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Original Article

Assessment of the Effects of Extremely Low Frequency Electromagnetic Fields on *Toxoplasma gondii*

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Abstract

Background: The effects of extremely low frequency electromagnetic fields (ELF-EMF) on *Toxoplasma gondii* have not been explained yet. The aim of this study was to assess the possible effects of ELF-EMF on growth, survival time and viability of *Toxoplasma gondii*. In addition, the life span of *Toxoplasma* infected animals was investigated.

Methods: Sixty adult male BALB/c mice were used for in vivo and in vivo experiments in Laboratory of Biopyhsics and Parasitology of Medical Faculty, Adnan Menderes University, Turkey, in 2010. During in vivo experiments, pulsed and continuous EMFs were applied for 5 d to the infected mice. During in vivo experiments, pulsed and continuous EMF was applied to the tachyzoites within peritoneal exudates for 8 h/d at 4 °C and the tachyzoites were then injected to mice. In both experiments, the number of *T. gondii* in peritoneal exudates was counted and *T. gondii* protein bands patterns were investigated with polyacrylamide gel electrophoresis and Western Blotting.

Results: Pulsed and continuous EMF exposure reduced the number of *T. gondii* tachyzoites in comparison to controls. However, no statistically significant differences were observed at the patterns of protein bands among the samples.

Conclusion: EMF exposure induces a decrease in the number of *T. gondii*. Further studies are required to understand the mechanism of EMF on intracellular parasites.

Introduction

Toxoplasma gondii is a common intracellular parasite capable of infecting almost all mammals, including humans and birds, all over the world. There are important morphological structures including tachyzoites, tissue cysts, and oocysts in *T. gondii*'s life cycle (1). Toxoplasmosis can cause serious symptoms mostly in brain, especially in immuno-compromised or congenitally infected patients (2).

Electromagnetic fields in different levels of intensity, frequency, energy and direction create changes in the biological balance of living organisms (3). Electromagnetic radiation affects ions (4), neurotransmitters (5), hormones and antibody binding sites on the surface of cell membrane (6). These effects alter transmembrane signals such as ion transport or electro-conformational changes of membrane proteins. The transmembrane signals can then initiate cellular processes, which result in altered protein synthesis, gene transcription, and cell proliferation (7-8).

Extremely low frequency (ELF) fields are electromagnetic fields with frequencies that are below 300 Hz. Power lines and electronic appliances are the main source of extremely low frequency electromagnetic fields (ELF-EMF). In recent years, researches have been focused on the determination of the biological effects of ELF-EMF on prokaryotes, such as nematode, ciliates, protozoan, bacteria etc. (9-11). Although, ELF-EMF applications (continuous or pulsed) have been shown to decrease growth rates at both low and high magnetic field values (12-14), the effects of ELF-EMF on *T. gondii* have never been completely demonstrated yet.

The purpose of this study was to investigate the effects of continuous and pulsed EMF on growth, survival time and viability of *T. gondii*. In addition, the protein profiles of ELF-EMF exposed *Toxoplasma* were investigated by SDS-PAGE and Western Blotting to better understand the alteration of membrane structure.

Materials and Methods

Animals

Sixty adult male BALB/c mice (Experimental Animal Center, Adnan Menderes University, Aydin, Turkey) weighing 25.20±0.22g were used in all experiments. The 12 h light/dark cycle was automatically controlled and the room temperature was thermostatically regulated to 22±1 °C. Animals had free access to standard laboratory feed and water *ad libitum*.

All procedures were performed with the approval of Animal Experimentation Ethics Committee of Adnan Menderes University.

Parasites

The tachyzoites of the RH strain of *T. gondii* were maintained by serial passages of peritoneal exudate with five day intervals (15). At the time of harvesting, peritoneal exudates, collected freshly from infected mice, were diluted in 10 ml of PBS. Then, $1x10^5$ parasites in PBS were inoculated intraperitoneally to the mice in our experimental groups (16, 17).

EMF Exposure System

Mice were simultaneously exposed either continuous EMF (CEMF) or pulsed EMF (PEMF) in north-south direction. Before and during the EMF applications, the EMF levels were measured by using a digital Gauss/ Teslameter (Model 7030, F.W. Bell, Syprus, and Orlando, US).

