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Effects of Electromagnetic Fields on Cellular Responses to Agents Causing Oxidative Stress and DNA Damage

Doctoral dissertation

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ABSTRACT

Increased human exposure to different types of electromagnetic fields (EMFs) has raised concerns about possible adverse health effects from such exposure. Widespread human exposure occurs both to extremely low frequency (ELF) magnetic fields (MFs) from the generation, distribution and use of electricity and to radiofrequency (RF) radiation from wireless communication. The exposure limits for EMFs are based on well-known biological effects that require field intensities much higher than those commonly found in human environment, and current research on biological effects is largely focussed on possible effects from fields below the exposure limits. ELF MFs have been classified as possibly carcinogenic to humans based on findings from epidemiological studies on residential ELF MF exposure and childhood leukaemia. However, experimental studies have not provided clear support for carcinogenic effects, and there is no known mechanism for such effects. Extensive research on bioeffects of RF EMFs has not produced consistent evidence of health risks at low field intensities, but there are still some data gaps. For both ELF and RF EMFs, possible carcinogenicity and genotoxicity have been among the main concerns. There is no known biophysical mechanism for DNA damage from weak EMFs, and most experimental studies have not found any genotoxic effects from EMFs alone. However, there is increasing evidence that relatively weak ELF MFs may enhance the effects of DNA-damaging agents. For RF EMFs, less data are available about combined effects with other agents. In this study, cellular effects of weak EMFs were studied in cell cultures, with particular focus on combined effects with agents that induce DNA damage and oxidative stress.

In this study, exposure to ELF MFs was found to modify responses to DNA damage both in yeast and mammalian cells. In yeast cells, simultaneous exposure to an ELF MF with a frequency of 50 Hz and magnetic flux density of 120 μT concurrent with ultraviolet (UV) radiation resulted in enhanced cell cycle arrest in the G_1-phase. Consistently with the increased cell cycle arrest, MF exposure enhanced the growth delay caused by UV-induced damage. In murine L929 fibroblasts, pre-treatment with a 50 Hz MF at 100 or 300 μT inhibited apoptosis and enhanced G_2/M-phase cell cycle arrest induced by menadione, a chemical that induces increased production of reactive oxygen species. Only pre-exposure to MFs affected responses to menadione; no MF effects were observed when MF exposure followed treatment with menadione. In fibroblasts, MF exposure did not alter responses to UV radiation in any exposure schedule.

No effects of 100 or 300 μT MFs (50 Hz) on UV radiation induced oxidative processes were observed in L929 cells by measuring ultraweak chemiluminescence (photon emissions). Also, exposure to a 300 μT, 50 Hz magnetic field had no effect on intracellular reduced glutathione level (which should decrease as a result of increased production of reactive oxygen species), although similar MF exposure altered subsequent responses to menadione.

Exposure to 872-900 MHz RF EMF with a pulse-modulated signal similar to that used in GSM mobile phones increased UV radiation induced apoptosis in yeast cells. In contrast, no enhancement of UV-induced apoptosis was observed in cells exposed to unmodulated RF EMF at identical exposure levels (0.4 or 3.0 W/kg).

The most important contribution of the present study is the suggestion that relatively weak EMFs may have measurable impacts on cancer-relevant biological processes such as apoptosis and cell cycle arrest. Effects were found when EMFs were studied as cofactors that modify cellular responses to other agents; no effects from EMFs alone were found. The measurements of cellular oxidative processes did not support the hypothesis that the effects of ELF MFs are explained by increased levels of reactive oxygen species, but the methods used had some limitations and more studies are therefore warranted. The observation of modulation-specific effects of RF EMFs is an interesting finding that should be confirmed in further studies. Overall, studying combined effects of EMFs with other agents appears to be a fruitful avenue for further studies on biological effects of weak EMFs.

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-To my lovely family-

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Kuopio, June 2009

Ari Markkanen

Abbreviations

4NQO	4-Nitroquinoline 1-oxide
ANOVA	Analysis of variance
BLM	Bleomycin
CDMA	Code division multiple access
CFU	Colony forming unit
CL	Chemiluminescence
CNS	Central nervous system
CPA	Cyclophosphamide
CW	Continuous wave
DNA	Deoxyribonucleic acid
DMBA	7,12 –Dimethylbenz(a)antracene
ECACC	European Collection of Cell Cultures
ELF	Extremely low frequency (0 – 300 Hz)
EMF	Electromagnetic field
EMS	Ethylmethanesulfonate
ENU	Ethylnitrosourea
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
GSH	Reduced glutathione
GSM	Global system for mobile communications
HFE	High field effect
Hz	Herz, cycles per second, the unit of frequency
IARC	International Agency for Research on Cancer
ICNIRP	International Commission on Non-Ionising Radiation Protection
IF	Intermediate frequency (300 Hz – 100 kHz)
IR	Infrared (300 GHz – 300 THz)
LFE	Low field effect
MDA	Malondialdehyde
MF	Magnetic field
MNNG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
MQ	Menadione
MX	3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone
NIEHS	National Institute of Environmental Health Sciences
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate, also known as TPA
PI	Propidium iodide

PS	Phosphatidylserine
RF	Radiofrequency (100 kHz – 300 GHz)
RFR	Radiofrequency radiation
ROS	Reactive oxygen species
SAR	Specific absorption rate
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks of European Commission
Sub G_1	Apoptotic cells in FACS analysis (hypodiploid DNA content)
t-BOOH	*tert*-butylhydroperoxide
TPA	Tumour promoter phorbol ester 12-O-tetradecanoylphorbol-13-acetate
UV	Ultraviolet
UVA	Ultraviolet A (320 – 400 nm)
UVB	Ultraviolet B (280 – 320 nm)
UVC	Ultraviolet C (240 – 280 nm)
VIS	Visible light (400 – 750 nm)

List of original publications

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Markkanen A, Juutilainen J, Lang S, Pelkonen J, Rytömaa T, Naarala J.

Effects of 50 Hz magnetic field on cell cycle kinetics and the colony forming ability of budding yeast exposed to ultraviolet radiation.

Bioelectromagnetics 22:345 - 350, 2001.

II Markkanen A, Juutilainen J, Naarala J.

Pre-exposure to 50 Hz magnetic fields modifies menadione-induced DNA damage response in murine L929 cells.

International Journal of Radiation Biology 84:742 – 751, 2008.

III Markkanen A, Naarala J, Juutilainen J.

No effect of 50 Hz magnetic fields on UV-induced radical reactions in murine fibroblast cells.

Manuscript.

IV Markkanen A, Penttinen P, Naarala J, Pelkonen J, Sihvonen A-P, Juutilainen J.

Apoptosis induced by ultraviolet radiation is enhanced by amplitude-modulated radiofrequency radiation in mutant yeast cells.

Bioelectromagnetics 25:127 - 133, 2004.

Contents

1 INTRODUCTION

The question whether electromagnetic fields (EMFs) cause biological effects that are harmful to human health, is still open. In our everyday environment, we are continuously surrounded by structures and appliances that emit in the extremely low frequency (ELF) range of the electromagnetic spectrum, such as power lines and household appliances. In addition, increasing emissions in the radiofrequency (RF) part of the electromagnetic spectrum result from the use of wireless communication devices such as mobile phones and their base stations. Several investigations have reported a multitude of biological effects of EMFs, from whole organisms down to the cellular level. However, the extrapolation of the observed biological effects to specific human health effects and diseases is not clear.

Expert groups have concluded that ELF magnetic field (MF) exposure is possibly carcinogenic to humans (NIEHS 1998, IARC 2002, WHO 2007, SCENIHR 2009). This conclusion is mainly based on epidemiological studies on residential ELF MF exposure and childhood leukaemia.

Although a large number of reports have been published regarding biological effects caused by ELF MFs during the last ten years (see NIEHS 1998, IARC 2002, WHO 2007, SCENIHR 2009 for extensive reviews), there is still need for more research in this area to support adequate assessment of potential health risks of ELF MFs. Especially, there is insufficient understanding of interaction mechanisms that could explain biological effects of weak environmental fields. Numerous hypotheses have been put forward, but none is convincingly supported by biological experimental data.

There are many observations of cellular responses induced by ELF MFs *in vitro*. However, theoretical considerations suggest that ELF MFs are unlikely to induce DNA damage directly. Lack of direct carcinogenic effects is also supported by results from genotoxicity studies; most of them have not shown any DNA damage from exposure to MF alone, except for extremely strong fields (IARC 2002, WHO 2007). However, there is increasing evidence that MFs may interact with DNA-damaging agents (WHO 2007).

The widespread use of mobile phones has raised concerns about possible health effects of exposure to RF EMFs from such devices. National and international agencies have established safety guidelines for exposure to RF EMFs, and human exposure from mobile communication systems is below these guidelines. However, concerns remain about possible effects that might occur below the current guidelines.

Although many effects on biological systems exposed to low dose levels of RF EMFs have been reported in the scientific literature, there is no consensus in the scientific community about the existence of effects below current guidelines, and no known interaction mechanisms that could explain effects from weak fields. Like in the case of ELF MFs, possible carcinogenic and genotoxic effects have been among the main questions concerning exposure to RF fields. Both theoretical considerations and experimental evidence indicate that direct DNA damage caused by weak RF fields is not likely. However, less data are available about the possibility that RF fields enhance the effects of DNA damaging agents.

In this study, cellular effects of weak EMFs (ELFs and RFs) were studied in cell cultures, with particular focus on combined effects with agents that induce DNA damage and oxidative stress.

2 REVIEW OF THE LITERATURE

2.1 Physics and biophysics of electromagnetic fields

Electromagnetic radiation is energy flow in the form of electric (E) and magnetic (H) fields that make up electromagnetic waves. In such a wave, time-varying electric and magnetic fields are mutually linked with each other at right angles and perpendicular to the direction of motion. An electromagnetic wave is characterized by its intensity and frequency (f) of the time variation of the electric and magnetic fields. In terms of modern quantum theory, electromagnetic radiation is the flow of photons through space at speed of light. Each photon contains a certain amount of energy, which increases with growing frequency (Jokela 2006). The electromagnetic spectrum can be divided into non-ionising and ionising radiations, depending on the capability to ionise molecules; only ionising radiation has sufficient photon energy to break chemical bonds. The spectrum of non-ionising radiation can be further divided into several categories according to frequency or wavelength: extremely low frequency (ELF) electromagnetic fields, intermediate frequency (IF) electromagnetic fields, radiofrequency (RF) electromagnetic fields, infrared (IR) radiation, visible (VIS) light, and ultraviolet (UV) radiation (Figure 1).

