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CYTOLOGICAL EFFECTS OF PULSED ELECTROMAGNETIC FIELDS AND STATIC MAGNETIC FIELDS INDUCED BY A THERAPEUTIC DEVICE ON *IN VIVO* EXPOSED RATS.

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Abstract

Background: There is a trend towards the use of magnetic fields in medicine. Pulsed electromagnetic fields (PEMFs) technology was based upon 20 years of fundamental studies on the electromechanical properties of bone and other connective tissues. More recently, these magnetic fields have been used to treat several health conditions. There remains continuing concern that exposure to electromagnetic devices may cause adverse effects. The aim of the present study was to investigate the cytological effects induced in rats exposed in a patented medical device that uses PEMFs combined with static magnetic fields (SMFs).
Material and Methods: Thirty sexually mature 14-week-old male and female Sprague Dawley rats were distributed into three groups: (a) 5 males and 5 females (independently) exposed to PEMFs combined with SMFs, (b) animals treated with SMFs only, and (c) non-exposed animals. Acridine orange fluorescent-staining micronucleus test and male germ cells analysis were performed according to standardized techniques.
Results: A lack of evidence for alterations on micronucleus frequency, on polychromatic erythrocytes percentage, and on sperm counts and morphological characteristics of male germ cells were found in mature rats exposed to PEMFs medical device compared to non-exposed animals.
Conclusions: This study suggests that the applied magnetic field generated in a therapeutic device did not have any detectable cytotoxic or genotoxic effect in exposed rats. In view of these findings and the contradictory reports in the literature, it is necessary to carry out more research to help clarify the controversy concerning cytogenotoxic risk associated with therapeutic magnetic fields exposures.

Key words: Cytotoxicity, pulsed electromagnetic fields, static magnetic fields, micronuclei, sperm abnormalities.

Introduction

Electromagnetic fields (EMFs) have been used therapeutically, and have also been correlated with adverse health effects. The idea that electric and magnetic fields can be used for therapeutic purposes has existed since long before they were understood or were controllable. Although the biological effects of low-frequency electromagnetic radiation have been studied for many years, there is still no consensus on whether these effects are physiologically significant (Macklis, 1993; Miller and Green, 2010; Binhi, 2012).

Interest in the health effects of low-frequency EMFs was rekindled by a series of epidemiological studies done during the late 1970s and early 1980s (Jauchem and Merritt, 1991). By the way, Milham (1985) analyzed the occupational grouping of cancer deaths in adult white men who died between 1950 and 1982; an increased proportionate mortality ratio for leukemia and for non-Hodgkin's lymphoma was found. More recent studies in adult human beings have suggested increased occupationally associated EMFs risks for breast cancer, abnormal pregnancies, chromosomal abnormalities, congenital deformities, and several other health hazards (Ahlbom *et al*., 2001; Pearce *et al*., 2007; Hug *et al*., 2010). There is no doubt that bio-systems can be affected by EMFs at several levels. Actually, these fields are the most probable candidate to affect cellular interactions and it is possible to consider a diversity of cellular effects induced by electric and magnetic fields (Cifra *et al*., 2011).

Selected low-energy, time varying pulsed electromagnetic fields (PEMFs) have been used for the past 40 years to treat therapeutically resistant problems of the musculoskeletal system. Recently, diverse approaches using PEMFs technology were tested for therapeutic effects in several types of cancer (Rollan Haro *et al*., 2005; Grosel *et al*., 2006) with promising results.

Regarding the issue of potential genotoxic and cytotoxic effects of EMFs, there are many commercial machines that use several types of magnetic fields that are unproven or have not been properly tested. In view of this, we have undertaken the present study to further evaluate the cytological effects induced in rats exposed in a patented medical device (US patent number 6,235,251 B1). This machine uses pulsed electromagnetic fields (PEMFs) and static magnetic fields (SMFs). Although the diversity of cytological effects attributed to EMFs is large, we chose to confine our *in vivo* experimental investigation to single parameters, i.e., the analysis of micronuclei in bone marrow, sperm analysis, and morphological characteristics of male germ cells in exposed Sprague-Dawley rats.

