Absence of genotoxic potential of 902-MHz (GSM) and 1747-MHz (DCS) wireless communication signals: \textit{In vivo} two-year bioassay in B6C3F1 mice

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\textbf{Running title}: \textit{In vivo} genotoxicity of GSM and DCS signals in mice

\textbf{Key words}: Radiofrequency radiation, mobile phones, B6C3F1 mice, genotoxicity, peripheral blood, micronuclei.

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Abstract

Purpose: The aim of the present investigation was to determine the incidence of micronuclei in peripheral blood erythrocytes of B6C3F1 mice that had been chronically exposed to radiofrequencies (RF) used for mobile communication. Methods: ‘Ferris wheels’ were used to expose tube-restrained male and female mice to simulated environmental RF signals of the Global System for Mobile Communications (GSM, 902-MHz) or Digital Cellular System (DCS, 1747-MHz). RF signals were applied to the mice for 2 hours/day on 5 days/week for 2 years, at maximal whole-body-averaged specific absorption rates of 0.4, 1.3, and 4.0 W/kg body weight. Concurrent sham-exposed mice, cage controls, and positive controls injected with mitomycin C were included in this investigation. At necropsy, peripheral blood smears were prepared, and coded slides were stained using May-Grünwald-Giemsa or acridine orange. The incidence of micronuclei was recorded for each mouse in 2000 polychromatic and 2000 normochromatic erythrocytes.

Results and conclusions: There were no significant differences in the frequency of micronuclei between RF-exposed, sham-exposed, and cage control mice, irrespective of the staining/counting method used. Micronuclei were, however, significantly increased in polychromatic erythrocytes of the positive control mice. In conclusion, the data did not indicate RF-induced genotoxicity in mice after 2 years of exposure.
**Introduction**

Non-ionizing radiofrequency (RF) radiation in the frequency range used for wireless communication systems has a tremendous impact in modern society. The escalated use and the consequent exposure to RF resulted in increased concern regarding its potential adverse effects on human health, thus prompting concerted effort to investigate the issues related to RF-exposure. Some research priorities were identified: (1) additional large-scale animal studies to test the effects of long-term exposure to RF, (2) studies that examine effects on health other than cancer, such as memory loss and effects on the eye or inner ear, and (3) large-scale epidemiological studies in people exposed to RF [U.S. Government Accountability Office (GAO), 2001; Valberg et al., 2007; Jauchem 2008]. The above research needs have been addressed in a number of studies in several countries, some of which have been already completed, are ongoing or planned.

The European Commission through its fifth framework program, the Swiss and Austrian governments, the Global System for Mobile Communications (GSM) Association, and the Mobile Manufacturers’ Forum have been supporting research projects addressing human health-related issues of exposure to RF emitted from wireless communication systems. Among these projects, the program with the acronym PERFORM-A [EC Contract N° QLK4-CT-1999-01476 entitled: "In vivo Research on Possible Health Effects Related to Mobile Telephones and Base Stations (Carcinogenicity Studies in Rodents)"] addressed the potential carcinogenic effects of long-term exposure to wireless mobile communication signals in experimental animals. The PERFORM-A1 study, in particular, focused on the carcinogenic potential of RF exposure in male and female B6C3F1 mice, which were exposed to 902-MHz (GSM) or 1747-MHz (Digital Cellular System, DCS) RF for 2 hours/day on 5 days/week over a period of 2 years. Complete
histopathological examination was subsequently conducted to determine the incidence and severity of neoplastic/non-neoplastic lesions. Detailed data have been published previously (Tillmann et al. 2007).

Up to now, very few investigators have examined the potential genotoxic effects of chronic exposure to RF. Observations related to genotoxicity following RF exposure are considered important, since enhanced genetic damage is very often linked to carcinogenicity. Hence, the present genotoxicity study was appended to the PERFORM-A1 carcinogenicity study in mice. This combination offered the possibility to evaluate the extent of genetic damage following chronic exposure to RF 902 MHz (GSM) and 1747 MHz (DCS) and to correlate it with carcinogenicity. In the present investigation, the rodent micronucleus (MN) assay was used to determine the genotoxic potential of RF exposure, a standard in vivo genotoxicity test used for regulatory purposes in several countries (Auletta et al. 1993; Health Protection Branch Genotoxicity Committee, Canada 1993; Kirkland 1993; Sofuni 1993). Since MN arise from broken chromosomal fragments and whole chromosomes that are not incorporated into daughter cells at the time of cell division (due to disturbances in the spindle apparatus), the MN test can identify both clastogenic and aneugenic agents. Furthermore, it has been suggested that long-term studies using peripheral blood may evaluate MN in both, or either, normochromatic (NCE, mature) or polychromatic erythrocytes (PCE, immature), in contrast to the short-term bone marrow MN tests, where scoring is limited to PCE. The incidence of micronucleated PCE provides an index of damage induced within 72 h of sampling, whereas the incidence of MN in NCE at steady state provides an index of average damage during the 30-day period preceding sampling (Witt et al. 2000). Although RF and sham exposures were conducted over a period of 2 years, the incidence of MN was evaluated in NCE as well as in PCE in order to detect more acute, chronic, and delayed effects of RF exposure.
Methods