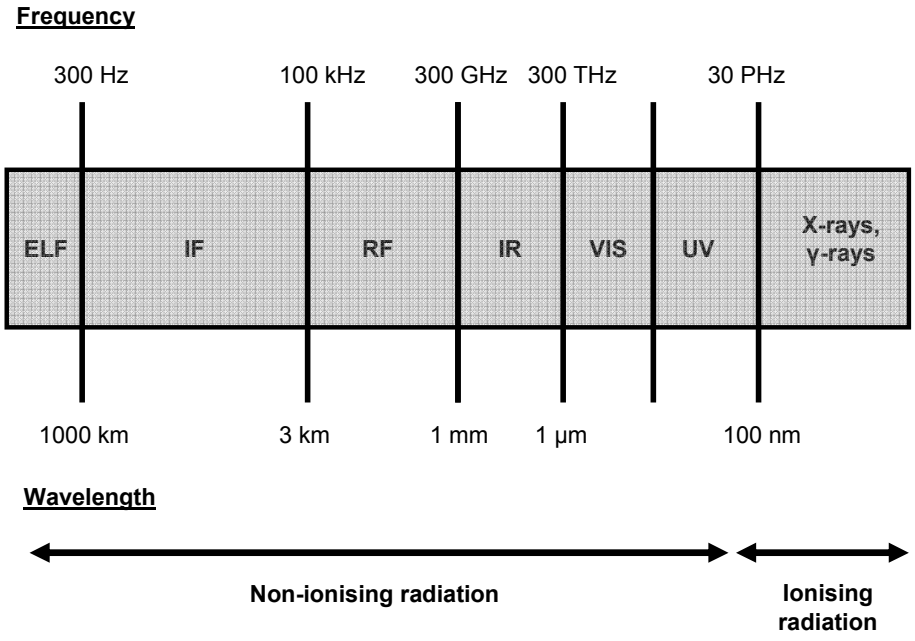

Figure 1. The electromagnetic spectrum

2.1.1 Extremely low frequency magnetic fields (ELF MFs)

Humans are exposed to 50 Hz MFs during electric power generation, transmission, and use of various household electric appliances. MF flux density around a conductor increases with increasing current in the conductor, and declines rapidly with distance from the conductor. MFs are capable of penetrating tissues and are not easily shielded by most materials. ELF MFs induce electric fields and currents in electrically conducting objects such as humans, animals or cell cultures. These induced currents cause adverse biological effects if they are strong enough (about 10 mT or higher at 50 Hz), which is the basis of guidelines for limiting human exposure to ELF fields (ICNIRP 1998).

A mechanism that is not based on induced currents has been described recently. The "radical pair mechanism" is a generally accepted way in which static and low frequency MFs can affect the chemistry of individual molecules at relatively low magnetic flux densities, generally increasing concentration of free radicals in low fields, below 1 mT, (low field effect, LFE) and decreasing them in high fields (high field effect, HFE) (Brocklehurst and McLauchlan 1996, Till et al. 1998, Timmel et al. 1998, Broclehurst 2002). Free radicals are normally short-lived reactive chemical species (atoms or molecules) that posses one or more unpaired electrons. Radicals are generated as a result of metabolic processes, e.g., in mitochondria and by various external exposures, such as ionising or UV radiation. The biological relevance of radicals is not limited to damage associated with high radical levels; they are also a part of normal cell physiology, including intracellular signal transduction (Finkel 2003). Effects of MF on radical level, in spite of the small magnitude of the effect (Timmel et al. 1998, Eveson et al. 2000), could potentially have multiple effects on biological functions. However, although the radical pair mechanism is theoretically well known, and has been experimentally demonstrated in cell-free biochemical systems (Eveson et al. 2000), its practical biological relevance is not very well known.

2.1.2 Radiofrequency electromagnetic fields (RF EMFs)

The widespread use of wireless communication devices such as mobile and cordless phones has increased human exposure to RF EMFs. The main sources of human exposure are the devices that are held next to the body (phones or other wireless devices); the network transmitters (base stations) generally cause only negligible exposure, because field intensity decreases rapidly with distance. Although the penetration of RF EMFs into biological tissues decreases with increasing frequency, the penetration depth is still high at the frequencies generally used for wireless communication (0.5-3 GHz), and RF fields at these frequencies can potentially cause biological effects in the deeper structures of the body.

RF radiation may interact with biological tissue through a number of mechanisms (reviewed in Sheppard et al. 2008). At low radiofrequencies, the induced currents may cause stimulatory effects similar to those of ELF fields, but at higher frequencies (above approximately 10 MHz) the thermal mechanisms are the most well-known: interactions between RF fields and biological tissue are likely to result in energy

transfer to the tissue and this will ultimately lead to an increase in its temperature and induce various biological effects. Thermal effects are the basis for limiting human exposure to high frequency RF EMFs (ICNIRP 1998).

So-called non-thermal mechanisms are those that are not associated with temperature changes. Several mechanisms for non-thermal RF interactions have been proposed, but there are no generally accepted mechanisms that would cause biological effects below the threshold for thermal effects (Sheppard et al. 2008, Adair 2003). One of the aspects of the discussion on possible non-thermal effects of RF EMFs is related on the modulation characteristics of the RF signal. Modulation of RF signals is necessary to make them carry information. It has been hypothesized that amplitude-modulated signals (which are common in wireless communication systems) would have biological effects different from those of unmodulated (continuous wave; CW) signals, but evidence for such modulation-specific effects is weak and there is a lack of plausible biophysical mechanisms for such responses at environmental field levels (Juutilainen and de Seze 1998, Foster and Repacholi 2004).

The radical pair mechanism, which was discussed above in connection with ELF MFs, is not relevant to RF fields; effects on radicals above 10 MHz and especially above 100 MHz are unlikely (Sheppard et al. 2008).

2.2 Biological effects of electromagnetic fields

2.2.1 ELF MF

2.2.1.1 Overview

It is well known that at high ELF MF levels (10 mT or higher), the induced electric fields can cause stimulation of excitable cells (such as muscle and nerve cells), and generate magnetophosphenes (visual sensations caused by MFs). Current research on ELF MFs is largely focused on studying whether there are any biological effects that occur below the thresholds for these well-established effects.

The strongest evidence for adverse health effects associated with weak ELF MFs has come from epidemiological studies, particularly from those reporting that childhood leukaemia is associated with residential ELF MFs of 0.3 – 0.4 μT (IARC 2002, WHO 2007). Associations of ELF MFs to other type of cancers, such as female breast cancer, adult brain tumours or adult leukaemia, are weaker and remain inadequate (IARC 2002, WHO 2007, SCENIHR 2009).

Experimental studies have not provide clear support for carcinogenicity of ELF MFs. Animal studies have generally found no evidence that weak ELF MFs alone could induce tumours (WHO 2007). This observation is supported by results from *in vitro* studies, which have generally shown that there are no genotoxic effects from weak ELFs alone, except for extremely strong fields (IARC 2002). However, there is increasing evidence that MFs may interact with DNA-damaging agents (IARC 2002, WHO 2007). Such combined effects of ELF MFs with other agents are reviewed in more detail below.

2.2.1.2 Combined effects with other agents

In a recent meta-analysis, Juutilainen et al. (2006) reviewed studies that have combined ELF magnetic fields with other physical and chemical agents. Results of co-exposure studies published 2006 or later are reviewed below and described in Table 1.

Table 1. Recent *in vitro* and *in vivo* studies on combined effects of ELF MFs and various cofactors.

Biological endpoint	MF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Genotoxicity						
DNA double strand breaks Viability ROS production	50 Hz 1 mT Exposure duration up to 96 h	Hydrogen peroxide	Probably before MF exposure *(unclear description)*	Human neuroblastoma cell line SH-SY5Y	Co-treatment induced increased DNA damage No co-effects on viability ROS production increased in co-exposure set up	Falone et al. 2007
Micronuclei, aneuploidy	60 Hz 0.8 mT Exposure for 28, 88, 180, or 240 h	Bleomycin	Cells exposed first to bleomycin during 3 h and then incubated further with MF exposure 28 – 240 h	Human fibroblast cells CCD-986sk	Co-exposure enhanced cytotoxicity of bleomycin	Cho et al. 2007
Microsatellite mutations	50 Hz 1 mT Exposure for 12 h	γ-radiation	γ-radiation before MF exposure	Glioma cell line UVW	Increased mutation frequency compared to ionising radiation alone	Mairs et al. 2007
DNA strand breaks	50 Hz 3 mT Exposure duration 30, 60 or 120 minutes	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) 4-nitroquinoline 1-oxide (4NQO)	Simultaneous exposure with ELF MF	Human peripheral blood leukocytes	MNNG induced increased DNA damage in co-exposure 4NQO induced decreased DNA damage in co-exposure	Villarini et al. 2006
Carcinogenesis						
Mammary tumours	50 Hz 100 µT Exposure duration 24 h/day 7 days/week 26 weeks	7,12-dimethylbenz(a)anthracene (DMBA)	Before MF exposure	*In vivo* Female Fischer 344 rats	MF exposure increased the mammary tumourigenesis compared to sham exposed rats	Fedrowitz and Löscher 2008

continued

Table 1. *(Continued)*

Biological endpoint	MF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Carcinogenesis (cont'd)						
Malignant lymphoma/lymphatic leukaemia	50 Hz 7 or 70 or 350 μT Exposure duration 22 h/day, 7 days/week, 30 weeks	DMBA	DMBA injected within 24 h after birth (before MF exposure)	*In vivo* CD-1 mice	MF exposure did not increased malignant lymphoma/lymphatic leukaemia	Negishi et al. 2008
Other studies						
Apoptosis	100 Hz 0.7 mT For exposure duration, see set up	X-rays I) 0,2,4,6,8,10 Gy II) 2 Gy	2 set ups: I) X-ray 1st, then EMF exposure 2x 30 min with a 12 h interval II) EMF exposure 6 x 30 min with a 12 h intervals, and finally X-ray exposure	Human hepatoma cell line BEL-7402	Apoptosis rate of cells exposed to X-rays significantly increased by EMF; several MF exposures caused significantly higher apoptosis rates than two MF exposures	Jian et al. 2009
Viability	60 Hz 14 mT cycled: 5 min on / 10 min off Duration of exposure 4 h	Heat (+ 53 °C)	After MF exposure for 10min	Salmonella enterica I, serovar Typhimurium (*S. enterica*, LT2) strain: TT22240	Viability of co-exposed cells were increased compared heat treated cells	Williams et al. 2006
Resistance to tobacco mosaic virus	10 Hz (25.6 or 28.9 μT) MF + static MF Exposure for 8 or 24 h	Tobacco mosaic virus (TMV)	TMV inoculation before or after MF exposure	Nicotiana tabacum (L.) cv. Samsun plant (TMV-resistant) = tobacco	Following ELF-MFs exposure, an increased resistance was detected	Trebbi et al. 2007

Genotoxicity was studied by Falone et al. (2007), who exposed human neuroblastoma SH-SY5Y cells to hydrogen peroxide (H_2O_2) combined with a 1 mT 50 Hz MF for different exposure times up to 96 h. In the co-exposed cells, DNA damage detected using the single cell gel electrophoresis (comet assay) was increased compared to cells treated with H_2O_2 alone. Also ROS production was found to be increased in the co-exposed cells compared to cells exposed to H_2O_2 alone, but viability was not affected by the MF exposure. Cho and co-workers (2007) studied the effect of bleomycin (BLM) together with a 60 Hz MF in human fibroblast CCD 986sk cells. The cells were first exposed to the cofactor and then incubated for different times under the influence of a 0.8 mT MF. The combined exposure was found to enhance the frequencies of micronuclei and aneuploidy induced by BLM. Mairs et al. (2007) found that a 1mT 50 Hz MF combined with γ-radiation induced more microsatellite mutations than γ-radiation alone in glioma cells. Villarini et al. (2006) studied the effect of two mutagens combined with MFs on human peripheral blood leucocyte cells. Cells were simultaneously exposed to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) or 4-nitroquinoline 1-oxide (4NQO) and a 50 Hz MF at 3 mT. Co-exposure to MF and MNNG increased DNA damage measured by alkaline comet assay compared to the cells exposed to MNNG alone. However, combined exposure to MF and 4 NQO decreased DNA damage compared to the cells exposed to the mutagen alone.

