (1 + \exp(-(V - V_{1/2})zF/RT))) + B$. R, T, F have their usual meanings. V_{1/2} denotes the half-activation voltage at which 50% of the maximal membrane conductance is observed. z gives the number of membrane bound equivalent gating charges. The parameter A denotes the maximal membrane conductance G_{max} of expressed and conducting rCx46-connexons hemi-channels and B the corresponding leak conductance of the oocyte. B is assumed to be voltage independent. For different experiments G(V) was normalised to the corresponding values

obtained at A=1 and B=0, respectively. A similar subtraction of the leak-current was considered in the experiments on paired oocytes.

2.9.5 Cell cultures

Granulosa cell line GFSHR-17 (rat) (Keren-Tal et al. 1993), HeLa cells (human), Chinese Hamster Ovary (CHO) cells and fibroblasts (human, Participant 3) were cultivated in Dulbecco's modified Eagle's medium F-12 Ham (DMEM-F12, Sigma Corp., USA) added with 10% fetal calfserum (FCS, Sigma Corp., USA) and 50 U/ml Penicillin, 50 µg/ml Streptomycin (Gibco BRL, G) (300 mosmol, pH 7.4). Both cell lines were incubated under an atmosphere containing 5% CO₂ at 37°C. The culture dishes (35 mm in diameter) for the measurement of the intercellular free calcium ([Ca²⁺]_i) contained six coverslips of 10 mm diameter. Under these conditions the cultured cells were ELF-EMF exposed (50 Hz sinusoidal, 4h - 24h, 1.0 mT, 5 min on/10 min off) or sham exposed. The culture dishes (50 mm in diameter) for the measurement of volume regulation and the Comet assay analysis for contained a coverslip of 25 x 50 mm diameter. Under these conditions the cultured cells were ELF-EMF exposed or sham exposed.

2.9.6 Measurement of [Ca²⁺]_i

Measurement of [Ca²⁺]_i was performed according to Grynkiewicz et al. (1985). For measurement of [Ca²⁺]_i the cells were loaded with fura 2-AM (5 µM and 1% DMSO) for 60 min under ELF-EMF exposure. Fura 2-AM was added to the bath during an off phase of ELF-EMF exposure, respectively. After the indicated time of exposure Fura 2-Amloaded loaded cells grown on a coverslip were transferred to an exposure-free superfusion chamber mounted on an inverted Axiovert (Zeiss, Germany) microscope. The cells were superfused with a bath solution containing (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1.5 MgCl₂, 5 glucose, 10 Hepes, (pH 7.4, adjusted with NaOH; 300 mosm) at 2 ml/min for 3 min to wash-off extracellular fura 2-AM and DMSO at room temperature. The cells were excited at 340 nm and 380 nm using a monochromator polychrome II (T.I.L.L. Photonics GmbH, Planegg; Germany) by a 75 W XBO xenon lamp and the corresponding fluorescence was registered with a digital CCD camera (C4742-95, Hamamatsu Photonics K.K.; Japan). The ratio of excitation at 340 nm to 380 nm was calculated and calibrated to determine [Ca²⁺]_i using the program Aquacosmos (Hamamatsu Photonics K.K.; Japan). Measurement of [Ca²⁺]_i was started about 10 min after completion of ELF-EMF exposure and recorded for 4-8 cells simultaneously. In a further series of experiments two additional stressors were applied after the period of ELF-EMF exposure, respectively. Either 200 µM H₂O₂ was added to the bath or 30 mM NaCl was replaced by KCl. The cells were superfused with the corresponding solution (2 ml/min) for 10 min. Thereafter the solution was replaced by the bath and the recording of [Ca²⁺]_i started.

2.9.7 Comet assay

The granulosa cell line of rat (GFSHR-17), HeLa cells (human) and Chinese Hamster Ovary (CHO) were cultivated as described above. The cells were ELF- and sham- exposed at various frequencies using the exposure parameters 5 min on/10 min off, 1.0 mT applied during 12 to 20 hours. After exposure the Comet assay was performed as described by Ivancsits et al. (2003a, b). The viability of the cells was determined by trypan blue and only slides containing cells with a viability of more than 90% were analysed. For each experiment 3000 nuclei are scored (1000 per slide) for exposed and sham exposed cells and classified into 5 categories according to material and methods of Ivancsits et al. (2002b). The results for DNA damage in the Comet assay are obtained from at least 2 independent exposure experiments. Analysis of exposed and sham-exposed cells was performed in a double-blind approach.

2.9.8 Measurement of cell volume regulation

For measurement of the cell volume the cells were trypsinised (0.25% Trypsin, pH 7.4), collected and centrifuged for 5 min at 500 xg after ELF exposure (18h expression time, 1.0 mT, 50 Hz, 5 min on/10 min off). The pellet was resuspended in 10 ml PBS (in mM: 140 NaCl, 2.7 KCl, 8 Na₂HPO₄, 1.5 KH₂PO₄, 300 mosmol, pH 7.4). For the volume measurements, 1-2 µl of the cell suspension were placed on a cover slip in an exposure-free superfusion chamber mounted on an inverted Axiovert (Zeiss, Germany) microscope. After 5 min, the cells adhered and 2-3 ml of PBS-solution (300 mosmol) were added to the

dish. The cells were superfused with the PBS-solution (300 mosmol) and the cell volume was recorded at time intervals of 30s about 40 minutes. The cell sizes were registered with a digital CCD camera (C4742-95, Hamamatsu Photonics K.K.; Japan). After 30 sec the 300 mosmol PBS-solution was replaced by a 250 mosmol PBS-solution (or 350 mosmol solution). This hypotonic (or hypertonic) solution was exchanged after 20 min by PBS-control (300 mosmol), again. The diameter of the cells of spherical shape (breadth, height) were measured and the corresponding rotationellipsoid volume determined. The time dependent volume $V(t)$ was subtracted by the basis volume V_0 , normalised to the maximal volume and the mean calculated.

2.10 Experiments with the human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

2.10.1 RF-EMF 900 MHz GSM signal exposure system dosimetry

Cells were irradiated with a simulated mobile phone microwave radiation in specially constructed exposure system, which is based on the use of high Q waveguide resonator operating in TE_{10} mode. The irradiation chamber has been placed vertically inside a cell culture incubator with two 55 mm-diameter glass Petri dishes placed so that the E-field vector was parallel to the plane of the culture medium. Temperature controlled water was circulated through a thin (9 mm) rectangular glass-fiber-molded waterbed underneath the Petri dishes. In all experiments reported here, cells were exposed for 1 hour to 900 MHz GSM signal at an average SAR of 2.4 W/kg. SAR values ranged from 1.8 W/kg to 2.5 W/kg depending on the area of the dish, what was caused by the non-uniform distribution of the RF-EMF radiation field. The average SAR level of 2.4 W/kg was selected because it is slightly above of the safety limit for the mobile phone microwave radiation emission as defined by ICNIRP (International Commission on Non-Ionizing Radiation Protection). RF-EMF signal was generated with EDSG-1240 signal generator and modulated with pulse duration 0.577 ms and repetition rate of 4.615 ms to match the GSM signal modulation scheme. Signal was amplified with RF Power Labs R720F amplifier and fed to the exposure waveguide via monopole type feed post. The SAR distribution in the cell culture and the E-field above the cell culture were determined using computer simulations (FDTD method). The simulations were done with commercial XFDTD code (Remcom, USA) with simulation grid size of $3 \times 3 \times 3 \text{ mm}^3$ in the main grid and $1 \times 1 \times 1 \text{ mm}^3$ in sub grid, consisting of the culture dishes and part of the waterbed. The maximum SAR was obtained in the centre of Petri dish, decreasing to about 6 dB at the edges of dish. Simulation results were verified with measurements. Electric field in the air above cell cultures was measured with a calibrated miniature Narda 8021B E-field probe. The measured E-field values differed less than 15% from the corresponding simulated E-fields. The SAR distribution was measured with small, calibrated temperature probes (Luxtron and Vitek) directly from the culture medium. The measurements were done at room temperature outside the incubator with increased culture medium height, in order to reduce the measurement uncertainty at air-medium boundary. The temperature was measured (Vitek probe, BSD Medical, USA) for 10 sec. in order to limit the effect of heat convection and conduction (Moros and Pickard, 1999). The Luxtron probe has lower temperature resolution (0.1°C) compared to Vitek probe (0.001°C) and thus the temperature had to be measured for 1 min. to achieve sufficient temperature rise (1°C). Due to these short measurement times the power fed to the chamber was increased up to 25 W and the resulting SAR value was afterwards scaled down to 1 W of input power. The measured SAR values at the centre of the culture medium (3-mm depth) were 2.5 W/kg (Luxtron, USA) and 5.0 W/kg (Vitek, USA). These values can be compared to the simulated value of 2 W/kg and 3.6 W/kg, respectively, with simulation parameters changed to correspond with the measurement situation. The measured values can be considered as the upper and lower limits of SAR due to measurement uncertainties described above and thus they validate the simulations. Waveguide resonator's water-cooling system was tested with long-term temperature measurements by using Luxtron probe. The temperature was recorded twice a minute over normal 1-hour exposure period at 2 W/kg. The temperature remained at $37 \pm 0.3^\circ\text{C}$ during the whole measurement time. Therefore, the reported biological effects are of non-thermal nature. Additionally, human endothelial cells were also exposed to GSM 1800 MHz radiation in talk and cw mode. See also 2.1.

2.10.2 Cell cultures and exposure

EA.hy926 and EA.hy926v1 cells (gift from Dr. Cora-Jean S. Edgell, North Carolina University at Chapel Hill, NC, USA) (Edgell et al. 1983) were grown Dulbecco's MEM, supplemented with antibiotics, 10% fetal bovine serum, L-glutamine and HAT-supplement. For experiments, cells were removed from culture flasks with trypsin, washed and seeded at density of 1.2×10^6 cells per 55 mm-diameter glass Petri dish (900 MHz GSM exposure) or seeded at density of 0.4×10^6 cells per 35 mm-diameter plastic Petri dish (1800 MHz GSM signal exposure). After overnight culture semi-confluent monolayers of EA.hy926 cells were exposed to sham or RF-EMF radiation. Cell cultures for sham and irradiation were prepared in the same kind of glass dishes, derived from the same batch of cells, were seeded at the same cell density and were grown for the same period of time before experiment. The only difference between irradiated and sham samples was that the irradiated dishes resided for 1-hour in incubation chamber with RF-EMF radiation turned-on whereas sham dishes resided in the irradiation chamber for the same period of time but with irradiation turned off.

2.10.3 ^{32}P -orthophosphate metabolic labelling

To determine changes in protein phosphorylation ^{32}P -orthophosphate was present in cell culture during the 1-hour sham or RF-EMF exposure. In experiments where the time-course of hsp27 phosphorylation was determined, ^{32}P -orthophosphate was present in cell cultures during the whole post-exposure incubation period. During the phosphorylation cells were incubated in culture medium consisting of phosphate-free DMEM that was supplemented with dialysed FBS and with ^{32}P - orthophosphate (NEN, Cat no. NEX-053s). Briefly, confluent monolayers of endothelial cells were washed twice with the pre-warmed (37°C) labelling medium that did not contain ^{32}P -orthophosphate, in order to wash away residual phosphates from the cell cultures. Thereafter, pre-warmed ^{32}P -orthophosphate-containing medium (5 mCi) was added to the cells and dishes were irradiated immediately for one hour. Following irradiation petri dishes were placed on ice, labelling medium was aspirated, cells were rinsed with cold PBS supplemented with protease/phosphatase inhibitors (1 mM PMSF; 0,4 mM orthovanadate) and cells were scraped and collected with ice cold PBS. From this point onwards orthovanadate was present in all solutions used to extract phosphoproteins. In experiments where the time-course of hsp27 phosphorylation was determined, ^{32}P -orthophosphate was present in cell cultures during the whole post-exposure incubation period (up to 5 hours).

2.10.4 2D-electrophoresis - for protein phosphorylation studies

Cells were harvested, washed once with ice cold PBS containing 1mM PMSF and lysed on ice for 10 minutes in buffer consisting of 9.5 M Urea, 2% CHAPS, 0.8% Pharmalyte pH 3-10 and 1% DTT. Lysates were cleared of debris by centrifugation 42000 xg at +15°C for 1 hour. The pellet containing insoluble debris was discarded and supernatant was collected and its protein concentration was measured with the Bradford method. Proteins in the lysates were separated using standard 2D-electrophoresis method - isoelectrofocusing (IEF) in the first dimension and SDS-PAGE in the second dimension.

1st-dimension isoelectric focusing

The 125 ug protein was applied to the groove of the re-swelling tray that contained 11 cm-long IPG strip with pH range of 3-10 (APBiotech, Sweden). The IPG strips were incubated overnight with the protein lysate solution. The proteins in IPG strips were separated by isoelectrofocusing (IEF) using the programmable power supply with the following protocol:

- 300 V, 1 W, 1 mA for 6 minutes at 20°C
- 3500 V, 1 W, 1 mA for 6 minutes at 20°C
- 3500 V, 1 W, 1 mA for 24 hours at 20°C

After completion of the IEF-separation the strips were equilibrated for 10 minutes on a rocking platform in solution-I consisting of urea (6 M), glycerol (30% w/v), SDS (2% w/v), DTT (100 mg/10 ml) in 50 mM Tris-HCl buffer pH 8.8 with a trace of bromphenol blue (migration marker). Thereafter, the strips were placed in a solution-II that contained iodocetamide (480 mg /10 ml) instead of DTT and equilibrated on a rocking platform for another 10 minutes at room temperature.

2nd-dimension SDS-PAGE

Equilibrated IPG strips were attached on the top of 8% SDS-PAGE gel with melted agarose to ensure firm contact. Gels were run with 40 mA/gel for ca. 2.5 hours at 4°C.

After completion of the electrophoretic separation gels were silver stained using Morrissey's modification of the Merril's method and images for computerised analysis were acquired into PC using the Bio-Rad GS-710 densitometer.

2.10.5 ³²P-autoradiography

2D-gels, containing metabolically ³²P-labelled phosphoproteins, were dried in gel dryer and used for autoradiography. Images generated on X-ray films were acquired into PC for computerised analysis using the Bio-Rad GS-710 densitometer.

2.10.6 2D-electrophoresis - protein expression screening

Immediately after the end of the exposure to 900 MHz GSM mobile phone radiation-like signal at the average specific absorption rate (SAR) of 2.4 W/kg cells were placed on ice, washed with ice-cold PBS and lysed with buffer consisting of: 7 M urea, 2 M thiourea, 4% chaps, 2% IPG buffer pH 3-10 NL, and 1% dithioereitol (DTT), 1 mM sodium orthovanadate and 1 mM PMSF. Protein concentration in lysates was measured using Bradford-method and 175 µg of total protein was used for 2-DE.

IEF was performed using IPGphor apparatus and non-linear pH 3-10 18 cm long IEF strips (Amersham Biosciences, Sweden). The samples were loaded using in-gel rehydration in a buffer containing 9 M urea, 2% chaps, 0.2% DTT, 0.5% IPG buffer pH 3-10 NL for 12 hours. IEF was run at 20°C using step-and-hold and gradient methods as follows: 30 V - 2 hrs, 100 V - 0.5 hrs, 300 V - 0.5 hrs, 600 V - 0.5 hrs, 1500 V - 0.5 hrs, 8000 V gradient 4 hrs, 8000 V - until the 65000 volt-hours were achieved.

For SDS-PAGE the IEF strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl pH 8.8, 2% SDS, and 10 mg/ml DTT for 15 min and then for another 15 min in the same buffer 25 mg/ml iodoacetamide replacing DTT. SDS-PAGE was run in 8% gel using Protean Ixi Multicell apparatus (Bio-Rad, UK) and a constant current of 40 mA/gel at 10°C.

After electrophoresis gels were fixed with 30% ethanol and 0.5% acetic acid overnight, washed with 20% ethanol and ddH₂O, sensitised with sodium thiosulfate (0.2 g/l), incubated in silver nitrate solution (2 g/l) and developed in a solution of potassium anhydride (30 g/l), 37% formaldehyde (0.7 ml/l) and sodium thiosulfate (0.01 g/l). The development was stopped with Tris (50 g/l) and acetic acid (0.05%) solution. Silver stained gels were stored in ddH₂O at 4°C. The gels were scanned using GS-710 densitometer (Bio-Rad, UK).

The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the Helsinki University, Finland. The spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with trypsin (Sequencing Grade modified Trypsin, promega, USA). The peptide mixture was concentrated and desalted using Millipore ZipTipTM µ-C18 pipette tips. The peptide mass fingerprints were measured with Bruker BiflexTM MALDI-ToF mass spectrometer in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The MALDI spectra were internally calibrated with the standard peptides, angiotensin II and adrenocorticotropin-18-39. The database searches were performed using ProFound (<http://prowl.rockefeller.edu/cgi-bin/ProFound>) and Mascot (<http://www.matrixscience.com>) searches.

2.10.7 Western blotting

Immediately after the completion of 2D-electrophoretic separation of protein lysates, gels were placed into the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS) and blotted onto PVDF membrane (Bio-Rad) using Novablot semi-dry blotting apparatus (APBiotech, Sweden). The transfer of proteins on the membrane was performed with current of 0.8 mA/cm² for 45 minutes at room temperature. Following transfer, the membranes were blocked overnight at 4°C in Tris-buffered-saline (TBS, pH7.4) containing 5% of non-fat milk proteins. The expression of Hsp27, MAP p38 kinase and protein phosphatase-1α was detected in western blot membranes by ECL method using specific polyclonal antibodies, peroxidase-conjugated second antibody and West Pico ECL kit (Pierce, USA).

2.10.8 Immunoprecipitation

Immunoprecipitation experiments were performed using cells that were metabolically labelled with ^{35}S -methionine (APBiotech, Sweden) as follows. Briefly, confluent EA.hy926 monolayers were washed twice with the pre-warmed (37°C) labelling medium (phosphate-free) to remove phosphates. After completion of washing, the pre-warmed ^{35}S -methionine-containing (2 mCi) labelling medium was added to culture dishes and cells were allowed to incorporate ^{35}S -methionine overnight. Then, ^{35}S -methionine-containing cultures were irradiated for 1 hour. After the end of irradiation dishes were placed on ice, cells scraped in 1 mM PMSF containing PBS and used in immunoprecipitation. In experiments where the time-course of protein expression changes was determined the ^{35}S -methionine was present in the cell cultures during the whole post-exposure incubation period (up to 8 hours). Harvested cells were lysed in ice-cold RIPA buffer. Lysates were centrifuged 10000 xg at 4°C for 10 minutes to remove debris and lysates' protein concentration was measured with the Lowry-Ciocalteu method. Samples containing 230 μg of proteins were placed in eppendorf tubes and pre-cleared with 2.3 μg of non-immune goat IgG (Santa-Cruz, USA; sc-2028) and 20 μl of recombinant-Protein-G-conjugated Sepharose-4B (Zymed, USA, Cat. no. 10-1242) at 4°C on a shaker for 30 minutes. After pre-clearing the Sepharose-beads were removed by centrifugation (1000 xg; 4°C ; 10 min) and selected proteins (hsp27, MAP p38 kinase, protein phosphatase-1 α) were immunoprecipitated with 2 μg of specific antibody and 20 μl of recombinant-Protein-G-Sepharose-4B-conjugate (overnight at 4°C on a shaker). Thereafter, beads were collected by centrifugation, washed 4 times with RIPA buffer, dispersed in the electrophoresis sample buffer, boiled on water-bath for 3 minutes and proteins released from the beads were resolved using 8% SDS-PAGE gel (40 mA/gel). Gels were stained with coomassie blue, dried between cellophane sheets and exposed with X-ray film for different periods of time to detect the ^{35}S -methionine labelled immunoprecipitated proteins.

2.10.9 cDNA Expression Arrays

Total RNA isolation

For the isolation of total RNA from RF- or sham-exposed cells we used NucleoSpin RNA II kit (Clontech, USA). Briefly, confluent cell cultures were directly lysed on glass culture dishes. RNA, from the cleared cell lysates, was directly immobilised in Spin columns provided by the manufacturer. After DNase treatment the total RNA was eluted from the columns and analysed for the possible remaining DNA contamination by PCR using b-actin primers against genomic DNA. RNA from several independent experiments were pooled and stored at -80°C for further use.

Probe synthesis and analysis of gene expression

For the synthesis of cDNA probes and differential analysis of gene expression we used Atlas Pure Total RNA Labelling System (Clontech) and Atlas cDNA Expression Arrays (Clontech), respectively. In this system mRNA was enriched from the total RNA by binding it to Streptavidin-biotin-oligo- (dT) coated magnetic beads. After enrichment of the mRNA they were reverse transcribed to radioactive cDNA probes directly when still bound to magnetic particles. Purified probes were hybridised with Atlas filters containing complementary cDNA spots and analysed by autoradiography. AtlasImage 2.0 software was used for the differential gene expression analysis of autoradiograms.

2.10.10 Cell cycle analysis

Cell cycle distribution among the EA.hy926 cells was detected by staining the DNA with propidium iodide followed by flow cytometry analysis. Briefly, cells were collected by centrifugation, washed once with phosphate-buffered saline (PBS) and fixed in 90% methanol on ice for 10 min. After fixation, the cells were washed twice with PBS and suspended in RNase solution in PBS (100 units/ml) and incubated at 37°C for 30 minutes. At this point the propidium iodide solution (10 mg/ml in PBS) was added to the cells in RNase solution, and the incubation was continued on ice overnight. Upon termination of incubation, cells were washed once with PBS and analysed by FACScan (Becton Dickinson, USA).

2.10.11 Caspase-3 activity

Caspase-3 activity was detected in non-fixed cells using CaspaTaq Caspase-3 Activity Kit (Intergen, USA). The active caspase-3 molecules are labelled in cells with a green fluorescent probe (FAM-DEVD-FMK) which only binds to the active caspase-3. Dead cells were excluded from the analysis by staining with propidium iodide that, when used with non-fixed cells, labels only cells with permeable membrane (necrotic or late apoptotic cells). The cell cultures were either sham- or RF-EMF-exposed with or without staurosporin (positive control of caspase-3 activation). Activity of caspase-3 was analysed either immediately after the exposure or 4h to 24h after exposure. Fluorescent content of the cells was analysed by flow cytometry with Lysys II software (Becton-Dickinson, USA).

2.10.12 Immunohistochemistry

A standard indirect immunofluorescence method was used for immunohistochemistry. Cells were washed twice with PBS and fixed in cold 3.7% paraformaldehyde in fixing buffer (0.1 M Pipes, 1 mM EGTA, 4% polyethyl glycol 8000, 0.1 M NaOH pH 6.9) overnight at 4°C. After fixing cells were rinsed twice with PBS, permeabilised with 0.5% Triton X-100 in fixing buffer 10 min, rinsed with PBS and permeabilised with 0.1% sodiumborohydride in PBS. After permeabilisation cells were rinsed with PBS and blocked with 5% BSA in PBS for 30 min. The primary antibody (Hsp27 StressGen, Canada) was incubated for an hour as well as the TRITC-labelled secondary antibody (DAKO, Denmark). After antibody incubations cells were rinsed with PBS and stained with Alexa Fluor 488 phalloidin for 30 min. Specimens were observed using a Leitz fluorescence microscope and computerized image acquisition system (Metafer, Germany)

2.10.13 Image analysis

Images of 2D-gels and X-ray films were analysed with the PDQuest 6.1.0/6.2.0 software (Bio-Rad, UK) or Phoretix 1D Advanced 5.0 (Nonlinear Dynamics, USA).

2.10.14 cICAT method

Protein Labelling and Purification

1 mg each of sample (talk and cw exposed) was labelled separately using the acid cleavable isotope-coded affinity tag (cICAT) reagent (Applied Biosystems, USA) following the vendors protocols as has been described (Burlingame). Briefly, the protein samples were separately reduced at cysteine residues using Tris (2-carboxyethyl) phosphine (TCEP, Pierce, USA), and the free sulfhydryl groups labelled using either the normal ($C^{13}(0)$) cICAT reagent, or the isotopically heavy ($C^{13}(9)$) cICAT reagent containing nine C^{13} atoms. The samples were then combined, enzymatically digested using trypsin (Promega, USA) and the resulting peptides were fractionated using strong-cation exchange (SCX) HPLC. The SCX HPLC was carried out on an Integral HPLC system (Applied Biosystems, USA) using a 2.1 mm x 250 mm polysulfoethyl A SCX HPLC column (PolyLC). The A buffer was 5 mM KH_2PO_4 /25% acetonitrile pH 3.0 and the B buffer was 5 mM KH_2PO_4 /25% acetonitrile pH 3.0 containing 300 mM KCl. The peptides were eluted and collected in one minute fractions using a gradient profile of 0-25% B over 30 minutes, followed by 25-100% B over 20 minutes, followed by washing of the column for 10 minutes at 100% B. Collected peptide fractions were affinity purified using avidin chromatography columns. The purified, labelled cysteine-containing peptides were then subjected to an acid incubation to cleave the biotin affinity tag from the peptides (Burlingame). The cleaved samples were then separated using offline microcapillary reverse-phase liquid chromatography (μ LC) with collection in one minute fractions onto a MALDI sample target using a 180 μ m x 15 cm reverse phased column home-packed with 5 μ m, 300 Å C18 material (Magic, Michrom Inc., USA) and an Ultimate capillary LC system coupled to a Probot sample spotter (Dionex, USA). The MALDI matrix α -cyanohydroxycinnamic acid (CHCA, Agilent, USA) was automatically added to the eluent at each spot on the sample plate.

Automated mass spectrometric analysis

After μ LC fractionation to the MALDI sample plate, the samples were analysed using an abundance-ratio dependent methodology on a oMALDI qTOF mass spectrometer (oMALDI Qstar, MDS-Sciex/Applied

Biosystems). First, a single-stage mass spectrum of each sample spot on the plate is acquired for 30 seconds at each spot. The relative intensity ratios, as well as singlet peaks for all of the detected, cICAT labelled peptides are then automatically calculated using an automated software algorithm. Those peptides showing $C^{13}(0):C^{13}(9)$ relative intensity ratios of >1.7 or <0.6 were outputted to an inclusion list for identification by tandem mass spectrometry (MS/MS). All those peaks identified as singlets (i.e. no matching $C^{13}(0)$ or $C^{13}(9)$ peak) were also selected for MS/MS analysis. The peptide masses contained in the inclusion list at each sample spot were then analysed by MS/MS analysis, using one minute data acquisition time for each peptide.

Sequence database searching and quantitative analysis

The acquired MS/MS data were searched against the human protein sequence database maintained at the National (USA) Cancer Institute using the search program Sequest. For all MS/MS data, the search was run with no enzyme constraint, or amino-acid composition constraint (i.e. only those sequences containing cysteine), and a mass tolerance of 0.1 Da was used for the precursor peptide mass. A differential mass addition to cysteine of 227.13 was indicated in the search parameters for the $C^{13}(0)$ reagent, and 236.16 for the $C^{13}(9)$ reagent. Only matches to peptide sequences containing cysteine were kept after the database search. The results were further statistically scored using a recently described statistical algorithm for validation of sequence database search results. Only those peptides having a confidence score of 0.85 or greater using this tool were considered to be accurate matches. The quantitative $C^{13}(0)/C^{13}(9)$ values determined by the automated software described above were matched to each identified peptide, and these ratios were each checked for accuracy by manual inspection of the raw mass spectral data.

2.11 Effects of ELF-EMF and RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

2.11.1 ELF-EMF and RF-EMF exposure setups

See 2.1

2.11.2 Cell cultures and RNA isolation

See reports of the REFLEX Participants who provided samples for this investigation.

2.11.3 RZPD cDNA arrays

The whole-genome Human Unigene RZPD-2 cDNA array contains about 75,000 cDNA clones (I.M.A.G.E. clone collection), the Mouse Unigene RZPD-1 array about 25,000 clones, each selected from UniGene clusters (group of Bernhard Korn (RZPD), see also: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene>). The cDNA products were PCR amplified by M13 forward and reverse standard primers and spotted in duplicates on 22 x 22 cm nylon membranes (mouse: 1 part, human: 3 parts) in a 5 x 5 pattern (group of Uwe Radelof (RZPD), see also Boer et al. (2001) Genome Res. 11, 1861-1870). Each 5 x 5 field contained 11 genes spotted in duplicates as well as the E.coli kanamycin gene (1 spot) and an Arabidopsis gene (2 spots) as "empty" spots for background subtraction during data analysis. For quality control, M13 forward and reverse primers were end labelled with ^{33}P gamma ATP and hybridised to each membrane to control of all filters of the same robot run were spotted even and complete. After quality control, the membranes were stripped and used for complex hybridisation after about 6 weeks. Only filters from the same robot run containing comparable concentrations of PCR products representing single genes or ESTs were used for hybridisation with the different samples to be compared. One individual hybridisation experiment was done on the same filter batch with all 4 samples. Repetitions, however, were performed with different filter batches to exclude biases caused by using filters from only one robot run.

2.11.4 Hybridisation of global cDNA arrays and image analysis

RNA was isolated from exposed and as a control sham-exposed cells from different cell lines (Table 1). RNAs coming from individual experiments were checked separately for degradation (28S/18S rRNA ratio 1.5-2.0) and concentration (at least 1 µg/µl in H₂O) with the Bioanalyzer (Agilent). Afterwards, RNAs from 2 individual exposures were pooled for each hybridisation sample in same concentrations. Hybridisation was performed according to Boer et al. (2001) with minor modifications: 10 µg of total RNA per sample was reversely transcribed using (dT)18 primer and 33P alpha-dCTP without amplification (Superscript II reverse transcriptase, Invitrogen) and purified. The labelled cDNA was hybridised with the arrays. The hybridisation solution contained 6 x SSC/5 x Denhardt's, as well as Cot-1 DNA (Invitrogen Co., Germany) and (dA)40 oligonucleotide for blocking. After exposition of the hybridised membranes, the PhosphorImager screens were scanned (Fuji FLA-3000, 100 µm resolution, Fuji BAS-reader software). The primary image analysis (estimation of nVol grey level values for each individual spot) was done by the help of the ArrayVision software package (Interfocus), which had been adjusted to the 5x5 array before. The background was corrected locally in each 5x5 field by subtracting the empty spot signal (average signal of 3 spots, see above). Normalisation was done via the average signal intensity (without empty spots) on the whole membrane. Two independent hybridisations were performed for each experiment (4 data points per gene because of spotting of each gene in duplicates).

2.11.5 Pre-processing (data cleaning) and Modified SAM method (and Selective SAM method)

Original expression profiling data ("control" data *ctrl*, related to gene expression without the applied EMF, and "exposed" *exp*, related to gene expression after EMF exposure) were normalised and the background was removed as mentioned above. For each experiment (Table 1) at least two hybridisations were performed. In a first pre-processing, signals were removed giving a zero or an infinite ratio ($ratio = exp/ctrl$), that could lead to a reduction of the available measurements for some genes.

Table 1. Gene expression profilings on human and mouse global cDNA arrays

Cell line	Exposition	Exposure experiments/ profiling	Array	Participant
ES-1	ELF/EMF: 50 Hz, 1 mT	4	Human	3
human primary fibroblasts	5 min ON, 10 min OFF, 24 h		Unigene RZPD-2	
ES-1	ELF/EMF: 50 Hz, 1 mT	4	Human	3
human primary fibroblasts	5 min ON, 10 min OFF, 15 h		Unigene RZPD-2	
SY5Y	ELF/EMF: 2 mT	4	Human	11
human neuroblastoma	5 min ON, 5 min OFF, 16 h		Unigene RZPD-2	
ES	ELF/EMF: 50 Hz powerline	3	Mouse	4
mouse embryonic stem cells	2.3 mT		Unigene RZPD-1	
NB69	RF/EMF: 1800 MHz (GSM Basic)	2	Human	5
human neuroblastoma	SAR 2 W/kg		Unigene RZPD-2	
	5 min ON, 10 min Off, 24h			
EA.hy926	RF/EMF: 900 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
EA.hy926	RF/EMF: 1800 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
EA.hy926	RF/EMF: 1800 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
T-lymphocytes	RF/EMF: 1800 MHz	2	Human	8
human, quiescent	DTX only		Unigene RZPD-2	
from peripheral blood	SAR 1.4 W/kg			
	10 min ON, 20 min OFF, 44 h			
	(RNA prepared in Heidelberg)			
U937	RF/EMF	5	Human	9
human lymphoblastoma	GSM-900 MHz		Unigene RZPD-2	
	2 W/kg, 1 h			
CHME5 (µglie)	RF/EMF	5	Human	9
human microglial	GSM-900 MHz		Unigene RZPD-2	
	2 W/kg, 1 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.0 W/kg			
	5 min ON, 5 min OFF, 24 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.3 W/kg			
	continuous waves, 24 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.3 W/kg			
	continuous waves, 24 h			

2.11.6 Biostatistics (Dr. Daniel Remondini, Participant 8)

The statistical analysis to find those genes that significantly changed their expression level between the ctrl and the exp state was done as follows: In order to increase the statistical significance of the test, we considered only those genes with $NG = 4$ “good” measurements (both for ctrl’s and exp’s). The genes of each experiment were kept separated in 3 different groups, related to each part (nylon membrane) they belonged to (“Part 1”, “Part 2”, “Part 3”). Normalisations and analysis were performed on each part separately, in order to avoid possible biases due to different behaviour of the arrays during hybridisation or scanning. Data were processed in order to evaluate and reduce possible artefacts due to the array reading procedure:

- as a first step, data were rescaled by means of a cubic root function in order to gather the data in a smaller interval: $ctrl' = \sqrt[3]{ctrl}$, $exp' = \sqrt[3]{exp}$;
- the averages of all 8 measurements for each gene were taken as a “reference” set. A scatter plot was made of each measurement (ctrl or exp) versus the reference set, and the resulting plot was fitted linearly. On the basis of the fit parameters a rescaling was performed on data if they were not on a Y=X curve.

The “interesting” genes were found by calculating a normalised difference $diff(i)$ between exp and ctrl values for each gene i :

$$diff(i) = \frac{E[exp(i)] - E[ctrl(i)]}{\sqrt{\frac{\sigma_{ctrl}^2}{NG-1} + \frac{\sigma_{exp}^2}{NG-1} + s_0}},$$

where “E[]” denotes “expected value of”, and s_0 is a correction term that removes possible divergences in the denominator, calculated as the median of the σ_i distribution for each gene (ctrl and exp distributions were merged). For a first selection, genes were considered outliers if they exceeded the threshold of 3σ calculated from the resulting distribution of $diff(i)$.

To increase the robustness of our selection, following the bootstrapping procedure as shown in [PNAS 2001, Vol. 98 no. 9, pg. 5116-5121], new datasets were generated as permutations of the original ones, and the same analysis was performed over these datasets, ranking the differences $diff_p(i)$ (p referring to the p -th permutation) from larger to smaller. A plot of $diff(i)$ vs. $E[diff_p(i)]$ was generated for each group of genes, and the interesting genes were chosen as those that significantly deviated from the $y=x$ line: the distance of $diff(i)$ from the $y=x$ line, defined as $dist(i)$, was to be larger than a threshold value $\Delta=1.2$.

Finally, the outlier genes selected for each part in each experiment were chosen from the intersection between the initial outliers ($|diff(i)| \geq 3\sigma$) and those from the bootstrapping procedure ($|dist(i)| \geq \Delta$). In this way the outlier selection results more selective than just applying the technique as shown in [PNAS 2001, Vol. 98 no. 9, pg. 5116-5121].

2.11.7 Data mining

The ratio of the spot-to-spot comparison was taken for further analysis, without values smaller than 0.001 or bigger than 1,000, respectively, which were eliminated before. Since each PCR fragment was spotted twice on each membrane, and each hybridisation experiment was performed twice, four ratios went into an own database tool (Martin Holst, access database program, Microsoft) to make an analysis in a non-statistical manner: All eight values coming from two hybridisations had to show the same tendency to be taken over in the final list showing clones appearing up- or down-regulated in both experiments. The clones of this list were connected to the gene ontology data via a Stanford database (<http://genome-www5.stanford.edu/cgi-bin/SMD/source//sourceBatchSearch>) according to IMAGE IDs. With the help of these data and a text query tool (Microsoft Excel, Matthias Schick) regulated genes belonging to certain gene families of interest could be extracted manually. The same procedure was applied on genes extracted by bio-statistical analysis.

3.0 RESULTS

The studies in REFLEX cover a wide range of frequencies within the spectrum of electromagnetic fields (EMF). Based on the assumption that possible biological effects may be generated by EMF in a different way dependent on their frequencies the results of the REFLEX project are reported separately for extremely low frequency electromagnetic fields (ELF-EMF) and radio frequency electromagnetic fields (RF-EMF).

3.1 Results in ELF-EMF research

3.1.1 Genotoxic effects

3.1.1.1 Human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and granulosa cells of rats (Participant 3)

Intermittent ELF-EMF exposure, but not continuous ELF-EMF exposure induced DNA strand breaks in human fibroblasts.

In the first set of experiments fibroblasts were continuously exposed to ELF-EMF at 1000 μ T for 24h. In these experiments significant differences in DNA-breaks between exposed and sham-exposed cells were observed neither with the alkaline nor with the neutral Comet assay. As Nordenson et al. (1994) reported positive genotoxic effects applying intermittent field exposure, our next experiments concentrated on exposures at different intermittence conditions. Intermittence of 5 min on/5 min off and 15 min on/15 min off revealed an increase of DNA-breaks in both, the alkaline and the neutral Comet assay, compared to sham-exposed cells, whereas at 5 min on field/25 min off field did not enhance the frequency of DNA-breaks (Tables 2, 3).

Table 2. Mean values of alkaline Comet tailfactors at different exposure conditions (n = 2), cell strain IH-9

Alkaline Comet Assay - different exposure conditions				
	exposed		sham	
	Comet tailfactor %	\pm SD [#]	Comet tailfactor %	\pm SD [#]
Continuous exposure (24h)	4.29	0.02	4.27	0.03
15'/15' on/off	6.47*	0.14	4.23	0.05
5'/5' on/off	6.98*	0.04	4.41	0.16
5'/10' on/off	7.47*	0.13	4.48	0.05
5'/15' on/off	6.68*	0.17	4.42	0.03
5'/20' on/off	5.90*	0.12	4.38	0.12
5'/25' on/off	4.27	0.04	4.23	0.03
1'/10' on/off	5.89*	0.19	4.21	0.14
3'/10' on/off	6.60*	0.06	4.19	0.22
10'/10' on/off	6.91*	0.07	4.24	0.07
15'/10' on/off	6.56*	0.15	4.11	0.08
25'/10' on/off	5.37*	0.05	4.21	0.04

[#] SD indicates standard deviation

* indicates significant differences (p < 0.05) exposed vs. sham

Table 3. Mean values of neutral Comet tailfactors at different exposure conditions (n = 2), cell strain IH-9

Neutral Comet Assay - different exposure conditions				
	exposed		sham	
	Comet tailfactor %	±SD [#]	Comet tailfactor %	±SD [#]
Continuous exposure (24h)	4.20	0.03	4.17	0.05
15/15' on/off	5.72*	0.01	4.25	0.04
5/5' on/off	6.09*	0.02	4.31	0.08
5/10' on/off	6.21*	0.01	4.35	0.07
5/15' on/off	5.66*	0.06	4.23	0.13
5/20' on/off	4.52	0.16	4.50	0.21
5/25' on/off	4.25	0.05	4.34	0.07
1/10' on/off	4.16	0.15	4.16	0.13
3/10' on/off	5.94*	0.05	4.20	0.06
10/10' on/off	6.19*	0.11	4.11	0.11
15/10' on/off	6.02*	0.03	4.21	0.10
25/10' on/off	5.44	0.01	4.15	0.01

[#]SD indicates standard deviation

* indicates significant differences (p < 0.05) exposed vs. sham

Based on these findings, we tried to find out the optimal exposure conditions for maximal effects on DNA strand break levels. We started with a fixed field-on time of 5 min and varied field-off times from 5 to 25 min. These experiments indicated that DNA strand break levels (SSB and DSB) culminated at an off-time of 10 min and reached control levels at extended off-times (Figure 7). Significant differences (p< 0.01) between exposed and sham-exposed cells were found at 5 min on/5 min off, 5 min on/10 min off, 5 min on/15 min off and 5 min on/20 min off intermittence for alkaline Comet assay and at 5 min on/5 min off, 5 min on/10 min off and 5 min on/15 min off intermittence for neutral Comet assay, but not at 5 min on/25 min off for both assays.

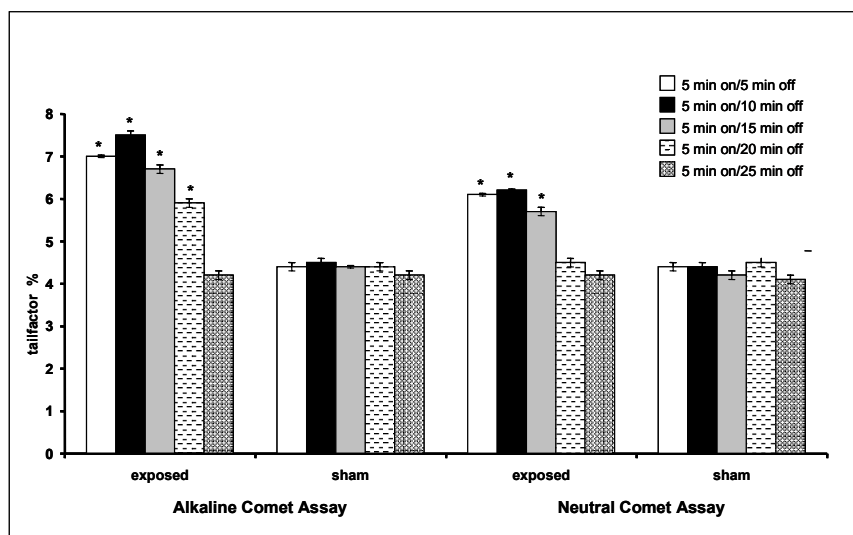


Figure 7. Alkaline and neutral Comet Assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 50 Hz sinus, 24h, 1000 µT, intermittent) - variation of off-time. * p<0.01 exposed versus sham-exposed

Subsequently, a fixed off-time of 10 min was chosen and on-times have been varied from 1 to 25 min. Again, the highest level of DNA strand breaks was obtained at an intermittence of 5 min on/10 min off (Figure 8). Comet tailfactors of exposed and sham-exposed cells differed significantly at each on-time in

alkaline Comet assay and at 3 to 15 min on in the neutral Comet assay. Solely the alkaline Comet tailfactors of 5 min on/10 min off, 5 min on/25 min off and 25 min on/10 min off-EMF exposed cells differed significantly to the other applied intermittence conditions. Since an intermittence of 5 min on/10 min off was able to induce the highest levels of DNA strand breaks in both alkaline and neutral Comet assay, further experiments were performed at 5 min on/10 min off.

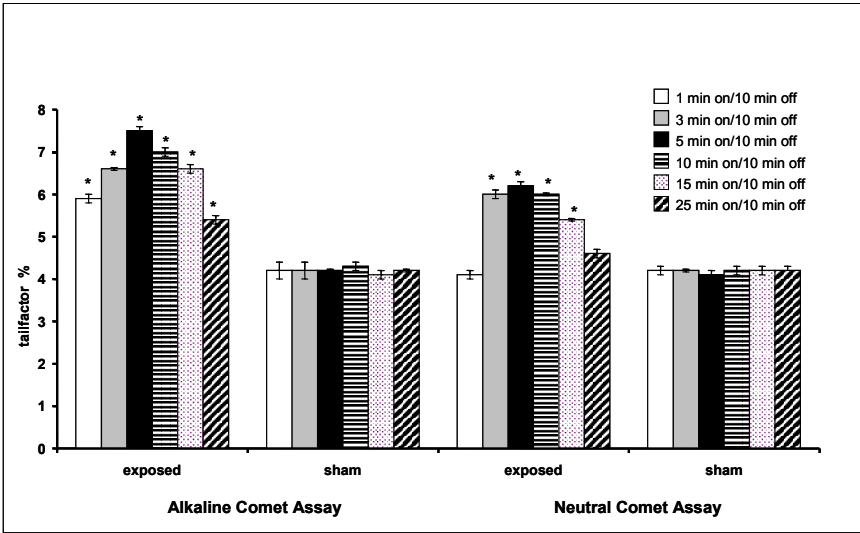


Figure 8. Alkaline and neutral Comet assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 50 Hz sinus, 24 h, 1000 µT, intermittent) - variation of on-time. * p < 0.01 exposed versus sham-exposed

ELF-EMF 50 Hz sinus generated a higher rate of DNA strand breaks in human fibroblasts than ELF-EMF powerline.

By comparing 50 Hz sinus to the 50 Hz powerline signal it was found out that at 50 Hz powerline Comet assay tailfactors were significantly lower than at 50 Hz sinusoidal (Figure 9). All further experiments in the ELF-EMF range were, therefore, carried out with 50 Hz sinus.

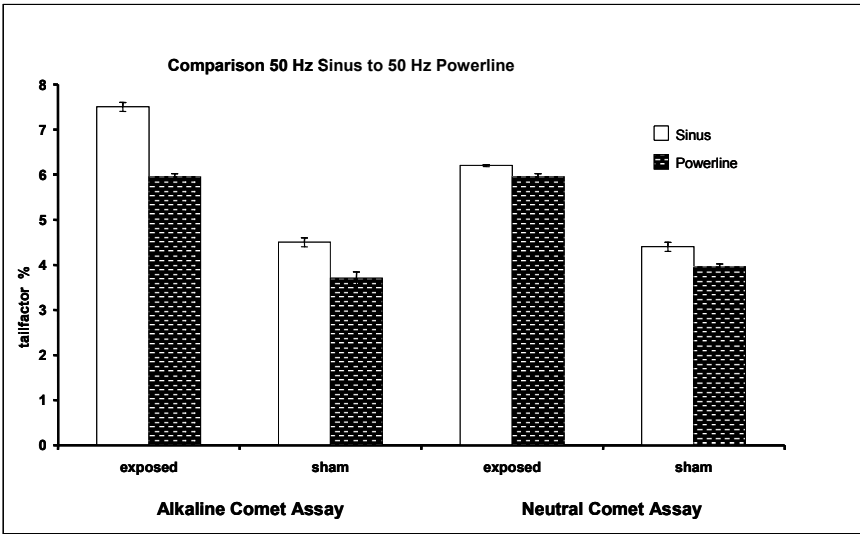


Figure 9. Alkaline and neutral Comet Assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 24 h, 1000 µT, intermittent)

Genotoxic effects were frequency dependent.

In order to investigate the frequency dependence of genotoxic effects of ELF-EMF (1 mT, 5 min on/ 10 min off) cultured human fibroblasts were exposed to different frequencies (3-550 Hz). Exposure time was set to 15 hours. Genotoxic effects were evaluated using the alkaline Comet assay. Figure 10 presents the tailfactors in exposed and sham exposed cells. Significant increases in DNA damage could be found at 3 Hz, 16 2/3 Hz, 30 Hz, 50 Hz, 300 Hz, and 550 Hz. Effects on strand break levels varied with the applied frequencies and could be ranked as follows: 50 Hz > 162/3 Hz > 3 Hz > 300 Hz > 550 Hz > 30 Hz. Quite obviously, the extent of induced DNA damage did not correlate with the applied frequency.

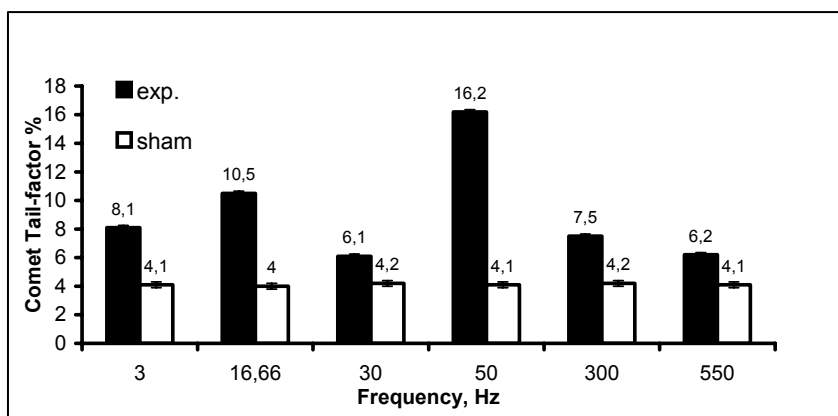


Figure 10. Alkaline Comet Assay tailfactors of ELF-EMF exposed and sham exposed fibroblasts (cell line ES-1, 15 hrs, 1000 μ T, intermittent) after variation of exposure frequency (3-550 Hz).

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was dependent on exposure time.

Alkaline and neutral Comet tailfactors increased with exposure time (1-24 hours, 1000 μ T, intermittent (5 min on/10 min off)), being largest at 15 hours (Figure 11). Comet assay levels declined thereafter, but did not return to basal levels.

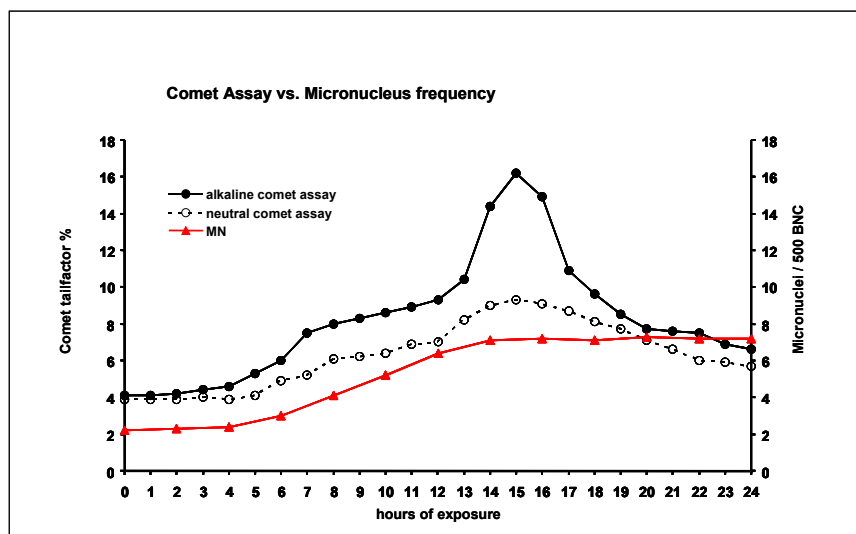


Figure 11. Influence of exposure time on formation of DNA single and double strand breaks and micronuclei in human fibroblasts (cell strain ES-1, 1 mT, 5 min on/10 min off cycles).

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was dependent on the age of the donors.

Fibroblasts from six healthy donors (ES1, male, 6 years old; AN2, female, 14 years old; IH9, female, 28 years old; KE1, male, 43 years old; HN3, female, 56 years old; WW3, male, 81 years old) revealed differences in response to ELF-EMF exposure (Table 4). Cells from older individuals exhibited a higher rate of single and double strand breaks and their break levels started to decline later than in cells from younger donors (Figures 12, 13).

Table 4. Alkaline and neutral Comet assay tailfactors of donors with different age (ES-1: 6, AN2: 14, IH9: 28, KE1: 43, HN3: 56, WW3: 81 years of age) - variation of exposure duration (basal-, maximum-, and end-levels)

	cell strain	hours exposure duration	Alkaline Comet Assay		Neutral Comet Assay	
			Comet tailfactor %	±SD [#]	Comet tailfactor %	±SD [#]
basal levels	ES1	0	4.112	0.018	3.901	0.006
	AN2	0	4.077	0.064	3.900	0.035
	IH9	0	4.223	0.047	4.161	0.148
	KE1	0	6.227	0.044	5.224	0.013
	HN3	0	6.802	0.018	6.313	0.064
	WW3	0	7.101	0.064	6.816	0.023
maximum levels	ES1	15	16.155	0.184	9.305	0.057
	AN2	15-16	16.501	0.004	9.394	0.134
	IH9	16	16.707	0.040	9.716	0.054
	KE1	18	17.300	0.064	10.462	0.277
	HN3	18-19	18.311	0.078	11.364	0.122
	WW3	19	18.517	0.069	12.822	0.076
and levels	ES1	24	6.611	0.017	5.742	0.023
	AN2	24	7.210	0.062	5.824	0.030
	IH9	24	7.511	0.017	6.127	0.054
	KE1	24	8.242	0.038	6.738	0.023
	HN3	24	8.718	0.008	6.761	0.006
	WW3	24	9.229	0.037	8.010	0.063

#SD .standard deviation

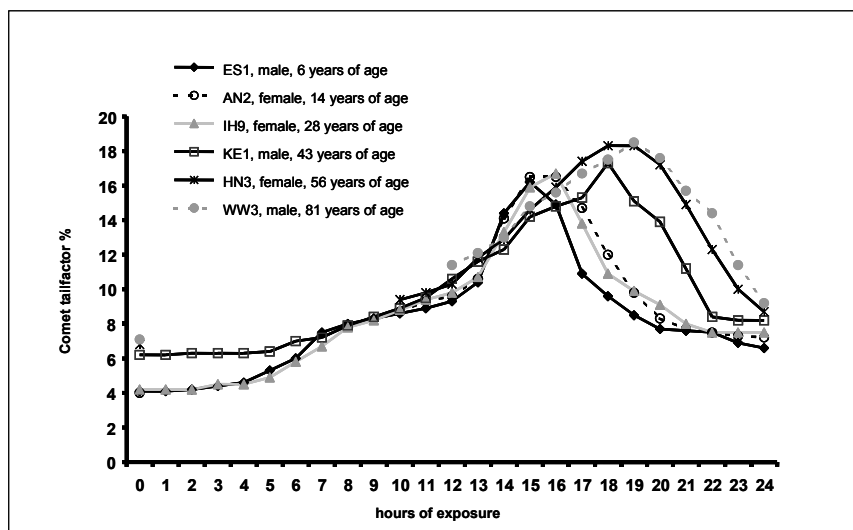


Figure 12. Alkaline Comet tailfactors of human diploid fibroblasts of donors with different years of age exposed to ELF-EMF (1 mT, intermittent 5 min on/10 min off) for 1-24 hours

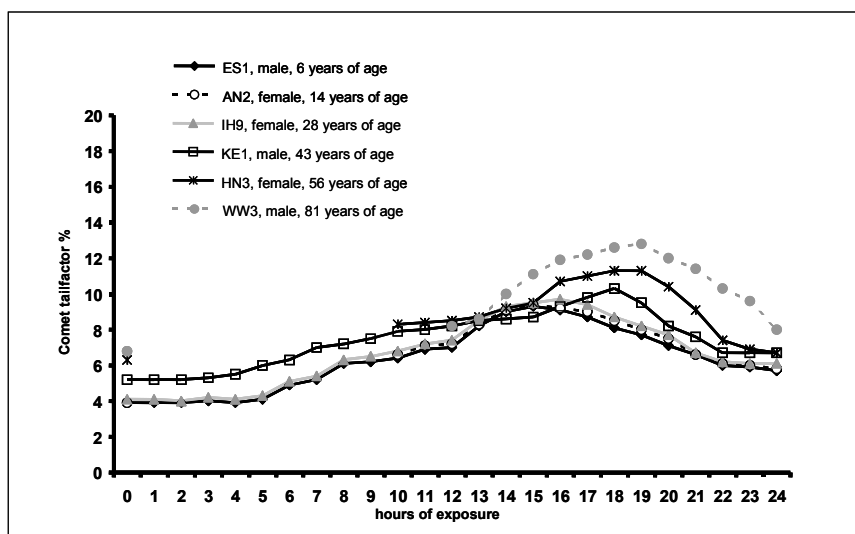


Figure 13. Neutral Comet tailfactors of human diploid fibroblasts of donors with different years of age exposed to ELF-EMF (1 mT, intermittent 5 min on/10 min off) for 1-24 hours

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was accompanied by a rise in micronuclei frequencies.

In addition, variation of exposure time from 2 to 24 hours revealed a time dependent increase in micronucleus frequencies. As shown in Figure 11, this increase became significant ($p < 0.05$) at 10 hours of ELF-EMF exposure. Thereafter, micronucleus frequencies reached a constant level, which was about 3-fold as compared to the basal levels (Figure 14).

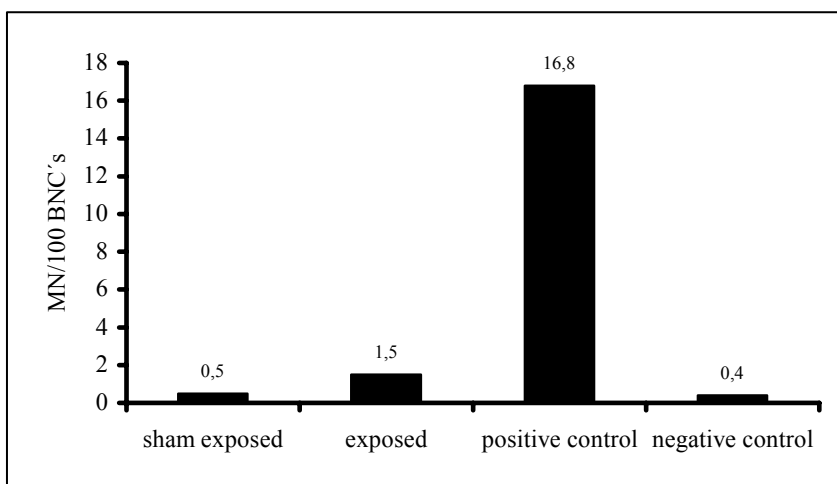


Figure 14. Micronucleus frequencies of ELF-EMF exposed (50 Hz, 1 mT, 15 h, 5 min on/10 min off) cultured human fibroblasts and controls (Vienna). Bleomycin 10µg/ml was used as a positive control.

ELF-EMF exposure did not diminish the number of fibroblasts in culture.

No differences in cell count between exposed and sham exposed cells at any exposure duration could be detected. Therefore, an elimination of cells by apoptosis and cell death during ELF-EMF exposure can probably be ruled out.

ELF-EMF exposure induced DNA strand breaks in human fibroblasts in a dose dependent way.

When magnetic flux density increased from 20 to 1,000 μT , a dose dependent rise in Comet assay tailfactors could be observed. At an exposure time of 24 hours a magnetic flux density as low as 70 μT produced significantly elevated ($p < 0.01$) alkaline and neutral Comet assay levels as compared to sham-exposed controls (Figure 13). At 15 hours of exposure genotoxic effects already occurred at 35 μT (Table 5, Figure 12). Using regression analysis, a significant correlation between Comet tailfactors and applied magnetic field (alkaline Comet assay: $r = 0.843$, $p = 0.004$; neutral Comet assay: $r = 0.908$, $p = 0.0007$), as well as between alkaline and neutral Comet assay could be found ($r = 0.974$, $p = 0.00001$).

Table 5. Mean values of alkaline and neutral Comet tailfactors at intermittent ELF exposure (5/10 on/off, 1000 μT , 24 h) (n = 2) dose response, cell line ES-1

μT magnetic flux density	Alkaline Comet Assay				Neutral Comet Assay			
	exposed		sham		exposed		sham	
	tailfactor %	$\pm\text{SD}^\#$	tailfactor %	$\pm\text{SD}^\#$	tailfactor %	$\pm\text{SD}^\#$	tailfactor %	$\pm\text{SD}^\#$
20	4.16	0.02	4.21	0.13	3.63	0.01	3.60	0.08
50	4.16	0.06	4.20	0.12	3.70	0.16	3.72	0.03
70	4.87*	0.03	4.28	0.02	3.99*	0.01	3.71	0.01
100	5.25*	0.06	4.28	0.05	4.32*	0.00	3.73	0.04
250	5.31*	0.02	4.25	0.07	4.24*	0.06	3.60	0.02
500	5.52*	0.01	4.22	0.01	4.48*	0.02	3.79	0.05
750	6.17*	0.08	4.26	0.11	5.08*	0.08	3.67	0.10
1000	6.50*	0.18	4.27	0.10	5.71*	0.01	3.79	0.16
2000	6.62	0.01	4.13	0.04	5.79*	0.05	3.70	0.01

[#] SD indicates standard deviation

• indicates significant differences ($p < 0.05$) exposed vs. sham

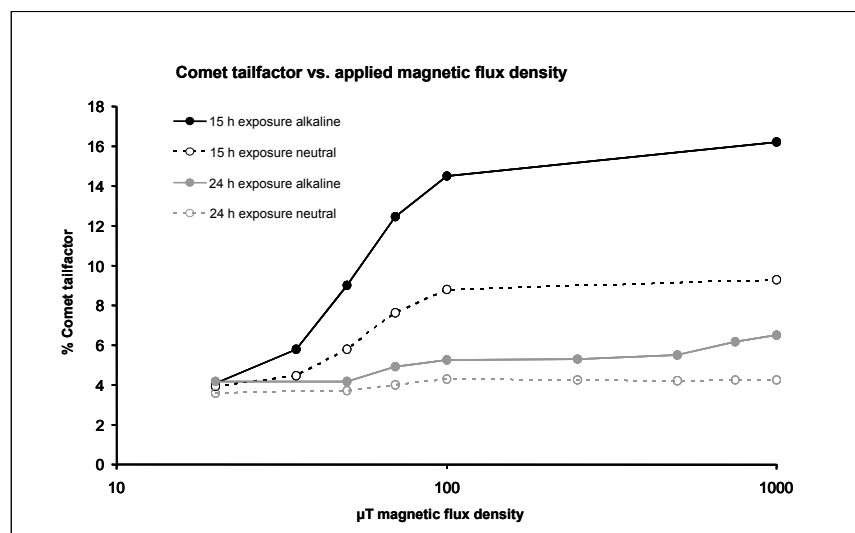


Figure 15. Dose dependent formation of DNA single and double strand breaks determined with Comet assay under alkaline and neutral conditions with cell strain ES-1 (exposure time 15 and 24 hours, 5 min on/10 min off cycles)

DNA strand breaks in human fibroblasts after ELF-EMF exposure were rapidly repaired.

After having demonstrated a time dependent relationship between alkaline and neutral Comet assay tailfactors and ELF-EMF exposure, the next aim was to find an explanation for the declining of the Comet assay levels after reaching the peak value. When exposure was terminated after 12 or 15 hours the Comet tailfactors returned to basal levels after a repair time of 7 to 9 hours (Figures 15, 16), comprising in a fast repair rate of DNA single strand breaks (< 1 hour) and a slow repair rate of DNA double strand breaks (> 7 hours). The marked Comet peak value between 12-17 hours disappeared when the Comet assay was performed at pH 12.1 instead of pH >13, thereby eliminating the cleavage of alkali labile sites in the DNA (Figure 18). The decline of Comet tailfactors after 15-20 hours of exposure could be prevented, when the cells were exposed in the presence of 10 µg/ml cycloheximide, an inhibitor of protein synthesis (Figure 18).

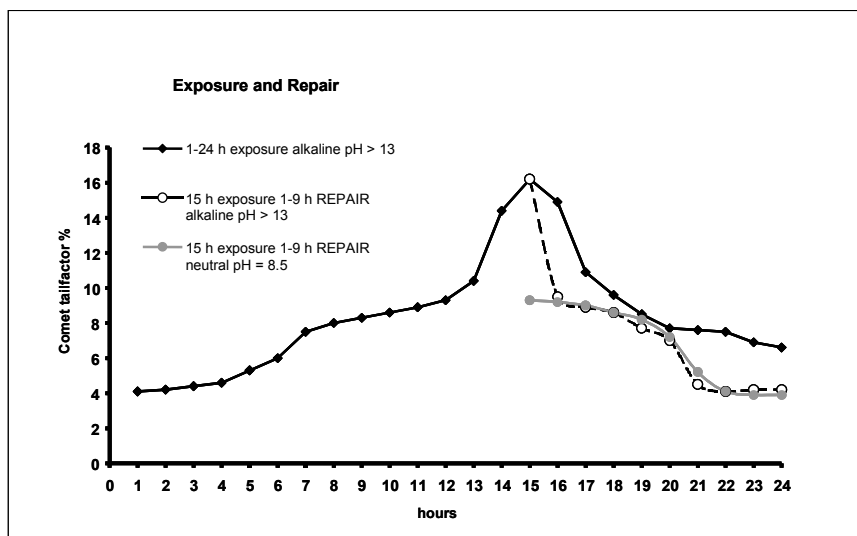


Figure 16. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after termination of ELF-EMF exposure (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline and neutral Comet assay - repair after 15 h ELF-EMF exposure

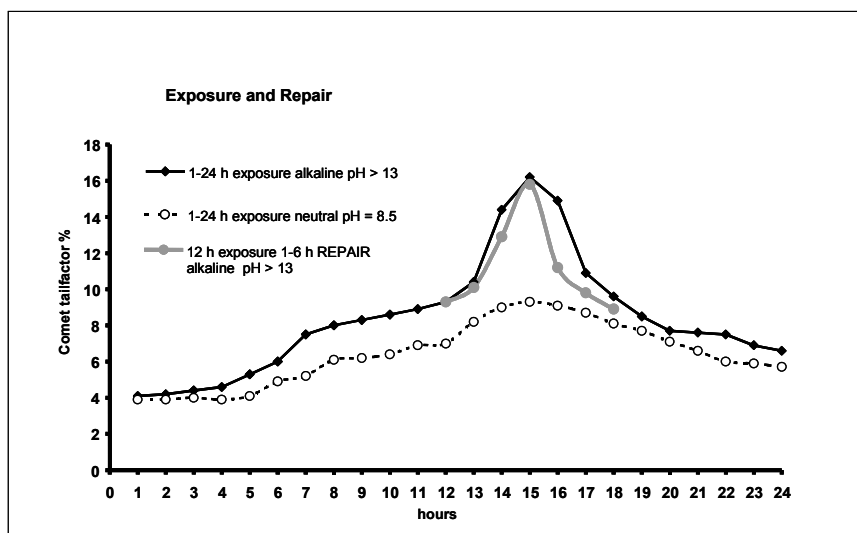


Figure 17. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after termination of ELF-EMF exposure (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline and neutral Comet assay - repair after 12 hours ELF-EMF exposure

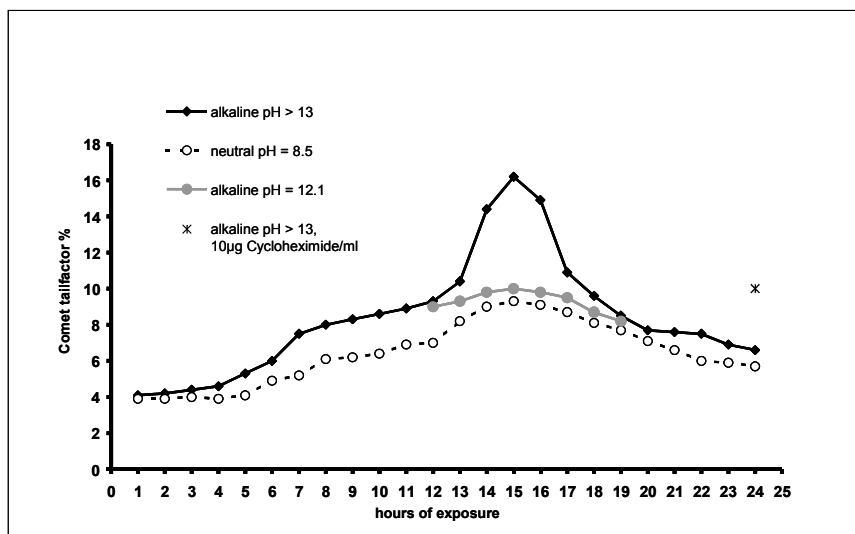


Figure 18. Comet assay of exposed human fibroblasts was performed at different pH (1 mT, intermittent 5 min on/10 min off)

DNA repair deficient cells react differently to ELF-EMF exposure.

Diploid human fibroblasts from patients with the genetically determined DNA repair defects Cockayne Syndrome, Ataxia Teleangiectatica, and Bloom Syndrome were obtained from Coriell Cell Repository (Camden, New Jersey, USA). The cells were cultured under standardized conditions and exposed (24 hours, 1 mT, 5 min on/10 min off) or sham exposed, and alkaline Comet assay was performed as described. As a result the Cockayne and Bloom Syndrome fibroblasts exhibited a similar pattern of genotoxicity as normal control fibroblasts, whereas the cells from a patient with Ataxia Teleangiectatica showed an almost threefold increased ELF-EMF induced Comet tailfactor as compare to normal cells (Figure 19).

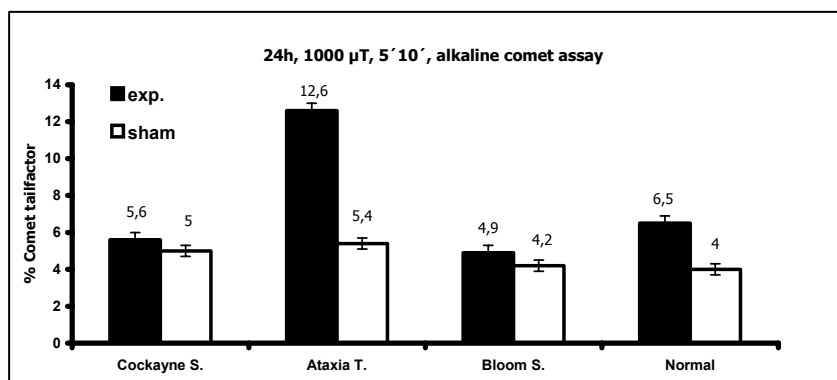


Figure 19. Alkaline Comet assay tailfactors of ELF-EMF exposed human fibroblasts from patients with various genetically determined DNA repair defects and normal controls.

Generation of DNA strand breaks through ELF/EMF was cell type specific.

ELF-EMF exposure (50 Hz sinusoidal, 1 mT, 5 min on/10 min off, 1-24 hours) of different human cell types (melanocytes, skeletal muscle cells, fibroblasts, monocytes, stimulated and quiescent lymphocytes) and of SV40 transformed rat granulosa cells revealed differences in induced DNA damage. Rat granulosa cells exhibited the highest DNA strand break levels and seemed to be most sensitive to intermittent ELF-

EMF exposure (Figures 20, 21). Human melanocytes also reacted, but not as strong as fibroblasts or rat granulosa cells. In contrast, stimulated or non-stimulated lymphocytes, monocytes and skeletal muscle cells did not respond at all.

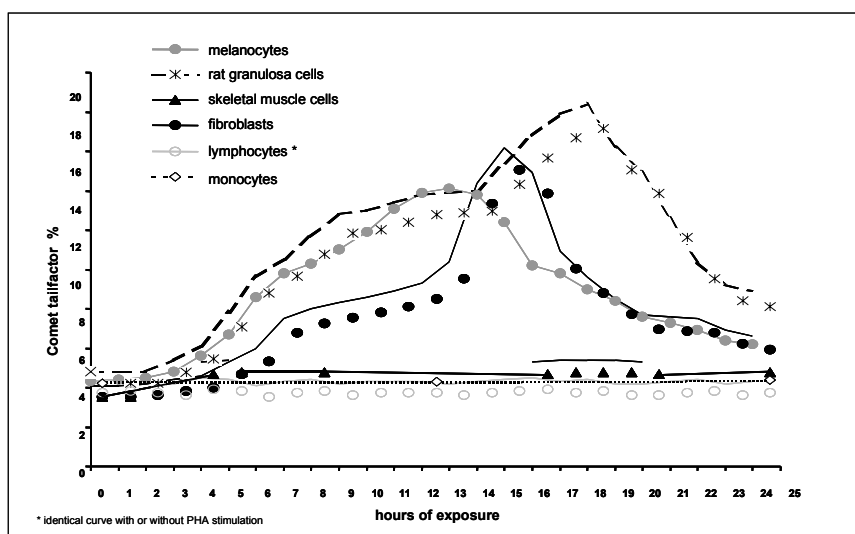


Figure 20. Alkaline Comet tailfactors of different human cell types (fibroblasts, melanocytes, monocytes, lymphocytes, skeletal muscle cells) and SV 40 transformed rat granulosa cells exposed to ELF-EMF (50 Hz sinusoidal, 1 mT, intermittent 5 min on/10 min off) for 1 to 24 hours.

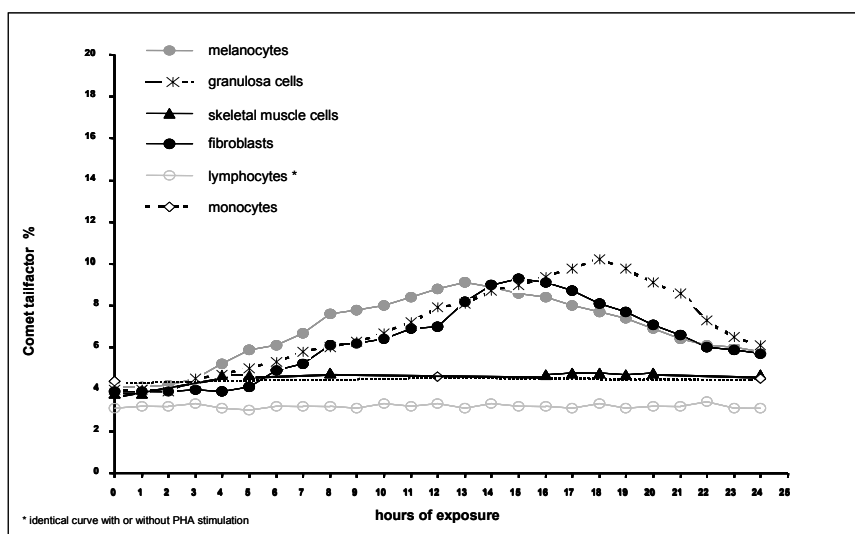


Figure 21. Neutral Comet tailfactors of different human cell types (fibroblasts, melanocytes, monocytes, lymphocytes, skeletal muscle cells) and SV 40 transformed rat granulosa cells exposed to ELF-EMF (50 Hz sinusoidal, 1 mT, intermittent 5 min on/10 min off) for 1 to 24 hours.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF and their repair were modified by UVC or heat stress.

To test a possible impact of ELF-EMF exposure on DNA repair, cells were subjected to combined exposures to ELF + UVC or ELF-EMF + heat stress. In the first set of experiments fibroblasts were pre-exposed to UVC (10 min., 1.2 kJ/m²). Subsequently, ELF-EMF exposure (50 Hz, sinus, 1000 μ T) was varied from 1-24 hours. Results of the alkaline Comet assay showed that DNA damage caused by UVC could be removed within 7 hours of ELF-EMF exposure (Figure 22). UV/ELF-EMF exposed cells

resulted in 50 % higher Comet assay levels than UV/sham exposed cells after 1 hour of ELF-EMF exposure. In UV/ELF-exposed cells DNA-damage was repaired very slowly, but the maximum at 15 hours ELF-EMF-exposure could not be detected any more. The results were similar with the neutral Comet assay, but DNA damage (DNA double strand breaks) was repaired within a shorter time (Figure 23)

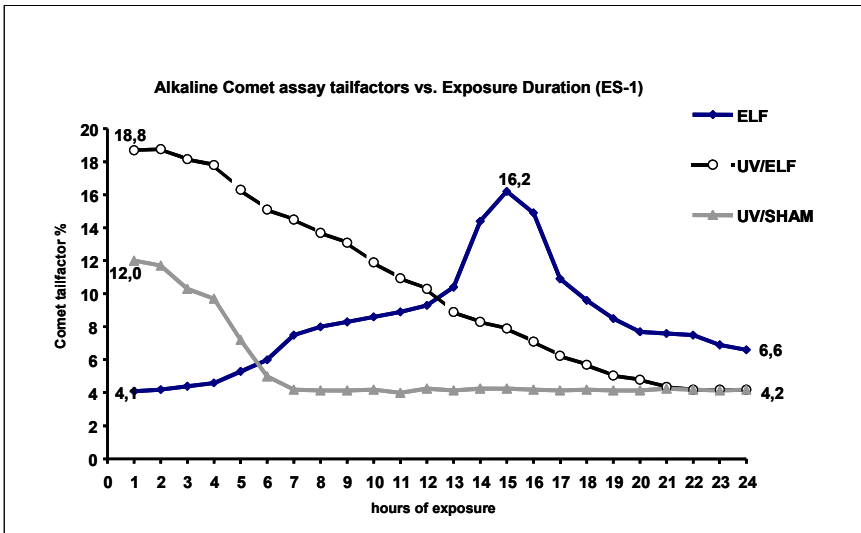


Figure 22. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after exposure with UV-C, ELF-EMF or UV C + ELF-EMF (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline Comet assay

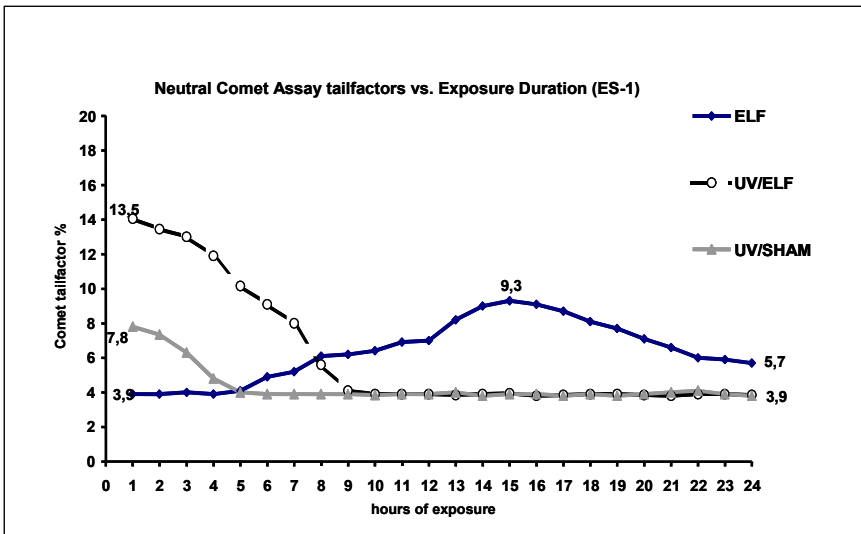


Figure 23. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after exposure with UV-C, ELF-EMF or UV C + ELF-EMF (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using neutral Comet assay

Based on the results with human fibroblasts, which suggest an induction of DNA repair upon intermittent ELF-EMF exposure, we concluded that pre-exposure to ELF-EMF would have a protective effect and diminish effects of additional exposures from other genotoxic factors. To check this assumption, fibroblasts were pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min on/10 min off, 1 mT) for 20 hours to ensure maximum induction of DNA repair. Subsequently, cells were either exposed to UVC (254 nm, 4.5

kJ/m^2 , 30 min) or to mild heat stress (38.5°C , 4 h). Recovery of DNA damage was evaluated using alkaline and neutral Comet assay. UVC-exposure produced 50 % higher DNA strand break levels than ELF-EMFs alone and DNA damage was completely repaired after 3 hours (Figure 24). DNA damage induced by mild thermal stress was even higher and persisted longer than 6 hours after exposure termination. Pre-exposure to ELF-EMF intensified and elongated UVC or temperature induced DNA damage. After 24 hours of recovery time ELF-EMF pre-exposed cells still exhibited higher DNA strand break levels and just about 50% of the initially induced DNA damage had been repaired after this time. The results were similar with the neutral Comet assay, indicating induction and repair of DNA double strand breaks (Figure 25).

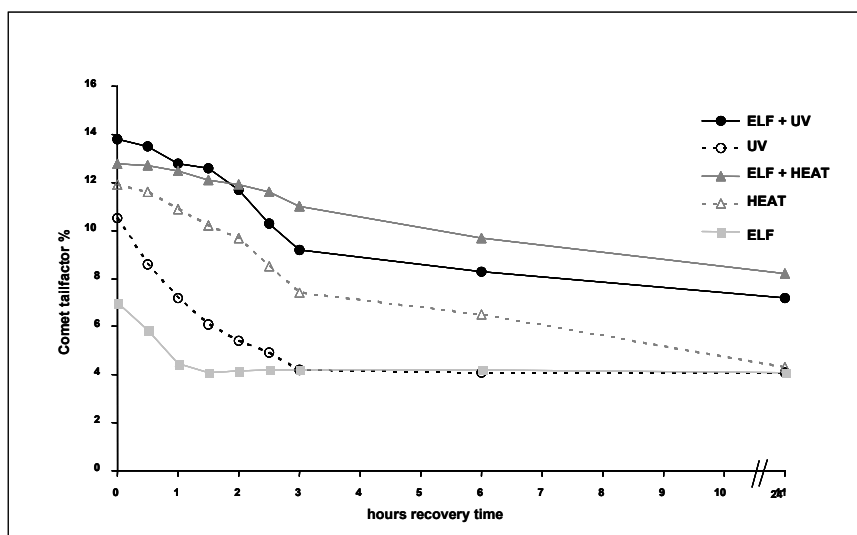


Figure 24. DNA damage and repair of cultured human fibroblasts pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min field-on/10 min field-off, 1 mT, 20 hours) and additionally exposed to UVC (254 nm, 30 min, 4.5 kJ/m^2) or mild thermal stress (38.5°C , 4 hours) evaluated using alkaline Comet assay.

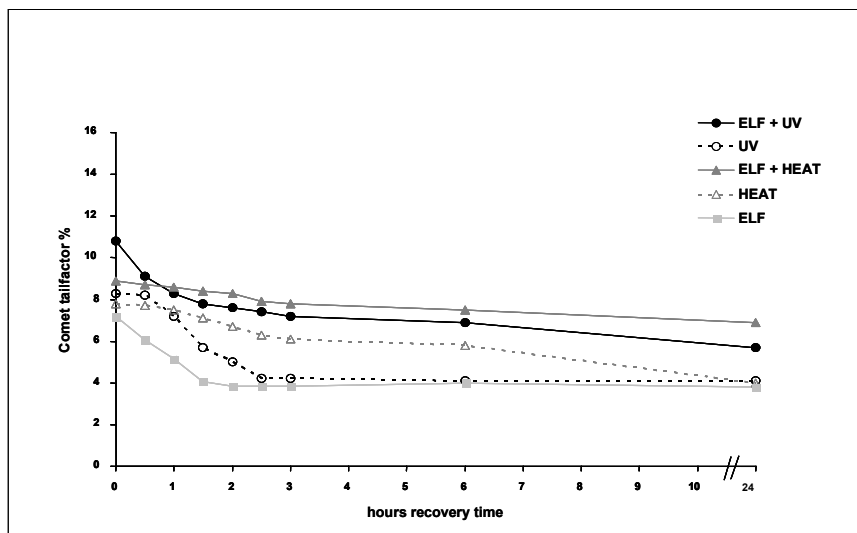


Figure 25. DNA damage and repair of cultured human fibroblasts pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min field-on/10 min field-off, 1 mT, 20 hours) and additionally exposed to UVC (254 nm, 30 min, 4.5 kJ/m^2) or mild thermal stress (38.5°C , 4 hours) evaluated using neutral Comet assay.

ELF-EMF generated chromosomal aberrations in human fibroblasts.

Chromosomal aberrations were evaluated at exposure conditions producing maximum effects in the Comet assay and in the micronucleus test (15 h, 1 mT, 5min on/10 min off). Five different types of aberrations were separately scored (gaps, breaks, rings, dicentric chromosomes, acentric fragments). Significant increases ($p < 0.05$) between exposed and sham exposed cells could be demonstrated for all types of aberrations (Table 6). Gaps were 4-fold increased, breaks 2-fold, and dicentric chromosomes and acentric fragments 10-fold. Translocations were evaluated using the fluorescence in situ hybridisation (FISH) technique. 1,000 metaphases were scored for each specifically labeled chromosome (1-22, X and Y) after ELF-EMF exposure (50 Hz, 24h, 5 min on/10 min off, 1 mT). No stable translocation in any of the 24,000 metaphases of ELF-EMF exposed cells could be detected (data not shown).

Table 6. Percentage of chromosomal aberrations induced by ELF-EMF exposure (50 Hz, 5' field-on/10' field-off, 1 mT, 15 h) in cultured human fibroblasts.

Types of aberrations	ELF-exposed (% \pm SD)	sham-exposed (% \pm SD)	p-value*
chromosome gaps	24.3 \pm 1 %	5.5 \pm 0.7 %	< 0.001
chromosome breaks	2.2 \pm 0.3 %	1.3 \pm 0.3 %	0.0015
ring chromosomes	0.1 \pm 0.07 %	---	0.0133
dicentric chromosomes	0.4 \pm 0.1 %	0.06 \pm 0.05 %	< 0.001
acentric chromosomes	0.3 \pm 0.07 %	0.02 \pm 0.04 %	< 0.001

^a A number of 1,000 metaphases were scored in each of five independent experiments. Results are expressed as percentage chromosomal aberrations per cell.

^b Significant differences ($p < 0.05$) as compared to sham-exposed controls using Student's t-test for independent samples

ELF-EMF did not alter the mitochondrial membrane potential in human fibroblasts.

The experimental settings in the present tests were based on conditions, which resulted in the highest inducible frequencies of these DNA strand breaks in human fibroblasts. Evaluating changes in the mitochondrial membrane potential after ELF exposure (50 Hz, 15 hours, 1 mT, 5 min on/10 min off) using JC-1, revealed no significant differences between exposed and sham-exposed fibroblasts.

3.1.1.2 Granulosa cells of rats, Chinese hamster ovary cells (CHO) and HeLa cells (Participant 7)

ELF-EMF exposure caused a significant increase of DNA strand breaks in cultured rat granulosa cells, CHO cells and HeLa cells.

The effect of ELF-EMF was analysed on the genomic level by use of the Comet assay. Especially the dependence on exposure time and frequency was analysed. Figure 26 shows that exposure to ELF-EMF at 16 2/3 Hz (5 min on/10 min off, 1.0 mT) caused a significant increase in single and double DNA strand breaks in cultured granulosa cells. The same result was obtained with CHO and HeLa cells (not shown). The data presented in Figure 26 indicate that the genotoxic effect at 16 2/3 Hz is time dependent with a maximum after about 18 hours of exposure, which resembles the results obtained at 50 Hz by Participant 3 (Ivancsits et al., 2003).

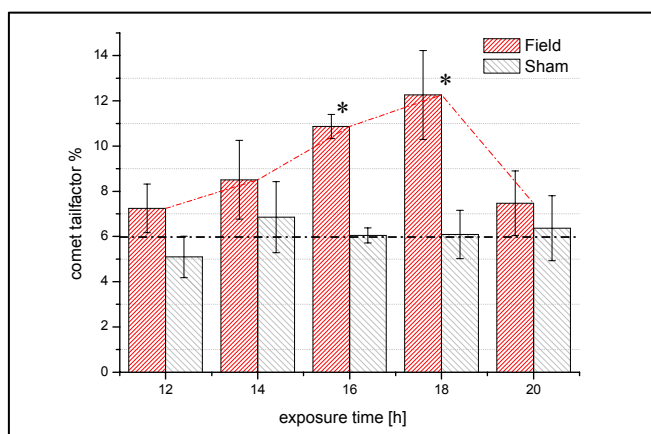


Figure 26. DNA damage of rat granulosa cells after exposure for 12 to 20 hours to ELF-EMF (16 2/3 Hz, 5 min on/10 min off, 1 mT) evaluated using alkaline Comet assay. For values of n see Material and Methods. (* $p < 0.05$)

To investigate the frequency dependence of the genotoxic effect of ELF-EMF (5 min on/10 min off) exposure of rat granulosa cells a constant exposure time of 18h was selected. Figures 27 and 28 present the tailfactors of exposed and sham exposed granulosa cells using the alkaline and neutral Comet assay. At the applied frequencies within the range of 8 Hz to 1000 Hz a significant frequency dependence was not observed for the rate of double DNA strand breaks as derived from the neutral Comet assay (Figure 28). The corresponding results of the alkaline Comet assay are presented in Figure 27. At 8 Hz, 16 2/3 Hz and 50 Hz an intensity of 1 mT could be applied (Figure 27a). A significant increase of DNA strand breaks was found at 16 2/3 Hz. Surprisingly, especially at 50 Hz the s.e.m. data of sham and ELF-EMF exposed cells differ significantly. The large error could be caused by a variable time dependent location of the maximum and/or the influence of the specific cell passage. Further experiments are under analysis to confirm the data presented in Figure 27a. At 1000 Hz the recorded DNA damage is significantly lower than observed at 16 2/3 Hz (Figure 27b). But it has to be noted that the maximal applied flux density was limited to 0.6 mT due to the used exposure system (Participant 10). Furthermore, DNA damage was measured at 20 μ T, which approximately corresponds to the maximal acceptable magnetic flux density as recommended by the 26. BlmSchV¹. Again a significant increase of DNA strand breaks was observed (Figure 27b).

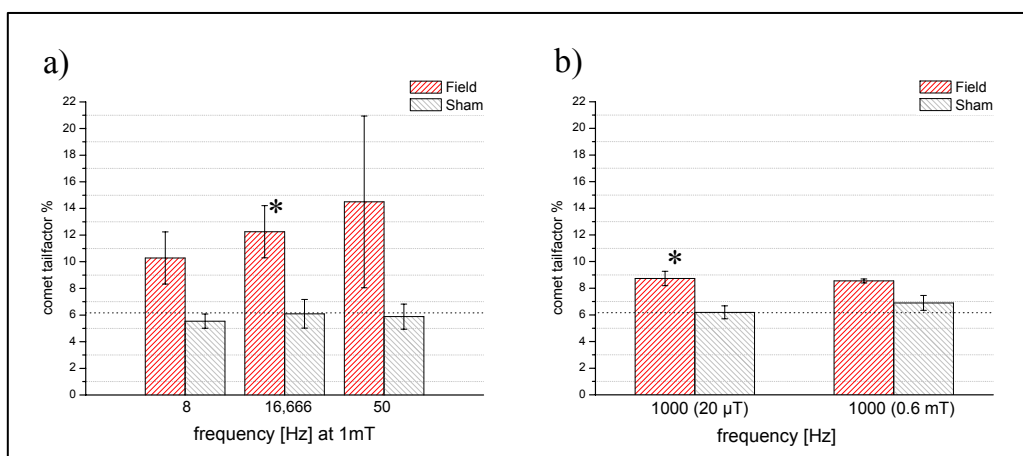


Figure 27. DNA damage of rat granulosa cells as function of frequency of ELF-EMF (5 min on/10 min off) after an exposure for 18 hours as derived by the alkaline Comet assay. **a)** In the frequency range of 8 to 50 Hz the flux density was set to 1 mT. **b)** At 1000 Hz the flux density was adjusted to 20 μ T and 0.6 mT (for further explanation, see text). For values of n see Material and Methods. (* $p < 0.05$)

¹ 26. Verordnung zur Durchführung des Bundes-Immissionschutzgesetzes (Verordnung über elektromagnetische Felder – 26. BlmSchV)

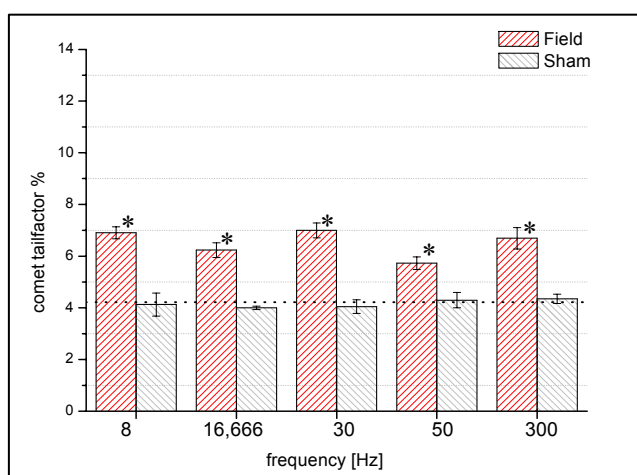


Figure 28. Double DNA strand breaks of rat granulosa cells as function of frequency of ELF-EMF (5 min on/10 min, 1 mT) after an exposure time of 18 hours as derived by the neutral Comet assay. For values of n see Material and Methods. (* p< 0.05)

3.1.1.3 Embryonic stem cells (ES) of mice (Participant 4)

Lack of effects on single and double strand break induction 0, 18, 24 and 48 hours after completion of a 6 or 48 hours ELF-EMF exposure.

The effects of ELF-EMF on the integrity of DNA strands in differentiating ES cell from EB outgrowths were studied. Two schemes were applied: (1) For ELF-EMF exposure (50 Hz Powerline, 2.0 mT, 5 min on/30 min off, 6 hours) the percentage of primary DNA damage was measured immediately after ELF-EMF exposure at the stage of neural differentiation (4+4d - 4+6d) and 18 hours after exposure using the alkaline and neutral Comet assay. (2). In the second set of experiments, the same ELF-EMF exposure conditions were applied for 48 hours instead of 6 hours, and the alkaline Comet assay was done immediately after exposure, while the neutral Comet assay was done 24 or 48 hours post exposure. No significant differences were observed in the induction of single or double DNA strand breaks between sham-exposed or ELF-EMF exposed neural progenitors.

3.1.1.4 Summary (Participant 1)

Our data indicate a genotoxic action of ELF-EMF in various cell systems. This conclusion is based on the following findings:

- Intermittent exposure to 50 Hz ELF-EMFs generated DNA single and double strand breaks in various cell systems such as human fibroblasts, melanocytes, granulosa cells of rats, Chinese hamster ovary cells (CHO) and HeLa cells, but not in human lymphocytes, monocytes, myelocytes and neural progenitors from mouse embryonic stem cells (see 3.1.1.1, 3.1.1.2 and 3.1.1.3).
- DNA damage generated by ELF-EMF in human fibroblasts was dependent on time and dose of exposure, on the age of the donors the cells derived from, and on the genetic background of the cells. A flux density of 35 μ T was high enough to significantly increase the number of DNA strand breaks (see 3.1.1.1)
- The increase in DNA strand breaks in human fibroblasts due to ELF-EMF exposure was accompanied by an enhanced formation of micronuclei which was also dependent on the exposure time (see 3.1.1.1).
- The DNA repair system in human fibroblasts which was strongly activated by ELF-EMF during exposure did not work error-free as shown by a significant increase of different types of chromosomal aberrations (see 3.1.1.1).
- Genotoxic effects were frequency dependent. Significant increases in DNA strand breaks were found, when an ELF-EMF of 3 Hz, 16 2/3 Hz, 30 Hz, 50 Hz, 300 Hz, 550 Hz and 1000 Hz was applied. The

effect was strongest with 50 Hz ELF-EMF and second strongest with 16 2/3 Hz (see 3.1.1.1 and 3.1.1.2).

3.1.2 Cell proliferation and differentiation

3.1.2.1 Human neuroblastoma cell line NB69 (Participant 5)

ELF-EMF promoted the growth rate of NB69 neuroblastoma cells.

Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells contain the neuroblast-specific protein β III-tubulin. However, these cells do not contain the neuroepithelial marker nestin, which is present in immature progenitors and in some neuroblastoma cells, nor the astrocyte-specific antigen GFAP. The cells remained in an undifferentiated state throughout the experimental period. Only the treatment with retinoic acid induced neurite extension accompanied by cell growth reduction.

Two series of experiments were carried out to analyse the cell growth response of NB69 cells to ELF-EMF. In the first series, the ELF-EMF administrated alone (42 hours) provoked a modest, though significant increase in the number of cells at day 5 postplating (5 dpp), both at 10- μ T (12% over controls, $**p<0.01$) and 100- μ T MFD (17 % increase over controls, $***p<0.001$) as shown in Figure 29A). Retinol (ROL) alone or in combination with ELF-EMF did not change significantly the cell growth (data not shown). The ELF-EMF exposure also provoked modest increases in the total DNA levels, the effect being statistically significant at 10 μ T (8 % over controls, $p<0.05$, Figure 29B). However no significant changes were observed in the protein or protein/DNA contents in the ELF-EMF exposed samples. Taken as a whole, these results indicate that exposure to 50 Hz ELF-EMF at 10 or 100 μ T promote cell growth in the NB69 human neuroblastoma cell line.

In the second series of experiments, the 42-hours exposure to ELF-EMF at a flux density of 100 μ T significantly increased cell growth (11 % over controls, $***p<0.001$, Figure 30A). This result confirms the growth-promoting response obtained in the first experimental series. However, such an effect was not observed, when the ELF-EMF exposure was maintained for a longer period of time, i.e. 90 hours (Figure 30B). The treatment with retinoic acid (RA) alone significantly reduced the cell number, both at 42 and 90 hours, when compared to untreated controls. Also, RA-treated cells did show the growth-promoting effects of a 100 μ T ELF-EMF, these samples demonstrating reduced growth rates when compared to unexposed controls: 35% reduction at the end of 42 hours-treatment ($p<0.0001$) and 57% reduction at the end of 90 hours-treatment ($p<0.0001$).

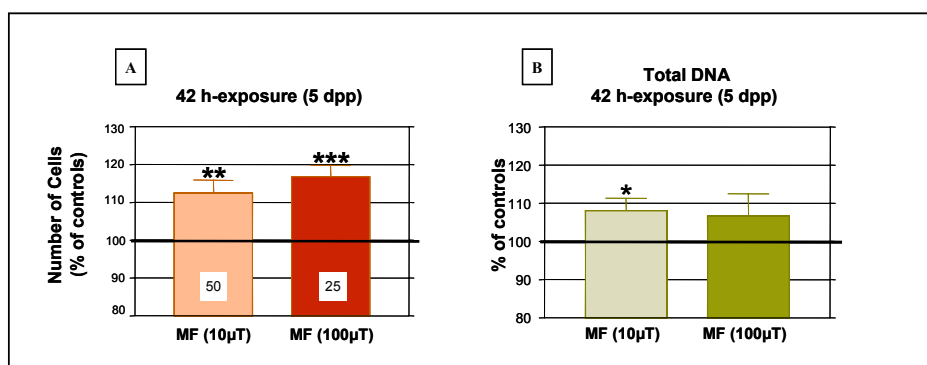


Figure 29. First series of experiments. A-Cell growth estimated by cell counting (Trypan blue exclusion) and B-Total DNA estimated by spectrophotometry.

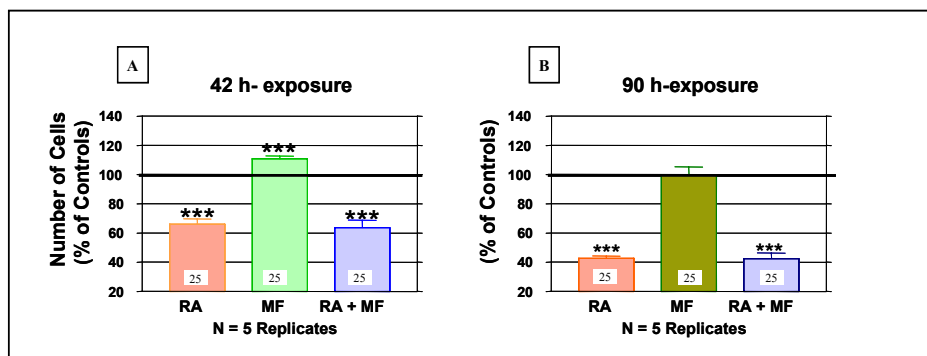


Figure 30. Second series of experiments: A-Number of cells at day 5 postplating (after 42 hours-exposure) and B-Number of cells at day 7 postplating (after 90 hours-exposure).

No experiment could be performed at a flux density of 2.0 mT following the 3 hours on/3 hours off exposure protocol, since the ohmic heat in the coils due to the electric current could not be compensated by the incubator resulting in an unstable ambient temperature. When the samples were exposed to 2 mT ELF-EMF in a 5min on/30 min off cycle, no effect was observed on the cell growth. Similarly, the cells did not respond to a 5 min on/30 min off cyclic exposure to a 100 μ T ELF-EMF. It is possible that the NB69 cell line requires a longer exposure cycle to show significant changes in the cell growth.

A growth-promoting effect of ELF-EMF in NB69 neuroblastoma cells was not observed after an extended exposure period.

As described above, the growth promoting effect of a 100- μ T EMF was not observed when the exposure was maintained for a longer period of time, i.e. 90 hours (Figure 30B). In NB69 cultures kept in control conditions, the number of cells peaks at day 6 and then decays (Figure 31). In the present experiments, long-term cultures (7 days postplating) reached a confluence stage close to saturation. This physiological condition could be the cause of the lack of response to ELF-EMF after long-term exposures between 3 and 7 days postplating.

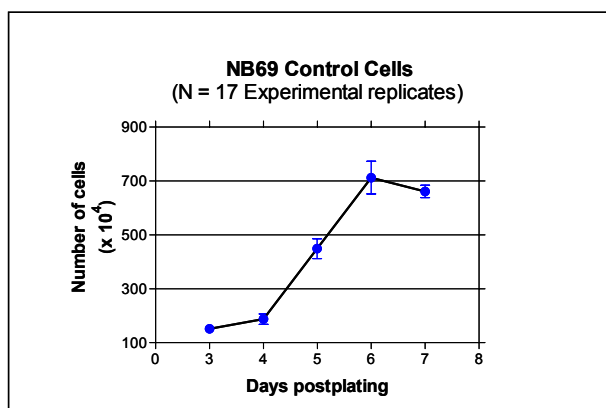


Figure 31. Growth pattern of NB69 cells: On day 7 the number of cells is reduced when compared to that at day 6. Long-term cultures (7 days postplating), reached a confluence stage close to saturation.

ELF-EMF did not counteract the retinoic acid-induced inhibition of cell proliferation in NB69 neuroblastoma cells.

To better characterise the potential ELF-EMF effects on the proliferation/differentiation rate, NB69 cells were treated with a chemical agent that inhibits proliferation and induces differentiation. All trans retinoic

acid (RA) promotes differentiation in NB69 cells, inducing outgrowth of long neurite-like processes and driving of the cell morphology along a neural pathway. As shown in Figure 30AB, the cell growth decreases after treatment with RA (2 μ M) for 5 or 7 days. This response to RA remained unchanged after exposure to the ELF-EMF during 42 or 90 hours.

ELF-MF enhanced the cellular proliferation rate NB69 neuroblastoma cells as revealed through analysis of cell proliferation markers (PCNA).

Studies of expression of cell proliferation markers (PCNA) reinforce the described effects of ELF-EMF on cell growth: To determine whether the above growth-promoting effect detected by Trypan Blue exclusion involves changes in cell proliferation, we searched for changes in the proliferating cell nuclear antigen (PCNA). PCNA immunolabelling shows that exposure to 10 μ T ELF-EMF significantly increases the proportion of PCNA-positive cells (24% increase; Figure 32). This effect was associated with an increase in the number of cells (15% increase), showing a significant linear correlation between both of the parameters, PCNA positive cells and total number of cells, at the end of 42 hours exposure ($p < 0.01$). These results confirm and reinforce the previous observations that a 42 hours exposure to ELF-EMF at a flux density of 10 μ T can modulate cell growth in NB69 cells. The mean value of PCNA positive cells in controls is $32 \% \pm 1,3$).

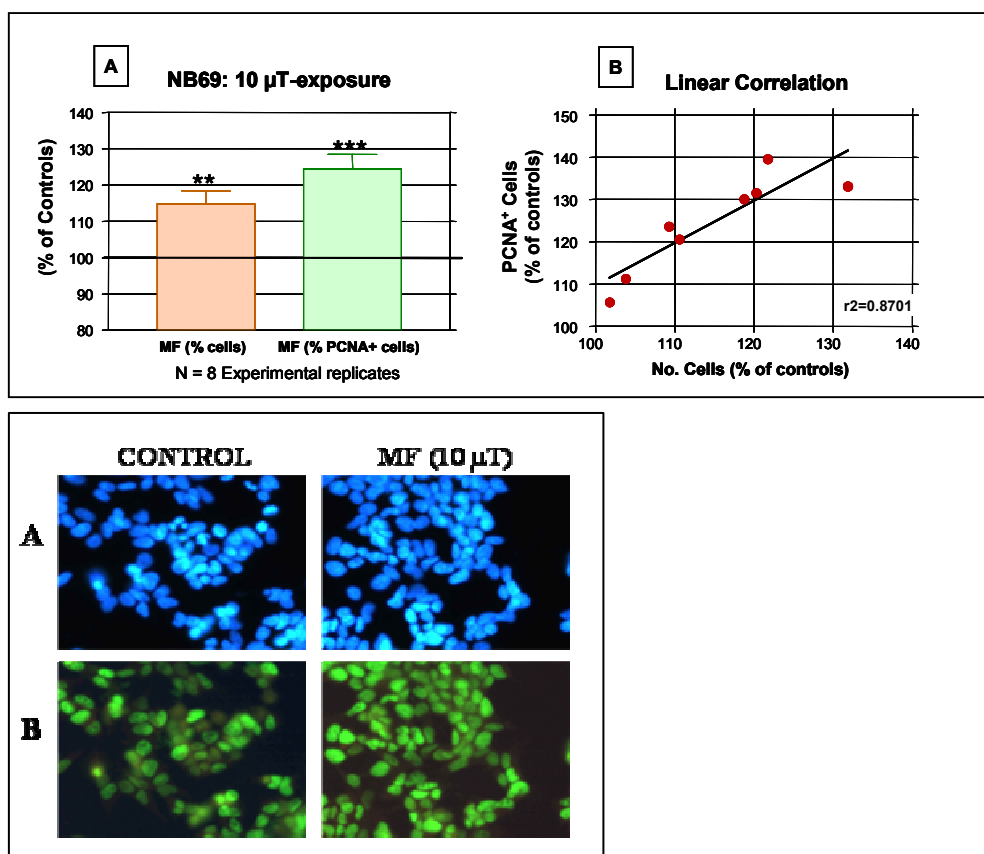


Figure 32. A) Growth response of NB69 to 10 μ T after a 42 hours exposure as revealed by Trypan (brown) and PCNA content (green). Student T' test: ** $p < 0.01$; *** $p < 0.001$. B) Linear correlation between these two parameters. Photomicrographs of NB69 cells. A: Hoechst-marked nuclei. B: PCNA labelling. The ELF-EMF exposure induced an increase in the number of cells expressing PCNA.

Neuroblastoma cell cultures contain two different phenotypes. One type is characterised by flattened, “sail-like” morphology and shows a strong adherence to the culture flask. These cells are called S-cells. The second phenotype corresponds to much smaller, triangular cells that adhere to the culture flask loosely. These cells, called N cells have a neuroblastic phenotype, are clonogenic and tumorigenic and

grow rapidly in the culture flask. We have observed that the relative proportion of both phenotypes evolves along the successive subcultures, which seems to significantly influence the response of the culture, as a whole, to the field exposure. In fact, young passages, having a high proportion of N cells, are particularly sensitive to ELF-EMF, whereas older passages, very rich in S-cells and with virtually no N cells, are not responsive to ELF-EMF exposure. Consequently, we conducted experiments with young passages, where the cells were exposed to a 100- μ T ELF-EMF during 63 hours (day 6 post-plating). A significant increase in the number of cells was observed in the exposed samples (9.7% over controls $p < 0.0001$, Figure 33A). The mean cell number in controls was $621.689 \pm 62.314 (\times 10^4)$. The increase in the number of cells was found to be associated with significant increases in the proportion of PCNA-positive cells. Figure 33B shows the percent of PCNA positive cells at days 5 and 6 post-plating (dpp). Only at 6 dpp significant changes in the number of PCNA positive cells were observed (31.7% over controls, $p < 0.01$, $N = 3$ experimental replicates). Those changes do not represent an ELF-EMF-induced increase in PCNA labelling, since the percent of PCNA positive cells in the control cultures spontaneously decreased between days 5 and 6 post-plating (Student T'test *, $p < 0.05$). Such a decrease did not occur in the exposed cultures. The present results indicate that the regulation of the kinetics of the cell cycle could be altered by ELF-EMF at 100 μ T. Provided that in the proliferating cell the PCNA levels are maximal at late G1 and S phases, it is possible that such phases of the cell cycle are implicated in the above described responses.

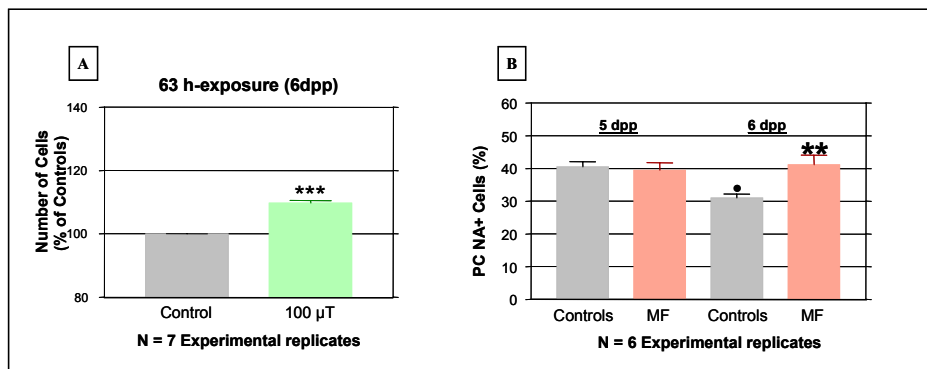


Figure 33. A) Percent of cells at the end of 63-hours exposure and/or incubation (6 day post-plating). B) Percent of PCNA positive cells at 5-6 days post-plating (dpp). In controls the percent of PCNA positive cells significantly decreases between the days 5 and 6 post-plating (Student T'test •, $p < 0.05$), whereas in exposed cells at 100 μ T this decrease did not occur).

ELF-EMF increased the DNA synthesis in NB69 neuroblastoma cells.

We also tested the BrdU incorporation into DNA. As shown in Figure 31, at the end of a 42-hours exposure (5 pp) to the 100 μ T ELF-EMF a significant increase of BrdU- positive cells was observed in the treated cultures (41 % over controls, Student's T test $p < 0.01$). Such an effect was followed (63-hours exposure, 6 pp) by a subsequent increase in the number of cells (9.7% over controls, $p < 0.001$, Figure 34). This response was accompanied by a significant reduction in the percent of spontaneously apoptotic cells (58.5 % of that in controls, $p < 0.05$).

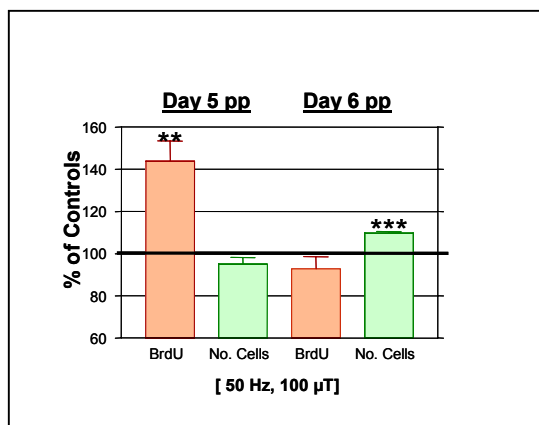


Figure 34. Cell growth in a total of 18 experiments: ELF-EMF effects at 5 days post-plating (42 hours exposure) or at 6 days post-plating (63 hours exposure): Red, percent of BrdU-positive cells; Green, Number of Cells analysed by Trypan blue exclusion. Student T'test: ** $p < 0.01$; *** $p < 0.001$.

ELF-EMF affected the cell cycle in NB69 neuroblastoma cells.

The experiments performed to test the DNA content and the cell cycle distribution by flow cytometry showed that at the end of day 5 post-plating (Figure 35A), the 42-hours exposure to 100 μ T ELF-EMF induces increases in the number of cells in G2-M phase of the cell cycle (28% over controls; $N = 3$ experimental replicates). The exposure also provoked a modest reduction of cells in G0-G1. However, no significant changes were observed in the number of cells in S-phase and in the number of total cells (Trypan Blue exclusion, Figure 352A). The flow-cytometry assay at the end of day 6 post-plating did not reveal EMF-induced changes in the cell cycle (G0-G1; S and G2-M), even though a significant increase in the number of alive cells was observed (16.7% over controls, $p < 0.05$; $N = 5$ experimental replicates, Figure 35B). These results confirm and reinforce our previous observations using other techniques, that 100 μ T 50-Hz ELF-EMF can promote cell growth in the NB69 cell line from a human neuroblastoma.

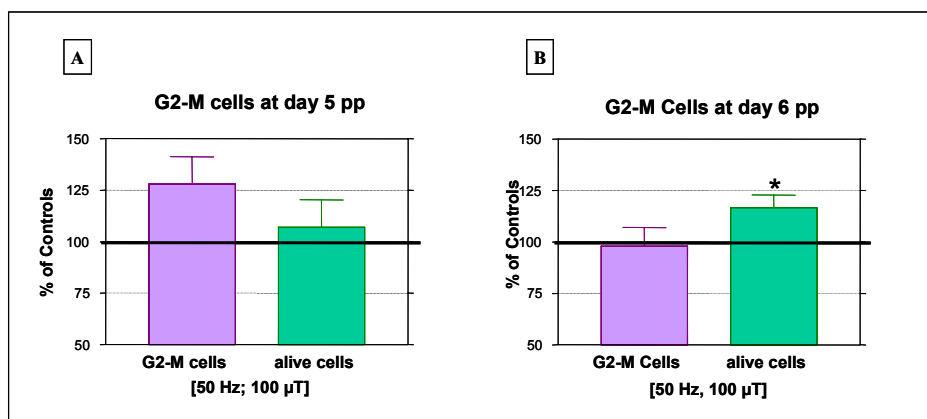


Figure 35. A) A 42 hours-exposure to a 100- μ T field provokes an increase in the percent of G2-M cells at day five postplating (5 pp). B) This increase was not observed one day later, after 63 hours- exposure, however, a significant increase in the number of alive cells occurred.

ELF-EMF diminished the spontaneous apoptosis in NB69 neuroblastoma cells.

In order to investigate the potential influence of 50 Hz ELF-EMF on apoptosis the percent of apoptotic cells was estimated with TUNEL-labelling after 63 hours of exposure. Also, the number of cells was

quantified by Trypan Blue exclusion. The results (Figure 36A) indicate that ELF-EMF of 50 Hz at a flux density of 100 μ T (3 hours on/3 hours off) induces a significant reduction in the spontaneous apoptosis of the NB69 cell line. This response was associated with an increase in the number of cells (9.7% over controls, $p < 0.001$, Figure 36B as we previously observed in experiments described above. Apoptosis was also determined through flow cytometry analysis; the results confirming a reduction at the end of 63 hours-exposure (data not shown).

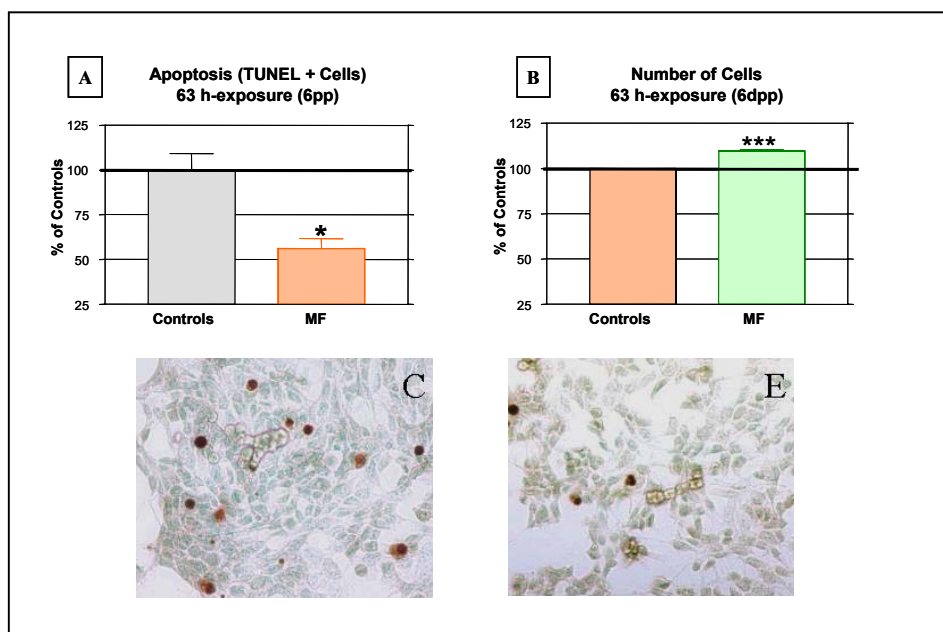


Figure 36. A) ELF-EMF (50 Hz, 100 μ T) induce a significant reduction in the spontaneous apoptosis of the NB69 cell line. B) This described response is associated with an increase in the number of cells. (* $p < 0.05$, *** $p < 0.001$, Student T' test, three independent experimental replicates). Photomicrographs showing TUNEL-positive cells in controls (C) and exposed (E) samples.

ELF-EMF altered the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB).

The results of a total of 8 experiments show that both, the labelling/cell in NB69 cells and the proportion of phospho-CREB positive cells increase after 60 min of exposure (35.4% over controls, $p < 0.01$, Figure 37 and photomicrograph). The percent of p-CREB positive cells in controls after 60 min of exposure was 32%. However, no differences were observed between ELF-EMF-exposed and controls samples after 30 or 120 min of exposure. These preliminary results suggest that the activation of p-CREB is involved in the previously described effects of 50 Hz 100 μ T ELF-EMF on cell growth/apoptosis. In additional experiments the analysis of Western confirms that the ELF-MF induced a short-time dependent activation of the transcriptional factor CREB, with a peak at 60 min followed by a recovering of the basal levels at 120 minutes of exposure (data not shown).

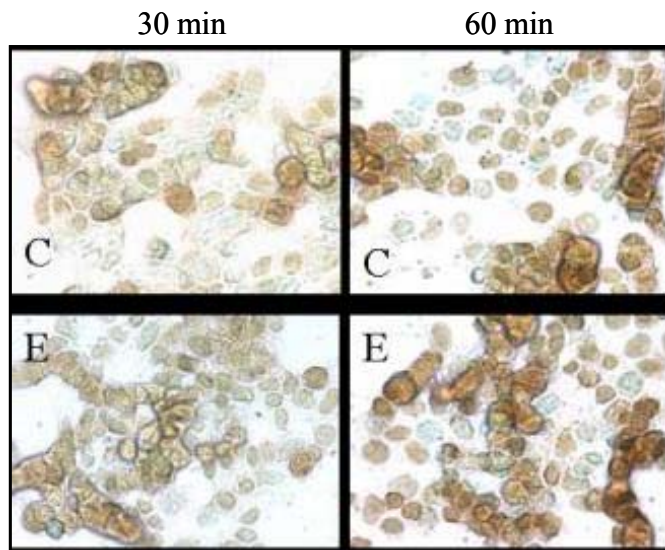


Figure 37. Immunocytochemistry for phospho-CREB. Changes in p-CREB positive cells showed a time-dependent response in the presence of the 100 μ T ELF-EMF. Photomicrograph showing the p-CREB labelling in brown and the counterstaining in green(methylgreen).

3.1.2.2 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

ELF-EMF accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes.

See 3.1.4.3

3.1.2.3 Human lymphocytes (Participant 8)

ELF-EMF exposure did not have any influence on proliferation, cell cycle and functionality of human lymphocytes.

The experiments with ELF-EMF (50 Hz) were performed at 50 μ T magnetic field intensity on cells from 20 donors. Cell proliferation, cell cycle together with membrane activation markers were studied on lymphocytes from young donors. Data obtained by all the experiments performed indicated that no significant differences exist on cell proliferation or DNA synthesis at any time during the continuous exposure up to 6 days, as well as on cell cycle during the continuous exposure up to 96 hours. Cell activation phase was studied on CD3+HLA-DR+ T lymphocytes and CD4+CD25+ T helper lymphocyte subpopulation, but also in this case no differences were found between cells exposed and not exposed.

3.1.2.4 Embryonic stem cells of mice (Participant 4)

ELF-EMF did not have any influence on the growth and neuronal differentiation of embryonic stem cells of mice.

See 3.1.4.1

3.1.2.5 Summary (Participant 1)

Our data show some influence of ELF-EMF on proliferation and differentiation of some, but not all cell systems investigated. This conclusion is based on the following findings:

- ELF-EMF at a flux density of 0.1 mT significantly increased the proliferation of neuroblastoma cells (NB69 cell line) after exposure for 42- and 63-hrs (see 3.1.2.1).
- ELF-EMF at a flux density of 0.8 mT accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes (see 3.1.2.2 and 3.1.4.3)

- ELF-EMF at a flux density of 2 mT did not have any influence on the growth and neuronal differentiation of embryonic stem cells of mice (see 3.1.2.4 and 3.1.4.1)
- ELF-EMF at a flux density of 0.8 mT did not have any influence on proliferation, cell cycle and activation of lymphocytes, either (see 3.1.2.3).

3.1.3 Apoptosis

3.1.3.1 Embryonic stem cells of mice (Participant 4)

ELF-EMF at a flux density of 2 mT up-regulated the transcript levels of the anti-apoptotic gene bcl2 and the growth arrest and DNA damage inducible gene GADD45 and down-regulated bax in ES cell-derived neural progenitor cells. This may indirectly influence the apoptotic process in neural progenitor cells.

See 3.1.4.1

3.1.3.2 Neuroblastoma cell line NB69 (Participant 5)

ELF-EMF at a flux density of 100 μ T inhibited the spontaneous apoptosis in NB69 neuroblastoma cells.

See 3.1.2.1

3.1.3.3 Human fibroblasts (Participant 3)

No differences in cell count between ELF-EMF exposed and sham exposed human fibroblasts at any exposure duration could be detected. Therefore a possible elimination of cells by apoptosis and cell death can probably be ruled out.

See 3.1.1.1

3.1.3.4 Summary (Participant 1)

Our data indicate that ELF-EMF may have some indirect effect on apoptosis in certain, but not all cell systems investigated. This conclusion is based on the following findings:

- ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the GADD45 gene and down-regulated the transcript levels of the bax gene by which the apoptotic process may be modulated (see 3.1.3.1 and 3.1.4.1).
- ELF-EMF at a flux density of 0.1 mT inhibited the spontaneous apoptosis in neuroblastoma cells in a way which is at present not well understood (see 3.1.3.2 and 3.1.2.1).
- ELF-EMF at a flux density of 1 mT did neither measurably affect the apoptotic process nor could a cytotoxic effect be detected in human fibroblasts in the course of a 24h exposure (see 3.1.1.1 and 3.1.3.3).

3.1.4 Gene and protein expression

3.1.4.1 Embryonic stem cells of mice (Participant 4)

ELF-MF exposure resulted in up-regulation of egr-1, c-jun and p21 transcript levels in p53-deficient, but not in wild type ES cells.

To analyse the effects of ELF-EMF, undifferentiated wild type (wt) and p53-deficient ES cells were exposed at different intermittence schemes and flux densities of 0.1, 1.0 and 2.3 mT for 6 and 48 hours, respectively (Table. 7). The exposition of ES cells to 5 min on followed by 30 min off cycles applied at

the high flux density of 2.3 mT resulted in a statistically significant up-regulation of *egr-1*, *p21* and *c-jun* mRNA levels in p53-deficient ES cells (Figure 38A and C), whereas wild type cells showed no variations in transcript levels compared to sham exposure and control cells (Figure 38A). In contrast, low flux densities of 0.1 and 1 mT ELF-EMF applied at 5 min on/30 min off intermittence cycles induced no significant effects on transcript levels indicating that a high flux density of ELF-EMF signals is necessary to affect mRNA levels of regulatory genes (Table 7).

Table 7. Conditions of the exposure of p53-proficient and deficient pluripotent embryonic stem cells embryonic stem cells to ELF-EMF and summary of the effects on transcript levels of regulatory genes.

Intermittent exposure (5min on/30 min off)			
6 hours ELF-EMF exposure ; wt, p53 ^{-/-}		48 hours ELF-EMF exposure; wt, p53 ^{-/-}	
0.1 mT	no ELF-EMF effect (n=3)	0.1 mT	no ELF-EMF effect (n=3)
1.0 mT	no ELF-EMF effect (n=3)	1.0 mT	no ELF-EMF effect (n=3)
2.3 mT	up-regulation of <i>egr-1</i> , <i>p21</i> and <i>c-jun</i> in p53 ^{-/-} cells (without recovery time, RT); no ELF-EMF effect after 18 h RT (n=6)	2.3 mT	no ELF-EMF effect (n=3)

Intermittent exposure (5min on/10 min off)			
6 hours ELF-EMF exposure; wt, p53 ^{-/-} ; without RT		6 hours ELF-EMF exposure; wt, p53 ^{-/-} ; 18h RT	
2.3 mT	no ELF-EMF effect (n=6)	2.3 mT	no ELF-EMF effect (n=6)

Continuous exposure			
6 hours ELF-EMF exposure; wt, p53 ^{-/-}		48 hours ELF-EMF exposure; wt, p53 ^{-/-}	
0.1 mT	no ELF-EMF effect (n=3)	0.1 mT	no ELF-EMF effect (n=3)
1.0 mT	no ELF-EMF effect (n=3)	1.0 mT	no ELF-EMF effect (n=3)

ELF-MF exposure of p53-deficient cells induced only short-term and transient effects on gene expression levels.

To elucidate, whether ELF-EMF induce short- or long-term responses, p53-deficient and wt ES cells were exposed to intermittent 5 min on/30 min off ELF-EMF signals for 6 hours. In parallel, the cells were analysed after a recovery time of 18 hours. No statistically significant effects could be seen after 18 hours recovery in control, sham- and field-exposed variants suggesting that ELF-EMF induced only an immediate transient response in p53-deficient cells (Figure 38B). These observations correlated with the results of the 48 hours ELF-EMF exposure to p53-deficient ES cells at early differentiation stage, where no ELF-EMF effects on transcript levels were found (data not shown, see Table 7).

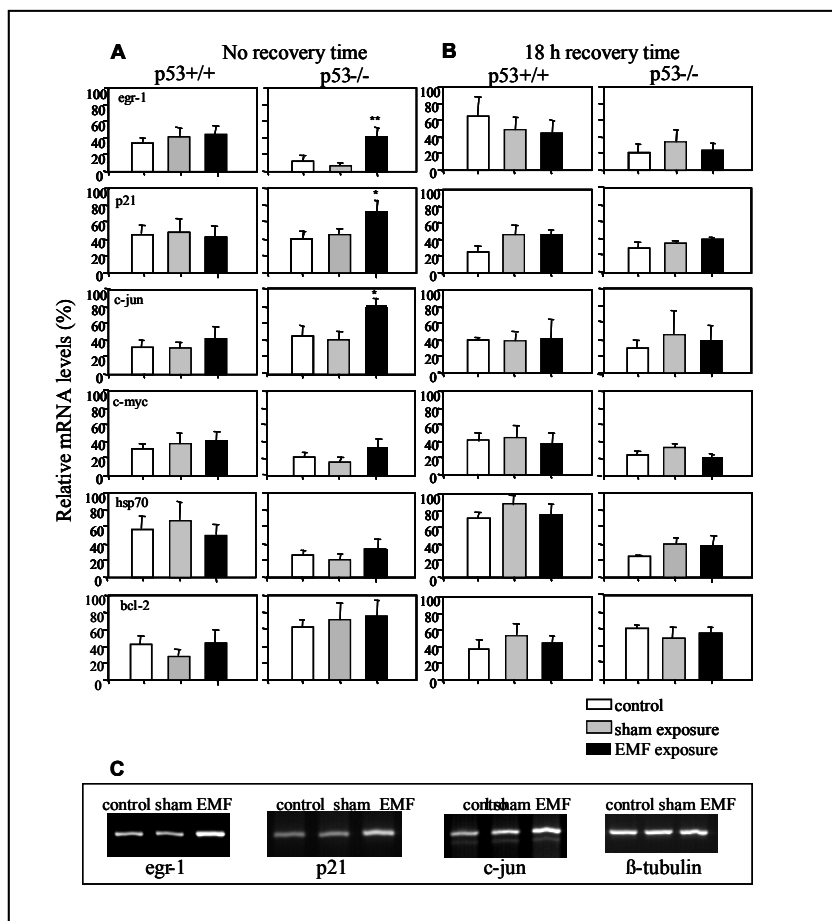


Figure 38. Relative mRNA levels of genes encoding egr-1, p21, c-jun, c-myc, hsp70 and bcl-2 in p53-deficient (p53^{-/-}) EC cell-derived embryoid bodies (EB) compared to wild-type (wt, p53^{+/+}) D3 cells immediately after 6h ELF-EMF (2.3 mT, intermittency 5 min on / 30 min off cycles) exposure (A) and after 18h recovery time (B) analysed by semi-quantitative RT-PCR. ELF-EMF exposure resulted in a significant, but transient up-regulation of egr-1, c-jun and p21 mRNA levels in undifferentiated p53-deficient (C), but not wt ES cells. Statistical significance was tested by Student's t-test for significance levels of 1% and 5% (**p<0.01; *p<0.05)

ELF-MF effects on transcript levels of regulatory genes in p53-deficient cells were dependent on intermittence cycles (on/ off cycle duration).

In addition, we analysed in wt and p53-deficient ES cells the influence of ELF-EMF signals applied at another intermittence scheme of 5 min on/10 min off for 6 hours with a flux density of 2.3 mT. We found that 5 min on/10 min off ELF-EMF signals with or without recovery time had no effects on the transcript levels of the investigated regulatory genes in both, wt and p53-deficient cells. Further, continuous ELF-EMF signals at flux densities of 0.1 and 1 mT were applied to wt and p53-deficient ES cells. We found no influence of continuous ELF-EMF on the mRNA levels of the regulatory genes included in the study (data not shown). Experiments with the highest flux density (2.3 mT) could not be performed with the continuous exposure protocol, because the generated ohmic heat of the coils could not be compensated by the incubator and would have resulted in an unstable ambient temperature.

ELF-EMF exposure up-regulated the transcript levels of bcl-2, the growth arrest and DNA damage inducible gene (GADD45) and down-regulates bax in ES cell-derived neural progenitor cells.

Elf-EMF (50 Hz-Power line, 2 mT, 5 min.on/30min. off, Table 8) was applied for 6 or 48 hours on neural progenitors (Table 8). Semi-quantitative RT-PCR analysis revealed no effect of ELF-EMF on transcript levels of genes involved in neuronal differentiation (nurr1, en-1) and on markers of differentiating

(nestin) or differentiated neuronal (TH) or astrocytic (GFAP) cells. In addition, we studied the effect of ELF-EMF on transcript levels of genes involved in the regulation of cell homeostasis (hsp70), cell cycle (p21) and anti-apoptosis (bcl-2). RT-PCR analysis revealed that, whereas transcript levels of p21 and hsp70 remain similar in sham and ELF-EMF exposed variants, a significant up-regulation of the growth arrest inducible gene GADD45 was observed at stage 4d+11d. (Figure 39). The quantitative RT-PCR (Q-RT-PCR) with specific primers and TaqMan probes showed that bcl-2 was first down-regulated at stage 4+7d ($p<0.05$), then up-regulated in the intermediate stage 4+11d ($p<0.01$) and at the terminal stage 4+23d ($p<0.05$). GADD45 was significantly up-regulated at stage 4+11d, then down-regulated at the terminal stage 4+23d (Figure 40). These studies were further substantiated by immunofluorescence analyses of neuronal markers. However, by immunofluorescence analysis, no changes in intracellular distribution and number of cells expressing neuronal markers (β III-tubulin, TH, GFAP) were observed (data not shown).

Table 8. Conditions of the exposure of neuronal progenitor cells to ELF-EMF and summary of the effects on transcript abundance, neural differentiation induction and DNA break induction.

Intermittent exposure (5min on/30 min off)			
48 hours, ELF-EMF (Power line, 50Hz)		6 hours, ELF-EMF (Power line, 50Hz)	
2.0 mT	up-regulation followed by down-regulation of GADD45 up-regulation of bcl-2 down-regulation of bax no effect on neural differentiation no effect on DNA break induction (n=6)	2.0 mT	no effect on DNA break induction (n=3)

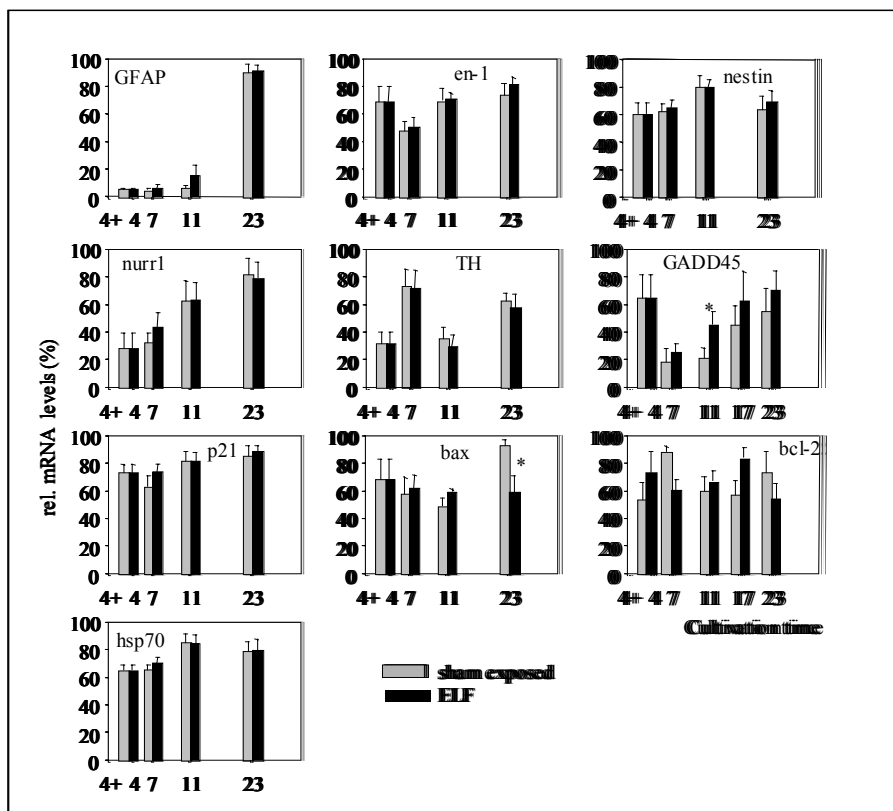


Figure 39. Relative mRNA levels of genes encoding the regulatory genes bcl-2, bax, p21, hsp70 and the genes involved in neuronal differentiation en-1, nurr1, TH, GFAP and nestin in ES-derived neural progenitors after 48 hours ELF (50Hz Powerline) EMF exposure (2.0 mT, intermittence 5 min ON/30 min OFF), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript up-regulation of GADD45 and down-regulation of bax. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for a significance level of 5% (*, p ≤ 0.05).

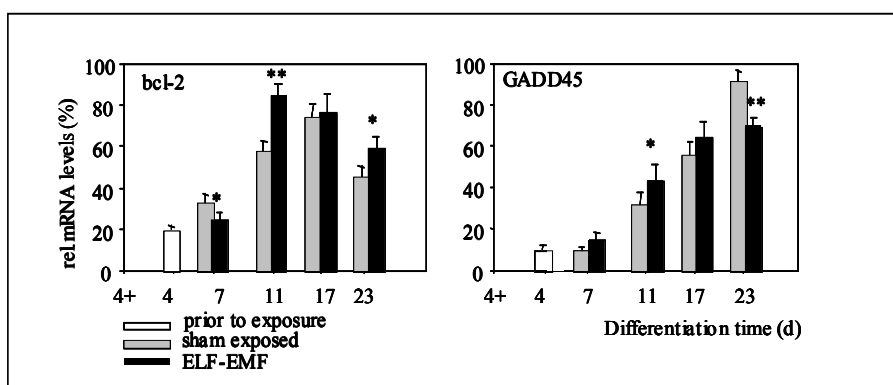


Figure 40.: Quantitative RT-PCR for estimation of relative mRNA levels of genes encoding the regulatory genes bcl-2 and GADD45 in ES-derived neural progenitors after 48 hours ELF (50Hz Powerline) EMF exposure (2.0 mT, intermittence 5 min on/30 min off), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript down-regulation followed by up-regulation of bcl-2, which correlated with up-regulation followed by down-regulation of GADD45. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for a significance level of 5% (*) and 1% (**, p < 0.01).

3.1.4.2 Human neuroblastoma cell line SY5Y (Participant 11)

In order to obtain cellular models to study ELF-EMF, we have characterised some neuroblastoma cell lines for their ability to express nAChRs and evaluate whether ELF-EMF can interfere with the expression of alpha3, alpha5 and alpha7 nAChR subunits, as well as with that of Phox2a, Phox2b and dopamine-beta-hydroxylase (DβH).

Three human neuroblastoma cell lines (SH-SY5Y, SK-N-BE and IMR32) have been analysed, by means of northern blot analysis, for the expression of neuronal acetylcholine receptor subunits. Due to the high degree of homology between different subunits, the experiments have been carried out with probes derived from the cytoplasmic portion, the least conserved region of nAChR subunits, in order to avoid cross-contamination. The results showed that these cells express the ganglionic type of nAChRs (alpha3, alpha5 and alpha7), but not alpha 4, mainly expressed in the CNS (data not shown and Fornasari 1997; Flora 2000). Furthermore only human neuroblastoma cell lines SY5Y and IMR-32 appeared to express either Phox2a, Phox2b or DβH (Flora, 2001 and data not shown), although with differences in the level of expression. As the SY5Y lineage shows higher expression of the three genes, we decided to use this as a model in all the experiments.

ELF-EMF did not affect the expression of nicotinic acetylcholine receptors (nAChRs) which represent the neuronal nicotinic system in human neuroblastoma cells.

At the beginning of our experiment we decided to use field strengths which are larger than the maximum real-world exposure and eventually scale-down, in the case of measurable effect, establishing the minimum threshold level to which ELF-EMF do not represent a risk to human health. Neuroblastoma cell line SY5Y was then exposed to ELF-EMF (50 Hz, powerline signal) continuously for 16 hours at flux densities of 2 mT and 1 mT and the expression level of human alpha 3, alpha 5 and alpha 7 nAChRs subunits analysed by means of Northern blotting. Figure 41 (panel A) shows the results obtained by three independent exposures at 2 mT (lanes 1, 4 and 5) together with that of sham-exposed cells (lanes 2, 3 and 6). The densitometric quantification of the mRNA level, however, has shown no effect on the expression level of nAChR subunits as compared to that of the sham-exposed cells set as 100%, when cells were exposed either at 2 mT or 1 mT (Figure 41), panel B and C respectively). We then decided to explore whether an intermittent exposure might be influent on the expression of the nAChR subunits tested. Exposing the cells to intermittent magnetic field (5 min on/5 min off), 2 mT and 1 mT flux density, for 16 hours did not affect the expression of the alpha3, alpha5 and alpha7 nAChR subunit genes (Figure 42, panel A and B respectively).

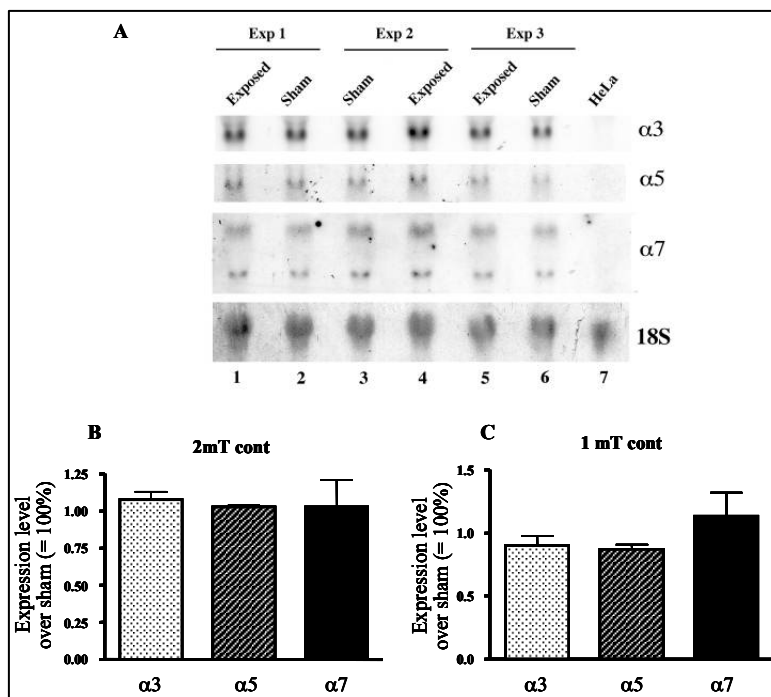


Figure 41. nAChR subunits expression upon exposure to 16 hours continuous ELF-EMF. 20 μ g of total RNA extracted from SY5Y cells exposed to 2mT and 1 mT continuous 50 Hz magnetic field, for 16 hours, was hybridised to cDNA probes corresponding to the human $\alpha 3$, $\alpha 5$ and $\alpha 7$ nAChR subunits. The expression level was normalised to that of 18S RNA. A, Northern blot analysis of total RNA extracted upon exposure to 2mT ELF-EMF. Here reported are the results of three independent experiments (Exp1, lanes 1-2; Exp 2, lanes 3-4; Exp 3, lanes 5-6). Lane 7, HeLa total RNA has been used as a negative control. B and C, Densitometric quantification of the expression level of nAChR subunits upon exposure to 2 mT and 1 mT continuous ELF-EMF, respectively. The data are the mean of three independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set equal to 100%.

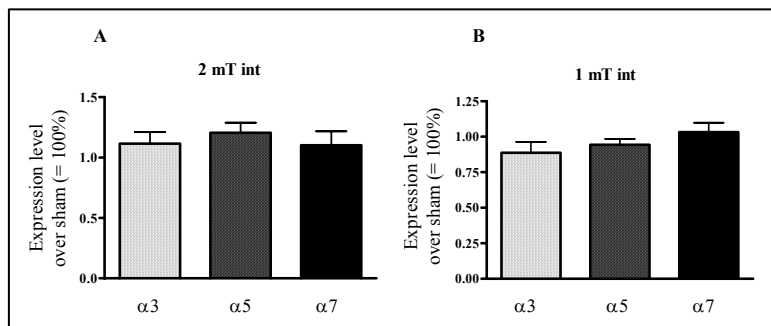


Figure. 42. nAChR subunits expression upon exposure to 16 hours intermittent ELF-EMF. 20 μ g of total RNA extracted from SY5Y cells exposed to 2mT and 1 mT continuous 50 Hz magnetic field, for 16 hours, was hybridised to cDNA probes corresponding to the human $\alpha 3$, $\alpha 5$ and $\alpha 7$ nAChR subunits. The expression level was normalised to that of 18S RNA. The data are the mean of three independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set equal to 100%. A, Densitometric quantification of the expression level of nAChR subunits upon exposure to 2 mT intermittent ELF-EMF. B, Densitometric quantification of the expression level of nAChR subunits upon exposure to 1 mT intermittent ELF-EMF.

As we were not able to measure any effect at the mRNA level, we wondered whether the exposure to ELF-EMF might have an effect at the level of receptor proteins. To this purpose we carried out radioligand assays on cells exposed to either continuous or intermittent 50 Hz ELF-EMF, flux densities of 1 mT and 2 mT for 16 hours, to assess the amount of protein functionally assembled in the receptors. The

binding was performed in the presence of radiolabelled ligands, ^3H -Epibatidine to quantitate alpha3-containing receptor and ^{125}I -alpha-bungarotoxin to quantitate alpha7-containing receptor. However, as shown in Figure 43, no change in the amount of either alpha3- or alpha7-containing receptor was detected under the same conditions used in Northern blot analysis of Figures 41 and 42, as compared to that of the sham-exposed cells set as 100%.

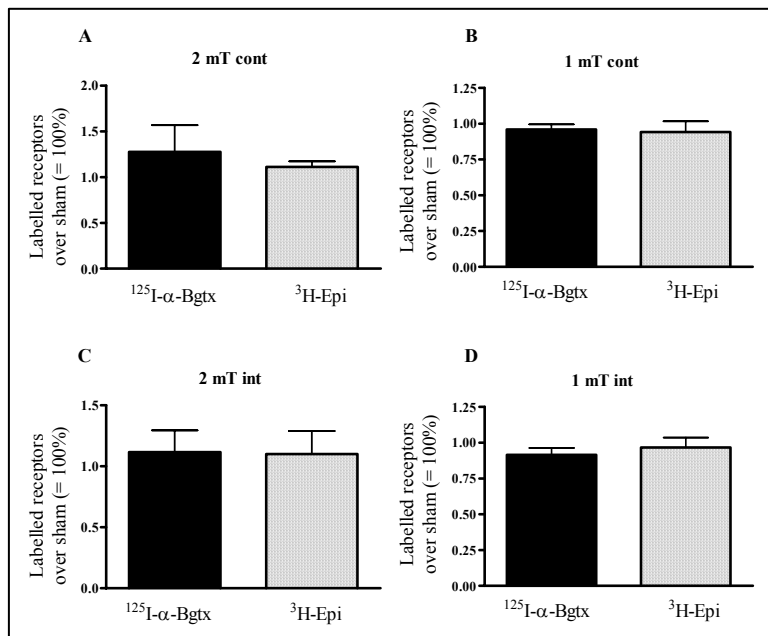


Figure 43. Quantitative analysis of the alpha3- and alpha7-containing receptors upon continuous and intermittent ELF-EMF exposure for 16 hours

The amount of labelled receptors were obtained from the binding of saturating concentration of ^3H -Epibatidine (grey bars) and ^{125}I -alpha-bungarotoxin (black bars) to the cell homogenates, performed in quadruplicate. The values are the mean of three independent experiments \pm and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%. A and B, continuous exposure to 1 mT and 2 mT ELF-EMF, respectively. C and D, intermittent (5 min on/5 min off) exposure to 1 mT and 2 mT ELF-EMF.

The experiments carried out until now have showed that the ELF-EMF does not influence the expression of nAChRs upon exposure of the cells to magnetic field with flux densities of either 1 mT or 2 mT for a relatively short period of time (16 hours). We then investigated whether the duration time of the exposure of SY5Y cells to ELF-EMF might affect the expression of the genes encoding the nAChR subunits, and especially, whether longer exposure to ELF-EMF might affect the expression of some of the genes. To answer this question two different exposure protocols have been used: a) 50 Hz powerline signal, flux density 1 mT, continuous exposure, duration 48 hours. The RNA or proteins were extracted immediately after the end of exposure (immediate recovery); b) 50 Hz powerline signal, flux density 1 mT, continuous exposure, duration 48 hours. The RNA or proteins were extracted 48 hours after the end of exposure (delayed recovery).

As shown in Figure 44, panel A, the expression of nAChR subunits, as measured at mRNA level, was again not affected by a prolonged exposure to the ELF-EMF followed by an immediate recovery of the cells (protocol a). Furthermore, no effect was detected at the level of receptor proteins (Figure 44, panel B). We then wondered whether the effect could be a delayed one, that is mediated by the activation of a cascade of second messengers that results in a change of gene expression. To test this hypothesis, cells were collected for RNA and protein analysis 48 hours after the end of the exposure (protocol b). The

results shown in Figure 44 seemed to rule out an indirect effect as neither the level of mRNA (Figure 45, panel A) nor of the receptor proteins (Figure 45, panel B) changed under these experimental conditions.

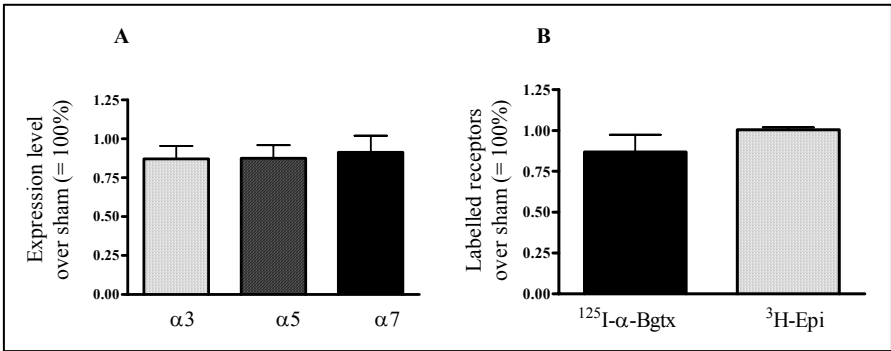


Figure 44. nAChR subunits expression and quantitative analysis of the alpha3- and alpha7 containing receptors upon continuous exposure to 1 mT ELF-EMF for 48 hours: Immediate recovery

Cells were exposed to 1 mT ELF-EMF for 48 hours and recovered immediately after the end of the exposure. A, Densitometric quantification of the expression level of nAChR subunits after Northern blot analysis. The data are the mean of five independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set as 100%. B, quantification of the alpha3- (grey bars) and alpha7-containing (black bars) receptors. The values are the mean of three independent experiments \pm and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%.

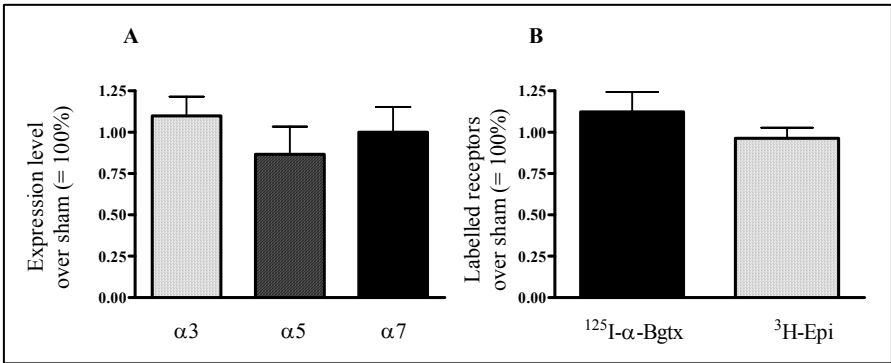


Figure 45. nAChR subunits expression and quantitative analysis of the alpha3- and alpha7 containing receptors upon continuous exposure to 1 mT ELF-EMF for 48 hours: Delayed recovery

Cells were exposed to 1 mT ELF-EMF for 48 hours and recovered 48 hours after the end of the exposure. A, Densitometric quantification of the expression level of nAChR subunits after Northern blot analysis. The data are the mean of five independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set as 100%. B, quantification of the alpha3- (grey bars) and alpha7-containing (black bars) receptors. The values are the mean of three independent experiments \pm and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%.

ELF-EMF did not affect the expression of markers of the catecholaminergic system in neuroblastoma cells.

In collaboration with Participant 1 we decided to investigate the influence of ELF-EMF on neurotransmitter release. In particular, the activity of the dopamine-beta-hydroxylase (D β H) which is a key enzyme in the synthesis of noradrenaline has been studied. Furthermore, we investigated possible modifications on the expression of two related homeo-domain transcription factors, Phox2a and Phox2b, that are relevant for the specification of the autonomic nervous system. Moreover, in noradrenergic cells, they are directly involved in the determination of the neurotransmitter phenotype by regulating the expression of D β H. As protocols, we applied the same exposure conditions used for the analysis of the human nAChR subunits. As shown in Figure 46, panels A and B, continuous exposure of SY5Y neuroblastoma cells to 1 mT and 2 mT 50 Hz ELF-EMF did not affect the expression level of either Phox2a, Phox2b and D β H genes, as compared to that of the sham-exposed cells set to 100%. Again we asked whether an intermittent exposure might have an effect on gene expression of these proteins. We then measured the mRNA level upon intermittent exposure (5 min on/5 min off) to 1 mT ELF-EMF, but no change was observed (Figure 47). Previous experiments have shown that an exposure of SY5Y cells for 48 hours at 1 mT flux density reduced the amount of mRNA of Phox2a, but not of Phox2b and D β H. We then exposed the cells under these conditions up to nine times independently.

