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Assessment of genotoxicity and genomic instability in rat primary astrocytes exposed to 872 MHz radiofrequency radiation and chemicals

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Abstract

Purpose: We examined genotoxicity, co-genotoxicity and induced genomic instability (IGI) in primary astrocytes exposed to radiofrequency (RF) radiation.

Materials and methods: Rat primary astrocytes were exposed to 872 MHz GSM-modulated or continuous wave (CW) RF radiation at specific absorption rates of 0.6 or 6.0 W/kg for 24 h. Menadione (MQ) and methyl methanesulfonate (MMS; only in genotoxicity experiments) were used as co-exposures. Alkaline Comet assay and flow cytometric micronucleus scoring were used to detect genetic damage.

Results: No IGI was observed from RF radiation alone or combined treatment with MQ. RF radiation alone was not genotoxic. RF radiation combined with chemical exposure showed some statistically significant differences: increased DNA damage at 6.0 W/kg but decreased DNA damage at 0.6 W/kg in cells exposed to GSM-modulated RF radiation and MQ, and increased micronucleus frequency in cells exposed to CW RF radiation at 0.6 W/kg and MMS.

Conclusion: Exposure to GSM modulated RF radiation at levels up to 6.0 W/kg did not induce or enhance genomic instability in rat primary astrocytes. Lack of genotoxicity from RF radiation alone was convincingly shown in multiple experiments. Co-genotoxicity of RF radiation and genotoxic chemicals was not consistently supported by the results.
Introduction

A very large fraction of the world’s population are exposed to radiofrequency (RF) radiation due to the widespread use of mobile communication technologies. Numerous studies have been performed to investigate possible health risks of RF radiation, but there are still uncertainties in the health risk assessment, and further research is motivated by the high number of exposed subjects. In their meta-analyses of genetic damage in mammalian cells, Vijayalaxmi and Prihoda (2008, 2012) found that there were statistically significant increases in some genotoxicity endpoints in certain exposure conditions. However, effects were observed mainly in studies with small sample size, and evidence of publication bias was found in the meta-analyses. As the photon energy of RF radiation is too low to cause direct genotoxicity, it is important to assess not only genotoxicity from RF radiation alone, but also the possibility that RF radiation enhances the effects of genotoxic physical and chemical agents (Juutilainen et al. 2007; Luukkonen et al. 2009, 2010; Verschaeve et al. 2006, 2010; Vijayalaxmi and Prihoda 2008, 2012), IARC (2013) classified RF electromagnetic fields as possibly carcinogenic to humans (Group 2B). Concerning studies on genotoxicity the IARC Working Group noted that short-term in vitro studies gave consistently positive results for DNA damage at high exposure levels. The Working Group considered that these findings were likely due to thermal effects, and concluded that there is weak evidence that RF radiation is genotoxic, a conclusion supported by other review papers (Verschaeve et al. 2010).

No studies have so far assessed whether RF radiation can induce genomic instability, a relatively recently discovered phenomenon potentially important for assessment of cancer risk. Induced genomic instability (IGI) can be defined as de novo appearance of delayed damage (chromosomal aberrations, mutations, micronuclei and apoptosis) observed in the progeny of exposed cells many cell generations after exposure (Morgan et al. 1996; Baverstock 2000). As
development of cancer requires accumulation of genetic damage, IGI is obviously important in carcinogenesis (Streffer 2010). IGI can be assessed using genotoxicity assays, but it is distinct from direct genotoxicity and appears to be induced and transmitted epigenetically (Baverstock 2000; Huumonen et al. 2014a). Although IGI was originally found in cells exposed to ionizing radiation, also non-genotoxic agents induce genomic instability (Korkalainen et al. 2012). With regard to electromagnetic fields, IGI has been observed in neuroblastoma cells exposed to a 50 Hz, 100 µT magnetic field (Luukkonen et al. 2014; Kesari et al. 2015).

In this study, we performed experiments to investigate genotoxicity, co-genotoxicity and IGI in cultured astrocytes exposed to RF radiation. Genotoxic effects or IGI in astrocytes would be highly relevant for assessing plausibility of increased brain cancer risk among mobile phone users, as gliomas originating from astrocytes are the most common primary brain tumors in humans. Furthermore, there is previous evidence that primary astrocytes may be more sensitive to RF radiation than secondary cell lines (Höytö et al. 2007), and increased DNA damage has been reported in rat primary astrocytes exposed to amplitude-modulated 900 MHz RF radiation (Campisi et al. 2010). To address the suggested modulation-specific effects of RF electromagnetic fields (Juutilainen et al. 2011), both continuous-wave (CW) and modulated fields were used. A GSM-type modulation (pulsed at 217 pulses/s) was used, as the suggested modulation-specific effects - if there are any - are more likely to be associated with this kind of clearly pulsed signal (Juutilainen et al. 2011). Micronuclei and DNA strand breaks (by the Comet assay) were measured to assess genotoxicity, and the same assays were used to evaluate IGI 36 days after exposure. Co-genotoxicity was assessed by combining RF exposure with two chemicals that cause DNA damage by different mechanisms. Menadione (MQ) increases the intracellular production of reactive oxygen species (ROS) and has been used in our previous experiments (Luukkonen et al. 2009, 2014), whereas methyl methanesulfonate (MMS) is an alkylating agent.
Materials and methods

