Original Article

Effects of Various Densities of 50 Hz Electromagnetic Field on Serum IL-9, IL-10, and TNF-α Levels

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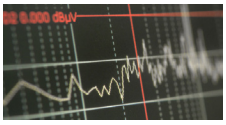

Abstract

Background: Extremely low-frequency electromagnetic fields (ELF-EMFs) are abundantly produced in modern societies. In recent years, interest in the possible effects of ELF-EMFs on the immune system has progressively increased.

Objective: To examine the effects of ELF-EMFs with magnetic flux densities of 1, 100, 500, and 2000 μT on the serum levels of interleukin (IL)-9, IL-10, and tumor necrosis factor-alpha (TNF-α).

Methods: 80 adult male rats were exposed to ELF-EMFs at a frequency of 50 Hz for 2 h/day for 60 days. The serum cytokines were measured at two phases of pre- and post-stimulation of the immune system by human serum albumin (HSA).

Results: Serum levels of IL-9 and TNF-α, as pro-inflammatory cytokines, were decreased due to 50 Hz EMFs exposure compared with the controls in the pre- and post-stimulation phases. On the contrary, exposures to 1 and 100 μT 50 Hz EMFs increased the levels of anti-inflammatory cytokine, and IL-10 only in the pre-stimulation phase. In the post-stimulation phase, the mean level of serum IL-10 was not changed in the experimental groups.

Conclusion: The magnetic flux densities of 1 and 100 μT 50 Hz EMFs had more immunological effects than EMFs with higher densities. Exposure to 50 Hz EMFs may activate anti-inflammatory effects in rats, by down-modulation of pro-inflammatory cytokines (IL-9 and TNF-α) and induction of the anti-inflammatory cytokine (IL-10).

Keywords: Interleukin-9; Interleukin-10; Tumor necrosis factor-alpha; Immunization; Electromagnetic fields

Introduction

In the past decades, researchers started to pay more attention to the effects of non-ionizing extremely low-frequency electromagnetic fields (ELF-EMFs) on biological system.[1] Some studies have indicated that exposure to ELF-EMFs has possible health hazards such as cancer, leukemia, neurodegenerative diseases, and infertility.[2-5] Despite potential negative impacts of ELF-EMFs, nowadays, they can effectively be used to diagnose and treat various diseases such as cancer, muscle regeneration, diabetes, arthritis, and neurological disorders.[1,6] These disorders can be caused by abnormal immune response to harmful invaders. During an immune

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system response, naive $CD4^+$ T cells can be committed to four major cells lineages, namely T helper (Th)1, Th2, Th17, and regulatory T (Treg) cells based on their cytokine production pattern.[7] Several studies reveal that effects of ELF-EMFs could be influenced by their frequency, density, and duration of exposure.[8-10]

Interleukin (IL)-9 effects on multiple cell types influence the development of immunity and inflammation. It has largely been regarded as a Th2 cytokine, however, under certain conditions, Tregs, Th1, Th17, and Th9 cells can also produce IL-9. IL-4 and signal transducer and activator of transcription 6 (STAT6) facilitate transcription of GATA binding protein 3 (GATA3), which is required for both Th2 and Th9 development.[11] IL-9 up-regulation is related to the pathogenesis of a number of diseases, including asthma,[12] collagen-induced arthritis,[11] experimental auto-immune encephalomyelitis (EAE), and multiple sclerosis (MS).[13] On the contrary, IL-9 deficiency suppresses acute and chronic colitis in patients with inflammatory bowel diseases (IBD).[14] IL-9 neutralization and IL-9 receptor deficiency decrease IL-6 producing macrophages and Th17 cells in the central nervous system (CNS) in an EAE mice model.[15]

