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Title: Effect of Short Time Exposure of Local ELF-MFs on Sleepiness Induced in Male Rats

Running title: ELF-MFs and Sleep Induction

Authors: Elnaz Azizi[1], Fatemeh Ayoobi[1], Ali Shamsizadeh[2], Amir Moghadam-Ahmadi[1], Seyed Ali Shafiei[3]*, Ali Reza Khoshdel[4], Mohammad Reza Mirzaei[5]

1. Non-Communicable Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

2. Physiology-Pharmacology Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

3. Neuroscience Research Center, Qom University of Medical Sciences, Qom, Iran.

4. Department of Biochemistry, Department of Biochemistry, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran.

5. Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

***Corresponding author:** Seyed Ali Shafiei, Neuroscience Research Center, Qom University of Medical Sciences, Qom, Iran. Email: sashafiei@muq.ac.ir

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Abstract

Introduction: Lack of high-quality sleep causes serious side effects like anxiety and changes in plasma concentration of oxalate. The current study aimed to investigate the impact of local extremely low frequency magnetic fields (ELF-MFs) on inducing sleep (sleepiness) and anxiety in male rats.

Methods: In this experimental study, 40 male rats were allocated in four groups (n=10). The ELF-MFs exposure (0, 10 and 18 Hz) was applied with intensity 200µT for three days (10 min/day). Sham-treated animal did not receive ELF-MF. Serum level of oxalic acid (OA) and sleepiness were measured both before first and after last exposure to ELF-MF or sham. Anxiety, sleepiness and OA were measured by using elevated plus maze, open-field test (OFT) and ELISA test, respectively.

Results: Comparison of oxalate levels between before and after exposure to ELF-MF revealed that ELF-MF (10 Hz) decreased the serum level of oxalate ($p<0.05$). Comparison of the percent of open:closed arm entry (in elevated plus maze) between before and after exposure to ELF-MF revealed significant differences. Also, frequency, velocity and distance moved were decreased in the open-field test.

Conclusion: Results of the present study demonstrated that ELF-MF with short time exposure may modulate the metabolism of OA and may modulate anxiety-like behavior or kind of induction of sleepiness in male rats.

Keywords: Magnetic field, ELF-MFs, Oxalic acid, Anxiety, Sleepiness

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1. Introduction

It is necessary for a person to have good sleep to feel happy during the day (Sadock & Sadock, 2011). Insomnia is a general health disorder associated with occupational problems, interpersonal problems, driving accidents, educational problems, difficulty working night shifts and anxiety (Bélanger et al., 2004; Husby & Lingjaerde, 1990; Lader, 1999; Okada et al., 2000). According to studies, insomnia may develop at any age, although it is more common in aging (Friedman, 2006). Reduced sleep duration in humans has been associated with risk for metabolic disorders, diabetes, obesity, including weight gain, and cardiovascular disease (Ayas et al., 2003; Di Milia et al., 2013; Schmid et al., 2015; Singh et al., 2005). The usual way to treat the patient's sleep disorders is drug therapy, but there are some non-pharmacological treatments such as cognitive behavioral therapy and proper diet (Morin et al., 2006).

Some studies showed that exposure to extremely low frequency magnetic fields (ELF-MFs) may affect cognitive functions (Akbarnejad et al., 2018; Shafiei et al., 2014; Sharma et al., 2017). Nevertheless, there are conflicting reports on the influence of MFs on sleep quality. For example, electromagnetic fields may affect sleep and cognation tasks in the rat (Dyche et al., 2012; Hassanshahi et al., 2017). Chronic exposure of pulsed ELF-MFs may be able to alleviate the sleep disorders of patients suffering from insomnia. (Pelka et al., 2001) and can affect sleep quality (Altpeter et al., 2006). On the other hand, Schmid et al. and Tworoger et al. did not report any significant effect on sleep quality of young women (Schmid et al., 2012; Tworoger et al., 2004), even in diabetic patients with sleep disorders (M. P. Wrobel et al., 2008) and sleep patterns of healthy people (Wagner et al., 2000). However, our latest studies showed that exposure to extremely low ELF-MFs can change EEGs recorded from some area of the scalp and may even influence reaction time in young healthy women (Ayoobi et al., 2017; Shafiei et al., 2012). A good night's sleep after ELF-MF exposure had been reported by some participants who have sleep problems. However, the evaluation of this issue was not the focus of this research (Amirifalah et al., 2011; Amirifalah et al., 2013). These studies have shown that local ELF-MFs may be become a useful therapeutic tool in the treatment of some psychological symptoms (Shafiei & Firoozabadi, 2014).