Cell culture

Rat primary astrocyte cell cultures derived from the brains of 1-3 days-old RccHan:WIST rats were used. We have previously used primary astrocytes to study biological effects of RF radiation, and the same preparation protocol was used in this study (Höytö et al. 2007). Cells were cultured in Dulbecco’s modified Eagle medium (containing 1.0 g/l glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50 mg/ml streptomycin. Cell cultures were maintained in cell culture flasks with a 75 cm² cell culture area (Nunc, Roskilde, Denmark) in a humidified incubator (Sanyo Electric co Ltd, Japan) with 5% CO₂ and a temperature of 37 ºC. Cells were detached by trypsinization (0.25% Trypsin in 0.02% EDTA in Ca²⁺- and Mg²⁺-free phosphate buffer saline (PBS)) and used for exposures at passage numbers from 4 to 14. Cells were plated on petri dishes (21.5 cm² Nunc, Denmark) 24 h prior to each exposure. The cell counts seeded per petri dish were 2.0x10⁵ for micronucleus assay and 2.5x10⁵ for Comet assay.

For the delayed time point, 2500 cells were seeded per Petri dish (21.5 cm² Nunc, Denmark) inside a glass cloning cylinder (volume 250 µl, SIGMA-ALDRICH, Inc. St. Louis, MO, USA). After a two-hour incubation, the cloning ring was removed and the cells were furthermore cultured 36 d after the end of the exposures. The cell culture medium was changed once a week. The cloning cylinder was used to avoid passages of cells during long incubation period as every passage causes stress to cells.

Exposure of the cells

Cells were exposed to 872 MHz RF radiation for 24 h at a SAR of 0.6 or 6.0 W/kg. Continuous wave (CW) or GSM-type modulation (pulsed at 217 pulses/s) were used. We used a waveguide
exposure system designed and built by the Radiation and Nuclear Safety Authority (STUK), Helsinki, Finland. A more detailed description of the exposure system and dosimetry has been published previously (Höytö et al. 2006).

Briefly, the exposure system contains two separate chambers, an aluminum exposure chamber with inner dimensions of 248 mm (wide) x 175mm (high) x 420 mm (length) and an identical sham exposure chamber. The chamber is a high Q waveguide resonator operating in the TE102 mode. The cell cultures on two Petri dishes are placed at the point of electric field maximum so that the electric field is perpendicular to the plane of the cell culture medium. The exposure chambers are equipped with a heat exchanger that uses water circulation to compensate for the energy absorbed from RF radiation, and keeps the cell cultures in isothermal conditions (37.0 ± 0.3 °C). The two Petri dishes are placed on the glass surface of the heat exchanger. The temperature of the incoming and outgoing water is continuously measured and adjusted by a temperature control unit. A temperature-controlled mixture of air and CO₂ (5% inside the chambers) is provided by ventilation from a modified cell culture incubator (Heraeus, HERAcell, Germany). Identical Petri dishes are always used and they are placed in a fixed position (within ±60 mm from the center line of the chamber, and center of the dishes 95 mm from the front wall of the chamber). With this sample placement, the exposure system provides a uniform specific absorption rate (SAR) distribution (±35% in the cell cultures). The input power was 0.83 W at SAR 0.6 W/kg and 8.33 W at SAR 6.0 W/kg. Continuous monitoring of the electric field in the chamber was done using a monitor post placed at the electric field maximum.

After the RF/sham exposure, cells were exposed to MQ (Sigma Chemical CO, St. Louis, MO, USA) or MMS (Sigma-Aldrich, USA/Germany). MQ exposure was conducted at 15 µM and MMS exposure at 40 µg/ml for three hours after the 24-h exposure to RF radiation. After the chemical exposure, cells were rinsed with PBS and fresh medium was changed or the cells were detached for the comet assay. To assess IGI cells were cultured 36 days after the exposures.
**Comet assay**

The alkaline Comet assay was used to measure DNA damage. Alkaline Comet Assay (pH > 13) detects DNA double-strand and single-strand breaks, single-strand breaks associated with incomplete excision repair sites, DNA-DNA/DNA-protein cross-links, and alkali labile sites.

