

Evidence of Genotoxicity Induced by 60 Hz Magnetic Fields on Mice Bone Marrow as Assessed by In Vivo Micronucleus Test

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Abstract: Modern life implies a constant exposure of living organisms to electromagnetic fields (EMFs) generated by human-made technology. The potential genotoxic effect induced in mice exposed to ELF-EMFs was investigated. The evaluated cytological endpoint included the frequency of micronucleated polychromatic erythrocytes (MN) in bone marrow. Three experimental conditions were arranged: (1) acute exposure of 72 h with three intensities of 60 Hz magnetic fields at 1.0, 1.5, and 2.0 mT, (2) expanded exposure of 10 days/8 h daily with three replications, and (3) a combined treatment of 72 h exposure to ELF-EMFs at 60 Hz and 2.0 mT plus 5 mg/kg of Mitomycin-C. For acute exposures, a positive control of MMC as a reference of clastogenic effect was used. Statistically significant differences indicative of ELF-EMF genotoxic effect were observed for MN frequency when compared ELF-EMF exposed and control animals at 1.5 and 2.0 mT exposure conditions. In addition, an antagonistic effect between ELF-EMF exposure and Mitomycin-C treatment in terms of MN frequency was observed for this co-exposure condition. In conclusion, the present study indicates the *in vivo* susceptibility of mammals to the genotoxicity potential of ELF-EMFs.

Key words: Micronuclei induction, 60 Hz sinusoidal magnetic fields, BALB/c mice, genotoxic potential

Introduction

Life organisms of industrialized areas are exposed to environmental electromagnetic fields and waves encompassing a very broad range of frequencies. In recent years, there have been a lot of publicity and controversy worldwide surrounding health hazards of exposures to some of these fields, particularly the extremely low frequency- electromagnetic magnetic fields (ELF-EMFs) at power line-frequencies (50-60 Hz). Interest in the health effects of ELF-EMFs was rekindled by a series of epidemiological studies done during the late 1970s and early 1980s (JAUCHEM

& MERRITT, 1991). Therefore, the potential hazards of exposure to ELF-EMFs are discussed in several epidemiological studies (AHLBOM et al. 2001, PEARCE et al. 2007, HUG et al. 2010).

There are many biological radiation targets, however the DNA molecule has received the greatest attention with respect to EMF damage, because of its relevance for cell function, proliferation, viability, mutation and cancer (BURDAK-ROTHKAMM et al. 2009, FOCKE et al. 2010, CIFRA et al. 2011, ZHU et al. 2016).

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A review by VIJAYALAXMI & OBE (2005) concluded that 22% of previous studies of ELF-EMF-induced genotoxicity indicated a genotoxic effect whereas 46% did not and 32% of the studies were inconclusive. The controversy about ELF-EMF genotoxic effects derives from the fact that many scientists believe ELF-EMF devices and power lines emit little energy and are therefore too weak to have any effect on cells. Also, the inconclusive nature of laboratory experiments and variability turns this concern very difficult. However, in 2002, the International Agency for Research on Cancer (IARC 2002) categorized extremely low frequency magnetic fields as “possibly carcinogenic to humans”; that was based on pooled analyses of epidemiological research that reported an association between exposure to low-level magnetic fields and several types of cancer.

Recently, DOMINICI et al. (2011) found a significantly higher frequency of micronucleus (MN) in human blood cells from welders that are occupationally exposed to ELF-EMF. Moreover, the increase in MN frequency was associated with a proportional increase in ELF-EMF exposure levels with a dose-response relationship. Earlier, YAGUCHI et al. (1999, 2000) demonstrated that exposure to ELF-EMF magnetic fields at 5, 50, and 400 mT could induce chromosomal aberrations or sister chromatid exchanges in mouse m5S cells. Also, LAI and SINGH (1997) found evidence of genotoxic effects attributed to these fields. They reported that a 2 h exposure of rats to a 60 Hz magnetic field at 0.1, 0.25 and 0.5 mT of magnetic flux densities caused a dose-dependent increase in DNA strand breaks in brain cells, indicating a clastogenic effect.