Then again, oxalic acid level has been shown to be a good marker of sleep deprivation (Aalim M Weljie et al., 2015). The primary sources of blood oxalate are vegetarian diet, ascorbic acid

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decomposition and endogenous synthetic pathways in the liver and erythrocytes (Hodgkinson & Zarembski, 1968). Endogenous substrates for oxalate synthesis include glycolate in peroxisome and hydroxyproline, and sleep deprivation of several days is related to hydroxyproline levels in mice (Koehl et al., 2006). Acute sleep deprivation eliminated oxalate from plasma via increased urinary excretion (Aalim M Weljie et al., 2015).

Therefore, due to the importance of inducing sleep and given the safety of extremely weak magnetic fields at very low frequencies, in this study we decided to evaluate the effect of ELF-MFs on sleep induction (sleepiness) in male rats by assessing behavioral tests and measuring oxalate acid density.

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2. **Material and method**

2.1 Subjects

This study were obtained from the Central Animal House, Rafsanjan University of medical Sciences, Iran. Forty male rats of the Wistar strain, (160–210 g) were distributed into four experimental groups (N=40: exposed (0, 10 and 18 Hz) n=10/group; sham exposed n=10) and were individually housed before and during experimental protocols. The animals were exposed to a stable temperature (22–24 °C) and a relative humidity of 45–55%. They were kept under a cycle of 12 h light and 12 h darkness for at least 2 weeks in our laboratory before starting experiments. Rodents are awake at night and sleep during the day. In order to adjust the sleep-wake cycle of rats, we inverted 12 hours of light and 12 hours of darkness for these animals in the laboratory so that they have time to wake up during the day and we can convenience of experiments. Animals were fed daily and cages cleaned.

2.2 ELF-MFs exposure

Helmholtz coils with 40 cm diameter and separated by a distance of 20 cm were used to generate a nearly uniform magnetic field. A dodecagon made of acrylic with 25 cm diameter and 15 cm height was used, located between two Helmholtz coils. The animal was placed in this box and a magnetic field with intensity 200 μT was applied for 10 min/day for three days. ELF-MFs exposure was performed at 9 am. The three groups (0, 10 and 18 Hz) were differentiated in terms of the transmitted frequency and in group four, all stages were performed like those of the previous groups, except that the set was turned off and there was no magnetic field (Fig. 1). Food and water were unavailable during exposures.

2.3 Open-field test

Anxiety-like behaviors and locomotor activity were measured with an open-field and behavioral tracking analysis system; we used a box 50 cm in height and 70 cm in diameter, with a video camera installed 2.5 m above the apparatus. Each rat activity was digitally monitored in the box and then using EthoVision XT software analyzed (version 7.1, Noldus Information Technology, Wageningen, The Netherlands). The interior area of the box was divided into inner and outer zones. First, the rat was placed in the center of the box and its activity was recorded for 5 min. Then the following behavioral parameters were scored: Velocity (cm/s); frequency; and total distance moved (cm) in the inner zone. In the end, after removing the rat from the open field, The experimental chamber was cleaned with diluted ethanol (10%) and dried (Crawley, 1999).

2.4 Elevated plus maze test

To measure the movement and anxiety of the rat used elevated plus maze test. It has comprised 2 open arms (50×10 cm) and 2 enclosed arms (50×10×40 cm) that extended from a common central platform (10×10 cm). A wooden apparatus was elevated 50 cm above floor level. Testing was conducted between 9.00 a.m. and 13.00 p.m in a quiet room. The rats were placed in the center of the maze facing an open arm individually and 5 min were allowed of free exploration. All sessions were filmed. The floor was cleaned after each test, with distillated water. Animals were tested before the first ELF-MF exposure and after the last ELF-MF exposure. Measurements were made from the time spent in central, open and closed parts of the maze. In the absence of anxiety, the animal moves more and has a great deal of activity in the open arm; if, on the contrary, it experiences anxiety and sleepiness, it will remain in the closed arm (Rodgers & Dalvi, 1997).

