

Radiofrequency electromagnetic fields (UMTS, 1,950 MHz) induce genotoxic effects in vitro in human fibroblasts but not in lymphocytes

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Abstract

Objective Universal Mobile Telecommunication System (UMTS) was recently introduced as the third generation mobile communication standard in Europe. This was done without any information on biological effects and genotoxic properties of these particular high-frequency electromagnetic fields. This is disconcerting, because genotoxic effects of the second generation standard Global System for Mobile Communication have been reported after exposure of human cells in vitro.

Methods Human cultured fibroblasts of three different donors and three different short-term human lymphocyte cultures were exposed to 1,950 MHz UMTS below the specific absorption rate (SAR) safety limit of 2 W/kg. The alkaline comet assay and the micronucleus assay were used to ascertain dose and time-dependent genotoxic effects. Five hundred cells per slide were visually evaluated in the comet assay and comet tail factor (CTF) was calculated. In the micronucleus assay 1,000 binucleated cells were evaluated per assay. The origin of the micronuclei was determined by fluorescence labeled anticentromere

antibodies. All evaluations were performed under blinded conditions.

Results UMTS exposure increased the CTF and induced centromere-negative micronuclei (MN) in human cultured fibroblasts in a dose and time-dependent way. Incubation for 24 h at a SAR of 0.05 W/kg generated a statistically significant rise in both CTF and MN ($P = 0.02$). At a SAR of 0.1 W/kg the CTF was significantly increased after 8 h of incubation ($P = 0.02$), the number of MN after 12 h ($P = 0.02$). No UMTS effect was obtained with lymphocytes, either unstimulated or stimulated with Phytohemagglutinin.

Conclusion UMTS exposure may cause genetic alterations in some but not in all human cells in vitro.

Keywords Comet assay · Micronucleus assay · Genotoxic effect · Radiofrequency electromagnetic fields

Introduction

The use of radiofrequency (RF) signals generated by radio and television transmitters and mobile communication systems is constantly increasing all over the world, and the exposure of people to these signals is a topic of controversial discussion throughout the industrialized countries since epidemiological and animal studies led to conflicting results regarding possible adverse health effects; among them and most important, the development of cancer (Repacholi 1998; IEGMP 2000; Kundi et al. 2004; ICNIRP 2004; Krewski et al. 2007; IEGEMF 2007). In addition, most recent results from a still ongoing population-based case-control study (German part of the international Interphone Study) point to a slightly increased risk of glioma or meningioma in mobile phone users for more than 10

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years (Schütz et al. 2006). In another epidemiological study also carried out as part of the Interphone Study in Finland (five northern European countries) an increased odds ratio of borderline statistical significance for glioma was found for more than 10 years of mobile phone use reported on the side of the head where the tumour was located, (Lahkola et al. 2007). Another evaluation of the same study in Sweden points to an increase of acoustic neuromas found in people who had used a mobile phone for more than 10 years (Schoemaker et al. 2005). Moreover, a pooled analysis of two case–control studies by Hardell et al. (2006) yielded an increased risk of malignant brain tumours for a cumulative lifetime cellular and cordless mobile phone use of more than 2,000 h. A recent meta-analysis of two cohort studies and 11 case–control studies with data on 10 and more years' use of mobile phone gives a consistent pattern of an increased risk for acoustic neuroma and glioma (Hardell et al. 2007).

Since somatic cell mutations represent the initial step in the process of carcinogenesis (Ames et al. 1973), the presence of electromagnetic field (EMF)-induced genetic alterations become a pivotal question. Although according to the present majority opinion the weight of evidence is against a genotoxic action of EMF (Meltz 2003; Vijayalaxmi and Obe 2004) such effects are demonstrated yet in a number of studies in vitro (REFLEX Final Report 2004; Diem et al. 2005; Ivancsits et al. 2002a) and in vivo (Lai and Singh 1996, 2004, Paulray and Behari 2006). These effects are in line with experimental evidence that RF-EMF induces changes in gene expression (Leszczynski et al. 2002; Lee et al. 2005; Czyz et al. 2004; Pancini et al. 2002). However, the controversy is ongoing, since results of the REFLEX project on genotoxicity could not be confirmed in two recent studies (Speit et al. 2007; Whitehead et al. 2006).

It was the aim of the present study to extend our investigation with cultured human diploid fibroblasts and lymphocytes exposed to Global System for Mobile Communication (GSM) signals which we carried out as part of the REFLEX project (Ivancsits et al. 2002a; Diem et al. 2005) to the exposure of the same cell lines to Universal Mobile Telecommunication System (UMTS) radiation. UMTS was introduced in Europe about 5 years ago without any preceding investigation and exclusion of adverse effects to the health of exposed people.

Materials and methods

Cell culture

Human diploid fibroblasts were initiated from skin biopsies from three healthy donors (ES-1, males, 6 years old; IH-9,

females 29 years old; HW-2, males, 53 years old) and maintained in culture and stored deep frozen as previously described (Ivancsits et al. 2002a, 2005). Cells were seeded into 35-mm Petri dishes (Nunc, Roskilde, Denmark) at a density of 8×10^4 cells/3 ml 4 h prior to exposure. Lymphocyte short-term cultures were initiated according to standard techniques from venous blood of three healthy female donors (22, 32, and 33 years old).

Comet assay analysis

Alkaline comet assay was performed according to Östling and Johanson (1984) with minor modifications by Singh et al. (Singh et al. 1988, 1991; Singh and Lai 1998) as described previously (Ivancsits et al. 2002a, b). Clear microscopic slides were used and the third layer of low melting agarose was left out.

8×10^4 cells were seeded into 35-mm Petri dishes (Nunc) and allowed to get attached to the bottom of the flask for 4 h prior to UMTS exposure. RF-exposed and sham-exposed cells (10,000–30,000) were mixed with 100 µl low melting agarose (0.5%, 37°C) and this cell suspension was pipetted onto 1.5% normal melting agarose pre-coated slides, spread with a cover slip, and maintained on a cold flat tray for about 10 min to solidify. After removal of the cover slip 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 the cells were lysed for 90 min 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 (1 mM Na₂EDTA, 300 mM NaOH, pH > 13 to a level approximately 0.4 cm above the slides. The slides were left in the solution for 40 min to allow equilibration and unwinding of the DNA before electrophoresis. Electrophoresis conditions were 25 V, 300 mA, 4°C, 20 min, field strength: 0.8 V/cm. All steps were performed under dimmed light to prevent the occurrence of additional DNA damage. After electrophoresis the slides were washed three times with Tris buffer (0.4 M Tris, pH 7.5) to neutralize, then air-dried, and stored until analysis. Comets were visualized by ethidium bromide staining (20 µg/ml, 30 s) and examined at 400× magnification using a fluorescence microscope (Axiophot, Zeiss, Germany). Five hundred DNA spots/comets per slide were visually classified into five categories corresponding to the amount of DNA in the tail according to Anderson et al. (1994) with modifications as previously described (Diem et al. 2002; Ivancsits et al. 2003). All evaluations were performed under blinded conditions and by the same investigator (E.K.). Results were expressed as “comet tail

factors” (CTF) calculated in accordance with Diem et al. (2002) and Ivancsits et al. (2002a). The different classification groups were not weighted equally due to the fact that they do not represent equal grades of DNA damage. CTF is calculated according to the following formula:

$$\text{CTF}(\%) = \frac{AF_A + BF_B + CF_C + DF_D + EF_E}{500}$$

with A being the number of cells classified to group A, F_A the average of group A (2.5% of fragmented DNA), B being the number of cells classified to group B, F_B the average of group B (12.5% of fragmented DNA), C being the number of cells classified to group C, F_C the average of group C (30% of fragmented DNA), D being the number of cells classified to group D, F_D the average of group D (67.5% of fragmented DNA), E being the number of cells classified to group E, and F_E the average of group E (97.5% of fragmented DNA). Due to the scoring of 500 cells, being about ten times the cells usually processed by computer-aided image analysis, standard deviations become very low. The coefficient of variation of the CTF in unexposed fibroblasts was 4.2%, as estimated from triplicates of negative controls out of 20 experiments ($n = 60$).