With regard to large exposures, a study of RAGEH et al. (2012) showed a significant four folds increase in the induction of bone marrow MN when newborn rats were exposed continuously to 50 Hz and 0.5 mT magnetic fields for 30 days, suggesting an association between DNA damage and ELF-EMF exposure. However, opposite results were obtained by ABRAMSSON-ZETTERBERG & GRAWÉ (2001) who found that an extended exposure of 18 days to 50 Hz, 14 μ T magnetic fields did not significantly change the frequency of micronucleated erythrocytes of adult and foetal mice.

Also, static magnetic fields (SMFs) have been studied as genotoxic agents as assessed by MN test. SUZUKI et al. (2001) observed a significant increased MN frequency in bone marrow cells of mice exposed *in vivo* to 3.0 and 4.7 T of SMF. They concluded that the increase of MN frequency was dose dependent. On the contrary, FATAHI et al. (2016) found no alteration in human blood lymphocyte MN frequency

after an *in vivo* and *in vitro* exposure to 7 T magnetic resonance imaging machine.

In view of these conflicting results, and the contradictory reports in the literature, we have undertaken the current study to further evaluate the possible genotoxic effect of 60 Hz sinusoidal magnetic fields on bone marrow cells of whole exposed mice by means of the micronucleus test.

Materials and Methods

Animals. Sexually mature, 12-week-old male BALB/c mice (25-30 g) were used. Animals were born and raised in our breeding colony. After a 10 day quarantine period, animals were then randomly distributed into experimental (ELF-EMF exposed) and control groups. This research project fulfilled all requirements of the University's Animal Care and Use for Research Protocol, which is based on the National Guidelines for Ethics and Biosafety under the General Law of Health for issues regarding Health Research, Ministry of Health, México City.

Treatment protocol. Three experimental conditions were arranged, giving a total of seven independent experiments. They included: (1) acute exposure of 72 h with three intensities of 60 Hz magnetic fields at 1.0, 1.5, and 2.0 mT, (2) expanded exposure of 10 days/8 h daily with three replications, and (3) a combined treatment of 72 h exposure to ELF-EMFs at 60 Hz and 2.0 mT plus 5 mg/kg of Mitomycin-C (a well-known genotoxic agent). For acute exposures, in all cases a positive control of MMC as a reference of clastogenic effect was used. The evaluated cytological endpoint included the frequency of micronucleated polychromatic erythrocytes in bone marrow. For acute exposures, twelve animals were used for each treatment regimen and controls. For expanded exposure, a total of six animals were used for each treatment and controls. Three independent experiments were done for this expanded exposure, giving a total of 18 animals analyzed. Following the recommendations from VIJAYALAXMI & OBE (2005) and due to the inconclusive nature of laboratory experiments and variability, we decided to perform three independent experiments at three different times and in three different laboratories: (A) our own, at Department of Exact Sciences and Human Development at Biological Sciences School, Universidad Autónoma de Nuevo León, México, (B) Pathology Department at the Medicine School, Universidad Autónoma de Nuevo León, México, and (C) Biomedicine Institute at the Instituto Mexicano del Seguro Social, México. For acute exposures, an exposure time of 72 h was chosen because it is

