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**Research article:**

**STUDIES OF EFFECTS OF GSM-900 MICROWAVE EXPOSURE  
ON DNA "MICRONUCLEUS" FORMATION IN MICE**

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### **Abstract.**

Possible genotoxic effects of microwave exposure from GSM-900 mobile telephones have investigated with *in vivo* micronucleus assay of mouse erythrocytes from CBA mice and GFAP knockout mice.

No significant change in the frequency of erythrocytes with micronuclei neither in the young (polychromatic PCE) or mature (normichromatic NCE) erythrocytes. There is, however, a tendency but not significant to increased MPCE in female mice after 35 days of exposure. There is a marked tendency to lower PCE-fraction in the exposed groups. When male and female is studied separately there is no significant difference. However, if the values are normalised to eliminate the sex-difference there is a significant lower fraction in the exposed mice. Another observation is lower weight of the exposed male.

If normalised data for both sexes are pooled there is an almost significant difference (95% level) in weight. We found a less pronounced difference in the CBE mice than in the GFAP experiment. Thus genotype might play a role in microwave exposure. Differences in exposure time and number of controls in GFAP and CBA experiment might influence the results. We observe a moderate decrease of formation of new erythrocytes in the exposed animals. This might fit the tendency of lower weight in the exposed animals and might indicate a general decreased cell-proliferation in the exposed animals.

**Keywords:** GSM-900, mobile telephones, microwave, erythrocytes (E), polychromatic (PCE) or mature normichromatic (NCE), micronuclei, CBA mice, GFAP knock-out mice

## 1. Introduction

In-vitro investigations on different cell systems provided evidence for a lack of direct genotoxic and mutagenic effects of continuous and pulsed microwaves at different power densities (Kerbacher et al., 1990). Additionally, no synergistic effect was found between the applied field and Mitomycin C, Adriamycin and Proflavin (Meltz et al., 1989, Meltz et al., 1990, Maes et al., 2006). No synergistic effect was found between moderate-power-radio-frequency electromagnetic radiation and Adriamycin on cell-cycle progression and sister chromatid exchanges (Ciaravino et al., 1991). However, a synergistic effect was found with Mitomycin C in a recent investigation of 954 MHz waves emitted by the antenna of a GSM base-station (Maes et al., 1993, Verschaeve et al., 1994, Maes et al., 1995). Indeed, a very reproducible increase in the frequency of Mitomycin C-induced sister chromatid exchanges were found in human Lymphocytes when the blood was first exposed to the microwaves (15 W power density, SAR= 1.5 W/kg). It was also shown that the microwaves alone may have a certain clastogenic (chromosome breaking) effect, but only in very precise circumstances of exposure (Maes et al., 1995). This effect was also studied for exposures to 450 MHz fields from a mobile telephone but the results were not very clear (Maes *et al.*, unpublished data). No effect was found in a limited sample of maintenance workers professionally exposed to microwaves of different frequency (Maes et al., 1995). An increased frequency of chromosome aberrations was also found in *in-vitro* experiments involving other cell types, microwave frequencies and cytogenetic endpoints (d'Ambrosio et al., 1992, Garajvrhovac et al., 1991, Garaj-Vrhovac et al., 1990, Leonard et al., 1983, Maes et al., 1993, Meltz, 1998).

Also in *vivo*, changes in metaphase counts and translocation numbers were observed even at low exposure levels. This was found in Balb/c mice that were exposed for two weeks to pulse modulated 9400 MHz microwaves, one hour a day for 5 days a week (Manikowska et al., 1979). In male CBA/CAY, mice exposed to 2450 MHz microwaves increased chromosome exchanges and other cytogenetic abnormalities were found in germ cells exposed as spermatocytes (Manikowska-Czerska et al., 1985). However, negative results were also found in other investigations in somatic cells (Huang et al., 1977) as well as in germ cells (Saunders et al., 1988,

Beechey et al., 1986). The alkaline comet assay is a sensitive method for assessing DNA single-strand breaks and/or alkali-labile sites in DNA. By using this method, DNA damage was found in Lymphocytes exposed to 954 MHz waves (Verschaeve et al., 1994) and in brain cells from rats being exposed *in vivo* to 2450 MHz waves at SAR values of 0.6 and 1.2 W/kg (Lai and Singh, 1995 ). The latter may be of particular importance regarding the possible, but to date totally unproved, association between microwaves (mobile telephones) and brain cancer. In a short pilot experiment in which "comets" were investigated in white blood cells of rats, no clear-cut effect could be ascribed to a 954 MHz microwave exposure over several weeks (Verschaeve et al., 1994). Further studies of RF-exposed individuals do indicate increased frequencies of genetic damage (e.g., chromosomal aberrations) in their lymphocytes or exfoliated buccal cells, a number of shortcomings, however, prevents any firm conclusion (Verschaeve, 2009).