2.5 Oxalic acid (OA) measurement

The comprehensive serum metabolic oxalic acid (OA) was measured by oxalate assay kit (Colorimetric-KA4532, Abnova, Taiwan), to evaluate the amount to which systemic metabolism is impacted by sleep restriction, Blood taken in the morning of one day before the first exposure

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to ELF-MF (before behavioral tests) and one day after the last exposure to ELF-MF (after behavioral tests). The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of OA in samples. OA is added to a monoclonal antibody enzyme well which is pre-coated with OA monoclonal antibody; this is subject to incubation, and then, OA antibodies labeled with biotin are added and combined with Streptavidin-HRP to form the immune complex. Next, incubation is carried out and the material is washed again to remove the uncombined enzyme. To close, Chromogen Solution A, B is added, and the color of the liquid changes into blue, and with the effect of the acid, the color finally becomes yellow. The chroma of color and the concentration of the substance OA of the sample were positively correlated.

2.6 Procedure

Figure 2 represents all of the protocol on different days. This protocol was implemented in different frequencies (0, 10 and 18 Hz), for these groups and also for the sham group, in which the signal generator did not produce any electrical signals and thus there were no ELF-MFs in the sham group.

2.7 Statistical Analysis

The obtained data were analyzed by SPSS software version 20.0. The quantitative data were reported as mean ± SD and qualitative data in percentages. Paired t-test was used to compare open-field cores, elevated plus maze, ELF-MF and mean oxalic acid before the first and after the last exposure. Differences among groups were compared using One-way ANOVA across all groups. The significance level in all tests was $P<0.05$.

3. Result

3.1 ELF-MF exposure and anxiety

The average presence of rats in the open arm decreased after the last ELF-MF exposure, and there were significant differences in the ELF-MF exposure 10 Hz ($p=0.03$) and ELF-MF exposure 18 Hz groups ($p=0.007$). On the other hand, the average presence of rats in the closed arm increased significantly after the last ELF-MF exposure in the ELF-MF exposure 18 Hz group ($p=0.01$) (Fig. 3).

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3.2 ELF-MF exposure and locomotor activity

Open-field test was used to determine anxiety-like behaviors and locomotor activity before and after ELF-MF exposure in different groups. For locomotor activity, the traveled distance of movements decreased after the last ELF-MF exposure and the traveled distances were significant between before the first and after the last ELF-MF exposure in the direct current (DC) magnetic field exposure, in which frequency was 0 Hz ($p=0.02$), ELF-MF exposure 10 Hz ($p=0.002$) and ELF-MF exposure 18 Hz groups ($p=0.000$). Also, the velocity decreased after the last ELF-MF exposure and was significant in ELF-MF exposure 0 Hz ($p=0.02$), ELF-MF exposure 10 Hz ($p=0.002$) and ELF-MF exposure 18 Hz groups ($p=0.000$). Frequency decreased after the last ELF-MF exposure and it was significant in all groups ($p=0.01$) except sham ($p=0.48$) (Fig. 4).

3.3 ELF-MF exposure and OA density

The serum levels of OA were measured by ELISA. The mean serum level of OA was 2.10 ± 0.56 nmol/µl before ELF-MF exposure, and it was significantly higher as compared after ELF-MF exposure (1.28 ± 0.47) in the wave 10 Hz group ($p<0.05$). No significant difference was observed among the other groups before and after ELF-MF exposure (Fig. 5).

All results are summarized in Table 1.

4. Discussion

Our previous human experiments suggested that exposure of 200 µT MFs with extremely low frequency may improve sleep disturbance (insomnia) that is caused by anxiety (Amiri Fallah et al., 2011; Amirifalah et al., 2013). In this study, procedures involving magnetic fields at very low frequencies were used to evaluate the impact of DC (0 Hz), 10 Hz, 18 Hz and 200 µT MF on sleepiness induction rates.

We suggest that the reduction in the frequency, velocity and distance moved in open-field tests after exposure (Fig. 4) and also the decrease of average presence of rats in the open arm (Fig. 3) were due to a kind of induction of sleepiness. Therefore, the findings of this study are consistent with those of our previous studies, confirming our hypothesis that the ELF-MFs can induce sleepiness (Table 1).