IL-10 is produced by immune cells including macrophages, T lymphocytes, and natural killer cells (NK). IL-10/IL-10 receptor (R) interaction starts an intracellular signaling pathway involving Junus kinase 1 (JAK1), tyrosine kinase 2 (Tyk2), and STAT3. STAT3 dimerization and nuclear translocation induce the expression of target genes. It can suppress the immune response by inhibiting nuclear factor kappa B (NF-κB) nuclear translocation, major histocompatibility complex class II (MHCII) expression, and pro-inflammatory cytokines production.[16] Owing to the anti-inflammatory actions of IL-10, this cytokine has potential for therapeutic effects. Exogenous IL-10 is able to down-regulate the enhanced pro-inflammatory cytokine release from the lamina propria mononuclear cells isolated from patients with Crohn's disease (CD).[17] IL-10 therapy for inflammatory bowel disease (IBD) is suggested by inhibition of IL-1β in organ cultures of intestinal biopsies taken from patients with ulcerative colitis.[18]

Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, is produced by many cell types including activated macrophages, NK cells, and T cells.[19] TNF-α can induce up-regulation of MHC molecules, IL-1, IL-6, and IL-8 expression, T cell proliferation, and interferon-gamma (IFN-γ) production. These effects can be attributed to NF-κB and mitogen-activated protein kinase (MAPKs) pathways including extracellular signal regulated kinase 1/2 (ERK), p38, and JNK activation, and can thus contribute to the pathogenesis of several inflammatory diseases.[20] Administration of anti-TNF antibody to rheumatoid arthritis (RA) mouse model leads to a reduction in inflammation.[21] Furthermore, deficiency of TNF-α and TNFR-1 or neutralization of TNF-α with monoclonal antibodies reduces EAE.[19]

The effects of 50 Hz EMFs have so far been published with focus on a field flux density of usually ≤100 μT. However, some appliances in use today may produce a field flux density of up to 100 μT. Some studies have investigated the effects of ELF-EMFs on cytokines levels without stimulation of the immune system;[22,23] several have been conducted on stimulated cytokines levels after exposure to ELF-EMFs.[24,25] In our previous studies, the effects of 50 Hz EMFs were investigated on some cytokines levels such as IFN-γ, transforming growth factor-beta (TGF-β), IL-4, IL-6, IL-9, IL-10, IL-12, and IL-17 in pre- and post-immunization phases.[26-28] The current study was conducted to determine if exposure to 50 Hz EMFs with flux densities of 1, 100,

500, and 2000 μT would alter serum levels of IL-9, IL-10, and TNF-α in two stages of pre- and post-immunization of adult male rats.

Materials and Methods

Exposure Devices

Electromagnetic exposure solenoids made of polyvinyl chloride (PVC) tubes two meters long and 40 centimeters in diameter, were used. The solenoids were fed with a 50 Hz sinusoidal power frequency current. The magnetic field flux densities of 1, 100, 500, and 2000 μT were produced in each solenoid by combination of different turns of wires, and electrical potential and currents. The mean background EMF density in the animal lab was around 0.07 (SD 0.03) μT. The densities of 50 Hz EMFs were measured in the middle of solenoids using an ELF-EMFs Survey Meter (HI-3604, Holaday Ind, USA).[26,29]

Animals and 50 Hz EMFs Exposure

Eighty adult male Wistar rats were purchased from animals facilities of Hamadan University of Medical Sciences. They were kept in standard plastic laboratory cages at a temperature of 21–22 °C and a relative humidity of 55%–65% with a 12:12-hour light:dark cycle. During the experiment, they were fed with standard pellets of food and provided with tap water *ad libitum*. After one week of adaptation to the laboratory conditions, the rats were weighed and randomly divided into five equal groups—a control group and four groups exposed to 50 Hz EMFs. The rats were grouped and randomized according to entry to the study; they had a mean weight of 202.5 (SD 7.5) g and 8 weeks old.