Study design and guidelines

The present investigation was performed as an add-on to the PERFORM-A1 mouse carcinogenicity study reported by Tillmann et al. (2007). The animal experiment was conducted at the Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM), Hannover, Germany. The protocol complied with the German Animal Welfare Act and was approved by the responsible local authority. The study considered guideline No. 453 of the Organization for Economic Co-Operation and Development (OECD) and was performed in compliance with the principles of Good Laboratory Practice (GLP, German Chemicals Law, § 19a, Appendix 1, June 28, 2002). The entire study was conducted ‘blind’ to all scientists involved. The staff of the Foundation for Research on Information Technologies in Society (IT’IS), Zurich, Switzerland, responsible for the technical aspects, for controlling and monitoring the RF exposures, were also not aware of the identity of the exposure groups. All data were ‘decoded’ after completion of the histopathological examinations in the PERFORM-A1 carcinogenicity study and the MN evaluations in the present add-on investigation. The incidence of MN was analyzed by independent investigators in separate laboratories, viz. the Fraunhofer ITEM and the University of Texas Health Science Center, San Antonio, USA (UTHSCSA).

Animal housing and maintenance

Young adult, specified pathogen-free B6C3F1/Crl BR male and female mice, 4 to 5 weeks of age, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in two separate rooms, one for 902-MHz (GSM) and another for 1747-MHz (DCS) experiments. The temperature in both rooms was maintained at 22 ± 2 °C with a relative humidity of 30-70%
and an airflow rate of 12-15 exchanges/hour. A time-controlled system provided 12-hour light and dark cycles. Mice were maintained in Makrolon® polycarbonate type II cages (22 x 16 x 14 cm, EBECO, Castrop-Rauxel, Germany) with absorbent softwood bedding throughout the study, except the daily RF-exposure period. Male mice were caged individually, while the females were housed two per cage. Except during exposure, all animals had free access to standard diet (Altromin 1324N, supplied by Altromin International, Lage, Germany) and drinking water from the Hannover city water supplier (Hannover, Germany).

Experimental design

For details of experimental design, exposure conditions, and exposure monitoring, see Tillmann et al. (2007). A total of 1170 mice (585 males and 585 females) were randomized by weight into groups using computer-generated numbers. Exposure group identities are given in Table I. Each group initially consisted of 50 + 15 males and 50 + 15 females. Fifty animals of each sex were used for the 2-year exposure study, while the other 15 animals per group were used for interim examinations (organ weights, hematology, gross pathology, and histopathology, but not MN induction) after a 12-month exposure period. The number of male and female mice per group was derived from the guidelines/bioassays that have been successfully utilized for decades in the testing of products in the chemical and pharmaceutical industries (National Toxicology Program, OECD, Environmental Protection Agency). Additionally, 30 males and 30 females were assigned as sentinel animals. All mice were acclimatized to the animal room conditions for about 4 weeks. A training program was initiated during this period to accustom the mice to the RF-exposure setup by gradually increasing the time during which the animals were restrained in tubes (similar to those regularly used for inhalation studies, see Figure 1).
Table I. Exposure groups, dose levels, and numbers of mice evaluated for micronucleus induction after 2 years of RF exposure.

<table>
<thead>
<tr>
<th>Exposure level</th>
<th>Sex</th>
<th>Frequency</th>
<th>Restraint duration (daily, 5 days/week)</th>
<th>Max. wb-SAR [W/kg]</th>
<th>Number of animals$^s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>m</td>
<td>Cage control</td>
<td>---</td>
<td>---</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>m</td>
<td>902 MHz</td>
<td>2 h</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>m</td>
<td>902 MHz</td>
<td>2 h</td>
<td>0.4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>m</td>
<td>902 MHz</td>
<td>2 h</td>
<td>1.3</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>m</td>
<td>902 MHz</td>
<td>2 h</td>
<td>4.0</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>m</td>
<td>1747 MHz</td>
<td>2 h</td>
<td>0.0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>m</td>
<td>1747 MHz</td>
<td>2 h</td>
<td>0.4</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>m</td>
<td>1747 MHz</td>
<td>2 h</td>
<td>1.3</td>
<td>44</td>
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<tr>
<td></td>
<td>f</td>
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</tr>
<tr>
<td>High</td>
<td>m</td>
<td>1747 MHz</td>
<td>2 h</td>
<td>4.0</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of animals ± SD: 39 ± 3.5

