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Effects of 2.45 GHz Non-Ionizing Radiation on Anxiety-Like Behavior, Gene Expression, and Corticosterone Level in Male Rats

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Abstract

Introduction: The effects of short-term and long-term exposures to 2.45 GHz radiofrequency electromagnetic radiation (RF-EMR) on anxiety-like behavior, corticosterone level, and gene expression were investigated. The goal of this study was to explore the effect of electromagnetic fields of 2.45 GHz on clinical signs such as body weight and anxiety-like behavior, including the elevated plus maze test and open-field test, and also on messenger RNA (mRNA) expression of Bax (Bcl2-associated x) and Bcl-2 (B-cell lymphoma 2) genes on the cognitive memory functions in an animal model of rats.

Methods: The animals were classified into eight groups, sham groups and exposed groups for short-term and long-term exposures to the same dose of RF-EMR for one hour daily. The Wi-Fi equipment in the sham control group was not turned on during the experiment. Both genes were further confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). The semi-quantitative PCR method of electromagnetic fields in the 2.45 GHz range impacted the expression of Bax and Bcl-2 genes in the rat's memory.

Results: The present study exhibited that short-term radiation could decrease the percentage of entry into the open arm and the percentage of time spent, while there were no substantial impacts on the long-term radiation effect. Our data support the hypothesis that short-term exposure worked as a systemic stressor, raising plasma corticosterone and changing glucocorticoid receptor expression in the hippocampus.

Conclusion: Additional research on this specific frequency and amount of radiation is required to discover strategies for protecting the nervous system from the detrimental effects of RF-EMR radiation.

Keywords: Non-ionizing electromagnetic radiation; Gene expression; Elevated plus maze; *Bcl-2*; *Bax* gene; Corticosterone.

Introduction

The worldwide usage of wireless fidelity (Wi-Fi) devices and mobile phones has increased enormously in recent years,[1] raising concerns about their potential adverse health consequences.[2-4] Mobile phones have been part of the lives of the majority of the world's population since the late 1990s. The 2.4 GHz frequency is used by Wi-Fi devices.[1] Hence, mobile phones and the wireless internet use non-ionizing electromagnetic radiation (NI-EMR).[5] The harmful effects of electromagnetic fields (EMFs) may have been documented in the early 1960s.[6] EMF can be a significant inducer of stress, and it mediates stress reactions employing stress proteins.[7] On the other hand, human research has indicated that heavy users may develop brain tumors.[8] Hence, many studies have shown that the 1.8 and 2.4 GHz frequency bands alter the biological, physiological, and behavioral influence of EMFs on people and animal models.[9,10] Additionally, human studies have been conducted to assess the effects of these radiations on anxiety, sleep, behavioral characteristics, and cognitive functioning.[1] Thus, individual measurements of anxiety can aid in the early identification of mental illness [11]. For example, Greenebaum and Barnes found that the pulse field of 2.45 GHz microwave radiation leads to cognitive impairment and memory loss.[12] However, some studies mention that pulsed EMFs induce axonal regrowth and accelerate the growth of regenerative neurites.[13,14] Therefore, it is vital

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for the public, especially young children who often use the wireless internet during their youth, to recognize the link between EMF and medical conditions, including neurological disorders and behaviors.[15] In conclusion, practically all studies assume that electromagnetic waves have distinct impacts on biological tissues, but it is difficult to determine whether these effects are thermal or non-thermal. EMFs are widely thought to have no non-thermal impact on cells, tissues, or biological organisms.[16] Generally for rodents' test, under certain situations, because the studied non-thermal effects are mostly generated by the EFM, detectable impacts can be anticipated with a SAR as low as 1 W/kg.[17] However, based on the National Toxicology Program, the lowest exposure level used for rats was 1.5 W/kg.[18]

Traditionally, rodents' anxiety-like behavior has been assessed by observing their responses to an innovative, potentially life-threatening environment.[19] The elevated plus maze (EPM) test was performed to assess the impact of EMF exposure on anxiety. The EPM test is perhaps the most frequently used animal model of anxiety, along with the open field.[6,20-22] Female rats show less anxiety when subjected to EPM.[23] Two adjacent arms are bounded by high walls (closed-arms), while the other two are unbounded (open arms). When rats are kept in this device, they show a significant preference for closed arms, exhibiting a fear response to open arms. This response is regarded as anxiety.[6] The hippocampus is a potential location for sensory and cognitive function, and the process has previously been recommended for stress protein expression.[7] Additionally, it plays a role in anxiety [23,24]. The hippocampus is sexually dimorphic and is implicated in anxiety and spatial memory development.[23] However, memory loss and degradation of learning skills are caused by neuronal apoptosis in the hippocampus and cortex.[25] Chronic stress or anxiety can persistently stimulate the hypothalamic-pituitary-adrenal (HPA) axis, which leads to increased glucocorticoids.[26] The activation of the HPA axis results in the production and secretion of glucocorticoids from the adrenal cortex. Glucocorticoid signaling is necessary for stress adaptation and survival.[27] As Albrechet-Souza et al mentioned, HPA axis activation was thought to be an adaptive reaction to stressful or difficult conditions in EPM.[28] Glucocorticoids, particularly cortisol in primates and corticosterone in rats, act on gene expression and regulation all across the body, affecting various physiological processes that prepare the organism for the alterations in energy and metabolism that occur during chronic stress conditions.[26,29] EPM, as an animal model for anxiety, stimulates the HPA axis, resulting in an increase in plasma corticosterone (CORT) levels.[30] Anxiety-like behavior in animals can be modeled in the open-field test (OFT).[31] A reduction in anxiety and/or increased exploration is indicated by increased activity or extended time spent in the open field center.[32]