The four experimental groups were exposed to 50 Hz EMF with magnetic field flux densities of 1, 100, 500, and 2000 μT measured at the center of the solenoids chambers for 2 h/day. The sham-exposed control rats were placed in a solenoid when they were not connected to electricity. Temperature and humidity were maintained constant during exposure using fan-controlled air circulation. All procedures were approved in advance by Ethical Committee for Hamadan University of Medical Sciences.[26,29]

Immunization Protocol

After one month of exposure, blood samples were collected from retro-orbital plexus of the rats. Sera were separated by centrifuge at 3500×g for 10 min. These sera were designated as pre-immunization phase and stored at -70 °C for cytokines analysis by enzyme-linked immunosorbent assay (ELISA). Then, each rat received 100 μg of human serum albumin (HSA) in 0.25 mL of phosphate buffer saline (PBS) mixed with an equal volume of incomplete Freund's adjuvant (IFA). The solutions were injected intraperitoneally (IP) on days 31, 44, and 58 of the exposure to EMF. The rats were immunized with HSA to activate naive $CD4^+$ T cells and to shift them toward functional patterns of Th1, Th2, Th17, and Treg subsets formation.[28] On day 61, at the end of the exposure period, the rats were sacrificed with

TAKE-HOME MESSAGE

- Non-ionizing extremely low-frequency electromagnetic fields (ELF-EMFs) has effect or side-effect on biological systems.
- Serum levels of IL-9 and TNF-α, as pro-inflammatory cytokines, were decreased due to 50 Hz EMFs exposure compared with the controls in the pre- and post-stimulation phases.
- Exposure to 1- and 100-μT 50 Hz EMFs may activate anti-inflammatory effects in rats, by down-modulation of pro-inflammatory cytokines (IL-9 and TNF-α) and induction of the anti-inflammatory cytokine (IL-10).

ether asphyxia. The animals' peripheral blood was collected from vena cava veins and centrifuged to isolate the sera, as described above. The serums were then designated as post-immunization phase and immediately stored at -70 °C for cytokines analysis.

Cytokines Assay

The concentrations of IL-9, IL-10, and TNF-α were measured in pre- and post-immunization phases in the sera of rats exposed to various densities of 50 Hz EMFs and control rats. Commercial ELISA kits (Eastbiopharm, China) were used according to the manufacturer's instructions to measure cytokines. All the samples were analyzed in duplicate. Intra- and inter-assay coefficients for the three cytokines measured were <10% and <12%, respectively. Sensitivities for IL-9, IL-10, and TNF-α assay were 0.53, 1.51, and 2.51 pg/mL, respectively.[4,29]

Statistical Analysis

All statistical analyses were performed with SPSS® for Windows® ver 16.0. One-way ANOVA was used to compare means of continuous variables among the study groups. Tukey's HSD was used as *post hoc* test. *Student's t* test for paired samples was used for comparison of mean levels of cytokines at pre- and post-immunization phases.

Results

Two rats (from 1 and 500 µT groups) died during the 60-day study period. The mean serum IL-9, IL-10, and TNF-α levels were changed significantly ($p<0.001$) among the studied groups at pre-immunization phase (Fig 1). The mean concentration of IL-9 in sham-exposed rats significantly ($p=0.001$) decreased in rats exposed to 100 µT. The mean concentration of TNF-α also significantly ($p<0.005$) decreased in