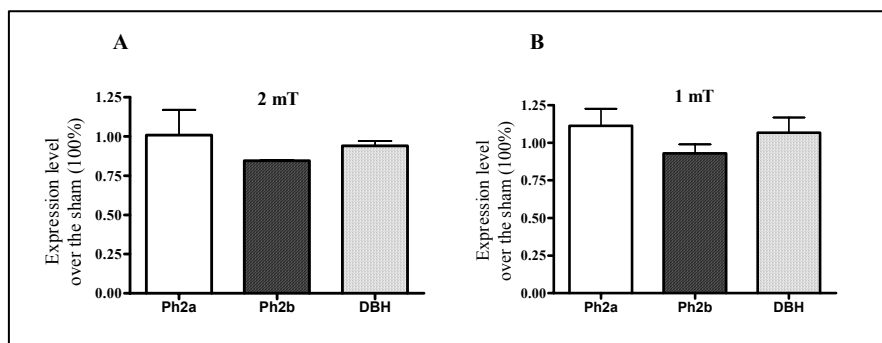


Figure 46. Noradrenergic phenotype specifying genes expression upon continuous exposure to ELF-EMF for 16 hours

20 μ g of total RNA extracted from SY5Y cells continuously exposed to 1mT and 2 mT 50 Hz ELF-EMF for 16 hours was hybridised to cDNA probes corresponding to the human Phox2a, Phox2b and D β H genes. The expression level was normalised to that of 18S RNA. A Densitometric quantification of the expression level of the three genes upon continuous exposure to 2 mT ELF-EMF. B, Densitometric quantification of the expression level of the three genes upon continuous exposure to 1 mT ELF-EMF. The data are the mean of five independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set as 100%.

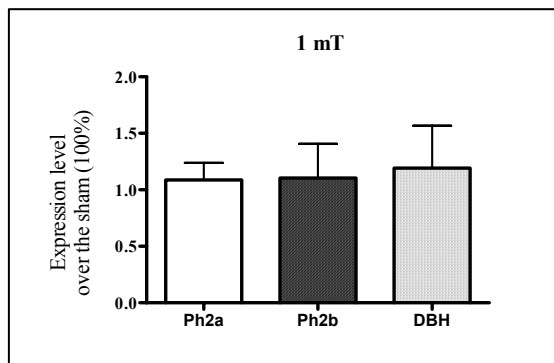


Figure 47. Noradrenergic phenotype specifying genes expression upon intermittent exposure (5 min on/5 min off) to ELF-EMF for 16 hours.

Densitometric quantification of the expression level of the three genes upon intermittent exposure to 1 mT ELF-EMF, after Northern blot analysis. The data are the mean of five independent experiments \pm S.E., expressed as a percentage of the sham-exposed sample set as 100%.

Statistical analysis has ruled out that, upon this exposure protocol, the expression of the noradrenergic specifying genes was affected, as shown in Figure 48, either harvesting the cells immediately after the end of the exposure (Figure 48, panel A) or 48 hours later (Figure 48, panel B). As no change was seen at the level of mRNA we asked whether the exposure to ELF-EMF might affect the expression of Phox2a and Phox2b at protein level. To this purpose we decided to measure, by western blot analysis, the protein level upon continuous exposure to 50 Hz ELF-EMF, flux density 1 mT, for 16 and 48 hours (Figure 49). Cells were harvested immediately after the end of the exposure (Figure 49, lanes 10-13 and lanes 6-9, respectively). Also protein extract from cells harvested 48 hours after the end of the continuous 48 hours exposure was tested (Figure 49, lanes 2-5). Densitometric analysis of the signal obtained for Phox2a (Figure 49, panel A) and Phox2b (Figure 49, panel B), normalised to that of the beta-tubulin, revealed that exposure of SY5Y cells to relatively short or longer period of time did not affect the expression of Phox2a and 2b, at protein level, as compared to that of the sham-exposed cells (Figure 49, panel C).

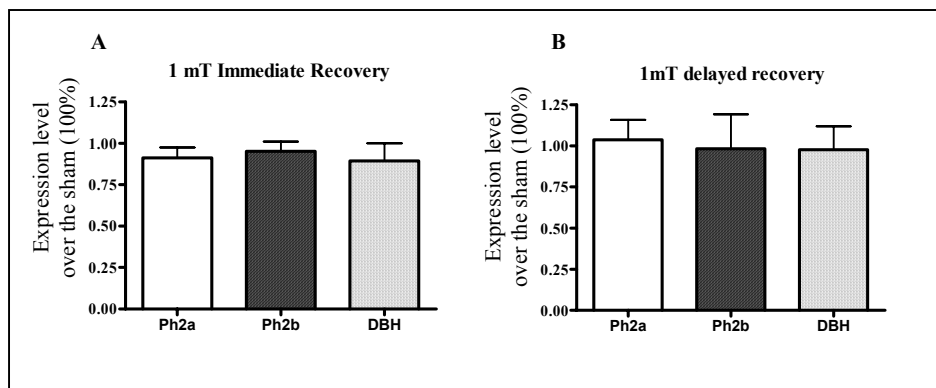


Figure 48. Noradrenergic phenotype specifying genes expression upon continuous exposure to ELF-EMF for 48 hours

SY5Y cells were continuously exposed to 1 mT 50 Hz ELF-EMF for 48 hours and collected either immediately (panel A) or 48 hours (panel B) after the end of the exposure. After northern blot analysis, the expression level of Phox2a, Phox2b and D β H genes was quantified by densitometric scanning of the autoradiogram. The data are the mean of six independent experiments \pm S.E. (Phox2b and D β H) and nine independent experiments (Phox2a), expressed as a percentage of the sham-exposed sample set equal to 100%.

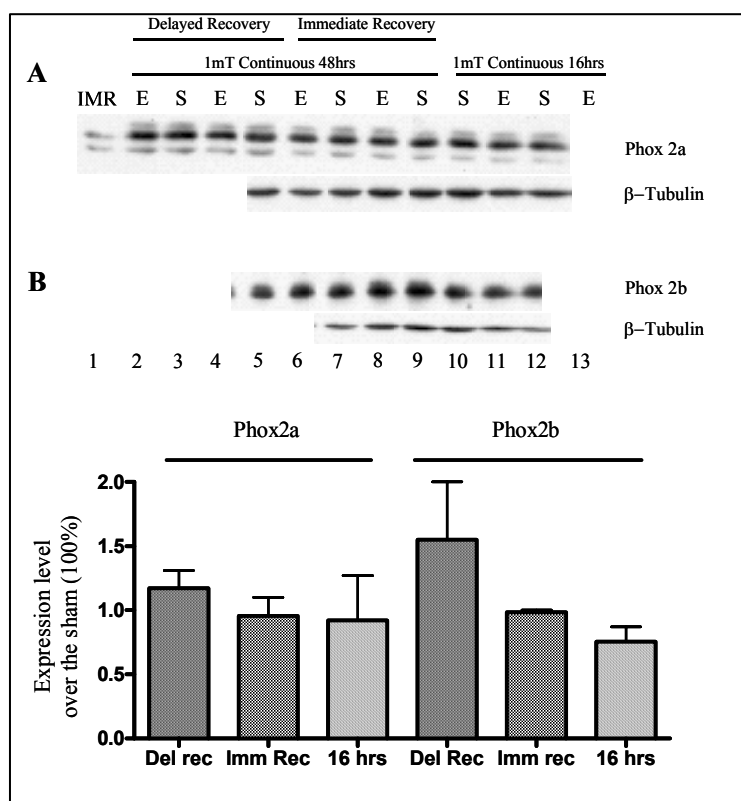


Figure 49. Western blot analysis of Phox2a and Phox2b expression upon continuous exposure to 1 mT ELF-EMF. 20 µg of total protein extract were size-fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The expression of Phox2a and Phox2b was detected by incubation with anti-Phox2a (panel A) and anti-Phox2b antibodies (panel B). Lanes 2-9, samples from continuous exposure to 1 mT ELF-EMF for 48 hours recovered 48 hours (lanes 2-5) or immediately (lanes 6-9) after the end of the exposure. Lanes 10-13, samples from 1 mT continuous exposure for 16 hours. Lane 1, IMR 32 neuroblastoma cells nuclear extract used as a control. E= exposed, S= sham-exposed. The expression of Phox2a and Phox2b was normalised to that of β-tubulin. C, quantification of the expression level of Phox2a and Phox2b. The data are the mean of two independent exposures ± S.E. and are expressed as percentage of the sham-exposed samples set as 100%. Del Rec = recovery 48 hours after the end of the exposure; Imm rec = recovery immediately after the end of the exposure; 16 hours = samples were exposed for 16 hours to 1 mT continuous ELF-EMF.

3.1.4.3 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

In higher vertebrates, heart formation is a complex phenomenon that starts at early stages of embryogenesis, prior to the end of gastrulation, with commitment of anterior lateral plat mesoderm cells to cardiogenic lineage. Studies in different organ systems have shown that tissue-specific transcription factors which control the expression of differentiation markers are also regulators of cellular differentiation. Basic helix-loop-helix proteins such as the myogenic factor are key regulators of skeletal muscle differentiation, while the erythroid cell-specific zinc finger protein GATA-1 is crucial for erythroid cell differentiation. It is now becoming evident that inactivation of the mouse homologue of the *Drosophila melanogaster* homeobox gene tinman, the homeobox gene *Nkx2.5* or *Csx* affects heart morphogenesis (Biben 1997, Lints 1993). Moreover, the GATA-4 protein, a member of the GATA family of transcription factors, has been found to be restricted to the hearts and to characterise very early stages of heart formation during embryonic development (Grepin 1995).

ELF-EMF affected the expression of cardiogenic genes in murine embryonic stem cells (GTR1).

In the first step of our study we looked at the effects of ELF-EMF (0.8 mT, 50 Hz sinusoidal) on the expression of cardiogenic genes in mouse embryonic carcinoma (EC) cells (P19 cells). Despite the encouraging results obtained in our pilot experiments, in a subsequent set of ten separate experiments P19

cells exposed to ELF-EMF desultorily underwent a gene program of cardiogenesis and revealed structural and functional cardiomyocyte features. Only in 2 experiments, exposure to ELF-EMF primed the expression of both GATA-4 and Nkx-2.5 genes, and led to the appearance of alpha-myosin heavy chain (MHC) and myosin light chain-2V (MLC), two cardiospecific transcripts. A representative RT-PCR analysis of cardiogenic and cardiac specific gene expression from ELF-EMF responsive cells is shown in Figure 50).

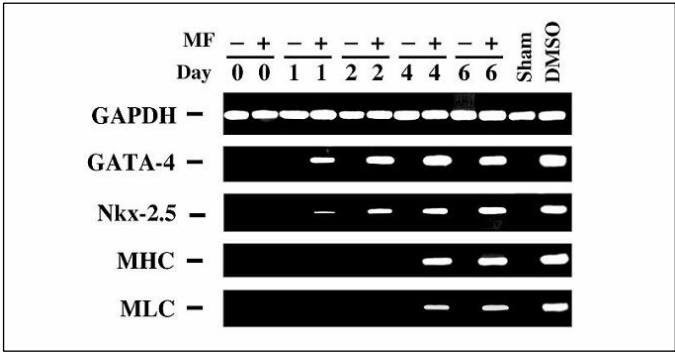


Figure 50. RT-PCR analysis of cardiogenic and cardiospecific transcripts in P19 cells exposed to ELF-EMF (continuous exposure, 4 days). MHC, alpha-myosin heavy chain. MLC, myosin light chain-2V.

We reasoned that the lack of data reproducibility of these results could be due to the consistent dilution of the myocardial phenotype within multiple non-myocardial cells encompassed by the P19 model of cell differentiation. To circumvent this problem, we decided to change the biological model, by using a line of pluripotent embryonic stem (ES) cells engineered for a gene trapping selection of a virtually pure population of ES-derived cardiomyocytes. RT-PCR analysis of targeted transcripts in unexposed cells indicated that, differently from undifferentiated LIF-supplemented cells, EBs expressed both GATA-4 and Nkx-2.5 mRNA (Figure 51). The expression of these cardiogenic genes resulted to be further enhanced in puromycin selected, ES-derived cardiomyocytes (Figure 52). Figures 51 and 52 show that ELF-EMF exposure remarkably increased GATA-4 and Nkx-2.5 gene expression in both EBs and cardiomyocytes, as compared to unexposed GTR1 cells. Interestingly, in both groups of cells ELF-EMF also increased the expression of the prodynorphin gene, an endorphin gene that has been recently shown to play a major role in orchestrating ES cell cardiogenesis (Ventura 2003(a), 2003(b), 2000). These responses were further inferred by the quantitative analysis of mRNA levels as shown in RNase protection experiments (Figure 53). Interestingly, nuclear run-off analyses of GATA-4 gene transcription indicated that the ELF-EMF action occurred at the transcriptional level (Figure 54). The activation of a program of cardiogenic gene transcription was also associated with the appearance of the cardiac specific transcripts alpha-myosin heavy chain and myosin light chain-2V (Figure 55).

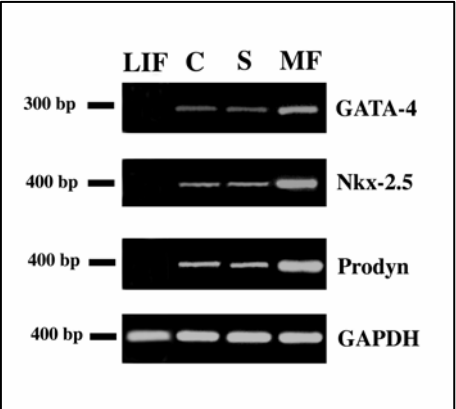


Figure 51. ELF-EMF was applied from the time of LIF removal and EBs were collected after 3 additional days. C: control EBs; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

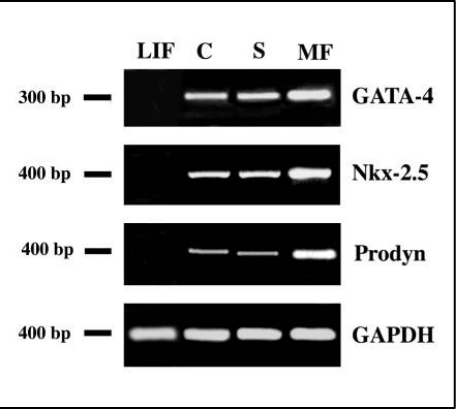


Figure 52. Effect of ELF-EMF (MF) on Cardiogenic gene expression in puromycin-selected cells. ELF-EMF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control puromycin-selected cardiomyocytes; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

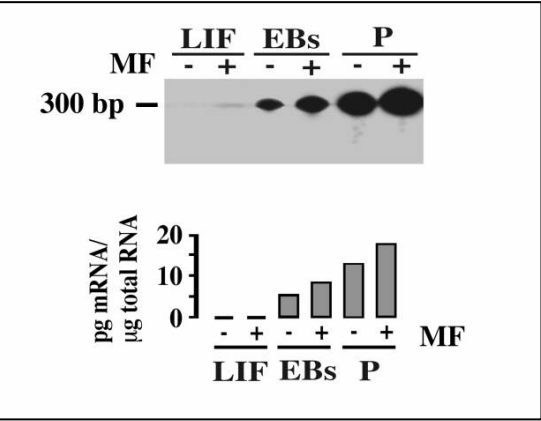


Figure 53. RNase protection analysis of GATA-4 mRNA expression in GTR1 ES cells cultured in the absence or presence of ELF-EMF. LIF, undifferentiated GTR1 cells. EBs, embryoid bodies collected 5 days after LIF removal. P, puromycin-selected cardiomyocytes: puromycin was added at day 8 following LIF removal. Each group of cells was cultured in the absence (-) or presence (+) of ELF-EMF. Autoradiograms are representative of 3 separate experiments.

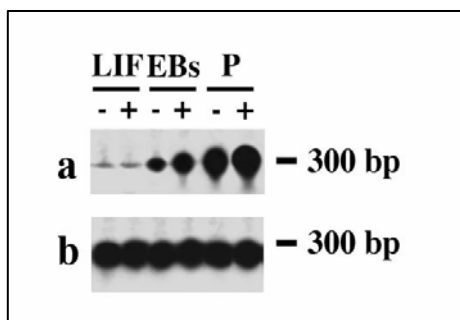


Figure 54. Nuclear run-off analysis of GATA-4 gene transcription in isolated ES cell nuclei. Nuclei were isolated from undifferentiated GTR1 cells (LIF), from EBs collected 5 days after LIF removal (EBs) or from puromycin-selected cardiomyocytes (P): puromycin was added at day 8 following LIF removal. Each group of cells was exposed in the absence (-) or presence (+) of ELF-EMF (MF). Puromycin was added at day 8 following LIF removal. Row a, GATA-4 gene transcription. Row b, cyclophilin gene transcription (cyclophilin was assessed as a constant gene for control). Autoradiograms are representative of 3 separate experiments.

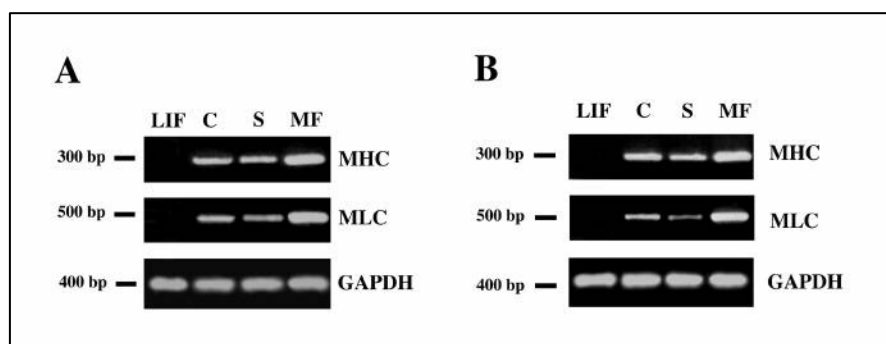


Figure 55: Effect of MF on the expression of cardiac specific genes. A: ELF-EMF (MF) was applied from the time of LIF removal and EBs were collected after additional 3 days. B: MF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control cells; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

Exposure of GTR1 ES cells to ELF-EMF after LIF removal and throughout 4 days of puromycin selection for an overall period of 10 days from LIF withdrawal was able to increase the yield of ES-derived cardiomyocytes: the number of beating colonies reached 170.44 ± 28.0 % of the control value, estimated in cardiomyocytes selected from untreated cells (mean \pm SEM of 4 separate experiments).

We finally investigated whether the transcriptional responses evoked by ELF-EMF may encompass genes that are essential for the specification of non-myocardial lineages. Noteworthy, the expression of MyoD, a gene involved in skeletal myogenesis was not affected in both EBs and puromycin selected cells (Figure 56), while the expression of neurogenin1, a neuronal specification gene, was slightly enhanced only in EBs (Figure 56).

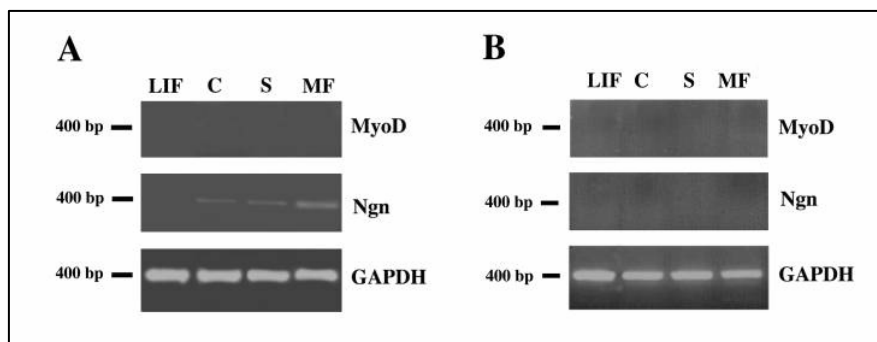


Figure 56. Effect of ELF-EMF (MF) on the expression of genes promoting non-myocardial lineages. A: MF was applied from the time of LIF removal and EBs were collected after additional 3 days. B: MF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control cells; S: sham. MyoD and neurogenin1 (Ngn) are skeletal muscle and neuronal specification genes, respectively. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

3.1.4.4 Membrane currents of oocytes of *Xenopus laevis* expressing rCx46 (Participant 7)

*ELF-EMF did not significantly affect the leak-current of oocytes of *Xenopus laevis* expressing hemi-channels of rCx46.*

During expression of hemi-channels, composed of the connexin rCx46, the oocytes were exposed to ELF-EMF (50 Hz). As suitable parameter for functional integrity of an oocyte the leak-current was selected which was electrophysiologically measured at voltage-clamp. A representative experiment of a sham exposed oocyte is shown in Figure 57. Figure 57a) shows membrane currents recorded at depolarising test potentials starting from a holding potential of -90 mV. The figure indicates that the rCx46-mediated current becomes activated by depolarising test potentials above about -10 mV. Figure 57b) shows the corresponding leak subtracted steady-state current values (I_{ss}) as function of driving voltage ($V - V_{rev}$) where V_{rev} denotes the corresponding reversal potential. The corresponding steady-state current values were derived at the end of the applied test pulse.

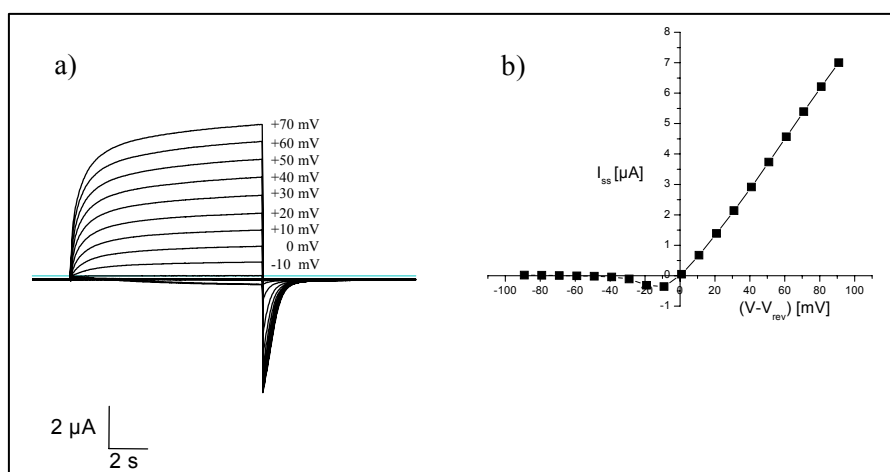


Figure 57. Voltage-dependent current activation of sham exposed *Xenopus* oocytes expressing rCx46 after an expression time of 16 hours. **a)** Representative voltage-jump current-relaxations at given test potentials. The common holding potential was set to -90 mV. The dotted line denotes the zero current level. **b)** Leak subtracted steady-state current amplitudes (I_{ss} , ■) derived from the data in a) as function of driving voltage ($V - V_{rev}$)

The leak-current was derived at test potentials in the range of -70 mV to -100 mV using a constant holding potential of -90 mV. A comparison of leak-currents for exposed and sham exposed oocytes is given in Figure 58. Figure 58a shows the leak-current for oocytes which were continuously exposed for 16 hours at 2.3 mT and Figure 58b) the corresponding leak-current after an intermittent exposure (5 min on/10 min off) at 1.0 mT for 16h. A significant influence of ELF-EMF exposure on the leak current could not be observed for the different exposure conditions.

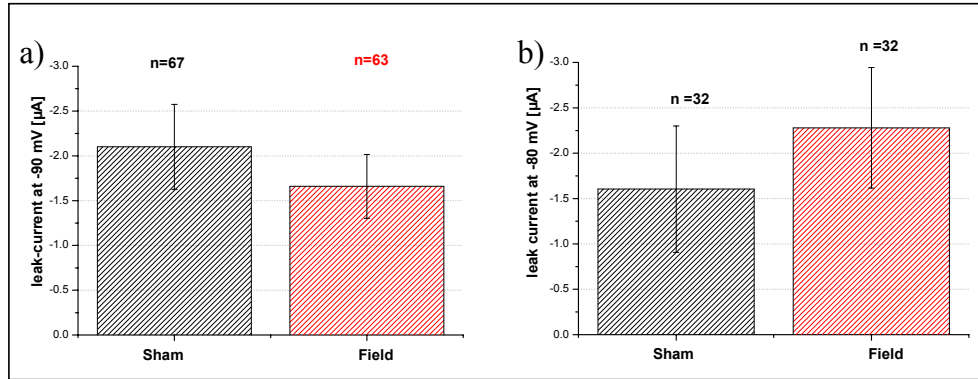


Figure 58. Leak-currents of single oocytes after expression of rCx46- hemi-channels at different holding potential. **a)** Leak current amplitudes at holding potential -90 mV of sham and ELF-EMF (2.3 mT, 16 hours) continuously exposed oocytes. **b)** Leak-currents at holding potential -80 mV of sham and ELF-EMF (1.0 mT, 16 hours, 5 min on/10 min off) exposed oocytes. Data are given as mean ± s.e.m. n denotes the number of different oocytes.

No significant influence of ELF-EMF on the number of expressed and conducting hemi-channels composed of rCx46 in oocytes.

The expression level of hemi-channels composed of rCx46 was estimated from the number of conducting hemi-channels which corresponds to the mean steady-state current amplitude (I_{ss}) and/or the maximal membrane conductance G_{max} at depolarising test potentials. Expression of endogenous hemi-channels was suppressed by injection of the corresponding anti-sense. Figures 59a, 60a and 61a show the relationship I_{ss} vs ($V - V_{rev}$) for different oocytes for the selected exposure condition. For clearer presentation $G(V)$ was normalised to a maximal value of $G(V)$ which is obtained at $V = +50$ mV (Figures 59b, 60 and 61b). A significant influence of ELF-EMF exposure on the number of expressed and conducting hemi-channels of rCx46 could not be read from the analysed data. This finding is also reflected in the frequency distribution of G_{max} for sham and exposed oocytes (Figure 59c).

No significant influence of ELF-EMF on the voltage-dependent gating properties of rCx46 expressing oocytes

A possible effect of ELF-EMF exposure on the voltage dependent gating properties of conducting hemi-channels of rCx46 was analysed. I_{ss} vs ($V - V_{rev}$) was measured and the corresponding relation $G(V)$ vs ($V - V_{rev}$) derived. The latter relationship could be fitted by a simple Boltzmann equation. The fit yields as essential parameter the number of apparent equivalent voltage gating charges z . z was determined for the different exposure conditions. As can be read from Figure 59a) a significant effect of ELF-EMF exposure on the voltage-dependent gating which is reflected in the apparent number of equivalent charges z (Figure 59b) was not observed.

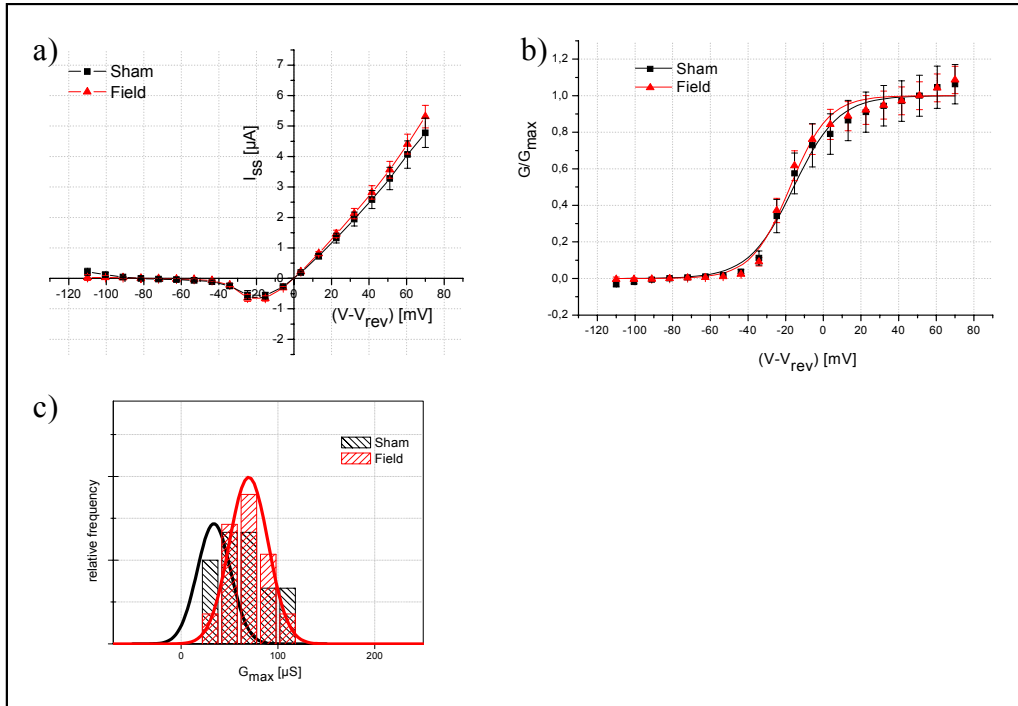


Figure 59. Voltage dependence of macroscopic rCx46-mediated membrane current and corresponding conductance after continuous ELF-EMF exposure for 16 hours at 2.3 mT. **a)** Mean \pm s.e.m. of leak subtracted steady-state current amplitudes (I_{ss}) as function of $(V-V_{rev})$ in the absence and presence of ELF-EMF exposure (sham \blacksquare , $n = 14$; field \blacktriangle , $n=15$). **b)** Mean \pm s.e.m. of corresponding normalised membrane conductance $G/G_{max} = G(V)/G(V=+50 \text{ mV})$ as function of $(V-V_{rev})$ in the absence (\blacksquare , $n = 14$) and presence of ELF (\blacktriangle , $n = 15$). The solid lines show fits of the data by a simple Boltzmann function (for details see Materials and Methods). The derived parameters are $z(\text{sham}) = 2.11 \pm 0.17$; $z(\text{field}) = 2.45 \pm 0.23$; $V_{1/2}(\text{sham}) = (-15.76 \pm 1.06) \text{ mV}$; $V_{1/2}(\text{field}) = (-17.66 \pm 1.07) \text{ mV}$. **c)** Distribution of relative frequency of $G_{max} = G(V=+50 \text{ mV})$ in the absence ($n = 14$) and presence ($n = 15$) of ELF-EMF. Solid lines present the Gauss distribution with the parameters: $G_{max, \text{mean}}(\text{sham}) = 64.2 \mu\text{S}$, corresponding standard deviation (sd) $\text{sd}(\text{sham}) = 7.8 \mu\text{S}$ and $G_{max, \text{mean}}(\text{field}) = 69.8 \mu\text{S}$, $\text{sd}(\text{field}) = 20.0 \mu\text{S}$

To investigate an influence of field intensity on the results presented above, the experiments were repeated at 1.0 mT and the EMF-ELF was intermittently applied (5 min on/10 min off) for 16 hours. The corresponding results are given in Figure 60. Again, a significant effect of ELF-EMF on the number of expressed hemi-channels of rCx46 (Figure 59a) as well as their voltage dependent gating properties was not observed (Figure 60b).

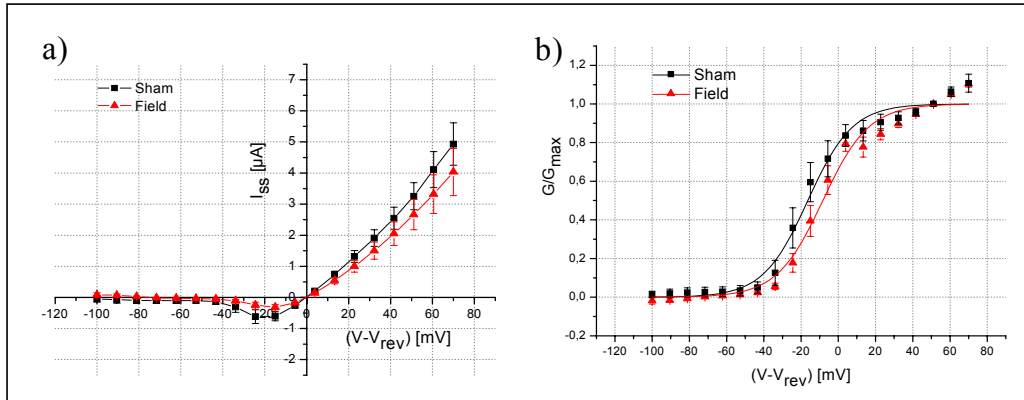


Figure 60. Voltage dependence of rCx46-mediated membrane currents and corresponding membrane conductance after intermittent ELF-EMF exposure (5 min on/10 min off) for 16 hours at 1.0 mT. **a)** Mean \pm s.e.m. of leak subtracted steady-state current amplitudes (I_{ss}) as function of ($V-V_{rev}$) in the absence and presence of ELF-EMF exposure (sham \blacksquare , $n = 5$; field \blacktriangle , $n = 5$). **b)** Mean \pm s.e.m. of normalised membrane conductance $G/G_{max} = G(V)/G(V = +50 \text{ mV})$ as function of driving voltage ($V-V_{rev}$) in the absence (\blacksquare , $n = 5$) and presence of ELF-EMF (\blacktriangle , $n = 5$). The solid lines present fits of the data by a simple Boltzmann function, respectively (for details see Materials and Methods). The derived parameters are $z(\text{sham}) = 2.08 \pm 0.19$; $z(\text{field}) = 2.02 \pm 0.20$; $V_{1/2}(\text{sham}) = (-16.39 \pm 1.29) \text{ mV}$; $V_{1/2}(\text{field}) = (-8.32 \pm 1.38) \text{ mV}$

In a further series of experiments ELF-EMF at an intensity of 2.3 mT was intermittently (5 min on/10 min off) applied for 16 hours. The corresponding results are given in Figure 61. A significant effect of ELF-EMF exposure on the number of expressed hemi-channels of rCx46 (Figure 61a) was not observed. The data indicate a decrease of z after ELF-EMF exposure which appears not be significant (see legend of Figure 61b).

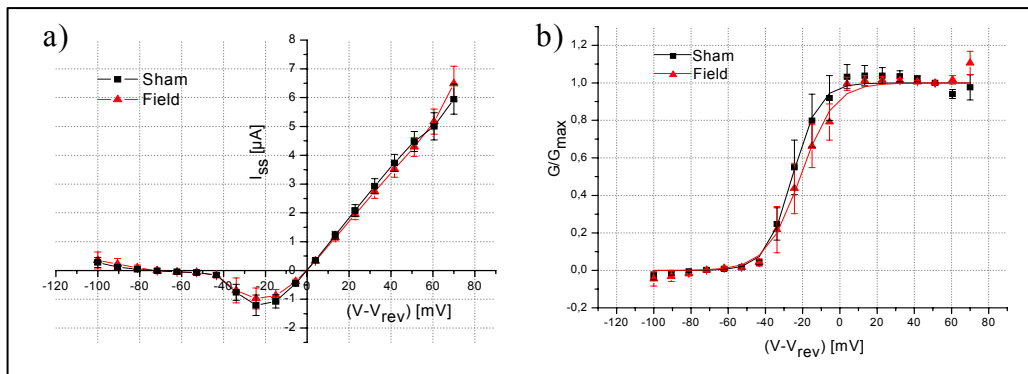


Figure 61. Voltage dependence of macroscopic rCx46-currents (I_{ss}) and corresponding membrane conductance (G) after intermittent exposure (5 min on/10 min off) for 16 hours at 2.3 mT. **a)** Mean \pm s.e.m. of leak subtracted steady-state current amplitudes (I_{ss}) as function of ($V-V_{rev}$) in the absence and presence of ELF-exposure (sham \blacksquare , $n = 4$; field \blacktriangle , $n = 5$). **b)** Mean \pm s.e.m. of normalised membrane conductance $G/G_{max} = G(V)/G(V = +50 \text{ mV})$ as function of ($V-V_{rev}$) in the absence (\blacksquare , $n = 4$) and presence of ELF-EMF (\blacktriangle , $n = 5$). The solid lines show fits of the data by a simple Boltzmann function (see Material and Methods). The derived parameters are $z(\text{sham}) = 3.54 \pm 0.26$; $z(\text{field}) = 2.77 \pm 0.24$; $V_{1/2}(\text{sham}) = (-25.51 \pm 0.59) \text{ mV}$; $V_{1/2}(\text{field}) = (-21.31 \pm 0.88) \text{ mV}$

For a more detailed analysis of the voltage dependent gating properties the kinetics of rCx46-mediated current activation was considered. The time dependent current activation could be described by a sum of two exponential functions: $I(t) = a_0 + a_1 \cdot \exp(1 - \exp(-t/\tau_1)) + a_2 \cdot \exp(1 - \exp(-t/\tau_2))$. The corresponding time constants of activation τ_1 and τ_2 were obtained from corresponding fits to the experimental data and the

results are presented in Figures 62a-c. The figure indicates that ELF-EMF exposure does not influence the voltage dependent time constants of channel activation significantly.

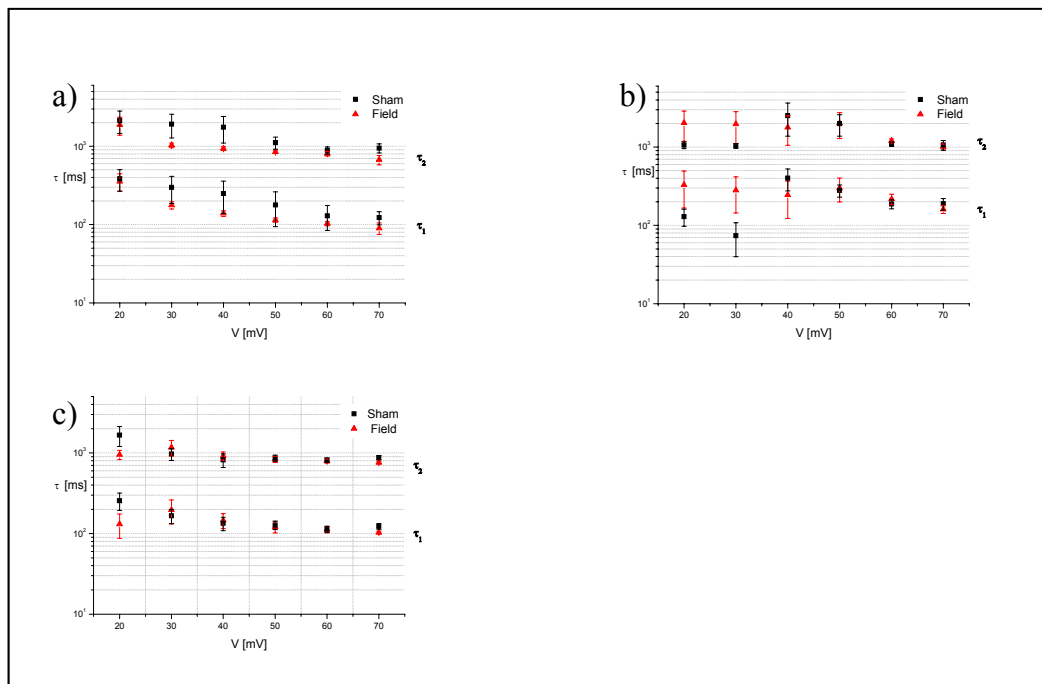


Figure 62. Time constants of voltage-dependent current activation. Time constants of current activation were plotted as function of voltage. Each point represents mean \pm s.e.m of five different oocytes. The time constants of activation were obtained by fitting the time course of current activation by a sum of two exponential functions (see text). **a), b)** and **c)** present the time constants τ_1 and τ_2 at different exposure conditions: **a)** 2.3 mT, 16 hours continuous, **b)** 2.3 mT, 16 hours intermittent (5 min on/10 min off) and **c)** 1.0 mT, 16 hours intermittent (5 min on/10 min off).

No significant influence of ELF-EMF on the reversal potential of rCx46-mediated membrane current in oocytes.

Finally, the reversal potential V_{rev} of the rCx46-mediated membrane current was considered at different exposure conditions (Figure 63). The reversal potential is mainly determined by the expressed and conducting hemi-channels composed of rCx46, but also includes the contribution of all electrogenic transport systems. A field induced shift of the reversal potential would indicate a change of the intrinsic voltage sensor of the channel or by variation of the intracellular ion composition. No significant effect on the reversal potential of rCx46-mediated membrane current could be observed.

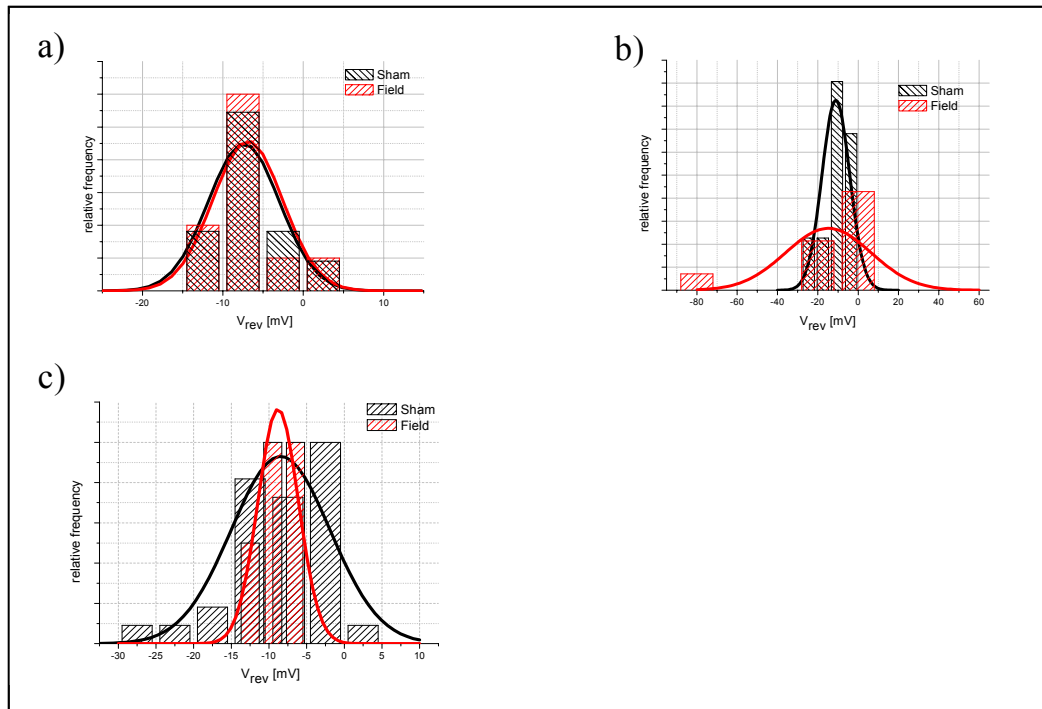


Figure 63. Relative frequency of reversal potential (V_{rev}) of conducting hemi-channels composed of rCx46 for different exposure conditions during an expression period of 16 h. The data were derived from the corresponding experiments given in Fig. 59. **a)** 1.0 mT, intermittent exposure: $n = 11$ for sham exposed and $n = 10$ for ELF-EMF exposed oocytes; **b)** 2.3 mT, permanent exposure: $n = 18$ for sham exposed and $n = 20$ for ELF-EMF exposed oocytes; **c)** 2.3 mT intermittent exposure: $n = 5$ for sham exposed and $n = 4$ for ELF-EMF exposed oocytes. Solid lines present the corresponding Gauss distributions using the parameters mean of relative frequency ($V_{rev, mean}$) and standard deviation (sd): **a)** $V_{rev, mean}(sham) = -6.9$ mV, $sd(sham) = 4.4$ mV, $V_{rev, mean}(field) = -7.4$ mV, $sd(field) = 4.4$ mV; **b)** $V_{rev, mean}(sham) = -11.1$ mV, $sd(sham) = 6.9$ mV, $V_{rev, mean}(field) = -14.8$ mV, $sd(field) = 2.1$ mV; **c)** $V_{rev, mean}(sham) = -8.8$ mV, $sd(sham) = 2.1$ mV, $V_{rev, mean}(field) = -8.5$ mV, $sd(field) = 0.7$ mV

A slight but not significant influence of ELF-EMF on the gating properties of hemi-channels expressed in *Xenopus* oocytes dependent on the external calcium concentration was observed.

The expression level of rCx46 in single were characterised by detailed biophysical analysis of corresponding voltage-jump current relaxation experiments. In parallel the gating by external Ca^{2+} concentration was characterised. A significant influence on the rCx46 mediated membrane conductance, the corresponding half-activation voltage ($V_{1/2}$) and the number of apparent equivalent gating charges (z) of the rCx46-hemi-channels in exposed and sham-exposed *Xenopus laevis* oocytes could not be observed for intermittently applied ELF-EMF at 50 Hz powerline signal (1.0 mT, 5 min on/10 min off) after an exposure time of 14 hours, 17 hours and 20 hours, respectively. Since it is known that external calcium significantly modulates the voltage dependent gating behaviour of expressed hemi-channels composed of rCx46, the experiments were repeated at various external calcium concentrations. The results indicate an influence by ELF-EMF exposure, but the differences are not significant (Figure 64). The membrane conductance and the gating parameters of exposed oocytes expressing rCx46 are smaller than those of sham exposed cells after an exposure time of 14 hours and 20 hours, respectively.

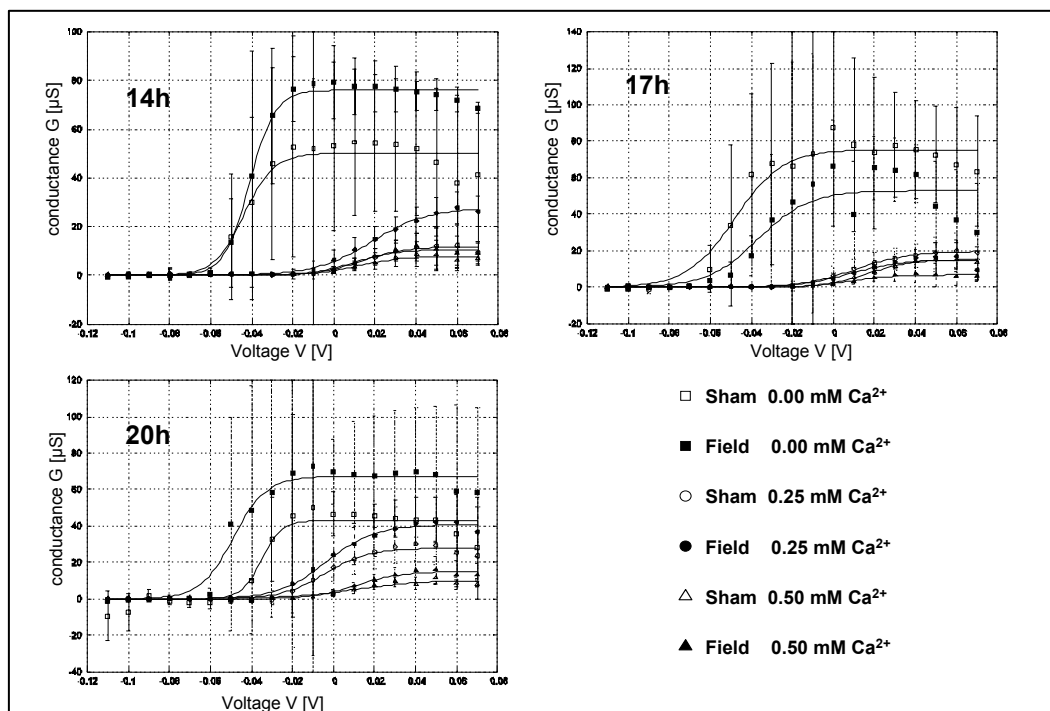


Figure 64. Conductance of hemi-channels composed of rCx46 expressed in oocytes after intermittent exposure (5 min on/10 min off) for 14 hours, 17 hours and 20 hours at 1.0 mT in the presence of 0.0 mM ($n = 2-6$), 0.25 mM ($n = 2-4$) and 0.5 mM ($n = 2-5$) Ca^{2+} in the bath. Closed symbols denote results of exposed oocytes and open symbols those of sham exposed oocytes.

ELF-EMF did not significantly affect the results of electrophysiological recordings of paired *Xenopus* oocytes.

The voltage-clamp experiments were repeated using paired oocytes. Paired oocytes expressing rCx46 form cell-to-cell channels (gap junctions) by head-to-head association of two hemi-channels which results in an increase of transjunctional conductance (G) between paired oocytes. Paired oocytes were intermittently ELF-EMF exposed (5 min on/10 min off) for 8 hours at 50 Hz powerline signal of 1.0 mT. The half-activation voltage ($V_{1/2}$) and the number of apparent equivalent gating charges (z) derived from the voltage-gating of junctional conductance of paired oocytes expressing rCx46 showed no significant change by ELF-EMF exposure (Figure 65). But the conductance of exposed paired oocytes is smaller than the conductance of sham exposed cell pairs. This finding is not significant on the basis of the 3 paired oocytes analysed so far.

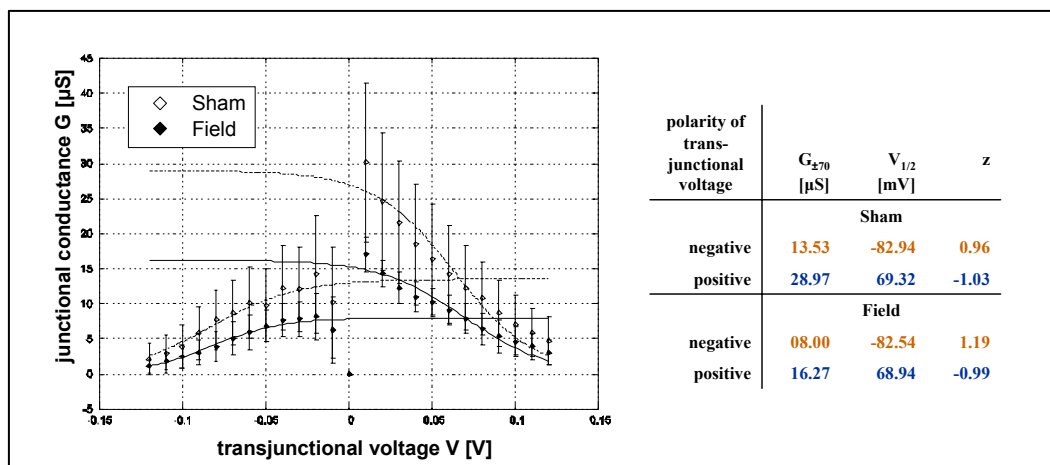


Figure 65. Mean junctional conductance (G) of paired oocytes expressing cell-to-cell channels composed of rCx46 as function of transjunctional voltage. The table summarizes the maximal conductance $G_{\pm 70} = G(V = \pm 70 \text{ mV})$ and the voltage-dependent gating parameters: the half-activation voltage ($V_{1/2}$) and number of apparent equivalent gating charges (z) of cell-to-cell channels after intermittent exposure (5 min on / 10 min off) for 8 hours at 1.0 mT, 50 Hz powerline (closed symbol: exposed cell pairs ($n = 3$); open symbol: sham exposed cell pairs ($n = 3$)). The parameter values were obtained by fitting the experimental data of G vs. V by a simple Boltzmann-distribution.

No significant influence of ELF-EMF on gap junctional coupling of rat granulosa cells was observed.

Gap junctional coupling by cell-to-cell channels of pairs of cultured granulosa cells was recorded after continuous exposure to ELF-EMF of 2.3 mT for 30 min. Figure 66a shows the maximal gap junctional conductance in the absence and presence of ELF-EMF exposure. The data were obtained as function of days in culture after passage, respectively. The corresponding mean gap junctional conductance of sham- and field-exposed cell all pairs is given in Figure 66b. No significant influence of ELF-EMF exposure on gap junctional coupling of rat granulosa cells was found.

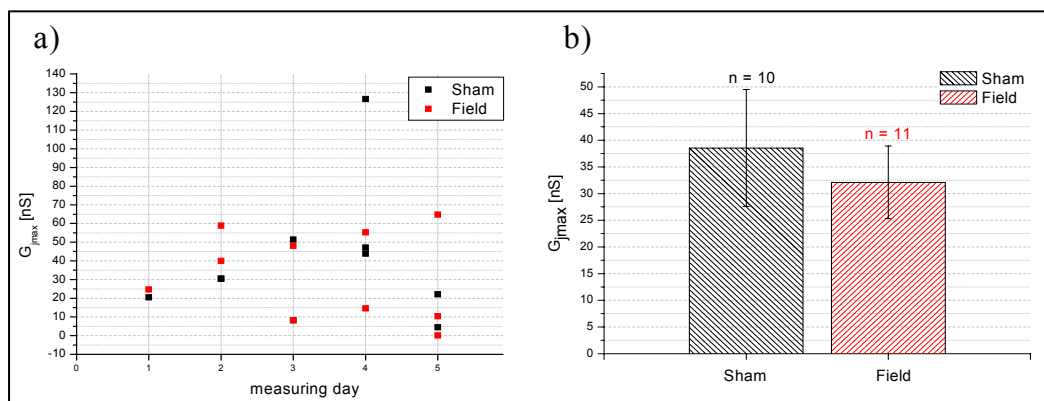


Figure 66. Gap junctional coupling of rat granulosa cell pairs as function of culture time in the presence and absence of ELF-EMF. **a.)** Maximal gap junctional conductance G_{jmax} of cultured pairs of granulosa cells in the absence (■, $n = 10$) and presence of ELF (■, $n = 11$) as function of time in culture. ELF-EMF was continuously applied with 2.3 mT for 30 min at room temperature, respectively. Measurements were performed by application of the double whole-cell patch clamp technique. **b.)** Mean \pm s.e.m. of gap junctional conductance measured at 1 to 5 days (see a)), in the absence ($n = 10$) and presence of ELF-EMF ($n = 11$). n denotes the number of different cell-pairs.

An effect of ELF-EMF on cytoplasmic free calcium of cultured human fibroblasts and granulosa cells of rats was not observed.

After exposure of fibroblasts for 5, 6, 7, 9, 10 and 11 hours to ELF-EMF $[Ca^{2+}]_i$ was recorded. Measurement of $[Ca^{2+}]_i$ was started 10 min after end of exposure and recorded up to 40 min under exposure –free incubation conditions. In Figure 67 $[Ca^{2+}]_i$ was followed after exposure for 11 h (Figure 67a) and 15h (Figure 67b). During the presented recording time no significant change of $[Ca^{2+}]_i$ was observed. The described experiments were repeated for a cultured granulosa cell line (not shown). The observed variability in the time course of $[Ca^{2+}]_i$ of some sham- and field exposed cells seems not to be significant. As in the case of fibroblasts a long-lasting influence of ELF-EMF on the time course and amplitude of $[Ca^{2+}]_i$ was not observed for cultured rat granulosa cells. For clearer presentation the results of $[Ca^{2+}]_i$ recorded for fibroblasts and rat granulosa cells are summarised in Table 9.

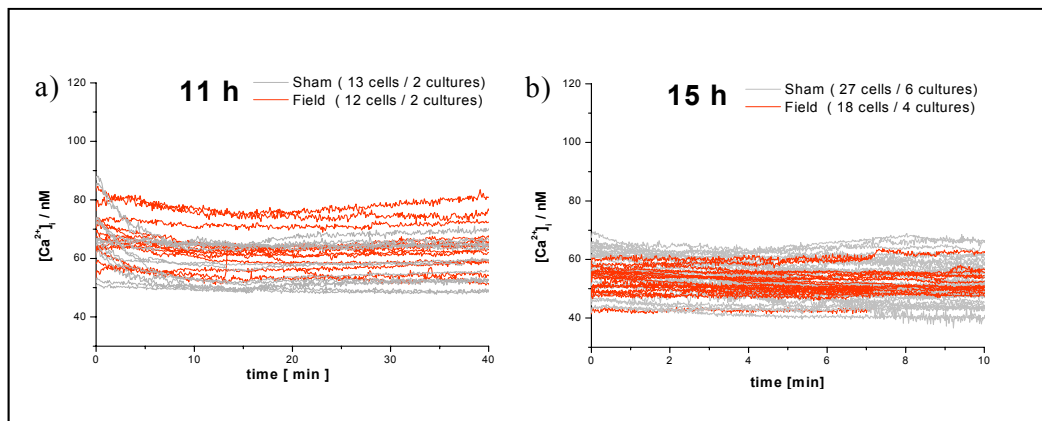


Figure 67. Time course of $[Ca^{2+}]_i$ in fibroblasts after ELF-EMF exposure at 50 Hz sinusoidal, 1.0 mT, intermittent (5 min on/10 min off) for **a)** 11 hours and **b)** 15 hours exposure time (grey curves denote sham- and red curves field-exposure)

Table 9. Summary of $[Ca^{2+}]_i$ data obtained on cultured fibroblasts and rat granulosa cells after ELF-EMF (5 min on/10 min off, sinusoidal 50 Hz, 1.0 mT) exposure

cell system	exposure time	7 h	9 h	11 h	15 h	17 h
Fibroblasts	sham	35 cells / 6 cultures	27 cells / 5 cultures	13 cells / 2 cultures	27 cells / 6 cultures	56 cells / 7 cultures
	field	37 cells / 6 cultures	15 cells / 3 cultures	12 cells / 2 cultures	18 cells / 4 cultures	14 cells / 3 cultures
		no ELF-EMF effect	no ELF-EMF effect	no ELF-EMF effect	no ELF-EMF effect	no ELF-EMF effect
Granulosa	exposure time	4 h	5 h	6.5 h	7.75 h	
	sham	11 cells / 1 culture	54 cells / 5 cultures	60 cells / 5 cultures	51 cells / 3 cultures	
	field	19 cells / 2 cultures	57 Cells / 5 cultures	47 cells / 3 cultures	7 cells / culture	
		no ELF-EMF effect	no ELF-EMF effect	no ELF-EMF effect	no ELF-EMF effect	
ELF-EMF stimulation: 50 Hz, sinusoidal, 1 mT (5 min on / 10 min off)						

In a further series of experiments the cells were exposed to an additional stressor added to the bath after the end of ELF-EMF exposure. Figure 65 shows the time course of $[Ca^{2+}]_i$ of fibroblasts during an additional exposure to 200 μM H_2O_2 in the bath. No significant effect on $[Ca^{2+}]_i$ could be found during a consecutive treatment by H_2O_2 . Application of another stress condition like cell- depolarisation by high external KCl (30 mM) also did not affect the time course and amplitude of $[Ca^{2+}]_i$ for ELF-EMF exposed cells (data not shown).

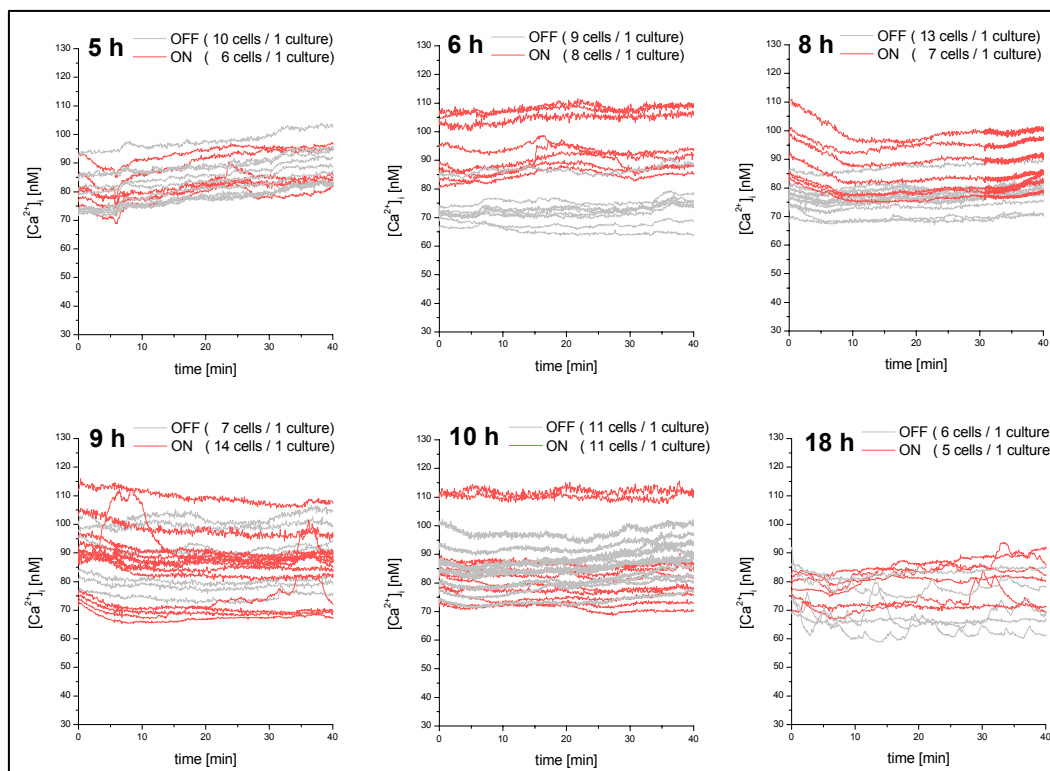


Figure 68. Time course of $[Ca^{2+}]_i$ in fibroblasts after end of ELF-EMF (5 min on/10 min off, 50 Hz sinusoidal, 1.0 mT) exposure for 5, 6, 8, 9, 10 and 18 hours which was followed by an addition of 200 μM H_2O_2 to the bath for further 10 min (grey curves denote sham- and red curves field-exposure). For further details see text.

A summary of $[Ca^{2+}]_i$ measurements on fibroblasts and rat granulosa cells after application of ELF-EMF followed by addition of H_2O_2 to the bath is given in Table 10. $[Ca^{2+}]_i$ was recorded in the presence of 200 μM H_2O_2 .

Table 10. Summary of $[Ca^{2+}]_i$ data obtained on cultured fibroblasts and rat granulosa cells after ELF-EMF (5 min on/10 min off, sinusoidal 50 Hz, 1.0 mT) exposure followed by addition of H_2O_2 to the bath.

cell system	stimulation	exposure time	5 h	6 h	8 h	9 h	10 h	18 h	
Fibroblasts	ELF-EMF and 200 μ M H ₂ O ₂	sham	10 cells / 1 culture	9 cells / 1 culture	13 cells / 1 culture	7 cells / 1 culture	11 cells / 1 culture	6 cells / 1 culture	
		field	6 cells / 1 culture	8 cells / 1 culture	7 cells / 1 culture	14 cells / 1 culture	11 cells / 1 culture	5 cells / 1 culture	
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	
	ELF-EMF and 30 mM KCl	exposure time	6.5 h	7.5 h	8.5 h	9.5 h			
		sham	7 cells / 1 culture	7 cells / 1 culture	6 cells / 1 culture	7 cells / 1 culture			
		field	5 cells / 1 culture	5 cells / 1 culture	10 cells / 1 culture	8 cells / 1 culture			
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect			
Granulosa	ELF-EMF and 200 μ M H ₂ O ₂	exposure time	5 h	6 h	7 h	8 h	18 h		
		sham	14 cells / 1 culture	14 cells / 1 culture	20 cells/ 2 cultures	14 cells / 1 culture	14 cells / 1 culture		
		field	5 cells / 1 culture	5 cells / 1 culture	10 cells / 1 culture	8 cells / 1 culture	14 cells / 1 culture		
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect		
ELF-EMF stimulation: 50 Hz, sinusoidal, 1 mT, 5 min on / 10 min off									

The volume regulatory response of granulosa cells appeared not to be influenced by ELF-EMF.

The volume regulatory response of cultured granulosa cells was studied after application of a hypotonic shock followed by a hypertonic shock. For clearer presentation for each experiment the volume change ($v(t)-v(t=0)$) of 10 cells was analysed as function of time and normalised to the maximal value v_{\max} , respectively. As exposure period again 18 h were selected, since after this exposure period rat granulosa cells show the maximal response on the genomic level. The volume regulatory behaviour of rat granulosa cells appears not to be influenced by ELF-EMF. In addition, there was no significant difference between exposed and sham exposed cells for hypotonic (Figure 69) as well as hypertonic conditions (not shown). The volume analysis was started 15 min after end of ELF-EMF exposure.

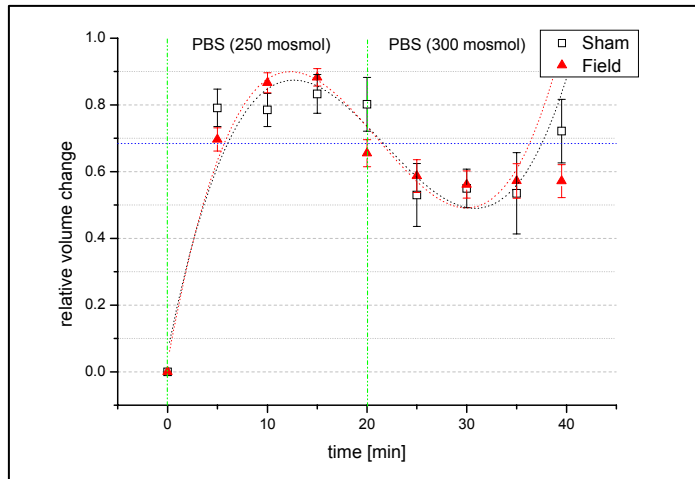


Figure 69. Relative volume change of granulosa cells (GFSHR-17) after addition of a hypotonic solution at $t = 0$. Prior to this treatment the cells were sham exposed ($n = 7$) or ELF-EMF exposed ($n = 24$) for 18 hours at 50 Hz, 1.0 mT, (5 min on/10 min off)). The mean \pm s.e.m. is given, respectively.

3.1.4.5 Whole-genome analysis of various cell lines exposed to ELF-EMF (Participant 12)

Altogether, 58 whole-genome analyses of 10 different cell lines (sham-exposed cells and control cells) were performed (Table 1). After primary data analysis, we only worked on genes which were reproducibly regulated in several experiments (see materials and methods) and which belonged to certain gene families (Table 11). We defined gene families which are potentially relevant for the cellular answer on EMF exposure: signal transduction, ion/electron transport, metabolism of energy/proteins, cell proliferation/apoptosis, immune answer/inflammation and extracellular matrix/ cytoskeleton. Each gene family was sub-divided in subgroups again, e.g. GTP proteins in the signal transduction family (Tables 11, 12). In a first step, we did not go into single genes, but simply counted genes up- or down-regulated in the different gene families. The total number of regulated genes in a certain gene family is not very meaningful, because the sizes of the gene families are of course very different. Therefore, the total numbers of genes on the human array belonging to a gene family are shown in the first column of Tables 11 and 12. Although a single gene might appear in different categories (e.g. all small G proteins are GTP binding proteins), the Tables give a good overview on what might happen in the cells after EMF exposure on the molecular level.

In human fibroblasts (Participant 3), a number of G proteins and calcium associated proteins involved in signal transduction seem to be strongly regulated. Genes involved in adhesion of cells and cytoskeletal genes appear strongly regulated in several hybridizations, although the variances in numbers between the experiments are high (Table 11).

The fibroblast experiments (Participant 3) were also assessed by bio-statistics (Participant 8: Dr. Remondini, Table 12): Mitochondrial and ribosomal genes appeared strongly regulated, also Ca-related genes, cell cycle, apoptosis, extracellular matrix, and the cytoskeleton. The overall number of significantly regulated genes is higher in the ELF-EMF treated fibroblasts than e.g. in endothelial cells

exposed to RF-EMF (Participant 6). This was expected, since the number of regulated genes in fibroblasts after ELF-EMF exposure is pretty uniform in the non-statistically evaluated gene numbers (compare Table 12 with Table 11). From the experiments with SY5Y human neuroblastoma cells (Participant 11) and mouse embryonic stem cells (Participant 4) it was not possible to extract bio-statistically significant data.

In detail, the following genes were extracted by bio-statistics so far:

Actin associated proteins (belong to cytoskeleton):

- Caldesmon (tropomyosin binding, actin binding. Activation of ERK MAP kinases lead to phosphorylation of caldesmon. Regulatory protein of the contractile apparatus): down-regulated (fibroblasts, participant 3).
- Gamma-actin: down-regulated (fibroblasts, Participant 3).
- "coactosin-like": down-regulated (fibroblasts, Participant 3).
- "actin-binding": down-regulated (fibroblasts, Participant 3).
- "procollagen-proline 2": down-regulated (fibroblasts, Participant 3).
- "actin modulating activity": up-regulated (fibroblasts, Participant 3).
- "actin-binding, calcium ion binding": down-regulated (fibroblasts, Participant 3).
- CD2-associated protein, actin binding: down-regulated (fibroblasts, Participant 3).
- Tropomodulin 3: actin binding down-regulated (fibroblasts, Participant 3).

Calcium (Ca²⁺)-associated proteins:

- protein phosphatase 4: down-regulated (fibroblasts, Participant 3).
- Thrombospondin (cell adhesion): down-regulated (fibroblasts, Participant 3).
- "EGF-containing fibulin-like..." (cell adhesion): down-regulated (fibroblasts, Participant 3).
- matrix metalloproteinase 2 MMP 2 (extracellular matrix, collagen metabolism): down-regulated
- follistatin (extracellular matrix, heparin binding): down-regulated (fibroblasts, Participant 3).
- SPARC (extracellular matrix, collagen binding): down-regulated (fibroblasts, Participant 3).
- ("myosin light polypeptide": up-regulated (fibroblasts, Participant 3).
- ("hypothetical protein": up-regulated (fibroblasts, Participant 3).

Extracellular matrix (ECM):

- thrombospondin (see Ca): down-regulated (fibroblasts, Participant 3).
- "EGF-containing..." (see Ca): down-regulated (fibroblasts, Participant 3).
- MMP2 (see Ca): down-regulated (fibroblasts, Participant 3).
- Connective tissue growth factor CTGF (cell adhesion, obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).
- Collagen XV (obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).
- Lysyl oxidase (also processed by bone morphogenetic protein 1 BMP1, obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).

Cytoskeleton (see also actin and calcium-associated proteins):

- "hypothetical protein": down-regulated (fibroblasts, Participant 3).
- "protein phosphatase 4, caldesmon): down-regulated (fibroblasts, Participant 3).
- "SH3 protein interacting with Nck": down-regulated (fibroblasts, Participant 3).
- "in kinesin complex": down-regulated (fibroblasts, Participant 3).

Ion transport:

- "potassium channel activity": down-regulated (fibroblasts, Participant 3).
- SLC12A5 KCl (potassium chloride) transporter: down-regulated (fibroblasts, Participant 3).
- SLC26A3 sulfate porter: down-regulated (fibroblasts, Participant 3).
- "ferric ion binding": down-regulated (fibroblasts, Participant 3).
- (ATP synthase, H⁺ transport): down-regulated (fibroblasts, Participant 3).
- ("H⁺ transporter): down-regulated (fibroblasts, Participant 3).
- "iron ion transport": down-regulated (fibroblasts, Participant 3).

Ribosomal proteins:

- 7 ribosomal proteins down-regulated, 3 ribosomal proteins up-regulated, 1 ribosomal protein up-regulated in profile nr.1, down-regulated in profile nr.2 (fibroblasts, Participant 3).

Table 11. Numbers of genes regulated within different gene families

Gene Family	total number of clones in Human Unigene RZPD-2	partner 3 fibroblasts Exp1 ELF up-regulated genes	partner 3 fibroblasts Exp2 ELF up-regulated genes	partner 3 fibroblasts Exp1 ELF down-regulated genes	partner 3 fibroblasts Exp2 ELF down-regulated genes	Gene "Superfamily"
Signal	2528	251	232	296	190	signal transduction
GTP	560	66	73	66	52	signal transduction
Small G	235	31	32	28	27	signal transduction
Jak	23	0	0	5	3	signal transduction
Rab	80	0	9	11	13	signal transduction
Ras	66	10	7	10	5	signal transduction
wnt	5	0	0	0	0	signal transduction
phosphatase	334	39	36	35	26	signal transduction
protein kinase	304	28	29	35	24	signal transduction
phospholipase	72	9	8	10	7	signal transduction
calcium	715	67	72	80	50	signal transduction
calmodulin	131	6	13	17	11	signal transduction
channel	348	31	25	36	22	ion/electron transport
voltage-gated	164	16	13	16	6	ion/electron transport
electron transport	423	52	52	57	37	ion/electron transport
ion transport	501	49	48	45	29	ion/electron transport
metaboli	1241	122	128	135	96	metabolism of energy/proteins
ATP	1234	113	112	157	82	metabolism of energy/proteins
mitochon	574	84	82	70	65	metabolism of energy/proteins
ribosom	254	47	48	32	39	metabolism of energy/proteins
translation	168	30	28	20	13	metabolism of energy/proteins
transcript	1991	201	190	228	136	metabolism of energy/proteins
cell cycle	478	46	52	54	43	cell proliferation/apoptosis/differentiation
apoptos	373	31	37	37	23	cell proliferation/apoptosis/differentiation
differentiat	177	14	21	22	11	cell proliferation/apoptosis/differentiation
immun	390	31	37	38	32	immune answer/inflammation/stress answer
inflamma	184	10	20	24	11	immune answer/inflammation/stress answer
stress	118	12	11	14	13	immune answer/inflammation/stress answer
peroxidase	32	6	5	4	6	immune answer/inflammation/stress answer
heat shock	188	4	7	5	3	immune answer/inflammation/stress answer
DNA repair	154	14	19	19	10	immune answer/inflammation/stress answer
early	8	2	2	0	2	immune answer/inflammation/stress answer
adhesion	573	46	49	53	43	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	19	34	31	16	extracellular matrix/cytoskeleton/adhesion
cytosk	529	45	50	47	42	extracellular matrix/cytoskeleton/adhesion
junction	129	11	10	13	10	extracellular matrix/cytoskeleton/adhesion
actin	494	32	32	38	32	extracellular matrix/cytoskeleton/adhesion

Table 12. Numbers regulated genes in different expression profiling experiments (bio-statistical analysis by Dr. Remondini/Participant 8)

Gene Family	total number of clones in Human Unigene RZPD-2	partner 3 fibroblasts ELF up-regulated genes	partner 3 fibroblasts ELF down-regulated genes	Gene "Superfamily"
Signal	2528	0	12	signal transduction
GTP	560	0	2	signal transduction
Small G	235	0	1	signal transduction
Rab	80	0	1	signal transduction
Ras	66	0	1	signal transduction
phosphatase	334	1	2	signal transduction
protein kinase	304	0	1	signal transduction
calcium	715	2	6	signal transduction
calmodulin	131	0	1	signal transduction
channel	348	0	2	ion/electron transport
voltage-gated	164	0	1	ion/electron transport
ion transport	501	0	7	ion/electron transport
electron transport	423	0	3	ion/electron transport
metaboli	1241	0	4	metabolism of energy/proteins
ATP	1234	2	7	metabolism of energy/proteins
mitochon	574	0	5	metabolism of energy/proteins
ribosom	254	3	7	metabolism of energy/proteins
translation	168	3	0	metabolism of energy/proteins
transcript	1991	2	9	metabolism of energy/proteins
cell cycle	478	1	4	cell proliferation/apoptosis/differentiation
apoptos	373	0	4	cell proliferation/apoptosis/differentiation
differentiat	177	0	2	cell proliferation/apoptosis/differentiation
immun	390	0	2	immune answer/inflammation/stress answer
DNA repair	154	0	0	immune answer/inflammation/stress answer
inflamma	184	0	1	immune answer/inflammation/stress answer
adhesion	573	2	3	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	2	5	extracellular matrix/cytoskeleton/adhesion
cytosk	529	1	6	extracellular matrix/cytoskeleton/adhesion
actin	494	1	4	extracellular matrix/cytoskeleton/adhesion
ijunction	129	0	0	extracellular matrix/cytoskeleton/adhesion

3.1.4.6 Summary (Participant 1)

Our data indicate that ELF-EMF may affect the gene and protein expression in various cell systems. This conclusion is based on the following findings:

- ELF-EMF at a flux density of about 2 mT up-regulated the expression of early genes, such as p21, c-jun and erg-1, in p53-deficient mouse embryonic stem cells, but not in healthy wild-type cells suggesting that the genetic background affects the responsiveness of the cells (see 3.1.4.1).
- ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the GADD45 gene and down-regulated the transcript levels of the bax gene by which the apoptotic process may be modulated (see 3.1.3.1 and 3.1.4.1).
- ELF-EMF at a flux density of 0.8 mT up-regulated the expression of cardiac specific genes in cardiomyocytes derived from embryonic stem cells thus promoting cardiogenesis (see 3.1.4.3).
- ELF-EMF did not affect the expression of neuronal genes in neuroblastoma cells (SY5Y) such as nAChRs, D β H, Phox2a and Phox2b, either at mRNA or protein level (see 3.1.4.2).
- ELF-EMF did not affect either the expression level of conducting hemi-channels composed of rCx46, nor their gating properties by voltage, pH, Ca²⁺ in *Xenopus laevis* oocytes (see 3.1.4.4).
- ELF-EMF appeared to regulate the expression of a series of genes and proteins in human fibroblasts such as mitochondrial and ribosomal genes as well as Ca-, cell cycle-, apoptosis-, extracellular matrix-, and cytoskeleton-related genes, although it must be considered that the variances observed between the various experiments was high (see 3.1.4.5).

3.2 Results in RF-EMF research

3.2.1 Genotoxic effects

3.2.1.1 Human HL-60 cell line (Participant 2)

Genotoxic effects of EMF may occur directly either by damage to chromosomes and/or by damage to DNA repair mechanisms. Indirect genotoxic effects may arise by various processes such as generation of oxygen radicals or impairment of radical-scavenging mechanisms. Direct and indirect genotoxic effects of defined RF-EMF were investigated in the human cell line HL-60.

A. Direct genotoxicity

RF-EMF increased the micronucleus frequency and the number in DNA strand breaks in HL-60 cells dependent on the energy of radiation as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

The effect of RF-EMF on the formation of micronuclei (MN) and DNA strand breaks was examined by use of the cytokinesis-block in vitro micronucleus assay and the alkaline Comet assay. To validate the MN assay and to prove the susceptibility of HL-60 cells to physical noxes, cells were exposed to ionising-irradiation. As shown in Figure 70, a dose-dependent induction of micronuclei in HL-60 cells was found for doses of exposure increasing from 0.5 to 3.0 Gy. Cell division was effected by ionising-irradiation at doses ≥ 1.0 Gy as shown in Figure 71, inferred from the ratio of BNC against mono-, bi-, tri- and tetranuclear cells (in %).

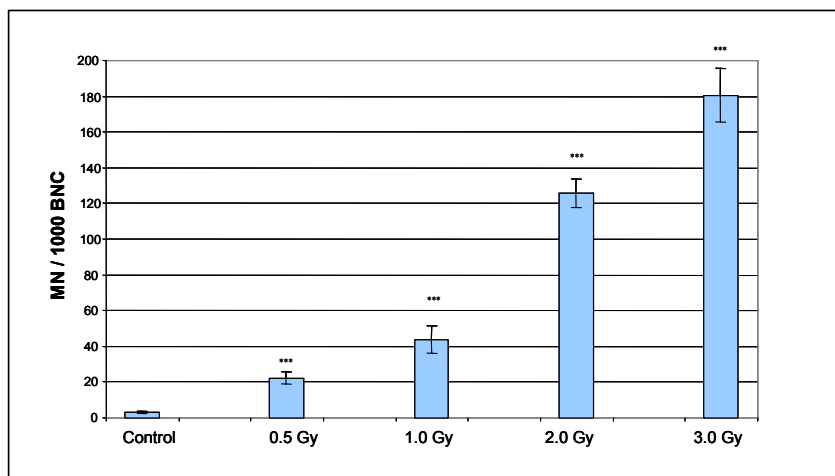


Figure 70. Effect of ionising-irradiation (6 MeV) on micronuclei formation in binucleated HL-60 cells. Each data point is based on at least three independent experiments. Each bar represents the mean \pm SD of results obtained in three independent experiments. *** $P < 0.001$ (Student's t-test, two-sided). All in all (mono-, bi-, tri-, and tetranucleated) 15000 cells were analysed.

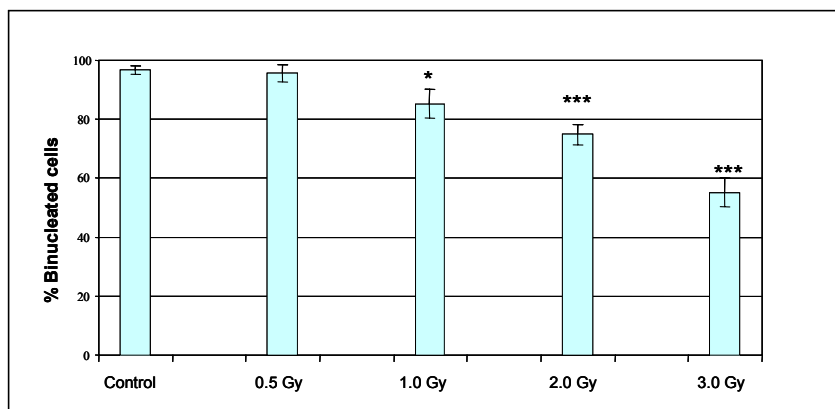


Figure 71. Effect of ionising-irradiation on HL-60 cell division. The number of binucleated cells relative to the number of mono-, bi-, tri-, and tetranuclear cells following ionising-irradiation (6 MeV) of HL-60 cells. Each bar represents the mean \pm SD in % of results obtained in three independent experiments; * p <0.05, *** p <0.001 (Student's t-test, two-sided).

In a series of experiments SAR levels ranging from 0.2 W/kg to 3.0 W/kg were examined in order to clarify whether the effects of RF-EMF exposure (1800 MHz, continuous wave, 24 h) on MN frequencies in HL-60 cells are energy dependent (Figure 72). Whereas at SAR of 0.2 W/kg, 1.0 and 3.0 W/kg MN frequencies were not changed in RF-EMF-exposed cells as compared to sham controls and incubator controls, MN frequencies were significantly increased at SAR of 1.3 W/kg and above. The maximum increase was noted at a SAR of 1.3 and 1.6 W/kg. This effect was approximately 66 % of the effect observed after 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2 s). At a SAR of 3.0 W/kg the MN frequency was similar to that found in sham-exposed cells. While MN frequencies of incubator controls were around 3.5, the MN frequency determined after RF-exposure at a SAR of 1.3 W/kg was 13.3 (approximately 3.8 fold higher). The MN frequency determined in cells after exposure to ionising-irradiation (0.5 Gy, exposure time: 5.2s), used as a positive control, was 22.3 ± 3.5 ($n=3$; 6.3 fold increase compared to control).

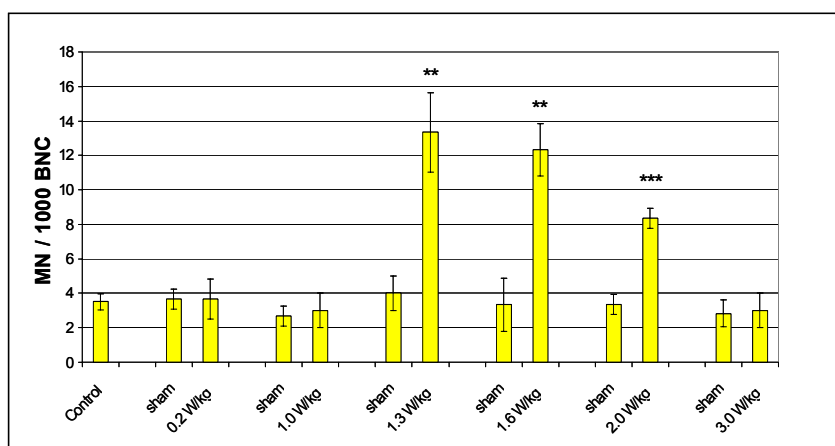


Figure 72. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-field (1800 MHz, continuous wave) for 24h ranging from SAR 0.2 to 3.0 W/kg, compared to control and sham-exposure. Each bar represents the mean \pm SD of results obtained in three independent experiments (except of control: $n = 11$). Each data point is based on at least three independent experiments except of the control with 11 independent experiments and on a total number of 11000 (control), 18000 (sham-exposed) and 18000 (RF-exposed) bi-nucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetra-nucleated) 47000 cells were analysed. The micronuclei frequency of BNC after exposure to ionising-irradiation (0.5 Gy, exposure time: 5.2s), which was used as a positive control, were on average 22.3 ± 3.5 ($n=3$). ** P <0.01; *** P <0.001 (Student's t-test, two-sided).

In order to compare micronuclei induction in cells exposed to RF-fields at different ranges of SAR, the average micronuclei frequencies (MN/1000 BNC) were calculated for the following groups: experiments performed at all SAR tested (range 0.2 W/kg to 3.0 W/kg, number of independent experiments n=18), experiments performed at SAR ranging from 1.0 W/kg to 2.0 W/kg (number of independent experiments n=12), and experiments at SAR of 0.2 W/kg and 1.0 W/kg (number of independent experiments n=6). While the calculated average of MN/1000 BNC in HL-60 cells at a SAR of 0.2 W/kg to 1.0 W/kg was not significantly different from that observed in sham-exposed controls, both groups ranging from 0.2 W/kg to 3.0 W/kg ($p<0.01$) or from 1.0 W/kg to 2.0 W/kg ($p<0.001$) exhibited a significant increase in micronuclei induction after RF-exposure as compared to sham-exposed controls (Figure 74A).

Previous experiments had clearly shown that RF-EMF exposure results in an increase of DNA strand breaks in HL-60 cells. In order to achieve a better understanding of whether these effects are energy dependent, additional experiments were performed applying RF-exposure (1800 MHz, continuous wave, 24h) at SAR of 0.2 W/kg to 3.0 W/kg. As shown in Figure 73 the effect of RF-EMF on DNA strand breaks at these exposure conditions exhibited a similar energy dependency as the effect of RF-EMF on micronucleus formation (Figure 72). RF-EMF exposure at a SAR of 1.0 W/kg and below had no effect on Comet formation in HL-60 cells (expressed as Olive Tail Moment OTM) as compared to control and sham-exposed cells. On the other hand RF-EMF at SAR of 1.3 W/kg and above caused a significant increase in DNA strand breaks. The maximum of this effect was observed at SAR 1.3 W/kg (OTM = 2.20 ± 0.16) and 1.6 W/kg (2.24 ± 0.10). At a SAR of 3.0 W/kg Comet formation in RF-EMF exposed cells (OTM 1.23 ± 0.12) was similar to that observed in sham-exposed cells (OTM 1.18 ± 0.03). While the Olive Tail Moment was around 1.0 in sham-exposed and incubator controls, the OTM determined after exposure at a SAR of 1.3 W/kg was approximately 2.2 fold higher (Figure 73). The OTM determined in cells after exposure to hydrogen peroxide (100 $\mu\text{mol/l}$, 1 h), used as a positive control, was 8.3 ± 1.3 (n=3; 8 fold increase compared to control).

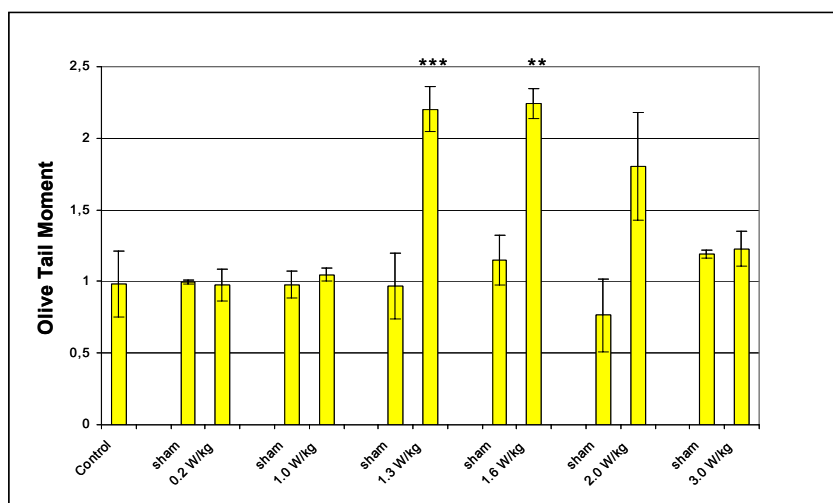


Figure 73. Comet formation in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave) for 24h ranging from SAR 0.2 to 3.0 W/kg, expressed as Olive Tail Moment, compared to control and sham exposure. Each bar represents the mean \pm SD of results obtained in at least three (except SAR 1.3 W/kg: n=4) independent experiments. The OTMs of the Comets after exposure to hydrogen peroxide (100 $\mu\text{mol/l}$, 1 h), which was used as a positive control, were on average 8.3 ± 1.3 (n=3). ** $P<0.01$; *** $P<0.001$ (Student's t-test, two-sided).

In order to compare Comet formation in cells exposed at different SAR ranges the average values of the Olive Tail Moments were calculated for the following groups: experiments performed at all SAR tested (range 0.2 W/kg to 3.0 W/kg, number of independent experiments n=18), experiments performed at SAR ranging from 1.0 W/kg to 2.0 W/kg (number of independent experiments n=12), and experiments at SAR of 0.2 W/kg and 1.0 W/kg (number of independent experiments n=6). While the calculated average of OTMs in HL-60 cells at SAR of 0.2 W/kg and 1.0 W/kg was not significantly different from that observed in sham-exposed controls, both groups ranging from 0.2 W/kg to 3.0 W/kg ($p<0.01$) or from 1.0

W/kg to 2.0 W/kg ($p < 0.001$) exhibited a significant increase in Comet formation after RF-exposure as compared to sham-exposed controls (Figure 74B).

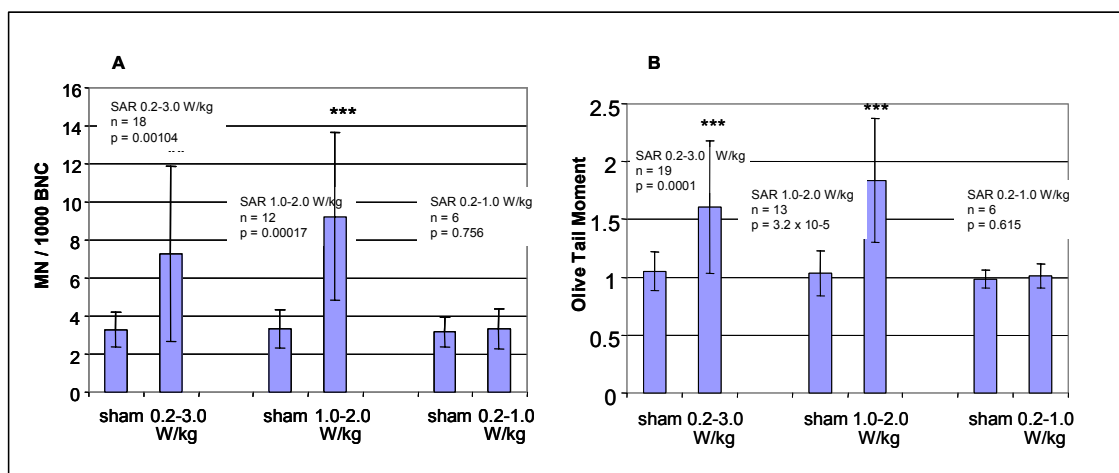


Figure 74. MN induction and Comet formation in HL-60 cells after RF-field exposure (1800 MHz, continuous wave, 24 h) over all SAR groups tested versus total sham, expressed as MN per 1000 BNC (A) and as Olive Tail Moment (B). Each bar represents the mean \pm SD of results obtained in indicated number of experiments. ** $P < 0.01$; *** $P < 0.001$ (Student's t-test, two-sided).

RF-EMF increased the micronucleus frequency and the number of DNA strand breaks in HL-60 cells dependent on the exposure time as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

Using the cytokinesis-block MN assay it was also investigated, whether the duration of exposure of HL-60 cells to RF-fields has an influence on MN induction (Figure 75). Short exposure periods (6 h) caused no or less pronounced effects compared to longer exposure periods of 24 and 72h. The level of the effect on MN frequency noted after RF-EMF exposure for 72h (MN/1000 BNC: 20.22 ± 2.08) was comparable to that observed after 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2 s) (MN/1000 BNC: 22.33 ± 2.48).

Furthermore, it was investigated, whether the duration of exposure of HL-60 cells to RF-EMF has an influence on Comet formation (Figure 76). Short exposure periods (2 and 6h) caused less pronounced effects compared to the longer exposure period of 24h. After 72h of exposure Comet formation was similar to that observed after short exposure times (2 and 6h).

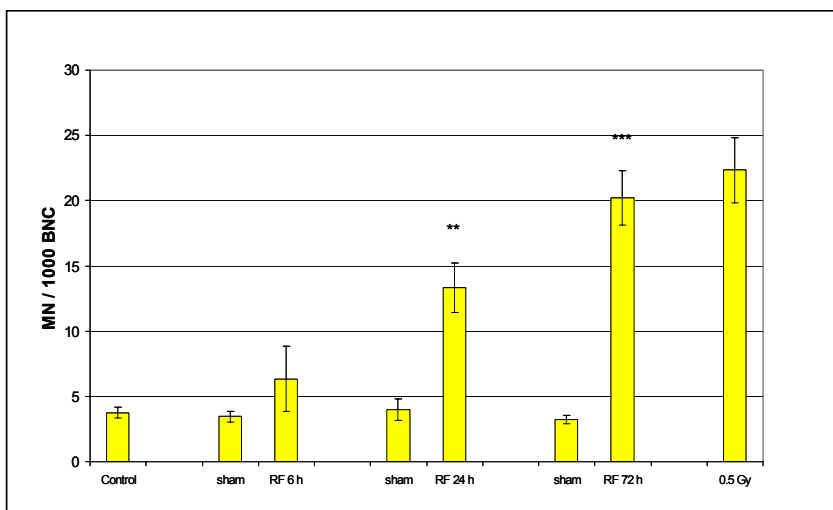


Figure 75. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-fields (1800 MHz, continuous wave, SAR = 1.3 W/kg) for 6, 24 and 72h, compared to control and sham-exposure. Positive control: 0.5 Gy ionising-irradiation (6 MeV). Each bar represents the mean \pm SD of results obtained in three independent experiments (except control: n = 4). Data points are based on a total cell number of 4000 (control), 9000 (sham-exposed), 9000 (RF-exposed) and 3000 (0.5 Gy-exposed, exposure time: 5.2s) binucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetranucleated) 25000 cells were analysed. ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

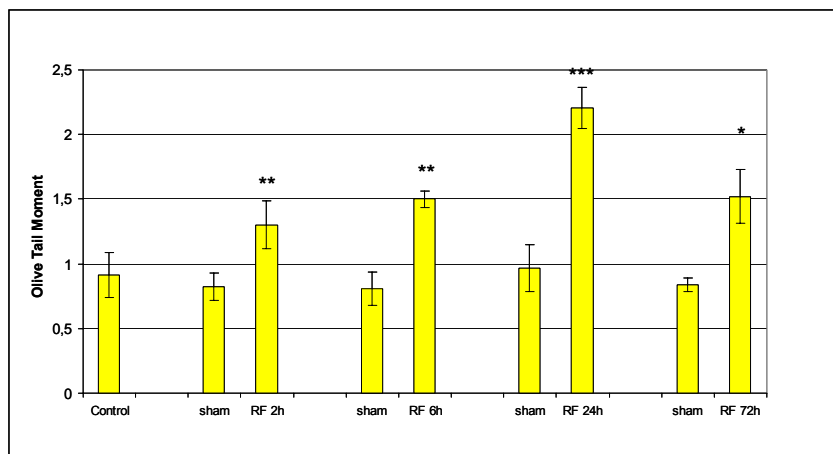


Figure 76. Comet formation in HL-60 cells after exposure to RF-fields (1800 MHz, continuous wave, SAR 1.3 W/kg) for 2, 6, 24 and 72h, expressed as Olive Tail Moment, compared to control and sham-exposure. Each bar represents the mean \pm SD of results obtained in at least three (except RF-field exposure, 24h with n=4) independent experiments. * P<0.05; ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

The effects of RF-EMF on genomic integrity of HL-60 cells were exposure-signal-dependent as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

In a further series of experiments it was studied, whether different RF-signals (1800 MHz, SAR 1.3 W/kg: continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) for 24h are capable to cause MN induction in HL-60 cells (Figure 77) The number of independent experiments was extended to at least three independent experiments for each of the different types of RF-signals at that SAR with the most pronounced effect (SAR 1.3 W/kg). Using the cytokinesis-block MN assay the different RF-signals had similar effects on MN induction as observed following continuous wave exposure. While the MN frequency of continuous wave-exposed cells was 13.33 ± 1.89 , the MN frequencies determined after different other RF-exposure signals were 16.11 ± 3.10 (C.W., 5 min on/10 min off), 13.22 ± 2.88 (GSM-

217Hz) and 17.66 ± 1.70 (GSM-Talk). The MN frequency determined in cells after exposure to ionizing-irradiation (0.5 Gy, 6 MeV, exposure time: 5.2 s), used as a positive control, was 22.3 ± 3.5 (n=3; 6.3 fold increase compared to control).

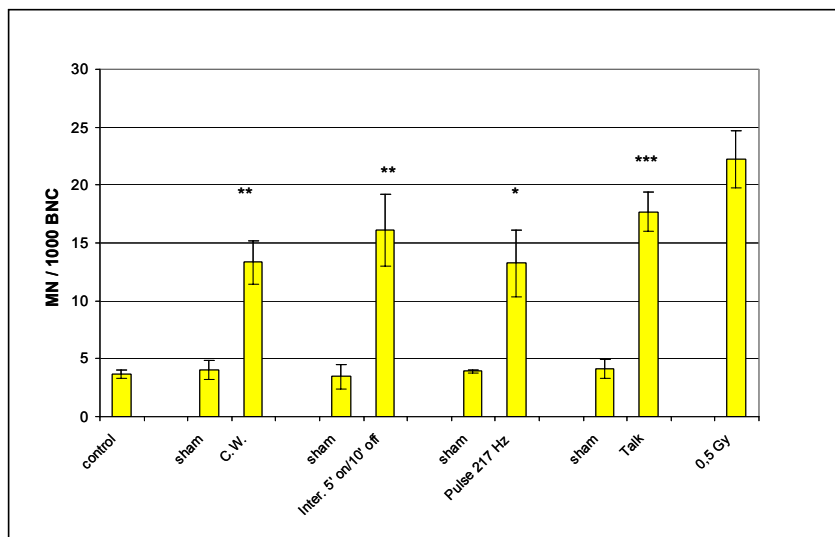


Figure 77. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-field (1800 MHz, SAR 1.3 W/kg, 24h) compared to control and sham-exposure for different signal modulations. Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Bars represent means \pm SD of three independent experiments (except control = 5). * $P < 0.01$; ** $P < 0.01$; *** $P < 0.001$ (Student's t-test, two-sided). Each data point is based on at least three independent experiments except the control with five and the positive control with three independent experiments and on a total of 5000 (control), 12000 (sham-exposed), 12000 (RF-exposed) and 4000 (0.5 Gy-exposed) binucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetranucleated) 32000 cells were analysed.

Calculation of the average numbers of micronuclei per 1000 BNC determined after exposure (SAR of 1.3 W/kg, 24h) to all RF-signals tested (continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) showed an increase in micronuclei induction as compared to sham-exposure at a significant level (number of independent experiments n=12, $P < 0.001$) (Figure 79A).

Using the Comet assay the different RF-signals had similar effects on Comet formation as observed after continuous wave exposure (Figure 78). While the OTM of continuous wave-exposed cells was 2.20 ± 0.16 , the OTMs determined after different other RF-exposure signals were 2.11 ± 0.05 (C.W., 5 min on/10 min off), 1.77 ± 0.01 (GSM-217Hz) and 2.26 ± 0.24 (GSM-Talk).

Calculation of the average value of Olive Tail Moments determined after exposure to all RF-signals tested (continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) showed a significant increase in Comet formation compared to sham-exposed controls (n=14, $P < 0.001$) (Figure 79B).

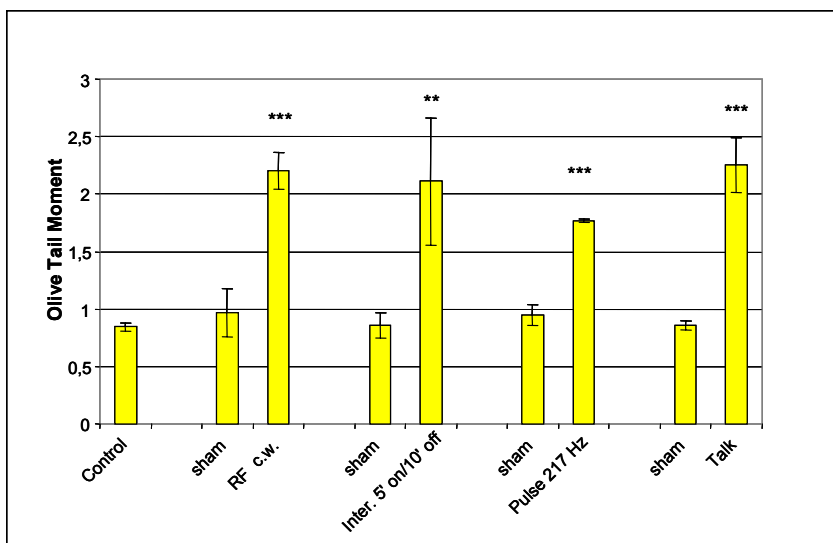


Figure 78. Comet formation in HL-60 cells after exposure to RF-fields (1800 MHz, SAR 1.3 W/kg) for different signal modulations, expressed as Olive Tail Moment, compared to control and sham exposure. Each bar represents the mean \pm SD of results obtained in at least three (except continuous wave and C.W., 5 min on/10 min off: n=4) independent experiments. ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

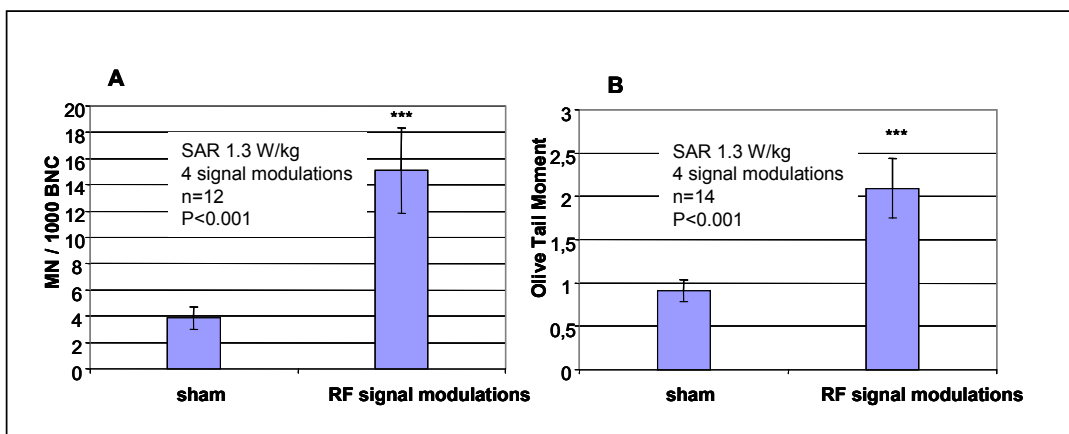


Figure 79. MN induction and Comet formation in HL-60 cells over all RF-field signal modulations at 1800 MHz, 24h, versus total sham, expressed as MN per 1000 BNC (A) and as Olive Tail Moment (B). Each bar represents the mean \pm SD of results obtained in indicated number of experiments. *** P<0.001 (Student's t-test, two-sided).

As shown by flow cytometric analysis RF-EMF increased the micronuclei frequency, but did not affect cell cycle.

The results of the flow cytometric analysis of MN induction in HL-60 cells following RF-EMF exposure at the SAR level of 1.3 W/kg tested above using the continuous wave signal, according to the method of Nüsse et al. 1984, 1997, parallel the results obtained using the cytokinesis-block MN assay. Figure 80 shows a representative flow cytometric analysis of MN induction for sham-exposed (A) and RF-exposed (B) HL-60 cells after exposure to RF (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h. The DNA distribution of micronuclei (marker M1) and nuclei (marker M2) is obtained by projection of the particles defined by their side scatter intensities as micronuclei and nuclei. The percentage of MN is higher in the RF-exposed sample than in the sham-exposed sample (4.1% MN versus 2.7% MN).

The quantitative results of MN content analysis in four independent experiments are presented in Table 13. In all HL-60 cell experiments the MN content of the RF-exposed samples is higher than in sham-exposed samples. Normalisation of MN content in sham-exposed cells to 100% revealed a significant induction of MN after exposure to RF-field by $138.2 \pm 18.4\%$ ($P < 0.01$; Student's t-test, two-sided). This result parallels those obtained using the microscopic analysis of MN frequencies.

Moreover, by means of flow cytometry the DNA-content of G1/G0, S and G2/M phase can be quantified by determining the fraction of each sub-population. The DNA-content distribution (ethidium bromide fluorescence) showed no differences between RF-field exposed and sham-exposed cells, indicating no influence of RF-EMF on cell cycle (Figure 80). Furthermore, cell cycle analysis demonstrated no accumulation of cells arrested in S and G2/M phase following exposure to RF fields. Additionally, for 24h RF-field exposure (1800 MHz, continuous wave, SAR 1.3 W/kg) no increase of the cell population in the sub G1 peak, which can be considered a marker of apoptotic cell death, was observed by flow cytometry.

Table 14 shows the data of DNA content distribution as percentage of gated cells for G1/G0, G2/M and S phase for RF-field exposed cells (continuous wave, SAR 1.3 W/kg, 24h), as compared to incubator control, sham-exposure and positive control hydrogen peroxide (100 $\mu\text{mol/l}$ for 1 hour). The distribution of G1/G0, G2/M and S phase in the incubator control was $51.9 \pm 4.2\%$, $18.3\% \pm 3.7$ and $19.4 \pm 1.7\%$; that of the positive control hydrogen peroxide: $28.4 \pm 12.1\%$, $9.1 \pm 5.2\%$ and $9.0 \pm 3.6\%$. Overall, the percentage of gated cells for the positive control was clearly lower than in all other conditions due to the fact, that here out of the gate analysed a high content of cellular debris was detected. This serves as a measure of cytotoxicity exerted by hydrogen peroxide. Sham-exposed and RF-exposed cells showed a similar DNA distribution as in the incubator control. No significant differences in DNA distribution of RF-exposed cells compared to sham-exposed cells were observed.

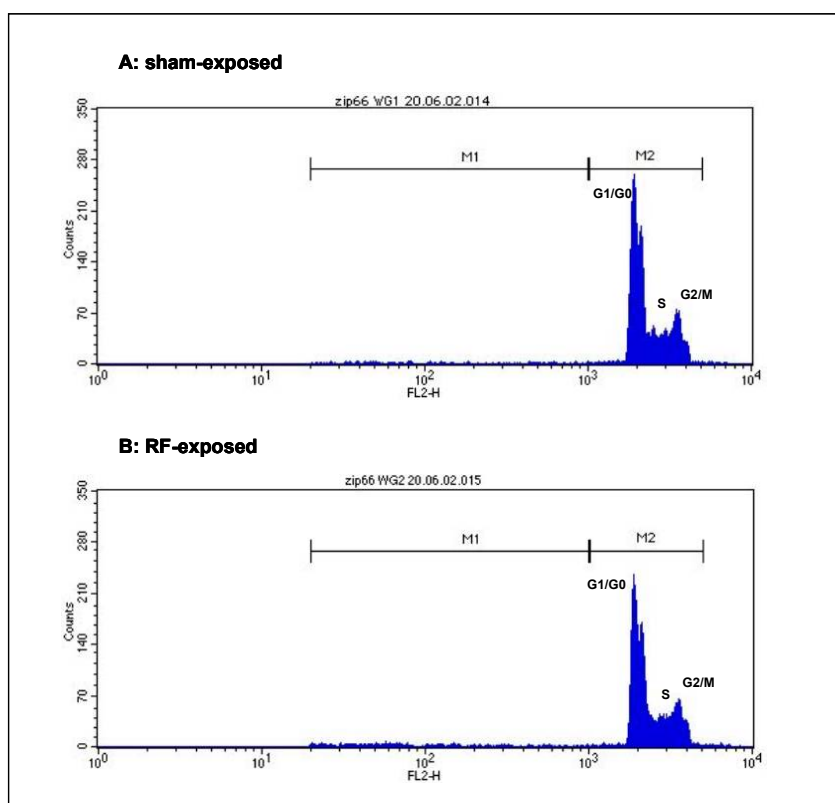


Figure 80. Flow cytometric analysis of micronuclei induction and determination of the proportions of cells in G1/G0, S and G2/M phases of the cell cycle after exposure of HL-60 cells to sham (A) or RF field (B, 1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h.

The diagrams show representative ethidium bromide fluorescence histograms of micronucleated cells/nuclei suspension after treatment with FACS solution I and II. The method was performed according to Nüsse and Kramer (1984), Nüsse and Marx (1997) and Wessel and Nüsse (1995). G1/G0, S and G2/M peaks are indicated. For measurement of MN the G1/G0 peak was adjusted to approximately 2000 relative fluorescence units (FL2-H). The sort window for counting the MN comprised the relative DNA fluorescence units from 20 to 1000 (M1). For quantitative determination of MN the ratio of events in M1 (micronuclei) was compared to the events in M2 (nuclei) and expressed as % MN. For this representative experiment out of four the results for sham-exposure is 2.7% MN (A) and for RF-exposure is 4.1 % MN (B).

Table 13. Quantitative flow cytometric analysis of micronuclei frequencies after RF-field exposure (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to sham-exposure.

No. of experiment	Content of MN [%]		
	Sham-exposed	RF-field exposed	Content of MN in RF-exposed cells rel. to sham-exposed cells (100%)
1	2.69	4.08	151.67
2	5.49	6.37	116.03
3	4.24	6.57	154.95
4	1.85	2.41	130.70
mean	3.57 ± 1.62	4.86 ± 1.98	138.23 ± 18.41 **

Data of column 4 are values of RF-exposed cells in percentage relative to the corresponding sham-exposed value.

** Significant difference between the content of MN of RF-exposed cells to sham-exposed cells at P<0.01 (Student's t-test, two-sided).

Table 14. DNA distribution and cell cycle analysis of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24 h, compared to control, sham-exposure and positive control hydrogen peroxide (100 µmol/l for 1 h). Data represent DNA content distribution as percentage of gated cells in G1/G0, G2/M and S phase.

Group	n	G1/G0 [%]	S [%]	G2/M [%]
control	3	51.90 ± 4.17	18.25 ± 3.65	19.37 ± 1.71
sham	4	54.98 ± 6.69	19.45 ± 3.50	18.43 ± 4.07
RF-field	4	52.94 ± 6.19	19.01 ± 3.54	20.06 ± 2.73
positive control H ₂ O ₂ (100 µmol/l for 1h)	3	28.37 ± 12.11	9.11 ± 5.17	8.96 ± 3.59

RF-EMF did not affect apoptosis as demonstrated by the Annexin V and TUNEL assay.

As the findings of structural alterations on the genomic level correlated with an external cellular stimulus do per se not prove a genotoxic effect, it has to be ruled out, that such changes are due to induction of apoptosis. Apoptotic cells typically undergo a series of structural changes: blebbing of the plasma membrane, condensation of the cytoplasm and intact organelles, and nuclear fragmentation. The most common biochemical property of apoptosis is the endonucleolytic cleavage of chromatin, initially to large fragments of 50-300 kilobase pairs and subsequently to monomers and multimers of 180-200 base pairs.

By establishing two flow cytometry methods for detection of apoptosis, Annexin V assay and TUNEL assay, a differentiation approach was included in the experimental strategy. By means of these two tests the detection of apoptotic changes at different stages in the apoptotic process became feasible. As a positive control for apoptosis induction by camptothecin, a topoisomerase I inhibitor, was used in the flow cytometry assays.

After initiation of apoptosis most cell types translocate phosphatidylserine (PS) from the inner plasma membrane leaflet to the cell surface. Once on the cell surface, PS can easily be detected by staining with a FITC conjugate of Annexin V, a protein that has strong natural affinity for phosphatidylserine. As

externalisation of phosphatidylserine occurs before nuclear changes, associated with apoptosis, take place, the Annexin V test detects apoptotic cells significantly earlier than do DNA-based assays.

Figure 81 shows a representative flow cytometric analysis of Annexin V staining following RF- (1800 MHz, 1.3 W/kg, continuous wave, 24h) and sham-exposure of HL-60 cells. As a positive control the apoptosis inducer camptothecin, a topoisomerase I inhibitor, was used to prove inducibility of apoptosis in the HL-60 cell system. The histograms show apoptosis associated Annexin V-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select sub-populations, the histograms for “gated” and “not gated” cells are presented.

The TUNEL method (Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) identifies apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer FITC-dUTP to the free 3'-OH of cleaved DNA. These labelled cleavage sites can then be detected by flow cytometry.

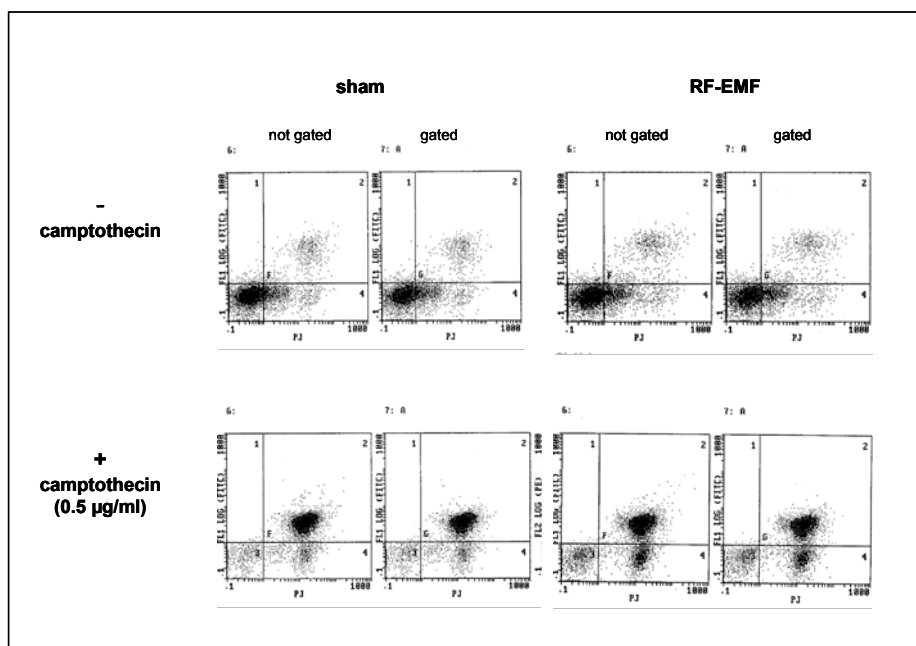


Figure 81. Flow cytometric analysis of RF- (1800 MHz, continuous wave, 1.3 W/kg, 24h) and sham-exposed HL-60 cells after staining with Annexin V-FITC (FL-1) and propidium iodide (PI, DNA content). The apoptosis inducer camptothecin is included as a positive control.

The histograms show apoptosis associated Annexin V-FITC (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select certain sub-populations, the histograms for “gated” and “not gated” cells are presented.

Figure 82 shows a representative flow cytometric analysis of TUNEL staining following RF-exposure (1800 MHz, , continuous wave, SAR 1.3 W/kg, 24h) and sham exposure of HL-60 cells. As a positive control the apoptosis inducer camptothecin, a topoisomerase I inhibitor, was used to prove inducibility of apoptosis in the HL-60 cell system. The histograms show apoptosis associated TUNEL-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that within the quantification procedure no differences in cell population analysed occurs, histograms for “gated” and “not gated” cells are presented (Figure 82).

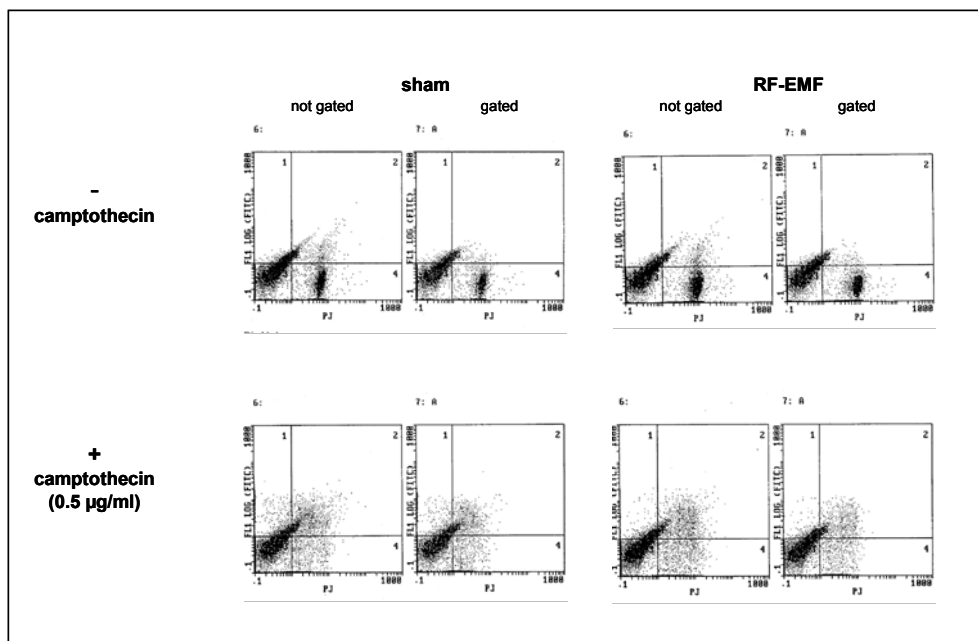


Figure 82. Flow cytometric analysis of RF- (1800 MHz, continuous wave, 1.3 W/kg, 24h) and sham-exposed HL-60 cells after labelling with TUNEL reaction mixture (Roche, Mannheim) for 1 hour at 37°C. The cells then underwent flow cytometric analysis in order to determine the number of green stains (representing apoptotic DNA fragmentation). DNA content analysis was performed on a Becton Dickinson FACScan by using the manufacturer's protocol. The apoptosis inducer camptothecin is included as a positive control.

The histograms show apoptosis associated TUNEL-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select certain sub-populations, the histograms for “gated” and “not gated” cells are presented.

Neither by the Annexin V assay nor by the TUNEL assay, apoptosis induced by RF-electromagnetic fields (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) could be detected in HL-60 cells. Moreover, HL-60 cells exposed to RF-field at SAR 1.3 W/kg and continuous wave signal for 24h show no induction of the cell population in the sub G1 peak, which can be considered a marker of cell death by apoptosis (Figure 80).

RF-EMF did not exert a cytotoxic effect on HL-60 cells.

The trypan blue vitality test did not reveal any cytotoxic effects on the HL-60 cells from any RF-field applied. The vitality of the exposed cells was on the same order of magnitude ($\approx 90\%$) as the cells of the sham-exposed and the incubator control. To exclude minor effects of RF-fields on viability of HL-60 cells, cell viability was examined spectrophotometrically by the MTT assay (Figure 83). Supplementary, also by this method no cytotoxic effect was detectable: absorbance $A_{570\text{nm}}$ (RF-exposed HL-60 cells) = 0.91 ± 0.13 ; $A_{570\text{nm}}$ (sham-exposed HL-60 cells) = 0.98 ± 0.15 .

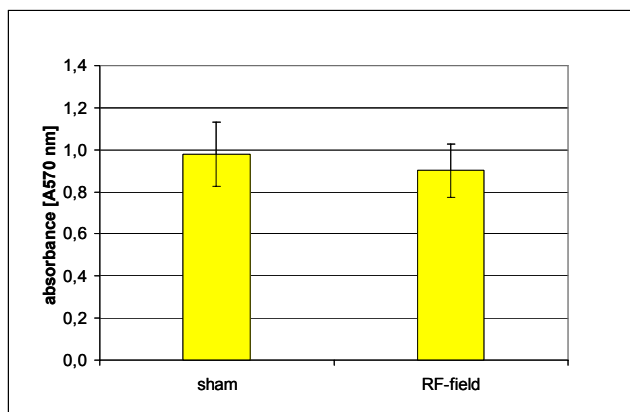


Figure 83. Viability of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) compared to sham exposure. Cell viability was evaluated by the MTT assay and reported as absorbance at 570 nm. Bars represent means \pm SD of 12 independent experiments.

In addition, the ratio of binucleated cells (BNC) against mono-, bi-, tri- and tetranucleated cells (% BNC) was determined as a measure of cell division and cell cycle progression. No effect of RF-EMF exposure on % BNC for different energies (Figure 84) or for different signal modulations (Figure 85) was found in comparison to sham-exposed or ionising-irradiated (0.5 Gy, 6 MeV, exposure time: 5.2s) HL-60 cells.

Additionally, no significant differences in DNA distribution of RF-exposed cells compared to sham-exposed cells were observed with respect to increased incidence of cellular debris as a measure of cytotoxicity (Table 14).

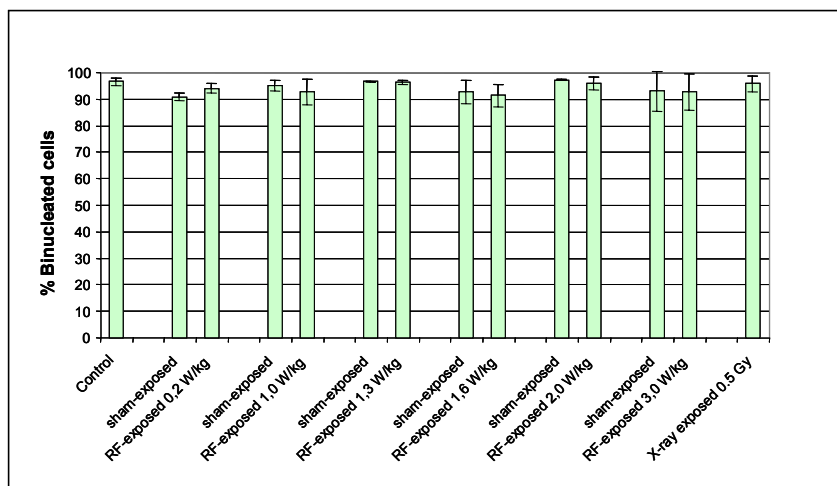


Figure 84. Effect of RF-field exposure on cell division. Shown is the number of binucleated HL-60 cells relative to the number of mono-, bi-, tri-, and tetra-nuclear cells (% BNC) following RF-field exposure (1800 MHz, continuous wave, different SAR levels, 24h). Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Each bar represents the mean \pm SD of results obtained in three independent experiments.

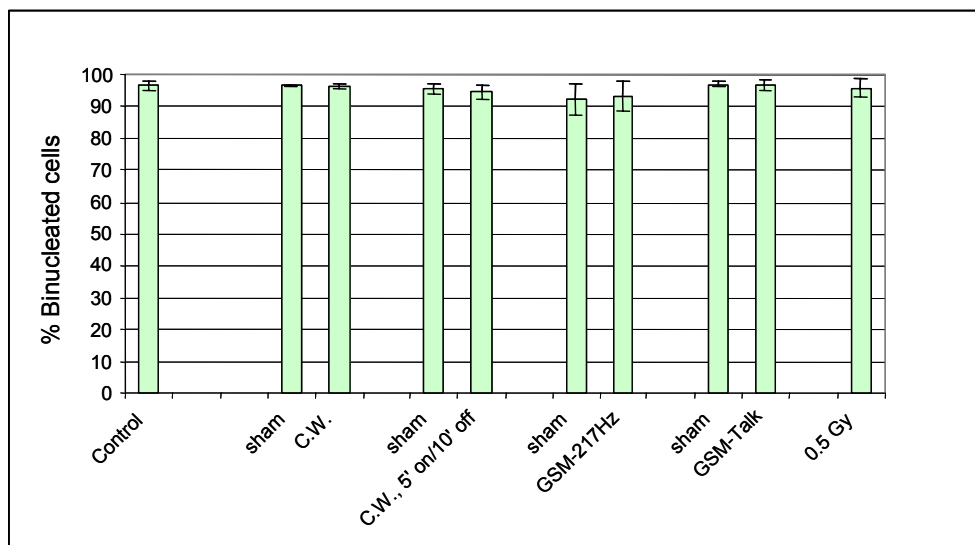


Figure 85. Effect of RF-field exposure on cell division. Shown is the number of binucleated HL-60 cells relative to the number of mono-, bi-, tri-, and tetranuclear cells (% BNC) following RF-field exposure (1800 MHz, SAR 1.3 W/kg, different signal modulations, 24h). Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Each bar represents the mean \pm SD of results obtained in three independent experiments.

Concludingly no in vitro cytotoxic effects of RF-EMF could be detected in RF-EMF-exposed and sham-exposed cells for the exposure conditions tested using either microscopic evaluation (trypan blue exclusion, % BNC), colorimetric MTT assay or flow cytometric analysis (nuclear ethidium bromide staining).

B. Indirect genotoxicity (by reactive oxygen species)

RF-EMF induced formation of reactive oxygen species as shown by flow cytometric detection of oxyDNA and rhodamine fluorescence.

It was the aim of these series of experiments to examine whether RF-EMF (1800 MHz at SAR 1.3 W/kg, 24h exposure) is capable to induce indirect genotoxic effects by affecting the generation and elimination of reactive oxygen species (ROS). For monitoring these ROS-formation and elimination steps, different assays, measuring nitric oxide, oxyDNA, oxidative DNA-damage via Dihydrorhodamine 123 (DHR123), lipid peroxidation, glutathione peroxidase activity, superoxide dismutase activity, have been established and were applied following RF-field exposure of HL-60 cells at that exposure condition with the most significant effect on DNA integrity (1800 MHz, continuous wave, 1.3 W/kg, 24h).

Nitric oxide (NOx)

Nitric oxide (NOx), was measured using the colorimetric Nitric Oxide Assay Kit, Calbiochem, Bad Soden, Germany. The data in Table 15 show the NOx production from HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 0.25×10^5 cells, in neither treatment group the detection limit of 1 $\mu\text{mol NOx/l}$ was exceeded. The results presented are the means of three independent experiments. Concludingly, with this assay no in vitro effect of RF-field exposure on NOx formation was detected for the exposure conditions tested.

Table 15. NO_x formation in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposure.

Group	NO _x [μmol/l]
control	< 1
sham	< 1
RF-field	< 1

0.75 x 10⁶ viable HL-60 cells/3 ml cell culture medium were cultivated for 24h at 37°C. After centrifugation, aliquots of culture media corresponding to 0.25 x 10⁵ cells were collected and analysed for nitric oxide (NO_x) by the colorimetric Nitric Oxide Assay Kit, Calbiochem, Bad Soden, Germany. The results presented are representative for three independent experiments.

Flow cytometric detection of oxidative DNA damage (oxy-DNA)

The presence of oxidised DNA (by a fluorescent probe, directly binding to 8-oxoguanine as the major oxidative DNA product) was indicated by a green/yellow fluorescence that could be detected using a flow cytometry system. In Figure 86 a partial augmentation (occurring as a shoulder on the right side of the signal, see arrow Figure 86 of FL-1 fluorescence intensity), indicating the presence of oxidised DNA, was observed for the RF-exposed signal (green) in contrast to sham-exposed signal (blue). Additionally, RF-exposed cells showed a significant shift to the left as compared to sham-exposed cells. Table 16 shows the data for the quantification of ROS levels using oxyDNA-FITC conjugate to stain 8-oxoGuanosine residues on oxidatively damaged DNA of HL-60 cells. RF-field exposure of HL-60 cells induced a mean increase of oxidative DNA damage of 21.7 ± 2.0 %.

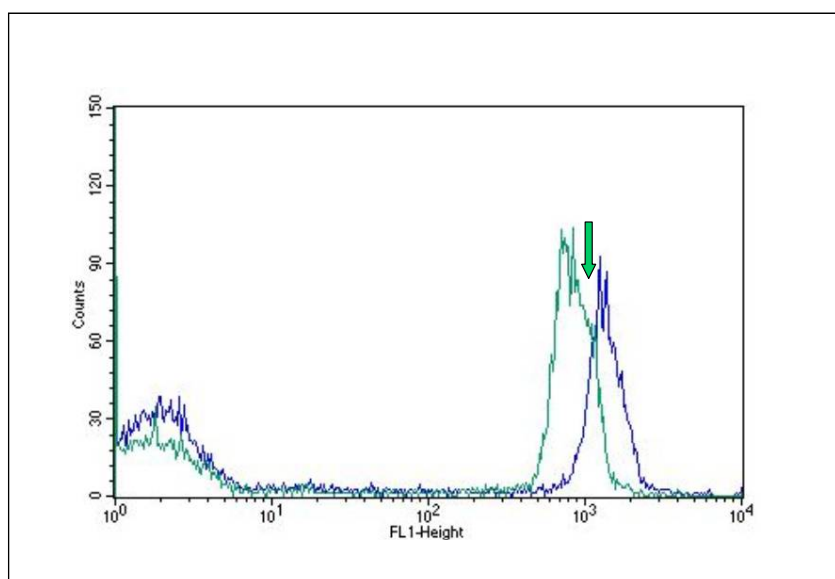


Figure 86. Flow cytometric detection of ROS levels using oxyDNA-FITC conjugate to stain 8-oxoGuo residues on oxidatively damaged DNA of HL-60 cells. The diagram shows the signal of oxidatively damaged DNA of RF-field exposed cells (green line) compared to sham-exposed cells (blue line). One representative histogram plot out of four independent experiments is shown. A partial augmentation (shoulder at the right side of the signal, indicated by arrow) of FL-1 fluorescence intensity was observed for the RF-exposed signal in contrast to sham-exposed signal.

Table 16. Quantification of ROS levels of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h using oxyDNA-FITC conjugate to stain 8-oxoGuo residues on oxidatively damaged DNA.

No. of experiment	% augmentation of fluorescence signal of RF-field exposed cells (area under curve AUC of shoulder at the right side of the signal)
1	21.38
2	24.44
3	19.80
4	20.98
mean \pm SD	21.65 \pm 2.0

0.75 x 10⁶ HL-60 cells/dish were sham- or RF-field exposed for 24 h. oxyDNA-FITC was used to stain 8-oxoGuo residues on oxidatively damaged DNA using the oxyDNA assay from Calbiochem-Novabiochem GmbH, Bad Soden, Germany. Oxidatively damaged DNA was quantified by determination of the area under the curve (AUC) of the shoulder at the right side of the signal fluorescence intensity (see arrow Figure 83) in RF-field exposed cells * Significant difference between the median of fluorescence intensity of RF-field exposed cells and sham-exposed cells at P<0.05 (n=4, Student's t-test, two-sided).

Oxidative DNA damage measured by DHR123 and flow cytometry

Cellular production of ROS was determined by measuring the rhodamine fluorescence of HL-60 cells, incubated in growth medium containing 5 μ mol/l dihydrorhodamine 123 (DHR123) for 24h at 37°C. DHR123 is a non-fluorescent reduced Rhodamine 123 (Rh123) derivative that is freely permeable through cell membranes. Intracellular oxidation converts DHR123 to the fluorescent Rh123, which is retained intracellularly by the mitochondrial potential.

Figure 87 displays the overlay fluorescence histograms for RF-field exposed cells (1800 MHz, continuous wave, SAR 1.3 W/kg), compared to sham-exposed cells after simultaneous incubation with DHR123 for 24h. The figure shows that, in RF-field exposed cells, the fluorescence intensity signal shifts to the right in comparison to the signal of sham-exposed cells. In contrast, treating cells with 100 μ mol/l H₂O₂ resulted in an even more pronounced shift of fluorescence signal. These shifts indicate enhanced fluorescence intensities and thereby increased production of intracellular ROS during RF-field exposure or H₂O₂ treatment of HL-60 cells.

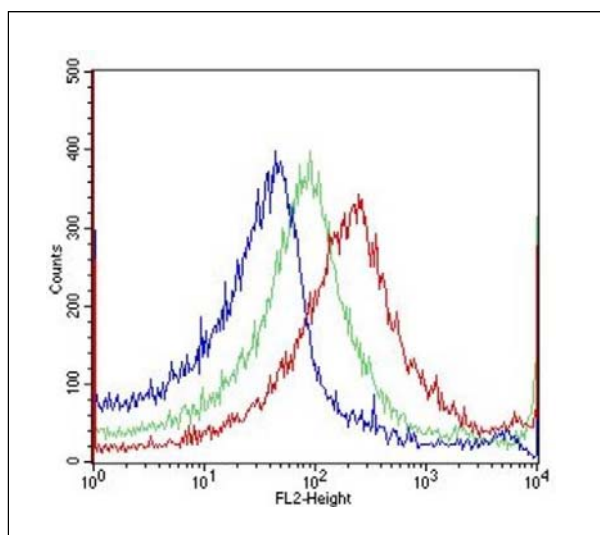


Figure 87. Fluorescence histograms for RF-field exposed (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) and sham-exposed HL-60 cells simultaneous treated with 5 μ mol/l dihydrorhodamine 123 (DHR123). Blue line represents sham-exposed sample, green line represents RF-field exposed sample and red line represents H₂O₂-treated positive control (100 μ mol/l for 1 hour). DHR123 reacts with intracellular ROS to form fluorescent Rh123, which is then retained by the mitochondria, enabling a flow cytometric assessment of cellular oxidant production.

By means of the DHR123 flow cytometry detection assay the shift of the signal can be quantified by determining the medians of the fluorescence intensities and the increase in rhodamine fluorescence for each population compared to sham, expressed in percent (Table 17). The data show that there is no difference in the level of oxidatively damaged DNA for control cells and sham-exposed HL-60 cells, expressed as the median of fluorescence intensity. The median values for control cells were 41.8 ± 5.8 and for sham-exposed cells 39.9 ± 8.5 (n=3). The values for the positive control (H_2O_2 , 100 $\mu\text{mol/l}$, 1h) were 230.6 ± 100.3 . In contrast, exposing cells to RF-fields resulted in a significant increase in median (75.5 ± 19.2 ; $P < 0.05$, n=3). The percentage increase of rhodamine fluorescence for RF-field exposed cells compared to sham-exposed cells is $17.8 \pm 9.7\%$, that of H_2O_2 -treated cells $31.9 \pm 12.4\%$ (Table 17).

Table 17. Detection and quantification of ROS levels with Dihydrorhodamine 123 after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to control, sham-exposed and H_2O_2 -treated HL-60 cells. For quantitative measurement the shift in median and the increase of fluorescence intensity was evaluated.

No. of exp.	Median of fluorescence intensity [units]				% increase of rhodamine 123 fluorescence relative to sham-exposure ^a	
	control	sham-exposed	RF-field exposed	H_2O_2 -treated	RF-field exposed	H_2O_2 -treated
1	39.95	30.51	95.60	149.89	28.98	43.12
2	37.18	42.17	57.26	199.02	12.35	18.53
3	48.26	46.98	73.65	342.89	11.97	33.91
mean	41.8 ± 5.8	39.9 ± 8.5	$75.5 \pm 19.2^*$	230.6 ± 100.3	17.8 ± 9.7	31.9 ± 12.4

^a differences of rhodamine 123 fluorescence (AUC, area under curve) of cells exposed to RF-field or those treated with H_2O_2 was determined and the values were plotted as percentage increase relative to sham-exposure.

* Significant difference between the median of RF-exposed cells to the median of sham-exposed cells and the median of RF-exposed cells to that of control cells at $P < 0.05$ (Student's t-test, two-sided).

Lipid peroxidation

Lipid peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit, Calbiochem, Bad Soden, Germany. Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, were estimated spectrophotometrically at 586 nm after reaction with a chromogenic reagent at 45°C. The absorbance values obtained for the samples were compared with a standard curve of known concentrations of MDA / 4-HNE (1 - 20 $\mu\text{mol/l}$). For MDA and 4-HNE the amounts of lipid peroxidation markers in all experiments and samples were below 1 $\mu\text{mol/l}$. Table 18 shows that there is no difference in the level of lipid peroxidation for RF-field exposed HL-60 cells, compared to control and sham-exposed cells (n=3).

Table 18. Lipid peroxidation (LPO) in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	amount of (MDA + HNE) [$\mu\text{mol/l}$]		
	exp. 1	exp. 2	exp. 3
control	< 1.0	< 1.0	< 1.0
sham	< 1.0	< 1.0	< 1.0
RF-field	< 1.0	< 1.0	< 1.0

Lipid peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit from Calbiochem, Bad Soden, Germany. Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, were estimated spectrophotometrically at 586 nm in an aliquot corresponding to 6×10^5 cells after reaction with a chromogenic reagent at 45°C. The results presented are means of three independent experiments.

Antioxidant enzyme activities

RF-EMF did not affect antioxidant enzyme activities of HL-60 cells (SOD and GPx activity).

To screen the possible effect of RF-EMF on endogenous antioxidant enzyme activity, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined in the HL-60 cells that were exposed to RF-fields (1800 MHz), continuous wave, SAR 1.3 W/kg for 24h. Positive controls as indicated in the assays by the manufacturer were included in the analysis.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem, Bad Soden, Germany. The data in Table 19 show SOD activities in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 4×10^5 cells, in neither treatment group the detection limit of 0.2 U/ml SOD activity was exceeded. The results presented are means of two independent experiments. Concludingly, no in vitro effect of RF-field exposure on SOD activity was detected for the exposure conditions tested.

Table 19. Superoxide dismutase (SOD) activity in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	SOD ₅₂₅ activity [U/ml]	
	exp. 1	exp. 2
control	< 0.2	< 0.2
sham	< 0.2	< 0.2
RF-field	< 0.2	< 0.2

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem, Bad Soden, Germany. The SOD-mediated increase in the rate of autooxidation of the reaction mixture was utilized to yield a chromophore with maximum absorbance at 525 nm. SOD activity was measured in an aliquot corresponding 4×10^5 cells (n=2). Detection limit for SOD activity is 0.2 U/ml.

Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity of cell homogenates was determined using a cellular Glutathione Peroxidase Assay Kit, Calbiochem, Bad Soden, Germany. The data in Table 20 show the GPx activity in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 1×10^6 cells, in neither treatment group the detection limit of 5.6 mU/ml GPx activity was exceeded. The results presented represent two independent experiments. Concludingly, no in vitro effect of RF-field exposure on GPx activity was detected for the exposure conditions tested.

Table 20. Glutathione peroxidase (GPx) activity in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	GPx activity [mU / ml]	
	exp. 1	exp. 2
control	< 5.6	< 5.6
sham	< 5.6	< 5.6
RF-field	< 5.6	< 5.6

Glutathione peroxidase (GPx) activity of cell homogenates was determined in two independent experiments using the cellular Glutathione Peroxidase Assay Kit from Calbiochem, Bad Soden, Germany. Cell homogenisate of 1×10^6 cells is added to a 1050 µl of a solution containing glutathione (GSH, 1mmol/l), GSH reductase (0.4 U/ml) and NADPH. The reaction is initiated by the addition of 350 µl of the diluted organic peroxide t-butyl hydroperoxide and the absorbance at 340 nm was recorded over a period of 5 minutes. The rate of decrease in the absorbance is directly proportional to the GPx activity in the cell homogenisate. Detection limit for GPx activity is 5.6 mU/ml.

Summarising, the endogenous antioxidant enzyme activities of HL-60 cells (SOD and GPx activity) were not altered by RF-field exposure compared to sham-exposure using the conditions of the assays described above. This screening approach revealed, that the analysis of antioxidant enzyme activities does not show enough methodological sensitivity for the amounts of ROS to be generated by RF-field exposure of HL-60 cells.

Indirect genotoxicity by modulation of cellular toxifying and detoxifying capacities

The generation of genotoxic effects through RF-EMF was inhibited by ascorbic acid.

In a further series of experiments it was examined, whether ascorbic acid as a free radical scavenger and inhibitor of reactive oxygen species is capable to inhibit MN induction and DNA damage by co-administration to RF-field-exposure (continuous wave, SAR 1.3 W/kg, 24h). The inhibition of micronuclei induction and Comet formation was measured by use of the cytokinesis-block in vitro Micronucleus assay and the Comet assay. In both tests systems, ascorbic acid effectively reduced the RF-field induction of micronuclei and DNA damage (Figures 85 and 86).

Figure 88 displays the inhibition of MN induction induced by RF-fields (continuous wave, SAR 1.3 W/kg, 24h) and simultaneous treatment of cells with ascorbic acid (AA, 10 μ mol/l) for 24h. MN frequencies for sham-exposed and RF-field exposed cells were 4.1 ± 0.2 and 11.6 ± 1.9 expressed as MN / 1000 BNC. After co-incubation of sham-exposed and RF-exposed HL-60 cells with ascorbic acid (AA, 10 μ mol/l) for 24h the frequencies were 4.3 ± 0.4 and 4.8 ± 1.9 . The MN frequency for the incubator control was 3.4 ± 0.4 . Data show, that ascorbic acid inhibits RF-field associated MN induction significantly ($n=3$, $P<0.05$). The inhibition resulted in an induction by factor 1.08 compared to sham.

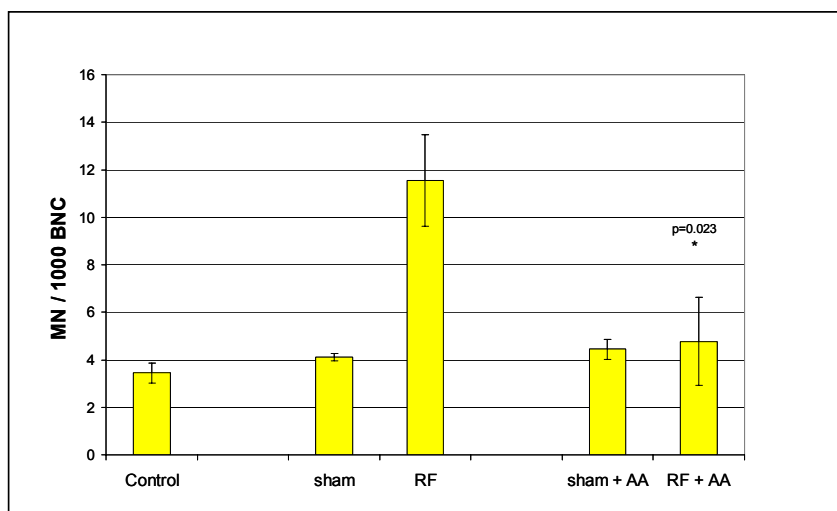


Figure 88. Effect of ascorbic acid (AA, 10 μ mol/l) on RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) induced MN frequencies in HL-60 cells, compared to control and sham-exposed cells. Each bar represents the mean \pm SD of results obtained in at least three independent experiments. Significant differences between RF-field exposure and sham-exposure with co-administration of AA is given by * $P<0.05$ (Student's t-test, two-sided).

Figure 89 displays the inhibition of DNA damage induced by RF-fields (continuous wave, SAR 1.3 W/kg, 24h) and simultaneous treatment of cells with ascorbic acid (AA, 10 μ mol/l) for 24h. The values of Olive Tail Moment for sham-exposed and RF-field exposed cells were 0.9 ± 0.1 and 2.0 ± 0.2 . After co-incubation of sham-exposed and RF-field exposed HL-60 cells with ascorbic acid (AA, 10 μ mol/l) for 24h the values were 1.0 ± 0.1 and 1.2 ± 0.03 . The OTM for the incubator control was 0.8 ± 0.05 . Data show, that ascorbic acid inhibits the RF-field induced DNA damage significantly ($n=3$, $P<0.01$). The inhibition resulted in an induction by factor 1.2 compared to sham. Additionally, no induction of cytotoxicity (trypan blue test), no alteration of cell medium pH value and no influence on cell growth or cell cycle progression was observed for ascorbic acid alone and for co-administration of ascorbic acid together with RF-field exposure over 24h.

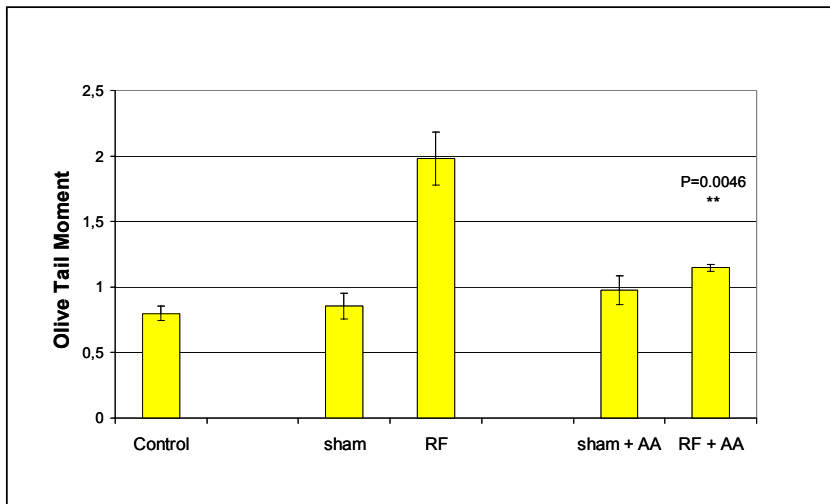


Figure 89. Effect of ascorbic acid (AA, 10 μ mol/l) on RF-field induced Comet formation in HL-60 cells (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to control and sham-exposed cells. Each bar represents the mean \pm SD of results obtained in at least three independent experiments. Significant differences between RF-field exposure and sham-exposure with co-administration of AA is given by ** $P < 0.01$ (Student's t-test, two-sided).

Concludingly, this observed inhibition of genotoxicity by ascorbic acid supports the hypothesis that the effect of RF-field on genomic integrity may be explained by the generation of free oxygen radicals.

3.2.1.2 Human fibroblasts and granulosa cells of rats (Participant 3)

RF-EMF generated DNA strand breaks in human fibroblasts and in granulosa cells of rats.

The influence of RF-EMF exposure on the generation of DNA strand breaks in cells of two different tissues (human fibroblasts, rat granulosa cells) was evaluated using alkaline and neutral Comet assay. Four different sets of exposure conditions were tested: continuous (1800 MHz, 2 W/kg), intermittent (5 min on/10 min off, 1800 MHz, 2 W/kg), pulse modulation (1800 MHz, 2 W/kg, amplitude 217 Hz, 5 min on/10 min off) and talk modulation (1800 MHz, 1.2 W/kg, DTX 66%, GSM basic 34%, continuous). Different exposure duration was applied (4, 16 and 24 hours).

An elevation of Comet assay levels in exposed cells compared to sham-exposed controls could be detected in each of these experiments, even at continuous exposure (Figure 90). This elevation became significant at 16 hours of exposure, but no significant differences between 16 and 24 hours could be detected. At intermittent, pulse modulation and talk modulation Comet assay levels were significantly higher than at continuous exposure. Human fibroblasts and granulosa cells responded equally to RF-EMF, albeit the latter exhibited higher basal and higher end levels (Figure 91). The Comet factors with neutral Comet assay were similar, albeit lower (Figures 92, 93). Dose response investigations with human fibroblasts, which were exposed intermittently (5 min on/10 min off) for 24 hours, revealed a dose dependent increase of the Comet tailfactor beginning already at a SAR of 0.3 W/kg with a peak level at 1.0 W/kg (Figure 94).

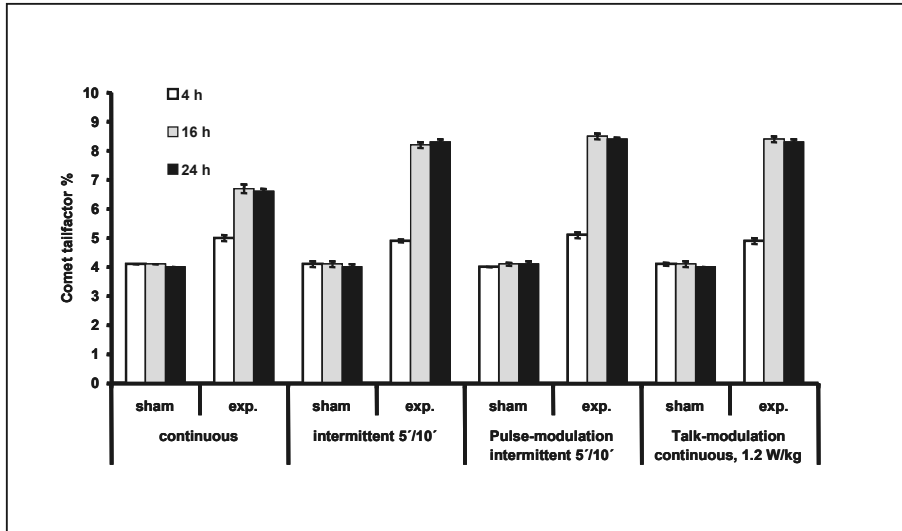


Figure 90. Influence of exposure time and of different exposure conditions on formation of DNA single and double strand breaks in human fibroblasts determined with Comet assay under alkaline conditions (cell strain ES 1, 1800 MHz, SAR 2 W/kg).

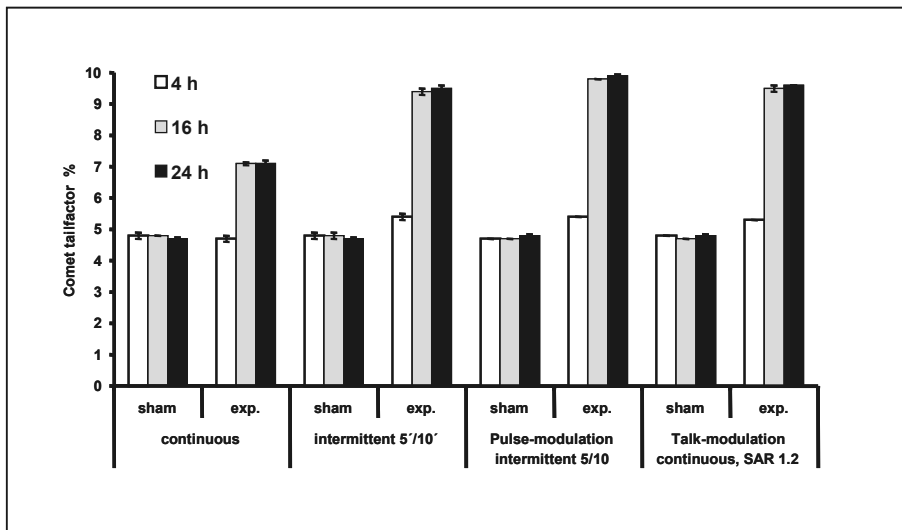


Figure 91. Influence of exposure time and of different exposure conditions on formation of DNA single and double strand breaks in granulosa cells determined with Comet assay under alkaline conditions.