Positive and negative controls were included in each set of experiments. Negative controls were cultured in the incubator as long as the corresponding exposure, slides of positive controls immersed in ice-cold phosphate-buffered saline were irradiated for 3 min using UV light (254 nm, 800 $\mu\text{W}/\text{cm}^2$).

Micronucleus assay

Micronucleus (MN) assay was performed according to Fenech (Fenech and Morley 1985; Fenech 1993). 8×10^4 cells were seeded into 35-mm Petri dishes (Nunc) and allowed to get attached to the bottom of the flask for 4 h prior to exposure. In order to block cytokinesis, Cytochalasin B (3 $\mu\text{g}/\text{ml}$; Sigma, St Louis, USA) was added 4 h before the first round of replication (this is 34 h after seeding) according to the protocol by M. Fenech (1993). A modified protocol was used for the time-dependent exposure when the cytochalasin B was added 52 hours after seeding of the cells. Termination of the cell culture was done 72 h after seeding. Subsequently, fibroblasts were treated with hypotonic KCl solution (0.075 M, 8 min) and fixed with a mixture of methanol:glacial acetic acid (1:1) added dropwise. Samples were stored at -20°C overnight. The fixation step was repeated subsequently and the suspension was dropped onto degreased slides. Slides were air-dried, stained with 4,6-diamidino-2-phenylindole (10 mg/l; Sigma, St Louis, USA) for 5 min, and washed in 0.9% NaCl solution. Micronuclei were examined at $400\times$ magnification using a fluorescence

microscope (Axiophot, Zeiss, Germany) and 1,000 binucleated cytokinesis-blocked cells were scored for MN according to criteria published by Lasne et al. (1984). All evaluations were performed under blinded conditions and by the same investigator (E.K.). The results were expressed as MN/500 BNC (binucleated cells) in order to allow a direct comparison to earlier results that have always been expressed as MN/500 BNC. Nuclear division index (NDI) was calculated according to Eastmond and Tucker (1989). Positive and negative controls were included in each set of experiments, both were maintained in the incubator for the same period, positive controls contained 25 μm Vincristin. Phytohemagglutinin (PHA) stimulated lymphocytes were exposed and analyzed essentially as described for fibroblasts

Micronucleus in situ hybridization assay

Micronucleus in situ hybridization was performed according to Farooqi et al. (1993) and to the protocol of the manufacturer of the “all human centromere probe” (Appligene Oncor, Illkirch, France). Air-dried slides were prepared as described and denatured in 70% Formamide/2 $\times$ SSC buffer, pH 7.0 at 72°C for 2 min. The “All human centromere probe” was denatured at 72°C for 10 min. Twenty microliters of denatured centromere probes were added to the slides. After overnight hybridization (37°C , humidified chamber) the slides were stained with 4,6-diamidino-2-phenylindole (10 mg/l; Sigma, St Louis, USA) for 1 min and evaluated microscopically as described. Between 1,300 and 2,100 binucleated cells/experiment were evaluated and the results were expressed as centromere-positive (c^+) MN/500 BNC.

Universal Mobile Telecommunication System

The third generation mobile system Universal Mobile Telecommunication System (UMTS) is a wideband code division multiple access system. The extremely low frequency (ELF) envelopes as emitted by UMTS handsets are dominated by the fast-transmit power control (TPC) with an update rate of 1,500 Hz, i.e., is a function of various environmental parameters. Ndoumbè Mbonjo Mbonjo et al. (2004) have proposed an UMTS test signal scheme with maximized short-term amplitude modulated (AM) variations in the MHz range. However, the ELF spectral power was not consequently maximized due to the small crest factor for their radio frequency (RF) envelope of only 2 dB. To overcome the shortcoming and to maximize the likelihood of biological responses we (N.K.) synthesized an UMTS test signal that maximizes both MHz and sub-kHz power variations.

Table 1 summarizes the settings for the proposed UMTS frequency division duplex mode test signal. The center frequency of the uplink of 1,950 MHz is used as the transmit carrier frequency. Six 960 kbit/s data channels are applied, because this configuration results in the strongest short-term AM variations. The channel data is represented as 32-kbit (8.5 ms) pseudo noise sequences. As the scrambling code has only a low effect on the AM variations, a long code was chosen because short codes will only be applied for multiuser detector base stations. The TPC waveform is composed of deep fades, compressed modes and a Physical Random Access Channel/Physical Common Packet Channel (PRACH/PCPCH) procedure. The TPC function has a periodicity of 1 s. Inner loop power controlled fades with a dynamic range of 24 dB are composed as zick/zac functions with a step size of 3 dB (maximum step size for open loop power control). The fades are arranged within a $1 - 1/2^n$ seconds scheme, $n = 0, \dots, 6$, leading to strong 1 Hz components (signal periodicity) but also to various higher components defined by the temporal

distances between the individual fades. Pulsed signal structures are integrated due to their sharp power gradients: A compressed mode of three slots occurs in every fade, and PRACH/PCPCH channels were simulated with a sequence consisting of two preambles together with an open loop power controlled two frame data segment. Spectral power is distributed over the entire ELF region and is composed as a sum of 1-Hz spectral components.

UMTS exposure setup

An exposure apparatus for uniform exposures of cell monolayers with bandwidths of larger than 5 MHz at 1,950 MHz was developed and provided by the ITIS-foundation (Foundation for Information Technologies in Society, Zurich, Switzerland; <http://www.itis.ethz.ch>). The setup was based on two short-circuited R18 rectangular waveguides, both of which were placed inside a commercial incubator (BBD 6220, Kendro, Vienna, Austria) to ensure constant environmental conditions for cultured cells (37°C, 5% CO₂, 95% humidity). Two sets with six 35-mm diameter Petri dishes each were exposed simultaneously per waveguide chamber (one set exposed and the other sham-exposed). For loading the setup, the shortcut could easily be removed and the cell dish holder could be placed inside positioning the Petri dishes in the H-field maxima ensured by the appropriate insets for the Petri-dish holders. Exposure was controlled by field sensors, temperature sensors for the air environment, and by an optimized air-flow system based on ventilators with a common inlet for both waveguides (Schuderer and Kuster 2003; Schonborn et al. 2001). The signal unit was designed for generation of the UMTS test signal. The system could be used for exposure of cell monolayers up to 17 W/kg with less than 26% non-uniformity of the specific absorption rate (SAR). The system allowed for exposure with a temperature load of the medium less than 0.03°C/(W/kg). The temperature difference between the waveguides did not exceed 0.1°C. For further technical details of the exposure apparatus and dosimetry see Schuderer (2005).

To enable blind experimentation, a computer randomly determined which of the two waveguides was exposed. This setting, which could neither be controlled nor ascertained by the experimenter, was stored in an encoded file and uncovered by the ITIS foundation in Zurich via e-mail in exchange with the transmission of results.