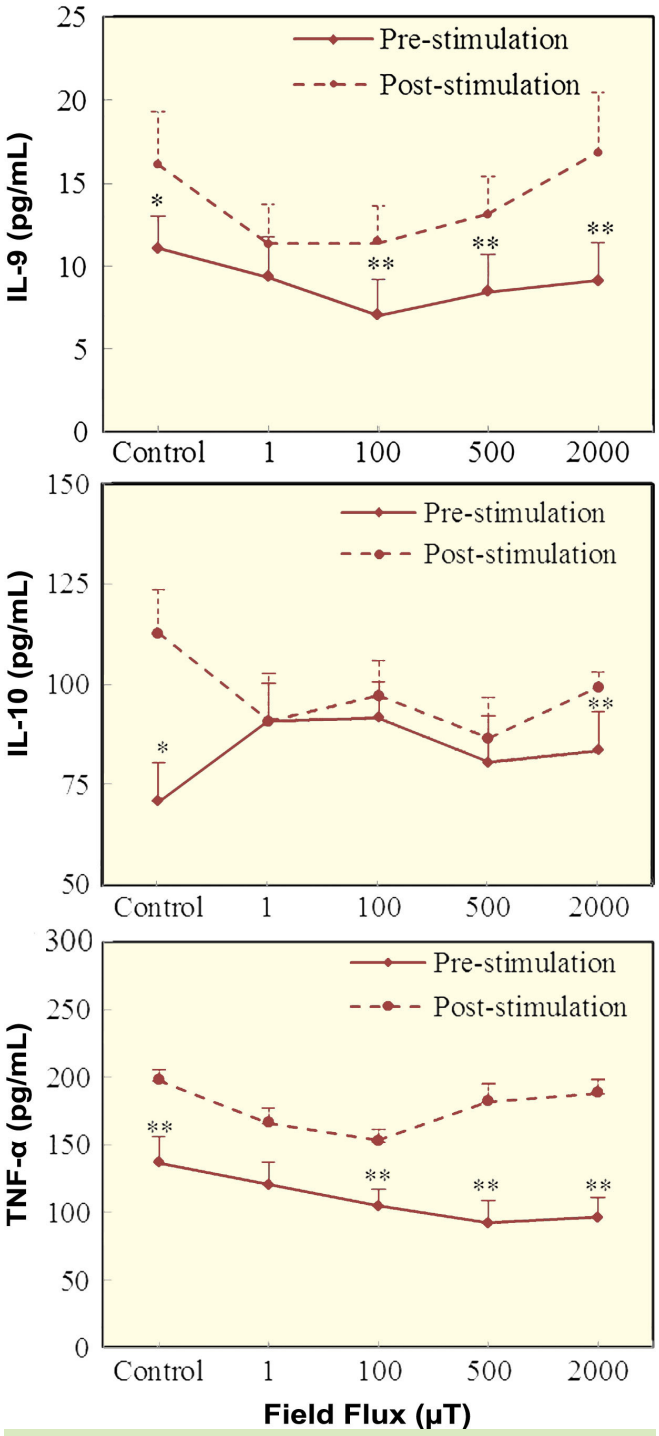

Figure 1: Serum levels of IL-9, IL-10, and TNF-α in pre- and post-immunization phases after exposure to various magnetic field flux densities of a 50 Hz EMF. Error bars represent SD. Comparison was made between pre- and post-immunization phases (*p<0.05, **p<0.01).

rats exposed to ≥100 μT compared with the control group (Fig 1). The mean levels of IL-10, however, significantly ($p<0.05$) increased in rats exposed to 1 and 100 μT compared with the control group (Fig 1).

The levels of IL-9, IL-10, and TNF-α significantly ($p<0.001$) changed post-immunization after ELF-EMFs exposure (Fig 1)—IL-9 level decreased in 1- and 100-μT groups; so did TNF-α in 100-μT group; IL-10 did not change (Fig 1). In other words, the effects of ELF-EMFs exposure were lost due to time and/or HSA injection.

The levels of IL-9 and TNF-α in the control group and 100-, 500-, and 2000-μT groups significantly increased in the post-immunization phase compared to the pre-immunization phase. The levels of IL-10 also increased in the control group and 2000-μT group in the post-immunization phase (Fig 1). These could be attributed to time and/or HSA injection.

An attempt to model the effects of various magnetic field flux densities on the serum levels of IL-9, IL-10, and TNF-α showed that a parabolic curve model had an acceptable fit. It seems that lower densities of ELF-EMFs would have higher effects on the serum levels of the studied cytokines.

Discussion

In our study, the serum levels of IL-9 and TNF-α decreased in both pre- and post-immunization phases due to exposure to 50 Hz EMFs; the level of IL-10 increased only in the pre-immunization phase. Comparison of pre- and post-immunization cytokines levels in each group showed that the levels were increased in the post-immunization phase, particularly for IL-9 and TNF-α.

