

Research Paper: Comparing the Effects of Long-term Exposure to Extremely Low-frequency Electromagnetic Fields With Different Values on Learning, Memory, Anxiety, and β -amyloid Deposition in Adult Rats



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ABSTRACT

Introduction: Extremely Low-Frequency Electromagnetic Fields (ELF-EMFs) have gathered significant consideration for their possible pathogenicity. However, their effects on the nervous system's functions were not fully clarified. This study aimed to assay the impact of ELF-EMFs with different intensities on memory, anxiety, antioxidant activity, β -amyloid (A β) deposition, and microglia population in rats.

Methods: Fifty male adult rats were randomly separated into 5 groups; 4 were exposed to a flux density of 1, 100, 500, and 2000 microtesla (μ T), 50 Hz frequency for one h/day for two months, and one group as a control group. The control group was without ELF-EMF stimulation. After 8 weeks, passive avoidance and Elevated Plus Maze (EPM) tests were performed to assess memory formation and anxiety-like behavior, respectively. Total free thiol groups and the index of lipid peroxidation were evaluated. Additionally, for detection of A β deposition and stained microglia in the brain, anti- β -amyloid and anti-Iba1 antibodies were used.

Results: The step-through latency in the retention test in ELF-EMF exposure groups (100500 & 2000 μ T) was significantly greater than the control group ($P < 0.05$). Furthermore, the frequency of the entries into the open arms in ELF-EMF exposure groups (especially 2000 μ T) decreased than the control group ($P < 0.05$). No A β depositions were detected in the hippocampus of different groups. An increase in microglia numbers in the 100, 500, and 2000 μ T groups was observed compared to the control and one μ T group.

Conclusion: Exposure to ELF-EMF had an anxiogenic effect on rats, promoted memory, and induced oxidative stress. No A β depositions were detected in the brain. Moreover, the positive impact of ELF-EMF was observed on the microglia population in the brain.

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Highlights

- ELF-EMFs have gathered significant consideration for their possible pathogenicity.
- ELF-EMFs' effects on the nervous system's functions were not clarified yet.
- Positive impact of ELF-EMF was observed on the microglia population in the brain.

Plain Language Summary

ELF-EMFs effects on human health are a considerable concern. Studies revealed the adverse effects of ELF-EMF in neurological disorders such as Alzheimer's Disease (AD). Anxiety could be an early manifestation of AD. There is a correlation between occupational exposure to ELF-EMF and AD. Recently the researchers interested in the study of the effects of ELF-EMFs on the human body. Some studies examined the molecular mechanisms and the influence of ELF-EMFs on the biologic mechanisms in the body. Also, Microglia act in the Central Nervous system (CNS) immune responses; over-activated microglia can be responsible for devastating and progressive neurotoxic consequences in neurodegenerative disorders. This study aimed to evaluate the memory, anxiety, antioxidant activity, β -amyloid deposition, and frequency of the microglial cells exposed to microtesla (μ T) and 2000 (μ T) ELF-EMFs.

1. Introduction

Exremely Low-Frequency Electromagnetic Fields (ELF-EMFs) are in the frequency ranges of 1-300 (Hz) originating from electricity sources. ELF-EMFs effects on human health are a considerable concern with controversy (Patruno, Tabrez, Pesce, Shakil, Kamal, & Reale, 2015). Some studies revealed the adverse effects of ELF-EMF in neurological disorders such as Alzheimer's Disease (AD) (Jelenković, Janać, Pesić, Jovanović, Vasiljević, & Prolić 2006; Osera et al., 2015). Anxiety could be an early manifestation of AD. Moreover, there is a correlation between occupational exposure to ELF-EMF and AD. However, some restrictions affect this meta-analysis study's outcomes, such as duration and time windows of exposure, biologic mechanisms for this association, interactions between EMFs exposure, and established risk factors for AD (García, Sisternas et al., 2008). Increasing anxiety may be an early warning sign of AD (Komaki et al., 2014). Many studies support a high incidence of anxiety in patients with dementia (Ferretti, McCurry, Logsdon, Gibbons, & Teri, 2001). Therefore, the study of the effects of ELF-EMFs on the human body has gained significant interest recently, mainly because of the possible effects on memory and behavior.

