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Evaluation of *bax*, *bcl-2*, *p21* and *p53* genes expression variations on cerebellum of BALB/c mice before and after birth under mobile phone radiation exposure

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ABSTRACT

Objective(s): The increasing rate of over using cell phones has been considerable in youths and pregnant women. We examined the effect of mobile phones radiation on genes expression variation on cerebellum of BALB/c mice before and after of the birth.

Materials and Methods: In this study, a mobile phone jammer, which is an instrument to prevent receiving signals between cellular phones and base transceiver stations (two frequencies 900 and 1800 MHz) for exposure was used and twelve pregnant mice (BALB/c) divided into two groups (n=6), first group irradiated in pregnancy period (19th day), the second group did not irradiate in pregnancy period. After childbirth, offspring were classified into four groups (n=4): Group1: control, Group 2: B1 (Irradiated after birth), Group 3: B2 (Irradiated in pregnancy period and after birth), Group 4: B3 (Irradiated in pregnancy period). When maturity was completed (8-10 weeks old), mice were dissected and cerebellum was isolated. The expression level of *bax*, bcl-2, p21 and *p53* genes examined by real-time reverse transcription polymerase chain reaction (Real-Time RT- PCR).

Results: The data showed that mobile phone radio waves were ineffective on the expression level of bcl-2 and p53 genes) $P>0.05$). Also gene expression level of *bax* decreased and gene expression level of *p21* increased comparing to the control group ($P<0.05$).

Conclusion: From the obtained data it could be concluded that the mobile phone radiations did not induce apoptosis in cells of the cerebellum and the injured cells can be repaired by cell cycle arrest.

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Introduction

Radiofrequency (RF) wave used in daily life equipment's like mobile phones, televisions, microwave ovens, base stations, radio, wireless. Radiofrequency and microwaves usually exist in the environment so every person exposed to these electromagnetic waves. These days, using of mobile communication systems has rocketed across the world (1). The electromagnetic fields (EMFs) emitted from cellular phones with the frequency ranges of 800 to 2000 MHz that classified into the radio-frequency (RF) spectrum. In the Global System for Mobile Communications (GSM), 900 and 1800 MHz RF-EMF is most widely used frequencies (2, 3). The RF wave power that expressed by cell phones are below 1 watt (4). The greatest power output from a mobile phone is regulated by the mobile phone standard in each country. In many systems, the cell phone and the base station check reception quality and signal strength and the power level increase or decrease automatically (5). The rate of absorbed energy by the human body measured by the specific absorption rate (SAR), The SAR, indicating the heat energy absorbed by the head of a cell phone user (6). The possible health risk of mobile phones to the human organs has been raised, especially in the Brain. Many scientists have warned about the harmful effects of the EMFs. The World Health Organization (WHO) has apportioned a high preference to research on possible adverse effects of mobile phone exposure on the brain, that it is the main concern with regards to the effects of RF-EMF, because the brain is the sensitive organ to physical stimuli, especially during development (2, 3, 7-9). These stimuli may be due to the increment of ROS production, mitochondrial functions, homeostasis,

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upregulated heat shock protein expression, and finally, gene expression changes in the brain (10, 11). However, the available evidence is not sufficient to report any definite conclusions, and future investigations still need to fully explore the detailed mechanisms. Due to the proximity of the mobile phone device to the head, the brain is exposed to relatively high specific absorption rates (SAR), therefore, with this regard cerebellum is the sensitive parts of the brain and it is the primary center of motor coordination in the CNS. The development of the human cerebellum begins in the uterus and continues for several years postnatal. The cerebellum is easily damaged because the Purkinje cell is quite sensitive. Cerebellar damage makes disorders in fine movement, equilibrium, posture, and motor learning (12, 13).

In this study for more assessing that these non-ionizing electromagnetic fields are a stressor for the fetal brain, we evaluated the *bax*, *bcl-2, p21* and *p53* genes as a stress marker, because these genes play key roles in cell cycle and apoptosis path (14).

There are three apoptosis pathways: the extrinsic pathway, intrinsic pathway and perforin-granzyme pathway (14, 15). Many factors can incite or inhibit the intrinsic pathway. The most typical modifiers of these pathways are *bcl-2* and *p53* gene families (15, 16).