$^#$, decoded exposure levels after completion of the study; $^s$, evaluation of micronuclei was performed on survivors only, after 2 years of exposure; m, male; f, female; wb-SAR, whole-body specific absorption rate; SD, standard deviation.
**RF exposure, 902 MHz (GSM) and 1747 MHz (DCS)**

The exposure signal and system were described earlier in detail (Tillmann et al., 2007). In brief, mice restrained in tubes (i.e. all animals except cage controls and sentinel) were sham-exposed or exposed to RF for 2 hours/day on 5 days/week over a period of 2 years. The RF signals simulated exposure from GSM (902 MHz) and DCS (1747 MHz) handsets. The exposure units were supplied by IT’IS with assurances for RF transmission, dosimetry, and continuous monitoring. The main equipment consisted of “Ferris wheels” (see Figure 1), signal generator (Rhode & Schwarz, Munich, Germany), amplifiers (LS Electronic, Spanga, Sweden), and electronic control and monitoring devices (SPEAG, Zurich, Switzerland). The “Ferris wheel” concept was developed by Balzano et al. (2000) and adopted and optimized by IT’IS for uniform whole-body exposure of mice. Briefly, the “Ferris wheels” consisted of two parallel, circular, stainless steel metal plates, which were placed 117 mm apart with a conical (GSM) or bi-conical (DCS) antenna in their center and stainless steel posts forming a cylindrical cavity of 755 mm radius. A “Ferris wheel” could house up to 65 mice. The position of the animals was optimized for maximum uniform exposure by using a radius (center of wheel to center of the tubes) of 700 mm for GSM exposure and of 670 mm for DCS exposure. In order to maintain a symmetrical load, missing animals were replaced by conical plastic tubes filled with 36 ml of liquid simulating the dielectrical properties of muscle tissue in mice at the corresponding RF frequencies.
Figure 1. Mouse exposure setup. Presented is one of the “Ferris wheels” developed by IT’IS and a restraint tube with a dielectric stopper.

All applied signals were compliant with the definitions of the GSM or DCS signaling standards and were designed to simulate all exposure conditions (low-frequency power envelope) as they occur during the use of GSM/DCS mobile phones at maximized time-averaged exposure. Each exposure session (duration 2 h) was divided into three phases of 40 min each. Each slot was modulated with a random code. In the first phase non-discontinuous transmission (DTX) mode (“GSM Basic”) was applied simulating the exposure conditions during continuous talking, i.e.,
one active slot per basic frame while each 26th basic frame was idle. The second phase, “GSM Talk”, simulated a conversation, i.e., by temporal switching between the non-DTX (average time active: 2/3) and DTX (average time active: 1/3) modes. The third phase, “GSM Environment”, simulated exposure during a conversation. This included GSM features such as non-DTX, DTX, power control, handovers, etc. according to their statistical occurrence. The target whole-body-averaged SAR during "GSM Basic" for the "high exposure" group was 4 W/kg body weight. Since the maximum slot average power was kept constant, the exposure during “GSM Talk” was 2.7 W/kg and during “GSM Environment” 1.1 W/kg body weight, respectively. All exposure levels were reduced by a factor of 3 for the "medium exposure" group and a factor of 9 for the "low exposure" group. The rational, the signal and the monitoring techniques are described in detail previously (Kainz et al., 2006).

For each RF frequency, four “Ferris wheel” exposure units were used, allowing to simultaneously expose the three power levels and shame. The thermal threshold and breakdown levels revealed that the high-dose level was close to, yet below, the thermal threshold (Ebert et al. 2005). The spatial peak and organ-averaged SAR (relative to the whole-body average values) in the mice ranged from 0.18 to 1.9 for 902-MHz GSM and from 0.14 to 3.3 for 1747-MHz DCS. A new methodology was proposed to obtain comprehensive dosimetric information for whole-body, peak spatial SAR, as well as the averaged values for the most important organs. For each of the values the uncertainty as well as the instant and life-long variations were determined (Kuster et al., 2006). During the 2-years exposure period the position of any tube on the wheel was moved clockwise by one port on a weekly basis. With this rotation scheme all mice were positioned on each of the exposure compartments for a similar duration over the course of the study. Therefore, in cumulated terms of the dose received by the mice, any differences in the exposure signal within and between wheels were minimized. The whole-body exposure as well as the organ-
specific averaged SAR were several magnitudes higher than those of humans during phone or base station exposure (Tillman et al. 2007 and Table II). However, the tissues in the closest vicinity of the mobile phones may have been exposed to values of comparable magnitude.