In CEMF exposure setup, 50 Hz continuous EMF was generated by a pair of Helmoltz coils, each having 154 turns, carrying a maximum of 5 amperes and having a resistance of 2.1 ohms, separated by a distance 40 cm equal to the radius of the coil with maximum flux density for I=5 amperes in Helmholtz array of 3.5 mT (Phywe, Germany). Picture of CEMF exposure setup were seen in Fig.1a.

PEMF exposure apparatus consisting of a pair of Helmholtz coils were placed opposite

to each other and in a signal generator (Igea, Carpi, Italy) (Fig.1 b). The parameters of the pulsed signal were as follows: the pulse duration=1.3 ms, intensity of magnetic field 2.3 mT, induced electric field= 2 mV, frequency= 75 Hz. Experiments were carried out at normal room temperature (22±1 °C). Each exposure cage was composed of plexiglass (10x12x10 cm) and housed five mice.

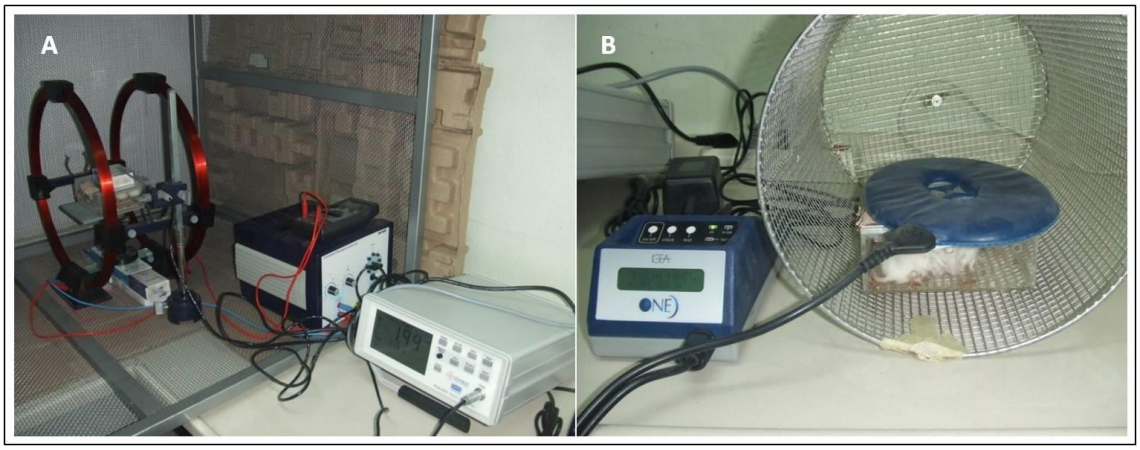

Fig. 1: System for generating ELF-EMF: (a) Helmholtz coil exposure set up. (b) Pulsed EMF exposure set up

Experimental procedure

Two groups of experiments, in vitro and in vivo, were performed in this study. The experimental procedures of these groups were explained as follows:

A- In vivo study

Tachyzoites in PBS ($1x10^5$ /ml) were inoculated into thirty mice via intraperitoneal injection. The mice were then randomly divided into three groups: group 1 was including 10 mice that were exposed to 50 Hz and 2 mT CEMF for 8 h per day for 5 d; group 2 was including 10 mice that were exposed to 75 Hz and 2.3 mT PEMF for 8 h per day for 5 days; and group 3 was the control group including 10 mice that were stayed in the same experimental condition for 5 d, but not exposed to EMF. At the end of the experiment, mice were sacrificed. Exudates with tachyzoites, collected from peritoneum of mice, were investigated with a hemacytometer and number of *T. gondii* was calculated. Then, the exudates were centrifuged at 3000 rpm for 5 min and washed with PBS five times. Numbers of tachyzoites in three groups were equalized to each other, and stored as frozen pellets at -20 °C for using SDS-PAGE and Western blotting.