Some studies examined the molecular mechanisms through which the influence of ELF-EMFs on the biologic mechanisms within the body. It has been demonstrated that ELF-EMFs can modulate oxidative stress (Cichoń, Bijak, Miller, & Saluk, 2017; Esmailpour, Sheibani,

Shabani, Mirnajafi Zadeh, & Akbarnejad, 2018). Changing the balance between ROS generation and exclusion has been suggested to play a critical function in neurodegenerative diseases (Soleimani et al., 2019). Furthermore, EMFs enhance cellular and molecular processes, neuronal differentiation, and neural marker expression (Cho et al., 2012). ELF-EMFs' role on behavioral changes has been reported in numerous studies; however, they are not obvious enough for judgments yet (Rauš Balind et al., 2016). Some evidence indicated that exposure to ELF-EMF stimulates β -amyloid peptide secretion (Del Giudice et al., 2007). They have also illustrated the effects of EMFs on the β -amyloid formation (Consales, Merla, Marino, & Benassi, 2012; Terzi, Ozberk, Deniz, & Kaplan, 2016). Sobel and Davanipour hypothesized that exposure to ELF-EMFs can increase β -amyloid peptides in the brain through modulating the Ca^{2+} channels (Sobel & Davanipour, 1996; D'Angelo, Cořtadini, Kamal, & Reale, 2015). The production of β -amyloid peptides is among AD's most remarkable neuropathological features (Tahmasebinia & Pourgholaminejad, 2017).

Microglial cells (microglia) act in the Central Nervous System (CNS) immune responses. Microglial cells may be the main target of the neurobiological effects of EMFs (Yang et al., 2010). Activated microglia are significant in the pathogenesis of AD (Yang et al., 2010). Over-Activated microglia can be responsible for devastating and progressive neurotoxic consequences in neurodegenerative disorders and excessive production of some factors, including superoxide, nitric oxide, and promote neuro-inflammations (Yang et al., 2010).

This study aimed to evaluate the memory, anxiety, antioxidant activity, β -amyloid deposition, and frequency of the microglial cells in adult rats exposed to 1-2000 microtesla (μ T) (1, 100, 500 and 2000 μ T) ELF-EMFs, frequency 50 Hz, one h/day for two months.

2. Methods

Animals and ethics statement

In this experiment, 8-10 weeks male adult Wistar rats weighing 200-250g were used. Animals were held in usual conditions (12:12 h light/dark cycle) at the fixed temperature at 23°C, with free access to food and water. The study rats were divided into 5 groups randomly: 4 groups exposed to a flux density of 1, 100, 500, and 2000 μ T, 50 Hz frequency for one h/day for 2 months, and one group as a control. The control group was without ELF-EMF stimulation. All experiments and technical procedures were done based on the ethical guidelines on the care of animal-laboratory of Hamadan University of Medical Sciences.

Magnetic field device

The exposure apparatus contained a solenoid coil of a length of 2 meters and a radius of 20 centimeters, and the turns were 1000 per meter. A copper wire with two millimeters diameter was used, and a 220 V and 50 Hz sinusoidal power frequency current was fed through the solenoid in the exposure system. A tunable resistance circuit was connected to the power source to set out the electric and magnetic fields. The used circuit generated an effective magnetic field in the range of 0–2000 microtesla (μ T), with a sinusoidal wave frequency of 50 Hz. The magnetic flux density was measured using a teslometer (HI-3604 Holladay, USA), and the density was adjusted by changing the coil current using an external resistance circuit (Salehi et al., 2013).

Learning and memory assessment: Passive Avoidance Learning (PAL) task

A passive avoidance test was carried out after 2 months of exposure to ELF-EMF stimulation. In brief, each rat was placed into a light compartment of a light-dark box. Rats during naturalization were allowed to freely travel the box for 5min with the sliding door between the light and dark compartments open (Gruden et al., 2016). Then, animals have come back to their home cages.

For conditioning, i.e., performed 2h after naturalization, animals were placed into the light compartment. When both hind limbs entered the dark box, the sliding door was closed, and an electrical foot shock (50-Hz square wave,

one mA for 3 s using a shock generator–scrambler) was delivered through the floor grid in the dark compartment. Animals were held in the light-dark box for 5min and then returned to their home cage. Tests were carried out 24h after the conditioning by representing the animals into the light compartment of the light-dark box. The step-through latency to the dark compartment (Step-Through Latency, STLR) and the Time spent in the Dark Compartment in the retention trial (TDC) was measured 24 h after the training for 300 s (Sakurai et al., 2008; Komaki et al., 2014; Moradkhani, Salehi, Abdolmaleki, & Komaki, 2015; Ganji, Salehi, Nazari, Taheri, & Komaki, 2017; Rebolledo-Solleiro et al., 2017; Khodamoradi et al., 2018).

Evaluating the anxiety-like behavior: Elevated Plus-Maze (EPM)

The maze used in this work had two open arms and two closed arms 51 lengths \times 10 cm wide crossing at the center perpendicular to each other, forming a plus shape and elevated 50 cm from the floor. Animals were placed on the central square of the maze facing an open arm at the beginning of the test. Moreover, they were allowed to explore the maze for 10 min. The number of entries to the open arms and the time spent in open arms were taken as an anxiety index (the higher the index, the lower the anxiety). The total number of arms (open + closed) entries was taken as a measure of locomotion. An entry was accounted for when the four paws of the rat were placed in the respective arm. Before each trial, the maze was cleaned with detergent and dried. The illumination level at the central square of the maze was 5.1 lux during testing (Komaki et al., 2014; Komaki et al., 2015; Rebolledo-Solleiro et al., 2017).