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Materials and Methods

Animals

Thirty sexually mature 14-week-old, male and female Sprague-Dawley rats were used, weighing 250-300 g for females, and 400-450g for males. Animals were born and raised in our breeding colony. After a 10-day quarantine period, animals were then randomly distributed into experimental (exposed) and control groups. At the time of experiments, the animals were housed at 25º C in standardized acrylic cages (five animals per cage) under a 12-12 h light/dark cycle, with free access to standard diet and tap water. This research project fulfilled all requirements of the principles for the Care and Use of Animals in Research and Teaching adopted by the American Physiological Society (APS), based upon humane care principles and approved by the APS Council on July 16, 2010.

Magnetic Field Exposure Facilities and Measurements

Magnetic fields were produced by a patented machine that combines PEMFs and SMFs exposure (US patent 6,235,251 B1). In brief, this device comprised permanent neodymium magnets arranged so that each magnet being adjacent to the magnetic north pole and magnetic south pole of an adjacent magnet respectively. Thus, the plurality of these magnets forms a ring of permanent magnets. The device further includes an electrically conductive wire wound substantially around the ring, and tubing wrapped around the ring of permanent magnets between windings of the wire. A cooling device introduces a flow of coolant through the tubing. This machine also includes a control circuit, connected to the wire, for selectively generating a coil current for passing through the wire.

Magnetic flux density (rms) was measured in the middle of the ring where the magnetic fields were homogeneous, by using an axial Hall-effect probe (Bell FW 6010 teslameter, Orlando Fl.) An oscilloscope (BK-Precision model 2120) was coupled to the system to monitor the resultant field. A pulsed 120 Hz square waveform electromagnetic field was then generated, with a maximum peak of 17.6 mT (rms), at the center of the exposure zone where cages were allocated. The waveform was sketched along with the measured fields. On the other hand, the static magnetic fields were measured with the aid of the teslameter, setting the apparatus in DC mode; this value was a maximum peak of 8.6 mT at the center of the exposure zone. The local geomagnetic field was measured by using an axial high sensitivity Hall probe (Integrity Design IDR-321 geo-magnetometer, Essex Jct., VT9); and the average value was 20 µT within the exposure room. Moreover, the local temperature in the exposure zone was measured setting the Bell FW 6010 teslameter in Temp mode. The temperature value was an average of 25.3 ± 0.5 ºC when the variable field was on and 24.9 ± 0.3 ºC without current in the coil: no statistically significant differences were observed between two conditions (Kolmogorov-Smirnov test for normality, followed by paired *t*-test).

Experimental Design

Three groups were considered for bioassays: (*a*) 5 male and 5 female rats (independently), exposed to 120 Hz PEMFs combined with SMFs for three consecutive days, two exposures of 50 min each with no-exposure intervals of 1 h between exposures, (*b*) 5 male and 5 female rats (independently), exposed only to SMFs, without current in the coil, and (*c*) non-exposed animals, including 5 males and 5 females without any magnetic treatment. These exposure schedules were included considering the fact that these schedules are the ones which device's manufacturer suggests for treating several health conditions in humans. During the no-exposure intervals, and for non-exposed animals, the cages containing the animals were placed in the same exposure room where any detectable EMF was measured. In the case of micronuclei analysis, both male and female animals were used. For germ-cell tests, only males were analyzed.

Micronucleus Assay

Animals were sacrificed at the end of exposure period. Following sacrifice, Acridine Orange fluorescent staining was applied to the micronucleus (MN) test, according to the original technique (Hayashi *et al*., 1983). In brief, bone marrow from both femurs was flushed with 0.5 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 600x *g* for 1-3 min and a small drop of re-suspended cells was placed on a clean microscope slide to make a thin smear. All smears were air-dried, fixed in absolute methanol and stained using Acridine Organge (Fisher Sci. Co., Fair Lawn, NY).

Coded slides were examined under X1000 magnification using a fluorescence microscope equipped with appropriate filters. Immature, polychromatic erythrocytes (PCE) were identified by their orange-red color, mature erythrocytes by their green color and the MN by their yellowish color. For each rat, 500 erythrocytes were examined to obtain the percentage of PCE. In addition, for each rat, 2000 consecutive PCE were examined to determine the incidence of MN. Decoding of the slides was done after completing the microscopic analysis.

Male Germ Cells Analysis

For sperm counts, orchiectomy was performed by open castration method. A midline or pre-scrotal incision was made and the testes were milked out on the incision site. The testicles were then exposed by incising the *tunica vaginalis*. The spermatic cord was exposed, ligated and incised. Semen samples were thereafter collected from the cauda epididymis according to a previously described procedure (Oyeyemi and Ubiogoro, 2005). The samples were analyzed immediately after collection. The spermatozoa were counted by hemocytometer using a Neubauer (Deep 1/10mm, LABART Germany) chamber, as described by Pant and Srivastava (2003).