Serum IL-9 level decreased in both pre- and post-immunization phases after exposure to 50 Hz EMFs. Although some studies have investigated the effects of 50 Hz EMFs on cytokine production, only a very few have been conducted on production of pro-inflammatory cytokine IL-9 after EMFs exposure. Since in some works, IL-9, as a Th17-derived cytokine, could contribute to inflammatory diseases and autoimmunity,[15] the effects of various densities of 50 Hz EMFs on Th17 cell signature cytokine IL-17 and related gene retinoid-related orphan receptor α (RORα) were reported in our previous works. It is shown that the effects of 50 Hz EMFs with 1- and 100-μT magnetic field flux density suppressed the serum level of IL-17 and expression of RORα, suggesting inhibitory effects of 50 Hz EMFs on inflammation.[28,30] In contrast, an *in vivo* study by Pena-Philippides, *et al*, investigated the effect of 50 Hz pulsed EMF with 0.1-μT magnetic field flux density was able to increase the expression of IL-9 and decrease the expression of IL-1α and TNF-α in ischemic brain damaged of C57BL/6 male mice. These effects indicated that 50 Hz pulsed EMF application could have a possible adjunctive treatment for stroke patients with down-regulation of major pro-inflammatory cytokines, IL-1α and TNF-α.[31] Another *in vivo* study demonstrates that exposure of tumor-bearing mice to 7.5 Hz EMFs with a field flux density of 400 000 μT reduces tumor growth and pro-inflammatory cytokine IL-6 production, while it does not change IL-9 production. It is suggested that the 7.5 Hz EMFs may be useful for therapy against cancer and inflammation.[32]

This study showed that the serum levels of TNF-α decreased in both pre- and post-immunization phases during exposure to 50 Hz EMFs. Similar to our results, an *in vitro* study indicated that exposure to 1000-μT 50 Hz EMFs decreased TNF-α and increased pro-inflammatory cytokines, IL-1β and IL-6, production by human peripheral blood mononuclear cells (h-PBMCs) after 72 hours of exposure. The increased IL-1β and IL-6 production may

demonstrate a complex feedback relationship between IL-6 and TNF-α, suggesting that 50 Hz EMFs might inactivate the immune system against inflammation.[33] Another study shows that when human fibroblast-like cell (h-FLC) cultures are exposed to 2250-μT 50 Hz pulsed EMFs, TNF-α and IL-1β production decreases on days 14 and 21 of the culture. These facts coincide with the clinical effects on patients with osteoarthritic undergoing 50 Hz pulsed EMFs irradiation.[34] Gottlieb, *et al*, report that TNF-α could induce upregulation of IL-1, IL-6, IL-8, and IFN-γ production.[35] In an *in vivo* study, lower plasma IL-1β and IL-6 were observed in 186 male workers who had long-term exposure to 5–32 000 Hz EMFs, suggesting that ELF-EMFs may affect the immune system and reduce inflammatory responses.[36] In addition, our previous study indicates that 50 Hz EMFs with a density of 100 μT decreases the rat serum level of IFN-γ, the signature cytokine for the Th1 involved in the pathogenesis of autoimmune disorders.[27] In contrast, Ikdea, *et al*, report that the levels of TNF-α is not significantly changed in h-PBMCs after a 6-hour exposure to 50 Hz 100- and 500-μT EMFs.[25] Furthermore, in one of our previous studies, it was shown that the level of IL-6 by the whole spleen culture and total blood culture of rats exposed to 50 Hz EMFs was not significantly different.[26]

Some studies have explained the mechanisms that may mediate expression or release of IL-9 and TNF-α. TNF-α is a pro-inflammatory cytokine demonstrated to coordinate the cellular responses and organize local inflammation. This pro-inflammatory response is most probably mediated by activated inflammatory cells, inducing endothelial cells to express leukocyte adhesion molecules and release of secondary cytokines and other inflammatory mediators.[37] Gounni, *et al*, conclude that expression and release of IL-9 are up-regulated by TNF-α in human peripheral blood eosinophils.[38] However, it can also be speculated that one of the mechanisms of IL-9 in the inflammation process may be mediated by affecting the release of TNF-α. Zhang, *et al*, indicate that IL-9 significantly up-regulates the TNF-α secretion of nucleus pulposus (NP) cells isolated from the host immune system.[39] It can therefore be proposed that IL-9 and TNF-α may form a positive feedback regulatory loop in the inflammation process. Our study also showed that serum cytokines concentrations increased in the post-immunization phase compared with the pre-immunization phase, particularly for IL-9 and TNF-α. It can be postulated that longer duration of 50 Hz EMFs would activate the immune system and produce high levels of IL-9, IL-10, and TNF-α. After immunization of rats with HSA, the macrophages and T cells were further activated to produce the high levels of these cytokines.