Carcinogenicity in animals was addressed in two studies. The effect of a 50 Hz MF at 100 μT on mammary tumours induced by 7,12-dimethylbenz(a)antracene (DMBA) were studied in female Fischer rats by Fedrowitz and Löscher (2008). The mammary tumourigenesis was found to be enhanced by the MF treatment. Negishi et al. (2008) studied combined effects of 50 Hz MFs and DMBA on lymphoma/leukaemia in CD-1 mice. No MF effect on DMBA induced malignant lymphoma/lymphatic leukaemia was found.

Exposure to a 100 Hz MF at 0.7 mT was found to enhance apoptosis induced by X-rays in human hepatoma cells (Jian et al. 2009). The effect was even more pronounced if ELF exposure was repeated several times and before X-ray exposure.

Williams et al. (2006) found the ELF MFs protected *Salmonella* bacteria against heat stress. A strong (14.6 mT) MF was used and it cycled 5 min on, 10 min off for 4 h. Exposure to the cofactor (heat, +53 ºC) was done after the MF exposure. The viability of MF-exposed bacteria was increased compared to the control cells exposed only to heat.

Trebbi et al. (2007) reported that exposure to a 10 Hz MF together with a static MF protected tobacco plants against tobacco mosaic virus, especially after 8 h of exposure. The 10 Hz MF was speculated to act as a resistance inducer.

Altogether, in all co-exposure studies performed after 2006, except in one experiment, combined effects of ELF MFs were reported. In the meta-analysis of Juutilainen and co-workers (2006) the percentage of positive studies was 91 % for *in vivo* studies and 68 % for *in vitro* studies with eukaryotic cells, so recent findings are consistent with the earlier studies on ELF MFs. Combined effects have been found with many different experimental models, cofactors and exposure schedules (MF first, cofactor first, or simultaneous exposure). This encourages further research on such effects, and indicates that combined effects are not linked only to some specific cells or cofactors. Three of the studies were done at MF levels that did not exceed the ICNIRP exposure limits for ELF MFs (100 μT for the public and 500 μT for workers; ICNIRP 1998), and two of them reported positive findings.

2.2.2 RF EMF

2.2.2.1 Overview

Increased temperature induced by RF radiation is known to cause many biological effects such as changes in biochemical reaction rates, alterations in biochemical and physiological processes *in vivo* and *in vitro*, thermoregulatory responses in animals, changes in animal behaviour, cell death, tissue damage, and burns (reviewed in Sheppard et al. 2008). However, recent research on RF EMFs has focused on studying whether there are other biological effects resulting from exposure to low-intensity RFs below these well-established effects. Of particular interest is the possible existence of health effects that might occur due to accumulation of multiple, long-term, low-intensity RF exposures.

One of the greatest concerns related to RF radiation is whether it is involved in carcinogenesis. Because widespread human exposure from the RF technology used in wireless communication is quite new, epidemiological studies are not yet able to exclude carcinogenic effect from exposures longer than ten years to such technologies. However, it seems unlikely that exposure to mobile phone radiation for less than ten years is associated with increased incidence of cancer (SCENIHR 2009). Results from animal and *in vitro* studies also support the view that weak RF EMFs alone are not carcinogenic or genotoxic (Heikkinen 2006b; SCENIHR 2009). However, only a limited number of studies are available on the possibility that RF EMFs might enhance the effects of known genotoxic or carcinogenic agents.

2.2.2.2 Combined effects with other agents

Heikkinen (2006b) reviewed studies on RF EMFs combined with other physical or chemical agents until 2006. Results of co-exposure studies published 2006 or later are reviewed below and described in Table 2.

Table 2. Recent *in vitro, ex-vivo,* and *in vivo* studies on combined effects of RF EMFs and various cofactors.

Biological endpoint	RF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Genotoxicity						
DNA strand breaks	900 MHz EMF pulsed (GSM modulated) Exposure for 24 h SAR 1 W/kg	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX)	Exposure for MX (1 h) after RF EMF exposure	Human dermal fibroblast (HD) Turner's syndrome fibroblast (TS)	No effects from RF EMF	Sannino et al. 2009
DNA strand breaks Formation of ROS	872 MHz EMF pulsed (GSM modulated) and continuous wave Exposure for 1h SAR 5 W/kg	Menadione	Simultaneous exposure for 1 h	Human SH-SY5Y neuroblastoma cells	CW RF radiation enhanced menadione-induced DNA damage and ROS production No effect from modulated RF radiation	Luukkonen et al. 2009.
DNA strand breaks (C) Chromosome aberration test (CA)	835 MHz EMF pulsed (CDMA modulated) Exposure for 24 h (CA) or 48 h (C, CA) SAR 4 W/kg	Ethylmethanesulfonate (EMS) CA Cyclophosphamide (CPA) C+CA 4-nitroquinoline 1-oxide (4NQO) C	RF exposure 48 h, clastogen treatment 4 h before comet assay; 6 h simultaneous exposure to chemical mixture and RFR and then additional 18 h or 42 h exposure for RFR.	L5178Y tk+/- mouse lymphoma cells (comet) Chinese hamster lung (CHL) cells (CA test)	RF exposure enhanced the clastogenic activity of the model clastogens in comet assay No effect on chromosome aberrations	Kim et al. 2008
Micronuclei	902.5 MHz EMF continuos, SAR 1.5 W/kg or 902.4 MHz EMF pulsed (GSM modulated), SAR 0.35 W/kg Exposure 1.5 h/day, 5 days a week for 78 weeks	X-ray	X-ray exposure three times, one week interval	Erythrocytes from *in vivo* study; Female CBA/S mice	No effect of combined exposures on micronucleus frequency were observed	Juutilainen et al. 2007

continued

Table 2. *(Continued)*

Biological endpoint	RF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Genotoxicity (cont'd)						
DNA strand breaks Chromosomal aberrations and sister chromatid exchange Micronuclei	935 MHz EMF (GSM modulated) Exposure for 24 h SAR 1 and 2 W/kg	X-ray	1 min X-ray exposure immediately before or after the RFR exposure	Human lymphocytes	No effect from RFR	Stronati et al. 2006
DNA strand breaks Chromosomal aberrations Sister chromatid exchanges	450, 900 MHz and other frequencies in the MHz or GHz frequency range (occupational exposure) Estimated exposure more than 1 h a day during at least 1 year (average 2.3 y) SAR not known	Blood lymphocytes cells were exposed to mitomycin C to investigate whether the occupational RF exposure changes the sensitivity of the cells to the chemical exposure	72 h exposure for SCE and 1 h exposure for comet assay	Peripheral blood lymphocytes from workers exposed to RF fields	No co-operative action was found	Maes et al. 2006
DNA strand breaks Frequency of micronucleated polychromatic erythrocytes	900 MHz EMF pulsed (GSM modulated) Exposure 2 h/day, 5 days/week for 104 weeks SAR 0.3 W/kg and 0.9 W/kg at nominal whole-body average	MX	MX expose through drinking water throughout the whole study	*In vivo* Female Wistar rats	No combined effects found	Verschaeve et al. 2006
Carcinogenesis						
Mammary gland tumours	902 MHz EMF pulsed (GSM modulated) Exposure 4 h/day, 5 days per week for 6 months SAR 0.4, 1.3 or 4W/kg	7,12-dimethylbenz(a)anthracene (DMBA)	Before RF exposure	*In vivo* Female Sprague-Dawley rats	No significant effects of RF EMF on mammary tumourigenesis	Hruby et al. 2008

continued

Table 2. *(Continued)*

Biological endpoint	RF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Carcinogenesis (cont'd)						
Mammary gland tumours	902 MHz EMF pulsed (GSM modulated) Exposure 4 h/day, 5 days a week for 104 weeks SAR 0.4, 1.3 or 4W/kg	DMBA	Before RF exposure	*In vivo* Female Sprague-Dawley rats	No significant effects of RF EMF on mammary tumourigenesis	Smith et al. 2007
CNS tumours	1.95 GHz EMF (W-CDMA signal) Exposure 1.5 h/day, 5 days a week for 104 weeks SAR 0.67 or 2.0 W/kg at whole-body average	Ethylnitrosourea (ENU)	Before RF exposure	*In vivo* Fischer 344 rats	No significant effects found	Shirai et al. 2007
CNS tumours	860 MHz EMF (MiRS signal) Exposure 6 h/day, 5 days a week for 39 weeks SAR 0.27 – 0.42 W/kg whole-body average	ENU	Before RF exposure	*In vivo* Sprague-Dawley rats	No effects of RF EMF	Zook and Simmens 2006
Mammary gland tumours	900 MHz EMF pulsed (GSM modulated) Exposure 4 h/day, 5 days a week for 26 weeks SAR 0.44, 1.33 or 4.0 W/kg	DMBA	Before RF exposure	*In vivo* Female Sprague-Dawley rats	No significant effects on mammary tumourigenesis found	Yu et al 2006
Multiple tumours	900 MHz EMF pulsed (GSM modulated) Exposure 2 h/day, 5 days a week for 104 weeks SAR 0.3 W/kg and 0.9 W/kg whole-body average	MX	MX in drinking water throughout the whole study	*In vivo* Female Wistar rats	No effects from RF EMF	Heikkinen et al. 2006a

