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The Effect of Prenatal Exposure to 2.4 GHz Radio Frequency on the Histology and Expression of the osteocalcin and *RUNX2* Gene of the Forelimb in an NMRI Mouse

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Abstract

Introduction: Today the use of electromagnetic waves has dramatically increased in modern industrial societies. This study aimed to investigate the effect of prenatal exposure to 2.4 GHz wireless frequency on forelimb development in an NMRI mouse in vivo.

Methods: A total of 21 female mice weighing 25-30 g were included in the present study. They were randomly assigned to 3 groups, namely control (n=7), sham (n=7), and experimental (n=7). After mating, the experimental group was exposed to 2.4 GHz radio frequency at a distance of 20-30 cm from the device, 4 h/d until the delivery. The sham group was placed at a distance of 20-30 cm from the device every day without exposure to electromagnetic waves, and the control group had a pregnancy period without any stress and electromagnetic wave exposure. After giving birth, the forelimbs were isolated from the infants and examined by stereological studies and RT-PCR for the evaluation of osteocalcin and *RUNX2* gene expression.

Results: Although, at first glance, there was no macroscopic teratogen effect in forelimbs in all groups, via a stereological method, we showed that bone and cartilage volume decreased in the experimental group compared to the other groups. We also found that the experimental group had lower expression of the osteocalcin and *RUNX2* gene than the control and sham groups did. However, there were no significant differences between the control and sham groups in terms of bone and cartilage volume and gene expression.

Conclusion: Although teratogen effect of prenatal exposure to 2.4 GHz radio frequency on forelimbs was not demonstrated macroscopically, further studies showed negative effects on the forelimb bone, cartilage volume, and gene expression.

Keywords: Development; Electromagnetic fields; Forelimb; Prenatal; RUNX2.

Introduction

Currently, there are increasing concerns about the use of electronic devices generating electromagnetic fields (EMFs) in industrial societies because of the possible Adverse effects on human health.[1] Collectively, EMFs are divided into 2 types: (1) radio frequency/microwave radiation emissions (RF) that are used in wireless devices such as cordless phones, Wi-Fi and mobile phones, and (2) extremely low-frequency EMFs (ELF-EMFs) from electronic devices such as a radio, personal computers,

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and radios.[2] Although EMFs are considered as "ionizing radiation", they are packets of energy, being exposed to their excessive levels results in the elevated risk of some diseases in humans.[3] Similar to ionizing radiation, they can cause cellular damage (α, β, and γ particles).[3]

Fetuses and babies are prone to detrimental effects of environmental pollutions including cell phone radiation as a result of a high number of stem cells and low immunity-mediated resources.[4] Human stem cells are vulnerable to microwave exposure via altered gene expression.[5,6] Leukemia, asthma, behavioral problems of emotion and hyperactivity are some possible effects of the exposure of fetuses to EMFs.

In animal experiments, there are some studies reporting detrimental effects of gestational exposure of high magnetic fields on testis,[7,8] kidney,[9] ovary,[10] heart,[11] bone and muscle tissue [12] as well as teratogenic and behavioral effects on embryos,[13-17] although there are documents reporting no adverse effect of prenatal exposure of high magnetic fields on embryos.[18,19] Our surveys on available sources revealed that there is still a paucity of molecular studies regarding forelimb development following prenatal exposure of 2.4 GHz EMFs. It has been shown that the EMFs with a carrier frequency similar to that used by a microwave oven (about 2.45 GHz) may have positive effects in an exposed population.[3] In the present study, we evaluated forelimb development following prenatal exposure of 2.4 GHz radio frequency via stereological studies along with the expression level of osteocalcin (a marker for the bone formation process) and Runx2 (a key transcription factor for osteoblast differentiation).

Methods

Materials

The RNX-Plus lysis buffer, DEPC water, the TBE buffer, the DNA Ladder marker 50 bp, and Agarose were purchased from CinnaGene (I.R.I). cDNA synthesis kit and Isopropanol were obtained from Merck (Germany) and Thermo Scientific (USA) companies respectively. Master Mix and DNA Green Viewer were prepared from Ampliqon (Denmark) and Aria Toos (I.R.I.) companies respectively. Osteocalcin, RUNX2, and GAPDH primers were designed by Pishgam Company (I.R.I.). Ethanol, formalin, xylene, hematoxylin, eosin, and paraffin were purchased from Abcam Company (UK). M-MLV reverse transcription kit was obtained from Fermentas (Germany).