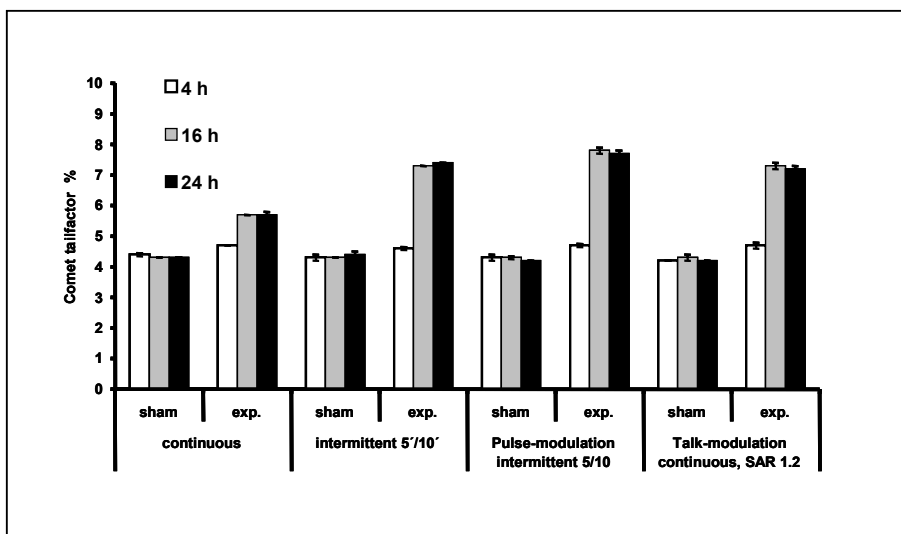


Figure 92. Influence of exposure time and of different exposure conditions on formation of DNA double strand breaks in human fibroblasts determined with Comet assay under neutral conditions (cell strain ES 1, 1800 MHz, SAR 2 W/kg).

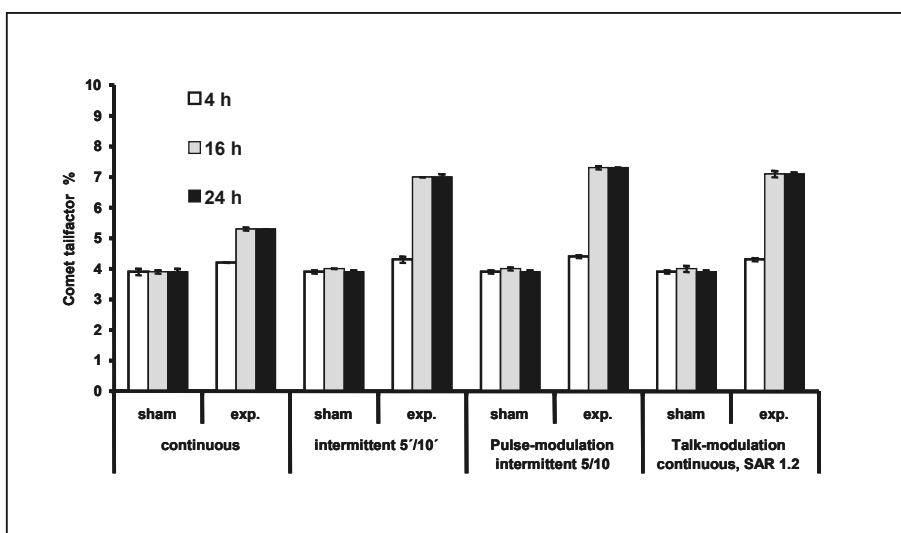


Figure 93. Influence of exposure time and of different exposure conditions on formation of DNA double strand breaks in granulosa cells determined with Comet assay under neutral conditions (1800 MHz, SAR 2 W/kg).

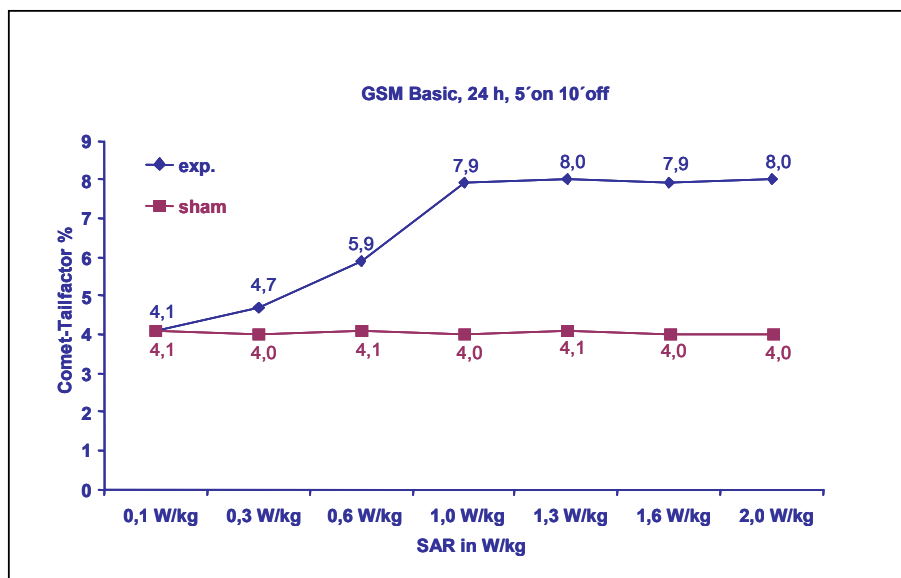


Figure 94. Dose dependent increase of DNA single and double strand breaks in human fibroblasts determined with the Comet assay under alkaline conditions

RF-EMF generated chromosomal aberrations in human fibroblasts.

Since we could demonstrate an induction of DNA strand breaks in human fibroblasts after RF-EMF exposure and due to the results of chromosome aberrations upon ELF-EMF exposure, we also evaluated the chromosome aberrations after RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15 hours). These preliminary experiments (500 metaphases scored) revealed in RF-EMF exposed fibroblasts a 10-fold increase in chromosome gaps, a 4-fold increase in chromosome breaks and very high incidences of dicentrics and acentric fragments (Table 21).

Table 21. Percentage of chromosomal aberrations induced by RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15h) in cultured human fibroblasts^a

types of aberration	RF-exposed (% ± SD)	sham-exposed (% ± SD)	p-value ^b
chromosome gaps	57.5 ± 2.1 %	4.8 ± 1.6 %	< 0.001
chromosome breaks	8.1 ± 0.7 %	1.7 ± 0.1 %	< 0.001
dicentric chromosomes	4.5 ± 0.7 %	---	
acentric fragments	1.5 ± 0.7 %	---	

^a a number of 1,000 metaphases were scored in each of five independent experiments. Results are expressed as percentage chromosomal aberrations per cell.

^b Significant differences (p<0.05) as compared to sham-exposed controls using Student's t-test for independent samples

RF-EMF induced micronuclei in human fibroblasts.

Cultured human fibroblasts were exposed to RF-EMF (GSM basic 1950 MHz, 15h, 2 W/kg) and micronuclei frequencies were evaluated. These results showed an induction of micronuclei in RF-EMF exposed fibroblasts (Figure 95a). The observed increase in micronucleus frequencies was about 20-fold compared to sham exposed cells or non-exposed controls, but not as high as in bleomycin (10 µg/ml, 17h) treated cells, which were used as positive controls.

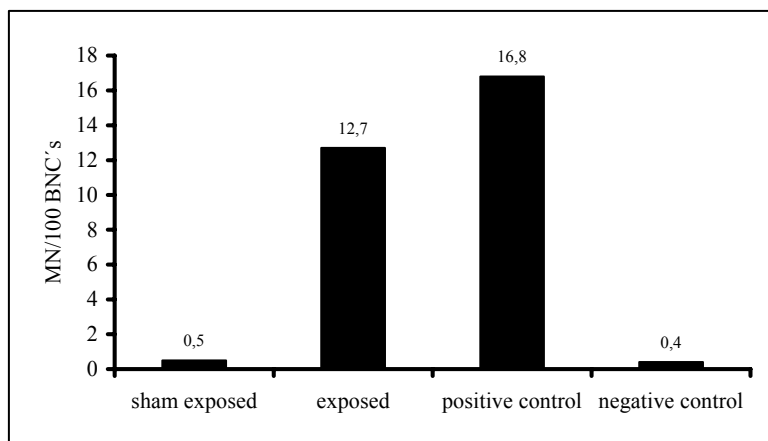


Figure 95. Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cell were used as a positive control.

Results on the influence of RF-EMF on the mitochondrial membrane potential were inconsistent.

Evaluating changes in the mitochondrial membrane potential after RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15h) using JC-1, revealed a significant decrease in the mitochondrial membrane potential in one experiment, which could not be reproduced..

3.2.1.3 Mouse embryonic stem cells (Participant 4)

RF-EMF affected double-strand DNA break induction in ES cell derived neural progenitors immediately after exposure.

We studied the possible effects of RF-EMF on the integrity of DNA strands in differentiating ES cell from EB outgrowths. Two schemes were applied: (1) For RF-EMF exposure (GSM signal 217-Hz, 1.71 GHz, 1.5 W/kg, intermittency 5 min on/30 min off, 6h), the percentage of primary DNA damage was measured in the alkaline and neutral Comet assay immediately after the RF-EMF exposure at the stage of neural differentiation (4+4d - 4+6d) and 18 hours after the RF-EMF exposure. No differences in the induction of single-strand breaks as measured by the alkaline Comet assay were observed 0 and 18 hours after exposure. The tailfactor was slightly, but significantly increased in the neutral Comet assay immediately after exposure ($p < 0.05$) (Table 26). In the second set of experiments, the same RF-EMF exposure conditions were applied for 48 hours instead of 6 hours, and the alkaline Comet assay was done immediately after exposure, while the neutral Comet assay was done 24 or 48 hours post exposure. However, no significant differences were observed in the induction of single- or-double DNA strand breaks between sham-exposed or EMF exposed neural progenitors after prolonged exposure (48h).

3.2.1.4 Summary (Participant 1)

Our data indicate a genotoxic action of RF-EMF in various cell systems. This conclusion is based on the following findings:

- RF-EMF exposure was able to induce DNA single and double strand breaks as well as an increase in micronuclei in HL-60 cells (3.2.1.1).
- The DNA damage generated by RF-EMF in HL-60 cells was dependent on the time of exposure, the field strength and the type of RF-EMF signals (3.2.1.1).
- The DNA damage in HL-60 cells probably resulted from an increase in free oxygen radicals induced during RF-EMF exposure (3.2.1.1).
- RF-EMF exposure at a SAR value between 0,3 and 2,0 W/kg produced DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals (3.2.1.2).

- RF-EMF exposure at a SAR value of 2 W/kg caused an increase in chromosomal aberrations in human fibroblasts demonstrating that the DNA repair was not error-free (3.2.1.2).
- RF-EMF exposure at a SAR value of 1,5 W/kg caused a slight, but significant increase in DNA double strand breaks in neural progenitor cells stemming from mouse embryonic stem cells (3.2.1.3).

3.2.2 Cell proliferation and cell differentiation

3.2.2.1 Human neuroblastoma cell line NB69 and neural stem cells (NSC) (Participant 5)

RF-EMF did not affect growth or viability of NB69 neuroblastoma cells and neural stem cells (NSC).

NB69 cells were exposed at day 3 postplating to GSM-Basic over a 24-hour period. After exposure, the cells were left to grow in the absence of field for an additional 24 hour lapse (5 days postplating). The field exposure was applied alone or in combination with bFGF. As it is shown in Figure 96, the GSM-Basic signal alone did not affect significantly cell growth or cell viability. The morphological analysis did not show significant differences between exposed and control groups, either (data not shown). The treatment with bFGF alone induces differentiation in NB69 cells, which exhibit significant increases in the number and the extension of processes per cell and in the cell size (see 3.2.4.2). An equivalent response was obtained after exposure to the combined treatment with bFGF plus GSM-Basic.

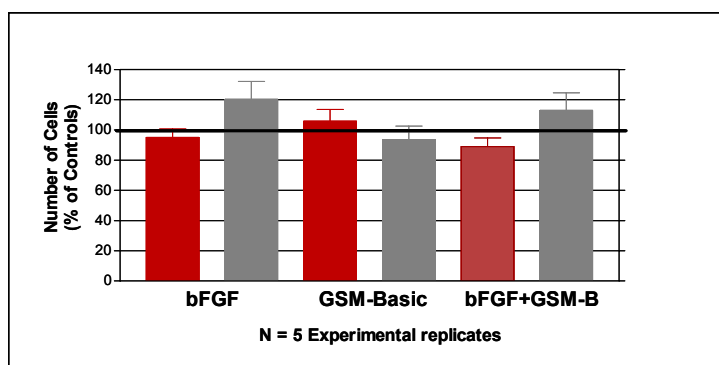


Figure 96. Number of living (red) and dead (grey) cells after a 24-h exposure to GSM-Basic signal followed by 24 additional hours of incubation in the absence of the RF-EMF. No significant changes were observed in the exposed samples when compared to the respective controls

NS cells were exposed at day 2 postplating to GSM-Basic over a 24-hour period. After exposure, the cells were left to grow in the absence of field for an additional 48-hour lapse (5 days postplating). At the end of this period, the samples were studied for cell growth and/or cell viability. The treatment did not affect cell growth (Figure 97A) and did not induce significant changes in the cells' morphology (data not shown). However, the cell size in the exposed samples was observed to be slightly, but not significantly augmented when compared to the respective controls (Figure 97B; image analysis of 60 microscope fields per condition of a total of 4 experimental replicates). The observed, slight reductions in the percent of dead cells, and in the increase of the cells' size after exposure to the GSM-Basic signal (Figure 97) could be due to an enhancement of the cell attachment to the substrate in the exposed samples. Such an attachment was microscope observed, though not image-analysis quantified (data not shown).

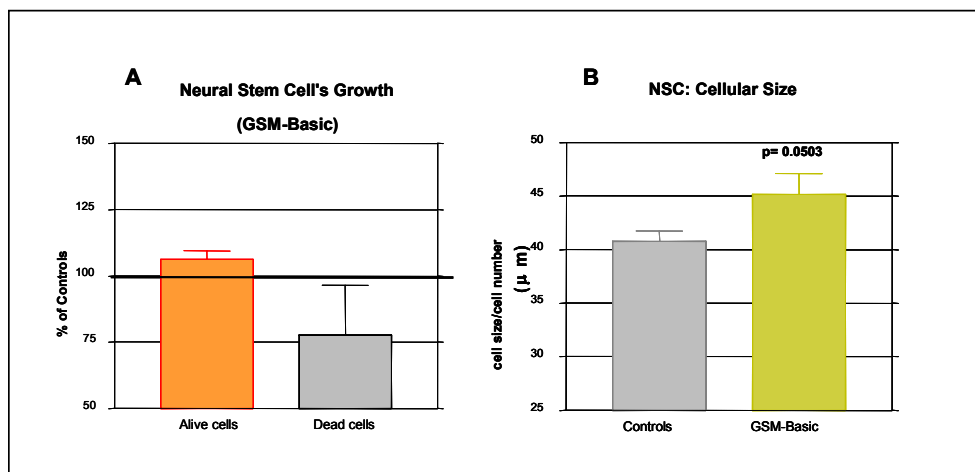


Figure 97. (A) Number of living and dead cells after a 21-h exposure to GSM-Basic signal followed by 48 additional hours of incubation in the absence of RF-EMF (5 days postplating). No significant changes were observed, even though a small reduction of dead cells occurs in the exposed samples. (B) The cell size in the exposed samples was observed to be slightly, but not significantly augmented when compared to the respective controls.

RF-EMF may affect the expression of FGF receptors in NB69 human neuroblastoma cells and in neural stem, potentially influencing cellular differentiation.

See 3.2.4.2

RF-EMF affected the differentiation of neural stem cells (NSC), but not of neuroblastoma cells (NB69).

The aim of the study was to determine whether the exposure of neural stem cells (NSC) to GSM-1800 signals (GSM-Basic signal, 21-hours, 5 min on/10 min off) can influence the evolution of the phenotypic differentiation at the middle term (6 additional days after exposure). As described in the methodology, the cells were exposed to RF-EMF at day 2 postplating. After the 21-hour exposure, the cells were grown for 6 additional days in the absence of the GSM stimulus. At the end of this period, the cells were immunostained with O1 for mature oligodendrocyte identification, GFAP for astrocytes and β -tubulin III for neurons. All experiments and analysis were conducted following blind protocols. The data (N= 5 experimental replicates) indicate that the GSM-Basic signal promotes marked morphological changes in differentiating oligodendrocytes and astrocytes derived from NSC (Figure 98).

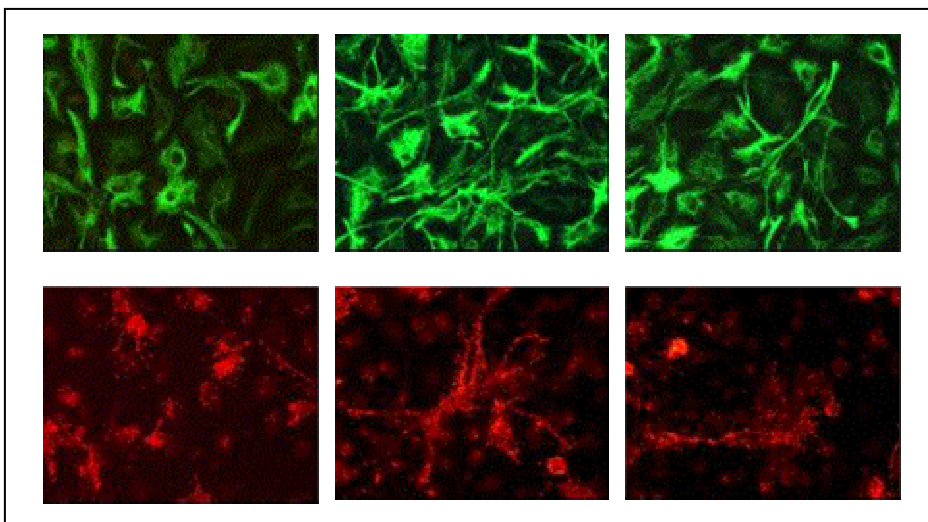


Figure 98. Photomicrographs of neural stem cells progeny. Astrocytes labelled with GFAP (green) and oligodendrocytes labelled with O1 (red) immunostaining: A, Control; B, and C, exposed to the GSM-Basic signal. The exposure increases cell extension in astrocytes and oligodendrocytes.

Similar experiments have been carried out to evaluate the phenotypic differentiation of NB69 cells treated with the above GSM-signal and exposure conditions, in the presence or absence of RA. As described in the methodology, the cells were exposed to the field at day 3 postplating, during a 24-hour period, and then grown for 2 additional days in the absence of GSM exposure. After that, the cells were analysed for expression of mature neuronal cell marker β -tubulin III, and for tyrosine hydroxylase (TH) marker. Exogenous, basic fibroblast growth factor was used as a positive control. The data show that the neuronal outgrowth of NB69 cells (Figure 99) and the percent of TH⁺ cells seem not to be altered by the exposure to the GSM-Basic signal. Only the treatment with bFGF promoted the neuronal microtubules network in these cells.

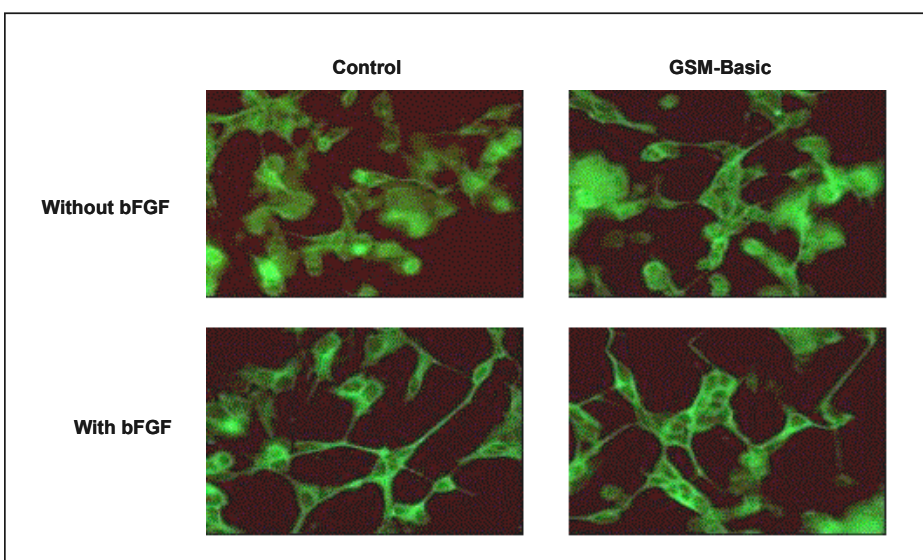


Figure 99. Photomicrographs of NB69 cells analysed 3 days after the exposure and/or incubation in the presence or absence of bFGF. Cells stained with anti-beta-tubulin antibody. The neuronal outgrowth of NB69 cells seems not to be altered by the exposure to the GSM-Basic signal. However, the treatment with bFGF promotes the neuronal microtubules network in these cells.

3.2.2.2 Human lymphocytes and thymocytes (Participant 8)

RF-EMF did not affect proliferation, cell cycle and activation of human lymphocytes.

The experiments with RF-EMF were performed at SAR 1,4-2,0 W/kg using different RF modulations or time exposure. Studies on cell proliferation were performed discriminating CD4+CD28+/- and CD8+CD28+/-lymphocytes subpopulation. Cells were exposed to Talk modulated RF-EMF (2 W/kg) and two intermittent types of exposure were applied: 1) 10 min on/20 min off for 44 hours; 2) 2 hours on/22 hours off for 72 hours. We performed experiments with cells from 6 donors using the former approach, from 11 donors using the latter approach. All cells were acquired and analysed after 72 hours and 120 hours of culture. A small increase (3%) of proliferating CD8+CD28+ T lymphocytes was observed in exposed cells both at 72 hours and 120 hours of culture. Since the differences observed are similar to the calculated standard error, we considered this effects not relevant. In Tables 22 and 23 data related to proliferating and not proliferating cells subsets after 72 hours and 120 hours of culture, respectively, are reported.

Table 22. T lymphocytes subsets exposed to talk modulated RF-EMF 2 hours on/22hours off for 72 hours. Data are reported as mean (% \pm s.e.) of all experiments performed

Lymphocyte subsets	Sham % \pm se	RF % \pm se	p
P CD4+CD28+	30.7 \pm 2	28.2 \pm 2	ns
P CD4+CD28-	0.9 \pm 0.4	0.7 \pm 0.3	ns
NP CD4+CD28+	13.0 \pm 2	12.4 \pm 2	ns
NP CD4+CD28-	1.8 \pm 0.9	0.8 \pm 0.3	ns
P CD8+CD28+	19 \pm 2	21 \pm 2	0.042
P CD8+CD28-	7.9 \pm 3	7.7 \pm 3	ns
NP CD8+CD28+	8.0 \pm 1	9.3 \pm 1	ns
NP CD8+CD28-	1.1 \pm 0.2	1.0 \pm 0.4	ns

P = proliferating; NP = non proliferating; ns = not significant

Table 23. T lymphocytes subsets exposed to Talk modulated RF-EMF 2 hours on/22 hours off for 72 hours and analysed at 120 hours of culture. Data are reported as mean (% \pm s.e.) of all experiments performed

Lymphocyte subsets	Sham % \pm se	RF % \pm se	p
P CD4+CD28+	61.0 \pm 2	62.1 \pm 2	ns
P CD4+CD28-	0.60 \pm 0.07	0.80 \pm 0.11	ns
NP CD4+CD28+	9.3 \pm 0.2	8.7 \pm 0.2	ns
NP CD4+CD28-	1.4 \pm 0.1	1.72 \pm 0.11	ns
P CD8+CD28+	32 \pm 4	35 \pm 4	0.048
P CD8+CD28-	5.4 \pm 3	4.3 \pm 2	ns
NP CD8+CD28+	5.4 \pm 0.7	4.7 \pm 0.9	ns
NP CD8+CD28-	0.9 \pm 0.3	0.8 \pm 0.3	ns

P = proliferating; NP = non proliferating; ns = not significant

Cell cycle analysis was performed in PBMCs exposed at three RF-EMF modulations and in the case of Talk signal also PBMCs from old donors were analysed. In all the cases observed no differences were found between exposure and control cells. Some slight differences (1-2%, sometimes increase and sometimes decrease) were observed when analysis of activation markers on CD4+ and CD8+ T lymphocytes were performed on both young and old donors. Since the effects were really small, we

performed from 5 up to 8 replications of T lymphocyte phenotypical analysis, using cells from the same donor.

Results obtained from replications did not confirm the data previously obtained, thus suggesting that such small significant effects must be considered at the noise level of the statistical analysis. Moreover, we performed a more sophisticated analysis on fluorescence intensity in order to verify if the number of molecular markers could be changed in RF exposed cells in comparison with sham exposed cells. We found that in T helper lymphocytes from elderly, but not from young donors, and exposed to Talk modulated RF-EMF, CD95 molecules shifted significantly their fluorescence from bright to dim, as reported in Figure 96. This effect means that in the exposed cells the number of molecular markers on membrane surface was slightly decreased (around 9%).

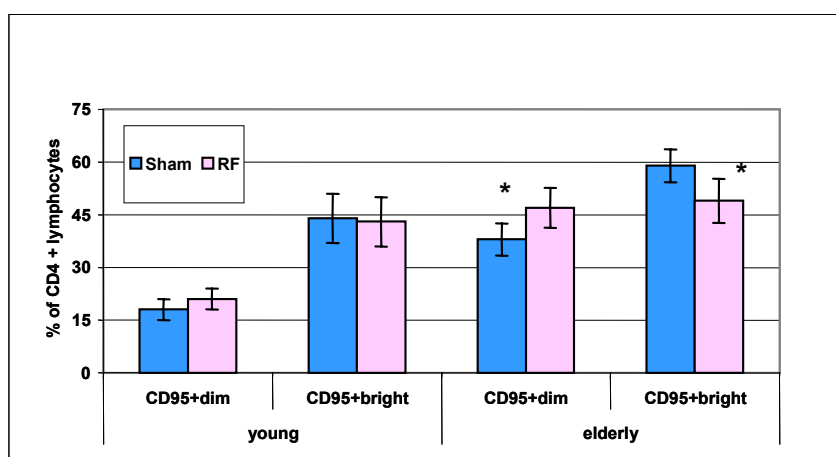


Figure 100. Analysis of fluorescence brightness of proliferating CD4+CD95+ from 10 young and 8 elderly donors, after sham and talk modulated RF-EMF exposure (2W/kg). * = $p < 0.05$. Data are represented as mean \pm s.e.

RF-EMF (DTX) may inhibit the production of IL-1beta in human lymphocytes, but did not affect the production of IL 6.

The results obtained showed no significant differences between cells sham-exposed or differently modulated RF-EMF exposed, except in the case of IL-1beta. Indeed, we found a decrease of IL-1beta production (around 13%) in CD3-stimulated PBMCs exposed to DTX modulated RF-EMF in comparison with sham-exposed cells. As demonstrated in Figure 101, the decrease observed was statistically significant on 6 experiments performed. This result was not found when PBMCs were exposed to Talk modulated RF-EMF or using the other stimulus.

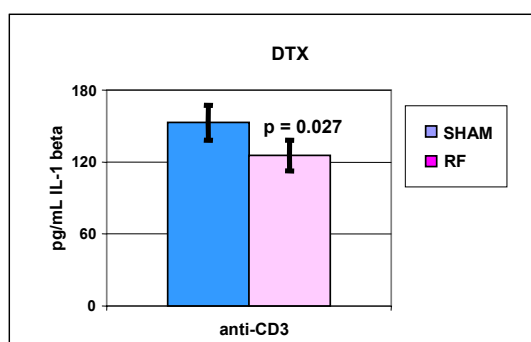


Figure 101. Effect of DTX modulated RF (SAR 1.4 W/kg) on IL-1 beta production in CD3-stimulated PBMCs.

RF-EMF did not affect thymocyte differentiation.

HTOC were performed in order to assess *in vitro* phenotypical differentiation and apoptotic phenomena due to negative selection, which usually occurs *in vivo* inside the thymus. Thus, different subsets of thymocytes were analysed such as CD71+CD4-CD8-, CD3-/CD4+CD8+, abTCR-/CD4+CD8+, gdTCR-/CD4-CD8-, CD3+CD4+CD8-, CD3+CD4CD8+, abTCR+CD4+CD8-, abTCR+ CD4-CD8+ cells. Each population represents a different phase of development, which was monitored before the exposure and at the end of culture, in presence or absence of RF-EMF. Thymocyte apoptosis was assessed with two different methods, in the same conditions, but the data obtained from 6 human thymus on thymocyte differentiation and apoptosis did not suggest positive results on both the endpoints. Actually, a small increase (4%) of double positive thymocytes (CD4+CD8+) was found in RF-EMF-exposed cultures in comparison with sham-exposed tissue fragments. Also in this case the effect is of the order of the standard error thus we consider these results irrelevant.

3.2.2.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF did not affect the cell cycle of HL-60 cells as shown by flow cytometric analysis.

See 3.2.1.1

RF-EMF did not affect the growth behaviour of HL-60 cells with respect to growth velocity and DNA synthesis.

See 3.2.1.1

Indicators for HL-60 cell growth were the proliferation rate, reflected by the cellular doubling time, and the synthesis of the enzyme thymidine kinase (TK). The enzyme thymidine kinase plays an important role in DNA synthesis. It has been well established that the cellular activity of thymidine kinase is correlated with the growth rate of cells (Johnson et al. 1982). Its relation to the cell cycle has been shown in previous studies (Chang 1990; Kit 1976; Pelka-Fleischer et al. 1987; Piper et al. 1980).

Cellular doubling time

Cellular growth behaviour with respect to growth velocity was assessed by determination of the cellular doubling time. Cellular doubling time of HL-60 cells following RF-field exposure (1800 MHz, 24h) for different SARs (continuous wave, 0.2, 1.0, 1.3, 1.6, 2.0 and 3.0 W/kg) and different signal modulations (continuous wave, C.W., 5 min on/10 min off, GSM-217Hz, GSM-Talk) was compared to controls and sham-exposure. No alteration of the cellular doubling time was observed for any of the different SARs or signal modulations tested (Figures 101, 102). The value of the doubling time for the control was 20.8 ± 2.8 h. Calculation of the average HL-60 doubling time after exposure to all SAR levels tested revealed a value of 22.0 ± 3.8 h (n=21) versus that for all sham-exposed cells: 21.6 ± 4.5 hours (n=21). On the other hand the calculation of the average HL-60 doubling time after exposure to all signal modulations tested at SAR = 1.3 W/kg revealed a value of 21.3 ± 4.2 h (n=14) versus that for all sham-exposed cells: 21.7 ± 4.5 h (n=14).

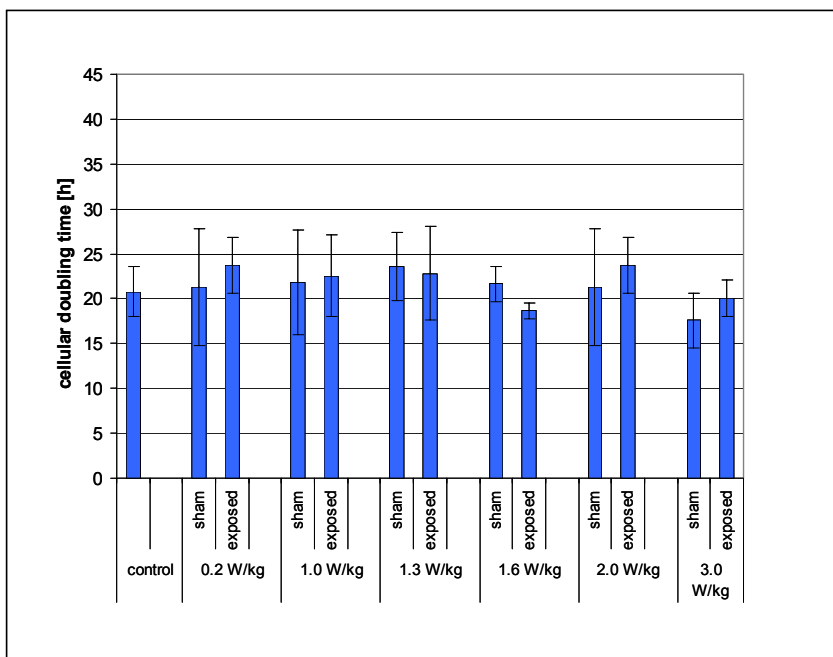


Figure 102. Effect of RF-field exposure (1800 MHz, continuous wave, SAR 0.2, 1.0, 1.3, 1.6, 2.0 and 3.0 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean \pm SD of results obtained in at least three independent experiments, except for control (n=6) and SAR 1.3 W/kg (n=6).

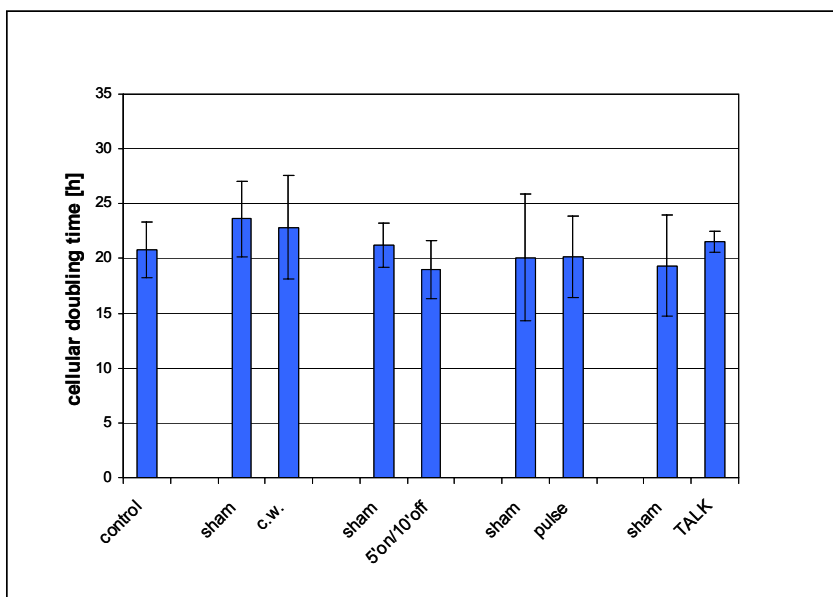


Figure 103. Effect of RF-field exposure (1800 MHz, different signal modulations, SAR 1.3 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean \pm SD of results obtained in at least three independent experiments, except for control (n=6), continuous wave (n=6) and GSM-Talk (n=2).

Thymidine kinase (TK) activity

Intracellular thymidine kinase (TK) activities were determined by radioenzyme assay with ¹²⁵I-deoxyuridine monophosphate as substrate (Prolifigen® TK-REA, AB Sangtec Medical, Bromma, Sweden). The level of radioactivity is directly proportional to the enzyme activity, the TK value is calculated from the standard curve and expressed as U/l.

TK activities of HL-60 cells following RF-field exposure (1800 MHz, continuous wave, 1.3 W/kg, 24 h) was compared to control and sham-exposure. Table 24 represent levels of thymidine kinase activities for two independent experiments. No changes in intracellular TK activities were found in HL-60 cells following RF-field exposure compared to control and sham-exposure. In summary, the growth behaviour of HL-60 cells with respect to growth velocity and DNA synthesis are not altered by RF-EMF exposure compared to control and sham-exposure using the assays described above.

Table 24. Thymidine kinase (TK) activity in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, 1.3 W/kg, 24h), compared to control and sham-exposed cells

Group	thymidine kinase activity [U/l]	
	exp. 1	exp. 2
control	121.4	126.8
sham	151.1	116.3
RF-field	121.9	118.4

3.2.2.4 Mouse embryonic stem cells (Participant 4)

RF-EMF did not induce cardiac differentiation of R1 ES cells and cardiac differentiation and proliferation of P19 EC cells, but may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

See 3.2.4.1

3.2.2.5 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) (see 3.2.2.1), R1-embryonic stem cells and embryonic cancer cells (P19) (3.2.2.4 and 3.2.4.1), human lymphocytes and human thymocytes (3.2.2.2) and HL-60 cells (3.2.2.3), though some effects on the differentiation process in neural stem cells were observed (see 3.2.2.1). These effects may be of indirect nature possibly through modulation of the expression of various genes and proteins. With respect to neural stem cells, RF-EMF may affect proliferation and differentiation via up-regulation of bcl-2 which mediates the apoptotic pathway and via inhibiting nurr-1 and TH transcription (see 3.2.2.4 and 3.2.4.1)

3.2.3 Apoptosis

3.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

RF-EMF did not affect apoptosis in neuronal cells.

Spontaneous apoptosis was found higher in sensitive primary nerve cells than in the human neuroblastoma cell line SH-SY5Y) (around 20% versus 10% using the DiOC₆ dye). A high percentage of spontaneous apoptosis in granule cells was found using Annexin V staining compared to DiOC₆ staining. This observed difference seems to be cell type-dependent and it appeared difficult, in this case, to

correlate information given by these two dyes. Besides this technical consideration, exposure of primary granule cells to GSM-900 at 2.0 W/kg for one hour did not induce apoptosis as shown by the time-kinetics up to 24 hours after exposure (Figure 104). The same observation was made with the SH-SY5Y cell line. To test longer time exposure, we decided to expose granule cells and SH-SY5Y to GSM-900 during 24 hours and to quantify apoptosis at the end of exposure. In these exposure conditions, no significant difference was observed between sham and exposed cells in both cases (Figure 105).

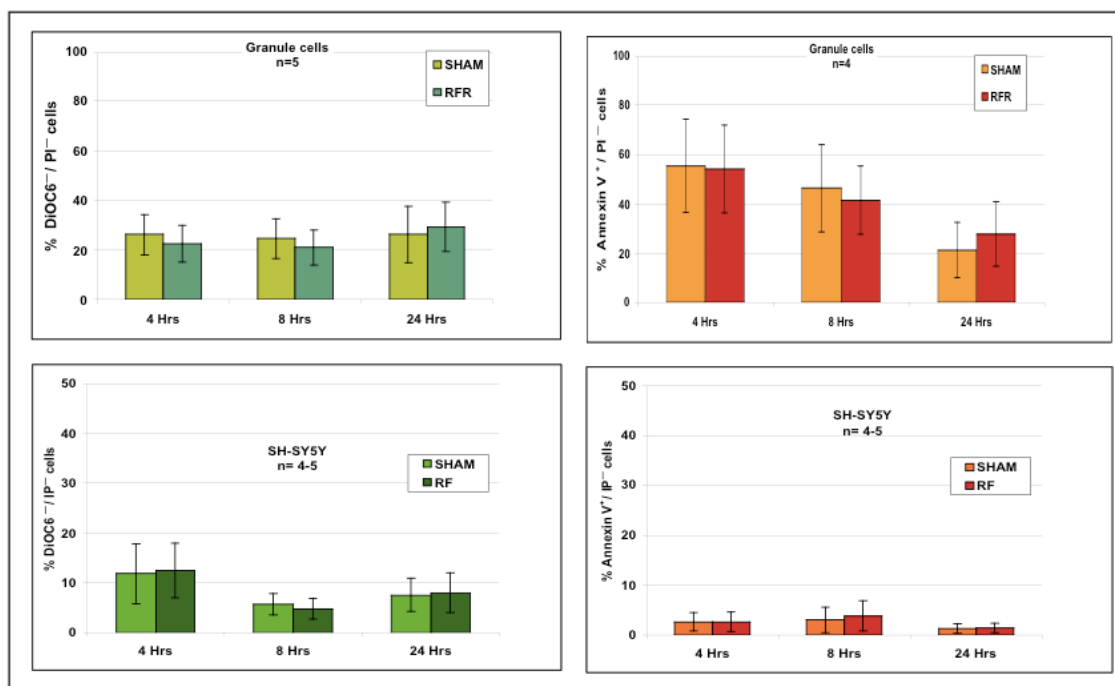


Figure 104. Effect of a one-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential (DiOC6⁺/PI⁻, left panel) and apoptotic cells (ANX⁺/PI⁻, right panel) after exposure of primary granule cells (upper panels) and human neuronal cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data are presented as the mean \pm SEM of 4 to 5 independent and blind experiments.

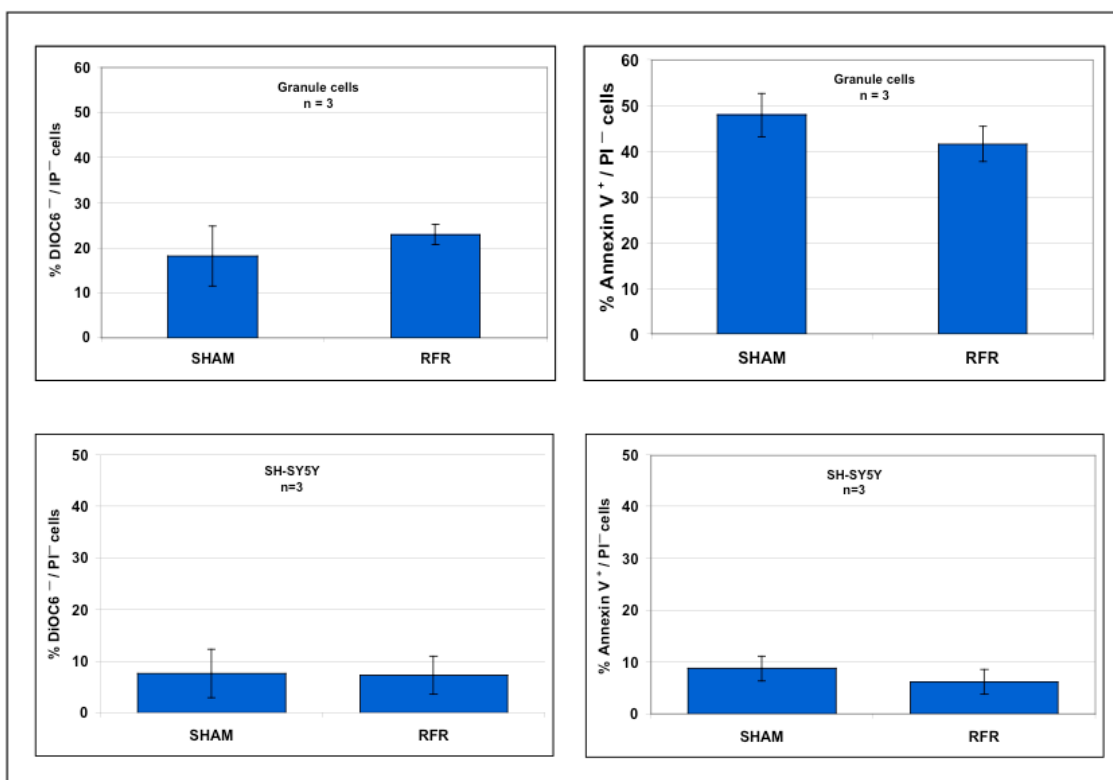


Figure 105. Effect of a 24-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential (DiOC6/PI⁻, left panel) and apoptotic cells (ANX⁺/PI⁻, right panel) after exposure of rat primary neurons (upper panels) and human SH-SY5Y neuroblastoma cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 24 hours. Apoptosis was measured immediately after exposure. Mean \pm SEM of 3 independent, blinded experiments are presented.

We conclude from our results that granule cells and SH-SY5Y cells are not sensitive to GSM-900 exposure for up to 24 hours.

RF-EMF did not affect apoptosis in astrocytic cells.

Primary cultures of astrocytes and human U87 glioblastoma cells were sham-exposed or exposed to GSM-900 for one hour at 2.0 W/kg and apoptosis was followed up during 24 hours in the conditions described previously. Figure 105 shows the data obtained using the primary culture and the U87 cell line. In the two cell types, no difference in the number of cells with depolarised mitochondrial potential or in AnnexinV-positive cells could be evidenced after exposure to GSM-900. An increase in apoptotic astrocytic cell population measured with DiOC₆ was noticed, correlated to the time spent in culture. Nevertheless, no significant difference was observed between sham- and GSM-900 exposed cells. In the exposure conditions tested, no demonstration of a significant effect of GSM-900 signal on primary astrocytes or glioblastoma U87 cells could be made.

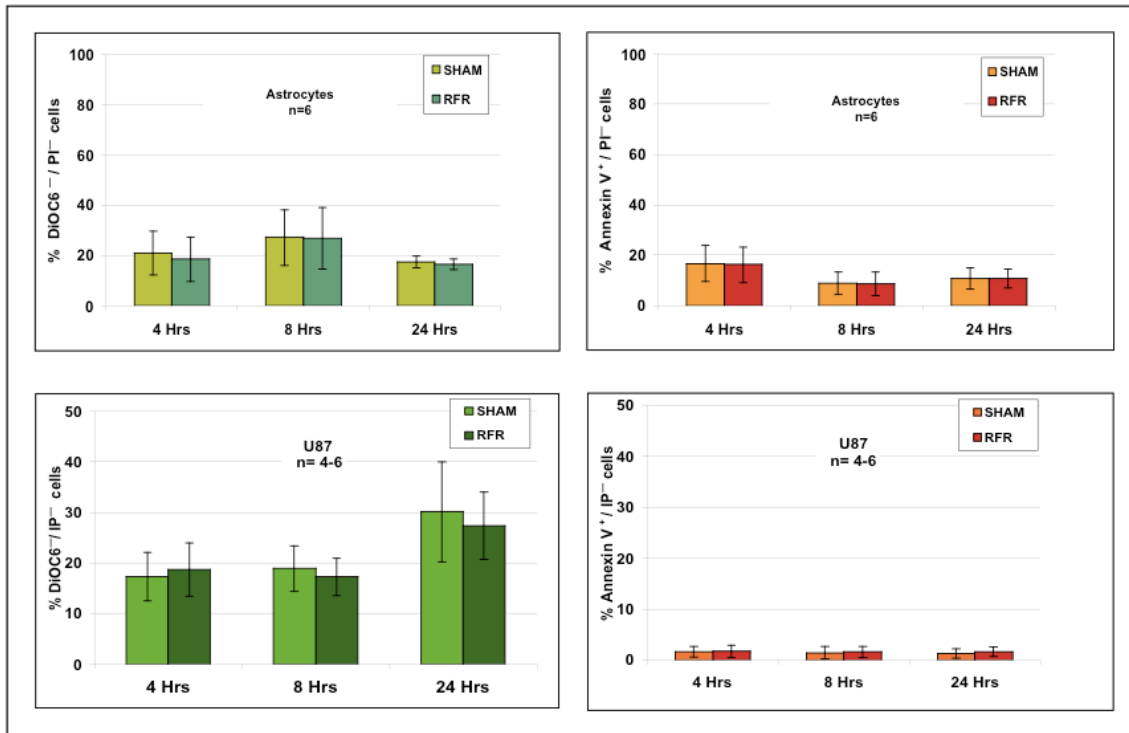


Figure 106. Effect of a 1-hour exposure to GSM-900 on apoptosis in astrocytic cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential (DIOC6/PI⁻, left panel) and apoptotic cells (ANX⁺/PI⁻, right panel) after exposure of primary astrocytes (upper panels) and human astrocytic cells (lower panels) to GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data from 4 to 6 independent experiments are presented as the Mean ± SEM.

In summary, an immediate or delayed effect of RF-EMF on apoptosis in rat primary cells and human cell lines could not be demonstrated.

RF-EMF did not influence apoptosis in immune cells.

The ability of the human U937 promyeloma cells to undergo apoptosis was tested by using camptothecin as a positive control (Figure 107). We show a significant 4- to 8-fold increase ($p < 0.01$), depending of the marker of apoptosis used, in U937 cells treated for 4 hours with CPT (4 µg/ml).

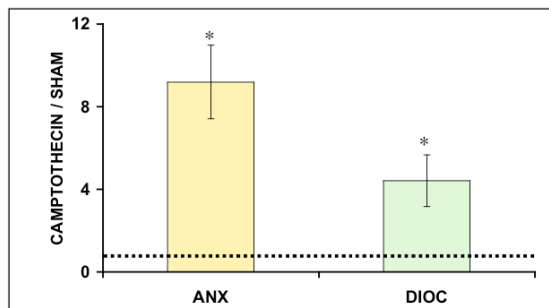


Figure 107. Effect of camptothecin treatment on apoptosis in human U937 cells. The results are expressed as the ratio of apoptotic cells (ANX⁺/PI⁻, left panel) and cells with depolarised mitochondrial transmembrane potential (DIOC-/PI⁻, right panel) in camptothecin-treated versus sham-exposed U937 promyeloma cells. U937 cells were sham-exposed for 1 hour and then treated for 4-hours with camptothecin (4 µg/ml). Data are presented as the mean ± SEM of 5 independent experiments. *= $p < 0.01$

Sham-sham experiments (n=3-4) showed that inter-incubator (incubator used for RFR exposure versus incubator used for sham-exposure) variation of U937 apoptotic cells was around 1.1 ± 0.5 after one hour and 1.2 ± 0.6 when the cells were placed back in a control incubator for 4 hours after the one hour spent in the dedicated incubators (Figure 108). The effect of CPT treatment was very similar when the samples were put for one hour in either dedicated incubator. We therefore concluded that both dedicated incubators were equivalent within a range of 50% of ratio variation and that an at least two-fold increase in apoptosis in RFR-exposed cells could be considered as significant.

No statistically significant influence of GSM-900 could be evidenced on spontaneous apoptosis of U937 cells (Figure 109), when they were exposed for one hour at a SAR of 0.7 W/kg and even at the highest SAR tested (2.0 W/kg). No difference could be detected either immediately after exposure or after a 4-hour resting period in a control incubator. Hence, no delayed effect of GSM-900 on apoptosis could be evidenced. A longer exposure duration, i.e. 48 hours, to GSM-900 at the lowest SAR tested (0.7 W/kg) was also not able to alter spontaneous apoptosis in the human cell line.

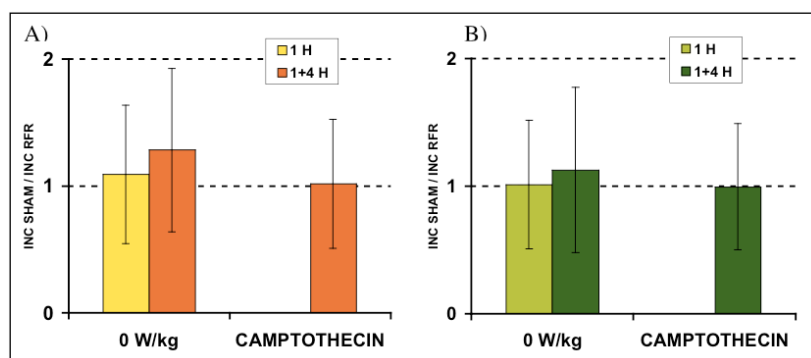


Figure 108. Comparison of occurrence of apoptosis in U937 cells in both dedicated incubators. Results are expressed as the ratio between apoptotic U937 cells in the incubator used for RFR exposure and apoptotic U937 cells in incubator used for sham-exposure. Sham-sham exposure lasted 1 hour and cells were harvested either immediately (1 H) or after an additional 4-hour resting period or camptothecin treatment (1+4 H). A) apoptotic U937 cells (ANX^+/PI^-) and B) U937 cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$). Data are presented as the mean \pm SEM of 3 to 4 independent experiments.

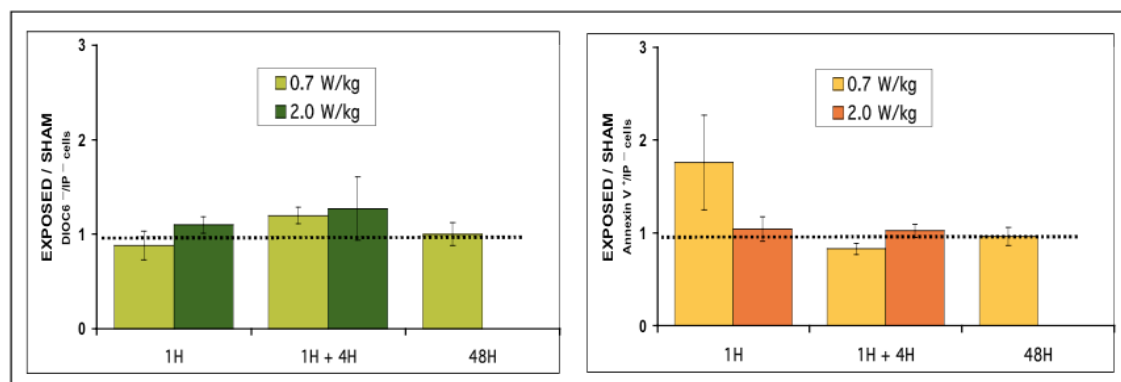


Figure 109. Effect of GSM-900 exposure on apoptosis in human U937 cells. Results are expressed as the ratio of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells ($Annexin V^+/PI^-$, right panel) in GSM-900- versus Sham-exposed U937 cells. U937 cells were exposed to GSM-900 at 0.7 W/kg (n=7) and 2.0 W/kg (n=6) for 1 hour, for 1 hour followed by a 4-hour resting period or for 48 hours. Data are presented as the mean \pm SEM.

RF-EMF did not influence chemically-induced apoptosis in immune cells.

When possible interaction between RFR and camptothecin was tested, we show that a one-hour treatment with GSM-900 at either 0.7 or 2.0 W/kg was not able to influence camptothecin-induced apoptosis (Figure 110).

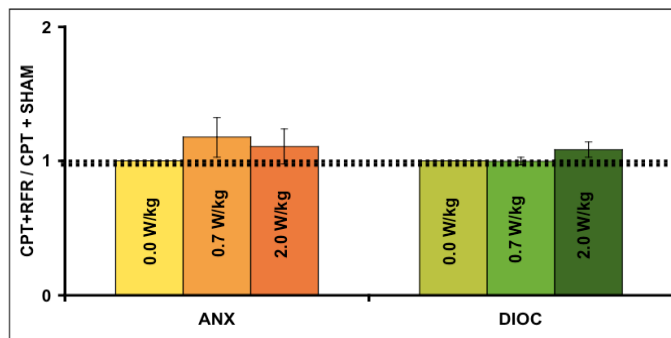


Figure 110. Effect of GSM-900 exposure on camptothecin (CPT)-induced apoptosis. The results are expressed as the ratio of apoptotic cells (ANX ⁺/PI ⁻, left panel) and cells with depolarised mitochondrial transmembrane potential (DIOC6 ⁺/PI ⁻, right panel) in GSM-900 plus camptothecin-treated versus GSM-exposed U937 promyeloma cells. U937 cells were sham-exposed or exposure to GSM-900 for 1 hour and then treated for 4-hour with camptothecin (4 µg/ml). The SAR level was 0.7 W/kg (n=7) or 2.0 W/kg (n=6). Data are presented as the mean ± SEM.

In summary, we showed no evidence for an immediate, cumulative or delayed effect of RF-EMF on apoptosis in a human monocytic cell line. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, our results strongly suggest that the apoptotic process is not a major biological target for GSM mobile telephony-related signals.

3.2.3.2 Human lymphocytes (Participant 8)

RF-EMF did not affect apoptosis in human lymphocytes.

Negative results were obtained studying spontaneous or dRib-induced apoptosis, when PBMCs were exposed at all the three signal modulations. Moreover, PBMCs from old donors were exposed to Talk modulated RF, but also in this case no effects on spontaneous or oxidative stress-induced apoptosis were found. These results were also confirmed by mitochondrial membrane polarisation, since no differences were noticed in dependence of the exposure and age of donor.

RF-EMF did not increase the Hsp70 level in human lymphocytes after induction of apoptosis.

dRib induces an increase of hsp70 in treated cells in comparison with untreated cells already detectable after 3h of treatment (unpublished data) up to 44 hours of treatment. When we studied RF-EMF effects (1800 MHz, GSM talk signal, 2 W/kg), we did not find any alteration of hsp70 gene product, after 44 hours of intermittent exposure, in PBMCs from 7 young donors, as showed in Figure 111. In this figure it is possible to notice the significant difference between the level of hsp70 in untreated cells versus the dRib-treated cells. Additional analysis were performed in separated lymphocytes and monocytes, but no differences were found between RF-exposed and sham-exposed cell populations.

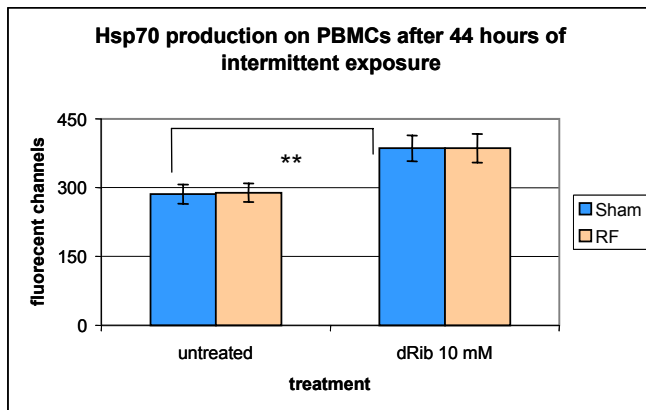


Figure 111. Hsp70 production in untreated and dRib treated PBMCs after 44hours of intermittent exposure. PBMCs were obtained from 7 donors and data are represented as mean of fluorescent channels \pm s.e. ** = $p < 0.01$, untreated PBMCs versus dRib treated PBMCs.

RF-EMF did not affect apoptosis in thymocytes.

Thymocyte apoptosis was assessed with two different methods, in the same conditions, but the data obtained from 6 human thymus on thymocyte apoptosis did not suggest positive results on both the endpoints. Actually, a small increase (4%) of double positive thymocytes (CD4+CD8+) was found in RF-EMF-exposed cultures in comparison with sham-exposed tissue fragments. Also in this case the effect is of the order of the standard error thus we consider these results irrelevant.

3.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF did not affect apoptosis in HL-60 cells as shown by flow cytometric analysis and the Annexin V and TUNEL assay.

See 3.2.1.1

3.2.3.4 Embryonic stem cells of mice (Participant 4)

RF-EMF exposure may influence the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors.

See 3.2.4.1

3.2.3.5 Human endothelial cell lines (Participant 6)

The RF-EMF-induced enhancement of hsp27 phosphorylation as well as the concomitantly RF-EMF-induced down-regulation of proteins of Fas/TNFA suggest that the anti-apoptotic pathway in RF-EMF exposed cell systems may be modified.

See 3.2.4.6

3.2.3.6 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on apoptosis in various cell systems such as brain cells and human monocytes (see 3.2.3.1), human lymphocytes and thymocytes (see 3.2.3.2), human endothelial cells (3.2.4.6) and HL-60 cells (see 3.2.3.3). On the other hand, an indirect effect on apoptosis through modulating the expression of various genes and proteins cannot be excluded at present. Up-

regulation of bcl-2 in differentiating embryonic stem cells (see 3.2.3.4 and 3.2.4.1) and of hsp27 in endothelial cells (see 3.2.4.6) both of which may affect the apoptotic process support such an assumption.

3.2.4 Gene and protein expression

3.2.4.1 Mouse embryonic stem cells (Participant 4)

Loss of p53 function rendered pluripotent ES cells sensitive to RF-EMF after prolonged exposure.

Exposure of undifferentiated cells to GSM-217 signals for 6 hours (Table 25) did not evoke any short-term modification of gene expression patterns neither in wt nor in p53^{-/-} ES cells (Figure 112A). On the other hand, long-term (48 hours) GSM-217 exposure of p53^{-/-} cells during EB development resulted in the up-regulation of transcript levels of 4 out of 6 analysed genes. Whereas c-jun, p21 and c-myc mRNA levels were only transiently up-regulated at early stages (days 2, 5 and 5+2 of EB differentiation, Figure 112B, a prominent induction of hsp70 levels in p53^{-/-} cells was observed throughout the differentiation period. The same experimental protocol was applied to analyse the influence of GSM signals simulating talking and listening phases during a typical conversation (GSM-Talk). However, no changes of gene expression patterns in both wt and p53^{-/-} cells were observed upon the exposure to GSM-Talk signals regardless of the protocol used. These observations indicate that the genetic constitution of cells determined by the p53 function affected cellular responsiveness to GSM-modulated EMF, whereas low frequency components characteristic for GSM-Talk modulation were not responsible for these effects.

Table 25. Conditions of the exposure of p53-proficient and deficient pluripotent embryonic stem cells to RF-EMF and summary of the effects on transcript levels of regulatory genes.

GSM-Basic			
1.5 W/kg (5min on/30 min OFF)			
p53 ^{+/+} ES cells		p53 ^{-/-} ES cells	
6h, (5min ON/30 min OFF) (n=3)	no EMF effect	6h, (5min on/30 min OFF) (n=3)	no EMF effect
48h, (5min on/30 min OFF) (n=3)	no EMF effect	48h, (5min on/30 min OFF) (n=3)	upregulation of hsp70, c-jun,c-myc and p21

GSM-Talk (33% GSM-Basic, 66% DTX)			
0.4 W/kg (5min on/30 min OFF)			
p53 ^{+/+}		p53 ^{-/-}	
6h, (5min on/30 min OFF) (n=3)	no EMF effect	6h – (5min on/30 min OFF) (n=3)	no EMF effect
48h,(5min on/30 min OFF) (n=3)	no EMF effect	48h (5min on/30 min OFF) (n=3)	no EMF effect

GSM – DTX (100% DTX)			
0.11W/kg			
p53 ^{+/+}		p53 ^{-/-}	
6h, (5min on/30 min OFF) (n=3)	no EMF effect	6h, (5min on/30 min OFF) (n=3)	no EMF effect

n- number of experiments

presence of 1% DMSO. The mRNA levels of regulatory genes were not affected by EMF exposition, and no significant differences were found between EMF-, sham-exposed and control variants of undifferentiated P19 cells, respectively.

Without DMSO induction, only low levels (5-10%) of spontaneous cardiac differentiation were found. By differentiation induction with DMSO during the first 48 hours of EB development, the differentiation of spontaneously beating cardiac cells was increased to a maximum level of 90% in both variants. Exposure of P19 cells to GSM-217 signals for 22 and 40 hours did not result in significant changes of cardiac differentiation suggesting that EMF had no effects on spontaneous or DMSO-induced cardiogenesis. Furthermore, undifferentiated EC cells were EMF- (GSM-217) and sham-exposed at a SAR value of 2.0 W/kg for 22 or 40 hours, and the lengths of cell cycle phases were analysed by flow cytometry. In both cases, no differences in the distribution of cells in G1, S or G2/M phases were observed between sham- and EMF-exposed variants.

RF-EMF exposure may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

We used an experimental protocol that has been shown to be efficient for differentiation induction of ES cells into the neural lineage (Figure 6) for our experiments aimed at defining the influence of RF-EMF (1.71 GHz, 1.5 W/kg, 5 min on/30 min off) as shown in Table 26. Neural progenitors were analysed at stage 4+4d to 4+6d, where nestin-positive cells were detected in ca. 60-80% of the cells. The exposure conditions applied for 6 or 48 hours are shown in Table 23. The analysis of hsp70, bax and p21 mRNA levels after RF-EMF exposure of neural progenitor cells did not provide evidence of gene expression changes. Bcl-2 was significantly up-regulated after the RF-EMF exposure at the terminal stage 4+23d (Figure 109, $p<0.01$). In addition, we investigated the effect of RF-EMF exposure on GADD45 transcript levels and found that GADD45 mRNA levels were significantly up-regulated at the same stage of differentiation at 4+23d (Figure 113, $p<0.05$). The data on the influence of RF signals on gene expression pattern in differentiating neuronal cells revealed statistically significant down-regulation of nurr-1 at stage 4d+11 and TH at terminal stage 4d+23d, but no clear shifts in transcript levels of the tissue specific genes GFAP, Nestin and En-1 (Figure 113). Quantitative RT-PCR with specific primers and TaqMan probe confirmed the up-regulation of GADD45 at 4+23d stage, but not for bcl-2. According to the Q-RT-PCR data nurr-1 was down-regulated both at stage 4+7d and 4+11d, but the decrease in mRNA levels was statistically significant for stage 4+7d (Figure 114).

Table 26. Conditions of the exposure of neuronal progenitor cells to PL-MF or RF-EMF.

Intermittent exposure (5min on/30 min off)			
48h, GSM 217 Hz (1.71 GHz)		6h, GSM 217 Hz (1.71 GHz)	
1.5 W/kg	Alterations of GADD45 transcript levels Down-regulation of nurr-1. No effect on DNA break induction (n=7)	1.5 W/kg	Low, but statistically significant induction of double-strand DNA breaks (n=7)

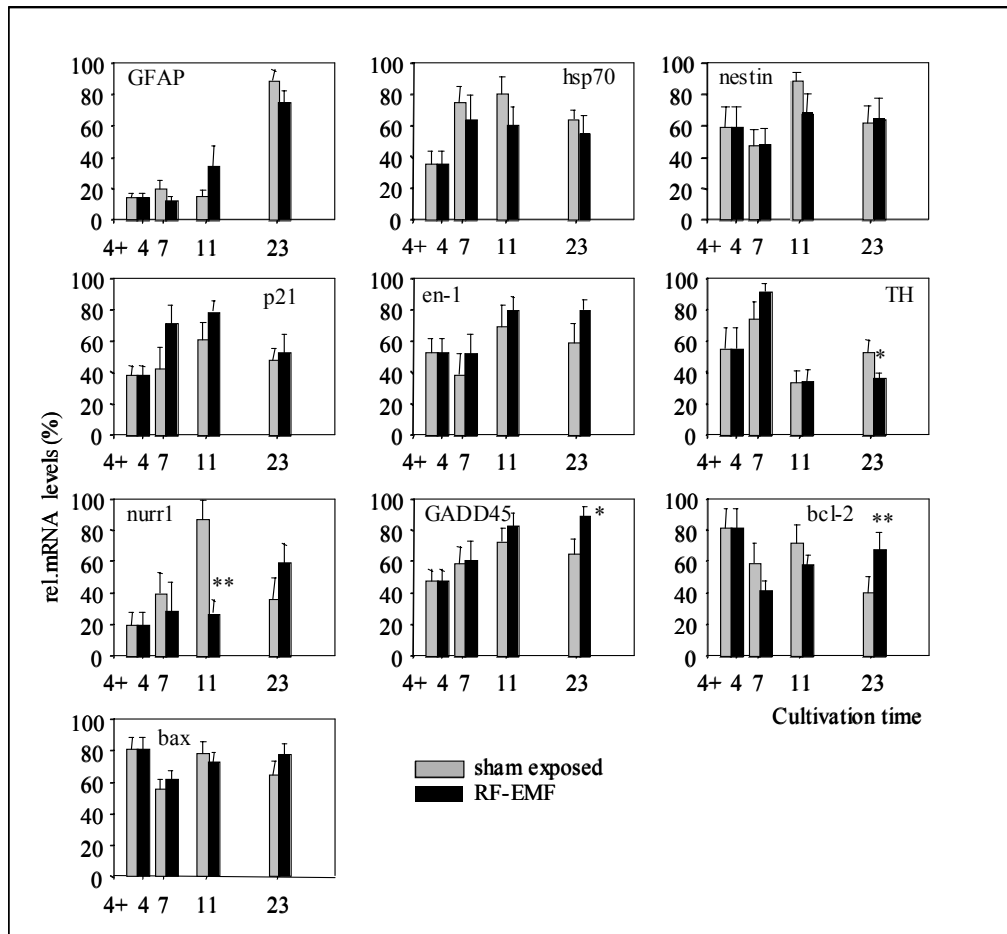


Figure 113. Relative mRNA levels analysed by semi-quantitative RT-PCR of genes encoding bcl-2, p21, c-jun, hsp70, c-myc and egr-1 in p53-deficient (p53^{-/-}) and wild-type (p53^{+/+}) D3 ES cell-derived EBs and EB outgrowths after 6 (A) and 48 hours (B) exposure to GSM-Talk. No effects of GSM-Talk on gene expression levels in ES cells were observed.

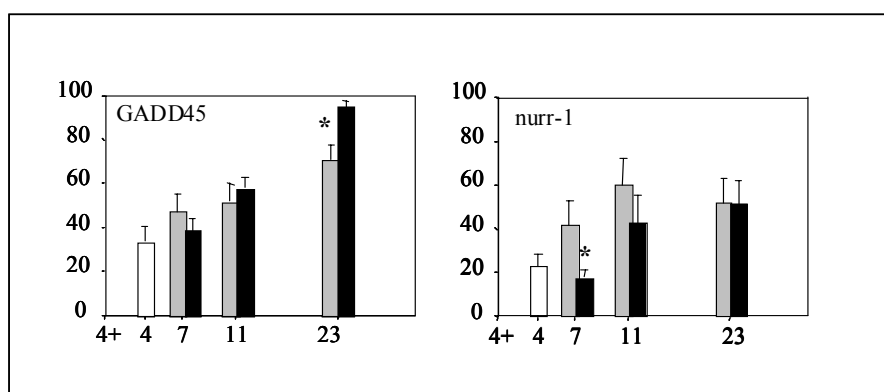


Figure 114. Quantitative RT-PCR with specific primers and Taqman probe for estimation of relative mRNA levels of the growth arrest and DNA damage inducible gene GADD45 and the nurr-1 gene involved in neuronal differentiation in ES-derived neural progenitors after 48 hours RF-EMF (GSM signal 217-Hz) exposure (1.5 W/kg, 1.71 GHz, intermittency 5 min ON/30 min OFF), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript down-regulation, followed by up-regulation of GADD45 and down-regulation of nurr-1 at stage 4+7d. Error bars represent standard deviations. Statistical significance was tested by the Student's two-tailed paired t-test for a significance level of 5% (*, $p < 0.05$).

3.2.4.2 Human neuroblastoma cell line NB69 and neural stem cells (NSC) (Participant 5)

RF-EMF (GSM-CW and GSM-Basic) interfered with the expression of FGF receptors in NB69 human neuroblastoma cells.

In a first experiment the cellular response to a chemical promoter of differentiation was characterised. Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells contain the neuroblast-specific protein β III-tubulin, but not the neuroepithelial marker nestin, which is present in immature progenitors and in some neuroblastoma cells (Kashima et al. 1995). Untreated NB69 cells remain in an undifferentiated state. Immunocytochemistry for FGFR1-3 revealed the three types of receptors in the human neuroblastoma cell line. On day 3 after plating approximately 70% of cells expressed R1, whereas FGFR3 and FGFR2 were present in a smaller proportion of cells, 30% and 20%, respectively (Figure 115, grey colour). Basic fibroblast growth factor (bFGF), which induces morphological changes including neurite extension at a 20-ng/ml concentration, was used as a positive control for the subsequent EM treatments. This growth factor reduced the number of NB69 cells expressing FGF receptors R1, R2 and R3. Such an effect was accompanied with changes in the cellular morphology associated to differentiate phenotypes. These changes included increased neural outgrowth, neural microtubule network and cell surface. As shown in this Figure 115, the 24-hour treatment with bFGF induced a consistent reduction in the percent of FGFR positive cells for the 3 receptors tested ($p < 0.0001$; ANOVA).

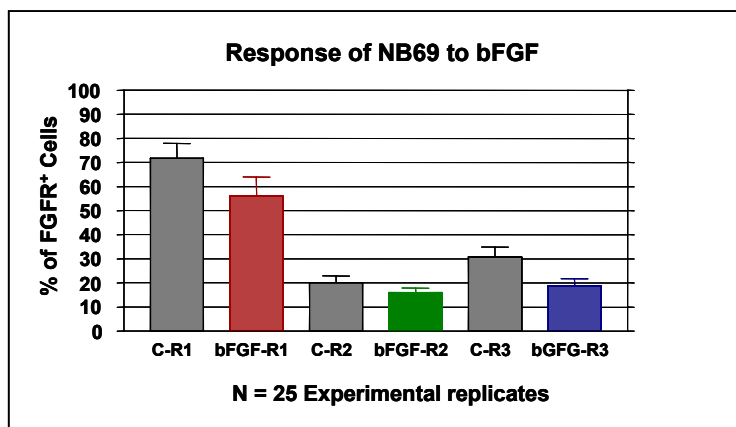


Figure 115. Expression of FGF Receptors 1, 2 and 3 in NB69 cells after a 24-hours treatment with 20-ng/ml bFGF. See text for details.

In the following experimental series, the cellular responsiveness to a differentiation-promoter was confirmed through treatment with bFGF, which induced a reduction of the percent of cells expressing FGF receptors R1, R2 and R3. Such an effect was associated with cell enlargement and neurite arborisation. In contrast, the treatment with GSM-Talk (SAR of 2 W/kg) signal alone (N= 4 experimental replicates), does not modify significantly the normal expression of the FGF protein receptors R1, R2 and R3 in NB69 cells (Figure 116). However, the results of the combined treatment bFGF + GSM-Talk signal show that the EMF seems to antagonize the significant reduction of FGFR-2 expression induced by bFGF. This indicates that the GSM-Talk signal might interfere with some of the cellular responses to bFGF.

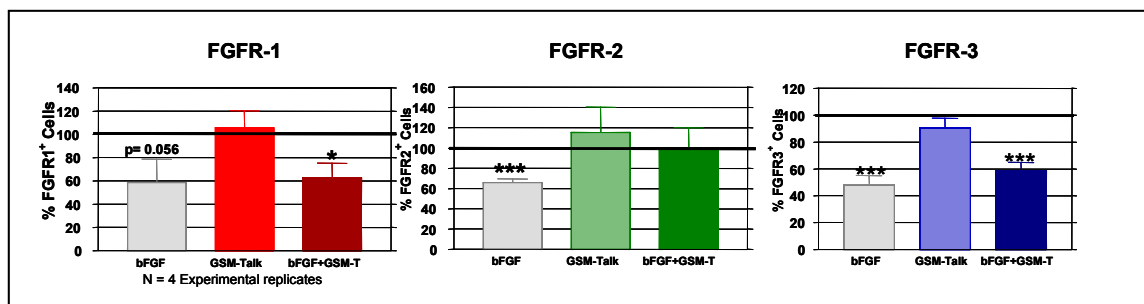


Figure 116. Response of NB69 cells to GSM-Talk signal. Percent of cells expressing FGF Receptors R1, R2 and R3. The treatment with bFGF induced significant reductions in the percent of cells expressing the receptors. Data normalised over the respective controls. (*, $p < 0.05$; **, $p < 0.01$; ANOVA followed by Student's T test).

When administered alone, the exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls, Figure 117) and photomicrographs of NB69 cell cultures) without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by bFGF at a 20 ng/ml concentration. However, unlike bFGF, the exposure to GSM-Basic alone did not provoke changes in the cellular morphology. Provided that, as described previously, the GSM-Basic treatment does not induces significant changes in the total cell number or the cell viability, the present results indicate that the GSM-Basic-induced effect on FGFR-1 is not due to a reduction in the number of cells, but to a loss of expression of this receptor.

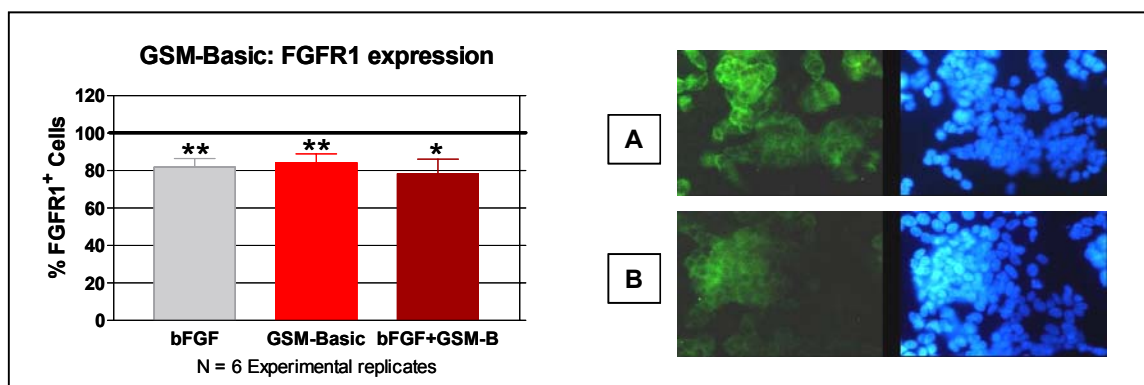


Figure 117. Photomicrographs of NB69 cell cultures: A, control; B, exposed to GSM-Basic signal. Immunocytochemistry for FGFR1. The image analysis showed a statistically significant reduction in the protein expression in the exposed cells (left). The matched fields (right) show the total cells stained with Hoechst.

The treatment with RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal (Figure 118A), whereas the exposure to GSM-DTX signal at a lower SAR (1 W/kg) did not modify significantly the normal expression of the FGF protein receptors R1 in NB69 cells (Figure 118B).

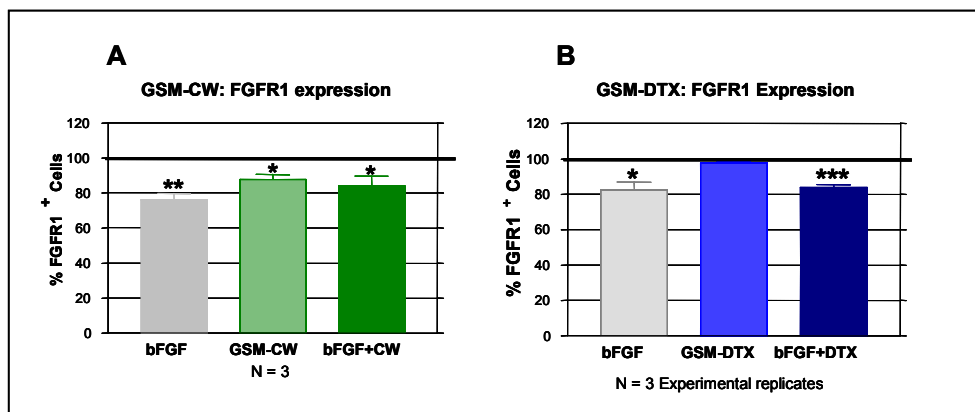


Figure 118. FGFR1 expression in NB69 cells after exposure to: (A) RF-CW signal, 2 W/kg and (B) GSM-DTX signal, 1 W/kg. Only RF-CW induced a significant response. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA followed by Student's T test for unpaired data).

Taken together, the results on the expression of FGFRs in NB69 cells exposed to GSM-1800 MHz signals and RF-CW signals suggest that (1) these cells are sensitive to low-SAR signals, (2) the cellular response does not seem to be dependent on the tested low-frequency modulation and (3) the observed response on FGFR1 could be indicative of a EMF-induced promotion of cell differentiation. However, additional studies on the expression of differentiation markers have to be done to confirm this hypothesis. (4) The GSM signal does not seem to interfere significantly with the cellular response to bFGF.

RF-EMF affected the expression of FGF receptors in neural stem cells (NSC).

In order to enhance expansion of NS precursor cells, neurospheres were seeded onto adherent substrate and treated with the mitogen epidermal growth factor (EGF) during the first 3 days in culture. After this period the EGF was withdrawn, and cells grew in a defined medium, which promoted differentiation processes to neurons, astrocytes and oligodendrocytes. Between 2 h and 3 days cultures comprised mainly nestin-positive, undifferentiated precursors. At later stages, the total number of cells dropped, paralleling to a gradual loss of nestin content, and an enhancement in the differentiation processes of neurons, oligodendrocytes and astrocytes.

Immunocytochemistry for FGFR1-3 identified the three types of receptors in the progeny of EGF-expanded NSCs. During the first day after plating, approximately 70% and 50% of the precursors expressed FGFR1 and FGFR2, respectively, whereas FGFR3 was restricted to a less abundant population. At 3 days and thereafter the number of cells exhibiting FGFR1 and FGFR2 decreased gradually, so that at 3 days the percent of FGFR-1 was 35% and at 9 days postplating only approximately 15% of the cell population was immunopositive for this receptor. FGFR1 immunostaining was preferentially localised in the cytoplasmic compartment, FGFR-3 was found in the cytoplasmic and/or nuclear compartments, and FGFR2 was frequently confined to the nucleus. In situ hybridisation studies on the third day postplating showed high levels of FGFR1 mRNAs in NSC.

On the basis of our previous data showing that the GSM-Basic signal induces a reduction in the percent of cells expressing FGF-R1 in human neuroblastoma cells, we tested firstly this signal (2 W/kg) on NSC. Like in NB69 cells, a significant decrease in FGFR1-positive NS cells was also observed after exposure to the GSM-Basic signal (50% reduction with respect to controls, Figure 119). Western blot analysis for FGFR1 confirmed this effect (data not shown). The study also indicates that the response of the neural stem cells seems to be dependent on the age of the culture (Figure 120).

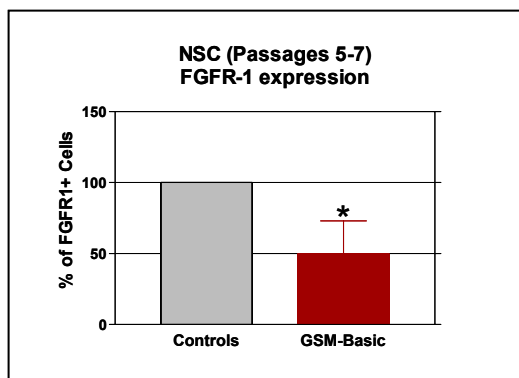
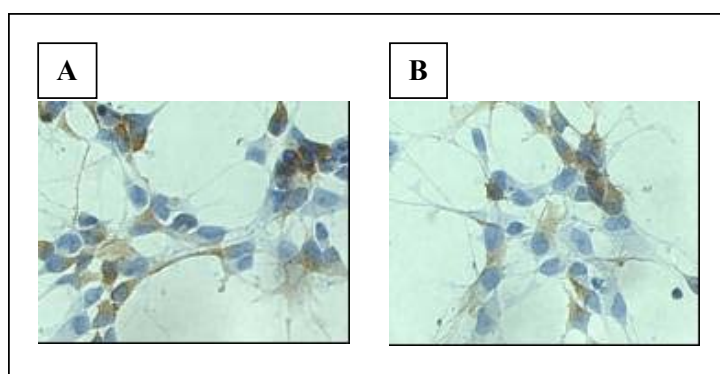


Figure 119. Percent of FGFR-1 positive labelling vs. total cell number normalised over their controls, quantification by the program for image analysis (IPWIN-3). Data represent the mean \pm SEM of 3 independent experiments, done in duplicate (two coverslips), for the different treatment conditions. Student T-test * $p < 0.05$.



Photomicrographs of Neural Stem Cells, A, control and B exposed to GSM-Basic signal. Immunocytochemistry for FGFR1-positive cells (brown).

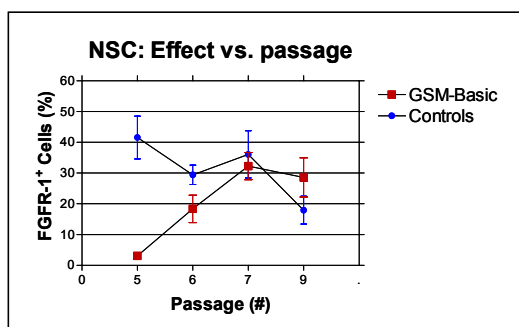


Figure 120. NSC exposed to GSM-Basic signal. The percent of FGFR-1 positive cells seems to be dependent on the passage number.

RF-EMF did not affect gene expression of FGF Receptor-1 in NB69 neuroblastoma cells and in neural stem cells (NSC)

In situ hybridisation studies were conducted on neural stem cells and in NB69 cells exposed for 21 hours (5 min on/10 min off) to the GSM-Basic signal. The objective is to evaluate potential EMF effects on gene expression of FGFR1. All experiments were conducted following blind protocols. The results on both cell types showed no differences in FGFR1 mRNA-expression between controls and exposed samples. An image-analysis study confirmed this result in NSC (Figure 121). Taken together, the

described effects on NB69 and NSC (FGFR1 protein-expression and FGFR1 mRNA-expression), suggest that the GSM-Basic signal can modulate FGFR1 protein translation without affecting protein transcription.

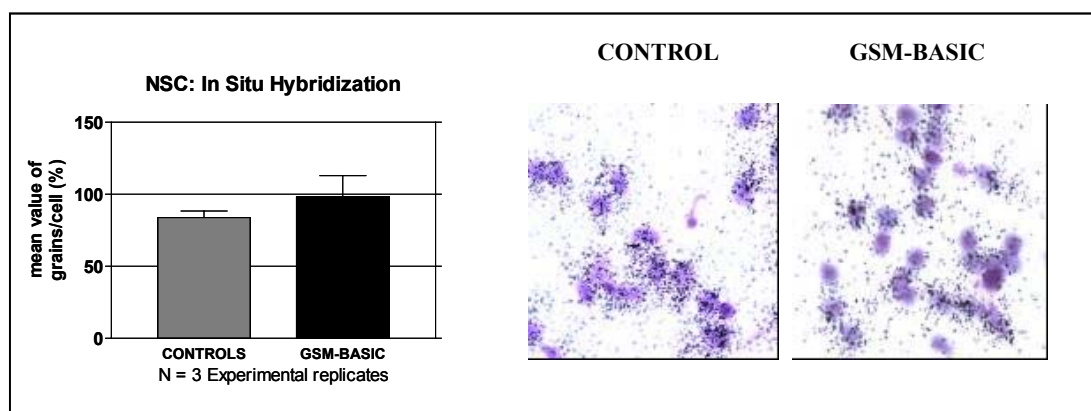


Figure 121. Quantification of in situ hybridisation staining by image-analysis technique (IPWIN 3.0) of the number of grains/cell. The photomicrographs show the developmental pattern of FGFR1 in NSC processed with Radiolabelled Probe Specific for Transcript of FGFR1.

3.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF exposure reproducibly up- and down-regulated protein expression in HL-60 cells (41 proteins showed to be up-, 1 protein to be down-regulated and 14 proteins appeared to be de-novo expressed).

The proteome screening approach included analysis of the entire HL-60 protein expression pattern by means of 2-D polyacrylamide gel electrophoresis (2D-PAGE). After having established the technique for HL-60 cells, cells were exposed to RF-fields at selected conditions in repeated independent experiments in order to obtain reproducible information on changes in the cellular protein pattern, correlated with RF-EMF-exposure.

HL-60 cells were exposed at 1800 MHz, continuous wave, SAR 1.3 W/kg, 24h, or were sham exposed in repeated independent experiments. Additionally, incubator controls were run and analysed for their protein expression pattern by 2D-PAGE. Cell samples were partly analysed as described above, partly stored at -80 °C for further analyses. Comparison of protein pattern after 2D-PAGE showed that optimal reproducibility is achieved when the 2-D separation step is performed in one series with identical reagent batches. In order to be able to perform statistics, appropriate numbers of comparable 2D-gels are required, also to have enough material for protein identification.

Figures 122, 123, and 124 show representative high-resolution 2-dimensional polyacrylamide gels (23 x 30 cm) for each of the conditions described above (incubator control, sham-exposure and RF-field exposure).

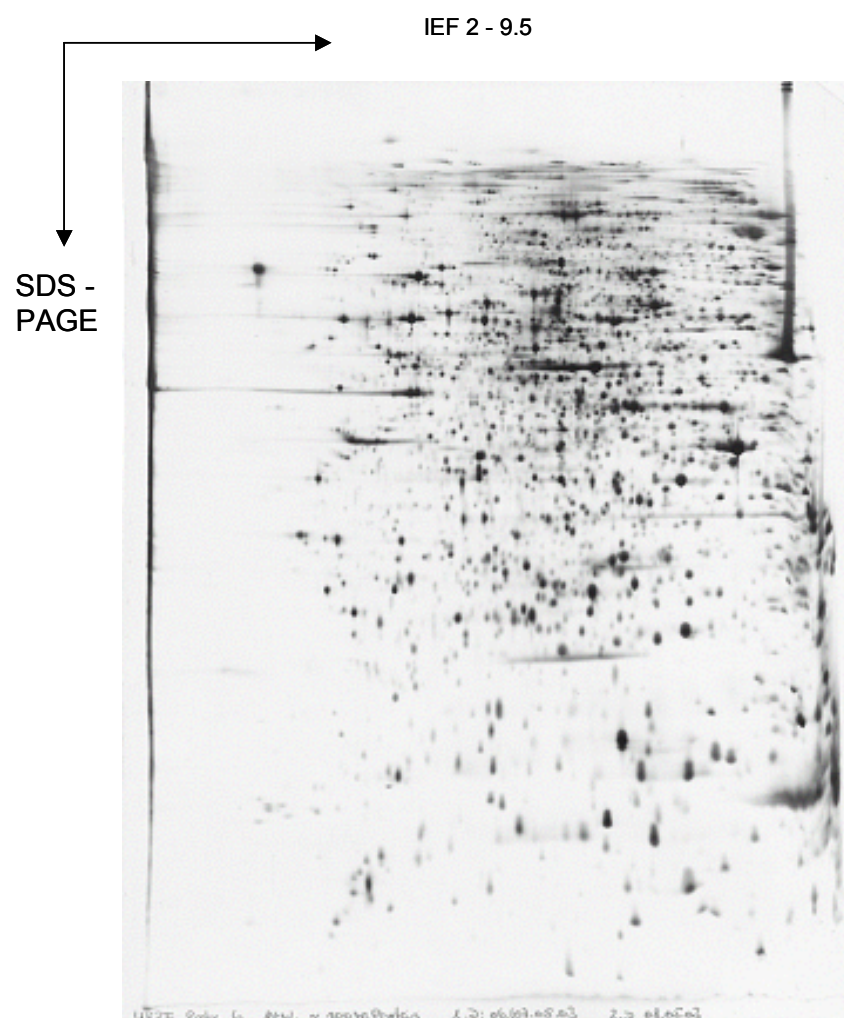


Figure 122. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of incubator control HL-60 cells (whole cell lysate). Incubation time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

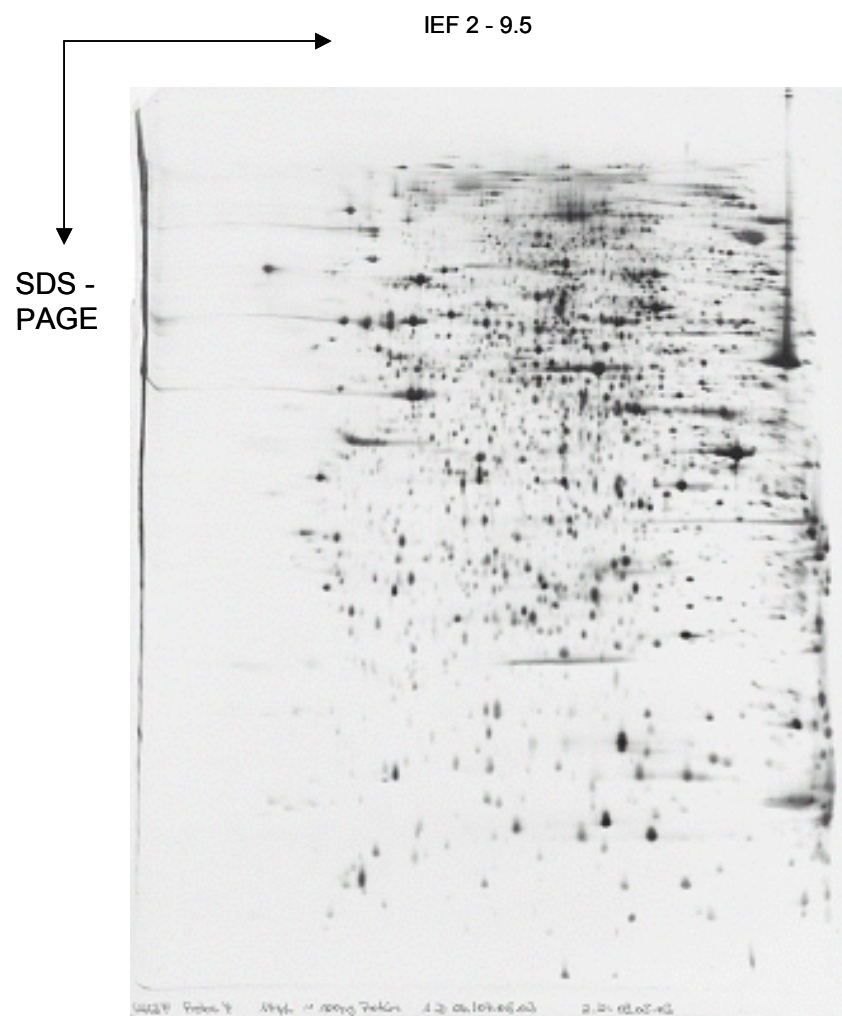


Figure 123. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of sham-exposed HL-60 cells (whole cell lysate). Exposure time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

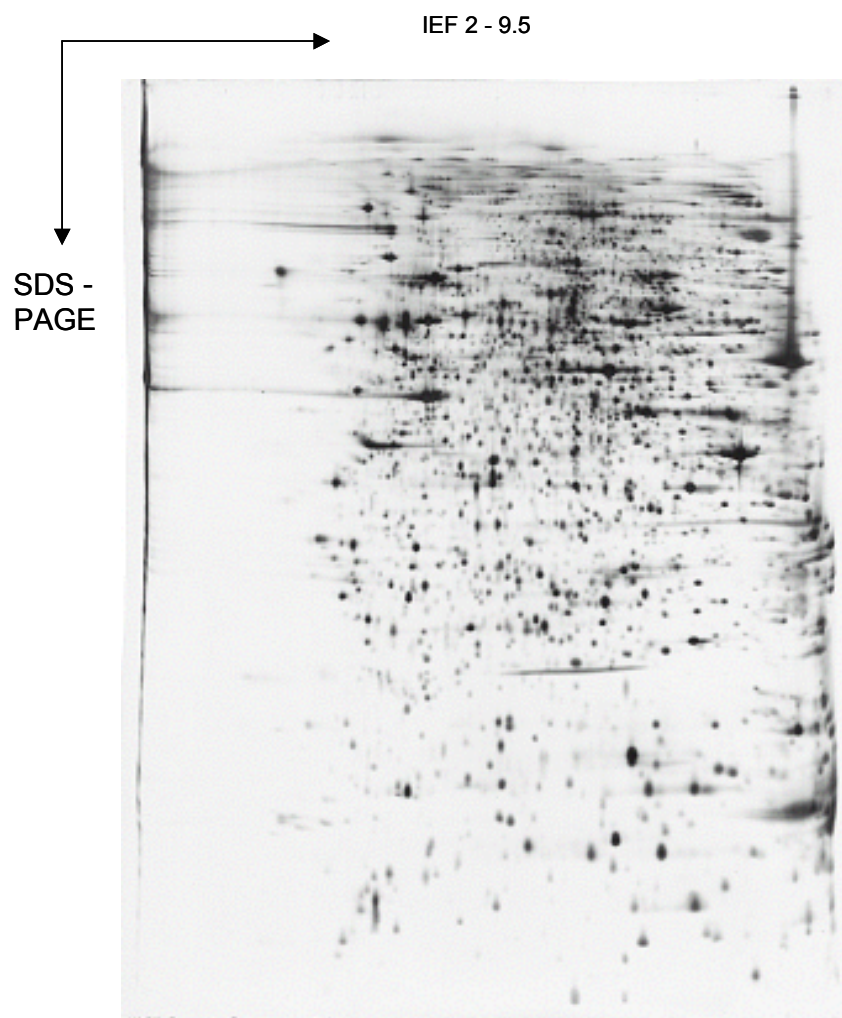


Figure 124. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of RF-exposed HL-60 cells (whole cell lysate). Exposure characteristics: 1800 MHz, continuous wave, 1.3 W/kg, 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

Following digitalisation, in a second analysis step the qualitative and quantitative comparison of protein expression was performed by use of Proteom Weaver image analysis program. Figure 125 shows a representative comparative 2D-gel, in which expression differences between RF-field exposed and sham-exposed HL-60 cells are marked. Expression differences were quantified. In Table 27 (a-c) proteins up-or down-regulated and those that have disappeared after RF-field exposure are listed.

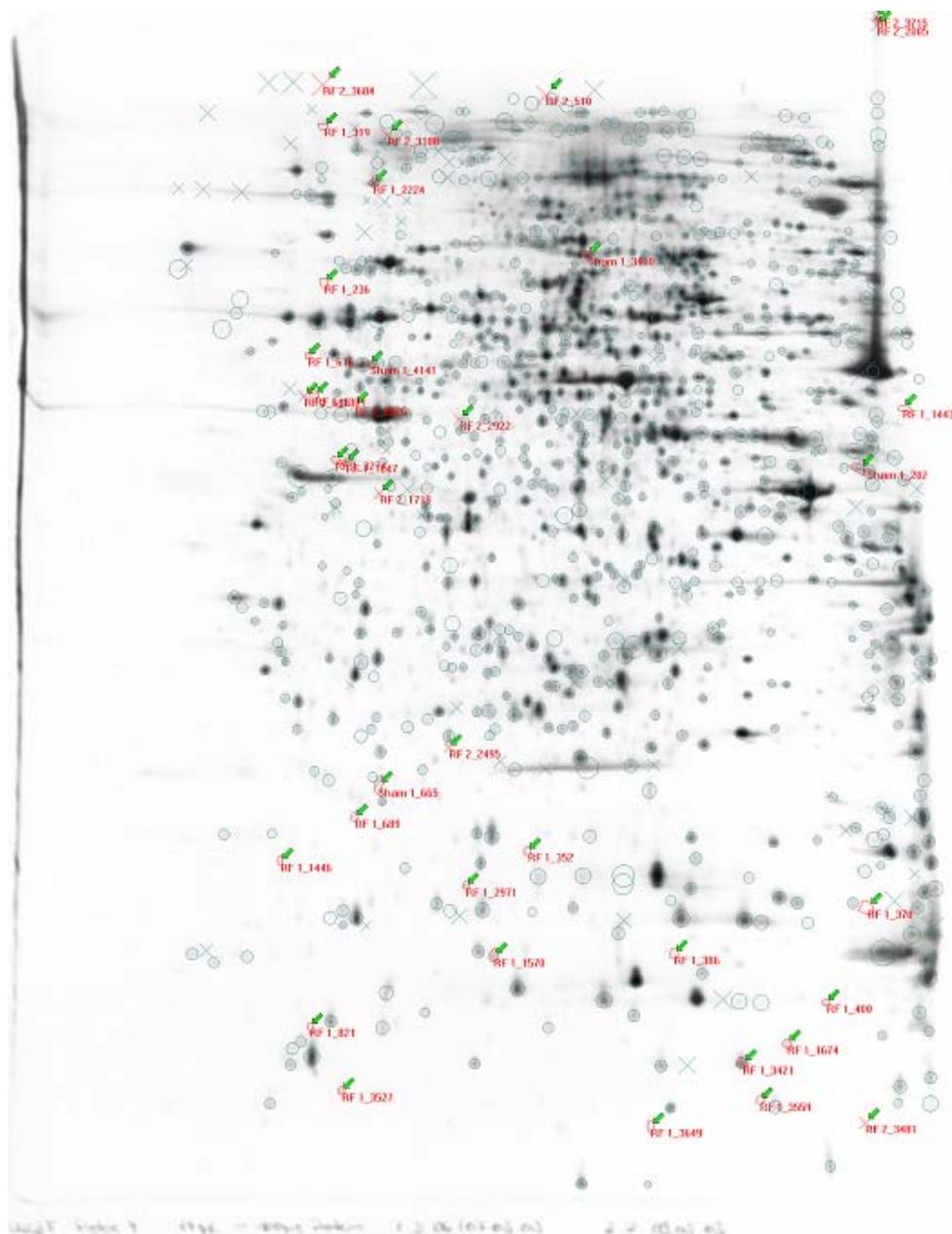


Figure 125. Representative qualitative and quantitative comparison of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profiles of sham-exposed and RF-exposed HL-60 cells (whole cell lysate).

Exposure conditions: see above. Green arrows mark different proteins of sham-exposed cells compared to RF-field exposed cells (green arrows and red circles: up-regulated proteins; green arrows and red crosses: newly expressed or disappeared proteins).

Table 27. List of proteins in 2-DE patterns of HL-60 cells, differing between RF-field exposed and sham exposed cells. Exposure conditions: see above

a: List of proteins up-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).

sham		RF-field	

b: List of proteins down-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).

sham		RF-field	

c: List of newly expressed or disappeared proteins in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow) .

sham		RF-field	

Overall, 56 polypeptides of HL-60 cells are influenced in their expression under RF-EMF. Reproducibly, 41 proteins showed to be up- and 1 protein to be down-regulated following RF-field exposure. 14 proteins appeared to be de-novo expressed after RF-field exposure of HL-60 cells.

By use of these lists identification strategies are further performed. They will include in gel-cleavage, identification of selected proteins by mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS). Further identification of selected proteins will include immunoblotting and functional protein assays.

3.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

T lymphocyte gene expression analysis was performed in collaboration with Participant 12 and Dr. Daniel Remondini in Bologna. The results suggest that no differences in gene expression are found between

quiescent T lymphocytes exposed to RF-EMF (DTX only) in comparison with sham-exposed cells. This finding did not suggest any significant interaction of RF-EMF with gene profile expression.

3.2.4.5 Brain cells of different origin (Participant 9)

RF-EMF exposure did not affect expression and activity of the inducible nitric oxide synthase (iNOS or NOS2) in nerve cells.

A basal level of NOS₂, probably due to the SVF deprivation, was detected in C6 cells, although inter-experiment's variation could be seen. A 48-hour treatment with LPS plus CK increased the expression of the enzyme by a factor 5 (Figure 126)

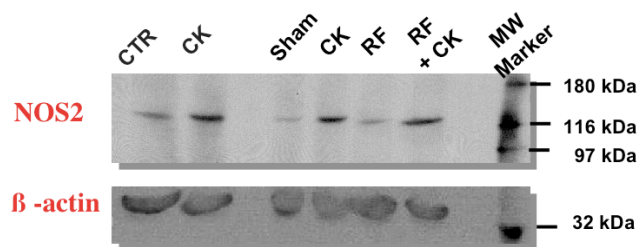


Figure 126. Representative blot for the detection of NOS₂ (upper blot) and β-actin (lower blot) proteins.

Sham/sham experiments showed that a 15-20% inter-incubator variation had to be expected, so that a more than a 30% difference between sham- and RF-exposed data would be considered as a significant biological effect for both NOS₂ expression and nitrite accumulation (Figure 127).

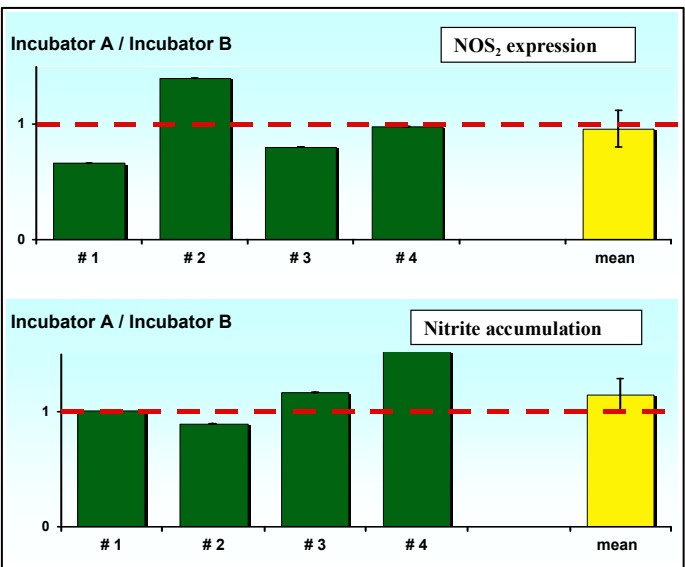


Figure 127. Expression of NOS₂ protein and nitrite accumulation (culture medium) in C6 cells in four sham/sham experiments (#1 to #4). Data are given as the ratio ± SEM between the levels found in the incubator A used for RF exposure and incubator B used for sham-exposure.

Exposure to GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours was shown to not alter the expression of NOS₂ compared to sham exposure. Co-exposure to GSM and LPS plus cytokine was ineffective in modifying the effect of LPS plus cytokine treatment on NOS₂ expression (Figure 128).

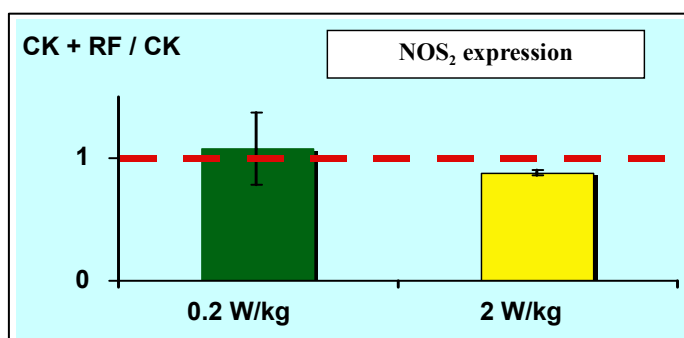


Figure 128. Expression of NOS₂ protein in C6 cells exposed to frame-GSM-900 at 0.2 and 2 W/kg for 48 hours. Data are given as the ratio \pm SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

Nitrite accumulation in culture medium was used as a marker of NOS₂ activation. No nitrite accumulation was shown in sham-exposed samples. Although inter-experiment variability, treatment with the cocktail of LPS plus CK led to a significant nitrite accumulation ($p < 0.001$). As shown in Figure 129, a mean 20-fold increase in nitrite accumulation was measured after 48 hours of LPS plus CK treatment.

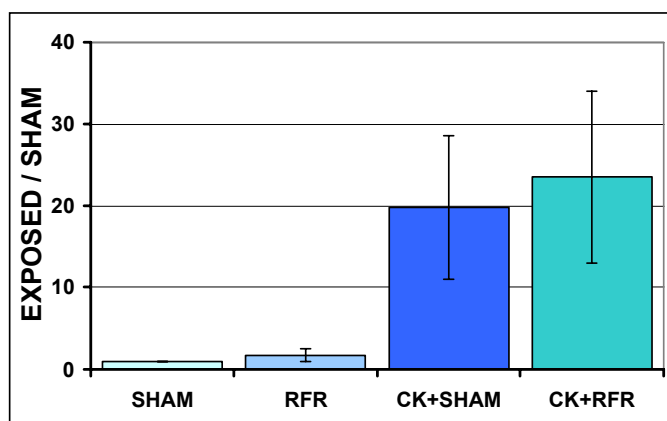


Figure 129. Nitrite accumulation in culture medium of C6 cells sham-exposed or exposed to GSM-900 at 0.2 W/kg and /or treated with CK+LPS (CK). In all cases, treatment duration was 48 hours. Data are given as the ratio \pm SEM between the levels found in treated- versus sham-exposed samples.

No significant effect of GSM-900 exposure was detected on nitrite accumulation. When co-exposures to GSM-900 and LPS + CK treatment were performed, no modulation of chemically-induced NOS₂ expression. Overall, exposure to GSM-900 did not modulate CK+LPS-induced nitrite accumulation (Figure 130).

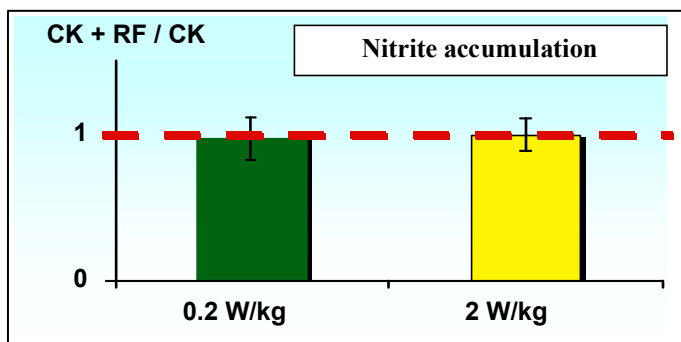


Figure 130. Nitrite accumulation (culture medium) in C6 cells exposed to frame-GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours. Data are given as the ratio \pm SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

No evidence of an effect of RF-EMF (GSM-900) exposure on spontaneous expression and activity in C6 cells was obtained from our experiments. However, one can note that only strong treatments (serum deprivation plus long duration of chemical treatment) are shown to increase NOS₂ expression in C6 cells. It is noteworthy that most papers in the literature looked at the mRNA but not at the *protein* level as we did in the present work. Two SAR levels of GSM-900 were tested. Even at the highest SAR level of 2 W/kg corresponding to the public exposure limit recommended by the EU Commission, GSM-900 exposure was not shown able to influence NOS₂ expression or activity in activated C6 cells. Taken together, RF-EMF at a low SAR level were not identified as a stimulus for C6 cells activation.

RF-EMF (GSM-900 signals) did not affect heat shock protein expression in nerve cells.

When used as a positive control, heat shock (43°C for 20 min) increased expression of hsp70 in all nerve cell cultures, i.e. human U87 astrocytoma cells, rat C6 glioma cells and human SH-SY5Y neuroblastoma cell lines. However, when exposed to GSM-900 for 24 hours, none of the cell line showed a significant change in expression of hsp70 (Figures 131, 132). Altogether, our data show that exposure to ELF-EMF does not seem to be able to induce Hsp70 expression in rat and human nerve cells.

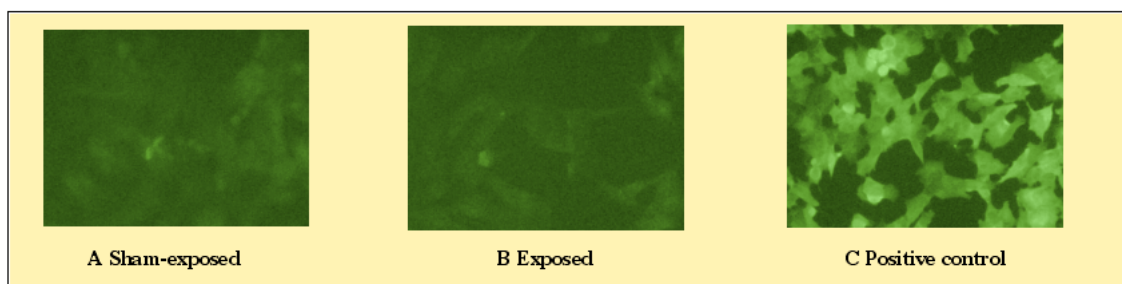


Figure 131. Fluorescent Hsp70 immunolabelling after sham-exposure or exposure of human SH-SY5Y neuroblastoma cells to frame GSM-900 signal at 2 W/kg for 24 hours (A and B) or to heat shock (43°C, 20 min) (C).

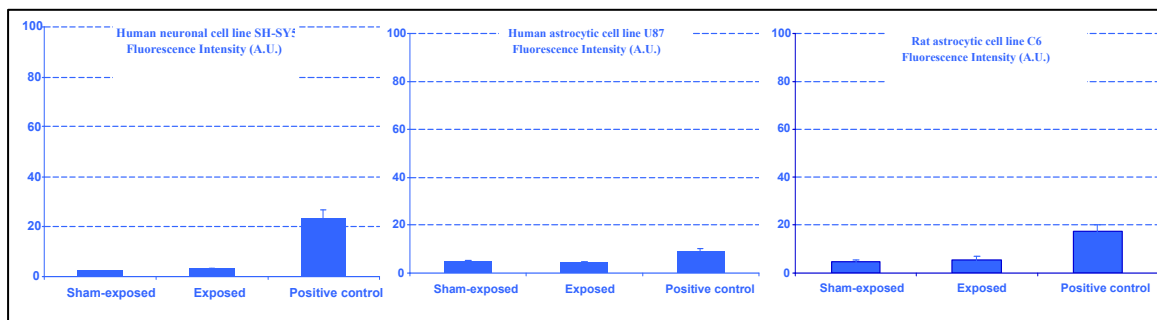


Figure 132. Effect of GSM-exposure (2 W/kg, 24h) or Heat Shock exposure (43°C, 20 min) (Positive control) on hsp70 expression in three different cell lines. Results are expressed in Fluorescence Intensity (A.U). Data from 3 independent experiments are presented as the Mean \pm SEM

GSM-900 microwave exposure did not affect hsp27 expression in human endothelial cell line EA.hy926.

The hsp27 expression was measured using the immunofluorescence technique. Qualitative analysis did not allow for detecting any difference in fluorescence intensity in RFR exposed cells versus sham cells. Our quantitative results obtained after fluorescence image analysis of hsp27 expression in EA-hy926 revealed that no significant difference was observed between sham-or exposed cells in both cell lines (Figures 133, 134)

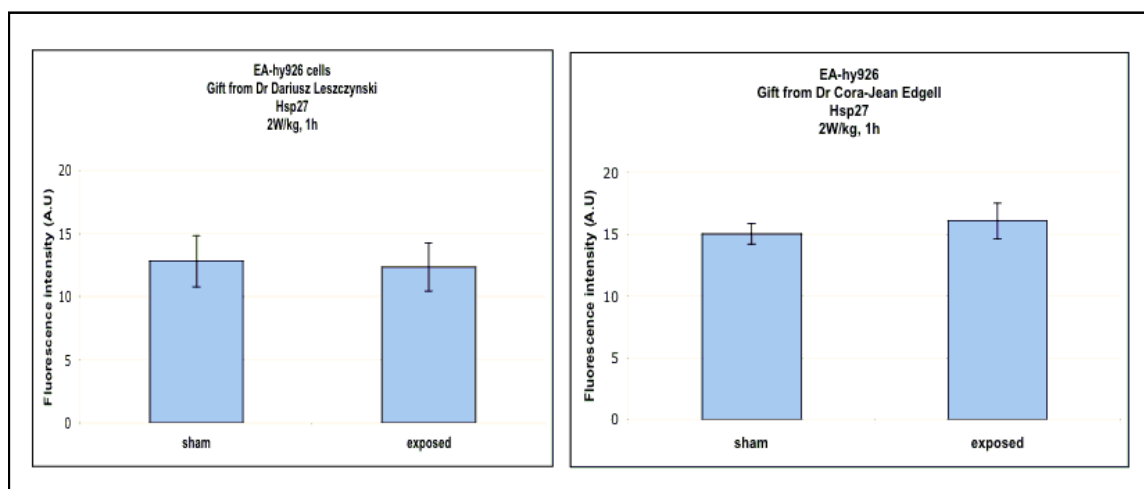


Figure 133. Effect of GSM-exposure (2 W/kg, 1 hour) on hsp27 in EA-hy926 cell lines given by Participant 6 and Dr Cora-Jean Edgell. Results are expressed as the fluorescence Intensity (A.U). Data from 5 independent experiments are presented as the Mean \pm SEM.

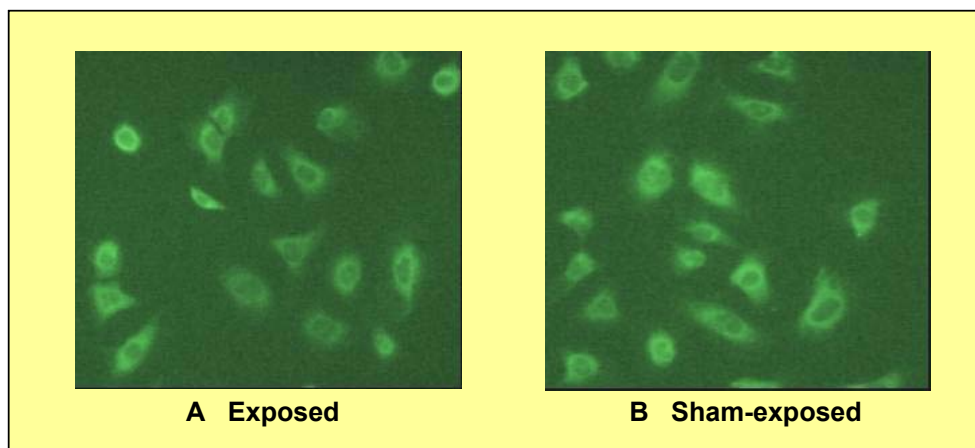


Figure 134. Fluorescent hsp27 immunolabelling after exposure(A) or sham-exposure(B) of EA-hy926 cell lines given by Participant 6 to GSM-900 signal at 2 W/kg for 1 hour.

A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression. However, with our method we were unable to confirm previous data on hsp27 expression in endothelial cell lines (Leszczynski et al., 2002). Therefore, we can not conclude that RFR induce stress response.

No conclusive data was obtained on the effect of RF-EMF exposure on Hsp27 expression in rat brain.

Table 28 shows results obtained on hsp27 expression in rat brains in the pilot experiment.

Results obtained on samples treated without perfusion show non-specific labelling disturbing the analysis. Results obtained on perfused brains show acceptable background noise. Image analysis obtained on perfused brains reveal conflicting and opposite results within groups preventing to draw conclusions.

Table 28. Hsp27 expression in rat brains

Experimental procedure	Background noise	Hsp27 labelling	Exposure conditions
Perfusion	-	+	Control
Perfusion	+	-	Control
Perfusion	-	-	Sham-exposed
Perfusion	-	+	Sham-exposed
Perfusion	-	-	Exposed
Perfusion	+	+/-	Exposed
Without perfusion	+	+	Control
Without perfusion	+	+	Control
Without perfusion	+	+/-	Sham-exposed
Without perfusion	+	++	Sham-exposed
Without perfusion	+	+	Exposed
Without perfusion	+	+/-	Exposed

- : negative labelling; + : positive labelling; +/- : negative or positive labelling depending of the area; ++ : clear positive labelling

This pilot study did not allow us to draw conclusion on results obtained *in vitro* on hsp27 expression but it gave information on technical methodologies and on the number of animals to use.

RF-EMF (GSM-900) exposure weakly affected gene expression in immune cells.

This investigation was carried out in cooperation with Participant 12. Criteria for the selection of significantly altered gene expression was an exposed over sham ratio less than 0.5 for a significant decrease and more than 2.0 for a significant increase. Using these criteria, over 15588 human genes were detected, changes in expression of about 50 genes were significant corresponding to 0.3% of total number of detectable genes. Genes shown to be altered after RF-EMF exposure (increase or decrease) are known to be involved in signal transduction, ion electron transport, metabolism of energy and proteins, cell proliferation, apoptosis or differentiation, immune answer, inflammation, stress answer, extracellular matrix, cytoskeleton, adhesion and DNA repair. The largest modification in RNA expression corresponded to genes related to signal transduction (linked with GTP or calcium) and energy metabolism. Only a few genes involved in apoptosis or stress response were detected and show no significant sensitivity to RFR exposure. Concerning our purpose to investigate modification of genes involved in inflammatory response and processes, one gene corresponding to a component of major histocompatibility complex class II and another acting as plasminogen activator were altered by RF-EMF. Finally, the largest increase of expression (30 fold increase), after mobile phone exposure, concerned genes described to participate in amine oxidase (copper containing) activity. This enzyme is involved in cell growth and proliferation but also in immune regulation.

3.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski 2001). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold and co-workers (Aebersold et al. 2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses (Figure 135).

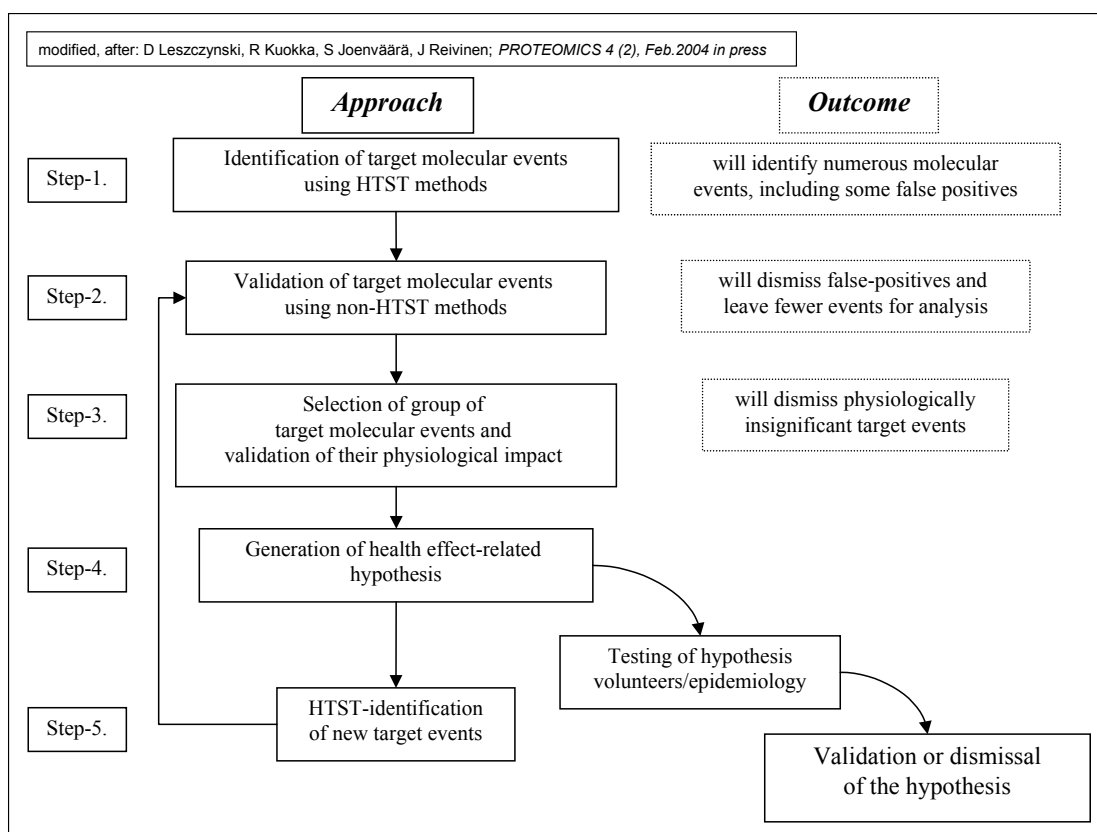


Figure 135. Scheme of experimental procedure which execution will elucidate new RF-EMF induced molecular events that might affect cell physiology. Events of magnitude sufficient to alter cell physiology could be then examined for their potential impact on the organ/whole body physiology in attempt to predict the extent of eventual health hazard.

A. The 5-step feasibility study

Step-1: HTST-identification of target molecular event

Firstly, we have determined the extent of cell response to RF-EMF (Leszczynski et al. 2002). This has been done by analysing global changes in the pattern of protein phosphorylation. As an experimental model we have used cultures of human endothelial cell line EA.hy926 (Edgell et al. 1983). Cells were exposed for 1 hour to 900MHz GSM mobile phone simulating signal at an average specific absorption rate (SAR) of 2.4 W/kg (Deli et al. 1995) that is slightly above the European safety limit (SAR=2.0 W/kg). In order to be able to determine changes in protein phosphorylation, the ^{32}P -labelled orthophosphate was present in the cell cultures during the 1 hour RF-EMF exposure period. Immediately after the end of exposure cells were harvested; proteins extracted and separated using standard two-dimensional electrophoresis (2-DE). Using PDQuest software (Bio-Rad, UK), some 1266 different protein spots were identified in silver-stained 2-DE gels (Figure 136A). Using autoradiography it was possible to determine that among the 1266 proteins spots, in non-irradiated control exposed cells, were detected some 110 phosphoproteins (Figure 136B), whereas in exposed cells were detected some 372 phosphoproteins (Figure 136C). The observed broad change in the pattern of global protein phosphorylation has suggested that cells respond to RF-EMF and that possibly any of the hundreds of phosphoproteins that have altered their phosphorylation status could, at least potentially, affect cell physiology. By using western blot or mass spectrometry, to identify the phosphoproteins present in the 2DE spots, it might be possible to find variety of protein targets that could be used in examining effects of mobile phone radiation on cell physiology. With this approach, the selection of molecular targets for further studies would not be based only on deduction of potentially affected events but on the knowledge of the identities of proteins that indeed respond to RF-EMF. Thus, in the continuation of Step-1, using

simple western blot screening with antibodies directed against various stress response proteins, we have identified hsp27 as one of the phosphoproteins responding to RF-EMF. Hsp27 is continuously expressed in endothelial cells (Edgell et al. 1983). In 2DE-western blots it appeared as two spots of 27kDa molecular weight but with different isoelectric points (pI=5.7 and pI=6.1) (Figure 136D). Only the hsp27_{pI=5.7} isoform was phosphorylated and, following exposure to RF-EMF, the size of hsp27_{pI=5.7}-spot has increased (Leszczynski et al. 2002).

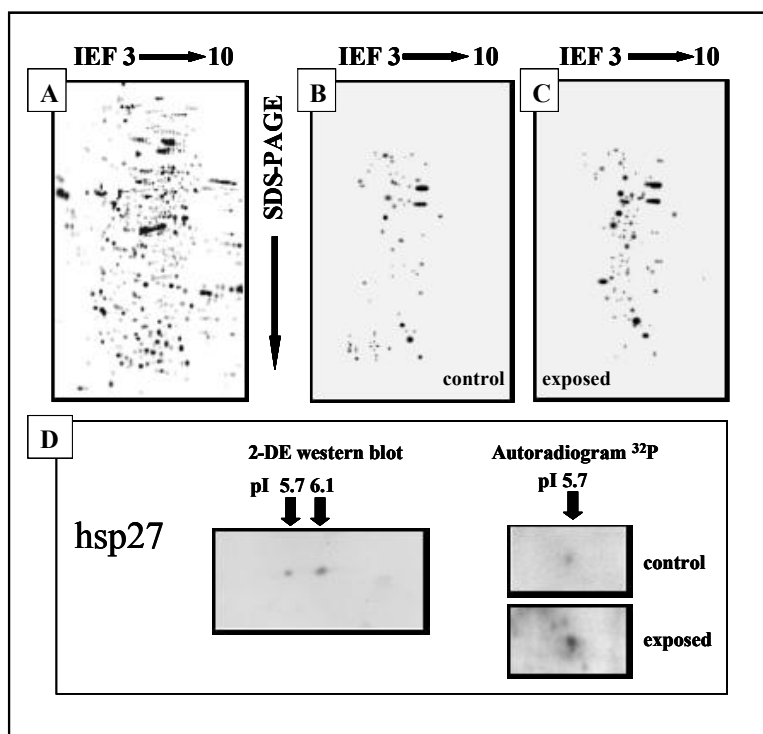


Figure 136. Identification of target molecular event for further studies – Step-1. Pattern of protein expression in EA.hy926 human endothelial cell line as determined by 2DE (panel A). Pattern of expression of ³²P-labeled phosphoproteins in control (panel B) and in exposed (panel C) cells. Hsp27 protein was identified, using 2DE western blot, as existing in EA.hy926 cells in two isoforms with different pI values (panel D-left). The pI 5.7 form was phosphorylated and its phosphorylation level has increased after RF-EMF exposure (panel D-right). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-2: Validation of target molecular event

The change in phosphorylation status of hsp27 was confirmed in several ways to assure the validity of this observation (Leszczynski et al., 2002):

- immunoprecipitation of phosphorylated hsp27 (Figure 137A),
- immunoprecipitation of p38MAPK (Figure 137B), an up-stream kinase indirectly involved in phosphorylation of hsp27,
- inhibition of hsp27 phosphorylation by introduction to cell cultures of inhibitor of hsp27-up-stream kinase p38MAPK (SB203580) and determining hsp27 phosphorylation status by immunoprecipitation (Figure 137C).

Thus, in the Step-2 was confirmed that hsp27 is the valid molecular target event of the RF-EMF and that it is justified to further examine impact of this change on cell physiology.

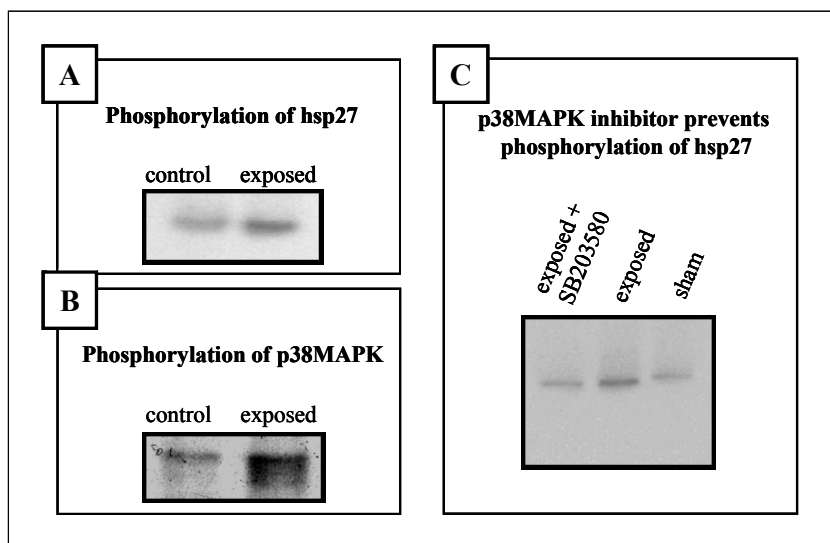


Figure 137. Validation of the target molecular event – Step-2. Increase in phosphorylation of hsp27 was confirmed by immunoprecipitation (panel A). Effect of p38MAP kinase on the RF-EMF-induced hsp27 phosphorylation was confirmed by determining, by immunoprecipitation, that p38MAPK is also activated by the RF-EMF exposure (panel B). As expected, presence of p38MAPK inhibitor, SB203580, in the culture medium during the exposure to RF-EMF, has prevented phosphorylation of hsp27 (panel C). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-3: Cellular response – validation of the physiological event

Observed by us phosphorylation and increase in expression of hsp27 (Leszczynski et al. 2002) is a well-established mechanism of cell response to a broad variety of stress stimuli (Rogalla et al. 1999). Therefore, the observed by us doubling of Hsp27 expression and 2- to 7-fold increase in amount of phosphorylated hsp27 in cells (Leszczynski et al. 2002) have suggested that EA.hy926 cells have recognised RF-EMF as an external stress factor and that they have launched an hsp27-dependent counter response. Phosphorylation of hsp27 has been shown to regulate polymerisation of F-actin and stability of made of this protein - stress fibers (Landry and Huot 1995). Thus, we have examined status of the stress fibers in exposed cells by staining F-actin with AlexaFluor-labelled phalloidin. As shown in Figure 136A, RF-EMF exposure has caused increase in cellular staining with phalloidin what indicates increase in stability of F-actin stress fibers. The stability of stress fibers, as determined by the pattern of staining with phalloidin-AlexaFluor, increased after 1 hour irradiation and did not decline during the 1 hour of post-irradiation incubation. Induction of the stability of stress fibers caused cells to shrink and visible cell shrinking was observed among the cells brightly stained with AlexaFluor-phalloidin (Figure 138A; middle and right panels). The increase in the stability of stress fibers was prevented in the presence of p38MAPK inhibitor SB203580 (Figure 138B). Also it was possible to observe that in cells expressing high levels of hsp27 (Figure 138C), the cell edges were brightly stained with phalloidin-AlexaFluor, what indicates re-localisation of F-actin stress fibers to cell ruffles whereas in cells expressing low levels of hsp27, network of stress fibers was seen throughout the whole cytoplasm but not in the ruffles. Such behaviour of hsp27 and stress fibers in cells exposed to RF-EMF is in agreement with the general pattern of cellular response to stimuli that activate hsp27-dependent stress response (Landry and Huot 1995).

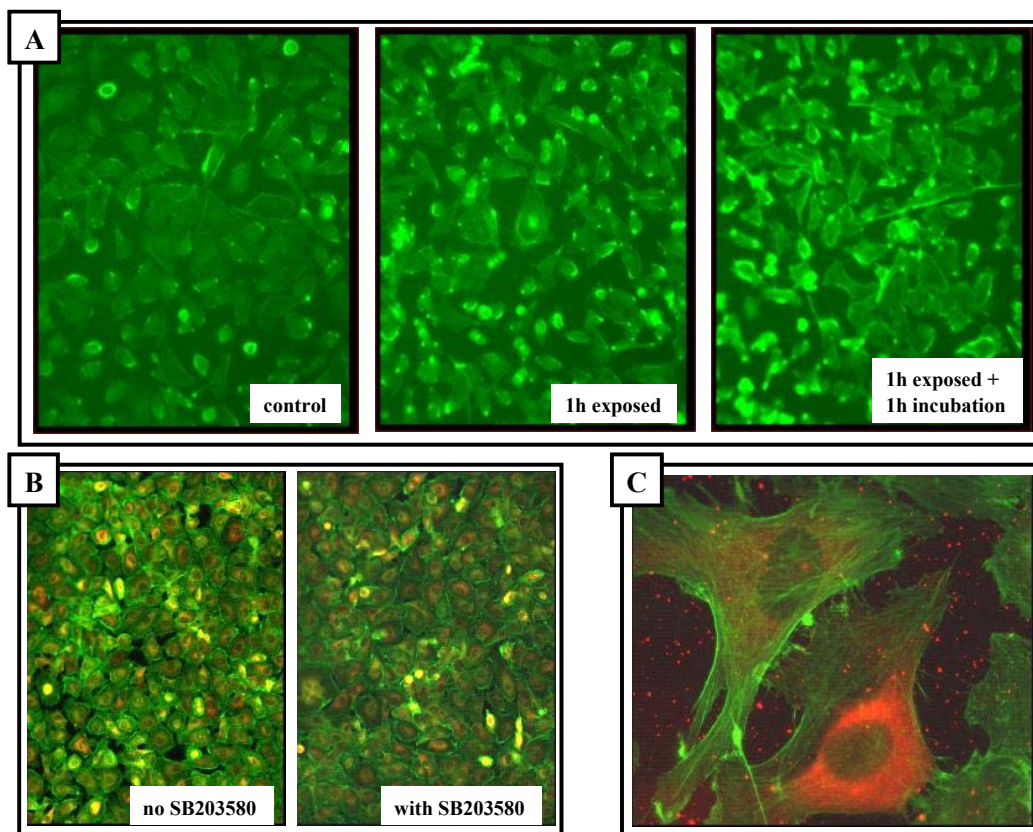


Figure 138. Cellular response to RF-EMF – validation of physiological event – Step-3. Exposure of cells to RF-EMF has caused increase in cell staining with AlexaFluor-labelled phalloidin (panel A). This suggests the increase in the expression/stability of F-actin, protein that forms cellular stress fibers. Rounding up is visible among the cells expressing highest F-actin content (the brightest staining with AlexaFluor-phalloidin). This effect persisted during the 1-hour post-exposure incubation of cells in control conditions. Presence of p38MAPK inhibitor, SB203580, in cell culture medium has prevented increase in AlexaFluor-phalloidin staining (panel B). Large magnification of cells shown in panel C demonstrates difference in distribution of AlexaFluor-phalloidin stained stress fibers (green colour) in cells with high (cell on the right) and low (cell on the left) content of hsp27 protein (indirect immunohistochemical staining; red fluorescence).

Step-4: Generation of hypothesis based on molecular and physiological events

The above results (Leszczynski et al. 2002) have formed basis and support for our working hypothesis (Figure 132, Step-4). Stabilisation of stress fibers and caused by it cell shrinking, when occurring in endothelial cells lining brain's capillary blood vessels, might be of importance for the functioning of blood-brain barrier. Stabilisation of stress fibers and cytoplasmic distribution of F-actin was previously shown to cause: (i) cell shrinkage (Landry and Huot 1995; Piotrowicz and Levin 1997a), that might lead to opening of spaces between cells, (ii) increase in the permeability and pinocytosis of endothelial monolayer (Deli et al. 1995; Lavoie et al. 1993), (iii) increase in formation of the so called “apoptosis-unrelated” blebs on the surface of endothelial cells (Becker and Ambrosio 1987), which eventually might obstruct blood flow through capillary blood vessels, (iv) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, to secrete larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997b) which might, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells and possibly led to the associated with cell's proliferative state - cell shrinkage and unveiling of basal membrane. Also, the activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome c), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which is activated by caspase-9. Thus, induction of the

increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells. Therefore, based on the known cellular role of over-expressed/phosphorylated hsp27 we have proposed a hypothesis (Leszczynski et al. 2002) that: the activation (phosphorylation) of hsp27 by mobile phone radiation might be the molecular mechanism (i) regulating increase in blood-brain barrier permeability, which would explain, observed in some animal experiments, increase in blood-brain barrier permeability, and (ii) regulating apoptosis through interference with the cytochrome c/caspase-9/caspase-3 pathway (Figure 139). Thus, it is possible that the RF-EMF might have effect on cytoskeleton-related and on the apoptosis-related cell functions. This notion supports and justifies further examination of cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

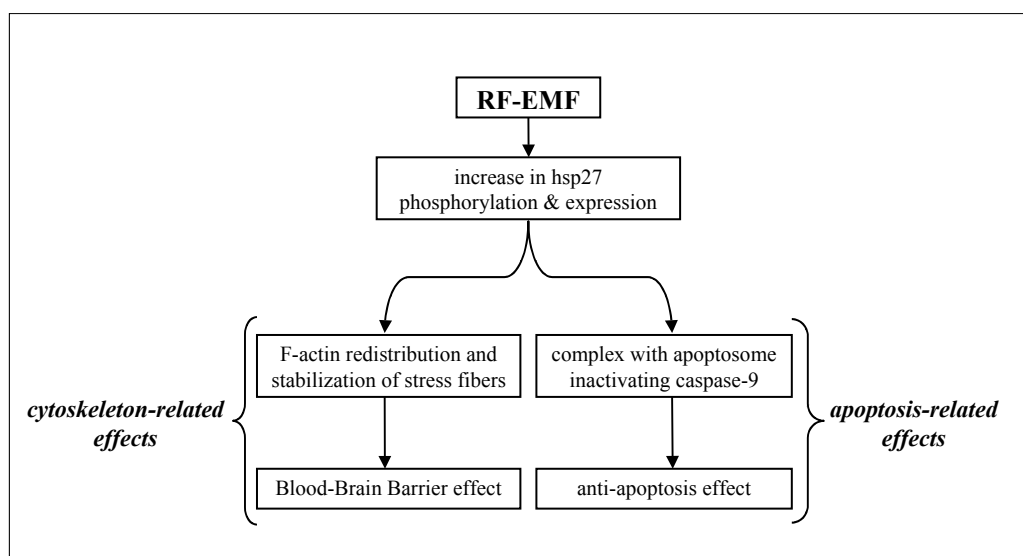


Figure 139. Hypothesis based on the molecular and physiological events – Step-4. Based on the known functions of hsp27 we have proposed that RF-EMF induced hsp27 phosphorylation might affect cell cytoskeleton and cell apoptosis. For full scheme and description of hypothesis see reference Leszczynski et al. 2002.

Step-5: HTST-identification of new target events, with support of hypothesis

Further experiments using HTST have revealed additional information pertinent to the cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

The suggested changes in the cytoskeletal proteins were detected using 2-DE separated proteins. Approximately 1300 protein spots were detected 2-DE. Comparison of the control and exposed samples revealed some 49 protein spots which were statistically significantly (student T-test, $p < 0.05$, $n = 10$) affected by the exposure (increased or declined expression). Few of the spots were selected for the mass spectrometry identification using the following criteria: spots needed to be (i) enough separate from the adjacent spots, (ii) sufficiently large and (iii) well focused in all dimensions. Cytoskeletal proteins vimentins (Figure 140) and tubulin (not shown) were identified by mass spectrometry among the proteins that responded to RF-EMF. The suggested interference with apoptosis was further examined using cDNA Expression Arrays (Clontech) and screening expression of 3600 different genes. Among the genes that were down-regulated in cells exposed to RF-EMF were numerous genes encoding proteins of Fas/TNF α -apoptotic pathway (Figure 141). This pathway was suggested as target for the RF-EMF induced phosphorylated hsp27. Therefore, concomitantly observed increase in hsp27 phosphorylation, that is anti-apoptotic event, and down-regulation of proteins of Fas/TNF α apoptotic pathway suggest that further studies aiming at elucidation of RF-EMF effect on cell apoptosis are justified.

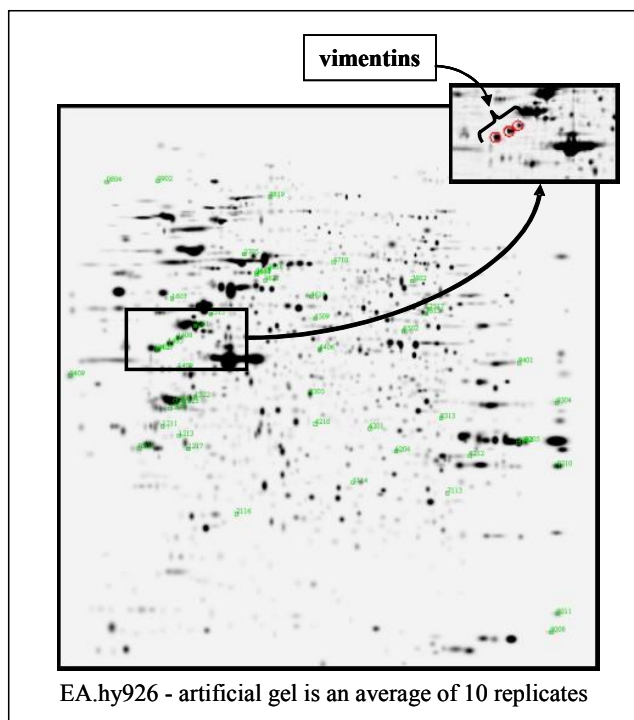


Figure 140. HTST-identification of new target events – cytoskeleton-related – Step-5. Using larger 2-DE gels (20x20 cm) and 10 replicates of each run we have identified some 49 proteins that, in statistically significant fashion, have altered their expression following RF-EMF exposure. Among the mass spectrometry identified spots were cytoskeletal proteins vimentins (inset) and tubulin (not shown).

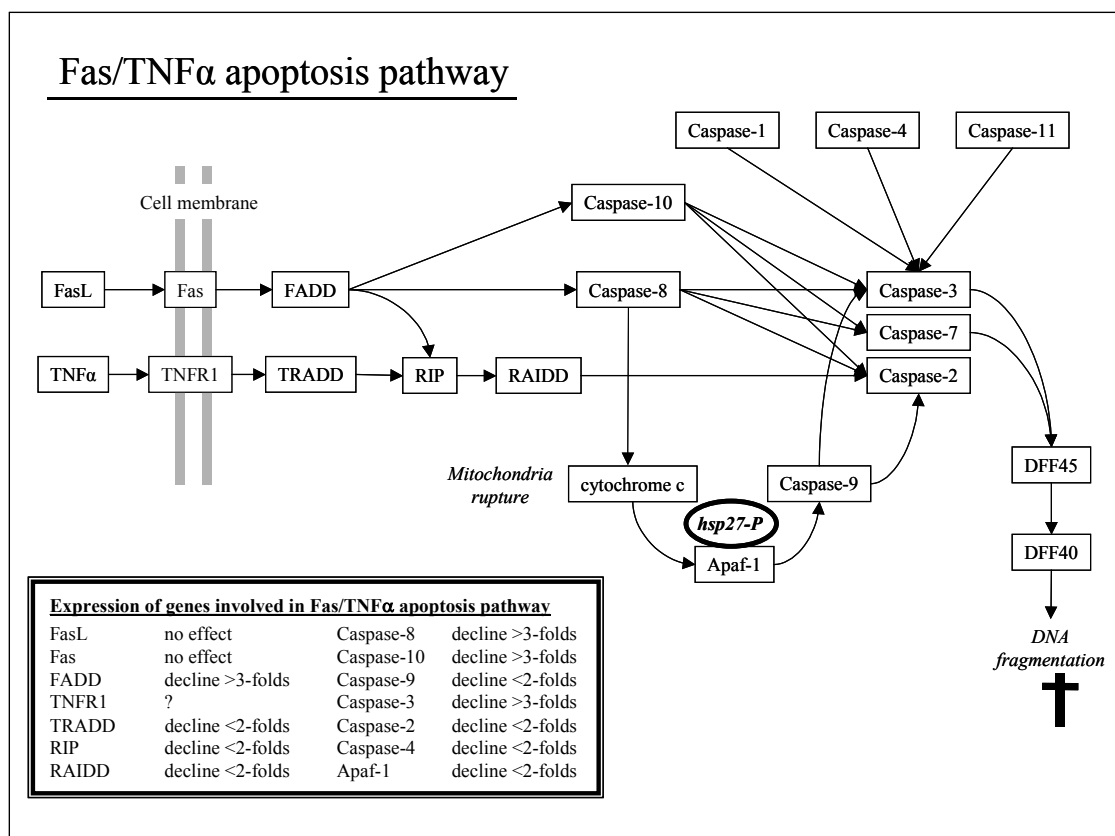


Figure 141. HTST-identification of new target events – apoptosis-related – Step-5. Analysis of RF-EMF-induced expression changes, using cDNA Expression Array for 3600 tumour-related genes, has revealed that the majority of genes that encode proteins forming Fas/TNF α apoptotic pathway are down-regulated (Table inset).

B. Genotype-dependent cell response to 900 MHz GSM radiation

We have compared response to mobile phone radiation of two human endothelial cell lines: fast proliferating EA.hy926 (Edgell et al., 1983) and its slow proliferating variant EA.hy926v1 (derived by sub-cloning from the EA.hy926 cell line).

Proteomics approach

Using 2-DE and MALDI-MS proteomics approach we have determined what proteins respond to the mobile phone radiation. Using PDQuest 6.2 software (Bio-Rad, UK) the 2-DE artificial gels (Figure 135) were generated from 10 independent protein samples from ten independent replicates of controls and irradiated cell cultures. The protein expression pattern in ten replicate control samples was then compared with the protein pattern in ten replicate irradiated samples. The normalised spot volumes of the proteins from control and exposed sample gels were statistically analysed using student t-test at the confidence level of 95%. The most striking observation was that the comparison in-between the two cell lines showed that their protein expression patterns are very different in spite of the closely related origin of both cell lines (Figures 142A, 142B). Only approximately half of all of the protein spots could be matched confidently between the cell lines. This difference in protein expression pattern might explain the observed differences in the growth rate between the cell lines. Because of the observed differences in the protein expression and proliferation between the cell lines, it was not a surprise that the response to the mobile phone radiation also varied between EA.hy926 and EA.hy926v1 cell lines. The comparison of the exposed and control samples has shown several tens of protein spots with radiation-induced statistically significant change in expression levels (t-test $p < 0.05$). In EA.hy926 cell line there were 38 of protein spots which expression was altered by the radiation exposure (Figure 142A) whereas in EA.hy926v1 cell line there were 45 differentially expressed protein spots (Figure 142B). The identity of

the all radiation-responding protein spots is being determined by MALDI-MS and will be reported in due time.

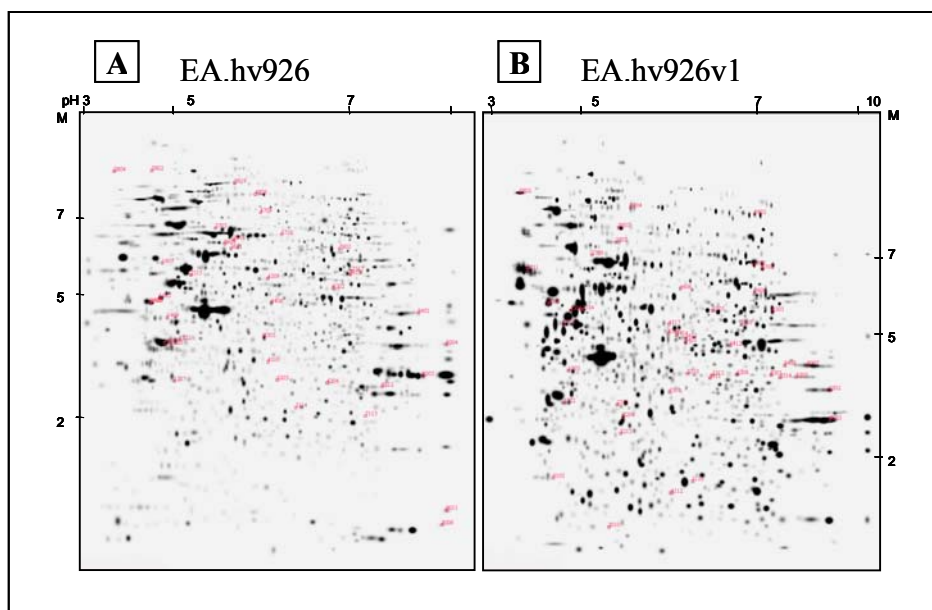


Figure 142. 2-DE gels of proteins extracted from human endothelial cell lines; EA.hy926 (A) and EA.hy926v1 (B). The 1st dimension IEF using pH gradient 3-10 NL, 2nd dimension 8% SDS-PAGE. Statistically significantly (t-test $p < 0.05$) differing spots in the cell lines are numbered using PDQuest SSP numbers.

(A) EA.hy926 cell line - 38 statistically significantly differing spots. Four spots: vimentin (1402 and 1405), isocitrate dehydrogenase 3 (NAD⁺) alpha (4305), and heterogeneous nuclear ribonucleoprotein H1 (4406), were identified using mass spectrometry.

(B) EA.hy926v1 cell line; 45 statistically significantly differing spots.

Few of the protein spots, which expression was statistically significantly altered by the irradiation, were identified using MALDI-MS (Figure 143). In order to increase probability of a single protein present in the single spot, the protein spots that were selected for MALDI-MS analysis had to fulfil the following requirements: (i) spots were well separated from other spots in both 2-DE dimensions, (ii) spots were sufficiently large (Figure 143A). The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the Helsinki University, Finland. The selected spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with a sequence-grade modified trypsin (Roche, France). The peptide mixture was concentrated and desalted using Millipore ZipTipTM μ -C18 pipette tips. The peptide mass fingerprints were measured with Bruker BiflexTM MALDI-ToF mass spectrometer in a positive ion reflector mode using α -cyano-4-hydroxycinnamic acid as a matrix. The database searches were performed using ProFound and Mascot searches. The protein spots that were identified with MALDI-MS were as follows (Figure 143):

- Protein spot 4305 - isocitrate dehydrogenase 3 (NAD⁺) alpha (Kim et al. 1995) is a subunit of the mitochondrial enzyme, which catalyses the conversion of isocitrate to 2-oxoglutarate in the citric acid cycle. The expression level of this protein was moderately down-regulated in the exposed samples having a ratio exposed vs. control 0.72 with the p-value of 0.03. The down-regulation of this protein might affect cellular energy production.
- Protein spot 4406 - heterogeneous ribonucleoprotein H1 (Honore et al. 1995) is a component of the heterogeneous nuclear ribonucleoprotein (HNRNP) complexes which provide a substrate for the processing events which pre-mRNAs go through before becoming functional mRNAs in the cytoplasm. The expression level of this protein is slightly down-regulated in the exposed samples with a ratio exposed vs. control 0.61 with the p-value 0.03. The potential down-regulation of this protein might affect protein translation process.

- Protein spots 1402 and 1405 - vimentin (Ferrari et al. 1986) is a protein component of class III-intermediate filaments. In EA.hy926 cells it was found to be expressed in at least two different iso-forms differing in molecular weight and isoelectric point. Both vimentin iso-forms were up-regulated; spot 1402 (experimental MW/pI ca. 47 kDa/4.4) by 2.5-fold with p-value of 0.006 and spot 1405 (experimental MW/pI ca. 48kDa/4.8) by 2.2-fold with p-value of 0.02.

