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Clastogenicity and aneuploidy in newborn and adult mice exposed to 50 Hz magnetic fields

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Abstract

Purpose: To detect possible clastogenic and aneugenic properties of a 50 Hz, 650 μT magnetic field.

Materials and methods: The micronucleus test with CREST (Calcinosis, Raynaud's phenomenon, Esophageal dismotility, Sclerodactility, Telangectasia) antibody staining was performed on liver and peripheral blood sampled from newborn mice exposed to an ELF (Extremely Low Frequency) magnetic field during the whole intra-uterine life (21 days), and on bone marrow and peripheral blood sampled from adult mice exposed to the same magnetic field for the same period.

Results: Data obtained in newborn mice show a significant increase in micronuclei frequencies. In absolute terms, most of the induced micronuclei were CREST-negative (i.e., formed by a chromosome fragment). However, in relative terms, ELF exposure caused a two-fold increase in CREST-negative micronuclei and a four-fold increase in CREST-positive micronuclei (i.e., formed by a whole chromosome). No significant effect was recorded on exposed adults.

Conclusions: These findings suggest the need for investigation of aneugenic properties of ELF magnetic fields in order to establish a possible relationship to carcinogenesis.

Keywords: *Magnetic fields, ELF, genotoxicity, micronuclei, CREST*

Introduction

Since Wertheimer and Leeper (1979) showed a possible relationship between electrical powerlines and childhood cancer, many studies have been carried out to investigate the biological effects of Extreme-Low Frequency (ELF) electromagnetic fields. However, the results obtained are contradictory and comparison between them is difficult (Berg 1999), because of the many differences in parameters (duration and periodicity of the exposure, flux intensity, endpoints investigated). Besides studies on teratogenesis (Levin 2003), tumor promotion (Lacy-Hulbert et al. 1998) and hematology (Bonhomme-Faivre et al. 1998, Ali et al. 2003), several authors have examined the genotoxic properties of ELF magnetic fields. Some studies have been performed on samples taken from individuals professionally exposed. Increases in micronuclei frequencies and chromosomal aberrations have been observed in lymphocytes of photocopying machine workers (Iravathy Goud et al. 2004), powerline operators and railwaymen (Nordenson et al. 1984, 1988, 2001, Skyberg et al. 1993, Valjus et al. 1993).

Laboratory studies (especially *in vitro*) are more abundant. Several works denied the hypothesis that ELF magnetic fields have genotoxic properties (for review, see Vijayalaxmi & Obe 2005). Svedenstål and Johanson (1998) did not detect any increase of micronucleated erythrocytes in adult mice exposed for 90 days to a 14 μT magnetic field; the same result was obtained by Abramsson-Zetterberg and Grawé (2001), using an identical field, both in adult and newborn mice; Maes et al. (2000) exposed human lymphocytes (up to 2500 mT) and found no significant effect on chromosome aberrations, sister chromatid exchanges and single-strand breaks. Moreover, McNamee et al. (2002) reported no significant effect on DNA strand breaks in cerebellar cells of immature mice exposed continuously to a 60-Hz magnetic field at 1 mT for 2 h; Testa et al. (2004) detected an absence of DNA damage (using several

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cytogenetic assays) in human blood cells exposed *in vitro* for 48 h to a 50-Hz, 1 mT magnetic field.

On the other hand, other works have detected positive results only in conditions of co-exposure with other mutagenic agents, such as static magnetic fields (Tofani et al. 1995, Miyakoshi et al. 2000), benzopyrene (Cho & Chung 2003, Moretti et al. 2005), X-rays (Ding et al. 2003) and vinblastine (Verheyen et al. 2003). These results led to the hypothesis that ELF magnetic fields are able to enhance, but not to start, a mutagenic event.

