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Efectos agudos de los campos electromagnéticos de 60-Hz sobre funciones de linfocitos y macrófagos murinos *ex vivo*, y crecimiento de células tumorales *in vitro*

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ORIGINAL ARTICLE

Acute effects of 60-Hz electromagnetic fields on *ex vivo* murine lymphocyte and macrophage functions, and *in vitro* tumor cell growth

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ABSTRACT. Electromagnetic fields (EMFs) have been associated with impairing health. There are data that associate EMFs exposure to incidence of cancer, but there are conflicting results between epidemiological and laboratory studies. Similarly studies on the effect of EMF on the immune system have produced variable results. In the present study, we evaluated the acute effects of 60 Hz EMFs exposure at 1.0 mT, on proliferation of murine thymic lymphocytes, production of nitric oxide and phagocytosis of *Candida albicans* by peritoneal murine macrophages, as well as the effect of 8 h/day of EMF exposure during 6 days on proliferation of murine lymphoma L5178Y-R cell growth. We observed that exposure to EMF did not alter lymphocyte and macrophage functions, and did not affect *in vitro* cell growth of the murine lymphoma cell line L5178Y-R.

Key words: Lymphocyte and macrophage function, electromagnetic field tumor cell growth.

RESUMEN. Los campos electromagnéticos (CEM) se han asociado con efectos para la salud. Existen datos que relacionan la exposición a CEM con incidencia de cáncer, sin embargo hay controversia entre los estudios epidemiológicos y aquellos de laboratorio. Por otra parte, investigaciones recientes muestran el efecto de CEM sobre el sistema inmune, con resultados variables. En el presente trabajo se evaluó el efecto de CEM de 60 Hz de frecuencia, a una exposición aguda (72 h), con una intensidad de 1.0 mT sobre la proliferación de linfocitos de timo, la producción de óxido nítrico y fagocitosis de *Candida albicans* por macrófagos peritoneales murinos, así como también el efecto sobre la proliferación de células de linfoma murino L5178Y-R expuestas 8 h diarias por un período de 6 días. Se observó que la exposición a CEM no alteró las funciones *ex vivo* de los linfocitos y macrófagos, y no afectó la proliferación de células tumorales *in vitro.*

Palabras clave: Macrófagos, linfocitos, campo electromagnético, crecimiento celular tumoral.

INTRODUCTION

Our civilization is continuously exposed to extremely low frequency (i.e. 50 or 60 Hz) electromagnetic fields (EMFs) produced by high-voltage transmission lines, video display terminals, electric blankets, or clinical nuclear magnetic resonance-imaging procedures. However, there is not convincing evidence to link EMFs to harmful effects on human health.[8]

A number of epidemiological studies have suggested an increased risk for cancer development, particularly leukemia and brain and breast cancers, due to residential or occupational exposure to EMFs.[2,20,21] EMFs might act as tumor promoters and interfere with DNA repair.[13] On the contrary, the majority of laboratory studies have indicated that non-ionizing radiation has no mutagenic effects.[15,17] In addition, recent studies have demonstrated that EMFs and magnetic fields (MFs) can influence immune function. In this regard, it was reported an overall *in vivo* immunopotentiation of humoral and cell-mediated immune responses in rats exposed to static MFs.[9] In addition, it was demonstrated that EMFs and MF caused lymphocyte suppression,[18,19,22] or increase in lymphocyte proliferation.[5,7]

In the present study, we evaluated the acute effects of 60-Hz EMF on *ex vivo* immune functions, and against *in vitro* lymphoma L5178Y-R growth. We found that EMFs did not alter *ex vivo* functions of lymphocytes and macrophages, and did not affect *in vitro* tumor cell growth.

MATERIAL AND METHODS

Reagents, culture media, and cell line: Penicillin-streptomycin solution, L-glutamine, and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). Lipolysaccharide (LPS) from *Escherichia coli* serotype 0128:B12, fetal bovine serum (FBS), methanol, phosphate buffer saline (PBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), sodium dodecyl sulfate (SDS), dimetilformamide (DMF), and Giemsa stain were purchased from Sigma Chemical Co. (St. Louis, MO). The murine lymphoma cell line L5178Y-R was obtained from The American Type Culture Collection (ATCC) (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution (complete RPMI 1640 medium).