Animals

A total of 21 female mice weighing 25-30 g were included in the present study. All animals were maintained in the animal house with a constant room temperature of 23–26ºC, free access to food and water, and a 12-hour light-dark cycle (on at 6:00 and off at 18:00). Animals were randomly divided into control, sham, and experimental groups. Two female mice were kept overnight in the laboratory cage with a male mouse. In the next morning, the vaginal plugs was evaluated and considered as first day of pregnancy. Afterward, the experimental group was exposed to 2.4 GHz radio frequency at a distance of 20-30 cm from the device 4 hours every day until the delivery. To study the effects of electromagnetic waves due to the winding of the device itself as well as daily stress due to the displacement of the cages, the sham group was placed at a distance of 20-30 cm from the device 4 hours every day without any electromagnetic waves exposure, and finally the control group had a pregnancy period without any stress and exposure to electromagnetic waves. After the birth-giving, one baby from each mother was chosen randomly and sacrificed. Right forelimbs were fixed in 10% formalin/PBS for using in stereological studies and left forelimbs were stored in -80°C freezer for RNA extraction.

The Magnetic Field Exposure System

The exposure apparatus was a wireless router (CISCO, EA6300V1, China) that applied 2.4 GHz wireless frequency for 21 days and 4 h/d.

Histological and Stereological Studies

Fixed samples were processed using a tissue processor device as follows: (1) dehydration using alcohol with ascending concentration (50%, 70%, 80%, 90%, and 100% (×3)); (2) clearing with xylene (×2); (3) impregnation using paraffin (×2). Then, the tissues were embedded in paraffin and sectioned in 5-μm slices. The paraffin sections were mounted onto albumin glue-coated slides and stained with H&E. After that, all specimens were visually inspected by using a light microscope (Nikon E 200, Japan) with a magnification of 10x and were photographed by using a camera (Nikon DS-Fi1, Japan). For stereological studies, the Cavalieri's method for the estimation of bone and cartilage volume was performed. Briefly, after sectioning the tissue, the first section for evaluation was chosen randomly. After 50 μm, the next section was taken and this method was continued until the number of sections arrived at 10 in each sample. Finally, a point-counting grid was applied to estimate the area of the bone and cartilage of the forelimb. The following formula was used to measure the total volume of the cartilage and bone used the Cavalieri's principle:

$V=\Sigma P\times a/p\times t$

In this formula *t* is the distance between the sampled sections. The ΣP is estimated using the point-counting method.[20,21] The *a/p* stands for the area associated with each point projected on the limb bud. A sample of H&E staining with a point-counting grid is presented in Figure 1.

RNA Extraction

At first, 50 mg of forelimb tissue was homogenized in a 1.5 μL micro-tube and lysed using a 500 μL RNX-Plus lysis

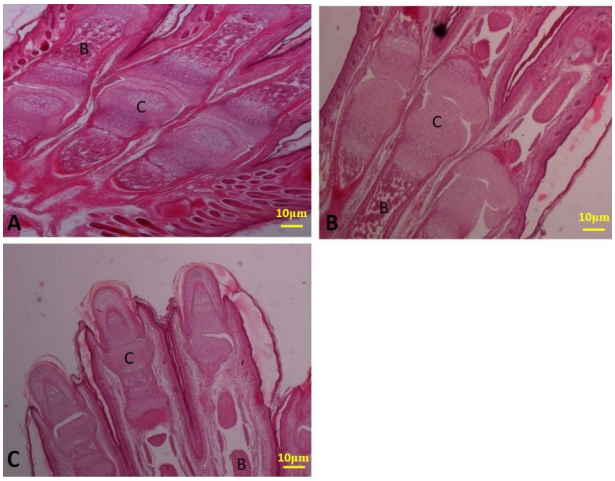

Figure 1. The Light Micrographs of the Studied Groups (H&E Staining). (A) Sham group; (B) control group and (C) experimental group.