The *bcl-2* gene identified in the B-Cell Follicular lymphoma for the first time (16) and in this type of lymphoma, *bcl-2* was found to cause more longevity (15). *bcl-2* members regulate apoptosis by controlling the mitochondrial membrane potential and preventing the release of cytochrome-c into the cytoplasm from mitochondria. This situation is thought to increase the possibility of precipitation for malignity by increasing longevity in the cell (17, 18). *p53* gene arrests cell cycle at the late of G1 phase in the case of DNA damage and spares time for DNA repair. Meanwhile, if DNA is repaired *p53* diminishes and then the cycle will be completed. If the repair attempt is unsuccessful, *p53* leads the cell to apoptosis (15, 19). Therefore, if *p53* gene is damaged, the risk of tumor genesis will increase due to the increased amount of DNA damage (15).

Preliminary studies have shown that RF-EMFs may lead to the brain cell damages, which depends on numerous factors such as the specific absorption rate (SAR), duration and the frequency of EMF exposure. Furthermore, the effects of microwave exposure on the biochemistry, morphology, and neuropathology of the brain have been reviewed on the frequency and intensity ranges used in current mobile communication systems.

A research in 2014 showed the effects of mobile phone radiation to the gene expression of bcl2 and *p53* on the brain cells of rats. This study showed that electromagnetic waves have a sensible effect on the gene expression level related to the apoptosis (20). In another study, the effect of mobile phone radiation on gene expression in the cortical neurons has been reviewed. The results showed an increasing rate of the *bax* gene comparing to the low amount of *bcl2* (21). The effect of radio frequency waves on the mouse brain in 2012 has been investigated. The results of this study showed changes in the gene expression levels of pro-apoptotic and anti-apoptotic (22). In 2014 some researchers showed the effect of mobile phone exposure on rat neurons. The results show the chromatin compaction and an increasing of cells apoptotic (23). In 2015 has been shown the association between gene expression and mobile phone exposure on the BALB/c mice hippocampal. The study found no significant contrast on gene expression (24). These results strongly showed that RF-EMFs may have some effects on the brain. There are many other studies under investigation and the possible effects of long-term use of mobile phones need further investigations.

This study focused on the effect of mobile phones on cerebellum gene expression in pregnant mice, their fetuses, offspring and mature mice.

Materials and Methods

Animals

Five males and 25 females BALB/c mice weighing 24-28 g were obtained from the animal center of the Mashhad University of Medical Sciences. For mating two females with a male were placed into the cage overnight. Vaginal smear was performed the next morning, and this was designated as the zero days of pregnancy if sperm were detected. twelve pregnant mice were housed and maintained at room temperature 21±2 °C, with good ventilation, a relative humidity of 65%-70% and 12 hr:12 hr light/dark cycle (lights on at 7:00 am) with enough food and water. The study was conducted by using two groups of mice; each group comprised 6 pregnant animals. In one of the group, pregnant mice were exposed from the first day (E1) of gestation when a vaginal plug was observed, thus indicating that the mating had happened. The whole body exposure on continuous days from E1 to E19. The gestation period of the mouse is about 19–20 days (25). The second group was not exposed during E1 to E19 days. After child birth, in each group, 8 offspring were selected and divided randomly into the control and the exposure groups.

Finally, the study was conducted using four groups of mice; each group was comprised of four offspring. One group served as a control and three groups B1, B2 and B3 were exposed to the Jammer.

Mice were classified into four groups as mentioned below.

Control group: consist of four mice without exposing from the embryonic to maturity period.

B1 group: consist of four mice without exposing in the embryonic and exposing from the birth to maturity period.

B2 group: consist of four mice exposing in the embryonic till maturity period.

B3 group: consist of four mice exposing in the embryonic and without exposing from the birth to maturity period.

In this way, B1, B2, and B3 groups were exposed to radiation within their home cage for two hr per day until the maturity era according to the Table 1. Irradiation two hr per day was selected according to the previous studies (26-28).