For sperm morphology analysis, smears were prepared from epididymis following the previously described method of Wyrobeck (1979). Spermatozoa preparations were stained using 1% Eosin Y die/1 h (Fisher Sci. Co., Fair Lawn, NY) and examined under high power magnification (X1000). One hundred cells per slide, 10 smears for each animal, yielding a total of 5000 cells per group, were evaluated in a blind way, for the presence of bi-cephalic or biflagellate forms, and abnormalities in head shape such as enlarged and amorphous head were scored and expressed as a percentage of sperm morphology abnormalities.

All chemicals were supplied by Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

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Statistical Analysis

The statistical differences were calculated among groups by using analysis of variance for normal distributions. In the case of the percentage of sperm morphology abnormalities, the data obtained were first transformed by using the arcsine function. After that, an analysis of variance for normal distributions and the correspondent parametric Tukey test for establishing individual differences were performed; the normality of the data was determined by the Kolmogorov-Smirnov test (p<0.05). All analyses were done using the SPSS package version 15.0. Differences were considered to be significant when the probability values were lower than 0.05.

Results

The design of the present study evaluated the cytotoxic and genotoxic effects of magnetic fields exposure generated in a patented therapeutic machine that combines PEMFs and SMFs exposure. Both male and female Sprague-Dawley mature rats were analyzed for the micro-nucleated polychromatic erythrocyte frequency and PCE percentages. On the other hand, sperm counts and sperm morphology abnormality percentages were evaluated in male rats. Figure 1 shows the obtained results for the frequency of micronuclei from bone marrow of exposed rats. No differences were found among groups in both, (independently exposed) male and female animals, indicating no clastogenic effect induced by exposure to PEMFs or SMFs (p> 0.05). By the way, Figure 2 shows the percentages (grouped means) of polychromatic erythrocytes (PCE) from three analyzed groups. These values showed no statistically significant differences among groups, for male and female animals (p>0.05). These results indicated that immature erythrocytes (PCE) were no altered by exposure to both PEMFs and SMFs.

Regarding the germ cells analysis, no alterations were found in either the sperm counts (Fig. 3), and morphological characteristics of spermatic cells (Fig. 4) when compared the grouped means among groups (p>0.05).