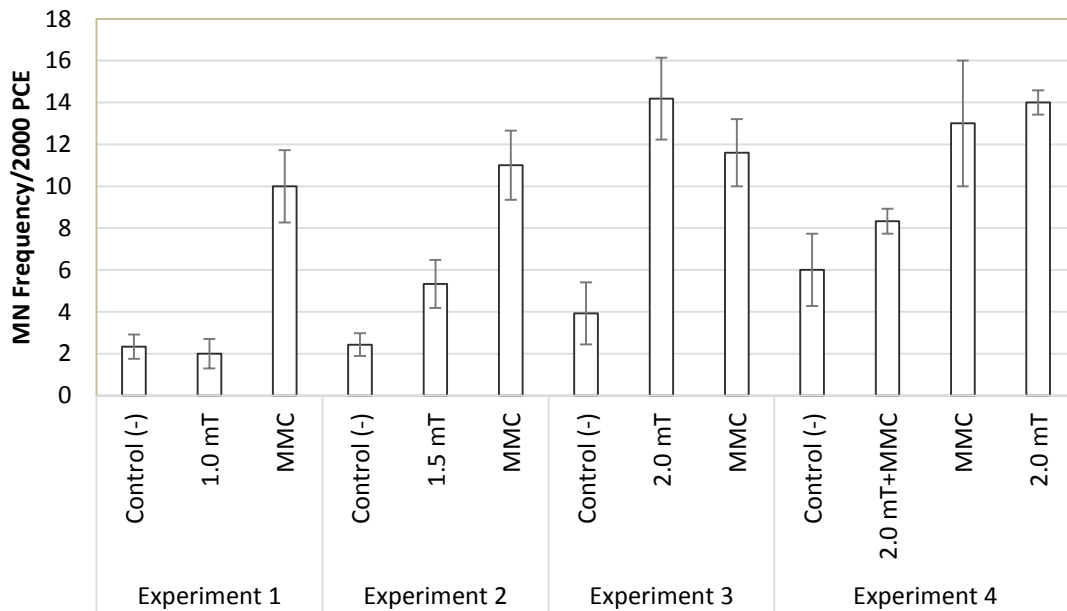


Fig. 1. Effect of ELF-EMF on frequency of MN/2000 PCEs

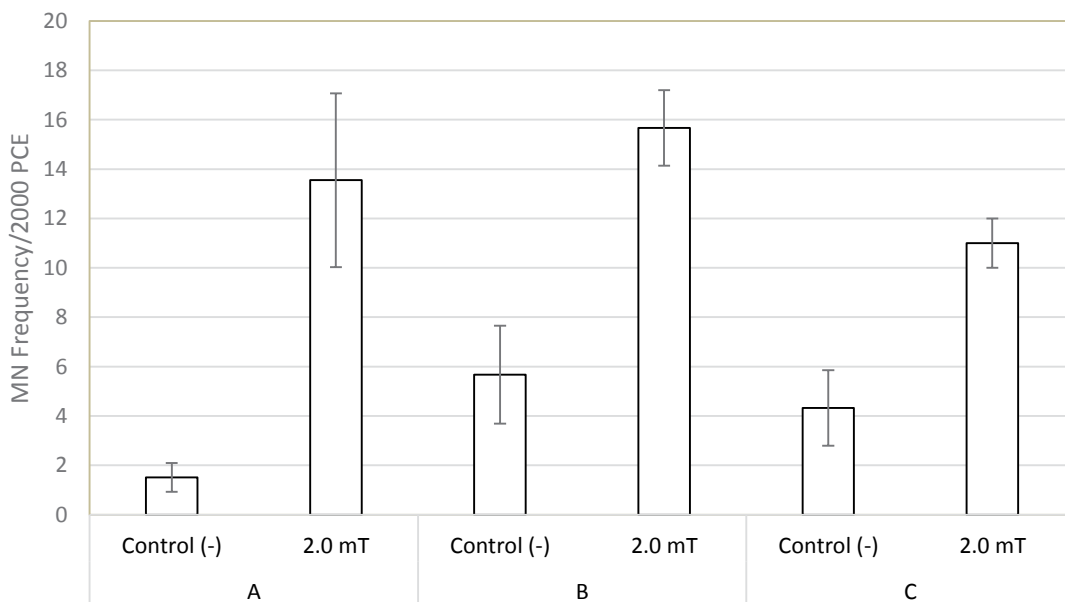


Fig.2. Effect of 10 days/8h daily ELF-EMF exposure on frequency of MN/2000 PCEs in bone marrow cells of mice

generally accepted that the period of differentiation from stem cells to mature erythrocytes in mice is ≈ 72 h (FILMANOWICZ & GURNEY, 1961). In the case of the expanded exposure (10 days/8 h daily), a labour criterion was adopted.