Micronuclei are membrane-bound, chromatin-containing bodies separated from the main nucleus in the cell. They appear in the cytoplasm of eukaryotic cells after exposure to a variety of genotoxic agents. The presence of micronuclei in cells indicates damage that may lead to earlier death of the cell or to a mutant phenotype, characteristic of e.g. a neoplastic transformation. Assays detecting micronuclei have become increasingly important methods in genetic toxicology. This method has been used to study the genotoxic effect of very low doses of ionising radiation exposure in rodents (Abramsson-Zetterberg et al., 1996, Abramssonzetterberg et al., 1995, Abramsson-Zetterberg et al., 1999). In the present study we have used this method to investigate possible genotoxic effects of GSM-900 microwave exposure in mice.

## **2. Material and methods**

### **2.1 Microwave exposure**

The GSM (Global System for Mobile communication) phone at the 900 MHz band has a maximum output power of 2W. With a duty factor of 1/8 (switched "on" one second every eight second) this leads to a time average of 0.25W output leaving the antenna. The maximum power absorbed is thus of the order of one tenth of a Watt.

In the present study we expose the whole animal in a TEM-cell with the unique characteristic of having both linear amplitude and phase response versus frequency. Thus it lends itself to extremely broad band sweep frequency testing using a variety of wave forms including CW and pulsed (or modulated) exposure fields. These fields can be accurately generated in the TEM-cell without the distortion that is typically introduced when conventional antennas are used to establish impulse test fields (Martens et al., 1993).

The cell is enclosed in a wooden box that supports the outer conductor and central plate. The outer conductor is made of brass-net and is attached to the inner walls of the box. The centre plate, or septum, is constructed of aluminium and is held up by Teflon braces which are attached at the inner side walls. To allow access to the inside of the cell both ends can be removed. The inside of the cell is ventilated through 18 holes (diam. 18 mm) in the side walls and top of the box and the brass-net allows air to circulate. These holes are also used for examination of the interior during exposure. Probes for monitoring temperature inside the cell or test object are inserted through these holes.

The test system consists of four TEM-cells. A GSM telephone is hooked up to a terminal program on a computer that controls the power and repetition of pulses. A power splitter divides the power from the GSM generator into two equal parts that are fed into two of the four cells. The output from the cells is terminated in a 50 Ohms dummy load. Both forward and reflected average powers are measured, with Bird model 43 power meters, at the inputs and outputs of the cells.

## 2.1 Experimental animals

For study of micronuclei in peripheral blood, we have utilized CBA mice originated at the Swedish University of Agriculture, Uppsala, Sweden. Peripheral blood was taken from the CBA mice after they have been exposed daily to GSM-900 microwaves during 1 month (Dec 1997-Jan 1998).

We have also utilized a newly generated "knock-out" mouse model (Pekny et al., 1995). By the use of gene targeting, Pekny and his collaborators have created mice deficient for glial fibrillary acidic protein (GFAP), which is an astrocyte-specific protein that forms intermediate filaments in the cytoskeleton of astrocytes (Fuchs and Weber, 1994). The disruption of GFAP gene was performed via targeted mutation in E14 embryonic stem cells. Clones were injected into C57BL6J blastocysts, which were subsequently transferred to foster mothers and allowed to develop normally, reach adulthood and reproduce. However, contrary to normal mice and to other GFAP-deficient mice derived from other strains of ES cells, the GFAP-deficient mice utilized by us develop spontaneous tumours, both lymphoblastic (non-Hodgkin) lymphomas and Anaplastic rapidly growing tumours diagnosed as malignant Schwannomas (Pekny et al. 1996). A plausible explanation for the tendency to develop tumours could be that a mutation (e.g. during the embryonic stem cell manipulation) took place close to a GFAP locus and then usually segregates with the disrupted GFAP gene.

The GFAP rats were exposed in the TEM Cells for about 8 hours during 43 days from November 16 1995 to July 9 1996. The total time of exposure was 1143 hours.