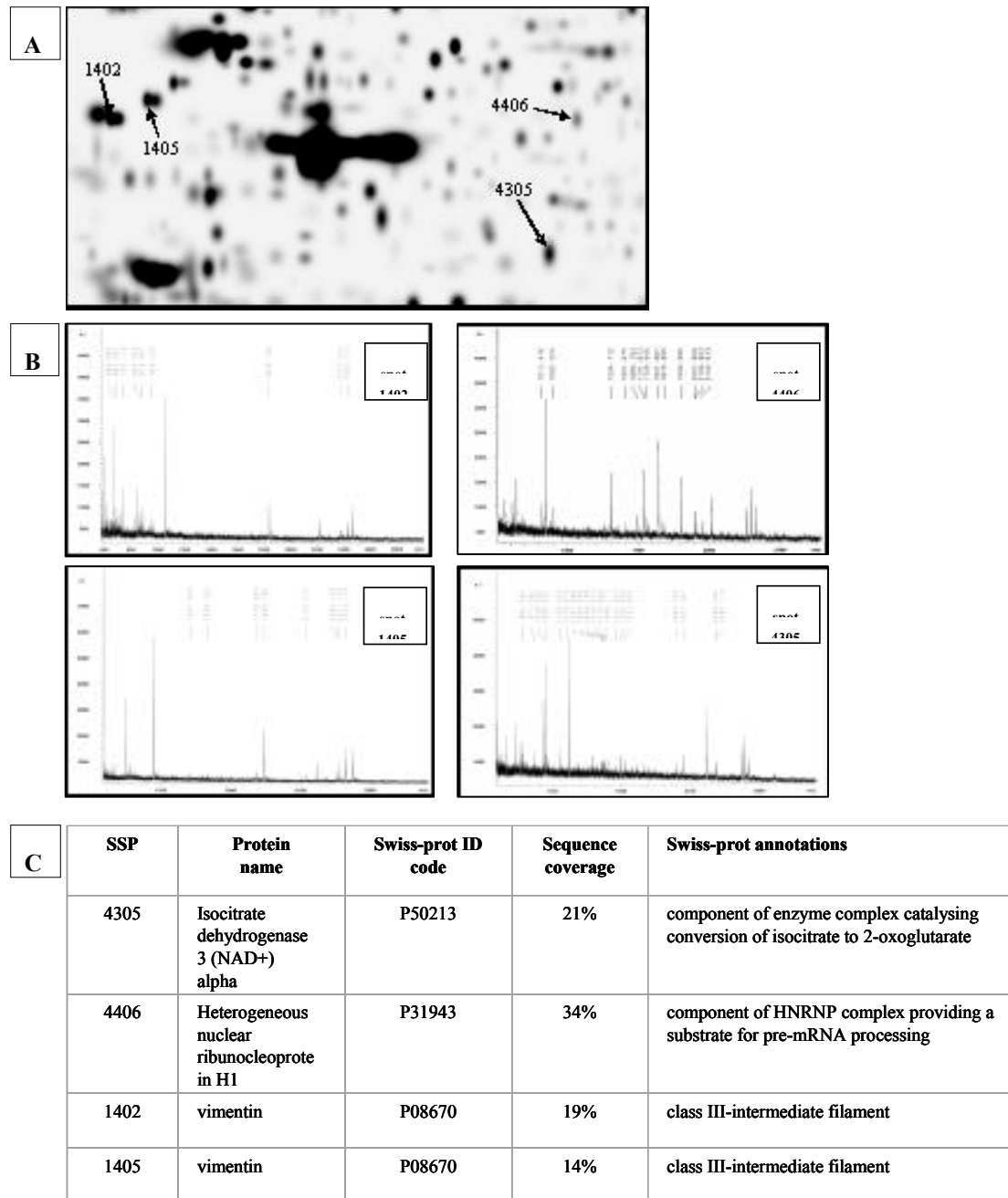


Figure 143. (A) Fragment of the 2-DE gel of EA.hy926 cells with marked spots that were identified with MALDI-MS. (B) MALDI-MS spectra showing peptide finger prints of the four identified protein spots. (C) Table summarising the properties of the identified proteins.

Alterations in the vimentin expression suggest that some form of cytoskeletal response might be taking place in cells exposed to the mobile phone radiation. This notion agrees with our earlier observation of the effect of the mobile phone radiation on the stability of F-actin stress fibers (Leszczynski et al. 2002; Leszczynski et al. 2004). Changes in the vimentin expression observed in 2-DE were further confirmed by SDS-PAGE and western blotting and by cell staining using indirect immunofluorescence method. For SDS-PAGE/western blotting a standard protocol was used. Briefly, the cell lysates were separated using 7.5% SDS-PAGE, blotted to PVDF-membrane, blocked with 5% non-fat dry milk and exposed to the primary vimentin antibody (Zymed, USA) and the secondary antibody containing a HRP-conjugate (Dako, Denmark). The signal was detected using enhanced chemiluminescence (Pierce, UK). For immunocytochemistry cell were fixed in 3% paraformaldehyde, membranes were permeabilised in 0.5% Triton X-100 and as a primary antibody was used vimentin antibody (Zymed, USA) and the secondary antibody was TRICT-conjugated (Dako, Denmark). The images were captured using a Leitz fluorescence microscope and computerised image acquisition system (Metafer, Germany).

SDS-PAGE and western blot have confirmed that EA.hy926 cells express two iso-forms of vimentin. The higher molecular weight form (experimental MW ca. 57 kDa) was present both in control and in irradiated cells and its expression was not affected by the irradiation (Figure 144A). The lower molecular weight vimentin (experimental MW ca. 48 kDa) was not detectable in the non-irradiated cells but was expressed in the irradiated cells (Figure 144B). Indirect immunohistochemistry staining of vimentin has shown the change in the distribution pattern of the vimentin filaments after the exposure to the mobile phone radiation (Figure 143C, 143D). Together, the observed changes in the vimentin expression suggest that the mobile phone radiation might potentially alter cell physiology by affecting cellular cytoskeleton.

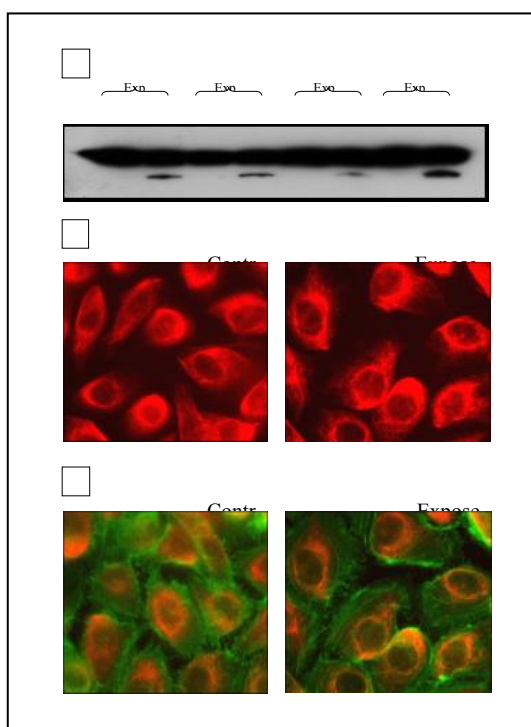


Figure 144. (A) Western blot-detected expression of vimentin in non-irradiated EA.hy926 cells (C-lanes) and in exposed cells (RF-lanes). MW ca. 57 kDa and ca. 48 kDa. Four separate experiments are shown. (B) Immunostaining of vimentin in non-exposed and in exposed EA.hy926; red colour – vimentin. Note diffuse-like staining for vimentin in non-exposed cells as compared with more filament-like expression in exposed cells. (C) Immunostaining of vimentin (red colour) and F-actin stress fibers (green colour) in non-exposed and in exposed EA.hy926. F-actin was detected with phalloidin-AlexaFluor. Note diffuse-like staining for both vimentin and F-actin in non-exposed cells as compared with more filament-like expression in exposed cells.

Transcriptomics approach

Using cDNA Expression arrays (Clontech, USA) we have determined that number of genes increased/declined expression in both cell lines following the exposure to mobile phone radiation (900 MHz GSM). Most strikingly, genes that were up regulated in one of the cell lines were down-regulated or not affected in the other cell line (Tables 29, 30, and 31). It suggests that the cell response might depend on the genotype.

Table 29. Genes which were up-regulated in EA.hy926 cell line following to the exposure to the 900 MHz GSM.

Gene name	EA.hy926 (fast proliferating)		EA.hy926v1 (slow proliferating)	
	Ratio RF/sham	Difference RF-sham	Ratio RF/sham	Difference RF-sham
Increased expression in EA.hy926				
proliferating cell nucleolar antigen P120; NOL1	29,71	18734	0,38	-6343
Homo sapiens mRNA for beta 2-microglobulin	14,70	3895	0,50	-5312
MCM7 DNA replication licensing factor; CDC47 homolog; p1.1-MCM3	12,18	9029	0,98	-172
zinc-finger protein (ZNFT7) (fragment).	8,43	15053	0,46	-2512
chloride conductance regulatory protein ICLN; nucleotide-sensitive chloride channel 1A; chloride ion current inducer protein (CLCI); reticulocyte PICLN	5,37	16944	0,79	-1686
HHR23A; UV excision repair protein protein RAD23A	4,42	20499	0,66	-7485
ferritin heavy chain (FTH1); FTHL6	4,30	6961	0,93	-2435
CD166 antigen precursor (activated leukocyte-cell adhesion molecule) (ALCAM).	4,09	3184	0,70	-2869
nucleolar phosphoprotein B23; nucleophosmin (NPM); numatrin	3,89	50540	0,97	-1699
annexin IV (ANX4); lipocortin I; calpactin II; chromobindin 9; phospholipase A2 inhibitory protein	3,68	7176	4,20	15254
N4-(beta-N-acetylglucosaminy)-L-asparaginase precursor (EC 3.5.1.26) (glycosylasparaginase) (aspartylglucosaminidase) (N4-(N-acetyl-beta-glucosaminy)-L-asparagine amidase) (AGA).	3,63	13266	0,55	-1060
glial growth factor 2 precursor (GGFHPP2); neuregulin; heregulin-beta3 + neu differentiation factor + heregulin-alpha	3,53	21908	0,70	-3070
serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit (PP-1A)	3,31	7521	1,08	1402
flavin reductase (EC 1.6.99.1) (FR) (NADPH-dependent diaphorase) (NADPH-flavin reductase) (FLR) (biliverdin reductase B) (EC 1.3.1.24) (BVR-B) (biliverdin-IX beta-reductase) (green heme binding protein) (GHBP)	3,17	15395	0,77	-2827
cytochrome c	2,97	12337	0,82	-2047
DNA-directed RNA polymerase II 19 kD polypeptide (EC 2.7.7.6) (RPB7).	2,88	12043	0,88	-1360

Table 30. Genes which were down-regulated in EA.hy926 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926 (fast proliferating)		EA.hy926v1 (slow proliferating)	
	Ratio RF/sham	Difference RF-sham	Ratio RF/sham	Difference RF-sham
Decreased expression in EA.hy926				
pyruvate kinase M2 isozyme (PKM2)	0,33	-6273	0,90	-2490
RAD51-interacting protein	0,31	-3405	0,79	-510
glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	0,29	-3486	1,59	599
glutathione S-transferase A1 (GTH1; GSTA1); HA subunit 1; GST-epsilon	0,29	-4095	0,36	-2545
early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225	0,28	-4223	0,54	-1932
caspase-3 (CASP3); apopain precursor; cysteine protease CPP32; YAMA protein; SREBP cleavage activity 1; SCA-1	0,27	-5844	1,04	79
calpain 2 large (catalytic) subunit; M-type calcium-activated neutral proteinase (CANP)	0,27	-3464	0,95	-1210
ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25	0,26	-6905	1,10	730
alpha-actinin 1 cytoskeletal isoform; F-actin cross linking protein	0,25	-5276	0,82	-2597
ras-related protein RAB-11B; YPT3	0,24	-2870	0,79	-3643
DNA ligase I; polydeoxyribonucleotide synthase (ATP) (DNL1) (LIG1)	0,22	-2969	0,38	-2497
ATP synthase lipid-binding protein P2 precursor (EC 3.6.1.34) (ATPase protein 9) (subunit C)	0,19	-4520	0,80	-1486
EDF-1 protein	0,14	-6951	0,68	-2931
coatamer delta subunit; delta-coat protein; delta-COP; archain (ARCN1)	0,14	-4280	n/a	n/a
nuclear transport factor 2 (NTF-2) (placental protein 15) (PP15).	0,14	-2836	0,65	-2155
fascin (actin bundling protein).	0,14	-16072	0,54	-6852
neurogranin (NRGN); RC3	0,11	-18728	0,86	-2175
MYLE	0,11	-4959	0,81	-539
sepiapterin reductase (EC 1.1.1.153) (SPR).	0,10	-3467	0,63	-1123
caspase-8 precursor (CASP8); ICE-like apoptotic protease 5 (ICE-LAP5); MORT1- associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease (FADD-like ICE; FLICE); apoptotic cysteine protease MCH-5	0,05	-3359	1,90	1080

Table 31. Genes which were affected in EA.hy926v1 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926v1 (slow proliferating)		EA.hy926 (fast proliferating)	
	Ratio RF/sham	Difference RF-sham	Ratio RF/sham	Difference RF-sham
Increased expression in EA.hy926v1				
procollagen C-proteinase enhancer protein precursor.	9,58	10373	1,09	5589
HOMER-3.	9,45	3027	0,98	-25
T-lymphoma invasion and metastasis inducing TIAM1	5,55	6554	0,01	-1335
elafin precursor (elastase-specific inhibitor) (ESI) (skin-derived antileukoproteinase) (SKALP).	5,33	10720	1,53	12919
mitochondrial matrix protein P1 precursor; p60 lymphocyte protein; chaperonin homolog; HUCHA60; heat shock protein 60 (HSP-60); HSPD1	4,43	8459	1,00	-89
proteasome component C8; macropain subunit C8; multicatalytic endopeptidase complex subunit C8	4,06	5029	1,10	5254
special AT-rich sequence binding protein 1 (SATB1); MAR/SAR DNA-binding protein	3,48	11151	0,99	-222
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	3,12	2805	0,64	-1344
ras-related protein RAP-1B; GTP-binding protein SMG p21B	3,04	4820	63,80	2512
phospholipase A2; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ); 14-3-3 protein zeta/delta; protein kinase C inhibitor protein 1 (KCIP1); factor activating exoenzyme S (FAS)	2,93	4033	1,14	767
Decreased expression in EA.hy926v1				
tuberin; tuberous sclerosis 2 protein (TSC2)	0,33	-7082	0,42	-16846
KIAA0115; dolichyl-diphosphooligosaccharide protein glycosyltransferase 48-kDa subunit precursor; oligosaccharyl transferase 48-kDa subunit; HA0643	0,27	-3494	0,19	-1968
sodium channel beta-1 subunit precursor (SCN1B)	0,27	-2842	0,00	-1846
embryonic growth/differentiation factor 1 (GDF1) + UOG-1	0,21	-3542	0,00	-702
SH3P18 SH3 domain-containing protein	0,05	-3389	0,00	-949

C. Comparison of the effect of CW and modulated RF-EMF on protein expression

Using cICAT method combined with liquid-phase chromatography and mass spectrometry we have compared protein expression changes in cells exposed either to continuous wave or to radiofrequency modulated (“talk” signal) RF-EMF (1800 MHz GSM). The cICAT reagent labelled samples were analysed using an automated mass spectrometric approach in which those proteins showing abundance differences between the two conditions being compared were selectively identified. In total, 58 unique proteins were identified and determined to show significant changes in abundance using this approach. These proteins were selected for identification by MS/MS analysis based upon the criteria that the measured abundance ratios ($C^{13}(0)/C^{13}(9)$) were either >1.7 or <0.60 . The average abundance ratio for all detected cICAT reagent labelled peptide pairs ($n=1476$) was 1.26 ± 0.38 , indicating that the vast majority of proteins within the two samples did not change in abundance. Peptides detected as singlets (i.e. having no corresponding $C^{13}(0)$ or $C^{13}(9)$ signal) were also selected for MS/MS analysis. The threshold abundance ratio values were selected based on the following criteria: 1) In relation to previously described errors of quantitative measurements using the ICAT reagent, these values represent conservative estimates of significant abundance changes; 2) These values are significant outliers relative to the average and median $C^{13}(0)/C^{13}(9)$ values for this dataset. The average $C^{13}(0)/C^{13}(9)$ value for all detected cICAT reagent labelled peptides ($n = 1476$) was 1.26 ± 0.38 , and the median was 1.19. The threshold values for significant changes in abundance are therefore well outside the standard deviation for this dataset. Furthermore, the fact that the average and median values are close to one indicates the accuracy of the quantitative measurements used here, as it is expected that the majority of proteins will be constitutively represented, giving ratios close to one.

In conclusion it appears that the “talk” signal has caused increase in expression of a variety of proteins whereas CW did not (Table 32). It suggests that the modulation might have impact on cell response to RF-EMF.

Table 32. List of proteins induced by “talk” signal but not by the CW signal.

Protein name	Accession No.	C ¹³ (0)/C ¹³ (9)	Confidence score
serine-threonine protein kinase	NP_055212	1.9	0.996
GP:AF090929_1		1.8	0.912
RING finger protein 20	AAK58539	2.0	0.958
Homo sapiens cDNA FLJ20303	AK000310	2.4	0.957
hypothetical protein FLJ20420	NP_060282	2.0	0.984
fatty-acid synthase	PIR:G01880	2.2	0.993
hypothetical protein DKFZp434G171.1	T42678	1.9	0.855
hypothetical protein DKFZp564N1563.1 (2)	T46270	0.5	0.921
Serine/threonine protein phosphatase 2A	Q15173	1.8	0.950
Beta-adaptin 1	Q10567	2.0	0.813
Actin-like protein 2	O15142	2.0	0.993
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (2)	P16615	1.9	0.944
CD59 glycoprotein precursor (6)	P13987	2.4	0.995
Chloride intracellular channel protein 1	O00299	2.7	0.997
Cellular nucleic acid binding protein (2)	P20694	2.2	0.980
Cofilin, non-muscle isoform	P23528	1.7	0.990
Coatmer alpha subunit	P53621	2.4	0.997
Coatmer beta subunit	P53618	2.0	0.993
Cleavage and polyadenylation specificity factor	Q9P210	1.9	0.994
Cyclophilin A (3)	P05092	2.2	0.975
Destrin (Actin-depolymerizing factor)	P18282	1.7	0.990
Aspartyl aminopeptidase	Q9ULA0	1.8	0.993
D-dopachrome tautomerase	P30046	1.7	0.952
Elongation factor 2	P13639	2.0	0.982
Alpha enolase	P06733	2.1	0.981
Fatty acid synthase (2)	P49327	2.1	0.975
Filamin A (2)	P21333	2.5	0.975
FL cytokine receptor precursor	P36888	1.9	0.970
Follistatin-related protein 1 precursor Q12841	Q12841	1.7	0.942
PROTEIN KINASE C SUBSTRATE	P14314	1.7	0.984
Transducin beta chain 1	P04901	1.9	0.993
Transducin beta chain 2	P11016	1.8	0.964
Guanine nucleotide-binding protein beta subunit-like protein (3)	P25388	2.9	0.998
Stress-induced-phosphoprotein 1	P31948	2.2	0.993
Pyruvate kinase, M1 isozyme	P14618	1.9	0.983
LAM2_HUMAN, partial CDS	AAC34573	0.6	0.994
Galectin-1 (3)	P09382	1.9	0.996
Myosin heavy chain, nonmuscle type A	P35579	0.6	0.996
Myoferlin (2)	Q9NZM1	2.9	0.940
NHP2-like protein 1 (3)	P55769	3.6	0.980
Nitric-oxide synthase, endothelial (2)	P29474	2.0	0.990
Purine nucleoside phosphorylase (2)	P00491	1.9	0.997
40S ribosomal protein S27a	P14798	3.0	0.974
Heterogeneous nuclear ribonucleoprotein D0	Q14103	3.0	0.984
Heterogeneous nuclear ribonucleoprotein K	Q07244	2.6	0.880
Heterogenous nuclear ribonucleoprotein U	Q00839	2.1	0.992
ribosomal protein S2 (2)	P15880	1.8	0.940
40S ribosomal protein S3a	P49241	2.1	0.991
Splicing factor, arginine/serine-rich 9	Q13242	2.0	0.986
Tubulin beta-2 chain	P05217	2.8	0.998
T-complex protein 1, theta subunit (2)	P50990	4.9	0.995
Transcription intermediary factor 1-beta	Q13263	2.3	0.995
Thioredoxin	P10599	2.1	0.935
Hypothetical UPF0123 protein BK223H9.2	Q9UH06	2.1	0.994
Splicing factor U2AF 35 kDa subunit (2)	Q01081	2.4	0.945
Ubiquitin-activating enzyme E1	P22314	1.8	0.963
Zinc finger protein 147	Q14258	3.7	0.995
Nuclear pore complex protein Nup133	Q8WUM0	2.3	0.914

3.2.4.7 Whole-genome analysis of various cell lines exposed to RF-EMF (Participant 12)

Altogether, 58 whole-genome analyses of 10 different cell lines (sham-exposed cells and control cells) were performed (Table 1). After primary data analysis, we only worked on genes which were reproducibly regulated in several experiments (see materials and methods) and which belonged to certain gene families (Table 33). We defined gene families which are potentially relevant for the cellular answer on EMF exposure: signal transduction, ion/electron transport, metabolism of energy/proteins, cell proliferation/apoptosis, immune answer/inflammation and extracellular matrix/cytoskeleton. Each gene family was sub-divided in subgroups again, e.g. GTP proteins in the signal transduction family (Tables 33, 11). In a first step, we did not go into single genes, but simply counted genes up- or down-regulated in the different gene families. The total number of regulated genes in a certain gene family is not very meaningful, because the sizes of the gene families are of course very different. Therefore, the total numbers of genes on the human array belonging to a gene family are shown in the first column of Tables 33 and 11. Although a single gene might appear in different categories (e.g. all small G proteins are GTP binding proteins), the tables give a good overview on what might happen in the cells after EMF exposure on the molecular level.

Although appearing regulated in all experiments, mitochondrial genes, ribosomal genes and cell cycle genes especially showed a high rate of regulation in the some RF-EMF experiments (U937 human monocytic cells and microglia cells, Participant 9; HL-60 human hematopoietic leukemia cells, Participant 2).

Moreover, the bio-statistical analysis of RF-EMF experiments (Participant 8, Dr. Remondini) allowed some interesting conclusions from the experiments with HL-60 cells (Participant 2), endothelial cells (Participant 6) and U937 cells (Participant 8, Table 34). Again, the regulation of mitochondrial and ribosomal genes was evident with this analysis. Most of the regulated genes in endothelial cells appear in the groups of ATP-associated genes (energy metabolism), transcription, and cytoskeleton. Remarkably, compared to ELF-EMF experiments, we find more up-regulated genes in RF-EMF experiments (Table 34, see also Table 12). However, the results do have to be interpreted in more detail, because down-regulation of a special gene does not mean that the respective process is down-regulated as well (for example, down-regulation of Bcl-2 might lead to up-regulation of apoptosis).

For T-lymphocytes (Participant 8) and microglia cells (Participant 9) the bio-statistical analysis did not reveal significant data.

In detail, the following genes were extracted by bio-statistics so far:

Actin associated proteins (belong to cytoskeleton):

- Caldesmon (tropomyosin binding, actin binding. Activation of ERK MAP kinases lead to phosphorylation of caldesmon. Regulatory protein of the contractile apparatus): down-regulated (endothelial cells, Participant 6).
- Gamma-actin: down-regulated ((endothelial cells, Participant 6, and U937 cells, Participant 8)
- "coactosin-like": down-regulated (endothelial cells, Participant 6)
- "actin-binding": down-regulated (endothelial cells, Participant 6)
- "procollagen-proline 2": down-regulated (endothelial cells, Participant 6)
- "actin modulating activity": up-regulated (endothelial cells, Participant 6)
- "actin-binding, calcium ion binding": down-regulated (endothelial cells, Participant 6)
- CD2-associated protein, actin binding: down-regulated (endothelial cells, Participant 6)
- Tropomodulin 3: actin binding down-regulated (endothelial cells, Participant 6)

Calcium (Ca²⁺)-associated proteins:

- Ca: "hypothetical protein" (actin-binding): down-regulated (endothelial cells, Participant 6)
- "hypothetical protein": down-regulated (endothelial cells, Participant 6)
- voltage-gated Ca channel: up-regulated (perhaps up-regulated, because Ca goes down? Endothelial cells, Participant 6)

Cytoskeleton (compare also actin and calcium-associated proteins):

- "hypothetical protein": down-regulated (endothelial cells, Participant 6)
- "protein phosphatase 4, caldesmon): down-regulated (endothelial cells, Participant 6)
- "SH3 protein interacting with Nck": down-regulated (endothelial cells, Participant 6)
- "in kinesin complex": down-regulated (endothelial cells, Participant 6)

Table 33. Numbers of genes regulated within different gene families

Gene Family	total number of clones in Human Unigene RZPD-2																				Gene "Superfamily"
	partner 8 U937 monocytes RF up-regulated genes	partner 8 microglia cells RF up-regulated genes	partner 8 U937 monocytes RF down-regulated genes	partner 8 microglia cells RF down-regulated genes	partner 6 endothelial cells P1 RF 900MHz up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp1 up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 up-regulated genes	partner 6 endothelial cells P1 RF 900MHz down-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 down-regulated genes	partner 2 HL-60 RF ON/OFF up-regulated genes	partner 2 HL-60 RF continuous waves Exp1 up-regulated genes	partner 2 HL-60 RF continuous waves Exp2 up-regulated genes	partner 2 HL-60 RF ON/OFF down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes						
Signal	2528	149	40	##	45	190	176	92	160	149	149	91	153	162	190	155	72	signal transduction			
GTP	560	37	15	45	14	42	58	24	49	40	51	20	43	51	49	38	18	signal transduction			
Small G	235	16	5	20	3	14	17	10	23	15	19	8	17	18	21	18	7	signal transduction			
Jak	23	2	0	1	1	1	4	2	1	4	0	1	4	2	1	1	0	signal transduction			
Rab	80	3	1	11	3	6	6	4	6	4	9	0	5	5	6	3	5	signal transduction			
Ras	66	4	2	7	0	4	4	1	6	4	7	2	6	6	7	2	2	signal transduction			
wnt	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	signal transduction			
phosphatase	334	24	6	21	7	24	31	17	23	20	25	19	18	26	19	29	9	signal transduction			
protein kinase	304	19	6	19	4	19	24	16	27	15	16	18	16	25	23	11	13	signal transduction			
phospholipase	72	6	1	7	1	6	4	5	1	2	4	1	3	7	7	5	2	signal transduction			
calcium	715	40	6	39	14	56	51	27	45	30	45	20	45	44	45	35	13	signal transduction			
calmodulin	131	8	2	6	1	8	6	1	11	8	9	6	5	8	11	10	4	signal transduction			
channel	348	12	1	18	7	12	12	8	16	12	11	12	28	11	16	13	6	ion/electron transport			
voltage-gated	164	3	0	6	2	7	5	3	3	5	4	5	12	9	7	2	3	ion/electron transport			
electron transport	423	25	11	29	6	35	44	20	37	17	34	25	19	36	26	18	15	ion/electron transport			
ion transport	501	22	8	32	11	35	21	13	25	18	21	26	39	29	27	37	14	ion/electron transport			
metaboli	1241	80	21	81	15	98	124	68	91	71	86	66	87	110	114	64	39	metabolism of energy/proteins			
ATP	1234	81	27	92	24	75	116	49	111	77	83	86	102	104	111	82	47	metabolism of energy/proteins			
mitochon	574	50	10	63	19	55	61	32	67	46	55	49	51	76	64	66	24	metabolism of energy/proteins			
ribosom	254	39	14	39	19	32	35	15	30	25	32	33	31	23	26	37	13	metabolism of energy/proteins			
translation	168	21	2	13	9	18	14	12	25	14	18	18	12	12	21	14	17	metabolism of energy/proteins			
transcript	1991	116	39	##	41	172	142	78	144	120	129	122	143	138	138	130	75	metabolism of energy/proteins			
cell cycle	478	34	11	39	14	42	46	28	46	37	39	44	49	42	46	22	23	cell proliferation/apoptosis/differentiation			
apoptos	373	29	8	26	10	34	36	12	18	31	29	18	30	39	32	30	10	cell proliferation/apoptosis/differentiation			
differentiat	177	17	2	20	1	14	11	8	5	6	13	6	7	9	12	7	4	cell proliferation/apoptosis/differentiation			
immun	390	19	5	26	7	22	44	21	25	19	24	19	31	30	29	17	18	Immune answer/inflammation/stress answer			
inflamma	184	8	1	6	3	15	15	8	13	13	9	10	11	6	12	14	2	immune answer/inflammation/stress answer			
stress	118	5	6	7	2	8	16	5	8	5	13	5	8	12	10	11	4	immune answer/inflammation/stress answer			
peroxidase	32	2	2	7	0	3	4	2	5	3	16	1	4	4	4	5	3	immune answer/inflammation/stress answer			
heat shock	188	2	2	4	2	3	5	4	6	6	2	3	3	2	4	6	1	immune answer/inflammation/stress answer			
DNA repair	154	10	3	17	4	11	15	6	13	16	15	7	13	12	17	7	8	immune answer/inflammation/stress answer			
early	8	0	0	1	0	0	1	0	0	1	2	1	0	0	2	1	1	immune answer/inflammation/stress answer			
adhesion	573	30	5	28	9	42	44	19	42	28	38	19	34	31	32	28	14	extracellular matrix/cytoskeleton/adhesion			
extracellular matrix	226	14	4	7	5	12	12	7	7	8	8	5	11	10	11	10	8	extracellular matrix/cytoskeleton/adhesion			
cytosk	529	33	9	36	21	39	41	19	33	37	43	24	30	46	48	32	21	extracellular matrix/cytoskeleton/adhesion			
junction	129	0	3	11	5	3	10	2	9	3	10	5	5	7	4	8	2	extracellular matrix/cytoskeleton/adhesion			
actin	494	35	7	35	19	42	39	24	38	41	40	28	30	34	44	40	21	extracellular matrix/cytoskeleton/adhesion			

Table 34. Numbers regulated genes in different expression profiling experiments (bio-statistical analysis by Dr. Remondini/Participant 8)

GeneFamily	total number of clones in Human Unigene RZPD-2	partner 6 endothelial cells RF up-regulated genes	partner 6 endothelial cells RF down-regulated genes	partner 2 HL-60 cell RF up-regulated genes	partner 2 HL-60 cell RF down-regulated genes	partner 8 U937 cells RF up-regulated genes	partner 8 U937 cells RF down-regulated genes	Gene "Superfamily"
Signal	2528	4	9	1	0	1	1	signal transduction
GTP	560	1	2	1	0	0	0	signal transduction
Small G	235	0	1	0	0	0	0	signal transduction
Rab	80	0	1	0	0	0	0	signal transduction
Ras	66	0	0	0	0	0	0	signal transduction
phosphatase	334	0	1	0	0	1	0	signal transduction
protein kinase	304	0	2	0	0	0	0	signal transduction
calcium	715	1	2	0	0	0	0	signal transduction
calmodulin	131	0	1	0	0	0	0	signal transduction
channel	348	1	0	1	0	1	0	ion/electron transport
voltage-gated	164	1	0	0	0	0	0	ion/electron transport
ion transport	501	1	0	0	0	0	1	ion/electron transport
electron transport	423	1	1	0	0	1	0	ion/electron transport
metaboli	1241	3	0	0	0	2	0	metabolism of energy/proteins
ATP	1234	1	4	0	0	0	1	metabolism of energy/proteins
mitochon	574	2	1	0	0	0	0	metabolism of energy/proteins
ribosom	254	0	1	0	0	1	4	metabolism of energy/proteins
translation	168	0	0	0	0	0	0	metabolism of energy/proteins
transcript	1991	1	6	3	0	2	0	metabolism of energy/proteins
cell cycle	478	0	3	1	0	0	0	cell proliferation/apoptosis/differentiation
apoptos	373	1	0	0	0	0	0	cell proliferation/apoptosis/differentiation
differentiat	177	0	1	1	0	0	0	cell proliferation/apoptosis/differentiation
immun	390	0	0	0	0	0	0	immune answer/inflammation/stress answer
DNA repair	154	0	1	0	0	0	0	immune answer/inflammation/stress answer
inflamma	184	0	0	0	0	1	0	immune answer/inflammation/stress answer
adhesion	573	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	1	0	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
cytosk	529	0	5	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
actin	494	0	4	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
junction	129	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion

3.2.4.8 Summary (Participant 1)

Our data indicate an effect of RF-EMF on gene and protein expression in various cell systems. This conclusion is based on the following findings:

- RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in embryonic stem cells deficient of the p53 gene (3.2.4.1)
- RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats (3.2.4.2).
- RF-EMF exposure at a SAR value of 1.3 W/kg up- or down-regulated the expression of various genes and proteins in HL-60 cells and in endothelial cells of human origin (3.2.4.3, 3.2.4.6, 3.2.4.7).
- RF-EMF exposure at a SAR value of 2.4 W/kg activated the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (3.2.4.6).
- RF-EMF exposure at a SAR value of 2.4 W/kg changed the global pattern of protein phosphorylation in endothelial cells of human origin with possible consequences for the signal transduction pathway (3.2.4.6).
- RF-EMF exposure at a SAR value at 2 W/kg did not significantly affect gene expression in human lymphocytes, although a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.4, 3.2.4.5).
- RF-EMF exposure at a SAR value of 2 W/kg did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) and the expression of hsp27 and hsp70 in nerve cells (3.2.4.5).
- The increased expression of hsp27 in endothelial cells (EA.hy926) after RF-EMF exposure as described in 3.2.4.6 could not be reproduced in another laboratory where slightly different methods were used (3.2.4.5).

4.0 DISCUSSION

4.1 Results obtained after ELF-EMF exposure

4.1.1 Genotoxic effects

4.1.1.1 Human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and granulosa cells of rats (Participant 3)

Intermittent ELF-EMF exposure generated DNA strand breaks in various but not all cell lines.

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a reproducible increase in DNA strand breaks in cultured human cells. These findings are in accordance with some recent studies with whole-body exposure of rodents to ELF-EMF which revealed DNA single- and double-strand breaks in the brain (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the majority of the studies investigating genotoxic effects of 50/60 Hz electromagnetic fields (McCann et al. 1993, 1998; Murphy et al. 1993; Moulder 1998) have reported a negative outcome on genotoxicity. Our results from tests with continuous exposure of fibroblasts to EMF corroborate these findings. Subjecting cells continuously to a constant field probably may induce adaptive mechanisms, protecting the genome from harmful influences. A regular change of environmental conditions might interfere with such mechanisms and lead to DNA damage. The extent of damage would depend on the duration of exposure and the time of recovery.

It is highly unlikely, that the observed genotoxic damage is caused non-specifically by spots of increased temperature within the cell layer as a secondary effect of the electromagnetic field. If so, the damage would increase with a prolongation of on-time during the intermittent exposure and would be largest at continuous exposure. The largest effects, however, are obtained at 5 min on/10 min off cycles, and continuous exposure had no effect at all. Therefore, we conclude, that the observed induction of DNA-single and double strand breaks is a direct consequence of an intermittent exposure to ELF-EMF.

Environmental exposure to continuous ELF-EMF is rather exceptional. Different electrical household devices (hair dryer, razor, vacuum cleaner) reaching peak values up to 1 mT are often used for a short period of time (5-10 min), producing a variety of exposure levels. To date, we could make out only one study dealing with genotoxic effects of ELF-EMF at intermittent exposure. This was done by Nordenson et al. (1994), who found a significant increase of chromosome aberrations in human amniotic cells (50 Hz, 30 μ T, 20 s on-off). However, these results have not been corroborated by other studies as yet.

Genotoxic effects of ELF-EMF varied with exposure time.

We observed a time dependent increase of DNA breaks up to 15 to 19 hours of ELF-EMF exposure and then a decline to a steady state level of about 1.5 fold of the base line. This unexpected finding can be explained, if the exposure activates DNA repair processes and this activation takes a time of 10 to 12 hours. After this time the DNA damage is repaired then at an enhanced rate, which leads to a reduction of DNA breaks albeit not to a normalisation. This explanation is experimentally supported by the observation, that the single strand DNA breaks (alkaline conditions) are repaired after approximately 30 minutes, and double strand breaks 7 to 9 hours after finishing the exposure. Removal of damaged DNA-bases by induced repair enzymes (glycosylases) may lead to a temporary increase of abasic sites in the DNA (Friedberg et al. 1995). Abasic sites (alkali-labile sites) result in DNA single strand breaks after alkaline treatment (Tice et al. 2000). The alkali-labile sites generated after ELF-EMF exposure are therefore detected as peak at hour 12 to 17 at Comet assay conditions of pH > 13, but not of pH 12.1, the latter not being able to cleave the alkali sensitive sites.

It is well known, that the repair of single strand breaks is a fast and almost error free process, while the repair of more complex DNA damage (i.e. DNA double strand breaks) by homologous recombination, single strand annealing or non-homologous end joining require more time and are error prone in part (Van den Bosch et al. 2002). Therefore DNA double strand breaks may affect the integrity of the genome leading to cell death, uncontrolled cell growth or cancer (Van Gent et al. 2001).

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a time dependent increase in micronuclei in cultured human fibroblasts. These findings are in accordance with Simko et al. (1998a/b), who could demonstrate an ELF-EMF-induced formation of micronuclei in human amnion and in human squamous cell carcinoma cells. In contrast, the greater part of studies performed as yet using different cell types did not point to direct clastogenic effects of ELF-EMFs (Livingston 1991; Scarfi et al. 1991, 1994; Paile et al. 1995), but they propose epigenetic or co-clastogenic mechanisms in combination with other genotoxic exposures (Lagroye et Poncy 1997; Cho and Chung 2003; Simko et al. 2001b). Micronucleus formation can either result from chromosomal non-disjunction due to damage of kinetochore proteins or from acentric fragments secondary to DNA double strand breaks. Since we did not use kinetochore antibodies to differentiate between these two possible mechanisms, the cause for the micronuclei induction remains an open question. At extended exposure times micronucleus frequencies reached a constant level, which is not in contrast to the results found in Comet assay since micronuclei cannot be repaired.

ELF-EMF produced DNA strand breaks in human fibroblasts in a dose dependent way.

We could demonstrate a dose dependent relationship between alkaline and neutral Comet assay tailfactors and applied magnetic flux density. The guidelines of the International Commission on Non-Ionizing Radiation Protection (ICNIRP 1998) are 500 μ T during workday for occupational exposures and 100 μ T for 24 h/day for the general population. The on-set of genotoxic effects in our tests was at a magnetic flux density as low as 35 μ T at 15 hours and 70 μ T at 24 hours of exposure, being well below these proposed guideline values. Moreover, these guidelines are dealing with continuous EMF exposure. No proposal how to handle intermittent exposures has been made by the ICNIRP as yet.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was related to the age of the donors.

Our findings of significant differences in basal DNA single and double strand break levels in fibroblasts of donors of different age are consistent with studies of several species and tissues (Mullaart et al. 1988; Holmes et al. 1992; Zahn et al. 1996; Diem et al. 2002). In addition, we here report differences in response to ELF-EMF exposure in relation to donor age, which point to a higher susceptibility of older donors to the genotoxic action of ELF-EMF. This could be interpreted by a later on-set of DNA repair. These findings are in agreement with age-related increases of DNA damage and mutations as a result of a reduced DNA repair capacity (Wolf et al. 2002; Bohr 2002; Cabelof et al. 2002; Ben Yehuda et al. 2000; Goukassian et al. 2000). Observations of an altered gene activity during ageing were reported for the rat brain, heart, and liver (Salehi et al. 1996; Goyns et al. 1998) and human fibroblasts (Linskens et al. 1995). This decline may be due to a reduction in chromatin associated RNA polymerase II activity (Rao and Loeb 1992), to mutation-induced changes in binding activity of transcription factors (Sheerin et al. 2001), or due to a decline in protein synthesis secondary to a decrease in the amount and activity of certain elongation factors (Shikama et al. 1994). Changes in the availability of proteins or enzymes may be critical if proteins of DNA repair machinery are affected.

Effects of ELF-EMF were cell type specific.

Our results point to cell type specific reaction and to differences in sensitivity of different tissues to ELF-EMF exposure. We could identify three responder (human fibroblasts, human melanocytes, transformed rat granulosa cells) and three non-responder cell types (human lymphocytes, monocytes and skeletal muscle cells). Up to date a plausible mechanism for these findings is mere speculation, but these data propose an epigenetic, indirect action of intermittent ELF-EMF. The observed cell specific response can not be explained by age-related effects, since the non-responding skeletal muscle cells are derived from the oldest donor.

In our experiments we exposed dividing and quiescent lymphocytes to ELF-EMF, and in both cases no induction of DNA strand breaks could be observed. Isolated monocytes did not respond either. The other cell types used were cells in the log-growing phase and some of them showed genotoxic effects, whereas dividing skeletal muscle cells did not react. Therefore, it is not likely that the observed effects could be due to differences in response between proliferating and non-proliferating cells. In addition, the observed effects can not be attributed to differences between adherent cells or suspension cultures, since there are non-responder cell types in both cases.

Based on the results with human fibroblasts, which suggest an induction of DNA repair upon intermittent ELF-EMF exposure, we speculate, that the effects reported here, may reflect differences in DNA repair

capacities between different tissues. This explanation, however, requires further assessment, e. g. evaluation of repair kinetics.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF and their repair were modified by UVC or heat stress.

During ELF-EMF exposure UVC induced DNA-damage was repaired very slowly, although the maximum at 15h ELF-exposure could not be detected any more. The results were similar with the neutral Comet assay, but DNA damage (DNA double strand breaks) was repaired within a shorter time. These results suggest that ELF-EMF-exposure might impair and/or delay the onset of repair of DNA damage.

In regard to studies on repair kinetics, the exposure time dependent extent of DNA damage implicated an induction of DNA repair upon intermittent ELF-EMF exposure. We concluded that pre-exposure to intermittent ELF-EMF would have a protective effect and reduce genotoxic actions of additional exposures. In contrast to our assumption, pre-exposure to intermittent ELF-EMF for 20 hours resulted in an additive genotoxic effect of combined exposures and a reduced repair rate of UVC or heat stress induced DNA damage. A protective effect of ELF-EMF exposure could not be confirmed by these results. In contrast, they suggest an impairment or delay of DNA repair mechanisms due to ELF-EMF exposure.

Recently, Robison et al. (2002) have demonstrated that pre-exposure to ELF-EMF for 4 to 24 hours can decrease DNA repair rate and protect human HL-60 cells from heat induced apoptosis. Miyakoshi et al. (2000) showed that strong ELF-EMF for 2 hours can potentiate X-ray-induced DNA strand breaks in human malignant glioma cells, whereas others (Whitson et al. 1986; Frazier et al. 1990; Cantoni et al. 1996) found no evidence that ELF-EMF could inhibit repair of DNA damage induced by ionising radiation or UV light using different human cell types. However, in these experiments ELF-EMF exposure was not performed prior to UV or X-ray exposure, but afterwards. ELF-EMF preconditioning of cells may evoke different reactions. In addition, responses of the cells could differ with ELF-EMF exposure duration, applied exposure protocol (continuous vs. intermittent) or used cell type. Anyhow, these experiments may not overrule our theory of an induction of DNA repair upon ELF-EMF exposure, since repair processes are very complex and different mechanisms may engaged in the repair of UV or thermal stress induced DNA damage.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was dependent on the genetic background of cells.

We concluded that the cupola-shaped time dependent pattern of DNA breaks in the Comet assay mirrors the action of repair processes. This is supported by the more than two fold increased rate of DNA breaks in DNA repair deficient fibroblasts from a patient with Ataxia Telangiectasia after 24 hours of exposure. The increased DNA breaking rate seen in fibroblasts from this patient and in fibroblasts from older donors points to the significance of the genetic background regarding the response to ELF-EMF-exposure.

Generation of DNA strand breaks in human fibroblasts by ELF-EMF was dependent on the frequency of ELF-EMF.

Although intermittent ELF-EMF induced DNA strand breaks in the Comet assay at a broad frequency range between 3 and 550 Hz, there are noteworthy peak effects at 50 Hz and 16.66 Hz, these representing the commonly used frequencies of alternating current in Europe. However, this has been tested as yet at intermittent 5 min on /10 min off cycles only and may be different under changed intermittent conditions.

ELF-EMF generated chromosomal aberrations in human fibroblasts.

Structural chromosome aberrations result from breakage and abnormal rearrangement of chromosomes. They can be classified either to stable or unstable aberrations, depending upon their ability to persist in dividing cell populations. Unstable aberrations are ring chromosomes, dicentric chromosomes or acentric fragments, whereas stable aberrations, which result from repair processes, consist of balanced translocations or other symmetrical rearrangements. At exposure conditions producing maximum effects in micronucleus test and in Comet assay, we observed significant increases in gaps, breaks, ring chromosomes, dicentric chromosomes and acentric fragments, but not of translocations. These results are in accordance with studies performed by Nordenson et al. (1994) and Khalil and Quassem (1991), who applied intermittent or pulsed field ELF-EMF exposure. Several other studies performed at continuous ELF-EMF exposure could detect an increase in chromosomal damage (Jacobson-Kram et al. 1997; Galt et al. 1995; Scarfi et al. 1991; Paile et al. 1995).

The fate of a cell carrying a chromosomal aberration is crucial for the assessment of a possible cancer risk. Cells with unstable aberrations like rings, dicentrics or acentric fragments will be committed to apoptosis or cell death, whereas cells with repairable DNA damage like chromosomal gaps or breaks may survive. The repair process itself can lead to translocations, thereby creating a stable mutation. Surprisingly this could not be detected in 24-times 1,000 metaphases, when each chromosome had been separately painted.

Although no significant differences in cell numbers could be detected between ELF-EMF exposed and sham-exposed cells, a possible elimination of cells carrying non-stable chromosomal aberrations is not contradictory to these previous findings. Cell numbers were assessed directly after ELF-EMF exposure termination, whereas for evaluation of chromosomal aberrations, cells were maintained in culture. In addition, the total fraction of cells with non-stable aberrations in exposed cells was 0.8%. The method used for assessment of cell numbers (Coulter counter) is too imprecise to detect such a low number of cells.

Since experimental analyses have shown, that DNA double strand breaks are the principal lesions to produce chromosomal aberrations (Bryant 1998; Natarajan and Obe 1978; Obe et al. 1992), the induction of micronuclei and chromosomal aberrations is in good agreement with the previous demonstration of DNA strand breaks.

ELF-EMF did not influence the mitochondrial membrane potential.

Hitherto, response of $\Delta\Psi_m$ to ELF-EMF exposure has not been assessed and no data on EMF-induced modifications of the membrane potential of cells are available. Effects of electric fields on membrane ATPases (optimal ranges: 5 - 30 Vcm⁻¹, 10 Hz - 1 MHz) have been reported by several groups (Tsong 1992). Short pulses of electric field (100 μ s decay time) of several kVcm⁻¹ have been used to trigger ATP synthesis in rat liver submitochondrial particles. The electric field-induced ATP synthesis was abolished by inhibitors of the F₀F₁-ATPase, oligomycin, *N,N'*-dicyclohexylcarbodiimide, venturicidin and aurovertin, but occurs independently of components of the mitochondrial electron transport chain. In low field experiments (<75 Vcm⁻¹) Tsong showed a dependence of ATP yield on the field strength and frequency of the alternating current (AC) field. Effects of AC fields on the activity of Na⁺,K⁺-ATPase, the enzyme principally responsible for establishing ion gradients across the cell membrane, have also been reported by Blank (1992). Under normal conditions *in vitro*, the enzyme in weak electric fields has a decreased ability to split ATP (100 Hz, threshold for effects of 5 μ Vcm⁻¹ estimated by extrapolation). When the enzyme activity was inhibited to less than half its optimal level by ouabain or low temperature, an increase in ATP splitting was observed. The greatest effects appear to be in the extremely low frequency range that includes 50 Hz.

Our data do not indicate an influence of intermittent ELF-EMF exposure of human fibroblasts on $\Delta\Psi_m$. Although these results cannot rule out rapid alterations of $\Delta\Psi_m$, we consider it to be unlikely that ELF-EMF-induced formation of DNA strand breaks is mediated via significant intracellular changes which affect $\Delta\Psi_m$.

4.1.1.2 Human fibroblasts and granulosa cells of rat (Participant 7)

The genotoxic effects induced by ELF-EMF are not reflected by physiological functions like volume regulation and free cytoplasmic Ca²⁺-concentration.

The experiments have been performed on two different cellular levels, the genomic and the cellular level using cultured granulosa cells of rat (GFSHR-17) and human fibroblasts. On the genomic level the neutral and alkaline Comet assay has been applied to evaluate ELF-EMF (5min on/10 min off, 1 mT) induced effects on DNA single- and double-strand breaks. In parallel, the effects were compared with those obtained on the cellular level by analysis of volume regulation (Ngezahayo et al., 2003) and cytoplasmic free Ca²⁺ concentration (Pilger et al., submitted). Since Participant 3 observed no effect during permanent ELF-EMF exposure on the occurrence of DNA strand-breaks, but for intermittent exposure (Ivancsits et al. 2002a; Ivancsits et al. 2003b), we followed this exposure protocol.

The results of the alkaline Comet assay indicate that intermittent exposure to ELF-EMF induced a significant increase of single- and double-strand breaks in rat granulosa cells (Figure 26) with a maximum after exposure for 16h to 18h. The maximum is followed by a decline of DNA damage in the time range

of a few hours which can be attributed to the onset of DNA repair mechanisms (Ivancsits et al. 2002a). It should be noted that a similar time course of DNA damage was observed at an exposure frequency of 16 2/3 Hz for rat granulosa cells. The time course of DNA damage on rat granulosa cells is similar as reported for cultured human fibroblasts (Ivancsits et al. 2002a; Ivancsits et al. 2003b), CHO and HeLa cells, but appears to be more pronounced. Therefore, it seems to be reasonable to suggest that the sensitivity of cultured cell lines to ELF-EMF (intermittently applied) depends significantly on the cell type.

In addition the frequency dependence of DNA damage was studied. The quantity of DNA single-strand breaks appears to depend on the frequency of the applied ELF-EMF with a maximum at lower frequency within the applied sequence of frequencies (8 Hz, 16 2/3 Hz, 50 Hz, 1000 Hz) (Figure 27). Surprisingly, virtually no frequency dependence is found for the results of the neutral Comet assay (Figure 28).

The important question arises whether the ELF-EMF effects observed on the genomic level are reflected in a change of the macroscopic cellular behaviour, especially in basic regulatory physiological functions. As marker of physiological cell functions the regulatory volume decrease/increase of rat granulosa cells was considered. In response to a constantly applied hypotonic shock rat granulosa cells swell due to water influx like an osmometer and shrink thereafter to the original cell volume within the time scale of an hour. According to this physiological regulatory behaviour a hypertonic shock causes cell shrinkage. The results show no significant influence of ELF-EMF exposure at the additional stress condition caused by a non-isotonic bath medium (Figure 69). It could be argued that, since for technical reasons the regulation of cell volume was studied 10 min after the end of ELF-EMF exposure for 18h, the DNA repair mechanisms act significantly and thereby bias the results. But the time span for DNA repair after an exposure time of 18h occurs in the range of hours (Figures 16, 17), whereas the experiments focused to volume regulation were started 10 min after end of ELF-EMF exposure. Therefore it can be concluded that a significant increase of DNA single- and double-strand breaks by ELF-EMF exposure is virtually not reflected in a change of regulatory volume decrease/increase of granulosa cells. As second macroscopic cellular parameter the time course of free cytoplasmic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$) was studied by fluorescence-spectroscopy after ELF-EMF exposure for 5h, 6h, 7h, 8h, and 18h. $[\text{Ca}^{2+}]_i$ was recorded in the absence (Figure 67, Table 9) and presence of a further stress factor, the exposure to H_2O_2 containing bath media (Figure 68, Table 10)). Also for this cellular parameter no significant influence of ELF-EMF exposure could be observed. In co-operation with Participant 3 a further cell-culture system, human fibroblasts, were used. In parallel to the results showing ELF-EMF induced DNA strand-breaks, the corresponding free Ca^{2+} -concentration was recorded. Also for this cell system the observed ELF-EMF induced DNA strand-breaks are not reflected in a change of the cellular level of free cytoplasmic Ca^{2+} (Figures 66, 67) or the mitochondrial potential (see also ref. Pilger et al., submitted). In contrast to our findings Tonini et al. (2001) reported a fast, within the time range of minutes, and significant increase of $[\text{Ca}^{2+}]_i$ in a cultured neuroblastoma cell line by ELF-EMF exposure at 50- to 60-Hz and 0.12 μT (0.24 μT). Surprisingly, the observations were made at continuous ELF-EMF exposure. The various findings could be related to the specific cell type, the different set-up used for ELF-EMF exposure or the method applied for analysis of $[\text{Ca}^{2+}]_i$.

4.1.1.3 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not induce the formation of DNA strand breaks in embryonic stem cells.

The potential to induce primary DNA damage by ELF-EMF was analysed by the Comet assay, as a consequence of up-regulation of the DNA-damage inducible gene GADD45 after ELF-EMF exposure (4.1.3.1). In addition, it was shown by Participant 3 that the exposition of human fibroblasts to ELF-EMF results in the increase in DNA breaks suggesting a possible direct mutagenic effect (Ivancsits et al., 2002). A correlation has been described between up-regulation of GADD45, bcl- X_L , and increased DNA damage as determined on the basis of the alkaline Comet assay in human preneuronal cells (Santiard-Baron et al. 2001). However, we did not observe significant effects of 6h or 48h intermittent ELF-EMF exposure on single- and double-strand DNA break induction in the alkaline and neutral Comet assay. One reason for our negative results (as compared to the data presented by Participant 3 on human fibroblasts, e.g.) could be the different intermittency scheme of exposure, which was applied (5 min on/30 min off by Participant 4 vs. 5 min on/10 min off by Participant 3). Actually Ivancsits et al. did not observe effect by using an intermittency scheme of 5 min on/25 min off, but only for shorter off time durations. However, by RF-EMF exposure of ES cells using 5 min on/30 min off cycles, we found a low, but significant

increase in double-strand DNA breaks, suggesting that the EMF frequency has significance for the DNA damaging effects.

4.1.1.4 Summary (Participant 1)

As discussed by Participant 3 there has been sporadic literature concerning *in vitro* studies which demonstrate that ELF-EMF may possess a genotoxic potential (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the energy impact to the genome of living cells exposed to ELF-EMF had been calculated to be too low to generate DNA damage. Since the mainstream literature contradicted the assumption of genotoxic effects (McCann et al. 1993; McCann et al. 1998; Murphy et al. 1993; Moulder 1998), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were systematically investigated and confirmed in 4 laboratories, of which two were not members of the REFLEX consortium, support the view that ELF-EMF causes genotoxic effects in certain, but not all cell systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings of genotoxicity caused by ELF-EMF are hard facts. DNA single and double strand breaks were observed in human fibroblasts exposed to ELF-EMF at a flux density as low as 35 μ T, which is far below the presently valid safety limit. Increases in micronuclei and chromosomal aberrations were found at higher flux densities (3.1.1.1). These effects, although striking in fibroblast from normal donors and donors with a known repair deficiency, were not observed consistently in all cell types, e.g. in human lymphocytes. This suggests that the genetically determined defence mechanisms of cells play a decisive role as to whether or not the cells respond to ELF-EMF exposure. The question arises why the genotoxic potential of ELF-EMF was not confirmed many years ago when suitable biochemical methods became available the first time. One explanation may be that most of the experiments were carried out with lymphocytes which seem to be resistant to ELF-EMF, and that in experiments with different cell systems the exposure time and the exposure conditions may have been inadequate.

As already stated, for energetic reasons ELF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to ELF-EMF is too low for damaging their DNA, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes. Participant 3 observed in its most recent experiments that the increase of DNA strand breaks in human fibroblasts after ELF-EMF exposure can partly be inhibited by oxygen radical scavengers. This finding speaks for the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by ELF-EMF. This assumption is further supported by results obtained by Simko et al., who measured an increase of free oxygen radicals in macrophages derived from murine bone marrow after exposure to ELF-EMF at a flux density of 1 mT (Simko et al. 2001) and by Lupke et al. who observed an increase of free oxygen radicals in monocytes derived from umbilical cord blood and in a human monocytic leukaemia cell line also after exposure to ELF-EMF (50 Hz) at a flux density of 1 mT (Lupke et al. 2004). Into the same direction hint the results of Zymslony et al., who assessed the effects of ALF-EMF (50 Hz, 40 μ T) on the oxidative deterioration of DNA in rat lymphocytes after *in vitro* irradiation by UVA (Zymslony et al. 2004). The free radical hypothesis is further supported by the studies of Lai and Singh (2004) who found that brain cells of rats after whole body-exposure to ELF-EMF (60 Hz) at very low flux densities (0.01-0.25 mT) for 2-48 hrs showed increases in DNA single and double strand breaks, and that these increases could be blocked by pre-treating the animals with the free radical scavengers melatonin, N-tert-butyl- α -phenylnitron and Trolox (a vitamin E analogue). The work of Lai and Singh, which must still be reproduced by other independent research groups, deserves special attention, since the DNA damage reported by them was observed in the brain of whole-body exposed animals, not in isolated cells as in the REFLEX study.

Based on the data of the REFLEX project it must be assumed that ELF-EMF is able to damage the genome in certain, but not all cell systems after exposure *in vitro*. The work of Lai and Singh suggests that these effects might also be seen after exposure *in vivo*. The genotoxic effects of ELF-EMF may be best explained by an ELF-EMF induced increase of intracellular free radicals within the exposed cells and by the genetic background of the exposed cells. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through ELF-EMF *in vivo* as suggested by Lai and Singh (2004) needs to be further clarified.

4.1.2 Cell proliferation and differentiation

4.1.2.1 Human neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF enhanced proliferation and reduces spontaneous apoptosis of NB69 neuroblastoma cells.

The described results indicate that 42- or 63-hour exposure to 50 Hz magnetic fields at 10 or 100 μ T can increase proliferation and reduce spontaneous apoptosis in human neuroblastoma cells. Initial evidence obtained through cell counting (Trypan blue exclusion) was subsequently confirmed through PCNA labelling, 5-bromo-2'-deoxyuridine (BrdU) labelling for identification of DNA-synthesizing cells, and flow cytometry. The modest, though statistically significant increase in the total number of cells in response to a 100- μ T field estimated by the Trypan blue exclusion is consistent with the observed increase in the number of PCNA positive cells. This is also consistent with the increased numbers of cells in G2-M phase and of BrdU positive cells observed 24 hours before the increase in the number of cells was detected. The present data also indicate that a 50-Hz EMF at 100 μ T can induce changes in the activation of the transcriptional factor CREB in a time-dependent manner.

A number of experimental studies investigating proliferative effects of EMF using in vitro or in vivo models (Kavet 1996), have provided limited evidence that ELF-EMF can represent a growth stimulus. Kwee and Raskmark (1995) have reported that a 24-h exposure to 50 Hz MF at 80 μ T significantly increases the proliferation of transformed human epithelial amnion cells and K14 skin fibroblast cells. Wei et al. (2000) have reported that 60 Hz MF (30-120 μ T, 3-72 h exposure) can induce proliferation in human astrocytoma cells and strongly strengthen the effect of two chemical agonists.

Some studies, however, have reported effects that are in apparent contrast to those described above. For instance, Conti et al. (1983) and Cleary (1993) have reported reduced 3 H-thymidine incorporation into lymphocyte DNA after exposure to 2.0 - 7.0 mT, 50-Hz magnetic fields. It has been proposed that several physical and biological variables, including different field parameters, exposure protocols, cell types and physiological conditions (degree of differentiation or activation) may account for the conflicting results reported in the literature (see for instance Schimmelpfeng and Dertinger 1997). In fact, the cellular response to the fields seems to be strongly dependent on biological parameters (Simko et al. 1998a/b; Wei et al. 2000). In addition, there is experimental evidence that specific combinations of AC/DC fields interact with biological systems (Blackman et al. 1994; Trillo et al. 1996) and the key to affecting proliferation of cells in a consistent manner might lie in the simultaneous control of the AC field amplitude and frequency, and the AD/DC field intensity ratio (Yost and Liburdy 1992; Blackman et al. 1985a,b; Trillo et al. 1996; Bauréus Koch et al. 2003).

Also, a recent study by Pirozzoli et al., (2003) has shown that the apoptosis induced by camptothecin in neuroblastoma cells (LAN-5) can be prevented by a 24-h exposure to 50 Hz, 1 mT MF. In addition, the cells respond to the stimulus with an increase in the proliferation index after seven days of continuous exposure to the field. In our cellular model (NB69) and under our experimental conditions, a 63-h exposure to 50 Hz, 100 μ T MF significantly reduces the spontaneous rate of apoptosis while increasing proliferation in an extent that is similar to that reported by Pirozzoli et al.

The present data on PCNA, a protein that has been reported to be peak in proliferating cells at late G1 and S phases (Oue et al. 1995), indicate that the normal regulation of the PCNA positive cells is altered by the exposure to 50 Hz, 100 μ T MF. In the MF-exposed samples the percent of PCNA-positive cells does not differ significantly from that at day 5 post plating, while in the control groups a significant reduction of PCNA positive cells was observed on day 6. These data are consistent with previous results reported by Cridland et al. (1999) on normal human fibroblasts showing a modest though significant increase in the length of the G1 phase when exposed to 50 Hz, 20 and 200 μ T MF

The mechanism of interaction between ELF-EMF and NB69 neuroblastoma cells is not known yet.

Regarding the mechanism of interaction of magnetic fields that could underlay the herein described responses of NB69, the mobilization of cellular Ca^{2+} or some Ca^{2+} -regulatory process have been proposed as pre-eminent targets of the MF stimuli (Tonini et al. 2001). Also, in a recent work Zhou et al. (2002) have reported that ELF MF at 100 μ T induced a time-dependent activation of CREB DNA binding in HL-60 cells. The effect was dependent on both the extracellular and intracellular Ca^{2+} , which suggests that ELF-EMF can activate CREB DNA binding through calcium-related signal transduction pathways. Similarly, in the present study, the activation of CREB was found to be influenced by the MF stimulus in

a time-dependent manner. Although additional research is needed to determine whether or not calcium is involved directly in the observed response of NB69 cells to 50-Hz MF, the present results are consistent with such a possibility. Further work is also necessary to determine the gene transcription pattern resulting from the increase of CREB activation after exposure to MF. Such an information would be crucial to identify the mechanism(s) by which MF interact with human neuronal cells in vitro. This hypothesis is not in contradiction with the recent results reported by Ivancsits et al. (2002b) and the studies by this group included in the REFLEX project. Their results showed that 50 Hz MF induced a dose dependent and time dependent DNA-single and double-strand breaks, with responses at a magnetic flux density as low as 35 μ T.

4.1.2.2 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not exert any influence on neuronal differentiation of embryonic stem cell.

We could not find evidence that under our experimental conditions, ELF-EMF exposure of ES cell derived neural progenitors affected the neural differentiation process, because we did not observe effects on transcript levels of genes involved in neuronal and glial differentiation (nestin, en-1, nurr1, tyrosine-hydroxylase and GFAP). Immunofluorescence analysis did not show any changes in the intracellular distribution and number of cells expressing neuronal markers (β III-tubulin, TH, GFAP).

4.1.2.3 Human lymphocytes and embryonic stem cells (Participant 8)

ELF-EMF did not affect proliferation, cell cycle and activation of lymphocytes.

Since the immune System has a key role in contrasting diseases, possible damages induced by exposure of immune cells, such as lymphocytes, could represent a great risk for human health. Thus, the objectives were to determine if different EMF exposures were able to modify human lymphocytes functionality and gene expression using appropriate in vitro tests. Moreover, since immune system efficiency is modified with ageing, a group of elderly donors was enrolled in order to study possible EMF effects age-related. On the whole, the results obtained show no differences between sham- and ELF-EMF exposed lymphocytes for most of the endpoints studied. Obviously, ELF-EMF is not able to modify proliferation, cell cycle and cell activation, which are fundamental phases of lymphocyte function. Negative results are extremely important for evaluations on human health risk.

ELF-EMF activated the expression of cardiac genes in embryonic stem cells thus enhancing their cardiac differentiation.

ELF - EMF were able to promote the differentiation of mice embryonic stem cells into a specific cardiac cell lineage, selectively promoting the expression of fundamental genes involved in the orchestration of cardiac differentiation. At the end of the differentiation process the expression of typical cardiac genes revealed that a specific direction of differentiation into a cardiac phenotype took place, which was also demonstrated by lack of expression of genes related to other cell lineages (e.g., skeletal muscle cells, neuronal cells, etc.).

4.1.2.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.2.1) the findings reported in the literature about a possible influence of ELF-EMF on the proliferation and differentiation of various cell systems in vitro are controversial. Just recently, Lisi et al. (2004) demonstrated that exposure to ELF-EMF (50 Hz, 1 mT) triggered the differentiation of human pluripotent embryonic stem cells. In the REFLEX project, no data were obtained which suggest a major effect of ELF-EMF on cell proliferation and differentiation in human fibroblasts (4.1.1.1), embryonic stem cells (4.1.2.2), human lymphocytes (4.1.2.3) or neuroblastoma cells (3.1.4.2). On the other hand, some influence of ELF-EMF on proliferation and differentiation in certain cell systems cannot be excluded (4.1.2.1).

Participant 5 (3.1.2.1, 4.1.2.1) observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed to ELF-EMF for 63h at a flux density of 50 or 100 μ T. This observation is in line with the results of a recent study by Tokalov et al. (2003) and Tokalov and Gutzeit (2003) who reported that ELF-EMF alone does not have

any effect on the proliferation of HL-60 cells, while it protects heat shock treated HL-60 cells from becoming apoptotic. Quite obviously, ELF-EMF enabled heat shock treated HL-60 cells to escape the cell cycle arrest and to re-enter the normal cell cycle thus allowing the cell to continue the proliferation process. The authors explained this phenomenon by an ELF-EMF induced release of hsp-proteins which are thermo- or cytoprotective.

An answer of what may be the reason for the sporadically observed, but until now not unambiguously confirmed influence of ELF-EMF on cell proliferation and differentiation, may be provided by the REFLEX findings on gene and protein expression. As found by Participant 8 (3.1.4.3, 4.1.2.3), ELF-EMF accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes. Further evidence for the validity of such an assumption comes from Participants 3 and 12 (3.1.4.5), who observed in human fibroblasts a remarkable influence of ELF-EMF on the expression of various genes, among them genes regulating Ca-metabolism, cell cycle, apoptosis, extracellular matrix, and cytoskeleton.

Of course, even if there is a relationship between ELF-EMF exposure and an acceleration of cell proliferation and differentiation in in vitro studies through the proposed mechanisms, it is at present not possible to draw any conclusion for the in vivo effects on man and animal.

4.1.3 Apoptosis

4.1.3.1 Mouse embryonic stem cells (Participant 4)

ELF-EMF altered the expression of bcl-2, bax and GADD45 gene in ES-cell derived neural progenitor cells.

In our experiments with ELF-EMF exposed wild-type mouse ES derived neural progenitors, we showed by Q-RT-PCR analysis significant changes in the transcript levels of the anti-apoptotic bcl-2 gene and the related pro-apoptotic bax gene. The biological significance of this finding and its relevance to the in vivo situation is not yet known. Apoptotic cell death is regulated by members of the bcl-2 family for differentiating mouse embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Bcl-2 over expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour, 2002). Bcl-2 and bax mRNA transcripts in the hippocampus were significantly but transiently upregulated following the administration of the potent neurotoxin domoic acid (Ananth et al. 2001).

Another gene, whose expression was affected after ELF-EMF exposure, was the 'growth arrest DNA-damage' inducible GADD45 gene. The members of the GADD protein family are considered to play an important role in maintaining genomic stability and to regulate cell cycle activity (Chung et al. 2003).

Our results, which demonstrate changes of bcl-2, bax and GADD45 transcript levels indicate that ELF-electromagnetic signals could be perceived in ES cell-derived neural progenitors as environmental stress signals. These signals may trigger cellular responses for the maintenance of cellular homeostasis via alterations of genes that control cell cycle and apoptotic cell death.

In summary, we may conclude that exposure of ES-derived neural progenitor cells to magnetic fields simulating 50Hz power line ELF-EMF may influence transcript levels of genes encoding proteins of the bcl-2 family involved in apoptosis and the p53 responsive growth arrest and DNA damage inducible GADD45 gene. Since the fundamental processes of programmed cell death and cell cycle regulation are closely related to processes underlying cell transformation, the association of ELF-EMF with early stages of carcinogenesis cannot be excluded yet. Further investigations in vivo using genomics analyses and animal studies after EMF exposure have to be performed.

4.1.3.2 Neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF inhibited spontaneous apoptosis in neuroblastoma cells.

Environmental electromagnetic fields (EMF) such as those from electric power transmission and

distribution lines have been associated with increased risk of childhood leukaemia, cancer of the nervous system and lymphomas (Ahlbom et al. 2001; De Roos et al. 2001). In vitro studies of EMF effects have attempted to find an explanation to the epidemiological data and to determine the possible mechanism for cancer risk. Recent evidence has suggested that a common property shared by a number of known and suspected tumour promoters, is their ability to block the process of apoptosis (Jaattela et al., 1999). Therefore, one possible mechanistic explanation for the apparent effect of weak ELF magnetic fields would be their expression of tumour-promoting activities by interfering with the regulation of apoptosis. We have addressed this hypothesis by testing the effects of a 50 Hz 100 μ T MF on apoptosis in the human neuroblastoma cell line.

Our data indicate that the field exposure can significantly inhibit spontaneous apoptosis of NB69 cells as revealed through TUNEL assay. This response was associated with significant increase in the number of cells as well as in BrdU incorporation into ADN. Besides, the immunoreactivity for Bcl-2 protein in exposed samples was also significantly increased at 60 min of exposure with respect to controls (data not shown). Regulation of apoptosis is delicately balanced by signalling pathways between apoptosis-promoting factors such as p53 and caspases, and antiapoptotic factors such as Bcl-2 and MDM2. Several lines of evidences have shown that the functional interaction between these factors play important roles in the control of cell growth and apoptosis.

Previous studies investigating changes in susceptibility to apoptosis after EMF exposure have reported both reduced (Simko et al. 1998b; Fanelli et al. 1999; Ding et al. 2001; Kumlin et al. 2002; Robison et al. 2002) and increased susceptibility (Ismael et al. 1998; Tofani et al. 2001; Mangiacasale et al. 2001; Liu et al. 2003). Other studies concerning DNA repair after EMF exposure have reported no effects (Cossarizza et al. 1989a; Frazier et al. 1990; Cantoni et al. 1996). In the majority of the studies reporting effects on apoptosis, cancer cells were exposed to MF ($B > 100 \mu$ T) after apoptosis induction by radiation or chemical treatments. The cellular susceptibility to such MF-driven apoptosis has been reported to be dependent on the cell type, the presence of genetic abnormalities, cell physiology and the MF exposure time. Cancer cells frequently have decreased cell death as a primary mode of increased cell proliferation. Attention has been focused on the expression of the p53 gene, which induces either a stable arrest of cell growth or apoptosis. The final outcome of the different mechanism of action of p53 is to maintain the genomic stability of the cell. Thus, the absent of this protein or their inactivation contributes to genomic instability, the accumulation of mutations and increased tumorigenesis. In the study by Czyz et al. (2004a, included in the present report) the exposure to 50 Hz EMF at 2.3 mT results in up-regulation of *egr-1*, *c-jun* and *p-21* transcript levels in p53-deficient, but not in wildtype embryonic stem cells. These data indicate that loss of p53 may also affect the sensitivity of cells to external stress factors, such as EMF.

On the other hand, it has been reported (Tian et al. 2002b) that X-ray irradiation followed by 60 Hz EMF exposures can affect cell cycle distribution and transiently suppress apoptosis in *xrs5* cells, which show a defect in rejoining of DNA double-strand breaks. The effect has been proposed to be exerted through EMF-induced decrease in the levels of caspase-3, p21, p53 and phospho-p53 and by increasing Bcl-2 expression. Our present results show that a 50 Hz 100 μ T MF induces changes in the cell cycle together with a reduction of spontaneous apoptosis associated with increased Bcl-2 expression in NB69 cells. It is possible that a MF action on p53 and Bcl-2 is responsible for the effects on growth and apoptosis observed in our study. In addition we have investigated possible EMF-induced changes in the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB). CREB appears to be a primary transcriptional activator of the antiapoptotic gene Bcl-2 (Francois et al. 2000). Inhibition of CREB activity induces apoptosis in sympathetic neurones (Riccio et al. 1997) while CREB overexpression inhibits apoptosis induced by okadaic acid (Walton et al. 1999). Our data show that EMF exposure significantly increases the percent of p-CREB positive cells after 60-minute exposures. These results suggest that CREB may also be involved in the above-described effects of 50 Hz, 100 μ T EMF on growth/apoptosis of NB69 cells.

4.1.3.3 Human fibroblasts (Participant 3)

ELF-EMF may not affect the apoptotic process in human fibroblasts after intermittent exposure for 24 hours at a flux density of 1 mT.

No differences in cell count between exposed and sham exposed human fibroblasts after any exposure duration could be detected. Therefore, an elimination of cells by apoptosis and cell death during ELF-EMF exposure can probably be ruled out (3.1.1.1).

4.1.3.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.3.2), data reported in the scientific literature on possible effects of ELF-EMF on the apoptotic process are inconsistent. In many studies available to date, inhibition of apoptosis, enhancement of apoptosis and no effect at all have been reported. Most recently, Lai and Singh (2004) found a significant increase both in apoptosis and in necrosis in brain cells of rats after in vivo exposure to ELF-EMF which they explained by an increase in free radicals. Kim et al. (2004) demonstrated that apoptosis in testicular germ cells of mice can be induced by continuous exposure to ELF-EMF (60 Hz, 0.1 and 0.5 mT). The REFLEX findings did not show a significant effect of ELF-EMF on apoptosis in human fibroblasts (3.1.1.1, 4.1.3.3), embryonic stem cells (3.1.4.1, 4.1.3.1), human lymphocytes (3.1.2.3) and neuroblastoma cells (3.1.2.4).

On the other hand, some influence of ELF-EMF on the apoptotic process cannot be excluded at present. Participant 5 observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 μ T (3.1.2.1, 4.1.2.1). A similar phenomenon was also reported by Tokalov and Gutzeit (2003) and Tokalov et al. (2003), who did not observe any direct effect of ELF-EMF on apoptosis in HL-60 cells either, while ELF-EMF protected heat shock treated HL-60 cells from becoming apoptotic, thus enabling cells arrested in the cell cycle to continue the proliferation process.

An answer of what may be the cause for the sporadically observed, but probably not systematically enough studied influence of ELF-EMF on apoptosis may be provided by the REFLEX findings on gene and protein expression. As found by Participant 4 (3.1.4.1, 4.1.3.1), ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the bcl-2 and the GADD45 gene and down-regulated the transcript levels of the bax gene thus influencing cellular processes, which may result in an enhancement of the anti-apoptotic pathway. Further evidence for the validity of such a hypothesis comes from Participants 3 and 12 (3.1.4.5), who observed a remarkable influence of ELF-EMF on the expression of various genes, including those that regulate cell cycle and apoptosis.

From the physiological point of view, inhibition as well as promotion of apoptosis may be induced by ELF-EMF dependent on the type of cell exposed, its genetic background, its immediate metabolic stage and the pattern of exposure. The mechanisms may follow different routes. It may be possible, that two counteracting mechanisms balance out each other which would result in a zero outcome. Taken together, even if a relationship between ELF-EMF exposure and an inhibition or promotion of apoptosis in in vitro experiments were proven, it would in no way be possible to draw any conclusion for the in vivo situation in man and animal.

4.1.4 Gene and protein expression

4.1.4.1 Mouse embryonic stem cells (Participant 4)

Short-term high intensity exposure to ELF-EMF signals may cause a transient up-regulation of immediate early response and regulatory genes in p53-deficient ES cells.

It was found that a high flux density of 2.3 mT of 50 Hz ELF-EMF signals applied to p53-deficient ES cells at an intermittency scheme of 5 min on/30 min off induced a significant up-regulation of transcript levels of the immediate early growth response gene *egr-1*. This upregulation was paralleled by a transient upregulation of mRNA levels of the cyclin kinase inhibitor p21 and the AP-1 component c-jun in p53-deficient, but not in wt ES cells. This finding confirms our observation that loss of p53, affects the sensitivity of cells to external stress factors, such as GSM-signals. A correlation between loss of p53

function and external stress-induced expression of *egr-1* has also been described by Zhang and Chen (2001), who reported experimental evidence for UV-induced *egr-1* expression in p53-deficient mouse cells, whereas the effect was suppressed by functional p53. Our data indicate that a similar *egr-1*-dependent pathway may be triggered upon ELF-EMF exposure.

The role of c-jun and p21 in these processes has not been clarified so far. Egr-1 cooperates with c-jun in the regulation of DNA synthesis and cell survival in response to ionizing radiation (Hallahan et al. 1995). p21 is implicated in G1 arrest following ionizing radiation-induced DNA damage (Brugarolas et al. 1995). Therefore, one could speculate that the tumor suppressor p53 may be involved in the maintenance of cellular homeostasis of ES cells in response to external stress. However, there are also other data showing that despite abundant quantities of p53 in ES cells, the p53-mediated response is inactive, because of a predominantly cytoplasmic localisation and sequestration of p53 (Aladjem et al. 1998). In spite of this, undifferentiated ES cells are sensitive to DNA damage, because they activate a p53-independent apoptotic response. According to Sabapathy et al. (Sabapathy et al. 1997), the balance between positive and negative regulators of the cell cycle is critical for ES cell differentiation and, if disturbed by exogenous factors, this could lead to the activation of a tumorigenic pathway.

The nature of gene-expression responses to ELF-EMF was short-term only.

In our experiments, cellular responses to ELF-EMF signals were observed only immediately after the end of the 6h exposure and disappeared after an 18h recovery time. Similarly, a 48h exposure to ELF-EMF did not result in gene expression-related responses throughout the differentiation process. These results indicate a short-term nature of cell responses to ELF-EMF and the existence of pathways compensating potential stress-evoked effects of ELF-EMF.

There is some indication that threshold of field flux density exists for ELF-EMF biological effects.

We further investigated the influence of the signal strength and the quality of ELF-EMF exposure on cellular reactions in the ES cell system. Our data indicate the existence of threshold values of field flux density that are needed to evoke biological effects by ELF-EMF. Modifications of transcript levels in p53-deficient cells were observed only upon exposure to ELF-EMF signals applied at a high (2.3 mT) flux density, whereas weaker fields did not cause gene expression-related responses.

ELF-EMF effects in p53-deficient cells were dependent on intermittency cycles (on/off cycle duration).

The exposure protocols of ELF-EMF signals involving either intermittent (on/off cycles) or continuous exposure affected the responses of ES cells. Only an intermittency scheme of 5 min on/30 min off ELF-EMF signals exerted effects on transcript levels, whereas intermittency signals of 5 min on/10 min off exposure or continuous exposure showed no effects on transcript levels of ES cells. These findings demonstrate that a specific intermittency scheme of ELF-EMF exposure may be a critical factor to determine the interference of electromagnetic fields with biological systems (Murphy et al. 2002).

The mechanism of action induced by ELF-EMF exposure of living cells is not yet known.

Several hypothetical models have been proposed to explain the mechanisms of interference of ELF-EMF with biological systems, such as an induction of electric currents by acceleration of ions, resonant interactions involving driving vibrations or orbital transitions in biomolecules (Valberg et al. 1997), biochemical reactions involving free radicals (Brocklehurst and McLauchlan 1996; Eveson et al 2000) or direct interactions of EMF with moving electrons within DNA (Blank 1997). It was also suggested that external oscillating fields cause forced vibrations of free ions of the cellular surface and distort the gating of electro-sensitive channels on the plasma membrane. This would explain, why pulsed electromagnetic fields could have a higher biological activity than continuously applied fields (Panagopoulos et al. 2000, 2002). According to another model (Binhi and Goldman 2000), specific 'windows' of the electric-field frequency and amplitude might be predicted. These properties of ELF-EMF could explain the positive results of certain exposure schemes with a specific on/off cycle (in our case, 5 min on/30 min off) and the lack of biological effects at other experimental conditions.

4.1.4.2 Neuroblastoma cells (SY5Y cell line) (Participant 11)

The function of neuronal nicotinic receptors in the brain

Neuronal nicotinic receptors (nAChRs) are a family of ligand-gated cationic channels expressed both in the peripheral and central nervous system where they play a fundamental role in synaptic transmission. At the periphery nAChRs are expressed in post-ganglionic neurons of the autonomic nervous system (Wang et al. 2002 and references therein). In the CNS they seem to be located predominantly at the presynaptic and preterminal parts of the axons where they control the release of a number of different neurotransmitters, such as glutamate, GABA and dopamine (Wonnacott 1997).

nAChRs are composed of different subunits: so far nine ligand binding subunits, alpha 2 - alpha 10, and three structural subunits, beta2 - beta4, have been cloned from different species (Wang et al. 2002). Different combinations of alpha and beta subunits can form different receptor subtypes with their own pharmacological and biophysical characteristics. Neuronal nAChRs are involved in a number of functional processes including cognition, learning and memory (Jones et al. 1999). Alterations in the expression and/or activity of nAChRs have been implicated in different neurological disorders. For instance, mutations in the alpha4 or beta2 subunits produce in humans the autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al. 1995; De Fusco et al. 2000). Roles for the alpha7 subunit have been suggested in Alzheimer's disease (Dineley et al. 2001) and schizophrenia (Lindstrom 1997; Freedman 1999; Freedman et al. 2000). It has been recently shown that the expression of alpha7 is increased in a well-established mouse model of Alzheimer's disease, whereas the beta-Amyloid (1-42) peptide binds with high affinity to alpha7, suggesting a pathogenetic role for this receptor subtype (Grassi et al. 2003).

Insights into the functional role of nAChRs and their possible involvement in neurological disorders have been obtained by means of knock-out mice (Cordero-Erausquin et al. 2000). By this approach, it has been possible to show that the absence of the beta2 subunit as well as the hyperactivity of the alpha7 subunit are conditions sufficient to promote neurodegeneration. Epidemiological studies have shown that exposure to electromagnetic fields (EMF) might be responsible for neurodegenerative diseases such as Alzheimer's (Sobel et al. 1995, 1996). In light of the role of nAChRs in physiological and pathological conditions, we wondered whether EMF might affect the expression of these molecules. With this aim, we have characterized some neuronal cell lines for their ability to express nAChRs. We have identified some human neuroblastoma cell lines that are currently used to evaluate whether extremely low frequency EMF (ELF-EMF) can interfere with the expression of alpha3, alpha5 and alpha7 nAChR subunits. The expression of these subunits has been studied both at mRNA level by Northern blotting, and at protein level by radioligand assays, upon exposure to different protocol settings.

The function of the catecholaminergic system in the brain

The catecholaminergic system is very relevant for many brain functions. Moreover, in the periphery, catecholamines, in particular norepinephrine, are released by the post-ganglionic neurons of the autonomic nervous system, representing the main neurotransmitters of the orthosympathetic division. In collaboration with Participant 1, we decided to investigate the expression of Dopamine beta-hydroxylase (DBH), the limiting enzyme for the synthesis of norepinephrine, in order to investigate whether ELF-EMF might modify its expression, therefore interfering with autonomic functions as it has been reported in some papers (Kim et al. 2002). With this aim, we carried out Northern blot analyses with RNA extracted from neuroblastoma cells exposed to ELF-EMF.

Finally, we have also been investigating the effects of ELF-EMF on the expression of two transcription factors, Phox2a and Phox2b. These homeodomain proteins are the main regulators of the expression of Dopamine beta-hydroxylase (Yang et al. 1998). In particular, they are responsible for the development of all three divisions of the autonomic nervous system (Lo et al. 1999; Stanke et al. 1999). Indeed Phox2b KO mice fail to develop the whole autonomic nervous system (Pattyn et al. 1999), whereas Phox2a mice show an apparently less severe phenotype, but die the day of birth (Morin et al. 1997). Furthermore, preliminary results from our laboratory have shown that they seem to play a role in the regulation and maintenance of the expression of nAChR alpha3 subunit gene (Flora, personal communication). In order to understand whether ELF-EMF can interfere with the expression of these transcription factors, therefore affecting the formation and function of the autonomic nervous system, we have been carrying out Northern blot experiments to evaluate possible variation in the expression of Phox2a and Phox2b mRNA.

ELF-EMF did not affect the expression of neuronal genes such as nAChRs, DBH, Phox2a and Phox2b, either at mRNA or protein level.

A human neuroblastoma cell line, SY5Y, was used in all the experiments, as it expresses the ganglionic-type nAChR subunits alpha3, alpha5 and alpha7 as well as DBH, Phox2a and Phox2b genes. Cells were

exposed by means of the ELF-EMF generator, setup by Participant 10, under different exposure protocol. The intensity of the electromagnetic field applied was always higher (2 mT and 1 mT) than that of a real life situation, in order to highlight possible, if any, macroscopic effect on gene expression due to ELF-EMF exposure. The duration time of the exposure varied from a relatively short period of time (16h) to a longer period (48h), in order to investigate a time-dependent effect upon exposure to EMF. Finally, the type of exposure, intermittent (5 min on/5 min off) rather than continuous was chosen in order to mimic different kinds of situations that may be encountered during the life-time of an individual. The cells were always collected immediately after the end of the exposure for gene expression analysis, except in one case (1 mT continuous exposure for 48h), when the cells were harvested 48 hours after the end of the exposure, in order to investigate possible indirect effects on the expression of nAChR subunits, DBH, Phox2a and Phox2b, due to the activation of second messenger cascades. We found that exposure of human neuroblastoma cells to continuous (magnetic field intensity of 2 mT and 1 mT) and intermittent (2 mT and 1 mT) low-frequency EMF either for a relatively short period (16h) as well as a longer period (48h) does not seem to influence the expression of neuronal genes for nAChRs, DBH, Phox2a and Phox2b, either at mRNA or protein level. In order to validate these negative results, every exposure condition was tested in at least three to nine independent experiments.

4.1.4.3 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

ELF-EMF up-regulated the expression of cardiac specific genes thus promoting cardiogenesis.

The exposure of EC (P19) cells to ELF-EMF yielded conflicting results and poor reproducibility of the data. On the contrary, the development of a model of in vitro cardiogenesis based on “gene trapping” selection of cardiomyocytes from pluripotent (GTR1) cells provided a potentially homogenous and reproducible approach to assess whether ELF-EMF may afford developmental decisions (i.e. cardiogenesis) in ES cells. In this ES cell model, ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF-EMF-exposed cells. Failure of ELF-EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of ELF-EMF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis.

4.1.4.4 rCx46 in oocytes of *Xenopus laevis* (Participant 7)

The influence of ELF-EMF exposure on the expression of rCx46 in single and paired oocytes of *Xenopus laevis* was analysed. Especially the expression level as well as the corresponding regulatory properties of conducting hemi-channels and cell-to-cell channels (Bruzzone et al., 1996) were studied. ELF-EMF exposure neither significantly influenced the expression level of conducting hemi-channels composed of rCx46 (Figures 59 to 61), nor their gating properties by voltage, pH, Ca²⁺ (Figure 64). A similar result was found for cell-to-cell channels, which could be formed by pairing of oocytes expressing rCx46 (Figure 65). This finding is in contrast to the observation that in general ELF-EMF exposure causes a decrease of cell-to-cell coupling (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003), but different regulatory mechanisms were suggested. It was proposed that ELF-EMF increases Ca²⁺-influx which in turn inhibits gap junctional coupling in synovial fibroblasts (Marino et al. 2003). But in osteoblast like cells such an increase of Ca²⁺ was not observed, despite the finding that ELF-EMF induced a decrease of gap junctional coupling (Yamaguchi et al. 2002). In contrast to further reports (Lohmann et al. 2003; Zeng et al. 2003) the authors showed that ELF-EMF does also not effect the distribution of the corresponding membrane protein connexin (Cx43) between the cytoplasmic and the membrane pool. Therefore, a change in the state of Cx-phosphorylation was considered as target of ELF-EMF exposure causing a decrease of cell-to-cell coupling (Yamaguchi et al. 2002, see also Lacy-Hulbert et al. 1998). By ELF-EMF exposure of oocytes expressing rCx46 cytoplasmic free Ca²⁺ and/or signal transduction pathways involved in protein phosphorylation also of rCx46 virtually remain unchanged. This conclusion can be drawn from the unchanged behaviour of the leak-current of the oocytes in the absence and presence of

ELF-EMF (Figure 58). The leak-current includes the sum of all electrogenic transport systems which are known to partially depend on cytoplasmic free Ca^{2+} and protein phosphorylation. At present the origin for the different response of cell systems expressing Cx43 (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003) and oocytes expressing rCx46 at ELF-EMF exposure remains unsolved.

4.1.4.5 Whole-genome analysis of various cell lines exposed to ELF-EMF (Participant 12)

If we look on the numbers in Table 12, it is obvious that members of some gene families are regulated predominantly. Moreover, repetitions of experiments with the same cell line and the same exposure conditions look more similar than repetitions with different cell lines or different exposure conditions. This might tell us that obviously something is happening on the gene-expression level after ELF-EMF exposure. Otherwise, if we only would see experimental variances (differences in experimental procedures, cell cycle stages, etc.), we would expect about the same numbers in each experiment, or higher similarities between same experiments as between different cell lines.

The results with the different cell lines obviously have not the same quality. For example, the results of profiling 1 and 2 of the fibroblasts (Participant 3) seem to be more similar than between the experiments between cells with differentiating potential. Also genes of different gene families react differently on certain influences. Whereas for example structural proteins like cell adhesion proteins are regulated slowly, certain proto-oncogenes like c-fos, c-fos or actin can be regulated within 10 to 30 min of growth factor addition (Quantin and Breathnach 1988). Moreover, the situation is different here from, for example, a disease situation with a certain defect in a single gene. We deal with environmental influences here, which are complex and variable. Even adaptation to the electromagnetic fields after some hours due to changes in gene expression cannot be excluded.

How the potential molecular changes after ELF-EMF exposure are regulated, remains speculative. However, if we look on the genes extracted by the bio-statistical analysis in more detail, some interesting points become obvious: A remarkable number of members of the actin cytoskeleton and associated proteins are down-regulated (also in RF-EMF experiments). Remarkably, in ELF-EMF treated cells (Participant 3) the actin-associated proteins obviously down-regulated seem to be regulated by Ca, and several Ca regulators were also down-regulated in our experiments. This would mean that the actin cytoskeleton as far as regulated by Ca is down-regulated. In addition to Ca^{2+} -associated proteins, proteins associated with other cations like Fe^{+} , K^{+} , and H^{+} are down-regulated. More experiments will be necessary for showing if these proteins might be involved in signalling or energy metabolism after ELF-EMF exposure.

4.1.4.6 Summary (Participant 1)

From the REFLEX data, the conclusion must be drawn that ELF-EMF may affect gene and protein expression in various cell systems. Based on the results of the genome analysis of human fibroblasts as carried out by Participant 12 (3.1.4.5), ELF-EMF appears to regulate the expression of a series of genes and proteins such as mitochondrial and ribosomal genes and Ca-, cell cycle-, apoptosis-, extracellular matrix-, and cytoskeleton-related genes. In particular, a number of G proteins and calcium associated proteins involved in signal transduction seem to be strongly regulated by ELF-EMF. Since the variances between the experiments were high, the significance of these findings is limited. Participant 4 observed a transient up-regulation of early response and regulatory genes only in embryonic stem cells deficient for p53 and not in wild type cells after ELF-EMF exposure. This suggests that the genetic background affects the responsiveness of the cells (3.1.4.1, 4.1.4.1). Participant 8 found that ELF-EMF up-regulates the expression of cardiac specific genes in cardiomyocytes derived from embryonic stem cells thus promoting cardiogenesis (3.1.4.3, 4.1.4.3). All these findings were obtained after ELF-EMF exposure at rather high flux densities. It remains, therefore, an open question whether or not these in vitro results are of any significance for the real life exposure of man and animal.

The REFLEX data on gene and protein expression due to ELF-EMF exposure are in line with the results of a series of studies already published in the literature. Goodman et al. (1994) and Lin et al. (2001) reported increased hsp70 transcript concentrations in HL-60 cells after exposure to weak ELF-EMF (60 Hz). Tokalov and Gutzeit (2004) observed an increase in several heat shock proteins in HL-60 cells after

exposure to ELF-EMF (50 Hz, 60 μ T, 30 min), which was comparable to that after exposure to heat (41°C, 30 min) or X-ray (200 kV, 5 Gy). Most recently, Zeng et al. (2004a) and Xu et al. (2004) demonstrated that ELF-EMF (50 Hz, 0.4 mT, 24h) altered the signal transduction-related protein expression in human breast cancer cells (MCF-7). Mannerling et al. (2004) who studied the hsp70 expression in several human cell lines reported an increased expression after exposure to ELF-EMF (50 Hz, 0.1 or 0.2 mT, up to 24h). Of course, the expression of genes and proteins induced by ELF-EMF may again be dependent on the type of cell exposed, its genetic background and its immediate metabolic stage and, of course, the pattern of exposure. The available data indicate that the flux density (threshold) at which effects on gene and protein expression are at first found is in the range or not far above the presently valid safety levels of 100 μ T for the general public or 400 μ T for the workplace.

4.2 Results obtained after RF-EMF exposure

4.2.1 Genotoxic effects

4.2.1.1 Human promyelocytic cell line HL-60 (Participant 2)

Discussion on potential health effects of using mobile telephones has focused on possible cancer-enhancing effects. It seems quite clear that any cancer-related effects of radiofrequency electromagnetic waves cannot be based on direct genotoxic effects, since the energy level is not high enough to damage DNA. Most of RF-field studies concluded that RF-field exposure is not genotoxic or mutagenic. With respect to DNA strand breaks, there is no replicated evidence for DNA and/or repair damage due to RF-field exposure (Lai versus Malayapa, examples see literature cited). On the other hand, some studies have shown that radiofrequency-field/microwave (RF-fields/MW) radiation and extremely low frequency (ELF) fields cause increased DNA strand breakage and chromosome aberrations. This has been shown in cell lines (Phillips et al. 1998), human blood (Verschaeve et al. 1994), animals (Lai and Singh 1995, 1996a/b, 1997a/b/c, 2004) and living human beings (Fucic et al. 1992; Garaj-Vrhovac 1999). The basic strategy in our studies was to test whether RF-EMF are able to alter DNA integrity (MN induction and DNA strand breakage), cell proliferation, cell cycle kinetics and/or apoptosis using the promyelocytic leukaemia cell line HL-60 testing different SAR levels, exposure times and signal modulations. For the experiments a highly standardized exposure system setup was provided by Participant 10. This setup enabled the exposure of suspensions of cells with a highly standardized temperature constancy, an inhomogeneity of SAR of less than 30% and an efficiency of more than 20 W/kg per Watt input power. All experiments were performed blinded, i.e. not knowing, which of the waveguides was exposed to the RF-field and which was the sham control.

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to the induction of single and double DNA strand breaks.

DNA damage through RF-EMF was evaluated immediately after exposure using the alkaline single cell gel electrophoresis assay (Comet assay). RF-fields at 1800 MHz, continuous wave exposure for different exposure times caused the induction of single and double DNA strand breaks in HL-60 cells. No significant difference was seen between exposed and sham exposed cells at a SAR of 0.2 to 1.0 W/kg. An increase in the steepness of the dose response relation is observed between SAR 1.0 W/kg and 1.3 W/kg. A less expressed increase is observed between 1.6 W/kg and 3.0 W/kg (Figure 73).

Two other laboratories have recorded that RF-field/MW produced significant DNA strand breaks. Verschaeve et al. (1994), who used a GSM cell phone signal to expose human and rat peripheral blood lymphocytes, found significantly increased strand breaks at high, but non-thermal exposure levels. Phillips et al. (1998) exposed Molt-4 T-lymphoblastoid cells with cell phone radiation in the SAR range 0.0024 W/kg to 0.026 W/kg. A 2-hour exposure to these low levels of cell phone radiation significantly increased or decreased the DNA damage. Decreased DNA damage is evidence of increased repair that is, of course, evidence of damage (Meltz 1995). In some other studies the observations of significant increase in DNA single and double strand breaks in brain cells of rats whole body exposed to 2.45 GHz RF-field (Lai and Singh 1995, 1996a/b, 1997a/b) were not confirmed using rodent and human cells

exposed in vitro and in vivo to RF-fields (Malayapa et al. 1997, 1998; Maes et al., 1997; Vijayalaxmi et al., 2000; Li et al., 2001).

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to an increase in micronuclei.

Micronuclei are easily measured under day light microscopy. They consist of small amounts of DNA that arise in the cytoplasm when chromatid/chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis. We have used the conventional cytokinesis-block MN assay to assess induction of cytogenetic damage in HL-60 cells after exposure to RF fields. RF-EMF exposure on HL-60 cells at 1800 MHz, 24h, continuous wave, at the given experimental conditions, caused a significant increase of micronuclei induction in the same SAR-dependent manner as observed for the induction of DNA strand breaks. Whereas at a SAR of 1.0 W/kg no significant difference of micronuclei frequencies was noted compared to sham controls, a clear increase was observed at SAR of 1.3 W/kg and 1.6 W/kg, and, less expressed at a SAR of 2.0 W/kg and 3.0 W/kg (Figure 72).

Induction of both, micronuclei and Comet formation, by RF-EMF was dependent on the time of exposure. A short exposure period of 6 hours caused no increase in MN frequencies compared to longer exposure times of 24 and 72 hours, respectively. Exposure to a 1800 MHz magnetic field at SAR of 1.3 W/kg for 72 hours produces a similar micronucleus frequency in HL-60 cells as that caused by 0.5 Gy ionising radiation (exposure time: 5.2 s), i.e., an average of 22 MN per 1000 BNCs (Figures 75, 76). However, it is not likely that the two entities cause MN induction by similar mechanism and produce the same types of DNA damage. In contrast to these findings, Comet formation already started after short exposure periods of 2 and 6 hours, respectively, with a maximum after 24 hours, and a clear decline occurred towards a longer exposure period of 72 hours. Mechanistically, this finding may be explained by DNA repair phenomena in the case of the DNA strand breakage in contrast to MN induction.

On the other hand, data from several other studies have indicated in primary human lymphocytes an absence of significant differences in the incidence of CA, SCE and MN between RF-EMF-exposed and sham-exposed cells (Vijayalaxmi et al. 1997, 2001a/b; Bisht et al. 2002). The significant increase and a weak effect on sister chromatid exchanges in RF-field exposed human blood lymphocytes reported by Maes et al. (1996, 1997) was not confirmed in their own subsequent investigation (Maes et al. 2001). Some positive findings occurred under conditions in which RF exposure elevated the temperature (Manikowska-Czerska et al. 1985; Sarkar et al. 1994; Varma and Traboulay 1997).

RF-EMF-associated increase of DNA strand breaks and micronuclei (1800 MHz, 1.3 W/kg, 24h) in HL-60 cells was signal-independent.

Interestingly, DNA strand breaks and MN induction were similarly induced by different RF-EMF signal modulations including CW exposure, CW intermittent exposure (5 min on/10 min off), 217 Hz pulse modulation and GSM Talk each at 1800 MHz, SAR 1.3 W/kg for 24h (Figure 77, 78).

RF-EMF induced formation of reactive oxygen species as shown by flow cytometric detection of oxyDNA and rhodamine fluorescence.

ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH^\cdot) and singlet oxygen (1O_2), continuously generated from the mitochondrial respiratory chain, own a powerfully oxidative potential. ROS are capable of attacking lipids, nuclear acids and proteins, resulting in certain degrees of oxidative damage. The total ROS level in resting HL-60 cells, however, was directly measured in the present study, by flow cytometric detection of Rh123 and the oxidized nucleotide 8-oxoguanosine (Figures 86, 87). Detecting the ROS level by flow cytometry has been a novel approach with the characteristic of fastness, convenience and reproducibility and, to our knowledge, has not been frequently reported before. DHR123, one of common ROS captures, is membrane permeable. It is oxidized intracellularly by ROS to become fluorescent Rh123, which is pumped into mitochondria and remains there. After a period of accumulation it is then detectable by flow cytometry (e.g., Gao et al., 2002). The probe used in the Calbiochem OxyDNA Assay kit is specific for 8-oxoguanine, which, as part of the oxidized nucleotide 8-oxoguanosine, is formed during free radical damage to DNA and is thus a sensitive marker for differences of ROS levels (de Zwart et al. 1999; Kasai 1997; Cooke 1996) in HL-60 cells after exposure to RF-EMF compared to control and sham-exposed cells (Figure 86).

If an involvement of free radicals in the mechanism of RF-EMF induced DNA strand breaks in HL-60 cells could be shown, this would have an important implication on effects to cell integrity due to RF-EMF

exposure. The “free radical hypothesis” stating that EMF increase free radical activity has been proposed by various researchers (Grundler et al. 1992; Reiter 1997; Lai and Singh 2004). Involvement of free radicals in human diseases, such as cancer and atherosclerosis, have been suggested (Beckmann and Ames 1997). Free radicals also play an important role in aging processes (Reiter 1995).

Co-administration of ascorbic acid, a free radical scavenger, inhibited the effects of RF-EMF on HL-60 cells and may, thus, decrease DNA damage without affecting cellular growth.

Two plausible biological mechanisms involving free radicals have to be discussed for the RF-EMF effect. The first involves increased free radical formation and activity and genetic damage as a response to RF-field exposure. The second involves increased free radical activity and genetic damage because of an induced reduction of free radical scavenger, e.g. reduced SOD activity or melatonin (Reiter 1994). Indications were found in our investigations for increased free radicals activities and a correlation with genetic damage (Figures 86 to 89). Cells possess efficient antioxidant defence systems, mainly composed of the enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, which can scavenge the ROS excessive to cellular metabolism, and make ROS level relatively stable under physiological conditions. Under the conditions used in our experiments, endogenous antioxidant enzyme activities of HL-60 cells (SOD and GPx activity) did not show pronounced alterations following RF-field exposure as compared to sham-exposure. Therefore, the first proposed mechanism mentioned above seems to be dominant.

In summary, the findings of an increase of micronuclei induction as well as Comet formation in HL-60 cells after exposure to RF-EMF at the conditions stated above indicate, that RF-EMF might generate genotoxic effects. The results obtained clearly show that RF-EMF under distinct exposure conditions cause DNA damage in human HL-60 cells. Since on the basis of these data RF-EMF have to be regarded as potentially genotoxic, it is pivotal to clarify first the molecular mechanisms involved in these potentially clastogenic effects in forthcoming experiments and secondly the biological consequences of DNA damage induced by RF-EMF, in particular the relevance for inducing mutations and changes in cellular signalling cascades. Responsive to the European Commission’s suggestions, additional independent verification experiments of the results obtained so far have to be conducted in the same cell line and other cell types, which are normal or similar in the metabolic process. Studies on indirect genotoxicity (e.g., reactive oxygen species, oxy-DNA, DNA repair) of RF-EMF on HL-60 cells, have to be extended. Studies on potential changes in gene expression profiles with respect to DNA repair have to be continued in co-operation with other groups.

4.2.1.2 Human fibroblasts and granulosa cells of rats (Participant 3)

RF-EMF generated DNA strand breaks in granulosa cells of rats and DNA strand breaks and chromosomal aberrations in human fibroblasts.

We could demonstrate an induction of DNA single and double strand breaks upon RF-EMF exposure in human diploid fibroblasts and in rat granulosa cells in culture. This induction depended on exposure duration as well as on the applied signal and could be determined in cells of different tissues. Based on the findings which we obtained with ELF-EMF, we also used for the intermittent RF exposure an “on” duration time of 5 minutes and an “off” duration time of 10 minutes. In contrast to ELF-EMF, RF-EMF induced DNA strand breaks also under continuous exposure conditions. However, the effects were more pronounced under intermittent exposure conditions at 5 min on /10 min off cycles.

The identification of the processes which lead to this DNA breakage will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study started already at an SAR of 0.3 W/kg which is far below 2 W/kg, the highest level allowed by the European safety limits. This suggests that the currently allowed radiation emission levels for the mobile phones, are clearly not sufficient to protect from biological effects. We have demonstrated, that the effect of ELF-EMF depends on the cell type and the on and off duration times used in research. The negative effects of RF-EMF reported in the literature (McNamee et al. 2002a, b; Tice and Hook 2002; d'Ambrosio and Scarfi 2002a), however, are based on lymphocytes and continuous exposure.

As with Elf-EMF, RF-EMF exposure of human fibroblasts induced also an increase in micronuclei and an even higher incidence of chromosome gaps, chromosome breaks, dicentric and acentric fragments, which was 10-fold after ELF-EMF exposure as compared to control cells and 100-fold after RF-EMF

exposure. The RF-EMF results regarding chromosomal aberrations are of preliminary nature, but they are in line with the results obtained after ELF-EMF exposure. The evaluation of the micronuclei carried out in our laboratory was reproduced blindly with coded slides in two independent laboratories that do not belong to the REFLEX consortium (Universities of Ulm and Kaiserslautern, Germany).

RF exposure revealed a significant decrease in the mitochondrial membrane potential in one experiment, which could not be reproduced. The RF induced formation of DNA strand breaks could not be related to changes in the membrane potential.

4.2.1.3 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exposure of ES-derived neural progenitor cells induced a low transient increase of double DNA strand breaks measured by the neutral Comet assay.

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to test the induction of primary DNA damages. It has been shown previously that EMF exposition of human HL-60 cells resulted in an increase of DNA breaks, suggesting a direct mutagenic effect (Ivancsits 2002). In addition, a correlation was found between up-regulation of GADD45, of the bcl-2 family member bcl-X_L, and an increased amount of early DNA damage measured by the alkaline Comet assay in human preneuronal cells exposed to the amyloid protein (Santiard-Baron 2001). Therefore, we used the alkaline and neutral Comet assay to detect single, and double-strand DNA breaks, resp., in neural progenitors derived from murine pluripotent ES cells after RF-EMF exposure. Under our experimental conditions, 6 hours exposure to GSM signals induced a low transient increase of double-strand DNA breaks, whereas ELF-EMF did not induce a significant DNA damage. Our finding suggests that genotoxic effects of RF-EMF, at least *in vitro*, could not be excluded.

4.2.1.4 Summary (Participant 1)

As discussed by Participant 2 (4.2.1.1) there is sporadic literature about *in vitro* studies demonstrating that RF-EMF may possess a genotoxic potential (The Royal Society of Canada 1999; Stewart Report 2000). Since the energy impact on the genome of living cells exposed to RF-EMF was calculated to be too low to cause DNA damage and since the mainstream literature contradicted the assumption of genotoxic effects (Moulder et al. 1999; Meltz 2003), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were elaborated in a hitherto unknown systematic approach and confirmed in four laboratories, of which two were not members of the REFLEX consortium, support the view that RF-EMF causes genotoxic effects in certain, if not all cellular systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings on genotoxicity caused by RF-EMF are hard facts. RF-EMF exposure at a SAR value below 2 W/kg induced an increase in DNA single and double strand breaks as well as in micronuclei in HL-60 cells. The DNA damage was dependent on the time of exposure, the field strength of RF-EMF and the type of RF-EMF signals. There is some indication that the effects may be caused via an increase in free oxygen radicals generated by RF-EMF (3.2.1.1, 4.2.1.1). RF-EMF exposure between SAR values from 0.3 to 2.0 W/kg made also DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals. This increase of DNA-strand breaks in human fibroblasts was accompanied by an increase in micronuclei and in chromosomal aberrations thus demonstrating that the DNA repair was not error-free (3.2.1.2, 4.2.1.2). In addition, RF-EMF exposure at a SAR value of 1.5 W/kg caused a slight, but significant increase in DNA double strand breaks in embryonic stem cells of mice (3.2.1.3, 4.2.1.3).

As already stated, for energetic reasons, RF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to RF-EMF is too low for a DNA damage, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes in the course of RF-EMF exposure. In their experiments Participant 2 observed an increase of free radicals in HL-60 cells after RF-EMF exposure. With the oxygen radical scavenger ascorbic acid it was possible to inhibit the generation of DNA strand breaks and of micronuclei during RF-EMF exposure (3.2.1.1, 4.2.1.1). This findings support the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by RF-EMF during

exposure. This possibility is further strengthened by the observation of Lai and Singh (1997a,b), who demonstrated that the increase in single and double DNA strand breaks in brain cells of RF-EMF exposed rats can be blocked with radicals scavengers. A final conclusion whether or not this finding is indisputable is still pending, since an increase in DNA strand breaks at the same model could not be confirmed by another research group (Malyapa et al. 1997, 1998).

Taken together, RF-EMF is able to damage the genome at least in certain cell systems after exposure in vitro. As with ELF-EMF, the genotoxic effects of RF-EMF may be best explained indirectly by an RF-EMF induced intracellular increase in free radicals. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through RF-EMF in vivo as suggested by the work of Lai and Singh (1997a,b) needs further clarification.

4.2.2 Cell proliferation and differentiation

4.2.2.1 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EMF did not affect cell growth of NB69 and neural stem cells.

A short-term (24h) exposure to the GSM-Basic signal does not modify the cell growth of NB69 cells and NSC. However, as described in 4.2.4.2, this signal induced in both cell a reduction in the proportion of cells expressing FGFR1. Signalling through fibroblast growth factor receptors (FGFRs) is essential for many cellular processes, including proliferation and differentiation (Kovalenko et al., 2003) and nervous system development (Oh et al. 2003). Our results indicate that in the selected exposure conditions, the GSM-basic signal does not induce changes in cell proliferation. Also, the short-term response induced by this GSM-signal on FGFR1 does not seem to be related to changes in cell growth.

4.2.2.2 Human lymphocytes and thymocytes (Participant 8)

RF-EMF may not affect proliferation, cell cycle, apoptosis and activation of human lymphocytes and thymocytes.

The immune system plays a decisive role in health and disease. Therefore, it was important to find out whether or not RF-EMF affect the immune system. Lymphocytes were exposed to RF-EMF at 1800 MHz with three different signals, such as GSM basic, Talk modulated and DTX only (SAR 1.4 - 2 W/kg). The in vitro tests were chosen in order to study the following endpoints: 1) cell proliferation; 2) cell cycle; 3) expression of membrane receptors on T lymphocytes, 4) spontaneous and induced apoptosis; 5) mitochondrial membrane potential (MMP) modifications in induced and spontaneous apoptosis; 6) cytokine production; 7) Hsp70 levels in induced and spontaneous apoptosis; 8) thymocyte development and apoptosis; 9) T lymphocyte gene expression.

On the whole, the results obtained suggest that no differences exist for the most endpoints studied in RF-EMF exposure. Only some slight differences were observed in PBMCs; in particular, CD8+CD28+ appeared increased in exposed cultures, but the difference (3%) of the order of the calculated standard error did not indicate a relevant effect from a biological point of view. Actually, in a previous work we found that 900 MHz (SAR 76 mW/kg) RF seem to slightly decrease lymphocyte proliferation when these cells are low-stimulated (Capri et al., accepted 2004); thus our results suggest that RF effects on lymphocyte proliferation are frequency-dependent. However, the literature on this field is still scanty. Some groups showed different effect on cytolytic T lymphocyte proliferation (Cleary et al. 1996) and some groups did not found significant effects on mitotic indices between RF-exposed and sham-exposed lymphocytes (Vijayalaxmi et al. 1997).

A more interesting result appears the decrease of CD95 molecules on membrane surface of stimulated CD4 helper T cells, from elderly donors, which was found when cells were exposed to Talk modulated RF in comparison with sham exposed cells. Due to the importance of this receptor in the regulation and homeostasis of immune response, these results deserve further evaluations to confirm this decrease (around 9%) on CD4⁺ helper T lymphocytes from elderly and not from young donors.

An important observation was the observed decrease (around 13%) of IL-1 β production; this effect was found only in low-stimulated PBMCs exposed to DTX RF and suggest that a possible cell target of RF-EMF are monocytes rather than lymphocytes. Also this effect deserves further investigation in order to confirm possible interactions of RF-EMF exposure with human monocytes. Data in the literature are really scanty. A recent study, performed in vivo, demonstrated a transient increase of interferon- γ (IFN- γ) in mice exposed to GSM-modulated 900 MHz in mice exposed 2 hours/day for 1, 2 and 4 weeks in a TEM cell (Gatta et al. 2003)

Negative results are extremely important for evaluations on human health risk. RF-EMF exposure is obviously not able to interfere with cell cycle, spontaneous or chemically- induced apoptosis, mitochondrial membrane polarisation and cell activation. Negative results were also obtained on thymocyte development. This last result is extremely important, since it was observed in conditions very near to what happens in vivo. Moreover, results from gene expression of quiescent T lymphocyte confirm the absence of significant changes due to RF-EMF exposure.

4.2.2.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF generated genotoxic effects in HL-60 cells within a narrow energy window without affecting cell proliferation, cell progression and apoptosis.

Using the MTT assay, the annexin V assay, the TUNEL assay, cell counting, determination of cellular doubling time and thymidine kinase activity, it could be shown that the RF-field at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not effect cell viability and cell growth, and did not induce apoptosis in HL-60 cells. These findings are in substantial agreement with previous literature reports on effects of RF-EMF in HL-60 cells and other human cells (e.g., Hambrook et al. 2002, Higashikubo et al. 2001). In contrast to the present results, induced cell proliferation and apoptosis have been reported in various other cell types after exposure to EMF (Blumenthal et al. 1997, Philips et al. 1997, Ismael et al. 1998, Kwee and Raskmark 1998, Simko et al. 1998, Velizarov et al. 1999).

4.2.2.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells.

Several in vitro studies report negative effects of high frequency EMF on cell cycle, gene expression and differentiation (Fritze et al. 1997; Cain et al. 1997; Goswami et al. 1999; Ivaschuk et al. 1997), DNA and chromatin structure (ICNIRP 1996; Repacholi 1998) and rat embryo development (Klug et al. 1997). In contrast, several reports described positive effects by high-frequency EMF exposure on the length of cell cycle phases, proliferation and gene expression levels in mammalian cells (Cleary et al. 1996; Czerska et al. 1992; Goswami et al. 1999; Lai and Singh 1996a; Sarkar et al. 1994). These studies, however, were performed with different experimental models, carrier frequencies (835 MHz to 2.45 GHz versus 1.71 GHz used in our study) and modulation schemes, and therefore, are not comparable. Moreover, positive RF-EMF effects were often observed at relatively high average SAR values (Cleary et al. 1996; Czerska et al. 1992; Fritze et al. 1997), which suggests that they could arise from RF-EMF-evoked thermal effects. In our studies, GSM signals were applied under conditions of the ICNIRP safety limit using an experimental set-up that enabled precise temperature control (Schönborn et al. 2000), and any temperature increase as a consequence of EMF exposure (Laurence et al. 2000) can be excluded.

For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test (EST, (Spielmann et al. 1997) using cardiac differentiation of ES cells as endpoint has been established. Therefore, for a further specification of the effects of GSM-217 signals, we analysed EMF exposure during the process of cardiac differentiation. GSM-217 EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells. These data present evidence that wild-type ES cells are not sensitive during cardiac differentiation to EMF. However, this finding is in contrast to EMF-induced effects in ES-derived neural progenitors.

The differentiation process in cells is affected by RF-EMF exposure, when applied at the neural progenitor stage.

The intact nervous system might be very sensitive to induced electric fields and currents, due to the high level of spontaneous activity and the greater number of interacting neurons. It has been suggested that induced current densities above 10 mA/m² may have effects on some central nervous system functions (Saunders and Jefferys 2002). Because of the special public concern for neurotoxicity due to EMF exposure, we used an experimental protocol successful at selectively differentiating ES cells into the neural lineage (Rolletschek et al. 2001). It provides a tool to investigate in vitro neuropathogenic effects of environmental factors during early development. We exposed the cells to EMF during the differentiation stage when the first neural nestin-positive progenitors appear. This developmental stage is presumably very sensitive to environmental factors. In our experiments, we observed RF-EMF effects on neural differentiation. Among the investigated transcripts (the mRNA levels of the neuronal genes TH, Nurr1 and en-1, and the astrocyte-specific gene GFAP) we observed a statistically significant down-regulation of nurr at 4d+11d and TH at the terminal stage 4d+23d. This might indicate a delayed neural differentiation and would correlate with the up-regulation of the growth arrest gene GADD45 at terminal stage. The significant up-regulation of GADD45 at the terminal stage 4d+23d was also confirmed by quantitative RT-PCR with TaqMan probe. Bcl-2, whose transcript levels were found increased in our study, has also been shown to be involved in neuronal differentiation and axonal regeneration (Daadi et al. 2001). Human teratocarcinoma-derived neurons expressed bcl-2 in 85% of the implanted neurons after transplantation into the rat striatum. In addition, the in vitro induction into the neuronal lineage resulted in an up-regulation of bcl-2 expression. The authors suggested that neuronal differentiation could be mediated at least partially by bcl-2 (Daadi et al. 2001).

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to measure the eventual induction of primary DNA damages (4.2.1.3)

4.2.2.5 Summary (Participant 1)

As discussed by Participant 4 (4.2.2.4), the results on possible effects of RF-EMF on cell proliferation and differentiation in vitro which are reported in the literature (The Royal Society of Canada 1999; Stewart Report 2000) are controversial. The REFLEX data do not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) and neuronal stem cells (3.2.2.1, 4.2.2.1), embryonic cancer cells (P19) (3.2.4.1, 4.2.2.4), human lymphocytes and human thymocytes (3.2.2.2, 4.2.2.2) and HL-60 cells (3.2.2.3, 4.2.2.3). In neural progenitor cells only some effect on the differentiation process was observed at a SAR level of 1.5 W/kg (3.2.4.1, 4.2.2.4). Quite obviously, whether or not living cells respond to RF-EMF exposure in vitro may depend on the type of the cell, its genetic background, its metabolic state and, of course, on the exposure conditions.

An answer of what may be the reason for the sporadically observed, but until now not confirmed influence of RF-EMF on cell proliferation and differentiation may be provided by the REFLEX findings on gene and protein expression. As shown by Participant 5, RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum (3.2.4.2, 4.2.4.2). Participant 3 (3.2.4.3, 4.2.4.3), Participant 6 (3.2.4.6, 4.2.4.6), and Participant 12 (3.2.4.7, 4.2.4.7) observed, that RF-EMF enhanced the expression of various genes among them ribosomal and mitochondrial genes, ATP related genes and genes encoding calcium-associated proteins and cell cycle proteins.

Of course, the relationship between RF-EMF exposure and the acceleration or inhibition of cell proliferation and differentiation in vitro caused by alteration of gene and protein expression is not proven yet. Should that be shown one day, it is to be found out, whether such cellular events occur also in vivo in RF-EMF exposed man and animal. The most recent data of Weisbrot et al. (2003), who observed an increase in the numbers of off-springs, an elevation of the hsp70 levels, an increase in serum response element (SRE) DNA-binding and an induction of the phosphorylation of the nuclear transcription factor, ELK-1, in *Drosophila melanogaster* after RF-EMF discontinuous exposure (900/1900 MHz, 1.4 W/kg) during the 10 day developing period, speak in favour of such an assumption.

4.2.3 Apoptosis

4.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

There is no indication that apoptosis is affected in nerve and immune cells after exposure to GSM-like RF-EMF.

Beside the importance of the apoptotic process in cellular homeostasis, only a few papers are available in the literature on the effects of ELF-EMF on apoptosis and almost no data were published on the interaction of RF fields with the apoptotic process. Thus, one of our objectives within the REFLEX programme was to investigate the potential role of environmental electromagnetic fields, specifically GSM-900 radiofrequency radiation (RFR) on the apoptotic process in critical cell types.

Briefly, apoptosis or programmed cell death plays a central role both in development and homeostasis of multicellular organisms (Skulachev 2002). A dual physio-pathological role of cellular apoptosis has been described (Rossi and Gaidano 2003). On the one hand, apoptosis is a major mechanism of protection against genotoxic agents since potential cancer cells are removed by apoptosis. On the other hand, dysregulation in the apoptotic pathways is involved in different pathologies since excessive apoptosis can contribute to diseases such as AIDS or neurodegenerative diseases (Olney 2003) whereas default in apoptosis is involved in cancer or autoimmune diseases (Burns and el-Deiry 2003). Moreover, inducing apoptosis in apoptosis-resistant tumour cells may lead to therapeutic applications (Tolomeo and Simoni 2002) while preventing apoptosis in apoptosis-sensitive cancer cells may be deleterious.

Because the phone is close to the head when in use, brain cells represent a major potential target for RFR emitted by the phones. Furthermore, one of the most critical cell types in the central nervous system is primary neurons. For our studies, we chose rat granule cells. Granule cells of the cerebellum constitute the largest homogeneous neuronal population of mammalian brain. Cerebellar granule cells are a model of election for the study of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection (Contestabile 2002). We failed to detect any influence of GSM-900 exposure on apoptosis in this highly critical cell type. All other nerve cell types tested, i.e. SH-SY5Y neuroblastoma cells, human U87 astrocytoma cells and rat C6 glioma cells - a priori less critical than granule cells - were not shown to be sensitive to GSM-900 exposure for up to 24 hours. Hence, no demonstration of an immediate or delayed effect of RFR on apoptosis in nerve cells has been made in rat primary cells and human cell lines. We conclude from our results that nerve cells do not represent a major target, in terms of apoptosis, for RFR emitted by mobile phones.

Because of the role of the immune system for cell homeostasis, cells from the immune system were to be tested. No evidence for an immediate, cumulative or delayed effect of RFR on apoptosis was shown in a human monocytic cell line. Gene expression experiments gave some confirmation that RF-EMF (GSM-900) had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, in our experiments, no substantial effect of exposure to RF-EMF (GSM-900) on spontaneous apoptosis of nerve and immune cells was found. No delayed effect could be evidenced either. When tested, interaction between GSM-900 exposure and pro-apoptotic chemicals could not be evidenced.

The results from the REFLEX programme strongly suggest that the apoptotic process may not be a major biological target for GSM mobile telephony-related signals. The REFLEX programme is contributing to most of our current knowledge on the effects of RF fields on cellular apoptosis. Two papers have been very recently published on that topics. Hook et al. (2004) found no evidence of programmed cell death in Molt 4 human lymphoblastoid cells after exposure to 4 different American signals for up to 24 hours at SAR ranging from 0.0024 and 3.2 W/kg. Markkanen et al. (2004) in Finland reported that 900-MHz CW or GSM-modulated RF fields at a SAR of 0.6 W/kg did not induce apoptosis in a control yeast strain and in its temperature-sensitive mutant of *cdc48* (apoptosis strain). When yeast strains were pre-exposed to UV, GSM-900 only was able to enhance the UV-induced apoptosis in the mutant yeast strain only. In the REFLEX programme, no significant effect on spontaneous apoptosis was detected in cells from the immune system (human peripheral blood mononuclear cells, human U937 cells) and in EA.hy926 human endothelial cells exposed to RFR-fields (GSM-900 and GSM-1800). No delayed effect (time kinetics) after RFR exposure was demonstrated.

In the present body of work, the status (transformed or non-transformed) of cells used did not influence the effect of RF-fields. The signalling pathways involving bcl-2 was not affected in either p53^{+/+} or p53^{-/-} embryonic stem cells tested after exposure to RFR-fields. The activity of caspase3 was not altered in EA.hy926 cells. In all systems tested, intermittence in the signal did not elicit apoptosis. Data also suggest that for the exposure conditions tested, field effects were not substantially affected by the cell genetics (embryonic stem cells), or the age of the donor (human peripheral blood mononuclear cells).

Hence, no effects of GSM signals (GSM-900 and GSM-1800) have been detected on spontaneous apoptosis of mature and embryonic stem cells in the various groups involved in REFLEX, even in conditions reported to modify other biological endpoints (for instance, an increase of hsp27 expression was detected in EA.hy926 human endothelial cells, 4.2.4.6). However, the expression of the bcl-2 anti-apoptotic gene was shown to increase in murine differentiating embryonic stem cells after exposure to GSM-1800, which correlated with changes in the process of neural differentiation (down-regulation of certain neuronal genes, 4.2.2.4). This needs to be further investigated in order to understand the potential relevance for human health.

We then focused our research on the investigation of some interaction between RF-EMF and known pro-apoptotic drugs. Extension of studies on the expression of apoptosis-related genes was also performed. The data show that cells from the immune and the nervous systems did not exhibit any sensitivity in a concomitant or successive treatment with apoptogenic chemicals and GSM signals, by contrast to cells from the endothelium. However, even in that cell type, GSM-900 was shown to interact with only one chemical (polyHema) over the two chemicals tested. The effect observed was a partial prevention of chemically-induced apoptosis. In this cell line, a weak decrease in pro-apoptogenic genes after exposure to GSM-900 was correlated with the former effect observed. These data on endothelial cells have still to be independently replicated.

Moreover, apoptosis-related genes were shown only weakly affected after exposure to RFR when compared to other gene families such as the ribosomal-related genes (Participant 12). Compared to data of the Juutilainen group, experiments performed within the REFLEX consortium used mammalian cells instead of yeast cells. This, as well as the nature of apoptogenic agent (chemical versus physical), may account for the discrepancy observed in the interaction experiments.

These data suggest that, except for murine differentiating stem cells, low-level RFR are not able to interfere with the spontaneous integrative apoptotic process. If confirmed, interaction with pro-apoptotic chemicals is suggested to be highly dependent of the cell type and the chemical agent used.

4.2.3.2 Human lymphocytes (Participant 8)

RF-EMF may not affect apoptosis in human lymphocytes.

1800 MHz RF (GSM basic, Talk and DTX modulated; SAR 1.3 - 2 W/kg) is not able to modify spontaneous and chemical-induced apoptosis, when human PBMC were exposed 10 min on and 20 min off for 44 hours. This result was also confirmed using cells from old donors (GSM basic, SAR 2 W/kg), since their cells could result differently susceptible to undergo apoptosis (Salvioli et al. 2003). Data were further confirmed by the analysis of mitochondrial membrane potential, which was not affected by RF in all the conditions tested. Negative results were also obtained analysing thymocyte apoptosis during their differentiation. On the basis of these data we can conclude that these types of exposures do not affect apoptotic process, even if this is not established for longer or chronic exposures. (4.2.2.2)

4.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

Using the annexin V assay and the TUNEL assay, it could be shown that RF-EMF at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not affect cell viability and cell growth, and did not induce apoptosis. (4.2.2.3)

4.2.3.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF affected the bcl-2 –mediated anti-apoptotic pathway in differentiating embryonic stem cells.

An up-regulation of bcl-2, bax and GADD45 transcript levels was observed after exposure of ES-derived neural progenitors at specific stages of differentiation to high frequency and extremely low frequency electromagnetic field. We studied the gene expression levels of regulatory genes like hsp70, p21 and apoptosis-related genes of the bcl-2 family (the anti-apoptotic bcl-2 and the pro-apoptotic bax gene). These regulatory genes were pre-selected after our previous experiments with undifferentiated p53-deficient ES cells, where we found significantly increased transcript levels of p21 and hsp70 (Czyz 2004a) after RF-exposure and of p21 after ELF-EMF exposure (50 Hz Powerline) (Czyz 2004b). Therefore, we analysed the effects of EMF on apoptosis-related genes in wild-type (wt) embryonic stem cells at the stage of neural differentiation. Our data demonstrated up-regulation of the transcript levels of bcl-2 in neuronally differentiated ES cells at terminal stages for RF-EMF. However, the biological significance of this finding and its relevance to the situation in vivo has to be clarified.

Apoptotic cell death is executed by caspases and can be regulated by members of the bcl-2 family as reported for differentiating murine embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Studies applying the model of central axotomy in mouse have shown a degeneration of up to 70% of nigral neurons post transection due to the activation of c-jun, but bcl-2 over-expression leads to a reduced phosphorylation state of c-jun in transected neurons and protection against cell death (Winter et al. 2002). Bcl-2 over-expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour et al. 2002). In other studies, in situ hybridisation revealed a rapid and transient increase in bcl-2 mRNA in neurons following de-afferentation (Wilkinson et al. 2002).

In the RF-EMF experiments, we extended our study by including the analysis of mRNA levels of the growth arrest DNA-damage inducible (GADD45) gene and found a significant up-regulation at the terminal stage of differentiation (at 4+23d). The members of the GADD protein family are considered to play important roles in maintaining genomic stability and in regulating the cell cycle (Chung et al. 2003). The phenotype of GADD45-deficient mice is similar to the phenotype of p53-deficient mice, including genomic instability and sensitivity to radiation induced carcinogenesis (Hollander et al. 1999). GADD45 was found to promote G2/M arrest thus inhibiting entry of cells into S-phase and allowing genomic DNA repair in keratinocytes (Maeda et al. 2002). These findings suggest that GADD45 is a component of the p53 pathway that maintains genomic stability, albeit damage-induced transcription of the GADD45 gene is supposed to be mediated by both p53-dependent (Kastan et al. 1992) and p53-independent mechanisms (Jin et al. 2001). Our results, which demonstrate an up-regulation of bcl-2 and GADD45 mRNA levels indicate, that electromagnetic signals are, probably, perceived in embryonic stem cell-derived neural progenitors as environmental stress signals at defined stages of differentiation. Such signals may trigger cellular responses for maintenance of the cellular homeostasis via mobilization of the mechanisms of DNA repair and protection against apoptotic cell death.

4.2.3.5 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF may affect the hsp27 mediated anti-apoptotic pathway in human endothelial cells.

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that “should” die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation (6.2.4.6).

4.2.3.6 Summary (Participant 1)

As discussed by Participant 9 (4.2.2.4), knowledge on a possible influence of RF-EMF on the apoptotic process in living cells *in vitro* is rather poor. In the two most recent studies no such effect of RF-EMF was observed (Hook et al. 2004; Markkanen et al. 2004). The REFLEX data did not reveal a significant influence of RF-EMF on apoptosis of various cell types such as brain cells and human monocytes (see 3.2.3.1 and 4.2.3.1), human lymphocytes (3.2.3.2, 4.2.3.2) and HL-60 cells (3.2.1.1, 4.2.3.3). On the other

hand, an indirect effect on apoptosis via the bcl-2 or hsp27 mediated anti-apoptotic pathway which was detected in differentiating embryonic stem cells (3.2.4.1, 4.2.3.4) and in endothelial cells, respectively (3.2.4.6, 4.2.3.5), cannot be excluded at this time.

Of course, based on the data on gene and protein expression obtained in the REFLEX project, an effect of RF-EMF on the apoptotic pathway, either through inhibition or promotion, seems to be possible. Whether or not cell cultures respond to RF-EMF may depend on the type of cell exposed, their genetic background, their metabolic state and, of course, on the pattern of exposure. But taken together, even if a relationship between RF-EMF exposure and an inhibition or promotion of apoptosis in *in vitro* experiments were proven, it would in no way be possible to draw any conclusion for the *in vivo* situation in man and animal.

4.2.4 Gene and protein expression

4.2.4.1 Mouse embryonic stem (ES) cells (Participant 4)

The genetic constitution of early differentiating embryonic stem cells may play a role on their responsiveness to differently modulated RF-EMF.

In ES cells deficient for the tumour promoter p53, a permanent up-regulation of mRNA levels of the stress response gene hsp70 paralleled by a slight and temporary increase of p21, c-jun and c-myc expression was found in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. On the other hand, wt ES and EC cells exposed to GSM-217 signals revealed no effects on gene expression, cardiac differentiation and proliferation. This would indicate, that the genetic background of stem cells may potentially influence the response of early differentiating cells to GSM signals dependent on the modulation schemes, whereas wt cells analysed in this study remained insensitive to GSM-modulated EMF.

p53^{-/-} mice are highly susceptible to the development of spontaneous tumours, in particular, of malignant lymphomas at early age (Attardi and Jacks 1999; Sigal and Rotter 2000). p53 is required for G1 arrest in response to DNA damage and is involved in apoptosis (Attardi and Jacks 1999) via modulating versatile regulatory genes. In our studies using mouse p53^{-/-} ES cells, hsp70 mRNA levels were continuously elevated after 48h EMF exposition. Heat shock proteins act as chaperons whose expression is activated or up-regulated in response to external stress (Beere and Green 2001). Hsp70 has also been defined to regulate homeostasis in response to external stress during early embryo development (Luft and Dix 1999), while up-regulated hsp70 levels were observed in tumour cells and are correlated with metastases and poor prognosis (Zylicz et al. 2001). A potential involvement of heat shock proteins in cell responses to EMF was reported recently: up-regulated hsp27 protein levels and a transient increase of hsp27 phosphorylation were found in human endothelial cells (Leszczynski et al. 2002).

However, if EMF act as inducers of cellular transformation processes, effects on expression levels of other early response genes should be expected. Indeed, we observed an up-regulation of c-myc, c-jun and p21 mRNA levels in p53^{-/-} cells upon GSM-217 exposure. The same genes were previously shown to be affected by environmental factors, such as UV or X-irradiation in various systems (Amati et al. 1993; Angel et al. 1988; Jean et al. 2001). Because in our model, the shifts in gene expression were low and transient, it is conceivable that EMF signals, while affecting gene expression pattern in p53^{-/-} cells, do not induce permanent cellular transformations in wt cells.

The response of early differentiating cells to RF-EMF is dependent mainly on the carrier frequency of the modulation schemes.

Contrary to GSM-217 signals, which elicited cellular responses in p53^{-/-} ES cells, GSM-Talk modulation exerted no effects on gene expression in our model. This indicates that low frequency components generated by GSM-Talk (2 and 8 Hz) do not promote the action of EMF signals in our cell system. In contrast, time-averaged SAR values (1.5 W/kg for GSM-217 vs. 0.4 W/kg for GSM-Talk) may comprise a factor determining the biological activity of EMF. Furthermore, it cannot be excluded that the modulation scheme (time distribution of high SAR pulses) may also play a role in evoking biological

responses, because slot-averaged SAR values remained similar between the analysed modulation schemes.

The exposure duration may also influence the biological responses to RF-EMF.

In our in vitro studies, up-regulated transcript levels of regulatory genes in p53^{-/-} ES cells were observed after 48 hours exposure to GSM-217 signals, whereas a short-term 6h exposure exerted no effects.

The parameters of genetic constitution, carrier frequency and exposure duration in determining the response of biological systems to RF-EMF have been proposed by in vivo studies using Eμ-Pim1-transgenic mice predisposed to develop spontaneous lymphomas. Repacholi et al. observed an increase in tumour formation after long-term, 18 months exposure of Eμ-Pim1-transgenic mice to 900 MHz EMF (Repacholi et al. 1997), which positive data suggest that mobile phone radiation-induced events may be hazardous to cells deficient in cell repair when occurring frequently over long periods.

It has been shown previously that the exposition of human HL-60 cells resulted in an increase in DNA breaks, suggesting a possible direct mutagenic effect (Ivancsits et al. 2002). In addition, there is a report about a correlation between up-regulation of GADD45, of the member of the Bcl-2 family bcl-X_L, and an increased amount of early DNA damage as measured by the alkaline Comet assay in human preneural cells exposed to the amyloid protein (Santiard-Baron et al. 2001). Therefore, we used the alkaline and neutral COMET assay to detect single, and double-strand DNA breaks respectively, in the neuronal progenitors derived from murine pluripotent ES cells after ELF or RF-EMF exposure. Under our experimental conditions, however, we could not find a clear evidence of increased single-strand DNA break induction in the alkaline Comet assay. A low but significant increase in double-strand DNA breakage was observed only after a short (6h) RF-EMF exposure in the neutral Comet assay.

In summary, we found that RF-EMF simulating GSM signals caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in ES cells deficient for p53 in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. Here again we found that the genetic constitution (loss of p53 function) could alter the responsiveness of ES cells and render them sensitive to high frequency EMF, while wild-type cells were irresponsive. However, we did not observe any distinct direct genotoxic effects as measured by the Comet assay.

In ES cell-derived neuronal progenitors we found indication of growth arrest and effects on apoptosis (a significant up-regulation of the growth arrest and DNA damage inducible gene GADD45, the proapoptotic bax and the antiapoptotic gene bcl-2 mRNA levels), which correlated with changes in the process of neural differentiation (down-regulation of the neuronal genes Nurr1 at stage 4d+11d and TH at 4d+23d).

4.2.4.2 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum.

In NB69 cells, this response is similar to that induced by exogenous treatment with 20 ng/ml of bFGF, and by the combined treatment with bFGF plus EMF. In this biological system, an induction of morphological changes (increases in cell size and cell extensions) is also observed after 24 hours of treatment with bFGF. Such morphological changes are accompanied with a reduction in the proportion of cells expressing FGFR1-3 receptors. However, in cultures exposed to the GSM-Basic for 24 hours the effect on FGFR1 was not associated to changes in the cells' morphology. Evidence exists that treatment of olfactory neuroblastoma cells with bFGF deregulates FGFR1 prior to differentiation (Nibu et al. 2000). It is possible that in our NB69 cells the response induced the GSM-Basic signal on FGFR1 can also be associated to a promotion of differentiation in long-term cultures. Additional work using differentiation markers for neuronal NB69 cells has to be done to verify the above hypothesis.

On the other hand, in neural stem cells, the 24h-exposure also induced a reduced expression of receptor FGFR1, and further significant changes in the cell morphology were observed six days later, in the absence of the GSM-exposure. The oligodendrocytes showed an advanced developmental stage with respect to controls. Similarly, the astrocytes showed longer cell-processes. The morphology of the neuronal progeny of NSC was not significantly changed by the exposure to the GSM-Basic signal.

Evidence exists that treatment of a human astrocytoma cell line, U-87 MG, with 835 MHz electromagnetic radiation induces alterations in F-actin distribution and cell morphology (French et al. 1997). Their astrocytes showed a similar response to that observed in our precursors lineage exposed to the GSM-signal: an increased cell spreading. Taken together, the present data suggest that the reduction of cells expressing FGFR1 induced by signal could be linked to a promotion of the differentiation of non-neuronal populations.

The changes in FGFR1 induced by RF-EMF are dependent mainly on the carrier frequency.

Both, basic and CW signal induced similar changes on FGFR1. This suggests that the cellular response is not dependent on the tested low-frequency modulation, but on the carrier frequency. The signals used in advanced telecommunication systems such as global system for mobile communications (GSM) and universal mobile communications system (UMTS) include extremely low frequency (ELF) amplitude modulation or pulse modulation components. There is a lack of scientific data on the possible health implications of such modulations. A recent study (Huber et al. 2002) has reported that a 30-minute exposure to 900 MHz, 1 W/kg GSM signal can influence the electrical activity of the brain, both before and after sleep onset, in young male test subjects. Both sleep and waking EEG changes were observed only with pulse modulated-EMF. Also, it has been reported a statistically significant micronucleus effect in peripheral blood cultures following 15-minute exposure to phase modulated field (Gaussian minimum shift keying, GMSK), 1.748 GHz, at SAR \leq 5 W/kg (d'Ambrosio et al. 2002b). However, the micronucleus frequency result was not affected by CW exposure. No changes were found either in cell proliferation kinetics after exposure to both CW and phase modulated fields. In our present work we investigated whether fibroblast growth factor receptors (FGFR) could be influenced by the modulation of the GSM-signals. NB69 cells were exposed to GSM-Talk, GSM-Basic and CW signals, 2 W/kg SAR or to DTX-signal, 1W/kg SAR. The exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a significant decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls) without affecting significantly the number of cells expressing receptors R2 and R3. The effect on R1 was equivalent to that induced by basic fibroblast growth factor (bFGF) at a 20 ng/ml concentration. The exposure to RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal, whereas the exposure to GSM-Talk signal at the same SAR (2 W/kg) or to DTX-signal (1 W/kg SAR) did not modify significantly the normal expression of the FGF protein receptors R1. Our results indicate that the ELF modulation components resulting from the GSM signals shape (2, 8 and 217 Hz) and higher harmonics are not critical for the EMF-induced changes in FGFR-1 expression. Provided that the Talk mode is a temporal change between GSM Basic (66%) and DTX only (34%) our data together with those from Partner 4 indicate that the exposure duration could also be a critical factor for the herein described response. Future studies may also examine dose-response relationships by varying the exposure time and the specific absorption rate.

4.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF modulates the gene and protein expression in HL-60 cells.

Applying high resolution two-dimensional polyacrylamide gel electrophoresis to the HL-60 cell system, more than 4000 protein spots can be differentiated on the silver stained protein map. These spots were detected and the master gel image was calibrated. Clear differences in protein expression have been found for RF-field exposed HL-60 cells as compared to control and sham-exposed cells. The quantitative comparison has been completed. Further strategies in the future will include structural and biochemical identification of proteins significantly altered following RF-field exposure, beside mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS) and immunoblotting/functional protein assays, also by comparative studies with reference databases. Clarification of changes in protein expression after exposure to RF-fields will help to understand molecular pathomechanisms.

4.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

1800 MHz RF (DTX modulated, SAR 1,4 W/kg) is not able to modify gene expression profile when quiescent T lymphocyte are exposed 10 min on/20 min off for 44 hours. This result, obtained in collaboration with Participant 12 by means of micro-array technique, was expected because lymphocytes

were not stimulated and represents a first step toward a further evaluation in low-stimulated and RF-exposed T lymphocytes; future analyses should clarify the presence of potential gene targets for RF exposure in primary human cells.

4.2.4.5 Brain cells of different origin, human immune cells and human endothelial cell lines (Participant 9)

There is no indication that expression and activity of the inducible Nitric Oxide Synthase (iNOS or NOS₂) is affected in nerve cells after exposure to RF-EMF.

Under pathological conditions, nitric oxide, NO, can act as a neurotoxic agent (Leist and Nicotera 1998; Brown and Bal-Price 2003). A variety of stresses are known to induce neuronal cell death via NOS₂ (or inducible NOS, iNOS) activation and NO production in stimulated astrocytic cells. Hence, activated astrocytes may be involved in the pathogenesis of neurodegenerative diseases. Our goal was to determine whether exposure to a GSM-900 signal could activate C6 glioma cells by increasing the activity of the iNOS enzyme. A potential synergistic effect of such radiofrequency radiation (RFR) on cytokine-induced NO production was also investigated.

A few data are available in the literature on the effect of RF-EMF on nitric oxide, NO, production. Over three identified papers, only one used RF-EMF compatible with mobile telephony. Miura et al. (1993) reported that 10 MHz RFR (10 kHz bursts) caused an increase in NO production in rat cerebellum extracts. Using ultra-wideband pulses, Seaman et al. (2002) recently showed no influence of RFR in the Ultra Wide Band (UWB) range in RAW 264.7 macrophages except when nitrate was added to the culture medium. Paredi et al. (2001) showed a tendency for higher nasal NO levels in humans exposed to GSM-900 for 30 minutes that was due to skin heating experienced by the phone's users. As it has been clearly shown that heating from mobile phone microwaves is negligible, this effect was probably due to the battery's heating. Based on our data, GSM-900 did not appear to be able to alter chemically- induced activation in mammalian astrocytes and thus appeared unlikely to influence tumour cells characteristics and neuronal cells' viability via NO pathways.

There is no indication that expression of heat shock proteins is affected in nerve cells after exposure to RF-EMF.

The first objective was to determine whether exposure to GSM-900 microwaves could influence the expression of hsp70 proteins in neuronal and glial cell lines as reported in an endothelial cell line by Participant 6.

In response to environmental disturbances, cells respond by expressing heat shock proteins. Our study focuses on the 70-kDa family, which is the major form of stress proteins found in the brain (Pavlik et al. 2003) and on hsp27 that is expressed in endothelial cells (Loktionova et al. 1996).

Our data showed that exposure to GSM-900 microwaves were not able to induce hsp70 expression in rat and human nerve cells. These data are not in agreement with results of recent research showing that the expression of heat shock protein (hsp) may be induced in response to radiofrequency radiation exposure at non-thermal levels in different models (de Pomerai et al. 2000, in worms; Kwee et al. 2001, in human amnion cells; Leszczynski et al., 2002 in human EA-hy926 endothelial cells; Weisbrot et al. 2003, in *Drosophila*). Thus, a common feature on the effect of GSM-900 at low SAR (about 2 W/kg) on the expression of hsp cannot be drawn. Different cell types could behave differently to exposure to a GSM-900 signal or different members of the hsp family could show a different sensitivity to exposure to GSM signals. Moreover, we can note that, so far, none of the "positive" effects have been independently replicated.

We failed to independently confirm that expression of heat shock proteins is affected in EA-hy926 cells after exposure to GSM-like RF-EMF.

The second objective was to confirm the data of Participant 6 on hsp27 in the EA-hy926 cells. Hsp27 is indeed the major form of stress proteins that is expressed in endothelial cells (Loktionova et al. 1996). Our results obtained by fluorescent image analysis in the two cell lines tested differed from those obtained in one of them by Participant 6 after western blotting experiments. A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression since the sensitivity reported is 1-10 ng for Western Blot and less than 1 ng for ELISA.

Here, with our semi-quantitative method and statistical analysis we were unable to confirm previous data on hsp27 expression in endothelial cell lines. Exposure set-up used in both groups also differed (water bath versus air cooling, homogeneity of SAR distribution at the cells level, ...etc), which probably imply different dosimetric features. Whether this could account for the discrepancies observed could be determined (Participant 10). Meanwhile, we cannot conclude yet that RFR induce stress response. Hence, no implication for health hazard can be drawn at the moment based on Hsp expression in mammalian cells after low level RFR exposure.

The possible effect of low-level RFR on the expression of hsp is quite controversial. While some laboratories reported effects in mammalian cells (Leszczynski et al. 2002; Kwee et al. 2001) or simple organisms (de Pomerai et al. 2000; Weisbrot et al. 2003) as stated above, we (present work), as other groups (Cleary et al. 1997; Tian et al. 2002a; Miyakoshi et al. 2003) could not observe any effect in mammalian cells at SAR up to 10 W/kg. To date, no clear and satisfactory explanation can be given. However, we do think that the investigation of hsp expression after RFR exposure in *in vivo* mammalian models will help and enlighten the debate. Some but still sparse data are available, showing that heat shock proteins could not be induced in rat brain below 7 W/kg (Fritze et al., 1997).

There is some indication that gene expression is affected in immune cells after exposure to RF-EMF.

These data were obtained in collaboration with Participant 12 (4.2.4.7). First, these results gave a confirmation that GSM-900 had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. Then, only a few genes among several thousand tested genes were shown altered after RFR exposure (increase or decrease) in two human immune cell lines. The largest modification in RNA expression corresponded to genes related to signal transduction and energy metabolism. Finally, amine oxidase activity-related genes experienced the largest changes after exposure to mobile phone-like RFR. This later gene is coding for an enzyme which is involved in cell growth and proliferation but also in immune regulation. This gene-profiling analysis showed that RFR can influence some biological processes and gave us trails for further investigations such as looking at energy metabolism in cells exposed to RFR using spectroscopic NMR. Comparing gene profiling obtained in different cell types may provide a “signature” for environmental RF-EMF exposure. It is however still unclear if and how those changes in gene expression can be related to human health.

Reported gene profiling after ethanol stress in yeast (Alexandre et al. 2001) showed that about 6% of the yeast genome were experiencing changes (about 3% upregulated and 3% downregulated). This represents 20 fold more genes than what was found affected after exposure to RF-EMF (0.3%). Genes identified were mainly involved in energetic metabolism, protein destination, ionic homeostasis and stress response with more than 10 hsp members. Stronger criteria were used in Alexandre et al. (2001) for significance of changes in gene expression compared to ours. When compared with the effect of a strong stress agent such as ethanol (in yeast), RF-EMF affected only a few genes in the human immune cell lines we tested. Whereas genes involved in energy metabolism seem to be a common feature for both types of exposure, none of the genes identified in human immune cells after RFR exposure belonged to the stress response family. Based of these comparisons, RF-EMF may be identified as a weak environmental stress, if any.

4.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF induce cellular stress response.

Observed in this study changes in protein phosphorylation and activation of p38MAPK/hsp27 stress response pathway agree with the earlier studies suggesting that mobile phone RF-EMF radiation induces cellular stress response at non-thermal power level. In vitro, Cleary et al. (1997) claimed that RF-EMF exposure has no effect on stress proteins. However, because identification of stress proteins was based solely on their molecular weight in liquid chromatography, the exact identity of proteins claimed to be stress proteins remains unclear. Fritze et al. (1997b), using rat model, have shown increase in expression of stress protein hsp70 in brains of animals exposed for 4 hours to RF-EMF (890-915 MHz) at SAR of 1.5 W/kg. Daniells et al. (1998) and de Pomerai et al. (2000) have shown that overnight in vivo irradiation of nematode worms with RF-EMF (750 MHz) at SAR of 0.001 W/kg cause increase in expression of heat shock protein. Kwee et al. (2001) have shown induction of stress protein hsp70, but not hsp27, in cultures of transformed human epithelial amnion cells exposed for 20 min to RF-EMF (960 MHz) at SAR of 0.0021W/kg.

Because of the known broad spectrum of physiological processes that are regulated by stress proteins (Tibbles and Woodgett, 1999), and by hsp27 in particular, it is here hypothesized that mobile phone radiation-induced activation of hsp27/p38MAPK-dependent cellular stress response might: (i) lead to the development of brain cancer due to inhibition of cell apoptosis and (ii) cause increased permeability of blood-brain barrier due to stabilization of endothelial cell stress fibers (Figure 138).

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that “should” die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation. Hsp27, stress protein shown in this study to be affected by mobile phone radiation exposure, is a member of a family of small heat shock proteins that is ubiquitously expressed in most of cells and tissues under normal conditions in form of large-molecular complexes. In response to stress occurs rapid phosphorylation of hsp27 on serine residues (in human cells Ser-78 and Ser-82) what leads to dissociation of the large-molecular complexes into smaller units (Kato et al. 1994). Various stress factors have been indicated as inducers of changes in expression (accumulation) and/or phosphorylation (activity) of hsp27 (Ito et al. 1995; Deli et al. 1995; Garrido et al. 1997; Huot et al. 1997; Tibbles and Woodgett 1999). Activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome c), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which, in order to become active, has to be proteolytically cleaved by caspase-9. Thus, induction of the increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells what, in favourable circumstances, could help clonal expansion of the transformed/damaged cells - a prerequisite for the tumour development. Furthermore, hsp27 in particular was shown to be responsible for the induction of resistance of tumour cells to death induced by anti-cancer drugs (Huot et al. 1996; Garrido et al. 1997). Thus, it appears possible that RF-EMF induced changes in hsp27 phosphorylation/expression might affect not only tumour development but also its drug-resistance.