Nevertheless, there has been an increase in the number of papers detecting genotoxic properties of ELF magnetic fields alone, both with *in vivo* and *in vitro* exposure. Lai and Singh (1997a, 1997b, 2004) found that rats exposed (either for 2 or 48 h) to a 60-Hz sinusoidal magnetic field at intensities of 10, 100 and 500 μT showed increases in DNA single- and double-strand breaks in their brain cells. Similar results were obtained by Svedenstål et al. (1999) with brain cells of CBA mice exposed for 14 days to 500 μT. Yokus et al. (2005) found a significant increase in 8-hydroxy-2′-deoxyguanosine (indicative of oxidative DNA damage) in plasma of rats exposed to 970 μT for 50 days. Another study, conducted on mouse m5S cells, detected a significant, dose-dependent increase of chromatid-type chromosomal aberrations at 5, 50 and 400 mT (Yaguchi et al. 2000). Ivancsits et al. (2002, 2003a, 2003b) reported an increase in DNA single- and double-strand breaks in human fibroblasts intermittently (5′ on/10′ off) exposed to a 50-Hz magnetic field at 1 mT. Pasquini et al. (2003) observed an increased frequency of micronuclei in Jurkat cells exposed for 24 h to 5 mT (50 Hz) magnetic fields. Wolf et al. (2005) found in HL-60 leukemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts, a peak in DNA strand breaks and in the formation of 8-hydroxy-2′-deoxyguanosine after 24 and 72 h of exposure to 0.5 and 1.0 mT magnetic fields. Finally, Winker et al. (2005) revealed a time-dependent increase of micronuclei in human diploid fibroblasts, which became significant after 10 h of intermittent exposure (5′ on/10′ off) at a flux density of 1 mT.

Although the studies on the possible damage to genome integrity caused by ELF magnetic fields are numerous, works on aneuploidy induction are very few: two in condition of co-exposure with a well-known spindle poison (vinblastine) and another after exposure to X-rays. In the latter, Ding et al. (2003) found that a 5 mT, 60 Hz magnetic field significantly increased CREST (Calcinosis, Raynaud's phenomenon, Esophageal dismotility, Sclerodactility, Telangectasia)-positive micronuclei in CHO cells after exposure to X-rays. Verheyen et al. (2003) detected a significant increase of micronuclei (without distinguishing between CREST-positive and -negative) in human lymphocytes exposed to vinblastine and 80 or 800 μT. Mailhes et al. (1997), instead, found an increase in hyperploidy in murine oocytes.

The problem of the possible aneugenic effects of ELF magnetic fields has not been sufficiently investigated, although chromosome loss has been linked to myelodysplastic syndromes (Boultwood & Fidler 1995, Wyandt et al. 1998), solid tumors (Sandberg 1990) and leukemia (Neben et al. 2003).

The micronucleus test (Schmid 1975) is a simple *in vivo* assay used to detect cytogenetic damage induced by chemical and physical mutagens. Micronuclei are produced after chromosome breakage (clastogenic damage) or spindle disturbance (aneugenic damage); in the first case, the micronucleus contains a chromosome fragment, in the second case a whole chromosome. Conventional microscopic analysis, however, cannot discriminate micronuclei for their aneugenic or clastogenic origin. This can be achieved detecting the presence or absence of centromere proteins, through immunofluorescent staining with CREST antibodies (Degrassi & Tanzarella 1988, Miller & Adler 1990).

The aim of this work was to detect the possible clastogenic and aneugenic damage in newborn mice exposed to an ELF magnetic field during the whole intra-uterine life and in adult mice exposed for the same amount of time. In order to do this, the micronucleus test with CREST staining was performed on erythrocytes from liver (or bone marrow for adults) and peripheral blood. The liver is the main hematopoietic organ from the 11th day of gestation until the first days after birth (Keller et al. 1999, Udroiu et al. 2006), whereas in adult life this role is played by the bone marrow. While micronucleated erythrocytes from the hemopoietic organ reflect genotoxic damage which occurred in the last 48 h, those from peripheral circulation reflect events that occurred during the last 35 days (Schlegel & MacGregor 1982, Luke et al. 1988). Finally, it must be underlined that newborn mice, because of their accelerated erythropoiesis, are extremely sensitive to the induction of micronuclei (Bishop et al. 2004).

Materials and methods

Four female mice (CD-1 Swiss) were individually caged and exposed during pregnancy to 50 Hz, 650 μT magnetic field generated by a solenoid working 24 h per day, and 38 newborn mice were exposed until day three after birth (for a total of 21 days of exposure), when they were sacrificed.

The solenoid was 0.8 m in length and 0.13 m in radius, with 552 turns of 2.5 mm^2 copper wire, wound in two layers in continuous forward-backward

fashion around a cylinder of PVC. It was supplied by 50 Hz main power through a transformer. A voltage of 6.5 V (rms) has been applied to obtain a flux density of 650 μT (rms) at the centre of the solenoid. The field was uniform between $\pm$ 5% in the volume where the mice were exposed. The solenoid was not shielded for the electric field, as the induced electric field was negligible due to the low voltage used.