Human cultured fibroblasts: time and SAR dependent exposure

In general each set of experiments consisted of positive control, negative control, and exposure within each of the

Table 1 Specifications for the proposed UMTS test signal

<i>High frequency parameters</i>	
Transmit frequency	1,950 MHz
Signal bandwidth	5 MHz
<i>WCDMA parameters</i>	
Duplex mode	FDD
Chip rate	3.84 MHz
Number of data channels	6
Data channel bit rate	960 kbit /s
Data source	Pseudonoise, 2^{15} bit
Data channel spreading codes	$C_{4,0}$ – $C_{4,3}$
Data channel weighting factor	1
Control channel structure	Pilot, TFCI, FBI, TPC bits
TFCI, FBI, TPC	All bits 1
Control channel spreading code	$C_{256,0}$
Control channel weighting factor	1
Scrambling code	$C_{\text{long},64000}$
Pulse shaping filter	RRC, roll off 0.22
<i>Low frequency parameters</i>	
Signal periodicity	1 s
Inner loop power control	Step size 3 dB
Open loop power control	Step size 12 dB
Fading dynamic range	24 dB
Fading zigzag functions	Every $1 - 1/2^n$ s ($n = 0, \dots, 6$)
Envelope ratio peak/average	27.7
<i>Pulsed components</i>	
Compressed mode sequence	Three slots idle, every fade
PRACH/PCPCH sequence	Two preambles, two frames data part

two waveguides of the exposure setup (field exposed and sham-exposed dishes, since for each set of experiments it was unknown which waveguide was then assigned by the computer to carry the field). Three of those sets of experiments have been done independently for each individual data point, each including three dishes. This procedure was followed in an identical way for the both ascertainment methods, Comet assay and Micronucleus assay. These experiments have all been performed using UMTS 1,950 MHz continuous exposure. The diploid human fibroblast cell strain ES-1 has been used for all experiments concerning SAR dependence, time dependence, and the influence of intermittent exposure. The total number of independent exposure experiments here amounts to 93.

For the determination of SAR dependency the cells were exposed for 24 h to five different SAR levels: 0.05, 0.1, 0.5, 1.0, 2.0 W/kg. An MN evaluation using fluorescent anti-centromere antibodies was performed once at each SAR level.

For the determination of time dependency the cells were exposed at an SAR level of 0.1 W/kg for seven different exposure times: 4, 8, 12, 16, 20, 24, 48 h. An MN evaluation using fluorescent anti-centromere antibodies was performed once at each exposure time.

Exposure intermittency

Four different schemes of intermittent exposure have been tested: 5'on-10'off; 5'on-10'off; 10'on-10'off; 10'on-20'off with 16 h total duration. The CTF was used as an endpoint. Again, each sham-exposed and UMTS-exposed sample, and each negative and positive control were tested three times for each of the different intermittencies, and three slides have been prepared for each data point of each individual set of experiments (altogether 12 independent exposure experiments have been performed here).

Exposure of lymphocytes and fibroblasts from different donors

The UMTS effect was tested with three diploid human fibroblast cell strains from different donors and short-term lymphocyte cultures from three donors. The lymphocyte cultures were used either unstimulated (comet assay only) or stimulated with PHA according to standard techniques. We used the optimized exposure conditions obtained in the preceding experiments on time and SAR dependence and on the influence of intermittency; this means cells were exposed to 0.1 W/kg for a total of 16 h with an intermittency of 5'on-10'off. The comet assay was used with three fibroblast strains, three unstimulated lymphocyte cultures,

and three PHA-stimulated lymphocyte cultures. The micronucleus test was used with the three fibroblast strains and the PHA stimulated lymphocytes. Again each sham-exposed and UMTS-exposed sample, and each negative and positive control were tested three times for each of the cell cultures, and three slides have been prepared for each data point of each individual experiment. The total number of independent exposure experiments here amounts to 45.

Statistical analysis

Statistical analysis was performed with STATISTICA V. 5.0 package (Statsoft, Inc., Tulsa, USA). The statistical significance of CTF and MN differences between exposed cells and negative plus sham-exposed cells were calculated for each data point using the Mann–Whitney–Wilcoxon non-parametric test. A difference at $p < 0.05$ was considered statistically significant.

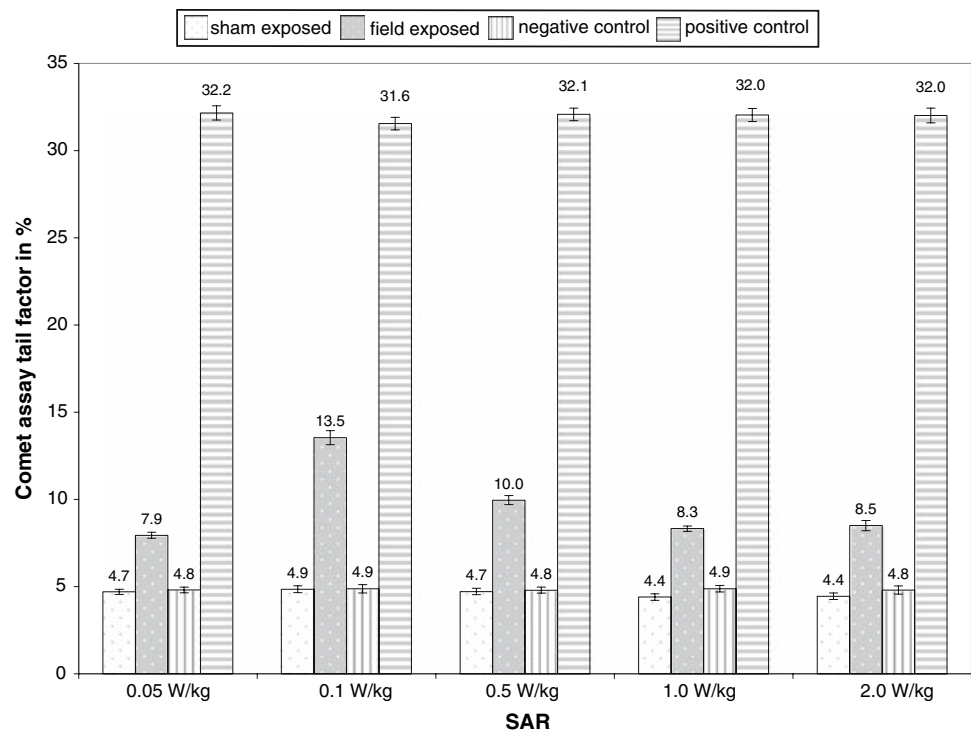
Results

Universal mobile telecommunication system exposure for 24 h induces an increase of the CTF in human cultured fibroblasts. There is a significant ($P = 0.02$) elevation of CTF already at SAR = 0.05 W/kg and the maximal effect was observed at 0.1 W/kg. Higher SAR did not result in a further increase of CTF (Fig. 1). The consistently decreased (but still significantly elevated, $P = 0.02$) UMTS effect at SAR levels ≥ 0.5 W/kg is difficult to explain and is confined to the comet assay results. The EMF-induced DNA damage in the comet assay was not caused by a preferential increase of D cells and E cells (Table 2) as it had been described with apoptotic cells (Olive and Banath 1995).

Using the formation of micronuclei as an endpoint we obtained a significant elevation of MN also at 0.05 W/kg ($P = 0.02$) and a maximal effect at 0.1 W/kg, the latter remaining constant at the same level up to 2.0 W/kg (Fig. 2). The analysis of micronuclei for the presence of centromeres by immunofluorescence microscopy revealed that the UMTS-induced elevation of MN is exclusively based on formation of acentric fragments and not on chromosomal non-disjunction. In contrast, the positive control, which was exposed to 25 μ m Vincristin contains almost exclusively centromere-positive MN as expected, because Vincristin is a mitotic poison and impairs the function of the spindle.

The time dependency was studied at SAR of 0.1 W/kg and revealed, that the CTF started to increase after 8 h of incubation ($P = 0.02$) with a maximal response after 20 h, and remained constant thereafter up to 48 h (Fig. 3).