Induction of the increase of the permeability of blood-brain barrier by RF-EMF exposure, which has been suggested by some animal and in vitro studies, is one of the controversial health issues that came up in relation to the use of mobile phones. It has been already established that, at thermal levels of exposure, microwave radiation causes increase in the permeability of blood-brain barrier (for review see Jokela et al. 1999; The Royal Society of Canada Report 1999, Stewart Report 2000, Zmirou Report 2001). However, the effect of non-thermal RF-EMF exposure on blood-brain barrier is still unclear. Some studies have suggested that mobile phone radiation, at non-thermal exposure levels, increases permeability of blood-brain barrier *in vivo* (Salford et al. 1994) and *in vitro* (Schirmacher et al. 2000), whereas others suggested lack of such effect (Fritze et al. 1997a; Tsurita et al. 2000). However, the no-effect claimed by Fritze et al. (1997a) is not so straight forward as suggested by the authors because they reported induction of stress response and increased permeability of the blood-brain barrier immediately after the end of irradiation. This effect was short lasting and, because of it, was considered by the authors as insignificant. Also, it remains unclear what would be the blood-brain barrier response to the repeated exposures to mobile phone radiation because the effect of repeated exposures was not examined. The increased blood-brain barrier permeability due to increase of endothelial pinocytosis was suggested by Neubauer et al. (1990) who have demonstrated increase in pinocytosis of cerebral cortex capillaries that were exposed to 2.45 GHz microwave radiation. Finally, the recently reported study by Töre et al. (2001) has shown that 2 hour exposure of rats to RF-EMF (900 MHz) at SAR of 2W/kg (averaged over the brain) causes increase in the permeability of blood-brain barrier. However, the molecular mechanism and the cellular signalling pathways that are involved in the induction of blood-brain barrier permeability are still unknown. We propose that the induction of hsp27 phosphorylation and increased expression by RF-EMF exposure, shown in this study to occur in vitro in human endothelial cells, might be the molecular signalling event that triggers the cascade of events leading to the increase in blood-brain barrier permeability. Phosphorylated hsp27 has been shown to stabilize endothelial cell stress fibers due to the increased actin polymerisation (for review see Landry and Huot 1995). The stabilisation of stress fibres was shown to cause several alterations to endothelial cell physiology: (i) cell shrinkage and opening of spaces between cells (Landry and Huot 1995; Piotrowicz and Levin 1997), (ii) increase in the

permeability of endothelial monolayer (Deli et al. 1995), (iii) increase in pinocytosis (Lavoie et al. 1993), (iv) formation of apoptosis-unrelated blebs on the surface of endothelial cells which may obstruct blood flow through capillary vessels (Becker and Ambrosio, 1987), (v) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, secretion of larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997) which could, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells leading to, the associated with proliferative state - cell shrinkage and unveiling of basal membrane. Occurrence of these events in brain capillary endothelial cells could lead to de-regulation of the mechanisms controlling permeability of blood-brain barrier. Furthermore, in addition to blood-brain barrier effects, the stabilization of stress fibres in endothelial cells may affect apoptotic process - it has been shown that the apoptosis-related cell surface blebbing is prevented by the stabilised stress fibres (Huot et al. 1998).

The proposed hypothetical molecular mechanism for the possible role of mobile phone radiation in development of brain cancer and in increasing permeability of the blood-brain barrier, although a hypothesis, it is reasonably supported by the evidence concerning both effects of microwaves on stress response and effects of hsp27 (increased expression and activity) on cell physiology. Proving or disproving of this hypothesis using in vitro and in vivo models will provide evidence to either support or to discredit the existence of some of the potential health risks that were suggested to be associated with the use of mobile phones.

The recently published hypothesis of French et al. (2001) of the possible effect of chronic/frequent exposure to mobile phone radiation that would induce abnormally high levels of stress proteins in cells still requires experimental confirmation that, indeed, repeating exposures to RF-EMF radiation could cause such an increase. On the other hand, proposed by us hypothetical mechanism of the mobile radiation effect on the brain relies on the single-exposure-induced transient increases in hsp27 phosphorylation and expression. We suggest that the transient effects, induced by repeated exposures, might, by chance of timing coincidence, led to survival of damaged/transformed cells and temporarily increase permeability of the blood-brain barrier. These events, when occurring repeatedly (on daily basis) over the long period of time (years) could become a health hazard because of the possibility of accumulating of brain tissue damage. Furthermore, our hypothesis suggests that other, than RF-EMF, cell-damaging factors might play a co-participating role in the tumour development caused by mobile phone radiation.

Finally, in addition to the p38MAPK/hsp27 stress pathway-induced effects, the extent of the global change of the pattern of protein phosphorylation observed in our study suggests that it is likely that multiple signal transduction pathways might be affected by the RF-EMF exposure. Identification of these pathways will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study were induced by RF-EMF irradiation at non-thermal levels, with SAR values set at the highest level that is allowed by the European safety limits. This suggests that the presently allowed radiation emission levels for the mobile phones, although low, might be sufficient to induce biological effects. However, determination of whether these effects might cause any significant health effects requires further studies.

5-step feasibility study of applying proteomics/transcriptomics to mobile phone research.

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski et al. 2004). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold et al. (2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST

approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses. The obtained by us results clearly demonstrate that by using HTST it is possible to identify RF-EMF-induced molecular events that might alter cell physiology. Even though the increase in expression/phosphorylation of the examined hsp27 protein was very modest (ca. 2-3 folds increase) it was possible to determine impact of this event on cell physiology. Whether any impact on organ (e.g., brain) or whole body will be exerted by this change remains to be determined by in vivo studies. Although the use of discovery science-approach employing HTST will not provide direct evidence of health hazard or its absence, it will be essential in unravelling of possibly all biological effects exerted by RF-EMF exposure. Further elucidation of the physiological significance of these biological effects for the health and well-being, in short- and long-term exposure conditions, will allow determination whether any health hazard might be associated with the use of mobile phones at the presently allowed radiation safety levels.

Use of HTST to determine genotype-dependent and modulation-dependent cellular responses.

Our study has shown that proteomics transcriptomics and might be an efficient tool when searching for the proteins and genes responding to a weak stimulus, like the mobile phone radiation. In this pilot study we have found several tens of protein and gene targets of the mobile phone radiation. Functions of the few of the MALDI-MS-identified protein spots suggest possibility of the effects of the mobile phone radiation on such physiological functions as (i) cellular energy production, (ii) protein translation, and (iii) cytoskeleton-dependent processes (e.g. cell size, shape and cell-cell interactions). Potential effects on these processes were supported by the evidence gained with cDNA arrays. Further studies will be needed to determine whether there is any impact of these changes on cell physiology.

The other major finding of the study is the observation that the exposure of cells to the continuous-wave microwaves ("CW-signal" 1800 MHz GSM) does not induce changes in protein expression whereas radiofrequency modulated microwaves ("Talk-signal" 1800 MHz GSM) induces broad changes in protein expression. Analysis of changes in expression of some 1500 proteins using cICAT method combined with liquid chromatography and MS/MS identification of proteins has revealed several tens of affected proteins. Importantly, using other methods such as 2-DE and cDNA arrays the same cytoskeleton-related genes/proteins were detected as being affected by RF-EMF exposure up. It means that with two different proteomics approaches we have observed similar protein changes what strengthens the validity of our observations.

4.2.4.7 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The elevated turnover of ribosomal proteins and proteins involved in energy metabolism allows the hypothesis that the cellular turnover is increased after RF-EMF exposure. To prove this hypothesis for RF-EMF treated HL-60 cells (Participant 2), a very interesting additional comparison was performed: In the 1800 MHz continuous wave experiments (2 expression profiles, 4 hybridisations) we used two controls instead of one: One control in each experiment was a sham-exposed control as usual. Cells from another incubator, neither exposed nor sham-exposed, served as a second control. After both experiments we performed comparisons between both controls as well as between sham-exposed and RF-exposed cells (each comparison with 8 data points per gene, from 2 expression profiling experiments, 4 hybridisations). After going through all investigated gene families listed in Tables 30 and 31, with some gene families we found remarkable differences between the control comparison and the sham-RF comparison (Figure 144). As expected, again ribosomal and mitochondrial genes are much more upregulated in the sham-RF comparison than in the "blinded" comparison (Sham-Ctrl). But there are also other gene families showing the same tendency, as for example ATP related genes, genes encoding calcium-associated proteins and cell cycle proteins.

The increasing ribosomal turnover might lead to cell growth and, in the end, to mitosis and cell proliferation, respectively. This hypothesis has not been confirmed so far by the BrdU-incorporation assay, because no significant increase of DNA synthesis, and therefore, an increase in cell proliferation, could not be detected (Participant 2). The same is true for the analysis of protein mass and the MTT assay for the detection of mitochondrial activity (Participant 2).

However, the changes in ribosomal protein synthesis are not so strong that we would expect a very strong increase in cell cycle progression. Ribosomal transcription rates have been found to vary by up to a factor of four (Derenzini et al. 2001; Leary and Huang 2001). It is known from stimulation by growth factors, that an increase of ribosomal activity not necessarily leads to significant changes in cell cycle (Stefanovsky et al. 2001; Bodem et al, 2000). We should investigate the cell cycle distribution after RF-EMF exposure in more detail. Also the analysis of the 45S precursor rRNA by real-time RT PCR or the analysis of the RNA polymerase I and associated proteins would help us to figure out if ribosomal transcription is elevated, which is a pre-requisite for an increase of ribosomal proteins (Stefanovsky et al. 2001; Jacob and Gosh 1999). Compared to the ELF-EMF results (Participant 3) the results with RF-EMF seem to be more uniform. The comparison of HL-60 profiling 2 and 3 for example (Participant 2), shows a much lower number of reproducibly regulated genes than after the analysis of only one experiment (compare Figure 139). If the reason for this is the differentiating potential of the cells (U937 cells of Participant 9, HL-60 cells of Participant 2, T-lymphocytes of participant 8), or the exposure conditions (homogenous ELF-EMF field with or without on/off cycles versus RF-EMF GSM talk signal), or both, remains to be elucidated. More cell lines, each exposed to different fields (ELF, RF), would have to be investigated to draw more reliable conclusions.

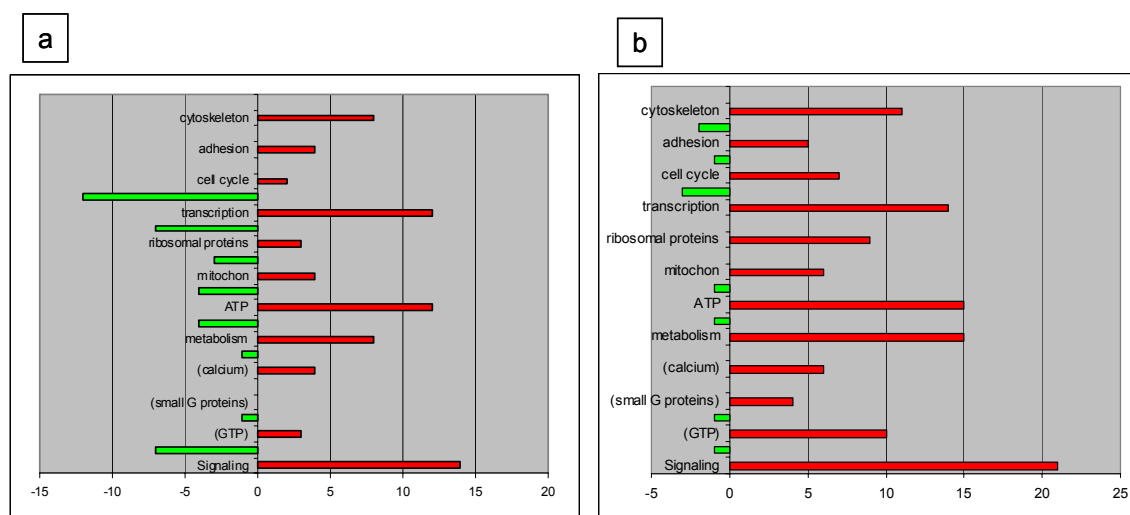


Figure 144. Numbers of regulated genes after RF-EMF exposure sorted according to different gene families. a, comparison between batches of control HL-60 cells (not exposed vs. sham exposed). Red: Genes appearing up-regulated in sham-exposed. Green: Genes appearing down-regulated in sham-exposed. b, comparison between sham-exposed (ctrl) and RF-EMF exposed HL-60 cells. Red: Genes showing up-regulation in RF-EMF. Green: Genes showing down-regulation in RF-EMF.

4.2.4.8 Summary (Participant 1)

Scientific work on gene and protein expression due to RF-EMF exposure using in vitro cell cultures and animal models is still in its early stages and as far as already published difficult to interpret (Independent Expert Group on Mobile Phones 2000). From the REFLEX data the conclusion can be drawn that RF-EMF may affect the gene and protein expression in various cell systems. RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of the p21 and c-myc genes and a long-term up-regulation of the hsp70 gene in p53 deficient embryonic stem cells (3.2.4.1, 4.2.4.1). RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats obviously without affecting protein transcription (3.2.4.2, 4.2.4.2). RF-EMF exposure up- or down-regulated the expression of various genes and proteins in HL-60 cells (1800 MHz, 1.3 W/kg) and in endothelial cells of human origin (900 MHz, 2.0 W/kg) (3.2.4.3, 3.2.4.6, 3.2.4.7, 4.2.4.3, 4.2.4.6, 4.2.4.7). RF-EMF exposure at a SAR value of 2.0 W/kg activated the p38MAPK/hsp27 stress response pathway and changed the global

pattern of protein phosphorylation in endothelial cells with possible consequences for the signal transduction pathway (3.2.4.6, 4.2.4.6).

Not unexpected, the available literature is controversial (Stewart Report 2000). While Lee et al. (2004) observed an alteration of gene expression in HL-60 cells after exposure to RF-EMF of 2450 MHz and Zeng et al. (2004a) an alteration of protein expression in human breast cancer cells (MCF-57) after exposure to RF-EMF of 1800 MHz, no such effects were found by Miyakoshi et al. (2004), who studied the influence of RF-EMF (1950 MHz) on the expression of hsp27 and hsp70 in human glioma cells (MO54). Opposite to the finding of Participant 6 (3.2.4.6, 4.2.4.6), no significant increase in hsp27 expression in endothelial cells was observed by Participant 9 who used a slightly different method (3.2.4.5, 4.2.4.5). Since the hsp27 expression was significantly increased in one laboratory, while this increase was near to significance in the other laboratory, this discrepancy seems to be neglectable. Furthermore, RF-EMF exposure (1800 MHz) at a SAR value of 1,4 W/kg did not affect gene expression in human lymphocytes (3.2.4.4, 4.2.4.4) and after RF-EMF exposure (900 MHz) at a SAR value of 2 W/kg only a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.5, 4.2.4.5). Finally, RF-EMF exposure did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) in nerve cells (3.2.4.5, 4.2.4.5).

The outcome of experiments following the genomics and proteomic approach may essentially depend on the cell system investigated and the RF-EMF signal used. Of course, the question remains as to whether or not these alterations in gene and protein expression are within the normal physiological range and if that is the case, they are without any biological relevance.

5.0 CONCLUSIONS

5.1 Conclusions based on the findings obtained in ELF-EMF research

5.1.1 Human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

These are the conclusions that Participant 3 draws from their findings:

1. The data strongly indicate a clastogenic potential of intermittent electromagnetic fields, which may lead to considerable chromosomal damage in dividing cells. However, the induced DNA damage did not persist in form of stable translocations.
2. The induced DNA damage was not based on thermal effects and arouses consideration about environmental safety limits for ELF-EMF exposure.
3. The effects were clearly more pronounced in cells from older donors, which could point to an age-related decrease of DNA repair efficiency of ELF-EMF induced DNA strand breaks.
4. In addition, three responder and three non-responder cell types could be identified, which could in part explain different results in reaction to ELF-EMF reported in the literature so far.
5. Fibroblasts from a donor with the genetically DNA repair defect Ataxia Telangiectasia had a more than two fold increase rate of ELF-EMF induced DNA breaks.
6. Between 3 and 550 Hz the largest DNA breaking effects were seen at 16.66 and 50 Hz, the most commonly used frequencies of alternating current in Europe.
7. Taken together, the results suggest that the observed effects of EMF exposure are caused by indirect mechanisms and are not inflicted due to changes in mitochondrial membrane potential.

5.1.2 Human neuroblastoma cell line NB69 and human hepatocarcinoma cell line HepG2 (Participant 5)

Our present results confirm preliminary observations that a 42- or 63-hour exposure to 50 Hz, sine wave MF at 10 or 100 μ T (3 hours on/3 h off exposure cycle) can induce changes in the cell growth of NB69 human neuroblastoma cells. The data indicate that such an effect is exerted through an increase in cell proliferation, as revealed by BrdU-incorporation and flow cytometry.

1. In contrast, a 50-Hz MF at 2000 μ T magnetic flux density, 5 min on/30 min off exposure cycle, did not affect significantly cell growth on the NB69 line. Thus, our cells were not responsive to these exposure parameters, which have been reported to be effective on differentiating neural embryonic stem cells (Participant 4). Additional experiments exposing NB69 cells to a 100 μ T field in a 5 min on/30 min off cycle showed no significant responses. This indicates that the exposure cycle is crucial to eliciting a detectable cellular response.
2. In the NB69 line, the results on PCNA labelling show that at day 6 post-plating the percent of PCNA-positive cells in samples exposed to a 50-Hz, 100- μ T field is significantly increased when compared to controls. Actually, the percent of PCNA positive cells significantly decreases in controls between the days 5 and 6 post-plating, whereas such a decrease did not occur in exposed cells. The results suggest that the MF could impair the normal cell cycle regulation through alterations in the late G1 and S-phases.
3. We have also investigated the response of a different human cancer line, the HepG2 human hepatocarcinoma cell line (data not shown), with a growth pattern different from that of the NB69 line. Fifty-Hertz magnetic fields at 10 or 100 μ T elicited similar responses in both cell lines, consisting of significant increase in the number of cells at days 5 postplating. In HepG2, the melatonin, at a 10 nM concentration, inhibited the growth-promoting effect induced by the field (Cid et al., 11th International Congress of IRPA, 2004). In the HepG2 line, the growth effect became even stronger when the exposure was maintained until day 7 post-plating, whereas in the NB69 line, an equivalent extension of the exposure period results in a loss of the effect. The differential responses in both cell lines could be due to the fact that, in control conditions, NB69 cultures become saturated at day 7 post-plating and, consequently, their capability to respond to any stimulus is strongly impaired.

4. A 50-Hz, sine wave MF at 100- μ T (3h on/3h off exposure cycle) induces a significant reduction in the spontaneous apoptosis of the human neuroblastoma cell line NB69. This response was associated to an increase in the total number of cells. The data suggest that both responses are a consequence of an effect of the field on cell cycle regulation.
5. In NB69 cells, a 50-Hz, sine wave MF at 100- μ T (3h on/3h off exposure cycle) alters the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB) in a time-dependent manner. The results suggest that the activation of p-CREB is involved in the above described effects of this field on cell growth/apoptosis.

5.1.3 Human lymphocytes (Participant 8)

On the whole, data obtained indicate no response of human PBMCs to ELF-EMF exposure. Thus the conclusions are that ELF-EMF do not affect proliferation and cell activation, two fundamental phases of lymphocyte function. Since previous works indicated that pulsed ELF-EMF may interfere with human lymphocyte functionality (Cossarizza et al. 1989a/b, 1991, 1993), future experiments could be addressed to investigate the role of pulsed signal in biological systems in comparison with the negative results obtained with A.C. 50Hz ELF-EMF.

5.1.4 Mouse embryonic stem cells (Participant 4)

1. ELF-EMF signals at a high flux density are capable to transiently increase transcript levels of the regulatory genes *egr-1*, *p21* and *c-jun* in ES cells deficient for the tumour suppressor *p53*.
2. The intermittency scheme of the ELF-EMF signals may play a critical role for changes in transcript levels of some regulatory genes.
3. The genetic constitution of pluripotent embryonic stem cells determined by loss of *p53* function can influence ELF-EMF-related cellular responses, whereas wild-type cells are insensitive. It remains to be elucidated, whether ELF-EMF-induced changes of expression levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
4. ELF-EMF exposure of ES-derived neural progenitor cells may influence transcript levels of genes of the *bcl-2* family and the *p53*-responsive growth arrest and DNA damage inducible gene *GADD45*. This finding is an indication that ELF-EMF may affect, at least transiently, fundamental cellular processes including programmed cell death and cell cycle regulation.
5. Alkaline and neutral Comet assay failed to demonstrate a clear effect on the induction of single- and double-strand DNA breaks after ELF-EMF exposure of ES cell derived neural progenitors.

5.1.5 Experiments with embryonic stem cells of mice during cardiac differentiation (Participant 8)

In the ES cell model (GTR 1), ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF-EMF-exposed cells. Failure of EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of MF with *GATA-4*, *Nkx-2.5* and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis. This work represents, in our opinion, a first step toward an extensive investigation concerning the influence of EMF on the expression of a sequence of genes specifically involved in cell differentiation, and in particular the differentiation into a cardiac phenotype, using genomic and post-genomic techniques.

5.1.6 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

The results clearly demonstrate that, under the discussed exposure conditions, the expression of major components of the cholinergic and catecholaminergic systems is unresponsive to environmental exposure to ELF-EMF.

5.1.7 *Xenopus laevis* oocytes, human fibroblasts and granulosa cells of rats (GFSHR-17 cell line) (Participant 7)

1. For the applied three exposure protocols (50-Hz powerline, 1.0 mT or 2.3 mT continuously applied for 16 h; 50-Hz powerline, 1.0 mT and 2.3 mT intermittently (on/off: 5 min/10 min) applied for 16h the data indicate that the expression level as well as the voltage dependent gating of rCx46-connexons is not significantly affected. Since we could previously show that protein kinase C dependent phosphorylation processes affect the voltage-dependent gating of rCx46-connexons (Ngezahayo et al. 1998), a significant interaction of ELF-EMF on proteinphosphorylation can be neglected. The formation of cell-to-cell channels composed of two rCx46-hemi-channels, respectively, between a pair of mechanically contacting oocytes indicates an effect of ELF-EMF exposure. Exposure virtually suppresses the formation of cell-to-cell channels, but the effect is not significant on the level of three experiments analysed so far. The known $[Ca^{2+}]_o$ -dependent gating property of hemi-channels appears not to be influenced by ELF-EMF exposure.
2. Continuous ELF-EMF exposure at high flux intensity 2.3 mT for 30 min did not significantly influence gap junctional coupling (cell-to-cell channels) of cultured pairs of rat granulosa cells as explored by the double whole cell patch-clamp technique.
3. The presented data indicate that intermittent exposure (5 min on / 10 min off) to ELF-EMF (50 Hz, 1 mT) neither generates a long lasting effect on the time course of $[Ca^{2+}]_i$ in cultured fibroblasts nor granulosa cells. This finding appears to be independent of an exposure for 5 to 18h. The corresponding observation of Ivancsits et al. (2003b), of a time dependent increase/decrease of DNA strand breaks with a maximum at about 15h, therefore seems not to be reflected in a corresponding long lasting change of $[Ca^{2+}]_i$. It is interesting to note that such a long lasting effect is also not found for the mitochondrial potential of fibroblasts. ELF-EMF exposure followed by exposure to further stressors, like 200 μ M H_2O_2 or 30 mM KCl, also caused no significant change of $[Ca^{2+}]_i$.
4. Exposure experiments show no significant influence on volume regulatory mechanisms of granulosa cells. Further studies of intracellular signal transduction pathways should allow to understand the unsolved question whether significant effects of ELF-EMF on the genomic level are reflected on the cellular level. At present significant changes of cellular properties could not be derived from the analysed cellular parameters.
5. ELF-EMF exposure of cultured granulosa cells shows a significant time dependent increase of double DNA strand breaks with a maximum at about 18 h as observed by the neutral comet assay. This time dependence was also observed at 8 Hz, 16.66 Hz, 30 Hz, 50 Hz and 300 Hz. Therefore, it appears likely that for the chosen ELF-EMF exposure protocol the observed increase of double DNA strand breaks is not frequency dependent. But, the results of the alkaline comet assay indicate a frequency dependent effect of ELF-EMF exposure on the sum of double and single DNA strand breaks. The data obtained for the granulosa cells, after ELF-EMF exposure at 50 Hz, by the alkaline comet assay are comparable with those obtained by participant 3.

5.1.8 Effects of ELF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.1.9 Summary (Participant 1)

The ELF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. ELF-EMF had genotoxic effects on primary cell cultures of human fibroblasts and on other cell lines. These observations were made in two laboratories within the REFLEX consortium (Participants 3 and 7) and confirmed by two other laboratories from outside the REFLEX project. ELF-EMF generated DNA strand breaks at a significant level at a flux density as low as 35 μ T. A strong positive correlation was observed between both the intensity and duration of exposure to ELF-EMF and the increase in single and double strand DNA breaks and micronuclei frequencies. Surprisingly this genotoxic effect was only found when cells were exposed to intermittent ELF-EMF, but not to continuous exposure. Responsiveness of fibroblast to ELF-EMF increased with the age of the donor and in the presence of specific genetic repair defects. The effect also differed among the other types of cells examined. In particular, lymphocytes from adult donors were not responsive. Chromosomal aberrations were also observed after ELF-EMF exposure of human fibroblasts.
2. ELF-EMF at a flux density of 10 and 100 μ T increased the proliferation rate of neuroblastoma cells (Participant 5) and at a flux density of 0.8 mT it enhanced the differentiation of mouse stem cells into cardiomyocytes (Participant 8). In contrast to these results, no clear-cut and unequivocal effects of ELF-EMF on DNA synthesis, cell cycle, cell differentiation, cell proliferation and apoptosis were found in the many other cell systems under investigation.
3. ELF-EMF inhibited the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 μ T (Participant 5). In contrast to these results, no clear-cut and unequivocal effects of ELF-EMF on the apoptotic process were found in the many other cell systems under investigation.
4. ELF-EMF at a flux density of about 2 mT up-regulated the expression of early genes, such as p21, c-jun and egr-1, in p53- deficient mouse embryonic stem cells, but not in healthy wild-type cells (Participant 4) and, in addition, may affect the expression of genes and proteins in a variety of other cell systems. The results of the whole genome cDNA micro-array and proteomic analyses indicate that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participant 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that ELF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

5.2 Conclusions based on the findings obtained in RF-EMF research

5.2.1 Human promyelocytic cell line HL-60 (Participant 2)

1. Different SAR levels have been examined with respect to the effect on comet formation and micronuclei induction in HL 60-cells. Comparing RF-EMF exposure (1800 MHz, continuous wave, 24h) at SAR levels ranging from 0.2 W/kg to 3.0 W/kg indicate that both effects appear to be energy dependent. Whereas at SAR of 0.2 W/kg and 1.0 W/kg both, comet formation and micronucleus frequency, were not significantly different from that observed in sham-exposed control cells, comet formation as well as micronucleus frequency were significantly increased at SAR of 1.3 W/kg, 1.6 W/kg and 2.0 W/kg. The maximal effect was observed at a SAR of 1.3 W/kg. At higher SAR levels from 2.0 to 3.0 W/kg micronucleus frequencies and comet formation were less expressed as compared to the effect noted at a SAR of 1.3 W/kg.
2. In order to extend the statistical basis of evaluation average numbers of micronuclei (micronuclei per 1000 BNC) in different experimental groups were calculated comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the number of micronuclei was increased at a significant level ($p < 0.001$) as

compared to sham-exposed controls, while in group (iii) micronuclei numbers per 1000 BNC were not significantly different from that observed in sham-exposed controls.

3. Likewise, in order to extend the statistical basis of evaluation average values of Olive Tail Moments as a measure of comet formation were calculated in different experimental groups comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the comet formation was increased at a significant level (group (i) $p < 0.01$; group (ii) $p < 0.001$) as compared to sham-exposed controls, while in group (iii) comet formation was not significantly different from that observed in sham-exposed controls.
4. Experiments on the influence of the duration of exposure showed that short exposure period (6h) caused no (MN) or less (Comet) pronounced effects on micronuclei induction and comet formation as compared to longer exposure periods of 24h. While micronucleus frequencies were further increased after exposure for 72h, comet formation after 72h of exposure was less expressed as compared to 24h exposure.
5. Experiments on the influence of RF-signals showed that at a SAR level of 1.3 W/kg all RF-signals tested, i.e. continuous wave (C.W.), C.W. 5 min on/10 min off, GSM-217 Hz, and GSM-Talk exhibited similar effects on micronuclei induction and on comet formation.
6. By applying sequential approaches for the detection of reactive oxygen species (ROS) in HL-60 cells, an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could be clearly demonstrated by flow cytometric detection of the oxidized nucleotide 8-oxoguanosine (oxy-DNA assay) and the fluorescent Rhodamine 123 (DHR 123 assay), respectively.
7. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) had no effect on the cellular doubling time and the activity of the enzyme thymidine kinase of HL 60 cells, indicating that RF-EMF exposure does not influence cellular growth rates.
8. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) did not induce apoptosis in HL-60 cells.
9. Within the investigated SAR energy ranges RF-EMF under the in-vitro conditions used are genotoxic in HL-60 cells without affecting cell-cycle distribution cell proliferation or cell progression.
10. The partial-body SAR for any 10-gram tissue like for example the head as exposed region to mobile phone electromagnetic fields should not exceed 2 W/kg according to the Radio-Radiation Protection Guidelines. Notably, our findings on genotoxic effects of RF-fields in HL-60 cells have been shown for SAR levels below these acceptable partial-body SAR levels.
11. These results on genotoxicity in the HL-60 cell line cannot be transferred automatically to other cells, especially to primary cells, and definitely not to whole organism.
12. Clear differences in protein expression have been shown for RF-exposed HL-60 cells as compared to control and sham-exposed cells. This indicates that, as also demonstrated by genetic profiling, RF-EMF exposure has an influence on as well the transcriptional as the translational level in these cells. Clarification of changes in protein expression with respect to functional analysis will help to understand molecular pathomechanisms.

5.2.2 Human fibroblasts and granulosa cells of rats (Participant 3)

Our results imply a genotoxic action of RF-EMFs below proposed radiation safety levels.

1. RF-EMFs were able to induce DNA single and double strand breaks in human fibroblasts and SV40 transformed rat granulosa cells. In contrast to ELF-EMF, genotoxic effects were also observed at continuous exposure.
2. In addition, the decline of DNA strand break levels at elongate exposure (16-24 h), which was found in ELF-EMF exposed cells, could not be demonstrated after RF-EMF exposure. These results could point to differences in mechanisms between the genotoxic action of RF and ELF-EMF
3. Differences in genotoxic effects between different cell types after EMF exposure could be found in RF as well as in ELF-EMF exposed cells.
4. RF-EMF exposure of human fibroblasts was able to induce higher incidences of chromosome aberrations than which was found in ELF-EMF exposed cells.
5. No effects of RF-EMF exposure on mitochondrial membrane potential could be observed. These findings are in accordance with the results obtained with ELF-EMF.

5.2.3 Human lymphocytes and thymocytes (Participant 8)

On the whole, the data obtained indicate a very low response of human PBMCs and no response of thymocytes to RF-EMF exposure. Concerning PBMCs, some results suggest a possible effect on the number of CD95 surface molecules in stimulated T lymphocytes from aged donors. Moreover, other results seem to indicate a greater susceptibility to RF of monocytes with respect to lymphocytes, as demonstrated by a decrease of IL-1 β cytokine, specifically produced by monocytes, in RF-exposed cultures. Future work could be addressed to analyse further effects on these type of human cells.

5.2.4 Human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

1. When administered alone, the exposure to the GSM-Basic signal at a 2W/kg SAR induced a decrease in the number of cells expressing the fibroblast growth factor receptor-1 (FGFR-1), both in NB69 cells and NSC, without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by 20 μ g/ml bFGF. Since the GSM-Basic treatment did not affect significantly the total cell number or the cell viability, the above data indicate that RF-induced effect in FGFR-1 is not due to a reduction in the number of cells, but to a loss of the cellular expression of receptor-1.
2. The results also indicate that the exposure to GSM 1800-CW signals at a 2 W/kg SAR induced effects on the expression of FGFR-1 equivalent to those described above for the GSM 1800-Basic signal. No significant effects on the expression of FGFR-1 were observed after exposure to GSM 1800-Talk and DTX signals at 2 W/kg SAR and 1 W/kg SAR, respectively. The data obtained with the different GSM-signals suggest that the cellular response is not dependent on the tested low-frequency modulation.
3. The exposure to the GSM-basic signal induces specific, morphological changes in oligodendrocytes and astrocytes derived from neural stem cells, at day 9 post-plating. These results are indicative that GSM-basic radiation at SAR = 2 W/kg can promote differentiation in NSC. The effect would be exerted through short-term changes in the expression of FGF receptor-1. In contrast, the GSM-Basic signal does not influence cytodifferentiation in NB69 cells or in the neuronal progeny of NSC, as revealed with anti-beta-tubulin antibody.

5.2.5 Brain cells of different origin and human monocytes (Participant 9)

1. Our results strongly suggest that the spontaneous apoptotic process is not a biological target for GSM mobile telephony-related signals. This was shown in different primary cells and cell lines from both nerve and immune systems.
2. Based on the expression and activity of inducible nitric oxide synthase (NOS2) in an astrocytic cell line, GSM-like signals did not “activate” the inflammatory process in nerve cells.
3. No evidence was found of effects of GSM-like signals on heat shock proteins in different mammalian nerve cells. Replication of the previously reported increase in hsp27 expression in a human endothelial cell line after exposure failed.
4. Based on the whole data set, our conclusion is that exposure to low-level GSM-900 signal is unlikely to lead to neurodegeneration or to favour tumour development via pathways involving apoptosis, nitric oxide or heat shock proteins.

5.2.6 Mouse embryonic stem cells (Participant 4)

1. Our present data suggest that currently applied GSM radiation levels under certain circumstances might induce biological effects, at least in cells generated from embryonic stem cells in vitro.
2. The genetic constitution of pluripotent embryonic stem cells determined by loss of p53 function influences RF-EMF-related cellular responses at the level of gene expression, whereas wild-type cells are insensitive. It remains to be elucidated, whether RF-EMF-induced changes of mRNA levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
3. RF-EMF exposure of ES-derived neural precursor cells influences the bcl-2 mediated anti-apoptotic pathway, affects the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1.

4. Short exposure to RF-EMF could induce double-strand DNA breaks in ES-derived neural progenitor cells (as measured by the neutral Comet assay).

5.2.7 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

1. RF-EMF appears to be recognized by the cells as an external stress factor because it in response to exposure phosphorylation status of several hundreds proteins was altered either up or down; identification of these proteins will be done in due time.
2. RF-EMF appears to be a weak inducer of cellular stress response because it increases expression and phosphorylation of heat shock protein-27 (hsp27) - a known marker of cellular stress response.
3. RF-EMF induced phosphorylation of Hsp27 appears to be regulated by the activation of up-stream stress kinase p38MAPK.
4. RF-EMF-induced hsp27 activation appears to affect down-stream physiological processes in cell - stabilization of F-actin stress fibers what, in turn, alters cell size and shape (causes rounding-up of cells).
5. Using cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins we have determined that the cellular skeleton appears to be a target of RF-EMF exposure as changes in gene/protein expression of some dozen cytoskeletal proteins were induced by RF-EMF exposure.
6. RF-EMF-induced phosphorylation of hsp27 is followed by translocation of hsp27 to cell nucleus where it appears to interfere with the gene expression processes.
7. RF-EMF causes changes in the expression of several tens of genes and proteins as determined by high-throughput screening technologies - cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins.
8. RF-EMF-induced changes in gene and protein expression appear to be dependent on the cell genotype/phenotype what suggests that some cell types might be more and some less responsive to RF-EMF exposure.
9. RF-EMF induced changes in protein expression appear to be modulation dependent since RF-EMF exposure caused changes whereas CW-EMF did not.
10. the ability of RF-EMF to induce cellular stress response indicates that cells recognize this radiation in spite of it low energy but the induction of stress response per se can not be considered as any indicator of potential health risk.
11. We have practically demonstrated that the use of high-throughput screening methods of transcriptomics and proteomics is useful tool in determining the potential targets of RF-EMF exposure in cells.

5.2.8 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.2.9 Summary (Participant 1)

The RF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. RF-EMF produced genotoxic effects in fibroblasts, HL-60 cells, granulosa cells of rats and neural progenitor cells derived from mouse embryonic stem cells (Participants 2, 3 and 4). Cells responded to RF-EMF exposure between SAR levels of 0.3 and 2 W/kg with a significant increase in single and double strand DNA breaks and in micronuclei frequency (Participants 2 and 3). Chromosomal aberrations in fibroblasts were also observed after RF-EMF exposure (Participant 3). In HL-60 cells an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could clearly be demonstrated (Participant 2).
2. No clear-cut and unequivocal effects of RF-EMF on DNA synthesis, cell cycle, cell proliferation, cell differentiation and immune cell functionality were found in the cell systems under investigation. (Participants 2, 3, 4, 5, 6, 8). There is some indication that RF-EMF may affect the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1 in neural progenitor cells (Participant 4).
3. No clear-cut and unequivocal effects of RF-EMF on apoptosis were found in the cell systems under investigation was observed (Participants 2, 3, 4, 5, 6, 8 and 9). There is some indication that RF-EMF may have some influence on the bcl-2 mediated anti-apoptotic pathway in neural progenitor cells (Participant 4) and on the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (Participant 6) which may in turn exert an inhibitory effect on apoptosis.
4. RF-EMF at a SAR of 1.5 W/kg down-regulated the expression of neuronal genes in neuronal precursor cells and up-regulated the expression of early genes in p53-deficient embryonic stem cells, but not in wild-type cells (Participant 4). Proteomic analyses on human endothelial cell lines showed that exposure to RF-EMF changed the expression and phosphorylation of numerous, largely unidentified proteins. Among these proteins is the heat shock protein hsp27, a marker for cellular stress responses (Participant 6). The results of the whole genome cDNA micro-array and proteomic analyses indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participants 2, 6 and 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that RF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

6.0 EXPLOITATION AND DISSEMINATION OF RESULTS

6.1 Coordination (Participant 1)

a. Scientific publications, meetings, interviews, and round tables

Adlkofer F et al.: Brochure presenting the REFLEX project, June 2001 (*for distribution*)

Adlkofer F et al.: Oral presentation of results. Bundesamt für Strahlenschutz, Salzgitter/Germany, June 21-22, 2001, p. 18-19

Adlkofer F et al.: Oral presentation of the project. EBEA, Helsinki/Finland, Sep 6-8, 2001. Proceedings, p. 54-56, 269-270

Adlkofer F et al.: Oral presentation of results. EU/Japan/Korea/US workshop on EMF, mobile telephony and health. Brussels/Belgium, Oct 29-30, 2001, Proceedings, p. (*not numbered*)

Adlkofer F et al.: Oral presentation of results. Institut für Zoologie, Technical University of Dresden/Germany, April 2, 2002

Adlkofer F et al.: Oral presentation of results. COST281/EBEA Forum, Rome/Italy, May 2-5, 2002

Adlkofer F et al.: Oral presentation of the project.. 24th BEMS Meeting, Quebec City/Canada, June 23-27, 2002, Proceedings, p. 91-92, 95, 98-100

Adlkofer F et al.: Oral presentation of the project and of results. PIERS, Cambridge/Mass/USA., July 1-5, 2002. Proceedings, p. 498

Adlkofer F et al.: Oral presentation of results. Cursos de Verano Universidad de Malaga, Ronda/Spain, July 22-26, 2002

Adlkofer F et al.: Oral presentation of the project. Biological Effects of EMF, Rhodes/Greece, Oct 7-11, 2002. Proceedings, p. 514-522

Adlkofer F, Rüdiger HW, Wobus AM: DNA-Doppelstrangbrüche bei intermittierender Exposition. Diskussionsbeitrag. Deutsches Ärzteblatt, Nov 15, 2002, p. 3114-3115

Adlkofer F et al.: Oral presentation of results. Elektromagnetische Felder in der Umwelt, Umweltministerium Nordrhein-Westfalen, Dortmund/Germany, Nov 28, 2002

Adlkofer F et al.: Oral presentation of results. The EMF Biological Research Trust, London/UK, Jan 16, 2003

Adlkofer F et al.: Oral presentation of results. WHO EMF Project, Research Coordination Meeting, Geneva/Switzerland, June 12-13, 2003

Adlkofer F et al.: Oral presentation of the project. 25th BEMS Meeting, Maui/Hawaii, June 22-27, 2003. Proceedings, p. 127, 135 - 136

Adlkofer F: Interview. Television feature on “Elektrosmog”, ARD Germany, Aug 7, 2003

Adlkofer F et al.: Oral presentation of results. 3rd Int. EMF Seminar, Guilin/China, Oct 13-17, 2003. Proceedings, p. 23 - 24

Adlkofer F et al.: Oral presentation of results. O₂ Telecommunication Company, München/Germany, Oct 22, 2003

Adlkofer F et al.: Oral presentation of results. Die Umwelt-Akademie e.V., München/Germany, Dec 5, 2003

Adlkofer F: Interview. Bayer. landwirtschaftliches Wochenblatt, Heft 5, Jan 2004, p. 48

Adlkofer F: Interview. life + sciences, Heft 1, Feb - April 2004, p. 30-31

Adlkofer F et al.: Oral presentation of results. Bündnis 90/Die Grünen, Bavarian State Parliament, München/Germany, April 2, 2004

Adlkofer F: Round Table Discussion. Bayer. Akademie der Wissenschaften, München/Germany,

April 29, 2004

Adlkofer F et al.: Oral presentation of results. EMF-NET, Brussels/Belgium, April 30, 2004

Adlkofer F et al.: Oral presentation of results. BUND, 3. Rheinland-Pfälzisch-Hessisches Mobilfunk-symposium, Mainz, June 12, 2004. Tagungsband, p. 33 - 49

b. Posters

Adlkofer F et al.: Poster presentation. EBEA, Helsinki/Finland, Sep 6-8, 2001

Adlkofer F et al.: Poster presentation. An Environment for Better Health Conference, Arhus/Denmark, May 8-11, 2003

6.2 Experiments with the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. The results obtained by participant 2 have been subsequently actualised and reported in the usual scientific manner. These reports included confidential Annual Reports as progress reports to the European Commission (1st, 2nd, 3rd Annual Report) and public presentations at the following scientific meetings: BEMS 2002 (Radiofrequency EMF and DNA strand breaks), BEMS 2002 (RF-EMF genotoxic effects), PIERS 2002 (1800 MHz radiofrequency exposition of human HL-60 cells induces DNA strand breaks as measured by the alkaline comet assay), BEMS 2003 (Genotoxic effects of RF-EMF on cultured cells in vitro), Deutscher Ärztekongress 2002 (Workshop in German), Deutscher Ärztekongress 2003 (Workshop in German). Peer-reviewed publications have been prepared and will be submitted after the end of the project.

a. Scientific papers

in preparation:

Schlatterer K., Gminski R., Tauber R., Fitzner R. (2004) Radiofrequency (1800 MHz) electromagnetic fields cause DNA strand breaks and micronuclei formation in HL-60 human promyelocytic cells.

b. Scientific meetings

Fitzner R, Gminski R, Schlatterer K (2004) 1800 MHz radiofrequency electromagnetic fields cause energy-dependent genotoxic effects in human promyelocytic HL-60 cells. Session 14: Non thermal biological effects of EM Fields used for mobile communication. Progress In Electromagnetic Research Symposium (PIERS 2004), Pisa, March 28-31, 2004 (oral presentation)

Fitzner R (2004) In-vitro-Untersuchungen an HL-60-Zellen – Einfluss niederfrequenter Magnetfelder (50 Hz; 162/3 Hz). Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

Schlatterer-Krauter, K (2004) Einfluss hochfrequenter Elektromagnetfelder des Mobilfunks – Erbgut-veränderungen direkt oder indirekt verursacht. Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

c. Posters

Fitzner R, Gminski R, Schlatterer K (2004) Radiofrequency electromagnetic fields (1800 MHz) induce elevated production of reactive oxygen species in human promyelocytic HL-60 cells. Poster presentation. Bioelectromagnetics Society 26th Annual Meeting, 20-24th June, Washington, 2004

6.3 Experiments with human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

a. Scientific papers

published:

Ivancsits S, Diem E, Rüdiger HW, Jahn O (2002) Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. *Mutation Res* 519: 1-13

Ivancsits S, Diem E, Jahn O, Rüdiger HW (2003) Intermittent extremely low frequency electromagnetic fields cause DNA damage in a dose dependent way. *Int Arch Occup Env Health* 76: 431-436

Ivancsits S, Diem E, Jahn O, Rüdiger HW (2003) Age-related effects on induction of DNA strand breaks by intermittent exposure to electromagnetic fields. *Mech Age Dev* 124: 847-850

submitted:

Diem E, Jahn O, Rüdiger HW. Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells in vitro. *Mutation Research*

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Chromosomal damage in human diploid fibroblasts by intermittent exposure to extremely low frequency electromagnetic fields. *Mutation Research*

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Cell type specific genotoxic effects of intermittent extremely low frequency electromagnetic fields. *Mutation Research*

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Intermittent exposure to extremely low frequency electromagnetic fields increases the genotoxic sensitivity to UV-light or mild thermal stress in cultured human fibroblasts. *J Toxicol Envir Health*

Pilger A, Ivancsits S, Diem E, Steffens M, Kolb HA, Rüdiger HW. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts. *Radiat Envir Biophysics*

b. Scientific meetings

Wiener Forum Arbeitsmedizin, April 2000 Vienna, "Elektrosmog – Neue Untersuchungen zur genotoxischen Wirkung elektromagnetischer Felder" Oswald Jahn Oral presentation

26th International Conference of Occupational Health (ICOH), 27th August-1st September 2000, Singapore, In vitro evaluation of genotoxic potential of low EMF of 50 Hz" Oswald Jahn, Eva Valic, Elisabeth Diem, Hugo W. Rüdiger Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 27-28th September 2002, Vienna. Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Biologische Wirkung elektromagnetischer Felder“ Oral presentation

Bioelectromagnetics Society 24th Annual Meeting, 23rd-26th June, Quebec, 2002

H.W. Rüdiger, S. Ivancsits, E. Diem, A. Pilger, F. Bersani, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro” Oral presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung, 17-20th March 2003, Mainz. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Dosisabhängige Induktion von DNA-Strangbrüchen nach niederfrequenter elektromagnetischer Bestrahlung“ Oral presentation

Bioelectromagnetics Society 25th Annual Meeting, 22-27th June, Maui, 2003

H.W. Rüdiger, S. Ivancsits, E. Diem, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro” Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 19-20th September 2003, St. Pölten. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Induktion von chromosomalen Aberrationen durch niederfrequente elektromagnetische Felder“ Oral presentation.

6th Congress of European Bioelectromagnetics Association (EBEA), November 13–15th 2003, Budapest. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger “In vitro genotoxic effects of extremely-low-frequency electromagnetic fields” Oral presentation

c. Poster

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 28-29th September 2001, Salzburg, Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Gentoxische Wirkung

von elektromagnetischen Feldern“ Poster presentation

Conference on RF interactions with Humans: Mechanisms, Exposure and Medical Applications, 27-28th February 2003, London. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung, 17-20th March 2003, Mainz. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger “Induktion von chromosomalen Schäden durch niederfrequente elektromagnetische Felder“ Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. A. Pilger, S. Ivancsits, E. Diem, M. Steffens, H.A. Kolb, H.W. Rüdiger „Intermittierende Belastung mit 50 Hz ELF-EMF bewirkt keine Veränderungen des mitochondrialen Membranpotentials und freien Kalziums in humanen Fibroblasten“ Poster Presentation

6.4 Embryonic stem cells (Participant 4)

a. Scientific papers

published:

Jaroslav Czyz, Kaomei Guan, Qinghua Zeng, Teodora Nikolova, Armin Meister, Frank Schönborn, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. High frequency electromagnetic fields affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells. *Bioelectromagnetics* (2004) 25: 296-307.

Jaroslav Czyz, Teodora Nikolova, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Non-thermal effects of power-line magnetic fields (50 Hz) on gene expression levels of embryonic stem cells – the role of tumour suppressor p53. *Mutation Research* (2004) 557(1): 63-74.

submitted:

Teodora Nikolova, Jaroslav Czyz, Alexandra Rolletschek, Przemyslaw Blyszczuk, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Electromagnetic fields affect the transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells. Submitted to *Environ Health Persp.*

Part of our results were presented by Participant 8 of the REFLEX Project at the International BEMS 2003 Conference in June, 2003 in Maui, Hawaii.

6.5 Experiments with the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

For our REFLEX studies a specific software for the analysis of immunocytochemical images was developed in collaboration with Escuela Universitaria de Ingeniería Técnica Industrial (Erasmus-Socrates arrangement between Madrid-Belgium).

Part of the results has been presented in different meetings: BEMS, EBEA, and 2nd International Workshop on Biological Effects of EMF.

a. Scientific papers:

published:

Carlos Platero, Kristof Verbiest, Alejandro Úbeda, M-Angeles Trillo, Jaime Gosálvez, and Javier Bartolomé. Platform opened for the processing and management of biomedical images. XXI Jornadas of

Automática 1-7. ISBN: 84-699-3163-6 (2000)

Carlos Platero, M-Angeles Trillo and Alejandro Úbeda. Processing of biomedical images for the study of the potential influence of GSM electromagnetic radiation on neural stem cells. XXIII Jornadas of Automática 1-7. ISBN: 84-699-8916-2 (2002)

in preparation:

M-Angeles Trillo, M-Antonia Cid, M-Antonia Martinez, Vicente-J. Garcia, Alejandro Úbeda and Jocelyne Leal. Influence of 50 Hz magnetic fields on the proliferation and apoptosis of human neuroblastoma cells *in vitro*”.

M.A.Trillo, G. Alegría, M.A. Martínez, D. Reimers, E. Bazán, A. Úbeda, Jürgen Schuderer and J. Leal. Influence of RF fields (GSM signals, 1800 MHz) on the expression of FGFR1 by NB69 human neuroblastoma cell line and neural stem cells from rat embryonic nucleus striatum”.

M.A. Trillo, M.A. Martínez, M.A. Cid A. Úbeda and J. Leal. 50 Hz sinus wave magnetic field at 100 μ T activates phosphorylated cyclic adenosine monophosphate response-element binding protein (P-CREB) in NB69 human neuroblastoma cell line.

6.6 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

a. Scientific papers

published:

Leszczynski D, Joenväärä S, Reivinen R, Kuokka R. Non-thermal activation of hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer- and blood-brain barrier-related effects. Differentiation 70, 2002, 120-129

Leszczynski D, Nylund R, Joenväärä S, Reivinen J. Applicability of Discovery Science-Approach to Determine Biological Effects of Mobile Phone Radiation. Proteomics 4, 2004, 426-431

Nylund R, Leszczynski D. Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation. Proteomics, 4, 2004, 1359-1365

submitted:

Leszczynski D. Mobile phone radiation and blood-brain barrier: The available scientific evidence is insufficient to support or dismiss claims of an effect.

in preparation:

Nylund R, Griffin T, Maereker Ch, Schuderer J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line might be frequency modulation-dependent

Nylund R, Toivo T, Sihvonen AP, Jokela K, Schuderer J, Kuster N, Landry J, Leszczynski D. Mobile phone radiation-induced activation of cellular stress response induces cytophysiological effects.

Nylund R, Reivinen J, Leszczynski D. Cellular response to mobile phone radiation is proteome- and genotype-dependent

Leszczynski D. Induction of Cellular Stress Response by Mobile Phone Radiation: Possible mechanism behind the effects – a molecular biologists perspective. invited review for IEEE Transactions

Nylund R, Toivo T, Sihvonen AP, Schuderer J, Jokela K, Kuster N, Leszczynski D. Mobile phone radiation-induced Hsp27 stress response in human endothelial cell line EA.hy926 is a non-thermal effect.

b. Scientific meetings and reports for the media

invited lectures:

Harvard University, Boston, MA, USA, 15.11.2000, Proteomics: a novel approach to determine health effects of mobile phone radiation.

Centre for Immunology at St. Vincent's Hospital, Sydney, Australia, 11.02.2002, Possible Effects of Mobile Phones on Brain - Should We Be Afraid?

Telstra Laboratories, Melbourne, Australia, 12.02.2002, Mobile Phones and Health Risk: Why Do We Know So Little?

Department of Physics, Sydney University, Sydney, Australia, 15.02.2002, Mobile Phones, Cancer and Blood-Brain Barrier: A Possible Molecular Mechanism.

Zhejiang University, School of Public Health, Hangzhou, China, 10.10.2003; Biological effects of mobile phone radiation.

Brooks AFB, San Antonio, TX, USA, 3.12.2003; Application of transcriptomics and proteomics in search for the potential health effects of EMF.

invited presentations at the conferences:

24th Annual Meeting of Bioelectromagnetics Society, Quebec City, Canada, 23-27.06.2002, Effect of mobile phone radiation on gene and protein expression.

27th General Assembly of the International Union of Radio Science (URSI), Maastricht, The Netherlands, 17-24.08.2002, Effect of GSM mobile phone radiation on blood-brain barrier: Use of proteomics approach to define the hypothetical molecular mechanism.

COST 281 Seminar "Subtle Temperature Effects of RF-EMF", 12-13.11.2002, London, UK, Indirect evidence of non-thermal biological effects induced by mobile phone radiation in vitro.

FGF & COST 281 Workshop on "Genetic and Cytogenetic Aspects of RF-Field Interaction", 24-27.11.2002, Löwenstein, Germany, Mobile phone radiation-induced gene expression might be cell genotype-dependent.

Proteomica Symposium, University of Madrid, 4-8.02.2003, Cordoba, Spain, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields.

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields. (Plenary talk)

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Cellular response to mobile phone radiation appears to be cell genotype-dependent.

WHO & ICNIRP & China Health Ministry, 3rd International EMF Seminar in China: Electromagnetic Fields and Biological Effects, 14-17.10.2003, Guilin, China, Discovery science and mobile phone safety: a need for the new research approach. (Keynote talk)

FGF & COST281 Workshop "The Blood-Brain Barrier (BBB) - Can it be influenced by RF-field interactions?", 3-6.11.2003, Reischensburg, Germany, Mobile Phone Radiation and Blood-Brain Barrier: The available scientific evidence is insufficient to dismiss or to support claims of a health risk in humans.

6th Meeting of the European BioElectromagnetics Association, Budapest, Hungary, 12-16.11.2003, New research approach in EMF research - proteomics and transcriptomics (Plenary talk)

IEEE ICES (SCC-28) meeting, San Antonio TX, USA, 4-7.12.2003; Use of high-throughput screening techniques to determine biological effects of mobile phone radiation.

26th Annual Meeting of the Bioelectromagnetics Society, Washington, DC, USA, 20-25.06.2004; Biological effects of EMF: do they exist and what might be their biophysical mechanism - a molecular biologists perspective.

6.7 rCx46 in oocytes of *Xenopus laevis* and human fibroblasts and granulosa cells of rats (Participant 7)

a. Scientific papers

submitted:

Pilger A., Ivancsits S., Diem E., Steffens M., Kolb H.-A., Rüdiger H. W. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts *Radiation and Environmental Biophysics*.

in preparation:

Steffens M., Enders O., Behnsen J., Kolb H.-A. Effects of intermittent 50 Hz electromagnetic field on gap junctional coupling of paired *Xenopus laevis* oocytes expressing rCx46.

Steffens M., H.-A. Kolb; Effects of intermittent 50 Hz on conducting connexons of rCx46 expressed in oocytes of *Xenopus laevis*.

Steffens M., Kolb H.-A. Frequency dependent induction of DNA strand breaks by intermittent exposure to extremely low frequency electromagnetic field in various cultured cell lines.

b. Scientific meetings and reports

Steffens M., Kolb, H.-A. Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electromagnetic radiation. *Pflügers Arch.* 443 (Plenary Lectures, Oral Sessions, Poster Sessions, Symposia) : S280 (2002)

Wobus A.M., Trillo M.A., Ubeda A., Kolb H.-A. Effects of ELF-and RF-EMF on cell proliferation and cell differentiation. 25th Annual Meeting of the BEMS, Maui, USA (June 2003). *Proceedings*: p. 133

Kolb, H.-A. Die Angst vor dem Strom. *Neue Presse*. Hannover (September 05, 2003)

Kolb, H.-A. Biologische Wirkungen ELF- und RF- elektromagnetischer Felder (EMF) (Nicht-ionische Wirkungen). 1. Nationaler Kongress Elektromog-Betroffener, Biel, Switzerland (22.11.2003)

Kolb, H.-A. Interview with Südwestfunk: Wirkung von EMF auf biologische Systeme. Hannover (July 07, 2004)

c. Poster

M. Steffens, H.-A. Kolb; Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electromagnetic radiation. The Physiological Society Scandinavian Physiological Society, Deutsche Physiologische Gesellschaft (81st Annual Meeting), Tübingen, Germany (15–19 March 2002)

6.8 Experiments with human lymphocytes and thymocytes and with mice embryonic stem cells during cardiac differentiation (Participant 8)

a. Scientific papers

published:

Capri M, Scarcella E, Bianchi E, Fumelli C, Mesircas P, Agostini C, Remondini D, Schuderer J, Kuster N, Franceschi C, Bersani F (2004) 1800 MHz radiofrequency (mobile phones, different Global System for Mobile communication modulations) does not affect apoptosis and heat shock protein 70 level in peripheral blood mononuclear cells from young and old donors. *Int J Radiat Biol*, Vol 80, No 6: p. 389 - 397

Ventura C, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D, Bersani F (2004) Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. *The FASEB J*, published online Oct 26, 2004.

in preparation:

Magnetic fields and cell fate specification in embryonic stem cells. In preparation for *Science*

b. Scientific meetings

Effects of ELF-EMF on gene expression of various cell lines. 25th Annual BEMS Meeting 2003, Abstract book, p. 131

6.9 Experiments with brain cells of different origin and human monocytes (Participant 9)

a. Scientific papers

in preparation:

Poulletier de Gannes F. et al., Effects of GSM-900 radiofrequency radiation on apoptosis in brain cells. (Ready to be submitted to Radiation Research)

Lagroye I. et al., GSM-900 signal does not affect iNOS expression in rat C6 glioma cells (in preparation for Radiation Research)

Lagroye I. et al., Apoptosis in U937 after exposure to 217 Hz-modulated GSM-900 radiofrequency radiation. (in preparation for Bioelectromagnetics)

Poulletier de Gannes F. et al., Expression of heat shock proteins in brain cells after exposure to GSM-900 radiofrequency radiation (in preparation for International Journal of Radiation Biology)

b. Scientific meetings

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002)

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Leszczynski, D., Billaudel, B., Czyz, J., Dulou, P.-E., Guan, K., Haro, E., Joenväärä, S., Kuokka, R., Lagroye, I., Meister, A., Reivinen, J., Veyret, B., Wobus, A.M., Zeng, Q. Effects of mobile phone radiation on gene and protein expression in vitro. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Lagroye I., Bersani F., Billaudel B., Capri M., Czyz J., Dulou P.-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Do ELF or RF fields affect the apoptotic process? Data from the REFLEX programme. 24st Annual Meeting of the BEMS, Québec, Canada (June 2002).

Lagroye I., Poulletier de Gannes F., Haro E., Billaudel B., Dulou P.-E., Veyret B. Effect of GSM-900 radiofrequency on apoptosis of immune and nervous cells. 27ème assemblée générale de l'URSI, Maastricht, Pays-Bas, (August 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E., Billaudel B., Capri M., Dulou P.-E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B., Do GSM signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E., Billaudel B., Capri M., Dulou P.-E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B. Do GSM-900 signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological Effects of Electro-magnetic Fields, Rhodes, October 7-11, 2002, page 404-408.

Adlkofer F., R. Tauber, H.W. Rüdiger, A.M. Wobus, A. Trillo, D. Leszczynski, H.-A. Kolb, F. Bersani, I. Lagroye, N. Kuster, F. Clementi, C. Maercker, Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field Exposure Using Sensitive in vitro Methods (REFLEX), 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Poulletier de Gannes F., I. Lagroye, E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Heat shock proteins as sensors of nonthermal effects? Subtil effects of temperature, Cost 281 meeting, London, UK, (November 2002).

Lagroye I., Bersani F., Billaudel B., Capri M., Czyz J., Dulou P.-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poulletier de Gannes F., Reivinen J., Schuderer J., Veyret B., Wobus A.M., Zeng Q. Effects of ELF- and RF-EMF on the apoptotic process. Abstract for the BEMS 25th annual meeting - Maui, Hawaii, June 22-27, 2003, page 134.

Poulletier de Gannes F., I. Lagroye, E. Haro, M. Taxile, P.-E. Dulou, B. Billaudel, B. Veyret, Effects of GSM-900 on apoptosis in brain cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Lagroye I., Haro E., Billaudel B., Veyret B. The effect of GSM-900 radiofrequency radiation on camptothecin-induced apoptosis in human U937 lymphoblastoma cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Poullétier de Gannes, F., Sanchez, S., Lagroye, I., Haro, E., Dulou, P.-E., Billaudel, B., Veyret, B. In vitro and in vivo studies of the effects of GSM-900 microwave exposure on heat shock proteins in the brain and skin. 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

Lagroye I, Bersani F, Billaudel B, Capri M, Czyz J, Dulou P-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poullétier de Gannes F. Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Effects Of ELF And RF Fields On Apoptosis In Different Cell Lines. . 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

F. Poullétier de Gannes, S. Sanchez, I. Lagroye, E. Haro, B. Billaudel, B. Veyret. Effects of GSM-900 microwave exposure on heat shock proteins: *in vitro* and *in vivo* studies on different models in PIOM laboratory. COST 281bis workshop on "Influence of RF Fields on the Expression of Stress Proteins". April 28-29 2004, STUK Helsinki, Finland.

F. Poullétier de Gannes, I. Lagroye, S. Sanchez, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on hsp27 expression in EA-hy926 endothelial cells: a replication study. 26th Annual Meeting of the BEMS, Washington DC, USA (June 2004).

6.10 Provision of exposure setups and technical quality control (Participant 10)

Exploitation

The success of the exposure setups developed under the umbrella of REFLEX have resulted in additional demands for similar setups being used in further European research programs, e.g. PERFORM B.

Dissemination

a. Scientific papers

published:

J. Schuderer, T. Schmid, G. Urban, N. Kuster, "Novel High Resolution Temperature Probe for RF Dosimetry", Physics in Medicine and Biology, vol 49, pp. N83-N92, 2004.

J. Schuderer, T. Samaras, W. Oesch, D. Spät, N. Kuster, "High Peak SAR Exposure Unit with Tight Exposure and Environmental Control for In Vitro Experiments at 1800 MHz", IEEE Transactions on Microwave Theory and Techniques, vol 52, No 8, 2004: 2057-2066

J. Schuderer, D. Spät, T. Samaras, W. Oesch, N. Kuster, "In Vitro Exposure Systems for RF Exposures at 900 MHz", IEEE Transactions on Microwave Theory and Techniques, vol 52, No8, 2004: 2067-2075

J. Schuderer, W. Oesch, N. Felber, N. Kuster, "In Vitro Exposure Apparatus for ELF Magnetic Fields", Bioelectromagnetics, in press, 2004.

J. Schuderer, "EMF Risk Assessment: *In Vitro* Research and Sleep Studies", Dissertation, ETH, 2003

J. Schuderer, N. Kuster, "The Effect of the Meniscus at the Solid/Liquid Interface on the SAR Distribution in Petri Dishes and Flasks", Bioelectromagnetics, vol. 24, pp.103-108, 2003.

submitted:

J. Schuderer, U. Lott, N. Kuster, "UMTS In Vitro Exposure System and Test Signal for Health Risk Research", Bioelectromagnetics, submitted 2004

in preparation:

W. Oesch, J. Schuderer, N. Kuster, R. Mertens, R. Adey, "Selection of Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", in preparation, 2004.

b. Scientific meetings

J. Schuderer, T. Samaras, W. Oesch, N. Nikoloski, D. Spät, N. Kuster, "Electromagnetic Field Exposure of Cells at 900 and 1800 MHz: Requirements, Dosimetry and Performance Comparison of Different Setups", FGF & COST281 Workshop on the Influence of RF Fields on the Expression of Stress Proteins, April, Helsinki, Finland, pp. 21-22, 2004.

J. Schuderer, W. Oesch, U. Lott, N. Kuster, "In Vitro Exposure Setup for Risk Assessment Studies with UMTS Signal Schemes at 1950 Mhz", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, p. 68, 2003.

J. Schuderer, W. Oesch, R. Mertens, U. Frauenknecht, N. Kuster, "Exposure Systems, Dosimetry and Quality Control", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, pp. 127-128, 2003.

T. Samaras, J. Schuderer, N. Kuster, "Temperature Distributions Inside Cell Cultures Exposed to Electromagnetic Fields In Vitro", Cost 281 Management Committee Meeting, London, GB, Nov. 12-13, 2002.

J. Schuderer, T. Schmid, G. Urban, N. Kuster, "Novel High Resolution Temperature Probe for Micro-dosimetry", 27th General Assembly of the International Union of Radio Science, Maastricht, Netherlands, August, paper No. 2110 (2.p), 2002.

J. Schuderer, W. Oesch, N. Kuster, "In Vitro Exposure Setup for ELF Magnetic Fields Enabling Flexible Signal Schemes and Double Blind Protocols", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 105-106, 2002.

J. Schuderer, W. Oesch, R. Mertens, U. Frauenknecht, N. Kuster, "Exposure Systems and Dosimetric Quality Control in the REFLEX Project", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 93-94, 2002.

R. Mertens, W. Kainz, N. Kuster, "Simulating Environmental GSM Features for Use in Bioexperiments", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, p. 105, 2002.

J. Schuderer, R. Mertens, W. Oesch, U. Frauenknecht, N. Kuster, "Flexible and Efficient In Vitro Exposure Setup for Risk Assessment Studies at 1800 MHz Enabling any Modulation Scheme from Sub-Hz up to 15MHz and Double Blind Protocols", 23rd Annual Meeting of the Bioelectromagnetics Society, St. Paul, Minnesota, USA, p. 26, 2001.

N. Kuster, W-R. Adey, "Criteria for Selecting Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", 23rd Annual Meeting of the Bioelectromagnetics Society, St. Paul, Minnesota, USA, p. 24, 2001.

J. Schuderer, N. Kuster, "The Effect of the Meniscus at the Solid-Liquid Interface on the SAR Distribution in Petri Dishes and Flasks", Millenium Workshop on Biological Effects of Electromagnetic Fields, Heraklion, Greece, pp. 203-207, 2000.

c. Posters

W. Oesch, H-U. Gerber, N. Kuster, "Requirements for Controlling & Monitoring Software of Expoure Systems in (Double-)Blinded Bio Experiments", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 152-153, 2002.

d. Reports in the general media

J. Schuderer, "EMF Risk Assessment: In Vitro Research and Sleep Studies", Diss. ETH 15347, 2003.

N. Kuster, "Latest Progress in Experimental Dosimetry for Human Exposure Evaluations and for Characterization and Optimization of Exposure", in "Communication Mobile – Effects Biologique", ed. Claude Legris, CADAS, Académie des Sciences, Paris, France, pp. 63-69, 2001.

6.11 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

a. Scientific papers

The results described in this final report will be *submitted* for two publications in international scientific journals.

Benfante R., Antonini R.A., Gotti C., Moretti M., Kuster N., Schuderer J., Clementi F., and Fornasari D. – “Extremely low-frequency electromagnetic field (ELF-EMF) does not affect the expression of $\alpha 3$, $\alpha 5$ and $\alpha 7$ nicotinic receptor subunit genes in SY5Y neuroblastoma cell line”. Manuscript in preparation

Antonini R.A., Benfante R., Flora A., Kuster N., Schuderer J., Adlkofer F., Clementi F., and Fornasari D. – “The expression of D β H (dopamine- β -hydroxylase) and noradrenergic phenotype specifying genes Phox2A and Phox2B is unresponsive to exposure to extremely-low-frequency electromagnetic field (ELF-EMF)”. Manuscript in preparation

6.12 Effects of EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

a. Scientific publications

submitted:

Schlatterer K, Gminski R, Hermann S, Tauber R, Fitzner R, Maercker C. Gene expression profiling identifies differences in ribosome biogenesis of human promyelocytic leukemia HL-60 cells following exposure to 1800 MHz radiofrequency electromagnetic fields.

in preparation:

Remondini D, Leszczynski D, Nylund R, Ivancsits S, Rudiger HW, Bersani B, Maercker C. The bio-statistical analysis of micro-array data give indications for the induction of calcium-related signaling pathways after exposure of primary fibroblasts and endothelial cells to electromagnetic fields.

Kuokka R, Griffin T, Maercker C, Schuderer J, Reivinen J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line: Microwave modulation might be the cause of biological response.

b. Oral presentations at scientific meetings and round tables

Maercker C, Czyz J, Ivancsits S, Ruediger HW, Jahn O, Diem E, Pilger A, Rolletschek A, Schuderer J, Kuster N, Guan K, Trillo A, Bazán E, Reimers D, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Adlkofer F, Wobus AM (2002) Gene expression profiling studies on global cDNA arrays show sensitivity of human and mouse cell lines to extremely-low frequency (ELF-EMF) and radiofrequency (RF-EMF) exposure. 24th annual BEMS Meeting, Quebec, Canada.

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Maercker C (2004) Genomics and proteomics approaches in EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

Maercker C (2004) Invited participant in a round table discussion about research needs in European EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

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c. Posters

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7.0 POLICY RELATED BENEFITS

7.1 Studies on the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. Our results obtained with human promyelocytic HL-60 cells have made a substantial addition to the data base relating to genotoxic and phenotypic effects of RF-EMF in vitro. Its value lies in providing new data that will enable mechanisms of RF-EMF effects to be studied more effectively (e.g. ROS effects) than in the past. Proteomics studies should be extended to identify possible, potential biological and molecular markers. Furthermore, our data provide new information that will be used for risk evaluation by WHO, IARC and ICNIRP.

7.2 Studies on human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant3)

Based on our findings we propose the suitability of the comet assay, micronucleus test and evaluation of chromosomal aberrations for monitoring and surveillance of EMF exposed subjects. Due to possible cell specific differences in response to EMF the biological material chosen for biomonitoring could be crucial. Our findings arouse concern about environmental threshold limit values and protective measures regarding EMF exposure in particular with respect to older individuals or people suffering from repair syndroms. The observed activation of DNA repair could display beneficial health effects and could be applied for medical treatment.

7.3 Studies on mouse embryonic stem cells (Participant 4)

Our research results confirmed subtle biological effects emanating from both extremely low frequency fields (simulating the magnetic components of 50 Hz power line fields) and high-frequency (RF) electromagnetic fields (EMF) simulating GSM-modulated schemes. The effects were dependent on the genetic constitution of the cells, and especially the transcription of apoptotic/anti-apoptotic related genes was shown to be affected. Neural progenitor cells appeared to perceive EMF at certain stages of differentiation as external stress signals, which may activate at least, a bcl-2 mediated anti-apoptotic pathway.

1. Important for the quality of life and for human health could be the improvement of products emitting EMF. In this context, 'no effect-levels' were observed with regard to flux density, which can be used to determine threshold values more precisely.
2. The analysis of transcript levels affected by EMF should be extended by genomics and proteomics studies in animals, but also in human populations, to identify further potential molecular markers which may serve as "EMF-responsive bio-indicator".
3. Cell biological studies should be continued to elucidate the molecular processes that may be affected by EMF, especially in the context of carcinogenesis.

7.4 Studies on the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

The described results indicate that a human neuroblastoma cell line can be sensitive to the in vitro exposure to power frequency, sine wave EM fields at magnetic flux densities that are equal to or lower than the exposure threshold (100 μ T) recommended by ICNIRP and UE for the general public. The effects, which include changes in the cells' proliferation, apoptosis or the cellular response to growth factors, among others, were found to be dependent on the ELF-EMF density, the exposure time, the cell passage or the cell cycle. The same human cancer cell line, as well as neural stem cells from rat's embryonic nucleus striatum, was found to be sensitive to the in vitro exposure to GSM-1800 signals at a SAR of 2 W/kg, the exposure threshold recommended by ICNIRP and UE for the general public. The effects included changes in the expression of fibroblast growth factor receptor-1, accompanied or not with changes in the cellular morphology linked to a potential promotion of non-neuronal precursors of NSC progeny. No differential responses were detected when the cells were exposed to GSM signals with

different ELF modulation patterns, suggesting that the observed effects reflect a cellular sensitivity to the RF carrier wave, rather than to the ELF modulation.

1. These data identify cellular mechanisms of response to specific parameters of exposure to ELF and RF electromagnetic fields that are ubiquitous in today's human environment. Such information can significantly contribute to the establishment of adequate strategies for the protection against non-ionising radiation in public, residential or occupational environments.
2. The study of the cellular mechanisms of response to ELF and RF EMF should be extended to properly identify the biophysical phenomena underlying the potential health effects of the exposure to environmental, non-ionising radiation. In this context, two types of studies are of crucial interest. A) Studies aimed to identify potential markers or bio-indicators of EM sensitivity; B) Studies on human cancer cells and the in vitro response to chemicals that could prevent the EMF effects.

7.5 Studies on the human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

We have found that 900 and 1800 MHz GSM radiation at SAR of 2.0-2.4W/kg causes activation of stress response in human endothelial cell line. The stress response was followed by a physiological response on a single cell level. Cellular stress fibers were stabilized what was followed by the changes in cellular size and shape (cells contraction). Also we have observed effect on other cytoskeletal proteins, in particular on vimentin and formed of it - vimentin filaments. In respect of cell apoptosis we have observed decline in the expression of nearly all proteins involved in Fas/TNF α -dependent apoptosis pathway. This suggests that the mobile phone radiation might have some potential to prevent apoptosis of cells - a possibility that is currently being explored in further research. All-in-all, results of our research suggest that cells recognize mobile phone radiation as an external stress and this in spite of the very low energy of the exposure. Part of the results of our research has been already published in three articles (one in Differentiation 2002; two in Proteomics 2004). These results, although directly can not be used for prediction of any health hazard, they are available for the scientific evaluation of the potential risks associated with the use of mobile phones and for recommendations of further research needs. The remaining experimental data obtained by us within REFLEX is in process of submission for publication (3 manuscripts) and will be available for the scientific community in 2005.

7.6 Studies on rCx46 in oocytes of *Xenopus laevis* and human fibroblasts and granulosa cells of rats Participant 7)

On the level of DNA we found significant evidence for DNA damage by ELF-EMF for cultured human fibroblasts and granulosa cells of rats. The findings were strongly related to the exposure protocol. At intermittent ELF-EMF exposure maximal effects were observed after 16-18 h of exposure independently on the applied frequency in the range of 8 Hz to 300 Hz.

The effects appeared not to be reflected on the cellular level of free cytoplasmic calcium. Also cellular studies on the expression level of connexin 46 in oocytes of *Xenopus laevis* showed no significant effect on ELF-EMF exposure. Therefore, it is tempting to suggest that significant effects by ELF-EMF exposure on the genomic level appear not to be reflected on the cellular level. But it has to be taken into account that the methods which are applied to study DNA damage are quite different and most probably more sensitive than those for studying cellular parameters.

7.7 Studies on embryonic stem cells during cardiac differentiation and human lymphocytes and thymocytes (Participant 8)

Studies on embryonic stem cells open a totally new perspective: on one side, the possibility to study in a reproducible way the effects of ELF-EMF on cell differentiation and in particular on cardiogenesis; on the other side, the possibility to direct in some way the differentiation processes of stem cells into specific cell phenotypes.

The more or less negative results studies on human lymphocytes and thymocytes are of paramount importance for risk evaluation since they show that the immune system cells are nearly insensitive to ELF and RF EMF exposure.

7.8 Studies on brain cells of different origin and human monocytes (Participant 9)

We have found no evidence of biological effects of GSM-like signals on mammalian immune and nerve cells. The endpoints were apoptosis and the expression of stress- or inflammation induced proteins. Protocols included exposures at SAR levels corresponding to the public limit for local exposure (2 W/kg) and prolonged exposure duration (24 to 48 hours) that represent a “worst-case” exposure condition. These in vitro results will add to the database, on which the next scientific evaluation of RF-EMF health effects will be based. Although the present findings do not suggest a need for a revision of the local exposure limits to RF-EMF (1999/519/CE), more investigations on animal models of neurodegenerative diseases are needed.

7.9 Provision of exposure set-ups and technical quality control (Participant 10)

Our research is basically aimed at guaranteeing appropriate exposure setups and thorough quality control of the engineering aspects of the various experiments. High technical quality control is of special interest, since the variability of experiments within REFLEX is rather broad. Solid risk assessment will contribute to the future development of new communications technologies.

7.10 Studies on the human neuroblastoma cell line SY5Y (Participant 11)

Our results fit in a scientific debate around the contribution of ELF-EMF on brain neurodegenerative diseases, with particular emphasis on Alzheimer’s disease (AD). Epidemiological studies showed that workers with likely electromagnetic field exposure may have an elevated risk of AD. On the other hand, experimental studies employing animal models failed to confirm these observations. In our studies we demonstrate that, at molecular level, the cholinergic system, which is one of the most affected neurotransmission system in AD, did not undergo any modification in the expression of relevant nAChR subtypes.

7.11 cDNA array analysis (Participant 12)

The micro-array technique is a state-of-the-art tool to investigate changes in gene expression and therefore molecular defects in human cells. Whereas in the field of medicine this kind of technique is on the way to get a diagnostic standard for certain diseases (e.g. cancer), it is not common so far for the detection of environmental effects. With our study we have shown that the whole-genome analysis is a suitable method to detect potential molecular effects of EMF. Since the different labs participating in the REFLEX project have worked with the same exposure setups (Participant 10) and we have done all hybridisations and data analyses in the same way, we have created a platform which can work with comparable material (RNA) of different cell lines and different experimentators. A quality control of the RNA (test for degradation, concentration) allowed us to make experiments with very different cell lines under comparable conditions and therefore to produce reliable results. This is a big difference to other assays (e.g. microscopical analysis), which strongly depend on people and software. In the near future, whole-genome approaches might support or even replace other measurements of the effects of electromagnetic fields or related environmental influences. Since the technique is very sensitive and specifically applicable for human cells, it also should be applied for in vivo studies in upcoming projects.

7.12 Summary (Participant 1)

The policy related benefits of the REFLEXproject consist in the fact that new knowledge has been generated independent of whether one likes it or not. Biological effects of extremely low-frequency (ELF) and radio-frequency (RF) electromagnetic fields (EMFs) the exposure to which is constantly increasing especially in Europe with its high density of population and industry and with the omnipresence of EMFs in infrastructures and consumer products have become a topic of public concern. This is due to the fear of people that based on the many conflicting research data a risk to their health cannot be excluded with some certainty. Therefore, the overall objective of REFLEX was to find out whether or not the fundamental biological processes at the cellular and molecular level support such an assumption. For this purpose, possible effects of EMFs on cellular events controlling key functions, including those involved

in carcinogenesis and in the pathogenesis of neurodegenerative disorders, were studied through focussed research. Failure to observe the occurrence of such key critical events in living cells after EMF exposure would have suggested that further research efforts in this field could be suspended and financial resources be reallocated to the investigation of more important issues. But as clearly demonstrated, the results of the REFLEX project show the way into the opposite direction.

The REFLEX project has made a substantial contribution to the data base on biological effects of both ELF-EMF and RF-EMF on in vitro cellular systems. The study was designed to investigate whether or not EMF exposure below the energy density reflected by the present safety levels generates in vitro critical cellular events. Gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis) that are caused by or result in an altered gene and protein expression profile are such critical events, the convergence of which is required for the development of chronic diseases. Genotoxic effects and a modified expression of numerous genes and proteins after EMF exposure could be demonstrated with great certainty, while effects on cell proliferation, cell differentiation and apoptosis were much less conclusive. Since all these observations were made in in vitro studies, the results obtained neither preclude nor confirm a health risk due to EMF exposure, but they speak in favour of such a possibility. Because of their fundamental character the findings will be presented to WHO, IARC and ICNIRP. It will be up to these organisations to make use of them for risk evaluation, in combination with findings from animal and epidemiological studies.

A major European added value of REFLEX consists also in the fact that the need for further research and especially how it should look alike have clearly been demonstrated. Furthermore, the outcome of the project should stimulate the research and development departments of the electrical, electronic, and telecommunication industry to make use of the methods developed in order to better adjust the state of technology to the conditions of life, and prompt the European governments to ensure multidisciplinary EMF research in order to take care, that the solution of the presently existing problem of uncertainty about a possible health risk for the people in Europe and beyond due to EMF exposure will not be postponed in the far future.

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ANNEX I - Results submitted after deadline

A) ELF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to ELF-EMF (50 Hz, 1 mT, 15h, 5 min on/10 min off) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.1.1.1) were re-evaluated under blinded conditions.

- a) Prof. Guenter Speit, Medizinische Fakultät/Humangenetik, University of Ulm, Albert-Einstein-Allee 7, 89081 Ulm, Germany

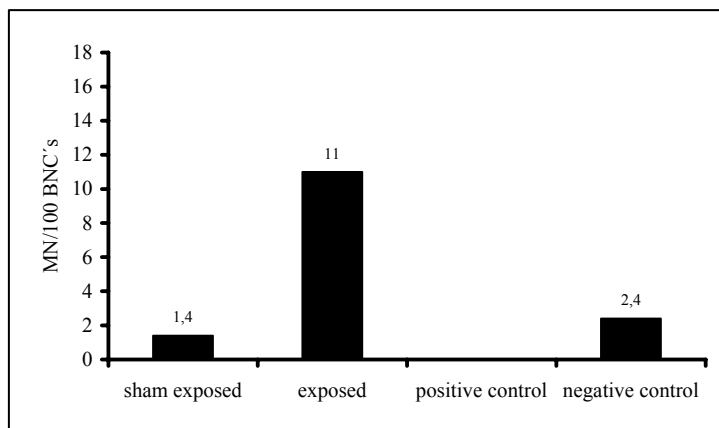


Figure 1: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated cells (10µg/ml) were used as a positive control.

- b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universität Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

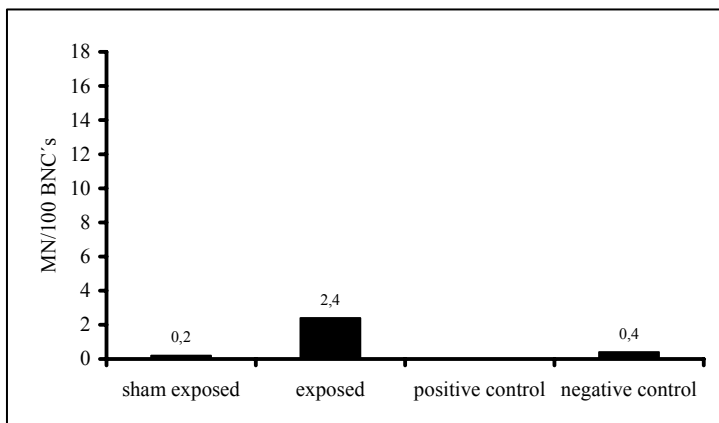


Figure 2: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated fibroblasts (10 µg/ml) were used as a positive control.

ELF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 6 to 10-fold as compared to sham exposed cells and negative controls. Although basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely higher values. The variability in micronucleus frequencies between the laboratories maybe due to the fact that different staining techniques were applied: Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GEMSA. In addition, the laboratories in Ulm and Kaiserslautern scored 500 binucleated cells only instead of 2,000 processed in Vienna.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after ELF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and the different a numbers of scored cells.

2) Evaluation of genotoxic effects on human fibroblasts following intermittent exposure to 50 Hz powerline magnetic fields by Participant 8 (Bologna/Naples)

Some experiments related to the evaluation of the induction of genotoxic effects following intermittent (5 min field on/10 min field off) exposures to 50 Hz ELF magnetic fields, 1 mT field intensity, were carried out on human diploid fibroblasts (ES-1). In particular, to evaluate the induction of DNA single strand breaks the alkaline comet assay was applied following 15, 18 and 24 hours exposures, while the induction of micronuclei (MN) was measured following 24 h exposure.

Moreover, positive controls were also provided by treating cells with hydrogen peroxide (Alkaline Comet Assay) or Mitomycin-C (MMC; micronucleus assay) at several concentrations.

The results obtained do not indicate induction of genotoxic effects, neither in terms of comets induction nor in terms of MN frequency increase. On the contrary, positive controls showed an increase in DNA damage, as expected.

Experimental conditions and cytogenetic analysis

Human diploid fibroblasts (ES-1) were cultured in DMEM containing 10% FBS, 20 mM Hepes buffer, 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Cells were supplied with fresh culture medium every 48 h and splitted once a week. Cells were received at passage 6 and a master bank was established with cells at passage 8. For experiments, cells from passages 10-16 were used.

Preliminary experiments were devoted to find the dose of chemicals to be used to provide positive controls for both the assays employed. Dose-response curves were set up by treating ES-1 cells with H₂O₂ ranging from 25-100 µM final concentration for 30 minutes (alkaline comet assay) and with MMC ranging from 0,005 – 0,05 µg/ml final concentration for the whole culture period (MN assay). For both chemicals a dose-dependent increase in DNA damage was detected, even for the lowest doses tested. However, the best compromise between induced damage and cell survival was found at 50 µM H₂O₂ and 0,025 µg/ml MMC and these concentrations were used for the experiments.

A second set of preliminary experiments was devoted to define the cell cycle duration of ES-1 cells to block cytokinesis for micronucleus test: it resulted of 28 h (data not shown).

The exposures were carried out at 50 Hz (1 mT field intensity) and the signal used was powerline. Several exposure durations were tested, as reported below.

Alkaline Comet Assay

Ten independent experiments (5 by exposing cells for 24 h, 2 by exposing cells for 18 h, 3 by exposing cells for 15 h) were carried out by setting up 8 cultures each: 4 to be exposed and 4 to be sham exposed.

Moreover, positive controls were provided by treating cultures for 30 min with hydrogen peroxide (H₂O₂, 50 µM). 24 hours before the experiments, 50000 cells/3 ml complete medium were seeded into 35 mm Petri dishes (Corning, cat. 430165). Following the intermittent exposure cultures were processed for the comet assay as described in details in (1). Slides were stained, just before the analysis, with ethidium

bromide (12 µg/ml) and images of 1000 randomly selected cells (250 from each of four replicated cultures) were analyzed by a computerized image analysis system (Delta Sistemi, Rome, Italy) fitted with a Leica DM BL fluorescence microscope at 250 X magnification. This system acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters. DNA damage was evaluated by calculating the tail factor, as reported in (1). Moreover, tail moment, comet moment and percentage of tail DNA were also measured.

Cytokinesis-block Micronucleus Assay

4 independent experiments were carried out by exposing cells for 24 h. For each experiment 4 cultures were set up: 2 to be exposed and 2 to be sham exposed. Positive controls were provided by adding MMC (0,025 µg/ml) 24 h after the seeding.

50000 cells/5 ml complete medium were seeded in slide flasks (NUNCLON, cod. 170920, 9 cm² growth area) and recovered for 24 h. To block cytokinesis, 4 hours before the end of the first cycle (48 h after seeding), Cytochalasin-B (3 µg/ml final concentration) was added and at the end of the second replication cycle (80 h after seeding), cells were incubated for 30 min at 37°C with hypotonic solution (KCl 0,075 M) and fixed for 10 min (methanol 80% in distilled water). Air dried slides were stained for 8 min (10% Giemsa in phosphate buffer pH 6.8). The intermittent MF exposure was carried out during the first 24 hours following the recovery.

MN were scored in binucleated cytokinesis-blocked (CB) cells with well preserved cytoplasm by using a light microscope, and for each experiment their frequency was evaluated in 2000 cells (1000 cells for each duplicate slide). The results were expressed as micronucleated binucleated (MNB) cells per thousand binucleated cells.

The morphological criteria for MN scoring in binucleated cells were similar to those reported by Fenech for human lymphocytes (2). By classifying 500 cells according to the number of nuclei, the binucleate cell index (BCI) and the cytokinesis-block proliferation index (CBPI) were evaluated for each culture, as reported in (3) and (4), respectively.

Statistical analysis

Differences between treated and untreated samples (sham exposed vs. exposed; sham exposed vs. positive controls) were tested by using the two tailed paired Student's t test. P values lower than 0.05 were considered as statistically significant.

RESULTS

Comet assay

Following 24 h intermittent exposure (5 min on/10 min off) to 50 Hz powerline MF, human diploid fibroblasts did not show statistically significant differences in all the parameters investigated when sham-exposed samples were compared to exposed ones. The results obtained are reported in table 1 as mean ± standard error of 5 independent experiments. Same results have been obtained when cultures exposed for 18 h (2 experiments) and 15 h (3 experiments) were compared to their own sham exposed cultures, as shown in table 2 and 3, respectively.

On the contrary, when sham exposed cultures were compared to H₂O₂ -treated samples, a significant increase in all the parameters investigated was detected following treatments of 30 minutes (p<0.05). The results obtained are reported in figure 1 as mean ± standard error of all the parameters investigated.

Micronucleus assay

Following 24 h exposure no genotoxic effects were detected by comparing sham-exposed with exposed samples. The proliferation index (CBPI) also resulted not affected by the exposure. The results are reported in table 4 (4 independent experiments), where data related to MMC treatments are also shown. By comparing sham-exposed with MMC treated cultures a statistically significant increase in MN frequency was detected, together with a decrease of cell proliferation and of percentage of binucleated cells (p<0.05 in all cases).

DISCUSSION

The data here reported do not support the hypothesis that intermittent exposures to 50 Hz MF (powerline signal) induce genotoxic effects in human diploid fibroblasts.

Concerning the alkaline comet assay, our finding is also supported by the results obtained by treating cells with hydrogen peroxide as positive control, where an increase in all the comet parameters investigated has been detected. Moreover, the cells investigated showed an high sensitivity to 30 min treatment already at 25 μ M final concentration (data not shown), while most of the data reported in literature on several cell types indicate that 30 min treatments at concentrations between 50 and 100 μ M are needed to induce a statistically significant effect.

Concerning the data on the induction of micronuclei (MN), also in this case we have not found genotoxic effects induced by the field. On the contrary, MMC treatments at doses of 0,025 μ g/ml resulted cytotoxic (CBPI: 1,18 vs. 1,45 in treated and untreated cultures, respectively) while a dose of 0,033 μ g/ml is necessary to induce MN increase in human peripheral blood lymphocytes without affecting cell proliferation.

The data here reported refer only to the exposures performed with the powerline signal: experiments devoted to test the sinusoidal signal are in progress.

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Table 1 – mean values \pm standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 24 h to 50 Hz powerline magnetic field. Results of 5 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.65 \pm 0.98	6.36 \pm 0.93
Comet Moment	1.86 \pm 0.43	2.01 \pm 0.49
% DNA	3.8 \pm 0.97	4.12 \pm 1.12
Tail Moment	1.10 \pm 0.32	1.29 \pm 0.36

Table 2 – mean values \pm standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 18 h to 50 Hz powerline magnetic field. Results of 2 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	6.24 \pm 0.25	6.11 \pm 0.26
Comet Moment	2.32 \pm 0.04	2.25 \pm 0.04
% DNA	4.37 \pm 0.22	4.20 \pm 0.22
Tail Moment	1.56 \pm 0.05	1.54 \pm 0.11

Table 3 – mean values \pm standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 15 h to 50 Hz powerline magnetic field. Results of 3 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.23 \pm 0.26	5.20 \pm 0.63
Comet Moment	1.76 \pm 0.08	1.68 \pm 0.18
% DNA	3.07 \pm 0.29	3.08 \pm 0.68
Tail Moment	0.89 \pm 0.03	0.88 \pm 0.15

Table 4 – MN frequency, proliferation index (CBPI) and binucleate cell index (BCI) in cultures sham-exposed, exposed for 24 h and positive controls. Data are reported as mean \pm SD of 4 independent experiments. * Sham-exposed vs. MMC treated cultures: $p < 0.05$.

Parameters	Sham-exposed	MF-exposed	MMC (0.025 μ g/ml)
MN/1000 CB cells	0.45 \pm 0.06	0.43 \pm 0.07	2.35 \pm 0.35*
CBPI	1.45 \pm 0.02	1.43 \pm 0.02	1.15 \pm 0.01*
% BCI	45.2 \pm 2.45	43.3 \pm 2.01	15.0 \pm 1.41*

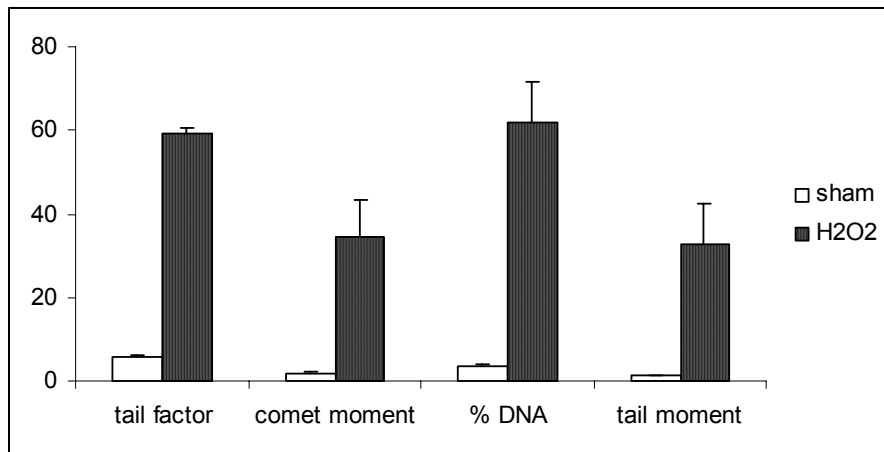


Figure 1 – mean values \pm standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts treated for 30 min with 50 μ M hydrogen peroxide compared to sham-exposed samples.

B) RF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to RF-EMF (GSM basic 1950 MHz, 15h, 2 W/kg) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.2.1.2) were re-evaluated under blinded conditions.

- a) Prof. Guenter Speit, Medizinische Fakultät/Humangenetik, University of Ulm, Albert-Einstein-Allee 7, 89081 Ulm, Germany

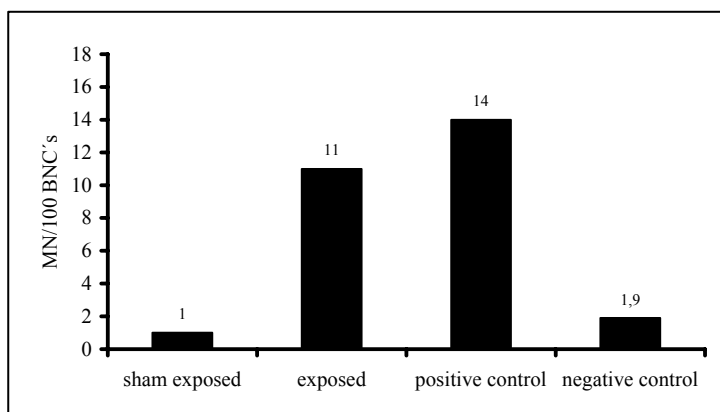


Figure 1: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were used as a positive control.

- b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universität Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

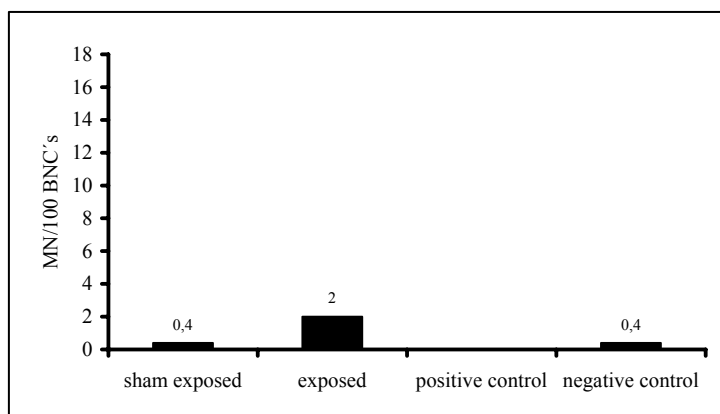


Figure 2: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were as a positive control.

RF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 5-fold compared to sham exposed cells and negative controls. Although, basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely lower levels. These variability could be attributed to different staining techniques. Laboratories in Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GEMSA. Positive controls could not be evaluated, due to a too low number of assessable cells.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after RF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and a various number of scored cells.

ANNEX II

Technical support (Participant 10)

OBJECTIVES

To be able to compare the results of investigations carried out in the different laboratories and to ensure the conclusiveness of the data obtained in the studies, it is of the utmost importance that the conditions of exposure to EMFs be strictly controlled. Therefore, the objective are:

- Evaluation of existing setups
- Development of an optimised ELF setup
- Development of an optimised RF setup (900 MHz)
- Development of an optimised RF setup (1800 MHz)
- Comprehensive dosimetry ELF setup
- Comprehensive dosimetry RF setups
- Technical quality control during the entire period of exposure

Detailed information about the applied exposure systems and their dosimetry are given in

- [Schuderer et al., 2004a] for the ELF setup
- [Schuderer et al., 2004b] for the RF setup (GSM)
- [Schuderer et al., 2003] for the RF setup (UMTS)
- [Schönborn et al., 2000] for the waveguide setup of Participant 4
- [Laval et al., 2000] and [Schuderer et al., 2004c] for the Wire-patch cell of Participant 9
- [Toivo et al., 2001] and [Schuderer et al., 2004c] for the resonator setup of Participant 6
- [Kuster et al., 2002] for the choice of the exposure signals

REQUIREMENTS

The requirements for the exposure systems are formulated in Kuster and Schönborn (2001). In particular, the following parameters need to be fulfilled:

ELF setup:

- large loading volume with uniform exposure
- high dynamic (μT - mT) and frequency ranges (subHz - kHz)
- enabling complex signals and intermittent exposure
- good isolation between exposure and sham
- identical atmospheric parameters for exposed and sham cells (preferably placed in the same incubator)
- blinded exposure protocols by a computer-controlled random decision maker
- continuous monitoring of all environmental and technical parameters in order to detect any malfunctions
- evaluation of possible artefacts such as parasitic E-fields, temperature loads, vibrations, etc.

RF setup:

- peak SAR exposure: >100 W/kg
- maximum time averaged SAR exposure for thermal load < 0.1°C: > 2 W/kg
- deviations from uniformity of exposure: < 30%
- variability of exposure: < 10%
- loading volume with uniform exposure: min. 50 cm²
- flexible signal unit with high dynamic range enabling complex modulation such as:
 - continuous wave (CW)
 - pulse or sinusoidal modulation at any frequency and repetition rate
 - GSM DTX (discontinuous transmission mode)
 - GSM non-DTX
 - GSM talk (temporal changes between non-DTX and DTX)
 - UMTS signal schemes
- flexible intermittent exposure protocols: seconds to hours
- good isolation between exposure and sham: > 30 dB
- identical atmospheric parameters for exposure and sham: $\mu T < 0.1^{\circ}C$
- blinded exposure protocols
- continuous monitoring of exposure and environmental parameters
- detailed numerical modelling including meniscus
- evaluation of SAR distribution and experimental verification by dosimetric measurements
- uncertainty and variability analysis for SAR
- evaluation of the temperature load
- reliability, user-friendliness and self-detection of malfunctions
- minimal setup cost

MATERIAL AND METHODS

Overview

High-end exposure setups for ELF, RF-GSM and RF-UMTS were developed and fully characterized with respect to exposure parameters, design and possible artifacts.

Since the budget did not allow to equip every laboratory with these setups, existing setups were evaluated with respect to their suitability.

Evaluation of Existing Setups

In order to assess the performance of the already existing setups, the exposure systems in the laboratories of Participants 2 - 9 were evaluated with respect to dosimetric performance and characterization. Possible modifications to improve the performance were also evaluated.

In addition to the newly developed exposure systems, it was decided to use five existing setups (Participants 4, 5, 6, 8, 9) and to modify and improve the two RF setups of Participants 4 and 9. Similar methods for dosimetry and characterization as described for the newly developed RF setups were used.

ELF setups:

- *Helmholtz coils (Participant 5):* A pair of Helmholtz coils is placed inside a μ -metal shield; exposure and sham are kept in different incubators; and sinusoidal B-fields (50 Hz) up to 0.1 mT can be applied.
- *4-coil system (Participant 8):* Two unshielded 4-coil systems are arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied.

Both setups provide acceptably uniform B-fields, and no further modification and optimisation was applied.

RF setups:

- *Waveguide setup (Participant 4):* The waveguide setup of Participant 4 is operated at 1710 MHz and allows the exposure of eight 60 mm diameter Petri dishes. The original system [Schönborn et al., 2000] was enhanced with
 - a new signal unit, allowing complex GSM modulation
 - field sensors to monitor the exposure
 - temperature sensors to monitor the incubator environment
 - an optimised air flow system to reduce temperature differences between both chambers
- *Wire-patch cell (Participant 9):* The Wire-patch cell is an open radiating setup operated at 900 MHz. The setup is based on a 150 mm x 150 mm parallel plate configuration (distance 29 mm), short-circuited at the edges by four plots [Laval et al., 2000]. Eight 35 mm Petri dishes (placed inside a 60 mm dish with distilled water) are arranged symmetrically around the central coaxial feed. Since the WP cell is an open setup, exposure and sham groups need to be placed in two different incubators. For the purpose of REFLEX, the original system was enhanced by
 - distance keepers for the Petri dishes,
 - optimised Petri dish loading (to reduce the thermal load, distilled water instead of cell medium is used for the 60 mm dish),
 - E-field sensors for monitoring and regulation
 - A computer-controlled signal unit, allowing complex GSM modulation
- *Resonator setup (Participant 6):* The resonator setup consists of a short-circuited waveguide chamber at 900 MHz [Toivo et al., 2001]. Four 60 mm diameter Petri dishes are exposed in a standing wave E-field maximum in E-polarization. The dishes are placed on a glass plate which is water-cooled from below. In this way a temperature stability of $\pm 0.3^{\circ}\text{C}$ over the range from 0 – 10 W/kg average SAR is achieved. No modification of the resonator setup was performed.

Development of ELF Exposure Setup

An ELF setup was developed and four copies were installed in the laboratories of Participants 3, 4, 7 and 11. The following methods have been applied to achieve an optimised design for the ELF exposure system:

- Two coil chambers are placed inside the same incubator to guarantee identical environmental parameters for exposure and sham groups. A fan system serves for enhanced atmospheric exchange between coil chambers and incubator.
- μ -metal shielding is applied for the coils in order to provide sufficient sham isolation.
- E-field shielding of the exposure area is applied to remove parasitic E-fields, generated by the voltage drop over the inductive coils.
- Elastically damped dish holders are used to minimize the coupling of the mechanical vibrations to the Petri dishes.
- Numerical field calculation was used to optimise the 4-coil system within the μ -metal shielded exposure chamber. Optimisation parameters were size of the coils, number of windings and distance between the windings. B-field uniformity was used as an optimisation target.
- Exposure control is realized by monitoring and feedback regulation of the coil currents.
- Complex ELF signals can be applied by using an arbitrary function generator together with a custom-made current source to generate any signal with a point length of 16000 points, a point resolution of 12 bit and frequencies up to 1.5 kHz.
- A power-line signal was defined as the maximum accepted distortion for low- to medium-voltage power systems by the IEC (spectral components up to 1250 Hz are present).
- Environmental monitoring is applied with temperature sensors inside the chambers and by controlling the fan system (current measurement).
- Computer control allows blind protocols and easy handling of the system.

Development of RF Exposure Setup (GSM)

An RF setup (GSM) was developed and four copies were installed in the laboratories of Participants 2, 5, 6 and 8. The following methods have been applied to achieve an optimised design for the RF exposure system:

- The study from Schönborn et al. (2001) showed that the most suited setup with respect to highly uniform cell monolayer exposures should be based on waveguides.
- A waveguide setup was numerically analysed by using the FDTD method. The length of the waveguides was optimised, so that the system is operated at a fundamental resonator mode at 1800 MHz. In this way, superior power efficiency can be achieved.
- The required uniformity and maximum SAR is achieved for a fan-cooled cell monolayer exposure in E-polarization, which provides minimum temperature load.
- Exposure control is realized by field sensors.
- Environmental control is realized by placing the waveguides inside an incubator and using fans for atmospheric exchange. A common air inlet of the fan system was realized in order to reduce temperature differences between both waveguides.
- Environmental monitoring is applied with air temperature sensors and fan monitoring.
- Low variability is realized with a field sensor feedback regulation and by using dish holders together with distance keepers to provide defined positions of the Petri dishes with respect to the incident fields.
- Flexible signal schemes and blinded protocols are realized with a computer-controlled signal unit. AM modulation of the RF generator is applied via an arbitrary function generator and additionally via software commands. Temporal changes between different modulation modes like GSM DTX and non-DTX is realized with a GSM frame unit, blanking the output of the RF amplifier.

Development of RF Exposure Setup (UMTS)

An RF setup (UMTS) was developed and installed in the laboratory of Participant 3. Similar methods as for the RF setup (GSM) have been applied to achieve an optimised design for the UMTS exposure system:

- Similar to the GSM setup, two waveguides are used, equipped with field and temperature sensors and an optimized fan cooling system.
- Due to the different carrier frequency at 1950 MHz, new positions for the Petri dishes inside the waveguide are necessary. Because of the 5 MHz bandwidth of the UMTS signal, a broadband coax-to-waveguide adapter was required.
- The signal unit was updated by a UMTS signal generator. The fast power control of the signal is realized by AM modulation of the RF generator with a fading function stored on the arbitrary function generator.
- A UMTS test signal was defined, which represents worst-case exposure with respect to ELF spectral content. The signal is based on closed loop power controlled fades and additionally covers compressed mode and an open loop power controlled sequence for the physical random access channel.

Dosimetry ELF Exposure Setup

The following methods for dosimetry of the ELF setup were applied:

- *Numerical B-field characterization:* Mathematica V4.1 was used for analytical calculation of the B-field distribution as resulting from the 4-coil configuration.
- *Experimental B-field characterization:* A 3-axis Gaussmeter (FH49, Magnet-Physik, Germany) was used to measure the B-field distribution inside the exposure chamber.
- *Uncertainty and variability:* Uncertainty of the dosimetric assessment and exposure variability were analyzed for the applied numerical and experimental methods.
- *Induced E-field characterization:* The distribution of the induced E-fields within the cell medium was assessed by calculation.
- *Artefact characterization:*
 - *Parasitic E-fields:* A Wandel and Goltermann EFA-3 sensor system was used to determine the electric fields inside the exposure chamber as produced by the setup.
 - *Temperature:* Temperature was measured inside the cell medium with a SPEAG T1V3 probe as well

as for several positions inside the exposure chamber with Thermometrics Pt100 temperature sensors.

- *Vibrations*: To assess the acceleration resulting from coil vibrations, a Wilcoxon Research accelerometer Model 728T equipped with an amplifier unit P704T was applied.

Dosimetry RF Exposure Setup (GSM, UMTS, Wire-Patch Cell)

The following methods for dosimetry of the RF setups were used:

- *Numerical field simulation*: The FDTD simulation platform SEMCAD was applied for a full 3D electromagnetic field analysis.
- *Numerical modelling*: High resolution numerical models including menisci at the solid/liquid interfaces have been used to achieve realistic modelling. SAR extrapolation to the monolayer was applied to compensate discretization error in the strong SAR gradients.
- *Experimental verification*: Simulation was experimentally verified using the DASY3 near-field scanner equipped with 3-axis free space E- and H-field probes (SPEAG EF3DV2, H3DV6) and a dosimetric E-field probe with a diameter of only 1 mm [Pokovic et al., 2000].
- *Uncertainty and variability*: The uncertainty of the SAR assessment was evaluated with respect to the applied numerical and experimental methods. Possible variability of SAR values was additionally evaluated.
- *Thermal load*: The thermal load for the exposed group was assessed by measurement and simulation: A SPEAG T1V3 temperature probe was used for a single-point measurement of the temperature response of the medium (probe was fixed in the temperature maximum). Additionally, a coupled electro-thermal FDTD simulation was used for the 3D assessment of the temperature distribution as a function of exposure duration.

Quality Control and Maintenance

Quality control is ensured by the analysis of the exposure data as recorded from the monitoring unit and stored within an encoded file. All experimental settings and software commands are saved together with the sensor data for field exposure and environment. The monitoring sampling rate is 0.1 Hz. Decoding of the data files can only be provided by a dedicated software and is done by the quality assurance group after biological evaluation. A detailed report of all exposure parameters is then provided.

Furthermore the controlling and monitoring software is able to self-detect malfunctions and responds with warnings or abortions if required (tracing and handling of 60 errors).

For the RF setups, the ambient ELF-fields in the different laboratories were determined for several positions within the incubator and laboratory using a Wandel & Golterman EFA-3 sensor system.

RESULTS

Dosimetry ELF Exposure Setup

The performance of the ELF setup can be summarized by:

- Dynamic range for B-field amplitude (50 Hz): 0.02 – 3.6 mT_{rms}
- Dynamic range for B-field frequency: mHz – 1500 Hz
- Nonuniformity of B-field: < 1%
- Uncertainty for B-field assessment: 4.3%
- Variability of exposure: 1.6%
- Loading volume: 3500 cm³
- Parasitic E-fields (50 Hz): < 1 V/m
- Vibrations:
 - < 0.1 m/s² for elastically damped holder
 - < 1 m/s² for non-damped holder

- Signal schemes:
 - Sinusoidal 3 –1000 Hz
 - 50 Hz power-line signal (components up to 1250 Hz)
 - Arbitrary intermittency
- Exposure control and monitoring: provided by current measurements (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (GSM)

The performance of the RF setup (GSM) can be summarized by:

- Dynamic range for peak SAR: 0.01 W/kg to > 100 W/kg
- Nonuniformity of SAR: < 30%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 20%
- Variability of exposure: 5.1%
- Loading surface for cell monolayers: 60 cm²
- Signal schemes:
 - Continuous wave
 - 217 Hz pulse modulation
 - GSM non-DTX
 - GSM DTX
 - GSM Talk
 - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (UMTS)

The performance of the RF setup (UMTS) can be summarized by:

- Dynamic range for peak SAR: 0.01 W/kg to > 200 W/kg
- Nonuniformity of SAR: < 26%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 18%
- Variability of exposure: 1.9%
- Loading surface for cell monolayers: 60 cm²
- Signal schemes:
 - Continuous wave
 - UMTS test signal (maximized ELF spectral content)
 - 217 Hz pulse modulation
 - GSM DTX
 - GSM non-DTX
 - GSM Talk
 - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)

- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: $< 0.1^{\circ}\text{C}$)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Quality Control and Maintenance

Quality Control:

- In the course of the REFLEX project, approximately 1800 *in vitro* experiments have been performed. Each of these experiments is documented with a dosimetric evaluation report covering the time courses and statistics for the field values, air temperatures and fan currents as well as all experimental settings.
- Average ambient ELF B-fields in the incubators of laboratories 2, 3, 4, and 8 are:
 - Participant 2: $B = 0.3 \pm 0.2 \mu\text{T}_{\text{rms}}$
 - Participant 4: $B = 3.2 \pm 2.0 \mu\text{T}_{\text{rms}}$
 - Participant 5: $B = 3.5 \pm 2.2 \mu\text{T}_{\text{rms}}$
 - Participant 8: $B = 2.8 \pm 1.9 \mu\text{T}_{\text{rms}}$

Maintenance:

Maintenance and assistance was provided in the course of the project for:

- installation of the setups
- handling of the setups
- exchange of several Pt100 temperature probes
- exchange of some RF dish holders
- exchange of one malfunctioning ELF current source
- exchange of one malfunctioning RF generator
- provision of software updates
- evaluation of data files

DISCUSSION

All tasks except development of an optimised RF setup at 900 MHz have been fully solved. More setups than initially planned needed to be developed, since the quality of the setups available in the laboratories were not sufficient to meet the requirements of the project. In order to stay within the budget, the consortium decided to develop a new RF setup only for 1800 MHz and use already available setups for 900 MHz (setups of Participants 6 and 9). Furthermore, the setup of Participant 9 was updated to allow complex GSM modulation. In addition to the required deliverables, a novel UMTS exposure system was developed.

CONCLUSIONS

High-end exposure systems for conducting *in vitro* laboratory studies in several European research institutes were realized and characterized. These systems have already become standard exposure setups for bioexperiments around the world.

An ELF exposure system that allows flexible signal and intermittent exposure schemes has been developed and characterized. It is easy to handle due to automated software control. Coil currents, chamber temperatures and fan speed are continuously monitored and allow the experimental history to be traced with 10 s resolution. B-field and E-field distributions were characterized. The B-field shielding of the 4-coil configuration considerably enhances the uniformity of the field distribution, and a highly efficient E-field shielding inhibits strong parasitic electric fields generated by the coils. Temperature differences between exposed and sham-exposed cells are kept below 0.1°C . The vibration load on the

exposed Petri dishes is sensitive to mechanical resonance; however, a mechanically isolated and elastically damped dish holder limits this to less than 0.1m/s^2 , which is no more than twice the background vibration of the sham setup.

The waveguide-based, computer-controlled RF (GSM) setup enables the exposure of cell monolayers with excellent efficiency $> 20\text{ W/kg/W}$. The flexible signal unit allows the generation and control of complex modulated signals, e.g. temporal changes between different GSM operation modes (DTX/non-DTX). Exposure field strength and environmental parameters (air temperature, fan system) are continuously monitored. Due to the field regulation, exposure variability is kept below 10%. A coupled electro-thermal FDTD analysis was performed and resulted in a nonuniformity of SAR of $< 30\%$. The temperature load was assessed by measurement and simulation, and a maximum temperature increase of less than 0.03°C was found. No localized temperature hot “spots” are generated within the cell medium. All simulations were verified by dosimetric measurements.

An exposure setup allowing the blinded exposure of cell monolayers to UMTS signal schemes was developed and dosimetrically analysed. Cells can be exposed to up to 17 W/kg/W with less than 26% nonuniformity of SAR. The temperature load for the exposed cells is less than $0.03^\circ\text{C/(W/kg)}$. The UMTS specifications have been analysed in order to identify ELF spectral components in the signal. These mainly result from inner loop power control; however, pulsed signal structures due to compressed mode and PRACH/PCPCH procedures also contribute to the ELF components. A test signal is proposed which is compliant to the 3GPP FDD modulation specifications and is optimised for maximized ELF spectral power (1 Hz harmonics).

Quality control for the entire duration of the project is ensured due to automatically generated data files. Exposure field strength, temperature, fan currents and all settings and computer commands are stored in the data files with a sampling rate of 0.1 Hz. Evaluation reports are available for every experiment performed in the REFLEX consortium.

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Attachment 11



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Article: Effect of coherence time of the applied magnetic field on or
Author: Litovitz TA;Krause D;Mullins JM
NLM Unique ID: 0372516 Verify: PubMed
PubMed UI: 1872866
ISSN: 0006-291X (Print) 1090-2104 (Electronic)
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EFFECT OF COHERENCE TIME OF THE APPLIED MAGNETIC FIELD ON ORNITHINE DECARBOXYLASE ACTIVITY

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Received June 6, 1991

Skepticism over the possibility of weak electromagnetic fields affecting cell function exists because endogenous thermal noise fields are larger than those reported to cause effects. Four-hour exposure to a 55- or 65-Hz field approximately doubles the specific activity of ornithine decarboxylase (ODC) in L929 cells. To test the idea that the cell discriminates against this thermal noise because it is incoherent, partial incoherence was introduced into the applied field by shifting the frequency between 55- to 65-Hz at intervals of $\tau_{coh} - \delta\tau$ where τ_{coh} is a predetermined time interval and $\delta\tau \ll \tau_{coh}$ varies randomly from one frequency shift to the next. To obtain the full ODC enhancement, coherence of the impressed signal must be maintained for a minimum of about 10s. For $\tau_{coh} = 5.0$ s a partial enhancement is elicited, and at 1.0s there is no response. Unfortunately coherence times of this duration are too short to solve the thermal noise puzzle. © 1991 Academic Press, Inc.

Concern over possible adverse health effects resulting from exposure to electromagnetic fields (EMF) has generated an increasing effort to determine how fields interact with biological systems. Results from cell culture studies have documented alterations in cell metabolism after exposures to extremely low frequency fields (1). Such data make it clear that EM fields interact with cells and affect their metabolism, but, neither the mechanisms of the interaction nor the long term biological consequences of such responses are understood. Many of the reported EMF effects have been obtained with applied time varying magnetic fields as low as 1 μ T with associated induced electric fields below 1 μ V/cm. The magnitudes of such fields are well below the random thermal noise fields generated by the thermal motion of ions in and about the cell (2)(3). It is, thus, a mystery as to how cells can detect, and respond to them.

Because an important characteristic of thermal noise fields is their incoherence, we have explored the possibility that the cell's signal transduction mechanism might demand a certain degree of coherence in the applied fields before it would respond to them. In this way the thermal field would be ignored by the cell. We have explored this concept experimentally by asking whether, during exposure, a time varying EMF must maintain coherency over some

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minimum interval to elicit a cellular response. The coherence time is loosely defined as the time interval over which we can reasonably predict the frequency, phase, and amplitude of the field. The biological endpoint selected for this purpose was the EMF-induced enhancement of specific activity for the enzyme ornithine decarboxylase (ODC) in murine L929 fibroblasts. The effect of the signal coherence time was examined for 60 Hz magnetic fields.

METHODS

Logarithmically growing cultures of murine L929 cells, maintained in Eagle's minimum essential medium with 5% fetal bovine serum, were plated 24 hr prior to magnetic field exposure. To avoid serum stimulation of ODC activity, the culture medium was not changed before experiments were begun. ELF exposures were conducted using incubator-housed Helmholtz coils to produce sinusoidal, 60 Hz horizontal magnetic fields of 1 to 100 μ T. Four 25 cm² flasks of cells were used for each exposure and to serve as controls four identical flasks were placed in an incubator chamber adjacent to that housing the Helmholtz coils. At the end of exposure cells were harvested by gentle scraping, washed with phosphate buffered saline and stored as frozen pellets. Ornithine decarboxylase activities were assayed by the procedure of Seely and Pegg (4) modified by addition of 0.2% Nonidet P-40, 50 μ g/ml leupeptin, and 50 μ M pyridoxal-5-phosphate to the cell lysis buffer. Results of each set of experiments are expressed as the mean ratio of the enzyme activities of exposed cultures to those of the corresponding controls (\pm SEM).

A computer program which interfaced with a function generator was used to determine the ELF frequency and also the time interval for which a given frequency was maintained. At user-selected intervals (henceforth termed coherence times, or τ_{coh}) the frequency of the ELF field signal was alternately shifted from 55 Hz to 65 Hz (see Figure 1). Coherence times of the exogenous fields were varied from 0.1 to 50 s. The phase of successive intervals was randomized by inserting a small uncertainty in τ_{coh} . Thus the time between frequency shifts was actually $\tau_{\text{coh}} - \delta t$ where $\delta t \ll \tau_{\text{coh}}$ and is a random time which varied between 0 and 0.05 s.

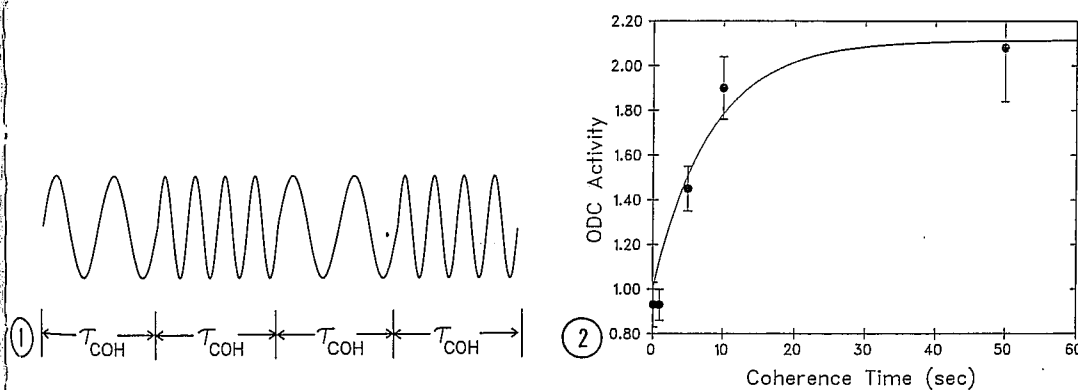


Figure 1. A plot demonstrating the partially coherent waveform created by shifting frequencies from 55- to 65- Hz at intervals of time, $\tau_{\text{coh}} \pm \delta\tau$, where $\delta\tau$ is a random number ($\ll \tau_{\text{coh}}$) varying between 0 and .05 s.

Figure 2. Plot of the enhancement of ODC activity (exposed/control) as a function of the coherence time, τ_{coh} , of the applied field. The solid line is the best fit to the mathematical function given by Eq. 1 where τ_{cell} is found to be 8.2 s. The experimental points shown represent a minimum of six different exposures.

RESULTS AND DISCUSSION

Cultures were subjected to a series of exposures to 60 Hz magnetic fields of 1, 10 or 100 μ T, for times ranging from 1 to 8 hr. The enhancement of ODC activity was measured in terms of the ratio of exposed/control activity. Maximal enhancement of ODC activity (2.04 ± 0.21) was produced by 4 hr exposure to a magnetic field of 10 μ T. The associated induced electric field was approximately .04 μ V/cm. Comparable enhancements of ODC activity (1.79 ± 0.20 , 2.10 ± 0.35) were obtained with frequencies of either 55 or 65 Hz. Using 4 hour exposures, 10 μ T fields, and frequencies shifting alternately between 55- and 65-Hz, we varied the coherence times from 0.1 to 50.0 s.

The results are plotted in Figure 2. They show that application of fields for four hours but with coherence times of 10 or 50 s did produce enhancements in ODC activities. The amount of enhancement was (within experimental accuracy) the same as that observed after exposures which were coherent for the full four hours of exposure. In contrast, for coherence times of 0.1 or 1.0 s no enhancement of ODC activity was observed. A 5 s coherence time produced a level of enhancement (1.54 ± 0.06) that was intermediate between control values and those obtained with τ_{coh} of 10 s or longer.

The ratio of exposed/control ODC activity, [ODC], plotted in Figure 2 was fit to the function,

$$[ODC] = 1 + 1.26(1 - e^{-\frac{\tau_{coh}}{\tau_{cell}}}) \quad (1)$$

with best fit value of $\tau_{cell} = 8.2 \pm 3$ s. Thus there appears to be some fundamental time constant, τ_{cell} associated with the cell signal transduction mechanism. For the cell to respond to an ELF signal it is necessary for the exogenous field to maintain coherence for a minimum time interval greater than about several seconds, with full response requiring an interval greater than about 10.0 s. Some sort of signal averaging thus appears to function in producing field-induced enhancement of ODC activity by L929 cells.

We now consider whether this coherence phenomenon will be sufficient to account for the ability of cells to discriminate against the thermal noise caused by thermal fluctuations in the position of nearby ions. To determine the thermal electrical field noise we use the Johnson-Nyquist expression where the time average noise voltage V_{KT} and electric field E_{KT} are expressed as,

$$\overline{V_{KT}^2} = \frac{4\rho kT\Delta\nu}{d} ; \text{ where } E_{KT} = \frac{\sqrt{\overline{V_{KT}^2}}}{d} \quad (2)$$

In these expressions ρ is the resistivity of tissue, $\Delta\nu$ is the band width of the cell signal transduction mechanism, d is the diameter of the cell, k is the Boltzmann constant, and T is the

absolute temperature. Following Adair () we use these expressions assuming that $d = 20 \mu\text{m}$, $\rho = 2 \Omega\cdot\text{m}$, and $\Delta\nu = 100 \text{ Hz}$. This predicts that $E_{kT} \approx .02 \text{ V/m}$. Thus we find that the thermal noise field is about 5,000 times larger than the magnitude of the 60 Hz electric fields induced in this experiment.

How does the requirement of a minimum value of τ_{coh} affect the signal-to-noise calculation? Weaver and Astumian (2) have suggested that if signal averaging is present the minimum detectable field would be given by the expression,

$$E_{\text{min}} = \frac{E_{kT}}{\sqrt{\nu \tau_{\text{avg}}}} \quad (3)$$

where ν is the frequency of the applied signal and τ_{avg} is the time over which the cell averages the signal. If we assume that $\tau_{\text{avg}} \approx \tau_{\text{cell}}$ the minimum detectable field is still over 100 times larger than the applied fields used in this experiment. In fact, to obtain an improvement in signal-to-noise of 10,000, the averaging process would have to last for about 10^6 seconds (i.e. > 100 hours) which is clearly an unreasonable averaging requirement.

Even though our results do not explain the signal-to-noise puzzle, the necessity for a minimum coherence time will have to be accounted for in any model proposed for the mechanism by which cells detect an applied EM field. In addition to EMF frequency and time of exposure, coherence time must be considered an important factor in determining the cellular response.

ODC is a critical enzyme, required for DNA replication and cell proliferation, and so modification of its enhancement by an applied field is of general interest for questions of EMF exposure. We suggest, however, that the coherence phenomenon noted in these experiments is likely of more widespread consequence, and that other biological responses with demonstrated EMF sensitivity will display comparable coherence dependence. Indeed a similar effect has been observed in studies of EM induced abnormalities in chick embryos (5).

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Attachment 12

Protein and DNA Reactions Stimulated by Electromagnetic Fields

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The stimulation of protein and DNA by electromagnetic fields (EMF) has been problematic because the fields do not appear to have sufficient energy to directly affect such large molecules. Studies with electric and magnetic fields in the extremely low-frequency range have shown that weak fields can cause charge movement. It has also been known for some time that redistribution of charges in large molecules can trigger conformational changes that are driven by large hydration energies. This review considers examples of direct effects of electric and magnetic fields on charge transfer, and structural changes driven by such changes. Conformational changes that arise from alterations in charge distribution play a key role in membrane transport proteins, including ion channels, and probably account for DNA stimulation to initiate protein synthesis. It appears likely that weak EMF can control and amplify biological processes through their effects on charge distribution.

Keywords Electric fields; Magnetic fields; Charge transfer; Protein; Hydration energy; DNA.

The Problem

Mark Twain once defined common sense as the sense that tells you the earth is flat. For most people, that line generally evokes a guilty smile. We know the earth is not flat even as our senses deceive us into believing that it is. In the study of biological effects of electromagnetic fields (EMF), we know that we do not usually perceive effects of these fields. However, we also know that biochemical and physiological measurements show profound effects of EMF on living cells. As scientists, we try to let science guide our common sense.

To put EMF in perspective, we know that of the four fundamental physical interaction forces, EM forces are those that mainly affect living systems. One would expect that biological responses to EM forces evolved over time in optimizing the ability of cells to survive. However, it appears that biological systems are unusually sensitive to EMF in frequency ranges that are unlikely to have been experienced by

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living systems before the advent of modern technology. Obviously, EMF must affect the same systems and reactions as were affected by other factors that played a role in the adaptation of living systems.

One of the other factors is easy to pinpoint, an ability to influence molecular interactions with water. Water is an essential component of living systems, so much so, that the search for life beyond Earth is essentially a search for the water needed to sustain it. Water has many unusual properties, among which is an ability to interact with and dissolve ions and many biopolymers. Because water hydrates molecules and forms solutions, chemical forces play a major role in biological systems. Of course, hydration forces are ultimately electromagnetic, e.g., water dipoles interacting with ions and the charged groups on proteins, but their effects are easier to describe in chemical terms and using thermodynamic properties. Natural biopolymers such as proteins and nucleic acids in solution are hydrated, and changes in charge distribution can lead to changes in molecular conformation. Such structural changes are generally accompanied by changes in hydration and very large changes in heat and entropy.

EMF interact with molecules to cause changes in charge distribution, but when considering biological mechanisms, we must also focus on the cell as the functional unit and on the ultra-thin (~ 10 nm) cell membrane that surrounds the cell and controls traffic in and out of the cell. The cell is sustained by biochemical reactions, many of which involve electron transfer, while cell functions are generally carried out by membrane components and involve ions. In this review, we shall consider electron and ion transport processes in solution and across membranes. We shall also discuss the effects of EMF on two major classes of biopolymers, proteins involved in transport across membranes, and the DNA in the cell nucleus that can be stimulated to initiate protein synthesis. Charge transfer due to EMF is a likely triggering mechanism in both biopolymers. The overall effect occurs in a two-step process, in which EMF move charges within the biopolymers, and the perturbations cause the biopolymers to change their conformation to accommodate the changes in charge distribution. Many of the biological examples discussed, e.g., the multi-subunit proteins, hemoglobin and Na,K-ATPase, and the DNA that initiates stress protein synthesis, are from studies carried out in this laboratory. Recent reviews describe EMF mechanisms in Na,K-ATPase (Blank, 2005) and in DNA (Blank and Goodman, 2007).

Electron Transfer in Chemical Reactions

Electric and magnetic fields exert a force on static and moving charges, and accelerate them. The largest effects of the fields are on electrons, the unit negative charges, because of their high charge to mass ratio. At the sub-atomic level, the Born-Oppenheimer Approximation assumes that electrons respond instantaneously compared to protons and heavier atomic nuclei. Even weak EMF in the low-frequency range can affect the rates of electron transfer reactions between molecules. A $10\text{ }\mu\text{T}$ magnetic field exerts a very small force of only $\sim 10^{-20}$ newtons on a unit charge, but this force can move an isolated electron more than a bond length, ~ 1 nm, in ~ 1 nanosecond.

Effects on electrons in chemical reactions were detected indirectly in studies of the effects of electric and magnetic fields on the Na,K-ATPase (Blank, 2005). Each field, studied separately, accelerated the reaction when the enzyme was relatively

inactive. By assuming that the same charge was affected in the two fields, one could estimate the velocity (v) and determine the nature of the charge (q) that was critical in the action of this enzyme. If both fields exerted the same force at the threshold, we can equate the electric (E) and the magnetic (B) forces:

$$F = qE = qvB. \quad (1)$$

From this $v = E/B$, the ratio of the threshold fields, and by substituting the measured thresholds (Blank and Soo, 1992, 1996), $E = 5 \times 10^{-4}$ volts/m and $B = 5 \times 10^{-7}$ T ($0.5 \mu\text{T}$), we obtain $v = 10^3$ m/s. This very rapid velocity, similar to that of electrons in DNA (Wan et al., 1999), indicated that electrons were probably involved in the ATP splitting reaction and the ion transport mechanism of the Na,K-ATPase (Blank, 2005). An electron moving at a velocity of 10^3 m/s crosses the enzyme ($\sim 10^{-8}$ m) before the 60 Hz field has had a chance to change. This means that even though a low-frequency sine wave signal was used, the effective stimulus was actually a repeated DC pulse. This is true in all low-frequency studies that involve effects on fast moving electrons.

The magnitudes of the threshold fields that affect the Na,K-ATPase are in the very low range of mV/m electric field and μT magnetic field. The very small force of $\sim 10^{-20}$ newtons on an electron and the very small dimensions and short times, calculated above, are relevant at the molecular level for the proteins and DNA that we consider in later sections. The small magnitudes also suggest boundary conditions on the responses that can be expected from weak fields. In essence, they question the possibility of direct effects of such weak fields on much more massive ions and molecules. There just is not sufficient EMF energy to cause significant movement of ions, especially if they are hydrated. Ions are affected by the much larger DC electric fields involved in physiological membrane processes, a subject treated below.

In the search for weak fields that can cause biological effects, we realized that weak DC magnetic fields are also unlikely to affect physiological processes for the same reasons. The ability of DC magnetic fields to affect lifetimes of free radical pairs (Steiner and Ulrich, 1989) only occurs at field strengths that are several orders of magnitude higher than the AC magnetic field thresholds mentioned earlier and other studies to be discussed. This review is focused on the effects of the low levels of EMF, comparable to those in the environment, that are apt to influence biological processes, so the effects of DC magnetic fields will not be considered.

Electrons are not usually invoked in the mechanism of the Na,K-ATPase, so it was necessary to demonstrate the effects of magnetic fields on electrons in known electron transfer reactions. This was done by studying electron transfer from cytochrome C to cytochrome oxidase (Blank and Soo, 1998) and in the oxidation of malonic acid (Blank and Soo, 2003), also known as the Belousov-Zhabotinsky (BZ) reaction. In both of these reactions, as well as in the Na,K-ATPase reaction, the following was true:

- Magnetic fields accelerated the rate of the reaction at very low thresholds. The experimentally determined threshold values were Na,K-ATPase ($0.2\text{--}0.3 \mu\text{T}$), cytochrome oxidase ($0.5\text{--}0.6 \mu\text{T}$), BZ reaction ($<0.5 \mu\text{T}$).
- In all three cases, magnetic fields were most effective when the intrinsic chemical forces were low, showing that EMF competes with the intrinsic chemical forces driving the reactions. To emphasize the fact that EMF will affect a reaction only

when the intrinsic chemical forces are weak, a recent study reported no effect of magnetic fields on the BZ reaction (Sontag, 2006) under conditions where the chemical forces swamped the magnetic forces. The magnetic fields were only applied well after the reaction was under way and the chemical forces had already set the oscillatory pattern of the reaction.

It was interesting that the two enzymes studied showed frequency optima close to the reaction turnover numbers, Na,K-ATPase, 60 Hz; cytochrome oxidase, 800 Hz, suggesting that the EMF were interacting optimally when in synchrony with the molecular kinetics (Blank and Soo, 2001). As we shall see in a later section, this is not true for magnetic field interactions with DNA, which are stimulated in both the power frequency and radio frequency ranges (Blank, 2007). EMF interactions with DNA do not appear to involve electron transfer reactions with well-defined kinetics. There are no other frequency data on enzymes to add to this list; studies on the enzyme ornithine decarboxylase (Byus et al., 1987) were done at 60 Hz only. While there are very few examples from which to generalize, it is reasonable to expect frequency optima only where electron transfer reactions have well-defined kinetics.

There are additional frequency data for DNA that should be mentioned, but the experiments are quite different from the above studies and the results cannot be compared. The studies involved stimulation of DNA in striated muscle to produce specific muscle proteins by stimulating (electrical) action potentials in the attached nerves. The stimulation of DNA will be discussed in detail in a later section, but the electric fields associated with the action potentials are likely to stimulate electron movement in DNA of the muscle nuclei (Blank, 1995). The two frequencies studied in muscle, high (100 Hz) and low (10 Hz) frequency, were chosen to correspond to the frequencies of the fast muscles and slow muscles that are characterized by different contraction rates and different proteins. In the experiments, either the fast or slow muscle proteins were synthesized at the high- or low-frequency stimulation rates corresponding to the frequency of the action potentials. This clear frequency dependence on electric fields was to be expected from the muscle physiology, but it is unlikely to have come from particular electron transfer reactions as in cytochrome oxidase. It is more probable that an entire region of DNA, coding for multiple proteins, was activated simultaneously.

Many of the biochemical charge transfer reactions that occur in living cells are oxidation-reduction reactions, but by and large, they have not been the concern of biologists interested in EMF mechanisms. It is the electrochemists who study electron transfer mechanisms at electrode surfaces driven by electric fields, and who ask such questions as the number of steps in a reaction, number of electrons transferred per step, rate of each step, etc. Those concerned with biological EMF mechanisms are oriented towards cell function and focus on physical chemical processes involving membranes and ions, the topic of the following section.

Cell Membranes and Ion Transfer

The functional unit in physiological systems, the cell, is surrounded by an ultra-thin (~10 nm) cell membrane having the basic structure of a phospholipid bilayer. The bilayer serves as a matrix in which many different functional elements (e.g., enzymes, channels, transporters) are embedded in varying amounts in different tissues. In the

red cell, a relatively inactive cell, the functional elements constitute about half of the membrane (Blank et al., 1979), while in active synaptic vesicle membranes there is twice as much protein by weight as lipid. A diagram of a synaptic vesicle membrane is on the cover of the November 17, 2006 issue of *Cell*.

Cells are sustained by biochemical reactions, many of which involve electron transfer, but the charge transport processes in many cell functions (e.g., nerve, muscle conduction) primarily involve ions and the much more energetic electric fields needed to transport them. This accounts for the focus on ions and electric fields as triggers of physiological processes. The word trigger is appropriate. Electric fields transfer relatively small amounts of charge that cause changes in the membrane, which then allow the normal ion gradients to cause much larger changes in the cell. This will become clearer when we discuss the effects of electric fields on ion gradients across membranes and on ion channels in membranes.

Ion transport differs from electron transport in many ways. Ions are much more massive, have both positive and negative charges, and are stable in solution. In ion transport studies carried out in electric fields, cations and anions move in opposite directions and at different speeds because of their different sizes and degrees of hydration. These differences lead to significant *ion concentration changes* due to ion transport across ion selective membranes.

Living cells have compositions that differ markedly from the surrounding solutions, so natural membranes normally separate solutions having very different ionic compositions and concentrations. K is the main intra-cellular cation and Na is the main extra-cellular cation, so large ionic gradients exist across cell membranes (see bold faced symbols K and N in Fig. 1). Most cell membranes are cation selective, and differences in the rates of diffusion of K and Na across membranes lead to membrane potentials of about 100 mV. Ionic leaks are compensated by 'ion pumps', such as the Na,K-ATPase to be discussed in a later section, so the steady-state potentials are known as resting potentials. When nerves or muscles are activated, the changes in membrane potential are called action potentials.

Because of the differences in steady-state concentrations across the membrane, and because the permeability of the membrane to K is normally much greater than to Na, small currents due to applied electric fields can cause large changes in the ionic concentrations at the membrane surfaces. Take the examples given in Fig. 1, of an electric field across a cation selective membrane that separates a cell from its surrounding solution. Both solutions contain the cations sodium (N) and potassium (K), shown with N higher outside and K higher inside, as normally distributed in cells and with the symbols for the steady state N and K in proportion to the concentrations. (The anions are the same concentration on both sides and assumed not to cross the cation selective membrane.)

An ion current, indicated by arrows, will be carried by both ions, but in different proportions because of the steady-state ion concentrations across the membrane. In the top panel for an outward current, the major part of the current is carried by K. In the bottom panel for an inward current, the major part of the current is carried by N. The main result of a sustained DC current flow in either direction, shown in italic symbols, is a decreased cation gradient across the membrane for each cation. This means that a depolarizing current that normally stimulates a nerve and causes sodium ion flux actually decreases the concentration gradient (i.e., the chemical driving force) of the sodium ions that start the action potential. The decreased cation gradients across the membrane also decrease the membrane potential and affect

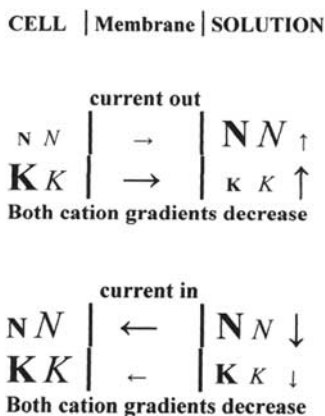


Figure 1. Changes in sodium (N) and potassium (K) concentrations at the surfaces of a cation selective membrane due to the current flow in outward and inward directions, as indicated by the arrows in the membrane. The relative sizes of the symbols N and K in the solution compartment indicate the relative concentrations in the two solutions, and the sizes of the arrows indicate the relative magnitudes of the current. The bold symbols represent the steady-state concentrations and the italic symbols show the concentrations after current flow in the two different directions. The upper diagram is for current out of the cell, when cations in the solution increase, and the lower diagram is for current into the cell, when cations in the solution decrease. Current in either direction leads to a reduction in the concentration gradients of both cations.

the distribution of charge across the membrane. As discussed in a later section, because of a direct effect on the charges as well as an indirect effect due to lowering the membrane potential, a depolarizing current opens ion channels, which are the major contributor to the increased ion fluxes. The depolarizing currents also have a direct effect through the changes in ion concentration at the membrane surfaces.

The changes in concentration at the membrane surfaces persist there, because they are dissipated slowly by diffusion into the solution. Such changes were demonstrated when the actual concentration of ions at a surface was measured by transporting surface active ions across liquid/liquid interfaces. The surface active ions carried the direct (DC) current and also indicated their presence at the interface by changes in interfacial tension (Blank and Feig, 1963). The concentration changes during current flow were significant and relatively long lived.

Intuitively, one expects that passing an alternating current (AC) through a cell might leave no net effect, because the processes during the initial half of the cycle would be canceled in the second half, when the electric field is reversed. However, it is easy to see from Fig. 1 that for cation selective cell membranes with cation gradients across them, the effects of AC on cation concentrations are additive. When considering an entire cell, the inward current directed into one side of a cell appears to be balanced by an outward current on the other side. Here again, we see from Fig. 1 that the effects on both sides of a cell are in the same direction. Cation gradients are reduced on both sides.

Because the effects on the cation concentrations are additive, even small AC electric fields lead to significant changes over time. The effects of AC currents through a simple theoretical model membrane showed that the concentrations do not increase indefinitely because of diffusion away from the surface and binding

reactions with fixed charges at the membrane surface. The effects varied with the AC frequency (Blank and Blank, 1986), depending upon the ion binding constants to fixed counter charges on the surface and ion mobilities in solution. It has been known for a long time that AC currents across nerves can reduce and block their activity. AC apparently decreases the ion gradients to the point that they can no longer drive the action potentials.

The fixed charges at a membrane surface not only can bind to the ions near the surface layer, but the change in surface charge can affect ion transport through the surface. To study the effect of the charge on a surface on the ability of ions to diffuse across the boundary, Miller and Blank (1968) used charged monolayers to show that the rate of ion transport is controlled by the charge on the surface. The effects of charged surfaces on the ability of ions to cross an interface could be explained by the expected ion concentration changes in the surface region, e.g., fixed positive charges reduce concentrations of adjacent cations and increase anion concentrations.

These studies show that ions at membrane surfaces may be important for understanding biological ion transport across membrane dimensions and in millisecond time scales. Actually, the surface concentration of ions at an axon membrane surface in the steady state is comparable to the magnitude of the ionic flux during an action potential. The number of ions stored at an axon membrane surface, having a capacitance of 10^{-6} farads/cm² and a resting potential of 100 mV, is about $\sim 10^{-12}$ ions/cm². The magnitude of the ion flows in an action potential is also about $\sim 10^{-12}$ ions/cm² of nerve axon membrane surface.

When discussing ion concentration changes at membrane surfaces and changes in polarization across membranes, it is important to realize that there is a major difference between the characteristic response times of chemical systems and electrical systems. In transient or non steady-state membrane processes, the two driving forces for ionic movement, the chemical potential for diffusion and the electrical potential for migration, change at very different rates. A membrane can be depolarized quite rapidly, with time constants on the order of 1–10 *microseconds*, while chemical potentials readjust at much slower rates with time constants of about 1 *millisecond*, characteristic of diffusion processes over distances on the order of cell diameters. It is therefore possible to generate unbalanced chemical gradients for short periods of time by manipulating membrane (electrical) potentials. The disparity in the response times of the two forces that drive ions across membranes can lead to unusual transient ionic fluxes.

Biological systems add an additional complication to the changes expected in physical systems, i.e., changes in ion concentration at surfaces due to depolarizing currents and due to the great disparity between the rates of change in concentration and electrical potential. In biological systems there are voltage-dependent ion channels that open when depolarized. This topic will be discussed in greater detail in a later section.

An analysis of the ion flows in excitable membranes, called the Surface Compartment Model (Blank, 1987), showed what happens when all of these factors occur in the layers of solution immediately adjacent to the membrane surfaces, specifically:

- the changes in ion concentration due to depolarizing currents (Fig. 1), ion flows under electrochemical forces (described by the same equations that apply to ions in solution), and any ion exchange between Na and K that occurred with fixed surface charges at the membrane surfaces due to changes in concentration;

- the disparity between the rates of change in ion concentration by diffusion and migration, and the much faster changes in electrical potential;
- the effects due to voltage-dependent ion channels that open by a charge transfer process shown in Fig. 3, to be discussed in detail later. The ion channels that had been incorporated into an empirical description of ion transport across membranes were complicated functions of time, while these were dependent on charge distribution.

The Surface Compartment Model was able to show that these factors could account for the unusual ionic fluxes seen in excitable membranes. It also showed how the apparent selectivity of channels could vary with different rates of opening. This description of ionic fluxes in excitable membranes offered insights into factors that contribute to the unusual fluxes and the apparent ion selectivity in channels.

It is obvious that the electrical activity that drives nerves and muscles utilize mechanisms that take advantage of ionic gradients that are normally present in living systems. These ionic gradients are built up by the action of membrane enzymes like the Na,K-ATPase and are fueled by the energy from the splitting of ATP. Consequently, it takes relatively little energy to trigger an action potential and take advantage of the energy stored in the ionic gradients across cell membranes. The ion fluxes that evoke an action potential are very weak stimuli by comparison. However, it does take energy to open the voltage gated ion channels and various transporters in the membrane. This source of energy, triggered by changes in charge, is the conformational energy stored in chemical structures. This is a probable explanation for the way ion channels are stimulated to open by depolarizing currents, and also for the way very weak EMF can stimulate responses in DNA, both of which require considerable energy.

Proteins and Hydration Energy–Hemoglobin Equilibria

The energetics of intermolecular interactions and interactions with water as a solvent determine membrane structure, as well as the changes that occur when perturbed by applied EMF. Among the early attempts to understand the energetics of chemical structures and their relation to chemical properties, Langmuir (1916) showed that the surface tension of a pure liquid could be derived from information about the interaction energy between molecules. Vaporizing a liquid breaks all bonds between molecules, while molecules at a liquid surface are not completely surrounded and miss interactions with the missing neighbors. It is the missing interactions that give rise to the surface tension. The unbalanced energy at a surface requires molecules to have extra energy to get to the surface, and that the liquid minimizes the energy and the surface area. Langmuir's success in relating surface tension to heat of vaporization indicated that nearest neighbor interactions account for most of the energy, and that the change in surface free energy (i.e., the surface tension) is a good approximation to the total free energy change.

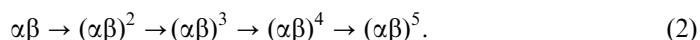
The situation in aqueous solutions is more complex, but we have estimated the total free energy change of a molecule in solution from the changes in surface area when interacting with water. In aqueous solutions, the interactions with water are quite energetic and have a profound influence on equilibria, especially those involving proteins. Lauffer (1975, 1989) characterized the aggregation of multi-subunit

protein molecules in aqueous media using the short-hand phrase “entropy driven” to summarize the energetics of the interaction. The aggregation is spontaneous (i.e., the free energy change, ΔF , is negative) and it occurs with an evolution of heat (i.e., the enthalpy change, ΔH , is positive). The negative ΔF together with the positive ΔH means that there is a large positive entropy change. Hence, “entropy driven”. The large increase in entropy is due to release of many water molecules when the hydrated proteins come into contact after releasing their bound water. The increase in ΔH is another consequence of the release of water from protein surfaces and the aggregation of the protein subunits.

This description is correct but incomplete, because aggregation is very dependent on pH while hydration is not. The pH affects molecular charge, since it is well known that proteins disaggregate as the charge increases, and they aggregate as the charge decreases. Two often quoted examples are hemoglobin (Fanelli et al., 1964) and tobacco mosaic virus protein (Klug, 1979). It is possible to extend Langmuir’s approach to include an effect of charge. The aggregation of multi-subunit proteins with a decrease in molecular charge can be formulated as a simple relation between molecular charge and the area of the protein molecule in contact with aqueous solvent. The basic idea is that proteins in aqueous media minimize their surface free energy by decreasing contact with the water *and* decreasing charge. For this reason, decreases in charge drive the protein toward aggregation. However, when there is an increase in charge, the two driving forces compromise and there is an increase disaggregation. The repulsive forces between charges would increase the surface free energy, and this can only be reduced by an increase in area. Disaggregation spreads the charges and lowers the repulsion between them.

This simple model using surface free energy to account for the influence of charge on subunit assembly was shown to apply quantitatively to the protein, hemoglobin (Hb) as a function of surface charge (Blank and Soo, 1987). The actual study, the disaggregation of the Hb tetramer $(\alpha\beta)^2$ into 2 dimers $(\alpha\beta)$, where α and β are protein subunits, showed that the concentration of hemoglobin dimers increased linearly with surface charge as the pH varied from the isoelectric point. As the Hb tetramers were disaggregating, the increasing charge was being spread over an increasing protein/water interface, and the surface charge density (total charge/total molecular area) remained constant.

The same surface free energy model could also account for the unusual effects of increasing concentration of Hb on the viscosity of solutions (Blank, 1984) if one assumes that the increase in viscosity with Hb concentration is due to aggregation into larger particles. The same forces that drive the aggregation of dimers to tetramers should continue because of the same loss of area upon aggregation:



At the point where the chain becomes long enough to close upon itself, there should be a steep change in the equilibrium. The closing of the chain means that an added $\alpha\beta$ has caused two links, with double the loss of interfacial area and double the free energy change. A closed chain would also account for the steep increase in viscosity, since a chain where the ends are joined is no longer as flexible and behaves more like a rigid rod.

The relation between changes in interfacial area and the free energy change enabled a semi-quantitative estimate of the energy change due to the changes in molecular shape when Hb is oxygenated. The conformational changes in Hb, documented by X-ray crystallography, enabled estimation of the interfacial area. The charge on the Hb at different pH's could be determined from titration studies, such as those in the study of disaggregation. The data enabled calculation of the acid and alkaline Bohr effects, the names given to the variation of the oxygenation equilibrium constant with pH and ionic strength (Blank, 1975).

The success of the surface free energy model in calculating the acid and alkaline Bohr effects demonstrated the predictive value of the relation between changes in surface free energy and the equilibrium constant. This idea also led to understanding the physical meaning of the empirical Hill coefficient that is widely used as a measure of cooperativity. By using the surface free energy model to estimate conformational changes (e.g., Hb), it was possible to show that the changes in free energy that affect the equilibrium constant are simply related to the Gibbs surface excess, a fundamental property in surface chemistry (Blank, 1989). According to the surface free energy model, the Hill coefficient is not empirical and is not constant. It varies with the degree of reaction, has a maximum value at the half way point, and is definitely equal to unity at both extremes. The approach to unity has been observed in the reaction between Hb and oxygen (Paul and Roughton, 1951).

The surface free energy model is a way to estimate the energy changes due to the hydration of nascent hydrophilic surfaces of biopolymers, such as proteins and nucleic acids, in terms of the surface free energies of newly formed surfaces. To make calculations, one needs estimates of surface areas and surface charge, so it has been relatively easy to apply these ideas to many properties of Hb, a well-characterized molecule. The model has also related the conformational changes of voltage-gated channel proteins (Blank, 1987, 1989) to the depolarizing currents that transfer charge across a channel, and the conformational changes of the Na,K-ATPase (Blank, 2005) and other membrane transporters to the charge movement when ATP splits. The same effects of EMF on charge movement may account for the ability of EMF to cause DNA to initiate protein synthesis (Blank and Goodman, 2007). These are examples of biological amplification that are related through the ability of small charge movements to stimulate large structural changes utilizing the energy stored in biopolymer conformation. The following three sections are devoted to Na,K-ATPase, ion channel proteins, and DNA.

