Available online at www.medicinescience.org

ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2020;9(4):929-34

1800MHz Radiofrequency electromagnetic radiation: Does it affect heat shock genes expression levels in the rat brain?

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Received 18 June 2020; Accepted 29 July 2020
Available online 08.10.2020 with doi: 10.5455/medscience.2020.06.112

Abstract

The brain could recognizethe cell phone radiation as environmental stress and may alter stress-related gene expression levels. Heat shock proteins (HSP) are a family of proteins that are produced by cells in response to the exposure of stressful conditions. We investigated whether exposure to 1800MHz Radiofrequency Electromagnetic Radiation (RF-EMR) recognize the expression levels of some HSPs (Cryaa, Crybb, Hsp20, Hsp25, Hsp70) in mature rat's brain tissue. The experimental group was exposed to 1.800MHz RF-EMR in restrainer for eight weeks with 2 h/day. The specific absorption rate (SAR) was 0.06 W/kg. The sham group was kept in restrainer but not exposed to radiation. The Control group was kept under their condition. Whole brain was homogenized.ViA7 Real-Time-PCR software was used to measure CT values of the genes and ΔΔCT values were also calculated. Cryaa (p=0.02), Crybb (p=0.01), and Hsp20 (p<0.001) genes expression levels were decreased in the exposed group according to the sham and control group. However, Hsp25 (p=0.069) and Hsp70 (p=0.329) genes expression levels were not altered. The brain can recognize RF-EMR as a stress factor and alter some HSPs levels in brain tissue. These changes may lead to neurobiological deficits in prolonged exposure.

Keywords: Electromagnetic radiation, heat-shock proteins, gene expression

Introduction

Radiofrequency electromagnetic field (RF-EMR) at 1800 MHz has been used in cell phone communication systems in some countries and has; therefore, raised wide concerns regarding the potential adverse effects on humans. The human exposure to cell phone radiofrequency electromagnetic field has increased in recent years, especially its effects on the brain due to the proximate distance of the mobile phone to the head. Experimental and retrospective studies on the potential effects of RF-EMR to the brain have shown contradictory results regarding the increased permeability of the blood-brain barrier (BBB) and cause brain tumors [1,2]. Also, other experiments have shown that electromagnetic radiation can affect the nervous system. It can cause insomnia, tiredness, and headache [3], and affect cognitive performance such as attention, learning, and memory capacity [4,5].

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Long-term exposure to mobile phones can also cause histopat hological damages and biochemical alterations in brain tissue. RF-EMR exposure has been shown to alter melatonin levels and antioxidant enzyme levels (SOD (Superoxide Dismutase), GPx (Glutathione Peroxidase), CAT (Catalase)), creatine kinase activity, MDA (Malondialdehyde)level, content of DNA double-strand breaks, protein kinase C, and histone kinase activities, has also been reported to induce changes in the level of expression of certain genes [6,7].

Gene expression is a unique way for cells to adapt to external changes. Investigations showed that cell phone radiation exposure may change gene expression levels in different cells including neuronal and non-neuronal cells in the brain [7]. RF-EMR acts as a stress factor and affects the stress molecular pathways. These molecular pathways include heat shock proteins (Hsps). Hsps take part in several biological processes, from adaptation to stressful conditions, apoptosis, cell cycle, cell differentiation, and cellular malignancy [8, 9]. Hsp70 has a neuroprotective role in the nervous system, and it is also known to be induced by cellular stress [11]. Hsp20 is highly expressed in long-lived cells like the brain [12]. A few studies showed that Hsp70 [13] and Hsp20 [14] expression levels can increase in different cell types with RF-EMR. However, some of