Fig. 1 Specific absorption rate dependent increase of the comet assay tail factor after 24 h exposure. *Figures and bars* represent mean and standard deviation of three independent exposures/SAR level, using fibroblasts of donor ES-1. Negative, positive, and sham-exposed controls were performed for each exposure. The CTF increase was statistically significant ($P = 0.02$) for all SAR levels, calculated for each individual SAR using three EMF exposed versus the combined values of three sham-exposed plus three negative controls



Identical experiments using the formation of micronuclei as an endpoint revealed a significant elevation of MN after 12 h ($P = 0.02$), with a maximal elevation after 16 h,

which remained constant at this level thereafter (Fig. 4). Again the elevated MN do not represent entire chromosomes but acentric fragments, as shown with fluorescent

Table 2 Percentage of nuclei visually classified as A–E according to the proportion of DNA in the “tail” of comets (indicating degree of DNA fragmentation), after exposure of fibroblasts of donor ES-1 to various SAR levels as indicated in Fig. 1

Exposure conditions			A cells <5%	B cells 5–20%	C cells 20–40%	D cells 40–95%	E cells >95%	Comet tail factor (%)
$n = 3$	0.05 W/kg	Exposed	379.9 ± 2.03	81.7 ± 3.11	23.7 ± 2.18	5.2 ± 0.62	9.5 ± 0.68	7.9 ± 0.17
		Sham	440.2 ± 2.84	40.8 ± 2.76	15.0 ± 1.21	2.5 ± 0.90	1.5 ± 0.90	4.7 ± 0.15
		Positive cont.	11.7 ± 3.64	178.6 ± 3.86	227.3 ± 4.01	40.7 ± 3.88	44.2 ± 2.86	32.2 ± 0.41
		Negative cont.	449.1 ± 2.76	43.8 ± 2.46	15.6 ± 1.25	2.7 ± 0.78	1.8 ± 0.88	4.8 ± 0.16
$n = 3$	0.1 W/kg	Exposed	301.3 ± 6.34	128.3 ± 4.19	32.8 ± 3.02	7.9 ± 0.80	29.7 ± 2.08	13.5 ± 0.40
		Sham	436.9 ± 3.80	43.6 ± 3.03	15.4 ± 1.56	2.7 ± 1.29	1.4 ± 0.79	4.9 ± 0.19
		Positive cont.	12.2 ± 3.17	175.6 ± 3.75	224.1 ± 3.79	42.0 ± 3.45	44.2 ± 2.45	31.6 ± 0.36
		Negative cont.	441.4 ± 2.77	40.8 ± 2.78	16.0 ± 1.12	2.8 ± 0.98	2.0 ± 0.94	4.9 ± 0.24
$n = 3$	0.5 W/kg	Exposed	370.8 ± 2.31	72.3 ± 2.71	31.6 ± 1.82	9.2 ± 1.03	16.2 ± 1.12	10.0 ± 0.26
		Sham	439.6 ± 3.43	41.5 ± 2.93	15.5 ± 1.78	2.2 ± 0.75	1.2 ± 0.72	4.7 ± 0.18
		Positive cont.	11.5 ± 2.84	179.9 ± 3.23	225.9 ± 3.63	42.1 ± 2.89	44.3 ± 2.79	32.1 ± 0.36
		Negative cont.	448.7 ± 2.74	41.0 ± 2.73	16.1 ± 1.37	2.4 ± 0.82	1.9 ± 0.79	4.8 ± 0.17
$n = 3$	1.0 W/kg	Exposed	398.3 ± 5.19	46.8 ± 3.58	37.1 ± 3.99	9.3 ± 1.56	8.7 ± 0.71	8.3 ± 0.15
		Sham	444.3 ± 5.55	39.2 ± 4.51	12.9 ± 1.25	2.3 ± 0.78	1.2 ± 1.05	4.4 ± 0.19
		Positive cont.	11.2 ± 3.27	179.6 ± 3.45	228.1 ± 3.49	40.9 ± 3.39	43.9 ± 2.53	32.0 ± 0.37
		Negative cont.	450.1 ± 2.72	44.1 ± 2.49	16.0 ± 1.19	2.3 ± 0.81	2.1 ± 1.07	4.9 ± 0.19
$n = 3$	2.0 W/kg	Exposed	402.8 ± 4.02	44.0 ± 3.74	30.5 ± 1.45	14.8 ± 1.11	8.0 ± 1.04	8.5 ± 0.29
		Sham	453.2 ± 2.29	28.5 ± 1.51	14.8 ± 0.62	2.3 ± 0.62	1.3 ± 0.98	4.4 ± 0.19
		Positive cont.	11.8 ± 3.27	180.0 ± 3.89	227.2 ± 3.39	44.6 ± 3.51	44.0 ± 2.28	32.0 ± 0.42
		Negative cont.	441.1 ± 2.36	42.9 ± 2.37	16.1 ± 1.29	2.4 ± 0.79	2.0 ± 0.91	4.8 ± 0.23

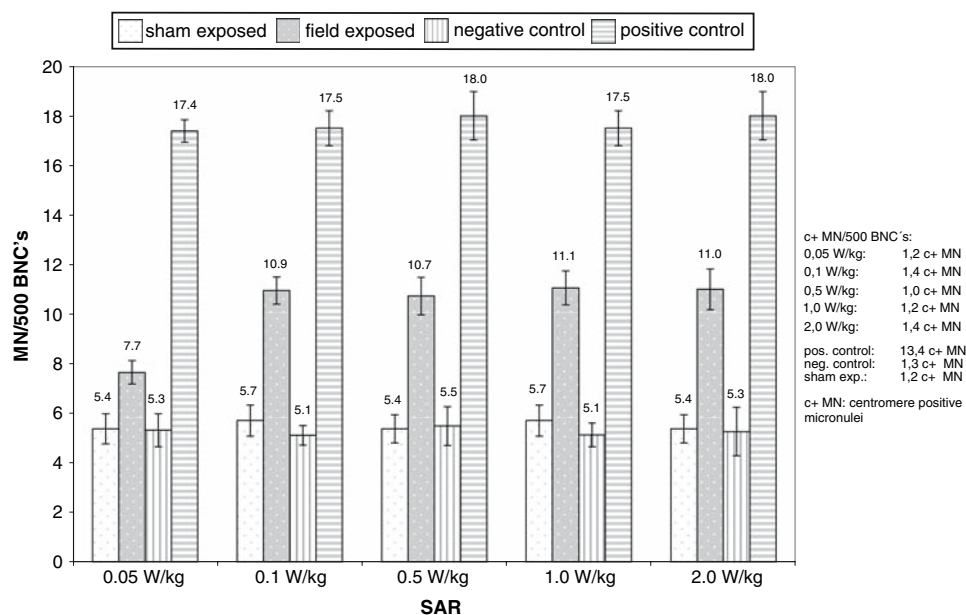


Fig. 2 Specific absorption rate dependent formation of micronuclei (MN) after 24 h exposure. *Figures and bars* represent mean and standard deviation of three independent exposures/SAR level, using fibroblasts of donor ES-1. Negative, positive, and sham-exposed controls were performed for each exposure. The MN increase was statistically significant ($P = 0.02$) at all exposure levels, calculated