Furthermore, molecular studies discovered that RF fields might affect different elements of biological activity.[33] Our previous study discovered that *ACTH* gene expression in irradiated and control rat brain tissue exposed to extremely low frequency considerably increased on the first day compared to controls after irradiation in rats.[27] This contrasts with findings by Regalbuto et al who indicated that 2.45 GHz radiofrequency fields did not have any detectable biological impacts at the cellular or molecular level (*in vitro*) under the exposure settings and conditions examined.[2] However, Lee et al observed that the RF fields at 2.45 GHz can affect gene expression by a non-thermal method in a cultured HL-60 cell line. They indicated that the functional categorization of the impacted genes shows that the upregulated genes and the cell cycle genes amongst the downregulated genes are connected to apoptosis. There was no significant rise in heat shock gene expression.[34] These results were extended previously by Mashevich et al, who showed that the genotoxic impact of electromagnetic radiation is induced by a non-thermal mechanism.[33] The *Bcl-2* family of proteins regulates intrinsic activation of an apoptotic tract in response to cellular stressors such as damage to DNA, ɣ-irradiation, activation of the oncogene, and withdrawal of the growth factor.[35] They suppress (e.g., *Blc-2*, *Bcl-XL*) or induce apoptosis (e.g., *Bad*, *Bax*) during neuronal development.[36,37] This family has anti-apoptotic and pro-apoptotic characteristics, but *Bcl-2*, in particular, is essential for cell survival.[38] It suggests that manipulating the action of the Bcl-2 family pharmacologically may be beneficial in treating human neurological disorders such as strokes and neurodegenerative disorders.[39] Mitochondrial activity and expression of *Bcl-2* can be linked with anxiety and mood disorders.[38] The *Bax* gene is expressed in the brain and has been identified as a pro-apoptotic homolog of *Bcl-2*.[25] Although apoptosis is a critical defense mechanism that protects organisms from pathogens, it also contributes to the development of neurological illness[40] and injury.[37] It has long been established that the ratio of *Bax*/*Bcl-2* affects whether a cell dies or survives following apoptotic stimulation[41] in the nervous system.[25] As a result, it is not surprising that pro-survival Bcl-2 proteins are overexpressed in various cancer forms.[35] Finally, given the documented effects of EMFs on gene expression, the prospective impacts of EMFs on anxiety are an intriguing issue. Therefore, the present study aimed to assess whether short-term and long-term exposures to 2.45 GHz influences behaviors, especially anxiety-like behavior, plasma CORT responses, and gene expression in male rats.

Materials and Methods

Animals

Fifty-six Wistar male rats, 5-6 weeks old, with a range of body weight of 180-250 g, were used for this study. The

rats were housed on a 12-hour light-dark cycle and were provided with commercial rat chow at the Iran University of Medical Sciences Experimental Animal Center. Before handling the rats, they were acclimatized to the laboratory conditions for one week. All tests were performed following the guidelines established by the Malek-Ashtar University Ethics Committee, the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). We made every attempt to alleviate the pain.

Experimental Design

The animals were categorized into the following two experiments at random:

A) Experiment 1: Influence of 2.45 GHz EMF on rats after EPM test exposure and determining CORT levels in rats exposed to 2.45 GHz
   Sham control groups (7 days in the short term; 30 days in the long term) with no radiation, and research groups (7 days in the short term; 30 days in the long term) with radiation ($n = 8$ each).
B) Experiment 2: Effect of EMF at 2.45 GHz on gene expression
   Sham control groups (7 days in the short term; 30 days in the long term) with no radiation, and research groups (7 days in the short term; 30 days in the long term) with radiation ($n = 6$ each).

EMF Application and Exposure System

The 2.45 GHz electromagnetic radiation device for live animals incorporated a computer, microwave signal sources, and a two-dimensional moving loading platform. The microwave output of a horn antenna has a power range of 1–200 W. The electromagnetic radiation device includes a power amplifier (ZHL-16W-43+, USA) to amplify the signal generated and a signal generator (the Agilent HP 83732B, USA), which can generate signals in the frequency bandwidth range from 10 MHz to 20 GHz. A horn antenna S-band (model LB-OH-320-10-C-NF) with dimensions of 257×124×164 mm, which can operate in the frequency range of 2 to 4 GHz, a power meter, a radiation box (20×30 cm), and a power density measuring device were used. Electromagnetic waves (pulsed modulation) with a frequency of 2.45 GHz and a density of 4 mW/cm^2, pulse width (2 ms), transmit power of 81 mW, and pulse repetition frequency of 500 Hz were irradiated from above on the surface of the radiation cage. The spacing between the horn antenna and the tested animals was 25 cm. We were aware and concerned that our rats equally received the EMF under the horn antenna.