Mice were housed individually in clear polycarbonate cages in order to prevent any electromagnetic disorder. In this study, male mice were selected due to their less hormonal changes. Also, the mice were freely exposed in the cage in order to decrease stress factors during radiation, sufficient amount of food and water was given to them. The effects of EMF exposure on body weight change and the parturition also was monitored.

At the end of the experiment, when the offspring reached maturity (8-10 weeks old), mice were anesthetized by the IP ketamine 100 mg/kg and xylazine 20 mg/kg and the brains were removed and cerebellums were quickly separated. The tissues were frozen at -80 ^{o}C.

Experiments were conducted in accordance with the proposal and were approved by the Institute Ethical and Research Advisory Committee of the Mashhad University of Medical Sciences.

RF-EMF exposure

For all experiments, we used a mobile phone jammer for exposure which is an instrument to prevent receiving signals between cellular phones and base transceiver stations.

This device produces the waves with the equivalent frequency and intensity like a cell phone. Maximum output power at the distance of two meters from four antennas CDMA, GSM, DCS and PHS is 2 Watts (33 dB). In this study used two antennas 900 and 1800 MHz GSM. The distance between the jammer at 900 and1800 MHz frequency and the cages were approximately 2 m. The electromagnetic quantities were measured with Aaronia portable spectrum analyzer (Aaronia AG, Germany).

RNA isolation and real-time reverse transcription polymerase chain reaction

Total RNA was extracted from the cerebellum of mice using Manual TriPure method (TriPure RNA Minikit. Germany). For reverse transcription (RT), first strand complementary DNA (cDNA) was synthesized from RNA by using a cDNA Synthesis Kit (RevertAidTm First Strand cDNA Synthesis Kit, Fermentas,) according to the manufacturer's instructions. After RT at 42 °C for 60 min, polymerase chain reaction (PCR) was performed using a PCR-101 Taq Master Mix (Genet Bioscience, Germany) according to the manufacturer's protocol. The specific primers pairs were used in this study are listed in Table 3. After an initial denaturation step of 3 min at 94 °C, 35 cycles of amplification for *bax*, *bcl-2*, *P21* and *p53* primers pair, respectively, were carried out. Each cycle included a denaturation step, 30 sec at 94 °C; an annealing step, 30 sec at 62 °C; and an elongation step, 45 sec at 72 °C. Final elongation temperature was 72 °C for 5 min. Relative levels of gene expression were measured by SYBR Green PCR kit. Real-time PCR was performed on a Step One™ real-time system (applied bio systems) using SYBR® Master Mix (Takara).

The fold-change in gene expression was calculated using the melt curve method and was normalized to endogenous *GAPDH*. Subsequently, the relative gene expression levels were calculated with reference to the control. The primers for 28S used in real-time RT-PCR were designed based on its cDNA sequence. cDNA from all control samples were mixed and then diluted to 0.1, 0.01 and 0.001 to generate a standard. Reactions were run in duplicate in a spectrofluorometric thermal cycler (Step One™ Real-Time PCR System) and prepared in 48-well plates.

Statistical analyses

Statistical analyses were performed by comparing the exposed groups with the control group using SPSS 21 software. The real-time RT-PCR data were evaluated using Kolmogorov - Smirnov analysis for all samples. If the result showed a significant difference among the groups, the data would analyze by the multiple comparison procedures of one–way ANOVA and Tukey tests. *P-value* less than 0.05 were considered as significant.

Table 1. Oligonucleotide primers used for real - time RT-PCR analysis

Gene	Forward primer	Reverse primer	length (bp)
bcl-2	5′- GTGGATGACTGAGTACCT -3′	5′- CCAGGAGAAATCAAACAGAG -3′	118
bax	5′- CTACAGGGTTTCATCCAG -3′	5′- CCAGTTCATCTCCAATTCG -3′	133
p21	5′- CTTGCACTCTGGTGTCTG -3′	5′- CTTGGAGTGATAGAAATCTGTCA-3′	107
p53	5′- GTATTTCACCCTCAAGATCC- 3′	5′- TGGGCATCCTTTAACTCTA -3′	84
GAPDH	5′- GAGAAACCTGCCAAGTATG -3′	5′- GGAGTTGCTGTTGAAGTC -3′	123