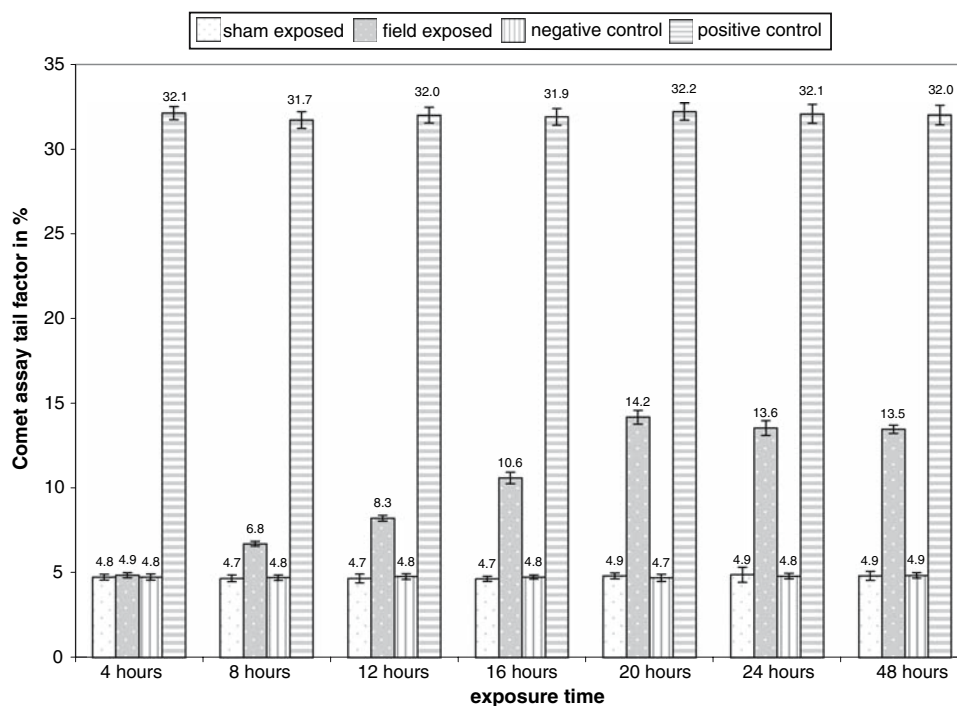
for each individual SAR using three EMF exposed versus the combined values of three sham-exposed plus three negative controls. The constant number of centromere-positive MN at all exposure levels including negative control and sham-exposed control indicates an MN formation by centromere negative chromosome fragments

centromere antibodies as described, just in contrast to the Vincristin-treated positive control.

Since 50-Hz ELF-EMF was reported to induce an increased CTF only when the exposure was performed

discontinuously with an intermittency of 5'on-10'off (Ivancsits et al. 2002a), we studied the influence of various intermittencies on the UMTS induction of CTF (Fig. 5). Intermittent exposure resulted in a mean CTF increase of

Fig. 3 Time-dependent increase of the comet assay tail factor at SAR = 0.1 W/kg, using fibroblasts of donor ES-1. The CTF increase became statistically significant after 8 h ($P = 0.02$) calculated for each individual time point using three EMF exposed versus the combined values of three sham-exposed plus three negative controls



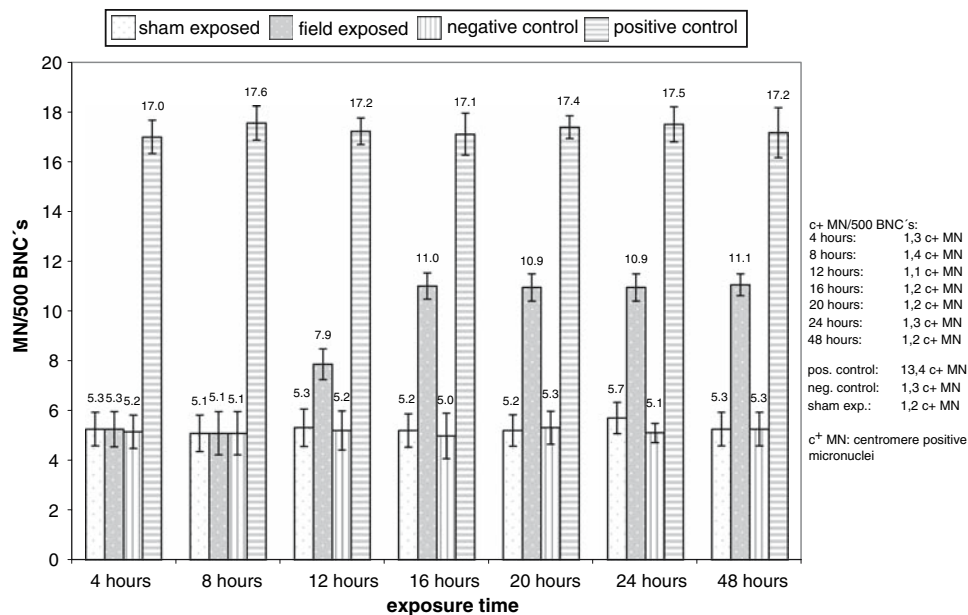


Fig. 4 Time-dependent formation of micronuclei (MN) at SAR = 0.1 W/kg, using fibroblasts of donor ES-1. Figures and bars represent mean and standard deviation of three independent exposures/time point. Negative, positive, and sham-exposed controls were performed for each exposure. The MN increase became statistically significant after 12 h ($P = 0.02$) calculated for each individual time

point using three EMF exposed versus the combined values of three sham-exposed plus three negative controls. The constant number of centromere-positive MN at all exposure time points including negative control and sham-exposed control indicates an MN formation by centromere-negative chromosome fragments

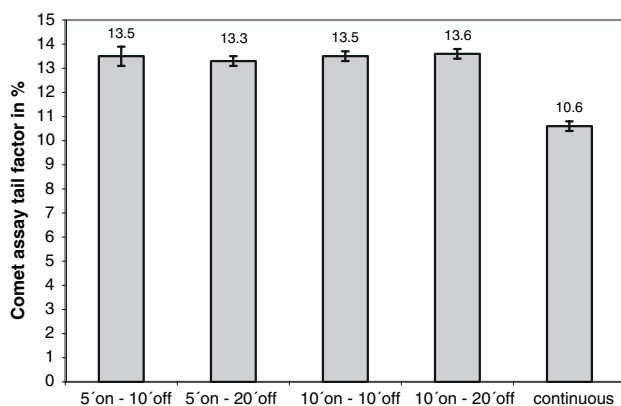


Fig. 5 Influence of various intermittency schemes on the comet assay tail factor as compared to continuous exposure (data taken from Fig. 3), using fibroblasts of donor ES-1. Figures and bars represent mean and standard deviation of three independent exposures. Sixteen hours of exposure and 0.1 W/kg was used in all cases

2.9 independently from the pattern of intermittency in a range between 5'on-10'off and 10'on-20'off.

Subsequently, an UMTS effect on three different fibroblast cultures and three different short-term lymphocyte cultures was examined under identical exposure conditions. In all cases an exposure time of 16 h, a SAR of 0.1 W/kg, and an intermittency of 5'on-10'off have been applied, according to the experimental conditions, which had shown