Behavioral Analysis

Elevated Plus Maze Test

An EPM was used to examine the animals' anxiety-like behaviors for one hour each day for one week (short-term exposed group) and four weeks (long-term exposed group). This test was conducted to assess rats' anxiety-like behavior due to their innate distaste for open and elevated places. The tool comprised two closed arms and two open arms, constructed from acrylic material and positioned 50 cm above the floor. The EPM was made of a central platform (10 cm × 10 cm), two opposite open arms (50 cm × 10 cm × 1 cm height), and two closed arms (50 cm × 10 cm × 40 cm height).[42,43] At the start of each test, the animal was put in the center, facing open arms, and given five minutes to wander freely. The motion path of the animals was captured using a digital camera (Mobile Datum Information Technology Co., Ltd., Shanghai, China). In addition, the percentage of open arm entries [open entries/(open + closed entries) 100] and the proportion of time spent in open arms [(open time/300) 100] were calculated. The maze was wiped using an alcohol solution before each experiment.[42] The anxiety index was determined by Mazor and colleagues'[44] and Rao and colleagues' methods,[11] which considered the frequency and length of time spent in the open arms in proportion to the overall quantity of time spent exploring the apparatus (the higher the index, the lower anxiety). The anxiety index was computed using the formula below:

$$\text{Anxiety index} = 1 - \left(\frac{\text{Open - arm time}}{\text{Total time}}\right) + \left(\frac{\text{Open - arm entries}}{\text{Total entries}}\right) / 2$$

Open-Field Test

Anxiety is thought to be measured by the amount of time spent in the middle of the open field.[31] Quantifying motor activity was measured using Plexiglas open field boxes (90×90 cm^2 with a 42-cm height). Black lines were painted on the cardboard on the box floor, splitting the floor into 18 cm × 18 cm squares. Gridlines, consisting of four 11-cm distances from each wall, divided open fields into centers and surroundings. Based on dependent measures, time spent in the center and distance traveled in the center divided by total distance traveled were measured. After each video recording was scored by an observer blinded to the treatment regimen, the number of squares crossed and those reared were recorded. Following the test, each rat was returned to its cage.

Biochemical Evaluation

Measurement of corticosterone

Chloroform was used to anesthetize rats, and blood samples were taken directly from their hearts. The blood serum was separated by centrifugation at 1600 g over a period of 10 minutes, and the CORT level in plasma was measured according to the manufacturer's instructions using the Corticosterone ELISA Kit (Cat # KA0468). The absorbance of the samples was determined using an ELISA reader set to 450 nm.

Molecular Study

RNA Preparation and cDNA Microarrays

Rats were anesthetized intraperitoneally with a combination of xylazine (5 mg/kg) and ketamine (80 mg/kg) and then put in the supine posture prior to being killed. The skull bone was separated, and the whole brain was extracted using blood vessel forceps. The surface fascia and brain tissue of the hippocampus were removed using ophthalmic surgical forceps. The whole hippocampus was then extracted carefully. Occasionally, liquid nitrogen (N_2) was injected into the grinder during the grinding process to prevent the tissue from freezing. Total RNA was extracted from pooled hippocampi (6 rats per sample) of EMF- or control group animals using the RNA Clean-up kit (Macherey-Nagel, Düren, Germany) and purified using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Table 1 shows primer sequences used in the polymerase chain reaction (PCR).

The *β-actin* gene, *Bcl-2*, and *Bax* genes were used to detect gene expression (SinaClone Co.). Quantitative real-time RT-PCR was conducted in one step using the One Step GoTaq® qPCR Master Mix (Promega) on an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). The real-time PCR reaction (25 µL) contained 10 µM forward primer, 10 µM reverse primer, 10 µL of Master Mix (1X), 0.4 µL of RT Mix (1X), 0.31 µL of Supplemental CXR Reference Dye, and nuclease-free water. The following conditions apply to the reaction: an initial 5 min (95°C), denaturation at 95°C (30 seconds), followed by 40 cycles of annealing, and extension at 60°C (30 seconds). The threshold cycle (Ct) was reported as a fold change compared to the control group using the $2^{-\Delta\Delta Ct}$ technique.[45,46] BestKeeper determines the standard deviation (SD) and stability value (SV) of selected reference genes using original data (CT values). Those with lower index values are considered highly stable.[47]

Table 1. The Primary Sequences of the Selected Genes

Gene	Sequence
β-actin	Forward: 5′-TCCTCCTGAGCGCAAGTAC-3′
	Reverse: 5′-CCTGCTTGCTGATCCACATCT-3′
Bcl-2	Forward: 5′-ATTGGGAAGTTTCAAATCAGC-3′
	Reverse: 5′-TCCTCTGTCAAGTTTCCTT-3′
Bax	Forward: 5'-GAGCTGCAGAGGATGATTGC-3'
	Reverse: 5'-AAGTTGCCGTCAGAAAACATG-3'

Statistical Analysis

All results are presented as the mean ± SD. All statistical analysis was analyzed using GraphPad Prism® 8.1 software for Windows (GraphPad Software, San Diego, CA, USA). Two-way repeated analysis of variance (ANOVA) was used to compare control groups and exposed groups between subjects, and then the Sidak's test was used to compare the results. Only when the *P* value was less than 0.05, 0.01, 0.001, and 0.0001 was it considered statistically significant.

Results

The findings of this study are given in two parts: one assessing anxiety levels in rats after irradiation and the other assessing gene expression in the brain. All the procedures are summarized in Figure 1.

Body Weight Measurement

The short-term and long-term exposed groups showed a significant reduction ($t = 4.174$; $df = 12$; $P = 0.0013$ and $t = 9.972$; $df = 14$; $PP < 0.0001$) in body weight at their endpoints, respectively, when compared to their control groups (Figure 2).