Serum sampling

Five mL peripheral serum samples were obtained from all animals. The serums were collected by centrifugation at 3000g for 15 min and stored at -80°C for the analysis of Total Antioxidant Capacity (TAC) (Tasset et al., 2012), Total Oxidant Status (TOS), free thiol groups, and index of lipid peroxidation (Malondialdehyde, MDA).

Measurement of Total Antioxidant Capacity (TAC)

TAC in serum samples using Ferric Reducing Antioxidant Power (FRAP) assay was measured. Reduced ferric tripyridyltriazine (Fe III-TPTZ) to a blue-colored Fe II-TPTZ by biological antioxidants occurs and is measured in this assay. The change of sample absorbance at 600nm was compared with the absorbance change of an identified standard ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Heydari et al., 2015; Asadbegi et al., 2018).

Measurement of Total Oxidant Status (TOS)

To measure serum TOS, the oxidation of ferrous ion to ferric ion, i.e., accompanied by several oxidant species in acidic pH, was used. The ferric ion was determined by xylenol orange. Briefly, 225 μ L of reagent 1 (150 μ M xylenol orange, 140mM NaCl, & 1.35M glycerol in 25mM H₂SO₄ solution, pH 1.75) was mixed with 35 μ L of the sample. The absorbance of each sample was read using a spectrophotometer at 560nm (sample blank) (Salehi et al., 2015; Asadbegi et al., 2018).

Measuring the Tissue Transglutaminase Antibody (TTG) level of the serum

The concentration of total serum thiol or sulfhydryl groups (thiol) were calculated using the described Ellman methods (Ellman, 1959), and the technique was modified by Hu (Hu, 1994). The method was applied after manual spectrophotometric optimization processes to an automated analyzer. The obtained sample and reagent-1 were mixed. After 90 seconds, reagent-2 was added. Thiols interacted with 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB) and formed a highly colored anion (5-thio-2-nitrobenzoic acid) with a maximum peak at 412 nm (Asadbegi et al., 2018).

Measuring the MDA

To evaluate lipid peroxidation, MDA, as the lipid peroxidation product, was measured. It was determined by reaction with thiobarbituric acid. 1.0mL of 20% trichloroacetic acid and 1.0mL of 1% thiobarbituric acid reactive substances with 100 μ L of the supernatant was mixed and incubated at 100°C for 80min. The cooled solution was centrifuged at 3000g for 20min, and the absorbance of the supernatant was measured at 532 nm (Salehi et al., 2015; Asadbegi et al., 2018).

Tissue preparation and immunohistochemistry

Rats were anesthetized after 2 months with ketamine and xylazine intensely and transcardially perfused with 150-200 ml PBS followed by 4% paraformaldehyde. The hippocampus of rats was harvested and fixed with paraformaldehyde. Fixed tissues were used for histological approaches. Tissue samples were embedded in paraffin (Merck, Germany), and 5 mm paraffin sections were transversely cut. After deparaffinization and antigen retrieval, sections were rinsed in PBS, treated with blocking solution, and incubated with recombinant anti-Iba1 antibody (Abcam, US) and anti-beta amyloid antibody (Abcam, US) overnight at 4 °C. The next day, after

washing with PBS, sections were incubated with FITC-conjugated goat anti-rabbit IgG (OriGene, Germany) as a secondary antibody for 2 hours at room temperature. Eventually, The sections were counterstained with DAPI for 45 min and cover-slipped with entellan (Alizadeh et al., 2015). The other sections were incubated in HRP goat anti-rabbit IgG (Abcam, US). After 3-times washing in PBS, using the avidin-biotin-peroxidase method (ABC Standard kit, Vectastain, VectorLabs), the antibody binding sites were visualized. Diaminobenzidine (DAB) was used as a chromogenic substrate. The sections were counterstained with hematoxylin and cover-slipped with entellan (Alizadeh et al., 2015; Simorgh et al., 2019).

SPSS was used for statistical analysis. The results were expressed as Mean \pm SD. One-Way Analysis of Variance (ANOVA) and Tukey's post-hoc test were used to compare the mean scores of the different study groups. P<0.05 was considered as the minimum level of significance.

3. Results

Learning and memory assessment: Passive Avoidance Learning (PAL) task

Exposure to the magnetic fields on the delay in entering the dark area at the reminder stage (STLr: Step-Through Latency in the retention test)

One-way ANOVA and Tukey's post hoc test data declared that exposure to magnetic fields significantly enhanced (P<0.05) the time of arrival in the dark area 24 after training in groups of 100 (24.6 \pm 8.7), 500 (20.1 \pm 6.5) and 2000 (14 \pm 5.1) microtesla (μ T), compared with the control group (2.8 \pm 0.9; Figure 1-A).