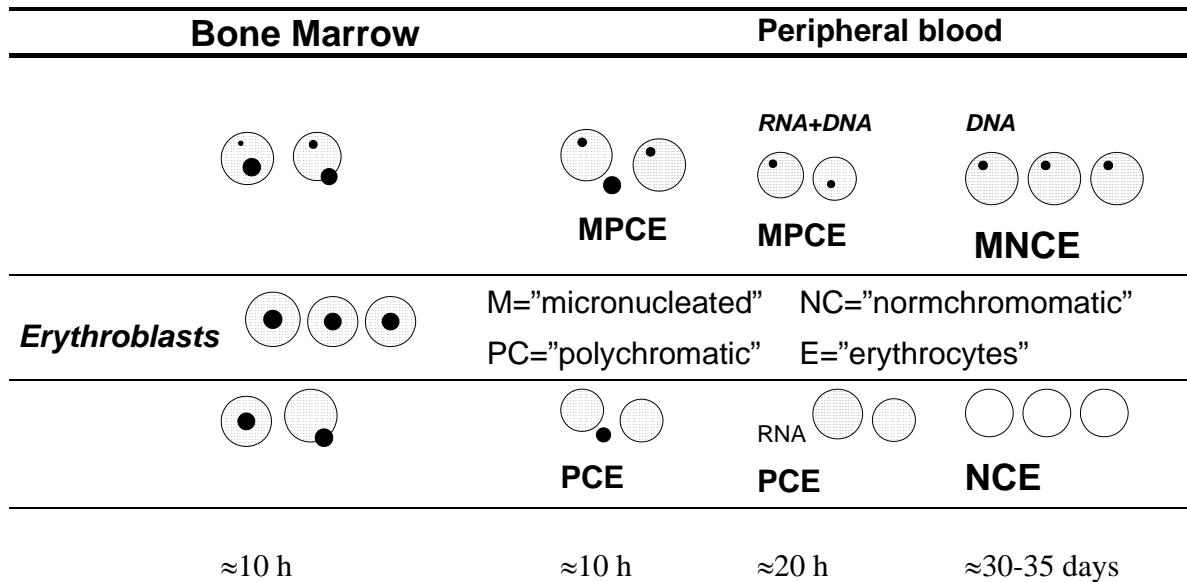
## 2.2 The *in vivo* micronucleus assay with mouse erythrocytes.

Erythrocytes are formed from stem cells called erythroblasts located in the bone marrow. Erythrocytes or red blood cells are formed by successively cell divisions as shown in **Table 1**.



**Table 1.**

Erythrocytes are formed from stem cells called erythroblasts located in the bone marrow. Erythrocytes or red blood cells are formed by successively cell divisions as shown in the table below.



From stem cell to erythrocyte, there are about seven cell divisions. The cells become progressively smaller and the chromatin of their nuclei more and more condensed. During the later cell cycles, the erythroblasts synthesise haemoglobin. It takes about ten hours from the last cell division until they appear as polychromatic erythrocytes (PCE) in the bone marrow (or spleen). The newly produced PCE contain RNA, which makes them distinguishable from the mature normchromatic erythrocytes (NCE). In order to distinguish micronucleated polychromatic erythrocytes (MPCE) and PCE from NCE, two frequently used dyes Giemsa (Staining DNA) and May Günwald (staining RNA), are used in the classical protocol for manual scoring. The relationship between the number of PCE and the number of NCE reflects the proliferation of erythroblasts. In the bone marrow of control CBA mice, usually more than 50% of the erythrocytes are PCE. In the peripheral blood the percentage of PCE is about 2%, and in the spleen about 8-10%, with a larger variation.

By using a flow cytometer equipped with two lasers and staining the micronuclei (DNA) with Hoechst 33342 and PCE (containing RNA) with Thiazole Orange it is possible to discriminate between NCE and PCE as well as MPCE and MNCE (Grawe et al., 1992).

Because a main nucleus in the erythrocyte is normally absent the presence of micronuclei is the

only positive nucleotide material in the erythrocyte. It has been shown that the *in vivo* rodent micronucleus assay has a very high sensitivity (Morita et al., 1997b, Morita et al., 1997a).

By using an automated analysis it is possible study presence or the absence of RNA (PCE or NCE) and the presence or absence of DNA (MPCE/MNCE or PCE/NCE). When all data from the experiment are collected, the number of events defined as PCE, NCE, MPCE and MNCE are determined.

Blood was collected from the lightly anesthetized mice. In total, about 50  $\mu$ l was drawn from the orbital-vein into heparinised tubes using a thin Pasteur pipette. The erythrocytes were then purified. Five  $\mu$ l of the collected blood were layered on 1 ml of a 65% *Percoll* (Pharmacia Biosystem, Uppsala, Sweden) solution in a tube. The tubes were centrifuged for 20 min at 600g. From each animal three parallel samples were obtained. The supernatant, including platelets and the majority of nucleated cells, was aspirated, leaving a pellet of erythrocytes and some nucleated cells.