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Ayoobi et al. (2017) examined the effect of ELF-MF on sleepiness induction in healthy students, especially females. They used 200 µT MF (3 min duration) at three frequencies (18, 14 and 10 Hz), applied to the skull in areas C4, Cz and C3, respectively, on 32 males and 31 females. The results of their study showed that exposure to local ELF-MFs may influence minimum reaction time in young healthy females (Ayoobi et al., 2017). Their findings are consistent with the results of this study.

However, some studies had inconsistent findings, like Kurokawa et al. (2003). They evaluated the effects of continuous exposure to 50 Hz, 20 µT MF on 20 subjects for 55 min. The reaction time in time perception tasks did not change with ELF-MFs exposure (Kurokawa et al., 2003). In the Kurokawa study, the magnetic field used was 10 times less than the magnetic field of this study.

Generally, reduced time spent in the central area in the open-field test (Prut & Belzung, 2003) and reduced time spent in the open arms plus maze (Lister, 1987) have been established as an index of increased anxiety behavior. Some animal studies indicated that high intensity and chronic exposure to ELF-MF may induce anxiety-like behavior in rodents (Kitaoka et al., 2013; Liu et al., 2008) and in this study we expect them to be sleepy because of short exposure.

Hi et al. (2011) conducted an experimental study and showed that 4 hr/day chronic exposure to ELF-MF has a significant effect on the anxiety level and on the spatial memory of adult rats. However, 1 hr/day exposure to ELF-MFs had no effect in any test (L. H. He et al., 2011). ELF-MF exposure of rats 4h/day increased the anxiety-like behaviors in the open-field test and the elevated plus maze test without their altering locomotor activity but had no effect was observed in the light/dark box test. Moreover, ELF-MF exposure of 1h/day had no effect in any test. Increased exposure to ELF-EMF may have a direct and significant relation with anxiety (Hosseinabadi et al., 2019).

Generally, in short time exposure, there is no significant evidence showing that ELF-MF exposure has an effect on anxiety induction. Furthermore, the results of the meta-analyses have shown that the exposure of ELF-MFs has no considerable effect on cognitive functions (Barth et al., 2010)

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Ouyang et al. (2017) measured plasma concentrations of oxalic acid in free-ranging wild birds' blood as a biomarker for sleep restriction based on some earlier study (A. M. Weljie et al., 2015). The findings showed that oxalic acid levels reduce strongly in birds that have their highest activity levels at night. They assumed it is likely that night activity may cause sleeplessness and, as a result, a decrease in the concentration of oxalic acid (Ouyang et al., 2017).

Our results suggest that the activity of rats and oxalate acid concentration reduced after ELF-MF exposure (Fig. 3). ELF-MF is assumed to cause severe anxiety in rats, resulting in oxalate decrease (Table 1). This is consistent with results of the plus maze test (Fig. 1) and the reduction of velocity, frequency and in the open-field test (Fig. 2). However, such a hypothesis is completely incompatible with the evidence obtained in previous our human research, which showed that healthy female participants experienced good sleep after the ELF-MF exposure. That result of Fig. 2 (plus maze) can be attributed to sleepiness.

In 2011, Akhtary et al. analyzed the effects of very low frequency electromagnetic fields on learning, memory and pseudo-anxiety behaviors in giant white laboratory mice. Field groups lived for 28 days in special fiberglass cages with magnetic field of 10 or 100 µT. The anxiety was measured by elevated plus maze test. The results showed that there was no significant difference among the groups (Akhtary et al., 2011). It can be concluded that a 10 µT field, with chronic conditions, could not induce anxiety. These findings are similar to the results of the present study.

In another study, 60 diabetic patients were divided into two groups, and 32 of them were subjected to a magnetic field with a frequency of 180 to 195 Hz with an intensity of about 100 µT for 20 min, 5 times per week. The results showed that ELF EMF had no effect on sleep disorders and serum level oxalate (M. Wrobel et al., 2008).