The intensity of the field we adopted in the experiment has been selected in agreement with the following considerations. We would test the effect of an industrial alternate magnetic field provided at the reference level assumed as international standards for the protection of the public against non-ionizing radiations. Since European Union (EU) Recommendation 1999/519 indicates the value of 2 mA/m^2 as basic limit for the induced current density in the human body for people exposed to the industrial alternate (under 1 kHz) magnetic field (EU Council Recommendation, 1999), we arranged the magnetic flux of the solenoid to get the same value for the induced current density in the mouse body.

For a sinusoidal field the following relationship can be assumed between the induced current density J and the magnetic flux density B:

$$\mathcal{J} = \pi \cdot f \cdot B \cdot R \cdot \sigma$$

where the frequency is $f = 50$ Hz and an homogeneous conductivity is taken $\sigma = 0.2$ S/m (CENELEC (European Committee for Electrotechnical Standardization) 1995). For determining the radius R, the length and the width of the mouse body has been measured. For a comparison the human thorax width and depth have been deduced from NASA-SRD-3000 ''Man-systems integration standards'' (50th percentile) (NASA 1995). The transverse sections of the mouse body and human thorax have been modelled by means of an ellipse whose axes are 88 mm and 26 mm, 392 mm and 250 mm respectively. The ellipse areas are 1,797 mm^2 and 76,969 mm^2 respectively. The square root of the reverse ratio of these areas, equal to 6.5, is the ratio of the magnetic flux density levels which induce the same current density in the mouse body and in the human thorax transverse sections.

Applying the above formula, ICNIRP (International Commission on Non-Ionizing Radiation Protection) Guidelines (1998) estimates equal to 100 μT the magnetic flux density for the 50 Hz industrial frequency like the one able to induce a current density equal to the basic limit value (2 mA/m^2) in the human thorax. To obtain this current density, which in man is induced by a 100 μT magnetic field, we used a magnetic flux density of 650 μT for mice exposures, given the above ratio of 1:6.5.

Another four female mice were kept unexposed during pregnancy and 36 newborn mice were sacrificed at day 3 after birth. Positive control was carried out exposing five 3-day-old mice to X-rays (3 Gy), which were sacrificed 24 h later. Exposure to X-rays was performed in a Gilardoni apparatus (Gilardoni, Milano, Italy; 250 kV, 6 mA, 3 mm Al filter) at a 0.5 Gy/min dose rate for 6 min.

Fifteen adult mice (CD-1 Swiss) were caged in groups of 3 or 4 of the same sex and exposed for 21 days to 50-Hz, 650 μT magnetic field and sacrificed at the end of the exposure. Another 15 adult mice were kept unexposed for 21 days as controls. Positive control was carried out exposing six adult mice to X-rays (3 Gy), which were sacrificed 24 h later.

This experimentation was approved by the animal use and care Committee of the University ''La Sapienza'' Roma.

The temperature and the relative humidity of the animal room were 20–22°C and 40–50% respectively. Artificial lighting was from 8 am to 8 pm and commercial pellets and tap water were available *ad libitum* throughout the experimental period. The temperature inside the coils was the same as in the room.

Liver (from newborns) and bone marrow (from adults) cells were flushed out with foetal calf serum with 25 mM EDTA (ethylenediaminetetraacetic acid). Cell suspensions were gently pipetted and centrifuged at 600 g for 5′. The pellet was resuspended and smeared on clean slides. Blood smears were prepared by collecting it from jugular veins. All slides (bone marrow, liver and peripheral blood) were fixed in absolute methanol and maintained at -20°C until staining.

The smears were stained by incubation in antikinetochore antibody (Antibodies Inc., Davis, CA, USA), followed by FITC (fluorescein isothiocyanate)-conjugated rabbit anti-human (Sigma Immunochemicals, St Louis, MO, USA) as a secondary antibody and FITC-conjugated goat anti-rabbit (ICN, Irvine, CA, USA) as a tertiary antibody. The slides then were counterstained by immersion in 2.5 μg/ml 4′-6′-diamidino-2-phenylindole (Sigma-Aldrich, St Louis, MO, USA) for 10 min and mounted in a mixture of propidium iodide (Sigma-Aldrich, St Louis, MO, USA), 1 μg/ml for blood, 2.5 μg/ml for liver, in antifade solution (Vector Laboratories, Burlingame, CA, USA).

Micronuclei were scored at 1000x magnification using a Zeiss Axiophot (Zeiss, Germany) microscope with ultraviolet light (359 nm excitation filter, 441 nm barrier filter) and classified for kinetochore staining (494 nm excitation filter, 523 nm barrier filter). The samples were coded and scored blindly by the same analyst. Micronuclei frequencies were determined counting 2000 erythrocytes per animal.