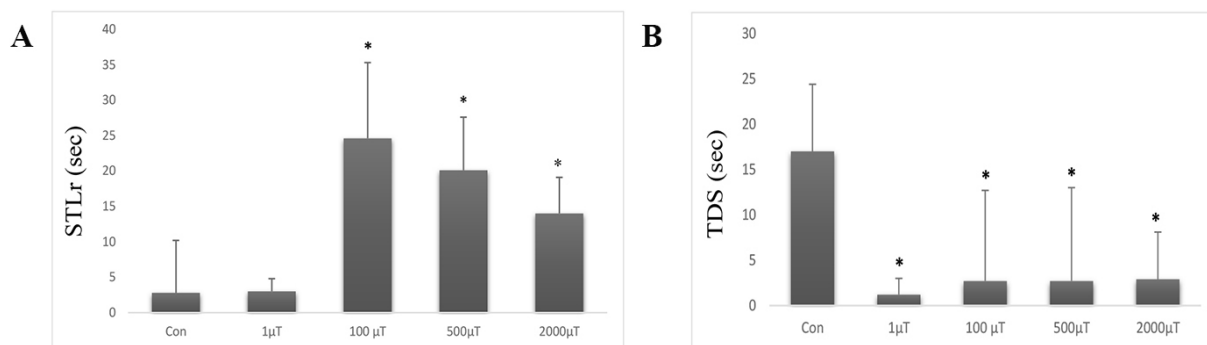
The effect of exposure to the magnetic fields on the presence in the dark area at the reminder stage (TDC: The Time spent in the Dark Compartment)

One-way ANOVA and Tukey's post hoc test data revealed that the contact with the magnetic fields caused a significant decrease (P<0.05) in the time of presence in the dark area at the reminder stage in groups 1 (1.2 \pm 0.8), 100 (2.7 \pm 1.1), 500 (2.7 \pm 0.9) and 2000 (2.9 \pm 1.2) μ T, compared to the control group (17 \pm 7.4; Figure 1-B).

Evaluating the anxiety-like behavior: EPM

Exposure with the magnetic fields on the percentage of Open Arms Entries (%OAE) in the EPM

Exposure to the magnetic fields caused a significant decrease (P<0.05) in the frequency of open arms entries in



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Figure 1. The effect of exposure to magnetic fields on the delay in entering the dark area at the reminder stage (A) and on the presence in the dark area at the reminder stage (B). Con: Control group, 1μT: exposure group with magnetic field 1μT, 100μT: exposure groups with 100μT magnetic field, 500μT: exposure group with 500μT magnetic field, 2000μT: exposure group with magnetic field 2000μT (*Significant when compared with the control group).

the elevated plus-maze in 500 (26.1±4.6%) and 2000 (18.8±8.1%) μT groups, compared with the control group (43.1±6.4%; [Figure 2-A](#)).

Exposure with the magnetic fields on the percentage of time spent in open arms in the EPM (%OAT)

One-way ANOVA and Tukey’s post hoc test data results suggested contact with magnetic fields caused a significant decrease (P<0.05) in the percentage of time spent in open arms in the elevated plus-maze in 1 (3±1.5%), 100 (4.5±1.3%), 500 (1.8±0.4%), and 2000 μT (0.7±0.27%) groups, compared with the control group (38±14.7%). As illustrated in [Figure 2-B](#), the ELF-EMFs groups indicated a significant difference from the control group.

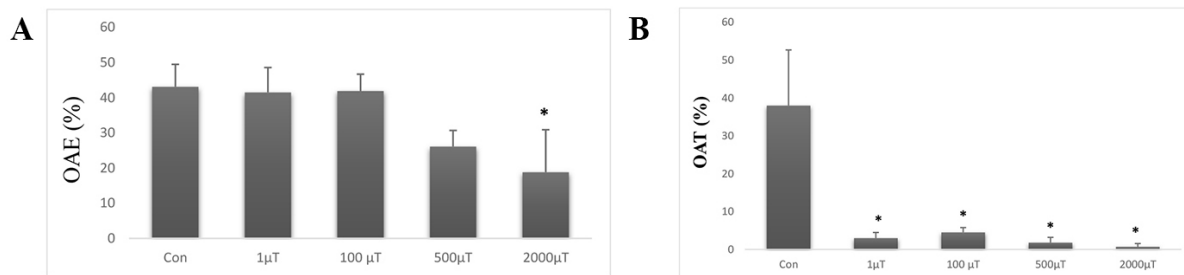
Total antioxidant capacity, total oxidant status, free thiol groups, and the index of lipid peroxidation

The effects of magnetic fields exposure on Total Antioxidant Capacity (TAC)

The present research results demonstrated that the contact with the magnetic fields caused a significant increase (P<0.05) in the TAC in the serum of 500 and 2,000μT groups, compared to the control group ([Figure 3-A](#)).