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Rev Latinoam Microbiol 2001; 43 (3): 130-134

Animals. Six- to eight-week old Balb/c male mice (Harlan Sprague Dawley Inc., Indianapolis, IN) were used. They were kept in a pathogen- and stress-free environment at 24 ± 2°C, under a light-dark cycle (light phase, 09:00-21:00), and given water and food *ad libitum*.

Exposure to EMF. The exposure system consisted of a selenoid with 552 turns of enamel insulated copper wire caliber 14, coil on a plastic matrix of a diameter of 27 cm and a length of 71 cm. This selenoid was fed by a 60-Hz alternate electric current which was connected to a variable autotransformer (STACO Inc. model 500B, Dayton, Ohio) with an entrance of 120 volts and 8 ampers and an exit of 120 volts and 12 ampers. The autotransformer was passed by a step-down transformer that allowed delivering of the required amperage to produce the magnetic intensity utilized in this study (1.0 mT, T=Tesla, is the density of magnetic flux unit). To assure the geometry of exposure, a separator with polyvinyl chloride (PVC) tube and acrylic was assembled to allow the animals or cell cultures to be located at the same position inside the selenoid. This is relevant considering that EMFs biological effects are directly associated with the induced electric field on the samples, and this electric field depends on the radium of the selenoid.[11] A gausimeter Bell model FW 6010 was used to measure the applied EMFs. This was coupled to an oscilloscope (model 2120 BK-precision, Miami, FL) to verify the generation of a 60-Hz alternate magnetic field. In addition, during the experiments, the EMFs were monitored with a probe coil calibrated with the gausimeter (1.7 mV of alternate current detected in the probe coil corresponded to 1.001 mT of magnetic flux density). As a control, we used a sham selenoid similar to the one utilized for the treatments, but not connected to the electric current.

Cell preparation and culture. Mice were killed by cervical dislocation immediately after 72 h of EMF exposure. Macrophages were obtained by peritoneal lavage with cold RPMI 1640 medium; next, thymus was removed, and single-cell suspensions were prepared by disrupting the organ in the same culture medium. Macrophage and thymic cell suspensions were washed two times with RPMI 1640 medium, suspended and adjusted to 2×10^6 and 1×10^7 cells/ml in AIM-V medium respectively. We changed the culture medium at this step, because it was reported that serum activates macrophages,[1] and AIM-V medium has been demonstrated to support cell culture.[10]

T cell proliferation assay. T cell proliferation was determined by a colorimetric technique. Cell suspensions (100 µl) were added to flat-bottomed 96-well plates (COSTAR, Corning Inc, NY) containing triplicate cultures (100 µl) of AIM-V medium (unstimulated control) or the mitogen Con A at submaximal and maximal concentrations of 0.6, 1.2, and 2.4 µg/ml. After incubation for 44 h at 37°C in an atmosphere of 5% CO_2-95% air, 20 µl of MTT (0.5 mg/ml final concentration) were added to all wells, and cultures were incubated for additional 4 h. After the incubation period, plates were centrifuged. One hundred microliters of supernatants were then removed followed by addition of 100 µl of cell lysing buffer (20% SDS in 50% of both DMF and water) to all wells, and cultures were incubated for additional 16 h. Optical densities at 540 nm were then determined in a microplate reader (BIO TEK Instruments Inc.).

Nitrite determination. Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production by resident or activated cells. Cell suspensions (100 µl) were added to flat-bottomed 96-well plates (COSTAR, Corning Inc.) and incubated for 2 h. Cell cultures were then washed once with RPMI 1640 to remove non-adherent cells, and adherent cells were incubated in 200 µl AIM-V medium per well in the presence or absence of LPS (20 ng/ml) for 72 h. After incubation, supernatants were obtained, and nitrite levels were determined with the Griess reagent as previously reported,[3,6] using $NaNO_2$ as standard. Optical densities at 540 nm were then determined in a microplate reader (BIO TEK Instruments Inc.).