B- In vitro study

RH strain tachyzoites were placed into the wells. Then the wells containing tachyzoites were randomly divided into three groups and each well contained approximately $1x10^5$ tachyzoites: in Group A, the wells were exposed to 50 Hz and 2 mT CEMF for 8 h in a day at +4 °C; whereas in Group B; the wells were exposed to 75 Hz and 2.3 mT PEMF for 8 hours in a day at +4 °C; and in control group (Group C), the wells were not exposed to EMF but stayed at +4 °C for 8 h in a day. After the EMF exposure, each group of tachyzoites was randomly inoculated intraperitoneally into the normal ten healthy mice. In order to determine the effect of EMF, the number of *T. gondii* tachyzoites in peritoneal fluid were counted with a hemacytometer and life span of the animals were determined at the end of the five days. Then, the exudates were centrifuged at 3000 rpm for 5 min and washed with PBS for five times. Numbers of tachyzo-

ites in three groups were equalized to each other, and stored as frozen pellets at -20 °C for using SDS-PAGE and Western blotting.

SDS-PAGE

All the samples (n=60) were analyzed on SDS-PAGE gels (10% separation gel, 5% stacking gel). A suspension containing $1x10^5$ parasites per ml was mixed with an equal volume of SDS-PAGE sample buffer with 8-16% Tris-Glycine (Novex, US) and loaded onto SDS-PAGE gels. Standard molecular weight markers, including 14-97 kDa (Santa Cruz, US) were also loaded onto SDS-PAGE gels. Electrophoresis was carried out at 100 V (gel electrophoresis apparatus, BioRad). Gels were stained with Silver Staining Kit (SilverQuest™, Invitrogen).

Western blotting

After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gels were applied to a sheet of nitrocellulose paper (0.2-pLm pore size) and electrophoresed for 2 h with a transfer apparatus. After electrophoresis, nitrocellulose paper was blocked with 3% casein (pH 7.4) overnight at 4 °C. On the following day, the strips were washed three times in a solution of 1xTBS (Tris Buffer Saline). Strips were then incubated with sera (*Toxoplasma* positive control sample) diluted by 1:100 in 1xTBS for 1 h at room temperature. Then, the nitrocellulose was washed for three times in 1xTBS and incubated with anti-human IgG-alkaline phosphatase conjugate (Sigma) diluted by 1:1000 for 1 hour. The strips were demonstrated with the chromogenic substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Statistical Analysis

Statistical analyses were conducted by SPSS 14 software (Chicago, IL, USA). Data were expressed as means ± standard error of means (SEM) of at least three independent experiments performed. Comparisons between groups were performed by using Kruskal-Wallis or Mann-Whitney U test. P <0.05 were considered statistically significant.

Results

In vivo study

The number of parasites in exudates of mice was lower in the CEMF-exposed group {Group 1, (1212.5±258.47) $x10^4$} than in the control group {Group 3, (1790.6±177.78) $x10^4$} but the decrease was not statistically significant (p=0.1557). Moreover, the number of parasites in the PEMF-exposed group {Group 2, (740.63±113.68) $x10^4$} was found to decrease significantly (P<0.01) in comparison to that of control group (Fig. 2). In addition, the number of parasites was observed to be significantly lower in Group 2 in comparison to Group 1 (P<0.05).

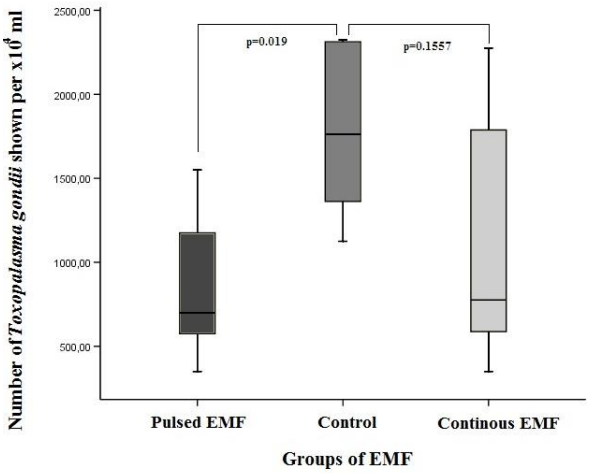

Fig. 2: The number of *T.gondii* obtained from in vivo EMF measurements. Comparison of the effects of ELF-CEMF (50Hz, 2mT) and ELF-PEMF (75Hz, 2.3mT) on the growth of *T. gondii*

In vitro study

For the tachyzoites-infected mice, the number of parasites was lower in the PEMF-exposed group {Group B, (317.5±32.5) $x10^4$} than in the control group {(982.5±220.89)$x10^4$} (P<0.05). The number

of parasites was also decreased in the CEMF-exposed group {Group A, (597.5±96.71) $x10^4$} in comparison to that of the control group (Group C), but the decrease was not statistically significant *(P*=0.4955) and no differences were observed in the life span of the animals (Fig. 3).