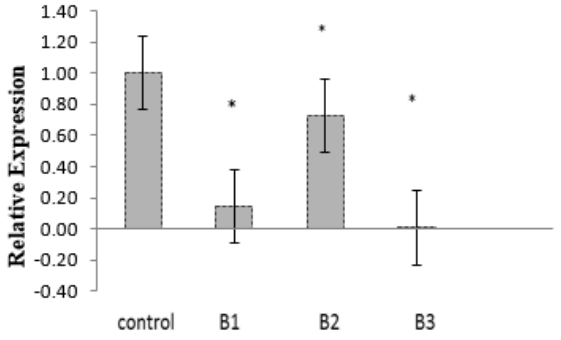

Figure 1. The expression level of *bax* gene in control and radiation groups: B1 (Irradiated after birth), B2 (Irradiated in pregnancy period and after birth), B3 (Irradiated in pregnancy period). Data are shown as the means ±SEM
* denotes significant difference at $P<0.05$ level when compared with the control group. Shift in *bax* mRNA expression after RF-EMF exposure as detected by real-time PCR

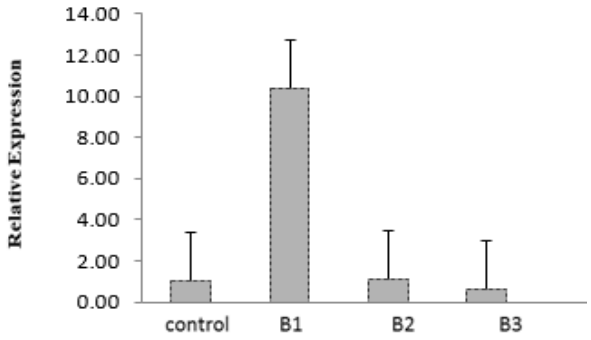

Figure 2. The expression level of *bcl-2* gene in control and radiation groups: B1 (Irradiated after birth), B2 (Irradiated in pregnancy period and after birth), B3 (Irradiated in pregnancy period). Data are shown as the means ±SEM
No shift in *bcl-2* mRNA expression after RF-EMF exposure as detected by Real-time PCR

Results

In this study, we examined the gene expression levels in the cerebellum after whole body exposure of BALB/c mice to a mobile phone. Four genes (*bax, bcl-2, p21* and *p53*) were quantified in independent samples from the four groups (control ,B1, B2, and B3) by quantitative real-time PCR.

Effects of 900 and 1800 MHz RF-EMF exposure on bax gene expression level

As shown in Figure 1, there were statistically significant differences on *bax* gene expression level between exposed and control groups. bax levels in the cerebellum tissue decreased significantly 2 hr after EMP compared with the control group ($P<0.05$).

Effects of 900 and 1800 MHz RF-EMF exposure on bcl-2 gene expression level

As shown in Figure 2, there were no statistically significant differences on *bcl-2* gene expression level between exposed (B1, B2, and B3) and control group ($P>0.05$).

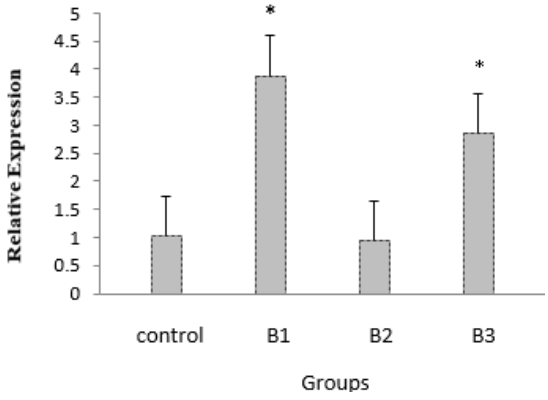

Figure 3. The expression level of *p21* gene in control and radiation groups: B1 (Irradiated after birth), B2 (Irradiated in pregnancy period and after birth), B3 (Irradiated in pregnancy period). Data are shown as the means ±SEM.* denotes significant difference at $P<0.05$ level when compared with the control group. mRNA expression of the cell cycle related genes detected by Real-time PCR

Effects of 900 and 1800 MHz RF-EMF exposure on p21 gene expression level

As shown in Figure 3, there were statistically significant differences on *p21* gene expression level between exposed and control groups. *p21* levels in the cerebellum tissue increased significantly 2 hr after EMP in two groups (B1 and B3) compared with the control group ($P<0.05$). There were no statistically significant differences on *p21* gene expression level between B2 and control group ($P>0.05$).