Phagocytosis assay. Macrophage suspensions at a concentration of 2×10^6 cells/ml were plated on 24 × 60 mm glass coverslips (Fisher Scientific, Pittsburgh, PA) placed inside 100 × 15 mm polystyrene Petri dishes (Fisher Scientific). After 2 h of incubation at 37°C, non-adherent cells were removed, and adherent cells were incubated whit a suspension of live *Candida albicans,* ATCC strain 32354, at a yeast to macrophage ratio of 5 to 2, in AIM-V medium in a final volume of 6 ml. After 1 h of incubation at 37°C, yeast's were removed, and macrophage monolayers were extensively washed whit PBS and air-dried. Cells were then fixed in methanol, and stained with Giemsa stain.[4] The coverslips were removed and mounted onto glass slides (Fisher Scientific). The percentage of macrophages ingesting one o more yeasts were determined by counting at least 100 cells under an oil-immersion objective.

Tumor cell proliferation assay. To determine the *in vitro* effect of EMF on tumor cell growth, L5178Y-R cells at a concentration of 5×10^4 cells/ml in complete RPMI 1640 medium, were exposed to EMFs (1.0 mT) for 8 h at day during 6 days. Cell suspensions were maintained at 37°C in an atmosphere of 5% CO_2-95% air for the remaining 16 h. One hundred-microliter aliquots of tumor cell suspensions were obtained at the beginning of the experiment and every 24 h immediately after EMF exposure, during the 6 days of treat-

Rev Latinoam Microbiol 2001; 43 (3): 130-134

ment. Tumor cells were then incubated in flat-bottomed 96-well plates with MTT (at a final concentration of 0.5 mg/ml) by additional 4 h. Next, 100 µl of cell lysing buffer were added to all wells, and plates were incubated for additional 16 h. Optical densities at 540 nm were then determined in a microplate reader (BIO TEK Instruments Inc.).

Statistical analysis. The results were expressed as mean ± SEM of triplicate determinations for each treatment from 3 independent experiments. Statistical significance was determined by Student's *t*-test ($P \leq 0.05$) and analysis of variance.

RESULTS

Acute effect of EMFs on T cell proliferation: Sixty-Hz EMF at 1.0 mT did not significantly alter T cell proliferation (Fig. 1).

Acute effect of EMFs on macrophage functions: As shown in Fig. 2, 60-Hz EMF at 1.0 mT did not significantly affect nitric oxide production, and did not influence phagocytosis of *Candida albicans* by peritoneal macrophages (Fig. 3).

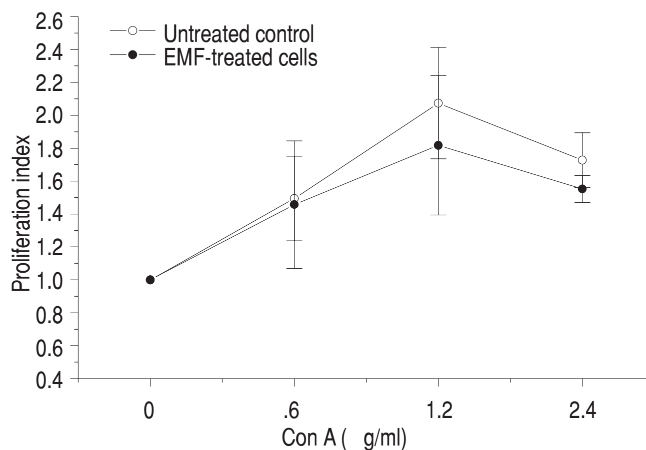

Figure 1. *Acute effect of EMFs on T cell proliferation. Mice were exposed to 60-Hz EMFs during 72 h or sham-treated, after which thymuses were removed and thymic cells were adjusted to 1 x 10*^{7} *cells/ml. The suspensions were then incubated in the presence or absence of Con A for 44 h at 37ºC. Cell proliferation was then determined by a colorimetric technique using MTT, as described in the text. The results were expressed as mean ± SEM of triplicate determinations for each treatment from 3 independent experiments. The proliferation index was calculated dividing the optical density for cell proliferation induced by Con A between the optical density of untreated control (0.295 ± 0.114) for each experimental group. There was not significant difference between EMF-treated animal thymic cell responses and those of untreated control animal cells at all Con A concentrations (P values equal to 0.94, 0.66, and 0.41 at 0.6, 1.2, and 2.4 mg/ml of Con A respectively). However, there was a significant difference (P > 0.01) between responses of Con A-treated cells from control and EMF-treated groups and those of cells not treated with Con A.*