The effects of exposure to the magnetic fields on Total Oxidant Status (TOS)

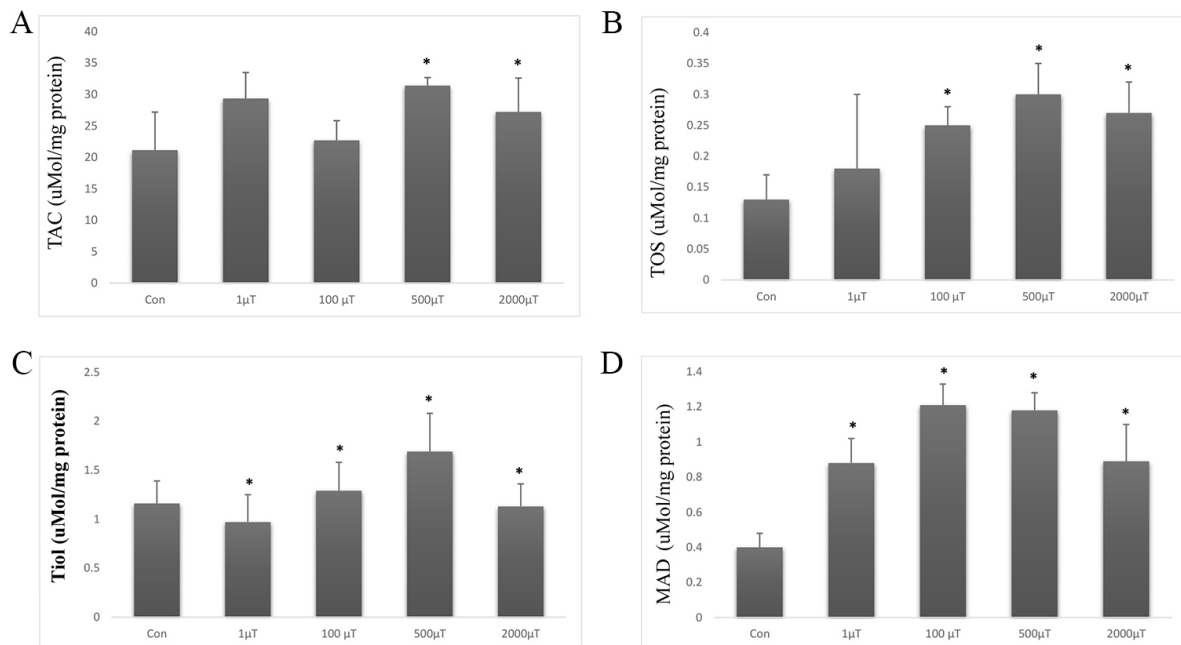
Findings revealed that in the ELF-EMFs groups, exposure to the magnetic fields caused a significant increase (P<0.05) in TOS in the serum of 100, 500, and 2000 μT, compared with the control group ([Figure 3-B](#)).



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Figure 2. The effect of exposure to magnetic fields on the percentage of open arms entries

A: The Elevated plus-maze; and B: On the percentage of time spent in open arms in the Elevated plus-maze. Con: Control group; 1μT: exposure group with magnetic field 1μT; 100μT: exposure groups with 100μT magnetic field; 500μT: exposure group with 500μT magnetic field; 2000μT: exposure group with magnetic field 2000μT. *Significant when compared with the control group.



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Figure 3. The effects of magnetic fields exposure on TAC

A: The effect of exposure to magnetic fields on serum levels of TAC (total antioxidant capacity); B: The effect of exposure to magnetic fields on serum levels of TOS (total oxidant status); C: The effect of exposure to magnetic fields on serum levels of thiol groups; D: Effect of exposure to magnetic fields on serum levels of Malondialdehyde (MDA). Con: Control group, 1 μT: exposure group with magnetic field 1 μT, 100 μT: exposure groups with 100 μT magnetic field, 500 μT: exposure group with 500 μT magnetic fields, 2000 μT: exposure group with magnetic field 2000 μT (* significant when compared with the control group).

The effects of exposure to the magnetic fields on the serum level of free thiol groups

The data indicated that exposure to the magnetic fields caused a significant decrease ($P < 0.05$) in serum levels of thiol groups in 1 μT group and a significant increase ($P < 0.05$) in groups of 100, 500, and 2000 μT, compared with the control group (Figure 3-C).