Membrane Transport Proteins—Na,K-ATPase

Many of the biological transport systems embedded in membranes are multi-subunit proteins that can open to both sides of a membrane in alternate conformations. This process enables the binding of substances to one side of the protein and subsequent release to the other side after a conformation change. The opening of a transporter creates new protein water interfaces and involves changes in binding of the subunits with each other, the water and the bilayer lipids. Similar reactions occur when the protein opens on the other side. If the two open states of the protein on opposite sides of the membrane were of approximately the same energy, it would minimize the energy required for the transport. In transport, the conformation change is usually triggered by the energy released from the ATP splitting reaction.

This type of transport mechanism has been documented for many different substances. A short list of recent articles includes studies on β -galactosidase and glucose-6-phosphate (Locher et al., 2003), various drugs (Dong et al., 2005; Yin et al., 2006; Reyes and Chang, 2005), zinc (Lu and Fu, 2007), metal chelates (Pickett et al., 2007), and vitamin B12 (Hvorup et al., 2007). The mechanism is best known from its association with the Na,K-ATPase, the enzyme that “pumps” Na and K ions against their gradients across cell membranes.

The Na,K-ATPase is probably the best studied of this class of transporters, known as ABC (ATP Binding Cassette) transporters, and as such it offers insights into how ATP driven conformation changes can occur in bilayer structures. The lipid bilayer membrane is stable because of hydration forces, and the term hydrophobic interactions used to describe these forces indicates that the lipid molecules interact with each other and avoid contact with water molecules. Exposing bilayer lipid molecules to water is energetically unfavorable, so membrane transport mechanisms utilize multi-subunit proteins in the bilayer that have hydrophobic areas that can interact with lipid molecules in the bilayer and hydrophilic areas that can interact with water at the surfaces. Because of their compositions, transporters can flip their conformations from inner-face-open-to-water to outer-face-open-to-water to enable the transfer of molecules by expanding the hydrophilic areas and contracting the hydrophobic and vice versa. In the Na,K-ATPase the different conformations are determined by the binding of Na, K and ATP.

The Na,K-ATPase is composed of two polypeptide chains (α and β) that extend through the bilayer in the form of a tetramer ($\alpha_2\beta_2$). The ATPase activity resides in the α chain and is directly influenced by the ion concentrations in contact with the two sides of the enzyme (Skou, 1957; Tonomura, 1986; Lauger, 1991; Jorgensen et al., 2003). The Na,K-ATPase is activated when sodium ions bind on the inside surface and potassium ions on the outside surface. In a complete cycle, the catalytic unit splits ATP on the inside surface, and for each ATP molecule split, 3 Na⁺ ions move from inside out and 2 K⁺ ions from outside in.

The enzyme complex has two conformations, E₁ when Na⁺ ions (and ATP) are bound on the inside, and E₂ when K⁺ ions are bound on the outside. The ion binding sites are not fully accessible to ion exchange with the surrounding solutions in the two conformations (Rephaeli et al., 1986; Glynn and Karlish, 1990). Potential sensitive dyes show charge shifts at specific points in the ATP-splitting cycle (Buhler et al., 1991). A release of Na⁺ ions accompanied a rapid movement of charge when binding sites open to the outer surface in the presence of Na⁺ ions (Hilgemann, 1994). These data suggest that conformational changes of the Na,K-ATPase and charge shifts within the protein are involved in the mechanism. The effects of applied low frequency electric and magnetic fields on Na,K-ATPase function, presented earlier, provide additional evidence of rapid charge movement that contributes to the conformation change after the enzyme has reacted.

The key to the conformation change is the rapid shift of charge across the enzyme. Figure 2 illustrates changes in a protein channel that starts with an asymmetric charge distribution. The outside surface is normally negatively charged, and the charged groups interact with water. This expanded area of contact with water is open to the outside. A significant shift in charge causes the channel to shift from an inside facing channel to an outside facing channel. If the charges crossing the enzyme are electrons, they cross very rapidly to the opposite side of the enzyme, and the ratio of charged hydrated area and uncharged unhydrated area remains virtually unchanged. With

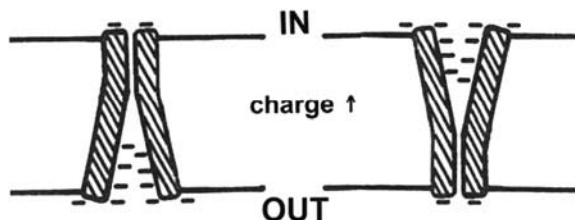


Figure 2. Changes in a protein channel that starts with an asymmetric charge distribution, and there is a large and rapid shift in charge as indicated by the arrow. The outside surface is initially negatively charged, and an expanded area of contact with water faces the outside. A large shift in charge causes the channel to change from an inside facing channel to an outside facing channel. If the charges crossing the enzyme are electrons, they cross very rapidly to the opposite side of the enzyme, and the ratio of charged hydrated area and uncharged unhydrated area remain virtually unchanged and with virtually no net change in energy.

virtually no net exothermic aggregation or endothermic disaggregation, the conformation change probably occurs with a minimum of energy change.

It is not generally accepted that ATP splitting and the accompanying ion transport involve electron transfer. However, it is quite clear from EMF measurements discussed earlier that there is a rapid flow of charge through the enzyme, resulting from the enzyme reaction. This flow of charge could trigger the sequence of conformation changes that are part of the cation transport mechanism (Blank, 2005). The effective concentrations of non-specific cation inhibitors of the Na,K-ATPase were related to the redox potentials (Britten and Blank, 1973), suggesting involvement of an electron transfer step. Many observations associate electrons with the ATPase reaction. In mitochondrial function, the ATP synthase catalyzes the same reaction and is directly coupled with electron transport. In the ATP synthase, it is possible to stop the flow of electrons in the electron transport chain with inhibitors, or to reverse the flow of electrons by changing the concentration of substrates. The electron transport chain can also be made to go in reverse when ATP is hydrolyzed and electrons are fed into the chain.

In line with the known reversibility of ATPases in mitochondria, Garrahan and Glynn (1967) were able to reverse the Na,K-ATPase reaction in red cells to generate ATP. They did this by creating a supernormal K ion gradient, thus hyperpolarizing the membrane. When the membrane potential changed from -15 mV to -85 mV, they were able to approximately double the ATP concentration from ADP and phosphate normally present. The increase in membrane potential makes the region near the catalytic portion of the Na,K-ATPase on the inner surface of the membrane more negative. The increase in H ion concentration near the enzyme would be expected to drive the reaction toward making more ATP. In any case, the experiment clearly shows the tight coupling between the ATPase reaction and ion flow across the membrane. It also shows their similar reversibility to charge flow, the Na,K-ATPase to ion flow and the mitochondrial ATP synthase to electron flow.

Charge Transfer and Ion Channel Function

In the section on ion transfer, the transient ion flows in excitable membranes were described in terms of concentration changes in the layers of solution immediately

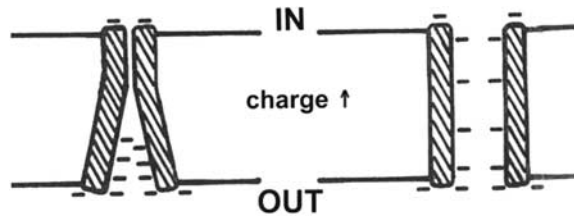


Figure 3. Changes in a protein channel that starts with an asymmetric charge distribution, and a large portion of the charge shifts rapidly, as indicated by the arrow, to spread across the length of the protein in the bilayer. If the charges crossing the enzyme are electrons, they can spread out very rapidly. The shift in charge is sufficient to open a hydrophilic channel and create a conduit for ions from inside to outside solutions. This implies that the charged parts of the protein that interact strongly with water create a continuous aqueous path. Because there is a change in the ratio of charged hydrated area and uncharged unhydrated area, this process must result in a significant change in energy. The distribution of charge depends on the membrane polarization, and if the charge movement is reversed by repolarization, the channel closes.

adjacent to the membrane surfaces. These thin regions were referred to as surface compartments and the equations describing the processes as the surface compartment model (Blank, 1987). The main processes were variations in Na and K ion concentrations due to depolarizing currents, ion exchange between ions in solution and those bound to fixed surface charges at the membrane surfaces, and the very different rates of ion concentration changes by diffusion and changes in electrical potential. Voltage-dependent ion channels that open and close depending on changes in charge distribution were included in the description, but a fuller discussion was deferred until after the section on transport mechanisms in the lipid bilayer.

The discussion of voltage dependent ion channels is easier to understand following the section on multi-subunit protein transporters that flip from inner-face-open-to-water to outer-face-open-to-water. Proteins like the Na,K-ATPase can apparently negotiate these changes with a minimum change in hydration energy by keeping the ratio of hydrated and unhydrated protein surfaces relatively constant during the charge transfer. However, this does not appear to be possible with the opening of an ion channel, where the whole length of a hydrophilic pathway through the bilayer must be open to enable the continuous flow of ions. Figure 3 shows a protein channel that starts with an asymmetric charge distribution, and where a large portion of the charge spreads across the length of the protein in the bilayer. If the charges are electrons, they can spread very rapidly. The change in the ratio of charged hydrated area and uncharged unhydrated area must result in a significant change in energy, and the energy change must be reversed when channel returns to its resting state, i.e., closes.

The surface free energy model can relate the opening of voltage gated channel proteins (Blank, 1987, 1989) to charge transfer due to the depolarizing currents, and it also provides a way to evaluate the energy changes that occur. The process shown in Fig. 3 assumes that the gating currents in excitable membranes transfer charge across the protein, and this changes the energetics of the channel protein to favor opening a channel. Since disaggregation is endothermic and aggregation exothermic, the model predicts an initial cooling as protein contacts water on channel opening, followed by heating on channel closing. The thermal changes should be quite large because of the

nature of hydration interactions and the protein surface areas involved. As described below, thermal changes occur, but not quite as predicted by Fig. 3.

Thermal measurements are generally difficult, especially when the changes are rapid and the systems small, as with nerves. It is always difficult to get an accurate measurement of temperature change when the action potentials in nerves are faster than the response time of the thermal sensors. Also, action potentials involve the opening and closing of two sets of channels at different rates. There are Na channels that enable the initial rapid depolarization, and K channels that account for the slower repolarization phase but that may open at the same time. The effect of an overlap of opening and closing on the temperature sensor further complicates the analysis. To add to the difficulties, even the easiest nerves to study contain many axons that conduct action potentials at different rates, so there is some interference because of slow and fast conducting axons. Analyzing these data is an unenviable challenge.

Despite the difficulties, thermal measurements have been made and analyzed, and there is agreement about the observations. In excitable membranes, the heat associated with excitation of nerve (Howarth et al., 1968) or electric organ (Keynes and Aubert, 1964) shows three distinct phases during an action potential. There is an initial, short-lived warming phase followed by a longer cooling phase of comparable amplitude and a still longer warming phase having the largest amplitude and most probably associated with recovery mechanisms. The net heat evolved is actually small in comparison with the initial heating and cooling, suggesting that the net heat is a measure of the dissipation due to the flow of ions down electrochemical gradients, and the chemical bond energy used to restore the ionic gradients.

It is difficult to interpret the measurements in terms of channel protein interactions, because there are multiple sources of thermal changes. These include current flow during the action potential, discharging and recharging the membrane capacitor, ion pumping during recovery, etc. The major changes of heat appear to be due to reversible processes, and the discharging and recharging of the membrane capacitor can account for about half of the reversible heat change observed. The changes in hydration energy during channel opening and closing are another source that may account for the reversible changes. It would be hard to find another source for the large negative heat, which is a major unexplained aspect of the process.

We can estimate the energy changes from channel opening and closing, assuming that the number of sodium channels per unit area of membrane is the same as in unmyelinated C fibers of rabbit vagus nerve (Howarth et al., 1968) of 110 nmol/kg wet weight. C fiber diameters range from 0.4–1.2 μm , so assuming an average diameter and a density of Na channels comparable to the squid axon (Levinson and Meves, 1975), it is possible to estimate the measured heats per gram from the estimated positive heat of 25 $\mu\text{cal/g}$ and the negative heat of 22 $\mu\text{cal/g}$. If all of the ΔH were due to the reactions of the proteins in the channels, the negative heat is a better measure of the largely reversible ΔH for channel opening and closing. In that case, the reversible channel process involves a ΔH of about 6 kcal/mole of channel protein (molecular weight 270 kD), or about .02 cal/g of channel. The ΔH for the aggregation of tobacco mosaic virus protein (Klug, 1979) is about 0.7 cal/g. This implies that only about 3% of the protein surface is involved in the reactions affecting channel opening and closing. Since the discharging and recharging of the capacitor can account for about half of the reversible heat change observed, only $\sim 1\%$ of the protein surface can account for the unexplained heat.

The measured heats appear of reasonable magnitude, but the sequence is at odds with what would be expected if the simple model depicted in Fig. 3 were the only source of heat exchanged. One expects the positive ΔH for channel closing to coincide with the falling phase of the action potential, and channel opening should be associated with a negative ΔH or heat absorption. The channel is certainly more complicated than the model in Fig. 3, and so are the thermal changes. The heat evolved during the discharging of the membrane capacitor is simultaneous with the heat absorbed during channel opening. The two are also opposed during repolarization and channel closing. Furthermore, the discharging is much faster than the recharging. Undoubtedly, the thermal measurements are missing a large part of the heat exchanged, and the heat changes associated with channel opening and closing are therefore much greater than we have estimated and involve a much larger fraction of the protein surface.

In the absence of an all-inclusive and accurate analysis of all the thermal contributions to the measurements, it is nevertheless clear that an action potential is accompanied by:

- a net heat evolution as one would expect in a dissipative process;
- a reversible heat due to discharging and recharging the membrane capacitor; and
- a reversible heat of channel opening and closing due to the hydration energy associated with a small part of the protein surface.

A recent article accounts for the unexplained heat changes during an action potential by suggesting the possibility of soliton propagation in the membrane lipids as the source (Jackson, 2005; Heimbürg and Jackson, 2006). The authors point out that this idea can also account for the well-known Meyer-Overton correlation between the effective concentrations of a wide range of anesthetics and their oil/water partition coefficients. The Meyer-Overton correlation is not a particularly good test, because many theories predict that correlation. In a review on anesthesia, Vandam (1966) referred to two then popular new theories of anesthesia—Pauling's clathrate formation theory and Miller's dissociation pressure of hydrates—and pointed out that any theory based on weak interactions between anesthetics and other molecules is bound to correlate with the Meyer-Overton data.

A better counter argument to the soliton proposal is probably invoking Ockham's razor rather than a detailed discussion and evaluation. Simply stated, voltage-gated ion channels are acknowledged by all to be clearly associated with the action potential, and the properties of these essential proteins may be able to account for the thermal observations without the need to turn to the properties of the matrix in which the channels are embedded. It could be that some of the optical properties ascribed to the lipids by Heimbürg and Jackson are also associated with the much larger structures that appear to be parts of channels, such as the cytoplasmic components of the K channel (Long et al., 2005). Certainly, the observed changes in the thermodynamic properties are to be expected from the protein channels.

Electromagnetic Field Stimulation of DNA

One of the earliest biological effects of EMF to be described was the ability to stimulate biosynthesis (Goodman et al., 1983; Goodman and Henderson, 1988). Since those early experiments, it has been shown that EMF in both extremely low frequency

(ELF) and radio frequency (RF) ranges stimulate protein synthesis. This means that even the weak EMF in the ELF range have made DNA come apart to initiate protein synthesis. So it is no surprise that EMF can cause dose dependent, single and double strand breaks in DNA at higher field strengths and higher frequencies (Lai and Singh, 1997; REFLEX Report, 2005; Ivancsits et al., 2005; Winker et al., 2005).

The data suggest that weak EMF produce strains in DNA that can cause the chains to separate, and if the strains are large enough, cause the chains to break. Since DNA is held together by H-bonds, and since EMF are most likely to act on electrons, EMF probably act on electrons in the H-bonds to weaken the bonds. Electrons could also be affected in the H-bonds that flicker in water at a frequency $\sim 10^{15}$ Hz, and that would be expected to do so in aqueous solutions as well (Fecko et al., 2003; McGuire and Shen, 2006). This would create many transient protons and electrons in and around the DNA solution that can be accelerated by EMF.

In research focused on the stimulation of a specific stress protein, hsp70 (Goodman and Blank, 1998; Blank and Goodman, 2002, 2004), it has been possible to identify specific DNA sequences in the promoter of this protein that are needed for the EMF response (Lin et al., 1999, 2001). This was clearly demonstrated when the EMF responsive DNA sequences were transfected into the promoter of a reporter gene, and the reporter gene responded to EMF (Lin et al., 2001). The EMF responsive DNA sequences on the promoter contain sites with bases CTCT that appear to be essential. CTCT bases have low electron affinities, so electrons would be more easily displaced. Also, the CTCT are pyrimidines, and when the H-bonds split between CTCT and the GAGA (purines) bases on the complementary chain, there is a smaller smoother area that would make it easier to disaggregate.

When electrons are displaced by EMF, it can be shown that there is a favorable energy balance in the DNA disaggregation that enables the process to proceed. Strong reactions between the newly exposed DNA surfaces and water contribute to the energetics of the process. Blank and Goodman (2007) estimated the energies associated with the changes, and showed that the aggregated and disaggregated DNA structures can have equivalent energies. A simple model of disaggregation due to an increase in charge at a local site shows that an increase in area lowers the increased charge density, and that DNA cleavage would be optimal for short segments and low initial charge. The essential CTCT sites identified on the promoter may be sites of DNA cleavage or sites from which electrons have been displaced. In DNA, the initial charge can fluctuate, since electrons in DNA are not localized and are able to move as a result of the random fluctuations in H-bonded networks. This would mean that the area of DNA exposed to water molecules also fluctuates, on a slower time scale, and that some fluctuations may produce large temporary increases in local charge density. At that point, the two DNA chains would come apart to create more surface in contact with water.

The method to estimate the energy change at the DNA site associated with the response to EMF uses the same criterion as in the disaggregation of multi-subunit proteins due to charging. In proteins, where Q is the initial charge and A the area of protein exposed to water, we found that the surface charge density, Q/A , remained constant while both Q and A increased (Blank and Soo, 1987). In DNA, Q is the initial charge due to partially ionized phosphate groups and A the initial area of a DNA segment exposed to water. We assumed the surface charge density, Q/A , remained constant while both Q and A increased. This way the tendency to minimize

the surface and to spread the charge over the maximum surface (thereby minimizing the repulsion between charges) was balanced. The separation of the DNA chains enables initiation of transcription.

If ΔA is the extra area that opens up to water when 1 charge is added to a segment having an initial charge, Q , we can set the charge density before equal to the charge density after a split

$$\frac{Q}{A} = \frac{Q + 1}{A + \Delta A}. \quad (3)$$

From this,

$$\Delta A = \frac{A}{Q} = \frac{1}{\text{charge density}}. \quad (4)$$

This means it is easier to open up a larger ΔA if one starts with a larger A , but not so large as to minimize the effect of adding one charge. Also, the fractional increase in open area will be greater as the charge density decreases. In any case, the opening must be large enough to allow access to the transcription enzymes. The optimal segment size may be the four base pair CTCT that was found to be associated with the EMF response.

The stimulation of DNA by magnetic fields is related to the physiological mechanism in striated muscle, where electric fields (not EM fields) associated with action potentials stimulate the DNA in muscle nuclei to synthesize muscle proteins *in vivo* (Blank, 1995). The effect is due to the electric field stimulus, since there is a clear relation between the muscle proteins synthesized and the frequency of the action potentials. Under normal physiological conditions, an action potential along a muscle membrane creates an electric field estimated at ~ 10 V/m (Blank and Goodman, 2004). In striated muscle, this electric field drives the currents across the DNA in nuclei that are normally adjacent to the membrane carrying the action potential, and the DNA is stimulated to synthesize different muscle proteins in response to the frequency of the action potentials. The magnitude of electric field provides a large safety margin in muscle, since fields as low as 3 mV/m stimulate HL60 cells, and the threshold electric stimulus for the Na,K-ATPase is even lower, at ~ 0.5 mV/m (Blank and Soo, 1992).

This model based on an ability to displace charges in DNA can account for observations on activation of DNA by either electric or magnetic fields. The same effects should be stimulated by a wide range of frequencies. ELF and RF frequencies have been shown to stimulate stress protein synthesis (Blank, 2007) and because of the relation to H-bond fluctuation frequencies described earlier, there is reason to believe that frequencies up to $\sim 10^{15}$ Hz would be effective (Blank and Goodman, 2007).

The Proposed Mechanism in Perspective

EMF do not have sufficient energy to directly affect large protein and DNA molecules, but even weak electric and magnetic fields can cause changes in charge distribution that trigger large structural changes in proteins. Electric and magnetic fields can move both ions and electrons, but they require very different energies

because of the different masses of the charged particles. The electric fields that normally affect ions in physiological systems are orders of magnitude stronger than the magnetic fields that affect electrons. Yet, both initial reactions cause changes in charge that couple with chemical forces and provide sufficient energy to trigger physiological processes. Much of the energy in biopolymer conformations is in the form of hydration energy, and this energy can drive many of the physiological processes stimulated by EMF. The similar effects on DNA when stimulated at high or low frequencies suggests that the biological mechanisms utilize the hydration energy stored in molecular conformations, even when strong EMF forces are available.

Biological systems tend to be energy efficient even when large energy stores are available to drive these processes. The chemical changes in biopolymers triggered by charge movements frequently involve conformational changes between structures of approximately equal energy. Also, biological systems appear to use a wide range of frequencies to drive these processes. The few biochemical reactions that show a frequency dependence (Blank and Soo, 1998b) suggest synchronization of the signal with the kinetics of the reaction. On the other hand, EMF stimulation of stress protein synthesis occurs in many cells with a wide range of frequencies (Blank, 2007).

The purpose of this review has been to develop an understanding of possible biological mechanisms of EMF based on experimental results. However, it is important that the proposals should also be considered in the context of a more general discussion in the EMF literature. In the past, a frequent criticism of experimental EMF studies describing biological changes has been the absence of a mechanism to account for the effects of weak EMF. The absence of a theoretical framework was often presented as an indication that the results were not possible. Despite the clear experimental evidence of repeatable biological effects, this point of view was made to sound plausible by the relatively large energy demands of the biological phenomena ascribed to stimulation by weak EMF. The present proposal indicates a huge energy source that can account for many biological phenomena, including those stimulated by EMF.

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Attachment 13

Original Contributions

ELECTRICAL WIRING CONFIGURATIONS AND CHILDHOOD CANCER

NANCY WERTHEIMER¹ AND ED LEEPER

Wertheimer, N. (Dept. of Preventive Medicine, U. of Colorado Medical Center, Box C-245, Denver, CO 80262), and E. Leeper. Electrical wiring configurations and childhood cancer. *Am J Epidemiol* 109:273-284, 1979.

An excess of electrical wiring configurations suggestive of high current-flow was noted in Colorado in 1976-1977 near the homes of children who developed cancer, as compared to the homes of control children. The finding was strongest for children who had spent their entire lives at the same address, and it appeared to be dose-related. It did not seem to be an artifact of neighborhood, street congestion, social class, or family structure. The reason for the correlation is uncertain; possible effects of current in the water pipes or of AC magnetic fields are suggested.

electricity; electromagnetic fields; leukemia; neoplasms

Electrical power came into use many years before environmental impact studies were common, and today our domestic power lines are taken for granted and generally assumed to be harmless. However, this assumption has never been adequately tested. Low level harmful effects could be missed, yet they might be important for the population as a whole, since electric lines are so ubiquitous. In 1976-1977 we did a field study in the greater Denver area which suggested that, in fact, the homes of children who developed cancer were found unduly often near electric lines carrying high currents.

In our modern power delivery systems, high-tension wires carrying current at

voltages up to several hundred kilovolts (kv) deliver power to distribution substations where the voltage is stepped down, resulting in proportionately higher current in the medium-voltage (usually 13 kv, wire-to-wire) primary lines. These latter radiate out from the substation to distribute power through a neighborhood. Then, at the local transformer, the voltage of the primaries is stepped down once more to produce the 240 volt current which is carried along the secondary wires to service drops which bring the power to the customer's house. The current flow will always be greatest in the wires directly issuing from the substation or the transformer. At these points the voltage has been stepped down and "transformed" into current. And it was particularly homes close to these transforming points that were over-represented among our cancer cases.

Because our findings appeared to relate to high current rather than voltage, we looked into the magnetic fields induced by current flow. Magnetic fields penetrate the human body (and buildings, etc.) readily. They are not easily shielded, but

Received for publication May 11, 1978.

Abbreviations: AC, alternating current; HCC, high-current configuration; Hz, hertz; LCC, low-current configuration.

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The authors thank the Colorado Department of Vital Statistics and Dr. John Cobb of the University of Colorado Preventive Medicine Department for their facilitation of this research.

TABLE 1
Daytime measurements of 60 Hz magnetic fields (in RMS gauss) in Colorado in 1976-1977

	75 cm above ground, under wires				75 cm above ground over buried plumbing which serves:		
	Large primaries (N = 64)*	High tension (N = 22)*	Thin primaries (N = 51)*	First spant secondaries (N = 84)*	Second spant secondaries (N = 73)*	First spant homes (N = 160)*	Second spant homes (N = 104)*
Maximum measurements	.035	.020	.008	.005	.004	.013	.008
Median measurements	.007	.0033	.0022	.0017	.0009	.0015	.0010
% >.0030 gauss	73.4%	54.5%	35.3%	20.2%	6.8%	22.5%	10.4%

* N = no. of sites studied.

† First span secondaries are those nearest the transformer; second span wires are further "down-stream" from the transformer (see text). First- and second-span homes are homes near the respective types of secondaries.

they can be cancelled by balancing the currents that produce them. Such cancellation occurs in electric wires, where the return current tends to balance the supply current. However, the cancellation is imperfect because the wires are often separated in space and, more importantly, because some of the return current does not flow through the wires at all, but returns instead through the ground, and particularly through the plumbing system to which most urban electrical systems are grounded at each house.

This results in a locally imbalanced current, both in the distribution wires and in the plumbing. That imbalanced current produces a 60 hertz (Hz) magnetic field which, though small (table 1), is nonetheless orders of magnitude larger than the 60 Hz field found in nature (about 10^{-8} gauss (1)). The ground-current flows not only in the street plumbing, but also through the pipes in the house. Current which enters the plumbing at one house can flow through several homes before it returns to the distribution wires, because the plumbing provides a continuous, low-resistance path between houses.

The ground-current produces a magnetic field within the house (localized near the plumbing) which appears to be related roughly to the types of wiring configurations nearby (see table 1). This relationship between wires and plumbing is to be expected because, other things being equal, the greatest unbalanced current tends to occur where the total current in the wires is greatest, and the unbalanced portion of the current must detour through ground paths such as the nearby earth and plumbing.

A number of household appliances and power tools also produce magnetic fields, but in comparing the fields from appliances with those from power lines, it is important to note that most appliances present approximately a magnetic dipole source, with fields falling off roughly as the inverse cube of the distance, while a

wire with unbalanced current will have a field falling off only as the inverse of the distance. For instance:

	1 cm	15 cm	1 m	3 m	30 m
Electrical drill	13 gauss	.12	.001	<.0001	<.0001
Electrical range (4 burners on high)	1 gauss	.04	.0015	<.0001	<.0001
Wire carrying 15 amperes	3 gauss	.2	.03	.01	.001

In the literature there are listings of 60 Hz magnetic fields produced by appliances which appear quite high. These should not be misinterpreted: They are apparently due to the use of measurements taken "as close as possible" to the appliance. Our measurements indicate that magnetic-field exposure to the whole body from normal use of household appliances rarely exceeds .001 to .002 gauss for any extended period, while the ambient fields in a house due to nearby distribution wires or plumbing may sometimes reach those levels, or more, for hours or days at a time. If magnetic-field exposure is responsible for our finding, it may be that, above some minimum threshold, duration of continuous exposure is more important than strength of exposure *per se*. There is some precedent for such a threshold effect in the literature on direct current (DC) magnetic fields (2).

Our field measurements showed that, on the average, those types of wires associated with cancer in our study exhibited high magnetic fields (compare tables 1 and 3). However, the readings varied considerably over time; and because our observations were all made in good weather and during work-day hours when domestic current is minimal, because current-flow had most probably altered since the time of our subjects' residency, and because it was rarely feasible to go close to the house to take a measurement, no attempt was made to take systematic measurements at our study homes. Rather this study is based on the *potential* current flow suggested by differ-

ent wiring configurations (nearness and size of wires, closeness to origin of current, etc.).

Experimental work on physiologic effects of low-level, extremely low frequency magnetic fields is limited. It has been recently reviewed (3). Among the positive reports are decreased mitosis in slime molds (4), decreased growth of seedlings (5) and chicks (6), decreased *in vitro* growth of embryonic tissue cells (7), and a number of behavioral and physiologic changes in rats (8). All these results are for fields considerably higher (.5–30 gauss) than the 60 Hz fields generally found near power lines; however, the findings reported often appear to be unrelated to dose over the range studied. Prolonged exposure to the .001–.1 gauss range most pertinent to wiring effects has not been explored experimentally.

Two studies suggest that a relatively strong AC (alternating current) field may interfere with growth of implanted tumors in animals (9, 10) except where the tumor tissue is exposed to the field *before* implantation. In this latter condition, tumor "takes" were increased (9).

To explore occupational exposure to AC magnetic fields, we analyzed data from a USPH publication on occupation by cause of death (11). All those occupational categories which seemed likely to include men frequently exposed to AC magnetic fields were grouped together and found to have, as a group, a cancer rate significantly higher than the total population. The "exposed" categories included: power station operators; stationary engineers; linemen and servicemen, telephone, telegraph and power; motor-men, street, subway and elevated railway; electricians; and welders and flame cutters. The

standard mortality ratio for cancer for these categories combined was 115, a significant increase over the ratio of 100 for all occupations ($\chi^2 = 24.5, p < .0001$). For other "natural causes" of death this same group showed a standard mortality ratio of 102 ($\chi^2 = 1.8$, not significant). While this crude analysis in itself proves nothing, it underlines the fact that the harmlessness of AC magnetic fields is still unproven.

METHODS

Our cases consisted of persons dying of cancer in Colorado before age 19 in the years 1950–1973, who also had a Colorado birth certificate. Only subjects with addresses occupied from 1946–1973 in the greater Denver area were used. Controls for these cases consisted of next Denver-area birth certificates, chosen both from the files organized by birth-month and county (*file 1 controls*), and from the alphabetical search-listings, which list all Colorado births alphabetically within several wide spans of years: 1939–1958, 1959–1969, and 1970–1974. These latter were called *file 2 controls*. If the next birth certificate was that of a sibling it was skipped.

Birth addresses were those listed on the birth certificates. "Death" addresses were obtained for both cases and controls by searching for parents in city directories

for the two years just prior to diagnosis of the case. For cases who could not be traced, the address on the death certificate was used. For controls, if the *file 1 control* could not be traced, the *file 2 control* with most similar birth date who could be traced was used. There were no significant differences in the proportion of "high-current configurations" (HCC's as defined below) shown by the *file 1 controls* used (21 per cent HCC), the *file 2 controls* used (23 per cent HCC) and the unused extra controls (25 per cent HCC), so it seems unlikely that our method of selecting controls biased our findings.

In all, 344 cases met our criteria. Thirty-nine of these were born before 1946, and 33 had a birth address which was lost because it had been demolished or was not adequately specified. Only death addresses were analyzed for these 72 cases and their respective controls. Similarly, 16 cases had no usable death address, so only birth addresses were used for these cases and their controls. Table 2 gives a summary of how many persons and how many addresses were available for cases and controls.

The procedure was simply to visit the birth and "death" addresses of each case and each control, and to draw a small map of the electrical wires and transformers in the vicinity. Primary (13 kv) wires were categorized as either "large-gauge" (built

TABLE 2

Distribution of persons and addresses available for analysis, for cases and controls, in a study of electrical wiring configurations and childhood cancer in Colorado in 1976–1977

Residential status	Cases		Controls	
	Persons*	Addresses*	Persons	Addresses
Stable	109	109	128	128
Moved, birth and death addresses available	147	294	128	256
Only birth address	16	16	16	16
Only death address	72	72	72	72
Totals	344	491	344	472

* Tables 3, 4, and 9 present data on total addresses, tables 5, 6, 7, 8, and 10 present data on total persons. Tables presenting data on persons are generally broken into total persons with an available birth address ($N = 272$) and totals with an available death address ($N = 328$).

to carry high currents) or "thin" depending on whether they were clearly larger than the secondary wires. Distances were measured from the part of the house nearest the wires to the wires, with a rollatape.

Three types of homes, because of their proximity to high-current wires, were considered to have "high-current configurations" (HCC's): 1) homes less than 40 meters from large-gauge primaries or an array of six or more thin primaries; 2) homes less than 20 meters from an array of 3–5 thin primaries or from high-tension (50–230 kv) wires; and 3) homes less than 15 meters from "first span" secondary (240 volt) wires. *First span secondaries* were defined as those secondaries which issued directly from the transformer and had not yet lost any current through a service drop occurring beyond the transformer pole. The span of secondary wires separated from any transformer by at least one intervening service drop (ignoring those drops directly attached to the transformer pole) were called *second span secondaries*. First span wires will have more current running through them than second span wires because the first span must carry current for all the drops that mark its distal end *plus* whatever current the second span requires.

All other configurations were considered "low-current configurations" (LCC's). In addition, where first span wires could be seen to be carrying current to no more than two single family homes, on the average (on both sides of the block), those wires were called *short first span* wires and, because they carried current for so few homes, they were always considered LCC's, regardless of distance. Houses situated beyond the pole at the end of a secondary line ("end poles" in tables 3 and 4) were considered the extreme example of LCC homes, because they had no distribution wires at all running past them.

Since the Denver area has been growing fast, many new primary wires have been installed to accommodate increased power demands. Many of these new installations are of a style easily distinguished from older wires. For addresses occupied before 1956 (20 years prior to our field work) we noted that only 59 per cent of the primary wires found near our study homes were of the "old fashioned" types which had been in use at the time of our subjects' occupancy. (Actually 71 per cent of the primary wires observed near pre-1956 case addresses were "old fashioned," but only 49 per cent of the wires near pre-1956 control addresses were of the older types that could have been in use in those early years.) Where the more modern wiring was observed, we could not tell whether it represented new installations or replacement wiring, but we did know that it could not have been there in its present form in the pre-1956 years. Therefore, we decided to treat all primary wires seen near homes occupied before 1956 as unreliable, and to code such homes strictly according to their more stable secondary-wire configurations.

This adjustment did not critically affect our findings. Proximity to primary wires was most strongly associated with cancer for recent addresses, and the association (as expected) was weaker in the older data. But the association was still significant when all years were considered and no adjustments made: For birth addresses, 31 per cent of the 272 cases and 22 per cent of the 272 controls had homes near (unadjusted) primaries, a difference significant beyond the .025 level by Chi-square. For death addresses the figures were 29 per cent of 328 cases and 19 per cent of 328 controls, significant beyond the .01 level.

RESULTS

General configurations. Table 3 shows how many cancer and control homes exhibited the various wiring configura-

tions. It can be seen that the most striking difference between cases and controls was found for subjects who had only one address from birth to death. This might be because, for subjects who moved, the effects of configurations at one address were diluted by effects of configurations at other addresses.

Table 4 indicates that the greater the exposure to current expected from a given wiring configuration, the greater the excess of cancer found in homes where that configuration was observed.

Type of cancer. The breakdown according to type of cancer (table 5) shows a fairly similar excess of HCC's in cancer cases for all categories but one, the death addresses of cases with "other tumors."

Such a wide association with different types of cancer is not characteristic of known carcinogens such as ionizing radiation; thus the broad association observed here suggests that the HCC-cancer relationship may not be a causal one. The most likely alternatives are that it is due to some artifact, or that it reflects some effect of HCC's on the body's general ability to resist cancer.

Onset age. As table 6 shows, the HCC-cancer relationship was observed in both young and older subjects. The fact that the relationship held for the birth as well as the death addresses of older subjects would seem to suggest that the effects of HCC exposure can be long delayed. However, a closer look at the data showed that

TABLE 3
Wiring configurations at the homes of cancer cases and controls, Colorado, 1976-1977

Type of configuration*	Stable residence:		Moved residence:			
	Case	Control	Birth address		Death address	
			Case	Control	Case	Control
Substation <150 m†	2	0	2	0	2	0
Large primaries <40 m	14	6	14	13	38	17
High tension <20 m	0	0	0	1	1	0
Thin primaries <20 m	13	10	11	4	17	11
1st span secondaries <15 m	19	10	26	11	23	20
Total HCC's	48	26	53	29	81	48
1st span secondaries >15 m	33	43	53	57	66	51
"Short" first span wires	6	11	9	4	11	19
Second span secondaries	20	33	40	40	51	66
End poles	2	15	8	14	10	16
Total LCC's	61	102	110	115	138	152
(% HCC)	(44.0)	(20.3)	(32.5)	(20.1)	(37.0)	(24.0)
	$\chi^2 = 14.4$		$\chi^2 = 5.4$		$\chi^2 = 7.6$	
	$p < .001$		$p = .02$		$p < .01$	

† All six cases within 150 m of a substation were also less than 40 m from large primaries.

* HCC = high-current configuration; LCC = low-current configuration.

TABLE 4
Cancer related to the amount of current expected from different wiring configurations, Colorado, 1976-1977

Wiring configuration	Expected current	Total addresses:		
		Case	Control	% cases
Substation	Very high	6	0	100.0
Other HCC	High	176	103	63.1
LCC except end poles	Low	289	324	47.1
End poles	Very low	20	45	30.8

TABLE 5
Wiring configurations and type of cancer, Colorado, 1976-1977

Residence	Type of wiring configuration*	Leukemia		Lymphoma		Nervous system tumors		Other	
		Case	Control	Case	Control	Case	Control	Case	Control
Birth address	HCC	52	29	10	5	22	12	17	9
	LCC (% HCC)	84 (38.2)	107 (21.3)	21 (32.3)	26 (16.1)	35 (38.6)	45 (21.1)	31 (35.4)	39 (18.7)
Death address	HCC	63	29	18	11	30	17	18	17
	LCC (% HCC)	92 (40.6)	126 (18.7)	26 (40.9)	33 (25.0)	36 (45.5)	49 (25.8)	45 (28.6)	46 (27.0)

* HCC = high-current configuration; LCC = low-current configuration.

23 (66 per cent) of the 35 older cases born at HCC's were also living at a HCC (usually the same address) within two years of their cancer onset. Only three (20 per cent) of the 15 older controls born at HCC's were living at a HCC within two years of the "death" date. Thus the HCC-cancer relationship observed in the birth addresses of older subjects can be largely attributed to a HCC residence near the time of cancer onset, and there is no need to posit a long-delayed effect of HCC's.

Urban-suburban differences. Since cancer may show a different incidence in urban and non-urban areas, it seemed important to rule out the possibility that a difference in urbanization between cases and controls was the significant variable in this study, and simply carried the HCC differences with it, spuriously. This seemed unlikely, intuitively, because the field work was done one neighborhood at a time, and on none of the 22 days of field work did the individual day's results fail to show a preponderance of HCC's in the case addresses.

A more formal survey shows that, although there was a slight excess of suburban addresses in the controls, it was not statistically significant. Furthermore, the cases showed more HCC's than the controls independently in three areas: in old Denver, in the more recently developed Denver areas (as estimated from a planning department publication (12)), and in the Denver suburbs (see table 7).

Socioeconomic class. The literature reports an excess of leukemia in families of higher socioeconomic class (13). Our data, dealing with all types of childhood cancer, show only an insignificant trend in this direction. It seemed possible that our method of choosing controls might have biased our control group against lower-class controls, since only controls who could be traced in directories were used. However, a check on the discarded controls showed that upper and lower

class controls were discarded equally often, while Class III controls were somewhat disproportionately retained. There was no significant difference in the per cent of discarded and retained controls showing a HCC and, as table 8 shows, the association between HCC's and cancer was observed within each social-class group. It therefore seems unlikely that some spurious relationship to social class explains our findings.

Family pattern. The literature reports an excess of first siblings and older mothers among children with leukemia (14). In our total sample of childhood cancer cases, a trend towards both more first siblings and older mothers was noted, but neither was statistically significant. Furthermore, the HCC-cancer relationship holds to approximately the same degree within each maternal-age

and sibling-order category tested, so we see no clue in these variables as to why the relationship between HCC's and cancer should exist.

Traffic congestion. A recent report (15) suggests that cancer may occur unduly often near heavy-traffic routes. Our data did show a mild excess of case-addresses near such routes; case-addresses were more likely than control-addresses to be found within 40 meters of streets having a daily traffic count of 5000 vehicles or more on the 1960 Department of Highways traffic map. However, once again, a significant excess of HCC's in cancer cases was found independently for addresses on heavy-traffic routes and for other addresses. (For heavy-traffic routes, 53 per cent of 74 case-addresses showed HCC's against 30 per cent of 48 control-addresses; for other locations, 35 per cent

TABLE 6
*Wiring configurations and cancer onset age, Colorado, 1976-1977**

Residence	Type of wiring configuration†	Cancer onset 0-5 years		Onset 6-18 years	
		Case	Control	Case	Control
Birth address	HCC	66	40	35	15
	LCC	103	129	68	88
	(% HCC)	(39.1)	(23.7)	(34.0)	(14.6)
Death address	HCC	68	37	61	37
	LCC	105	136	94	118
	(% HCC)	(39.3)	(21.4)	(39.4)	(23.9)

* Case-control differences are significant by Chi-square ($p < .01$) for each category in the table.

† HCC = high-current configuration; LCC = low-current configuration.

TABLE 7
*Wiring configurations in different neighborhoods of cancer cases and controls in Colorado in 1976-1977**

Residence	Type of wiring configuration†	Old Denver		Newer Denver		Suburban	
		Case	Control	Case	Control	Case	Control
Birth address	HCC	42	26	27	9	32	20
	LCC	77	91	40	44	54	82
	(% HCC)	(35.2)	(22.2)	(40.3)	(17.0)	(37.2)	(19.6)
Death address	HCC	49	24	35	19	45	31
	LCC	62	77	49	55	88	122
	(% HCC)	(44.1)	(23.8)	(41.7)	(25.7)	(33.8)	(20.3)

* Case-control differences are significant by χ^2 ($p < .05$ or better) for each category in the table.

† HCC = high-current configuration; LCC = low-current configuration.

TABLE 8

Father's occupational class at subject's birth, related to wiring configurations at birth residences of cancer cases and controls, Colorado, 1976-1977*

Type of wiring configuration†	Classes I and II		Class III		Classes IV and V	
	Case	Control	Case	Control	Case	Control
HCC	19	9	49	30	33	16
LCC	34	41	98	111	39	65
(% HCC)	(35.8)	(18.0)	(33.3)	(21.3)	(45.8)	(19.8)
	$\chi^2 = 3.2$		$\chi^2 = 4.7$		$\chi^2 = 10.8$	
	$p < .10$		$p < .05$		$p \approx .001$	

* Class categories follow the schema provided in "Mortality by Occupation Level and Cause of Death," Vital Statistics Special Reports 53, #5, 1963, and are as follows: Class I: Professional. Class II: Technical, Administrative and Managerial. Class III: Clerical, Sales, and Skilled Workers. Class IV: Semi-skilled Workers. Class V: Laborers.

† HCC = high-current configuration; LCC = low-current configuration.

TABLE 9

Increase of cancer cases within 40 meters of heavy-traffic routes, as related to the presence or absence of nearby primaries, Colorado, 1976-1977*

Type of subject	Near primary wires		Not near primaries	
	Traffic routes	Other locations	Traffic routes	Other locations
Cases	32	84	42	333
Controls	9	53	39	371
(% cases)	(78.0)	(61.3)	(51.9)	(47.3)
	$\chi^2 = 3.3$		$\chi^2 = 0.4$	
	$.05 < p < .10$		Not significant	

* "Nearby" primaries here means that the primaries were near enough to the house to qualify it as a high-current configuration (HCC).

of 417 case-addresses showed HCC's against 21 per cent of 424 control-addresses).

In fact, the excess cancer we found on heavy-traffic routes seems to be related to the frequent presence on such routes of primary wires carrying especially high currents. Table 9 shows that the excess of cancer cases on high-traffic routes occurred to a significant extent *only* where primary wires were nearby.

Sex distribution. Many cancers, including leukemia, occur more frequently in males than females. This is reflected in our data where 57 per cent of our cases were males, as compared to 49 per cent of the controls. The excess of HCC's among cases was significant for both males and females when the sexes were analyzed separately, but the trend was stronger in

the males; 51 per cent of the 197 male cases had a HCC at their birth- or death-address, or both, while 45 per cent of the 147 female cases had such an address. This compares with only 28 per cent of the 168 control males and also 28 per cent of the 176 control females.

It is interesting that significant male excess among our cancer cases appeared to be confined to two categories: 1) cases whose birth address had a lower current configuration than the death address, and 2) cases with stable address who developed cancer after at least one year of postnatal life at a residence situated near primary wires (table 10).

Because these two categories were chosen from a number of ways we might have categorized the data, they must remain suspect until a replication confirms or

TABLE 10
Sex distribution of cancer cases in a study of electrical wiring configurations and childhood cancer in Colorado in 1976-1977

Type of address	Males	Females	% male	Significance*
Birth address had lower current configuration than death address	28	14	66.7	$\chi^2 = 4.0, p < .05$
Stable residence at HCC† involving primary wires	22	4	84.6	$\chi^2 = 11.1, p < .001$
Other cases with any HCC address	56	48	53.8	$\chi^2 = 0.5$, not significant
Other cases with no HCC address	91	81	52.9	$\chi^2 = 0.5$, not significant

* An expected value of 50 per cent male was used to calculate the chi-squares.

† HCC = high-current configuration.

disputes them. However, we chose these categories for a reason: We hypothesized that males might be excessively susceptible to HCC's at all ages, including prenatally. (It is of interest here that male rats appear especially susceptible to experimental magnetic fields (8, p. 182) (16), as do embryos (17).) If males are more susceptible, they might frequently be aborted when pregnancy occurs at a HCC, but pregnancy at a LCC would allow the most susceptible males to be born and then to develop cancer later when exposed to a situation with higher current nearby. This hypothesis is consistent with the male excess in category 1 above.

Category 2 is presumed to provide a potentially similar situation: Where primary wires are found running near a house (in 1976), it is always possible that these wires were first installed or were "beefed up" at some time *after* the subject's birth. Or if they were present all along, the current they carry may sometimes have increased with time. If any of these things happens, the postnatal current flow near the house will be increased over the prenatal flow, even without a change in residence. Should this happen, the susceptible male who escaped abortion during pregnancy might develop cancer, and this would explain the male excess in category 2.

Substations. Power carried at higher voltage is stepped down to produce increased current at two points in our electrical distribution system: at the distribution substation, and again at the neighborhood transformers. As indicated, cancer cases were found in excess close to the "first span" wires issuing from the transformers. An even stronger trend was found for substations.

None of the 702 control addresses visited (including our unused extra controls) was within 150 meters of a substation. This is to be expected since probably less than one home in 1000 in the Denver area is that near a substation. What is surprising is that six of 491 case addresses were found within 150 meters of a substation and, in each case, less than 40 meters from the large primary wires issuing from that substation. These six are shown in table 3. Each cancer case had lived at the substation address within three years or less of his illness. Although these numbers are small, they are striking.

Blind studies. It should be noted that our Denver-area study, being exploratory, was not done blind. This could lead to error, although our observations were reasonably unambiguous. To check just how reliable our coding was, an assistant observed and coded 70 case and 70 control addresses randomly chosen from those previously coded by the principal inves-

tigator. The assistant did not know the case-control status of the addresses she coded. Her coding agreed with ours in 128 (91 per cent) of the 140 instances. In five of the 12 instances of disagreement, the assistant's judgment favored the hypothesis of a HCC-cancer correlation, while ours did not. In seven instances, the reverse was true.

Also, a blind study was done (for birth addresses only) in Colorado Springs and Pueblo. This study showed the same correlation as the Denver study, similar in degree but less significant due to the smaller numbers; 32 per cent of the 65 cases and 18 per cent of the 65 controls showed HCC's. The correlation was strongest for cases with onset before six years of age, possibly because many of the older cases had been gone from their birth addresses for many years before their cancer onset.

DISCUSSION

It is not clear *how* residence near a HCC might affect the development of cancer, but several possibilities should be considered:

1) Some association of both cancer and HCC's with a third factor may spuriously account for our correlation. Although we found no indication of such a third factor in our analyses of social class, neighborhood, congested streets, or family make-up, the possibilities have not been exhausted.

2) The magnetic fields produced by wire currents may somehow directly "cause" cancer. There is, however, no independent evidence or theoretical understanding which seems to support this possibility. The evidence concerning mutagenic effects of extremely low frequency magnetic fields, for instance, is ambiguous, but probably negative (18).

3) Carcinogenic activity may be associated with some *indirect* effect of the HCC's. For example, fields around power

lines might change the distribution of some ambient environmental carcinogen, such as particles which emit ionizing radiation. (However, the fields near domestic wires are too weak to make this seem probable.) Or the increased current flowing in the plumbing might locally affect the drinking water. (There is often a small amount of lead in copper water pipes, for instance, due to imperfect soldering. And lead in the water supply is correlated with cancer, at least geographically (19). However, it is not clear that AC current in pipes could affect this small amount of lead enough to make a difference.)

4) AC magnetic fields might affect the development of cancer indirectly, through some effect on physiologic processes. It is conceivable, for instance, that contact-inhibition of cellular growth, or the basic immune reaction of recognizing "self" from "not self," involves electrical potentials occurring at cell surfaces. Against an electromagnetic background different from that provided during evolution, any such cell mechanism might be altered.

Whatever the basis for our observed correlation, it should be emphasized that, although the risk of cancer appears to be increased for children living near HCC's, it is rarely increased by a factor of more than two or three. Therefore, if in the general population one child in 1000 is likely to get cancer before age 19, no more than two or three in 1000 living near a HCC would be expected to get it. The practical significance of the correlation, if any, lies in the high prevalence of HCC's, not in any very high risk posed by most HCC's.

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Attachment 14

A Pooled Analysis of Magnetic Fields, Wire Codes, and Childhood Leukemia

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We obtained original individual data from 15 studies of magnetic fields or wire codes and childhood leukemia, and we estimated magnetic field exposure for subjects with sufficient data to do so. Summary estimates from 12 studies that supplied magnetic field measures exhibited little or no association of magnetic fields with leukemia when comparing 0.1–0.2 and 0.2–0.3 microtesla (μT) categories with the 0–0.1 μT category, but the Mantel-Haenszel summary odds ratio comparing $>0.3 \mu\text{T}$ to 0–0.1 μT was 1.7 (95% confidence limits = 1.2, 2.3). Similar results were obtained using covariate adjustment and spline regression. The study-specific relations appeared consistent despite the numerous methodologic differences among the studies. The association of wire codes with leukemia varied considerably across studies, with odds ratio estimates for very high current *vs* low current configurations ranging from

0.7 to 3.0 (homogeneity $P = 0.005$). Based on a survey of household magnetic fields, an estimate of the U.S. population attributable fraction of childhood leukemia associated with residential exposure is 3% (95% confidence limits = –2%, 8%). Our results contradict the idea that the magnetic field association with leukemia is less consistent than the wire code association with leukemia, although analysis of the four studies with both measures indicates that the wire code association is not explained by measured fields. The results also suggest that appreciable magnetic field effects, if any, may be concentrated among relatively high and uncommon exposures, and that studies of highly exposed populations would be needed to clarify the relation of magnetic fields to childhood leukemia. (Epidemiology 2000;11:624–634)

Keywords: childhood neoplasms, electromagnetic fields, environmental exposure, leukemia, magnetic fields, wire codes.

The question of health effects of extremely low-frequency electromagnetic fields (EMFs) remains an unset-

tled topic.¹ The National Institute of Environmental Health Sciences funded our research team to conduct a pooled analysis of those studies of EMF and childhood leukemia for which original data could be obtained. We felt that a direct analysis of individual study data would allow a more reliable evaluation of interstudy differences in results (heterogeneity). It also could allow more reliable evaluation of dose-response relations and effects on public health than could a combination of summaries from studies, especially in light of the very different analyses presented in the published reports. The present paper reports our analyses.

Subjects and Methods

STUDIES

From literature searches, we identified 24 studies^{2–25} that presented data on household EMF or power-supply wiring information and childhood leukemia. To be eligible for inclusion in our pooled analysis, the study had to have obtained quantitative magnetic field measures for individual subjects or enough information to approximate Wertheimer-Leeper wire codes.¹ Nineteen studies^{2–16,22–25} had eligible data. Five articles reporting four studies^{22–26} appeared after our initial search in 1998; investigators in two of those studies^{22,23} supplied data in time for inclusion here. One study group¹⁶ refused our data request. Two studies^{8,15} were conducted using identical methods within the same

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This research was supported by Grant R03 ES-08920 from the National Institute of Environmental Health Sciences. Southern California Edison Co. supported previous work that contributed to this report.

Submitted September 13, 1999; final version accepted May 30, 2000.

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TABLE 1. Description of Studies in Pooled Analyses [All Are Case-Control Studies (Verkasalo Nested in Cohort)]

First Author	Location	Measurements*	Matching Factors†
Coghill ²	England	Direct	Age, sex
Dockerty ²³	New Zealand	Direct	Birth quarter, sex
Fajardo-Gutiérrez ³	Mexico	WC	Age, sex
Feychting ⁴	Sweden	Calc; some direct	Birth year, sex, diagnosis year, parish, transmission-line corridor
Fulton ⁵	Rhode Island	WC	Birth year
Green ²⁴	Ontario	WC‡	Birth year, sex
Linnet ⁶	Eastern U.S.	Direct; some WC	Age, race, RDD
London ⁷	Los Angeles	Direct; WC	Age, sex, race; some friend, RDD
McBride ²²	Canada	Direct; WC	Age, sex, area
Michaelis ⁸	Germany	Direct	Birth date, sex; some by locale
Olsen ⁹	Denmark	Calc	Birth year, sex, diagnosis date
Savitz ¹⁰	Denver	WC; some direct	Age, sex, RDD
Tomenius ¹¹	Sweden	Direct	Age, sex, birth district
Tynes ¹²	Norway	Calc	Birth year, sex, municipality
Verkasalo ¹³	Finland	Calc	Age, sex
Wertheimer ¹⁴	Denver	WC	Birth date; some by county

* Calc = magnetic field exposure calculated from configuration and electric load data; direct = direct magnetic field measurements; WC = wire code.

† RDD = random-digit dialing.

‡ Only wire code data from published report used here. Green *et al*^{24,25} also obtained magnetic field data.

country and treated as one study.⁸ Fulton *et al*⁵ and Tomenius¹¹ published analyses that used residence as the analysis unit, but we used individual-level exposures from their data. We thus had anonymous records on individual subjects from 15 distinct studies.

Table 1 summarizes the studies included here. All are case-control studies. Verkasalo *et al*¹³ initially conducted and reported a person-time cohort study. They supplied data from an unpublished case-control study nested within their cohort, based on all cases observed in the cohort plus ten controls for each case, and which obtained additional covariate data; the controls were age-sex matched but otherwise randomly sampled from the cohort. The two Swedish studies^{4,11} had a small overlap in source populations and so share a few cases, but this overlap could not be identified from the available data. Most studies had geographic restrictions on their source populations beyond those shown in Table 1; some had restrictions to areas near or crossed by high-voltage lines.^{4,12,13}

PRIMARY MEASURES

Twelve studies supplied magnetic field exposure estimates for some or all individuals. For four Nordic studies,^{4,9,12,13} we used estimates calculated by the original investigators from measured proximity to power lines and historical current-supply data. For eight studies,^{2,6–8,10,11,22,23} we used estimates based on direct measurements (measured magnetic fields at the front door of the residence,¹¹ measured fields in the child's bedroom,^{2,7,8,23} averaged fields in several

rooms,^{6,10} and averaged personal and house measures²²).

Some studies^{4,6–8,10,22} supplied more than one type of magnetic field measurement. For example, there were normal- or low-power measurements, spot and 24-hour measurements, mean and median values, data from the residence at diagnosis, and data from other residences. There is as yet no measure of magnetic field exposure that is known to be biologically the most relevant. In the absence of such knowledge, it would be best to examine a number of different measures. This was indeed done in several studies, but it raises multiplicity problems that are difficult to deal with statistically in even a single study. For a pooled analysis of the studies here, there would be more than 100 combinations of measures (although we did not have all measures for all of the

studies).

To avoid multiplicity issues and to keep our task manageable, we defined our target measure to be a child's time-weighted average exposure up to 3 months before diagnosis. When we had several measures from a study, we used a measure that, based on earlier work,^{27–29} seemed likely to provide the best approximation to this target. In particular, we preferred calculated historical fields or averages of multiple measurements rather than spot measurements when there was a choice. Table 2 summarizes the measurements used from each study. We also conducted analyses of each supplied measure and a

TABLE 2. Magnetic-Field Measures Used in Primary Analyses

First Author	Summary Measure Description*
Coghill ²	Nighttime (8:00 pm to 8:00 am) recordings in child's bedroom
Dockerty ²³	Arithmetic mean of 24-hour recordings in child's bedroom
Feychting ⁴	Average of calculations based on distances, phases, and loads of above-ground lines
Linnet ⁶	Time-weighted household mean based on typical child activity patterns and 24-hour child bedroom measurements and spot measurements in kitchen and family room; front door measurement when these data were not available; includes multiple homes covering 70% or more of the reference period (up to 5 years before diagnosis date)
London ⁷	Arithmetic mean of 24-hour recordings in child's bedroom
McBride ²²	Time-weighted mean based on 48-hour personal monitoring plus predictions from perimeter measurements
Michaelis ⁸	Arithmetic mean of 24-hour recordings in child's bedroom
Olsen ⁹	Average of calculations based on distances; phases; and loads of 50–400-kV transmission lines, cables, and substations within areas calculated as potentially having $\geq 0.1 \mu\text{T}$ exposure
Savitz ¹⁰	Arithmetic mean of low-power spot measurement in three or more locations (child's bedroom, parent's bedroom, other room occupied by child ≥ 1 hour/day, front door)
Tomenius ¹¹	Maximum uniaxial value outside front door of single-family homes and apartments
Tynes ¹²	Average of calculations based on distances, phases, and loads of above-ground lines ≥ 11 kV
Verkasalo ¹³	Average of calculations based on distances, typical line configuration, and loads of overhead 110–400-kV lines

* For details see original reports.

limited sensitivity analysis of summaries based on revisions of initial choices.

All North American studies^{3,5-7,10,14,22,24} obtained wire code data. Wire codes from two studies^{5,14} were recalculated from original data on distances to type of distribution line. Wire codes from one study³ were in a unique three-level form.

OTHER INFORMATION

Studies varied considerably in the covariates available for control and in their completeness of exposure and covariate information. One study¹¹ supplied no covariate data and so was excluded from covariate-adjusted analyses. Several studies^{4-9,12,22,23} supplied at least one socioeconomic variable on some or all subjects. One important ecologic covariate available for all studies was location; studies in North America involved 60-Hz fields with 110–125-V home supply, whereas all other studies involved 50-Hz fields with 220–240-V power. Thus, all comparisons of 60-Hz *vs* 50-Hz fields are also comparisons of 110–125-V *vs* 220–240-V systems and of North America *vs* other locations.

There are several discrepancies between the data we report and those in some published reports.^{6,7,10,14,22,23} Some differences arose because we did not impose exclusion criteria used by certain authors. For example, we included ten Down-syndrome subjects excluded by Linet *et al*⁶ because we could not identify such subjects in other studies and we could not identify any bias that would justify such an exclusion. Other differences arose from postpublication corrections or additions to the study data by the original investigators and from our use of exposure measures and cutpoints different from those used in the original publications; these differences led to especially large upward changes for the Tomenius¹¹ and McBride *et al*²² estimates. A few small discrepancies were unresolved; no such discrepancy appeared capable of producing more than negligible differences in summary results.

Coghill *et al*² and Linet *et al*⁶ restricted their cases to acute lymphoblastic leukemia (ALL). Because about 80% of childhood leukemias are ALL, and because not all datasets distinguished leukemia subtypes, we conducted no analysis restricted to ALL.

STATISTICAL METHODS

Data were analyzed using inverse-variance weighted (Woolf), Mantel-Haenszel, and maximum-likelihood (ML) tabular methods, and using ML logistic regression.^{30,31} (Inverse variance methods were included because they are common in meta-analysis.) All *P*-values were derived from score statistics or deviance (log likelihood-ratio) statistics.³⁰ For magnetic field exposures, dose response was examined using category indicators and splines in logistic models.³¹⁻³³ All results were adjusted for study: tabular analyses were always stratified on study, and all regressions included indicators for study.

All magnetic field measurements were converted into units of microtesla (μ T). Only two studies^{6,7} had more than four cases above 0.4 μ T; therefore, for categorical magnetic field analyses, values above 0.3 μ T were combined in a single category to ensure cell counts large enough for all statistical procedures. To avoid the trend distortions and power loss associated with percentile-category boundaries,^{33,34} we used equally spaced boundaries below the 0.3- μ T cutpoint. We combined low-exposure wire codes (UG = underground, VLCC = very low current code, and OLCC = ordinary low current code) into a single "LCC" low-current reference category for comparison with the two high-exposure wire codes (OHCC = ordinary high current, and VHCC = very high current). Previous results indicate that the three low-current categories do not correspond to meaningful differences in EMF exposure or childhood leukemia risk.^{3,5-7,10,22,24,35} Furthermore, in three studies,^{5,6,14} the proportions of subjects with a UG or VLCC code were too small to yield efficient estimates using those codes as reference category; in another,⁶ UG and VLCC were combined in the supplied data; and in another,³ low-current codes had been combined in data collection.

Complications arose in accounting for the variety of matching protocols used. Most studies matched on certain covariates (typically sex, age or birth date, and some sort of geographic unit). Many studies experienced some failures to match, leading to fewer subjects available for matched analyses than unmatched analyses. Several considerations led us to focus on unmatched analyses with analytic control for matched covariates. First, this choice provided the most subjects for analysis. Second, this choice avoided further efficiency loss due to the type of analysis overmatching documented by Brookmeyer *et al*.³⁶ Third, this choice also helped avoid small-sample bias away from the null due to sparse matched-set counts in study-specific analyses³⁷; although we would expect the unmatched analyses to suffer some small bias toward the null, we thought this possibility preferable to a potentially large bias away from the null due to sparse data. Fourth, results from matched analyses were less stable but exhibited the same patterns seen in the unmatched analyses.

Results for Magnetic Fields

CATEGORICAL ANALYSES

Table 3 displays the distribution of magnetic field measurements among the studies supplying such measurements. There are extensive differences among the studies, ranging from Olsen *et al*⁹ (which has only 0.5% of cases and controls above 0.1 μ T) to Linet *et al*⁶ (which has more than one-third of measured subjects above 0.1 μ T). Values above 0.3 μ T are relatively infrequent in all studies. The differences appear associated chiefly with location rather than with measurement method (direct *vs* calculated). Distributions in North American studies tend to be much higher than those in European studies, probably reflecting differences in power systems (for example, more overhead wires and lower household

TABLE 3. Study-Specific Distributions of Magnetic-Field Data

First Author	Magnetic-Field Category (μ T)						Total	No Measure*
	≤ 0.1	$>0.1\text{--}\leq 0.2$	$>0.2\text{--}\leq 0.3$	$>0.3\text{--}\leq 0.4$	$>0.4\text{--}\leq 0.5$	>0.5		
Cases								
Coghill ²	48	5	2	0	1	0	56	0
Dockerty ²³	72	9	3	1	1	1	87	34
Feychting ⁴	30	1	1	2	0	4	38	0
Linet ⁶	403	152	41	20	13	9	638	46
London ⁷	110	30	5	9	4	4	162	68
McBride ²²	174	77	32	11	1	2	297	102
Michaelis ⁸	150	17	3	3	3	0	176	0
Olsen ⁹	829	1	0	0	0	3	833	0
Savitz ¹⁰	24	7	2	3	0	0	36	62
Tomenius ¹¹	129	16	5	0	0	3	153	0
Tynes ¹²	146	2	0	0	0	0	148	0
Verkasalo ¹³	30	1	0	0	1	0	32	3
Controls								
Coghill ²	47	9	0	0	0	0	56	0
Dockerty ²³	68	13	1	0	0	0	82	39
Feychting ⁴	488	26	18	10	2	10	554	0
Linet ⁶	407	144	41	17	5	6	620	69
London ⁷	99	28	6	2	2	6	143	89
McBride ²²	194	96	28	5	3	3	329	70
Michaelis ⁸	372	29	7	4	0	2	414	0
Olsen ⁹	1,658	3	2	2	0	1	1,666	0
Savitz ¹⁰	155	28	10	3	2	0	198	67
Tomenius ¹¹	546	119	24	4	2	3	698	21
Tynes ¹²	1,941	25	7	5	4	22	2,004	0
Verkasalo ¹³	300	9	6	4	0	1	320	30

* No measure for a residence at or before time of diagnosis (cases) or corresponding index date (for controls).

voltage in North America), per capita electricity consumption,³⁸ and grounding practices. The higher distribution in Feychting and Ahlbom⁴ compared with the other Nordic studies reflects the fact that the source population was restricted to children dwelling within 300 meters of high-voltage lines⁴ (although Verkasalo *et*

*al*¹³ imposed a 500-meter limit and Tomenius¹¹ restricted subjects to census wards with transmission lines).

Table 4 displays odds ratio estimates computed directly from the raw counts underlying Table 3 and summary estimates assuming common odds ratios for each analysis category. The study-specific and summary esti-

TABLE 4. Study-Specific Odds Ratio Estimates and Study-Adjusted Summary Estimates, Magnetic Field Data (Reference Category, $\leq 0.1 \mu\text{T}$)

First Author	Magnetic Field Category (μT)					
	>0.1–≤0.2		>0.2–≤0.3		>0.3	
	Estimate	95% CL	Estimate	95% CL	Estimate	95% CL
Coghill ²	0.54	0.17, 1.74	No controls		No controls	
Dockerty ²³	0.65	0.26, 1.63	2.83	0.29, 27.9	No controls	
Feychting ⁴	0.63	0.08, 4.77	0.90	0.12, 7.00	4.44	1.67, 11.7
Linet ⁶	1.07	0.82, 1.39	1.01	0.64, 1.59	1.51	0.92, 2.49
London ⁷	0.96	0.54, 1.73	0.75	0.22, 2.53	1.53	0.67, 3.50
McBride ²²	0.89	0.62, 1.29	1.27	0.74, 2.20	1.42	0.63, 3.21
Michaelis ⁸	1.45	0.78, 2.72	1.06	0.27, 4.16	2.48	0.79, 7.81
Olsen ⁹	0.67	0.07, 6.42	No cases		2.00	0.40, 9.93
Savitz ¹⁰	1.61	0.64, 4.11	1.29	0.27, 6.26	3.87	0.87, 17.3
Tomenius ¹¹	0.57	0.33, 0.99	0.88	0.33, 2.36	1.41	0.38, 5.29
Tynes ¹²	1.06	0.25, 4.53	No cases		No cases	
Verkasalo ¹³	1.11	0.14, 9.07	No cases		2.00	0.23, 17.7
Study-adjusted summaries*						
Woolf	0.96	0.81, 1.14	1.08	0.80, 1.45	1.83	1.34, 2.49
MH	0.95	0.80, 1.12	1.06	0.79, 1.42	1.69	1.25, 2.29
Study + age + sex adjusted†						
MH	1.01	0.84, 1.21	1.06	0.78, 1.44	1.68	1.23, 2.31
Spline‡	1.00	0.81, 1.22	1.13	0.92, 1.39	1.65	1.15, 2.36

95% CL = 95% confidence limits.

* MH = Mantel-Haenszel; maximum-likelihood summaries differed by less than 1% from these summaries; based on 2,656 cases and 7,084 controls. Summary tests: 3-degree-of-freedom (df) MH categorical $P = 0.01$; 1 df Mantel trend $P = 0.06$ (from continuous data).

† Excludes Tomenius *et al*¹¹ (no covariate data); based on 2,484 cases and 6,335 controls with age and sex data; 3-df MH categorical $P = 0.01$; 1 df Mantel trend $P = 0.04$ (from continuous data).

‡ Estimates comparing odds at category means (0.14, 0.25, and 0.58 vs 0.02 μT) from a quadratic logistic spline with one knot at 0.2 μT , plus age and sex terms.

mates tend to show little or no association of fields below $0.3 \mu\text{T}$ with leukemia, but all studies with cases and controls in the $>0.3 \mu\text{T}$ category exhibit positive associations for that category. The differences across studies were within chance variation (deviance $P = 0.42$ using exposure categories in Table 4), as were differences between studies with different measures [ML odds ratios for $>0.3 \mu\text{T} = 1.70$ from studies with calculated fields and 1.68 from studies with direct measurement; 95% confidence limits (95% CL) for ratio of odds ratios = $0.46, 2.22$] or different field frequencies (ML odds ratios for $>0.3 \mu\text{T} = 1.97$ from studies with 50-Hz fields and 1.58 from studies with 60-Hz fields; 95% CL for ratio of odds ratios = $0.66, 2.36$).

The Tomenius data¹¹ included no covariate and so were excluded from covariate-adjusted analyses. The penultimate line of Table 4 shows the age-sex-study-adjusted Mantel-Haenszel estimates. The exclusions and adjustments had negligible effect, and odds ratio differences across age and sex categories (not shown) were within chance variation. Table 5 summarizes categorical analyses upon restriction to subjects with no missing data. Neither restriction nor adjustment for available covariates changed the qualitative result that there was little or no association evident below $0.2 \mu\text{T}$, but some positive association was evident above $0.3 \mu\text{T}$.

TREND ANALYSIS

The final line of Table 4 displays estimated odds ratios from a logistic model fit to individual-level magnetic field data using a quadratic spline for field along with age, squared age, and sex terms. The spline has a single knot at $0.2 \mu\text{T}$ (the middle category boundary) and so has one linear and two quadratic magnetic field terms; the model thus uses 3 degrees of freedom for field, the same number of degrees of freedom as in the four-category analysis. The spline estimate under each category is the leukemia odds ratio comparing the mean field measure in that category with the mean field measure in the $\leq 0.1 \mu\text{T}$ category and is thus a continuous-data analogue of the categorical summary estimate. Unlike the categorical analysis, the spline analysis imposes a smooth dose-response relation between field level and leukemia. Nonetheless, the spline results are similar to the categorical results: there appears to be little or no association below $0.2 \mu\text{T}$ but some association comparing high with low exposures; furthermore, differences among covariate-specific curves (not shown) were within chance variation.

Figure 1 displays a graph of the "floated" case-control ratios³⁹ fit by the spline model, along with pointwise confidence limits. This figure is a plot of the fitted odds of being a case *vs* being a control in our studies. Assuming these odds are proportional to the underlying childhood leukemia rates, this plot is an estimate of the *shape* of the curve relating leukemia rates to magnetic fields under the spline model.³⁹ The vertical axis corresponds to geometric mean case-control ratios rather than to odds ratios, but ratios of different points on the curve

equal the model-fitted odds ratios³⁹; for example, the ratio of the curve heights at 0.58 and $0.02 \mu\text{T}$ (the means of the >0.3 and $\leq 0.1 \mu\text{T}$ categories) is 1.65 , equal to the final odds ratio in Table 4. We caution against focusing on the central curve, however, because the data are compatible with a wide range of trends, including nonmonotonic, linear, and exponentially increasing shapes. For example, the strictly increasing trend above $0.1 \mu\text{T}$ is *not* a statistically stable feature, in that curves that plateau or even decline above $0.6 \mu\text{T}$ also fit the data well.

INFLUENCE AND SENSITIVITY ANALYSES

As with covariate adjustment, neither single-study deletions nor alternative choices for the exposure measure altered results qualitatively, nor did deletion of large field values (for example, the five subjects above $2.0 \mu\text{T}$, all controls from Tynes and Haldorsen¹²). Although the highest-category estimates and the fitted curve varied considerably with category-boundary and model choices, these choices also did not alter the basic qualitative results.

Use of alternatives among the supplied exposure measures produced only small differences in the summaries; we did not have all measures from all studies, however. Missing data varied with choice of measure, and this variation sometimes had more influence on estimates than the choice of measure. Two studies^{4,13} supplied calculated yearly exposure of children; we used these data to construct alternative-exposure measures that might arguably approximate more closely our target than the measures used in the original reports and in our analysis above. Use of these alternatives had little effect on the study-specific odds ratios below $0.3 \mu\text{T}$ but raised the >0.3 -*vs*- ≤ 0.1 odds ratio to 5.9 (95% CL = $2.0, 17$) for Feychting and Ahlbom⁴ and to 10 (95% CL = $1.4, 74$) for Verkasalo *et al.*¹³ Some of this increase may only be increased small-sample bias³⁷ due to reduction in numbers above $0.3 \mu\text{T}$. In any event, use of these alternatives changed the summaries by only a few percent.

The calculated-field measures from the Nordic studies were based on high-voltage lines and did not include contributions from sources such as in-home wiring and appliances.^{4,9,12,13} The effect of the latter omissions is not straightforward to assess, because fields are vector additive and so may even destructively interfere with one another, depending on the relative orientation and phase of the contributions from different sources. One study⁴ supplied spot measurements as well as calculated fields on 24 of 38 cases and 344 of 554 controls. These dual measurements permitted instrumental-variable corrections⁴⁰ for estimates from the calculated fields in the Nordic studies. Because these corrections involve strict assumptions and require extensive technical description,⁴⁰ they were not used in Tables 3 and 5, and we omit details. The main result was that odds ratio estimates from the Nordic studies^{4,9,12,13} were corrected toward the null. Nonetheless, because these studies contributed so few cases at the higher exposure levels, the corrections had only a small effect on the overall summary estimates.

TABLE 5. Study-Specific Odds Ratio Estimates and Study-Adjusted Summary Magnetic Field Estimates from Data Restricted to the 2,078 Cases and 5,516 Controls with Complete Covariate Data, without and with Covariate Adjustment* (Reference Category, $\leq 0.1 \mu\text{T}$)

First Author	Magnetic Field Category (μT)					
	$>0.1\text{--}\leq 0.2$		$>0.2\text{--}\leq 0.3$		>0.3	
	Estimate	95% CL	Estimate	95% CL	Estimate	95% CL
Restricted, no covariate adjustment						
Coghill ²	0.30	0.06, 1.52	No controls		No controls	
Dockerty ²³	0.65	0.24, 1.78	3.05	0.31, 30.1	No controls	
Feychting ⁴	0.63	0.08, 4.77	0.90	0.12, 7.00	4.44	1.67, 11.7
Linet ⁶	1.06	0.81, 1.40	0.99	0.63, 1.58	1.70	1.01, 2.87
London ⁷	1.08	0.58, 2.01	1.07	0.28, 4.12	1.82	0.75, 4.43
McBride ²²	0.88	0.61, 1.28	1.30	0.75, 2.25	1.45	0.64, 3.27
Michaelis ⁸	1.45	0.78, 2.72	1.06	0.27, 4.16	2.48	0.79, 7.81
Olsen ⁹	1.03	0.09, 11.4	No cases		4.13	0.37, 45.7
Savitz ¹⁰	1.68	0.66, 4.30	1.30	0.27, 6.29	3.89	0.87, 17.4
Tynes ¹²	1.11	0.26, 4.74	No cases		No cases	
Verkasalo ¹³	1.13	0.14, 9.25	No cases		2.04	0.23, 18.0
MH*	1.02	0.85, 1.23	1.10	0.81, 1.51	1.87	1.35, 2.60
Restricted and covariate adjusted						
Coghill ²	0.28	0.06, 1.44	No controls		No controls	
Dockerty ²³	0.66	0.24, 1.81	2.83	0.29, 27.9	No controls	
Feychting ⁴	0.60	0.08, 4.54	0.80	0.10, 6.22	4.57	1.72, 12.1
Linet ⁶	1.07	0.81, 1.42	0.96	0.61, 1.52	1.67	0.99, 2.82
London ⁷	1.02	0.55, 1.89	0.98	0.25, 3.75	1.82	0.75, 4.44
McBride ²²	0.85	0.59, 1.23	1.24	0.72, 2.14	1.40	0.62, 3.18
Michaelis ⁸	1.24	0.66, 2.33	0.93	0.24, 3.64	2.02	0.64, 6.37
Olsen ⁹	1.03	0.09, 11.4	No cases		3.74	0.34, 41.4
Savitz ¹⁰	1.78	0.70, 4.57	1.27	0.26, 6.17	4.08	0.91, 18.2
Tynes ¹²	1.12	0.26, 4.78	No cases		No cases	
Verkasalo ¹³	1.13	0.14, 9.25	No cases		2.05	0.23, 18.1
MH*	1.01	0.82, 1.25	0.94	0.65, 1.37	2.06	1.40, 3.01

95% CL = 95% confidence limits; MH = Mantel-Haenszel.

* Excludes Tomenius *et al*¹¹ (no covariate data). Covariate adjustment is for age and sex, plus social and economic variables in nine studies.^{4,6-9,12,13,22,23} Covariate-adjusted summary: 3-degrees-of-freedom Mantel-Haenszel categorical $P = 0.01$.

The dip in the curve in Figure 1 below $0.1 \mu\text{T}$ is mostly attributable to the Danish data,⁹ in which exposures below $0.1 \mu\text{T}$ were effectively set to 0 when calculating averages, and which contributed about one-quarter of the subjects in the $\leq 0.1 \mu\text{T}$ category. When this study was deleted, the dip disappeared, but the curve remained mildly sigmoidal.

NONCONTRIBUTING STUDIES

Myers *et al*¹⁶ reported only one case and two controls for "non-solid tumors" above $0.1 \mu\text{T}$; exclusion of this study could not have influenced our results to an important degree. Most of the data from the much larger study by Green *et al*²⁴ were neither presented in categories that could be combined directly with our categories nor broken into analysis categories above $0.15 \mu\text{T}$; the estimates in this study varied considerably with the measure and adjustment used, but all had wide confidence intervals and were statistically compatible with our results. Crude data from a personal-monitoring substudy by Green *et al*²⁵ produced odds ratios of 1.20 (95% CL = 0.59, 2.41), 1.76 (95% CL = 0.82, 3.80), and 0.71 (95% CL = 0.18, 2.88) comparing $0.1\text{--}0.2$, $0.2\text{--}0.3$, and $>0.3 \mu\text{T}$ with $\leq 0.1 \mu\text{T}$, reflecting the small numbers in this substudy. The U.K. Childhood Cancer Study group²⁶ reported birthdate-sex-socioeconomic status-adjusted odds ratios for total leukemia of 0.78 (95% CL = 0.55, 1.12), 0.78 (95% CL = 0.40, 1.52), and 1.68 (95%

CL = 0.40, 7.10) comparing categories of $0.1\text{--}0.2$, $0.2\text{--}0.4$, and $>0.4 \mu\text{T}$ with $\leq 0.1 \mu\text{T}$; our pooled data yielded age-sex-study-adjusted ML estimates of 1.01 (95% CL =

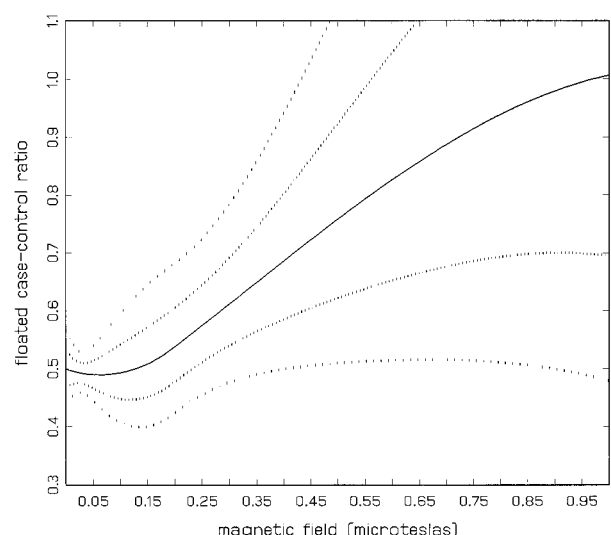


FIGURE 1. Floated case-control ratios³⁹ from 3-degree-of-freedom quadratic-logistic spline model fit to pooled magnetic field data, with adjustment for study, age, and sex. Inner dotted lines are pointwise 80% confidence limits; outer dotted lines are pointwise 99% confidence limits.

TABLE 6. Distribution of Residential Magnetic Field Measurements in Electric Power Research Institute Survey of U.S. Homes⁴¹ (N = 987) (Categories Exclude Lower Boundary)

Category (μ T)	No. of Homes in Category	%
≤ 0.05	437	44.2
0.05–0.1	277	28.1
0.1–0.2	173	17.5
0.2–0.3	55	5.6
0.3–0.4	20	2.0
0.4–0.5	8	0.8
0.5–0.6	7	0.7
0.6–0.75	6	0.6
over 0.75	4	0.4

Median = 0.06 μ T, mean = 0.09 μ T, and maximum = 1.01 μ T.

0.84, 1.21), 1.25 (95% CL = 0.96, 1.61), and 1.60 (95% CL = 1.03, 2.48) using the same categories.

ATTRIBUTABLE-FRACTION ANALYSIS

We estimated the excess fraction of U.S. childhood leukemia incidence that would be attributable to magnetic field exposures above 0.05 μ T, under the assumption that the dose-response estimate in Figure 1 represents the causal effects of fields. To estimate the U.S. population distribution of field exposure, we used data from a utility-based cluster-sampled survey conducted by the Electric Power Research Institute (EPRI).⁴¹ The data we obtained (Table 6) comprised spot field measurements averaged across rooms within each of 987 homes sampled from residences served by 301 EPRI utilities, which together served about 67% of U.S. homes.⁴¹

When these data were combined with the spline function in Figure 1 using a model-based attributable-fraction formula,⁴² we obtained a population attributable-fraction estimate of 3% for the effect of magnetic fields greater than 0.05 μ T (95% CL = –2%, 8%). The estimate is nearly the same if one uses any reference level up to 0.15 μ T (rather than 0.05 μ T), reflecting the fact that 90% of surveyed homes are in the 0–0.2 μ T range, in which the fitted ratios exhibit little variation. The wide confidence interval reflects the uncertainty about the distribution of exposure, as well as the considerable uncertainty about dose response. We further caution that our estimate refers only to effects of ambient residential fields and excludes effects of unmeasured personal field sources such as electric blankets.

We did not have survey data for Europe, but given the low Northern European exposures seen in Table 3, we would expect a correspondingly lower attributable-fraction estimate for Northern Europe.

RESULTS FOR WIRE CODES

Table 7 displays the distribution of wire codes among the studies supplying such codes, as well as data from Table V of Green *et al.*²⁴ As with fields, there are extensive differences among the studies, ranging within the U.S. from 15% with OHCC or VHCC codes in Linet *et al.*⁶ to nearly 50% with those codes in London *et al.*⁷ These differences reflect well-documented differences in power-grid architecture within the United States.^{1,41}

Table 8 displays odds ratio estimates computed directly from the raw counts underlying Table 7, and the corresponding covariate-adjusted estimates. Summary estimates are omitted because of the extensive unexplained heterogeneity among the study-specific results; for example, the VHCC odds ratios are less than 1 in three studies and more than 2 in three others (homogeneity $P = 0.005$). We found no covariate that accounted for the large variation in results, but deletion of Wertheimer and Leeper¹⁴ increased the homogeneity P -value to 0.11; no other single-study deletion increased the homogeneity P -value above 0.04. Eliminating Wertheimer and Leeper¹⁴ and Fulton *et al.*⁵ (the two earliest studies) yielded summary ML odds ratios of 1.02 (95% CL = 0.87, 1.22) for OHCC and 1.50 (95% CL = 1.17, 1.92) for VHCC based on 1,457 cases and 1,962 controls from six studies^{3,6,7,10,22,24} (deviance $P = 0.005$ for wire code; homogeneity $P = 0.15$).

As with fields, confounder adjustment had little effect on the wire code results beyond reducing the number of subjects, resulting in less stable estimates and more pronounced heterogeneity. For example, adjustment changed the Savitz *et al.*¹⁰ estimate of the VHCC odds ratio from 2.6 (95% CL = 0.92, 7.5) to 3.8 (95% CL = 1.2, 12); this change was entirely due to the deletion of

TABLE 7. Study-Specific Distributions of Wire-Code Data

First Author	Wire Code				No Measure
	VLCC*	OLCC	OHCC	VHCC	
Cases					
Fajardo-Gutiérrez ³	13†		92	82	0
Fulton ⁵	7	67	33	10	0
Green ²⁴ §	82	41	26	6	46
Linet ⁶	180	120	91	25	268‡
London ⁷	34	66	71	43	16
McBride ²²	152	77	83	39	48
Savitz ¹⁰	32	38	21	7	0
Wertheimer ¹⁴	4	86	53	13	7
Controls					
Fajardo-Gutiérrez ³	20†		102	65	0
Fulton ⁵	8	126	65	26	0
Green ²⁴ §	172	81	65	14	74
Linet ⁶	179	117	93	27	273‡
London ⁷	37	87	54	24	30
McBride ²²	157	77	105	23	37
Savitz ¹⁰	108	103	46	8	0
Wertheimer ¹⁴	17	107	26	6	7

VLCC = very low current code; OLCC = ordinary low current code; OHCC = ordinary high current code; VHCC = very high current code.

* VLCC includes underground (UG).

† Low-current categories not distinguished; translated as “baja” = LCC (low current code), “mediana” = OHCC, “alta” = VHCC.

‡ Subjects in Linet *et al.*⁶ had to meet a “residential stability” criterion to be wire coded.

§ Taken from Table V of Green *et al.*²⁴

TABLE 8. Study-Specific Odds Ratio Estimates and Study-Adjusted Summary Estimates without and with Restriction and Covariate Adjustment, Wire-Code Data [Reference Category: LCC (OLCC + VLCC + UG)]

First Author	Wire Code			
	OHCC		VHCC	
	Estimate	95% CL	Estimate	95% CL
Without restriction or adjustment				
Fajardo-Gutiérrez ³	1.39	0.65, 2.95	1.94	0.90, 4.19
Fulton ⁵	0.92	0.55, 1.52	0.70	0.32, 1.52
Green ^{24*}	0.82	0.50, 1.36	0.88	0.33, 2.35
Linnet ⁶	0.97	0.69, 1.34	0.91	0.52, 1.61
London ⁷	1.63	1.05, 2.53	2.22	1.26, 3.91
McBride ²²	0.81	0.57, 1.14	1.73	1.00, 2.99
Savitz ¹⁰	1.38	0.77, 2.46	2.64	0.92, 7.54
Wertheimer ¹⁴	2.81	1.63, 4.83	2.99	1.09, 8.15
With restriction and adjustment†				
Fajardo-Gutiérrez ³	1.41	0.66, 2.99	2.05	0.95, 4.43
Fulton ⁵	0.79	0.40, 1.53	0.54	0.21, 1.41
Linnet ⁶	0.99	0.70, 1.41	0.92	0.51, 1.66
London ⁷	1.46	0.91, 2.35	2.25	1.21, 4.20
McBride ²²	0.79	0.56, 1.12	1.55	0.89, 2.68
Savitz ¹⁰	1.52	0.82, 2.83	3.77	1.22, 11.7
Wertheimer ¹⁴	2.84	1.65, 4.89	3.10	1.14, 8.47

OLCC = ordinary low current code; VLCC = very low current code; UG = underground (LCC combines these three categories); OHCC = ordinary high current code; VHCC = very high current code.

* Computed from Table V of Green *et al.*²⁴

† Excludes Green *et al.*²⁴ (which was not in our database); restricted to subjects with covariate data; covariate adjustment is for age and sex, plus social or economic variables in four studies.^{3-7,22}

15 cases and 23 controls without covariate data. Adjusted results in our three-level format could not be computed from Green *et al.*²⁴ but their own adjustment produced little change in their estimates.^{24, Table V} Fajardo-Gutiérrez supplied additional data on wiring configurations that allowed one of us (W. T. K.) to construct an alternative approximation to the Wertheimer-Leeper wire code in this study.³ This alternative coding produced OHCC and VHCC (vs LCC) odds ratios of 1.5 (95% CL = 0.80, 2.9) and 1.2 (95% CL = 0.80, 1.9), which appear less consistent with other studies than the odds ratios from the original coding (Table 8).

Four studies^{6,7,10,22} recorded both magnetic fields and wire codes, allowing us to examine these exposures together (Table 9). Because these analyses involve only a

fraction of all subjects and because fields and codes are strongly associated (mean fields of 0.09 for LCC, 0.13 for OHCC, and 0.19 for VHCC), the results are even more unstable. Nonetheless, the associations seen with fields and codes entered into the same model were similar to the associations seen with separate models for the measures.

Discussion

For brevity and on scientific grounds, we restricted this report to analyses specified as *a priori* relevant to the main study question: Are magnetic fields or wire codes consistently associated with childhood leukemia? Our prior restrictions were meant to avoid analyses that “capitalize on chance” (small numbers and unstable estimates) either to reinforce or refute a particular hypothesis. Such restrictions are especially important in dose-response analyses of magnetic fields because of suggestions that the entire topic of EMF research is a product of

unconstrained data dredging.⁴³

Purely categorical dose-response analyses (that is, those conducted without regard to ordering, spacing, or smoothness constraints) can almost always be made to yield nonmonotone patterns by using categories small enough so that category-specific estimates become unstable. To avoid such problems, we supplemented our initial categorical analyses with smooth regression analyses (splines) rather than with smaller categories. We believe that dose-response modeling is important in the present context because, even upon pooling, there are still too few data to reject any plausible dose-response shape, especially above 0.2 μT . In particular, the data appear to be statistically consistent with anything from

TABLE 9. Summary Odds Ratio Estimates Based on 850 Cases and 1,004 Controls from Four Studies with Both Magnetic Field Measurements and Wire Codes^{6,7,10,22} (Reference Categories: $\leq 0.1 \mu\text{T}$ and LCC)

	Estimates from Logistic Regression* with					
	Magnetic Field Alone		Wire Code Alone		Field and Wire Code	
	Estimate	95% CL	Estimate	95% CL	Estimate	95% CL
Field (μT)						
0.1–0.2	1.08	0.86, 1.35			1.02	0.81, 1.29
0.2–0.3	1.10	0.76, 1.60			1.01	0.69, 1.48
>0.3	1.52	0.99, 2.33			1.38	0.89, 2.13
P value†		0.27				0.55
Wire code						
OHCC			1.15	0.92, 1.44	1.13	0.90, 1.42
VHCC			1.65	1.15, 2.35	1.58	1.18, 2.28
P value†				0.02		0.04

LCC = low current code; OHCC = ordinary high current code; VHCC = very high current code.

* Includes study indicators.

† From deviance tests of all categories.

curves that are nearly flat to curves that rise and then fall at high exposures to curves that rise faster than exponentially.

We had planned to use available information to impute magnetic field values for subjects having only wire codes, on the basis of information relating codes to field measurements.^{10,35} Nonetheless, because of the heterogeneity among wire code results and doubts about the accuracy of the imputation, we decided to forego those analyses.

One interesting result from our analysis is resolution of an apparent "wire code paradox." It has been remarked that wire codes show more consistent associations with childhood cancers across studies than do magnetic fields. The paradoxical element arose in part from the presumption that wire codes were a proxy for fields and thus should show less consistent associations if fields have an effect. An examination of our tables suggests that, after allowing for statistical variability, wire codes in fact show less consistent associations with childhood leukemia than do magnetic fields. Nonetheless, adjustment for measured fields does not reduce the association of wire codes with childhood leukemia (Table 9). Perhaps only fields are biologically relevant, but errors in the field measures are so large that wire codes pick up much of the field effect; another possibility is that both measures only reflect effects of some biologically relevant exposure that is missing from our data.

One can of course raise many criticisms of the individual studies, which would increase the already large uncertainty in our results. For example, confounding effects of socioeconomic status, residential mobility, residence type, viral contacts, and traffic density have been raised as possible explanations for the observed associations.⁴⁴⁻⁵¹ These confounding hypotheses are themselves problematic. First, a confounding explanation requires the confounder to have an effect considerably larger than the observed association, as well as a strong association with exposure.^{30,Ch. 2} These attributes have not yet been demonstrated for the hypothesized confounders across the different populations that display positive associations. Adjustment for recorded socioeconomic and housing factors produced only small changes in the field-leukemia association, but our data on such factors are incomplete and we have only limited data on other potential confounders. Some results suggest that traffic-density effects may be large enough to partly explain the associations seen here.⁴⁴⁻⁴⁷ We thus recommend that future studies obtain data on traffic density and ambient pollution levels, as well as details of socioeconomic status and residence history.

Biases due to measurement errors are undoubtedly present in and vary across all of the studies, but their assessment is not wholly straightforward. One problem is that there is no agreed-upon definition of the target exposure, although it is often thought of as some sort of average or cumulative exposure during some biologically relevant time before leukemia diagnosis. Only under fairly restrictive conditions^{40,52} can one be certain that the net bias due to such error will be toward the null.

Unfortunately, there is little or no evidence to establish such detailed attributes of the errors, and there is no basis for assuming such attributes are the same across studies and measures. For example, although some U.S. studies have found clear associations between fields measured at the front door, average magnetic fields in the home, and personal exposure to children^{27,53} and another U.S. study found some repeatability of spot measures over extended time periods,⁵⁴ these associations are not large enough to ensure that the measures would tend to exhibit similar associations with childhood leukemia. Furthermore, the associations are imperfect enough to indicate that probably all of the measures suffer considerable error as proxies for any biologically relevant exposure measure (if one exists). One study suggested that electric rather than magnetic fields may be the relevant exposure.² Other studies conflict with this suggestion, however, insofar as the electric-field associations with childhood leukemia reported in those studies tended to be null or smaller than the reported magnetic-field associations.^{7,10,23,25}

Selection biases may be present in the studies, but for most there is little evidence that would establish their magnitude or even their direction with any certainty. Some studies reported low response rates (for example, field measurements were obtained on only half the identified potential controls in McBride *et al*²²), and accurate response rates cannot be determined for all studies. Whether such problems have led to serious bias remains a matter of speculation; the limited evidence from U.S. studies appears conflicting (for example, contrast Savitz *et al*^{10,p.35} with Hatch *et al*⁵¹ and Savitz and Kaune⁵⁵).

Given the preceding considerations, it seems reasonable to suppose that measurement and validity differences are responsible for some of the variation in study-specific results. Those considerations also raise a serious criticism of our analysis, in that we pooled different magnetic field measures without demonstrating that all of the measures are comparable or combinable. Indeed, it is highly implausible that the measures we used (or any other choices among available measures) reflect common underlying exposure and error distributions. Furthermore, our criteria for choosing measures when we had a choice are not compelling (for example, minimize missing data), and one could reasonably argue in favor of other choices⁵⁶ (although not without dispute^{57,58}). We expected that measure heterogeneity would lead to extra variation among the study-specific results, so we are all the more surprised that the observed variation was limited. We caution, however, that other choices could lead to very different degrees of variation; our results may not even be typical of what would be seen upon trying all defensible choices (although exploring the full range of choices would not indicate which choice is most valid). These problems should further expand the considerable uncertainty apparent in our results.

Another meta-analytic issue is that of publication bias. Because of the publicity surrounding the topic, we speculate that the data in small unpublished studies (if any exist) would have little influence on the results, and

that all large studies of this topic get published. Unfortunately, there are as yet too few published studies of fields or wire codes and childhood leukemia to support a reliable analysis of this bias,^{59,60} and current methods for analyzing the bias are not well suited for relations that require several degrees of freedom to summarize.

Our attributable-fraction estimate is subject to further criticism through its dependence on the EPRI survey.⁴¹ The survey measurements are of residential fields and therefore exclude sources such as school exposures and electric blankets; this exclusion error probably increases with age, especially upon school entry. Furthermore, selection bias could have been introduced because the survey homes were not limited to homes with children. Nonetheless, we think our estimate shows that any population effect of fields is probably much too small to detect via ecologic or time-trend studies; large ecologic variation or trends in leukemia rates would more likely be due to ecologic or temporal confounding than to real EMF effects.

In light of the above problems, the inconclusiveness of our results seems inescapable; resolution will have to await considerably more data on high electric and magnetic-field exposures, childhood leukemia, and possible bias sources. It also appears to us that, if an effect exists below 0.2 μT , it is probably too small to reach consensus about it via epidemiologic investigation alone. In contrast, both our categorical and trend analyses indicate that there is some association comparing fields above 0.3 μT to lower exposures, although there are as yet insufficient data to provide more than a vague sense of its form and its possible sources. We believe individual-level studies that focus on highly exposed populations would be needed to clarify this association. Such populations might be found in densely settled areas of some industrialized countries, such as Japan.⁶¹ Even in these countries, efficiency might be improved by restricting the source population to locales containing transmission lines, as was done in some Scandinavian studies.

Acknowledgments

We thank Jack Sahl for scientific contributions at the inception of this project; A. Ahlbom, K. Ebi (EPRI), A. Fajardo-Gutiérrez, M. Feychting, L. Kheifets, R. R. Neutra, J. M. Robins, D. Savitz, J. Schüz, P. Verkasalo, and N. Wertheimer for helpful reviews; and the referees for their criticisms.

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Attachment 15

A pooled analysis of magnetic fields and childhood leukaemia

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Summary Previous studies have suggested an association between exposure to 50–60 Hz magnetic fields (EMF) and childhood leukaemia. We conducted a pooled analysis based on individual records from nine studies, including the most recent ones. Studies with 24/48-hour magnetic field measurements or calculated magnetic fields were included. We specified which data analyses we planned to do and how to do them before we commenced the work. The use of individual records allowed us to use the same exposure definitions, and the large numbers of subjects enabled more precise estimation of risks at high exposure levels. For the 3203 children with leukaemia and 10 338 control children with estimated residential magnetic field exposures levels < 0.4 µT, we observed risk estimates near the no effect level, while for the 44 children with leukaemia and 62 control children with estimated residential magnetic field exposures ≥ 0.4 µT the estimated summary relative risk was 2.00 (1.27–3.13), *P* value = 0.002. Adjustment for potential confounding variables did not appreciably change the results. For North American subjects whose residences were in the highest wire code category, the estimated summary relative risk was 1.24 (0.82–1.87). Thus, we found no evidence in the combined data for the existence of the so-called wire-code paradox. In summary, the 99.2% of children residing in homes with exposure levels < 0.4 µT had estimates compatible with no increased risk, while the 0.8% of children with exposures ≥ 0.4 µT had a relative risk estimate of approximately 2, which is unlikely to be due to random variability. The explanation for the elevated risk is unknown, but selection bias may have accounted for some of the increase. © 2000 Cancer Research Campaign

Keywords: EMF; cancer; childhood leukaemia; meta-analysis; pooled analysis; epidemiology

It is now twenty years since Wertheimer and Leeper (1979) published the first study suggesting an association between residential exposure to extremely low frequency magnetic fields (EMF) and childhood cancer. Ever since, this has been a controversial issue with the findings from several, but not all, subsequent epidemiological studies being consistent with an association, particularly with respect to residential exposure and childhood leukaemia (Portier and Wolfe, 1998). However, many of the reports have been based on small numbers of exposed cases, and despite intense experimental research no known biophysical mechanism to explain an effect has been established.

We conducted a pooled analysis based on primary data from nine studies on EMF and childhood leukaemia, addressing three specific questions:

1. Do the combined results of these studies indicate that there is an association between EMF exposure and childhood leukaemia risk, which is larger than one would expect from random variability?

2. Does adjustment for confounding from socioeconomic class, mobility, level of urbanization, detached/not detached dwelling, and level of traffic exhaust change the results?
3. Do the combined data support the existence of the so-called wire code paradox, that is, a stronger association between proxy measures of EMF and cancer than between direct measurements and cancer?

METHODS

The original plan for this project was to include all European studies that addressed the question of an association between EMF and childhood leukaemia and were based on either 24 or 48 hour magnetic field measurements or calculated fields. At the time five such studies were reported (Feychting and Ahlbom, 1993; Olsen et al, 1993; Verkasalo et al, 1993; Tynes and Haldorsen, 1997; Michaelis et al, 1998). In addition, a nationwide childhood cancer study was in progress and near completion in the UK (UKCCS, 1999). Since we were not aware of any other European study to be published in the near future, the inclusion of the UK study would give us a complete set of European studies. We felt that if we could also incorporate new studies from non-European countries this pooled analysis would be up to date and presumably stay current for several years. We were aware of three more studies in other parts of the world with compatible information that were all nearly

Received 23 May 2000

Revised 16 June 2000

Accepted 16 June 2000

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Table 1 Relevant characteristics for studies included in the pooled analysis

	Subjects			Exposure measures			Matching variables		Potential confounders								
	Cases	Controls	Year of diagnosis	Long measurements	Calculated fields	Wire codes	Sex	Year of birth	Area of diagnosis	Detached or other house	Mobility quintile	Study specific (no. of groups)					
												Common	Measure of social status				
												Social group	Mother's education	Income	Urbanisation	Car exhaust	Other
Canada	272	304	1990–94	✓		✓	✓	✓	✓	✓	✓		3		2		
Denmark	833	4746	1968–86		✓		✓	✓			✓	5			4		
Finland ^a	29	1027	1974–93		✓		✓	✓							2		
Germany	175	409	1992–95	✓			✓	✓	✓	✓	✓	2			3	2	2 ^c
New Zealand	86	80	1990–93	✓			✓	✓			✓		5		2		
Norway	148	572	1965–89		✓		✓	✓	✓	✓	✓	6			2		
Sweden	36	508	1960–85		✓		✓	✓	✓	✓	✓	4			2	3	
USA ^b	595	530	1989–94	✓		✓	✓	✓	✓	✓	✓			6	4		
UK	1073	2224	1992–96	✓			✓	✓	✓			7					

Specification of exposure information selected for the pooled analysis

Canada	Latest home inhabited before diagnosis for which a 24-hour bedroom measurement was available (may not be same home for long measurement & wire code)
Denmark	Latest home inhabited before diagnosis for which a calculated field was available
Finland	Calculated field for 12 months prior to diagnosis was provided especially for this exercise (may be average of values for more than one home)
Germany	Latest home inhabited before diagnosis (was home at diagnosis for almost all individuals)
New Zealand	Home inhabited at diagnosis
Norway	Latest home inhabited before diagnosis in which child lived in the power line corridor, field calculated for entire period
Sweden	Latest home inhabited before diagnosis in which child lived in the power line corridor, field calculated for entire period
USA	Latest home inhabited before diagnosis for which a record was available (may not be same home for long measurement & wire code)
UK	Home inhabited at diagnosis (UKCCS selection meant that the child must have lived there for previous 12 months)

^aCase control data generated from the original cohort; ^bacute lymphoblastic leukaemia only; ^cEast/West Germany.

completed or recently completed, so we could include those too (Linnet et al, 1997; Dockerty et al, 1998, 1999; McBride et al, 1999). Table 1 lists the studies and their relevant characteristics. A fourth study was also near completion in Ontario, Canada, but it was decided that since this study did not provide 24-hour indoor measurements, or anything similar to it, the exposure information in this study was not similar enough to justify inclusion (Green et al, 1999a,b). In effect, all large-scale published studies with extended indoor measurements or calculated fields were included in the pooled analysis with the exception of a few studies that were not population based.

The primary analyses reported here were all discussed and agreed upon prior to the commencement of the work. This included diagnostic categories, exposure definitions, time period for evaluation, cut points, confounders, and statistical methods. In addition certain analyses were done to confirm that the findings from these primary analyses were not dependent on these specifications and yet other analyses were done with an exploratory purpose.

This pooled analysis focused on childhood leukaemia, even though several of the studies also included other cancer diagnoses. The US study included only acute lymphocytic leukaemia (ALL). We did analyses both for total leukaemia and for ALL, but for brevity the more detailed results are given for total leukaemia. There was some variation with respect to age groups in the studies, and we decided to use the age interval 0–14 years.

Since we wanted the data to be as consistent as possible across studies, the data that we used from a particular study were

sometimes different from those that formed the basis for the original publication from that study. This was particularly the case with the exposure variables (Table 1). In effect, the study-specific results that we report in this article differ to various degrees from the results as reported in the original publications. These differences are biggest for the US study. Compared with the published results of the US study, the pooled analysis included fewer cases and controls (34 cases and 90 controls were excluded because 24/48-hour measurements were missing), limited the study period to the year prior to diagnosis rather than the five years immediately prior to diagnosis, restricted the number of residences for which measurements were utilized to one per subject rather than all homes resided in during the five years immediately prior to diagnosis, and used geometric means rather than arithmetic means.

In studies with long magnetic field measurements (24/48-hour), these were chosen as the primary exposure measure. The publication from the Canadian study uses personal measurements, but to achieve consistency with the other studies we chose to use the in-home measurements instead. In the UK, a two-phase measurement strategy was used, according to which 48-hour measurements were conducted when either a shorter measurement (108 minutes) or a characteristic of the residency indicated that EMF exposure was elevated. These measurements were all treated as long measurements because almost all elevated readings would come from 48-hour measurements. None of the adjustments to the measured exposure that were presented in the UKCCS analysis were used in the pooled analysis. (It should be noted that these adjustments had negligible effect.)

As a summation of all measurements for one subject, over the 24/48 hours, most of the centres used arithmetic means. We decided, however, to use geometric means from all studies, because they are less affected by outliers. For comparison we also analysed the data using arithmetic means. Therefore, each centre provided the geometric means as well as the arithmetic means, regardless of what they used in their original publication.

All centres without long measurements had calculated fields, i.e., calculations of magnetic fields based upon distance between the subject's home and the nearby power line, line characteristics, and load on the line. For these centres calculated fields were evaluated as the primary measure.

We also analysed wire-codes (i.e., a proxy measure of residential magnetic field level, based on the distance and configuration of nearby power lines) for all North American studies. These were classified and analysed according to the original Wertheimer–Leeper scheme (Wertheimer and Leeper, 1982). We also developed a European version of the wire-code, but eventually decided that the differences between the North American and the European distribution systems were too large to make this meaningful. The wire-code analyses, therefore, only included the North American studies.

With respect to the reference time for exposure characterization, there was considerable variation across studies. Residential measurement data were available for various periods from birth to diagnosis. We decided to aim for the average exposure during the last year prior to diagnosis for the cases and the corresponding age for the controls. We achieved this by using the exposure information for the home at the time of diagnosis for the cases and the home lived in by the matched control at the same age; when this information was unavailable we used instead the latest time period prior to diagnosis (Table 1). The reasons were that all studies could provide exposure data specified in this way and that exposure close to date of diagnosis is relevant to the hypothesis that EMF, if anything, would act as a promoter.

All studies utilized a matched case-control design, although the matching variables were not the same in all studies (Table 1). In Finland the original publication reported findings from a cohort study, but in preparation for this pooled analysis a control group was selected and the data were evaluated using a matched case-control design with 3 additional years of follow-up. Because we wanted to use as many as possible of the cases and controls to increase the flexibility of the analysis, we decided to ignore the matching. Instead we included adjustment for age and sex in all analyses, with age classified into one-year groups up to five years of age and then into five year groups. In all analyses, the measurement studies were also adjusted for socio-economic status, according to centre-specific definitions (Table 1). In addition, we adjusted for residence in the eastern or western part of the country in Germany.

One of the aims of this study was to test whether adjustment for any available covariate would have an effect on the summary relative risk estimates. In addition to the covariates included in the basic model, the following factors were available: socioeconomic status, mobility, level of urbanization, detached/not detached dwelling, and level of traffic exhaust. All of these variables were not available in all studies (Table 1). For socioeconomic class, level of urbanization, residential mobility, and traffic exhaust, the basic information and the definitions varied between centres as described in Table 1.

To estimate a summary relative risk across centres, a logistic regression model was applied to the raw data, with centres represented by dummy variables. We did this for measurement studies and calculated field studies separately but also across all studies. In the primary analyses, exposure was categorized in the four levels: $< 0.1 \mu\text{T}$; $0.1\text{--}<0.2 \mu\text{T}$; $0.2\text{--}<0.4 \mu\text{T}$; $\geq 0.4 \mu\text{T}$ and entered into the model with the use of dummy variables. The wire-code analyses were treated correspondingly. In addition, a similar analysis but with continuous exposure was conducted, the results of which are reported as relative risks per $0.2 \mu\text{T}$ intervals. This continuous analysis was also the basis for a likelihood ratio test of homogeneity of effects across studies.

RESULTS

Table 2 gives the absolute numbers of subjects by case/control status, study, and exposure level. In total there are 3247 cases and 10 400 controls. The UK provided by far the largest number of cases, while Denmark had the largest number of controls. In the highest exposure category ($\geq 0.4 \mu\text{T}$) there were 44 cases and 62 controls, with the largest number of cases from the USA and the largest number of controls from Sweden. Out of the 3247 cases, 2704 (83%) are ALL cases. The US study was restricted to ALL, which explains why the US numbers are the same in the left and right panels of Table 2.

In Table 3 we summarize the primary results for total leukaemia. For each centre the relative risks are estimated by exposure level and with adjustment for the basic potential confounders. Some of the studies are based on small numbers, particularly the highest exposure categories, and in some instances there are zero cases or controls. Although some of the centre-specific relative risk estimates are of little interest in themselves, particularly in the higher categories, all studies still provide information for the summary measures. The last column of the table gives the results of the logistic regression analysis with continuous exposure. The homogeneity test based on the continuous analysis across all nine centres resulted in a χ^2 with eight degrees of freedom of 10.7 corresponding to a P value of 0.22. The interpretation is that the variation in point estimates between the studies, is not larger than one would expect from random variability. We compared results for matched versus unmatched analyses to confirm that ignoring the matching did not introduce a bias. Because the results were similar, we only report the unmatched results.

Across the measurement studies, the summary relative risk is estimated at 1.87 (95% CI: 1.10–3.18) in the highest exposure category, with a corresponding P value of 0.01. The two lower categories have estimates close to unity. For the calculated fields studies the summary measure for the top exposure category is 2.13 (0.93–4.88), with a P value of 0.04.

In the very last line of Table 3, we give the summary relative risk estimate across all studies, regardless of whether the study is a measurement study or a calculated field study. We consider this an analysis based on the exposure measure that is closest to the specified magnetic field measurement and time period of study defined for the pooled analysis. The relative risk estimates in the two intermediate exposure categories are near the no effect value, while in the top category ($\geq 0.4 \mu\text{T}$) the relative risk estimate is 2.00 (95% CIs: 1.27–3.13), with a P value of 0.002. The continuous analysis gives a relative risk estimate per $0.2 \mu\text{T}$ of 1.15 (1.04–1.27) with a test for trend P value of 0.004.

Table 2 Absolute numbers of childhood leukaemia cases and controls by study and exposure level

Measurement studies										
Leukaemia cases	< 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total	ALL cases < 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total
Canada	174	56	29	13	272	151	50	26	12	239
Germany	156	12	5	2	175	130	10	5	2	147
New Zealand	76	6	4	0	86	64	5	3	0	72
UK	1018	38	13	4	1073	859	34	10	3	906
USA	418	111	49	17	595	418	111	49	17	595
Total	1842	223	100	36	2201	1622	210	93	34	1959
Controls	< 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total					
Canada	215	53	26	10	304					
Germany	380	21	6	2	409					
New Zealand	72	8	0	0	80					
UK	2099	91	26	8	2224					
USA	386	95	44	5	530					
Total	3152	268	102	25	3547					
Calculated fields studies										
Leukaemia cases	< 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total	ALL cases < 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total
Denmark	830	1	0	2	833	596	0	0	2	598
Finland	27	0	1	1	29	25	0	1	1	27
Norway	140	6	2	0	148	92	5	2	0	99
Sweden	27	3	1	5	36	17	1	0	3	21
Total	1024	10	4	8	1046	730	6	3	6	745
Controls	< 0.1	0.1–0.2	0.2–0.4	≥ 0.4	Total					
Denmark	4736	2	8	0	4746					
Finland	991	19	10	7	1027					
Norway	542	13	7	10	572					
Sweden	438	30	20	20	508					
Total	6707	64	45	37	6853					

Table 3 Total leukaemia. Relative risks (95% CI) by exposure level and with exposure as continuous variable (RR per 0.2 μ T) with adjustment for age, sex, and SES (measurement studies) and East/West in Germany. Reference level: < 0.1 μ T. Observed (O) and expected (E) case numbers \geq 0.4 μ T, with expected nos. given by modelling probability of membership of each exposure category based on distribution of controls including covariates.

Type of study	0.1–< 0.2 μ T	0.2–< 0.4 μ T	≥ 0.4 μ T	O	E	Continuous analysis
Measurement studies						
Canada	1.29 (0.84–1.99)	1.39 (0.78–2.48)	1.55 (0.65–3.68)	13	10.3	1.21 (0.96–1.52)
Germany	1.24 (0.58–2.64)	1.67 (0.48–5.83)	2.00 (0.26–15.17)	2	0.9	1.31 (0.76–2.26)
New Zealand	0.67 (0.20–2.20)	4 cases/0 ctrls	0 cases/0 ctrls	0	0	1.36 (0.40–4.61)
UK	0.84 (0.57–1.24)	0.98 (0.50–1.93)	1.00 (0.30–3.37)	4	4.4	0.93 (0.69–1.25)
USA	1.11 (0.81–1.53)	1.01 (0.65–1.57)	3.44 (1.24–9.54)	17	4.7	1.30 (1.01–1.67)
Calculated fields studies						
Denmark	2.68 (0.24–30.45)	0 cases/8 ctrls	2 cases/0 ctrls	2	0	1.50 (0.85–2.65)
Finland	0 cases/19 ctrls	4.11 (0.48–35.1)	6.21 (0.68–56.9)	1	0.2	1.15 (0.79–1.66)
Norway	1.75 (0.65–4.72)	1.06 (0.21–5.22)	0 cases/10 ctrls	0	2.7	0.78 (0.50–1.23)
Sweden	1.75 (0.48–6.37)	0.57 (0.07–4.65)	3.74 (1.23–11.37)	5	1.5	1.31 (0.98–1.73)
Summary						
Measurement studies	1.05 (0.86–1.28)	1.15 (0.85–1.54)	1.87 (1.10–3.18)	36	20.1	1.17 (1.02–1.34)
Calculated fields studies	1.58 (0.77–3.25)	0.79 (0.27–2.28)	2.13 (0.93–4.88)	8	4.4	1.11 (0.94–1.30)
All studies	1.08 (0.89–1.31)	1.11 (0.84–1.47)	2.00 (1.27–3.13)	44	24.2	1.15 (1.04–1.27)

In the measurement studies, because several of the relative risk estimates were higher when geometric rather than arithmetic means were employed the data were reanalysed using arithmetic means. Although the summary relative risk for all measurement studies was still elevated 1.59 (1.04–2.45), it was lower than that obtained when the analysis was based on geometric means.

While the primary categorical analyses were based on the pre-determined cut off points, we evaluated the robustness of the results by also using other cut off points. With 0.3–<0.4, 0.4–<0.5 and \geq 0.5 μ T as the three highest categories we found, across all studies

and for total leukaemia, relative risks of 1.60, 2.54 and 1.75, respectively.

The largest studies and therefore the studies that carry most of the weight in the summations are those from the US, Canada, and the UK. If the US study were to be excluded, the summary estimate for the highest exposure category would be reduced from 2.00 to 1.68 (1.00–2.83; $P = 0.03$). The exclusion of Canada would increase the summary estimates to 2.14 (1.27–3.61), while exclusion of the UK study would increase it to 2.29 (1.41–3.74). Table 3 also gives the expected number of cases in the highest category under the null

Table 4 Acute lymphocytic leukaemia. Relative risks (95% CI) by exposure level with adjustment for age, sex, and SES (measurement studies) and East/West in Germany. Reference level: $< 0.1 \mu\text{T}$.

Measurement studies	0.1– $<0.2 \mu\text{T}$	0.2– $<0.4 \mu\text{T}$	$\geq 0.4 \mu\text{T}$
Canada	1.33 (0.85–2.07)	1.44 (0.79–2.60)	1.65 (0.68–4.01)
Germany	1.29 (0.58–2.89)	2.19 (0.62–7.71)	2.21 (0.29–16.7)
New Zealand	0.71 (0.21–2.44)	3 cases/0 ctrls	0 cases/0 ctrls
UK	0.89 (0.59–1.34)	0.87 (0.42–1.84)	0.88 (0.23–3.39)
USA	1.11 (0.81–1.53)	1.01 (0.65–1.57)	3.44 (1.24–9.54)
Calculated fields studies			
Denmark	0 cases/2 ctrls	0 cases/8 ctrls	2 cases/0 ctrls
Finland	0 cases/19 ctrls	4.31 (0.50–37.2)	6.79 (0.74–62.6)
Norway	2.25 (0.78–6.55)	1.49 (0.30–7.45)	0 cases/10 ctrls
Sweden	0.88 (0.11–7.19)	0 cases/20 ctrls	3.46 (0.84–14.3)
Summary			
Measurement studies	1.07 (0.87–1.31)	1.15 (0.84–1.56)	1.95 (1.14–3.35)
Calculated fields studies	1.42 (0.58–3.45)	0.84 (0.25–2.81)	2.23 (0.88–5.65)
All studies	1.08 (0.88–1.32)	1.12 (0.84–1.51)	2.08 (1.30–3.33)

Table 5 Summary relative risks. (95% CI) for total leukaemia by exposure level based on best available measure with adjustment for potential confounders. Germany also includes East/West adjustment.

	0.1– $<0.2 \mu\text{T}$	0.2– $<0.4 \mu\text{T}$	$\geq 0.4 \mu\text{T}$
All studies but Finland			
Age, sex	1.07 (0.88–1.29)	1.11 (0.84–1.47)	1.91 (1.21–2.99)
Age, sex, SES	1.08 (0.89–1.31)	1.10 (0.82–1.46)	1.92 (1.22–3.02)
All studies but UK			
Age, sex, SES	1.18 (0.94–1.48)	1.15 (0.84–1.58)	2.28 (1.40–3.71)
Age, sex, SES, Urban	1.13 (0.90–1.42)	1.09 (0.79–1.50)	2.24 (1.37–3.67)
All studies but UK, Denmark, Finland, and NZ			
Age, sex, SES	1.20 (0.96–1.52)	1.15 (0.83–1.58)	1.97 (1.19–3.25)
Age, sex, SES, type of dwelling	1.21 (0.96–1.52)	1.15 (0.83–1.59)	1.97 (1.19–3.26)
All studies but UK and Finland			
Age, sex, SES	1.19 (0.95–1.49)	1.13 (0.83–1.55)	2.20 (1.34–3.61)
Age, sex, SES, mobility	1.18 (0.94–1.48)	1.14 (0.83–1.56)	2.20 (1.34–3.61)
Sweden and Germany			
Age, sex, SES	1.37 (0.71–2.64)	1.28 (0.47–3.51)	3.30 (1.24–8.81)
Age, sex, SES, car exhaust	1.36 (0.70–2.63)	1.27 (0.46–3.49)	3.24 (1.22–8.63)

Reference level: $< 0.1 \mu\text{T}$.

hypothesis. The total number of excess cases across all studies is 20, the largest number being contributed by the US study.

We then restricted these analyses to ALL. Since the ALL cases make up as much as 83% of all cases and since the controls are the same, the ALL results must be similar to the total leukaemia results. The results in Table 4 show that this is indeed the case, but in the highest exposure category the ALL relative risks are somewhat higher than for total leukaemia.

We also looked separately at other leukaemia to see whether the observed excess risk was restricted to the ALL group. The summary relative risk for other leukaemia was 1.42 in the highest exposure category, but based on only 4 exposed cases.

Next we addressed the issue of a possible effect of adjustment for more covariates. The results of this analysis are given in Table 5. In addition to the centres using different definitions of potential confounders we also faced the problem that all centres did not have data on all potential confounders. When we adjusted for a particular confounder we therefore included only those studies that have data on that confounder. Because of the centre specific differences in relative risks we could not compare the adjusted results calculated from only a subset of the studies to the basic model

results calculated from all the studies. Therefore, in Table 5 we present results with and without adjustment for a potential confounder for the group of studies that the estimates are based upon. As can be seen in Table 5, for none of the potential confounders does the adjustment result in anything but minor changes in any of the relative risk estimates.

The final issue is the so-called wire-code paradox. Table 6 has the results according to wire-code categories including a summary estimate for the two North American studies. In the table we also give magnetic field levels for each wire code category. The relative risk for the highest wire-code category is 1.24 (0.82–1.87) so these analyses do not provide evidence for the existence of such a paradox.

DISCUSSION

We did not find any evidence of an increased risk of childhood leukaemia at residential magnetic field levels $< 0.4 \mu\text{T}$. We did, however, find a statistically significant relative risk estimate of two for childhood leukaemia in children with residential exposure to EMF $\geq 0.4 \mu\text{T}$ during the year prior to diagnosis. Less than 1%

Table 6 Total leukaemia. Relative risks (95% CI) by wire-code with adjustment for age, sex, SES (local definitions) and mobility, number of subjects, and EMF levels based on subset of subjects with measurement on home used in wire code analysis.

North American studies	UG/VLCC ¹	OLCC ²	OHCC ³	VHCC ⁴
Canada	1	0.98 (0.66–1.46)	0.75 (0.52–1.10)	1.59 (0.90–2.82)
Case/control	151/154	77/77	83/105	39/23
USA	1	1.03 (0.73–1.44)	1.04 (0.71–1.51)	0.87 (0.47–1.61)
Case/control	177/173	119/115	88/87	24/26
All North American studies	1	1.01 (0.78–1.30)	0.89 (0.68–1.16)	1.24 (0.82–1.87)
EMF level, median in controls	0.04	0.05	0.08	0.11

¹Under ground/very low current configuration; ²Ordinary low current configuration; ³Ordinary high current configuration; ⁴Very high current configuration.

of subjects were in this highest exposure category. The results did not change following adjustment for the potential confounders. In addition, the existence of the so-called wire-code paradox could not be confirmed.

Earlier analyses of the hypothesis of an association between EMF and cancer have sometimes been criticized on the grounds that the findings might be a consequence of so-called data dredging. In order to avoid this and because this work has been a collaborative effort of a rather large group of investigators we specified which primary analyses we planned to do and how to do them before we commenced the analysis; this was before the results of several of the individual studies were known.

The fact that we had access to the raw data from each study gave us two substantial advantages. First, it allowed us to make the data from the various centres as compatible as possible, which was particularly important for the exposure variables. For example, it made it possible to use the same cut-off points in all studies, to use geometric means of the measurements, and to focus on exposure during the year preceding diagnosis. Second, we could arrange data in ways that were of little interest in themselves for some of the individual centres because of small numbers, but still of considerable interest for the total material. In particular this made it possible to analyse, in a consistent way, higher cut-off points than the commonly used 0.2 µT.

For the measurement studies, the findings may have reflected effects of selection bias due to non-participation. Differences were observed in several measures of socioeconomic status between cases and controls, particularly in the US study, with controls generally characterized by higher socioeconomic status than cases. In a recent analysis, Hatch et al found that exclusion of partial or non-cooperative participants from analyses of either in-home magnetic field measurements or wire-codes tended to increase the risk estimates for childhood leukaemia in the US study (Hatch et al, 2000). This was confirmed in the UK study in which there was a moderate association between a deprivation index and measured magnetic fields (UKCCS, 1999). This suggests that at least some of the elevation of risk estimates arose from differential participation of cases and controls.

Exposure measurements from both calculated and measured field studies are subject to error. Time-weighted average in a single 24- or 48-hour period immediately prior to diagnosis may not represent typical levels or the proper metric at the time period that is relevant for assessing risk of leukaemia, if any, and may not reflect the exposure of a child living in the home. Calculated fields are also averages over time and do not take individual characteristics of homes into consideration. Since elevated risk appears to be confined to only the small fraction of children who are highly

exposed and since we have no basis for determining the pattern of measurement errors in each study, we cannot reliably infer the underlying risk function that would be consistent with the observed risk pattern.

One feature of our results is the high degree of consistency between the group of studies with measured fields and the group of studies with calculated fields. This may be of significance when considering potential confounders because in the calculated fields studies, the dominant source of exposure is high voltage power lines, while in the measured fields studies internal sources (such as ground currents, household wiring, and exposures from electrical appliances) may predominate. In effect one would not expect the same confounders to be operating in these two types of studies. This may also be of significance when considering selection bias problems, because the calculated fields studies are using population registries in a way that makes selection bias a small issue. In this comparison between the measurement studies and the calculated fields studies, one must keep in mind, however that the calculated fields studies are small and based only on a total of 8 cases with exposure in the highest exposure category.

One of our goals was to see whether controlling for as many putative confounders as possible would change the results, but none of the covariates that we had access to changed the results in any substantial way when included in the models. On the other hand, none of these is an established risk factor for childhood leukaemia. Indeed, knowledge about risk factors for childhood leukaemia is very limited so one cannot exclude the possibility that adjustment for some other variable would have an effect. For the moment we can only conclude that mobility, traffic exhaust, type of dwelling, and urban/rural residency are not important confounders when studying EMF and childhood leukaemia.

An interesting finding in our analysis relates to the so-called wire-code paradox. In an earlier review, an expert committee noted on the basis of the earlier studies that there is a stronger association between markers for EMF exposure and leukaemia risk than between direct measurements and leukaemia risk (National Research Council, 1996). Our data based on subsequent studies do not support this. In fact, the two North American studies show no evidence of increased risk associated with residing in homes in high wire-code categories. It is also worth noting that the measured magnetic fields are low in all the wire-code categories. The reasons for the elevated risk estimates for high wire-code categories in the earlier North American studies are unclear, although considerable potential for bias has been noted for both studies carried out in Denver (Portier and Wolfe, 1998).

The results of numerous animal experiments and laboratory studies examining biological effects of magnetic fields have

produced no evidence to support an aetiological role of magnetic fields in leukaemogenesis (Portier and Wolfe, 1998). Four lifetime exposure experiments have produced no evidence that magnetic fields, even at exposure levels as high as 2000 μT , are involved in the development of lymphopoietic malignancies. Several rodent experiments designed to detect promotional effects of magnetic fields on the incidence of leukaemia or lymphoma have also been uniformly negative. There are no reproducible laboratory findings demonstrating biological effects of magnetic fields below 100 μT .

Our results have clear implications for future studies. The level of significance that we see for the excess risk at high exposure makes chance an unlikely explanation. Future studies will be of use only if the operation of selection bias and confounding can be adequately addressed, and if there are sufficient numbers with exposure over 0.4 μT .

In summary, for exposure up to 0.4 μT our data demonstrate relative risks near the no-effect level. For the very small proportion (0.8%) of subjects with exposure above 0.4 μT , the data show a two-fold increase, which is unlikely to be due to random variability. The explanation for the elevated risk estimate is unknown, but selection bias may have accounted for some of the increase.

ACKNOWLEDGEMENTS

We are indebted to the following scientists for input during the planning: Ben Armstrong, Anssi Auvinen, Ray Cartwright, Gerald Draper, Elisabeth Hatch, Kauko Heikkilä, Klea Katsouyanni, Ruth Kleinerman, Markku Koskenvuo, Corrado Magnani, Rolf Meinert, Sam Pattenden, Eero Pukkala, Alberto Salvan, Joachim Schuez, Lorenzo Simonato, and Sholom Wacholder. The study was supported by funding from the Commission of the European Communities, Contract no. BMH4-CT96-0186 and the Swedish Medical Research Council.

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Attachment 16

Published in final edited form as:

JAMA. 2009 January 28; 301(4): 393–403. doi:10.1001/jama.2009.7.

Genome-wide interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia

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Abstract

Context—Pediatric acute lymphoblastic leukemia (ALL) is the prototype for a drug-responsive malignancy. Although cure rates exceed 80%, considerable unexplained interindividual variability exists in treatment response.

Objective—Using a genome-wide approach, to assess the contribution of inherited genetic variation to therapy response and to identify germline single nucleotide polymorphisms (SNPs) associated with risk of minimal residual disease (MRD) after remission induction chemotherapy.

Design, Setting, and Patients—We performed a genome-wide interrogation of 476,796 germline SNPs to identify genotypes that predicted MRD in two independent cohorts of children with newly diagnosed ALL: 318 patients on St. Jude trials Total XIIIIB and XV and 169 patients on a Children's Oncology Group (COG) trial P9906.

Main Outcome Measures—MRD at the end of induction therapy, measured by flow cytometry.

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Author Contributions: Dr. Relling had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Yang J, Cheng, Relling; *Acquisition of data:* Campana, Downing, French, Devidas, Pui, Bowman, Willman, Borowitz, Relling; *Drafting of the manuscript:* Yang J and Relling; *Critical revision of the manuscript for important intellectual content:* Yang J, Cheng, Yang W, Pounds, Trevino, French, Evans, Pui, Camitta, Borowitz, Carroll, Hunger, Relling; *Statistical analysis:* Cheng, Yang W, Pei, Cao, Fan, Pounds; *Obtaining funding:* Campana, Evans, Borowitz, Carroll, Davies, Relling; *Study supervision:* Relling.

Results—There were 102 SNPs associated with MRD in both cohorts ($P \leq 0.0125$), including 5 SNPs in the interleukin 15 (*IL15*) gene. A high proportion, 21 of these 102 SNPs, also predicted hematologic relapse ($P < 0.05$). Of 102 SNPs, 21 were also associated with antileukemic drug disposition, generally linking MRD eradication with greater drug exposure. In total, 63 of 102 SNPs were also associated with early response, relapse, or with drug disposition.

Conclusions—Host genetic variability affected treatment response for childhood ALL, and germline variants may exert their effects on MRD by effects on leukemic cell biology and on host disposition of antileukemic drugs.

Introduction

The past three decades have witnessed steady improvements in treatment of pediatric acute lymphoblastic leukemia (ALL), with cure rates increasing from less than 10% in the 1960s to over 80% today. Such drastic advancement was partly derived from the identification of presenting clinical features (e.g. molecular subtype, leukocyte count, age) predictive of treatment outcome and subsequent implementation of risk-adapted therapy.^{1,2} The assessment of decreasing disease burden in response to therapy by sequential monitoring of minimal residual disease (MRD) status has now been integrated into risk stratification.³⁻⁵ MRD assays provide a direct assessment of early treatment response and are predictive of final treatment outcome, even after adjusting for other prognostic factors.⁶⁻⁹

Response to treatment varies during the 4-6 week phase of remission induction therapy, as exemplified by changes in early sequential MRD assays.^{4,8,9} Thus, some patients exhibit drastic depletion of their leukemia cells (from 100% to less than 0.01% leukemia cells in the bone marrow) within only 2-3 weeks of induction therapy, while others exhibit high levels of residual leukemia even after 4-6 weeks of therapy.

This interindividual variation in treatment response in cancer can arise from both tumor- and host-related factors; however, most prior studies focused on the former. Gene expression profiling of diagnostic leukemic blasts has identified tumor cell genetic features associated with outcome^{10,11} and drug resistance in childhood ALL.¹²⁻¹⁵ Much less is known about host genetic factors associated with cancer cure rates.¹⁶⁻¹⁹

Taking a global approach to identify host genetic factors that affect treatment response in ALL, we interrogated 476,796 germline single nucleotide polymorphisms (SNPs) for their association with MRD at the end of remission induction therapy. We studied two independent cohorts: children with newly diagnosed ALL treated on protocols at St. Jude Children's Research Hospital (St. Jude) and through the Children's Oncology Group (COG). We discovered 102 SNPs that were significantly associated with end-of-induction MRD in both cohorts. Further functional analyses indicated that many of these host genetic variations were likely to influence treatment response via affecting host disposition of antineoplastic drugs.

Methods

Patients

Two cohorts of patients were included (Table 1S), with approval of the Institutional Review Board. From St. Jude Children's Research Hospital Total Therapy protocols XIIIIB and XV, 371 children with newly diagnosed ALL had available germline DNA (i.e. collected at remission) and evaluable MRD status at the end of induction therapy. Of the ALL patients enrolled on the Children's Oncology Group (COG) P9906 study, 227 children had germline DNA and evaluable end-of-induction MRD status. The actual number of patients included in specific analyses is described below.

Treatment and MRD assessment

There were common and unique elements to the eligibility and treatment for the St. Jude and COG cohorts (Supplemental Fig. 1S), with details described elsewhere,^{20,21} (<http://www.acor.org/pedonc/diseases/ALLtrials/9906.html>). Common elements included daily prednisone, weekly vincristine, weekly daunorubicin, thrice weekly asparaginase, and intrathecal therapy including methotrexate. After 28 days of therapy, St. Jude patients received additional therapy with cytarabine plus etoposide (Total XIIIB) or cytarabine plus cyclophosphamide and 6-mercaptopurine (Total XV). MRD was studied in bone marrow at days 19 and 46 by flow cytometry, with the latter time point corresponding to the end of induction treatment.⁸ In contrast, COG patients finished the induction phase after 28 days of therapy, and MRD status was assessed using flow cytometry at day 8 (in blood) and at the end of the induction phase at day 28 (in bone marrow).⁷ For St. Jude, MRD status was categorized as negative ($<0.01\%$), positive ($\geq 0.01\%$, but $<1\%$), and high positive ($\geq 1\%$). In COG, MRD classification was nearly identical: negative ($\leq 0.01\%$), positive ($>0.01\%$, but $\leq 1\%$), and high positive ($>1\%$).

Diagnostic immunophenotype and molecular subtype analyses were performed as described.^{7,8}

Genotyping, genotype imputation and quality control

DNA (500 ng) was digested with restriction enzymes (XbaI and Hind III for 100K SNP chip, and StyI and NspI for 500K SNP chip), amplified, labeled and hybridized to the Affymetrix GeneChip Human Mapping 100K and 500K Sets according to the manufacturer's instructions.

SNP genotypes were coded according to the number of B alleles in the genotype call as determined using BRLMM,²² with the AA, AB, BB genotype calls coded as 0, 1, or 2, respectively. For genotypes that were not called by the BRLMM algorithm, we imputed the number of B alleles based on signal intensity and consistency with expected genotypes based on linkage disequilibrium,²³⁻²⁵ whenever possible.

SNPs with minor allele frequency (MAF) $< 1\%$ or call rates $< 95\%$ (i.e. the number of samples with definitive genotype call at this SNP is $< 95\%$ of the total number of samples typed for this SNP) were excluded (Fig. 1A); patient samples that failed to achieve 95% call rates (i.e. samples for which fewer than 95% of interrogated SNPs were successfully typed) were excluded (Fig. 1A and details in Supplemental Methods).

Genome-wide association analysis for MRD

MRD was treated as an ordinal variable, i.e. 1 for negative, 2 for positive, and 3 for high-positive, as defined above. In order to minimize confounding effects, patients with ALL subtypes (i.e. *E2A-PBX1*, *MLL* rearrangements, *BCR-ABL* ALL) that strongly predicted MRD and that differed in frequency between the two cohorts were excluded from the MRD analyses (Supplemental Table 1S). The final analysis included 476,796 SNPs, 318 St. Jude and 169 COG patients (Table 1 and Figure 1A).

SNPs associated with the end-of-induction MRD were identified by a three-step analysis (Figure 1B). Our goal was to find SNP genotypes that were associated with MRD in both cohorts—those that might be generalizable across treatment regimens for ALL. In step 1, we computed the statistical significance for each SNP genotype's association with MRD in each cohort separately. Rank (Spearman's) correlation was used for the test statistic, in order to account for both the ordinal nature of MRD and the gene dosage effect of genotypes. The P value was computed by a permutation-asymptotic hybrid method (see Supplemental Methods). An additive model was assumed, although the trend test is also reasonably robust to moderate

deviation from additivity.²⁶ In step 2, we determined the threshold for statistical significance by estimation of the false discovery rate (FDR) and an internal validation in each cohort. Using the P values obtained in step 1, in each cohort, FDR levels were estimated on a grid of per-test significance levels (P value cutoffs).²⁷ An internal validation (see Supplemental Methods) was then performed in each cohort. Based on the FDR estimates and the internal validation, a specific significance threshold ($P \leq 0.0125$) was chosen for each cohort to declare a set of SNPs for further investigation. In step 3, we used the COG MRD cohort to validate the top ranked SNPs ($P \leq 0.0125$) discovered in the St. Jude MRD cohort, and vice versa (bidirectional validation), using a rank-based inference procedure (Supplemental Methods). The 102 overlapping SNPs satisfying the significance threshold determined in step 2 (FDR estimation and internal validation) and step 3 (bidirectional validation) were prioritized for further bioinformatics and biological investigation, and analyses of association with additional relevant phenotypes.

Operating characteristics of the Spearman rank correlation test were determined via a simulation study (Supplemental Methods and Fig. 6S). The genotypes associated with MRD were also assessed by a pooled analysis that combined evidence across the two independent cohorts to provide a combined P-value for each SNP (Supplemental Methods). The FDR and the false positive report probability²⁸⁻³² (FPRP) for prioritized SNPs were estimated.

All statistical and computational analyses were performed using S-plus (Insightful Corp., Seattle, WA), R (www.r-project.org) and SAS (SAS Institute, Cary, NC).

Association of MRD SNPs with additional phenotypes

Antileukemic response: The relationship between the 102 overlapping MRD SNP genotypes and two additional leukemia response phenotypes was analyzed, in order to prioritize SNPs and to minimize the risk of false discoveries.

Patients were categorized into super responders, responders, and poor responders, based upon consideration of MRD status at two time points during the induction phase. MRD status was dichotomized as negative or positive, as defined above. Super responders were MRD-negative at the both early (day 8 in COG, day 19 in St. Jude) and later (day 28 in COG, day 46 in St. Jude) time points; responders were MRD-positive at the early time point but became MRD-negative at the later time point; and poor-responders had positive status at the later time point. The association between SNP genotypes and this MRD responsiveness phenotype was assessed by rank correlation in all evaluable patients in separate analyses of St. Jude (n=304) and COG (n=154).

The cumulative incidence of hematologic relapse (including isolated and combined hematologic plus extramedullary relapses) as a function of SNP genotypes in the combined St. Jude and COG cohorts was analyzed by Gray's test. Isolated central nervous system (CNS) relapse, isolated testicular relapse, combined CNS and testicular relapse, other relapse, lineage switch, second malignancy, and death in remission were incorporated in the analyses as competing events. Excluding individuals with *E2A-PBX1*, *MLL* rearrangements, or *BCR-ABL* ALL, 416 St. Jude and 180 COG patients were included in this analysis, overlapping with but not identical to the MRD cohorts as defined in Figure 1 and Table 1. Of these patients, 33 in St. Jude and 35 in COG experienced hematologic relapse (isolated and combined). St. Jude patients were divided into 4 strata according to their treatment protocol and risk classification, and COG patients formed the 5th stratum. Fine and Gray's cumulative incidence hazard regression model³³ was used to confirm the directional association with relapse for SNPs that achieved $P < 0.1$ in the Gray test.

Pharmacokinetic studies: Three pharmacokinetic phenotypic data sets were available from a subset of St. Jude patients for antileukemic agents used during remission induction. Patients in these three data sets overlapped with, but were not identical to, those studied in the primary St. Jude cohort for MRD.

The first data set included plasma clearance of etoposide determined on day 29 of remission induction therapy, in 101 patients enrolled on St. Jude Total XIIIIB.³⁴ Although etoposide was a component of induction therapy for only a subset of the St. Jude MRD cohort and none of the COG cohort, its elimination is mediated via *CYP3A*³⁵ and P-glycoprotein,³⁶ a common mechanism of elimination that also affects prednisone,^{34,37} vincristine,^{38,39} and anthracyclines,^{40,41} which were given to all patients in both cohorts.

The second data set included methotrexate plasma clearance in 319 patients treated on St. Jude Total XIIIIB²⁰ and Total XV²¹ protocols who received intravenous methotrexate as part of the early induction therapy. Although only a subset of the St. Jude MRD cohort and none of the COG MRD cohort received intravenous methotrexate, all patients in both cohorts received intrathecal methotrexate, which is known to distribute from cerebrospinal fluid to blood and exert a systemic antileukemic effect.⁴²⁻⁴⁴

The third data set included intracellular methotrexate polyglutamate accumulation in ALL blasts at 44 hours after receiving up-front methotrexate in 330 patients treated on St. Jude trials.^{45,46} Again, although intravenous methotrexate was given to and methotrexate polyglutamates were measured in only a subset of the St. Jude MRD cohort and none of the COG cohort, all patients in the MRD cohorts were exposed to methotrexate systemically via intrathecal injections.

The relationship between SNP genotypes and pharmacokinetic variables was analyzed using linear regression.

Results

Identification and validation of genomic loci associated with end-of-induction MRD

A total of 588,920 SNPs were genotyped in germline DNA of 371 St. Jude and 227 COG patients. After quality control procedures were applied (Supplemental Methods and Table 1S), 476,796 SNPs were evaluated in 318 St. Jude and 169 COG patients (Fig. 1A and Table 1). We analyzed the association between germline SNP genotypes and MRD status independently in the St. Jude and COG cohorts (Fig. 1B). A P value threshold of 0.0125 was established based on false discovery rate (FDR) estimates and an internal validation inference (Supplemental Methods and Figure 2S). Through a rank-based bi-directional validation, a significant impact of germline variation on MRD identified in the St. Jude cohort was validated in the COG cohort ($P=2.2 \times 10^{-6}$), and that identified in the COG cohort was validated on the St. Jude cohort ($P<10^{-11}$) (Supplemental Methods).

In total, 102 SNPs exhibited significant concordant association with end-of-induction MRD ($P \leq 0.0125$) in both the St. Jude and COG cohorts, with odds ratios ranging from 0.072 to 0.613 (median = 0.462) and from 1.63 to 7.42 (median = 2.18) (Supplemental Table 2S). Among these 102 SNPs, 50 were annotated to genes. Because 45 SNPs were clustered at 15 genomic loci by linkage disequilibrium (pair-wise $r^2 > 0.5$), these 102 SNPs represented 72 unique genomic loci (Supplemental Fig. 3S). A SNP in the *ST8SLA6* gene ($P=9.6 \times 10^{-8}$, combined cohort P value) had the strongest association with MRD but had no significant flanking SNPs and a relatively low MAF of 4% (Fig. 2, chromosome 10). The next highest ranked SNP (rs17007695) was in the *IL15* locus (Fig. 2, chromosome 4, Supplemental Table 2S) and was notable for strong ($P=8.8 \times 10^{-7}$, combined cohort P value) and comparable association with

MRD in both the St. Jude ($P=4.4\times 10^{-4}$) and COG cohorts ($P=2.3\times 10^{-4}$). Moreover, this SNP was flanked by four *IL15* SNPs (rs17015014, rs10519612, rs10519613, and rs35964658) that were also associated with MRD in both cohorts (Fig. 3A and Supplemental Table 2S), and these 5 SNPs were in linkage disequilibrium with each other (pair-wise r^2 from 0.48 to 0.97). Half of the St. Jude patients with the CC genotype, 35.6% of those with the CT genotype, and only 15.8% of patients with the TT genotype at the *IL15* SNP rs17007695 had detectable MRD at the end of induction therapy, with a similar trend observed in the COG cohort (Figure 3B). The CC genotype at *IL15* germline SNP rs17007695 was weakly associated ($P=0.0701$) with a higher *IL15* expression in leukemic blasts, and overexpression of *IL15* was associated with MRD in both cohorts ($P=0.0342$ in St. Jude and $P=0.0035$ in COG, Figure 4S).

All 102 SNPs remained significantly associated with MRD after adjustment for race, gender, leukocyte count at diagnosis, age and ALL subtypes (Supplemental Table 2S). To further explore possible confounding effects by race, we also examined the SNP vs. MRD associations in each major racial group. For instance, the GG genotype at rs13106616 was similarly associated with a lower risk of MRD across three race groups, although the allele frequency differed significantly by race (Supplemental Figure 5S). We also assessed the false positive report probability (FPRP) for these 102 SNPs and 82 (80.4%) exhibited $FPRP < 0.5$ (Table 2S), a level associated with replicated associations in other contexts.^{26,28-32}

Genome-wide association analysis for MRD using the 2-stage “discovery and validation” strategy—In addition to the bidirectional validation described above, we also present a genome-wide analysis for SNPs associated with end-of-induction MRD by following the “discovery and validation” approach. In the discovery stage, we computed the statistical significance for each SNP genotype’s association with MRD in the “discovery” cohort (St. Jude), estimating permutation-asymptotic hybrid P values for association with MRD as detailed in the Supplement. A P value threshold of 7×10^{-4} was arrived at by balancing the levels of false negative and false positive errors using the profile information criterion (Supplemental Figure 7S);²⁷ 624 SNPs met this threshold. In the second stage, these SNPs were then tested in the “validation” cohort (COG). Of these, 39 exhibited concordant associations at $P \leq 0.05$, significantly more than what would be expected by chance ($P=0.021$, Fisher’s exact test), and these are highlighted in Supplemental Table 2S. When the P value threshold was set at 0.0125 for the discovery cohort (St. Jude), 8635 SNPs met this cutoff, 330 of which were validated in the COG cohort with $P \leq 0.05$, exceeding what would be expected by chance ($P=1.8\times 10^{-9}$, Fisher’s exact test).

Relation of MRD-associated SNPs to other antileukemic response phenotypes

Although end-of-induction therapy MRD is highly predictive of long-term treatment outcome, the early reduction of leukemic burden during therapy is also informative.⁴⁷ Thus, nearly all patients with negative MRD at early time points (day 19 in St. Jude and day 8 in COG) remained leukemia-free. We examined which of the 102 overlapping SNPs could also distinguish patients who responded early (super responders, $n=145$ in St. Jude and $n=26$ in COG) vs. those who remained MRD-positive at the end of induction therapy (poor responders, $n=59$ in St. Jude and $n=52$ in COG), vs. individuals who were MRD-positive at the early time point but MRD-negative later (responders, $n=100$ in St. Jude and $n=76$ in COG). Of the 102 overlapping SNPs, 40 (40%) were also associated ($P<0.05$) with early response in both cohorts (Supplemental Table 3S).

Of the 102 SNPs, 21 were significantly associated with hematologic relapse ($P<0.05$) by stratified Gray’s test and in a cumulative incidence hazard regression model ($P<0.05$). For instance, there was a monotonic relationship between the number of copies of the C allele at

rs1486649 (an intergenic SNP) and the risk of hematologic relapse (Figure 4A and B, Supplemental Table 3S).

Relation of MRD-associated SNPs with antileukemic drug pharmacokinetics

To understand mechanisms by which host genetic variation might affect treatment response, we tested whether the 102 overlapping SNP genotypes were related to antileukemic drug disposition (Supplemental Table 3S). In total, 21 of the 102 MRD-related SNPs exhibited significant association with antileukemic agent pharmacokinetics, with 3 SNPs predicting more than one pharmacokinetic phenotype. Eight of 102 SNPs were associated with clearance of methotrexate (at $P < 0.05$); all 8 genotypes associated with positive MRD and greater drug clearance. Ten of the 102 SNPs were associated with the pharmacokinetics of etoposide, with 7 of 10 associating with positive MRD and greater drug clearance. Similarly, 6 of the 102 SNPs were significantly associated with the leukemic cell accumulation of methotrexate polyglutamates, with 5 of 6 associating with positive MRD and lower methotrexate polyglutamates. Thus, of 24 significant associations, 20 were directly consistent with a pharmacokinetically intuitive association with MRD, i.e. lower drug exposure translated into a higher level of MRD. Specific genotypes linked higher methotrexate clearance (decreased drug exposure) (Fig. 5A), lower accumulation of methotrexate polyglutamates in the leukemic blasts (Fig. 5B), and greater clearance of etoposide (Fig. 5C) with a higher frequency of MRD.

Comment

Eradication of malignant cells by chemotherapy is a composite phenotype which depends not only on the somatically acquired characteristics of the malignant cells but also upon inherent patient characteristics. Childhood ALL has long served as a prototype for a malignancy that is curable with drugs. Early assessments of MRD strongly predict cure rates, and are used to modify therapy.^{3-9,48} Eradication of MRD is affected by genetic characteristics of the blasts (e.g. the presence of the Philadelphia chromosome or the *TEL/AML1* translocation) and by host characteristics such as age.^{7,8} Using a candidate gene approach, a few germline genetic variations have been shown to affect the level of MRD,^{16,49} but this has not been previously assessed on a genome-wide level. Herein, we used an agnostic genome-wide interrogation to identify 102 germline genetic variations that affected the level of residual leukemia in two independent cohorts of patients, and found that a high proportion (63 of 102 SNPs or 61.7%) also affected early response, relapse risk, or antileukemic drug disposition.

One of the strongest signals from the genome-wide scan came from 5 SNPs located in and around the *IL15* gene, a proliferation-stimulatory cytokine.^{50,51} *IL15* can protect lymphoid tumors from glucocorticoid-induced apoptosis *in vitro*.⁵² and *IL15* expression in ALL blasts has been linked to both CNS involvement at diagnosis and an increased risk of CNS relapse.⁵³ Both higher *IL15* gene expression ($P = 0.0342$ in St. Jude and $P = 0.003$ in COG) and germline SNP genotypes were associated with an increased risk of positive MRD at the end of induction therapy (Supplemental Figure 4S), and we found a trend ($P = 0.0701$) towards a significant relationship between *IL15* germline SNP genotypes and *IL15* gene expression in ALL leukemic blasts. Several of the *IL15* SNPs that predicted MRD have been associated with enhanced *IL15* transcription/translation efficiency *in vitro*.⁵⁴ Thus, it is plausible that germline genetic variation in *IL15* plays a role in treatment response in childhood ALL via affecting *IL15*'s function or quantity in ALL blasts, and the fact that *IL15* SNPs were prominent from unbiased genome scans in two independently-treated cohorts points to its importance in determining ALL response, either as a prognostic marker or as a therapeutic target.