The frequencies of micronucleated erythrocytes were analysed with the Shapiro-Wilk's test but

resulted in an abnormal distribution. Therefore, as suggested by Mitchell and Brice (1986), the Kolmogorov-Smirnov statistical analysis was used to calculate the levels of significance for comparisons between frequency distributions of total micronucleated erythrocytes (ME), CREST-positive micronucleated erythrocytes (ME+) and CREST-negative micronucleated erythrocytes (ME−). The level of significance was established at $p < 0.05$. All analysis was carried out using STATISTICA 6.0 package (StatSoft, Tulsa, OK, USA).

Results

Table I shows the mean frequencies of ME, ME+ and ME− in the different groups of newborn mice. In peripheral blood, the mean frequencies of ME in the ELF-exposed group ($x = 4.42 \pm 2.66$) and in the X-irradiated group ($x = 31.60 \pm 3.83$) were significantly higher (both $p < 0.001$) than that of the non-exposed one ($x = 2.07 \pm 1.68$). In particular, the mean frequencies of ME+ in the ELF-exposed group ($x = 0.66 \pm 0.51$, representing 14.88% of ME) and in the X-irradiated group ($x = 1.10 \pm 0.42$, representing 3.48% of ME) were significantly higher ($p < 0.001$ and $p < 0.025$, respectively) than those of the non-exposed group ($x = 0.15 \pm 0.26$, representing 7.38% of ME). Also, the mean frequencies of ME- in the ELF-exposed group ($x = 3.76 \pm 2.63$) and in the X-irradiated group ($x = 30.50 \pm 3.94$) were significantly higher ($p < 0.025$ and $p < 0.001$, respectively) than those of the non-exposed one ($x = 1.92 \pm 1.76$).

In erythrocytes of the liver, the mean frequencies of ME in the ELF-exposed group ($x = 0.76 \pm 0.38$) and in the X-irradiated group ($x = 25.40 \pm 4.02$) were significantly higher ($p < 0.005$ and $p < 0.001$, respectively) than those of the non-exposed group ($x = 0.42 \pm 0.33$). However, the differences between the mean frequencies of both ME+ and ME− in the ELF-exposed group ($x = 0.13 \pm 0.22$ and $x = 0.63 \pm 0.36$, respectively) and those in the non-exposed group ($x = 0.05 \pm 0.16$ and $x = 0.36 \pm 0.33$) were not statistically significant. In the X-irradiated group, instead, the mean frequencies of both ME+ ($x = 1.70 \pm 1.40$) and ME− ($x = 23.70 \pm 3.17$) were significantly higher ($p < 0.005$ and $p < 0.001$, respectively) than those of the non-exposed group.

Finally, the ME mean frequency in peripheral blood were significantly higher than in liver both in the ELF-exposed ($p < 0.001$) and in the non-exposed group ($p < 0.001$).

Table II shows the mean frequencies of ME, ME+ and ME− in the different groups of adult mice. In peripheral blood, only the difference between the mean frequency of ME in the X-irradiated group ($x = 9.17 \pm 1.40$) and the non-exposed one ($x = 1.63 \pm 0.72$) resulted in statistical significance ($p < 0.001$). Also, the mean frequency of ME- in the X-irradiated group ($x = 8.75 \pm 1.21$) resulted in statistically higher values ($p < 0.001$) than that of the non-exposed one ($x = 1.50 \pm 0.60$).

In bone marrow, the difference between the mean frequencies of ME in the ELF-exposed group and those of the non-exposed one was not statistically different. The mean frequencies of ME, ME+ and ME− in the X-irradiated group were significantly higher than those of the non-exposed group.

To compare the micronuclei frequencies of newborn mice with those from adults, only data from peripheral blood can be put side by side. Only in the exposed groups, the difference between the ME mean frequency of the newborn mice and that of the adults, resulted in statistical significance ($p < 0.001$).