Comet assay was performed as described previously (Luukkonen et al. 2011) except for pre-coating of the slides, which were stored in a freezer (-20 ºC) after pre-coating and taken to room temperature 24 h before using them in analysis. For DNA damage analysis, samples were placed on ice immediately after exposure, and the cells were detached from Petri dishes with 1.5 ml of 0.25% Trypsin in 0.02% EDTA in PBS. After detaching the cells, samples were suspended in PBS and 15 µl (approximately 1.5x10^3 cells) of the cell suspension was pipetted in 75 µl of 0.5% low melting point agarose. After careful mixing 80 µl of the suspension was layered onto a microscope slide (pre-coated with a thin layer of 1% normal melting point agarose), immediately covered with a coverslip and kept on ice for 5 min to solidify the agarose. The coverslips were removed carefully and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% sodium lauroyl sarcosinate, 1% Triton X-100, pH 10) and incubated for 1 h at +4 ºC in the dark. The slides were then placed in a horizontal electrophoresis unit (Gibco-BRL, Horizon 20x25, Gaithersburg, USA) for 25 minutes, allowing DNA to unwind in the electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH 13, +4ºC). The electrophoresis was run for 30 min at 24 V (0.66 V/cm) and 380 mA. After electrophoresis, slides were neutralized (3x5 min) with Tris buffer (0.4 M, pH 7.5) and fixed in 96% ethanol for 1 min.

For the analysis, slides were coded and stained with 20 mg/ml ethidium bromide. The analysis of 50 (if the number of nuclei was low) or 100 nuclei per slide was performed with a fluorescence microscope (Axio Imager.A1, Carl Zeiss, Göttingen, Germany) using the Comet assay
IV (Perceptive Instruments, Haverhill, UK) image analysis software. Olive tail moment (OTM; a measure of tail length × a measure of DNA in the tail) was used as the parameter of DNA damage.

**Micronucleus formation**

After exposures, cells were incubated further for 144 h to allow micronuclei to form during the subsequent cell divisions. To assess IGI cells were cultured 36 days after the exposures. The number of micronuclei and relative cell number was assessed using flow cytometry (Bryce et al. 2007). Cells were stained as described previously (Luukkonen et al. 2011). In brief, ethidium monoazide (EMA, 8.5 µg/ml, Invitrogen Corporation/Molecular Probes, Eugene, OR, USA) was used to stain nuclei from cells with compromised cell membrane (necrotic and apoptotic cells). The second staining with SYTOX Green (0.4 µM, Invitrogen Corporation/Molecular Probes, Eugene, OR, USA) after lysing the cells tinted all chromatin. Thus, micronuclei and nuclei from healthy cells were stained only with SYTOX green and we were able to distinguish them from the nuclei and pieces of nuclei from dying cells stained also with EMA. Fluorescent counting beads (Peak Flow, Green Flow cytometry reference beads, 6 µm; Invitrogen Corporation, Eugene, OR, USA) were added into all samples, which allowed calculation of relative cell number from nuclei to beads ratio. This was used to monitor cell survival; results from experiments with very low cell survival are not reliable, and they were rejected. The samples were analyzed using a flow cytometer (BD FACSCanto II™ flow cytometer, Becton Dickinson, San Jose, CA) equipped with BD FACSDiva software v.8.0.1 (Becton Dickinson, San Jose, CA) and a target was to acquire 15 000 gated events from each sample.
**Statistical analysis**

All experiments were repeated 3-8 times. The data were analysed using repeated measures ANOVA with Tukey’s post-test. The analyses were performed using GraphPad Prism v. 5.03 (GraphPad Software Inc., La Jolla, CA, USA) and differences were considered as statistically significant if p < 0.05.

**Results**

**Genotoxicity**

Measurements of immediate genotoxicity after exposure to RF radiation alone resulted in highly consistent results, showing no evidence of increased DNA damage (Table 1) or micronuclei (Table 2), independent of SAR level and modulation.