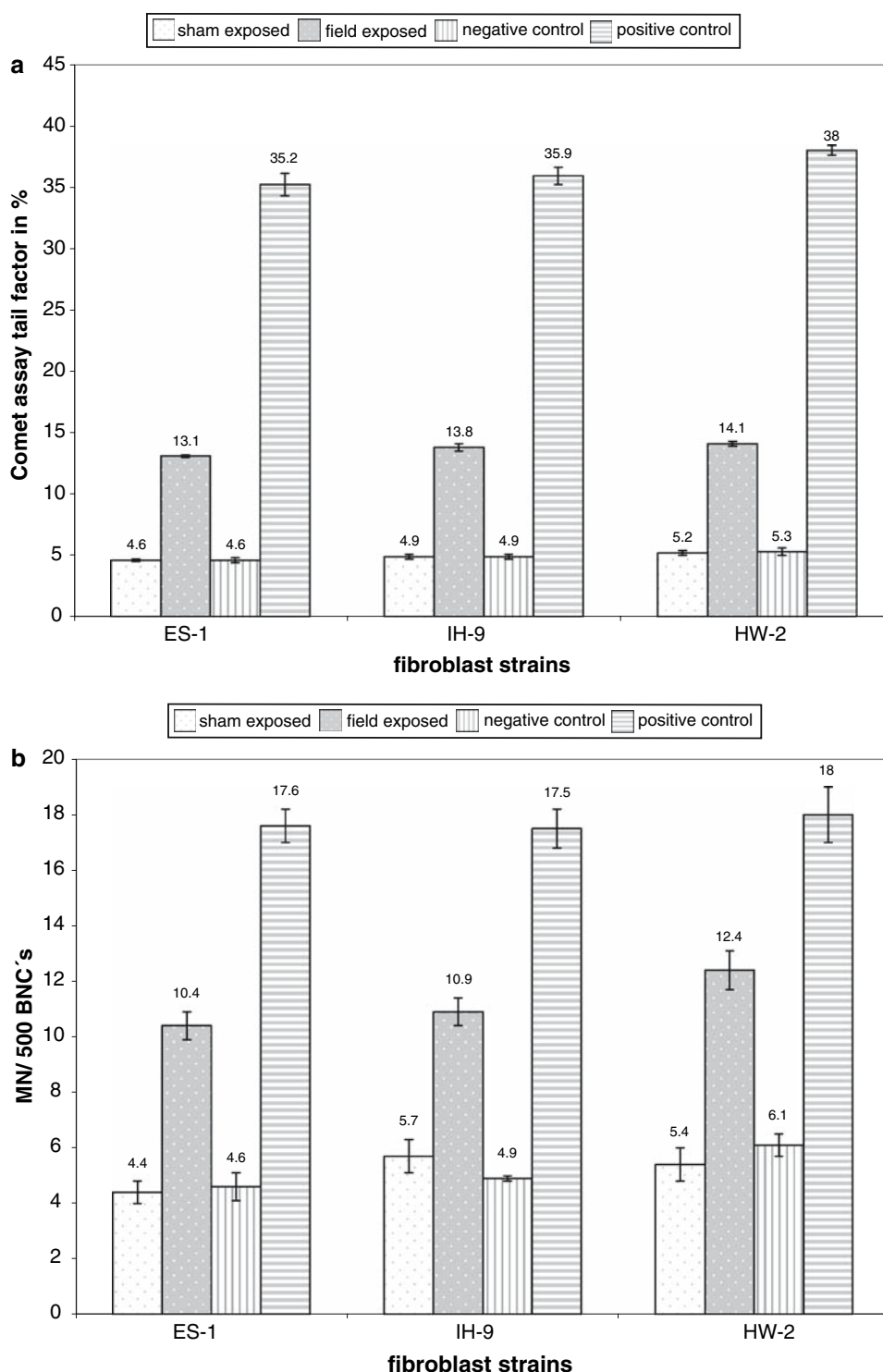
the largest effects in the precedent experiments. Both end-points comet and micronucleus formation were used with fibroblast cultures and PHA-stimulated lymphocyte cultures. The unstimulated lymphocytes were tested with the comet assay only. The three different fibroblast strains reacted similarly to UMTS exposure with both comet assay (Fig. 6a) and micronucleus test (Fig. 6b), the oldest donor (HW-2) being slightly more sensitive than the others. In contrast, the three lymphocyte cultures—PHA-stimulated (Fig. 7a, b) or unstimulated (Fig. 8)—failed to show any response to the UMTS exposure.

The NDI was determined with ES-1 fibroblasts using the exposure conditions 16 h, 0.1 W/kg, 5'on-10'off. NDI values (sham-exposed 1.99; field exposed 2.01; negative control 1.98; positive control 1.75) indicated that EMF exposure did not affect cell proliferation at least under standard conditions.

Discussion

The EMF-induced effects in the Comet assay are in agreement with earlier findings with human cultured fibroblasts using 1,800 MHz GSM exposure (Diem et al. 2005). However, the UMTS effects reported in this manuscript are already visible at considerable lower SAR

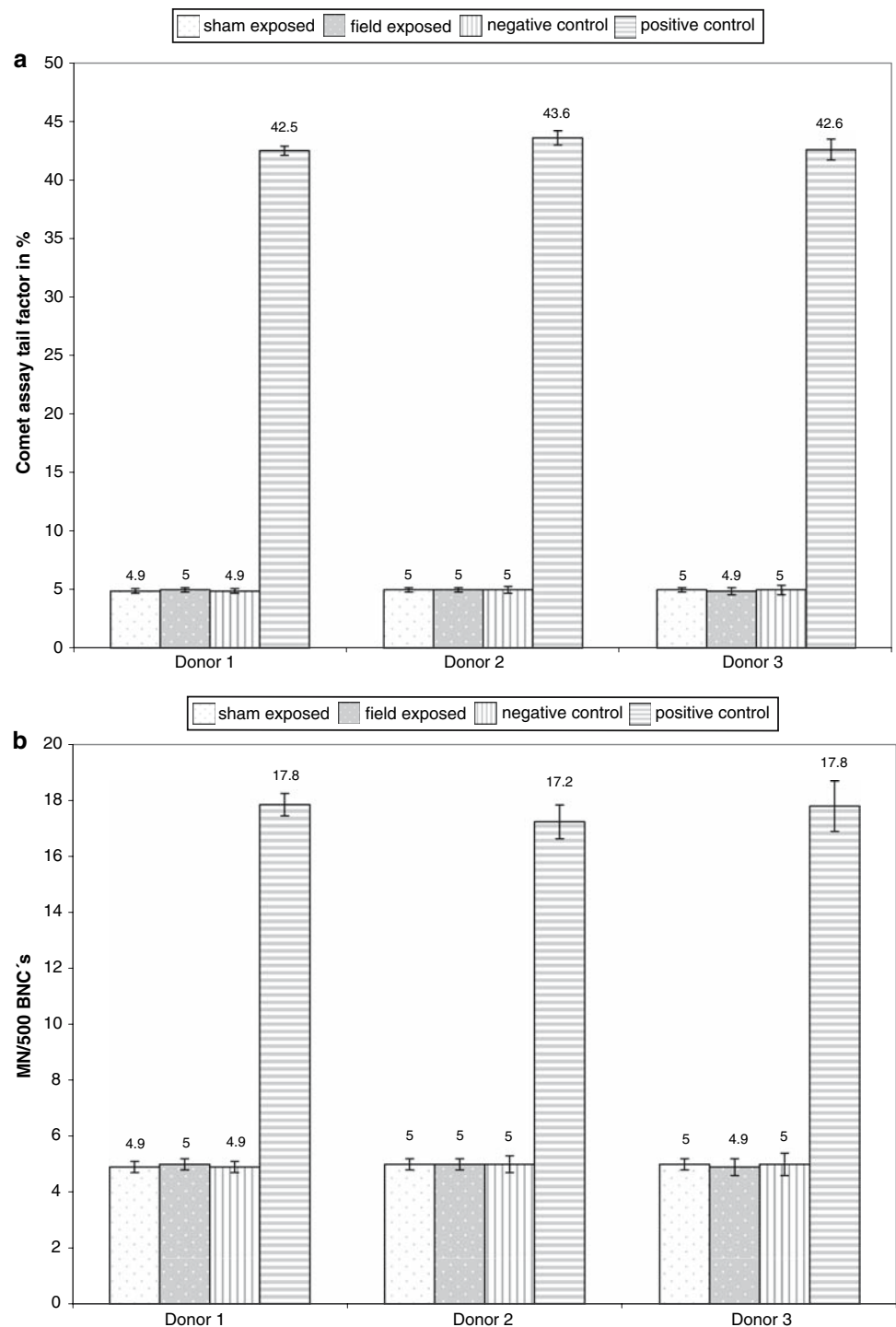
Fig. 6 a Radiofrequency electromagnetic field (RF-EMF)-induced increase of CTF in cultured fibroblasts from three donors. *Figures and bars* represent mean and standard deviation of three independent exposures. Sixteen hours of exposure and 0.1 W/kg was used in all cases, and negative, positive, and sham-exposed controls were included in each exposure. **b** RF-EMF-induced MN. Cells and exposure conditions identical as described for Fig. 6a. CTF and MN increases were each calculated for each fibroblast strain against the three negative plus three sham-exposed controls and were statistically significant for all strains ($P = 0.02$)



levels. In order to evaluate a genotoxic effect of UMTS exposure in vitro, two different test systems have been used which refer to different genetic endpoints: The comet assay measures fragmentation of nuclear DNA, which may occur either during various physiologic processes (as replication, repair, or apoptosis) or as a consequence of genotoxic events. The comet assay can be applied to non-dividing

cells. The micronucleus test detects errors in the distribution of chromosomal material during mitosis. This can be a whole chromosome after non-disjunction (due to a toxic influence on the spindle apparatus) or a chromosome fragment. The demonstration that the RF-EMF generated MN did not contain a centromere therefore points to chromosome fragments as the mode of origin and indicates