continued

Table 2. *(Continued)*

Biological endpoint	RF exposure	Cofactor	Set up for cofactor	Cell line / animals	Co-effects	Reference
Other studies						
Apoptosis DNA-fragmentation Reduced cellular glutathione level (GSH) Lipid peroxidation Proliferation Viability	872 MHz EMF pulsed (GSM modulated) and continuous wave Exposure for 1 or 24 h SAR 5 W/kg	Menadione Tert-butyl-hydroperoxide (t-BOOH)	Simultaneous exposure to chemical and radiation for 1 h, except in caspase-3 and DNA fragmentation for 24 h	Human SH-SY5Y neuroblastoma cells Murine L929 fibrosarcoma cells	GSM-modulated RF radiation enhanced menadione-induced caspase-3 activity in L929 cells and t-BOOH induced lipid peroxidation in SH-SY5Y cells	Höytö et al. 2008b
Formation of ROS	900 MHz EMF pulsed (GSM modulated) and continuous wave Exposure for 10 or 30 min SAR 0.3 or 1 W/kg	MX	Simultaneous exposure for 10 or 30 min to RFR and MX	Murine L929 fibrosarcoma cells	No indication that RF exposure in combination with MX induced formation of ROS	Zeni et al. 2007
Cochlear functionality	900 MHz EMF continuous wave 2 h/day, 5 days a week for 4 weeks at a local SAR 4 W/kg in the ear	Gentamicin	Gentamicin was injected approximately 1 h prior to EMF exposure from the first day of exposure through 2 weeks	*In vivo* Male Sprague-Dawley rats	No evidence of a combined effect of RF-field exposure and gentamicin on the auditory system	Parazzini et al. 2007
Apoptosis/necrosis	1800 MHz EMF (GSM-DTX modulated) Exposure for 12 h SAR 2 W/kg	Phorbol-12-myristate-13-acetate (PMA) Gliotoxin	Simultaneously exposed for 12 h	Human Mono Mac 6 cells	No combined effects detected	Lantow et al. 2006

Sannino et al. (2009) exposed human dermal fibroblast and Turner's syndrome fibroblast cells to a 900 MHz GSM-modulated EMF for 1 or 24 h at 1 W/kg and to the environmental mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). No effects of RF radiation on MX-induced DNA damage measured by the alkaline comet assay were found. Luukkonen and co-workers (2009) exposed human fibroblast (SH-SY5Y) cells to menadione and to 872 MHz continuous wave (CW) or GSM modulated EMFs at 5 W/kg for 1 h. Effects on DNA strand breaks were studied by the alkaline comet assay. Menadione-induced DNA-damage was enhanced by the CW signal but not by the GSM-modulated RF EMF. Also menadione-induced intracellular ROS production was found to be enhanced by CW RF radiation but not by GSM-modulated radiation at identical SAR level. In a recent study with mouse lymphoma cells (L5178Y tk+/-), cyclophosphamide (CPA) or 4-nitroquinoline 1-oxide (4NQO) were used to study possible co-genotoxic effect of 835 MHz code division multiple access (CDMA) modulated signals at 4 W/kg for 48 hrs (Kim et al. 2008). In comet assay, RF EMF was found to potentiate the effects of both clastogens (CPA and 4NQO). In the same study, no RF EMF effects were found on chromosome aberrations induced by ethylmethanesulfonate (EMS) or CPA after 24 or 48 hrs of exposure. Juutilainen and co-workers (2007) investigated micronucleus frequency in blood samples from two animal carcinogenicity experiments. In one of the studies female CBA/S mice were exposed to a 902.5 MHz continuous EMF at 1.5 W/kg or a 902.4 MHz GSM-modulated EMF at 0.35 W/kg after initial exposure to X-rays. The RF EMF exposures did not enhance the X-ray effects on micronuclei, but the study had limited power to detect combined effects because of the long time between the x-ray irradiation and determination of the micronuclei. Stronati et al. (2006) studied the effects of a 935 MHz GSM-modulated signal at 1 or 2 W/kg for 24 h on genotoxicity induced by X-rays on human lymphocytes. No effects of the RF EMFs on DNA strand breaks, chromosomal aberrations, sister chromatid exchanges or micronuclei were detected. In a recent study (Maes et al. 2006), human lymphocytes from workers who had been exposed to different RF sources were exposed to mitomycin C to investigate whether occupational RF exposure changes the sensitivity of cells to chemical exposure. No co-operative effects on DNA strand breaks, chromosomal aberrations or sister chromatid exchange were found. In an *in vivo* study with female Wistar rats (Verschaeve et al. 2006) GSM-modulated 900 MHz EMF at whole-body average SARs of 0.3 or 0.9 W/kg was combined with MX in drinking water. Effects on DNA strand breaks and micronucleated polychromatic erythocytes were studied. No RF EMF effects were found.

Several studies on co-carcinogenesis addressing different types of tumours in various animal models have been reported after 2006 (Hruby et al. 2008, Smith et al. 2007, Shirai et al. 2007, Zook and Simmens 2006, Yu et al. 2006, Heikkinen et al. 2006a). In none of them was RF EMF exposure found to have statistically significant effects on carcinogenesis induced by known carcinogens.

Effects of RF EMFs on cell death were studied by Höytö et al. (2008b) and Lantow et al. (2006). Höytö and co-workers (2008b) detected apoptosis by measuring caspase-3 activity in human SH-SY5Y neuroblastoma cells and murine L929 fibrosarcoma cells. Also internucleosomal DNA fragmentation measured by agarose gel electrophoresis, reduced glutathione (GSH), viability, proliferation and lipid peroxidation were measured. Menadione was used to induce oxidative stress and *tert*-butyl-hydroperoxide (t-BOOH) to induce lipid peroxidation. Cells were exposed to GSM-modulated or CW 872 MHz EMFs at 5 W/kg for 24 h. The GSM-modulated RF signal enhanced menadione-induced caspase-3 activity in

L929 cells, but not in SH-SY5Y. No effects on GSH levels were found. In SH-SY5Y cells GSM-modulated RF EMFs enhanced t-BOOH induced lipid peroxidation, but no effects were found in L929 cells. Lantow et al. (2006) investigated the effects of GSM-DTX modulated 1800 MHz field at 2 W/kg on apoptosis and necrosis induced by phorbol 12-myristate 13-acetate (PMA) or gliotoxin. No RF field effects on cell death detected by flow cytometry were found in human Mono Mac 6 cells.

Zeni et al. (2007) exposed murine L929 cells to 900 MHz GSM-modulated or CW EMFs at 0.3 or 1 W/kg for 10 or 30 minutes simultaneously with MX. No RF EMF effects on MX induced formation of ROS were found.

Parazzini and co-workers (2007) investigated the effects of RF radiation combined with gentamycin on the auditory system in male Spraque-Dawley rats. The rats were exposed to continuous wave 900 MHz radiation at 4 W/kg in the ear for 4 weeks; combined exposure to gentamycin lasted 2 weeks. No evidence of combined effects on the auditory system was reported.

In summary, all but one genotoxicity and carcinogenesis studies on combined effects after 2006 reported negative findings, using many different experimental designs and model organisms. In the literature review on combined effects by Heikkinen (2006b), positive findings were reported in 12 genotoxicity studies of 31 (39 %) and 5 co-carcinogenic studies of 14 (29 %). In the studies on other effects than genotoxicity or carcinogenesis, three positive findings were reported after 2006. However, these findings are isolated and inconsistent (one endpoint positive in one cell line, another endpoint positive in another cell line; some effects were found with a CW signal and some with a GSM-modulated signal).

Unlike studies on ELF MFs, the recent studies on combined effects of RF EMFs were predominantly negative and therefore support existence of such effects even less than the review by Heikkinen (2006b). It is unclear whether the few positive findings are due to chance or reflect true RF EMF effects, which might be weak and observable only in specific circumstances.

3 AIMS OF THE STUDY

The aim of the present work was to study possible cellular and biophysical effects of weak electromagnetic fields in cell cultures, with particular focus on combined effects with physical and chemical agents that induce oxidative and DNA damage.

The specific aims of the present study were as follows:

1. To investigate the effects of EMFs on viability and proliferation in UV-exposed cells (I, IV).
2. To study the effects of ELF MFs on cell cycle in UV- or menadione-exposed cells (I, II).
3. To study the effects of EMFs on apoptosis in UV- or menadione-exposed cells (II, IV).
4. To investigate the effects of different schedules of combined exposure to ELF MF and UV radiation or menadione (II).
5. To study the effects of ELF MF on oxidative reactions induced by UV radiation (III).

4 MATERIALS AND METHODS

4.1 Cell culture

4.1.1 Yeast cells (I, IV)

Experiments with *Saccharomyces cerevisiae* yeast cells were carried out with the haploid yeast strain *S. cerevisiae* SEy2101a (II) (obtained from professor Marja Makarow, Institute of Biotechonology, University of Helsinki, Finland) and *S. cerevisiae* cdc48-mutant KFy437 (III) and the corresponding wild-type (wt) control strain KFy417 (III) (both obtained from Dr. Frank Madeo, Institute of Molecular Biosciences, University of Graz, Austria). The yeast cells were cultured grown in a medium of YPD broth (1 % yeast extract, 2 % peptone, 2 % glucose) with or without agarose (II) or in medium of YEPD broth (1 % yeast extract, 2 % peptone, 4 % glucose, pH 5.8) with or without 2 % agarose (III).

4.1.2 Fibroblast cell line (II, III)

Murine fibroblast cell line (L929) was obtained from European Collection of Cell Cultures (ECACC), Salisbury, UK. Cells were cultured in plastic cell culture flasks in DMEM supplemented with 10 % (v/v) Fetalclone II, 50 U/ml penicillin, and 50 µg/µl streptomycin at +37 ºC in humidified 5 % CO_2, 95 % atmospheric air.

4.2 Exposure systems

4.2.1 System for simultaneous exposure to 50 Hz magnetic field and UV radiation (I, IV)

An exposure system was constructed for simultaneous exposure to UV radiation and 50 Hz magnetic field. On the upper part the system there was a level for petri dishes/cell flasks. A vertical magnetic field was generated by two 100 mm × 1 300 mm rectangular coils with 15 turns of copper wire in each at the level of dishes/flasks. The current was generated by a function generator B+K Precision model 3010 (Dynascan corp., Chicago, IL.) and amplified by GENELEC 1027B Power Amplifier (Genelec OY, Finland). UV-lamps were placed underneath the dishes/flasks in the way that the cells were exposed through the bottom of the dishes/flasks. The exposure system was originally devised to be used in a temperature controlled room (Study I), but it was also used for UV-exposure in other experiments in room temperature (+18 - +23 ºC) (Studies II, III, and IV).