The effects of exposure to the magnetic fields on Malondialdehyde (MDA) level

Our results declared that the exposure to the magnetic fields caused a significant increase ($P < 0.05$) in the levels of MDA in groups 1, 100, 500, and 2000 μT, in comparison to the control group (Figure 3-D).

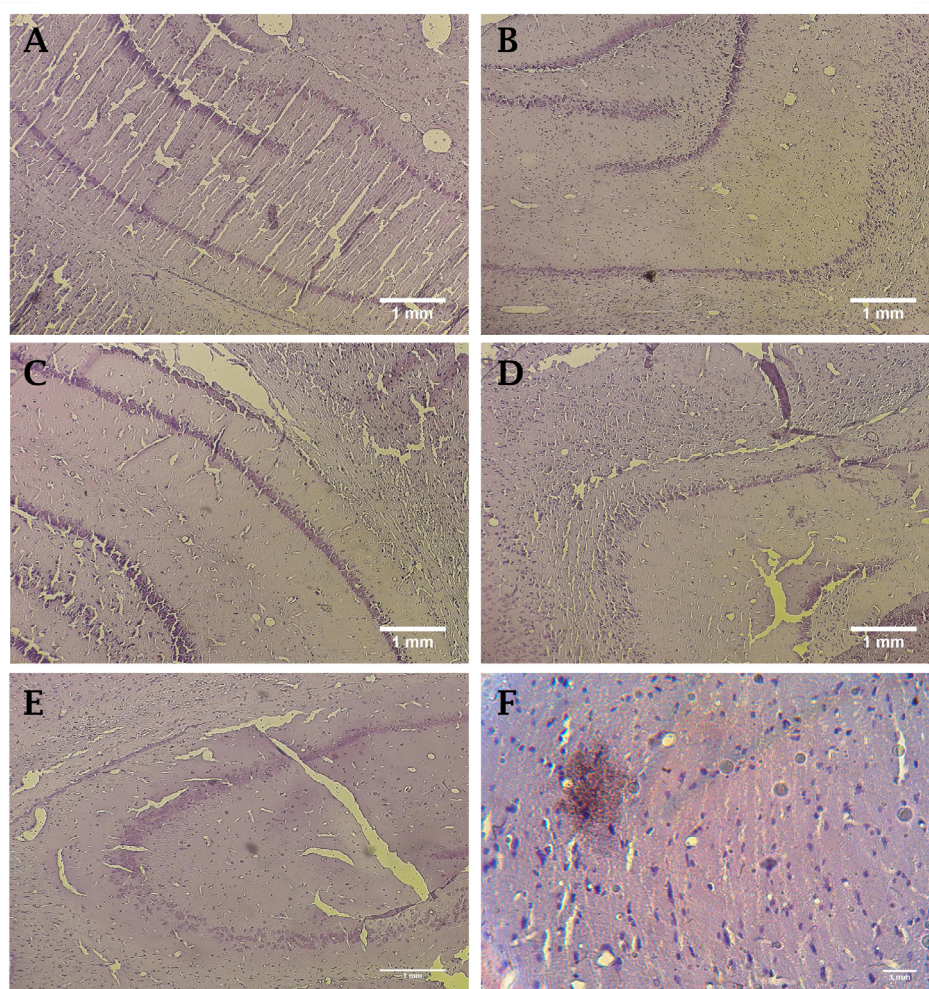
The effects of EMF on β-amyloid deposition and microglial cells in the brain

Brain immunohistochemistry analysis showed no β-amyloid deposition in experimental and control groups (Figure 4). Also, EMF exposure induces expression of Iba1 in 100, 500, and 2000 μT of the experimental groups (Figures 5 & 6).

5. Discussion

Reports on the biological effects of EMFs on the CNS have become critical issues in medical research (Grellier, Ravazzani, & Cardis, 2014). This study aimed to assay the impact of ELF-EMFs with different intensities on memory, anxiety, antioxidant activity, beta-amyloid (Aβ) deposition, and microglia population in rats.

The passive avoidance test showed an increase in the latency of entrance to the dark area and a reduction of the time spent in the dark area in the animals exposed to EMFs. Also, exposure to EMFs decreased the percentage of time spent in the open arm as an indicator of anxiety behavior. Studies on the effects of EMFs on memory are few and sometimes have controversy in findings. Most studies have focused on the role of EMFs on neurodegenerative diseases induction or therapeutic roles. In the previous study, we indicated that exposure to EMFs caused an enhancement in prolonged synaptic amplification (Komaki et al., 2014). Also, the effect of EMFs on the brain in the extensive epidemiological studies showed that various non-thermal microwave EMF exposures produce diverse neuropsychiatric effects such as anxiety (Pall, 2016).