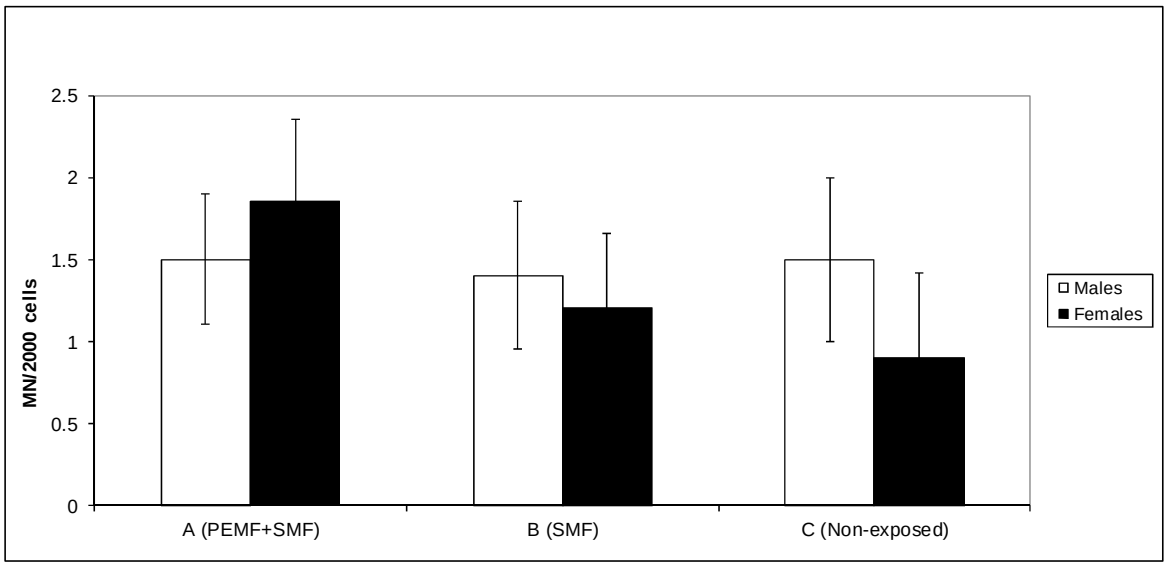

Figure 1: Frequency of micronuclei (MN) from bone marrow polychromatic erythrocytes of Sprague Dawley mature rats. (a) animals exposed to 120 Hz PEMFs combined with SMFs for three consecutive days, two exposures of 50 min each with no-exposure intervals of 1 h between exposures, (b) rats exposed only to SMFs, without current in the coil, and (c) non-exposed animals, including 5 male and 5 female rats without any magnetic treatment. No differences were found among groups in both male and female animals, indicating no clastogenic effect induced by exposure to PEMFs or SMFs (p> 0.05). Bars represent grouped means ± S.D.

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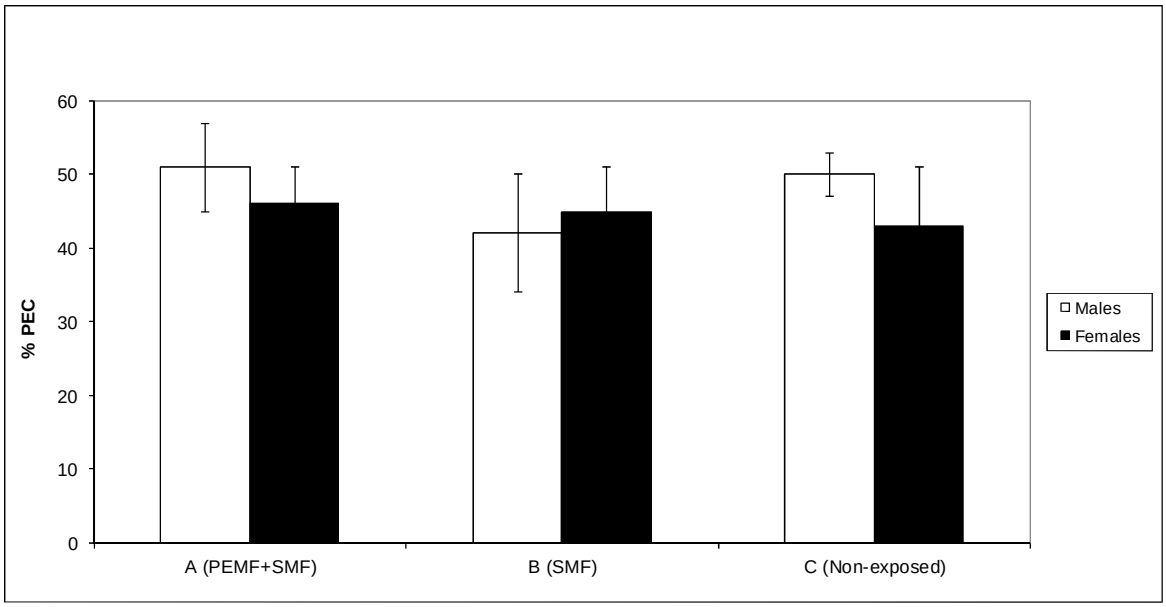

Figure 2: Percentage of polychromatic erythrocytes from bone marrow of Sprague Dawley mature rats. (a) animals exposed to 120 Hz PEMFs combined with SMFs for three consecutive days, two exposures of 50 min each with no-exposure intervals of 1 h between exposures, (b) rats exposed only to SMFs, without current in the coil, and (c) non-exposed animals, including 5 male and 5 female rats without any magnetic treatment. No differences were found among groups in both male and female animals, indicating no alteration on immature erythrocytes due to exposure to PEMFs or SMFs ($p > 0.05$). Bars represent grouped means ± S.D.