In our study, exposure to 50 Hz EMFs increased the serum level of IL-10 only in the pre-immunization phase. To date, no *in vivo* study has been conducted on the secretion of IL-10 after exposure to ELF-EMFs. Along with our results, an *in vitro* study shows that exposure to 1500-μT 75 Hz pulsed EMF stimulates the release of IL-10, while inhibits the release of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α by synovial fibroblasts (SFs).[40] Another study reports that exposure to 2250-μT 50 Hz pulsed EMF on h-FLCs culture enhances IL-10 production. These results suggest that IL-10 is a potent antagonist of some pro-inflammatory cytokines. Furthermore, ELF-EMFs might represent therapeutic effects for controlling the osteoarthritis-associated inflammation.[34] Kaszuba-Zwoinska, *et al*, report that exposure to 4000–5000-μT 50 Hz pulsed EMFs can increase IL-10 and decrease IFN-γ pro-inflammatory production stim-

ulated by phytohemagglutinin (PHA) or lipopolysaccharide (LPS) in h-PBMCs derived from healthy volunteers and patients with Crohn's disease. The authors believe that the ELF-EMFs could be an important step for non-invasive treatment of chronic inflammatory diseases.[41] On the contrary, an *in vitro* study by Ikdea, *et al*, shows that exposure to 50 Hz EMFs with a magnetic field flux of 2, 20, 100, and 500 μT does not affect IL-10 production by h-PBMCs.[23] Furthermore, TGF-β along with IL-10, the signature cytokines of Treg cells, suppress the immune system and play role in prevention of inflammatory diseases; TGF-β does not change after exposure to 50 Hz EMFs.[28] Some studies indicate that IL-10 has a negative feedback on IL-9 production. For instance, IL-10 is significantly increased in IL-9-/- EAE mice compared with their wild type counterparts following immunization with myelin proteolipid protein.[42]

The molecular mechanism underlying these effects is believed to be a free radical phenomenon. Free radicals influence the biological properties of molecules such as nucleic acid, DNA, and protein.[43] Exposure to 50 Hz EMFs with flux densities of 100 and 500 μT for 10 months, 2 hours/day on male Sprague-Dawley rats declines nitric oxide (NO) radical release. It is suggested that ELF-EMFs might have a negative feedback on NO pathway.[44]

We found that cytokine production was mostly changed at 100 μT field density and returned back to the normal level as the density increased; the relation between 50 Hz EMFs density and cytokine production was therefore not linear. Although these results seemed strange, some studies support this nonlinear relation between the EMF density and its effects. Chen, *et al*, exposed Friend erythroleukemia cells to 60 Hz EMFs with varying densities of 1, 2.5, 5, 10, 50, 100, and 1000 μT. They conclude that the maximal inhibition of differentiation occurs during exposure to 50 μT; it then increases with density enhancement.[45] Another study investigated the expressions of HSP27, HSP60, and HSP70 by human myeloid leukemia (HL-60) after exposure to 50 Hz EMFs in the range of 10–140 μT flux densities. Exposure to 60–80-μT 50 Hz EMF results in a significant induction in *HSP* genes expression; at higher intensities, the levels of gene expression are insignificantly decreased compared with the controls.[46]

One main limitation of our study was that it was performed in the background EMFs with a mean density of about 0.07 (SD 0.03) μT; elimination of the effects of these background radiation was not possible. We did also not assess other biological factors important in the immune response, including levels of free radicals, heat shock proteins, and mitogen-activated protein kinases.

In conclusion, our results would open new avenues for the treatment of inflammation. It seems that exposure to 50 Hz EMFs would affect the cytokine production, particularly at a flux density of 100 μT. Further studies are therefore required to address these questions.

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Conflicts of Interest: None declared.

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