Table II. Organ-averaged SAR at whole-body-averaged SAR of 4 W/kg body weight and the corresponding standard uncertainty and variations (Tillmann et al., 2007).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SAR_{organ} (group- and lifetime-average)*</th>
<th>Uncertainty (k=2)</th>
<th>Variations (instant) (k=1)</th>
<th>Variations (lifetime-averaged) (k=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSM (W/kg)</td>
<td>DCS (W/kg)</td>
<td>GSM (dB)</td>
<td>DCS (dB)</td>
</tr>
<tr>
<td>Blood</td>
<td>5.6</td>
<td>13.2</td>
<td>± 2.7</td>
<td>± 2.3</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>1.4</td>
<td>1.0</td>
<td>± 3.4</td>
<td>± 3.2</td>
</tr>
<tr>
<td>Skin</td>
<td>3.4</td>
<td>2.2</td>
<td>± 2.8</td>
<td>± 2.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.6</td>
<td>1.5</td>
<td>± 3.2</td>
<td>± 3.0</td>
</tr>
</tbody>
</table>

*Organ-averaged SAR were determined, applying the methodology of Kuster et al. (2006)

Positive control mice

Six of the sentinel mice (3 males and 3 females) received a single intraperitoneal (i.p.) injection of mitomycin C in aqueous solution (MMC, 1.0 mg/kg body weight, Sigma, Taufkirchen, Germany) at the end of the 2-year bioassay and were used as positive control animals for evaluation of MN induction in peripheral blood. MMC is a chemotherapeutic drug that has been shown to induce MN in mice (Vijayalaxmi et al. 1997). The positive control animals were sacrificed 48 h after MMC injection and peripheral blood smears were prepared.

Peripheral blood smears

All mice alive at the end of the 2-year RF-exposure period were included in this study. They were identical to the animals used for the carcinogenicity study by Tillmann et al. (2007). For the
number of included animals, which differed from the original number of 50 animals due to mortality during the 2-years exposure period, see Table 1. Because of the large number of animals, necropsies were completed between day 3 and day 19 after the last RF- or sham-exposure. Each day, between 1 and 7 mice from every treatment group were sacrificed. The mice were anesthetized with an overdose of carbon dioxide. For evaluation of MN induction peripheral blood was collected from the *Vena cava caudalis* and transferred into lithium-heparin-containing tubes (Sarstedt, Nümbrecht, Germany) to prevent clotting. Small drops of blood were then placed on clean microscope slides, and each drop was pulled behind a cover glass held at a 45° angle to prepare a thin smear over an area of 2-3 cm². One set of smears (at least 2 slides) was air-dried and another set (also at least 2 slides) was fixed in absolute methanol. Prior to analysis, slides were coded by combining exposure group and animal numbers.

*Staining of smears and MN evaluation*

Since RF and sham exposures were conducted over a period of 2 years, both acute and chronic effects were assessed using two different staining procedures to evaluate MN. One complete set of peripheral blood smear slides was air-dried and stained with May-Grünwald and Giemsa (both Merck, Darmstadt, Germany) (Schmid 1975) at the Fraunhofer ITEM. A light microscope (Photomicroscope III, Zeiss, Göttingen, Germany) was used to examine 2000 consecutive NCE to record the incidence of MN in each mouse. Another complete set of smears/slides was fixed in absolute methanol, air-dried, and mailed to UTHSCSA. Upon receipt, slides were stained with acridine orange (Sigma, St. Louis, MO, USA; 0.01 mg/ml of 0.2 M phosphate buffer, pH 7.4) as described previously (Vijayalaxmi et al. 1997). A fluorescence microscope (Carl Zeiss Inc., Thornwood, NY, USA) fitted with appropriate filters for the acridine orange stain was used to examine 2000 consecutive PCE to record the frequency of MN in each mouse. In addition,
10,000 consecutive erythrocytes per animal were examined to evaluate the proportion of PCE (% PCE) in peripheral blood and thus effects of RF exposure on blood formation. All evaluations were performed in a blinded manner. Data were decoded after completion of the whole PERFORM-A1 carcinogenicity study (see also "Study design and guidelines").

**Statistical analysis**

SAS software (2006), Version 9.1 for Windows was used for statistical analyses. The analysis of variance (ANOVA) test for repeated measures was used to assess significant differences in the incidence of MN between RF-exposed, sham-exposed, cage control, and positive control mice, and to compare between different RF frequencies (902 and 1747 MHz), maximal whole body SAR (0, 0.4, 1.3, and 4.0 W/kg), gender (male versus female mice), and all their interactions. The residuals were analyzed for homogeneity of variance and normality of distributions. Statistical significance was taken at a level of $p < 0.05$ for each effect. The Mann-Whitney Rank Sum test was also used for statistical analyses.