Monazzam et al. (2014) reviewed the quality of sleep and general health of employees who were exposed to very low frequency magnetic fields (ELF EMFs) in a petrochemical complex. They examined the relationship among the levels of ELF, sleep quality and the general health of 40 EMF exposed workers and 22 controls. The results showed that 28% of the subjects were in poor health and 61% had a sleep disorder. No significant difference was found among the case and control groups regarding sleep quality and general health (P <0.01) (Monazzam et al., 2014).

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In 2011, the effects of ELF-MFs on mouse anxiety levels were investigated. A magnetic field of 50 Hz was used with two groups of animals for 1 and 4 hr. Pseudo-anxiety behaviors were evaluated using open-field experiment and evaluated plus maze. Mice from the 4-hr exposure had more pseudo-anxiety behaviors and static motor activities followed by increased anxiety (L.-h. He et al., 2011). This difference can be due to the difference in the exposure time and frequency of the magnetic field in these two studies.

The limitations of our study were not measuring melatonin levels. Some reports suggest that melatonin production is slightly increased in exposed animals (Altpeter et al., 2006; Dyche et al., 2012). There are many studies in this field, but when we examined them, there was a significant discrepancy and even a negative one.

5. Conclusion

The results of this study showed that ELF-MF with short time exposure may modulate the anxiety-like behavior or kind of induction of sleepiness in male rats. This effect may be used to treat sleep disorders and requires further study.

6. Acknowledgement

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7. Declaration of interest statement

The authors declare there are no conflicts of interest

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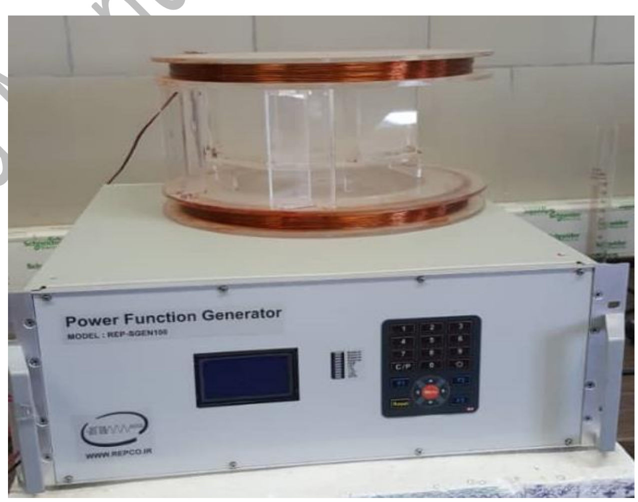

Figure 1: Schematic view of Helmholtz coils.

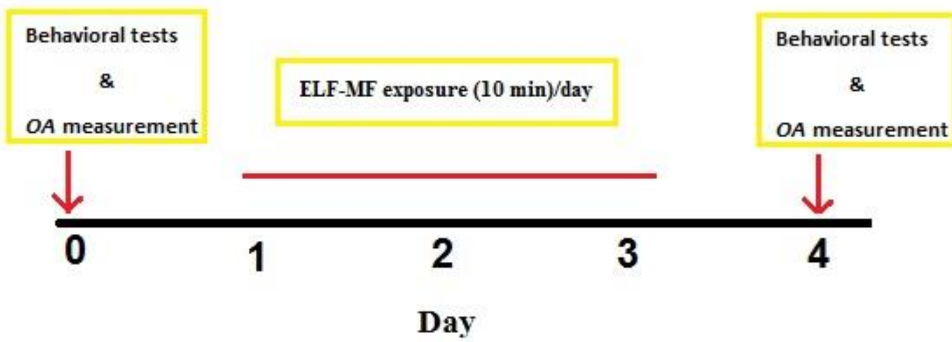

Figure 2: Schematic view of protocol of study on different days.