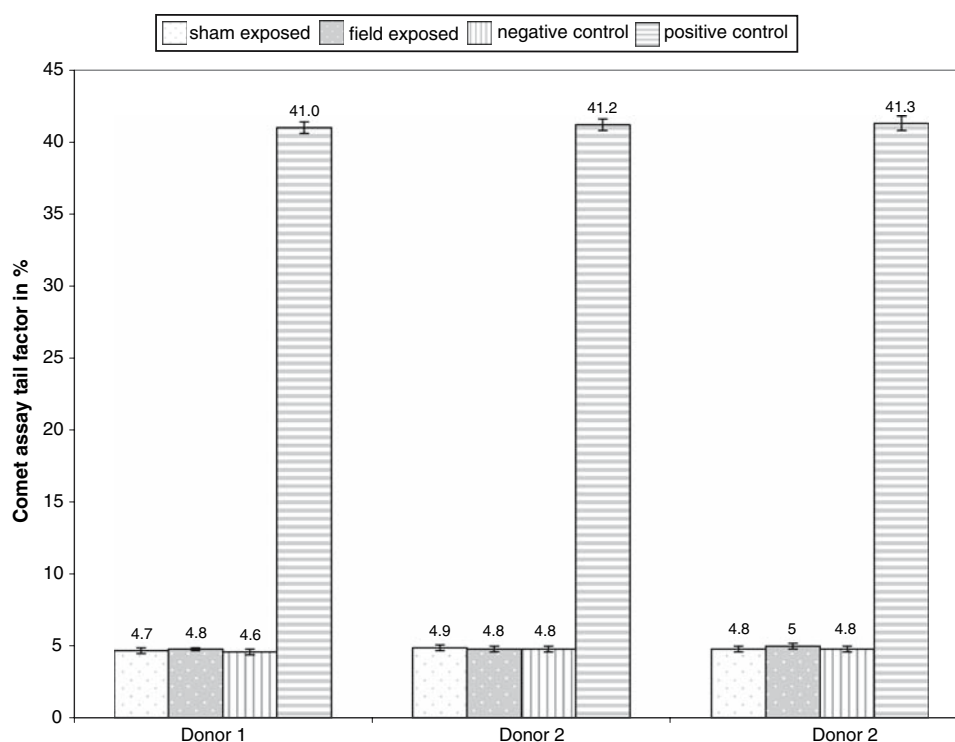
Fig. 7 Phytohemagglutinin stimulated human peripheral blood lymphocytes were unresponsive to RF-EMF with both endpoints after 16 h at 0.1 W/kg. **a** Comet tail factor. **b** Formation of micronuclei



a klastogenic effect. Formation of micronuclei represents always a serious genotoxic event that cannot be repaired. DNA fragmentation as assessed by comet assay, and chromosome fragmentation as assessed by micronucleus test do not necessarily go in parallel, but that they largely did so in our study can be considered as an additional strengthening of the conclusion that the UMTS exposure in our work may cause alterations of the genetic material.

Since a SAR of 0.05 W/kg, which consistently produced genotoxic effects in our in vitro study does not provide enough energy directly to break a chemical bond in DNA, we postulate an indirect mode of genotoxic action (as the generation or liberation of radicals or inactivation of repair processes). In addition, such an assumption would also provide an explanation of several puzzling observations: The time lag of 8–12 h from the beginning of exposure

Fig. 8 Radiofrequency electromagnetic field failed to increase CTF in unstimulated human peripheral blood lymphocytes after 16 h at 0.1 W/kg



until the increase of genotoxic indicators; the plateau effect after 16–20 h of exposure; and the plateau effect at SAR values >0.1 W/kg. Linearity with dose and time can only be expected for a direct and stochastic mode of action, whereas all indirect processes (being dependent on other factors as catalysis, receptors, speed of permeation, availability of a reactant etc.) usually follow a saturation pattern. Thermal factors are highly unlikely to explain the observed effects because temperature load of the culture medium is less than $0.03^{\circ}\text{C}/(\text{W/kg})$, and because of an increased effect during intermittent exposure.

Both genetic endpoints CTF and MN did not respond to UMTS exposure when human peripheral blood lymphocytes were used as a cell system. The same negative result was obtained with unstimulated and PHA-stimulated lymphocytes. This observation is in agreement with an earlier study on genotoxic effects of 50-Hz electromagnetic fields (ELF-EMF) using the alkaline and neutral comet assay as test systems (Ivancsits et al. 2005). In that study three responder (human fibroblasts, human melanocytes, and rat granulosa cells in culture) and three non-responder cell types (human lymphocytes, human monocytes, and human skeletal muscle cells in culture) could be identified. A recent study on ornithine decarboxylase activity after 872 MHz GSM exposure demonstrated a decreased activity in primary but not in secondary rat astrocytes (Höytö et al. 2007). This strongly points to the significance of the cell system used when investigating genotoxic effects of EMFs. The reason for this cell specificity is not known so

far. A clarification can possibly be expected from the elucidation of the (indirect?) mechanism, by which EMF exerts its genotoxic effects.

Although a genotoxicity of UMTS exposure is visible already at low SAR levels being within the range of emitted energy of a cell phone, the results must not be transferred forthwith to the in vivo situation. In particular, the time lag of 8–12 h from the beginning of in vitro exposure till the moment when the first effects became visible, and also the rapid reversibility of EMF effects as demonstrated with ELF-EMF (Ivancsits et al. 2002a, b) could mean, that an occasional use of a mobile phone may not pose a relevant genotoxic risk to humans.

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References

- Ames BN, Durston WE, Yamasaki E, Lee FD (1973) Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci* 70:2281–2285
- Anderson D, Yu T, Phillips B, Schmezer P (1994) The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the Comet assay. *Mutat Res* 307:261–271