these studies claimed that cell phone usage does not alter the Hsp70 gene [15,16]. Also, it has been shown that Hsp25 overexpression inhibits radiation-induced caspase-dependent apoptosis through the reduction of PKCd (Protein Kinase C delta)-mediated ROS (Reactive Oxygen Species) production in the exposed cells by radiation. Thus results reduced ROS-mediated apoptotic cell death [17]. Sanchez et al. reported that 900 MHz and 1800 MHz mobile phone exposure to hairless rat's skin cells (single exposure; 2 h/day Specific Absorption Rate (SAR): 0-5 W/kg; repeated exposure; 2 h/day for 5 days/week, for 12 weeks, SAR: 0, 2.5, 5 W/kg.) did not alter Hsp25 and Hsp70 gene expression levels [18]. The α-crystallins and β-crystallins are a superfamily of small heat-shock proteins (sHsp). sHsps have chaperone-like activity and could be over-expressed in several cell types under stress conditions. There are two α-crystallin genes, CRYAA and CRYAB, encoding αA- and αB-crystallins. The alternative systematic name of CRYAA is HSPB4. β-crystallins are subdivided into acidic (A) and basic (B) subunits, encoded by the CRYBA and CRYBB genes [19,20,21].

It is known that cell phone radiation has been recognized by cells as a stress factor. Also, it is known that Hsps expression levels are altered to protect the cells from cellular stress. In the present study, we aimed to show the effects of 1800 MHz cell phone exposure for 2 h/day along eight weeks on Cryaa, Crybb, Hsp20, Hsp25, and Hsp70 genes expression levels in Wistar Albino rat brain tissue.

Materials and Methods

Animals

Adult female Wistar Albino rats (8–12 weeks old and the average weight of 215±18 g) were supplied from the Animal Research Laboratory, Mersin University, Mersin, Turkey. Animals were fed with ad libitum and housed in acrylic cages in an animal room with a 12h/12h light/dark cycle. The temperature and relative humidity were maintained at 23±1°C and 50±5%, respectively. All of the animal measures were confirmed by the Mersin University Animal Experiments Ethics Committee (2014-HAYDEK-01).

The Nelder–Mead method was used for at least seven of the Wistar Albino rats, and a total of 21 rats were planned to be included in each group of female rats in the study [22]. Considering 20% loss, 27 rats in total were included in the study (n=9 rats per group). The rats were divided into three groups. The exposure and sham groups were kept restrained for 2 h/day for five days to adapt to the experimental conditions. The control group was kept at normal conditions during the experiment. The sham group was kept under the same conditions as the experimental group without exposure to RF-EMR, and the experimental group was exposed to 1800 MHz RF-EMR at an electric field 5-9 V/m (6.8±0.1), for 2 hours per day for eight weeks. Whole-body temperature measured before exposure and at the end of the 2-hour exposure under the experimental setup by inverting thermometer into the rectal cavity. The average body temperature was 36.1-37.2°C in all groups. No significant temperature rise occurs in the RF-EMR group.

After the last exposure, rats were sacrificed under anesthesia (ketamine 90 mg/kg, xylazine 10 mg/kg) immediately on ice and their whole brains were removed. Tissues were stored at −80°C until homogenization.

Electromagnetic Exposure and SAR Calculation

The electromagnetic radiation exposure system was designed by the Department of Biophysics according to previous studies [23] and RF-EMR control assessments were made by the Department of Electric and Electronic Engineering. An 1800 MHz GSM simulator (GSM-1800 CW2; Adapazari, Turkey) was used for the RF-EMR exposure. Once at the beginning and second at the end of the study, electric field measurements of the RF-EMR exposure system were measured with an Electrical Field Meter (PMM 8053 Portable Field Meter) by the Department of Electric and Electronic Engineering, Mersin University, Mersin. The Electric field measurement methods of RF-EMR exposed to the 1800 MHz GSM simulator was based on those used by Akar [23]. The restrainer used to expose the rats to the RF-EMR is illustrated in Figure 1.

The experimental group was placed inside to restrainer, and the rats were exposed to the EMF 5–9 V/m(6.8±0.1) for 2 h/day with 1 W at the same time every day. The specific absorption rate (SAR) was used for the dosimetry. The electric field measurements, which were used for the SAR calculation, were taken for the head, body, and tail of each rat during exposure for 1 min (Table 1). The SAR was calculated from the following formula according to a previous study [23]. The calculated SAR was 0.06 W/kg (Table 2).