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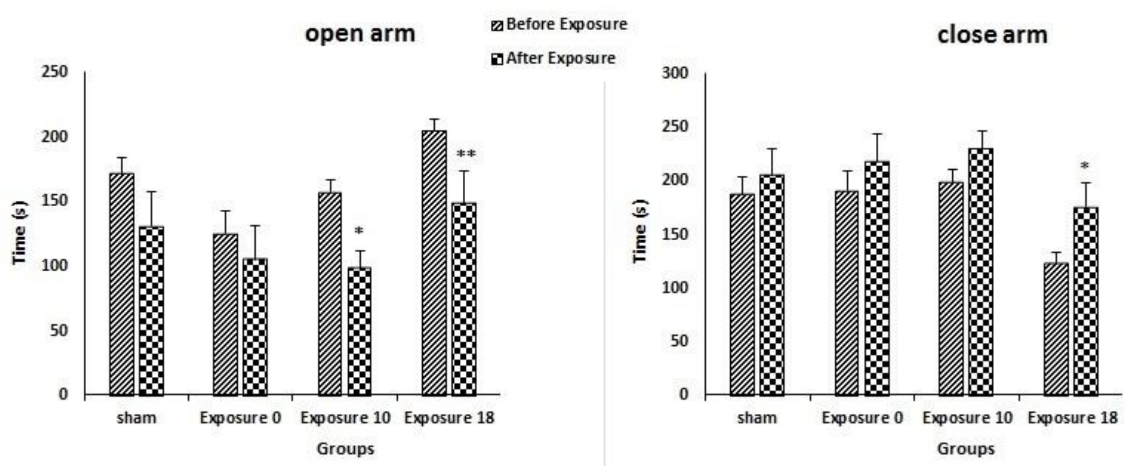

Figure 3: Elevated plus maze test in all groups. There was significant effect between the groups before the first and after the last ELF-MF exposures. * p<0.05, ** p<0.01.

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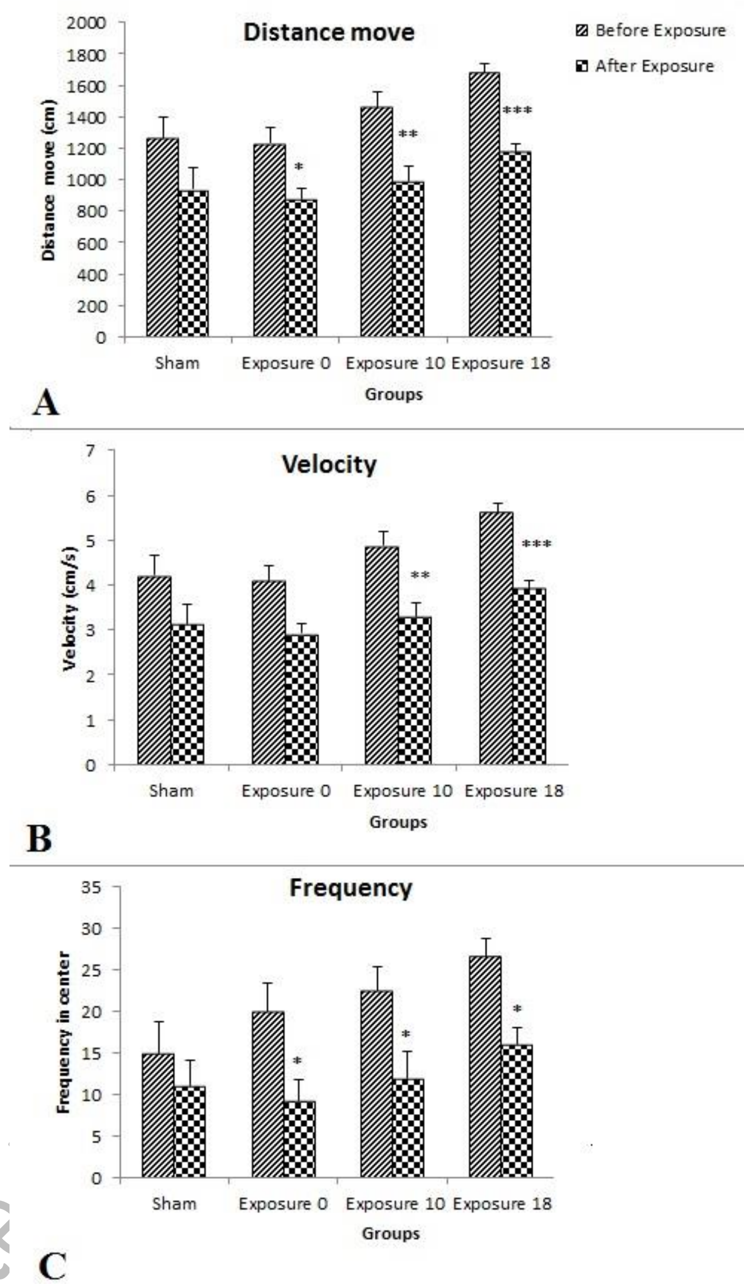

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Figure 4: Open-field tests in all groups. There was significant effect among the groups before the first and after the last ELF-MF exposures. * p<0.05, ** p<0.01, *** p<0.001.