- Czyz J, Guan K, Zeng Q, Nikolova T, Meister A, Schönborn F, Kuster N, Wobus A (2004) High frequency electromagnetic fields (GSM signals) affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells. *Bioelectromagnetics* 25:296–307
- Diem E, Ivancsits S, Rüdiger HW (2002) Basal levels of DNA strand breaks in human leukocytes determined by comet assay. *J Toxicol Environ Health A* 65:641–648
- Diem E, Schwarz C, Adlkofer F, Jahn O, Rüdiger HW (2005) Non-thermal DNA breakage by mobile phone radiation (1800 MHz) in human fibroblasts and transformed GFSH-R17 rat granulosa cells in vitro. *Mutat Res* 583:178–183
- Eastmond DA, Tucker JD (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ Mol Mutagen* 13:34–43
- Farooqi Z, Darroudi F, Natarajan AT (1993) Use of fluorescence in situ hybridization for the detection of aneuploids in cytokinesis-blocked mouse splenocytes. *Mutagenesis* 8:329–334
- Fenech M (1993) The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutat Res* 285:35–44
- Fenech M, Morley A (1985) Measurement of micronuclei in lymphocytes. *Mutat Res* 203:339–345
- Hardell LO, Carlberg M, Hansson Mild K (2006) Pooled analysis of two case-control studies on use of cellular and cordless telephones and the risk for malignant brain tumours diagnosed in 1997–2003. *Int Arch Occup Environ Health* 79:630–639
- Hardell LO, Carlberg M, Söderqvist F, Mild KH, Morgan LL (2007) Long-term use of cellular phones and brain tumours—increased risk associated with use >10 years. *Occup Environ Med* 64:626–632
- Höytö A, Juutilainen J, Naarala J (2007) Ornithine decarboxylase activity is affected in primary astrocytes but not in secondary cell lines exposed to 872 MHz RF radiation. *Int J Radiat Biol* 83:367–374
- ICNIRP (International Commission for Non-Ionizing Radiation Protection), Standing Committee on Epidemiology: Ahlbom A, Green A, Kheifets L, Savitz D, Swerdlow A (2004) Epidemiology of health effects of radiofrequency exposure. *Environ Health Perspect* 112:1741–1754
- IEGMP (2000) Mobile phones and health. Report of the independent expert group on mobile phones, Chairman: Sir William Stewart, National Radiation Protection Board, London
- IEGEMF (2007) Recent research on EMF and health risks. Fourth annual report from SSI's independent expert group on electromagnetic fields. Statens Stralskyddsinstitut, Stockholm
- Ivancsits S, Diem E, Rüdiger HW, Jahn O (2002a) Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. *Mutat Res* 519:1–13
- Ivancsits S, Pilger A, Diem E, Schaffer A, Rüdiger HW (2002b) Vanadate induces DNA strand breaks in cultured human fibroblasts at doses relevant to occupational exposure. *Mutat Res* 519:25–35
- 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 Environ Health* 76:431–436
- Ivancsits S, Pilger A, Diem E, Jahn O, Rüdiger HW (2005) Cell type specific genotoxic effects of intermittent extremely low frequency electromagnetic fields. *Mutat Res* 583:184–188
- Krewski D, Glickman BW, Habash RWY, Habbick B, Lotz WG, Mandeville R, Prato FS, Salem T, Weaver DF (2007) Recent advances in research on radiofrequency fields and health: 2001–2003. *J Toxicol Environ Health B* 10:287–318
- Kundi M, Mild K, Hardell LO, Mattsson MO (2004) Mobile telephones and cancer—a review of epidemiological evidence. *J Toxicol Environ Health B Crit Rev* 7:351–384
- Lai H, Singh N (1996) Single- and double strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. *Int J Radiat Biol* 69:513–521
- Lai H, Singh N (2004) Magnetic-field-induced DNA strand breaks in brain cells of the rat. *Environ Health Perspect* 112:687–694
- Lahkola A, Auvinen A, Raitanen J, Shoemaker MJ, Christensen HC, Feychting M, Johansen C, Klæboe L, Lönn S, Swerdlow AJ, Tynes T, Salminen T (2007) Mobile phone use and risk of glioma in 5 north European countries. *Int J Cancer* 120(8):1769–1775
- Lasne C, Gu Z, Venegas W, Chouroulinkov I (1984) The in vitro micronucleus assay for detection of cytogenetic effects induced by mutagen-carcinogens: comparison with the in vitro sister-chromatid exchange assay. *Mutat Res* 130:273–282
- Lee S, Johnson D, Dunbar K, Dong H, Ge X, Kim Y, Wing C, Jayatilaka N, Emmanuel N, Zhou C, Gerber H, Tseng C, Wang S (2005) 2.45 GHz radiofrequency fields alter gene expression in cultured human cells. *FEBS Lett* 579:4829–4836
- Leszczynski D, Joenväärä S, Reivinen J, Kuokka R (2002) Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: molecular mechanism for cancer- and blood-brain barrier-related effects. *Differentiation* 70:120–129
- Meltz M (2003) Radiofrequency exposure and mammalian cell toxicity, genotoxicity, and transformation. *Bioelectromagnetics* 24:196–213
- Ndombè Mbonjo H, Streckert J, Bitz A, Hansen V, Glasmachers A, Gencol S, Rozic D (2004) Generic UMTS test signal for RF bioelectromagnetic studies. *Bioelectromagnetics* 25:415–425
- Olive PL, Banath JP (1995) Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. *Exp Cell Res* 221:19–26
- Östling O, Johanson K (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Com* 123:291–298
- Pancini S, Ruggiero M, Sardi I, Aterini S, Gulisano F, Gulisano M (2002) Exposure to global system for mobile communication (GSM) cellular phone radiofrequency alters gene expression, proliferation, and morphology of human skin fibroblasts. *Oncol Res* 13:19–24
- Paulray R, Behari J (2006) Single strand DNA breaks in rat brain cells exposed to microwave radiation. *Mutat Res* 596:76–80
- REFLEX Final Report (2004) European Union Project QLK4-CT-1999-01574, Risk evaluation of potential environmental hazards from low frequency electromagnetic field exposure using sensitive in vitro methods. Final Report. <http://www.verum-foundation.de>
- Repacholi M (1998) Low-Level exposure to radiofrequency electromagnetic fields: health effects and research needs. *Bioelectromagnetics* 19:1–19
- Schoemaker MJ, Swerdlow AJ, Ahlbom A, Auvinen A, Blaasaas KG, Cardis E, Christensen HC, Feychting M, Hepworth SJ, Johansen C, Klæboe L, Lönn S, McKinney PA, Muir K, Raitanen J, Salminen T, Thomsen J, Tynes T (2005) Mobile phone use and risk of acoustic neuroma: results of the interphone case-control study in five North European countries. *Br J Cancer* 93:842–848
- Schönborn F, Pokovic K, Burkhard M, Kuster N (2001) Basis for optimization of in vitro exposure apparatus for health hazard evaluations of mobile communications. *Bioelectromagnetics* 22:547–559
- Schuderer J (2005) EMF risk assessment: “in vitro” research and sleep studies. In: Series in microelectronics. Hartung-Gorre Verlag, Konstanz

- Schuderer J, Kuster N (2003) Effect of the meniscus at the solid/liquid interface of the SAR distribution in Petri dishes and flasks. *Bioelectromagnetics* 24:103–108
- Schütz J, Böhler E, Berg G, Schlehofer B, Hettinger I, Schlaefer K, Wahrendorf J, Kunna-Grass K, Blettner M (2006) Cellular phones, cordless phones, and the risk of glioma and meningioma (Interphone Study Group, Germany) *Am J Epidemiol* 163(6):512–520
- Singh N, Lai H (1998) 60 Hz magnetic field exposure induces DNA crosslinks in rat brain cells. *Mutat Res* 400:313–320
- Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191
- Singh N, Tice R, Stephens R, Schneider E (1991) A microgel electrophoresis technique for direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides. *Mutat Res* 252:289–296
- Speit G, Schütz P, Hoffmann H (2007) Genotoxic effects of exposure to radiofrequency electromagnetic fields (RF-EMF) in cultured mammalian cells are not independently reproducible. *Mutat Res* 626:42–47
- Vijayalaxmi, Obe G (2004) Controversial cytogenetic observations in mammalian somatic cells exposed to radiofrequency radiation. *Radiat Res* 162:481–496
- Whitehead TD, Moros EG, Brownstein BH, Roto Roti JL (2006) Gene expression does not change significantly in C3H 10T(1/2) cells after exposure to 847.74 CDMA or 835.62 FDMA radiofrequency radiation. *Radiat Res* 165:626–635