The effect of prenatal exposure to 1800 MHz electromagnetic field on calcineurin and bone development in rats[1]

Adem Erkut[I], Levent Tumkaya[II], Mehmet Sabri Balik[III], Yildiray Kalkan[IV], Yilmaz Guvercin[V], Adnan Yilmaz[VI], Suleyman Yuce[VII], Erkan Cure[VIII], Ibrahim Sehitoglu[IX]

DOI: http://dx.doi.org/10.1590/S0102-865020160020000001

[I]Assistant Professor, Department of Orthopedic Surgery, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Intellectual and scientific content of the study; conception and design of the study; acquisition, analysis and interpretation of data; technical procedures; statistical analysis; manuscript preparation and writing.
[II]Associate Professor, Department of Histology and Embryology, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Intellectual and scientific content of the study, conception and design of the study, technical procedures, critical revision.
[III]Assistant Professor, Department of Orthopedic Surgery, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Intellectual and scientific content of the study, conception and design of the study, technical procedures, critical revision.
[IV]Associate Professor, Department of Histology and Embryology, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Conception and design of the study, technical procedures, final approval the manuscript.
[V]Assistant Professor, Department of Orthopedic Surgery, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Conception and design of the study, technical procedures.
[VI]Associate Professor, Department of Biochemistry, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Interpretation of data, conception and design of the study, technical procedures.
[VII]MD, Department of Internal Medicine, Kumru State Hospital, Ordu, Turkey. Conception and design of the study, technical procedures, critical revision.
[VIII]Associate Professor, Department of Internal Medicine, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Intellectual and scientific content of the study, conception and design of the study, technical procedures, critical revision, final approval the manuscript.
[IX]Assistant Professor, Department of Pathology, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey. Intellectual and scientific content of the study, conception and design of the study, technical procedures, critical revision.

ABSTRACT

PURPOSE: To investigated the effects of exposure to an 1800 MHz electromagnetic field (EMF) on bone development during the prenatal period in rats.

METHODS: Pregnant rats in the experimental group were exposed to radiation for six, 12, and 24 hours daily for 20 days. No radiation was given to the pregnant rats in the control group. We distributed the newborn rats into four groups according to prenatal EMF exposure as follows: Group 1 was not exposed to EMF; groups 2, 3, and 4 were exposed to EMF for six, 12, and 24 hours a day, respectively. The rats were evaluated at the end of the 60th day following birth.

RESULTS: Increasing the duration of EMF exposure during the prenatal period resulted in a significant reduction of resting cartilage levels and a significant increase in the number of apoptotic chondrocytes and myocytes. There was also a reduction in calcineurin activities in both bone and muscle tissues. We observed that the development of the femur, tibia, and ulna were negatively affected, especially with a daily EMF exposure of 24 hours.

CONCLUSION: Bone and muscle tissue development was negatively affected due to prenatal exposure to 1800 MHz radiofrequency electromagnetic field.

Key words: Electromagnetic Fields. Calcineurin. Bone Development. Muscle Cells. Rats.

Introduction

Even though there are attractive technological developments to improving human life, the exposure to environmental pollution and radiation may affect prenatal development in both humans and animals[1]. Various studies have reported that an electromagnetic field (EMF) may have side effects in various tissues and cells[2-4]. Although some studies have shown tissues with proliferative epithelial cells during and after pregnancy, reproductive cells and tissues of the hematopoietic system seem potentially sensitive to EMF exposure[5-7]. However, there are still few studies that have investigated prenatal bone development. According to recent studies, bone cells, including osteoblasts, osteocytes, and osteoclasts are considered radiosensitive and could die early in the postradiational period[8]. EMF is used in the treatment of osteoporosis and musculoskeletal disorders[9]. The stimulatory mechanisms that underlie EMF have been investigated in vitro in mesenchymal stem cells, osteoblasts, and osteoblast-like cells. Furthermore, EMF has been related to bone morphogenetic protein 2, transforming growth factor beta (TGF-β), insulin-like growth factor II, prostaglandins, nitric oxide synthase phosphorylation, and mitogen-activated protein kinase[10].