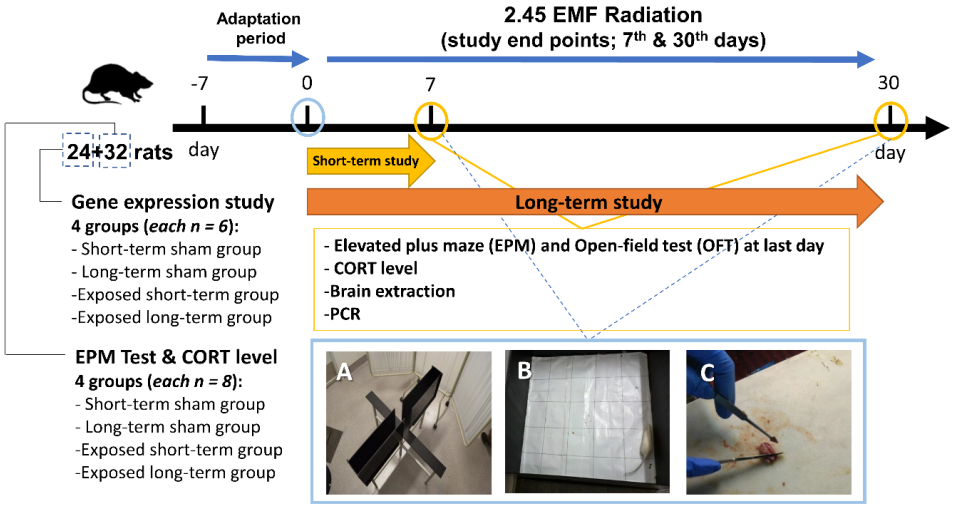

Figure 1. The Timeline of the Experimental Design; **A)** Elevated plus maze test (EPM); **B)** Open-field test (OFT); and **C)** Brain extraction.

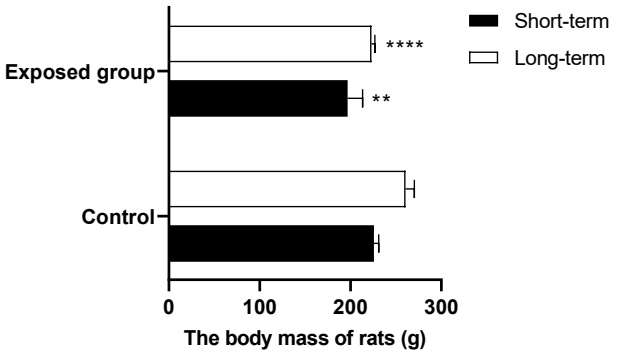

Figure 2. Body mass (g) at the end of the study. All results are given as mean ± SD, with ** $P<0.01$, *** $P<0.001$ as compared to the control group.

Microwave Radiation Effect on Anxiety-Like Behavior

Elevated Plus Maze Test

The EPM analyzed the anxiety-like behavior of rats after exposure. Figure 3A represents the percentage of entries in the open arms of control and 2.45 GHz exposed groups (short-term and long-term) in the EPM test. The percentage of entries in the short-term exposed group (6.38%) was significantly lower than the sham-exposed group (20.71%, $P<0.01$). However, sham groups were not exposed to EMF at 2.45 GHz. Figure 3B shows the percentage of time spent with open arms in the EPM test. The results are also the same as the percentage of entries. The short-term group (2.35%) significantly