4.2.1.1 UV exposure (I, II, III, IV)

Philips TL 40W/12 UVB-lamps (Philips, Holland) were used in Studies I, II, IV and UV lamps simulating the solar spectrum (Philips HP3136, Philips, Netherlands) were used in Study III.

The UV irradiation level was measured by MACAM spectroradiometer model SR9910 (Macam Photometrics LTD. Livingston, Scotland) and by Solar Light model 3D erythema UV & UVA intensity meter (Solar Light Co Inc., Philadelphia, USA). The calibration of the spectroradiometer has been checked against the national calibration standard at STUK - the Radiation and Nuclear Safety Authority (Helsinki, Finland).

4.2.2 ELF MF exposure system (II, III)

In Studies II and III, a two-coil exposure system was placed inside an incubator for 24-h ELF exposure of L929 cells. The dimensions of the coils were 340 × 460 mm, and the separation between the two coils was 220 mm. The current was generated by a Wavetek 5 MHz Function Generator model FG-5000 A (Wavetek, Germany), and amplified by a Peavey M-3000 Power Amplifier (Peavey Electronics corp., Meridian, USA).

4.2.3 RF EMF exposure system (IV)

Cell cultures were exposed to RF EMFs in a waveguide exposure chamber. The system consisted of two identical aluminium chambers (an exposure chamber and a control chamber) sited outside an incubator. Temperature-controlled air was circulated from the incubator by tubes and fans. The dosimetry of RF exposure was defined for use of two glass Petri dishes in each chamber. The temperature of the cell cultures was controlled by a bed of water under the Petri dishes; the temperature of the circulating water was regulated by a separate control unit. The RF signal source consisted of a signal generator SMY02 (Rohde & Schwarz, Germany), a Wideband RF Amplifier R720FC (RF Power Labs, USA), and a modulating unit. The RF power was measured by Directional Power Meter NAS (Rohde & Schwarz, Germany). Dosimetry for the exposure set-up was determined by FDTD calculations using commercial code XFDTD (RemCom Corporation, State College, USA) and by measuring the electric field profile inside the exposure chamber. Scaled to dissipated power within the chamber, the correlation between the measured electric field and the calculated field was excellent (maximum 5 % difference at the 869.5 MHz resonant frequency). The SAR in the Petri dishes was 2.5 W/kg/W ± 30 % (1 S.D.) for 1 W of dissipated power.

A pulse-modulated signal resembling that of the GSM mobile phone system was used for exposure, with pulse duration of 0.577 ms and pulse repetition rate of 217 Hz. One experiment was performed using a carrier wave frequency of 900 MHz and a specific absorption rate (SAR) of about 0.4 W/kg (0.1 - 0.5 W/kg). In another experiment a 872 MHz signal was used at a SAR of 3.0 W/kg (± 35 %). In both experiments, identical exposures (same frequencies and same time-average SAR values) were performed also using unmodulated (continuous-wave) signals.

4.3 Experimental design (I-IV)

Table 3. Design of the combined exposure experiments.

Study	EMF exposure	Cofactors	Exposure schedules / design
I	50 Hz MF, 120 μT	UVB, 175 J/m^2	1.) Simultaneous exposure for 10 min
II	50 Hz MF, 100 or 300 μT	UVB, 160 J/m^2 or Menadione, 150 μM	1.) Exposed first to UV or menadione for 20 or 60 minutes and then to MF for 24 or 48 h 2.) Exposed to MF for 24 hours, to UV or menadione for 20 or 60 minutes, and then exposed to MF for another 24 h 3.) Exposed to MF for 24 h, to UV or menadione for 20 or 60minutes, and then incubated for another 24 h without any exposures
III	50 Hz MF, 100 or 300 μT	Solar simulating UV, 240 J/m^2	1.) Simultaneus exposure for 1 h 2.) 24 h MF pre-exposure before UV exposure
IV	900 MHz EMF, 0.4 W/kg 872 MHz EMF, 3.0 W/kg	UVB, 250 J/m^2 + elevated temperature	1.) 20 min UV-exposure before RF exposure, and elevated temperature during RF exposure (1 h) and after RF exposure (24 h).

4.4 Viability and cell proliferation (I, IV)

The viability of yeast cells were analyzed by counting colonies on agar plates and cell density (effect on cell proliferation rate) was counted by a hemocytometer.

4.4.1 Colony forming ability (I, IV)

For CFU (colony forming units) analysis appropriate dilutions were made and yeast cells were plated on YPD (I) or YEPD (IV) agar plates to determine viable counts. Colony forming units were determined 48 and 72 h after plating from six (I) or eight (IV) replicates of agar plates.

4.4.2 Cell counting (IV)

The total cell number was counted using a cell counting chamber (Neubauer improved, Marienfeld, Germany) and an inverted phase-contrast microscope (Olympus CK2-TK,

Olympus Optical Co., Ltd, Japan).

4.5 Cell cycle kinetics (I, II)

In Study I, ethanol fixed (70 %) cells were stored at +4 °C until staining with propidium iodide (PI). The cells were washed with 50 mM sodium citrate buffer (pH 7.0), and incubated with 0.15 mg/ml of RNase A (Sigma, St. Louis, MO) overnight at +37 °C. Subsequently, the cells were stained with 8 μg/ml of PI for 30 minutes before flow cytometric analysis. Flow cytometric analysis was performed using Becton Dickinson FACScan cytometer and Cell Quest™ program for analysis of DNA content. PI fluorescence was collected on FL2 detector. A total of 6,000 - 10,000 cells were analyzed per sample and the flow rate was between 20 to 500 particles per second.

In Study II, the cells were resuspended into phosphate-buffered saline (PBS w/o Ca^{2+}, Mg^{2+}), fixed with ice-cold ethanol (70 % v/v), and stored at +4 °C until staining with PI. Subsequently, the cells were pelleted, suspended into PBS, and incubated with 0.15 mg/ml of RNase A for 1 h at +50 °C. PI was added to a final concentration of 8 μg/ml and the incubation was continued for 2 h at +37 °C in the cell culture incubator before flow cytometric analysis. Flow cytometric analysis was performed using Becton Dickinson FACSCalibur cytometer (Becton Dickinson, San Jose CA, USA) with an argon ion laser (488 nm) as the excitation source. Data were acquired using Cell Quest™ v 3.3 software (Becton Dickinson, USA) and Summit software version 4.2 (Dako, USA) was used for analysis of DNA content. Typical sample flow rates were between 100 to 400 particles per second. A total of 10,000 cells were analyzed per sample.

4.6 Apoptosis (II, IV)

In Study II, DNA content was analyzed by propidium iodide (PI) staining of permeabilized cells, where apoptotic cells can be identified as the cells containing fragmented DNA (number of hypodiploid cells as in sub G_1 peak) (Nicoletti et al. 1991, Darzynkiewics et al. 1992, Hughes and Mehmed 2003). The staining procedure and analysis is performed as above in the cell cycle analysis (cell cycle kinetics, study II).

In Study IV, apoptosis was investigated by using annexin V –FITC and propidium iodide (PI) staining utilizing flow cytometry. The use of the annexin V –FITC staining for quantification of apoptotic cells is based on the phenomenon that phosphatidylserine (PS) is exposed outer membrane during early apoptosis and on the ability of annexin V to bind to PS with high affinity. PI can penetrate into necrotic cells, but not viable or early apoptotic cells. For analysis, a total number of 1 x 10^6 yeast cells were washed once with Binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2,5 mM $CaCl_2$), harvested and cell walls were digested 2 h at +28 °C with 50 U/ml lyticase (SIGMA-ALDRICH CHEMIE GmbH, Germany) in the Binding buffer. The spheroplasts were harvested, washed once with Binding buffer and stained by using annexin V - FITC Kit (Bender Medsystems Diagnostics GmbH, Vienna, Austria). The spheroplasts were re-suspended in 195 μl of Binding buffer and 5 μl of annexin V-FITC was added. After incubation in staining solution for 10 minutes at room temperature (RT) in the dark, the spheroplasts were harvested, washed, and re-suspended in 190 μl of Binding buffer and transferred into FALCON® tubes (12 × 75 mm, Becton Dickinson

Labware, Franklin Lakes, NJ, USA). Propidium iodide (f.c. 1 μg/ml) was added and samples were incubated for 15 min at RT in the dark. Binding buffer was added for a final volume of 500 μl. Flow cytometric analysis was performed using Becton Dickinson FACSCalibur cytometer (Becton Dickinson, USA) with an argon ion laser (488 nm) as the excitation source and Cell Quest™ v 3.3 software for analysis of living, necrotic, and apoptotic cells. Typical sample flow rates were between 150 to 500 particles per second. A total of 10,000 cells were analyzed per sample.

4.7 Oxidative reactions (II, III)

Formation of oxygen free radicals was studied indirectly by measuring intracellular reduced glutathione level, which decreases as a result of high ROS level. Ultraweak chemiluminescence detection was used to study the effects of 50 Hz MF on UV radiation induced oxidative processes in living cells.

4.7.1 Measurement of intracellular reduced glutathione (GSH) (II)

In Study II, after the exposures, the cells were washed with Hank's-balanced salt solution (HBSS; 5.3 mM KCl, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 137 mM NaCl, 0.3 mM Na_2HPO_4, 5.6 mM glucose; pH 7.4), and loaded with 40 μM monochlorobimane (mBCl) in HBSS for 15 min in dark. The formation of a fluorescent GSH- mBCl complex was monitored at an excitation wavelength of 380 nm and an emission wavelength of 465 nm with a Perkin-Elmer HTS7000 Plus multiwell fluorometer (Perkin-Elmer, Norwalk CT, USA). Cell free wells, treated in the same way as the samples, served as blanks. Fluorescence values of blanks were subtracted from the fluorescence values of the cell samples.

4.7.2 Measurement of photon emissions (III)

A Wallac WinSpectral 1414 liquid scintillation counter (Wallac Oy, Turku, Finland) was used with the out-of-coincidence mode to measure ultraweak chemiluminescence (CL) of the cells. The wavelength range of the photomultiplier of the scintillation counter was 160 - 650 nm. The background values of CL signals (spontaneous CL) in mere culture media were subtracted from values of the control and exposed samples.

4.8 Statistical analysis

In Study I, the significance of the differences between experimental groups was evaluated by a paired t test. The CFU raw data was first normalized by taking logarithms from the total numbers of colony forming units.

In Studies II and IV, the significance of the differences between experimental groups was evaluated by the one-way analysis of variance (ANOVA) with Tukey's Post-Hoc -test for multiple comparisons using GraphPad Prism (GraphPad Software, San Diego, USA).