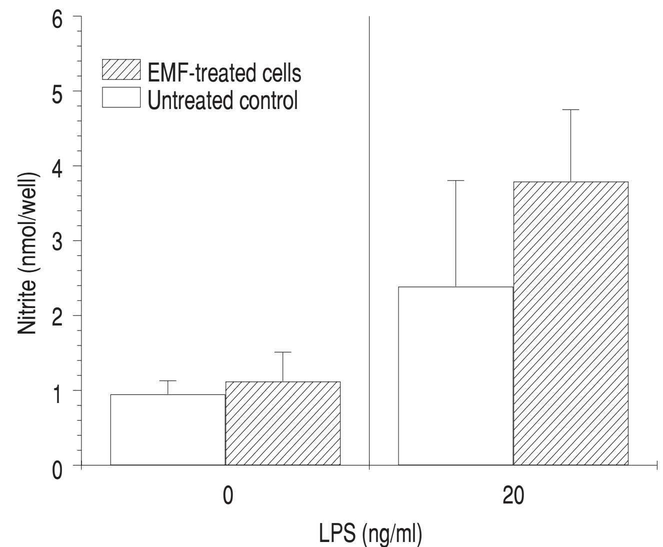

Figure 2. *Acute effect of EMFs on nitric oxide production by peritoneal macrophages. Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production. Were incubated 2 x 10*^{6} *macrophages/ml in the presence or absence of LPS for 72 h, as explained in the text. After incubation, supernatants were obtained, and nitrite levels were determined with the Griess reagent. Optical densities at 540 nm were then determined. The results were expressed as mean ± SEM of triplicate determinations for each treatment from 3 independent experiments. There was not significant difference between the response of untreated or LPS-treated macrophages from EMF-treated animals and that of control animal cells (P values equal to 0.710 and 0.44 for untreated and LPS-treated cells respectively).*

Effect of EMFs on *in vitro* tumor cell growth: Exposure of L5178Y-R cells to 60-Hz EMF at 1.0 mT, did not significantly alter its growth *in vitro* (Fig. 4).

DISCUSSION

Results from the present study indicated that 60-Hz EMFs (1 mT) did not affect *ex vivo* lymphocyte and macrophage functions, and *in vitro* L5178Y-R cell growth. Our results are related to a previous study reporting that static and sinusoidal magnetic fields of 1.0 to 100 mT did not alter nitric oxide production by murine macrophages.[16] In addition, exposure of mice to 60-Hz EMFs at 0.1 mT during 1 to 10 days produced inconsistent results on immune parameters including enhancing, suppressing and absence of effects.[14] We have recently reported that continuous exposure to 60-Hz EMF of 1.0, 1.5 and 2.0 mT, was capable to increase *in vitro* phytohemaglutinin-induced human lymphocyte cell proliferation, using an EMFs exposure device similar to that used in the present study.[7] The lack of effect of EMFs on *ex vivo* immune function