SDS-PAGE

The results of SDS-PAGE analysis yielded thirty bands ranging between 6 and 120 kDa. The numbers of the protein bands were the same in different groups, which were exposed to ELF-EMF with different intensities and frequencies. There was no significant difference among all samples (n=60) when the band numbers and staining properties were considered (Fig. 4a, 4b).

Western Blotting

Toxoplasma-specific antibody response was determined and no significant differences were observed among the samples. Typical western blot patterns against *T. gondii* were found in all samples. The patterns revealed a set of major surface antigen reacting bands with molecular weights of 30 kDa (SAG1), 22 kDa (SAG2) and 43 kDa (SAG3), 22-30 kDa dense granular proteins and 50-70 kDa rhoptry proteins with some minor bands (Fig.5) (18-22).

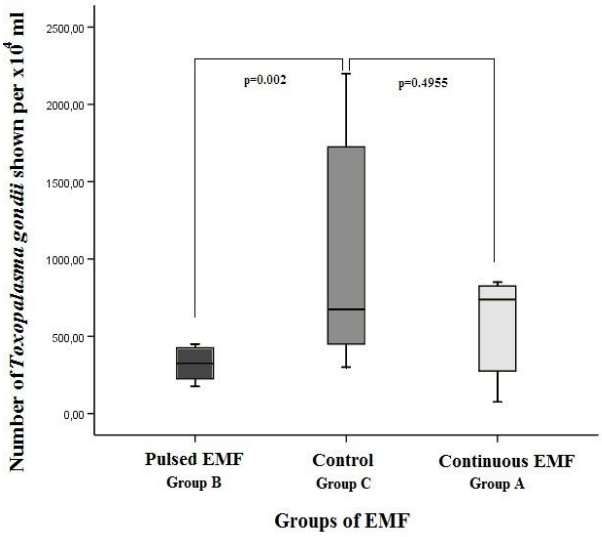

Fig. 3: The numbers of *T.gondii* obtained from in vivo ELF-EMF measurements. Comparison of the effects of ELF-CEMF (50Hz, 2mT) and ELF-PEMF (75Hz, 2.3mT) on the growth of *T. gondii*