In Study III, an exponential decay curve was fit with the data from each experimental run, using GraphPad Prism. The significance of the differences between experimental groups was

evaluated by comparing the mean values of k (decay constant) and Y_0 (initial value) by paired t tests.

In all Studies, a p-value lower than 0.05 was considered statistically significant.

5 RESULTS

The main results of this thesis are presented and discussed in the original publications I - IV. Here, a summary of the results is presented.

5.1 Effects of ELF MF studies

5.1.1 Colony forming ability (I)

In Study I, the cell viability was studied by counting CFUs on agar plates. The UV exposure induced growth delay up to 2 hours after UV irradiation. A significant difference was found in the number of CFUs between combined exposure to UV (175 J/m^2) and MF (120 μT) compared to UV only exposed yeast populations 7 hours after UV irradiation. Also at other time points the amounts of CFUs in the UV and MF co-exposed populations were lower than in the UV only exposed populations, although the differences were not statistically significant.

5.1.2 Cell cycle kinetics (I, II)

In Study I, the MF and UV co-exposed yeast populations had more efficient G_1-arrest at 60 min after UV irradiation than the UV only exposed populations. At that time point there was a very significant difference in G_1-phase and a significant difference in G_2-phase between UV+MF and UV only exposed populations. At 120 min after UV irradiation UV-induced G_2-arrest occurred. The G_2-arrest was more pronounced (although not statistically significant) in yeast populations exposed to UV+MF than in the UV only exposed ones.

In Study II, the UV exposure arrested cells in the S-G_2/M phases if UV exposure was done in the beginning of the exposure protocols. If the UV exposure was done in the middle of the experiments, cells were arrested in the G_1-phase. The exposure to MF at 100 or 300 μT after or before UV irradiation did not change the response to UV in any cell cycle phases of murine L929 cells. MQ exposure (150 μM) only arrested fibroblasts in the G2/M-phase efficiently. In the co-exposure with MQ, the MF at 100 μT pre-treatment triggered even more significant cell cycle arrest at the G_2/M-phase. In experiments with the stronger MF (300 μT), the response to the MF+MQ+MF exposure schedule was otherwise similar to that observed at 100 μT, but in this case also the proportion of G_1 cells was statistically significantly reduced. The MF+MQ+I exposure schedule at 300 μT produced qualitatively similar response on cell cycle (reduced counts of cells in G_1-phase, increased counts of cells in G_2/M-phase), but only the reduction of cells in G_1 was statistically significant.

5.1.3 Apoptosis (II)

In Study II, exposure to UV radiation induced 2.5 – 4.0 % apoptosis in L929 cells after 24 hours. At 48 hours after UV exposure, the proportion of apoptosis was increased to 23 - 25 %. The combined exposure to MF and UV had no effects on the number of apoptotic cells. In the MQ experiments, a one-hour MQ exposure alone resulted in 8 – 22 % apoptosis after 24 hours. After 48 hours, the proportion of apoptotic cells was increased to 15 – 50 %. MF

exposures at 100 or 300 μT after MQ exposure did not affect the proportion of apoptotic cells. However, if cells were first exposed to MF at 100 μT for 24 hours and then exposed to MQ, the proportion of apoptotic cells was significantly lower than in cells that were exposed to MQ only. At 300 μT, apoptosis was decreased significantly only in the exposure schedule that involved MF exposure both before and after the MQ exposure.

5.1.4 Oxidative reactions (II, III)

In Study II, we hypothesized that the ELF MF exposure would increase reactive oxygen species, which might result in decreased level of intracellular GSH. However, exposure of the cells to a 300 μT ELF for 24 h had no effect on intracellular GSH levels.

In Study III, exposure to 100 or 300 μT MFs did not affect the decay kinetics of photon emissions that were used for measuring oxidative processed induced by UV radiation. The initial photon emission level was in all exposure protocols slightly lower in the MF-exposed samples than in the UV only exposed samples, but this difference was far from statistical significance in spite of the high number of replicate experiments.

5.2 Effects of RF EMF studies

5.2.1 Cell proliferation and colony forming ability (IV)

In Study IV, none of the RF field exposures alone had significant effects on proliferation rate of yeast cells. The combined exposure to UV and RF radiations did not affect cell proliferation compared to UV only exposed cells.

The UV dose used (250 J/m^2) clearly reduced the number of reproductive live yeast cells compared to control cells that were exposed to sham-RF radiation and elevated temperature. None of the RF field exposures alone had significant effects on the number of viable yeast cells. At 900 MHz RF exposure (SAR 0.4 W/kg) the number of cdc48 colonies was lower in the UV+RF –exposed than in the UV only exposed cells in every single experiment with the GSM-modulated field. However this finding was not statistically significant.

5.2.2 Apoptosis (IV)

In the RF Study, the UV exposure alone clearly increased apoptosis of both yeast strains. The RF field exposures alone had no effects on programmed cell death.

The GSM-modulated RF exposure increased UV-induced apoptosis in cdc48-mutant yeast strain compared to the cells exposed to UV only. This was observed at both SAR levels. In contrast, no enhancement of UV-induced apoptosis was seen in the cells exposed to unmodulated RF fields.

5.3 Summary of co-exposure studies

The responses to EMF exposures observed in yeast and mammalian cells are summarised in Table 4.

Table 4. Main findings of four *in vitro* studies involving exposure to EMFs and selected cofactors.

Biological endpoint	Cells	EMF	Cofactor	Results	Study
Viability and cell proliferation	*S. cerevisiae* strain SEy2101a	50 Hz MF 120 μT	UVB	Fewer colony forming units in co-exposed cultures	I
	S. cerevisiae strain KFy437	872 / 900 MHz EMF GSM or CW SAR 0.4 or 3.0 W/kg	UVB + elevated temperature	No significant effects	IV
Cell cycle	*S. cerevisiae* strain SEy2101a	50 Hz MF 120 μT	UVB	Increased G_1-arrest at 60 min after UV-exposure	I
	L929 cells	50 Hz MF 100 or 300 μT	1.) UVB	1.) No effects	II
				2.) MF+MQ+MF at 100 μT: enhanced G_2/M-arrest	
			2.) Menadione (MQ)	MF+MQ+MF at 300 μT : enhanced G_2/M-arrest and reduced number of cells in G_1-phase;	
				MF+MQ+I at 300 μT: reduced number in G_1-phase only	
Apoptosis	L929 cells	50 Hz MF 100 or 300 μT	1.) UVB 2.) MQ	1.) No effects 2.) Decreased apoptosis	II
	S. cerevisiae strain KFy437	872 / 900 MHz EMF GSM or CW SAR 0.4 or 3.0 W/kg	UVB + elevated temperature	Increased apoptosis	IV
Oxidative reactions	L929 cells	50 Hz MF 100 or 300 μT	Solar simulating UV	No effects	III

6 DISCUSSION

The aim of this thesis was to study combined effects of electromagnetic fields, ELF MFs and RF EMFs, with physical and chemical agents that can induce oxidative and DNA damage in different eukaryotic cell models. More specifically, EMFs were evaluated as cofactors that possibly modify effects of UV radiation and menadione on viability, cell cycle, and apoptosis (I, II, IV). Possible effects of ELF MF on UV radiation-induced oxidative processes were also studied (III). The EMF exposures used were found to modify UV radiation effects on the viability of yeast cells (I, IV). Also combined effects with UV radiation and menadione were found on cell cycle (I, II) and apoptosis (II, IV). When EMF exposures were used alone, no effects were found (II, III, IV).

6.1 Effects of ELF MFs (I – III)

6.1.1 Effects on cell cycle (I, II)

Possible effects of 50 Hz MFs on cell cycle phases were studied with yeast and L929 cells. In both studies MFs were found to alter responses to other agents; such effects were observed in Study I in UVB-irradiated yeast cells and in Study II in L929 cells exposed to MQ.

In Study I, MF exposure enhanced the cell cycle arrest in G_1 (and possibly in G_2) checkpoint during the first cell cycle of the yeast cells compared to UV only exposed cells. Later on, no effects on cell cycle up to 7 h were seen. In *S. cerevisiae*, DNA damage or a replication block can result in checkpoint-dependent cell cycle arrest in G_1, S, or G_2/M phases to block cell proliferation until lesions are repaired; thus preventing damaged DNA from being inherited by daughter cells (Humprey and Pearce 2005). A possible interpretation of the finding is that the combined MF and UV exposure resulted in more extensive DNA damage than UV irradiation alone, which in turn caused more cells to be arrested in G_1 and G_2 –checkpoints. This hypothesis is supported by recent studies that have reported increased genotoxicity in cells exposed to MFs (Villarini et al. 2006, Mairs et al. 2007, Falone et al. 2007; see Table 1 on page 21).

In Study II, pre-treatment with MF before exposure to MQ caused more pronounced G_2/M checkpoint arrest than exposure to MQ alone. MQ is known to induce cell cycle arrest in S and G_2/M phases (Lamson and Plaza 2003). Again, a possible interpretation is that the more pronounced cell cycle arrest reflects increased DNA damage, and therefore the finding might indicate that the cells were sensitized during the 24 h pre-exposure to ELF MF and were then more vulnerable to MQ-induced DNA damage and responded with more pronounced cell cycle arrest.

Effects on cell cycle have been reported also by other researchers. Takashima et al. (2003) found that a 50 Hz MF at 30 mT inhibited G_1-arrest in UV-irradiated yeast cells. In that study cells were first exposed to UV and then to MF up to 48 hours. Tian et al (2002) also found inhibition of cell cycle arrest in G_1-phase in Chinese hamster ovary cells exposed to ionising radiation and a 60 Hz MF. In this study, cells were first exposed to X-rays and then to a 5 mT

MF for different durations (5, 10, or 24 hours). Richard et al (2002) found no effect of a 50 Hz MF on TPA induced cell cycle arrest in the G_1-phase. In this study, the cells were simultaneously exposed to TPA and MF at 1 mT for 36 hours. The differences between responses observed in these studies (inhibition of cell cycle arrest or no effects) and those observed in Studies I and II may be explained by differences in biological models, cofactors used, exposure schedules and other experimental details.

6.1.2 Effects on viability and apoptosis (I, II)

In Study I, cell viability was measured by counting CFUs from agar plates. The combined MF and UV exposure seemed to decrease yeast cell viability compared to UV only exposed yeast at 4, 6, and 7 hours after UV-irradiation, although the effect was statistically significant only at 7 hours after UV exposure. It is not known whether this decrease in CFUs resulted only from the cell cycle arrest observed (reduced proliferation) or whether there was also increased cell death. In another study that used yeast cells as a model organism, no MF effect on yeast cell viability (CFUs) was found with UV irradiation (Ager and Radul 1992). Compared to Study I, Ager and Radul exposed cells to UVC irradiation (in Study I, a UVB source was used) with lower UV dose, but much higher MF density. Also the exposure schedule was different; cells were first exposed to UV and then to MF. Additionally, there are other studies in which no effects on cell viability were found in co-exposure set-ups with MF and different agents (e.g. Ansari et al. 2000, Loberg et al. 2000, Tian et al. 2002). In the studies of Ansari et al. (2000) and Tian et al. (2002) exposure to the cofactor was done before MF exposure, whereas Loberg and coworkers (2000) exposed cells simultaneously to MF and the cofactors.