Effects of 900 and 1800 MHz RF-EMF exposure on p53 gene expression level

As shown in Figure 4, there were no statistically significant differences on *p53* gene expression level between exposed (B1, B2, and B3) and control group ($P>0.05$).

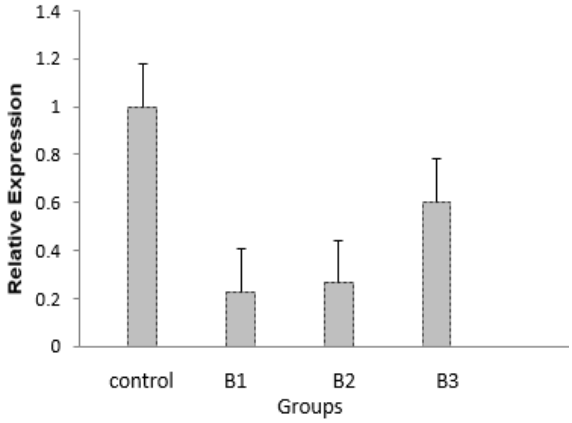

Figure 4. The expression level of *p53* gene in control and radiation groups: B1 (Irradiated after birth), B2 (Irradiated in pregnancy period and after birth), B3 (Irradiated in pregnancy period). Data are presented as the means ±SEM
No shift in *p53* mRNA expression after RF-EMF exposure as detected by Real-time PCR

Discussion

An electromagnetic field includes an electrical part and magnetic part. The electrical part is produced by stationary charges. The magnetic part is produced by moving charges, that both of them give biological effects, the magnetic field penetrates into the tissues more easily, so it has got the deleterious effect (29, 30). The mobile phones held close to the body and used regularly, so these devices can be the most dangerous sources of electromagnetic radiation (31). In reviews of reports and articles have not been done the same investigative research yet. Therefore, there is no reference baseline to compare the actual results. Apoptosis, as a programmed cell death, plays a key role in the maintenance of cell number homeostasis. disturbance of the apoptotic pathway can result from changes in the cell number homeostasis so that, leading to the development of cancer (32). The *bcl-2* gene belongs to a group of proto-oncogenes promoting cell survival by counteracting the process of apoptosis. The *bax* gene is an apoptosis-promoting member of the *bcl-2* gene family. *bcl-2* protein has the unusual property of increasing cell numbers by preventing apoptosis and the death-protective activity of *bcl-2* seems to be proportional to its expression level, whereas bax protein induces apoptosis when overexpressed in a variety of eukaryotic cells. *bcl-2* proteins are evolutionarily conserved regulators of apoptosis. The *bcl-2* family is composed of both proapoptotic and antiapoptotic family members. The best characterized anti-apoptotic proteins, *bcl-2* and *bcl-x*$_{L,\ L}$, appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing cytochrome c release and cell death initiation through the cell-intrinsic death pathway. In contrast, the pro-apoptotic family members *bax, Bid*, and *bax* have been shown to promote cytochrome c release (33, 34).

The finding of this study showed that *bax* protein was detectable in the three groups (B1, B2, and B3) and significantly down-regulated early after micro-wave exposure when compared with the control group. This indicated the *bax* is involved in the cellular apoptosis induced by mobile phone exposure.