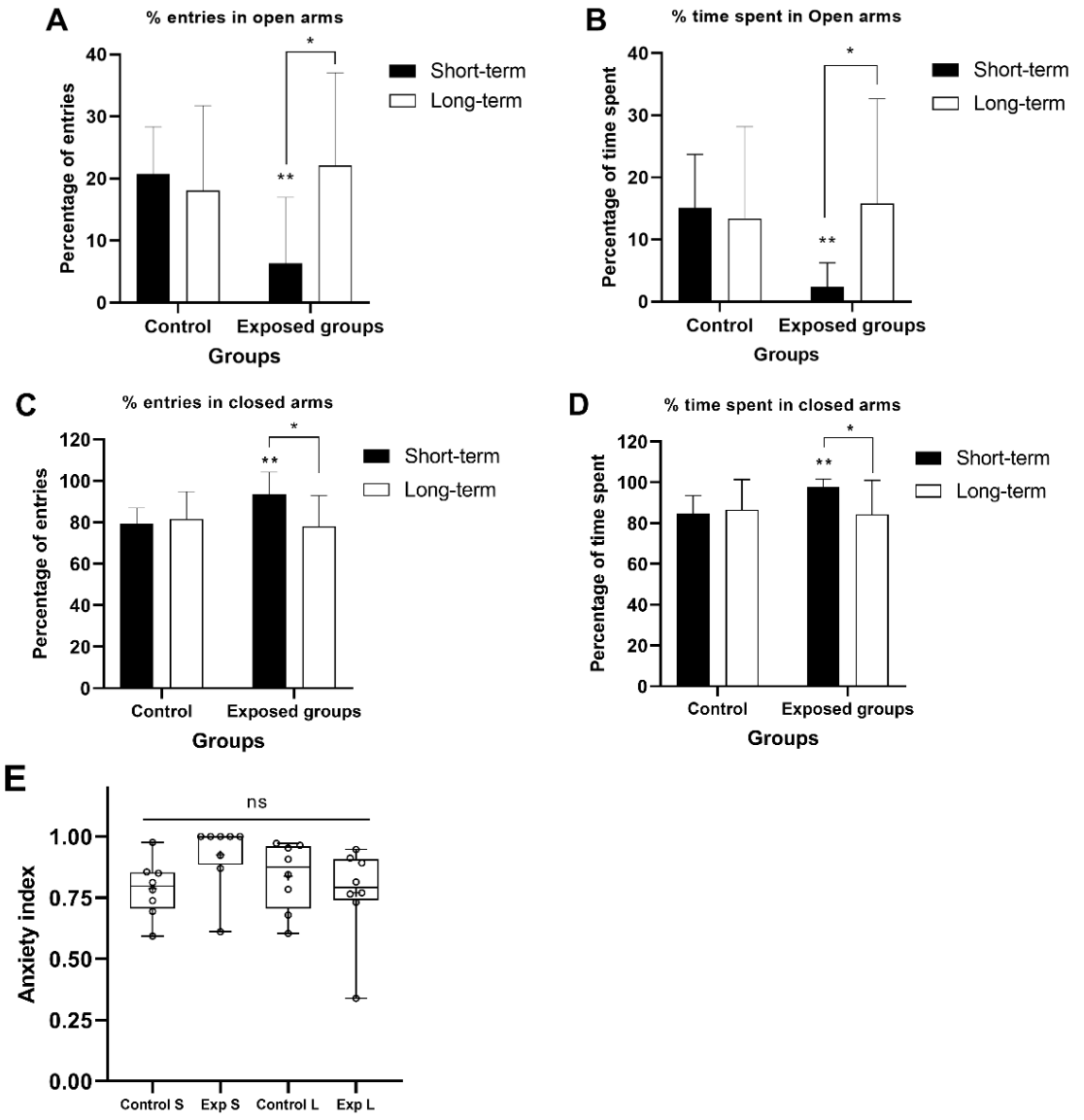

Figure 3. Effects of RF Electromagnetic Field Exposure on Anxiety-Like Behaviors in the EPM Test in Open Arms. (**A**) percentage of the entries into the open arms (within short-term groups: t=3.100, df=14, $P=0.0078$; within long-term groups: t=0.5622, df=14, $P=0.5829$), (**B**) percentage of the total time spent in the open arms (within short-term groups: t=3.808, df=14, $P=0.0019$; within long-term groups: t=0.3046, df=14, $P=0.7652$). (**C**) percentage of the entries into the closed arms (within short-term groups: t=3.100, df=14, $P=0.0078$; within long-term groups: t=0.5164, df=14, $P=0.6136$), (**D**) percentage of the total time spent in the closed arms (within short-term groups: t=3.808, df=14, $P=0.0019$; within long-term groups: t=0.2986, df=14, $P=0.7696$), (**E**) anxiety index, a correlation exists between the anxiety index and open arm times. Sham S: short-term sham group; Exp S: exposed short-term group; Sham L: long-term sham group; and Exp L: exposed long-term group. For anxiety index, the whiskers of the box plot were used. All results are given as mean ± SD, with ** $P<0.01$, *** $P<0.001$, and **** $P<00001$ as compared to the sham group, and * $P<0.05$ as compared with only exposed groups.

decreased compared with its sham group at 15.10% ($P<0.01$). Also, we found a significant difference within exposed groups ($P<0.05$). Regarding open arm entries, two-way ANOVA exhibited no significant differences in groups ($F_{(1,14)}=1.191$; $P>0.05$) and time exposure, including the short-term and long-term ($F_{(1,14)}=3.004$; $P>0.05$), and only groups–time exposure interaction ($F_{(1,14)}=5.947$; $P=0.028$) showed a significant difference. For time spent in open arms using two-way ANOVA, it showed that there was no significant difference in groups ($F_{(1,14)}=1.253$; $P>0.05$), time exposure ($F_{(1,14)}=2.197$; $P>0.05$), and groups–time exposure interaction ($F_{(1,14)}=3.648$; $P>0.05$).

Figure 3C exhibits the percentage of entries into closed arms in the EPM test. The percentage of entries in the short-term exposed group (93.61%) was significantly higher as compared to the sham short-term group (79.28%) ($P<0.01$). Also, Figure 3D shows the proportion of time spent in closed arms. Surprisingly, the short-term exposed group (97.65%) showed a significant increase compared to its sham short-term exposed group at 84.90% ($P<0.01$). There was a significant difference within the exposed groups ($P<0.05$). Regarding closed arm entries, two-way ANOVA exhibited no significant differences between groups ($F_{(1,14)}=1.309$; $P>0.05$) and time exposure ($F_{(1,14)}=3.223$; $P>0.05$), and only groups – time exposure interaction ($F_{(1,14)}=5.947$; $P=0.0306$) showed a significant difference. For time spent in closed arms using two-way ANOVA, it was shown that there was no significant difference in groups ($F_{(1,14)}=1.278$; $P>0.05$), time exposure ($F_{(1,14)}=2.200$; $P>0.05$), and groups–time exposure interaction ($F_{(1,14)}=3.656$; $P>0.05$). As illustrated in Figures 3B and 3D, rats spent significantly more time in the closed arms of the maze than in the open arms, which indicates a rise in anxiety mood in the exposed short-term group ($P<0.01$). In this study, the anxiety index showed a range of 0.3 to nearly 0.9, with a significant difference between the groups. All groups showed high anxiety index levels, with no significant difference based on one-way ANOVA using Tukey's test ($P>0.05$). However, the long-term exposed group indicated higher level among others.

Open Field Test

Figure 4A exhibits a number of entries in OFT. Two-way ANOVA showed a significant difference between time exposure ($F_{(1,28)}=82.22$; $P<0.0001$) and interaction between groups and time exposure ($F_{(1,28)}=98.10$; $P<0.0001$), but no significant difference was found in the groups ($F_{(1,28)}=3.174$; $P=0.0857$). For the percentage of time spent in the open field test (Figure 4B), two-way ANOVA revealed a significant difference between groups ($F_{(1,28)}=15.25$; $P<0.0001$) and time exposure ($F_{(1,28)}=106.0$; $P=0.0005$) and groups–time exposure interaction ($F_{(1,28)}=135.7$; $P<0.0001$). Also, there was a significant difference between the exposed groups ($P<0.0001$). The long-term exposed group showed a better result as compared to the short-term group.

Figure 4C shows the tracking path of rats in an open field test. It is considerably clear that rats in the exposed long-term group crossed the center of the open field more times than other groups.