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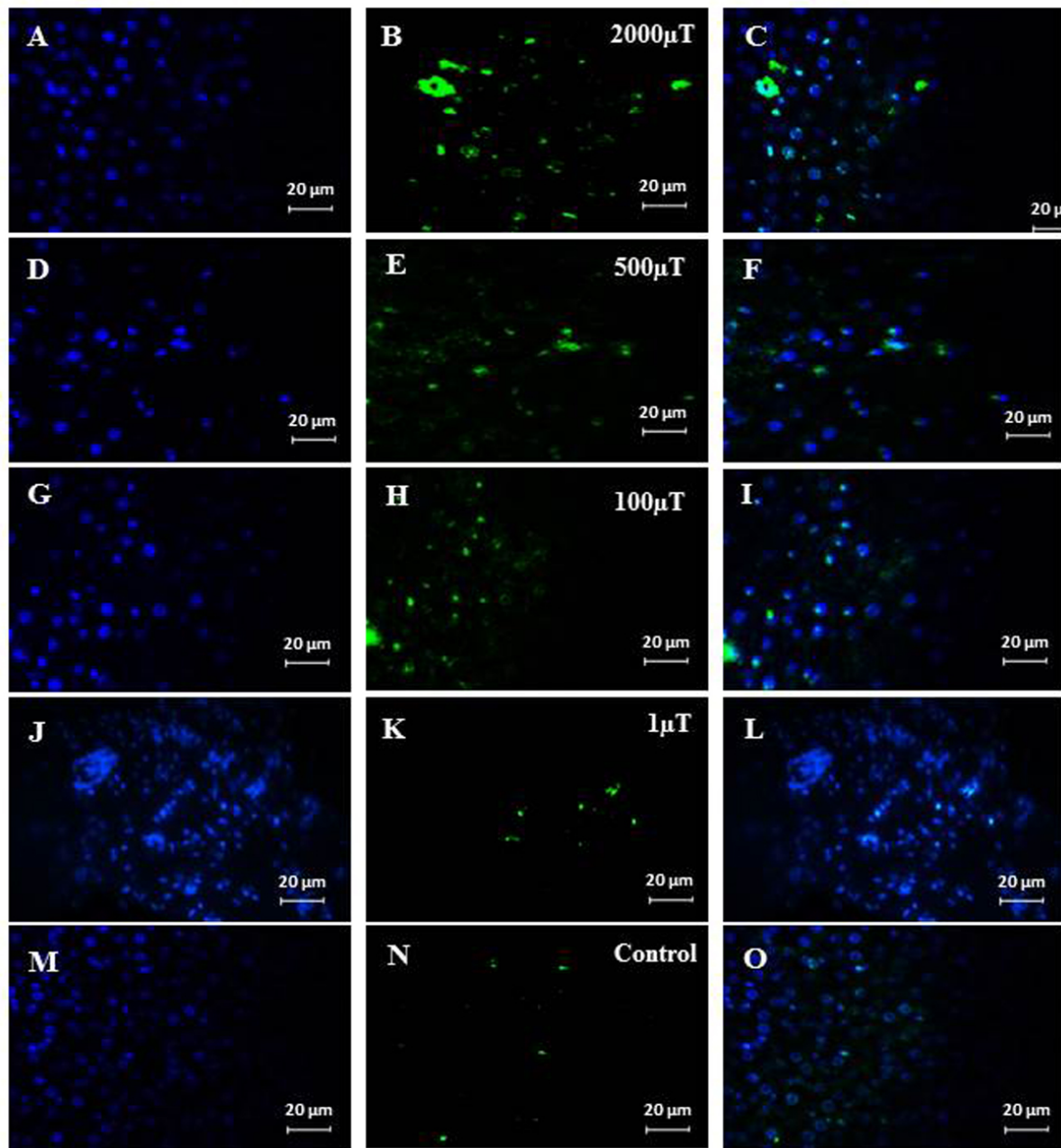
Figure 4. Immunohistochemistry images of β -amyloid staining using anti-beta amyloid antibody

A: Control group; B: 1 μ T group; C: 100 μ T group; D: 500 μ T group; E: 2000 μ T group; F: positive control group of β -amyloid.

Also, immunohistochemistry analysis indicated that exposure to EMFs does not induce deposition of β -amyloid peptides plaques. Numerous studies that reported the positive effects of ELF-EMFs on experimental animals found that exposure to 100 μ T/50 Hz ELF-EMF for 12 weeks induced no memory impairment in rats. There was no sign of Amyloid Precursor Protein (APP) formation in experimental groups or histopathological changes in the ELF-EMF exposure group (Zhang, Liu, Zhang, & Li, 2015).