**Results**

**Survivors**

In the present investigation, the incidence of MN in peripheral blood smears of B6C3F1 mice exposed for 2 years to RF (902 or 1747 MHz, 2 hours/day, 5 days/week) was analyzed as an add-on to the PERFORM-A1 mouse carcinogenicity study. As only the animals surviving the whole 2-year treatment period could be included, the final number of animals was smaller than the original 50 animals per group. The average number of animals analyzed per treatment group
amounted to 39 (range: 35 to 44 animals, see Table I). Mortality was higher in female than in male animals, but was obviously not influenced by RF treatment in both sexes.

Table III. Polychromatic erythrocytes (PCE) and incidence of micronucleated NCE and PCE in peripheral blood of mice chronically exposed to radiofrequency for 2 hours/day on 5 days/week over a period of 2 years.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Exposure level</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MN/NCE</td>
<td>MN/PCE</td>
</tr>
<tr>
<td>Cage control</td>
<td>---</td>
<td>4.4 ± 2.3</td>
<td>4.6 ± 1.2</td>
</tr>
<tr>
<td>902 MHz</td>
<td>Sham</td>
<td>4.4 ± 2.0</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>4.5 ± 2.3</td>
<td>4.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>3.3 ± 1.8</td>
<td>4.5 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4.2 ± 2.0</td>
<td>4.5 ± 2.0</td>
</tr>
<tr>
<td>1747 MHz</td>
<td>Sham</td>
<td>4.1 ± 2.0</td>
<td>4.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>3.2 ± 1.9</td>
<td>4.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>3.7 ± 1.9</td>
<td>4.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4.3 ± 2.3</td>
<td>4.1 ± 1.8</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1.0 mg/kg b.w.</td>
<td>7.7 ± 2.5</td>
<td>26.7 ± 6.1</td>
</tr>
</tbody>
</table>

Data represent group means ± standard deviation (SD) of the survivors only; underlined data: statistically significant increase, compared to the cage controls, $p \leq 0.05$, Mann-Whitney Rank Sum test. For number of animals see Table I. **MN/NCE**: micronucleated NCE, analysis of 2000 consecutive normochromatic erythrocytes per animal (Fraunhofer ITEM); **MN/PCE**: micronucleated PCE, analysis of 2000 consecutive polychromatic erythrocytes per animal (UTHSCSA); **% PCE**: fraction of PCE, analysis of 10,000 consecutive erythrocytes per animal (UTHSCSA).

**Polychromatic erythrocytes in peripheral blood**

As judged by PCE counts in peripheral blood, there was no toxic effect of RF exposure on blood formation. There were no differences in the proportion of PCE between cage controls/sham-exposed and RF-exposed animals, nor between male and female animals. The mean amount of PCE in peripheral blood of the animals (approximately 3%) was within the normal range for B6C3F1 control mice (for an example, see Witt et al., 2000). In contrast, positive control mice injected with 1 mg/kg body weight MMC demonstrated a clear reduction in the percentage of PCE to
2.0 ± 1.4 for male and 2.1 ± 1.5 for female mice, as compared to 3.2 ± 0.4% and 3.2 ± 0.6% PCE, respectively, in cage control animals (Table III).

**Micronuclei in polychromatic erythrocytes of the peripheral blood**

Analysis of MN in peripheral blood PCE of mice is an appropriate measure of treatment-induced clastogenic activity and mitotic damage. An increase in MN in peripheral blood PCE indicates an acute clastogenic and/or aneugenic event. By combining the mice from all necropsy times for each treatment group, there was no evidence of an RF-induced increase in the mean frequencies of micronucleated PCE, as compared to the sham-exposed and cage control animals, irrespective of the frequency or exposure level of RF treatment or the sex of the animal. As expected, however, 48 h after injection of the positive control MMC the incidence of micronucleated PCE was significantly enhanced to 26.7 ± 6.1 MN/2000 PCE (males) and 35.3 ± 2.1 MN/2000 PCE (females), compared to 4.6 ± 1.2 MN/2000 PCE (males) and 4.4 ± 1.4 MN/2000 PCE (females) for the cage control animals. The mean incidences of MN/2000 PCE are presented in Table III. Due to the short life-span of PCE and their rapid maturation to NCE, analysis of MN in peripheral blood PCE can only indicate acute genotoxic effects within 72 h after treatment. Thus, combining the animals from all necropsy times (3-19 days after the last RF exposure) may mask early genotoxic effects. We therefore compared MN frequencies in animals sacrificed 3 days, 10-11 days, and 17-18 days after the last RF exposure (see Figures 2A and B). Nevertheless, there was no significant increase in MN frequency due to RF exposure at all necropsy times (early, intermediate, and late).
Figure 2:

A

![Graph A](image1)