Results of Corticosterone Level

The results of the effect of EMFs on the secretion of CORT from plasma blood are presented in Figure 5. The findings of the present study showed a significantly increased serum CORT level (ng/mL) following exposure

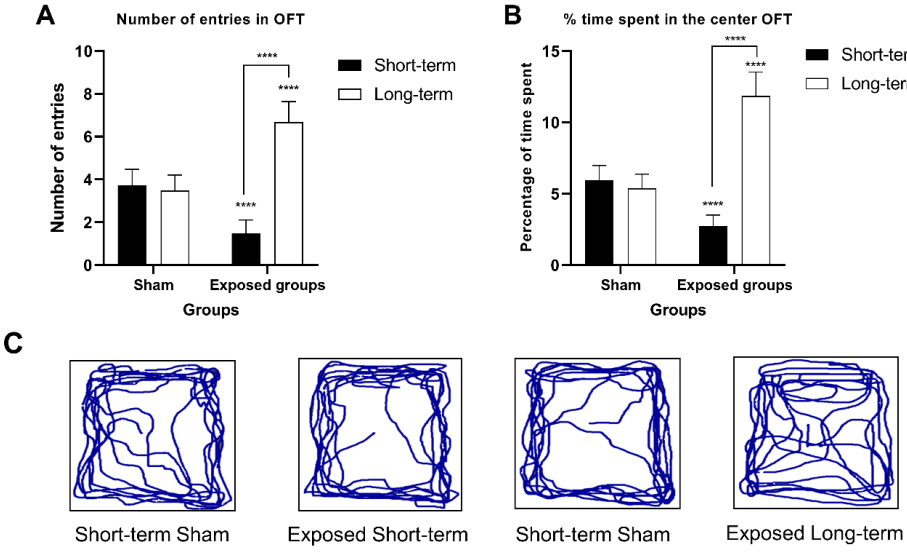

Figure 4. Comparison of Two Behavioral Parameters in the Open-Field Test on Tested Groups and Sham Groups. **(A)** the percentage of time spent in the center of OFT, **(B)** the number of entries to the center of OFT, and **(C)** representative images from the open field test tracking the pace of rats. Data represents the mean ± S.D. ($n=8$ per group). **** $P<0.0001$ compared to its sham group; a one-way ANOVA was used, followed by a student t test. No significant difference was found between the groups and the control group.

to the short-term exposed group compared with the sham short-term group ($F=3.781$; $P<0.0001$). However, according to the *t*-test, there was a significant difference within exposed groups in both short-term and long-term groups ($F=21.67$; $P<0.01$).

Based on two-way ANOVA using the Sidak's test, there was a significant difference in groups ($F_{(1,12)}=10.93$; $P=0.0063$) and time exposure (including short-term and long-term) ($F_{(1,12)}=12.50$; $P=0.0041$). Also, for interaction between groups and time exposures, there was a significant difference ($F_{(1,12)}=29.50$; $P=0.0002$). However, after long-term exposure, there was no significant difference ($P>0.05$).

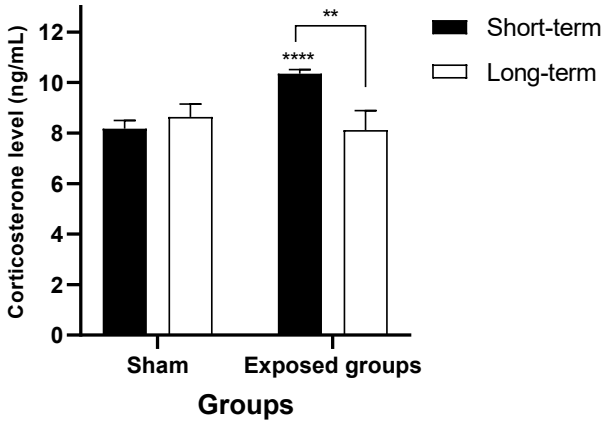

Figure 5. Comparison of Variations in Serum Corticosterone Levels in Rats in Exposed and Sham Groups in Short-term and Long-term Studies. For statistical analysis, Student's *t*-test was performed. Values are expressed as mean ± SD ($n=6$), ** $P<0.001$ (as compared within exposed groups), and **** $P<0.0001$ (as compared with its sham short-term group).

The Effect of EMF Exposure on Gene Expression

The amount of *Bax* and *Bcl-2* in the rat hippocampus was determined using RT-PCR to study pro-apoptotic and anti-apoptotic variations between the exposed group and the sham group. This study discovered that long-term exposure to EMF – as measured by comparing long-term exposed groups to long-term sham groups – reduces *Bcl-2* expression and increases pro-apoptotic *Bax* expression in the rat hippocampus. Fold change was measured after normalization of gene expression (data not shown). The statistical test indicated a rise in *Bax* gene expression in contrast with the *Bcl-2* gene (Figure 6). The *Bax* gene expression level in the brains of rats exposed to 2.45 GHz radiation in the short-term group was 1.695 compared to the sham short-term exposed group (1.165). The result for the long-term exposed group of *Bax* was 1.831 as