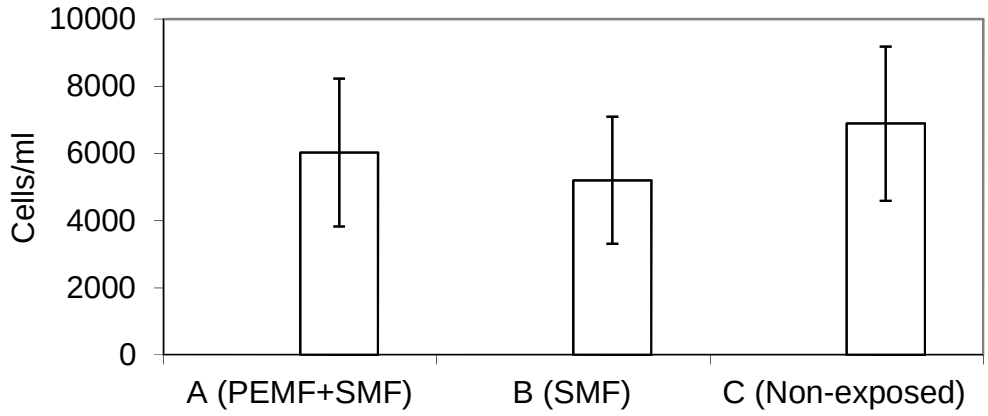

Figure 3: Effect of magnetic field exposure on sperm counts of Sprague Dawley mature rats. (a) animals exposed to 120 Hz PEMFs combined with SMFs for three consecutive days, two exposures of 50 min each with no-exposure intervals of 1 h between exposures, (b) rats exposed only to SMFs, without current in the coil, and (c) non-exposed animals, including 5 male rats without any magnetic treatment. No differences were found among groups, indicating no alteration on spermatozoa number due to exposure to PEMFs or SMFs ($p > 0.05$). Bars represent grouped means ± S.D.

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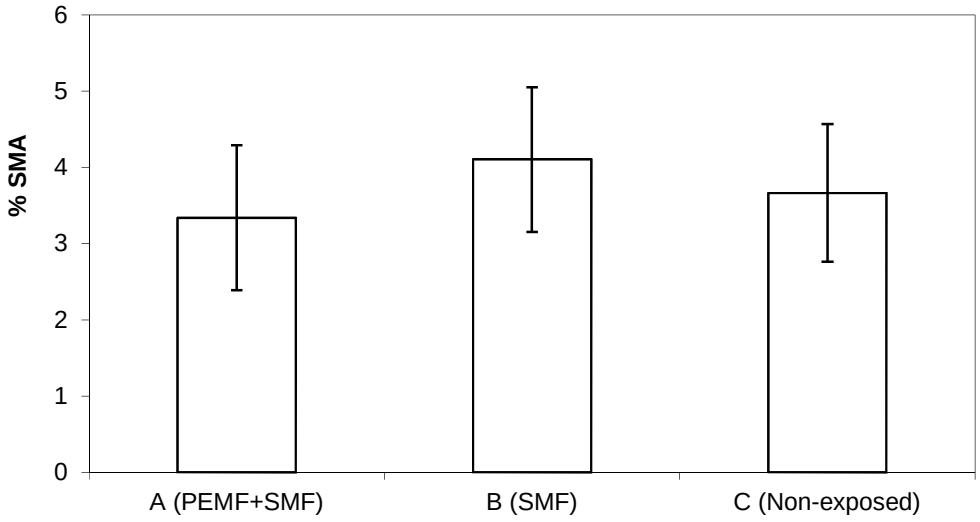

Figure 4: Percentage of sperm morphology abnormalities (SMA) from male germ cells of Sprague Dawley mature rats. (a) animals exposed to 120 Hz PEMFs combined with SMFs for three consecutive days, two exposures of 50 min each with no-exposure intervals of 1 h between exposures, (b) rats exposed only to SMFs, without current in the coil, and (c) non-exposed animals, including 5 male rats without any magnetic treatment. No differences were found among groups ($p > 0.05$). Bars represent grouped means ± S.D.

Discussion

Medical and biological effects of magnetic fields have been widely discussed during recent years. The question has been raised as to whether exposure to such fields causes genetic damage or other biological effects. On the other hand, since the early part of the twentieth century, attempts had been undertaken to treat or otherwise cure human ailments using magnetic field treatments. However, many researchers agree that life bodies could be adversely affected by exposure to electromagnetic radiation (Feychting *et al*., 2005). In the present study, we did not find any detectable genotoxic or cytotoxic effect induced in rats by an *in vivo* exposure to a pulsed-electromagnetic field therapeutic device.