The combined treatments with chemicals and RF radiation included the 6.0 W/kg SAR level only in combination with MQ treatment, as finding an effect was considered to be most likely with this combination (because of previous results with MQ). When RF radiation was combined with chemicals, three statistically significant differences were observed between RF radiation-exposed and sham-exposed cells. Exposure to MQ and GSM-modulated RF radiation at 6.0 W/kg resulted in increased DNA damage level compared to cells exposed to MQ alone (Figure 1). However, a decreased DNA damage level was observed at 0.6 W/kg. The effects of the chemicals (MQ and MMS) were statistically significant in all experiments. In the micronucleus experiments, no increase of micronuclei was observed in cells exposed to the combination that produced a statistically significant increase in the Comet assay (MQ and GSM-modulated RF radiation at 6 W/kg) (Figure. 2). The only statistically significant increase in micronucleus frequency was observed in cells exposed to CW RF radiation at 0.6 W/kg. The micronucleus data from the experiment with CW RF
radiation at 6.0 W/kg combined with MQ had to be rejected, as the relative cell survival was less than 40%, which was our pre-determined criterion for an acceptable experiment.

**Genomic instability**

Measurements of DNA damage and micronuclei were done 36 days after exposure to assess IGI. No statistically significant differences in DNA damage levels were observed in these experiments (Figure 3). The level of micronuclei showed high variation between replicate experiments, and relative values (in relation to the mean of the control group of each experiment) are therefore presented in table 3. There were no exposure-related differences. The data from the experiment with MQ combined with RF radiation at 6.0 W/kg had to be rejected because of too low cell survival.

**Discussion**

The results of this study showed lack of genotoxicity from RF radiation alone, consistently in many independent experiments with rat primary astrocytes, a glial cell type relevant to the development of the most common type of malignant brain cancer. This finding was independent of SAR level and presence/absence of pulse modulation in the RF signal. Importantly, the higher SAR level (6.0 W/kg) was identical to the highest whole-body SAR level used in the recent *in vivo* study that reported suggestive evidence of increased risk of glioma in male rats exposed to mobile phone-type RF radiation (NTP 2016). Although literature reviews and meta-analyses have generally concluded that there is little evidence that RF radiation is genotoxic (Vijayalaxmi and Prihoda 2008, 2012) there is some remaining uncertainty, particularly at high exposure levels that may induce thermal effects unless temperature is carefully controlled (Verschaeve et al. 2010; IARC 2013). The present study used an exposure system that effectively controls the temperature of the cell cultures, and the results do not support effects of RF radiation on DNA damage or micronuclei at levels up to 6.0 W/kg. Also, the present results do not support the previous findings of increased DNA damage (measured by the Comet assay) in rat primary astrocytes exposed to 900 MHz radiation amplitude-
modulated at 50 Hz (Campisi et al. 2010). Campisi et al. exposed the cells for 20 min at a power density of 0.26 W/m$^2$ (external electric field 10 V/m), but did not measure or calculate a SAR value; this makes it difficult to compare the two studies and to exclude the possibility of thermal effects in the exposure conditions used by Campisi et al.

Although the experiments involving chemicals were designed to study possible co-genotoxicity, they can also be discussed from the point of view of adaptive response, a phenomenon in which pre-exposure to a low dose of a toxic agent reduces the damage caused by subsequent exposure to a larger dose of the same or another toxic agent. Adaptive response induced by pre-exposure to RF radiation has been reported in human lymphocytes exposed to toxic doses of mitomycin-C or ionizing radiation and in rodents exposed to ionizing radiation (Vijayalaxmi et al. 2014). No consistent protective effect of the pre-treatment with RF radiation was observed in the present study, so the results do not support induction of adaptive response in rat primary astrocytes exposed to RF radiation. However, the chemical exposures were started immediately after the RF field exposure, while a certain time interval between the two exposures may be needed for optimal detection of an adaptive response (Vijayalaxmi et al., 2014).

To our knowledge, this was the first study that evaluated induction of genomic instability by RF radiation. There was no indication that IGI could result either from RF radiation alone or from combined exposure to RF radiation and MQ. This is an important finding, given the apparently important role of IGI in cancer (Streffer 2010). Studying genomic instability with primary astrocytes proved to be very challenging. These cells divide slowly, which means that a long-duration study is needed to assess genomic instability (=delayed genetic changes) in the distant progeny of the exposed cells. Because of the long duration of the study (36 d after exposure), the primary cell cultures apparently became unstable, as the genotoxicity measures showed large differences between replicate experiments even in the unexposed control cultures. Instability of the experimental system itself may have affected its ability to detect IGI, and further experiments with
other cell types are needed, possibly with secondary cells like in our previous studies on genomic instability induced by other agents (Korkalainen et al. 2012; Huumonen et al. 2014a, 2014b; Luukkonen et al. 2014; Kesari et al. 2015). It might also be useful to test whether RF radiation enhances genomic instability induced by other agents (e.g. MMS, ionizing radiation), although previous experiments with ELF magnetic fields showed that co-exposure was not needed for IGI (Luukkonen et al. 2014, Kesari et al. 2015).