As genome-wide interrogations for pharmacogenetics are still in their infancy, there are no published whole-genome data linking polymorphisms with anticancer drug response. We had the opportunity to couple the findings from our genome-wide SNP interrogation for MRD with

three relevant host pharmacokinetic phenotypes: systemic clearance of two antileukemic agents (etoposide and methotrexate) and intracellular disposition of the latter. Although 4-8 different antileukemic agents were used in these two cohorts, remarkably, 21 of the 102 MRD-predicting SNPs we identified were also significantly associated with disposition of these two antileukemic agents. Although many additional genetic variations would be expected to be specific for antileukemic drugs other than methotrexate and etoposide, and might therefore account for some of the remaining 81 MRD-predicting SNPs, several of the pathways involved in methotrexate disposition and etoposide disposition (<http://www.pharmgkb.org>) are likely to be shared by other antileukemic agents. Particularly for etoposide, whose disposition involves cytochrome *P4503A* metabolism and P-glycoprotein excretion, it is likely that there is overlap in the genetic determinants of its disposition with those affecting anthracyclines, glucocorticoids, and vincristine.^{34,37-41} The majority (83.3%) of the associations between SNP genotypes and drug disposition were pharmacologically intuitive, with genotypes that predicted increased drug exposure linked to lower levels of MRD. Together, these results suggest that more attention be given to details of drug administration and risk factors for rapid drug clearance, in addition to the considerable attention already placed upon better risk classification of ALL to tailor therapy intensity.

There was also a high proportion (21/102) of SNPs that were associated with not only MRD, but also with the risk of hematologic relapse in both cohorts. This high percentage is somewhat surprising in that the post-remission therapy (which would ultimately be expected to have a significant effect on relapse risk) differed substantially in the COG and St. Jude cohorts. This secondary analysis does lend credence to the hypothesis that we did identify true associations between SNP genotypes and poor response.

Like all risk features, genotypes that are informative for pharmacogenetic phenotypes are likely to be highly dependent upon therapy. For this reason, we purposefully chose two cohorts (St. Jude and COG) that had received somewhat different remission induction regimens, with slightly different time points for the primary phenotype (MRD), to identify polymorphisms more likely to have prognostic significance across multiple therapeutic regimens. The advantage of our bi-directional statistical approach is that the SNPs we identified may be more likely to have external validity for other patient groups; the disadvantage is that we might have missed SNPs more specific to the few elements of therapy that differed between the cohorts.

It is important to consider race, both from the standpoint of its possible effects on antileukemic drug efficacy⁵⁵⁻⁵⁷ and from its influence on germline SNP allele frequency.⁵⁸ The influence of race on ALL cure rates may be due to differences among races in the delivery of care, patient compliance, frequencies of poor-prognosis ALL subtypes, or to differences in allele frequencies for germline polymorphisms.¹⁶ We found good agreement between self-declared race and that determined using ancestry-informative SNPs, and the 102 MRD-associated SNPs remained significant after adjusting for ancestry (Supplemental Table 2S). Thus, population stratification was unlikely to have affected the SNP genotype/phenotype associations we discovered, consistent with other recent studies.^{59,60} The fact that SNP genotypes maintained their significance after adjusting for race, despite some cases of substantial differences in allele frequency by race (Supplemental Figure 5S), suggests that inherent differences in ALL prognosis among racial groups are partly influenced by differences in allele frequencies among racial groups, which could in the future lead to “race neutral” (but genomically-based) individualization of therapy.

We acknowledge that despite the fact that these SNP genotypes were associated with MRD in two independent cohorts, there is a danger of false negative and false positive findings, especially when sample size is relatively small. However, phenotypes of interest in pharmacogenetic studies (e.g. *CYP2C9/VKORC* for warfarin^{61,62} and *TPMT* for

thiopurine^{49,63}) may have effect sizes that exceed those likely to be observed for multigenic common diseases (e.g. diabetes and arthritis),²⁴ and therefore smaller sample size may suffice in the former. By identifying 102 SNPs based on evidence of association in two independent cohorts, and also by further validation of 62% of these SNPs (Supplemental Table 3S) as associating with the related phenotypes of relapse, “super response” at days 8 or 19, and antileukemic drug pharmacokinetics, we have further decreased the chance for false discoveries. The SNPs we identified may be in linkage disequilibrium⁶⁴ with the truly causative genetic variants that have not yet been interrogated directly by our genotyping platform (Supplemental Table 4S). Importantly, few of the 102 polymorphisms we identified have previously been suggested as candidates for affecting anticancer drug efficacy, and approximately half of the genomic variants are not annotated to genes at all, illustrating the need to further explore mechanisms by which germline genomic variation affects interindividual variability in antileukemic drug response.

Although the acquired genetic characteristics of tumor cells play a critical role in drug responsiveness, our results show that inherited genetic variation of the patient also affects effectiveness of anticancer therapy, and that genome-wide approaches can identify novel and yet plausible pharmacogenetic variation. Such variation may be factored into treatment decisions in the future, by placing additional emphasis on optimizing drug delivery to overcome host genetic variation, in addition to the current emphasis on tumor genetic variation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We are indebted to all patients and their parents who participated St. Jude Total Therapy protocols XIIIB and XV and COG P9906 study, clinicians and research staff at St. Jude and COG institutions, Drs. Jeannette Pullen and Andrew Carroll for assistance in classification of patients with ALL, Tianhe Zhang for his help in data analysis.

Funding/Support: This work was supported by CA093552-02, NCI CA 51001, CA 78224, CA21765, CA R37 36401, and the NIH/NIGMS Pharmacogenetics Research Network and Database (U01 GM61393, U01GM61374 <http://www.pharmgkb.org>) from the National Institutes of Health; American Lebanese Syrian Associated Charities (ALSAC); and by CureSearch.

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Table 1
Patient Characteristics and Relation to Minimal Residual Disease.

	St.Jude		COG	
	Patients (%) n=318	Relation to MRD ** (P value)	Patients (%) n=169	Relation to MRD ** (P value)
Race *	Caucasian	246 (77%)	110 (65%)	
	African	45 (14%)	8 (5%)	
	Other	27 (9%)	51 (30%)	0.179
Gender	Male	181 (57%)	118 (70%)	
	Female	137 (43%)	51 (30%)	0.132
Age at Diagnosis	<1 year	2 (1%)	0	
	1-10 years	236 (74%)	51 (30%)	
	>10 years	80 (25%)	118 (70%)	0.057
WBC at Diagnosis	<50k cells/ul	237 (75%)	94 (56%)	
	>50k cells/ul	81 (25%)	75 (44%)	0.155
Lineage	B-lineage	248 (78%)	169 (100%)	
	T-cell	70(22%)	0	NA
Molecular Subtype	<i>TEL-AML1</i>	61 (19%)	3 (2%)	0.45
	<i>BCR-ABL</i>	0	0	
	<i>E2A-PBX1</i>	0	0	
	<i>MLL</i>	0	0	
	<i>rearrangements No Common Translocation</i>	257 (81%)	166 (98%)	NA
End-of- induction MRD	<0.01%	257 (81%)	111 (66%)	
	0.01-1%	53 (17%)	36 (21%)	
	>1%	8 (2%)	22 (13%)	

WBC: white blood cell count; MRD: minimal residual disease, NA: non-applicable

*

Race was assigned based on germline genotype of ~600,000 SNPs, as described in the Supplemental Information.

**

Association between MRD (treated as a categorical variable, as defined in the Methods) and patient characteristics was assessed using a χ^2 test.

Attachment 17

Childhood Cancer in the vicinity of the Sutro Tower, San Francisco.

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19th September 2002

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Childhood Cancer in the vicinity of the Sutro Tower, San Francisco.

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Abstract:

The Sutro Tower is a prominent structure on an elevated site in San Francisco. Since 1973 it has provided radio and TV signals for the San Francisco Bay region. There have been long-standing concerns about the health effects of this high-powered transmitter located in the centre of a large urban population. The *a priori* hypothesis is that RF/MW radiation is a Ubiquitous Universal Genotoxic Carcinogen. This is based on a number of occupational studies and previous studies that have shown elevated cancer rates in residential populations living in the vicinity of radar and RF/MW broadcast towers. It is supported by many laboratory studies showing that ELF and RF/MW signals damage DNA. Thus it is predicted that at residential levels of RF/MW exposure cancer rates will increase in the vicinity of the Sutro Tower. This is tested by using the childhood cancer data-set from 1973-1988 with residential locations analysed to see if there is elevation of cancer and possible dose-response relationships. All of the analyses support and together confirm the hypothesis, and the radial patterns eliminate potential confounding factors.

Key Words: Childhood cancer, Genotoxicity, Radiofrequency and microwave radiation, exposure assessment.

Introduction:

The objective of this study is to test the *a priori* hypothesis by analysing the spatial distribution of childhood cancer to determine its relationships to the emissions of RF/MW radiation from the Sutro Tower. The Sutro Tower is 977ft high, on a hill that is 921ft high and the top of the tower is at 1898ft (577m) above sea level. The majority of the antennas are within 220ft (67m) of the top of the tower. With the land within 6 km of the tower varying from near the sea level to over 100m, the relative heights of the antennas above the ground vary from about 400 to 570m (1).

The Sutro Tower, built in the early 1970's, initially had VHF FM stations and VHF TV antennas (30 to 300 MHz). The TV channels 2, 4, 5 and 7 with frequencies below 180MHz had a total Effective Radiated Power (ERP) of 616kW. Broadcasting commenced on 4 July 1973. Later channels 9, 20, 32, 38, 44 and 66 were added. Channels above 13 are in the UHF range (0.3 to 3 GHz). The total UHF ERP was 18.3MW. FM radio stations <105MHz, totalled 54.7kW. The 997kW VHF ERP has provided chronic high exposure levels close to the tower site and an undulating declining signal strength with distance. Measurements recorded in 1988 showed that the nearest residential street to the east of the tower had RF/MW field intensities in the range 6 to 33 μ W/cm², and to the west they were 1 to 7 μ W/cm². The UHF signals are more focussed towards the horizon with near equal peak heights at several separated radial distances.

The radiofrequency/microwave (RF/MW) emissions from the Sutro Tower, in the heart of San Francisco, have been a long-standing public health concern. The Department of Public Health of the City/County of San Francisco carried out a survey of childhood cancer in 1988 (2). This showed that in the Noe/Eureka Valley, the suburb to the east of the Sutro Tower, the number of childhood cancer cases from 1973-85 were 21 when 11.8 was expected (OR=1.78, 95%CI: 1.1-1.53). The suburb to the west of the Tower, East Sunset, had a lower rate (OR = 1.47, 95%CI: 0.83-2.39). It is likely that the signals to the west of the tower are weaker than those to the east. Broadcast engineers usually design horizontal antenna patterns to send the signal towards the largest population regions so this may account for some of the variations in regional cancer rates.

Several other studies have found elevated adult and childhood cancer in residential populations living in the vicinity of broadcast RF/MW transmitters, (3-7). Other studies (8-10) report significant increases in residential cancer rates in association with chronic radar exposure. Therefore there is considerable scientific epidemiological evidence supporting the public's concern. Szmigielski and Richter et al. (11, 12) reported that higher occupational exposures produced higher risks of cancer, notably haemopoietic/lymphatic cancer and brain tumors.

Selvin, Schulman and Merrill (13) used a White Childhood Cancer data-set from the San Francisco area for the period 1973-1988, derived from the Surveillance, Epidemiology and End Results (SEER) cancer registry. They chose four cancer types: Brain Cancer, Leukaemia, Hodgkin's Lymphoma and Non-Hodgkin's Lymphoma. Together they amount to around 50% of all Childhood Cancers. Selvin et al was not an epidemiological study but a study to evaluate three statistical methods for clustering. The centre point for their study was the Sutro Tower. They used the childhood cancer data-set as a working example. Their method included significantly transforming the spatial data distribution according to population density. They then analysed their sample clustering methods assuming a linear radial RF/MW exposure pattern.

There are four problems with this work relative to an epidemiologic approach:

- (a) the spatial population density method distorts the spatial position of the cancer cases and therefore significantly alters their distance and their RF/MW exposure relationship from the tower;
- (b) the radial RF/MW exposure pattern is not linear but follows a complex undulating pattern that varies according to the mix of VHF and UHF antennas on the mast;
- (c) They also misplaced the position of the Sutro Tower to 1.2km further west; and
- (d) They failed to compare the San Francisco cancer rates with the mean SEER cancer rates for the period. Hence there are four large sources of error in the original paper when it is used to relate the childhood cancer incidence to the RF/MW radiation exposure from the Sutro Tower.

Table 1 shows that all cancer rates are elevated with RR between 1.62 and 2.6. The overall San Francisco childhood cancer rates are highly significantly doubled, compared with the mean SEER data for the period. The high cancer rate poses the

question as to why the childhood cancer rates in San Francisco are twice the average. Selvin et al. (13) used the Sutro Tower as the center-point of their clustering study. Thus it is relevant to use their data in a corrected and more appropriate epidemiological fashion to determine whether there is any evidence that the RF/MW radiation from the Sutro Tower contributed to the increase in childhood cancer. If it does, then it is highly probable that it also causes increases in adult cancer and many other human health effects that are shown in occupation studies of RF/MW exposures.

Table 1: San Francisco White Childhood Cancer numbers and rates 1973-88, Selvin et al. (10), compared with age-specific, time adjusted SEER means. Using the total childhood population of 50686. Rates are per 100,000 p-yrs.

Cancer Type	Case Number	Rate	SEER Rate	Rate Ratio	95%CI	p-value
Leukaemia	51	6.29	2.41	2.61	1.61-4.2	0.000048
Brain Cancer	35	4.32	2.66	1.62	0.98-2.72	0.059
Hodgkin's Lymphoma	26	3.21	1.35	2.39	1.28-4.45	0.0048
Non-Hodgkin's Lymphoma	11	1.36	0.83	1.64	0.64-4.24	0.3
Total Cancer	123	15.18	7.25	2.09	1.53-2.81	0.000002

Selvin et al. provided spatial maps of the four childhood cancer types they chose for children residing on the San Francisco Peninsula. These are used here to evaluate the possible contribution of the Sutro Tower RF/MW emissions to the elevated cancer rates.

The Hypothesis:

Several previous published studies have found higher cancer rates, especially leukaemia, in the close vicinity of radars and broadcast towers (2-12). Since far-field RF/MW exposure includes whole-body RF/MW exposure, it could be a Universal Genotoxic Carcinogen. Elwood (14) reviewed six occupational studies. Elwood's Table 3 supports the hypothesis, showing elevated cancer across many body organs in multiple independent studies. Since the whole world is exposed to short-wave radio and satellite RF/MW signals, then RF/MW is also a Ubiquitous substance. This is especially true for urban populations who are exposed to many radio, TV and mobile phone transmitters. Many previous studies provide a basis for the hypothesis. The hypothesis being tested by this spatial childhood cancer data around the Sutro Tower is that RF/MW is a Ubiquitous Universal Genotoxic Carcinogen.

Discussion:

The residential exposures are extremely low compared with international standards. The standards are based on avoiding heat and electric shocks during acute exposures. Residential exposures are quite small and do not involve RF/MW induced heat and shocks. Therefore there needs to be a plausible cellular carcinogenic mechanism that works at extremely low chronic exposure levels. A common plausible mechanism for such situations is a genotoxic carcinogen. A genotoxic carcinogen has no safe threshold because it damages DNA cell-by-cell, producing mistakes in the DNA repair, leading to enhanced cell death and cellular neoplastic transformation. Elevated cancer

rates are biologically plausible if the disease agent is genotoxic. RF/MW radiation significantly enhances chromosome aberrations in many studies (15-36) and direct DNA strand breakage (37-44).

The assessment of health effects is guided by Hill (45). Sir Austin Bradford Hill sets out his “viewpoints” for assessing the level of evidence for to move from association to causation. Primarily the factors are temporality, strength of association and dose-response relationship. A plausible biological mechanism is not necessary but can add support to the assessment.

Dolk et al. (5, 6) investigated cancer rates around a high-powered regional broadcast tower at Sutton Coldfield, Birmingham, followed up with a 20-site study. Their discussion of results recognizes the complexities of radiation patterns but specific patterns were not used to interpret their results, Cherry (46). Cherry identifies two general radiation and radial cancer patterns. Pattern A is low near the tower, rises to a broad, relatively flat multiple peak and then declines with further distance from the tower. Pattern B is high near the tower and declines with distance in an undulating fashion. Radiation Pattern A is produced by UHF signals and Pattern B by powerful VHF signals being present. For cancer rates to follow Pattern B, by being high near the tower, there needs to be a large human population a Pattern B radiation signal and an RF sensitive cancer type, e.g. leukaemia or brain cancer. Of the 21 sites studied, only one, Sutton Coldfield, has a high population living near a powerful tower with VHF signals. This produces a Pattern B for adult leukaemia, based on 6 leukaemia cases inside 1km. All other cancers at Sutton Coldfield follow a Pattern A, including All Cancer, Skin Melanoma and Bladder Cancer. Within 2 km of the Sutton Coldfield tower there were elevated rates of adult malignant and benign brain cancer, Skin Melanoma, Male and Female Breast Cancer, Colorectal, Prostate and Bladder Cancer. All other UK sites show Pattern A for Leukaemia, consistent with the combination of population and radiation patterns.

Accordingly, Cherry, based on logical RF/MW and cancer rate pattern matching, concluded that Dolk et al. showed an indicative causal effect as the cancer followed the population and exposure patterns. This study is based on these principles.

A follow-up Sutton Coldfield study, Cooper, Hemmings and Saunders (47) used the leukaemia rates for the period 1987-1994. They show only the 0-2km and 0-10km cancer rates. The results for All Leukaemia are summarised in Table 2.

Table 2: Summary of all Leukaemia results from the follow-up Sutton Coldfield radio and TV tower, (47).

Exposed Group	0-2km			0-10km		
	Cases	O/E	95%CI	Cases	O/E	95%CI
Adult Females	8	1.23	0.53-2.42	159	1.26	1.07-1.46
Adult Males	12	1.40	0.72-2.44	174	1.09	0.93-1.25
All Adults	20	1.32	0.59-2.92	114	1.19	0.97-1.40
Child Females	0			11	1.13	0.57-2.03
Child Males	1	2.34	0.07-13.03	15	1.04	0.59-1.73
All Children	1	1.13	0.03-6.27	26	1.08	0.71-1.59

Despite the relatively small numbers the All Adult and All Children cancer data shows a gross dose-response with higher rates in the 0-2km group than the 0-10km group.

The Sutro Tower has high-powered VHF and UHF transmitters and a dense population living near the tower. Using the above principles it is predicted that there should be Pattern B cancer patterns, especially for Leukaemia/ Lymphoma, Brain Cancer and All Cancer. The small data-set may limit the numbers in the near-tower area.

Methods:

The spatial childhood cancer data was obtained and the childhood residential population distribution was estimated using a detailed survey, to produce a radial cancer rate table within 0.5km radial rings. The first analysis involves the cancer rates very close to the tower, <0.5km and <1.0km. The second analysis uses broad rings, mainly 1km, from 0.1 to 5km and compares the cancer rates in the rings to the mean rate >5km. The third analysis uses a radial cumulative cancer RR rate from the tower moving out towards 5km in steps of 0.5km. The fourth analysis considers the radial RF/MW patterns derived from measurements and theoretical methods, and compares the cancer rates to the probable mean direct household exposure regime. Radial exposure patterns are non-linear, making log-linear trend analysis possibly more appropriate.

The 2x2 statistical analyses were carried out using EPI INFO 6 software package provided by the U.S. Center of Disease Control. Trend analysis uses linear and log-linear Least Squares Fit (LSF) with 2-tailed t-test significance. When the case sample size is 5 or more then Mantel-Haenszel statistics are used. If the case sample is smaller than 5 then the Yates Corrected Chi Squared and the Fisher Exact p-value are used.

Exposure-cancer rate comparisons were made using the Pattern A and B analysis of Cherry (46).

Personal Mean versus Direct Exposures:

Buildings, including residential homes, provide RF/MW signal blocking inside and in their "shadow". The extent of blocking varies with the type and thickness of the building materials. The actual internal exposure levels are much lower than the direct exposures used in this analysis. McKenzie, Yin and Morrell (48) took RF exposure measurements around TV masts in Sydney, Australia. At a residential site the direct signal strength measured at the roof level was $3\mu\text{W}/\text{cm}^2$. At the outdoor ground level it was $0.066\mu\text{W}/\text{cm}^2$ and inside the house it was $0.017\mu\text{W}/\text{cm}^2$. The shadow effects of trees and buildings between the tower and the site reduce the signal by a factor of 45. The building material also reduces the radiative signal strength by a factor of 175 times lower than the direct roof level signal. Since the indoor field strength is less than 0.6% and the ground level outdoor field strength is near 2 % of the direct exposure signal, a conservative estimate of the actual personal chronic childhood mean exposure is about 2-3% of the direct outside exposure. The estimate also allows for time away from home.

The adjustment factor would only alter the slope of the trend, increasing the RR gradient 30 to 50 times higher and not changing the intercept if it is close to zero.

Spatial Childhood cancer data-sets:

The cancer data-set provided by Selvin et al. (13) involves spatial distributions of four White Childhood (aged <21 years) Cancer incidence cases in San Francisco for the 16-year period 1973-1988. This data contained a total of 123 cases of childhood cancer from a population of 50,686 White Children. This provided Selvin et al. with sufficient cases to carry out their cluster analysis but it is a small data-set but adequate for a preliminary spatial epidemiological study. The data included 51 cases of leukaemia, 35 cases of Brain Cancer, 26 cases of Hodgkins Lymphoma and 11 cases of Non-Hodgkins Lymphoma cancer. Selvin et al. estimated that these categories of cancer cover close to 50 % of All Childhood Cancers.

The residential locations of the four types of cancer are provided on separate maps. Each map was enlarged. The cancer case locations for each type of cancer were determined using a computer linked digitizing pad. This formed the database of 123 cases with x and y co-ordinates, radial distance from the Tower and the cancer type, Figure 1 and Table 3.

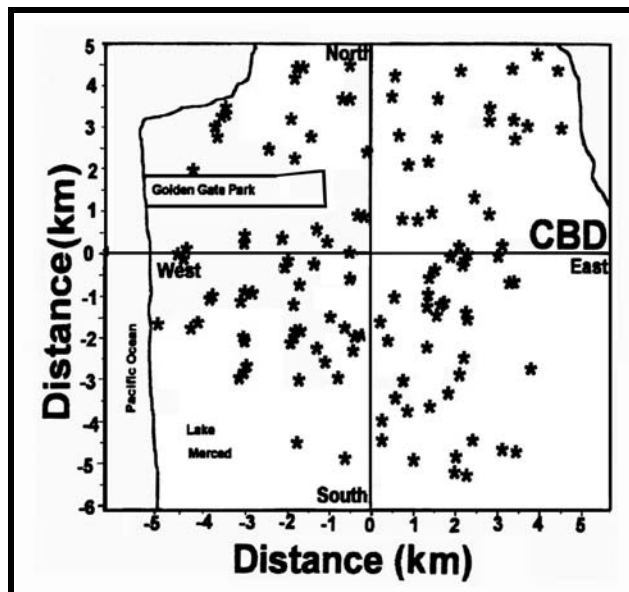


Figure 1: Spatial map of White Childhood (<21 years) Cancer for San Francisco, 1973-88, constructed from Figure 2(a) Selvin et al. (13). The Sutro Tower is at the intersection of the north-south and east-west lines.

All cancer incidence residential sites were placed on a detailed street map of San Francisco to confirm that they corresponded to residential locations. The spatial uncertainty, based on the ability to locate the centre of the mapped points and normal measurement uncertainty, is estimated as less than $\pm 100\text{m}$. However the original positions are based on the centre of the census tracts rather than actual residences. This adds about 100m to the position uncertainty of the actual residential location.

Table 3: Data analyzed into 0.5 km radial rings, centered on the Sutro Tower, including the estimated residential and the white, 0-<21, Childhood Cancer cases, 1973-1988. Total Cancer is the sum of the four cancers cited, amounting to about 50% of all Childhood Cancers (13).

Ring Range Km	RDF	Population Est.	Cancer cases			Total Cancer
			Brain Cancer	Leukaemia	Lymphoma	
0.1-<0.5	0.26	144	2	-	-	2
0.5-<1.0	0.44	750	1	2	2	5
1.0-<1.5	0.57	1625	4	6	0	10
1.5-<2.0	0.61	2430	3	8	2	13
2.0-<2.5	0.71	3610	3	6	6	15
2.5-<3.0	0.75	4700	3	6	7	16
3.0-<3.5	0.75	5515	3	3	4	10
3.5-<4.0	0.70	5980	5	6	7	18
4.0-<4.5	0.62	6000	4	2	3	9
4.5-<5.0	0.56	6060	4	6	4	14
>5.0	0.40	13872	3	6	2	11
Total		50686	35	51	37	123

For a public environmental health study, a total sample size of 123 cancer cases is considered quite small for a spatial analysis, especially when cancer sub-types are separately analysed. This small number means that unless the evidence in support of the hypothesis is extremely strong then it will be unlikely to produce significantly elevated cancer rates or significant dose-response relationships.

Childhood population spatial patterns.

The population and cancer data were analysed in a pattern of circular radial in rings with incremental diameters of 500m, giving 10 rings out to 5 km. The population and cases outside 5 km is used as the reference group. A detailed street map of San Francisco was used to identify the mean residential area densities in each ring, giving a Residential Density Factor (RDF) for each ring. In the radii of >5 km, the ocean becomes more and more dominant. Areas where few children are likely to live were identified and adjusted for, including the central business district (CBD), the port area, parks and reserves such as the Lincoln Park, Golden Gate Park and Harding Park/Lake Merced, and unpopulated areas, such as the San Miguel Hills, and low population areas such as the Presidio Reserve. Close to the tower, to the east is Twin Peaks Reserve and to the northwest is Mt Sutro. General residential density assessments were made in several site visits. The nearest highly-exposed residential property was at least 100m from the base of the tower.

The RDF for most of the rings is given in Table 3. The RDF varied from 26% very near the tower to a maximum of 75% in the 2.5 to 3.5km rings. Outside this the RDF drops off with the inclusion of the CBD, ocean, port area, reserves, etc, to decline to a mean of 56% in the 5km ring. Apart from the <0.5km and >5km rings the population estimates are rounded to the nearest 5. The area >5 km is the reference group area. It contains the ocean, the CBD and several reserves but it also contains several dense population

areas, including the area north of the CBD from China Town to Telegraph Hill (4 cancer cases). Along the southern border there is the Bayview District and Ingelside (6 cancer cases).

A possible confounding or bias factor could be the large antenna farm at the northwest end of the ridge of the San Bruno Mountains. This is about 2-3 km from the cluster of cancers in the reference group along the southern boundary of the San Francisco City/County, Figure 1. The group includes 4 Brain Cancers, 4 Leukaemias and 2 Hodgkin Lymphomas. Since multiple studies show that RF/MW exposure elevates the cancer rate it is likely that the cancer rate in the reference group is elevated because of this situation.

Beyond 5km the area is primarily ocean except for the band across the south that includes Harding Park and John McLean Park and a segment is outside San Francisco. It also includes the Central Business District (CBD). The >5km area was analyzed as three 0.5 km bands, giving a mean RDF = 0.4 and a population of 13872. Table 3 shows the 0.5km circular rings around the Sutro Tower, with estimated population and reported childhood cancer types data from Selvin et al.

Exposure Modelling and Assessment:

Cherry (46) outlined the relationship between radial cancer rates and radial exposure patterns around broadcast towers in relation to Dolk et al. (5,6). Both the population density and the RF/MW exposure patterns need to be considered in epidemiological studies. As mentioned above, there are two general patterns. Pattern A is low, rises to a broad undulating peak and then declines. Pattern B is high near the tower and declines, undulating, with distance. Pattern B is associated with VHF signals that produce a peak inside 1km. The shorter the wavelength (and higher the frequency) makes it easier to focus the UHF signal towards the horizon. AM and FM radio stations use frequencies from 500 kHz to about 300 MHz (Medium (MF), High (HF) and Very High Frequency (VHF) radiation). High-powered TV stations usually use 300-800 MHz, in the Ultra High Frequency (UHF) range.

Figure 2 shows two examples of VHF ground levels radial exposure patterns. The first is from 1933 around the Empire State Building in New York (49,50). Both graphs show logarithmic variation and undulating signal strength. In contrast to the VHF signals in Figure 2 the UHF antenna patterns are more focussed towards the horizon, Figures 3 and 4.

In Figure 3 the Relative Field peaks at 0.5, 3.5, 5.7, 7.9, 10.1 and 12.3°. With the assumption of the height of the antennae at 460m, these peak angles correspond to ground level positions at 52.7, 7.5, 4.6, 3.3, 2.6 and 2.1km from the tower. The actual exposure intensity is a function of the square of the Relative Field and the inverse square of the distance along a beam. This results in the ground level peaks being closer to the Tower, especially for the most remote peaks. These adjustments are taken into account by the radial UHF pattern in Figure 4. Figure 4 shows the main beam peaks at 12.5km and the major side-lobe peaks at 6, 4.5, 3.2, 2.2 and 1.1km.

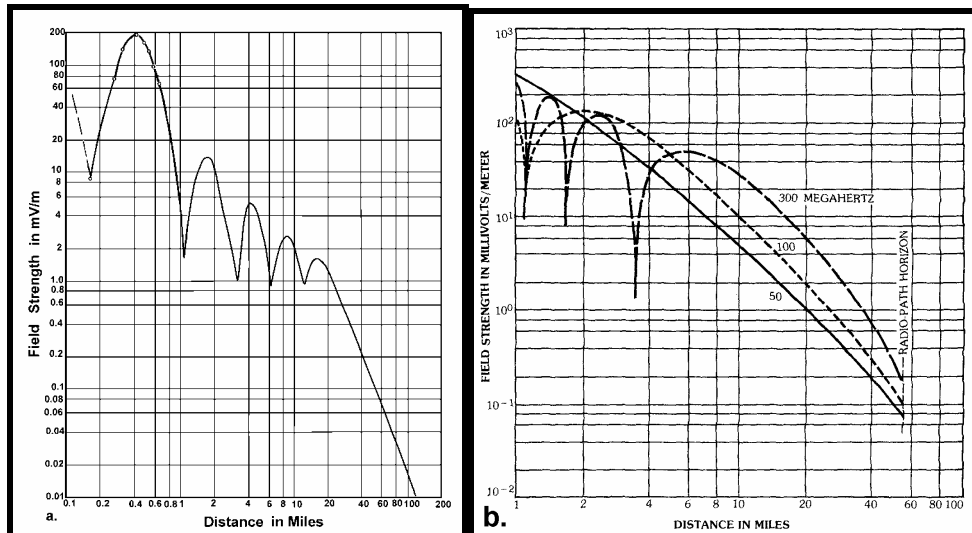


Figure 2: Ground level radiation pattern for (a) the 44 MHz (VHF) signal from the Empire State Building in New York City, Jones (12) by merging his figures 6 and 8, and (b) a theoretical set of 1 kW antenna at a height of 1000ft and a receiver at a height 30ft, Jordon (13). Field strength of 100mV/m=2.7nW/cm², 10mV/m=26.5pW/cm² and 1mV/m=0.27pW/cm².

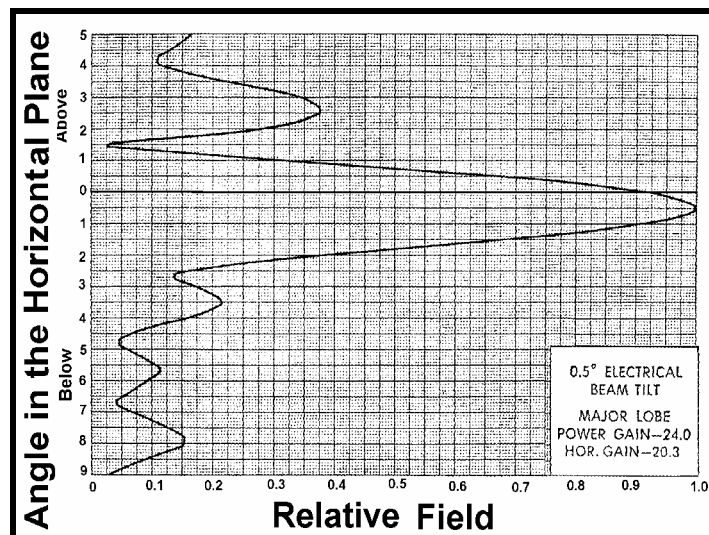


Figure 3: A typical Relative Field for a UHF RF/MW broadcast antenna from Hammett and Edison (1). The signal intensity is a function of the square of the Relative Field.

Because of the presence of powerful VHF signals, the Sutro Tower emissions follow a Pattern B radial shape. Hence the childhood cancer radial patterns are expected to also follow Pattern B if they are related to the RF/MW exposures from the Sutro Tower.

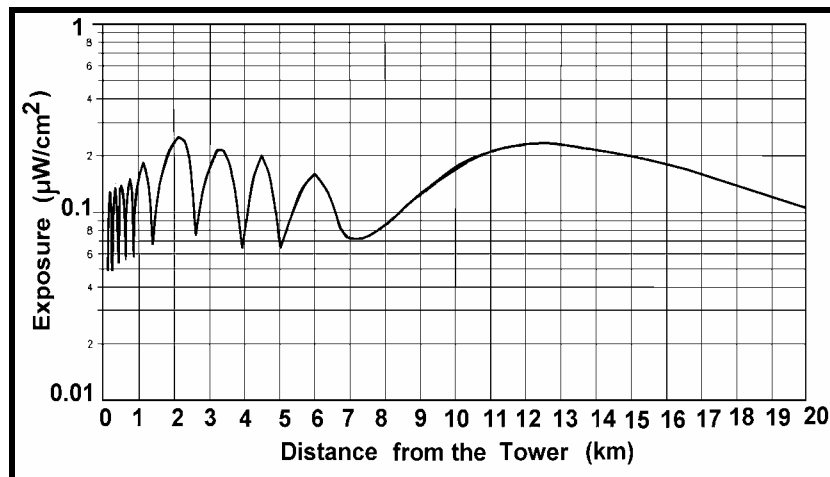


Figure 4: Ground level exposure for a typical UHF TV broadcast signal, from an antenna pattern (1), for an 18 MW EIRP transmitter at 460m AGL, for a flat horizontal surface.

Epidemiological Analysis Methods:

The highest exposures, due to the VHF signals are experience in residences within 100m to 1km of the tower. Hence cancer rates inside 500m and in the 0.5 to 1km ring are possible indications of the high RF/MW exposure. The data allows for a simple five 1km ring analysis for very high, high, middle, low and very low exposure, using the 2x2 analysis method to determine whether this reveals significant elevations and a dose-response. A radial cumulative analysis was used to determine if the radial distance from the Sutro Tower provides log-linear or linear correlation trends with cancer rates. A detailed radial ring analysis (0.5km ring bands) of cancer rates was compared with measured and theoretical radial RF/MW radiation patterns, to investigate the possible exposure-based dose-response characteristics.

The Sutro Tower broadcast facility was established in 1973 and the data in this study covers the period from 1973-88, following the producing of the public exposure to RF/MW from the Tower. The latency for childhood cancer is as short as 1 year. In the data-set involved 123 children, 21 of the children were diagnosed with cancer in the 0-2yr age range.

Results:

Close to Tower Childhood Cancer Rates:

The spatial cancer map, Figure 1, shows some circular patterns of high and low cancer rates and a high cancer rate in the immediate vicinity to the tower, <1 km. Within 500m of the tower there are 2 Brain Cancer cases, Table 3. Compared with the Brain Cancer rate in the very low exposure group (>5 km), this results in:

$$RR = 64.2, 95\%CI: 10.8-382, p=0.00103$$

The first 0.5km ring with at least one case of each cancer type is 0.5-1km. The cancer rates in the <1.0km ring are in Table 4.

Table 4: The Near Sutro Tower (<1km) Childhood Cancer rates compared with the remote >5km rates. (* Fisher Exact p-value for n<5).

Cancer Type	Cases	RR	95%CI	p-value
Brain Cancer	3	15.5	3.14-76.8	0.004*
Leukaemia	2	5.2	1.05-25.6	0.08*
Lymphoma	2	15.5	2.19-110	0.02*
Leukaemia/Lymphoma	4	7.8	2.34-25.7	0.0045*
All Cancer	7	9.9	3.84-25.4	<0.0000001

All cancer types are significantly elevated, except the lowest, Leukaemia, RR = 5.2, 95%CI: 1.05-25.6, n=2. For All Cancer the RR = 9.9, 95%CI 3.84-25.4, p<0.0000001. Brain Cancer (RR = 15.5) and Lymphoma (RR=15.5) are highly significantly elevated. The strength of the relationship of the All Cancer is classically causal, Hill (45). This occurs despite the very small sample size but the strength of the relationship is supported by several previous studies showing elevated cancer rates around broadcast towers.

Broad Ring (1km) Analysis:

The data set out in Table 3 was grouped into 1km rings out to 5km, with the cancer rates compared to the rates in the >5km remote ring, Table 5.

Table 5: The broad ring trend analysis with distance from the Sutro Tower, with Childhood Cancer rates relative to the remote >5km ring. The brackets show p-value adjusted for the single low data outlier.

Ring km	Brain Cancer		Leukaemia		Lymphoma		All Cancer	
	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
0.1-1	15.5	3.14-76.8	5.2	1.05-25.6	15.5	3.19-110	9.9	3.84-25.4
1-2	7.8	2.1-30.9	7.2	3.07-20.8	3.4	0.48-24.3	7.2	3.45-14.7
2-3	3.3	0.84-13.4	3.3	1.25-8.9	11.0	2.48-48.6	4.7	2.37-9.35
3-4	3.2	0.85-12.1	1.8	0.64-5.1	6.6	1.47-29.9	3.1	1.53-6.17
4-5	3.07	0.81-11.6	1.5	0.53-4.4	4.0	0.84-19.4	2.41	1.17-4.93
>5	1.00		1.00		1.00		1.00	
Trend p-value	0.03		0.02 (<0.005)		0.08 (<0.001)		<0.001	
Log/Lin Trend	p<0.001		0.05 (<0.03)		0.07 (<0.02)		<0.0001	

Table 5 shows significantly elevated childhood cancer rates in all 1km rings for All Cancer. For Brain Cancer all rates are significantly elevated for <2km and with a consistently declining with a significant linear trend, p=0.03, and highly significant log-linear trend, p=<0.001. Leukaemia and Lymphoma rates show quite variable patterns, especially for the small samples in ring <1km for Leukaemia and out to 2km for Lymphoma. They both show significant linear and log-linear trends, especially when the small sample outliers are removed. When all the data is combined to form the All Cancer trend, it is significantly elevated in all 1km rings and consistently declines with

distance. There is also a highly significant linear trend, $p=0.001$, and a log-linear trend, $p=0.0001$, Figure 5.



Figure 5: All Cancer around the Sutro Tower in a 1 km radial ring pattern, Log-Linear Trend $p<0.0001$.

These observations, through their strength of association, the level of significance and the dose-response relationships, especially for All Cancer, give considerable support to the hypothesis of an association between RF/MW exposure and risk.

Radial Cumulative Childhood cancer trends:

All methods used the group living further than 5km from the tower as the reference group. The cumulative incidence rate and Relative Risk was calculated from the 0.5km or 1km rings, in 0.5km increments out to 5km. This method is independent of the actual exposure assumptions since a uniform radial increment is used. The trend analysis results are summarized in Table 6.

Table 6: Trend p-values for the radial cumulative Childhood Cancers Relative Risk as a function of distance from the Sutro Tower, using 0.5 km increments, with linear, log-linear and inner ring outlier removed trends.

	Linear Original	Log-Linear Original	Linear Outlier Adj	Log-Linear Outlier Adj
Brain Cancer	<0.05	<0.005	<0.001	<0.0001
Leukaemia	<0.01	<0.02	<0.0001	<0.0001
Lymphoma	0.30	0.12	<0.005	<0.0001
Leukaemia/Lymphoma	<0.00001	<0.001	<0.00001	<0.001
All Cancer	<0.001	<0.0001	<0.0001	<0.00001

Table 6 shows a significant linear ($p<0.05$) and log-linear ($p<0.005$) cumulative trend for Brain Cancer, Figure 6.

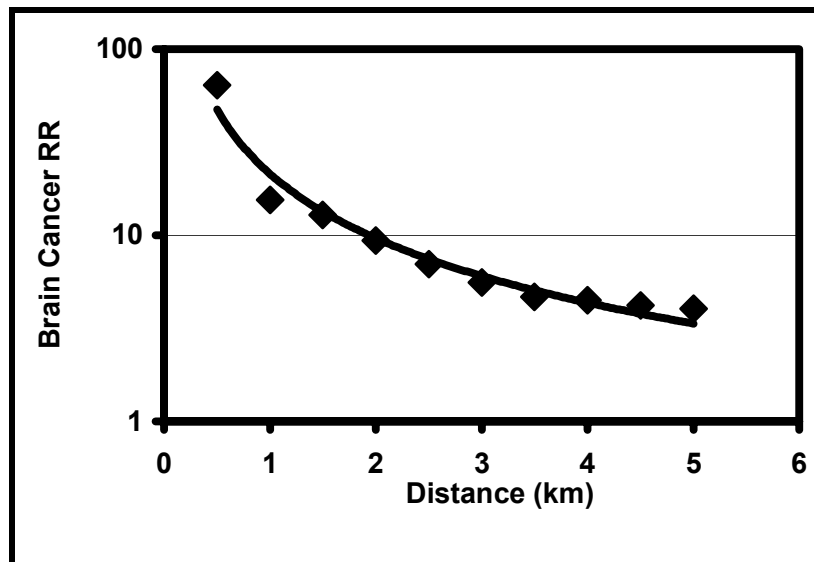


Figure 6: Radial cumulative Brain Cancer for childhood cancer in 0.5km radial segments around the Sutro Tower. Trends: linear $p = <0.05$, log-linear $p = <0.005$. The shown fitted line is a power law curve, $p < 0.00001$.

Inside 1.5km there were only 5 Leukaemia cases and 2 Lymphoma cases. Therefore Leukaemia and Lymphoma were combined for the radial analysis, Figure 7.

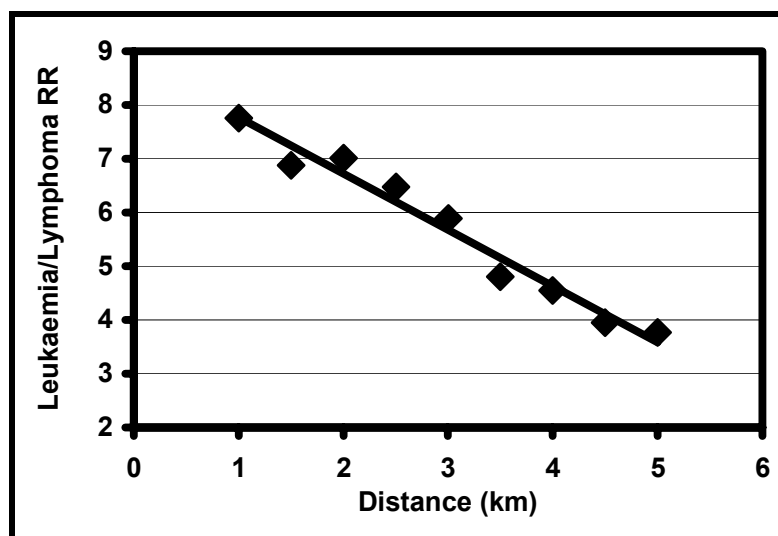


Figure 7: Radial cumulative Childhood Leukaemia/Lymphoma in 0.5km radial segments >1km, around the Sutro Tower. Trends: linear $p = <0.00001$ (shown curve), log-linear $p = <0.001$.

Removing the inner ring low sample outliers gives a highly significant trend, $p = <0.0001$. When all the data is combined to form the All Cancer set, both the linear and log-linear radial cumulative trends are highly significant, $p = 0.001$ and $p = 0.0001$, respectively, Figure 8.

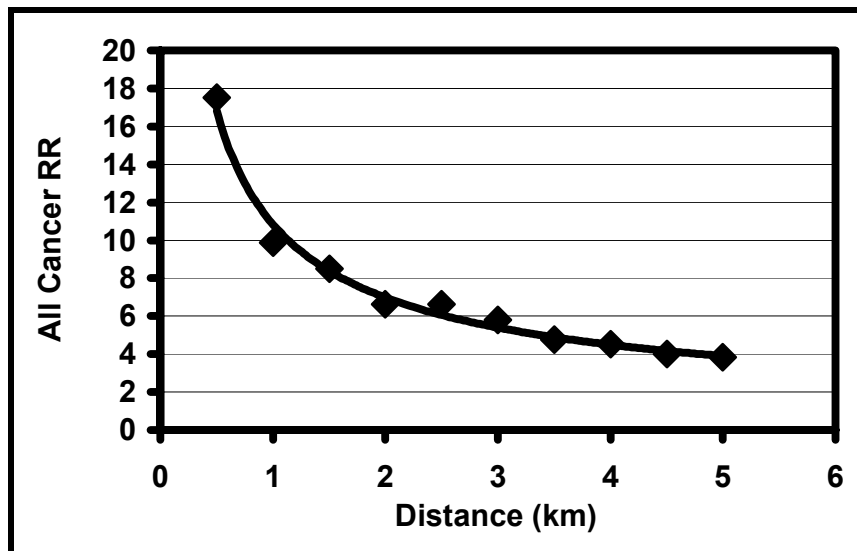


Figure 8: Radial Cumulative All Cancer for Childhood Cancer in 0.5 km radial segments >0.5km, around the Sutro Tower. Trends: linear $p = <0.001$, log-linear $p = <0.0001$ (shown curve).

Discussion of distance results:

The low sample size of cancer cases near the Tower produces lower RR rates inside 2km for Lymphoma and significant cumulative trends outside 2km. When all small sample outliers are removed the trends are all highly significant. The radial cumulative trend analysis shows that Brain Cancer, Leukaemia, Leukaemia/Lymphoma and All Cancer show totally significant radial cumulative trends that point towards the Sutro Tower as the primary cause of the elevated rates of Childhood Cancers in San Francisco.

The Sutro Tower radiation is ubiquitous over the whole study area and beyond. The only other genotoxic ubiquitous substance identified is the toxic air pollution from transport vehicle emissions. However, because the Sutro Tower is in a highly elevated site, surrounded by open land and suburban streets, the area has a small emission rate from vehicles and it is more well ventilated by wind. In contrast, the area outside 4 to 5 km contains many high vehicle volumes on busy roads and highways. Thus the transport spatial pattern is the reverse pattern of the radial cancer rates shown. Hence vehicle air pollution emissions are not a confounder of these results. There are no known sources of ionizing radiation or toxic water pollution specifically associated with living near the Sutro Tower.

This gives comprehensive, independent and direct support for the hypothesis and that the RF/MW radiation from the Sutro Tower is highly probably the source of elevated cancer rates in children living in San Francisco.

Actual and probable exposure patterns:

Figures 9 and 10 show the 0.5km Risk Ratios for Brain Cancer and All Cancer, respectively. They show high rates inside 1 km as shown in Dolk et al. (5) for Adult

Leukaemia, showing a Pattern B form, appropriate with having a high population density living close to tower and powerful VHF signals from the antenna.



Figure 9: The Childhood Brain Cancer Radial RR 0.5 km pattern around the Sutro Tower, showing a Pattern B consistent with the radiation Pattern B.

The All Cancer data only has brain cancer in the <0.5km ring, hence the RR is exceptionally low when it is included in the All Cancer data, Figure 10. Therefore the first point in the All Cancer data is reasonably treated as an outlier point.

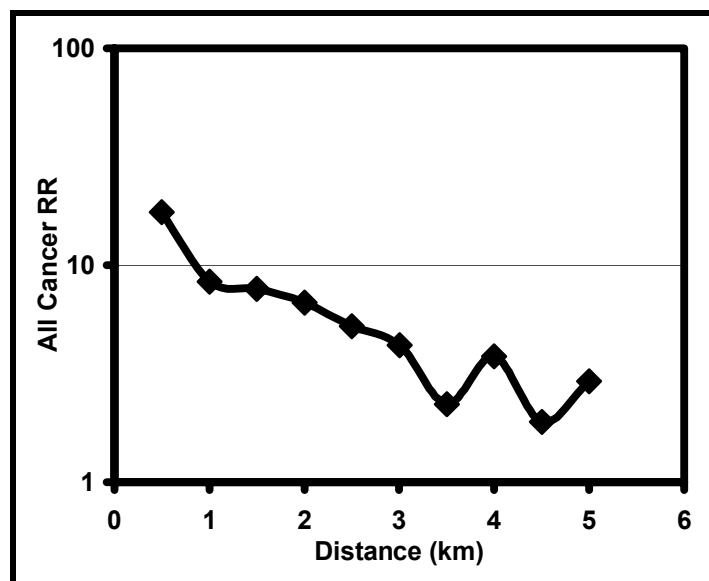


Figure 10: The All Cancer RR for Childhood Cancer in 0.5 km radial segments around the Sutro Tower, showing a Pattern B consistent with the radiation Pattern B.

Figure 11 sets out the 10 measured exposures in the 2 km radius, pointing to three peak and two minimum levels. The estimated exposure levels >2km are based on the UHF antenna pattern, Figure 4. The mixed VHF/UHF set of powerful antenna signals produce a Pattern B shape. Alternative exposure peaks are higher and lower because of the antenna vertical patterns, Figure 4. Because of the non-linear radial variation of exposure the patterns are presented with logarithmic exposures and RR levels. The

radial cancer rates are very similar to the broad shape of the exposure patterns. Both are Pattern B shapes. Hence the cancer radial patterns are close to the RF/MW probable radial pattern. Both Figure 9 and Figure 10 show a lower RR rate near 1km. This is associated with a lower intensity side-lobe, Figure 11.

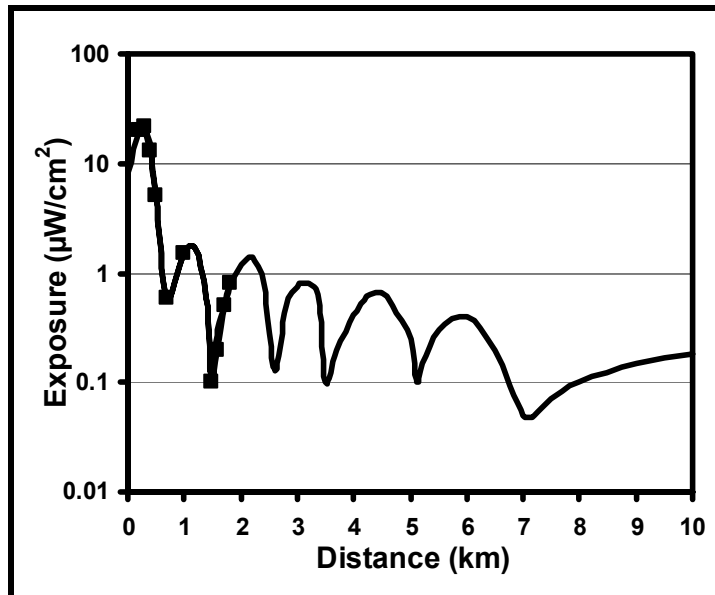


Figure 11: The probable radial RF/MW exposure pattern around the Sutro Tower estimated from 1988 measurements (circles) and the calculated radial exposure patterns from VHF and UHF antennas, Figures 2 and 4.

An exposure-based dose-response relationship is necessary for determining safety exposure standards, (51). Mean exposures for each 0.5km ring were taken from Figure 11. For the rings very close to the tower there are larger populations living in the outer half of the ring. Noting the logarithmic scale and the observation that within 500m of the tower the homes are generally in the range 300-500m, with more homes at the larger radius, giving a mean exposure estimate of $9\mu\text{W}/\text{cm}^2$. The 0.5km-ring mean direct exposure and the All Cancer rate was plotted in a dose-response graph, Figure 12, trend $p < 0.0001$.

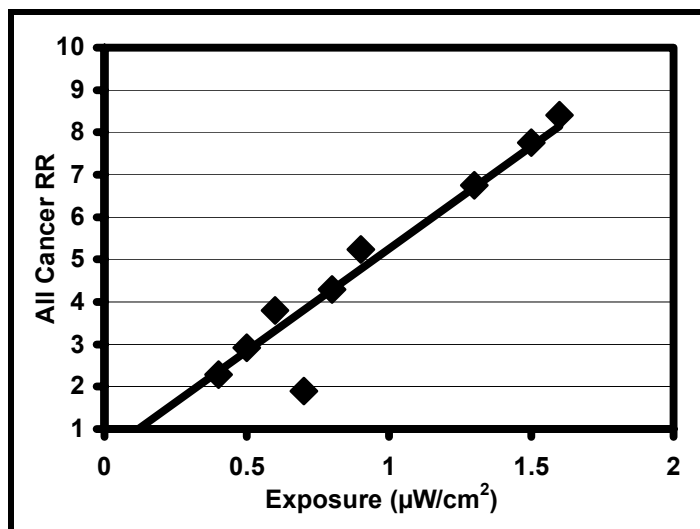


Figure 12: All Childhood Cancer as a function of measured and estimated RF/MW exposure in 0.5 km radial rings, ignoring the 9 $\mu\text{W}/\text{cm}^2$ outlier that only contained Brain Cancer. Trend $p < 0.0001$.

The trend line was fitted to ignore the outlier. The low exposure outlier near $0.7 \mu\text{W}/\text{cm}^2$ contains a very small population of Leukaemia/ Lymphoma cases. When it is removed then the trend has $p < 0.00001$. The Brain Cancer RF/MW exposure trend is extremely significant, $p < 0.00001$, Figure 13.

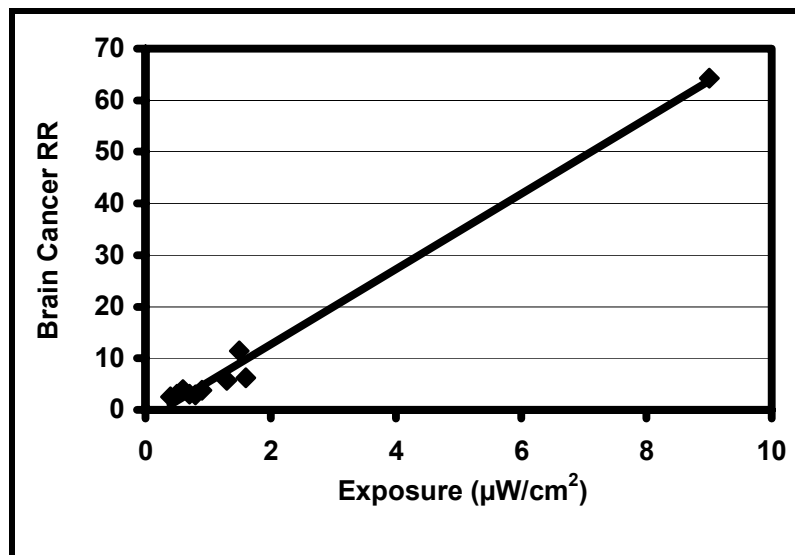


Figure 13: Childhood Brain Cancer as a function of measured and estimated RF/MW exposure in 0.5 km radial rings. Trend $p < 0.00001$.

The exposures used are the measured and estimated direct exposures. The actual chronic mean personal exposures are highly probably less than 2-3% of the direct exposure because of the shadow effects and household protection effects. All of the dose-response relationships are extremely significant and support the hypothesis that RF/MW is a Universal Genotoxic Carcinogen increasing the incidence of multiple cancer types with no safe threshold level.

This analysis shows that a fairly realistic approach accepts that the radial RF/MW radiation and cancer patterns vary in a logarithmic fashion with distance from the tower. This produces a linear exposure-based dose-response trend for cancer rates.

Recommendations for further research:

To further examine the hypothesis, this study should be extended to include all childhood cancers and all adult cancers. One data set could include the annual cancer age and site rates from 1960 to 2000 to evaluate the time series effects, including the introduction of the Sutro Tower exposure and in the later years the massive increase in other RF/MW sources throughout the city.

Spatial data for all cancer cases from 1970 to 2000 can be used to produce a time series of the spatial radial cancer rates. A detailed RF/MW spectrum exposure survey would assist the assessment of the spatial RF/MW exposures over time by considering

the introduction of each frequency from the Sutro Tower and any changes in the power output. It would also identify the range of frequencies and mean intensities of the over 2000 antennas now operating in San Francisco. The larger cancer and exposures data-sets would provide a basis to carry out a geographic analysis of the effects of hills, valleys and built-up areas.

Summary and Conclusions:

There are several previous studies showing elevated adult and childhood cancer around high-powered broadcast towers. Four previous studies also showed cancer associated RF/MW dose-response relationships (5-8). The previous epidemiological studies and the direct evidence of RF/MW genotoxicity was the basis of the hypothesis that RF/MW is a Ubiquitous Universal Genotoxic Carcinogen.

Close to the Sutro Tower there are very high and extremely significantly elevated childhood cancer rates where the mean exposure is relatively high but still a very small fraction of the Federal Communication Commission (FCC) standard. The five 1km-ring distance analysis shows dose-response relationships for each cancer type, and a highly significant linear or log-linear trends for the All Cancer, Leukaemia, combined Leukaemia/Lymphoma and Brain Cancer rates. The radial distance cumulative RR trends show cumulative show many significant trends that are extremely significant when small sample outliers are removed. The measured and theoretical radial exposure patterns are close to the 0.5km ring cancer radial rates in type B Pattern. The close similarity of the radial cancer and exposure patterns provides a basis to exclude confounder factors. These relationships result in highly significant exposure-based linear dose-response gradients, all pointing to the absence of a threshold for detectable risk.

Cherry (52) shows that the naturally occurring Schumann Resonance signal, with a mean field intensity of 0.1pW/cm^2 , is correlated with enhanced Human Cancer rates. Therefore every separate analysis in this study, the combined results taken together and with many previous studies, strongly support and confirm the hypothesis that RF/MW is a Ubiquitous Universal Genotoxic Carcinogen.

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Attachment 18

ELF magnetic fields, breast cancer, and melatonin: 60 Hz fields block melatonin's oncostatic action on ER⁺ breast cancer cell proliferation

Liburdy RP, Sloma TR, Sokolic R, Yaswen P. ELF magnetic fields, breast cancer, and melatonin: 60 Hz fields block melatonin's oncostatic action on ER⁺ breast cancer cell proliferation. J. Pineal Res. 1993;14:89-97.

Abstract: In this study we investigated whether a 60 Hz magnetic field can act at the *cellular level* to influence the growth of human estrogen-dependent breast cancer cells. Our experimental design assessed cell proliferation of a human breast cancer cell line, MCF-7, in the absence or the presence of melatonin which inhibits growth at a physiological concentration of 10⁻⁹ M. In three experiments, continuous exposure to average sinusoidal 60 Hz magnetic fields of 1.90 ± 0.01, 2.40 ± 0.70, and 2.53 ± 0.50 mG, or simultaneous exposure in matched incubators to average 60 Hz magnetic fields of 10.4 ± 2.12, 11.95 ± 2.73, and 11.95 ± 3.28 mG, respectively, had no effect on cell proliferation in the absence of melatonin. When MCF-7 cells were cultured in the presence of 10⁻⁹ M melatonin, an 18% inhibition of growth was observed for cells in a 2.40 ± 0.70 mG field. This effect was blocked by a 60 Hz magnetic field of 11.95 ± 2.75 mG. In a second experiment, a 27% inhibition of MCF-7 cell growth was observed for cells in a 2.53 ± 0.50 mG magnetic field, and this was blocked by a 60 Hz magnetic field of 11.95 ± 3.28 mG. These results provide the first evidence that ELF frequency magnetic fields can act at the *cellular level* to enhance breast cancer cell proliferation by blocking melatonin's natural oncostatic action. In addition, there appears to be a dose threshold between 2 and 12 mG. The mechanism(s) of action is unknown and may involve modulation of signal transduction events associated with melatonin's regulation of cell growth.

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Key words: melatonin—breast cancer—MCF-7
cells—60 Hz magnetic fields

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Received November 15, 1992;
accepted January 14, 1993.

Introduction

Exposure to ELF magnetic fields are reported to depress or time-shift melatonin secretion in animals [Wilson et al., 1990; Reiter and Richardson, 1990; Lerchl et al., 1991; Spears and Yellon, 1991; Stevens et al., 1992; Reiter, 1992] and such exposures are postulated to be a risk factor in human breast cancer epidemiology [Stevens, 1987; Tynes and Andersen, 1990; Demers et al., 1991; Matanoski et al., 1991]. The underlying mechanism(s) for eliciting such alterations in melatonin secretion *in vivo* are currently receiving much attention, and

the body of evidence supports the hypothesis that magnetic fields influence the pineal gland's control over the timed release of melatonin into the circulation. Once released into the circulation, melatonin itself acts at the cellular level and has natural oncostatic activity towards human estrogen-receptor-positive (ER⁺) breast cancer cells such as the MCF-7 [Hill and Blask, 1988; Blask, 1990; Cos and Blask, 1990; Cos et al., 1991]. This fact, plus the above findings that magnetic fields influence the release of melatonin into the blood stream, suggests that magnetic fields may play a significant role in breast cancer.

An experiment was designed to address a simple question: Do 60 Hz magnetic fields influence *in vitro* breast cancer cell proliferation, and is the presence of melatonin a modulatory factor in this process? The experimental design employed a well characterized cellular model system, the human breast cancer MCF-7 cell line [Hill and Blask, 1988; Blask, 1990; Cos and Blask, 1990; Cos et al., 1991], to evaluate any direct interaction between the magnetic fields and breast cancer cells, and to determine if melatonin mediated the interaction. A special feature of these *in vitro* experiments is that they relate to interactions at the cellular level which are distal to a magnetic field effect on melatonin release into the blood stream. Thus, the *in vitro* findings presented here complement important *in vivo* studies dealing with ELF magnetic fields and melatonin release into the blood stream. Preliminary results of this research were presented in abstract form [Liburdy et al., 1993b].

Materials and methods

Cells, hormones, and drugs

MCF-7 cells are derived from the pleural effusion of a mammary adenocarcinoma, and, when grown as a monolayer, they possess an epithelial-like morphology [ATCC HTB-22]. Melatonin-sensitive MCF-7 cells at passage #315 were a generous gift of Dr. David Blask of the Mary Imogene Bassett Hospital Research Institute, Cooperstown, NY. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM H-21), supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM) (UCSF Cell Culture Facility, San Francisco, CA), at 37°C in a humid atmosphere containing 5% CO₂. Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma Chemical Co. (Product # M-5250 [Lot # 22H7706] St. Louis, MO). Melatonin solution was prepared immediately before use by dissolving crystals in a minimum volume of ethanol and then adding media to achieve the appropriate concentrations.

Exposure systems

Two identical, double-wound, four-coil, Merritt exposure systems were fabricated at the Lawrence Berkeley Laboratory (LBL) for these studies [Merritt et al., 1983; Kirschvink, 1992]. Figure 1a and 1b illustrate the four-coil Merritt design in which there are four double-wound coils wrapped around a square plastic frame (35.5 cm/edge). With the

center axis of the coil system used as a reference line and the center point on this axis designating the origin, the four coils had the following spacing with respect to the origin: 16.7 cm, 4.23 cm, -4.23 cm, and -16.7 cm. Commercially available standard speaker cable with two parallel wire tracks was used for the double-wrap cable. Merritt's turns ratio of 26/11/11/26 was followed in winding these four coils (17.0 Ω , 6.57 mH) to optimize a large uniform exposure area in the center volume of the exposure system. Built into the circuit energizing these coils was a switch used to reverse the direction of the current in one of the wires in the speaker cable comprising the double-wound coils. When current is applied in the anti-parallel configuration (passive), the magnetic fields from the double-wound coils cancel, and when current is applied in the parallel configuration (active), a magnetic field is established. This feature enabled a true sham exposure to be performed in one incubator while a second coil system was operated simultaneously for exposure treatment in a second matched incubator. Each coil system was driven by identical signal generators available from Dynascan Corp., Chicago, IL (B&K Precision Model 3020). Each of the four coils comprising the exposure system was shielded by wrapping the wire bundles in two layers of heavy-duty aluminum foil, with a break of several inches, to eliminate the electric field components generated by the wire wrappings.

Field dosimetry was performed using commercially available fluxgate magnetometers. AC magnetic fields were measured using a Hewlett Packard Model 428B fluxgate meter (Cupertino, CA), calibrated by the Magnetic Field Measurements Group at LBL. DC magnetic fields were mapped using the above Hewlett Packard fluxgate meter, and a MAG-01 fluxgate magnetometer from Bartington Instruments, LTD (Oxford, UK). In addition, a FGM-3D1 fluxgate magnetometer from Walker Scientific, Inc. (Worcester, MA) was provided by Dr. Michael Yost of the School of Public Health, UC Berkeley. Active measuring volume of the HP and Bartington probes is a cylindrical volume of approximately 2.0 cm in length and 0.7 cm in diameter. DC field measurements using these three systems agreed to within 2–5%.

The coil systems were placed inside of two identical, water-jacketed Queue incubators (Queue Systems, Inc., Parkersburg, WV, Model 2710) maintained at $37 \pm 0.1^\circ\text{C}$. Identical commercial incubators were used in these studies, since the intensity of the ambient time-varying magnetic field flux density inside of incubators can vary considerably among manufacturers [Berglund et al., 1991;

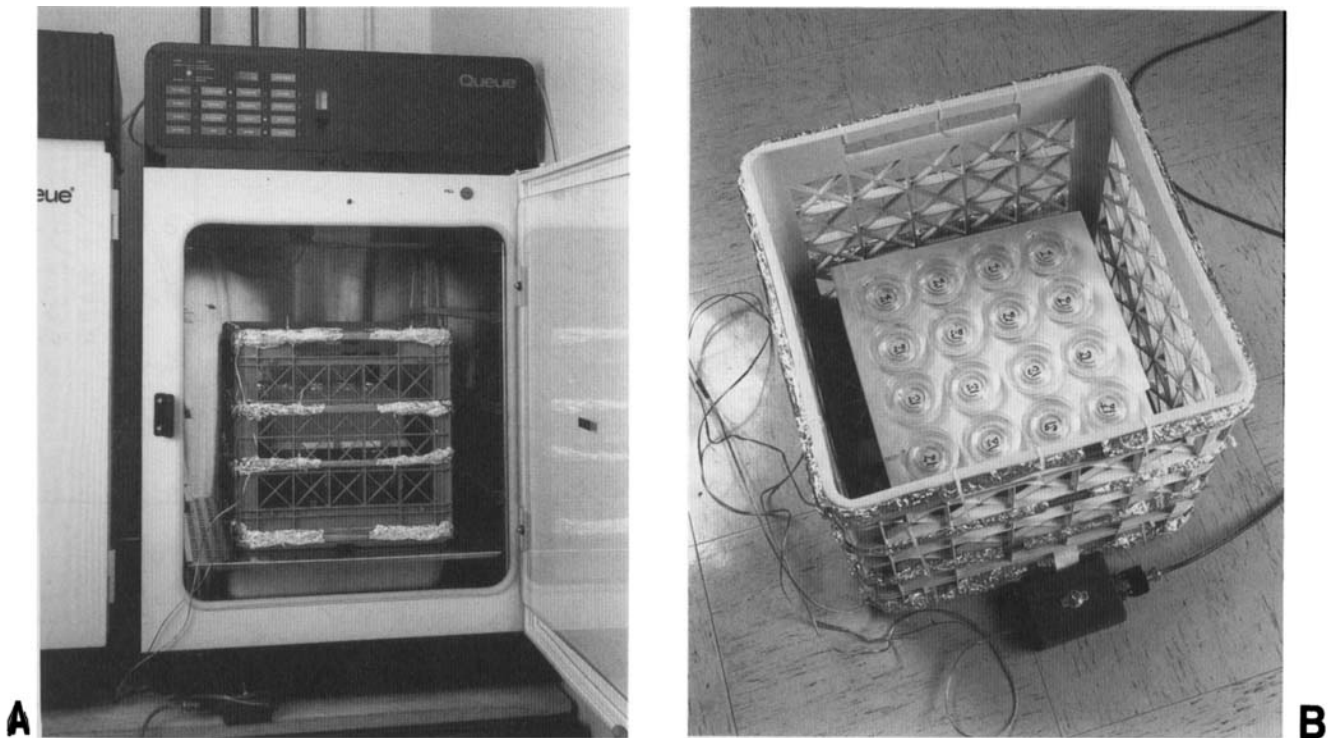


Fig. 1. Double-wound, four-coil Merritt exposure systems. (A) View of the exposure coil inside of a Queue Model 2710 incubator as used in these studies. (B) View of the exposure coil showing an array of plates positioned in the center plane of the coil. Two plastic frames were wound with double-wrap cable, and were energized in the parallel- or in the antiparallel-configuration so that identical current loads would result in a combined exposure magnetic field (parallel) or in canceled magnetic fields (antiparallel) for the exposure and the control treatment, respectively. Coils were shielded with aluminum foil to eliminate the electric field component. Magnetic field flux density was incident on the surface of the 35 mm tissue culture plates. (See text for details).

Yost and Liburdy, 1992]. When the above coils were operated, either in the active or passive mode, the total current load in the coils was approximately 6.57 mA and this corresponds to an ohmic power dissipation of 734 μ W. This did not result in significant heat loading inside the incubators; air temperature in both matched incubators during field operation was maintained to within $\pm 0.1^\circ\text{C}$, which is the nominal limit of temperature stability for these incubators. Three separate 60 Hz experiments were conducted in the studies presented here, and the inside of each incubator was extensively mapped before and after growth curves were completed. The average (mean \pm S.E.) of both measurements for the parallel (active) mode and antiparallel (passive) mode exposures, respectively, corresponded to (a) 10.4 ± 2.12 and 1.90 ± 0.01 mG; (b) 11.95 ± 2.75 and 2.40 ± 0.70 mG; (c) 11.95 ± 3.28 and 2.53 ± 0.05 mG. The Merritt coils used in these studies generate very uniform field values ($\pm 2\%$) over the central area where cells were placed [Kirschvink, 1992]. The variations about mean

values observed for these 60 Hz magnetic fields are attributed to the ambient background 60 Hz magnetic fields present in the laboratory during the seven month period in which these studies were conducted. DC fields were mapped at the same positions that 60 Hz fields were measured and values in incubator 1 and 2 were 142 ± 33 mG and 135 ± 6 mG, respectively. Plate positions in the exposure volume were systematically randomized during exposures, with matched active vs. passive exposures conducted simultaneously. Randomization of plates followed a pattern in which plates were rotated through exposure positions within the central volume of the coil, which was within the $\pm 2\%$ isocontour boundary of the Merritt coils [Kirschvink, 1992] on days during cell counting and feeding. Coils were left energized during the entire period of cell growth in these studies; thus, culture plates were removed for feeding and for the addition of melatonin with the fields continuously present. Cells were first placed in the energized active or passive coils after cell attachment (see below) when

melatonin was added. Time for feeding was kept to a minimum, and, on average, plates were out of the fields during feeding for approximately 5–10 minutes.

Culture techniques

MCF-7 cells were initially grown in 60 mm plates or T-75 flasks prior to experiments. Cells employed in these studies correspond to passage #318, 319, and 320. Cells were harvested in 0.2% EDTA phosphate buffer (2 g/L Na₂-EDTA, 8 g/L NaCl, 0.2 g/L KH₂PO₄, 1.15 g/L Na₂HPO₄), or with trypsin solution (0.50 g/L Trypsin, 0.5 g/L EDTA, 1.0 g/L glucose, and 0.58 g/L NaHCO₃) from the seeding vessel and diluted in DMEM. Cells were employed at seeding densities between $0.1\text{--}0.2 \times 10^5$ cells/35 mm culture dish in two ml of media. After cells became firmly attached, four hours after seeding, the plating medium was replaced with freshly prepared, melatonin-enriched medium (10^{-9} M) or DMEM alone. On alternate days, cells were harvested from plates in each treatment group by incubation with one ml of trypsin solution at 37°C for two min and counted using a hemocytometer; cell growth was expressed as cell/ml which is equivalent to cells/plate in these studies. Triplicate plates were used for each counting day. Fresh medium, with or without melatonin, as required, was fed to the remaining plates twice weekly. In these studies no gross morphological differences as revealed by transmitted light microscopy were noticed for MCF-7 cells grown in background compared to applied 60 Hz magnetic fields.

Statistical analysis

All data were tested for statistical significance using the multifactor analysis of variance program in Statgraphics (Manugistics, Inc., Rockville, MD, USA).

Results

Comparison of MCF-7 cell growth in matched incubators

We determined whether MCF-7 cells would exhibit identical growth curves in matched incubators in the presence of ambient background 60 Hz magnetic fields. The background magnetic field in the incubators was mapped and was found to fall within the range 1.9 ± 0.01 mG to 2.53 ± 0.50 mG. Field mapping in the laboratory room, as well as inside the incubators, indicated that the 60 Hz background field was similar throughout both incubators when unenergized or energized in the antiparallel mode. For these growth curves, cells at passage #319 were

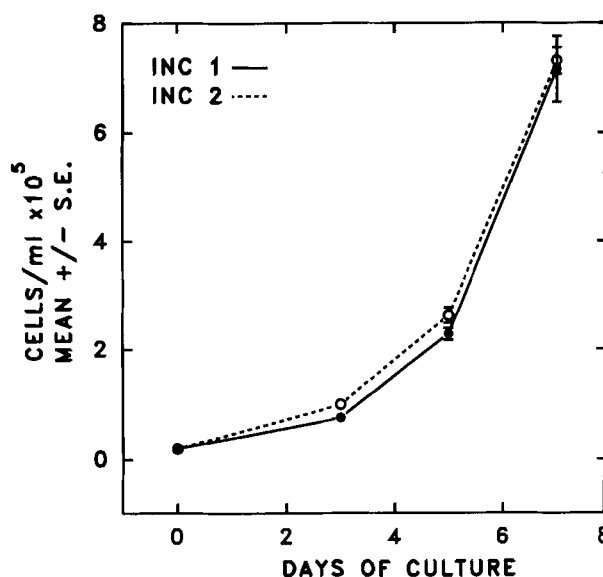


Fig. 2. Comparison of MCF-7 cell growth in matched incubators. Growth curves for MCF-7 cells from passage #319 for cells in matched Model 2710 Queue incubators 1 and 2. Cells were seeded at 0.15×10^5 /plate and cell proliferation was followed by hemocytometer count over seven days. Cells were positioned inside of the exposure coils with the coils unenergized (see text).

placed inside of the two incubators with the Merritt coils in place but unenergized, with the cells in the same position as used for exposure treatment. Figure 2 depicts growth curves for MCF-7 cells during simultaneous cell proliferation assays in incubators 1 (solid line) and 2 (dashed line). MCF-7 cells were seeded to attain exponential growth and to be 60–80% confluent by day 7; cell growth was observed to be identical in the matched incubators. This indicates that MCF-7 cells grow identically in the presence of ambient background magnetic fields in these two matched incubators.

60 Hz magnetic fields do not influence MCF-7 cell growth

Next we addressed the question of whether 60 Hz magnetic fields influence normal growth of MCF-7 cells in the absence of melatonin. Simultaneous cell proliferation assays were performed as described above for Figure 2, but with the coils in the matched incubators energized in either the antiparallel (passive) or in the parallel (active) configuration. Energization in the antiparallel configuration, which cancels the applied magnetic field, resulted in a similar magnitude ambient background magnetic field as those depicted in Figure 2. Three different growth curve experiments were conducted on different days with MCF-7 cells from different passages. Figures 3a, 3b, and 3c show typical expo-

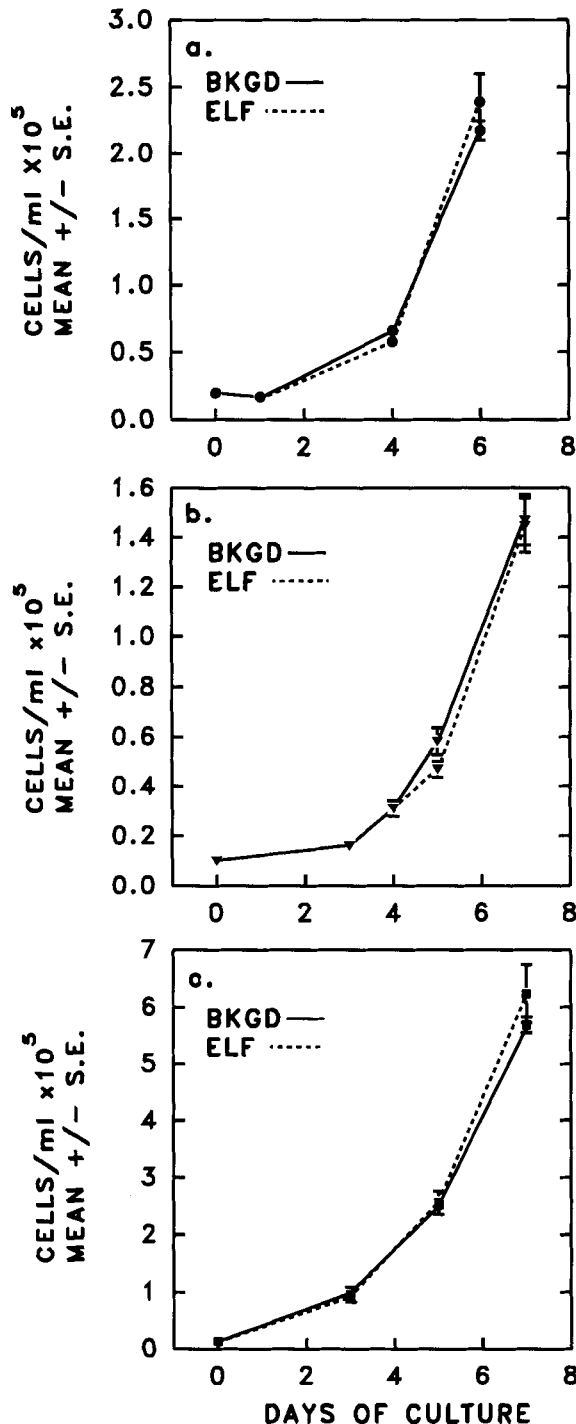


Fig. 3. 60 Hz magnetic fields do not influence MCF-7 cell growth (no melatonin). Cells from three different passages were characterized for cell proliferation in the absence of melatonin in three different experiments. Growth curves were performed as in Figure 2. Incubator 1 was used for 60 Hz (ELF) exposures and Incubator 2 was used for background (BKGD) 60 Hz field treatments. (a) Cells from passage #318 seeded at 0.3×10^5 /plate. BKGD: 1.90 ± 0.01 mG, ELF: 10.4 ± 2.12 mG. (b) Cells from passage #319 seeded at 0.15×10^5 /plate. BKGD: 2.40 ± 0.70 mG, ELF: 11.95 ± 2.75 mG. (c) Cells from passage #320 seeded at 0.137×10^5 /plate. BKGD: 2.53 ± 0.50 mG, ELF: 11.95 ± 3.28 mG.

nential growth curves for MCF-7 cells in the presence of the background or the applied magnetic field for cells at passage #318, #319, and #320, respectively. Background (BKGD) and applied magnetic fields were: (3a) 1.90 ± 0.01 mG and 10.4 ± 2.12 mG; (3b) 2.40 ± 0.70 mG and 11.95 ± 2.75 mG; and (3c) 2.53 ± 0.50 mG and 11.95 ± 3.28 mG. Comparison of the growth curves from these three experiments reveals that growth of MCF-7 cells is typically exponential and similar in the presence of the background 60 Hz field compared to that for the applied magnetic field. These data support the finding that cell proliferation of the MCF-7 cells employed here is not influenced by magnetic fields of 1.90–2.53 mG or 10.4–11.95 mG.

60 Hz magnetic fields block melatonin's natural oncostatic action on MCF-7 cell growth

Melatonin is oncostatic and inhibits the proliferation of MCF-7 cells when present in cell culture media at concentrations corresponding to the physiological range of 10^{-9} to 10^{-11} M [Cos and Blask, 1990]. We performed experiments to confirm melatonin's inhibition of MCF-7 cell growth. Growth curves for MCF-7 cells of passage #319 in the absence or in the presence of 10^{-9} M melatonin are depicted in Figure 4a. In these experiments, cells were placed in the incubator with the exposure coils energized in the antiparallel configuration (passive) so that a background 60 Hz magnetic field of 2.40 ± 0.70 mG was present. Cell proliferation revealed typical exponential growth over the 7-day period. When melatonin was present, an 18% inhibition of growth was detected at day 7; this inhibition was statistically significant ($P = 0.017$).

We note that MCF-7 cells display heterogeneity in their response to melatonin, and that some subclones can exhibit growth inhibition of up to 70% in the presence of 10^{-9} M melatonin between days 5–7 [Hill and Blask, 1988; Cos and Blask, 1990]. There is also evidence that the melatonin response is serum-dependent, which suggests that a factor(s) in serum is permissive for melatonin inhibition of MCF-7 cell growth. In addition, melatonin-sensitive MCF-7 subclones may exhibit different sensitivity to melatonin as passage number increases; this may reflect the fact that, as passage number increases, MCF-7 cells generally exhibit shorter doubling times with altered growth properties. Also, some MCF-7 subclones may exhibit no sensitivity to melatonin—this may depend on passage number, serum factors, or other unknown parameters. Sub-clone sensitivity of human cancer cell lines, such as the MCF-7 cell, to melatonin has

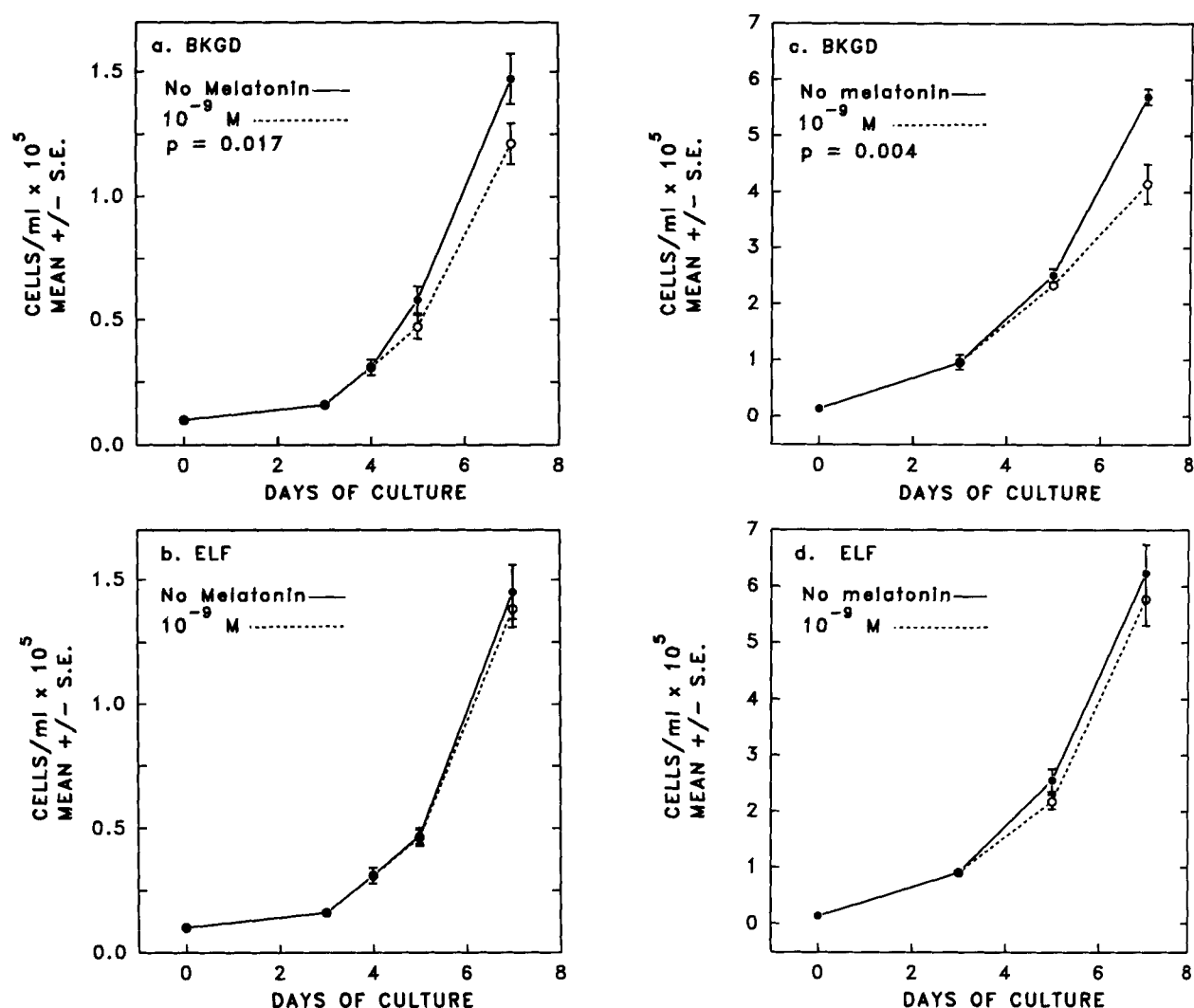


Fig. 4. 60 Hz magnetic fields block melatonin's action on MCF-7 cell growth. Cells from passage #319 were seeded at 0.15×10^5 /plate and grown in media containing either no melatonin or 10^{-9} M melatonin, in an incubator with a background (BKGD) 60 Hz magnetic field of 2.40 ± 0.70 mG, or in an incubator with a 60 Hz (ELF) magnetic field of 11.95 ± 2.75 mG. (a) Melatonin exhibits an 18% inhibition in MCF-7 cell proliferation at day 7 ($P = 0.017$), and (b) ELF magnetic fields block melatonin's growth inhibition of MCF-7 cells. Cells from passage #320 were seeded at 0.137×10^5 /plate and grown in media containing either no melatonin or 10^{-9} M melatonin in an incubator with a background (BKGD) 60 Hz magnetic field of 2.53 ± 0.50 mG, or in an incubator with a 60 Hz (ELF) magnetic field of 11.95 ± 3.28 mG. (c) Melatonin exhibits an 27% inhibition in MCF-7 cell proliferation at day 7 ($p = 0.004$), and (d) ELF magnetic fields block melatonin's growth inhibition of MCF-7 cells.

been noted by L'Hermite-Baleriaux and de Launoit [1992]. In all of the studies presented here, we employed MCF-7 cells that were responsive to melatonin, we have also identified our source of cells, as well as the passage number, the source of serum, and the media we used.

To address the question of whether an applied 60 Hz magnetic field can influence melatonin's natural oncostatic action, cells from the same passage #319 were placed in the matched incubator in the absence or presence of 10^{-9} M melatonin plus an applied 60

Hz magnetic field of 11.95 ± 2.75 mG, and cell growth was followed. These cell growth curves were obtained with the exposure coils energized in the parallel configuration (active). Figure 4b shows an exponential growth curve for the MCF-7 cells in absence of melatonin (solid line) that is superimposable with that obtained in the background field (compare with Figure 4a). This is consistent with results shown in Figure 3, in which magnetic fields alone do not alter MCF-7 cell growth. However, Figure 4b reveals that the 11.95 magnetic field in

the presence of melatonin at 10^{-9} M completely blocked melatonin's growth inhibition.

The above series of experiments was repeated using the same exposure coils, incubators, exposure conditions, and melatonin concentration (10^{-9} M), but with cells at passage #320. Figure 4c depicts MCF-7 cell growth in the absence or presence of melatonin in a background field of 2.53 ± 0.50 mG. Exponential growth was observed in the absence of melatonin. When melatonin was present a growth inhibition of 27% was observed at day seven that was statistically significant ($P = 0.004$). Simultaneously, in the matched incubator, MCF-7 cells were characterized for cell proliferation in the absence or presence of melatonin plus an applied ELF magnetic field of 11.95 ± 3.28 mG. These growth curves are presented in Figure 4d. Typical exponential growth was observed for cells in the absence of melatonin. However, when melatonin was present, the 60 Hz magnetic field was observed to block completely melatonin's growth inhibition.

In both of the experiments presented in Figure 4, the seeding density, melatonin, and serum were essentially equivalent, but the passage number was different (319 vs. 320). Cells at passage 319 displayed slower growth than cells from passage 320. The slower growth of cells at passage 319 may have contributed to an observed 18% growth inhibition of melatonin, compared to a 27% inhibition of growth for cells at passage 320.

Discussion

The findings presented here indicate that 60 Hz magnetic fields of 11.95 mG block melatonin's growth inhibition of MCF-7 breast cancer cells when melatonin is present at a physiological concentration of 10^{-9} M; 2.40 to 2.53 mG fields did not block melatonin activity (Figure 4). In the absence of melatonin, magnetic fields had no effect on MCF-7 cell growth (Figures 3 and 4). The above findings taken together indicate that (a) this *in vitro* effect occurs at the cellular level, (b) it involves an interaction that requires the presence of melatonin, and (c) a dose threshold appears to exist between 2 and 12 mG. These findings represent the first evidence for a *cellular level* response to ELF magnetic fields that is dependent on melatonin.

Several models have been proposed to elucidate the link between ELF magnetic field exposure and breast cancer incidence [Wilson et al., 1990; Reiter and Richardson, 1990; Reiter, 1992; Stevens et al., 1992]. These models suggest that the most important aspect of this link is a decrease in melatonin secretion in response to an *in vivo* magnetic field

exposure concomitant with enhancement in the production of prolactin and estrogen; the latter events are thought to increase the growth of susceptible breast epithelial cells [Cohen et al., 1978]. The study presented here deals with *in vitro* exposure of breast cancer cells and, therefore, relates to a subset of cellular events that occur distal to an *in vivo* effect on the pineal gland which regulates melatonin's secretion into the blood stream. The results reported here raise the possibility that a direct interaction between ELF magnetic fields, breast cancer cells, and melatonin may exist at the *cellular level*. These findings, therefore, complement important existing *in vivo* research [Wilson et al., 1990; Reiter and Richardson, 1990; Reiter, 1992; Stevens et al., 1992].

These cellular interactions most likely involve receptor activation and signal transduction pathways in the MCF-7 cell. In the tissue culture media employed in these studies, the relevant mitogen which triggers ER⁺-MCF-7 cell proliferation is estrogen. We confirmed the findings of Blask and colleagues that melatonin inhibits estrogen-dependent growth of MCF-7 cells [Cos and Blask, 1990]. Our finding that an ELF magnetic field blocks melatonin's action may be most simply explained by the magnetic field interfering with melatonin interaction with MCF-7 cells at a receptor binding site. It would be of interest to conduct binding studies using a compound that interferes directly with melatonin binding to MCF-7 cells, however a receptor for melatonin in the MCF-7 cell has not yet been identified. Melatonin has been postulated to exert its antiproliferative effect on human breast cancer cells through estrogen-dependent mechanisms as well as estrogen-independent mechanisms, based on melatonin's ability to block estradiol's mitogenic effect in MCF-7 cells in monolayer culture, and on estradiol's partial ability to rescue MCF-7 cells from melatonin's growth inhibition [Cos et al., 1991]. Thus, melatonin may interact with a variety of receptors in MCF-7 cells. Future studies should consider the possibility of estrogen receptor involvement.

There is experimental evidence for ELF magnetic fields altering signal transduction events. In *in vitro* studies with transformed HL60 cells, transcription and translation are altered in cell culture during exposure to ELF magnetic fields [Goodman and Shirley-Henderson, 1991]. Experimental evidence now exists for a 60 Hz field effect on one of the earliest signal transduction markers, $[Ca^{2+}]_i$, and was first reported for mitogen-activated thymic lymphocytes [Liburdy, 1992]. Alterations in $[Ca^{2+}]_i$ were not observed during 60 Hz field

exposure of resting cells, but approximately 100 seconds after addition of the lymphocyte mitogen Con-A, changes in $[Ca^{2+}]_i$ were detected. Alterations of an early ST marker like $[Ca^{2+}]_i$ are expected to occur at the cell surface and propagate down the signal transduction cascade to influence expression of such proto-oncogenes as c-MYC [Liburdy et al., 1993a]; this is a plausible biological mechanism that links ELF magnetic fields with the cell surface to subsequent gene expression events such as DNA synthesis and cell proliferation [Liburdy, 1992; Yost and Liburdy, 1992; Liburdy et al., 1993a]. To provide support for this interaction pathway, we have recently observed ELF-increased $[Ca^{2+}]_i$ and ELF-increased c-MYC mRNA induction in the same thymic lymphocytes [Liburdy, et al., 1993a]. The latter finding is consistent with c-MYC mRNA induction reported by Goodman and Henderson [1991] in HL60 cells. In addition, interesting studies by Luben [1993] indicate that pulsed 72 Hz magnetic fields are capable of inhibiting the activation of cAMP accumulation by β -adrenergic agonists in pineal cell cultures and in osteoblasts. They interpreted their findings to suggest that G-protein linked receptors on the cell surface are influenced by the fields, perhaps through small changes in the charge distribution of the cell membrane. The general feature that cell surface receptors involved in signal transduction are influenced by magnetic fields is consistent with the above lymphocyte studies (the T-cell receptor is G-protein linked) and the pineal gland studies (the β -adrenergic receptor is G-protein linked). Given these interactions and the findings presented above, we hypothesize that a mechanism for magnetic field action on MCF-7 growth may involve signal transduction events that (a) interfere with melatonin interaction with MCF-7 cells at the receptor level to alter subsequent signal transduction sequelae, and/or (b) activate other mitogen receptors, in the presence of melatonin, to overcome melatonin's anti-mitogenic action. The estrogen receptor is a possible candidate for the latter interaction, as discussed above, and future studies should address such issues.

Corollary issues that should also be investigated in future studies are (a) the possible role that serum factors may play in mediating melatonin-sensitive cell growth of MCF-7 cells in 60 Hz magnetic fields, and, (b) the effect of 60 Hz magnetic fields on MCF-7 cell growth at different melatonin concentrations. Since this study was conducted at a physiological dose of 10^{-9} M melatonin, studies are in progress over the concentration range corresponding to pharmacological (10^{-7} M) through

sub-physiological (10^{-11} M) doses of melatonin. This should yield important information concerning whether the blocking effect of 60 Hz magnetic fields on melatonin-sensitive MCF-7 cell growth represents an absolute decrease in melatonin activity or a shift of the melatonin dose response curve.

Acknowledgments

The helpful discussions and encouragement of Drs. Bary Wilson, James Morris, David Blask, and Russ Reiter in these studies are most gratefully acknowledged. We thank M. Wiesendanger for technical assistance in these studies. Research was supported in part by the Office of Energy Management, Utilities System Division, and the Office of Health and Environmental Research, of the Department of Energy, under contract DE-AC03-76SF00098.

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Attachment 19

Original Contribution

Residence Near Power Lines and Mortality From Neurodegenerative Diseases: Longitudinal Study of the Swiss Population

Anke Huss, Adrian Spoerri, Matthias Egger, and Martin Röösli for the Swiss National Cohort Study

Initially submitted May 5, 2008; accepted for publication August 25, 2008.

The relation between residential magnetic field exposure from power lines and mortality from neurodegenerative conditions was analyzed among 4.7 million persons of the Swiss National Cohort (linking mortality and census data), covering the period 2000–2005. Cox proportional hazard models were used to analyze the relation of living in the proximity of 220–380 kV power lines and the risk of death from neurodegenerative diseases, with adjustment for a range of potential confounders. Overall, the adjusted hazard ratio for Alzheimer's disease in persons living within 50 m of a 220–380 kV power line was 1.24 (95% confidence interval (CI): 0.80, 1.92) compared with persons who lived at a distance of 600 m or more. There was a dose-response relation with respect to years of residence in the immediate vicinity of power lines and Alzheimer's disease: Persons living at least 5 years within 50 m had an adjusted hazard ratio of 1.51 (95% CI: 0.91, 2.51), increasing to 1.78 (95% CI: 1.07, 2.96) with at least 10 years and to 2.00 (95% CI: 1.21, 3.33) with at least 15 years. The pattern was similar for senile dementia. There was little evidence for an increased risk of amyotrophic lateral sclerosis, Parkinson's disease, or multiple sclerosis.

dementia; neurodegenerative diseases; radiation, nonionizing

Abbreviations: ALS, amyotrophic lateral sclerosis; CI, confidence interval; ELF-MF, extremely low frequency magnetic field(s); ICD-10, *International Classification of Diseases, Injuries, and Causes of Death*, Tenth Revision.

Research on the long-term effects of extremely low frequency magnetic fields (ELF-MF) has focused on cancer since Wertheimer and Leeper (1) published their results on childhood cancer and wiring configurations in 1979. In 2001, the International Agency for Research on Cancer classified exposure to residential magnetic fields above 0.4 μ T as a "possible" cause of childhood leukemia (2). For noncancer endpoints, an initial report by Sobel et al. (3) on occupational ELF-MF exposure and Alzheimer's disease suggested that the risk could be substantial. Studies published subsequently have produced inconsistent results, but a recent meta-analysis (4) reported elevated risks in cohort, as well as case-control, studies. A recent review of the evidence for an association between ELF-MF and Alzheimer's disease by the World Health Organization (5) concluded that the available data were inadequate, and the topic was identified as a key research priority.

To our knowledge, no study has so far examined whether residential exposure from power lines is associated with an

elevated risk of neurodegenerative diseases. Even a small association could be of high public health relevance, since a considerable number of persons are exposed to these fields. For example, 9.2% of the Swiss population live within 600 m of a 220 or 380 kV power line. We used the Swiss National Cohort, a longitudinal study of the Swiss population (6), to investigate whether living in the vicinity of power lines was associated with mortality from neurodegenerative diseases such as Alzheimer's disease, senile dementia, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Parkinson's disease.

MATERIALS AND METHODS

Study population

The present analysis was based on the 2000 national census. Mortality data were available for the period 2000–2005,

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Table 1. Number of Deaths by Cause, Recorded in Swiss Mortality Data Between December 4, 2000, and December 31, 2005

	ICD-10 Codes	Total No. of Cases ^a	No. of Included Cases ^b	% of Total Cases	Mean Age at Death (Interquartile Range), years	Female Cases, %
All causes		294,833	282,378	96	78.2 (71.6–88.5)	51
Alzheimer's disease	G30	9,758	9,228	95	85.3 (80.0–90.5)	68
Senile dementia	G30, F00, F03	29,975	28,288	94	86.9 (82.7–91.7)	68
Amyotrophic lateral sclerosis	G12.2	759	744	98	70.3 (63.5–79.0)	46
Parkinson's disease	G20–21	6,994	6,683	96	83.7 (79.6–88.8)	48
Multiple sclerosis	G35	838	773	92	67.0 (57.9–77.4)	67
Cancer of the trachea, bronchus, or lung	C33–C34	14,384	14,281	99	70.0 (62.4–78.1)	26
Cancer of the esophagus	C15	2,119	2,101	99	70.5 (61.5–79.9)	24
Alcoholic liver disease	K70	3,356	3,303	98	63.4 (55.4–71.5)	28

Abbreviation: ICD-10, *International Classification of Diseases, Injuries, and Causes of Death*, Tenth Revision.

^a Deaths that could be linked to the census (refer to the text).

^b Excluded were persons with unknown building coordinates or who were under 30 years of age at the start of follow-up or death.

with causes of death coded according to the *International Classification of Diseases, Injuries, and Causes of Death*, Tenth Revision (ICD-10). Enumeration in the 2000 census is nearly complete: Coverage was estimated at 98.6% (7). Deterministic and probabilistic record linkages were used to link census records to a death record or an emigration record (6). Of death records of persons older than 30 years, 95.1% could be successfully linked to a 2000 census record. At present, the database includes follow-up data until December 31, 2005.

We excluded persons aged 29 years or less at the census, as well as persons with incomplete information on building coordinates. The database contains information on age, sex, marital status, education, and occupation, as well as additional variables describing, for example, the degree of urbanization of the area or building characteristics such as the number of apartments per building. The geo-coded place of residence of the participants (i.e., Swiss-grid coordinates extracted from the Swiss building registry) is also included in the census data. In general, these coordinates pinpoint a location within a few meters of the building's midpoint. Data from the 1990 census were used to identify the place of residence at that time. The 1990 and 2000 censuses additionally include information on whether individuals had lived at the same place 5 years before the census, that is, in 1985 or in 1995. We were thus able to identify persons who had lived at their place of residence for at least 5, 10, or 15 years.

Outcomes

We considered deaths from the following neurodegenerative diseases: Alzheimer's disease, senile dementia, ALS, Parkinson's disease, and multiple sclerosis. These diseases had to be listed on the death certificate as the primary or a concomitant cause of death. The recording of neurodegenerative diseases on death certificates might be related to socioeconomic position. We therefore included outcomes that are known to be related to socioeconomic position:

cancer of the trachea, bronchus, or lung; alcoholic liver disease; and all-cause mortality. The ICD-10 codes used are listed in Table 1.

Exposure

Exposure assessment was based on the distance of the place of residence to the nearest power line. We included all 220–380 kV power lines in Switzerland, over 5,100 km in total. We obtained geodata of the power lines from the Federal Inspectorate for Heavy Current Installations. Figure 1 illustrates localization of the power lines and buildings in Switzerland. We determined the shortest distance to any of the transmission lines and derived the number of persons living within the corridors around the power lines. We defined corridors of 0–<50 m, 50–<200 m, 200–<600 m, and 600 m or beyond. We determined exposure at the time of the 2000 census.

Information about the use of a building as a clinic or nursing home was available from a separate building record, which was completed by the owner of the building, and this information was then matched to the personal records of individuals. Some persons might live in a nursing home or clinic because of a neurodegenerative disease. Therefore, in order to obtain more appropriate exposure data for individuals living in such an institution in 2000, we used the exposure for the place of residence at the time of the 1990 census instead. Persons who lived in a nursing home or clinic in 1990 were excluded from the analysis.

Statistical analyses

We analyzed data using Cox proportional hazard models. We compared the risk of dying from neurodegenerative diseases across corridors and according to the duration of residence in exposed corridors (at least 5, 10, and 15 years). Person-years of observation were defined as the interval between December 4, 2000 (the date of the census), and death, emigration, or December 31, 2005.

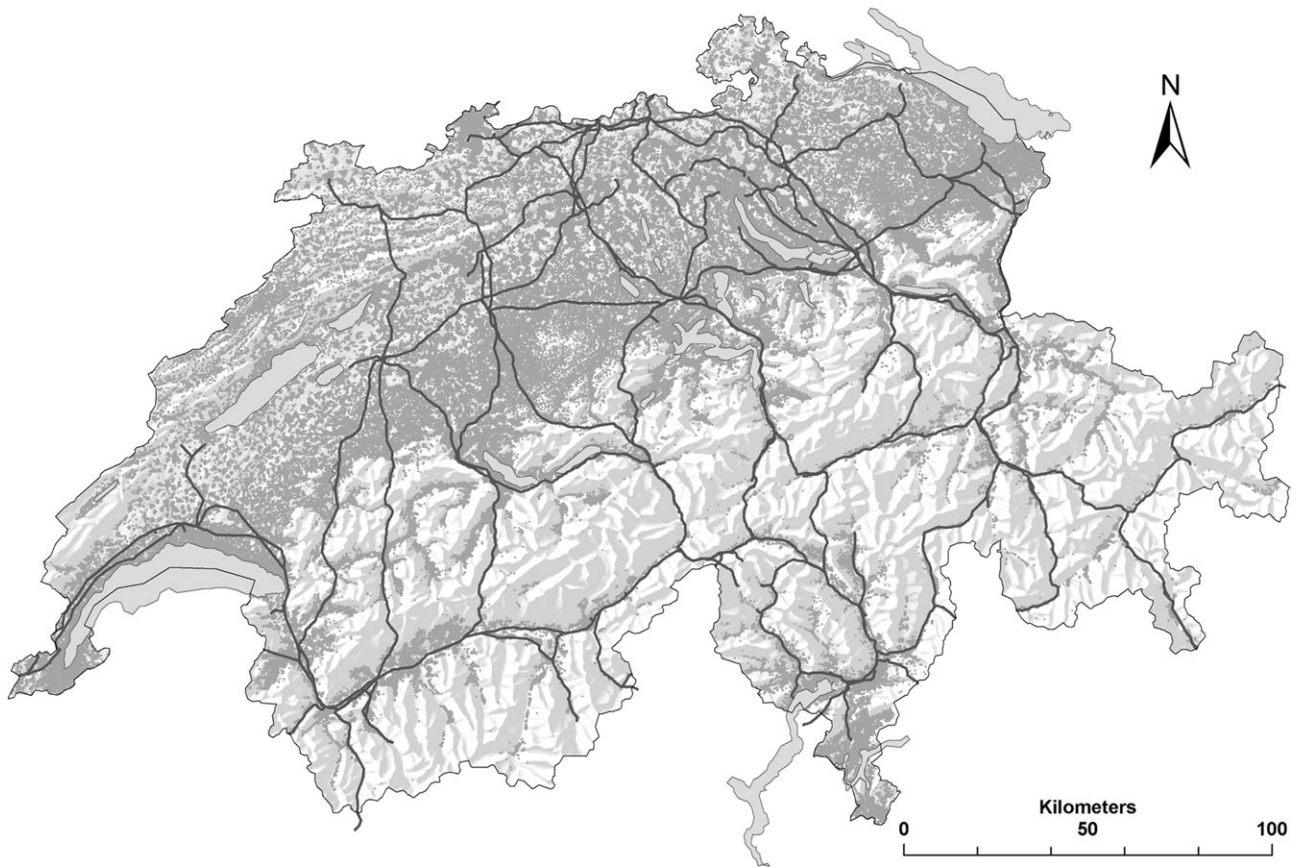


Figure 1. Power lines and buildings in Switzerland. Data sources: Federal Inspectorate for Heavy Current Installations, Fehraltdorf (power lines); Register of Buildings and Dwellings, Federal Statistical Office, Neuchâtel (building coordinates); and Federal Office of Topography swisstopo, Wabern (background map of Switzerland).

We used age as the underlying timescale in our models. All models were adjusted for sex; educational level (compulsory education, secondary level, and tertiary level); highest reported occupational attainment by code (4 levels extracted from the International Standard Classification of Occupations of 1988—1) legislators, senior officials, managers, and professionals, 2) technicians and associate professionals, clerks, service workers, and shop and market sales workers, 3) skilled agricultural and fishery workers, craft and related trades workers, plant, machine operators, and assemblers, and elementary occupations, and 4) no occupation reported); civil status (single, married, divorced, widowed); urbanization category (city, agglomeration, rural municipality); and language region (German, French, Italian). We also included the number of apartments per building into the model, a potential risk factor for magnetic field exposure due to indoor wiring (8).

Finally, because Alzheimer's disease might be associated with benzene exposure, we adjusted models for living within 50 m of a major road. We extracted proximity of the buildings to the "major road network" using data from the Swiss TeleAtlas database for this purpose. The major roads network includes motorways and motorway exits, as

well as "major roads of high importance": nearly 8,700 km with 7% of the population exposed to major roads in the 50-m corridor. In sensitivity analyses, we repeated analyses for persons aged less than 85 years, by sex, and examined whether results differed between deaths where Alzheimer's disease or senile dementia had been coded as the primary or concomitant cause of death.

We tested our models successfully for the proportionality assumption using Nelson-Aalen survivor functions and statistical tests based on Schoenfeld residuals. Data were analyzed by using Stata 9 (StataCorp LP, College Station, Texas) software. Results are presented as hazard ratios with 95% confidence intervals.

The Swiss National Cohort was approved by the cantonal ethics committees of Bern and Zurich.

RESULTS

Of the 7.29 million persons recorded in the 2000 census, 2.59 million were excluded because they were under the age of 30 years at the census. Furthermore, 39,871 persons with unknown building coordinates were excluded. The cohort

Table 2. Number of Deaths, Person-Years of Follow-up, and Hazard Ratios for Alzheimer's Disease and Senile Dementia Mortality According to Distance to Power Lines, Entire Study Population and Individuals With at Least 15 Years at the Identical Place of Residence, Switzerland, 2000–2005^a

Cause of Death	Distance to 220–380 kV Power Line, m	No. of Cases	No. of Person-Years	Crude		Adjusted	
				Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval
Entire study population							
Alzheimer's disease	0–<50	20	58,423	1.18	0.76, 1.83	1.24	0.80, 1.92
	50–<200	130	363,460	1.12	0.94, 1.33	1.13	0.95, 1.34
	200–<600	572	1,688,323	0.99	0.91, 1.08	1.02	0.94, 1.11
	≥600	8,506	20,711,618	1	Referent	1	Referent
Senile dementia	0–<50	60	58,423	1.19	0.92, 1.53	1.23	0.96, 1.59
	50–<200	371	363,460	1.06	0.96, 1.18	1.08	0.97, 1.19
	200–<600	1,702	1,688,323	0.98	0.93, 1.02	0.99	0.94, 1.04
	≥600	26,155	20,711,618	1	Referent	1	Referent
Individuals living at least 15 years at the identical place of residence							
Alzheimer's disease	0–<50	15	22,320	1.90	1.14, 3.15	2.00	1.21, 3.33
	50–<200	63	145,148	1.12	0.88, 1.44	1.15	0.89, 1.47
	200–<600	259	641,017	0.96	0.85, 1.09	1.00	0.88, 1.13
	≥600	3,861	7,698,419	1	Referent	1	Referent
Senile dementia	0–<50	33	22,320	1.40	0.99, 1.97	1.41	1.00, 1.98
	50–<200	169	145,148	1.00	0.86, 1.16	1.01	0.86, 1.17
	200–<600	819	641,017	1.00	0.93, 1.07	1.01	0.94, 1.09
	≥600	11,930	7,698,419	1	Referent	1	Referent

^a Cox proportional hazard models were based on either 4.65 million (entire study population) or 1.75 million (individuals with at least 15 years at the identical place of residence) people, with age as the underlying timescale, crude and adjusted for sex, educational level, occupational attainment, urban-rural area, civil status, language region, number of apartments per building, and living within 50 m of a major road.

thus consisted of 4.65 million persons. During the study period, 282,378 eligible and linked deaths from all causes were recorded, including 9,228 deaths from Alzheimer's disease, 28,288 deaths from senile dementia, 773 deaths from multiple sclerosis, and 6,683 deaths from Parkinson's disease (Table 1). The total number of person-years of follow-up was 22.82 million for the whole study population and 8.51 million for persons who reported living for at least 15 years at the identical place of residence (Tables 2 and 3).

The adjusted hazard ratio of Alzheimer's disease for persons living within 50 m of a 220–380 kV power line compared with that for persons who lived at a distance of 600 m or more was 1.24 (95% confidence interval (CI): 0.80, 1.92). There was little evidence of an increased risk beyond 50 m. Analysis by exposure duration revealed a dose-response relation with respect to years of residence in the vicinity of power lines: Persons living at least 5 years within 50 m had an adjusted hazard ratio of 1.51 (95% CI: 0.91, 2.51), which increased to 1.78 (95% CI: 1.07, 2.96) for persons with at least 10 years and to 2.00 (95% CI: 1.21, 3.33) for persons with at least 15 years (Figure 2; Table 2). These adjusted hazard ratios of 2.04 (95% CI: 1.06, 3.93) and 1.96 (95% CI: 0.88, 4.38) were similar for women and men, respectively, and for persons under 85 years of age (adjusted hazard ratio = 1.94, 95% CI: 0.97, 3.89).

For senile dementia, we observed the same pattern as with Alzheimer's disease, although associations tended to be weaker. For increasing exposure time in the vicinity of power lines, the adjusted hazard ratio increased from 1.23 (95% CI: 0.96, 1.59) for any exposure duration to 1.34 (95% CI: 0.98, 1.82) for persons with at least 5 years, to 1.36 (95% CI: 0.98, 1.89) with at least 10 years, and to 1.41 (95% CI: 1.00, 1.98) with at least 15 years of residence near the power line (Table 2). For both Alzheimer's disease and senile dementia, there was little evidence for a difference in effects between deaths coded as primary and deaths coded as concomitant cause ($P_{\text{interaction}} > 0.2$).

Parkinson's disease and ALS were not associated with residence in the proximity of power lines. The adjusted hazard ratio for any duration of exposure in the 50-m corridor was 0.83 (95% CI: 0.46, 1.49) for Parkinson's disease and could not be estimated (no case occurred in the 50-m corridor) for ALS. The adjusted hazard ratio for multiple sclerosis was 1.20 (95% CI: 0.30, 4.80). Similar results were obtained when restricting analyses to persons with at least 15 years at the same place of residence (Table 3).

No increased risk in the proximity of a power line was found for all-cause mortality, cancer of the lung, bronchus, or trachea, cancer of the esophagus, or alcoholic liver disease, for any duration of residence (data not shown) or when

Table 3. Number of Deaths, Person-Years of Follow-up, and Hazard Ratios for Amyotrophic Lateral Sclerosis, Parkinson's Disease, and Multiple Sclerosis Mortality According to Distance to Power Lines, Entire Study Population and Individuals With at Least 15 Years at the Identical Place of Residence, Switzerland, 2000–2005^a

Cause of Death	Distance to 220–380 kV Power Line, m	No. of Cases	No. of Person-Years	Crude		Adjusted	
				Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval
Entire study population							
Amyotrophic lateral sclerosis	0–<50	0	58,423				
	50–<200	10	363,460	0.88	0.47, 1.64	0.85	0.46, 1.59
	200–<600	39	1,688,323	0.74	0.54, 1.02	0.72	0.52, 1.00
	≥600	695	20,711,618	1	Referent	1	Referent
Parkinson's disease	0–<50	12	58,423	0.95	0.54, 1.67	0.87	0.50, 1.56
	50–<200	99	363,460	1.15	0.94, 1.40	1.06	0.87, 1.29
	200–<600	416	1,688,323	0.98	0.90, 1.09	0.92	0.84, 1.02
	≥600	6,156	20,711,618	1	Referent	1	Referent
Multiple sclerosis	0–<50	2	58,423	1.11	0.28, 4.43	1.19	0.30, 4.79
	50–<200	16	363,460	1.38	0.84, 2.26	1.45	0.88, 2.39
	200–<600	60	1,688,323	1.12	0.86, 1.45	1.16	0.89, 1.51
	≥600	695	20,711,618	1	Referent	1	Referent
Individuals living at least 15 years at the identical place of residence							
Amyotrophic lateral sclerosis	0–<50	0	22,320				
	50 –<200	7	145,148	1.05	0.50, 2.21	1.00	0.47, 2.11
	200–<600	29	641,017	0.97	0.66, 1.41	0.93	0.63, 1.35
	≥600	389	7,698,419	1	Referent	1	Referent
Parkinson's disease	0–<50	8	22,320	1.25	0.63, 2.51	1.15	0.57, 2.30
	50–<200	56	145,148	1.25	0.96, 1.63	1.14	0.87, 1.49
	200–<600	210	641,017	0.99	0.86, 1.14	0.93	0.81, 1.08
	≥600	3,006	7,698,419	1	Referent	1	Referent
Multiple sclerosis	0–<50	1	22,320	1.26	0.18, 8.98	1.35	0.19, 9.62
	50–<200	11	145,148	2.09	1.15, 3.82	2.19	1.19, 4.01
	200–<600	26	641,017	1.10	0.74, 1.65	1.14	0.76, 1.71
	≥600	299	7,698,419	1	Referent	1	Referent

^a Cox proportional hazard model based on 4.65 million and 1.75 million people, with age as the underlying timescale, crude and adjusted for sex, educational level, occupational attainment, urban-rural area, civil status, language region, number of apartments per building, and living within 50 m of a major road.

restricting analyses to persons with at least 15 years at the same place of residence (Table 4).

DISCUSSION

This large study of the entire Swiss population found that persons who lived within 50 m of a 220–380 kV power line were at increased risk of death from Alzheimer's disease, compared with persons who lived farther away from power lines. The risk increased with increasing duration of residence in the 50-m corridor. Notably, the risk declined rapidly with increasing distance, with only weak evidence for an increased risk beyond 50 m. A similar pattern was observed for senile dementia. In contrast, we found no consistent association for ALS, Parkinson's disease, or multiple sclerosis. Our study thus indicates a possible association

between ELF-MF exposure and risks of Alzheimer's disease and senile dementia.

Comparison with previous studies

Established risk factors for Alzheimer's disease include age and genetic factors (9). Controversy remains regarding environmental risk factors, including ELF-MF (10). The association between Alzheimer's disease and ELF-MF has generally been studied with respect to occupational exposures. Occupational exposures are typically about 0.5 μ T for electricians, some machine operators, or train drivers, above 1 μ T for some machine operators, and around 3 μ T for electrical power installers and repairers (11). In occupational settings, increased risks of Alzheimer's disease have been reported with magnetic field exposures at levels around 0.5 μ T (4). To our knowledge, an analysis of the potential

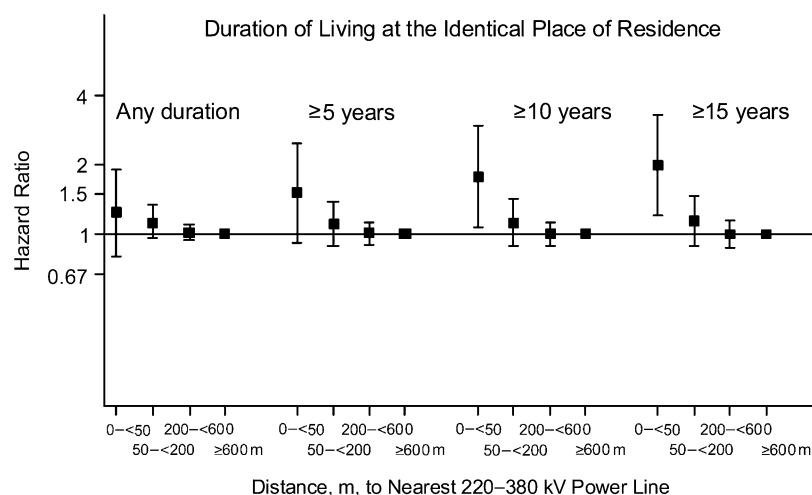


Figure 2. Mortality from Alzheimer's disease in relation to proximity to 220–380 kV power lines, Switzerland, 2000–2005. Cox proportional hazard models for persons in Switzerland who reported living at the place of residence at the time of the 2000 census or at the identical place of residence for at least 5, 10, or 15 years, using age as the underlying timescale, adjusted for sex, educational level, occupational attainment, urban-rural area, civil status, language region, number of apartments per building, and living within 50 m of a major road.

association of neurodegenerative diseases and residential exposure has not been reported in the scientific literature, even though ELF-MF exposure from power lines can be of the same magnitude as in occupational settings. In the

United Kingdom, propagation of magnetic fields at levels of about 0.5 μT at a distance of 50 m to a 275 kV line was reported (12). At maximum load, these levels could, however, be considerably higher. In Switzerland, the Federal

Table 4. Number of Cases and Hazard Ratios for Comparison Outcomes of Total Mortality, Alcoholic Liver Disease, Cancer of the Esophagus, and Lung Cancer According to Persons Who Reported Living at Least 15 Years at the Identical Place of Residence, Switzerland, 2000–2005^a

Cause of Death	Distance to 220–380 kV Power Line, m	No. of Cases	Crude		Adjusted	
			Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval
Total mortality	0–<50	341	1.11	1.00, 1.24	1.07	0.96, 1.19
	50–<200	2,144	1.01	0.97, 1.06	0.97	0.93, 1.01
	200–<600	10,104	1.02	1.00, 1.04	1.00	0.98, 1.02
	≥600	135,851	1	Referent	1	Referent
Alcoholic liver disease	0–<50	4	1.01	0.38, 2.70	1.11	0.41, 2.96
	50–<200	32	1.23	0.87, 1.75	1.31	0.92, 1.86
	200–<600	94	0.82	0.66, 1.01	0.87	0.70, 1.07
	≥600	1,409	1	Referent	1	Referent
Cancer of the esophagus	0–<50	1	0.37	0.05, 2.62	0.36	0.05, 2.55
	50–<200	16	0.88	0.54, 1.45	0.84	0.51, 1.38
	200–<600	77	0.94	0.75, 1.19	0.92	0.73, 1.16
	≥600	1,055	1	Referent	1	Referent
Lung cancer	0–<50	19	1.02	0.65, 1.59	1.00	0.64, 1.57
	50–<200	119	0.95	0.79, 1.14	0.94	0.78, 1.12
	200–<600	551	0.98	0.90, 1.07	0.99	0.90, 1.08
	≥600	7,248	1	Referent	1	Referent

^a Cox proportional hazard models, using age as the underlying timescale, crude and adjusted for sex, educational level, occupational attainment, urban-rural area, civil status, language region, number of apartments per building, and living within 50 m of a major road. The study population is the same as that for Table 3.

Office for the Environment estimated that, at full load, 1 μT would not be exceeded at a distance of 60–80 m from a 380 kV line and at 40–55 m from a 220 kV line (13).

For ALS, an association between the risk of ALS and employment in electrical occupations, which is related to both magnetic field exposure and the risk of experiencing electric shock, has been reported (14). The electric shock hypothesis would be consistent with our results, as we did not observe an association with residential magnetic field exposure. In the absence of a known biologic mechanism, the World Health Organization recently concluded that the available evidence on a possible association between ELF-MF and Alzheimer's disease, as well as ALS, was inadequate (5).

Of the few studies so far that evaluated magnetic field exposure and multiple sclerosis, none reported statistically significant increased risks, which is in line with the inconsistent results observed here (15–17). Also in line with previous studies, our results for Parkinson's disease provide little evidence for an association (18).

Strengths and limitations

This study combined the mortality register data with nearly complete population data from the 2000 census, complemented with information on duration of residence from the 1990 census. With the exception of persons emigrating from Switzerland, particularly older immigrants who tend to return to their countries after retirement, mortality data are also virtually complete. Record linkage failed in some instances, but this is unlikely to be associated with residence in the vicinity of power lines. Linkage success is very high in the age group above 30 years and highest in the age group between 65 and 85 years. Because mortality from neurodegenerative diseases is negligible in younger people, we restricted our analyses to persons aged 30 years or over. In sensitivity analyses, we excluded people aged 85 years or older and obtained virtually identical results.

The development of neurodegenerative disease, as well as its recording on death certificates, may be associated with socioeconomic position. The availability of data on education and occupation and other potential confounders on an individual level is an important strength of our study. This allowed us to adjust for several indicators of socioeconomic position, but this adjustment had only very small effects on our estimates. In addition, causes of death known to be associated with socioeconomic position were included for comparison but did not show an increased risk in the 50-m corridor.

There is no registry for neurodegenerative diseases in Switzerland, and we had to rely on information given on death certificates, where neurodegenerative diseases are known to be underreported (19–21). The degree of underreporting varies by disease. Death certification of cases of ALS and multiple sclerosis has been found to be reasonably accurate (22, 23). Underreporting of Alzheimer's disease, as well as senile dementia, is more common and increases with the age of the deceased (19, 21, 24–27). Mortality rates for Alzheimer's disease have been increasing since 1995, when a specific code was introduced in the ICD-10 system, in-

dicating that reporting of Alzheimer's disease on death certificates has become more complete in recent years. However, it is unlikely that the completeness of reporting is associated with living in the proximity of power lines.

The magnetic fields produced by power lines depend on a variety of factors, including the load characteristics, distance between conductors, and the placement of phases. Unfortunately, information on these characteristics was not available in our study. We acknowledge that the use of exposure corridors, without measurements or taking the load of the line and other factors into account, may have introduced Berkson-type error into the exposure assessment (28), and this could have reduced the power of our study. On the other hand, it is possible that our surrogate is not predictive for true exposures at all because other sources may be more important, for instance, at work or when travelling. This would imply that the observed association is due to another factor that could not be controlled for in the analysis. However, we believe that we allowed for the most important factors in the analysis, and we are not aware of other exposures that could plausibly explain the observed associations.

There is no consensus as to which exposures from overhead power lines are biologically relevant and should be measured (2). For example, ionized particles or contact currents may also be relevant (29–31). However, all of these exposures are associated with distance to a power line. We extended the corridors around power lines up to a distance of 600 m to make our results comparable with those of the study by Draper et al. (32). In contrast to their study, we found little evidence for an increased risk beyond 50 m. With respect to a potential mechanism, we can only speculate whether one of the mechanisms that have been proposed in the literature (5) might be of importance in the context of magnetic field exposure and neurodegenerative diseases. For example, induced electric fields in neural networks (electric fields induced in tissue by exposure to extremely low frequency electric and magnetic fields) have been reported to affect synaptic transmission in neural networks, as well as the radical pair mechanism (5). Increased free radical concentrations can cause oxidative damage to cellular components, which could play a role in the etiology of Alzheimer's disease.

Finally, underground cables that replace overhead power lines in some urban areas may represent an additional source of residential magnetic field exposure, but these were not considered in our study. In Switzerland, underground cables of 220–380 kV represent only around 0.8% of the grid, and we decided to omit cables from our analyses.

Public health implication

Assuming that the associations observed in this study are causal, what are the public health implications? Considering the relatively small number of cases of Alzheimer's disease and senile dementia diagnosed in the 50-m corridor (Alzheimer's disease: 20 of 9,164 (0.22%); senile dementia: 59 of 28,045 (0.21%)), it is clear that the public health impact appears limited. The true public health impact, however, is difficult to determine. Rates of Alzheimer's disease were reported to be from 2- to 8-fold higher if diagnoses were

based on clinical examination instead of death certificates (20, 24). In addition, Alzheimer's disease might go undiagnosed in another group of persons. Finally, although we found only weak evidence for an increased risk beyond 50 m, it is unlikely that there is an abrupt change in risk at 50 m. Nevertheless, our results do provide reassurance for the population living at distances of 50–600 m from a power line.

Conclusions

The results of our study support the hypothesis that magnetic field exposure plays a role in the etiology of Alzheimer's disease and senile dementia but not of ALS or other neurodegenerative diseases. Despite the large sample size covering the whole Swiss population, these findings must be interpreted with caution, because of the lack of known biologic mechanisms.

ACKNOWLEDGMENTS

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This work was supported by the Swiss National Science Foundation (grant 3347C0-108806).

The authors thank the Federal Inspectorate for Heavy Current Installations for providing them with the geodata of the power lines and the Federal Statistical Office whose support made the Swiss National Cohort and this study possible.

The members of the Swiss National Cohort Study Group are Felix Gutzwiller (Chairman of the Executive Board) and Matthias Bopp (Zurich, Switzerland); Matthias Egger (Chairman of the Scientific Board), Adrian Spoerri, Malcolm Sturdy (Data Manager), and Marcel Zwahlen (Bern, Switzerland); Charlotte Braun-Fahrländer (Basel, Switzerland); Fred Paccaud (Lausanne, Switzerland); and André Rougemont (Geneva, Switzerland).

The funding source had no influence on study design; collection, analysis, and interpretation of data; the writing of the report; or the decision to submit the paper for publication.

Conflict of interest: none declared.

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Attachment 20



IARC FINDS LIMITED EVIDENCE THAT RESIDENTIAL MAGNETIC FIELDS INCREASE RISK OF CHILDHOOD LEUKAEMIA

An expert scientific working group of the Monographs Programme of the International Agency for Research on Cancer (IARC) has concluded its review of health effects of static and extremely low frequency (ELF) electric and magnetic fields. Such fields include the earth's magnetic field, and also originate from electrical power transmission lines, electrical wiring in buildings, and electric appliances. Magnetic fields are measured in units of microTesla; the earth's static magnetic field, to which everyone is exposed, varies from 25 microTesla at the equator to 65 microTesla at the poles. Most research on health effects has been done on ELF magnetic fields with frequencies of 50 or 60 Hz.

Reports were first published in 1979 that childhood cancer might be associated with exposures to residential ELF fields. Numerous studies in many countries have been undertaken since then of possible increased cancer risks in children and adults from ELF magnetic field exposures. Special attention has focussed on leukaemia and on brain tumours, which early reports had suggested might be increased. IARC has now concluded that ELF magnetic fields are possibly carcinogenic to humans, based on consistent statistical associations of high level residential magnetic fields with a doubling of risk of childhood leukaemia. Children who are exposed to residential ELF magnetic fields less than 0.4 microTesla have no increased risk for leukaemia. Because of insufficient data, static magnetic fields and static and extremely low frequency electric fields could not be classified as to carcinogenic risk to humans.

However, pooled analyses of data from a number of well-conducted studies show a fairly consistent statistical association between a doubling of risk of childhood leukaemia and power-frequency (50 or 60 Hz) residential ELF magnetic field strengths above 0.4 microTesla. In contrast, no consistent evidence was found that childhood exposures to ELF electric or magnetic fields are associated with brain tumours or any other kinds of solid tumours. No consistent evidence was found that residential or occupational exposures of adults to ELF magnetic fields increase risk for any kind of cancer.

Studies in experimental animals have not shown a consistent carcinogenic or co-carcinogenic effects of exposures to ELF magnetic fields, and no scientific explanation has been established for the observed association of increased childhood leukaemia risk with increasing residential ELF magnetic field exposure.

Health effects of radiofrequency electromagnetic fields, which are produced by such sources as radio and television transmission towers, portable telephones, and radar, were not evaluated by the IARC working group. These exposures will be reviewed by the IARC Monographs Programme when research that is currently in progress has been published, most likely in 2005.

For further details of the Monographs evaluation, consult <http://monographs.iarc.fr>, under "Agents most recently evaluated," or inquire by e-mail to cie@iarc.fr

For further details of current research at IARC on electric and magnetic fields, inquire by e-mail to cardis@iarc.fr

For more general information, please contact Dr Nicolas Gaudin, Chief, IARC Communications (com@iarc.fr)

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Attachment 21



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IARC MONOGRAPHS

ULTRASHORT RADIATION, LOW-FREQUENCY ELECTROMAGNETIC FIELDS

VOLUME 102

IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS

International Agency for Research on Cancer



World Health
Organization

6. EVALUATION

6.1 Cancer in Humans

There is *limited evidence* in humans for the carcinogenicity of radiofrequency radiation. Positive associations have been observed between exposure to radiofrequency radiation from wireless phones and glioma, and acoustic neuroma.

6.2 Cancer in Experimental Animals

There is *limited evidence* in experimental animals for the carcinogenicity of radiofrequency radiation.

6.3 Overall Evaluation

Radiofrequency electromagnetic fields are *possibly carcinogenic to humans (Group 2B)*.

6.4 Rationale of the evaluation of the epidemiological evidence

The human epidemiological evidence was mixed. Several small early case-control studies were considered to be largely uninformative. A large cohort study showed no increase in risk of relevant tumours, but it lacked information on level of mobile-phone use and there were several potential sources of misclassification of exposure. The bulk of evidence came from reports of the INTERPHONE study, a very large international, multicentre case-control study and a separate large case-control study from Sweden on gliomas and meningiomas of the brain and acoustic neuromas. While affected by selection bias and information bias to varying degrees, these studies showed an association between

glioma and acoustic neuroma and mobile-phone use; specifically in people with highest cumulative use of mobile phones, in people who had used mobile phones on the same side of the head as that on which their tumour developed, and in people whose tumour was in the temporal lobe of the brain (the area of the brain that is most exposed to RF radiation when a wireless phone is used at the ear). The Swedish study found similar results for cordless phones. The comparative weakness of the associations in the INTERPHONE study and inconsistencies between its results and those of the Swedish study led to the evaluation of *limited evidence* for glioma and acoustic neuroma, as decided by the majority of the members of the Working Group. A small, recently published Japanese case-control study, which also observed an association of acoustic neuroma with mobile-phone use, contributed to the evaluation of *limited evidence* for acoustic neuroma.

There was, however, a minority opinion that current evidence in humans was *inadequate*, therefore permitting no conclusion about a causal association. This minority saw inconsistency between the two case-control studies and a lack of exposure-response relationship in the INTERPHONE study. The minority also pointed to the fact that no increase in rates of glioma or acoustic neuroma was seen in a nationwide Danish cohort study, and that up to now, reported time trends in incidence rates of glioma have not shown a trend parallel to time trends in mobile-phone use.

Attachment 22

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

Power Density (Microwatts/centimeter ² - uW/cm ²)		Reference
As low as (10 ⁻¹³) or 100 femtowatts/cm ²	Super-low intensity RFR effects at MW resonant frequencies resulted in changes in genes; problems with chromatin conformation (DNA)	Belyaev, 1997
5 picowatts/cm ² (10 ⁻¹²)	Changed growth rates in yeast cells	Grundler, 1992
0.1 nanowatt/cm ² (10 ⁻¹⁰) or 100 picowatts/cm ²	Super-low intensity RFR effects at MW resonant frequencies resulted in changes in genes; problems with chromatin condensation (DNA) intensities comparable to base stations	Belyaev, 1997
0.00034 uW/cm ²	Chronic exposure to mobile phone pulsed RF significantly reduced sperm count,	Behari, 2006
0.0005 uW/cm ²	RFR decreased cell proliferation at 960 MHz GSM 217 Hz for 30-min exposure	Velizarov, 1999
0.0006 - 0.0128 uW/cm ²	Fatigue, depressive tendency, sleeping disorders, concentration difficulties, cardio-vascular problems reported with exposure to GSM 900/1800 MHz cell phone signal at base station level exposures.	Oberfeld, 2004
0.003 - 0.02 uW/cm ²	In children and adolescents (8-17 yrs) short-term exposure caused headache, irritation, concentration difficulties in school.	Heinrich, 2010
0.003 to 0.05 uW/cm ²	In children and adolescents (8-17 yrs) short-term exposure caused conduct problems in school (behavioral problems)	Thomas, 2010
0.005 uW/cm ²	In adults (30-60 yrs) chronic exposure caused sleep disturbances, (but not significantly increased across the entire population)	Mohler, 2010
0.005 - 0.04 uW/cm ²	Adults exposed to short-term cell phone radiation reported headaches, concentration difficulties (differences not significant, but elevated)	Thomas, 2008
0.006 - 0.01 uW/cm ²	Chronic exposure to base station RF (whole-body) in humans showed increased stress hormones; dopamine levels substantially decreased; higher levels of adrenaline and nor-adrenaline; dose-response seen; produced chronic physiological stress in cells even after 1.5 years.	Buchner, 2012
0.01 - 0.11 uW/cm ²	RFR from cell towers caused fatigue, headaches, sleeping problems	Navarro, 2003

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

Power Density (Microwatts/centimeter ² - $\mu\text{W}/\text{cm}^2$)		Reference
0.01 - 0.05 $\mu\text{W}/\text{cm}^2$	Adults (18-91 yrs) with short-term exposure to GSM cell phone radiation reported headache, neurological problems, sleep and concentration problems.	Hutter, 2006
0.005 - 0.04 $\mu\text{W}/\text{cm}^2$	Adults exposed to short-term cell phone radiation reported headaches, concentration difficulties (differences not significant, but elevated)	Thomas, 2008
0.015 - 0.21 $\mu\text{W}/\text{cm}^2$	Adults exposed to short-term GSM 900 radiation reported changes in mental state (e.g., calmness) but limitations of study on language descriptors prevented refined word choices (stupified, zoned-out)	Augner, 2009
0.05 - 0.1 $\mu\text{W}/\text{cm}^2$	RFR linked to adverse neurological, cardio symptoms and cancer risk	Khurana, 2010
0.05 - 0.1 $\mu\text{W}/\text{cm}^2$	RFR related to headache, concentration and sleeping problems, fatigue	Kundi, 2009
0.07 - 0.1 $\mu\text{W}/\text{cm}^2$	Sperm head abnormalities in mice exposed for 6-months to base station level RF/MW. Sperm head abnormalities occurred in 39% to 46% exposed mice (only 2% in controls) abnormalities was also found to be dose dependent. The implications of the pin-head and banana-shaped sperm head. The occurrence of sperm head observed increase occurrence of sperm head abnormalities on the reproductive health of humans living in close proximity to GSM base stations were discussed."	Otitolaju, 2010
0.38 $\mu\text{W}/\text{cm}^2$	RFR affected calcium metabolism in heart cells	Schwartz, 1990
0.8 - 10 $\mu\text{W}/\text{cm}^2$	RFR caused emotional behavior changes, free-radical damage by super-weak MWs	Akoev, 2002
0.13 $\mu\text{W}/\text{cm}^2$	RFR from 3G cell towers decreased cognition, well-being	Zwamborn, 2003
0.16 $\mu\text{W}/\text{cm}^2$	Motor function, memory and attention of school children affected (Latvia)	Kolodynski, 1996
0.168 - 1.053 $\mu\text{W}/\text{cm}^2$	Irreversible infertility in mice after 5 generations of exposure to RFR from an 'antenna park'	Magras & Zenos, 1997
0.2 - 8 $\mu\text{W}/\text{cm}^2$	RFR caused a two-fold increase in leukemia in children	Hocking, 1996
0.2 - 8 $\mu\text{W}/\text{cm}^2$	RFR decreased survival in children with leukemia	Hocking, 2000
0.21 - 1.28 $\mu\text{W}/\text{cm}^2$	Adolescents and adults exposed only 45 min to UMTS cell phone radiation reported increases In headaches.	Riddervold, 2008

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

Power Density (Microwatts/centimeter ² - uW/cm ²)		Reference
0.5 uW/cm ²	Significant degeneration of seminiferous epithelium in mice at 2.45 GHz, 30-40 min.	Saunders, 1981
0.5 - 1.0 uW/cm ²	Wi-Fi level laptop exposure for 4-hr resulted in decrease in sperm viability, DNA fragmentation with sperm samples placed in petri dishes under a laptop connected via WI-FI to the internet.	Avendano, 2012
1.0 uW/cm ²	RFR induced pathological leakage of the blood-brain barrier	Persson, 1997
1.0 uW/cm ²	RFR caused significant effect on immune function in mice	Fesenko, 1999
1.0 uW/cm ²	RFR affected function of the immune system	Novoselova, 1999
1.0 uW/cm ²	Short-term (50 min) exposure in electrosensitive patients, caused loss of well-being after GSM and especially UMTS cell phone radiation exposure	Eltiti, 2007
1.3 - 5.7 uW/cm ²	RFR associated with a doubling of leukemia in adults	Dolk, 1997
1.25 uW/cm ²	RFR exposure affected kidney development in rats (in-utero exposure)	Pyrpasopoulou, 2004
1.5 uW/cm ²	RFR reduced memory function in rats	Nittby, 2007
2 uW/cm ²	RFR induced double-strand DNA damage in rat brain cells	Kesari, 2008
2.5 uW/cm ²	RFR affected calcium concentrations in heart muscle cells	Wolke, 1996
2 - 4 uW/cm ²	Altered cell membranes; acetylcholine-induced ion channel disruption	D'Inzeo, 1988
4 uW/cm ²	RFR caused changes in hippocampus (brain memory and learning)	Tattersall, 2001
4 - 15 uW/cm ²	Memory impairment, slowed motor skills and retarded learning in children	Chiang, 1989
5 uW/cm ²	RFR caused drop in NK lymphocytes (immune function decreased)	Boscolo, 2001
5.25 uW/cm ²	20 minutes of RFR at cell tower frequencies induced cell stress response	Kwee, 2001
5 - 10 uW/cm ²	RFR caused impaired nervous system activity	Dumansky, 1974
6 uW/cm ²	RFR induced DNA damage in cells	Phillips, 1998

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

Power Density (Microwatts/centimeter ² - uW/cm ²)		Reference
8.75 uW/cm ²	RFR at 900 MHz for 2-12 hours caused DNA breaks in leukemia cells	Marinelli, 2004
10 uW/cm ²	Changes in behavior (avoidance) after 0.5 hour exposure to pulsed RFR	Navakatikian, 1994
10 - 100 uW/cm ²	Increased risk in radar operators of cancer; very short latency period; dose response to exposure level of RFR reported.	Richter, 2000
12.5 uW/cm ²	RFR caused calcium efflux in cells - can affect many critical cell functions	Dutta, 1989
13.5 uW/cm ²	RFR affected human lymphocytes - induced stress response in cells	Sarimov, 2004
20 uW/cm ²	Increase in serum cortisol (a stress hormone)	Mann, 1998
28.2 uW/cm ²	RFR increased free radical production in rat cells	Yurekli, 2006
37.5 uW/cm ²	Immune system effects - elevation of PFC count (antibody producing cells	Veyret, 1991
45 uW/cm ²	Pulsed RFR affected serum testosterone levels in mice	Forgacs, 2006
50 uW/cm ²	Cell phone RFR caused a pathological leakage of the blood-brain barrier in 1 hour	Salford, 2003
50 uW/cm ²	An 18% reduction in REM sleep (important to memory and learning functions)	Mann, 1996
60 uW/cm ²	RFR caused structural changes in cells of mouse embryos	Somozy, 1991
60 uW/cm ²	Pulsed RFR affected immune function in white blood cells	Stankiewicz, 2006
60 uW/cm ²	Cortex of the brain was activated by 15 minutes of 902 MHz cell phone	Lebedeva, 2000
65 uW/cm ²	RFR affected genes related to cancer	Ivaschuk, 1999
92.5 uW/cm ²	RFR caused genetic changes in human white blood cells	Belyaev, 2005
100 uW/cm ²	Changes in immune function	Elekes, 1996
100 uW/cm ²	A 24.3% drop in testosterone after 6 hours of CW RFR exposure	Navakatikian, 1994
120 uW/cm ²	A pathological leakage in the blood-brain barrier with 915 MHz cell RF	Salford, 1994

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

Power Density (Microwatts/centimeter ² - uW/cm ²)		Reference
500 uW/cm ²	Intestinal epithelial cells exposed to 2.45 GHz pulsed at 16 Hz showed changes in intercellular calcium.	Somozy, 1993
500 uW/cm ²	A 24.6% drop in testosterone and 23.2% drop in insulin after 12 hrs of pulsed RFR exposure.	Navakatikian, 1994

STANDARDS		
530 - 600 uW/cm ²	Limit for uncontrolled public exposure to 800-900 MHz	ANSI/IEEE and FCC
1000 uW/cm ²	PCS STANDARD for public exposure (as of September 1,1997)	FCC, 1996
5000 uW/cm ²	PCS STANDARD for occupational exposure (as of September 1, 1997)	FCC, 1996
BACKGROUND LEVELS		
0.003 uW/cm ²	Background RF levels in US cities and suburbs in the 1990s	Mantiply, 1997
0.05 uW/cm ²	Median ambient power density in cities in Sweden (30-2000 MHz)	Hamnieri, 2000
0.1 - 10 uW/cm ²	Ambient power density within 100-200' of cell site in US (data from 2000)	Sage, 2000

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
0.000064 - 0.000078 W/Kg	Well-being and cognitive function affected in humans exposed to GSM-UMTS cell phone frequencies; RF levels similar near cell sites	TNO Physics and
0.00015 - 0.003 W/Kg	Calcium ion movement in isolated frog heart tissue is increased 18% (P<.01) and by 21% (P<.05) by weak RF field modulated at 16 Hz	Schwartz, 1990
0.000021 - 0.0021 W/Kg	Changes in cell cycle; cell proliferation (960 MHz GSM mobile phone)	Kwee, 1997
0.0003 - 0.06 W/Kg	Neurobehavioral disorders in offspring of pregnant mice exposed in utero to cell phones - dose-response impaired glutamatergic synaptic transmission onto layer V pyramidal neurons of the prefrontal cortex. Hyperactivity and impaired memory function in offspring. Altered brain development.	Aldad, 2012
0.0016 - 0.0044 W/Kg	Very low power 700 MHz CW affects excitability of hippocampus tissue, consistent with reported behavioral changes.	Tattersall, 2001
0.0021 W/Kg	Heat shock protein HSP 70 is activated by very low intensity microwave exposure in human epithelial amnion cells	Kwee, 2001
0.0024 - 0.024 W/Kg	Digital cell phone RFR at very low intensities causes DNA damage in human cells; both DNA damage and impairment of DNA is reported	Phillips, 1998
0.0027 W/Kg	Changes in active avoidance conditioned behavioral effect is seen after one-half hour of pulsed radiofrequency radiation	Navakatikian, 1994
0.0035 W/Kg	900 MHz cell phone signal induces DNA breaks and early activation of p53 gene; short exposure of 2-12 hours leads cells to acquire greater survival chance - linked to tumor aggressiveness.	Marinelli, 2004
0.0095 W/Kg	MW modulated at 7 Hz produces more errors in short-term memory function on complex tasks (can affect cognitive processes such as attention and memory)	Lass, 2002
0.001 W/Kg	750 MHz continuous wave (CW) RFR exposure caused increase in heat shock protein (stress proteins). Equivalent to what would be induced by 3 degree C. heating of tissue (but no heating occurred)	De Pomerai, 2000
0.001 W/Kg	Statistically significant change in intracellular calcium concentration in heart muscle cells exposed to RFR (900 MHz/50 Hz modulation)	Wolke, 1996

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
0.0021 W/Kg	A significant change in cell proliferation not attributable to thermal heating. RFR induces non-thermal stress proteins (960 MHz GSM)	Velizarov, 1999
0.004 - 0.008 W/Kg	915 MHz cell phone RFR caused pathological leakage of blood-brain barrier. Worst at lower SAR levels and worse with CW compared to Frequency of pathological changes was 35% in rats exposed to pulsed radiation at 50% to continuous wave RFR. Effects observed at a specific absorption (SA) of > 1.5 joules/Kg in human tissues	Persson, 1997
0.0059 W/Kg	Cell phone RFR induces glioma (brain cancer) cells to significantly increase thymidine uptake, which may be indication of more cell division	Stagg, 1997
0.014 W/Kg	Sperm damage from oxidative stress and lowered melatonin levels resulted from 2-hr per day/45 days exposure to 10 GHz.	Kumar, 2012
0.015 W/Kg	Immune system effects - elevation of PFC count (antibody-producing cells)	Veyret, 1991
0.02 W/Kg	A single, 2-hr exposure to GSM cell phone radiation results in serious neuron damage (brain cell damage) and death in cortex, hippocampus, and basal ganglia of brain- even 50+ days later blood-brain barrier is still leaking albumin (P<.002) following only one cell phone exposure	Salford, 2003
0.026 W/Kg	Activity of c-jun (oncogene or cancer gene) was altered in cells after 20 minutes exposure to cell phone digital TDMA signal	Ivaschuk, 1997
0.0317 W/Kg	Decrease in eating and drinking behavior	Ray, 1990
0.037 W/Kg	Hyperactivity caused by nitric oxide synthase inhibitor is countered by exposure to ultra-wide band pulses (600/sec) for 30 min	Seaman, 1999
0.037 - 0.040 W/Kg	A 1-hr cell phone exposure causes chromatin condensation; impaired DNA repair mechanisms; last 3 days (longer than stress response) the effect reaches saturation in only one hour of exposure; electro- sensitive (ES) people have different response in formation of DNA repair foci, compared to healthy individuals; effects depend on carrier frequency (915 MHz = 0.037 W/Kg but 1947 MHz = 0.040 W/Kg)	Belyaev, 2008
0.05 W/Kg	Significant increase in firing rate of neurons (350%) with pulsed 900 MHz cell phone radiation exposure (but not with CW) in avian brain cells	Beason, 2002

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
0.09 W/Kg	900 MHz study of mice for 7 days, 12-hr per day (whole-body) resulted in significant effect on mitochondria and genome stability	Aitken, 2005
0.091 W/Kg	Wireless internet 2400 MHz, 24-hrs per day/20 weeks increased DNA damage and reduced DNA repair; levels below 802.11 g Authors say "findings raise questions about safety of radiofrequency exposure from Wi-Fi internet access devices for growing organisms of reproductive age, with a potential effect on fertility and integrity of germ cells" (male germ cells are the reproductive cells=sperm)	Atasoy, 2012
0.11 W/Kg	Increased cell death (apoptosis) and DNA fragmentation at 2.45 GHz for 35 days exposure (chronic exposure study)	Kesari, 2010
0.121 W/Kg	Cardiovascular system shows significant decrease in arterial blood pressure (hypotension) after exposure to ultra-wide band pulses	Lu, 1999
0.13 - 1.4 W/Kg	Lymphoma cancer rate doubled with two 1/2-hr exposures per day of cell phone radiation for 18 months (pulsed 900 MHz cell signal)	Repacholi, 1997
0.14 W/Kg	Elevation of immune response to RFR exposure	Elekes, 1996
0.141 W/Kg	Structural changes in testes - smaller diameter of seminiferous	Dasdag, 1999
0.15 - 0.4 W/Kg	Statistically significant increase in malignant tumors in rats chronically exposed to RFR	Chou, 1992
0.26 W/Kg	Harmful effects to the eye/certain drugs sensitize the eye to RFR	Kues, 1992
0.28 - 1.33 W/Kg	Significant increase in reported headaches with increasing use of hand-held cell phone use (maximum tested was 60 min per day)	Chia, 2000
0.3 - 0.44 W/Kg	Cell phone use results in changes in cognitive thinking/mental tasks related to memory retrieval	Krause, 2000
0.3 - 0.44 W/Kg	Attention function of brain and brain responses are speeded up	Preece, 1999
0.3 - 0.46 W/Kg	Cell phone RFR doubles pathological leakage of blood-brain barrier permeability at two days (P=.002) and triples permeability at four days (P=.001) at 1800 MHz GSM cell phone radiation	Schirmacher, 2000
0.43 W/Kg	Significant decrease in sperm mobility; drop in sperm concentration; and decrease in seminiferous tubules at 800 MHz, 8-hr/day, 12 weeks, with mobile phone radiation level on STANDBY ONLY (in rabbits)	Salama, 2008

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
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Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
0.5 W/Kg	900 MHz pulsed RF affects firing rate of neurons (<i>Lymnea stagnalis</i>) but continuous wave had no effect	Bolshakov, 1992
0.58 - 0.75 W/Kg	Decrease in brain tumors after chronic exposure to RFR at 836 MHz	Adey, 1999
0.6 - 0.9 W/Kg	Mouse embryos develop fragile cranial bones from in utero 900 MHz The authors say "(O)ur results clearly show that even modest exposure (e.g., 6 min daily for 21 days" is sufficient to interfere with the normal mouse developmental process"	Fragopoulou, 2009
0.6 and 1.2 W/Kg	Increase in DNA single and double-strand DNA breaks in rat brain cells with exposure to 2450 MHz RFR	Lai & Singh, 1996
0.795 W/Kg	GSM 900 MHz, 217 Hz significantly decreases ovarian development and size of ovaries, due to DNA damage and premature cell death of nurse cells and follicles in ovaries (that nourish egg cells)	Panagopoulous, 2012
0.87 W/Kg	Altered human mental performance after exposure to GSM cell phone radiation (900 MHz TDMA digital cell phone signal)	Hamblin, 2004
0.87 W/Kg	Change in human brainwaves; decrease in EEG potential and statistically significant change in alpha (8-13 Hz) and beta (13-22 Hz) brainwave activity in humans at 900 MHz; exposures 6/min per day for 21 days (chronic exposure)	D'Costa, 2003
0.9 W/Kg	Decreased sperm count and more sperm cell death (apoptosis) after 35 days exposure, 2-hr per day	Kesari, 2012
< 1.0 W/Kg	Rats exposed to mobile phone radiation on STANDBY ONLY for 11-hr 45-min plus 15-min TRANSMIT mode; 2 times per day for 21 days showed decreased number of ovarian follicles in pups born to these pregnant rats. The authors conclude "the decreased number of follicles in pups exposed to mobile phone microwaves suggest that intrauterine exposure has toxic effects on ovaries."	Gul, 2009
0.4 - 1.0 W/Kg	One 6-hr exposure to 1800 MHz cell phone radiation in human sperm cells caused a significant dose response and reduced sperm motility and viability; reactive oxygen species levels were significantly increased after exposure to 1.0 W/Kg; study confirms detrimental effects of RF/MW to human sperm. The authors conclude "(T)hese findings have clear implicatiions for the safety of extensive mobile phone use by males of reproductive age, potentially affecting both their fertility and the health and wellbeing of their offspring."	De Iuliis, 2009
1.0 W/Kg	Human semen degraded by exposure to cell phone frequency RF increased free-radical damage.	De Iuliis, 2009

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
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Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
1.0 W/Kg	Motility, sperm count, sperm morphology, and viability reduced in active cell phone users (human males) in dose-dependent manner.	Agarwal, 2008
1.0 W/Kg	GSM cell phone use modulates brain wave oscillations and sleep EEG	Huber, 2002
1.0 W/Kg	Cell phone RFR during waking hours affects brain wave activity. (EEG patterns) during subsequent sleep	Achermann, 2000
1.0 W/Kg	Cell phone use causes nitric oxide (NO) nasal vasodilation (swelling inside nasal passage) on side of head phone use	Paredi, 2001
1.0 W/Kg	Increase in headache, fatigue and heating behind ear in cell phone users	Sandstrom, 2001
1.0 W/Kg	Significant increase in concentration difficulties using 1800 MHz cell phone compared to 900 MHz cell phone	Santini, 2001
1.0 W/Kg	Sleep patterns and brain wave activity are changed with 900 MHz cell phone radiation exposure during sleep	Borbely, 1999
1.4 W/Kg	GSM cell phone exposure induced heat shock protein HSP 70 by 360% (stress response) and phosphorylation of ELK-1 by 390%	Weisbrot, 2003
1.46 W/Kg	850 MHz cell phone radiation decreases sperm motility, viability is significantly decreased; increased oxidative damage (free-radicals) significantly decreased; increased oxidative damage (free-radicals)	Agarwal, 2009
1.48 W/Kg	A significant decrease in protein kinase C activity at 112 MHz with 2-hr per day for 35 days; hippocampus is site, consistent with reports that RFR negatively affects learning and memory functions	Paulraj, 2004
1.0 - 2.0 W/Kg	Significant elevation in micronuclei in peripheral blood cells at 2450 MHz (8 treatments of 2-hr each)	Trosic, 2002
1.5 W/Kg	GSM cell phone exposure affected gene expression levels in tumor suppressor p53-deficient embryonic stem cells; and significantly increased HSP 70 heat shock protein production	Czyz, 2004
1.8 W/Kg	Whole-body exposure to RF cell phone radiation of 900-1800 MHz 1 cm from head of rats caused high incidence of sperm cell death; deformation of sperm cells; prominent clumping together of sperm cells into "grass bundle shapes" that are unable to separate/swim. Sperm cells unable to swim and fertilize in normal manner.	Yan, 2007

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
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Reported Biological Effects from Radiofrequency Radiation at Low-Intensity Exposure (Cell Tower, Wi-Fi, Wireless Laptop and 'Smart' Meter RF Intensities)

SAR (Watts/Kilogram)		Reference
2.0 W/Kg	GSM cell phone exposure of 1-hr activated heat shock protein HSP 27 (stress response) and P38 MAPK (mutagen-activated protein kinase) that authors say facilitates brain cancer and increased blood-brain barrier permeability, allowing toxins to cross BBB into brain	Leszczynski, 2002
2 W/Kg	900 MHz cell phone exposure caused brain cell oxidative damage by increasing levels of NO, MDA, XO and ADA in brain cells; caused statistically significant increase in 'dark neurons' or damaged brain cells in cortex, hippocampus and basal ganglia with a 1-hr exposure for 7 consecutive days	Ilhan, 2004
2.6 W/Kg	900 MHz cell phone exposure for 1-hr significantly altered protein expression levels in 38 proteins following irradiation; activates P38 MAP kinase stress signalling pathway and leads to changes in cell size and shape (shrinking and rounding up) and to activation of HSP 27, a stress protein (heat shock protein)	Leszczynski, 2004
2.0 - 3.0 W/Kg	RFR accelerated development of both skin and breast tumors	Szmigielski, 1982
2 W/Kg	Pulse-modulated RFR and MF affect brain physiology (sleep study)	Schmidt, 2012

STANDARDS		
0.08 W/Kg	IEEE Standard uncontrolled public environment (whole body)	IEEE
0.4 W/Kg	IEEE Standard controlled occupational environment (whole body)	IEEE
1.6 W/Kg	FCC (IEEE) SAR limit for 1 gram of tissue in a partial body exposure	FCC, 1996
2 W/Kg	ICNIRP SAR limit for 10 grams of tissue	ICNIRP, 1996

Stress proteins, HSP, disrupted immune function	Brain tumors and blood-brain barrier
Reproduction/fertility effects	Sleep, neuron firing rate, EEG, memory, learning, behavior
Oxidative damage/ROS/DNA damage/DNA repair failure	Cancer (other than brain), cell proliferation
Disrupted calcium metabolism	Cardiac, heart muscle, blood-pressure, vascular effects

Attachment 23

Effects of zero magnetic field on the conformation of chromatin in human cells

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Received 12 February 1997; revised 17 April 1997; accepted 21 April 1997

Abstract

The effects of zero magnetic field on human VH-10 fibroblasts and lymphocytes were studied by the method of anomalous viscosity time dependencies (AVTD). A decrease of about 20% in the AVTD peaks was observed within 40 to 80 min of exposure of fibroblasts. This decrease was transient and disappeared 120 min after beginning of exposure. Similar kinetics for the effect of zero field was observed when cells were exposed 20 min and then kept at an ambient field. A 20% decrease of the AVTD peaks ($p < 0.005$ to 0.05) 40 to 70 min after 20 min exposure to zero field was reproduced in four independent experiments (out of four) with human lymphocytes from the same healthy donor. Contrary to the effects of zero field, irradiation of lymphocytes or fibroblasts with γ -rays resulted in significant increase of the AVTD peaks immediately after irradiation. We concluded that zero field and γ -rays caused hypercondensation and decondensation of chromatin, correspondingly. The effect of ethidium bromide served as a positive control and supported this conclusion. The effects of zero field on human lymphocytes were more significant in the beginning of G1-phase than in G0-phase. Thus, human fibroblasts and lymphocytes were shown to respond to zero magnetic field. © 1997 Elsevier Science B.V.