The pellet, mainly containing erythrocytes, was diluted in 40 ml PBS and transferred with pipette into a vigorously agitated tube containing 1.25 ml of fixative (a 1% solution of glutaraldehyde made in Sørensen's phosphate buffer, 1/20 M, pH 6.8 with 30 mg/ml sodium-dodecyl-sulphate included). The fixed cells were stored at 4\_C overnight. The fixative was aspirated and the cells were resuspended in a staining solution containing PBS, Hoechst 33342, and thiazole orange. The staining buffer was prepared by adding 1 ml Hoechst 33342 stock (Sigma, St Louis, MO, for DNA staining, 500mM in distilled water) and 50 ml thiazole orange stock (Molecular Probes, Eugene, OR for RNA staining, 1 mg/ml in methanol) to 100 ml PBS. The staining continued for 45 min at 37\_C. Samples were mixed every 15 min by inverting the tubes (Grawe et al., 1992, Grawe et al., 1993).

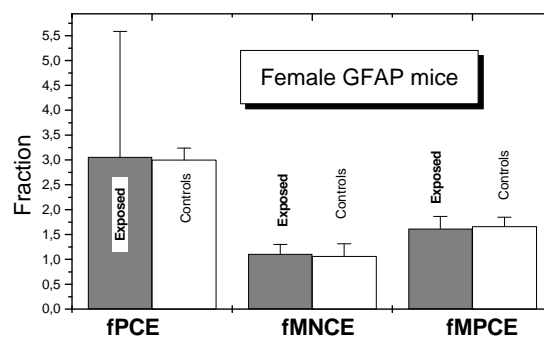
Flow cytometric analysis of the samples was performed with a dual laser FACStar Plus flow cytometer (Beckton Dickinson, Sunnyvale, CA) equipped with standard optics and with an argon ion laser operating at the 488 nm line, 200 mW as the primary laser, and a second argon ion laser operating at the 351–364 nm multiple UV lines at 100 mW (Grawe et al., 1992).

### 3. Results and discussion

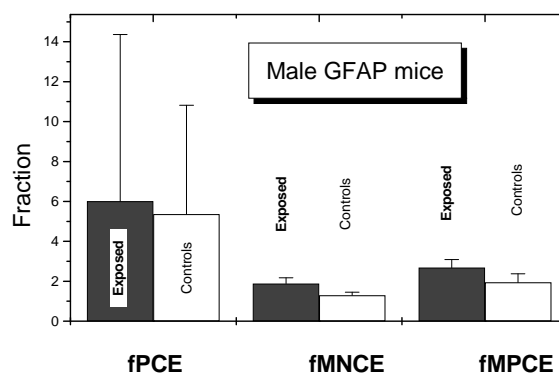
#### 3.1 GFAP mice exposed to GSM

Peripheral blood was taken from the GFAP mice exposed to GSM in several months.

The ratio between the average number of various types of micronucleus fPCE, fMNCE and fMPCE in exposed and controls is displayed in **Figure 1** (female) and **Figure 2** (male).



**Figure 1** The fraction of micronucleus in peripheral blood from female mice.



**Figure 2** The fraction of micronucleus in peripheral blood from male mice.

In **Table 2** the results are given, the relationship between exposed, and controls are given. In the exposed animals, there is an increase in micronucleus in all type of cells for males but for unchanged for females. The DNA in the stem cells for males seems to be more sensitive to GSM-900 microwave exposure than female.

**Table 2**

The results of the average number of various types of micronucleus fPCE, fMNCE and fMPCE in exposed and controls is displayed in and the relationship between exposed and controls are given.