Three statistically significant differences were found in experiments combining RF radiation exposure with subsequent exposure to MQ or MMS. Eleven combinations of RF radiation and chemicals were tested, so the observed rate of positive findings (3/11) exceeds the expected rate of false positive findings (on the average, 1/20 experiments will produce a p-value <0.05 just by chance). However, the positive findings were not internally consistent, which makes interpretation of the results difficult. In the Comet assay experiments, the statistically significant differences were seen when GSM-type modulation was combined with MQ, but the direction of the change was not consistent (increased DNA damage at 6.0 W/kg; decreased at 0.6 W/kg). The frequency of micronuclei was statistically increased when CW RF exposure (0.6 W/kg) was combined with MMS. It seems highly unlikely that the GSM-modulated signal would act only in combination with MQ, and the CW signal only in combination with MMS. Based on these considerations and data shown in Figures 1 and 2, the most likely interpretations are that either 1) all positive findings are due to chance or 2) the increased DNA damage in cells exposed to MQ and the GSM signal at 6.0 W/kg represents a true effect of high-SAR modulated RF field, and the two other statistically significant differences are chance findings. In our previous study, exposure to 872 MHz RF radiation at 1.5 or 6.0 W/kg was found to suppress ornithine decarboxylase (ODC) activity in rat primary astrocytes (Höytö et al. 2007). This finding was consistent in several independent experiments and indicates existence of a biological effect that could, in principle, lead to enhancement of MQ-induced oxidative damage in cells exposed to RF radiation due to decreased
levels of polyamines and their antioxidant activity (Höytö et al. 2007; Ha et al. 1998). However, the results of the present study are not consistent with the ODC study with regard to the effects of modulation: in the present study, only the GSM-modulated increased MQ-induced DNA damage, while ODC activity was identically affected by CW and GSM-modulated RF radiation (Höytö et al. 2007).

**Conclusions**

Exposure to CW or GSM-modulated 872 MHz RF radiation alone for 24 h at SAR levels up to 6.0 W/kg did not cause DNA damage or induce micronuclei in rat primary astrocyte. Evidence for combined effects with genotoxic chemicals was weak. No evidence was found of induction of genomic instability from RF radiation alone or in combination with chemical exposure.

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**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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References


Table 1. DNA damage expressed as olive tail moment (±SEM) in rat primary astrocytes exposed for 24 h to 872 MHz RF radiation at specific absorption rates (SAR) of 0.6 or 6.0 W/kg. GSM-type modulation (pulsed at 217 pulses/s) or continuous wave (CW) RF radiation was used, and the control cells were sham-exposed. n=3-8.

<table>
<thead>
<tr>
<th>SAR, W/kg</th>
<th>Sham</th>
<th>CW</th>
<th>Sham</th>
<th>GSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>1.56±0.13</td>
<td>1.45±0.10</td>
<td>1.28±0.09</td>
<td>1.04±0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.12±0.02</td>
<td>0.11±0.01</td>
<td>0.85±0.16</td>
<td>0.92±0.19</td>
</tr>
</tbody>
</table>

Table 2. Frequency of micronuclei (% ±SEM in rat primary astrocytes exposed for 24 h to 872 MHz RF radiation at specific absorption rates (SAR) of 0.6 or 6.0 W/kg. GSM-type modulation (pulsed at 217 pulses/s) or continuous wave (CW) RF radiation was used, and the control cells were sham-exposed. n=3-6.

<table>
<thead>
<tr>
<th>SAR, W/kg</th>
<th>Sham</th>
<th>CW</th>
<th>Sham</th>
<th>GSM</th>
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</thead>
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<tr>
<td>0.6</td>
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<td>0.55±0.09</td>
<td>0.58±0.11</td>
<td>0.62±0.12</td>
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<td>6</td>
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<td>0.63±0.06</td>
<td>0.51±0.08</td>
<td>0.52±0.12</td>
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</tbody>
</table>

Table 3. Induction of genomic instability: Relative (in relation to the mean of the control group of each experiment) frequency of micronuclei (% ±SEM) after 36 days incubation in rat primary astrocytes exposed for 24 h to 872 MHz RF radiation at specific absorption rates (SAR) of 0.6 or 6.0 W/kg followed by a 3-h exposure to menadione (15 µM). GSM-type modulation (pulsed at 217 pulses/s) was used. The control group was sham-exposed and thereafter incubated for 3 h without chemical exposure. n=3.

<table>
<thead>
<tr>
<th>SAR, W/kg</th>
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<th>MQ + RF</th>
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</tbody>
</table>