buffer. Following 15 minutes incubation in RT, another 500 µL lysis buffer after vortex for 15 seconds was added to the sample, and it was incubated again for 15 seconds in RT. Then, 200 µL of chloroform was added to the sample and centrifuged for 15 minutes with 1200 g at 4°C. Having collected the upper part of the solution in a new micro-tube, we added an equal amount of isopropanol to it and then it was centrifuged for 15 minutes with 1200g at 4°C. After that, we added 1mL alcohol 75% to the micro-tube and it was centrifuged for 8 minutes with 7500 g at 4°C. Finally, alcohol was discarded and allowed the RNA sediment to air dry. Extracted RNA was dissolved in 50 µL DEPC-treated water in 58°C for 10 minutes and its concentration was detected by spectrophotometer at the wavelengths of 260 and 280 nm.

cDNA Synthesis

Before cDNA synthesis, extracted RNA was incubated in DNAase I in 60°C for 30 minutes to get rid of any DNA contamination. 2 µg of total RNA was used for cDNA synthesis according to the protocol of M-MLV reverse transcription kit. Briefly, a combination of 2 µg of total RNA, 4 µL of the 5X sample buffer, 1 µL of the reverse transcriptase enzyme, 2 µL of dNTP, 2 µL of primer, 1 µL of RNase Inhibitor and 10 µL of DEPC-treated water were incubated in 42°C for 60 minutes and then in 70°C for 5 minutes.

Reverse Transcription-PCR Analysis

Specific primers were designed for amplifying RUNX2 and GAPDH genes (Table 1). The genes were amplified with the first cycle of denaturation at 95°C for 3 minutes and continued with 30 cycles of denaturation at 95°C for 30 seconds. Annealing the primers occurred for osteocalcin, RUNX2, and GAPDH genes at 60°C, 63°C, and 52°C respectively for 30 seconds. The extension was conducted at 72°C for 45 seconds, and final extension occurred at 72°C for 5 minutes.

Electrophoresis

The specificity of the RT-PCR results was confirmed by electrophoresis for a single band of the expected size of cDNA on the agarose gel. After preparing agarose gel 2%, DNA Green Viewer (a fluorescent substance) for the detection of DNA was added to the gel before casting. After loading the samples into the gel, electrophoresis was performed for 24 hours at 100 V. At the end of this time, the gel was slowly taken and put in the gel doc system and photographed using the camera. Finally, to have semi-quantitative data, the bands were analyzed using ImageJ software.

Statistical Analysis

Data analysis was conducted by SPSS 22. The normality of data was first evaluated via the Shapiro-Wilk test. A comparison of stereological data between groups was performed by a one- way ANOVA followed by LSD. Descriptive statistics (mean ± SD) were also employed to summarize the data, and $P \leq 0.05$ was considered as the significant level.

Results

Teratogenic is the result of the effects of prenatal exposure of 2.4 GHz radio frequency on forelimbs. All forelimbs of newborn mice were macroscopically examined for detecting any external abnormalities. In all group examinations, no external abnormality was observed.

The effect of prenatal exposure of 2.4 GHz radio frequency on the volume of bone and cartilages in the forelimbs, showed a significant difference in bone and cartilage volumes in the experimental group in comparison to other groups. As presented in Figure 2, bone volume in the experimental group was drastically lower than the control group ($P<0.001$) as well as the sham group ($P<0.01$). Cartilage also showed a lower significant volume in the experimental group than the other groups did ($P<0.01$) (Figure 3). The data revealed that exposing the forelimbs of the newborn mice to 2.4 GHz radio frequency increased the abnormalities of forelimb development.