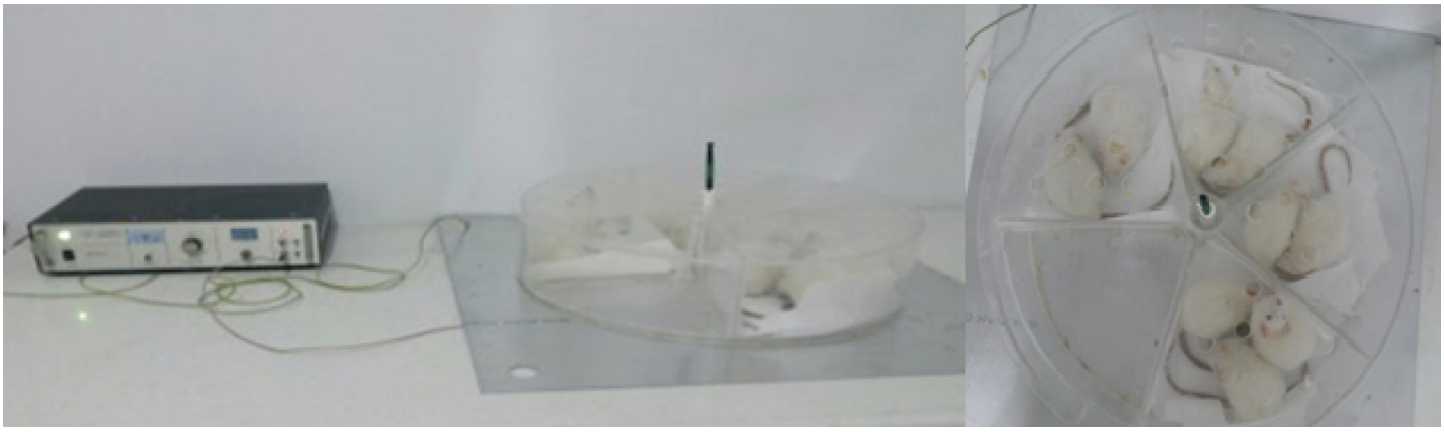

Figure 1. The RF-EMR (Radiofrequency Electromagnetic Field) exposure system. Signal Generator 1800 MHz Everest GSM Simulator 1800 CW2.

Table 1. Mean electric field values exposed to rats.

1800MHz						
Rat	Head d1ort (cm)	Head E1ort (V/m)	Body d2ort (cm)	Body E2ort (V/m)	Tail d3ort (cm)	Tail E3ort (V/m)
1	7	7.9	12	5.8	21	3.9
2	7	8.8	12	5.7	21	2.6
3	7	7.6	12	6.6	21	3.5
4	7	8.0	12	5.5	21	2.9
5	7	7.9	12	5.7	21	2.6
6	7	7.8	12	4.9	21	3.2
7	7	8.6	12	5.2	21	2.7
8	7	7.1	12	6.0	21	2.7

Moisture: %40-50, Light: 40Watt, Temperature: 22^{0}C
dort: Average distance to antenna, Eort:Mean electric field value exposed to rats.

Measurements were used in the following formula and the SAR value was calculated.

$$SAR = \sigma \cdot \frac{E_{RMS}^2}{\rho} \quad w/kg$$

E_{RMS}: Electric Field (the root means square electric field) (V/m)

σ: Average electrical conductivity (S/m)

ρ: Tissue bulk density (kg/m^3)

Table 2. Calculation of Electric Field and SAR.

	Tissue bulk density (kg/m3)	Average electrical conductivity (S/m)	Measured Average Electric Field Value (V/m)	SAR(W/kg)
Whole body	1040	1.389380	6.8±0.1	0.06

*Electrical conductivity values for 1800MHz frequency were obtained from http://www.fcc.gov/oet/rfsafety/dielectric.html.

Tissue Homogenization and Total RNA Isolation

Homogeneous brain tissues were prepared via a sterile lancet on the ice block in the shortest time possible. The homogenization was performed with the MagNA-Lyser Green Beads (Roche Life Science). The total RNAs were isolated from the homogenate with the Purelink RNA Mini Kit (Ambion, Thermo Fisher Scientific, USA).