Calcineurin (CN) is a protein *phosphatase* known as serine/threonine phosphatase and is present in all eukaryotes. In mammals, calcineurin has a role in the regulation of enzyme secretion. Alternatively, it is activated physiologically by Ca^{2+}/calmodulin during the differentiation of skeletal muscles and the development of osteoclasts in bone. The normal distribution of CN in tissues and its immunoreactivity have been investigated in various rat organs[11]. It has a widely proven distribution in the brain, kidney, testes, skeletal muscle, cardiac valves, splenic lymphocytes, and bone[12,13]. The development of the first bone cells starts early in the fetal stage. Some studies have shown that CN activation occurs in bone cells during the prenatal period. Additionally, CN activity has been reported to be required for biochemical and morphological differentiation of skeletal muscle myocytes[14]. Even though there are many studies that have investigated the effect of high EMF on organs and tissues, no studies have investigated the EMF effect on the development of bone trabeculae, bone marrow, and muscle tissue surrounding bones and joints during the fetal period.

In this study, we investigated the positive and negative effects of 6-, 12-, and 24-hour daily exposures to 1800 MHz radiofrequencies during the entire embryogenesis period on the histologic development of bone and musculoskeletal tissues. Furthermore, we also examined the effects of EMF exposure on CN levels.

Methods

This study was conducted in our University School of Medicine with the permission of the Local Ethics Committee of Experimental Animals on approval number: 2014/16). All procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Fifteen female and three male Sprague Dawley rats weighing 620–300g were used in this study. All of the animals were placed into special care units and were fed ad-libitum at a temperature of 20–23 ± 2°C and 50–55% humidity with a 12-hour dark: 12-hour light cycle. The 15 female rats were examined with vaginal smears during the estrous period. The rats were placed into three cages with each cage containing five females and one male. The next day, the male rats were taken out of the cages. The presence of sperm, as determined by the vaginal smear method, was detected with light microscopy[15]. Animals found to have sperm were considered pregnant at the first day. While 12 pregnant rats were exposed to EMF radiation during pregnancy via a digital signal generator (Anritsu MG3670 B type, Japan), four pregnant rats were not exposed to radiation.

The carrier frequency of the device was managed to be 1800 MHz, the modulation frequency was 217 Hz with a pulse width of 577 μsec and a maximum power of 2 W. Female rats delivered 120 pups, 40 of these newborn rats were males. The randomly selected groups were divided into Groups 1, 2, 3, and 4. The groups were designed as follows: Group 1 contained the newborn male rats (n = 10) from females in the control group that were not exposed to EMF during pregnancy; Group 2 contained newborn male rats (n = 10) from females exposed to 6 hours of daily EMF during pregnancy; Group 3 contained newborn male rats (n = 10) from females exposed to 12 hours of daily EMF during pregnancy; and Group 4 contained newborn male rats (n = 10) from females exposed to 24 hours of daily microwave radiation during pregnancy. The rats were cared for and fed in our unit for 60 days following birth without forming any special environment. At the end of 60th day, the body weights were 130–180 grams and their development was considered complete. Injections of 6 mg/kg of 2% xylazine hydrochloride (Rompun) followed by 75 mg/kg of ketamine hydrochloride (Ketalar) were given intraperitoneally

to induce a deep anesthesia, the rats were then sacrificed using the intracardiac perfusion method (4% formaldehyde).