In this study, decreasing of the *bax* gene expression in the three groups (B1, B2, and B3) showed that mobile phone radiation acted as a stress, but the duration and intensity of exposure did not induce apoptosis in the cerebellum. Considerable reduction of the *bax* gene expression in the B3 Group compared with the other groups indicated that in the embryonic period, cerebellum tissue is more sensitive than after the birth. During brain development and embryonic period, any environmental stimuli may disturb the cerebellum cells. In addition, it was revealed that in the B2 group which was exposed in longer duration, the *bax* gene expression was lower compared to the B1 and B3 groups. Maybe, it can be concluded, when mice exposed to the mobile phone for prolonged periods, have been caused radiation-resistance in the cerebellum cells. We found that 900 and 1800 MHz RF-EMF exposure has no effect on the expression of the anti-apoptotic related gene *bcl-2*.

The effect of 1900 MHz frequency EMW that generated by a mobile phone for 2 hr on neuron and astrocyte cell cultures investigated, the result showed a significant increase in the *bax* levels (35).

The application of 1950 MHz frequency and 5, 36 W/kg SAR EMW for 12, 24, and 48 hr on rat astrocyte cell cultures have been investigated. This study showed an increase in the *bax* levels and a considerable reduction in the *bcl-2* expressions just in the 48 hr application group (2). The result of this study is also opposed to the present study. These differences may be due to the different cell models, the exposure SARs, and the durations and the frequencies of exposure. However, to fully explore the details, further studies are needed.

In agreement with our findings, Yilmaz *et al.* demonstrated the effect of 900 MHz frequency and 0.29- 0.87 W/kg SAR ranges EMW for 20 min a day for 4 weeks in rats and they did not show a significant difference in the *bcl-2* expression levels (36).

The *p21* is a regulator of cell cycle progression at the G_1 and S phase.

In addition, *p21* was discovered as a senescent cell-derived inhibitor that can mediate cellular senescence. Expression of *p21* is mainly dependent on two factors; 1) stimulus provided 2) type of the cell. Growth arrest by *p21* can promote cellular differentiation. *p21*, therefore prevents cell proliferation (37). In this study increasing the rate of the *p21* gene expression in the B1 and B3 groups compared with the control group, indicating the effect of the mobile phone has been inserted stress in the genome. Therefore, by the increase of the *p21* gene expression, cell cycle arrest took place for repairing the damaged cerebellum cells.

The *p53* protein is a transcription factor playing a vital role in the regulation of cell growth, DNA repair, and apoptosis, in response to stressful conditions. *p53* has been shown to exert a tumor suppressor effect by inducing apoptosis, activating the cell cycle, stimulating cell differentiation and is involved in DNA repair pathways. *p53* gene arrests the cell cycle at the late of G1 phase in the case of DNA damage and spares time for DNA repair. Meanwhile, if DNA is repaired, *p53* diminishes and then the cycle will be completed. If the repair attempt is unsuccessful, *p53* leads the cell to apoptosis. Therefore, if *p53* gene is damaged, the risk of tumor genesis will increase due to the increased amount of damaged DNA (38, 39).

In the present study cerebellum cells did not show significant changes in the *p53* gene expression after exposure to 900 and 1800-MHz RF- EMF exposure. Therefore, we cannot conclude that the RF-EMF

exposure effects on the *p53* gene expression. In the studies that investigated the effect of mobile phones on the *p53* protein, a study similar to ours performed on rats. Dasdag *et al.* indicated that application of 900 MHz frequency and 0.25,1,2, and 4W/kg SAR EMW for 2 hr per day, during 10 months did not cause a significant change in the *p53* protein levels of cerebral tissues (40). Buttiglione *et al.* evaluated the effect of 900 MHz frequency and 0.25, 1, 2 and 4 W/kg SAR EMW for 24 hr on human amniotic cell cultures, they did not report significant changes in the activation of the *p53* protein (41). The result of these studies is also parallel to the present study.

Conclusion

The data suggest the EMW emitted by mobile phone can change the expression of the *bax* proteins, but cannot cause apoptosis in the cerebellum. The whole of gestation exposure for mobile communication microwaves can produce a measurable stress response of cerebellum region. The embryonic period is more sensitive than the other period of life. In addition, the mobile phone exposure effect on the expression level *p21* and cell cycle. To confirm the results of this study, more investigation is needed. Further studies should also be planned to evaluate the effects of EMW fields created by mobile phone on human fetuses.

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Conflict of interest

The authors have no conflict of interest to declare.

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