Gene Expression Analysis

cDNA (Complementary DNA) Synthesis

cDNA was synthesized from total RNA with high-capacity cDNA reverse transcription Kits (Ambion, Thermo Fisher Scientific, USA). A reaction mix of 20 µl included 2.0 µL 10× RT (Reverse Transcriptase) Buffer, 0.8 µL 25×Deoxynucleotide triphosphates (dNTPs) Mix (100 mM), 2.0 µL 10× RT Random Primers, 1.0 µL MultiScribe™ reverse transcriptase, 10.2 µL nuclease-free H_2O, 4 µL cDNA. The reaction was incubated at 25 °C for 10min, 37 °C for 120 minutes, 85 °C for 5 minutes, and the final hold was at 4 °C. The reaction was performed in a Techne Prime thermal cycler, Techne, UK.

Real-Time PCR

Real-time PCR was performed in a high-capacity real-time PCR System (ViA 7tm). A PCR mix of 20 µl included 10.0 µL TaqMan Gene Expression Master Mix (2×), 1.0 µL TaqMan Gene Expression Assay (20×), 2.0 cDNA template, 7.0 nuclease-free H2O. The thermal cycle conditions for the UDG (Uracil-DNA Glycosylase) incubation at 50 °C for 2 min, AmpliTaq Gold, UP enzyme activation at 95 °C for 10min, followed by 40 cycles of denaturing at 95 °C for 15 sec and were then annealed/extended at 60 °C for 1 min. The reference sample was made by mixing of the control group RNAs. Reactions were incubated in a 96-well plate. Actb (actin beta) was used as the endogenous control in Real-Time PCR. Cryaa (Rn00561064), Crybb1 (Rn00564028), Hsp20 (Rn00594138), Hsp25 (Rn01519180), and Hsp70 (Rn02532795) genes expression levels were determined with TaqMan Gene Expression Assays. All reactions were performed in triplicate. ΔΔCT values were calculated from the CT values with the ViiA™ 7 Software (Applied Biosystems).

Statistical Analysis

$2^{-\Delta\Delta CT}$ values were calculated and statistical analyses were performed.The Shapiro–Wilk test was used to determine whether all the parameters were normally distributed, and it was found that all parameters were normally distributed. Variance analyses were used to test the differences between the groups for each parameter. Homogeneity of the variance was determined using the Levene test. One-Way analysis of variance (One-way ANOVA) was used when the variance was homogenous, and the Welch test was used when it was not. The Tukey's test was used for multiple comparisons in the One-Way ANOVA and Games Howell test for multiple comparisons in the Welch test statistics. Descriptive statistics (mean±standard deviation) were calculated in each group for all parameters. Pearson's correlation coefficient was used for the differences between continuous measurements. The results for $p<0.05$ were accepted as statistically significant.

Results

After the analyses, the results showed that Cryaa, Crybb1, and Hsp20 gene expression levels were decreased in RF-EMR exposed group. Cryaa gene expression mean and standard deviation value was 0.232±0.100 in the exposed group, 0.495±0.170 in the sham group, 0.756±0.458 in the control group respectively. Cryaa gene expression levels were decreased in the sham and exposed group according to the control group ($p=0.02$). Also, there was a significant decrease in expression level in RF-EMR exposed group when compared with the sham group. The expression of this gene in stressed rats due to experimental conditions appears to be suppressed. RF-EMR also caused stress in rats. Therefore, the expression level of the Cryaa gene in the exposed group was more suppressed. Crybb1 gene expression mean and standard deviation value was 0.319±0.063 in the exposed group, 0.527±0.164 in the sham group and 0.651±0.196 in the control group. There was a similar change between the groups as well as the Crybb1 gene expression level, as was the Cryaa gene expression level ($p=0.01$).