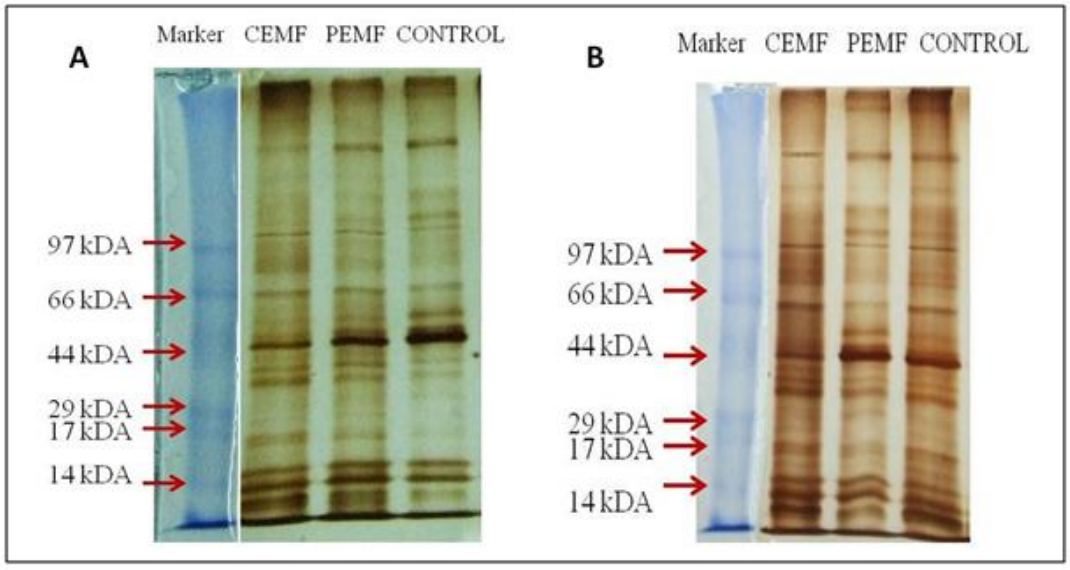

Fig. 4: Images of *T. gondii* protein bands colored with silver nitrate resolved with SDS-PAGE technique in the end of the (a) In vivo experiment (b) In vitro experiment

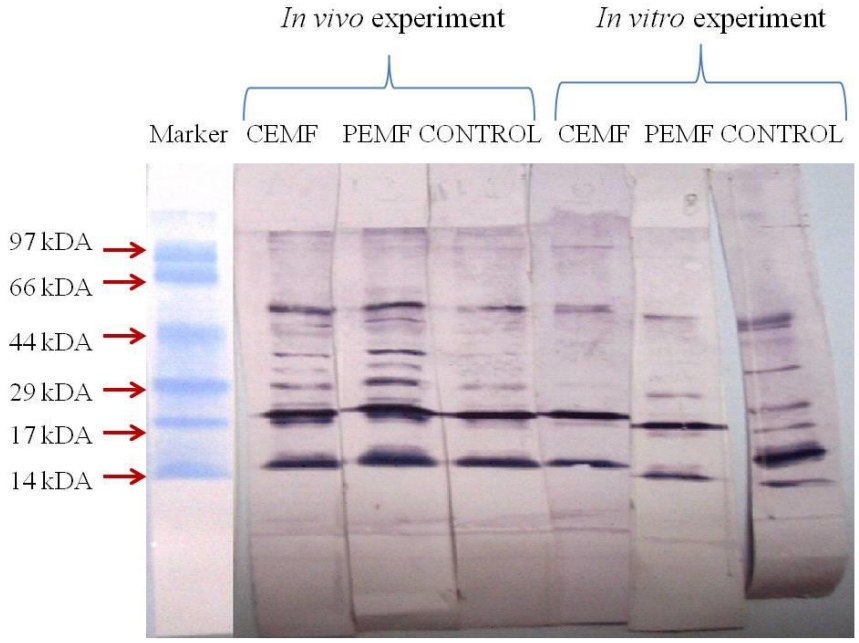

Fig. 5: Western blot images of control, continuous and pulsed ELF-EMF exposed groups

Discussion

Biological effects of ELF-EMF have been widely studied in various microorganisms such as *Escherichia coli, Staphylococcus aureus, Dictyostelium discoideum*, Kaposi's sarcoma-associated herpes virus, *Entamoeba invadens, Paramecium* etc. (23-27) however there is still lack of information about the effects of ELF-EMF on *T. gondii*. There are no previous reports investigating the effects of ELF-EMF on *T. gondii,* however the effects of EMF on other parasites have been reported. In this study, we observed that CEMF and PEMF exposures reduced the proliferation rate of tachyzoites in vivo and their viability in vitro experiments.

There are various ELF-EMF studies on microorganisms using different frequencies, exposure time and experimental conditions. ELF-EMF exposure changes the growth, life span, viability, virulence of the microorganisms (23, 28, 29). ELF-EMF exposure (1mT, 50 Hz, 12 h) on human glioma cells increased the mutation rate up to 3.75-fold compared to unexposed controls (30). In addition, human fibroblast cells treated with 1mT intermittent ELF-EMF for 2-24 h were significantly increased both chromosomal aberrations and micronucleus formation, and they suggested that intermittent ELF-EMF may lead to considerable chromosomal damage in dividing cells (31). No reproducible changes in the two-dimensional gel electrophoresis were observed in bacterial and yeast cells after exposure to ELF-EMF (32, 33). On another study, the yeast cells were treated with 50 Hz 1 mT EMF for 60 min, however no significant effects of ELF-EMF on the yeast proteome were determined by using 2-D Fluorescence Difference Gel Electrophoresis (34). In our study, SDS-PAGE analysis subsequent to ELF-EMF application at a dose of 2 and 2.3 mT did not appear to show a significant impact on protein bands of samples taken from infected peritoneal exudates.