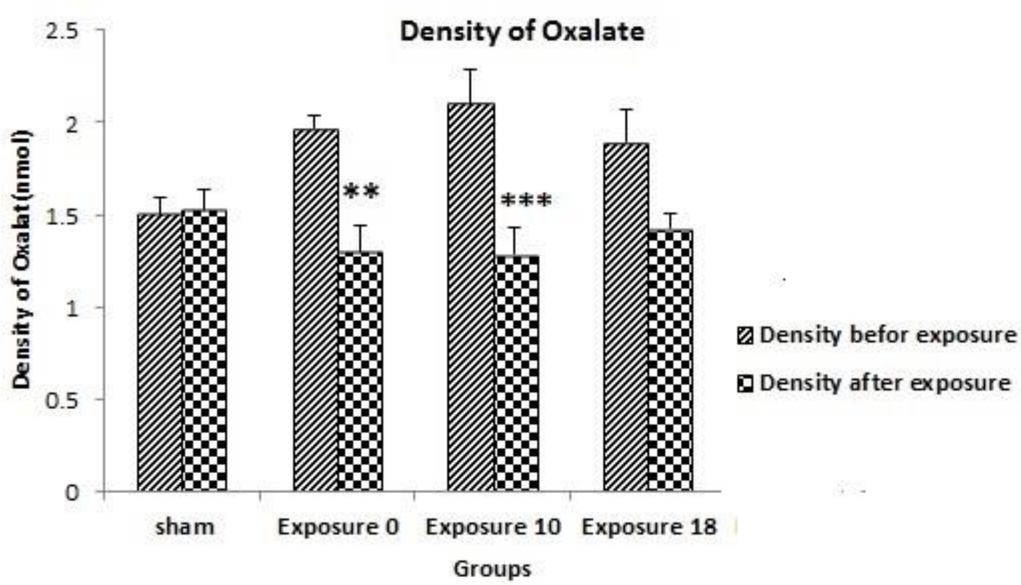

Figure 5: Comparison of serum levels of oxalic acid between before the first and after the last of the ELF-MF exposure groups. Serum OA levels in wave DC and wave 10 Hz group after ELF-MF exposure were significantly less than before the ELF-MF exposure. No significant difference was observed among the other groups. ** P<0.01, *** P<0.001.

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Table 1. **:** Comparison of all tests before the first and after the last ELF-MF exposures.

Tests		**Groups**			
		Sham	**Exposure 0**	**Exposure 10**	**Exposure 18**
Elevated plus maze test (s)					
Open arm	Before	171.22±35.68	124.65±55.49	156.67±28.66	204.44±27.28
	After	130.71±69.58	105.14±80.88	97.81±42.18 *	148.10±79.78 **
Close arm	Before	187.33±51.35	190.92±58.24	198.00±34.84	123.00±31.56
	After	205.22±78.35	217.51±81.17	229.71±53.21	175.71±71.53 *
Open-field test					
Distance moved (cm)	Before	1264.41±375.82	1233.51±257.88	1463.85±258.55	1683.34±174.31
	After	940.35±377.01	877.33±169.85 *	991.16±240.35 **	1179.85±154.59 ***
Velocity (cm/s)	Before	4.24±1.25	4.11±0.86	4.88±0.86	5.61±0.58
	After	3.13±1.26	2.92±0.57	3.30±0.80 **	3.93±0.52 ***
Frequency	Before	15.00±10.54	20.00±8.88	22.57±7.37	26.66±6.12
	After	11.00±8.49	9.29±6.75 *	12.00±8.14 *	16.11±5.58 *
Oxalic acid (OA)					
Density (n mol)	Before	1.51±0.24	1.96±0.20	2.10±0.56	1.89±0.53
	After	1.52±0.36	1.30±0.41 **	1.28±0.48 ***	1.42±0.22

* Significant difference between before the first and after the last ELF-MF exposures.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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