Keywords: Chromatin; Viscosity; Lymphocyte; Fibroblast; Magnetic field; Radiation; (Human)

1. Introduction

An increasing number of investigations has shown that weak (nonthermal) electromagnetic fields (EMF) affect biological systems ([1–3]; for review). Still, there is no accepted mechanism for such interaction

and some effects of EMF could not be reproduced under similar experimental conditions [4]. Some effects of electromagnetic fields were shown to be influenced by the static magnetic field (SMF) which can differ between laboratories due to different latitudes and ferromagnetic objects [5,6]. Usually, the SMF is considered as an important parameter during exposure to electromagnetic fields [5–11]. However, experimental data on the biological effects of weak SMF (comparable in field strength with geomagnetic fields) are very limited [12–14]. On the other hand, it is well known that many animals can detect the direction of the geomagnetic field and use it as an aid to navigation [15–17]. These observations suggest

Abbreviations: AVTD, anomalous viscosity time dependence; EMF, electromagnetic field; EtBr, ethidium bromide; ELF, extremely low frequency; PHA, phytohemagglutinin; SMF, static magnetic field

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that at least some cells or cellular structures are sensitive to weak changes in the SMF [12]. Zero magnetic field has been supposed to be an important reference in studies of effects of geomagnetic field on animals and man [18]. Changes in the ability to orient were described for animals, birds and fishes when magnetic field was compensated to zero [15–17]. Several different mechanisms have been considered to explain the biological effects of SMF [7,19–22]. The endogenous radicals have been regarded as primary targets for effects of magnetic fields, including SMF of geomagnetic values ([23], for review). Another theory was based on a possible modulation of high frequency oscillations in the cell nuclei [24]. A wave-like dependence of the SMF effect on magnetic flux density in the range of 0 to 114 μT was observed when the changes of anomalous viscosity time dependencies (AVTD) in lysates of *E. coli* cells were studied [24,25]. The largest effect was found when cells were exposed to zero magnetic field. In this study, the effects of zero magnetic field on human fibroblasts and human lymphocytes were examined using the AVTD method. For comparison, the AVTD effects of ionizing radiation at the same conditions of exposure were studied. A correlation between changes in AVTD and changes in chromatin conformation has been examined and discussed previously [10,26–28]. In this paper, we studied the AVTD effects of ethidium bromide (EtBr) which is known to act as a specific intercalating drug for DNA, changing the conformation of DNA and DNA-protein complexes [29]. Titration curve with EtBr served as a positive control for relationship of increase in the AVTD peaks with decondensation of chromatin and decrease in the AVTD peaks with chromatin condensation.

2. Materials and methods

2.1. Cells

Normal human diploid fibroblasts VH-10 were maintained at 5% CO_2 and 37°C in a humidified incubator as described previously [27]. Briefly, cells were seeded into 260 ml Nunclon flasks (Nunc, Denmark) and grown in Dulbecco's modification of minimal Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 90 IU/ml peni-

cillin, 90 $\mu\text{g}/\text{ml}$ streptomycin. Confluent cultures (12th–14th passage, 5 to 6 days after seeding, approximately 3 to 5×10^6 cells/flask) were used in experiments. The cells were trypsinised when reaching confluency and washed with Hanks solution (ICN Biomedicals, Inc., USA). Then cells were counted in a hemocytometer with trypan blue and diluted in RPMI 1640 medium with L-glutamine (ICN Biomedicals, Inc., USA), to a final concentration of 10^6 cells/ml. Viability was not less than 95% for all types of cells.

The human lymphocytes were obtained from peripheral blood of healthy donors by means of separation in Ficoll-Paque according to instructions of manufacturer (Pharmacia LKB, Sweden). The cells were diluted at a concentration of 1 to 1.5×10^6 cells/ml in RPMI 1640 medium or in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin (full medium). PHA (Flow Laboratories, UK) was used at a concentration of 20 $\mu\text{g}/\text{ml}$ to stimulate proliferation. We used 20 $\mu\text{g}/\text{ml}$ based on data about dependence of effects of magnetic fields on the PHA concentration [30] and observation that under this particular concentration the effect of magnetic field was observed in stimulated human lymphocytes [31].

2.2. Exposure

After dilution to a pre-set concentration, cells were preincubated 30 min before exposure. Preincubation was performed under the same conditions as exposure. Cells were exposed to zero magnetic field by means of compensation of ambient magnetic field using two pairs of Helmholtz coils. These coils were designed so that samples would be in a uniform field during exposure. Vertical coils were oriented in parallel to the horizontal ambient field before compensation. Horizontal (vertical) static magnetic field was applied by means of a pair of 100 turns, 19.6 (12.8) cm diameter Helmholtz coils mounted vertically (horizontally). Each coil had a resistance of 7.87 (5.23) Ω and an inductance of 4.77 (2.74) mH. The direct currents were supplied by two stabilized power supplies. The static magnetic fields were measured using a SAM 3 magnetometer (Dowty Electronics Ltd., UK) and calculated by means of measuring a

direct current. The measured and calculated values were equal with an accuracy better than 1%. Special precautions were taken to control the exposure of cells to ambient magnetic fields of extremely low frequency (ELF). As previously shown, weak ELF could result in the AVTD effects in *E. coli* cells [32]. The ELF magnetic field was measured by means of a Combinova MFM10 3-dimensional microteslometer. This field was up to 1.8 μT in our CO_2 -incubators. In order to exclude possible effect of this field, some experiments were performed without incubators at room temperature and ambient ELF magnetic field less than 0.15 μT . Preincubation was performed at the same temperature as exposure in each experiment.

The temperature was measured during exposure with an accuracy of 0.1°C. No measurable heating due to compensation of ambient magnetic field was observed during incubation of cells in Helmholtz coils. The control cells were concurrently subjected to the same manipulations except for exposure. Sham-exposed cells were run under the same conditions as exposed cells. The only difference was disconnection of wires between power supply and Helmholtz coils for sham-exposure. Control and sham-exposure were run in triplicate for each series of experiment. Control did not differ from sham exposure.

The cells were irradiated with ^{137}Cs γ -rays at a dose rate of 1 cGy/s. The cells were incubated after irradiation at room temperature or in a CO_2 incubator at 37°C as specified.

2.3. Cell lysis and AVTD measurements

Cell lysis was performed as described previously with some modifications [27]. VH-10 cells were lysed in polyallomer centrifuge tubes ($\varnothing 14$ mm, Beckman, USA) by the addition of a 3.2 ml lysis solution (0.25 M Na_2EDTA , 0.5% sarcosyl, 10 mM Tris-base, pH 7.15) to 0.3 ml of cell suspension. Tubes were kept in a water bath at 57°C for 10 min and then kept at 26°C for 2.5 h before AVTD measurements. The human lymphocytes were lysed by addition of 3 ml lysis solution (0.25 M Na_2EDTA , 2% sarcosyl, 10 mM Tris-base, pH 7.4) to 0.5 ml of a cell suspension. The lysates were kept at 26°C for 5 h before AVTD measurements. Ethidium bromide (EtBr) from Sigma (USA) was added to lysis solution if specified. The

lysis of cells was performed in darkness. The experimental and control samples were treated concurrently and exactly in the same way. The anomalous viscosity time dependencies were measured in lysates as described [27] using an AVTD-analyzer (Archer-Aquarius, Ltd., Russia). This method is based on radial migration of large DNA-protein complexes in a high-gradient hydrodynamic field of a rotary viscometer [33]. Radial migration of molecular complexes towards the rotating rotor causes anomalous changes of viscosity that can be registered by measuring the rotor rotation period as a function of time of rotation (Fig. 1). The rotor-tube was suspended in the cell lysate such that the rim of the rotor was at the meniscus of the solution. Then the rotor was set in rotation by a constant moment of force created by the electromagnetic field. After the rotation was switched on (time zero in Fig. 1), the viscosity of the lysate (period of rotation) increased due to the radial migration of the DNA-protein complexes. After the DNA had deposited on the external surface of the rotor, the period of rotation decreased to a value typical for the pure solvent. The dependence of viscosity on time after the start of the rotor is called anomalous viscosity time dependence. There are several shapes of the AVTD curves which can be realized during radial migration of DNA-protein complexes in Couette flow

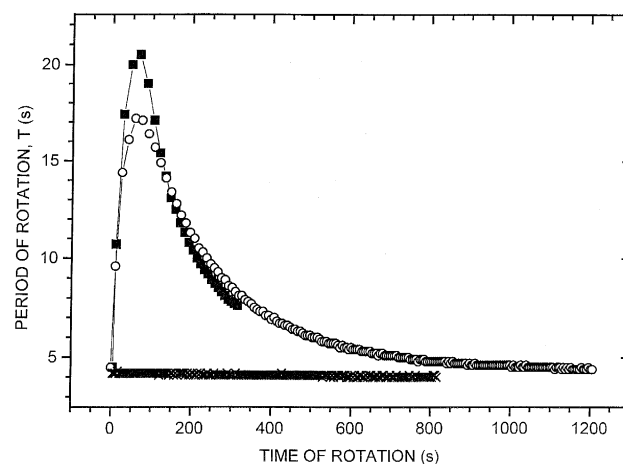


Fig. 1. Anomalous viscosity time dependence in the lysates of human lymphocytes which were lysed as described in Section 2 without (—○—) or with ethidium bromide in a concentration of 5 $\mu\text{g}/\text{ml}$ (—■—). The dependence of rotation period on duration of rotation for cell-free lysing solution is shown (—x—).

of a rotary viscometer [26]. The temperature of treatment as well as other conditions of lysis, including the time of incubation, were determined based on shape of the AVTD curve for control cells in order to get the optimal AVTD shape shown in Fig. 1. The AVTD depends on the chromatin conformational state which in turn is dependent on the DNA parameters such as molecular weight, micro-medium, and the number of proteins bound to the DNA. Each AVTD curve is a set of experimental points (period of rotation versus time of measurement) which are recorded by an IBM PC. The AVTDs were measured at the shear rate of 5.6 s^{-1} and shear stress of 0.007 N/m^2 . For each experimental condition, the AVTD was measured three times in different tubes. The maximum period of rotation (T_{max}) has been previously shown to be the most sensitive AVTD parameter [34,35]. The significance of differences between mean values in exposed samples $\langle T_{\text{max exp}} \rangle$ and control samples $\langle T_{\text{max con}} \rangle$ was evaluated with the Student's *t*-test in each experiment. Maximum relative viscosity (η) was used to determine the effect of exposure: $\eta = \langle T_{\text{max exp}} \rangle / \langle T_{\text{max con}} \rangle$. The summary data from several identical experiments was statistically analyzed using regression analysis, Student's *t*-test or paired Student's *t*-test. Results were considered as significantly different at $p < 0.05$.

3. Results

In first series of experiments with VH-10 human fibroblasts, two plastic tubes with 7 ml of cell suspension were preincubated for 30 min in CO_2 -incubator at $23 \pm 1 \text{ }^\circ\text{C}$. Then, one of these tubes was placed in another incubator with static magnetic field compensated to $0 \pm 0.1 \text{ }^\circ\text{C}$. Every 20 min of incubation, three aliquots of 0.3 ml were taken from each tube for cell lysis. After 40 min of cell incubation, statistically significant AVTD changes were observed in each of three independent experiments with maximum effect between 40 and 80 min of incubation. The summation of data produced the kinetics shown in Fig. 2. This effect was transient and disappeared 120 min after beginning of exposure (not shown). In subsequent experiments, the cells were incubated in zero field during 20 min and then returned to the same place in incubator with $23 \pm 1 \text{ }^\circ\text{C}$. Again, the

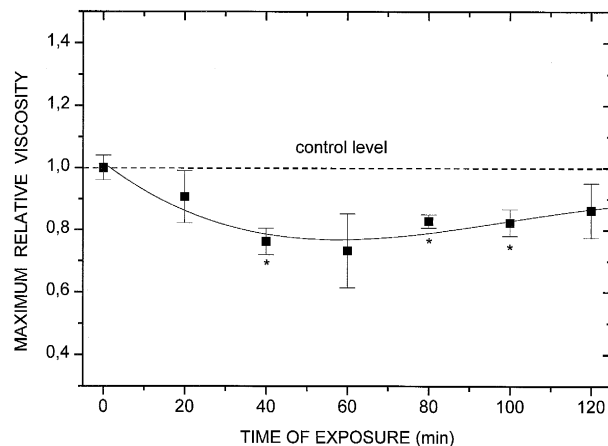


Fig. 2. Dependence of maximum relative viscosity on duration of VH-10 cell incubation in the magnetic field which was compensated to $0 \pm 0.1 \text{ }^\circ\text{C}$ by means of Helmholtz coils. The control fibroblasts were incubated at a magnetic field of $23 \pm 1 \text{ }^\circ\text{C}$. Mean of 3 independent experiments is given in each data point (* $p < 0.05$). Cubic polynomial regression was the best fit ($p < 0.05$) and shown in figure. Standard error is shown in all figures.

samples were taken every 20 min from control and exposed tubes. In each of three independent experiments, a statistically significant decrease in the AVTD peaks were observed with maximum effect between 40 and 80 min of incubation after exposure. The summary kinetics of the effect (Fig. 3) was similar to the one, obtained with continuous cell exposure to zero field (Fig. 2). The AVTD changes after irradiation of fibroblasts with 0.5 Gy of γ -rays were studied under the same incubation conditions to compare with the effect of zero field (Fig. 3). The AVTD peaks increased by 40% if cells were lysed immediately after irradiation. This effect disappeared after 20 min. Thus, the reverse response and differences in kinetics were observed after exposure of human fibroblasts to zero field as compared to 0.5 Gy. One possible artifact could be short-time decrease of temperature within 0.5°C which was observed after opening the doors of incubators. In order to test this possibility, the VH-10 cells were incubated at 36, 37 or 38°C for 20 min and then lysed for measurements. No significant temperature-induced difference in AVTD was observed (not shown). Another possible artifact of these experiments was the variation in the background ELF magnetic fields which are usually created by incubators when heating. Weak ELF fields have been shown to produce AVTD effects in cells

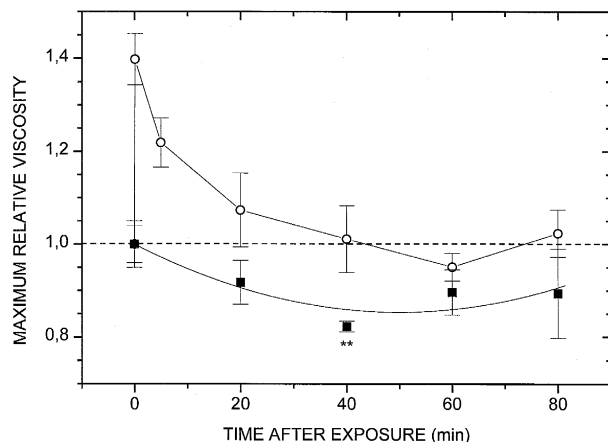


Fig. 3. Dependence of maximal relative viscosity on time of cell incubation after 20 min exposure to $0 \pm 0.1 \mu\text{T}$ (-■-) or 0.5 Gy of γ -rays (-○-). After dilution to a concentration of 10^6 cells/ml, the VH-10 cells, were preincubated at a SMF of $23 \pm 1 \mu\text{T}$, then exposed to zero field for 20 min (-■-) or 0.5 Gy of γ -rays (-○-), and again incubated at $23 \pm 1 \mu\text{T}$ before lysis. Means of 3 (-■-) or 2 (-○-) independent experiments are shown (* $p < 0.005$). Cubic polynomial regression ($p < 0.05$) is shown for the effect of zero field.

[32]. Variations in ELF magnetic flux density were 0.03 – $1.8 \mu\text{T}$ in the incubators during experiments. Nevertheless, no difference in AVTD was observed between cells which were simultaneously incubated in our incubators for 120 min and were taken for lysis every 20 min in three independent experiments (not shown).

In order to decrease the ELF background to less than $0.15 \mu\text{T}$, the experiments with human lymphocytes were performed without incubators, at room temperature. Fresh lymphocytes were diluted in RPMI 1640 medium, 0.5 ml distributed to test tubes, and kept for 30 min at an ambient magnetic field of $47 \pm 3 \mu\text{T}$. Then, cells were exposed to zero field during 20 min, three test-tubes in each exposure. The cells were kept at $47 \pm 3 \mu\text{T}$ within 10 to 180 min after exposure and then lysed. With the first donor we observed an effect of zero field with decrease of the AVTD peaks approximately 20% between 20 and 60 min ($p < 0.001$ to 0.05) after exposure. This statistically significant effect was reproduced in each of three subsequent experiments with the same donor. The summation of all experimental data from four independent experiments is given in Fig. 4. It is seen from Fig. 4, that effect of zero magnetic field had a

wave-like dependence on incubation time after exposure and the AVTD changes disappeared in 180 min after exposure. Parallel experiments were performed under the same conditions to compare effects of zero field and ionizing radiation. Significant increase in the AVTD peaks, about 70%, were observed immediately after irradiation of human lymphocytes with 0.5 Gy in 3 independent experiments (Fig. 4). Then the radiation-induced effect disappeared gradually due to repair processes. This repair was similar to that which has been observed previously in VH-10 cells at room temperature [27]. Again, the effects of zero field and ionizing radiation had opposite directions and significant differences in kinetics after exposure.

The following experiments with lymphocytes from two donors was designed to compare the sensitivity of G0- and G1-phases of cell cycle to zero field. The lymphocytes were preincubated in CO_2 -incubator at $23 \pm 1 \mu\text{T}$ in plastic tubes for 30 min after dilution at full medium. Then cells were exposed to zero field in G0-phase or stimulated by PHA for 2 h. Two tubes with equal volume of 7 to 10 ml of cell suspension were used in each cell cycle point tested. One of these tubes served as a control. Second one was placed in another incubator with static magnetic field

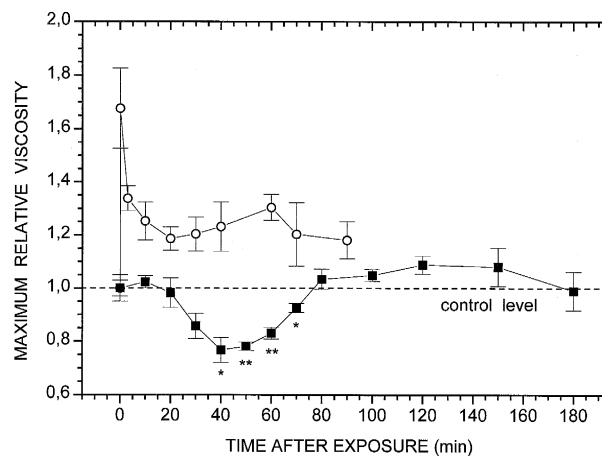


Fig. 4. Dependence of maximum relative viscosity on time after exposure of lymphocyte to zero magnetic field. The cells were preincubated 30 min at SMF of $47 \pm 3 \mu\text{T}$, exposed 20 min to $0 \pm 1 \mu\text{T}$ (-■-) or 0.5 Gy of γ -rays (-○-), and then incubated at $47 \pm 3 \mu\text{T}$ before lysis. All incubations and exposure was performed in RPMI 1640 medium at room temperature. Means of 4 (-■-) or 3 (-○-) independent experiments are given (* $p < 0.05$, ** $p < 0.005$).

compensated to $0 \pm 0.1 \mu\text{T}$. Every 10 or 20 min of incubation, 3 aliquots of 0.5 ml each were taken from both control and exposed tubes for cell lysis. For both donors, the decrease in AVTD was weak and statistically insignificant in G0. But lymphocytes displayed stronger sensitivity to zero field in G1-phase when exposure started in 2 h after PHA stimulation (Fig. 5). A significant decrease in the AVTD was observed within 20 to 80 min of exposure ($p < 0.01$, donor A; $p < 0.05$, donor B; paired t -test).

In two independent experiments with lymphocytes of the same donor, ethidium bromide in a concentration of 3 to 50 $\mu\text{g/ml}$ increased ($p < 0.01$ to $5 \cdot 10^{-4}$) the AVTD peaks (Fig. 1). The dependence of maximum relative viscosity on concentration was biphasic and had a maximum around 50 $\mu\text{g/ml}$ (Fig.

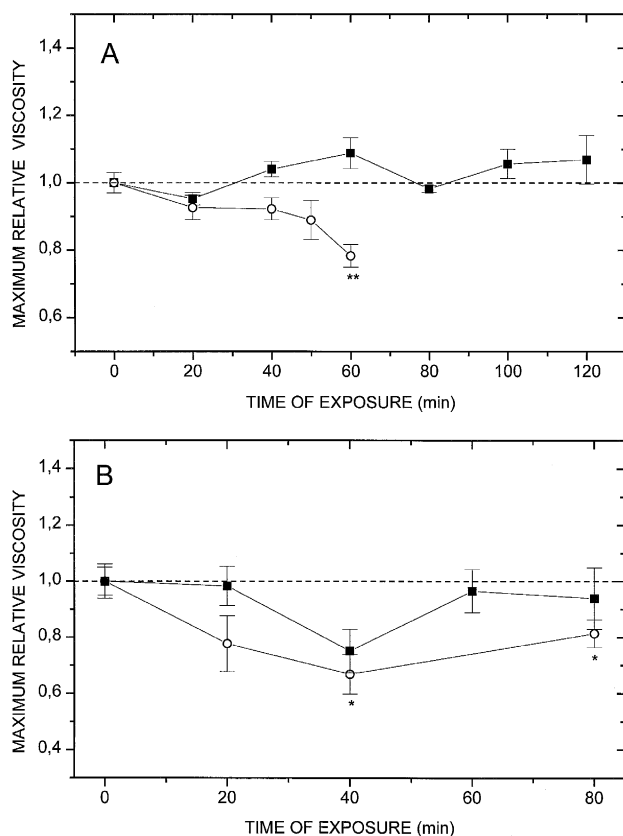


Fig. 5. Dependence of maximum relative viscosity on duration of exposure to zero magnetic field. The lymphocytes of 2 different donors (A and B) were exposed to the $0 \pm 1 \mu\text{T}$ magnetic field in G0- (■) or G1-phase (○) of cell cycle. The preincubation was performed in full RPMI medium at 37°C and a SMF of $23 \pm 1 \mu\text{T}$. Means of 3 measurements are given (* $p < 0.05$, ** $p < 0.005$).

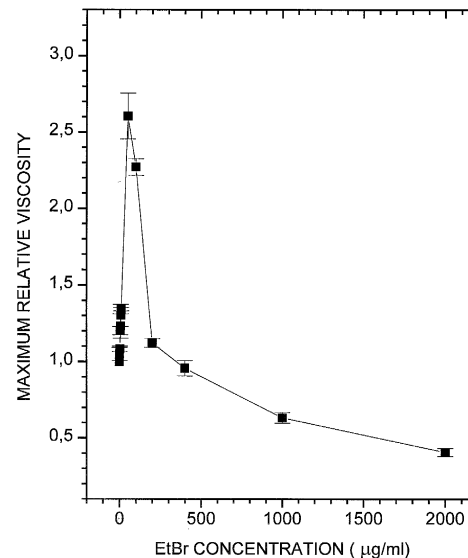


Fig. 6. Dependence of the maximum relative viscosity on the concentration of ethidium bromide during lysis of human lymphocytes. Ethidium bromide was added with lysis solution. Lysis was performed at darkness.

6). Then viscosity decreased and reached the control value at a concentration of 400 $\mu\text{g/ml}$. The further increase of the EtBr concentration resulted in significant decrease of the AVTD peaks below the control level.

4. Discussion

This study provided evidence that zero magnetic field caused significant changes of the AVTD in two different types of human cells. This has been previously shown for *E. coli* cells [24,25]. The biological significance of the AVTD changes induced by zero field remains to be investigated. According to the theory of radial migration, the migration of large DNA complexes towards a rotating rotor is determined by several parameters of chromatin conformation like as nucleoid rigidity, hydrodynamic radius, molecular weight, compactness [26,36]. Therefore, it seems likely that several processes leading to changes in chromatin conformation are involved, and the AVTD technique reflects a summation of these processes. A similar summation of the chromatin conformational changes may be produced by other techniques such as sedimentation in sucrose gradient [37] or halo assay [38] which respond to different proper-

ties of chromatin from cell lysates. Several experimental observations indicated relationship between the AVTD measurements and changes in chromatin conformation [27,28]. Gamma-rays induced chromatin decondensation as measured by viscoelastometry, sedimentation of nucleoids and the AVTD method with the maximum effects under the doses of 1 to 2 Gy which are equivalent to one single strand DNA break per domain of supercoiling in mammalian cells and cause complete relaxation of nucleoids [28,39]. Similar relaxation of nucleoids was induced by irradiation of cells and cell lysates as measured by increase in the AVTD peaks and as measured with classic viscometry technique [27,40]. Etoposide VP-16 produced the same AVTD increase as ionizing radiation [27]. This antitumor drug is known as a specific inhibitor of a topoisomerase-DNA interaction which results in trapping a covalently linked complex of the enzyme and 5' cleaved termini of the DNA molecule followed by changes in supercoiling of the domain. On the other hand, the AVTD peaks disappeared if lysates were subjected to agents that defragmented DNA, e.g. γ -rays at doses above 20 Gy or ultrasound.

EtBr changes the behavior of large DNA-protein complexes such as chromatin or nucleoids due to changes in a conformation of DNA which is supercoiled in cells and cell lysates [29]. If lysis is performed at 2 M NaCl and low Na₂EDTA concentration, the maximum of titration curve is observed at different concentrations of intercalator between 3 and 13 $\mu\text{g/ml}$ [29,38,41]. Despite these differences in maxima, the biphasic shape of titration curve is considered to reflect the release of negative supercoils in chromatin/nucleoids and the appearance of positive supercoils due to intercalation of EtBr to double helix of DNA [41]. In this study, the AVTD technique also showed the biphasic behavior of chromatin as was previously obtained by other methods such as the halo assay [38] or sedimentation of nucleoids [29,41]. The maximum around 50 $\mu\text{g/ml}$ in our experiments can be attributed to the difference in lysing solutions as far as interaction of intercalator with chromatin depends on salt conditions during lysis [41]. The considerable drop of the AVTD peaks at the high concentrations of 1 to 2 mg/ml can be caused by complete release of proteins in competition with EtBr followed by hypercondensation or degradation of

DNA [42]. The biphasic titration curve with ethidium bromide indicates relationship between the AVTD increase/decrease and chromatin decondensation/condensation.

At the molecular level, the AVTD changes can be caused both by DNA breaks and changes in DNA-protein interactions [27]. As it was shown with human lymphocytes and human fibroblasts, the AVTD effects had opposite direction and different kinetics for zero field and γ -rays. The difference in the AVTD effects of ionizing radiation and electromagnetic fields has been previously observed in *E. coli* cells [43]. The increase in the AVTD peaks after γ -irradiation of human cells is dealt with relaxation (decondensation) of chromatin due to DNA breaks. We suppose that hypercondensation of chromatin due to interactions of DNA with proteins and ions may be involved in response of cells to zero field. The changes in spectrum of proteins bound to DNA were shown while studying the effects of electromagnetic fields in *E. coli* cells [10,44]. Since the effects of zero magnetic field were observed with different cell types, this may indicate a common primary target such as ions [7,9], endogenous radicals [23] or domains of DNA supercoiling [24,25].

In all studied cases, the zero field effect was transient. Probably, this observation reflects the adaptive ability of cells to respond to weak changes of ambient magnetic fields. It is known, that some weak stimuli can affect the sensitivity of cells to subsequent influence of DNA-damaging agents. So-called adaptive response is observed when weak stimuli such as a primary low dose of ionising radiation (or chemical mutagens) was followed by exposure to a challenge high dose [45]. Zero magnetic field induced the same decrease of the AVTD peaks as was observed 40 to 60 min after exposure of human fibroblasts to low doses of γ -rays in the range of 0.5 to 2 cGy [27]. Under these conditions of exposure, significant changes in radiation-induced survival were found when human fibroblasts were irradiated with a challenge dose at time of maximum AVTD changes induced by primary low-dose irradiation [28]. One possible explanation of these observations could be the induction of changes in chromatin conformation which is considered to be important for sensitivity to genotoxic agents such as ionising radiation [46]. Weak EMF were also shown to modify the radiation-in-

duced damage [10,34]. In this context it would be interesting to study whether zero field can modify the sensitivity of cells to genotoxic agents.

In the present study, significant difference in response to zero field was observed between lymphocytes of tested donors. Such individual variability was also found in response of lymphocytes to other weak stimuli. For instance, the individual reactions of human lymphocytes to low-dose ionizing radiation were shown in an adaptive response as measured by chromosome aberrations [47] and with AVTD technique [35]. Individual changes in ability of stingrays to orient was observed by Kalmijn when magnetic field was compensated to zero [16]. Despite the individual variations between donors, we observed a reproducible response to the zero magnetic field with lymphocytes from the same donor. Individual variability should be taken into account in studies of adaptive reactions on magnetic fields. The chromatin conformation in G1-lymphocytes was more sensitive to zero magnetic field than in G0-phase. This observation is in agreement with data of other laboratories that mitogen-activation facilitates responses of lymphocytes to weak electromagnetic fields [8,30,48]. In conclusion, the present data suggest that the cells of different types may respond to zero magnetic field and the transient dependence of the response may reflect adaptive reactions of cells to changes in magnetic environment.

Acknowledgements

The authors are thankful to M. Tibäck for excellent technical assistance. We thank Dr. L.-E. Paulsson and Dr. G. Anger from Swedish Radiation Protection Institute for help with and providing the devices for magnetic fields measurements. This study was supported in part by the Swedish Council for Work Life Research, Svenska Elforsk AB, and Grant 95-04-12038a from the Russian Foundation for Fundamental Research.

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Attachment 24

The effects of radiofrequency fields on cell proliferation are non-thermal

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Received 30 November 1998; revised 14 December 1998; accepted 14 December 1998

Abstract

The number of reports on the effects induced by radiofrequency (RF) electromagnetic fields and microwave (MW) radiation in various cellular systems is still increasing. Until now no satisfactory mechanism has been proposed to explain the biological effects of these fields. One of the current theories is that heat generation by RF/MW is the cause, in spite of the fact that a great number of studies under isothermal conditions have reported significant cellular changes after exposure to RF/MW. Therefore, this study was undertaken to investigate which effect MW radiation from these fields in combination with a significant change of temperature could have on cell proliferation. The experiments were performed on the same cell line, and with the same exposure system as in a previous work [S. Kwee, P. Raskmark, Changes in cell proliferation due to environmental non-ionizing radiation: 2. Microwave radiation, *Bioelectrochem. Bioenerg.*, 44 (1998), pp. 251–255]. The field was generated by signal simulation of the Global System for Mobile communications (GSM) of 960 MHz. Cell cultures, growing in microtiter plates, were exposed in a specially constructed chamber, a Transverse Electromagnetic (TEM) cell. The Specific Absorption Rate (SAR) value for each cell well was calculated for this exposure system. However, in this study the cells were exposed to the field at a higher or lower temperature than the temperature in the field-free incubator i.e., the temperature in the TEM cell was either 39 or 35 ± 0.1°C. The corresponding sham experiments were performed under exactly the same experimental conditions. The results showed that there was a significant change in cell proliferation in the exposed cells in comparison to the non-exposed (control) cells at both temperatures. On the other hand, no significant change in proliferation rate was found in the sham-exposed cells at both temperatures. This shows that biological effects due to RF/MW cannot be attributed only to a change of temperature. Since the RF/MW induced changes were of the same order of magnitude at both temperatures and also comparable to our previous results under isothermal conditions at 37°C, cellular stress caused by electromagnetic fields could initiate the changes in cell cycle reaction rates. It is widely accepted that certain classes of heat-shock proteins are involved in these stress reactions. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Radiofrequency electromagnetic fields; Microwave radiation; Athermal effects; Cell proliferation; Microtiter plate culture

1. Introduction

The expansive growth in mobile communication in recent years has resulted in an increased exposure of the environment to weak radiofrequency (RF) electromagnetic fields. This has aroused a general interest in the possible effects of radiofrequency and microwave (MW) radiation on human health.

Consequently, the number of reports on RF and MW induced effects on various cellular processes is increasing rapidly. However, a satisfactory explanation by what biophysical and biological mechanisms these fields exert their biological effects has not yet been offered.

Unfortunately, the misconception still persists that RF and MW effects are solely the result of heat generation, contrary to the fact that a number of reported studies showed significant effects on various cellular activities in experimental systems under isothermal conditions [1–3].

However, if RF and MW effects were only due to increased temperatures, then it should be possible to detect changes in cellular activities depending only on temperature changes in the experimental system. And consequently, there should be no difference between the cellular activities of field exposed and non-exposed cells.

In a previous study, we showed that exposure of transformed human epithelial amnion cells (AMA) to a modulated RF field at 960 MHz at different power levels and exposure times, resulted in significant changes in cell

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proliferation [4]. In these experiments the cells were kept at a temperature of $37 \pm 0.1^\circ\text{C}$ during, before and after exposure.

Here, we report the results of experiments on the same cell line at the same RF field and exposure time, but at two different temperatures i.e., two degrees higher and lower than in the previous study.

2. Materials and methods

2.1. Chemicals

The following chemicals were used for the preparation of 1 l of medium in this study: 100 ml of Dulbecco's modified Eagle's medium $10 \times$ (#F0455), 40 ml of 7.5% NaHCO_3 solution in saline (#L1713), 10 ml of 200 mM glutamine solution (#K0282), supplied by Biochrom KG (Berlin, Germany), 100 ml of fetal calf serum (#04-001-1A), 10 ml of a penicillin–streptomycin solution (#03-031-1B) purchased by Biological Industries (Israel) and 740 ml of three-fold distilled water. Cell trypsinization was done by 2.5% trypsin solution (#25090-028) supplied by Gibco (Paisley, Scotland). Cell proliferation was determined by Celltiter 96 non-radioactive assay (#G5421, Promega, USA).

2.2. Cells

Transformed human epithelial amnion cells (AMA) were maintained as monolayer cultures in a humidified 5% CO_2 atmosphere at 37°C in a Queu incubator.

2.3. Microwave exposure

The EM field was generated by signal simulation of the Global System for Mobile communications (GSM), a 960

MHz carrier amplitude modulated with a 217 Hz square pulse of duty cycle 12%.

The cell cultures, growing in microtiter plates, were exposed in a specially constructed chamber, a Transverse Electromagnetic (TEM) cell. The TEM cell's analysis has been given previously [5]. A schematic diagram of the experimental set-up is shown in Fig. 1.

The electromagnetic field distribution in a TEM-cell exposure chamber is simulated by use of the finite difference time domain (FDTD) method. The Specific Absorption Rate (SAR) values for each cell well were obtained from the FDTD calculations of the EM field distribution in a microtiter suspension well [5–7]. The dielectric parameters were based on the composition of a mammalian cell [8,9].

The cell cultures were exposed in the TEM cell to a 960 MHz RF field at a power level, resulting in a SAR value of $2.1 \mu\text{W g}^{-1}$ in the cell culture, corresponding to a peak local induced E -field of 17 mV cm^{-1} . The exposure time was 30 min.

Static and AC magnetic fields, measured with a three-axis fluxgate meter Bartington Inst. model MAG-03 MC, in both the incubation and exposure incubators did not exceed $1 \mu\text{T}$ at 50 Hz.

2.4. Exposure conditions

The cells were grown in 50 ml flasks for 48 h until complete confluency. After trypsinization and appropriate dilution of the cultures with fresh medium, the cells were seeded into the two central rows (#6 and #7) of sterile 96-well microtiter plates (#655 180 Greiner, Germany), each well containing a volume of $100 \mu\text{l}$. Dilution of the seeding mixtures was adjusted so that cell confluency after 24 h of field-free incubation at 37°C never exceeded 40–50%.

After 24 h of field-free incubation of the plates at 37°C , the experiment was started (at zero time) by adding $10 \mu\text{l}$

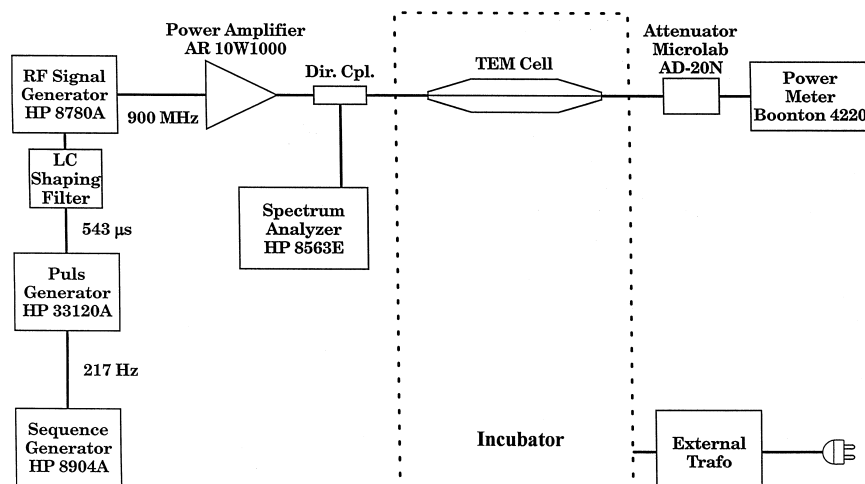


Fig. 1. Schematic diagram of experimental set-up of microwave exposure system.

of the Promega MTS reagent to the wells of row #6 of two microplates by means of an electronic Eppendorf pipette. The microplates were then transferred to another incubator (Heraeus), one plate was placed in the powered TEM cell and the other (control plate) 20 cm apart from it. The temperature in this incubator was maintained at either 35 or 39°C depending on the experimental design. After exposure, both microplates were transferred back to the field-free incubator at 37°C. Then the sham experiment was started in the same way, the two microplates were transferred to the exposure incubator and placed as described before, except that now the TEM cell was not powered. Afterwards, proliferation in all four microplates was allowed to continue for another 24 h in the field-free incubator. After this period, Promega MTS reagent was added to the wells of row #7. All absorbance measurements were done in a microplate reader (model 3550-UV, Biorad) at 490 nm (reference wavelength 655 nm) after 4 h of incubation with the reagent at 37°C.

2.5. Calculations

Cell proliferation was expressed as the ratio (D) of the absorbance values (A_f) of row #7 (24 h after exposure) to the absorbance values (A_o) of row #6 (before exposure) in percentage:

$$D = (A_f/A_o)100 \quad (1)$$

The reading (A) represents the average of six individual readings in wells B–G for rows #6 and #7, respectively. Therefore, growth change (ΔD) between exposed or sham and control cells can be simply presented by:

$$\Delta D = D_e - D_c \quad (2)$$

In order to decrease the effect of variation, a paired t -test was applied for the data statistical treatment based on comparing the obtained mean differences ΔD in the two sets of exposure vs. control and sham vs. control experiments at 35 and 39°C, respectively. Totally, 11 exposures and shams were compared with 11 simultaneous controls in both cases.

3. Results and discussion

In Table 1 the changes in cell proliferation, after exposure to an RF electromagnetic field for 30 min exposure times at two different temperatures, are shown. The RF field strength resulted in a SAR value of $2.1 \mu\text{W g}^{-1}$. The results of the corresponding sham experiments, under the same conditions, are also shown. Each result was based on the average of 11 independent experiments, of which each again was based on 12 individual absorbance readings with a 90% confidence interval.

We found that at both temperatures, there was a significant change in cell proliferation in the exposed cells in

Table 1

Percentage change in proliferation rate in AMA cells after exposure to 960 MHz microwave radiation

	N^a	T (°C)	Mean ΔD difference (%)	SD (%)	SEM (%)	P
Sham	11	35	2.36	8.07	2.43	0.355
Exposure	11	35	6.22	10.83	3.26	0.086 *
Sham	11	39	2.81	13.05	3.93	0.491
Exposure	11	39	6.26	10.32	3.11	0.072 *

^aEach point is the average of six individual absorbance measurements, i.e., 66 control and 66 exposed wells in 11 independent experiments.

*Significant at the confidence level of 90% (Paired sample t -test with $df = 10$).

SAR = $2.1 \mu\text{W g}^{-1}$, at two different temperatures for 30 min.

comparison to the control cells. On the other hand, the change in proliferation rate in the sham exposed cells was not significant at these temperatures.

Obviously, under our experimental conditions, a temporary change in temperature does not affect cell proliferation, whereas exposure to MW radiation results in a significant change. These changes were almost of the same order of magnitude as in our previous experiments isothermally at 37°C under the same experimental conditions.

From these experiments, it can be concluded that the changes in cell proliferation due to exposure to RF fields cannot be a result of heat generation, if any, from these fields. Of course, we cannot exclude that at much stronger field strengths, higher temperatures can be generated which may result in significant changes. However, it can be assumed that within the range of environmental RF field strengths and under isothermal conditions [3], temperature changes are not the cause of the biological effects due to field exposure.

Since the changes in cell proliferation due to MW radiation are of the same order of magnitude at both temperatures, there must be another mechanism that initiates the cell cycle reactions. The induction of stress to the cells could be related to an increased release of stress proteins, e.g., heat-shock proteins (hsp), which need not be induced by heat only. It has been shown that cells subjected to stress such as hyperthermia, toxic chemicals, pore formation, virus infection, respond in a number of ways — one is by increased synthesis of heat-shock proteins [10]. In a previous study, we found that exposure to low-frequency (ELF) electromagnetic fields, resulted in an increase of hsp-70 and hsp-90, which could not be obtained by a temperature raise only, even up to 40°C [11]. Others applying RF/MW radiation at much higher power levels and prolonged exposure times did not find a change in hsp level after exposure [12,13]. However, in these studies very long exposure times were used, in some cases up to 1 week. Electromagnetic field exposure causes only transient effects and long exposure times (hours, days) could result in a kind of adaptation, so that small changes may not be detected. Moreover, there is a very great

number of hsp's, all having different effects. In most of these experiments, the same class of hsp's has been used. It is not unlikely that only the synthesis of a specific hsp is induced by EM fields. So in future work, it will be necessary to extend the investigation to include a wider array of hsp's.

Acknowledgements

This research was supported by Novo Nordisk Denmark. S. Velizarov holds a post-doc grant from the Danish Government.

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Attachment 25

Environ Health. 2010; 9: 75.

PMCID: PMC3006375

Published online 2010 Nov 25. doi: [10.1186/1476-069X-9-75](https://doi.org/10.1186/1476-069X-9-75)


Association between exposure to radiofrequency electromagnetic fields assessed by dosimetry and acute symptoms in children and adolescents: a population based cross-sectional study

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Received 2010 Jun 9; Accepted 2010 Nov 25.

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Abstract

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Background

The increase in numbers of mobile phone users was accompanied by some concern that exposure to radiofrequency electromagnetic fields (RF EMF) might adversely affect acute health especially in children and adolescents. The authors investigated this potential association using personal dosimeters.

Methods

A 24-hour exposure profile of 1484 children and 1508 adolescents was generated in a population-based cross-sectional study in Germany between 2006 and 2008 (participation 52%). Personal interview data on socio-demographic characteristics, self-reported exposure and potential confounders were collected. Acute symptoms were assessed twice during the study day using a symptom diary.

Results

Only few of the large number of investigated associations were found to be statistically significant. At noon, adolescents with a measured exposure in the highest quartile during morning hours reported a statistically significant higher intensity of headache (Odd Ratio: 1.50; 95% confidence interval: 1.03, 2.19). At bedtime, adolescents with a measured exposure in the highest quartile during afternoon hours reported a statistically significant higher intensity of irritation in the evening (4th quartile 1.79; 1.23, 2.61), while children reported a statistically significant higher intensity of concentration problems (4th quartile 1.55; 1.02, 2.33).

Conclusions

We observed few statistically significant results which are not consistent over the two time points. Furthermore, when the 10% of the participants with the highest exposure are taken into consideration the significant results of the main analysis could not be confirmed. Based on the pattern of these results, we assume that the few observed significant associations are not causal but rather occurred by chance.

Background

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During the last decade the use of wireless communication devices has become very common in our daily life. On the other hand parts of the general population are afraid of potential negative health effects due to radiofrequency electromagnetic field (RF EMF) exposure caused by these wireless devices.

In Germany, 27% of the general population reported concerns about such potential health effects [1]. In this context, unspecific symptoms like headache, sleeping problems or difficulties in concentration were consistently attributed to self-reported mobile phone use [2,3] and to exposure to mobile phone base stations [4-7]. Most of the persons who are concerned about RF EMF exposure report symptoms to appear whilst being exposed, so acute effects on health and well-being are of special interest.

Children and adolescents are an important age group due to their high usage of mobile phones, and it is discussed if they might be more vulnerable to RF EMF. This potential for higher vulnerability is discussed due to their higher lifetime exposure (they start mobile phone use at an earlier age and use mobile phones more frequently than today's adults), their still developing nervous system and a greater conductivity of their brain tissue [8-10]. Recent studies indicated higher Specific Absorption Rate (SAR) values for children in comparison to adults [11,12], but this issue is still under discussion.

To date, only few studies investigated potential adverse health effects in young people. Two laboratory studies showed that a 900 MHz field did not have acute effects on cognitive functions in children and adolescents [13,14]. Epidemiological studies mainly investigated associations between self-reported exposure and perceived health of the participants. The results showed that self-reported mobile phone use was associated with poor perceived health [15-17]. One drawback of these studies is that the exposure assessment was based on self-reports of the participants. Earlier studies revealed that adolescents are not able to recall their mobile phone use over the past years accurately [18,19].

To avoid lack of valid exposure assessment we used personal dosimeters in our study. One advantage of these dosimeters is the possibility to assess all sources of individual exposure over the study period [20,21]. Until recently, there was only one epidemiological study in adults using personal dosimeters to assess the individual exposure, no data on children are so far available [22]. Therefore, the MobilEe study is the first epidemiological study using personal dosimetry for the assessment of exposure to RF EMF in children and adolescents, enabling objective assessment of exposure from all sources (mobile phones, DECT phones, base stations, WLAN) [23]. The aim of the study was to investigate a possible association between the individual exposure to such fields and acute health effects in children and adolescents.

Methods

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Study design

From 2006 to 2007 children (aged 8-12 years) and adolescents (aged 13-17 years) were randomly selected from the registration offices of four Upper Bavarian (South of Germany) cities with different population sizes (Munich, Augsburg, Rosenheim, Landsberg). Informed written consent was obtained from all participants.

Irrespective of their participation they were asked to fill in a short questionnaire to investigate possible differences between participants and non-participants.

Those who declared consent were invited to a local study centre where a computer-assisted personal interview (CAPI) was performed and data on participants' socio-demographic characteristics and potential confounders were collected. For children some data were collected by interviewing their parents (level of education, environmental worries).

During the 24-hour exposure measurement the participants filled in a diary recording acute symptoms at noon and in the evening before bedtime as well as the frequency of their own mobile phone use in the previous hours. Study methods have been described in detail elsewhere [23]. The study was approved by the Ethics Committee of the Medical Faculty of the Ludwig-Maximilians-University Munich (285/03).

Exposure assessment using personal dosimetry

Exposure was measured over 24 hours using a personal dosimeter (ESM-140, Maschek Electronics, Bad Wörishofen, Germany) covering exposure to mobile phones and their base stations (GSM 900 up and down link, GSM 1800 up and down link, UMTS up and down link), to cordless phones (DECT) and to WLAN (Wireless Local Area Network) [23]. The dosimeter was placed on the upper arm of the participants opposite to the side which they usually used to hold the mobile phone during phone calls.

During night time participants were asked to fix the dosimeter next to their bed on a bottle filled with water. Due to the physical characteristics of the dosimeter valid measurements can only be obtained if the dosimeter is moved. Therefore bedtime exposure levels were not considered to be a valid proxy of night time exposure and had to be excluded from the analyses [22].

For assessing overall exposure, a combination of frequency bands had to be used due to the dosimeter's low selectivity between up- and down-link channels. Furthermore, all values below the limit of determination (0.05 V/m) were replaced by half of the limit (0.025 V/m) [23]. For each of the seven frequency bands of the dosimeter the average of the squared field strength over the relevant time period (waking hours) was taken. Each of these averages was weighted by the inverse of the respective squared ICNIRP reference levels for the electric field strength. The square root of the sum of these seven weighted averages was multiplied by 100 to obtain a percentage of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) reference level [24]. The overall exposure was classified into quartiles for the main analysis [22].

Self-reported exposure (subjective exposure)

To compare the results to findings of previous studies we also collected data on self-reported exposure. Frequency of mobile phone use during the measurement day was assessed using a diary and dichotomized for the analysis: \leq five minutes vs. $>$ five minutes.

Endpoints

Using a paper-based diary, the following acute symptoms were taken into consideration and assessed twice during the 24-hour measurement (noon, in the evening before bedtime): headache, irritation, nervousness, dizziness, fatigue and concentration problems. The items were taken from the "Zerssen complaint list" [25] and assessed on a four-point Likert scale (heavy, moderate, weak, not at all). As only few participants reported heavy and moderate symptoms, the symptom variables were dichotomized. A symptom was considered present if it was reported with an at least weak intensity.

Potential confounders

Sociodemographic data like age and level of education were collected using questions from the German Health Interview and Examination Survey for Children and Adolescents (KIGGS) [26]. Environmental worries were assessed using the short form of the Environmental Worry Scale [27]. The scale consists of 12 questions about general and specific environmental worries (e.g. noise exposure, general environmental pollution). Participants were a priori classified in "not worried" and "worried" using the median as cut-off. This was done, because no standard values exist. For children, data of their parents were used, since there is no version of the Environmental Worry Scale for children.

Statistical analysis

Thirty measurements had to be excluded from the analysis due to technical errors. Multivariate analyses were done using logistic regression models adjusting for age, sex, level of education, study town and environmental worries stratified for children and adolescents. The potential confounding variables were defined a-priori and included in all analyses (complete-case-analyses).

We did separate analyses for the association between exposure during morning hours and the reported symptoms at noon, as well as for the association between exposure during afternoon and the reported symptoms in the evening.

In the main analysis, the potential association between measured exposure to RF EMF and acute symptoms was assessed. In a secondary analysis we used the self-reported mobile phone use of the participants during the measurement as exposure proxy.

Statistical analyses were carried out using SAS (SAS version 9.1; SAS Institute Inc., Cary, NC, USA).

Results

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Participation and descriptive data

Overall, 1498 children and 1524 adolescents participated in the interview and the measurement (52% of those invited). Fatigue was reported most frequently especially in the evening, followed by concentration problems and headache. For all acute symptoms, except for nervousness in the evening, the prevalence was higher in adolescents than in children (Table 1).

Table 1 Sociodemographic data and prevalence of acute symptoms of the participating children and adolescents		
	Children (n = 1498)	Adolescents (n = 1524)
	Prevalence n (%)	Prevalence n (%)
Sociodemographic data		
Sex, male	724 (48.3)	731 (48.0)
Age: 0-12 years respectively 13-19 years	791 (53.3)	963 (63.3)
Level of education of least 12 years of schooling ("grammar school")	939 (64.1)	771 (51.4)
Acute symptoms		
	Prevalence n (%)	Prevalence n (%)

Table 1
Sociodemographic data and prevalence of acute symptoms of the participating children and adolescents

The overall measured exposure to RF EMF was very low and ranged from a mean of 0.13% (all measurement values below the limit of determination) to a mean of 0.92% of the ICNIRP reference level per second during waking hours (Table 2). Only 2% of the children and 14% of the adolescents used their mobile phones more than five minutes in the afternoon. The duration of mobile phone use was higher in adolescents than in children and higher in the afternoon than during morning hours (Table 2).

Table 2 Measured and self-reported exposure of the participating children and adolescents		
	Children (n = 1498)	Adolescents (n = 1524)
Measured exposure levels	% ICNIRP-reference level: mean (standard deviation), range	
during waking hours	0.18 (0.08); 0.13 - 0.92	0.19 (0.08); 0.13 - 0.78
during morning	0.17 (0.08); 0.13 - 0.80	0.18 (0.07); 0.13 - 0.74
during afternoon	0.19 (0.08); 0.13 - 1.20	0.20 (0.07); 0.13 - 0.87
Self-reported exposure: % mobile phone use	n (%)	

Table 2
Measured and self-reported exposure of the participating children and adolescents

Association between self-reported and measured exposure

Measured exposure and self-reported exposure might be correlated. Therefore, we investigated if participants reporting ownership of a mobile phone and/or a long duration of use of a mobile phone have an exposure in the higher quartiles (measured exposure). Reporting a high exposure was significantly associated with the probability to have a measured exposure in quartile 2-4 in comparison to the reference category (1st quartile). Nevertheless, only 25% to 30% of the children and adolescents who reported ownership of a mobile phone or longer duration of use had a measured exposure in the highest exposure quartile (Table 3).

Table 3
Association between self-reported and measured exposure

Variable	Quartile	Children		Adolescents	
		n (%)	OR (95% CI)*	n (%)	OR (95% CI)*
Mobile phone ownership	1	122 (16.9)	1.0	295 (23.0%)	1.0
	2	188 (24.2)	1.8 (1.2-2.5)	320 (25.4%)	2.1 (1.3-3.4)
	3	189 (25.3)	1.9 (1.3-2.7)	322 (25.7%)	2.4 (1.4-4.0)

Table 3
Association between self-reported and measured exposure

Association between measured exposure and acute symptoms

Children with an exposure in the 4th quartile during the afternoon reported a statistically significant higher intensity of concentration problems in the evening (4th quartile 1.55; 1.02- 2.33) (Table 4).

Table 4
Results of the logistic regression models by measured and self-reported exposure for children

Symptoms	Quartile	Prevalence n (%)	OR (95% CI)*	Prevalence n (%)	OR (95% CI)*
Noon	Measured exposure during morning hours			> 5 minutes mobile phone use during morning hours*	
	1st	69 (17.6)	1.00	11 (28.7)	2.33 (0.98 - 5.94)
Headache	2nd	58 (15.9)	0.92 (0.52 - 1.62)		

Table 4
Results of the logistic regression models by measured and self-reported exposure for children

In adolescents, a significant association between measured exposure during morning hours and headache at noon (adjusted Odds Ratio 1.50; 95% CI: 1.03- 2.19 (4th quartile)) as well as exposure during the afternoon and irritation in the evening (4th quartile: 1.79; 1.23- 2.61) was observed (Table 5).

Table 5
Results of the logistic regression models by measured and self-reported exposure for adolescents

Symptoms	Quartile	Prevalence n (%)	OR (95% CI)*	Prevalence n (%)	OR (95% CI)*
Noon	Measured exposure during morning hours			> 5 minutes mobile phone use during morning hours*	
	1st	89 (23.7)	1.00	54 (35.1)	1.55 (1.05 - 2.29)
Headache	2nd	90 (23.9)	1.08 (0.72 - 1.62)		
	3rd	104 (28.3)	1.19 (0.83 - 1.70)		
	4th	112 (30.5)	1.58 (1.03 - 2.44)		

Table 5
Results of the logistic regression models by measured and self-reported exposure for adolescents

As self-reported exposure might be correlated with the measured exposure, a multivariate analysis adjusting for these variables (self-reported mobile phone use, self-reported distance to the next mobile phone base station) was additionally carried out. However, no statistically relevant differences in the results could be seen (data not shown).

Association between self-reported exposure and acute symptoms

No statistically significant association between the self-reported duration of mobile phone use and acute symptoms at noon or in the evening was observed for the children (Table 4). Adolescents using mobile phones more than five minutes during the morning hours reported statistically significantly more often headache (1.55; 1.05- 2.29), irritation (1.64; 1.10- 2.44) and fatigue at noon (1.8; 1.2- 2.6) (Table 5). No association was seen for exposure during the afternoon and acute symptoms in the evening.

Discussion

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The MobilEe-Study was the first study in children and adolescents using personal dosimeters to assess the individual exposure to RF EMF and to investigate a possible association between this exposure and acute symptoms.

The measured exposure levels were on average far below the current ICNIRP reference levels, which is in

accordance with the results of two previous studies using personal dosimeters for exposure assessment [22,28].

Regarding a potential association between measured exposure to RF EMF and acute symptoms, some of the observed results reached the level of statistical significance. As these results were only of borderline significance and not consistent over the two time points (morning, afternoon), we believe that the observed associations are due to chance or multiple testing. Taking multiple testing into account, none of these observed associations would have reached the level of statistical significance. The results of two previous studies in adults did not show associations between RF EMF exposure and acute symptoms [29,30], either. In a sensitivity analysis exposure was also considered as a binary cut-off (cut-off 90% percentile) to compare those 10% with the highest exposure to the remaining participants (data not shown). The observed results could not confirm the significant results of the main analysis.

When self-reported mobile phone use during the measurement was taken as exposure proxy, some statistically significant results at noon were observed for the adolescents. These results are in agreement with those of another epidemiological study that revealed an association between self-reported mobile phone use and the occurrence of negative health symptoms [3]. However, it has to be kept in mind that self-reported exposure is no valid proxy for the real exposure and that it is most likely that the observed associations are due to differential misclassification based on overestimation of self-reported exposure and subjective symptoms.

Children and adolescents with high self-reported exposure did not necessarily have high measured exposure. Although most of the results showed statistically significant associations between self-reported and measured exposure, about 70% of the participants would be miss-classified if one would use the self-reported exposure as a proxy for the real exposure.

The major advantage of this study is the use of a valid exposure assessment method to assess individual exposure to RF EMF. Personal dosimetry enables accounting for all sources of exposure, considers people's mobility and is convenient to handle for study participants [20,21]. In comparison to self-reported exposure it is more accurate and less prone to possible bias.

One disadvantage of the used dosimeter was the limited selectivity to differentiate between the frequency bands, e.g. the dosimeter cannot differentiate between GSM1800, DECT and UMTS [23]. Furthermore, it was not possible to differentiate between up- and downlink channels. Another available dosimeter, the DSP-090 (Satimo, in the past Antennessa, France) has a slightly better selectivity but is not suitable for children and adolescents due to its weight and size [21].

A second problem is the fact that the body of the participant influences the measured exposure values [31]. Comparisons with free field measurements showed that personal dosimeters may underestimate real exposure [32]. The dimension of this underestimation is likely to be the same for each participant and therefore should not influence the assignment to the exposure quartiles.

Measuring the night time exposure levels is another drawback of the used dosimeter. Our study participants placed the dosimeters near their beds, which resulted in a constant, but arbitrary measurement during the night. As shown in lab measurements, valid measurements can only be obtained if the dosimeter is moved and thus, we excluded the values. Of course young people spend some hours e.g. sitting in school, but in comparison to the fixed position of the dosimeter during the night we consider that although they are sitting the arm is moved sometimes. Therefore, we assume that the daytime measurements are valid.

The participation (52%) was reasonable considering that the children and adolescents had to carry the dosimeter for 24 hours. To analyse a possible bias caused by selective non-participation, we compared those who participated in the field study to those who did not. Parents and adolescents who had a higher level of education

and those who were concerned about mobile phone exposure were more likely to take part in the study (data not shown). It appears that primarily those parents and adolescents who were concerned about a possible association between RF EMF exposure and health took part in the study. We cannot rule out a preferential selection of these subjects in our study. Due to the objective exposure measurement a differential misclassification seems to be unlikely and therefore an overestimation of the results is also unlikely.

Due to the cross-sectional design of the study, exposure was only assessed for 24 hours and it might be that exposure during the study day may not be representative for a longer time period. To verify the representativeness of the measurements, 54 participants carried the dosimeter for five consecutive days. The results indicated that the exposure assessment on a single weekday reflects the typical average weekday exposure quite adequately. Between 20% and 57% of the participants were in exactly the same exposure quartile on two days of the week (perfect agreement). Highest complete agreement was always seen between two consecutive days. However, for weekdays, exposure categories differed by at most one exposure quartile for more than 80% of the population indicating that misclassification of exposure might result in an underestimation of the effect [23].

Conclusions

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In summary, we found some associations between measured exposure and acute symptoms. Due to inconsistencies between the part of the day and the fact that the results were not observed when taking the 10% of the participants with the highest exposure into consideration, we assume that these are caused by multiple testing. The results regarding self-reported exposure and symptoms are most likely due to differential misclassification and this fact emphasizes the necessity of a valid exposure assessment in epidemiological studies.

Abbreviations

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CAP: Computer Assisted Personal Interview; DECT: Digital Enhanced Cordless Phone; RF EMF: radiofrequency electromagnetic fields; GSM: Global System for Mobile Communications; ICNIRP: International Commission on Non-Ionizing Radiation Protection; UMTS: Universal Mobile Telecommunication System; WLAN: Wireless Local Area Network.

Competing interests

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The authors declare that they have no competing interests.

Authors' contributions

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SH was one of the principle investigators responsible for design, conduct and writing the manuscript. ST was also one of the principle investigators responsible for design and acquisition of data. CH made contributions to the statistics and to draft the manuscript. RK made contributions to draft the manuscript. KR made contributions to conception and design and also to analysis and drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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Dr. Anja Kühnlein helped with the coordination, data management and data analyses of the study and is thus gratefully acknowledged. We thank all participants, the study team and the study centres. This work was funded by the Federal Office for Radiation Protection (BfS, Oberschleißheim/Munich) as part of the German Mobile Telecommunication Research Programme (DMF).

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Attachment 26

Exposure to radio-frequency electromagnetic fields and behavioural problems in Bavarian children and adolescents

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Received: 2 October 2009 / Accepted: 23 November 2009 / Published online: 4 December 2009
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Abstract Only few studies have so far investigated possible health effects of radio-frequency electromagnetic fields (RF EMF) in children and adolescents, although experts discuss a potential higher vulnerability to such fields. We aimed to investigate a possible association between measured exposure to RF EMF fields and behavioural problems in children and adolescents. 1,498 children and 1,524 adolescents were randomly selected from the population registries of four Bavarian (South of Germany) cities. During an Interview data on participants' mental health, socio-demographic characteristics and potential confounders were collected. Mental health behaviour was assessed using the German version of the Strengths and Difficulties Questionnaire (SDQ). Using a personal dosimeter, we obtained radio-frequency EMF exposure profiles over 24 h. Exposure levels over waking hours were expressed as mean percentage of the reference level. Overall, exposure to radiofrequency electromagnetic fields was far below the reference level. Seven percent of the children and 5% of the adolescents showed an abnormal mental behaviour. In the multiple logistic regression analyses measured exposure to RF fields in the highest quartile was associated to overall behavioural problems for adolescents (OR 2.2; 95% CI 1.1–4.5) but not for children (1.3; 0.7–2.6). These results are mainly driven by one

subscale, as the results showed an association between exposure and conduct problems for adolescents (3.7; 1.6–8.4) and children (2.9; 1.4–5.9). As this is one of the first studies that investigated an association between exposure to mobile telecommunication networks and mental health behaviour more studies using personal dosimetry are warranted to confirm these findings.

Keywords Behavioural problems · Radio-frequency electromagnetic fields · Children · Adolescents · SDQ · Dosimetry

Introduction

Despite the increase of mobile phone use in the last decade and the growing concern whether radiofrequency (RF) fields adversely affect health and well-being, only few studies have been published that focussed on children and adolescents [1–5].

However, especially children and adolescents are important in the discussion of potential adverse health effects because of their possibly higher vulnerability to high-frequency electromagnetic fields. Young people might be more vulnerable due to their still developing nervous systems and the potential for higher cumulative RF exposure during their lifetimes [6–8].

The few studies that investigated a possible association between exposure to RF fields and health outcomes in children and adolescents focused mainly on perceived health. In these studies, self-reported use of mobile phones was associated with poor perceived health [1–3]. However, reporting bias and confounding might have been a problem.

One recent study examined the association between mobile phone exposure and behavioural problems. The

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authors of this analysis asked mothers about their use of mobile phones during pregnancy and the current mobile phone use of their children. In parallel, mental health behaviour was assessed using the Strengths and Difficulties Questionnaire (SDQ). The results of this study showed a statistically significant adverse effect of self-reported pre-natal and postnatal exposure to RF fields and behavioural problems in children [9]. Behavioural problems in children and adolescents are nowadays of special interest, as the number of children and adolescents being diagnosed with e.g. attention deficit hyperactivity disorder (ADHD) increases [10].

In the present analysis, we investigated the possible effects of exposure to RF EMF fields assessed using personal dosimetry on mental health behaviour in Bavarian (Southern Germany) children and adolescents.

Materials and methods

Study design and participants

The study took place in four Bavarian cities with different population sizes. Between 2006 and 2008 the participants were recruited randomly from the registration offices of these four towns and invited to participate in the study. Overall, 1,498 children (aged between 8 and 12 years) and 1,524 adolescents (aged between 13 and 17 years) were included.

All potential participants were invited in written form and asked to fill in a short-questionnaire irrespective of their participation in the study. The information from the questionnaire were used for a non responder analysis to assess potential selection bias. Those children and adolescents respectively their parents who declared consent were invited to a local study centre, where they completed a Computer Assisted Personal Interview (CAPI) on participants' well-being, socio-demographic characteristics and potential confounders. In case of the children one parent was also invited and completed an interview. After the interview the children and adolescents obtained the ESM-140 mobile phone dosimeter for a 24-h measurement. Further details of the design have been described earlier [11].

Study instruments

Mental health

During the interview 25 questions about mental health behaviour were asked following the German version of the Strengths and Difficulties Questionnaire (SDQ). For the

children and adolescents we used the self-report version of the SDQ. In case of the children, we also used a parental version. This questionnaire includes the same 25 items as in the self-report version for the children, but is completed by the parents.

The 25 items of the questionnaires are divided between 5 scales: emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems and prosocial behaviour. All scales comprises 5 items with three possible answer categories ("certainly true", "somewhat true", "not true") ranging between 0 and 2 points. Using the sum of the answer-values a range between 0 and 10 points can be reached for each of the 5 scales. To specify the overall difficulties score the four scales that refer to the behavioural problems are summed up to a score between 0 and 40 points. The prosocial scale is disregarded.

For each scale as well as for the total difficulties score the participants are classified as "normal", "borderline" or "abnormal" as described in [12–14]. Due to the small numbers of children and adolescents that were classified as "borderline", we dichotomised the outcome for the analysis ("normal" vs. "borderline/abnormal").

Potential confounding variables

Sociodemographic data [15], the four study towns, self estimated environmental worries and the self estimated general exposure to RF EMF fields taken from the CAPI were considered as potential confounders. Age and level of education were dichotomised:

Age

- 8–10 and 11–12 years (children)
- 13–15 and 16–17 years (adolescents)

Level of education

- at least 12 years of education (parents of the children)
- at least grammar school ("Gymnasium"; adolescents)

Environmental worries were assessed during the CAPI for the parents of the children and for the adolescents themselves with a short form of the environmental worry scale [16, 17]. The scale consists of 12 questions about general and specific environmental worries. Participants were divided into two groups ("not worried" and "worried").

Participants were also asked to estimate their general use of mobile and cordless phones (<nearly daily vs. at least nearly daily). Furthermore they should estimate the distance of their home to the next mobile phone base station (<500 m away vs. \geq 500 m away). These questions were taken from the questionnaire of the annual survey of the Institute for applied sciences (infas) [18].

Exposure assessment

Exposure was measured using the personal dosimeter ESM-140 (Maschek Electronics) and was assessed every second resulting in 86,400 measurements over 24 h. The following frequency ranges were covered [11]:

- GSM 900 (up and down link)
- GSM 1800 (up and down link) including Universal Mobile Telecommunication System (UMTS 2100) and Digital Enhanced Cordless Telecommunications (DECT)
- Wireless local area network (WLAN 2400) frequencies.

For the exposure assessment, a combination of the frequency bands had to be used as the dosimeter has a low selectivity between up- and down-link channels.

During nighttimes the dosimeter was fixed on a bottle filled with water. Due to the physical characteristics of the dosimeter valid measurements can only be obtained if the dosimeter is moved. Therefore night-time values were excluded and only exposure levels during individual waking hours were considered.

The overall exposure during these hours was summarized as average field strengths in percentage of the ICNIRP (International Commission on Non-Ionizing Radiation Protection) reference level [11].

Statistical analysis

For the analysis 30 participants had to be excluded due to missing interview data or technical errors of the measurements. Thus, data of 1,484 children and 1,508 adolescents could be used.

To analyze a potential association between exposure to RF fields and mental health, the mean percentage of the ICNIRP reference level during waking hours was used. Measured exposure was divided into quartiles enabling to assess a possible dose-response-relationship. We could not take exposure as a continuous variable, because too many values were below the limit of determination.

Multivariate analyses were done using logistic regression models stratified for children and adolescents. The models were adjusted for age, sex, level of education, self reported environmental worries, self reported general RF exposure and study place.

In case of the children, Odds Ratios for the concordant ratings between children and parents were calculated. Therefore, we restricted the analysis to those children where the self-assessment of the child was concordant with the parental report; discordant ratings were excluded.

As it might be that the observed outcome (behavioural problems) causes more extensive mobile phone use (reversed causality) we did a sensitivity analysis restricted

to children and adolescents who did not use a mobile phone.

Statistical analyses were carried out using SAS (SAS version 9.1; SAS Institute Inc., Cary, NC, USA).

Results

Descriptive data

Overall 6,386 children and adolescents were invited, of which 5,870 were eligible for the study. 4,452 persons (76%) answered the short questionnaire. 1,498 children (aged between 8 and 12 years) and 1,524 adolescents (aged between 13 and 17 years) participated in the interview and the exposure measurement (52% of those who were eligible for the study).

The adolescents and the parents of the children were more likely to have a higher level of education. Half of the children and nearly every adolescent (92%) owned a mobile phone. Most of the children (96%) and also the majority of adolescents did not use their mobile phone daily (77%). Regarding the use of a cordless phone, most of the children used the phone infrequently (72%), whereas 55% of the adolescents used the cordless phone at least nearly daily. Sixty-five percent of the adolescents and 67% of the parents of the children estimated the distance between their residence and the next mobile phone base station as less than 500 m (Table 1).

Exposure to mobile phone frequencies assessed using personal dosimetry was far below the reference limit and ranged from a mean of 0.13% (all measurement values below the limit of determination) to a mean of 0.92% of the ICNIRP reference level per second during waking hours. Median exposure was slightly higher for adolescents (0.19% of the reference level) than for children (0.18% of the reference level) (Table 1) [11].

Mental health behaviour

Children showed a slightly higher prevalence of abnormal overall behaviour (7%) than adolescents (5%) (Table 2). The same was seen for conduct problems and peer relationship problems. However, the prevalence of emotional problems was higher in adolescents (6%) than in children (3%). Four percent of the children and 5% of the adolescents were categorized as hyperactive.

Besides for peer relationship problems, parents assessed a higher prevalence of all mental health behavioural scales for their children than the children themselves (Table 2).

The overall agreement between children and their parents ranged between 75% (emotional problems) and 90% (peer relationship problems) (Table 3).

Table 1 Descriptive data of the children and the adolescents

Variable	Children prevalence <i>n</i> (%) <i>n</i> = 1,484	Adolescents prevalence <i>n</i> (%) <i>n</i> = 1,508
Age		
11–12 years respectively 16–17 years	693 (46.7)	545 (36.1)
Sex		
Male	724 (48.9)	731 (48.5)
Level of education		
At least 12 years of education resp. At least grammar school	939 (64.1) ^a	771 (51.4)
Mobile phone ownership	747 (50.4)	1506 (91.4)
General use of a mobile phone		
<Nearly daily	1419 (95.8)	1156 (76.9)
At least nearly daily	61 (4.2)	348 (23.1)
General use of a cordless phone		
<Nearly daily	1066 (71.8)	672 (44.6)
At least nearly daily	416 (28.2)	835 (55.4)
Self-estimated distance to the next base station		
<500 m	946 (67.1)	922 (64.7)
≥500 m	464 (32.9)	503 (35.3)
Exposure during waking hours (% ICNIRP- reference level)		
Range	0.13–0.92	0.13–0.78
Quartiles	0.15 0.17 0.20	0.15 0.17 0.21
Mean (standard deviation)	0.18 (0.06)	0.19 (0.06)

^a Data of the parents of the children

Table 2 Prevalence of abnormal behaviour assessed by the German version of the SDQ, stratified for age group and report version

Scale	Adolescents ^a <i>n</i> (%)	Children ^a <i>n</i> (%)	Parents of the children ^b <i>n</i> (%)
Overall behavioural problems	80 (5.3)	107 (7.2)	257 (17.4)
Emotional problems	86 (5.7)	51 (3.4)	387 (26.2)
Conduct problems	68 (4.5)	95 (6.4)	356 (24.1)
Hyperactivity	69 (4.6)	64 (4.3)	193 (13.1)
Peer relationship problems	72 (4.8)	109 (7.4)	93 (6.3)

^a Self-report version of the SDQ for children and adolescents

^b Parental version of the SDQ completed for their children

Association between exposure and mental health behaviour

Adolescents with an exposure in the highest quartile reported more frequently overall behavioural problems (OR 2.2; 95% CI 1.1–4.5) than adolescents with an exposure in the remaining three quartiles (Table 4).

Furthermore, an association between exposure to measured RF fields and conduct problems was seen both for the children (2.9; 1.4–5.9) and for the adolescents (3.7; 1.6–8.4). Restricting the analysis to those children whose

self-report was concordant with the parental report, the same tendency was observed. However, due to the lower sample size results were not statistically significant (Table 4). Neither for children nor for adolescents was an association between measured exposure and emotional problems, hyperactivity or peer relationship problems observed.

As measured exposure and subjective exposure (self-reported mobile phone and cordless phone use, self-estimated distance to the next mobile phone base station) might be correlated, we conducted also multivariate

Table 3 Accordance between data of the parents' report and self-report of the children

	Accordance (%)	
	Normal behaviour	Abnormal behaviour
Overall behavioural problems	79	4
Emotional problems	73	2
Conduct problems	73	3
Hyperactivity	84	22
Peer relationship problems	88	2

analyses without the variables for subjective exposure as potential confounders. No difference in the results could be seen (data not shown).

Discussion

This study is the first of its kind to investigate a possible association between exposure to RF EMF fields and well-being including mental health behaviour in children and adolescents using personal dosimetry for the exposure assessment.

In comparison to another German study, parents reported a similar prevalence of total difficulties for their children [19]. In two Norwegian studies that also used the self-report version in adolescents, the prevalence of behavioural problems was lower than in our study [20, 21]. This might be due to differences in the German and Norwegian cut-offs for the single questionnaire-scales or also due to cultural differences between the countries.

The SDQ is stated to be a reliable instrument for health behaviour problems when data of at least two informants can be used [22, 23]. As we collected the information of the children as well as information of one parent, we used these data for a sensitivity analysis. The results showed no association between exposure to RF fields and behavioural problems in children. Nevertheless, the results were comparable to those of the main analysis. Thus, we assume that the results for the adolescents were reliable as well, despite the fact that only data of the self-reported version of the SDQ was available for this group.

As the design of the study was cross-sectional, the measured exposure and the considered mental health behaviour outcomes were only assessed once. Therefore a misclassification concerning the outcome is possible. It might be the case that the participants over- or underreported possible behavioural problems. As the participants were not aware of their actual measured exposure level differential misclassification is unlikely. Thus, an underestimation of the exposure-response-relationship might be possible [24–26].

Considering the time dependent effort, participation in the study was quite good (52%). However, it was seen in a non-responder analysis that mainly those participated who were concerned about potential health effects due to RF EMF exposure. Thus a preferential selection bias might be possible. Due to the objectively measured exposure the participants were not aware of their true exposure over the study day and thus, an overestimation of the outcome is unlikely.

The overall exposure levels were on average less than 1% of the ICNIRP reference level. This result is in agreement with other studies [27, 28]. It would have been interesting to restrict the analysis to the three mobile phone bands. However, due to a low selectivity of the frequency bands—the 1,800 MHz frequency band included GSM 1800, UMTS and DECT—a differentiation was not possible.

Although exposure levels were low, an association between measured exposure and total behavioural problems for the adolescents was seen. Furthermore, we observed an association between measured exposure and conduct problems for children and adolescents. As we did not have data on parameters like e.g. family psychiatric history or data on pregnancy of the mother that are discussed as potential risk factors in the context of behavioural outcomes like hyperactivity [29] it might be that unmeasured confounding produced our results. Furthermore, up to now there is no known biologic mechanism to explain an association between exposure to radiofrequency fields and behavioural problems as observed in our study.

It might be that the observed outcome (behavioural problems) causes more extensive mobile phone use (reversed causality). We therefore tested for interaction between exposure and mobile phone use. The interaction terms did not reach the level of statistical significance for children and adolescents, indicating that the observed association was true independent of mobile phone use.

Up to now there is only one other study published, that investigated the association between exposure to cell phones and behavioural problems in children. The results of Divan et al. [9] indicated that reported mobile phone use might be associated with behavioural problems. The authors contribute that the results might also be produced by unmeasured confounders.

Conclusions

In summary, this is the first study that investigated a possible association between measured exposure to RF EMF fields and behavioural problems in children and adolescents. We observed an association between measured exposure and behavioural problems for the children and the

Table 4 Adjusted logistic regression for the association between exposure to mobile telecommunication networks and behavioural problems in children and adolescents

Exposure	Adolescents (<i>n</i> = 1508) ^a		Children (<i>n</i> = 1484) ^a		Concordant ratings children and parents ^a	
	Prevalence (abnormal behaviour) <i>n</i> (%)	OR (95% CI)	Prevalence (abnormal behaviour) <i>n</i> (%)	OR (95% CI)	Prevalence (abnormal behaviour) <i>n</i> (%) ^b	OR (95% CI)
Total behavioural problems						
1st Quartile	19 (5.0)	1.0	30 (8.1)	1.0	16 (5.3)	1.0
2nd Quartile	17 (4.5)	0.9 (0.5–1.9)	21 (5.7)	0.8 (0.4–1.5)	11 (3.7)	0.7 (0.3–1.7)
3rd Quartile	15 (4.0)	1.0 (0.5–2.1)	25 (6.7)	1.0 (0.5–1.9)	14 (4.4)	1.0 (0.4–2.3)
4th Quartile	29 (7.7)	2.2 (1.1–4.5)	31 (8.4)	1.3 (0.7–2.6)	15 (4.9)	1.0 (0.4–2.4)
Emotional symptoms						
1st Quartile	24 (6.4)	1.0	13 (3.5)	1.0	9 (3.3)	1.0
2nd Quartile	18 (4.8)	0.8 (0.4–1.7)	13 (3.5)	0.9 (0.4–2.0)	7 (2.6)	0.7 (0.2–1.9)
3rd Quartile	18 (4.8)	0.6 (0.3–1.3)	11 (3.0)	0.6 (0.2–1.5)	5 (1.8)	0.4 (0.1–1.4)
4th Quartile	26 (6.9)	1.1 (0.5–2.2)	14 (3.8)	0.7 (0.3–1.9)	10 (3.6)	0.7 (0.2–2.3)
Conduct problems						
1st Quartile	10 (2.7)	1.0	19 (5.2)	1.0	10 (3.7)	1.0
2nd Quartile	7 (1.9)	0.6 (0.2–1.7)	20 (5.4)	1.1 (0.5–2.3)	9 (3.1)	0.7 (0.2–2.1)
3rd Quartile	18 (4.8)	1.9 (0.8–4.5)	19 (5.1)	1.2 (0.6–2.5)	11 (3.9)	1.2 (0.4–3.4)
4th Quartile	33 (8.8)	3.7 (1.6–8.4)	37 (10.0)	2.9 (1.4–5.9)	19 (6.9)	2.3 (0.8–6.4)
Hyperactivity						
1st Quartile	12 (3.2)	1.0	18 (4.9)	1.0	6 (1.9)	1.0
2nd Quartile	18 (4.8)	1.7 (0.8–3.7)	13 (3.5)	0.7 (0.3–1.5)	4 (1.3)	0.5 (0.1–2.0)
3rd Quartile	20 (5.3)	2.0 (0.9–4.5)	12 (3.2)	0.6 (0.2–1.3)	8 (2.5)	1.3 (0.4–4.5)
4th Quartile	19 (5.1)	2.1 (0.9–4.8)	21 (5.7)	1.1 (0.5–2.6)	5 (1.6)	0.9 (0.2–3.5)
Peer relationship problems						
1st Quartile	21 (5.6)	1.0	27 (7.3)	1.0	7 (2.1)	1.0
2nd Quartile	18 (4.8)	0.8 (0.4–1.5)	22 (6.0)	0.8 (0.4–1.5)	7 (2.1)	1.0 (0.3–3.7)
3rd Quartile	13 (3.5)	1.1 (0.5–2.1)	25 (6.7)	1.0 (0.5–2.0)	9 (2.6)	1.7 (0.5–5.7)
4th Quartile	20 (5.3)	1.4 (0.7–2.7)	35 (9.4)	1.4 (0.7–2.7)	8 (2.5)	1.4 (0.4–5.3)

Adjusted for age, sex, level of education (in case of children the level of education of their parents), study town, environmental worries and the self estimated exposure to mobile phone frequencies

OR odds ratio, 95% CI 95% confidence interval

^a *n* differs between the different scales

^b Concordant ratings of the children and the parents

adolescents. Due to the fact that there is no known biological mechanism and behavioural problems were only assessed once, more studies are warranted to confirm the findings. A cohort study including a clinical confirmation of the diagnosis would be desirable to determine the temporal sequence of exposure to mobile telecommunication networks and mental health behaviour.

Acknowledgments This work was supported by the German Mobile Telecommunication Research Programme. Dr. Anja Kühnlein helped with the coordination, data management and data analyses of the study and is thus gratefully acknowledged. We thank all participants for their participation as well as the study team for the field

work. The participating towns are acknowledged for their support and the provision of rooms for the interviews. The authors declare they have no competing financial interests.

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Attachment 27

Effects of Everyday Radiofrequency Electromagnetic-Field Exposure on Sleep Quality: A Cross-Sectional Study

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Source: Radiation Research, 174(3):347-356.

Published By: Radiation Research Society

DOI: <http://dx.doi.org/10.1667/RR2153.1>

URL: <http://www.bioone.org/doi/full/10.1667/RR2153.1>

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Effects of Everyday Radiofrequency Electromagnetic-Field Exposure on Sleep Quality: A Cross-Sectional Study

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Mohler, E., Frei, P., Braun-Fahrländer, C., Fröhlich, J., Neubauer, G., Röösli, M. and the Qualiflex Team. Effects of Everyday Radiofrequency Electromagnetic-Field Exposure on Sleep Quality: A Cross-Sectional Study. *Radiat. Res.* 174, 347–356 (2010).

The aim of this cross-sectional study was to investigate the association between exposure to various sources of radiofrequency electromagnetic fields (RF EMFs) in the everyday environment and sleep quality, which is a common public health concern. We assessed self-reported sleep disturbances and daytime sleepiness in a random population sample of 1,375 inhabitants from the area of Basel, Switzerland. Exposure to environmental far-field RF EMFs was predicted for each individual using a prediction model that had been developed and validated previously. Self-reported cordless and mobile phone use as well as objective mobile phone operator data for the previous 6 months were also considered in the analyses. In multivariable regression models, adjusted for relevant confounders, no associations between environmental far-field RF EMF exposure and sleep disturbances or excessive daytime sleepiness were observed. The 10% most exposed participants had an estimated risk for sleep disturbances of 1.11 (95% CI: 0.50 to 2.44) and for excessive daytime sleepiness of 0.58 (95% CI: 0.31 to 1.05). Neither mobile phone use nor cordless phone use was associated with decreased sleep quality. The results of this large cross-sectional study did not indicate an impairment of subjective sleep quality due to exposure from various sources of RF EMFs in everyday life © 2010 by Radiation Research Society

INTRODUCTION

The possible effects of radiofrequency electromagnetic-field (RF EMF) exposure on health-related quality of life are of public health concern (1–3). The most often reported complaints related to RF EMFs are impairments of sleep quality (4, 5).

Several studies investigated the effect of short-term RF EMF exposure on sleep measures in a laboratory

setting, applying real and sham exposure randomly under well-controlled exposure conditions (6–8). Objective sleep measures derived from electroencephalography (EEG) were used in these laboratory studies. Overall, these studies showed no consistent association between RF EMF exposure and objective sleep measures (i.e. sleep architecture), but small differences for different frequency ranges in the EEG were observed repeatedly after exposure to RF EMFs. The primary aim of laboratory studies is to identify a possible biological mechanism of the effect of RF EMF exposure on sleep, if any exists. In general, laboratory studies are conducted with a relatively small number of participants and therefore have limited statistical power to investigate subjective sleep quality. Moreover, the unfamiliar environment of a sleep laboratory may prevent detection of subtle effects of RF EMFs on sleep quality, as has been reported by several individuals.

Epidemiological studies allow the examination of the association between RF EMFs and subjective sleep quality in a large population sample. The main challenge is to perform an appropriate exposure assessment. Until now, only a few studies were conducted. In early studies, associations between RF EMF exposure and subjective well-being or sleep quality were observed (9, 10). However, in these studies, simple exposure proxies like self-reported distance to mobile phone base stations were used, which have been demonstrated to be inadequate (11, 12). Information bias was also of concern in these studies and might have influenced the results. Additionally, selection bias might affect results in such cross-sectional studies if participation is related to both health and exposure status (13, 14). More recent studies on RF EMF exposure and sleep quality used spot measurements in the bedroom for exposure classification (15, 16). No differences in sleep quality (Pittsburgh Sleep Quality Index) or in other health outcomes (headache, SF-36 and health complaint list) were observed between individuals with high and low exposures. Although more sophisticated exposure assessment methods were used in these studies, it still is not

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clear how well such spot measurements represent long-term exposure to various sources of RF EMFs in our everyday environment. For these reasons, in our study, we used personal RF EMF exposure measurements and modeling of fixed-site transmitters (e.g. mobile phone base stations and broadcast transmitter) to develop a method to assess individual exposure (17).

Due to the unknown biological mechanism, it is unclear which aspect of exposure is relevant for sleep disturbances, if there are any. It is conceivable that exposure at the head, caused mainly by mobile and cordless phones, is most relevant (close to body sources). Alternatively, environmental sources like exposure from mobile phone base stations or broadcast transmitter, which in general cause lower but continuous whole-body exposures, might play a role (far-field environmental RF EMF exposure). RF EMF exposure might cause symptoms immediately, or the accumulated radiation might be more important. Additionally, psychological aspects appear to be important. Previous studies showed that subjective well-being and sleep quality can be impaired in people from concern or expectations if they think they are highly exposed to various sources of RF EMFs (3) (also called a *nocebo effect*).

The primary aim of this cross-sectional study was to evaluate whether environmental RF EMF exposure is associated with self-reported sleep quality. We also evaluated whether sleep quality is affected by other RF EMF exposure surrogates such as night exposure or use of mobile or cordless phones.

METHODS

In May 2008, 4000 questionnaires entitled “environment and health” were sent out to people aged between 30 to 60 years who were randomly selected from the population registries of the city of Basel (Switzerland) and from five communities in the surroundings of Basel. To minimize noneligibility due to language difficulties, only Swiss residents or people living in Switzerland for at least 5 years were selected. A reminder letter was sent out 3 weeks after the first invitation for participation. Nonresponders were contacted by phone 6 to 10 weeks after the first questionnaires were sent out, and they were asked a few key questions. Ethical approval for the study was received from the Ethical Commission of Basel on March 19, 2007 (EK: 38/07).

Written Questionnaire

The questionnaire addressed three issues: (1) sleep quality and general health status; (2) exposure-relevant characteristics and behaviors (17) such as owning a mobile phone, a cordless phone, and/or a wireless LAN and duration of cordless phone use and mobile phone use; and (3) socio-demographic factors such as age, gender, education, marital status and additional confounders like body mass index (BMI), physical activity, smoking behaviors and alcohol consumption.

Excessive Daytime Sleepiness and Self-Reported Sleep Disturbances

To assess subjective sleep quality, we used two sleep outcomes. Daytime sleepiness was determined by the Epworth Sleepiness Scale

(ESS), which assigns values ranging from 0 (no daytime sleepiness) to 21 (very excessive daytime sleepiness) (18). We calculated the ESS scores and created a new binary variable according to a previous study on insomnia indicating excessive daytime sleepiness (ESS score over 10) (19).

General subjective sleep quality was assessed by using four standardized questions from the Swiss Health Survey 2007 (20). The four questions on subjective sleep quality in the Swiss Health Survey asked about the frequency of difficulty in falling asleep, fitful sleep, waking phases during night, and waking up too early in the morning using a four-point Likert scale with categories “never”, “rare”, “sometimes” and “most of the time”. Out of these four questions, a binary sleep quality score (SQS) was calculated by adding up all items (ranging from 0 to 12) and defining a score of eight as having sleep disturbances (20).

Exposure Assessment

Our main hypothesis was that environmental whole-body exposure in everyday life may affect sleep quality. We developed a model for predicting personal exposure to environmental RF EMFs on the power flux density scale in mW/m^2 (17) in which we measured personal RF EMF exposure of 166 volunteers from our study area by means of a portable EME Spy 120 exposure meter. Volunteers carried the exposimeter and filled in an activity diary for 1 week (21). The exposimeter measured 12 different frequency bands of RF EMFs ranging from FM radio (frequency modulation; 88–108 MHz), TV (television, 174–223 MHz and 470–830 MHz), Tetrapol (terrestrial trunked radio police; 380–400 MHz), uplink in three frequency ranges (communication from mobile phone handset to base station; 880–915, 1710–1785, 1920–1980 MHz), downlink in three frequency ranges (communication from mobile phone base station to handset; 925–960, 1805–1880, 2110–2170 MHz), DECT (digital enhanced cordless telecommunications; 1880–1900 MHz), and W-LAN (wireless local area network; 2400–2500 MHz). In addition, we developed a three-dimensional geospatial propagation model in which the average RF EMF from fixed-site transmitters (e.g., mobile phone base stations and broadcast transmitters) was modeled for the study region (in- and outside of buildings) (22, 23). Based on this geospatial propagation model and on data from the exposimeter measurements, the relevance of potential predictors on exposure was examined in multivariable non-linear regression models. The following exposure-relevant factors were identified and included in the prediction model for environmental exposure in everyday life (17): owning a mobile phone, owning a wireless LAN at home, having the DECT base station in the bedroom, having a cordless phone at the place where one spends the most of their time during the day, house characteristics (window frame and type of house wall), hours per week in public transport and cars, percentage full-time equivalent spent at an external workplace, and exposure from fixed-site transmitters at home computed by the geospatial propagation model (22, 23).

To estimate exposure during the night, a separate night prediction model was developed. Ownership of a cordless phone base station in the bedroom, wireless LAN in the bedroom, house characteristics (type of house wall and window frame), and the modeled value of fixed-site transmitters were included in this specific prediction model.

We used the above-mentioned geospatial propagation model for modeling exposure from fixed-site transmitters at home (22) in mW/m^2 as well as in percentage of the ICNIRP (International Commission on Non-Ionizing Radiation Protection) (24) reference level according to method of Thomas *et al.* (28).

Finally, with respect to local exposure to the head, we used self-reported use of mobile and cordless phones per week as reported in the written questionnaire. Informed consent was also sought from participants to obtain operator data for their mobile phone use for the last 6 months from the three Swiss mobile phone network operators.

Sensitivity Analysis

To evaluate a placebo effect and information bias (which is also of concern in this area of research), we asked participants about their subjective exposure. They had to estimate their exposure compared to the Swiss population and to indicate whether they felt they were equally, less or more exposed in comparison to the average of the Swiss population. Geo-coded data were available for all study participants. This allowed us to calculate the distance from their residence to the next mobile phone base station as an additional exposure surrogate.

Nonresponder Analyses

To evaluate the extent of potential selection bias in our study, nonresponder interviews were conducted to gather information on general health status, socio-demographic factors and exposure-relevant behaviors and factors. One month after the reminder letter was sent out, we tried to contact all nonresponders. Information on age, gender and geo-coded addresses was available for all 4000 persons.

We calculated “selection bias factors” for different exposure proxies (i.e., owning a mobile phone, a cordless phone and/or a WLAN and distance to the next mobile phone base station) using the Greenland method (25) as was done by Vrijheid *et al.* (26). For these calculations we assumed that data from nonresponder phone interviews are representative for all nonresponders. Dividing the observed odds ratio by the bias factor yields the correct unbiased association between exposure and outcome. A bias factor of 1.0 indicates that there is no bias.

Statistical Analyses

For binary outcomes (ESS score and SQS), logistic regression models with three groups of exposure levels for all exposure proxies (<50th percentile, 50th to 90th percentile, >90th percentile) were performed. Mean average RF EMF exposures were calculated in mW/m² and converted to V/m. In addition, linear regression models were computed using the continuous score of both sleep scales. Separate analyses were done for each of the four questions of the Swiss Health Survey.

The models were adjusted for age, sex, body mass index (BMI), stress perception, physical activity, smoking habits, alcohol consumption, self-reported disturbance due to noise, living in urban or suburban areas, belief in health effects due to RF EMF exposure, education and marital status. Use of mobile and cordless phones was included in all models as an independent exposure measure. Missing values in the confounder variables were replaced with values of either the most common category (categorical variables) or with the mean value (linear variables) to ensure that all analyses were performed with an identical data set for the ESS and the SQS, respectively. Most missing values in confounder variables were observed in self-reported disturbance of noise [33 missing out of 1212 observations (2.7%)]. Stratified analyses and testing for interaction were done for people reporting as electrohypersensitive (EHS). We defined EHS individuals as those reporting as “electrohypersensitive” or those reporting adverse effects due to RF EMFs.

All statistical analyses were carried out using STATA 10.1 (StataCorp, College Station, TX).

RESULTS

Study Participants

Of the 4000 persons participating in the study, 237 were excluded due to noneligibility because of severe disabilities ($n = 27$), death ($n = 1$), incorrect addresses (n

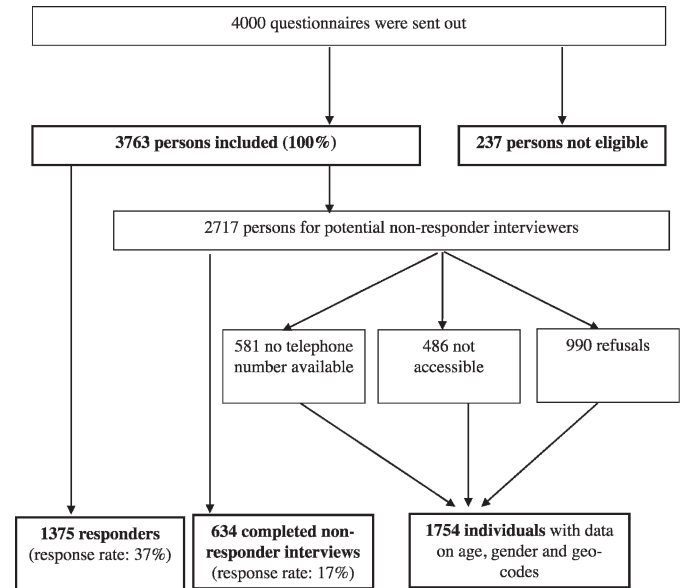


FIG. 1. Schematic illustration of the study design and response rate.

= 36), absence during study time ($n = 73$), or language problems ($n = 100$). A total of 1375 people completed the questionnaire. Detailed information on the response rate is illustrated in Fig. 1. Users of sleeping pills ($n = 81$) as well as night shift workers ($n = 82$) were excluded from all the analyses. The final analyses thus included 1212 participants. Due to missing values in exposure variables (mobile phone and cordless phone use) and in sleep quality scores (ESS and SQS), 1129 study participants remained for the analyses of excessive daytime sleepiness and 1163 study participants remained for the analyses of self-reported sleep disturbances. Characteristics of all study participants are listed in Table 1. The mean age (standard deviation) of study participants was 46 (± 9) years, and 39% of all responders lived in the city of Basel. There were more female (58%) than male participants. Ninety percent reported that they had a good or very good health status, which was comparable to the general Swiss population (87%).² The majority was married (60%) and of normal weight (BMI <25) (62%).

Seventy-eight percent of the study participants reported that they believed that there are people who develop adverse health effects due to RF EMF exposure, 18.2% assigned their own adverse health effects as being due to RE EMF exposure, and 8.1% reported that they were “electrohypersensitive”. Due to overlapping, 20.9% of our study population was electrohypersensitive according to our definition.

² National Statistical Institute (Switzerland) 2007; <http://www.bfs.admin.ch/bfs/portal/de/index/themen/14/02/01/key/01.html>.

TABLE 1
Characteristics and Results of Statistical Comparison of all Study Participants (including nonresponders)

	Participants (<i>n</i> = 1212) ^a	Percent	Nonresponders (<i>n</i> = 2388)	Percent	<i>P</i> value
Age (years)					0.05
30–40	319	26	719	30	
41–50	421	35	829	35	
51–60	472	39	840	35	
Sex					<0.05
Female	706	58	1190	50	
Male	506	42	1198	50	
Distance to the next mobile phone base station (percentage closer than 50 m)	45	4	165	7	<0.05
Health status ^{b,c}					<0.05
Very good	445	37	215	34	
Good	636	53	302	48	
Half-half	107	9	86	14	
Bad	12	1	18	3	
Very bad	3	0	8	1	
Educational level ^{b,c}					0.171
None	79	7	56	9	
Apprenticeship	591	49	320	51	
Higher education/University	542	45	255	40	
Owning a mobile phone ^{b,c}					<0.05
Yes	1049	87	572	90	
No	163	13	60	10	
Owning a cordless phone ^{b,c}					0.176
Yes	994	82	537	85	
No	213	18	96	15	
Owning wireless LAN ^{b,c}					0.931
Yes	492	41	259	41	
No	709	59	370	59	

^a After exclusion of nightshift workers (*n* = 82) and users of sleeping drugs (*n* = 81).

^b Nonresponder data only for a subsample of 634 nonresponders who answered a short nonresponder interview by phone (numbers in nonresponder analyses can vary due to missing data).

^c Data may not sum up to 100% due to missing data.

Level of Exposure

The predicted everyday life mean and median exposure was 0.18 V/m for all the included study participants. The cut-off point for 90th percentile was 0.21 V/m. The maximum predicted value was 0.33 V/m. The mean predicted exposure during the night was 0.06 V/m (median: 0.02 V/m, cut-off 90th percentile: 0.09 V/m, maximum: 0.33 V/m), and the mean exposure through fixed-site transmitters (geospatial propagation model) was 0.08 V/m (median: 0.04 V/m, cut-off 90th percentile: 0.12 V/m, maximum: 0.62 V/m). The mean level of exposure from fixed-site transmitters was 0.15% of the ICNIRP reference level. On average, study participants reported using their mobile phones 62.8 min per week and their cordless phones 75.1 min per week. Informed consent for objective data on mobile phone use from the network operators was obtained from 470 study participants. Those who gave informed consent reported that they used their mobile phone 46.5 min per week, while the operator data showed a

mobile phone use of 28.8 min per week (27). The Spearman rank correlation was 0.76 (95% CI: 0.71–0.83) for self-reported mobile phone use and the operator data.

The majority (64%) of the participants estimated that their exposure was similar to the average for the Swiss population, while 29% believed they were less exposed and 7% believed they were more exposed.

Excessive Daytime Sleepiness (ESS score)

The prevalence of excessive daytime sleepiness (ESS score > 10) was 29.5%. The results of the logistic regression models for crude and adjusted odds ratios (OR) are presented in Table 2. No statistically significant association between excessive daytime sleepiness and various exposure surrogates was observed. The analysis showed a tendency toward excessive daytime sleepiness for the highest-exposed group through fixed-site transmitters, although it was not statistically significant. This finding was confirmed when exposure

TABLE 2
Association between Excessive Daytime Sleepiness (Epworth Sleepiness Scale) and Different Exposure Surrogates
[odds ratios (OR) and 95% CI of the three exposure categories]

Excessive daytime sleepiness (<i>n</i> = 1129)	Exposure categories							
	< 50th percentile		50th–90th percentile			> 90th percentile		
	No. of cases ^a	OR	No. of cases ^a	OR	95% CI	No. of cases ^a	OR	95% CI
Far-field exposure								
Everyday life exposure								
Crude	180	1.00	153	1.10	(0.84–1.43)	25	0.77	(0.47–1.24)
Adjusted ^b	180	1.00	153	1.14	(0.83–1.57)	25	0.58	(0.31–1.05)
Exposure during night								
Crude	174	1.00	149	1.14	(0.87–1.48)	35	1.06	(0.68–1.65)
Adjusted ^b	174	1.00	149	1.05	(0.76–1.43)	35	1.21	(0.74–1.98)
Exposure through fixed-site transmitters								
Crude	170	1.00	142	1.07	(0.82–1.40)	46	1.86	(1.21–2.85)
Adjusted ^b	170	1.00	142	1.02	(0.74–1.39)	46	1.52	(0.93–2.50)
Close-to-body exposure								
Mobile phone use (self-reported)								
Crude	210	1.00	106	1.18	(0.89–1.57)	32	1.05	(0.69–1.64)
Adjusted ^b	210	1.00	106	1.24	(0.91–1.70)	32	1.03	(0.62–1.69)
Mobile phone use (operator data) ^c								
Crude	65	1.00	152	1.11	(0.72–1.70)	14	1.26	(0.63–2.54)
Adjusted ^b	65	1.00	152	1.30	(0.82–2.07)	14	0.91	(0.39–2.11)
Cordless phone use (self-reported)								
Crude	178	1.00	165	1.27	(0.98–1.65)	13	1.44	(0.71–2.90)
Adjusted ^b	178	1.00	165	1.30	(0.99–1.72)	13	1.65	(0.72–3.50)

^a Indicates number of people in the corresponding exposure group with an Epworth sleepiness score over 10.

^b Adjusted for age, body mass index, sex, physical activity, alcohol consumption, smoking habits, stress perception, urban/suburban, marital status, educational level, noise perception, belief in health effects due to radiofrequency electromagnetic-field exposure.

^c For a subsample of 453 subjects who consented to obtain data from the operator.

was calculated as a percentage of the ICNIRP reference level (adjusted OR for the 90th percentile: 1.62; 95% CI: 0.99–2.64). Similar results were found for linear regression models (data not shown).

Based on interaction tests, we found no indication that RF EMF exposure affects EHS individuals differently than non-EHS individuals ($P > 0.05$ for all exposure surrogates).

Self-Reported Sleep Disturbances (SQS)

Problematic sleep disturbances were reported by 9.8% of respondents. There was no evidence that having sleep disturbances was influenced by everyday life exposure, exposure through fixed-site transmitters or exposure during the night (Table 3). The OR for the top decile of exposed individuals according to the percentage of the ICNIRP reference value was 0.95 (95% CI: 0.47 to 1.90). Mobile phone and cordless phone use showed no statistically significant effects on having sleep disturbances, but tendencies toward fewer sleep disturbances with increased use of a mobile phone could be seen in the logistic (Table 3) and linear regression models (data not shown). However, analysis of a subsample with objective mobile phone operator data did not show such a tendency (Table 3).

The separate analyses of each item on the sleep quality score (falling asleep, fitful sleep, waking phases during night, waking up early in the morning) revealed no exposure–response association (data not shown). Interaction tests and stratified analyses for EHS and non-EHS individuals showed no difference between the two subgroups.

Sensitivity Analysis

An association between self-reported sleep quality and self-estimated exposure could indicate the presence of information bias or a placebo effect, or rather the development of symptoms due to concerns. In our study, we found some indications for the presence of a placebo effect (Table 4). People reporting to be less exposed to mobile phone base stations in comparison to the average population are less likely to suffer from excessive daytime sleepiness (Table 4). Correspondingly, people who lived closer than 50 m to the closest mobile phone base station had a higher risk for excessive daytime sleepiness, although it was not statistically significant. Self-reported sleep disturbances were increased in people claiming to be more exposed in comparison to the average population. These trends were most pronounced for self-estimated exposure to a mobile phone base

TABLE 3
Association between Self-Reported Sleep Disturbances (Sleep Quality Score) and Different Exposure Surrogates
[odds ratios (OR) and 95% CI of the three exposure categories]

	Exposure categories							
	< 50th percentile		50th–90th percentile			> 90th percentile		
	No. of cases ^a	OR	No. of cases ^a	OR	95% CI	No. of cases ^a	OR	95% CI
Self-reported sleep disturbances (<i>n</i> = 1163)								
Far-field exposure								
Everyday life exposure								
Crude	98	1.00	68	0.91	(0.65–1.28)	14	0.87	(0.48–1.60)
Adjusted ^b	98	1.00	68	1.11	(0.72–1.70)	14	1.11	(0.50–2.44)
Exposure during night								
Crude	88	1.00	76	1.14	(0.81–1.50)	16	1.01	(0.57–1.80)
Adjusted ^b	88	1.00	76	1.30	(0.85–1.98)	16	1.29	(0.66–2.53)
Exposure through fixed-site transmitters								
Crude	88	1.00	77	1.15	(0.82–1.62)	15	0.94	(0.52–1.69)
Adjusted ^b	88	1.00	77	1.16	(0.76–1.75)	15	1.09	(0.53–2.22)
Close-to-body exposure								
Mobile phone use (self-reported)								
Crude	124	1.00	41	0.71	(0.49–1.05)	13	0.71	(0.38–1.30)
Adjusted ^b	124	1.00	41	0.67	(0.43–1.02)	13	0.64	(0.31–1.28)
Mobile phone use (operator data) ^c								
Crude	42	1.00	30	0.91	(0.54–1.51)	5	0.60	(0.22–1.62)
Adjusted ^b	42	1.00	30	1.57	(0.89–2.78)	5	1.03	(0.32–3.30)
Cordless phone use (self-reported)								
Crude	102	1.00	66	0.80	(0.57–1.12)	8	1.51	(0.67–3.40)
Adjusted ^b	102	1.00	66	0.71	(0.49–1.03)	8	1.11	(0.44–2.78)

^a Indicates number of people in the corresponding exposure group with a sleep quality score over 8.

^b Adjusted for age, body mass index, sex, physical activity, alcohol consumption, smoking habits, stress perception, urban/suburban, marital status, educational level, noise perception, belief in health effects due to radiofrequency electromagnetic-field exposure.

^c For a subsample of 453 subjects who consented to obtain data from the operator.

station. Subjective exposure was not correlated to modeled mobile phone base station radiation (Spearman correlation coefficient: –0.01) or total everyday life exposure (Spearman correlation coefficient: 0.13).

Nonresponder Analysis

To evaluate a possible selection bias, we compared responders of the questionnaire with nonresponders. The nonresponder analyses, comparing all 1212 participants included in our analyses with the 2388 nonresponders, showed small differences between study participants and nonresponders (Table 1). Nonresponders were generally younger, and the participation rate for women was higher than for men. The distance between the closest mobile phone base station and place of residence was smaller for the responders. Some of the nonresponder information was available only for the nonresponders who participated in the telephone interviews (*n* = 634): Participants in these telephone interviews were more likely to be an owner of a mobile phone (90%) than full study participants (87%). Study participants who filled in the questionnaire were somewhat healthier than nonresponders. No difference was observed in educational level in owning a wireless LAN or cordless phone. The prevalence of nonresponders (telephone interviews) who reported that they were “electrohypersensitive” was 16%. In the

full study only 8% answered yes to the corresponding question (*P* < 0.0001).

In our selection bias factor, we found a bias factor of 0.79 for owning a mobile phone, 0.70 for owning a cordless phone, 0.95 for owning a W-LAN, and 1.33 for living within 50 m from a mobile phone base station. Thus we expect that in our study the exposure–response association for mobile and cordless phone use tends to be biased downward whereas the exposure–response association for fixed-site transmitter tends to be biased upward.

DISCUSSION

The aim of this study was to investigate the association between various RF EMF exposure surrogates and self-reported sleep quality. Neither everyday-life environmental RF EMF exposure nor exposure during night through fixed-site transmitters or from mobile and cordless phones was associated with excessive daytime sleepiness or with having sleep disturbances. We found some indication for placebo effects and information bias; this means that persons who assumed that they were exposed more than the average for the Swiss population reported that they suffered more often, although not statistically significantly, from sleep disturbances than participants who

felt that they were equally exposed as the average of the Swiss population.

Strengths

Our study is based on a large sample size. To our knowledge, our study used the most comprehensive exposure assessment method to date by considering exposure-relevant behavior and characteristics (prediction model) as well as modeling RF EMFs from fixed-site transmitters with a geospatial model (22). All relevant exposure sources of everyday life were included in the prediction model, and the feasibility and reproducibility of this exposure assessment method could be demonstrated (17). Using prediction models for exposure assessment instead of conducting spot or personal measurements, as has been done in other studies (15, 16, 28), is time- and cost-saving for large study populations and is expected to better represent all sources of RF EMF exposure in everyday life.

We included several exposure surrogates in our study. This allowed us to check for consistency and biological plausibility, because no biological mechanism has been established. In particular, we included both close-to-body sources and far-field sources. In addition to self-reported mobile phone use, we considered objective operator data on mobile phone use for a subsample who gave consent.

Limitations

The cross-sectional study design is one of our main limitations, in particular with respect to EHS individuals. EHS individuals may tend to avoid known sources of RF EMF exposure and are therefore expected to be less exposed. If so, a cross-sectional study, where outcome and exposure are measured at the same time, could not capture an increased risk. It could even result in observation of a protective effect from exposure (although this was not the case in our study). Conversely, people who did not attribute their own symptoms to EMF exposure were not expected to avoid exposure sources. Thus our cross-sectional study should reveal an association in nonhypersensitive individuals, if one is present, because RF EMF exposure is relatively constant over a few months (21). This means that present exposure is also representative of exposure a few months before. In this regard, it is also relevant that self-estimated exposure actually is not correlated to true exposure. This indicates that most persons are not aware of their most relevant exposure sources. Unawareness of the exposure status implies that information bias is unlikely in our study.

In our study, we did not take polysomnographic sleep measures. We were mainly interested in self-reported data on sleep quality and well-being, because a decrease in self-perceived sleep quality due to RF EMF exposure

is the most often stated concern of the population (3, 5). Subjectively perceived sleep quality is relevant to health because it is an established factor that influences personal well-being (29). Collecting more sophisticated sleep measures using electroencephalography (EEG) would require considerable additional effort in this large study population, and such an unfamiliar measurement procedure could mask subtle effects on self-perceived sleep quality.

The participation rate for the full study (whole questionnaire data) was 37% and was therefore lower than we had expected and lower than in the study of Kühnlein *et al.* (30) and similar to that of Thomas *et al.* (28). In recent years, a decreasing response rate has been a commonly observed phenomenon in epidemiological research (31). In our study people might have declined because we asked them to give their informed consent to provide objective data about their mobile phone use from the mobile phone operator companies. People may have felt that it was an invasion of their privacy. The main concern in having a low participation rate is selection bias. We made considerable effort to evaluate potential bias from nonparticipation. To be able to assess the risk of selection bias, we performed nonresponder interviews, and data on age, gender and geocodes were available for all 4000 persons. We were concerned that people attributing their sleep disturbances to mobile phone base stations or to RF EMFs in general would be more motivated to participate in our survey (32, 33). If these people live closer to a mobile phone base station than the average population, this could result in a bias, because distance is one parameter of our exposure prediction model. Interestingly, we found indications of the opposite but yielding the same possible bias: Study participants generally were healthier than nonresponders, and the proportion of persons living close to a mobile phone base station (<50 m) was smaller for participants than nonparticipants. Thus our selection bias modeling yielded a selection bias factor of 1.33 for living within 50 m of a mobile phone base station. According to this selection bias modeling our observed exposure-response associations for fixed site transmitter may be biased upward. Conversely, our exposure-response associations for mobile and cordless phone use may be biased downward.

Interpretation

The prevalence of excessive daytime sleepiness in our study was similar to previous studies in which 32.4% reported suffering from excessive daytime sleepiness (34). Prevalence of sleep disturbances was in our study even lower (9.8%) than observed in a study of a Swiss working population (20), where 19% of a relatively young Swiss working population suffered from disorders of initiating and maintaining sleep.

TABLE 4
Sensitivity Analysis to Evaluate the Possible Extent of Information Bias and Nocebo Effect: Association between Sleep Quality (excessive daytime sleepiness and self-reported sleep disturbances) and Subjective Exposure

Excessive daytime sleepiness (<i>n</i> = 1129)								
Subjective exposure categories								
equal ^a			lower			higher		
No. of cases ^b		OR	No. of cases ^b		OR	No. of cases ^b		OR
					95% CI			95% CI
Subjective exposure to all sources								
Crude	239	1.00	96	0.80	(0.60–1.06)	23	0.87	(0.52–1.47)
Adjusted ^c	239	1.00	96	0.78	(0.56–1.09)	23	0.84	(0.41–1.71)
Subjective exposure to mobile phone base station								
Crude	243	1.00	85	0.71	(0.53–0.95)	30	0.98	(0.62–1.59)
Adjusted ^c	243	1.00	85	0.67	(0.48–0.95)	30	0.83	(0.44–1.59)
Excessive daytime sleepiness (<i>n</i> = 1129)								
> 50 m			≤ 50 m					
No. of cases ^b		OR				No. of cases ^b		OR
					95% CI			95% CI
Distance to mobile phone base station (geo-coded)								
Crude	340	1.00	-	-	-	18	1.90	(1.00–3.59)
Adjusted ^c	340	1.00	-	-	-	18	2.06	(0.96–4.41)

^a Reference group includes also “don’t know” and missing values.

^b Indicates number of people in the corresponding exposure group with an Epworth sleepiness score over 10 or a sleep quality score over 8, respectively.

^c Adjusted for age, body mass index, sex, physical activity, alcohol consumption, smoking habits, stress perception, urban/suburban, marital status, educational level, noise perception, believe in health effects due to radiofrequency electromagnetic-field exposure.

We found no consistent evidence that RF EMF exposure is associated with subjective sleep quality. Our findings contradict early studies that used self-estimated distance to mobile phone base stations as exposure proxy (9, 10). This approach has been shown to be inappropriate for exposure estimation (12, 14, 35). Moreover, these early studies without objective exposure measures are likely to be affected by nocebo effects since we found some indication for such a bias in our study when using self-estimated exposure measures that were poorly correlated to true exposure levels. This was particularly pronounced with respect to self-estimated mobile phone base station radiation.

Our prediction models are developed and validated on the power flux density scale (mW/m²). In our prediction model for everyday life exposure, we added up contributions from different sources on the power flux density scale, based on the assumption that effects are not dependent on frequency. It has also been speculated in other studies that effects in the low-dose range maybe dependent on frequency, and another study weighted the exposure contributions according to the ICNIRP reference level (28). However, for exposure from a fixed-site transmitter, where we were able to compare both scales, we found a very high correlation (Spearman = 0.96), and the results of the epidemiological analyses were similar. This suggests that choice of the exposure scale is not crucial unless the effect is very frequency specific.

Our findings are in line with more recent cross-sectional studies on subjective sleep quality that used

spot measurements in the bedroom for exposure assessment (15, 16). This is probably an acceptable exposure proxy for environmental RF EMF exposure during the night, but it does not capture exposure during the day or exposure to close-to-body sources that one might be exposed to prior to sleep. However, such exposure may be relevant: Several studies indicated that exposure to a mobile phone prior to sleep affects EEG during the night (7, 8, 36, 37).

In addition to the cross-sectional studies on self-reported sleep quality and RF EMF exposure at home, two studies investigated sleep behavior at home using an experimental approach and recording polysomnographic sleep measures. In a German study of 394 individuals living within 500 m of a mobile phone base station, polysomnographic measures were recorded during five consecutive nights. A transportable mobile phone base station (GSM 900 and 1800) was installed and randomly turned on and off.³ Leitgeb *et al.* (38) recruited 43 volunteers who reported to be EHS. Polysomnography was applied during 9 nights (3 control nights, 3 nights with sham shielding, and 3 nights with true shielding). In both studies, polysomnographic measures were not related to exposure.

³ H. Danker-Hopfe, H. Dorn, C. Sauter and M. Schubert, Untersuchung der Schlafqualität bei Anwohnern einer Basisstation. Experimentelle Studie zur Objektivierung möglicher psychologischer und physiologischer Effekte unter häuslichen Bedingungen. Final report. Deutsches Mobilfunkforschungsprogramm, 2009.

TABLE 4
Extended

Self-reported sleep disturbances (<i>n</i> = 1163)							
Subjective exposure categories							
equal ^a		lower			higher		
No. of cases ^b	OR	No. of cases ^b	OR	95% CI	No. of cases ^b	OR	95% CI
116	1.00	49	0.92	(0.64–1.32)	15	1.23	(0.67–2.27)
116	1.00	49	1.05	(0.68–1.64)	15	1.47	(0.62–3.49)
109	1.00	47	1.08	(0.74–1.56)	24	1.99	(1.20–3.30)
109	1.00	47	1.16	(0.74–1.82)	24	1.61	(0.76–3.43)
Self-reported sleep disturbances (<i>n</i> = 1163)							
> 50 m				≤ 50 m			
No. of cases ^b	OR				No. of cases ^b	OR	95% CI
171	1.00	-	-	-	9	1.53	(0.72–3.25)
171	1.00	-	-	-	9	1.13	(0.41–3.04)

We evaluated various exposure proxies. Except in a subgroup analysis with non-sensitive individuals for excessive daytime sleepiness and cordless phone use, no statistically significant effects were found. Given the numerous tests performed, one statistically significant result can be expected by chance. Similarly, some of the observed exposure–response tendencies such as the decreased occurrence of sleep disturbances for the moderate user of cordless phones are probably due to chance or may be affected by selection bias. If there were a true exposure–response association in our large study population, we would have expected to see a consistent pattern in terms of outcome (i.e., similar effects for sleep quality or daytime sleepiness) or in terms of exposure sources (i.e., similar effects for close-to-body sources or for environmental sources). Nevertheless, the cross-sectional design is a limitation, particularly if one has the hypothesis that people avoid exposure if they are suffering from sleep disturbances. In our study we found no evidence for such a behavior, nor have recent reviews suggested that the ability to perceive RF EMF exposure actually exists (14, 39).

Overall, we found no indication that RF EMF exposure in our daily life impairs subjective sleep quality. In contrast to previous studies on that topic, we considered all relevant RF EMF sources of the everyday environment in our exposure assessment through consideration of various proxies that are relevant in everyday life.

ACKNOWLEDGMENTS

The study is funded by the Swiss National Science Foundation (Grant 405740-113595). It is part of the National Research Program 57 “Non-Ionising Radiation - Health and Environment”. Martin

Röösli is supported by the Swiss School of Public Health + (SSPH+). We thank Alfred Bürgi for calculating the exposure to fixed site transmitters for each study participant, Matthias Egger, Niklas Joos and Axel Hettich (QUALIFEX team) for inspiring discussions, Fabian Trees from the Swiss Federal Statistical Office for providing the geographical coordinates of the study participants, and the statistical department of Basel for providing the addresses of the study participants. Many thanks go to all study participants who volunteered for the study.

Received: January 22, 2010; accepted: April 13, 2010; published online: June 17, 2010

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Attachment 28

Personal Exposure to Mobile Phone Frequencies and Well-Being in Adults: A Cross-Sectional Study Based on Dosimetry

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The use of mobile phone telecommunication has increased in recent years. In parallel, there is growing concern about possible adverse health effects of cellular phone networks. We used personal dosimetry to investigate the association between exposure to mobile phone frequencies and well-being in adults. A random population-based sample of 329 adults living in four different Bavarian towns was assembled for the study. Using a dosimeter (ESM-140 Maschek Electronics), we obtained an exposure profile over 24 h for three mobile phone frequency ranges (measurement interval 1 s, limit of determination 0.05 V/m). Exposure levels over waking hours were totalled and expressed as mean percentage of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) reference level. Each participant reported acute symptoms in a day-long diary. Data on five groups of chronic symptoms and potential confounders were assessed during an interview. The overall exposure to high-frequency electromagnetic fields was markedly below the ICNIRP reference level. We did not find any statistically significant association between the exposure and chronic symptoms or between the exposure and acute symptoms. Larger studies using mobile phone dosimetry are warranted to confirm these findings. *Bioelectromagnetics* 29:463–470, 2008. © 2008 Wiley-Liss, Inc.

Key words: high-frequency electromagnetic fields; personal dosimetry; epidemiology; exposure assessment

INTRODUCTION

The use of mobile communication devices has increased in recent years. At the same time, there is growing public concern in several industrialized countries that high-frequency electromagnetic fields could cause adverse health effects even at exposure levels far below the reference level. In a recent study in Germany 30% of the participants reported concerns about such effects [Institute for Applied Social Sciences, 2005].

Mainly people living near mobile communication base stations are concerned about potential harmful effects of radiation [Hutter et al., 2004]. People relating their problems to mobile phones or base stations often report having unspecific symptoms like headache, restlessness, sleep disturbances, concentration and memory problems, absence of appetite as well as tinnitus [Hocking, 1998; Oftedal et al., 2000].

Despite these concerns, only few studies to date have examined health effects of exposure to mobile phone base stations. The main drawback of the published studies is lack of valid exposure assessment

[Santini et al., 2002; Navarro et al., 2003]. Hutter et al. [2006] have attempted to address this problem in their pilot study by measuring exposure to the base stations in the participants' bedrooms; however, the issue could not be fully resolved. Electromagnetic emissions of a mobile phone base station vary over time and indoor field strength strongly depends on the station's position,

The authors declare they have no competing financial interests.

Grant sponsor: Bavarian Ministry of the Environment, Public Health and Consumer Protection, Munich, Germany.

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Received for review 24 July 2007; Final revision received 24 January 2008

DOI 10.1002/bem.20414

Published online 4 April 2008 in Wiley InterScience (www.interscience.wiley.com).

making it difficult to capture accurately. In addition, bedtime exposure reflects only one part of the overall exposure.

In a laboratory study, Zwamborn et al. [2003] exposed people who reported sensitivity to radio-frequency waves (electrosensitive group) and a control group to a 1 V/m field at 900 and 1800 MHz (GSM: Global System for Mobile Communication), and at 2100 MHz (UMTS: Universal Mobile Telecommunications System). The study found a statistically significant association between exposure to UMTS and well-being in both groups. In contrast, no effect was seen for the GSM frequencies at 900 and 1800 MHz. However, a study in Switzerland did not confirm these results [Regel et al., 2006]. In another double-blind provocation study (900 MHz GSM mobile phone signal), Rubin et al. [2006] tested persons reporting electrosensitivity and controls but found no evidence of either the ability of electrosensitive people to detect such signals or of any increased symptom severity among them.

Until recently, the absence of personal dosimeters limited the feasibility in the general population of epidemiologic studies of the association between exposure to mobile phone frequencies and well-being in the general public [Spegel et al., 2006]. Such dosimeters were recently developed and, in our pilot study, shown to be suitable for epidemiologic studies [Radon et al., 2006].

We used personal dosimetry to investigate the level of exposure to mobile phone frequencies in a general population sample of adults living in Bavaria (southern Germany). In addition, the potential association between exposure to such fields and well-being of the participants is assessed.

MATERIALS AND METHODS

Study Design and Participants

The study took place in four Bavarian cities with different population size: Munich (~1300000 inhabitants), Freising (~43000 inhabitants), Ebersberg (~11000 inhabitants) and Grafing (~12500 inhabitants). The participants were recruited randomly from the registration offices of these four towns.

The study was nested within an ongoing study on noise exposure and well-being, which had a response rate of 40%. It took place from January 2005 until August 2006. Overall, 329 adults aged between 18 and 65 years were included. Written informed consent was obtained from all participants. Participants were invited to a local study centre, where they completed a Computer Assisted Personal Interview (CAPI) with

questions on chronic symptoms and potential confounders. After the interview the participants obtained a noise dosimeter for a 24-h measurement. From August 2005 to August 2006, all adult participants of this study were also asked to wear the ESM-140 mobile phone dosimeter (Maschek Electronics, Bad Wörrishofen, Germany, www.maschek.de). During the 24-h measurement, subjects were asked to complete a diary recording acute symptoms at noon and in the evening before bedtime. As an incentive, participants obtained a 25-Euro purchase voucher.

Irrespective of their participation in the field study, all subjects invited were asked to answer a short questionnaire to assess potential selection bias.

The study was approved by the Ethics Committee of the Medical Faculty of the Ludwig-Maximilians-University Munich (285/03).

Questionnaires

The following chronic and acute symptoms typically mentioned in the context of exposure to mobile phone frequencies were assessed: headache, neurological symptoms (e.g., tinnitus), cardiovascular symptoms (e.g., tachycardia), concentration problems, sleeping disorders and fatigue.

Chronic symptoms. The list of chronic symptoms during the last 6 months, assessed on a five point Likert scale (nearly daily, about three times a week, about two times a month, about once half a year, never) during the CAPI, was taken from the “Freiburger Beschwerdeliste” [Fahrenberg, 1975; Hiller, 1997]. The symptom “sleeping disorders” was defined by summarizing three questions relating to sleeping attitudes. This symptom was considered present if at least one of the three items was reported to occur at least twice per month. All other symptoms were also considered present if they occurred at least twice per month according to self-report.

Acute symptoms. The diary items were taken from the “Zerssen Beschwerdeliste” [Zerssen, 1976] and assessed on a four point Likert scale (heavy, moderate, barely, not at all). Concerning the “neurological symptoms” three questions relating to tinnitus, numbness in hands or feet and eyelid twitch were summarized. This symptom was considered present if it was reported with an at least moderate intensity for one of the three questions. The other symptoms were also considered present if they were reported with an at least moderate intensity.

Exposure Assessment

Exposure was measured using the personal dosimeter ESM-140 (Maschek Electronics, Table 1).

TABLE 1. Technical Data of the Maschek Dosimeter ESM 140

Frequency bands	
GSM 900 uplink	890–915 MHz
GSM 900 downlink	935–960 MHz
GSM 1800 uplink	1725–1780 MHz
GSM 1800 downlink	1820–1875 MHz
DECT	1880–1900 MHz
UMTS uplink	1920–1980 MHz
UMTS downlink	2110–2170 MHz
WLAN	2400–2485 MHz
Measurement range	0.01–5 V/m
Measurement cycle	0.5–10 s
No. of samples stored	130000
Size ($L \times W \times H$)	106 mm \times 47 mm \times 28 mm
Weight	76 g

For 24 h the dosimeter was placed on the upper arm of the participants which they usually used to hold the mobile phone or DECT phone during phone calls. During the measurement, subjects were asked to perform their routine daily tasks. At night, the participants placed the dosimeter next to their beds.

Exposure was assessed every second resulting in 86400 measurements over 24 h. The following frequency ranges were covered:

1. GMS 900 (up and down link).
2. GSM 1800 (up and down link) including Universal Mobile Telecommunication System (UMTS 2100) and Digital Enhanced Cordless Telecommunications (DECT).
3. Wireless local area network (WLAN 2400) frequencies.

We had to use a combination of frequency bands for the calculation because the dosimeter has a low selectivity between the up- and down-link channels.

Two of the nine available dosimeters were selected at random and tested at the Technical Inspection Agency South (TÜV Süd) to confirm the technical data given by the manufacturer and to investigate whether the different units of the dosimeters used in the study are comparable. The findings showed a good comparability of the different dosimeters. At exposure level of 0.5 V/m the relative standard deviation was between 4% (DECT) and 17% (GSM 1800). Furthermore, the findings proved the validity of the technical data provided by the manufacturer.

According to the results of these laboratory measurements, the limit of determination was set at 0.05 V/m. To classify the exposure all measured values that were below this limit were replaced by half of the

limit (0.025 V/m). This method is often used in the context of environmental epidemiology and the results seem plausible because all values have to be between 0 and the limit of determination [Hornung and Reed, 1990].

To calculate the exposure we first calculated the squared field strength, averaged over the waking hours, for the three frequency ranges. Thus, for example, the squared field strength $\bar{E}_{\text{GSM 900}}^2$ for GSM 900 has been calculated via the following formula:

$$\bar{E}_{\text{GSM 900}}^2 = \left(\frac{\sum_{\text{waking hours}} E_{\text{GSM 900 uplink}}^2(t)}{\text{duration of waking hours}} \right) + \left(\frac{\sum_{\text{waking hours}} E_{\text{GSM 900 downlink}}^2(t)}{\text{duration of waking hours}} \right)$$

The overall simultaneous exposure to multiple frequency fields was calculated by summation of the squared field strengths, averaged over waking hours, which are weighted by the inverse of squared ICNIRP reference level:

$$\text{Overall exposure\%} = \text{square-root} \left(\left(\frac{\bar{E}_{\text{GSM 900}}^2}{\text{limit}_{900 \text{ MHz}}^2} \right) + \left(\frac{\bar{E}_{\text{GSM 1800}}^2}{\text{limit}_{1800 \text{ MHz}}^2} \right) + \left(\frac{\bar{E}_{\text{WLAN}}^2}{\text{limit}_{2400 \text{ MHz}}^2} \right) \right) \times 100\%$$

Extracting the square-root over this sum returned an overall exposure in terms of field strengths percentage of the reference level.

Level of exposure during bedtime. As mentioned above, two dosimeters were selected at random and tested at the Technical Inspection Agency South (TÜV Süd). The engineers investigated whether the measured bedtime exposure was valid because during night-time the dosimeter was fixed at one position without movement. It was confirmed in the lab measurements that the measurement results of the dosimeter measurements depend on the direction in the field. Thus, valid measurements of the average exposure can only be obtained if the dosimeter is moved. Therefore, the bedtime exposure levels had to be excluded and only exposure levels during waking hours were used for the present article.

Statistical Analysis

To assess the potential association between exposure to mobile phone frequencies and well-being, exposure was divided into quartiles. The mean percentage of the ICNIRP reference level during

waking hours was used to analyze the association between exposure to mobile phone frequencies and chronic symptoms. The mean percentage of the ICNIRP reference level during morning hours (6 a.m.–12 p.m.) was considered relevant for the relationship between morning exposure and acute symptoms at noon. Finally, the mean percentage of the ICNIRP reference level during afternoon hours (12 p.m.–6 p.m.) was calculated to test the potential association between afternoon exposure and acute symptoms before bedtime.

χ^2 -tests were used to assess bivariate associations. Multivariate analyses were done using logistic regression models adjusted for age and sex. These potential confounders were defined a priori and included in all analyses. All models were calculated using complete case analysis. Statistical analyses were carried out using SAS (SAS version 9.1; SAS Institute Inc., Cary, NC).

RESULTS

Descriptive Data

Seventy-three of the 95 adults (77%) wore the dosimeter in Munich, 74 of 111 (67%) in Ebersberg, 118 of 144 (82%) in Freising and 64 of 85 (75%) in Grafing. Forty-seven percent of the subjects were male, 89% owned a mobile phone and 81% owned a cordless (Digital Enhanced Cordless Telecommunications) phone. Overall, 40% of the subjects reported concerns about possible health effects of exposure to mobile phone frequencies (Table 2).

To analyse a possible bias caused by selective non-participation, we compared the data from the short questionnaire of those who participated in the study to those from non-participants using χ^2 -tests. A significant difference was seen for the town of residence

($P < 0.001$) and mobile phone ownership ($P = 0.005$). Participants who wore the dosimeter were more likely to own a mobile phone (90.4%) than those who only took part in the noise-study (84.2%) or non-participants (81.6%). The percentage of non-participants in the two smaller towns, Grafing (12.7%) and Ebersberg (21.5%), was lower than in Munich (34.5%) or Freising (31.3%). No difference between participants and non-participants was seen concerning age, gender and education (data not shown).

Exposure

Exposure levels were far below the ICNIRP reference level and ranged from a mean of 0.13% (all measurement values below the limit of determination) to a mean of 0.58% of the ICNIRP reference level per second during waking hours. No difference was seen between morning hours (range: 0.13–0.56% of the ICNIRP reference level) and afternoon hours (range: 0.13–0.71% of the ICNIRP reference level) (Table 3). The mean and maximum exposure values for the three frequency bands during waking hours are described below (% ICNIRP limit):

GSM 900: mean 0.15; max. 0.47;
GSM 1800: mean 0.11; max. 0.49;
WLAN: mean 0.04; max. 0.10.

However, exposure varied by the size of the town of residence. Median exposure levels during waking hours were the highest in Munich and lowest in the smaller towns of Ebersberg and Grafing (Fig. 1).

Chronic Symptoms

The most frequently reported chronic symptoms were sleeping disorders (58%) and fatigue (21%),

TABLE 2. Sociodemographic Data of the Study Participants

Variable	Prevalence <i>n</i> (%) ^a
Age [years] (<i>n</i> = 322)	
18–35	121 (37.6)
36–45	83 (25.8)
46–55	57 (17.7)
56–65	61 (18.9)
Sex (<i>n</i> = 328)	
Male	155 (47.3)
Female	173 (52.7)
Possession of a mobile phone (<i>n</i> = 321)	
Yes	284 (88.5)
Possession of a cordless (DECT) phone (<i>n</i> = 320)	
Yes	260 (81.3)
Self-reported concerns about adverse health effects to EMF (<i>n</i> = 318)	
Yes	127 (39.9)

DECT, digital enhanced cordless telecommunications; EMF: electromagnetic fields.

^aData may not sum up to 100% due to missing data.

TABLE 3. Prevalence of the Chronic and Acute Symptoms and Results of the Logistic Regression Models by Exposure Quartiles (Odds Ratios and Corresponding 95% Confidence Intervals)

Outcome	Quartile 1		Quartile 2		Quartile 3		Quartile 4	
	Prevalence (%)	OR (CI) ^a	Prevalence (%)	OR (CI) ^a	Prevalence (%)	OR (CI) ^a	Prevalence (%)	OR (CI) ^a
Chronic symptoms								
Day-time ^b								
Headache (<i>n</i> = 318)	3 (3.7)	0.134–0.145	5 (6.2)	0.145–0.167	7 (9.1)	0.167–0.211	3 (3.8)	0.211–0.583
Neurological symptoms (<i>n</i> = 305)	3 (3.7)	1.0	2 (2.6)	0.5 (0.1–3.2)	6 (8.3)	1.9 (0.4–8.7)	2 (2.7)	0.6 (0.1–4.2)
Cardiovascular symptoms (<i>n</i> = 304)	4 (4.9)	1.0	4 (5.2)	1.4 (0.3–6.1)	7 (9.7)	3.3 (0.9–12.5)	5 (6.7)	2.4 (0.6–9.9)
Sleeping disorders (<i>n</i> = 318)	50 (61.0)	1.0	41 (50.6)	0.6 (0.3–1.2)	46 (59.7)	1.0 (0.5–1.9)	49 (61.3)	1.1 (0.5–2.1)
Fatigue (<i>n</i> = 305)	17 (20.8)	1.0	17 (21.8)	1.0 (0.5–2.2)	20 (27.8)	1.5 (0.7–3.4)	11 (14.7)	0.7 (0.3–1.8)
Acute symptoms								
Morning (6 a.m.–12 p.m.) ^{b,c}								
Headache (<i>n</i> = 317)	6 (7.7)	0.133–0.136	4 (4.9)	0.136–0.153	5 (6.2)	0.153–0.193	3 (3.7)	0.193–0.559
Neurological symptoms (<i>n</i> = 315)	14 (18.0)	1.0	8 (9.9)	0.5 (0.2–1.2)	6 (7.5)	0.4 (0.1–1.1)	9 (11.3)	0.5 (0.2–1.3)
Fatigue (<i>n</i> = 314)	18 (23.4)	1.0	13 (15.9)	0.7 (0.3–1.5)	13 (16.7)	0.7 (0.3–1.5)	9 (11.1)	0.5 (0.2–1.1)
Concentration problems (<i>n</i> = 314)	6 (7.7)	1.0	5 (6.3)	0.9 (0.3–3.3)	2 (2.5)	0.2 (0.02–1.4)	2 (2.5)	0.3 (0.06–1.8)
Afternoon (12 p.m.–6 p.m.) ^{b,c}								
Headache (<i>n</i> = 316)	3 (3.9)	0.129–0.147	6 (7.3)	0.147–0.168	6 (7.5)	0.168–0.218	9 (11.1)	0.218–0.713
Neurological symptoms (<i>n</i> = 314)	14 (17.7)	1.0	9 (11.0)	0.7 (0.3–1.7)	17 (21.8)	1.6 (0.4–7.0)	7 (8.9)	0.4 (0.2–1.2)
Fatigue (<i>n</i> = 318)	38 (47.5)	1.0	32 (39.0)	0.6 (0.3–1.1)	40 (50.0)	0.9 (0.5–1.7)	30 (37.5)	0.5 (0.3–1.0)
Concentration problems (<i>n</i> = 316)	5 (6.3)	1.0	8 (9.8)	1.4 (0.4–4.7)	10 (12.7)	2.0 (0.6–6.2)	8 (10.0)	1.4 (0.4–4.7)

CI, confidence interval.

^aOdds ratio adjusted for age and gender.^bMean% ICNIRP limit (classification of exposure in quartiles).^cThe participants were asked to complete a diary recording acute symptoms at noon (referring to the morning) and before bedtime (referring to the afternoon).

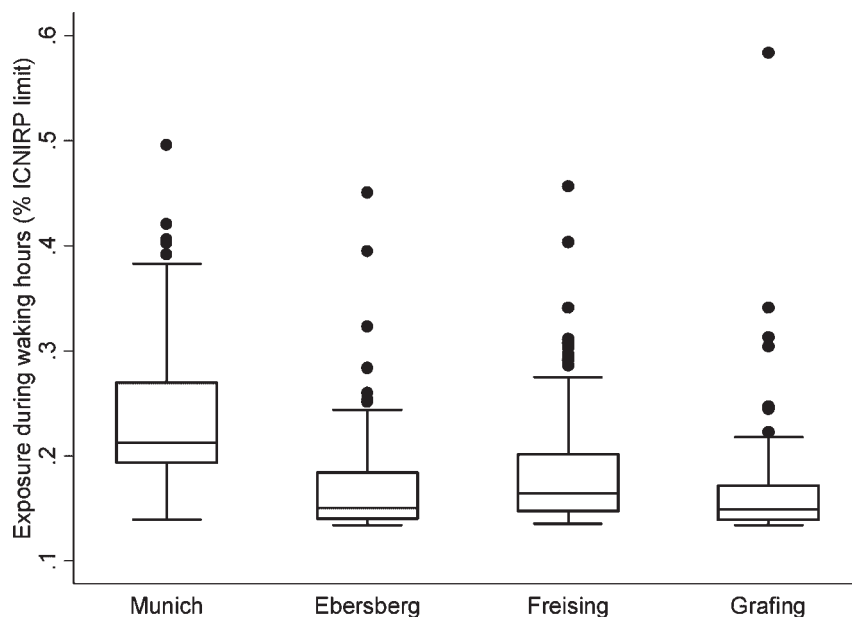


Fig. 1. Comparison of the mean % of the ICNIRP reference level during waking hours in the four study towns.

whereas only 4.2% of the subjects reported chronic neurological problems. No statistically significant association between exposure and chronic symptoms within the last 6 months was seen.

Acute Symptoms

The most frequently reported acute symptom was fatigue in the evening (43%); only 6% of the participants reported having headaches at noon. No statistically significant dose-dependent association between exposure during the morning hours and acute symptoms at midday or between exposure during the afternoon hours and acute symptoms in the evening was observed (Table 3).

Although not statistically significant, an inverse association between morning hours and fatigue at midday (odds ratio 0.5; 95% confidence interval 0.2–1.1) as well as between afternoon hours and fatigue in the evening (odds ratio 0.5; 95% confidence interval 0.3–1.0) was seen. In addition, concentration problems at midday were less frequently reported by participants in the highest exposure quartiles (not significant).

DISCUSSION

The aim of the study was to determine the level of exposure to mobile phone electromagnetic field frequencies and to examine the association between the exposure to mobile phone frequencies and the well-being of adults. Exposure levels were on average less than 1% of the ICNIRP reference level.

This is the first study to use personal dosimetry to assess individual exposure to mobile phone frequencies. This method enables accounting for all sources of exposure, including own and neighbour's use of mobile phones, mobile phone base stations and cordless phones in the home environment. Such estimates are not possible with stationary measurements or estimation alone [Schüz and Mann, 2000; Santini et al., 2002; Navarro et al., 2003; Neubauer et al., 2007]. Furthermore, it allows estimation of the personal exposure not only at the place of living but also at work and during leisure activities.

About 80% of the participants of an ongoing study on noise exposure also agreed to wear the mobile phone dosimeter. However, the ongoing study participants represented only 40% of those who were originally invited to participate. As this study was announced as a study on environmental noise exposure and well-being, a major selection bias with respect to interest in mobile phones and health is unlikely. Indeed, 40% of our participants were concerned about possible health effects of exposure to mobile phone frequencies compared with 30% reported in a recent questionnaire study in the German general population. Several studies have shown that the Bavarian residents tend to be more concerned about mobile phone exposure and health than people living in the north or east of Germany [Berg et al., 2006]. Nevertheless, we cannot rule out a preferential selection in our study of concerned

subjects. In addition, it is possible that the participants overestimated their symptoms during the measurement because they were more aware of their being measured. As the participants were not aware of their actual level of exposure, this bias might have resulted in non-differential misclassification of the outcome; thus, an effect underestimation might have occurred [Ahlborg, 1990; Kaye et al., 1994; Vrijheid et al., 2006].

Another limitation of our study was its relatively small sample size. This should be kept in mind when discussing the findings.

Measuring night-time exposure levels is a common difficulty of studies involving personal dosimetry. Our study participants placed the dosimeters near their beds, which resulted in a constant measurement during the night. As shown in the lab measurements, the dosimeter measurements depend on the direction in the field. Therefore, valid measurements can only be obtained if the dosimeter is moved. This has to be kept in mind in future studies.

A drawback of personal dosimetry is the limited selectivity to differentiate within the three frequency ranges. For this reason we calculated the cumulative overall exposure over all frequency ranges and obtained a mean value which is comparable to the ICNIRP reference level.

Regardless of the weighting scheme, personal dosimetry is a suitable tool to differentiate subjects with higher from subjects with lower overall exposure to mobile phone frequencies.

We a priori decided to analyse the association between exposure to mobile phone frequencies and well-being using exposure levels as quartiles. Due to the skewed distribution of exposure levels the exposure levels of the first and second quartiles were similar resulting in similar odds ratios underlining the plausibility of the results. There are a number of other ways of analysing the data, including use of exposure in tertiles or taking the logarithm of exposure levels as a continuous variable. One way of finding explorative cut off levels would be the use of LOESS-models. Such hypothesis-generating analyses might be done with larger data sets and have been omitted from the present analyses to avoid false positive results due to multiple testing.

Due to the results of the laboratory measurements we decided to replace all values that were below the limit of determination (0.05 V/m) by half of this limit instead of taking the measured values. One has to keep in mind that this could lead to more conservative results.

Since the measurement was limited to 24 h, only the status quo was assessed. However, it is possible that the individual exposure levels may not be representa-

tive. In a pilot test, one subject was asked to wear a dosimeter for seven consecutive days. Although this single within-subject comparison indicated a good reproducibility of the exposure levels it has to be confirmed in a larger sample of subjects [Radon et al., 2004].

Our analyses have only been adjusted for a limited number of potential confounders. Additional adjustment, for example, town of residence or mobile phone use did not change our results. Furthermore, a number of factors that might be associated with the unspecific symptoms under study (e.g., stress at work or coffee consumption) have not been assessed as the level of exposure was mainly driven by exposure to mobile phone base stations. Therefore, while these factors might be associated with mobile phone use they were not considered to be associated with exposure to mobile phone base stations.

Our findings indicate very low exposure levels. Maximum exposure during waking hours was 0.58% of the ICNIRP reference level, which is in agreement with some studies using stationary measurements [Hutter et al., 2006]. In addition, as in previous studies exposure was highest in the largest city indicating the plausibility of the results [Bergqvist, 2001]. However, our measurements cannot be compared with stationary measurements and the absolute values should be treated with caution.

Although not statistically significant, we found an inverse association between exposure and fatigue at midday and in the evening. A comparable association was found in our pilot study, where an inverse association between the exposure and fatigue was seen [Spegel et al., 2006]. A study which compared symptoms experienced by users of analogue and digital mobile phones indicated a significantly lower risk of fatigue in digital phone users [Sandström et al., 2001].

One might argue that tinnitus should not be included with neurological symptoms. However, as the prevalence of other neurological symptoms was extremely low (e.g., 1% for eyelid twitch) it would not have been possible to evaluate these alone. Restricting the analyses to tinnitus did not change the results (data not shown).

CONCLUSION

In summary, this study is the first to use personal dosimetry for the assessment of individual exposure to mobile phone frequencies, enabling objective assessment of exposure from all sources not possible with stationary measurements or estimation alone. We found no statistically significant association between exposure and chronic or acute symptoms. More research is

needed to clarify whether daily environmental EMF exposure may cause acute health problems.

ACKNOWLEDGMENTS

The assistance of Dr. Eder, Mr. Maschek, Mrs. Spiegel, Mrs. Meyer and Ms. Ehrenstein is gratefully acknowledged.

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