B

![Graph B](image2)
Figure 2. Influence of the time of sacrifice after the last RF exposure on micronucleus frequencies in peripheral blood PCE of RF-exposed B6C3F1 mice. Mice were sham-exposed or exposed to RF, peripheral blood smears were prepared, slides were stained with acridine orange, and PCE were analyzed as described in the "Methods" section. (A) 902 MHz: Each column represents mean ± SD of 8 animals (males and females combined) per group and time point. For data analysis, animals necropsied 3 days, 10 and 11 days, or 17 and 18 days after the last RF exposure were combined to evaluate early, intermediate, and late effects. (B) 1747 MHz: Each column represents mean ± SD of 5-9 animals (males and females combined) per group and time point. For data analysis, animals necropsied 3 days, 10 days, or 18 days after the last RF exposure were combined to evaluate early, intermediate, and late effects.

Micronuclei in normochromatric erythrocytes of the peripheral blood

Other than in rats and humans, micronucleated NCE are not selectively removed by the spleen from the peripheral blood of mice. As NCE exhibit a long life-span of greater than 30 days (Chaubey et al., 1993), increased frequencies of micronucleated NCE are therefore maintained in peripheral blood of mice at steady-state level for prolonged times. Thus, scoring of micronucleated NCE in peripheral blood of mice reflects average damage during at least the 30-day period preceding sacrifice. Due to the long life-span of NCE/micronucleated NCE in peripheral blood of mice, animals from all necropsy times per treatment were combined in the present investigation to analyze the chronic effect of RF exposure on DNA integrity. The mean incidences of MN/2000 NCE are presented in Table III. Analysis of NCE in the male animals demonstrated no significant differences between the RF-exposed and sham-exposed/cage control mice, and MN frequencies resembled those observed in PCE. Female mice, irrespective of the frequency (902 MHz and 1747 MHz) or exposure level used (low, medium, high), exhibited a consistently lower incidence of MN/2000 NCE as compared to the male animals. For example, the mean incidence of MN in the male cage controls amounted to 4.4 ± 2.3 MN/2000 NCE, whereas the mean frequency of micronucleated NCE in female cage controls was 2.3 ± 1.7 MN/2000 NCE. At 1747 MHz there seemed to be a slight exposure level-dependent increase in
micronucleated NCE from $1.7 \pm 1.3$ MN/2000 NCE in sham-exposed female animals to $2.6 \pm 1.6$ MN/2000 NCE at the high exposure level. However, it did not reach statistical significance and the incidence measured in sham-exposed females was unusually low compared to the cage control animals. As expected, both male and female animals exhibited an increased MN incidence of $7.7 \pm 2.5$ and $8.0 \pm 4.0$ MN/2000 NCE 48 h after injection of the positive control MMC. These incidences were significantly lower than those observed for peripheral blood PCE. Overall, there were no significant differences in MN frequencies between RF-exposed and sham-exposed/cage control mice, both in peripheral blood PCE and NCE.

**Discussion**

Induction of DNA damage in somatic cells can lead to the development of cancer and/or cell death. This is why in recent decades researchers have used several experimental techniques to investigate the extent of genetic damage in mammalian somatic cells exposed *in vitro* and/or *in vivo* to non-ionizing electromagnetic fields (Vijayalaxmi and Obe, 2004, Verschaeve, 2005; Vijayalaxmi and Obe, 2005; Vijayalaxmi and Prihoda, 2008).

There are very few peer-reviewed scientific publications addressing the genotoxic potential of long-term (subacute to chronic) *in vivo* studies with whole-body exposure to RF in experimental animals such as mice and rats. The exposures not only varied in magnitude, but also with respect to the signal (carrier frequency and modulation), and detailed dosimetric evaluations were not always provided. Although needed, chronic *in vivo* studies are quite rare. For these reasons, the present investigation was added to the PERFORM-A1 mouse carcinogenicity study (Tillmann et al., 2007), thus offering the possibility to determine in a high number of animals the genotoxic potential of chronic exposure to different environmentally relevant RF signals,
simulating exposure from GSM (902 MHz) and DCS (1747 MHz) handsets, and to directly correlate the results with the outcome of the carcinogenicity study. Irrespective of frequency or maximal whole-body-averaged SAR (0.4, 1.3, or 4.0 W/kg body weight during phase I, "GSM Basic") used, the results of the present study did not provide any evidence of RF-induced genotoxicity, which is in line with the lack of carcinogenic potential and RF-related death in the PERFORM-A1 main study and also with the absence of MN induction in a preceding short-term study (5 days/6 weeks) by Görlitz et al. (2005). Although higher slot-averaged whole-body SAR up to 33.2 W/kg were used in the study of Görlitz et al. (2005), incidence of MN in bone marrow PCE (5-days study), peripheral blood NCE (6-week study), keratinocytes, and spleen cells were not significantly different between sham- and RF-exposed mice.