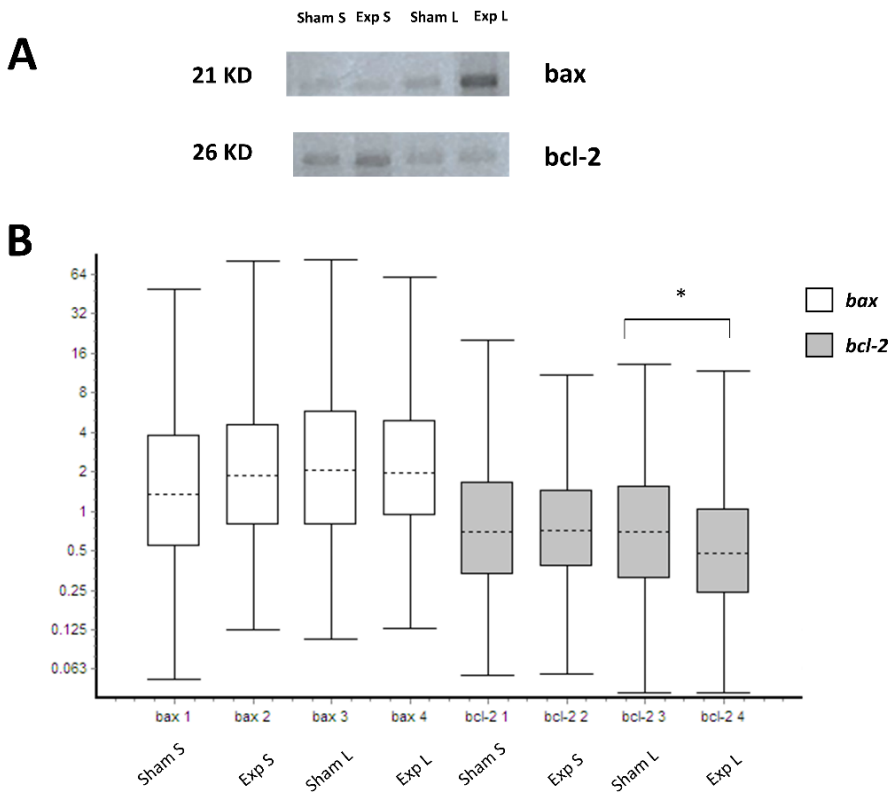

Figure 6. Expression Profiles of Candidate Genes in Various Experimental Conditions. **(A)** Electrophoresis analysis of PCR products amplifying genes (Bax and Bcl-2 proteins) from rat hippocampus tissue; **(B)** Box and whisker plot chart illustrating the expression ratio range from CT values for each experiment. Radiation at 2.45 GHz affects the relative gene expression of *Bax* and *Bcl-2* (apoptosis-related genes) in various groups (sham and exposed groups); Sham S: sham exposed group in the short term, Sham L: sham exposed group in the long term, Exp S: exposed group in the short term, and Exp L: exposed group in the long term. Whiskers represent the minimum and maximum observations. A significant decrease in *Bcl-2* gene expression was observed in the 2.45 GHz long-term group compared to the sham group; Student's *t* test was used for statistical analysis; Values are expressed as mean ± SD (n = 6); they are substantially different from the sham long-term exposed group (* $P<0.05$). The whiskers indicate the minimum and maximum expression ratio values, while the line within the box represents the median.

compared to its sham long-term exposed group (1.654). However, based on statistical results for the *Bcl-2* gene, there was a decrease in the expression of this gene in the brain of the 2.45 GHz radiation-exposed group in the short term and in the long term. In the long-term exposed group, a significant decrease ($P < 0.05$) was found at 0.423 as compared to the long-term sham group, which was 0.625 (Figure 5A).

Figure 6B indicates the diagram of expression of *Bax* and *Bcl-2* genes in a box-and-whisker plot. *β-actin* was used as our reference gene. The box presents the range of 50% of observations. The dotted line shows the middle expression of the gene. Based on the diagram, it can be concluded that increasing the duration of radiation at a frequency of 2.45 GHz in groups 1 to 4 caused no significant changes in the expression of *Bax* genes; however, we found decreased expression of the *Bcl-2* gene in both short-term and long-term groups. The long-term exposed group in *Bcl-2* (*bcl-2 4*) exhibited a significant difference ($P < 0.05$) from the sham long-term group (*bcl-2 3*). Both the sham long-term and exposed long-term groups exhibited down-regulated expression levels at 0.493 (0.161-1.474). As a result, it is necessary to identify and target the most appropriate reference gene to comprehend how to normalize gene expression in a particular experimental system.

These variations reached their maximum in the long-term sham and long-term exposed groups, with no significant difference. It was demonstrated that lowering the amount of an anti-apoptotic protein (*Bcl-2*) in the hippocampus in all groups, especially in the exposed short-term group ($P > 0.05$), was not linked with anxiety-like behavior in the EPM test in rats.

Discussion

Human exposure to radiofrequency and stress is unavoidable due to technology improvements and everyday unpleasant situations.[48] However, only a few experiments have been conducted on Wistar rats exposed to a Wi-Fi signal at 2.45 GHz.[48,49] The findings on anxiety behavior in EPM are also linked with emotion since the behaviors seen in EPM result from a conflict between the incentive to explore the labyrinth and the natural propensity to escape undesirable circumstances.[50] The present study demonstrated significant behavioral variations on the EPM apparatus in male rats exposed to EMF at 2.45 GHz than those in the sham groups. During acquisition trials, exposed animals may need more time to identify the escape platform, and they remained slower than the sham groups, especially in the short-term exposed groups. In addition, the proportion of entries and time spent significantly increased ($P < 0.01$) in closed arms, but in open arms in the short-term exposed group, we observed that the percentage of entries and time spent significantly decreased ($P < 0.01$). Reduced open-arm duration in the EPM is frequently associated with freezing or immobility behaviors that reflect anxiety levels.[11] On the other hand, the HPA axis would be activated in proportion to the degree of stress caused by the necessity of approaching the risk of the open arms and leaving the comfort of the enclosed arms.[28] It can be concluded that in the long-term exposed groups, the rats were adapted to the existing condition, and they did not show any significant difference ($P > 0.05$). The former study by Xiang et al found that the time spent in closed arms was much higher than open arms.[23] These findings corroborated Varghese and colleagues'[1] observation regarding the anxiety behavior, in which exposure of rats to EMF radiation at 2.45 GHz creates severe alterations in the brain, including impaired learning and memory, as well as the manifestation of anxiety behavior in rats; therefore, a decrease in brain antioxidant enzyme mechanisms such as superoxide dismutase, catalase, and decline in glutathione levels may linked to impaired he cognitive functions.[1] Thus, there is evidence that enhanced ROS generation by EMF is linked with cognitive impairment in animal models.[51] Another study by Yang et al[7] tested more than two thousand candidate genes against EMF exposure. Two heat shock proteins, HSP27 and HSP70, were significantly observed. Their results showed that EMF at 2.45 GHz induced a stress reaction in the rat hippocampus due to increasing these two genes in the hippocampus of rats. On the other hand, Sandrey et al found that EMFs have an essentially short-term influence on the body mass in rats.[52] However, our results showed a significant difference in the exposed groups compared with the sham groups regarding their body mass.