Electromagnetic field exposure conditions. A standardized magnetic field exposure facility of identical characteristics as one used in our previous works (HEREDIA-ROJAS et al. 2004; RODRÍGUEZ-DE LA FUENTE et al. 2012) was used. A coil was built by winding 552 turns of 1.3 mm diameter enamel insulated copper wire to form a cylindrical solenoid with a radius

of 13.5 cm and a length of 71 cm. This solenoid was connected to a step-down transformer and to a variable transformer that was plugged in to a 110 V AC (alternating current) source. Animals were allocated in the middle of this solenoid where the EMF was homogeneous, and kept at a temperature of $24 \pm 0.1^\circ\text{C}$ and 40% humidity. An equal number of sham-treated animals were used as controls and were placed in the same room into an EMF device of identical design as the one mentioned above, but it was turned off.

Magnetic flux density (rms) was measured using an axial Hall-effect probe (Bell FW 6010

Gaussmeter, Orlando, FL, USA). An oscilloscope (BK-Precision model 2120) was coupled to the system to monitor the resulting field. A 60 Hz alternating sinusoidal EMF was then generated. The EMF frequency content was nearly pure 60 Hz (<3% total harmonic distortion). The local temperature, inside the solenoids with baffles present but without animals, was measured setting the Bell FW 610 Gaussmeter in Temp mode. The temperature value average was 24.0 ± 0.103 when the field was on and $23.9 \pm 0.109^\circ\text{C}$ without current in the coils; no statistically significant differences were observed between two solenoids (Kolmogorov-Smirnov test for normality, followed by paired t-test).

In order to keep the geometry of exposure, a plastic separator was placed inside the solenoid to allow the placement of uncaged mice in predetermined zones where the rms value of the EMF was 1.0, 1.5 and 2.0 mT. A food container and a water bottle were also placed inside these compartments. Water and standard diet were offered *ad libitum*.

The electromagnetic field ambient background level was $<0.4\mu\text{T}$. Moreover, the local geomagnetic field was also measured, setting the Gaussmeter in DC mode and by using an axial high sensitivity Hall probe (Integrity Design IDR-321 geomagnetometer, Essex Jct., VT). The average value was $20\mu\text{T}$ within the exposure room.

Micronucleus test. Animals were sacrificed at the end of exposure period. Following sacrifice, the frequency of micronucleated erythrocytes in bone marrow was evaluated according to the procedure of SCHMIDT (1976). In brief, bone marrow from both femurs was flushed with 2.0 ml of foetal calf serum into a microfuge tube using a 1ml syringe fitted with a 22 G needle. The cells were concentrated by gentle centrifugation at 500g for 10 min and the supernatant was discarded. The pellet was re-suspended in a small amount of foetal calf serum and spread on clean microscope slides. Air dried smears were stained with 5% May-Grünwald-Giemsa for 12-15 min.

Coded slides were examined under X1000 magnification using a Leica DM2500 light microscope. For each animal, 2000 consecutive polychromatic erythrocytes (PCE) were scored to determine the frequency of MN. Decoding of the slides was done after completing the microscopic analysis.

2.5 Statistical analysis. The normality of the data was determined by the Kolmogorov-Smirnov test ($p<0.05$). The statistical differences were calculated among groups by using analysis of variance for normal distributions, and the correspondent parametric Tukey test for establishing individual differences. All analyses were done using the SPSS

package version 22.0. Differences were considered to be significant when the probability values were lower than 0.05.

Results

The present study aimed to evaluate the association between whole body exposure to ELF-EMFs and genotoxic hazard in bone marrow cells of mice. Three magnetic strengths of 1.0, 1.5, and 2.0 mT, and a combined treatment of 2.0 mT and 5mg/kg of Mitomycin-C were tested for MN frequency. Furthermore, the MN frequency was evaluated for an expanded (10 days/8 h daily) exposure condition. Although the diversity of biological effects attributed to ELF-EMF is large, we chose to confine our *in vivo* experimental investigation to a single parameter; the frequency of MN as a genotoxic effect.