Apoptosis induced by MQ was decreased after 24 h of MF exposure in Study II. This is an interesting finding, as apoptosis is a general cellular response to DNA damage, and an important protection mechanism that removes potential cancer cells. Magnetic field exposure has been reported to suppress apoptosis induced by other agents also in several other studies (Kumlin et al. 2002, Tian et al. 2002, Robison et al. 2002). The results of Robison et al. (2002) are of particular interest, because they resemble the results of Study II. Robison and co-workers reported that HL60 and Raji cells were protected from heat-induced apoptosis by 12 or 24 h pre-exposure to a 60 Hz, 150 µT MF. However, also MF exposure following apoptosis-inducing treatments has been reported to suppress apoptosis in other studies. Kumlin et al. (2002) reported that UV-induced apoptosis was inhibited in mouse skin by a 50 Hz, 100 µT MF, but the effect was independent on whether the 24 h MF exposure preceded or followed UV irradiation. Tian et al. (2002) reported inhibition of apoptosis resulting from a 24 h exposure to a 5 mT, 60 Hz MF following ionising radiation.

Also enhancement of apoptosis has been reported. Jian et al (2009) found that exposure to a 0.7 mT, 100 Hz MF increased apoptosis rate induced by X-rays in human hepatoma cells. The effect was significantly larger if cells were first exposed several times to the MF and before X-ray irradiation. Ding et al. (2000) reported that X-ray induced apoptosis was either increased or decreased by a subsequent 48 h exposure to a 5 mT, 60 Hz MF depending on the cell line.

The protective effect of ELF MF against apoptosis observed in Study II is supported by findings of other studies, but also opposite effects (enhancement of apoptosis) has been reported. Differences in the MF exposure variables, cofactors and biological models used

might well explain differences in the results.

6.1.3 Effects on oxidative reactions (II, III)

In Studies II and III, possible effects of MF on oxidative reactions were studied. In Study II, no effect on GSH concentration was seen from MF exposure alone. In Study III, no differences in photon counts were detected between UV and MF co-exposed cells and those exposed to UV radiation only. These negative findings do not support the hypothesis that 50 Hz MF exposure leads to increased cellular level of ROS, and alternative explanations may therefore be needed to explain the biological effects of ELF MFs observed in this work and other studies.

Although the results of studies II and III do not support MF effects on cellular ROS levels, definite conclusions are not possible because of limitations of the methods used. It is possible that, in Study II, cellular antioxidant defence has been sufficient to neutralize the increase of ROS production without any change in GSH content. Secondly, the type of radicals produced in Study III may not have been optimal for detecting the MF effects; according to the radical pair theory, both the magnitude and direction of the MF effect depends on the type (singlet or triplet state) of radical pairs produced (Eveson et al. 2000). Thirdly, in both Studies II and III, measurements were done from whole cell suspensions. According to the radical pair theory, MF most likely affects the recombination probability of radical pairs if the diffusive movement of radicals is restricted (Timmel et al. 1998). Substantial LFE might therefore occur only in smaller cell compartments, e.g., between membrane structures in cell organelles; the cell itself offers insufficient restriction for the movements of the radicals. Because of the multiple role of radicals in cellular physiology, significant biological effects might follow even from very localized changes in radical levels.

Effects of ELF MFs on oxidative reactions have been reported in a few studies (Regoli et al. 2005, Wolf et al. 2005, Zwirska-Korczala et al. 2005, Falone et al. 2007). Regoli et al. (2005) studied the responses of the land snails to 50 Hz MFs. Changes in activities of antioxidant enzymes were found following 10-day or 2- month exposures to MFs at 0.5 – 50 μT. The antioxidant α-tocopherol was found to prevent effects of 50 Hz MF (0.5 – 1.0 mT) on cell proliferation and DNA damage of HL-60 leukemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts (Wolf et al. 2005), which indirectly indicates involvement of ROS. Wolf and co-workers (2005) also reported increased levels of 8-OHdG adducts in all cell lines used and concurrently the intracellular ROS levels were increased in Rat-1 fibroblasts following exposure to MF. Antioxidative enzyme activities of 3T3-L1 cells were found to be altered after exposure to ELF MFs at 120 μT (Zwirska-Korczala et al. 2005). Also malondialdehyde (MDA) concentration, which is used as an indicator of lipid peroxidation, was significantly higher in MF exposed cells than control cells after 24 h of incubation. Falone et al. (2007) studied the effect of a 50 Hz, 1 mT MF on human neuroblastoma (SH-SY5Y) cells with or without H_2O_2. Enhanced ROS production was found in combined exposures, but also antioxidant activities were affected by ELF MF exposure alone.

Although no effects on radical processes were found in Studies II and III, the limitations of the methods used, existence of a plausible mechanism (the radical pair mechanism), and findings from other studies encourage further studies on the role of ROS in biological effects of MFs.

6.2 Effects of RF EMFs (IV)

6.2.1 Effects on viability and apoptosis (IV)

In Study IV, possible effects of RF EMFs on colony forming ability and apoptosis of yeast cells were studied. The use of yeast cells as a model was inspired by the findings of Madeo et al (1997), who reported apoptotic phenotypes in the cdc48 mutant yeast strain after heat stress.

In Study IV, there were no significant differences in CFUs between yeast cells exposed to combined UV and RF EMF treatment and those exposed to UV radiation only. However, apoptosis was increased in cells that were exposed to GSM-modulated RF EMF. This effect was consistently connected with the modulation of the RF field at both SAR levels and carrier frequencies used; the unmodulated fields showed a consistent lack of effects. This finding is interesting as it supports the hypothesis of specific (non-thermal) effects of amplitude-modulated RF fields. In the studies of Capri et al. (2004a, b), Merola et al. (2006), and Lantow et al (2006), no RF EMF effects were found on apoptosis induced by various cofactors. All these studies involved simultaneous exposure to RF EMF and the apoptosis-inducing agent. Höytö et al. (2008b) reported that apoptosis measured by caspase-3 activity was increased in L929 cells exposed simultaneously to MQ and a RF EMF for 24 h. Caspase-3 activity was increased by the GSM-modulated signal, but not by continuous wave EMF, and the effect was not observed in SH-SY5Y cells. In the same study, the GSM-modulated (but not continuous wave) field also enhanced lipid peroxidation induced by *tert*-butylhydroperoxide. In contrast to the effect on caspase-3 activity, this effect was observed only in SH-SY5Y cells, not in L929 cells.

The modulation-specific enhancement of apoptosis observed in yeast cells in Study IV is supported by the findings of Höytö et al. (2008b) in L929 cells. However, the latter results are difficult to interpret because of incoherent findings. Most other studies have generally used either only modulated or only CW signal, so direct comparison to the present Study is not possible. Additional studies would be useful to confirm the findings of Study IV.

6.3 General remarks of co-exposure studies

Humans are exposed simultaneously or sequentially to a mixture of multiple chemicals and physical agents including increasing levels of EMFs. This is one reason why studies on combined effects of EMFs and other environmental agents are useful for a complete understanding of the human health consequences of environmental exposure to EMFs.

Investigating combined effects is of interest also in assessing the risks of other environmental agents than EMFs, and many environmental pollutants have been found to be more toxic in co-exposure with other agents (e.g. Toyooka and Ibuki 2005, 2007). However, designing co-exposure studies is more difficult than traditional toxicological single-agent studies. There are many experimental variables, including selection of cofactors. For example in Study II, MF altered responses to MQ but not to UV irradiation. The UV source used in Study II (and I) was UVB lamp, which mostly induces direct DNA damage, although it also induces some free radical formation (Kulms and Schwarz 2002). Combined effects of UV radiation and

ELF MFs have been reported earlier with different UV sources, model organisms and MF exposure parameters (Ager and Radul 1992, Kumlin et al. 2002, Takashima et al. 2003), which may well explain differences in results. The finding of Study II showing different responses to two cofactors with similar cells and exposure circumstances indicates that, in future co-exposure studies, it may be useful to include more than one cofactor. Differences in the responses may provide important clues towards understanding the mechanism of biological effects of weak EMFs.

Another important variable in combined effects studies is selection of exposure schedule, which has been discussed previously by Juutilainen et al (2000, 2006). In Study II, a 24 h pre-exposure to ELF MF altered responses to MQ, while no effects were seen from MF exposures that followed MQ treatment. This finding was reproducible in several independent experiments, so it is unlikely to be a chance finding. On the other hand also simultaneous exposure to MF affected the responses to UV radiation in Study I, and positive findings have been reported from MF exposures that precede, follow or are simultaneous with other exposures (Juutilainen et al. 2006). In studies on RF EMFs combined with other agents, the most common approaches have been simultaneous exposure or RF EMF exposure following the cofactor (e.g. see Table 2 on page 25). The latter schedule (UV before RF) was also used in Study IV. Although it seems that most findings of combined effects studies with RF field exposure are negative, more variability in the exposure schedules with different cofactors are warranted for more complete assessment of the lack or existence of any effects.

All living organisms use numerous signal transduction systems to sense and respond to environmental changes, which allow the organisms to adapt to their environments. Protective effect of a preceding MF exposure, such as the suppression of apoptosis observed in Study II, resembles the so-called adaptive response. Adaptive response can be defined as improved resistance to stress damage after a prior exposure to lesser amount of stress. It was first described as a reduction in response to a high challenge dose of ionising radiation when a small dose was delivered before the challenge dose (Rigaud and Moustacchi 1996). Apart from ionising radiation, adaptive response has been reported also for chemical agents (Jeggo et al. 1977, Klaassen and Liu 1998, Schlade-Bartusiak et al. 2002) and UV radiation (Chouinard et al. 2002), and priming dose to one agent has been reported to reduce the response (genotoxicity or cell death) to a subsequent challenge dose to another agent (Vijayalaxmi and Burkat 1989, Flores et al. 1996, Anuszewska et al. 1997, Oliveira et al. 2000). At least on phenomenological level, the current findings in Study II can be classified as an adaptive response; the presumably mild stress associated with the 24 h MF exposure alters the response to a subsequent stronger challenge by MQ. Further studies designed based on the concept of adaptive response might be useful for understanding the biological effects of weak MFs.