Table 1. The Sequences of the Reverse and Forward Primers Used for RT-PCR

Gene Names	Forward	Reverse	Product Size (bp)
Osteocalcin	CTGACCTCACAGATGCCAAGC	AGATGCGTTTGTAGGCGGTC	210
RUNX2	GTGGAGATCATCGCGGACCA	GGTGAAACTCTTGCCTCGTCC	288
GAPDH	AGGTCGGTGTGAACGGATTTG	TAAGCAGTTGGTGGTGCAGG	458

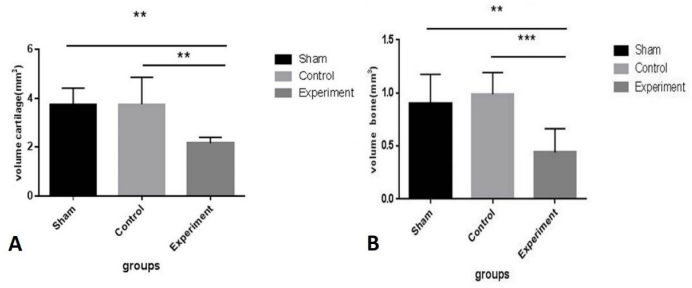

Figure 2. The graphs (A and B) showing the total volume of the cartilage and bone decreased significantly in the experimental group in comparison to the control and sham groups (**$P<0.01$, ***$P<0.001$).

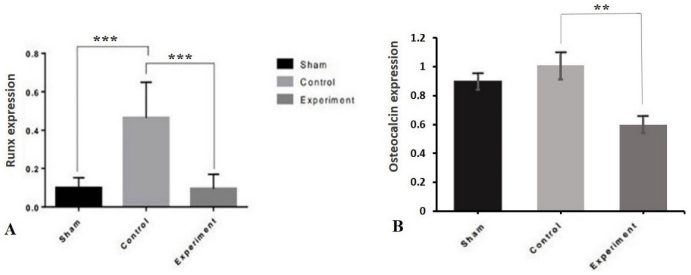

Figure 3. The graph shows the relative mRNA expression levels of osteocalcin and RUNX between different groups in the limb bud tissue; the relative mRNA expression of osteocalcin and RUNX in limb bud tissue decreased significantly after electromagnetic waves exposure in the experimental group in comparison to the control group (**$P<0.01$, ***$P<0.001$).

The effect of prenatal exposure of 2.4 GHz radio frequency on the expression of the osteocalcin and *RUNX2* gene was compared between the studied groups, the results of which revealed a significant difference between them. The relative expression of the osteocalcin and *RUNX2* gene in the experimental group was significantly lower than the other groups ($P<0.01$). The data revealed that exposing the forelimbs of the newborn mice to 2.4 GHz radio frequency caused reduction in the expression of the osteocalcin and *RUNX2* genes.

Discussion

Humans are exposed to the EMFs generated by such devices as industrial and household electrical supplies and electronic goods.[2] There are conflicting results regarding detrimental effects of EMFs on abortion and embryo development in human[22-26] and animals,[12,13,17] that show the requirement for further studies. In the current study, we showed that the histology and expression of the osteocalcin and *RUNX2* gene of mice forelimb were negatively impacted by prenatal exposure of 2.4 GHz radio frequency, whereas no teratogen effect was observed macroscopically.

The adverse effects of low-frequency EMFs on embryo development in the pre-implantation stage have been reported in the literature.[14,16] In mice, prenatal exposure to EMFs (50 Hz), 4 hours per day for 2 weeks, caused a lower significant number of blastocysts formation and increased DNA fragmentation.[16] Delayed cleavage of fertilized eggs in swine following exposure to EMFs (50 Hz) for 4 hours before ovulation is reported in other studies.[14] Besides, studies report the existence of no detrimental effects of low-frequency EMFs on development.[27-29] Accordingly, exposure of the pregnant mice to EMFs (50 Hz) did not affect the embryonic malformation, survival, and sex ratio compared to the control group.[27] Exposing males and females to EMFs (50 Hz) several weeks before mating showed no adverse effects on the fertility of both gametes and fetal development.[28]