When Rodriquez-De la et al., investigated 60 Hz EMF effect (1, 1.5 and 2 mT) on growth and differentiation of *E. invadens*, they determined an inhibiting effect on the growth of trophozoite cultures. In our study, we applied 2.3 mT of ELF-PEMF to control and *T. gondii* infected groups for a total of 8 h and demon-

strated the statistically significant reduction of *T. gondii* number in infected group ($P<0.01$). The decrease in the number of the cells is a result of an affected cell cycle (26). The alterations in intracellular calcium concentration have an impact on the signal transmission mechanisms affecting the genes taking part in growth (35). These two mechanisms may also help to explain the reduction in cell numbers in our study.

Paramecium, a protozoon with cilia, responds to environmental stimuli by changing its swimming attitude (36). Mean speed of Paramecium directional spin number increases depending on EMF dose (even at 0.5-2 mT). This result emphasizes that even low doses of EMF can affect the motility of protozoa (11). On the other hand, 72 Hz pulse EMF exposure increases *paramecium* cell division rate (37). This increase in cell division and motility of paramecium enables it to find host organisms, which in turn increase its virulence. For *T. gondii*, virulence depends on parasites gliding motility, host cell attachment and invasion of cells (37). In our study, we used the similar EMF dose that affects the virulence of paramecium; however, these EMF doses did not affect the virulence of *T. gondii.* Therefore, different mechanisms may play role for the virulence of *T. gondii.*

Delgado observed an inhibition of *Lactobacillus acidophillus* reproductively by applying 26 Hz and 40 Gauss (four mT) dose pulse EMF. Likewise, EMF shows a decrease in *T. gondii* numbers both in vitro and in vivo experiments even at lower doses (2 and 2.3mT) (38). Graham et al. (39) showed that growth of *Drosophila melanogaster* slows with the exposure of 1.5-μT and 80-μT magnetic field, but in our study, 50 and 75Hz EMF exposure did not make any change in weight of *T. gondii* infected mice.

Fojt et al. (40) have exposed *E. coli* and *S. aureus* to 50 Hz and 10 mT EMF less than 30 min. They have determined a decrease in colonizing unit number mostly in *E. coli* comparing with control group. ELF-PEMF treatment (2-250 Hz; 0.5-2.5 mT) decreased the growth rate of *S. aureus* (41). Our results also revealed a decline in the growth rate of *T. gondii* after ELF-PEMF exposure.

In the current study, EMF exposure also resulted in a reduction in cell number revealed by the alterations in *T. gondii* number in the peritoneal fluid of infected mice. Similar with our findings, Elmusharaf et al. (42) treated the bird infection caused by a protozoon named *Eimeria maxima* by EMF (5 μT/30 min/day; 21 days) and reported that EMF decreased the oocyst numbers of infected feces weight.

The environmental factors can provide the activation of stress genes (43). Fifty Hz electric field does not affect the viability or survival rate of *T. gondii* infected mice, but plays an inhibitory role on early phase of oxidative stress response (44). Moreover, electric field was shown to have no effect on life span of *T. gondii* infected mice, in our in vitro study no differences were also observed in the life span of the animals exposed to EMF. Although no significant changes in protein bands were observed in SDS-PAGE between two groups, 50 Hz CEMF seemed to be ineffective in reducing the number of *T. gondii* tachyzoites in infected mice, while 75 Hz PEMF could decrease the *T. gondii* number significantly.

Conclusion

Growth and viability of *T. gondii* were influenced by the exposure to low frequency electromagnetic fields, in the studied range. The most remarkable finding of our study was the decrease in number of parasites upon EMF exposure. This decrease in parasite number may also be related to virulence of *T. gondii*. These results emphasize that EMF has some effects on *T. gondii*, but these effects should be investigated by further studies using advance molecular techniques to figure out the role of signaling pathways. In addition, the relation in between virulence and ELF-EMF exposure should also be explained. Besides, these kinds of studies may also help to elucidate the mechanism of EMF on living systems.

Acknowledgments

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