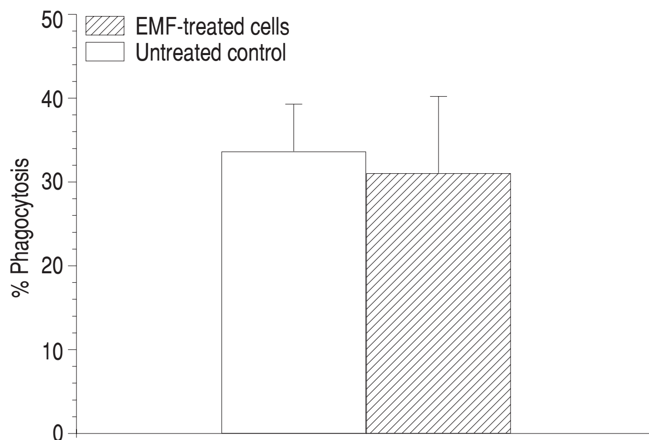

Figure 3. *Acute effect of EMFs on the phagocytosis of* Candida albicans *by peritoneal macrophages. Cultures of 2 x10*^{6} *macrophages/ml, from mice treated or not with 60-Hz EMFs at 1.0 mT during 72 h, were incubated with a suspension of* C. albicans, *as described in the text. After 1 h of incubation at 37ºC, yeasts were removed, and macrophages were then fixed in methanol and stained with Giemsa stain. The percentage of macrophages ingesting one o more yeast was determined by counting at least 100 cells under an oil-immersion objective. The results were expressed as mean* $\pm$ *SEM of triplicate determinations for each treatment from 3 independent experiments. There was not significance between the response of macrophages from EMF-treated animals and that of untreated control animal cells (P= 0.82).*

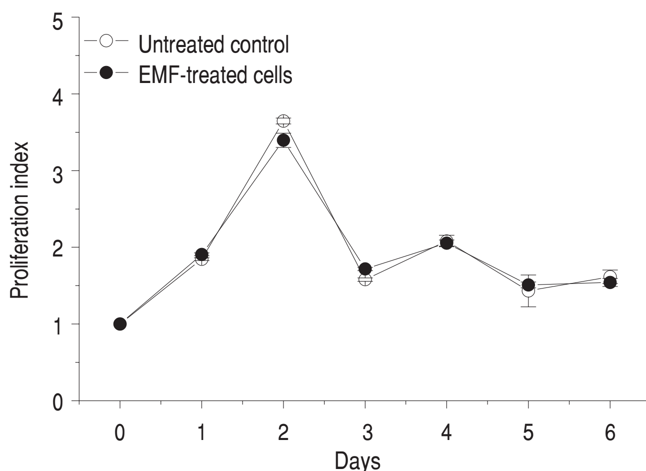

Figure 4. *Effect of EMFs on* in vitro *tumor cell growth. L5178Y-R cells at a concentration of 5 x 10*^{4} *cells/ml were exposed to EMFs (1.0 mT) for 8 h/day during 6 days. One hundred-microliter aliquots of tumor cell suspensions were obtained at the beginning of the experiment and every 24 h immediately after EMF exposure, during the 6 days of magnetic treatment. The cell suspension aliquotes were then incubated with MTT, and proliferation was determined. The results were expressed as mean* $\pm$ *SEM of triplicate determinations for each treatment from 3 independent experiments. There was not significant difference between EMF-treated tumor cell growth and that of untreated control tumor cells during all days of treatment (P > 0.5).*

might be related to a limited (not continuous) exposure to EMFs (cells were not exposed during the incubation time). This may have contributed to the recovery of the cells after EMFs treatment.

On the other hand, our results on *in vitro* tumor cell growth suggested an absence of effect by EMFs treatment. Our data agreed with other laboratory studies that reported no alterations on cancer growth following exposure to extremely-low frequency EMFs,[13,15] but differed from epidemiological studies showing a promoting effect of EMFs on cancer development.[8] This may be attributable to the existence of multiples not recognized factors capable to interact with EMFs to promote cancer development.

In general, EMFs have been reported to increase, decrease or have no effects on immune function[5,7,9,12,18,19,22] Such differences might be related to a number of factors including: a) type of exposure device to produce EMFs, b) treatment dose (exposure time, intensity and frequency of EMFs), c) exposure area (whole body *vs* specific body region), and d) target cell type and source (lymphocytes, macrophages, and tumor cells of murine, rat or human origins).

We have experiments underway to determine *in vitro*, *ex vivo* and *in vivo* acute and chronic effects of EMFs at intensities greater than 1.0 mT on immune functions and tumor cell growth. The World Health Organization has an international project to evaluate EMFs effects produced by electric and electronic devices, and power lines on human health.[23] Despite the large number of published works in recent years, there is no conclusive evidence supporting that exposure to EMFs is associated with human diseases.

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Rev Latinoam Microbiol 2001; 43 (3): 130-134

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