The effect of high-frequency EMFs on embryo development is a matter of interest to researchers today. Türedi et al described that prenatal exposure to 900-MHz EMFs causes an increased amount of malondialdehyde, superoxide dismutase, and catalase along with histopathological changes in heart tissue of rat babies.[11] The effects of prenatal exposure to 900-MHz EMFs have led to ovary impairments such as severe follicle degeneration, increased stromal fibrotic tissue, vasocongestion, and cytoplasmic vacuolization in granulosa cells, resulting in decreased follicle reservoirs.[11] Testis is demonstrated to be negatively affected by gestational exposure to 900-MHz EMFs. Some pathological alterations are a reduced diameter of seminiferous tubules, an increased apoptotic index, lipid peroxidation, DNA oxidation and also lower semen quality.[8,30] Altered biomarkers of oxidative damage and pathological changes in kidney, spleen and thymus tissues have been demonstrated in newborn rat exposing to 900-MHz EMFs prenatally.[9,30]

Moreover, there are several studies regarding whether prenatal exposure to 2.4 GHz radio frequency has beneficial or adverse effects. However, there is no consensus about the standardization of the magnetic field used. In most studies, a pulse with 150-300 microsecond intervals has been used.[11] The optimal application duration for EMFs has not been determined. Indeed, this period may vary according to the frequency and power of the magnetic field. All of the previous studies were on fracture healing and they were conducted via using an EMF with low energy.[14,15] Borhani et al[16] have used pulsed EMFs for 10 hours in their study. Whereas, Aldad et al[17] have made 2 different applications for four or 8 hours, and they have reported that there was no difference between the 2 groups and 4 hours of pulsed EMF application was sufficient.[17]

In the present study, no teratogen effect on forelimbs was observed macroscopically with prenatal exposure to 2.4 GHz wireless frequency. In line with our results, Ogawa et al demonstrated no significant difference of growth and external morphology and visceral and skeletal abnormalities in fetuses following exposure of pregnant rats to 1.95-GHz EMFs 90 min/ d.[18] Similarly, no observable morphological abnormalities were reported in pups exposing to 20 kHz prenatally.[19] Similar to our results, Poulletier de Gannes et al showed that repeated exposure to the 2450 MHz Wi-Fi wireless signal had no teratogenic effect on the prenatal and postnatal development.[31] In contrast, Saito et al discovered some skeletal abnormalities such as polydactyly, abdominal fissure, vestigial 13th rib, fused rib, and lumbar rib.[13] It should be noted that these studies are merely observational. Using stereological studies, we identified a lower volume of the bone and cartilage of the forelimbs in the experimental group than the control and sham groups. It was also revealed that the expression of the osteocalcin and *RUNX2* gene of the pup's forelimbs decreased significantly following gestational exposure to 2.4 GHz radio frequency. Erkut et al, in line with our results, described the reduced amount of cartilage, the reduced calcineurin activities, and also the increased number of apoptotic chondrocytes and myocytes in both bone and muscle tissues following prenatal exposure to 1800 MHz EMFs indicating its negative effects on bone and muscle tissue development.[12]

One of the key transcription factors for differentiation of osteoblast and bone formation is runt-related transcription factor 2 (Runx2).[32] Osteocalcin is a bone-specific protein considered as a marker for the bone formation process.[33] Bone loss and also impaired skeletal integrity and osteoblast differentiation can be induced by reactive oxygen species (ROS) accumulation.[34,35] Cell growth and proliferation inhibition, oxidative stress, ROS generation, and DNA breakdown result from several damaging effects of EMFs exposure at the cellular level.[1] The lower volume of the bone and cartilage as a result of the lower expression of the *RUNX2* gene may be due to the damaging effects of EMFs mentioned above.

To the best of our knowledge, this is the first report investigating the possible effects of prenatal exposure to 2.4 GHz radio frequency on forelimb development. Our findings collectively showed negative effects of 2.4 GHz radio frequency on the development of the forelimb's bone and cartilage. To better judge the effects of EMFs waves, more specialized tests are required.

Conclusion

In conclusion, our result indicated that exposing the forelimbs of the prenatal rats to 2.4 GHz radio frequency would negatively affect the development of osteogenesis, chondrogenesis and gene expression of osteocalcin and Runx. It is required that more ultrastructural parameters and molecular studies be concentrated on this topic.

Conflict of Interest

The authors have nothing to disclose.

Ethical Considerations

This study has been accepted by the ethical committee of Shahid Beheshti University of Medical Sciences (IR. SBMU. SM. REC.1394.163).

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