Rats were stressed due to experimental conditions. But also, cell phone radiation RF-EMR exposure caused extra stress in rats. Hsp20 gene expression mean and standard deviation values was 0.531 ±0.093 in the exposed group, 0.914±0.297 in the sham group, 0.878±0.172 in the control group and p-value was <0.001. Also, there was an association with gene expression levels and stress. Cell phone radiofrequency radiation was suppressed by the Hsp20 gene expression in the rat brain. However, there was no significant alteration of Hsp70 and Hsp25 gene expression levels (p>0.05). Hsp70 gene expression decreased in the exposed group according to the control group but the difference was not significant. However, Hsp25 gene expression level was increased in the exposed group (0.807±0.409) and the sham group (0,816±0.349) due to experiment conditions. It seems to be RF-EMR exposure was not affect the Hsp25 gene expression.

The mean and standard deviation values of the gene expression level and the p values of the genes in the three groups are summarized in Table 3. A comparison of the mean values in all groups is shown graphically in Figure 2. Also, the correlations between the parameters are detailed in Table 4. A strong positive correlation in the gene expression levels between the Cryaa and Crybb1 (r=0.694, p<0.001), Cryaa and Hsp20 (r=0.694, p<0.001), and Crybb1 and Hsp 20 (r=0.668, p<0.001).

Table 3. Gene expression mean and standart deviation values between the groups.

	1800MHz RF-EMF exposed group	Sham exposed group	Control group	P Value
	Mean ± SD.	Mean ± SD.	Mean ± SD.	
Cryaa	0.232 ± 0.100*	0.495 ± 0.170†	0.756 ± 0.458	**0.02**
Crybb	0.319 ± 0.063*	0.527 ± 0.164†	0.651 ± 0.196	**0.01**
Hsp 20	0.531 ± 0.093*	0.914 ± 0.297†	0.878 ± 0.172	**<0.001**
Hsp 25	0.807 ± 0.409	0.816 ± 0.349	0.465 ± 0.182	0.069
Hsp 70	0.830 ± 0.610	0.711 ± 0.677	1.050 ± 0.217	0.329

*: Difference from control group, †: Difference from exposed group.

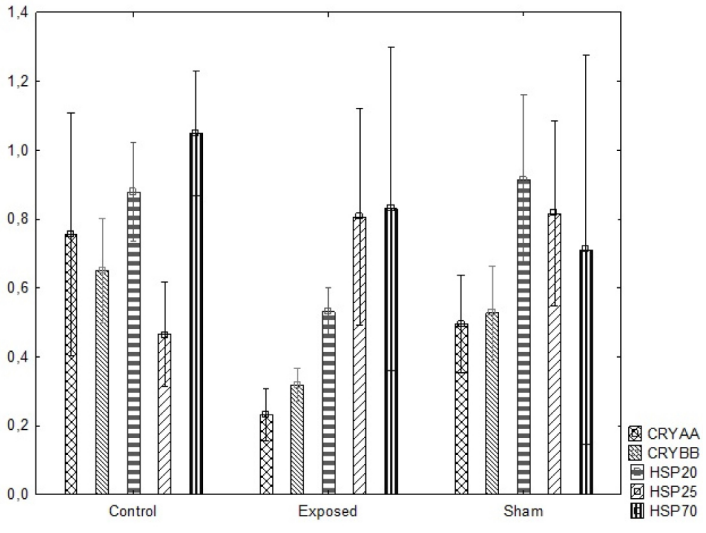

Figure 2. A graphical representation of the difference in the gene expression levels between the groups.

Table 4. The relationship between outcome variables

		crybb	hsp20	hsp25	hsp70
cryaa	r	0.653	0.694	-0.062	-0.083
	p	**<0.001**	**<0.001**	0.769	0.700
crybb	r		0.668	-0.094	0.161
	p		**<0.001**	0.654	0.454
hsp20	r			0.071	-0.002
	p			0.734	0.994
hsp25	r				0.110
	p				0.600

Discussion

Due to its usage, the brain is the most affected organ from cell phone radiation. RF-EMR can cause tissue heating especially in the skin, where a little amount of the brain warms up. It is believed that the temperature increase does not have a permanent effect on the brain tissue. The non-thermal effects on the cells of the brain induced by RF-EMR are seen as more important. In this study, we investigated whether the non-thermal effect of cell phone radiation is perceived as stress by brain cells. Therefore, we investigated heat shock proteins that respond rapidly to cellular stress. We preferred 1800 MHz radiofrequency radiation due to its widespread usage. We aimed to provide information about the cell phone radiation that could induce a stress response in the rat brain.