In-vitro studies revealed that exposure to ELF-EMFs influences the physiological function of neurons, such as increased oxidative stress, declined neuronal ion channels function and membrane receptors, inhibited neuronal proliferation, and induced apoptosis (Liu et al., 2015). Also, ELF-EMFs exposure has improved neurological scores (Suszyński et al., 2014), by increasing neurotrophic factor levels and declining oxidative damages (Tasset et al., 2012). Zhang et al. stated no associa-

tion between ELF-MFs exposure (100 μ T at 50 Hz) and AD. Furthermore, ELF-EMFs exposure does not influence the pathogenesis of AD (Zhang, Li, Wang, Lv, & Song, 2013). However, some studies signified the effects of ELF-EMF on the formation of β -amyloid plaques and AD. Several studies indicated that EMF leads to ROS formation in the cells and can promote the synthesis of β -amyloid protein precursors in an oxidative stress-mediated pathway (Kaplan et al., 2016). Our study revealed that long-term exposure to ELF-EMFs in the 4 intensities of 1, 100, 500, and 2,000 μ T induce oxidative stress in rats, significantly enhancing lipid peroxidation. Our results are consistent with the previous reports. Chu Lee et al. indicated that exposure to EMFs reduced the activity of the superoxide dismutase, glutathione content, and increased lipid peroxidation (Chu et al., 2011). Recently, few studies have been examined the EMFs effects on the state of oxidative stress in the whole organism. Following exposure to EMFs, the antioxidant system in rats showed



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Figure 5. Immunohistochemistry staining of microglial cells using anti-Iba1 antibody

A-M: Nuclei staining by DAPI; B-N: Iba1 antibody staining using an anti-Iba1 antibody; C-O: Merge.

a decrease in glutathione content and the activity of superoxide dismutase in the heart, liver, and kidney. However, the activity of catalase and serum MDA levels presented no significant difference as a serum peroxidation index, which indicates no induction of lipid peroxidation after exposure with EMFs (Martínez Sámano, Torres Durán, Juárez Oropeza, & Verdugo-Díaz, 2012). Furthermore, other studies indicated a significant increase in serum MDA and hydrogen superoxide and a decrease in

total glutathione and thiol free groups in the supernatant of the rats' heart tissues after 60 minutes of exposure to EMFs every day for two weeks (Ciejka, & Goraca, 2008; Goraca, Ciejka, & Piechota, 2010). According to previous studies and our findings, this inconsistency could be because of the differences between studied animal species, intensity, and duration of exposure.

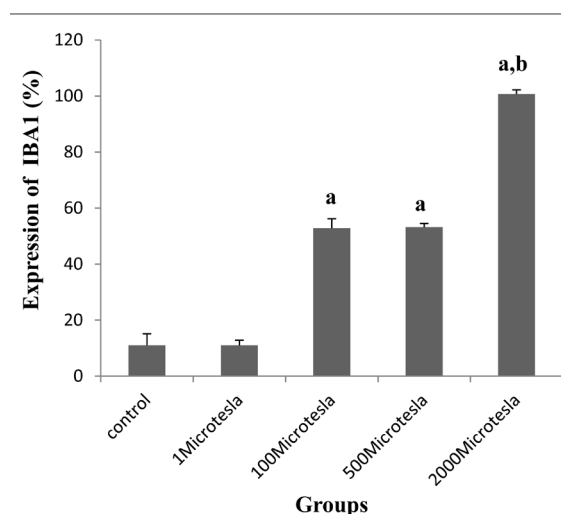


Figure 6. Column chart of the expression of Iba-1 in different groups

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A: Compared to control group; B: Compared to 100 μ T and 500 μ T groups.

Our results highlighted that EMF might affect microglial cells. We considered that high ELF-EMF could potentially induce microglial activation. Additionally, earlier studies have shown that the JAK-STAT signaling pathway is engaged in microglial activation (Kim, Park, Joe, & Jou, 2002; Huang et al., 2008; Yang et al., 2010). Electromagnetic pulse-exposed microglial cells shifted toward the pro-inflammatory instead of anti-inflammatory phenotype. These microglia enhanced the expression of inflammatory cytokines and the toll-like receptor-4 pathway, leading to neuronal death (Zhang et al., 2019). Histological experiments suggested that the number of microglial cells depends on the frequency of waves, and the highest frequency of microglia was seen in the 2000 μ T group. In this regard, the impact of long-term EMF on microglia markers caused elevation of Iba-1 in the brain of aged mice (Jeong et al., 2018). Duong et al. illustrated that exposure to EMF reduces oxygen-glucose deprivation-induced microglial cell death through reducing ROS. Thus, EMF protects human microglial cells from cell death (Duong & Kim, 2016). One study reported that EMF dramatically declined the phagocytic activity of microglial cells and enhanced the production of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and NO. Therefore, the EMF can cause neuroinflammation and lead to the promotion of neuronal damages.

5. Conclusion

Regardless of inducing oxidative stress and anxiety, our results indicated that exposure to EMFs improves

memory; it does not affect the formation of the amyloid precursor protein.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code: IR.UMSHA.REC.1396.17).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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