Histopathological methods

After weighing all of the rats, their bone tissue (femur, tibia, and ulna) and muscle tissue (quadriceps) were extracted and given code numbers before being incubated in a 10% formaldehyde solution. After standing in a fixative material for 24 hours, the bones were put in a Müller solution for 24–48 hours to be decalcified. Decalcification was completed by the addition of a 3% Müller solution. After standing in a fixative material for 24 hours, the muscle tissue was combined with the bone tissue and washed for 1–2 hours with running distilled water. They were then washed with an ethanol-xylene series (50–100%) before performing automated tissue processing (Citadel 2000, Thermo Fisher Scientific Shandon, England). The tissues were then embedded separately in paraffin. Tissues were cut into 4–6 μm thicknesses for routine hematoxylin-eosin staining. During light microscopic examinations with varying magnifications, suitable areas were photographed.

Sections that were cut into 4–6 μm thicknesses for immunohistochemical staining were incubated in xylene for 20 minutes, they were then allowed to stand for 10 minutes in an alcohol series (50–100%) before being put in a 3% H_2O_2 solution. After being washed with phosphate-buffered saline (PBS), the samples were heated with 700W in a citrate buffer solution for (heated four minutes (repeated five times) minutes and allowed to stand in a secondary blocker substance for 20 minutes. For every preparation to be stained with Anti-CN B (Code: ab136526, Abcam plc, Cambridge CB4 0FL UK), they were put into various dilutions (Anti-CN B 1/100) for 75 minutes. Diaminobenzidine (DAB) solution was used as a chromogen and Mayer's hematoxylin was used as a counterstain for 5 minutes. PBS was used as a negative controller. The preparations were photographed after being covered with a suitable substance. The results of the immunohistochemical stained tissues were subdivided into four categories according to the percentage of immunopositivity reaction as follows: mild (+), moderate (++), severe (+++), and very severe (++++). Two histologists and one pathologist blindly scored and evaluated the preparations with regard to statistical analysis.

Bone measurement methods

After being sacrificed, the animal's internal organs were extracted and the animal was fixed on an Xray board to clearly display their skeletal system. The anteroposterior Xrays were evaluated using a G.E. X-R 6000 device. They were then photographed before being sent to a digital photography program (Akgun PACS Viewer 4.0) to perform the measurements. The femur, tibia, quadriceps muscle, and sciatic nerve were extracted bilaterally for histologic evaluation. The means (with fallibility ± 0.01 mm) of the following distances were measured bilaterally using the Akgun PACS Viewer Program. Femur head diameters were measured from the physeal cartilage level, femur lengths were measured between the femur head and femur condyle, tibia lengths were measured between anterior eminence and ankle, ulna lengths were measured between the olecranon and wrist, and were *measured* the *cortical thickness* and medullary cavity in the distal area of the minor trochanter on radiographs.

Statistical analysis

Statistical analysis was performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA). All the data were evaluated and while nonparametric values are reported as medians ± standard deviation (SD), parametric values are reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the significant differences among the groups. As a post hoc test, multiple comparisons were performed using Tukey's HSD and LSD. For all the comparisons $p<0.05$ was considered statistically significant.

Results

The histopathological investigation of the bones of the control group revealed they had a normal histologic appearance for cartilage, bone trabeculae, and bone marrow. The cellular nuclei of chondroblasts, osteoblasts, chondrocytes, and osteocytes were rounded and flat. The mitotic activity of the isogenous groups had a normal appearance (Figure 1A). The histopathologic investigation of the bones of the group 2 exposed for six hours revealed that both the upper zone (resting cartilage) and lower zone (proliferative and hypertrophic cartilage) regions were relatively shorter than in the control group. There were decreasing bone thickness and loss of proteoglycan in the bone trabeculae. In the group 2, there was intensive eosinophilic infiltration surrounding chondrocytes of hyaline cartilage. There were no significant changes in the epiphyseal plate of the control group and 6-hour application group. The shortness found in the application