In the present study, the occurrence of MN, as a sensitive measure for both clastogenic and aneugenic events, was evaluated in both peripheral blood PCE and NCE to detect acute as well as chronic DNA-damaging effects of RF exposure. Due to the PCE migration time from bone marrow to peripheral blood and subsequent maturation to NCE, an increase in micronucleated PCE in peripheral blood only indicates acute DNA damage taking place within a narrow time-frame of about 2-3 days before sampling or genomic instability of hematopoietic stem cells in the bone marrow. In contrast, an increase in micronucleated NCE covers genotoxic activities during more than 3 weeks preceding sampling and is therefore an appropriate measure for subchronic and chronic studies with repeated exposures (Chaubey et al., 1993; Witt et al., 2000). Nevertheless, in the present study, neither PCE nor NCE (irrespective of early or late sampling after the last exposure) demonstrated an RF-mediated increase in the incidence of MN, thus speaking against a genotoxic potential of chronic whole-body RF-exposure in B6C3F1 mice.

To ensure validity of the method, some sentinel animals were administered the known clastogen MMC. As expected, these positive control animals exhibited an increased frequency of
MN in both peripheral blood PCE and NCE and a reduced PCE percentage. For NCE the MMC-induced increase in MN was significantly lower than that observed for peripheral blood PCE. However, sampling was performed 48 h after administration, and a time period of 48 h is too short to ensure complete maturation of micronucleated PCE to NCE. In Swiss mice, for example, the number of micronucleated NCE did not peak until 60 h after irradiation (Chaubey et al., 1993).

The spontaneous MN frequencies in peripheral blood NCE observed in the present study were within the range reported in other studies (Chaubey et al., 1993; Witt et al., 2000; Görlitz et al., 2005; Juutilainen et al., 2007) or even lower. Interestingly, cage control, sham-, and RF-exposed female animals all demonstrated lower MN incidences in peripheral blood NCE than male animals. This phenomenon, which is frequently observed for peripheral blood NCE (for example, see Witt et al., 2000 and Görlitz et al., 2005) and for bone marrow PCE, has often been interpreted as higher sensitivity and higher MN background levels in male animals (Marvournin et al., 1990). However, the reason(s) for this phenomenon is/are unclear, but may involve an enhanced rate of blood formation with inefficient enucleation or a lower DNA-repair capacity.

From the limited *in vivo* data concerning long-term whole-body exposure of mice to RF, there seems to be no clear evidence of genotoxic activity of repeated RF exposure. Nevertheless, there are a few subacute/subchronic *in vivo* studies with rats that indicate some genotoxic activity. For example, Trosic et al. (2002, 2004) and Trosic and Busljek (2006) demonstrated a significant increase in MN in peripheral blood PCE of male Wistar rats after 8 days and in bone marrow after 15 days of exposure to CW (continuous wave) RF of 2450 MHz (estimated whole-body SAR of $1.25 \pm 0.36 \ W/kg$, 2 h/day, 7 days/week for up to 30 days). The increase in both peripheral blood and bone marrow was small and not clearly exposure duration-related. These data are difficult to interpret, because (i) micronucleated PCE arise first in erythroid-lineage stem cells in
the bone marrow and then emerge into the circulating peripheral blood, not the other way around, and (ii) in rats, the spleen scavenges abnormal micronucleated PCE and NCE and hence it should only be possible to demonstrate a clear increase in MN by using the very young PCE fraction at high numbers (Wakata et al., 1998). Demsia et al. (2004) reported an about 3-fold induction of MN in bone marrow PCE of male and female Wistar rats mainly head-exposed to 912 MHz with peak spatial SAR (10g) of 0.42 W/kg (2 h/day on 30 consecutive days). MN induction was also observed in polymorphonuclear cells. The authors used rat bone marrow smears, stained with May-Grünwald and Giemsa (Schmid 1975), but did not devoid the slides of mast cell granules by, for example, cellulose columns. As mast cell granules stain identically to MN with the May-Grünwald/Giemsa stain (Romagna, 1988), the significance of this positive finding has to be further evaluated. Another positive finding was reported by Ferreira et al. (2006) in newborn Wistar rats exposed in utero to CW 834 MHz in a metallic box resulting in a not well defined exposure situation. Due to an about 2-fold induction of micronucleated PCE in peripheral blood, the authors concluded that under the experimental conditions used, there might be a genotoxic effect of RF exposure in hematopoietic tissue during embryogenesis.