Discussion

The aim of this work was to detect clastogenic and aneugenic damage in newborn and adult mice exposed to a 50-Hz, 650 μT ELF magnetic field. The results clearly show the accumulation of ME in the peripheral circulation, as expected in animals whose spleens do not remove micronuclei

Table I. Frequencies of ME in newborn mice.

	n	Mean ME+/1000E (min.–max.)	Mean ME-/1000E (min.–max.)	Mean total ME/1000E (min.–max.)
Peripheral blood				
Controls	36	0.15 (0–1)	1.92 (0–9)	2.07 (0–9)
ELF	38	0.66[c] (0–1.5)	3.76[a] (0–11)	4.42[c] (1–11.5)
X-rays	5	1.10[a] (0.5–1.5)	30.50[c] (25–35)	31.60[c] (26.5–36.5)
Liver				
Controls	36	0.05 (0–0.5)	0.36 (0–1)	0.42 (0–1)
ELF	38	0.13 (0–0.5)	0.63 (0–1.5)	0.76[b] (0–1.5)
X-rays	5	1.70[b] (0.5–4)	23.70[c] (18.5–27)	25.40[c] (19–29)

n = number of animals; ME = Micronucleated Erythrocytes; E = Erythrocytes; significance respect to sham: a: $p < 0.025$; b: $p < 0.005$; c: $p < 0.001$.

Table II. Frequencies of ME in adult mice.

	n	Mean ME+/1000E (min. – max.)	Mean ME-/1000E (min. – max.)	Mean total ME/1000E (min. – max.)
Peripheral blood				
Controls	15	0.13 (0 – 0.5)	1.50 (0.5 – 2.5)	1.63 (0.5 – 3)
ELF	15	0.30 (0 – 1)	1.57 (0.5 – 2.5)	1.87 (0.5 – 3.5)
X-rays	6	0.42 (0 – 1.5)	8.75[c] (7.5 – 10.5)	9.17[c] (7.5 – 11)
Bone marrow				
Controls	15	0.13 (0 – 0.5)	0.90 (0 – 2)	1.03 (0 – 2.5)
ELF	15	0.17 (0 – 1)	1.10 (0.5 – 2)	1.27 (0.5 – 2)
X-rays	6	0.92[a] (0.5 – 1.5)	21.83[c] (10 – 29)	22.75[c] (10.5 – 30.5)

n = number of animals; ME = Micronucleated Erythrocytes; E = Erythrocytes; significance respect to sham: a: $p < 0.025$; b: $p < 0.005$; c: $p < 0.001$.

(Schlegel & MacGregor 1984). In the X-irradiated mice, the accumulation of ME in peripheral blood was not detected because, following an acute exposure, the ME frequencies reach a peak after 24 h in the hemopoietical organ (liver in newborns and bone marrow in adults) and after 36 h in peripheral blood (Chaubey et al. 1993).

In newborn mice, ELF chronic exposure during the whole intra-uterine life (3 weeks to 50 Hz, 650 μT) caused a significant increase in ME frequency in the peripheral circulation. This is in contrast to the data obtained by Abramsson-Zetterberg and Grawé (2001). However, along with other dissimilarities in the experimental design, the intensity of the field (14 μT) and the time of sampling were different. In fact, the authors carried out the exposure for 18 days *in utero* and the animals were sacrificed after 35 days, a time exceeding the lifespan of a murine erythrocyte.

Our exposure did not induce a significant increase in ME mean frequencies in adult mice. This result agrees with those obtained by Svedenstål and Johanson (1998) and by Abramsson-Zetterberg and Grawé (2001), who both used a weaker magnetic field (14 μT).

The difference between the results we obtained in adults and those obtained in newborns, may be related to major sensitivity of the latter to genotoxic insults.

It is important to note that, in our experiment, a prolonged exposure to ELF magnetic fields caused a two-fold increase in circulating ME− and a four-fold increase in circulating ME+. A similar increase was also observed in the adults, even though it is not statistically significant. Although the absolute levels of induced micronuclei are not as high as those induced by well-known mutagens (ionizing radiation, for example), this data, if confirmed by other independent research laboratories, could indicate that ELF magnetic fields have different properties to damage the genome integrity.

Lai and Singh (1997b, 2004) showed that the effects of the ELF magnetic fields on the production of reactive oxygen species (ROS) determine their clastogenic properties. By the way, it should be noted that ROS interactions with the spindle apparatus can result in aneuploidy (Schuessler & Schilling 1984). Moreover, several authors found that ELF magnetic fields enhance the influx of Ca^{++} ions (Lacy-Hulbert et al. 1998) and this was associated with cell proliferation and carcinogenesis. However, Chiabrera et al. (1984) earlier proposed that Ca^{++} variations in the cytoplasm stimulate the depolarization of tubulin and Mailhes et al. (1997) underlined that alterations in calcium homeostasis have the potential to induce aneuploidy.

In conclusion, this work stresses the need to investigate the possible link between electromagnetic fields and aneuploidy, because the importance of the latter in relation to carcinogenesis.

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