group was not statistically significant. In the application group, bone marrow spaces were wider, the intensity of the trabecular structure was decreased and mildly eosinophilically stained. There was no change for osteocytes within the lacuna of bone trabeculae and these were observed to preserve their morphologic structure. While there were no decreases in the volume of both the medial femoral condyle and lateral femoral condyle of the application groups, there were scattered chondrocyte necrosis with few apoptotic cells due to the loss of proteoglycan (Figure 1B). The histopathologic investigation of both the upper zone (resting cartilage) and lower zone (proliferative and hypertrophic cartilage) regions of the bones of the 12-hour application group revealed lower distances than the control group with decreased growth distances. Additionally, shedding of the chondroblasts was observed on the surface of the cartilage. There was scattered thickness in the trabeculae. In the application group, hyaline cartilage and the surroundings of the chondrocytes of the isogenous groups were intensively eosinophilically stained, and there were intensive apoptotic cell clusters. There were no obvious changes between the epiphyseal plate of the control group and the 12-hour application group. The shorted distance of the upper zone and the lower zone in the application group was not statistically significant. In the application group, bone marrow spaces were wider, the amount of the adipocytes of the bone marrow were decreased, and the amount of the megakaryocytes were increased. The structures of the trabeculae were eosinophilically stained due to the increase in bone density. Even though osteocytes within the lacuna of bone trabeculae were basophilically stained, there was no decrease in their amount and their morphologic structures were preserved (Figure 1C). The histopathologic investigation of the 24-hour application group revealed a slimming of the upper zone (resting cartilage), whereas the distances of lower zone regions (proliferative and hypertrophic cartilage) were similar to the controls as well as the 6- and 12hour application groups. The volumes of the isogenous groups in the proliferative zone were decreased. However, the formation of structures of four and five chondrocytes was continued. Apoptosis was intensively increased due to the accumulation of glycogen in the chondrocytes within the hypertrophic zone of the controls and 6-hour application group. However, these levels were similar to the 12-hour application group. While the number of trabeculae increased, the thickness of the trabeculae was not distributed homogenously in the bone matrix. Hyaline cartilage and chondrocytes in the upper zone areas near the joint surface were intensively eosinophilic in the application group. There was a minimal insignificant difference in the epiphyseal plate between the controls and the 24-hour application group. Additionally, the distance increase in the application group was not statistically significant. In the application group, bone marrow spaces were wider, hematopoietic cells intensity was normal, and the intensity of the bone trabeculae was decreased. There was no decrease for osteocytes within the lacuna of bone trabeculae and they preserved their morphologic structure (Figure 1D). The histopathologic investigation of the bone tissues revealed that the resting cartilages of Groups 2, 3, and 4 were obviously decreased compared with the control group. The values of the proliferative hypertrophic cartilage and epiphyseal plate of the four groups were similar. Proteoglycan loss and chondrocytic apoptosis of Groups 3 and 4 were obviously higher than in Groups 1 and 2 (Table 1).