Fig. 1 shows the MN frequencies of four independent experiments. An increased MN frequency in 1.5 and 2.0 mT, 72 h-exposed animals was observed ($p<0.05$), whereas no variation in MN frequency was found for mice exposed to ELF-EMF, 72 h at 1.0 mT. Animals treated with 5mg/kg of Mitomycin-C showed higher MN frequencies when compared to negative controls, as expected. In addition, figure 1 also illustrates the genotoxic potential of ELF-EMF combined with the mutagen MMC (experiment 4). An opposite effect was observed when animals were co-exposed simultaneously to magnetic fields at 2.0 mT and 5 mg/kg MMC. These animals showed, in spite of the genotoxicity of the mutagen, a lower MN frequency when compared to animals treated with MMC alone ($p<0.05$).

On the other hand, Fig. 2 shows the MN frequency from an expanded 10 days/8 h daily at 2.0 mT exposure condition of three independent experiments. In all cases, a statistically significant increased MN frequency was observed in ELF-EMF exposed animals compared to their controls.

Discussion

Environmental exposure to ELF-EMFs generated by power lines and by domestic and industrial equipment is quite ubiquitous, especially for populations living in developed countries. Many researchers agree that life bodies could be adversely affected by exposure to this non-ionizing electromagnetic radiation (FEYTCING et al. 2005; BOWMAN et al. 2013). In the present study, we found evidence of a genotoxic effect induced by an *in vivo* exposure to 60 Hz at 1.5 and 2.0 mT magnetic fields on mice bone marrow cells. These results coincided with previous re-

ports that used the MN assay, indicating a genotoxic effect due to magnetic field exposure (SIMKÓ et al. 1998; CELIKLER et al. 2009). By the way, WINKER et al. (2005) claimed for a clastogenic potential of intermittent low-frequency EMFs, which may lead to considerable chromosomal damage in dividing human diploid fibroblasts. Furthermore, ERDAL et al. (2007) found an increased MN frequency in Wistar rat tibial bone marrow cells treated with a long term extremely low-frequency EMFs exposure, compared to non-exposed and acutely exposed animals. RAGEH et al. (2012) observed an increased MN frequency in bone marrow cells of new born rats indicating a clastogenic effect due to magnetic field exposure. More recently, UDROIU et al. (2015) found slight genotoxic damage when mice were exposed to 50 Hz, 65 μ T magnetic field, 24 h/day, for a total of 30 days, starting from 12 days post-conception as assessed by the MN test. On the contrary, there are several studies that indicate no genotoxic or cytotoxic effects attributed to magnetic field exposure (SCARFI et al. 1994, 1999, FRAHM et al. 2006; OKUDAN et al. 2010). Recently, ZHU et al. (2016) reported an absence of genotoxicity in a very sensitive model of human lens epithelial cells *in vitro*, examined by alkaline comet assay after an exposition to 0.4 mT ELF-EMF.

The controversy about ELF-EMF cytotoxic or genotoxic effects derives from the fact that many scientists believe ELF-EMF devices emit little energy and are therefore too weak to have any effect on cells. Also, the inconclusive nature of laboratory experiments turns this concern very difficult.

In the current study we did not find alterations in MN frequency when animals were exposed to 1.0 mT of magnetic flux density compared to their controls. However, earlier KHALKIL & QASSEM (1991) reported a statistically significant incidence of chromosomal aberrations when cultured human lymphocytes were exposed to a very similar intensity of 1.05 mT of pulsing 50 Hz magnetic fields. This magnetic field strength of 1.0 mT coincided with the threshold limit value (TLV) established for ELF-EMF since 1994 by the American Conference of Governmental Industrial Hygienists (ACGIH 1994). Later, in a study conducted by McMAHAN et al. (2002) there was a large discussion on this topic, and taking in account the opinion of scientists and other risk experts, they concluded that TLV for 60 Hz magnetic fields should be revised to be made stricter. Nevertheless, recently it has been also established that ELF-EMF exposure levels reaching 1.0 mT not induce breakage of DNA strands. Moreover, the DNA repair system is not perturbed by this exposure strength (LAMBROZO & SOUQUES 2012). Furthermore,