Data showed no modifications in micronuclei frequency and in PCE percentages of exposed animals. These results coincided with previous reports that used the MN assay, indicating no genotoxic or cytotoxic effects attributed to magnetic field exposure (Scarfi *et al*., 1994; Scarfi *et al*., 1999; Frahm *et al*., 2006; Okudan *et al*., 2010). Furthermore, it was observed that extremely-low frequency EMF exposure at 1.0 mT and 60 Hz did not enhance MN frequency by ionizing radiation in mouse embryonic fibroblast NIH3T3 cells (Jin *et al*., 2012). On the contrary, there are several reports with positive results suggesting 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.

The controversy about EMF cytotoxic or genotoxic effects derives from the fact that many scientists believe EMF medical devices emit little energy and are therefore too weak to have any effect on cells. Also, the inconclusive nature of laboratory experiments and the fact that there are no epidemiological studies of people exposed to such therapeutic machines 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.

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 extremely low frequency-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 at a flux density of 1.0 mT 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, the data obtained in this investigation suggest that exposure to pulsed EMFs or SMFs produced by the therapeutic device had no influence on sperm cells of exposed rats compared with the controls. The sperm counts were no altered by exposure, indicating no effect in cell cycle progression. These results agreed with those of Lundsberg *et al*. (1995) who found no association of occupational EMF exposure on sperm concentration among males. Even with higher frequency electromagnetic radiation, Aitken *et al*. (2005) observed that sperm number, morphology and vitality of the male germ line were not significantly affected when mice were treated with 900 MHz radio frequency electromagnetic radiation. In contrast, Furuya *et al*. (1998) found that the application of ELF-EMFs at 50 Hz with intensities ranging from 1.0

mT up to 100 mT affected the proliferative/differentiative capacity of mouse spermatogonia. Moreover, deleterious effects on spermatogenesis in mice exposed to a 1.5 T static magnetic field were reported by Narra *et al*. (1996). More recently, Ramadan *et al*. (2002) reported that exposure of mice to fractionated doses of high oscillating magnetic fields (20mT) caused a significant decrease in sperm count, and daily sperm production. Similarly, a significant reduction in sperm count was observed in adult Sprague-Dawley rats exposed to a 50 Hz and 25μT for 18 consecutive weeks (Al-Akhras *et al*., 2006) Furthermore, Hong *et al*. (2005) observed that 50 Hz EMFs may have the potential to induce DNA strand breakage in testicular cells and sperm chromatin condensation in exposed mice.

In regard to sperm morphology abnormalities, our results are related to those of Withers *et al*. (1985) who did not find alterations in sperm heads; however they exposed mice to 0.3 T static magnetic fields from a magnetic resonance device. By the way, Tablado *et al*. (1998) found that the size of sperm heads and the percentage of sperm with coiled tails or of sperm with abnormal mid-piece or tail were unaffected in mice exposed to a 0.7 T commercial permanent magnet. We have previously reported the lack of alterations on morphological characteristics of male germ cells in mice exposed to a 60 Hz and 2.0 mT magnetic fields (Heredia-Rojas *et al*., 2004). In contrast, Roychoudhury *et al*. (2009) found alterations of spermatozoa and fertilization rates in rabbits exposed to 50 Hz magnetic fields. For humans, it is accepted that there is evidence demonstrating that magnetic field exposure may have adverse effects on sperm quality (Li *et al*., 2010).

On the other hand, conflicting results have been obtained by Lorio *et al*. (2007) who observed significant increases in the values of kinematic parameters of spermatozoa after being exposed to an ELF-EMF with a square waveform of 5 mT and 50 Hz. On the contrary, a 5 mT sine wave (50 Hz) and a 2.5mT square wave (50 Hz) exposure did not produce any significant effect on sperm motility. These findings indicate that ELF-EMF exposure can improve spermatozoa motility, and that this effect depends on the field characteristics.

In conclusion, this *in vivo* study suggests that the applied magnetic fields generated in a therapeutic device did not have any detectable effect on MN frequency and PCE percentages of exposed rats and on sperm counts and morphological characteristics of male germ cells. However, with the results presented here, we are not supporting any therapeutic technique nor recommending the mentioned therapeutic device, rather we showed evidence for a lack of cytological effects induced by PEMFs and SMFs on exposed rats. In view of these findings, and the contradictory reports in the literature, it is necessary to carry out more research using various cell types and cytological endpoints under different experimental conditions to help clarify the controversy concerning the possible cytotoxic and genotoxic risk associated with a therapeutic magnetic field exposure.

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