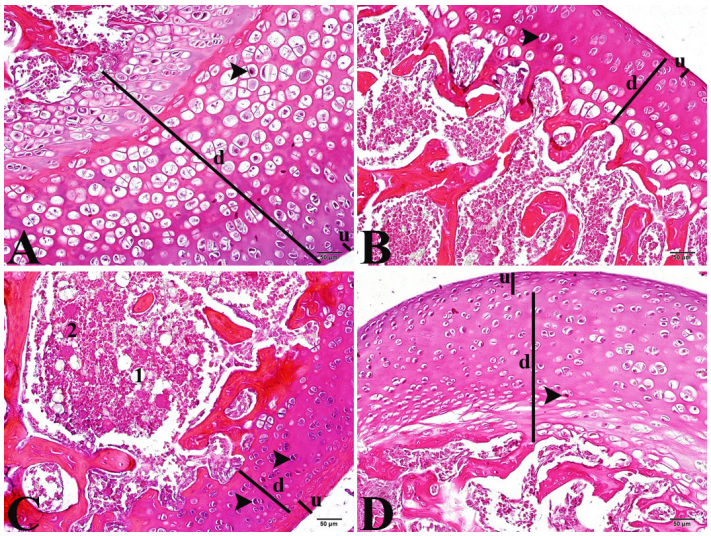

FIGURE 1 - Histopathologic examination of bone tissue by light microscopy, hematoxylin and eosin staining. (**A**) Group 1 (not exposed to electromagnetic field), u: upper zone of the femur head region, d: lower zone of the femur head region, arrowhead; apoptotic cells; (**B**) Group 2 (exposed to electromagnetic field 6 hours a day), u: upper zone of the femur head region, d: lower zone of the femur head region, arrowhead; apoptotic cells; (**C**) Group 3 (exposed to electromagnetic field 12 hours a day), u: upper zone of the femur head region, d: lower zone of the femur head region, 1: adipocytes, 2: megakaryocytes, arrowhead; apoptotic cells; (**D**) Group 4 (exposed to electromagnetic field 24 hours a day), u: upper zone of the femur head region, d: lower zone of the femur head region, arrowhead; apoptotic cells.

TABLE 1 - The evaluation of histopathologic results of bone tissues.

Groups n:10	Group 1 Median± SD	Group 2 Median± SD	Group 3 Median± SD	Group 4 Median± SD
Resting cartilage	4.00±0.42	3.00±0.42[a]	3.00±0.67[b]	3.00±0.32[c]
Proliferative and hypertrophic cartilage	4.00±0.48	4.00±0.48	4.00±0.48	4.00±0.32[c]
Epiphyseal plate	4.00±0.32	4.00±0.52	4.00±0.52	4.00±0.00[c]
Proteoglycan loss	1.00±0.32	2.00±0.48[a]	3.00±0.48[b,d]	3.00±0.32[c,e]
Apoptotic chondrocytes	1.00±0.48	2.00±0.92	3.00±0.48[b,d]	3.00±0.42[c,e]
Bone anti-calcineurin	4.00±0.42	3.00±0.57[a]	3.00±0.48[b]	2.50±0.70[c]

Group 1, control; Group 2, 6 h EMF applied; Group 3, 12 h EMF applied; Group 4, 24 h EMF applied. [a,b,c]$p<0.05$ *vs* group I; [d,e]$p<0.05$ *vs* group II.

Bone CN levels of Groups 2 and 3 were lower than in Group1, its level of Group 4 was lower than the other three groups (Figure 2).

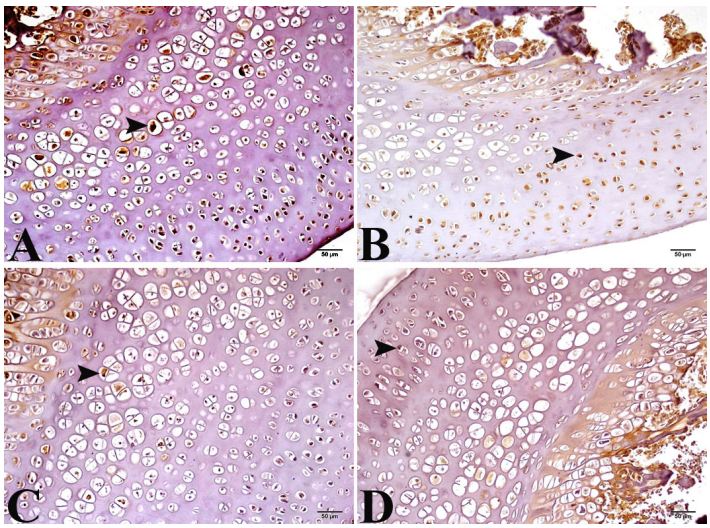

FIGURE 2 - Immunohistochemical staining of bone tissues with immunoperoxidase method; anti-calcineurine antibody staining. (**A**) Group 1 (not exposed to electromagnetic field), arrowhead; strong immunoreactivity; (**B**) Group 2 (exposed to electromagnetic field 6 hours a day), arrowhead; strong immunoreactivity; (**C**) Group 3 (exposed to electromagnetic field 12 hours a day), arrowhead; strong immunoreactivity; (**D**) Group 4 (exposed to electromagnetic field 24 hours a day), arrowhead; strong immunoreactivity