Electromagnetic fields can cause oxidative stress in the exposed cells which affect the electron distribution and their movement in the DNA. This electron distribution can cause damage in the DNA and gene structures, and also various cell protective mechanisms could be developed against this environmental stress [24,25]. RF-EMR is shown to activate chaperones that can prevent damage to native proteins and cell membranes. The heat shock proteins are highly conserved proteins that have chaperone activities that may protect the protein structures under stress conditions like heat and other environmental stress [26]. Also, Hsps play an important role that controls the balance of cell death or survival under cell stress conditions. When the cells are exposed to RF-EMR, specific molecular pathways induce the overexpression and accumulateone of the rapidly synthesized Hsps [1,8,10,21].

Several studies have investigated the effect of cell phone radiation and heat shock response in several tissues or cell lines. M. Lantow et al. determined that ROS production was significantly different under 1800 MHz cell phone radiation exposure (2 W/kg) in human monocytes compared to sham. Also, Hsp70 expression levels after 0, 1, and 2 h post-exposure at 2 W/kg for 1 h were not altered between the groups [27]. Weisbrot et al. examined the effects of 900/1,900 MHz cell phone radiation (60 min at 11 AM and 60 min at 4 PM for 10 days) (SAR=1.4 W/kg) on Drosophila melanogaster developing eggs. They found that the exposure of non-thermal radiation from the cell phone-induced phosphorylation of the nuclear transcription factor, ELK-1 (ETS transcription factor ELK1), increased the serum response element (SRE), and elevated Hsp70 protein levels within minutes [28]. Hussein et al. investigated the effect of 1800 MHz cell phone radiation on the rat's hippocampus and cerebellum tissues (SAR=0.6 W/kg, 2 h/day for three months). The immunohistochemical, biochemical, electron microscopy and histological investigations showed a significant reduction in

the antioxidant parameters (glutathione, superoxide dismutase, and glutathione peroxidase) and a significant elevation in MDA content in both regions of the exposed group. Also, degenerative changes in the hippocampus pyramidal cells, in the dark cells, and the cerebellar Purkinje cells with vascular congestion, overexpression of the cyclooxygenase-2 apoptotic gene, and DNA fragmentation were detected [29]. Kesari et al. reported elevated molecular, biological, and genetic changes in 45-day old male rat's brains which were exposed to 3G cell phone signals for 2 h/day for 60 days. They showed that in the exposed group's brain tissue, DNA strand breaks, micronuclei, caspase 3, and apoptosis activities were significantly increased ($P<0.05$). They also showed a transient increase in the phosphorylation of Hsp27, Hsp70, and p38 mitogen-activated protein kinase (p38MAPK) through the western blot technique [8]. Dasdag et al. investigated the effects of 900 MHz microwave (2 h/day for 10 months) on apoptotic glial cells and the status of oxidative stress in the adult male rat brain. Finally, they reported that cell phone use may alter apoptosis, total antioxidant capacity, and catalase release [30]. Miyakoshi et al. showed that 1950 MHz radiofrequency radiation did not affect cell proliferation and expression of Hsp27 and Hsp70 in a human glioma cell line [31]. Leszczynski et al. examined whether 900 MHz GSM mobile phone exposure altered the protein expression levels of p38MAPK (p38 Mitogen-Activated Protein Kinase) and Hsp27 in the human endothelial cell line EA.hy926 and they found transient changes. They claimed that RF-EMR exposure activates cellular signal transduction pathways, including the Hsp27/p38MAPK stress response pathway. Long-term activation of this pathway could cause brain tumors by inhibiting the cytochrome c/caspase-3 apoptotic pathway and cause an increase in blood-brain barrier permeability through the stabilization of endothelial cell stress fibers [32].