The first investigation on the genotoxic potential of chronic RF exposure in mice, also using MN induction as an endpoint, was reported by Vijayalaxmi et al. (1997). This study was appended to a primary investigation examining, in cancer-prone C3H/HeJ mice, the carcinogenic potential of chronic exposure to CW RF fields of 2450-MHz (average whole-body SAR of 1.0 W/kg; 20 h/day, 7 days/week over a period of 18 months). The final corrected results (Vijayalaxmi et al. 1998) indicated a small but statistically significant 0.5% increase in MN frequency in both bone marrow and peripheral blood PCE. As the MN incidences in both RF- and sham-exposed mice were still within the historical range for spontaneous MN in control mice
(similar age) and the small increase in MN was not correlated with carcinogenicity in the same mice (Frei et al. 1998), a real genotoxic effect was considered to be unlikely by the authors.

There are some more chronic studies which used MN induction as an endpoint and indicate a lack of genotoxic potential of RF exposure. In a recent chronic study, evaluation of MN induction was added to 2 long-term mouse bioassays with female CBA/S (78 weeks of exposure) and transgenic/non-transgenic K2 mice (52 weeks of exposure), investigating whether RF exposure enhances the carcinogenic effect of ionizing or ultraviolet light (UV) irradiation (Juutilainen et al. 2007). Different frequencies (902.5 MHz, 902.4 MHz, and 849 MHz), signal modulations ("Nordic Mobile Telephone" network, CW, speech-modulated GSM Basic, and speech-modulated "Digital Advanced Mobile Phone System" network) and whole-body-averaged SAR (1.5 W/kg, 0.35 W/kg, and 0.5 W/kg) were used. Nevertheless, MN incidence in both peripheral blood PCE and NCE was not altered by RF exposure, irrespective of RF frequency, modulation, SAR level, or mouse strain used, or application of preceding x-ray or parallel UV exposure. Besides evaluation of MN induction, Juutilainen et al. (2007) determined the percentage of PCE in peripheral blood, and, similar to our observations and the observations of Görlitz et al. (2005), found the PCE fraction to be not affected by RF exposure, thus indicating a lack of RF-mediated bone marrow toxicity. This was also in line with the 12-month interim examinations on hematology within the PERFORM-A1 study, demonstrating no RF-mediated changes (Tillmann et al., 2007).

Two additional chronic in vivo studies published by Vijayalaxmi et al. (2003) and Verschaeve et al. (2006) also pointed to a lack of genotoxicity and co-genotoxicity of RF exposure. Appended to a carcinogenicity study, Vijayalaxmi et al. (2003) investigated MN induction in the bone marrow of male and female Fisher rats. In this study, pregnant Fisher rats (from the nineteenth day of gestation) and their nursing offspring (until weaning) were exposed to a far-
field 1600-MHz iridium wireless communication signal followed by chronic head-only exposures of male and female offspring to a near-field 1600-MHz signal (2 h/day, 5 days/week, for 2 years). After 2 years, bone marrow was collected from all surviving rats and the incidence of MN/2000 PCE was evaluated. There were no significant differences between RF-exposed, sham-exposed, and cage control animals, but positive controls treated with MMC exhibited a significantly increased MN frequency. As observed also in the present study, there was good correlation between absence of genotoxicity and absence of carcinogenicity, as there was no significant increase in tumor development in the same rats (Anderson et al. 2004). Finally, Verschaeve et al. (2006) investigated the co-genotoxic effect of RF-exposure and a drinking water mutagen. Female Wistar rats exposed to 900 MHz (GSM Basic) for 2 hours/day on 5 days/week for 2 years (average whole-body SAR: 0.3 or 0.9 W/kg) in parallel received 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone. MN frequencies were evaluated after 3, 6, and 24 months of exposure in peripheral blood PCE, and in addition, DNA damage was assessed by the comet assay in white blood cells, liver, and brain. Interestingly, the data also did not provide any evidence of a genotoxic or co-genotoxic activity of RF exposure.

In conclusion, the present chronic study in B6C3F1 mice exposed to GSM (902 MHz) and DCS (1747 MHz) RF, including the most relevant extremely low frequency (ELF) amplitude modulation components of these signals, at 3 different maximal exposure levels (i.e., 0.4, 1.3, and 4.0 W/kg body weight during phase I, "GSM Basic") did not demonstrate acute, delayed, or chronic genotoxicity of RF exposure in peripheral blood erythrocytes. Seen that some subacute/subchronic in vivo studies have pointed to a tendency towards genotoxic activity of RF exposure, this discrepancy has to be further evaluated in terms of exposure technology and methodological aspects. However, bearing in mind all the chronic genotoxicity studies mentioned above, the overall data suggest that long-term chronic exposure to RF, especially to the
frequencies used for wireless mobile communications, does not induce excess genotoxicity in mice and rats. This is in line with a lack in carcinogenic potential of RF found in the same studies.
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References