Muscles of the control group had a normal histopathologic appearance, their striation structures were obvious, cellular nuclei were rounded and flat and had a normal histologic morphology (Figure 3A). Even though the muscles of the 6-hour application group were more edematous than the control group, the cellular structures were not more distorted (5%) and had morphologic appearances that were close to normal. The striations in the edematous regions were partially or totally disappeared and the myocytes had mild swellings. The nuclei of myocytes within the regions of decreased striations were pale and basophilically stained, their cytoplasm was mildly eosinophilically stained, and the nuclei of the control group were darkly stained (Figure 3B). The muscles of the 12-hour application group were more edematous than the control group and the amount of edema was greater than in the 6-hour application group.

While there was scattered fluid within the spaces and vascular endothelial shedding, cellular structures were beginning to swell and became distorted (15%). The histopathologic investigation showed the fibers to be intensive (75%) and to have a normal histologic appearance. The striations in the edematous regions were partially or totally disappeared and the myocytes were swollen. The myocytes within the regions of decreased striations were pale and their cytoplasm was mildly eosinophilically stained. Additionally, their shapes were rounded, they had karyolitic nuclei, and their nuclei were basophilically stained (Figure 3C). While the

muscles of the 24-hour application group were more intense and edematous than the control group, the edema in the 6- and 12-hour application groups were significantly increased. The cellular structures of the myocytes of the 24-hour application group were degenerated more than in the control group. Even though there were myocyte degenerations in the 6- and 12hour application groups, myocyte swelling of the 24-hour application group was similar to that of the 12-hour application group. Muscle fibers and striations of those cells with a normal histological appearance were closer to the central regions. Similarly, the striations in the edematous regions were partially or totally disappeared and edema fluid was present within regions of the swollen myocytes. In these regions of low striations, myocytes were pale, mildly eosinophilically stained, and their nuclei were scattered with dark stain. While apoptotic myocytes were present in the eosinophilically stained regions of this group, a decrease in the intensity was observed in the other groups (Figure 3D). The histopathologic investigation of the muscle tissue of Groups 3 and 4 revealed myocyte degeneration, endothelial swelling and shedding, edema, and an increased number of apoptotic myocytes, which were obviously more than in Groups 1 and 2 (Table 2).

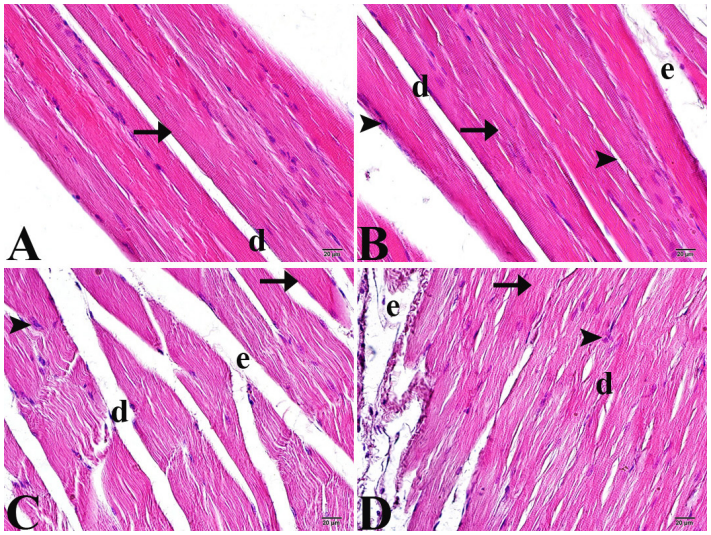

FIGURE 3 - Histopathologic examination of muscle tissue by light microscopy, hematoxylin and eosin staining. (**A**) Group 1 (not exposed to electromagnetic field), arrow; normal myocytes, d; degenerating cells; (**B**) Group 2 (exposed to electromagnetic field 6 hours a day), arrow; normal myocytes, arrowhead; pale staining myocytes, d; degenerating cells, e; edema; (**C**) Group 3 (exposed to electromagnetic field 12 hours a day), arrow; normal myocytes, arrowhead; pale staining myocytes, d; degenerating cells, e; edema. (**D**) Group 4 (exposed to electromagnetic field 24 hours a day), arrow; normal myocytes, arrowhead; pale staining myocytes, d; degenerating cells, e; edema