In summary, the co-exposure experiments have been found to be useful in studies on the biological effects of weak EMFs, and more such studies are warranted. The results of the present work have provided some basis for the design of future studies.

6.4 Methodological considerations

6.4.1 Cell lines used

For Studies II and III were selected the murine fibroblast L929 cell line, which has been earlier used in studies on EMF bioeffects (Litovitz et al. 1997; Penafield et al. 1997; Cress et al. 1999; Mullins et al. 1999; Desta et al. 2003). Additionally, our group has a lot of experience of using the L929 cell line in EMFs studies (Höytö et al. 2006, 2007a, 2007b, 2008a, 2008b). For co-exposure studies it is also crucial to find suitable model organisms which respond reliably to the cofactor used; cell lines which have been previously used in studies with EMF alone might not necessarily be the best ones. L929 cells have been previously used in studies involving exposure to menadione (Paul and Arrigo 2003). In all Studies I – IV, dose-response studies were done with the cofactors (UVB, UVA, MQ) before starting the combined exposures.

In addition to human or other mammalian cell lines, lower unicellular eukaryotic cells (e.g. yeasts) are widely used in *in vitro* studies (Barr 2003). *S. cerevisiae* is one of the most studied organisms and an excellent model for understand the eukaryote cell. For example, many of the mammalian cell cycle genes were first identified and studied in yeast cells (Hartwell et al. 1970). In 1997, Madeo and coworkers reported the first findings of characteristics of apoptosis in unicellular organisms. Since then, yeast cells have been used in studies of apoptosis by several other research groups (Bettiga et al. 2004, Fabrizio et al. 2004, Breitenbach et al. 2005, Bakkali et al. 2006, Eisenberg et al. 2007, Pereira et al. 2008, Perrone et al. 2008). The use of yeast cells in the present thesis (Studies I and IV) is well justified based on earlier studies of cell cycle (Tippins and Parry 1981, Siede and Friedberg 1990, Terleth et al. 1990, Ostroff and Sclafani 1995, Siede 1995, Pearce and Humprey 2001, Del Carratore et al. 2002) and apoptosis (Madeo et al. 1997, Ligr et al. 1998, Madeo et al. 1999, Frölich and Madeo 2000, Frölich and Madeo 2001, Laun et al. 2001, Madeo et al. 2002, Madeo et al. 2004).

6.4.2 Exposure to EMFs

In the Studies with 50 Hz MFs (I – III), the magnetic flux densities were between 100 – 300 μT, which are considerably higher than fields usually measured in urban environments. However, fields of this magnitude are found in some occupational environments (Jokela 2006). On the other hand, the flux densities used are quite low compared to other recently published co-exposure studies with 50-60 Hz MFs (Williams et al. 2006, Villarini et al. 2006, Mairs et al. 2007, Cho et al. 2007, Falone et al. 2007, Jian et al. 2009). The approach of using high level exposure is common in toxicological studies and aims at finding any effects that an agent may have regardless of real-life exposure situations. The exposure limits recommended by the International Commission on Non-Ionising Radiation Protection (ICNIRP 1998) are 100 μT for the general public and 500 μT for workers. Thus the flux densities used in the present work are relevant for investigating the adequacy of current exposure limits.

In Study IV, the SARs were 0.4 and 3.0 W/kg. Exposure limits for partial body exposure are 2 W/kg for general public and 4 W/kg for workers (ICNIRP 1998). In co-exposure studies involving exposure to RF EMF published after 2006 (Table 2 on page 25) the SAR levels were in most of cases 2 W/kg or below (Heikkinen et al. 2006a, Lantow et al. 2006, Stronati

et al. 2006, Verschaeve et al. 2006, Zook and Simmens 2006, Juutilainen et al. 2007, Shirai et al. 2007, Zeni et al. 2007, Sannino et al. 2009). The use of relatively low RF radiation exposure levels in this and other studies is related to the need to prevent effects from elevation of temperature resulting from absorption of RF energy. The need to prevent heating leads to the disadvantage that very high doses cannot be used in contrast to typical toxicological research, in which high doses are used to increase the effect size and thus the statistical power to detect any effects. On the other hand, an advantage of the use of low exposures is that it mimics real human exposure situations. In the present work, the exposure system involved cooling by circulating water, which allowed controlling temperature of the cell cultures. Therefore, it was possible to use a SAR of 3 W/kg (which is above the exposure limit for the general public) without temperature changes in the cell cultures.

Studies on bioeffects of EMFs have some specific challenges different from, e.g., toxicological studies with chemicals. Because even weak environmental fields are suspected to cause biological effects, evaluation of the background fields is crucial and measurements of ELF MFs in cell culture incubators in all culture maintenance phases are required (only ELF fields, the levels of background RF fields are not biologically meaningful). In Studies I – IV, ELF MF fields were measured in all incubators and different exposure set-ups, and the cell culture were placed so that the background MFs were the lowest level possible. Additionally, in Study III the static (geomagnetic) flux density was measured because also the geomagnetic field affects recombination of radical pairs (Timmel et al. 1998).

6.4.3 Assay methods

In Studies I and II, the effects on cell cycle phases were analyzed by flow cytometry and PI staining, a method that is widely used and an excellent technique for this purpose. In Study II, the same method was utilized for detecting the number of apoptotic cells simultaneously with cell cycle determination. The analysis of apoptosis by PI staining is based on the determination of fractional DNA content (as a sub G_1 peak), and it has been found to correlate well with other apoptosis assays (Nicoletti et al. 1991, Darzynkiewicz et al. 1992). This method is not without problems in the evaluation of apoptosis. The use of positive controls to set up proper acquisition parameters of the FACS is essential to discriminate true apoptotic cells from debris (Riccardi and Nicoletti 2006). Proper sample preparation, use of low flow rate and careful optimization by the operator during data acquisition are needed to increase the reliability of the analysis.

In Study IV, the effects on yeast cell apoptosis were evaluated by annexin V- FITC/PI assay and FACS. The annexin V affinity assay, which has found widespread use in measuring apoptosis *in vitro,* is a fast, simple, and sensitive method to detect PS externalization (a mark of early phase of apoptosis), and various kits are commercially available (Plenchette et al. 2004, Miller 2004, Ishaque and Al-Rubeai 2007). The method was chosen because Madeo and co-workers (1997) performed their experiments with yeast cells using the same method, and the TUNEL-staining may be unreliable (Pulkkanen et al. 2000). The drawback of this method is that fixation is not recommended after FITC–annexin V labelling (Plenchette et al. 2004, Miller 2004), which complicates the sample analysis; cells cannot be stored for batch analysis, which sets limits to experimental set-up. This method also requires careful handling of the cells to produce good results; rough handling might damage the cell membrane of all viable cells and allow saturation of the DNA with PI (Miller 2004). Therefore it is crucial that

same person is doing the staining procedure, and proper amount of controls are used to control staining procedure. However, not all cell types exhibit PS exposure during cell culture (Ishaque and Al-Rubeai 2007). Even if there is induction, it may only be confined to a small fraction of the apoptotic cells, thereby underestimating the level of apoptotic cell induction.

In Study II, intracellular GSH activity was measured by the fluorescent probe monochlorobimane (mBCl). GSH is a key antioxidant in most eukaryotic cells and is known to react with $OH^{\bullet}$, HOCl, $ONOO^{-}$, $RO^{\bullet}$, $RO_2^{\bullet}$, $CO_3^{\bullet -}$, $NO_2^{\bullet}$, carbon-centred radicals and $^1O_2$, but not $O_2^{\bullet -}$ radicals *in vitro* (Halliwell and Gutteridge 2007). The mBCl method is simple to perform and requires only a small number of cells, which makes it possible to use cultures simultaneously for additional assays. Sebastià et al. (2003) compared efficiency of four different GSH fluorescent assays and found that mBCl staining was useful for studying semiquantive GSH concentration in human neuronal cell cultures. However, like very often in *in vitro* studies, there are differences in sensitivities between different cell types. Berendji et al. (1999) found different GSH responses in three murine cells after nitrosative stress; murine L929 fibroblasts were the most resistant of the cell lines studied. Therefore, one complication of the interpretation of the findings of Study II is that the cell line used might be more resistant than some other cell lines to respond to oxidative challenge by reduced GSH.

In Study III, oxidative reactions after UV irradiation were evaluated using CL measurement. The attractiveness of the CL assay as an analytical tool is the simplicity of detection. The CL measurement provides a quick possibility to study the effects of oxidative processes with high detection sensitivity and without any fluorescent probes, which are commonly used to enhance the detectability of oxidative products. Disadvantages of fluorescent probes include that they are toxic to cells, some of them may require a catalyst for reaction, and some of them generate radicals themselves, which might produce misleading results (reviewed in Wardman 2007). These drawbacks are avoided by using CL method without probes. The disadvantage of the CL counting by liquid scintillation is that some radical emissions may be lost because of restricted wavelength sensitivity of photomultipliers (Cadenas and Sies 1984), but the fluorescent probes also have specific sensitivities to different reactive species. Some further improvements of CL measurements with scintillation counter would be useful; cells in the sample tubes settle down during the measurements (in Study III, measurement of one sample took 60 seconds), which decreases the sensitivity of the device.

7 CONCLUSIONS

In this Study, relatively weak EMFs were found to alter cellular responses, such as cell cycle arrest and apoptosis, to physical and chemical agents that cause DNA damage and oxidative stress. The EMFs alone did not induce any observable cellular responses.

Simultaneous exposure of yeast cells to a 50 Hz MF and UV radiation resulted in enhanced cell cycle arrest in the G_1-phase. Consistently with the increased cell cycle arrest, MF exposure enhanced the growth delay caused by UV-induced damage.

Pre-treatment with a 50 Hz MF was found to suppress oxidative stress induced apoptosis in murine fibroblast cells. The finding is potentially important and is supported by other studies reporting MF effects on apoptosis.

Results concerning 50 Hz MF effects on UV radiation induced oxidative processes and on intracellular GSH levels did not support the hypothesis that ELF MFs increase cellular level of ROS, and alternative explanations may therefore be needed to explain the ELF MF bioeffects observed in this and other studies. However, the methods used for detecting MF effects on oxidative processes have several limitations. Further studies are therefore warranted, especially because the radical pair mechanism is a plausible mechanism for biological effects at relatively low magnetic flux densities.

In yeast cells exposed to RF EMFs, UV-induced apoptosis was enhanced only by the pulse-modulated GSM-type signal. This finding supports the speculated modulation-specific biological effects of RF EMFs. Further studies are warranted to confirm the findings.

In summary, the findings in this Study indicate that low-level EMFs can influence cancer-relevant biological processes such as apoptosis and cell cycle arrest. Co-exposure experiments have been found to be useful for studying biological effects of weak EMFs, and further studies of this type are warranted.

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