it was observed that ELF-EMF exposure at 1.0 mT and 60 Hz did not enhance the MN frequency in mouse embryonic fibroblast NIH3T3 cells and human lung fibroblast WI-38 cells (JIN et al. 2012).

Regarding the issue that weak fields may have too little energy to cause genotoxic effect or DNA damage, it has been proposed that because low frequency electromagnetic radiation does not transmit enough energy to affect chemical bonds, it is generally accepted that ELF-EMFs are not capable of damaging the DNA directly (LUCERI et al. 2005). Nevertheless, several hypotheses have been put forward of how EMFs might affect the structure of DNA indirectly. Secondary currents and, hence, a movement of electrons in DNA might be induced (VALBERG et al. 1997). This may generate guanine radicals, which upon reaction with water may be converted to oxidative DNA damage (GIESE 2006). In a recent study, FOCKE et al. (2010) observed that exposure of human primary fibroblasts to a 50 Hz EMF induced a slight but significant increase of DNA fragmentation tested by the Comet assay. Moreover, they showed that EMF-induced responses in the Comet assay were dependent on cell proliferation, suggesting that processes of DNA replication rather than the DNA itself may be affected.

On the other hand, our results showed an antagonistic effect between ELF-EMF exposure and Mitomycin-C in terms of MN frequency. There are many reports attempting to investigate the possibility of antagonistic or synergistic effect by co-exposure conditions for a variety of radiations, chemicals and cytological endpoints. We have previously observed the same antagonistic effect between magnetic field exposure and Mitomycin-C when cell kinetics and mitotic rate were analyzed in human lymphocytes that were treated with magnetic fields of identical characteristics of those used here (HEREDIA-ROJAS et al. 2001) and also for chromosomal aberrations and sperm morphology in germ cells of mice (HEREDIA-ROJAS et al. 2004), at the same co-exposure conditions used in the present study. On the contrary, *in vitro* investigations of KERBACHER et al. (1990) and MELTZ et al. (1990) on different cell systems, provided evidence for a lack of an antagonistic or synergistic effect between continuous and pulsed microwaves at different power densities and Mitomycin-C, Adriamycin and Proflavin. CIARAVINO et al. (1991) also found no antagonistic or synergistic effect between moderated-power radio frequency electromagnetic radiation and Adriamycin on cell cycle progression. On the other hand, several studies have reported synergistic effects. CHEKHUN et al. (2013) reported an enhancement of genotoxicity of cisplatin

nanocomposite combined with an exposure to magnetic fields in MCF-7 human breast cancer cells determined by MN test and Comet assay. By the way, YOON et al. (2014) found that a 2.0 mT ELF-EMF exposure potentiated the expression of γ -H2AX and γ -H2A foci production when combined with ionizing radiation, but not when combined with hydrogen peroxide in non-tumorigenic human cell lines. More recently, XU et al. (2015) observed a synergistic inhibitory effect of static magnetic fields combined with antitumor drugs. They demonstrated that the growth of Hepa 1-6 cells treated with the static magnetic fields combined with cisplatin or adriamycin was significantly inhibited.

In conclusion, the present *in vivo* study suggests that 60-Hz magnetic fields can induce a clas-

togenic effect in mice after a magnetic field exposure at 1.5 and 2.0mT. In addition, an antagonistic effect between ELF-EMF and MMC, in terms of MN frequency was observed. Nevertheless, we believe that further experiments are required, recruiting more DNA damage endpoints i.e. Comet assay and chromosomal aberrations to corroborate and definitely resolve the controversy concerning the possible genotoxic risk associated with magnetic fields.

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