There were a small number of studies that showed miRNA elevation in the rat brain upon exposure to the cell phone microwave. Dasdag et al. investigated whether the 900 MHz cell phone microwave and 2.4 GHz Wi-Fi radiation altered miR-9-5p, miR-29a-3p, miR-106b-5p, miR-107, and miR-125a-3p in the rat brain. They determined that miR-106b-5p and miR-107 expression levels decreased in the 2.4 GHz Wi-Fi exposed group but only miR107 decreased in the 900 MHz cell phone exposed group. Researchers claimed that if this exposure prolonged, alteration of some miRNA expressions may lead to neurodegenerative diseases [33, 34].

In our experiment, Cryaa, Crybb1, and Hsp20 gene expression levels were significantly decreased in the exposed group according to the sham and control group. Also, there was a strong positive correlation between the Cryaa, Crybb1, and Hsp20 gene expression levels. We indicate that the expression levels of all three genes decreased together due to the cell phone RF-EMR exposure. Cryaa and Crybb1are small heat shock proteins, act as molecular chaperones. They have been believed for decades as the only organ-specific proteins and associated with cataract [35]. Interestingly Sparado et al. claimed that Crybb1 is the new candidate for schizophrenia. The authors observed a significant increase in the level of Crybb1 transcriptsin a set of fear conditioning experiments. Their results support that the Crybb1 take a role mediating fear and stress-associated responses with its repression appearing to reduce anxiety-like behavior [36]. In our results, cell phone exposure reduced the Crybb1 gene expression level in brain tissues of Wistar Albino rats. It could be related to reduced anxiety-like behavior and schizophrenia symptoms.

Hsp20 is known to act as a chaperon protein, binding to protein kinase 1 (PDK1) and allowing its nuclear transport. PDPK1 is a master kinase, which is crucial for the activation of AKT/PKB and many other AGC kinases including PKC, S6K, SGK. An important role for PDPK1 is in the signaling pathways activated by several growth factors and hormones. The increased expression level of this chaperone, can reduce the activation of the AKT/PKB molecular pathway and cause abnormal activation of growth factors. These changes may actbrain functions in long-term exposure.

On the other hand, there were no significant differences in Hsp25 and Hsp70 gene expression levels. Guptea et al. found strong evidence that the total Hsp25 gene expression level increased in the age-related brain regions [37]. In our study, adult rats were used; therefore, the Hsp25 ratio may be high in all groups. The level of Hsp25 gene expression may be increased in all regions of the brain in adult rats depending on age. Therefore, there may be no difference between the groups.

The Hsp70 is found in very little amounts in the normal brain [38]. Studies with different cell types have yielded conflicting findings; however, brain tissue has not yet been investigated [12-15]. With this experiment, the relationship between the cell phone radiation and Hsp70 protein in rat brain tissue was investigated for the first time. We found that the Hsp70 gene did not increase in the brain tissue following exposure to non-thermal external stimuli. Also, there was a decreased expression level in the RF-EMR group (0.830 ± 0.610) according to the control group (1.050 ± 0.217) but it was not statistically significant. 1800MHz RF-EMR exposure may have suppressed the mRNA expression of Hsp70 in rat brain tissue. This exposure could change the expression of some miRNAs which inhibits Hsp70 gene expression. HSP pathways and miRNAs associations should be investigated in further studies.

However, some limitations should be noted. First, we measured the whole-body temperature of the rats and there was no rise. The only head temperature should be calculated to show a non-thermal effect on the brain. Second, measuring protein levels of Cryaa, Crybb and Hsp20 could have supported the results. Protein synthesis is regulated through posttranscriptional mechanisms. Crystallin's and heat shock protein levels may not change at the same rate as their mRNA levels. Also,it should be noted that the earliest response of cells to environmental stress is to synthesize mRNA from related genes.

In conclusion, thebrain could recognize the 1800MHz cell phone radiofrequency radiation as a stress factor. It could change some heat shock genes expression levelsas a response to protect their cellular functions. Abnormal expression levels of stress genes may affect the brain functions on prolonged exposure. Some neurological disorders symptomsmay be affectedby these changes, also brain tumors may occur.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

This study was supported by the Research Fund of Mersin University in Turkey with Project Grant Number: 2015-AP4-1216.

Ethical approval

All of the animal measures were confirmed by the Mersin University Animal Experiments Ethics Committee (2014-HAYDEK-01).

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