	fPCE %	fMNCE ‰	fMPCE ‰	fPCE %	fMNCE ‰	fMPCE ‰
	<b>Female exposed</b>			<b>Male exposed</b>		
	1.83	0.87	1.29	2.43	1.96	2.9
	7.17	1.19	1.55	2.46	1.99	3.07
	2.77	1.01	1.66	2.98	1.52	1.96
	0.41	1.38	1.98	2.85	1.73	2.45
	3.11	1.1	1.61	2.28	1.78	2.86
				23.06	2.35	2.84
<b>Average E</b>	<b>3.06</b>	<b>1.11</b>	<b>1.62</b>	<b>6.01</b>	<b>1.89</b>	<b>2.68</b>
<b>Sd</b>	<b>2.53</b>	<b>0.19</b>	<b>0.25</b>	<b>8.36</b>	<b>0.28</b>	<b>0.41</b>
	<b>Female controls</b>			<b>Male controls</b>		
	2.68	1.01	1.49	2.86	1.4	1.93
	3.2	0.88	1.9	15.01	1.46	1.84
	3.17	0.93	1.53	4.33	1.18	1.67
	2.94	1.43	1.72	2.36	1.1	1.62
				2.24	1.37	2.68
<b>Average C</b>	<b>3.00</b>	<b>1.06</b>	<b>1.66</b>	<b>5.4</b>	<b>1.3</b>	<b>1.9</b>
<b>Sd</b>	<b>0.24</b>	<b>0.25</b>	<b>0.19</b>	<b>5.5</b>	<b>0.2</b>	<b>0.4</b>
<b>E/C</b>	<b>1.02</b>	<b>1.04</b>	<b>0.97</b>	<b>1.12</b>	<b>1.45</b>	<b>1.38</b>
<b>t-test E versus C p-value</b>	<b>0.960</b>	<b>0.766</b>	<b>0.781</b>	<b>0.880</b>	<b>0.002</b>	<b>0.019</b>

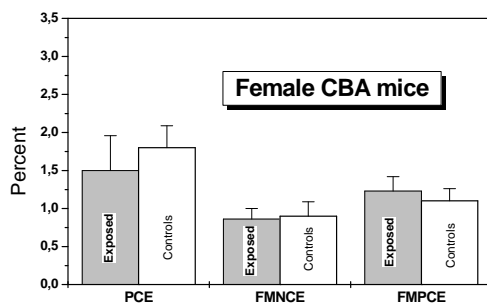
For MNCE and MPCE, there is a highly significant increase in the micronuclei formation for

exposed males. The ratio of 1.45 for fMPCE in male corresponds to the cytotoxic effect from 4 days continuous exposure with a total absorbed dose of 15 mGy ionising radiation (662 keV gamma-radiation). (Abrahamsson-Zetterberg et al. 1997). The total exposure time with GSM-900 microwaves in the TEM-cell was 1143 hours. Thus if the cytotoxic effect is cumulative it corresponds to a dose-rate of ionising radiation in the order of 13  $\mu$ Gy/h. Assume we use the mobile telephone during 100 hour per year. Then the cytotoxic effect of GSM-900 microwaves would correspond to an absorbed dose of ionising radiation in the order of 1 mGy per year that is in the same order as the natural radiation background (excluding radon).

### 3.2 CBA mice exposed to GSM

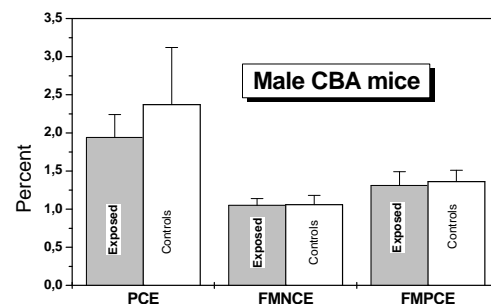
Peripheral blood was taken from the CBA mice exposed to GSM in 1 month.

The percentage the average number of various types of micronucleus PCE, MNCE and MPCE in exposed and controls is displayed in **Figure 3** (female) and **Figure 4** (male).



**Figure 3**

Percentage of micronucleus in peripheral blood from female CBA mice.



**Figure 4**

Percentage of micronucleus in peripheral blood from male CBA mice.

There is no significant change in the frequency of erythrocytes with micronuclei neither in the young (polychromatic PCE) or mature (normichromatic NCE) erythrocytes. There is, however, a tendency but not significant to increased MPCE in exposed female mice after 35 days.

There is a marked tendency to lower PCE-fraction in the exposed groups. When male and female is studied separately there is no significant difference. However, if the values are normalised to eliminate the sex-difference there is a significant lower fraction in the exposed mice.

Another observation is lower weight of the exposed male. If normalised data for both sexes are pooled there is an almost significant difference (95% level) in weight.

#### 4. Discussion and Conclusion

There is a less pronounced difference in the CBE mice than in the GFAP experiment. Previous experiments with ionising radiation there is no difference in the sensitivity of producing micronuclei in erythrocytes between CBA and SCID mice, although SCID mice has a decreased ability to repair DNA strain breaks.

Thus, the genotype might play a role in microwave exposure. The exposure time that is different in GFAP and CBA experiment might influence the results. There were some problems with fewer controls in the GFAP experiment that might play a role in the experiment.

We observe a moderate decrease of formation of new erythrocytes in the exposed animals. This might fit the tendency of lower weight in the exposed animals and might indicate a general decreased cell-proliferation in the exposed animals.

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