A study conducted by Yu-Hong et al found that EMF produces some behavioral issues in rats, including a substantial reduction in the capacity to acquire memory and a significant increase in activity in rats exposed to EMP for three months.[53] Another study using the EPM test by Jadidi et al showed that male and female mice were more anxious when exposed to mobile phone jammer radiation at 900 MHz and 900+1800 MHz frequencies respectively.[54] Cosquer et al showed that exposure to 2.45 GHz, which has been shown to increase the number of benzodiazepine receptors in the rat brain, did not affect anxiety responses measured in the EPM test.[6] However, their results contrasted with Othman et al, who mentioned that the Wi-Fi signal and constraint stress affect the brain and cognitive functions, particularly in the EPM test. In comparison, they could not find a link in synergistic effects on the brain between the Wi-Fi signal and restraint stress.[48] These findings align with conclusions made by Obajuluwa et al who showed that prolonged exposure to Wi-Fi may have negative consequences, including neurodegenerative disorders, as evidenced by substantial changes in *AChE* gene expression and several neurobehavioral indicators linked with brain injury.[15] As a point of human study, pregnancy-induced oxidative

stress and DNA damage in the cord blood and placenta have been linked to mobile phone use.[3]

Furthermore, in our study, the rate of Bcl-2 protein expression in the hippocampus, which indicates the activation of the mitochondrial apoptosis pathway, was significantly different ($P < 0.05$) in the exposed long-term group compared to the long-term sham group. It was found that mice overexpressing the *Bcl-2* gene displayed decreased anxiety-like (fear) behavior in the context of anxiety disorders.[55] Our findings also indicate that the lower expression of *Bcl-2*, its inhibitor impact on *Bax*, has been eliminated and leads eventually to *Bax* overexpression. Based on our main results, during long-term exposure to 2.45 GHz, apoptosis induction was induced by the up-regulation of *Bax* and down-regulation of *Bcl-2*. Hence, both exposed long-term and short-term groups downregulate *Bcl-2* expression. To some extent, in short-term exposure, anxiety levels, which we observed in our results in Figures 2 and 3, significantly decreased.[11] However, the opposite of this event happened in long-term exposure. Our findings contrast with those by Cobb et al[56] and Cassel et al,[57] who recently showed that 2.45 GHz had no impact on maze performance.

The findings of the current study indicate a markedly elevated serum CORT level in the short-term exposed group compared to the long-term exposed group. In this study, CORT secretion appears to have lacked an inhibitory effect in the long term. Although the earlier study had shown a connection between plasma CORT response and exploration of the open-arm, substantial increases in CORT with limited exposure of the closed arms have also been reported.[30] However, the capacity of the animals to sustain a high level of CORT after repeated exposure to the plus maze was the clearest sign that they are under the influence of stressful events.[58] However, Veenit et al mentioned that their results did not support the assumption that exposure to higher levels of CORT in peri-puberty affects anxiety behaviors of the animals in adulthood.[59] Additionally, plasma CORT levels measured following EPM testing were significantly correlated with the degree of risk assessment.[28] Therefore, the current findings support a positive correlation between plasma CORT levels and risk assessment in rats subjected to the EPM after exposure to EMFs at 2.45 GHz in the short-term period. However, this EMF can alter some gene expression, especially *Bcl-2*, as an anti-apoptotic gene in the short term and in the long term.

The limitation of this study is that during the experiment, the rats were not allowed to move freely more than 20 cm away from the EMF antenna, and this could alter their behavior in the EPM and OFT and their CORT levels.

Conclusion

The findings of the present study suggest that exposure to EMFs may act as an external stimulus, altering the activation of stress-related genes and eliciting an anxiety reaction in the rat hippocampus. For the anxiety-like behavior study, the results showed that short-term radiation could reduce the percentage of entries into the open arm and the percentage of time spent in this arm, while the effect of long-term radiation did not show any significant effects. In this study, we found that long-term exposure to EMF—long-term exposed groups compared to the long-term sham group—decreases the expression of *Bcl-2* and increases the expression of the pro-apoptotic *Bax* gene in the rat hippocampus. Thus, the current data confirm the existence of a positive connection between plasma CORT levels and risk appraisal in rats exposed to the EMFs at 2.45 GHz.

Author Contributions

SMS and MS designed the study; MT collected behavioral and CORT data; ZP performed molecular study; MT, ZP and AMC analyzed the data; and AMC wrote and edited the manuscript.

Conflicts of Interests

The authors declare no conflict of interest.

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