TABLE 2 - The evaluation of histopathologic results of muscle tissues.

Groups n:10	Group 1 Median± SD	Group 2 Median± SD	Group 3 Median± SD	Group 4 Median± SD
Myocytes degeneration	1.00±0.52	1.00±0.32	2.00±0.57[b,d]	3.00±0.57[c,e,f]
Swelling of Myocytes	1.00±0.52	1.00±0.42[a]	3.00±0.48[b,d]	3.00±0.48[c,e]
Shedding of endothels	1.00±0.52	1.00±0.42	2.00±0.32[b,d]	3.00±0.00[c,e,f]
Edema	1.00±0.47	2.00±0.48[a]	3.00±0.00[b,d]	4.00±0.42[c,e,f]
Apoptotic myocytes	0.00±0.52	2.00±0.32[a]	3.00±0.48[b,d]	4.00±0.52 [c,e,f]
Muscle anti-calcineurin	4.00±0.42	3.00±0.67	2.00±0.52[b,d]	2.00±0.67[c,e]

Group 1, control; Group 2, 6 h EMF applied; Group 3, 12 h EMF applied; Group 4, 24 h EMF applied. [a,b,c]$p<0.05$ *vs* group I; [d,e]$p<0.05$ *v*. group II; [f]$p<0.05$ *vs* group III.

Muscle levels of CN in Groups 3 and 4 were lower than in Groups 1 and 2 (Figure 4). The weight of Group 4 rats was lower than the other groups. Except for the femoral head, all femur measurements of Group 4 were lower than the other groups (Figure 5). Tibia measurements were not obviously different among the groups. The length of the ulna of Group 4 was lower than the other groups. All these results are shown in Table 3.

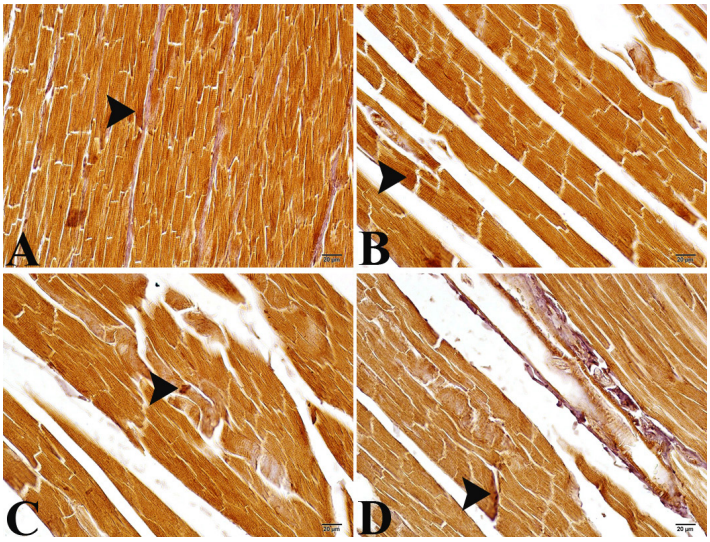

FIGURE 4 - Immunohistochemical staining of muscle tissues with immunoperoxidase method; anti-calcineurin antibody staining. (**A**) Group 1 (not exposed to electromagnetic field), arrowhead; strong immunoreactivity; (**B**) Group 2 (exposed to electromagnetic field 6 hours a day), arrowhead; strong immunoreactivity; (**C**) Group 3 (exposed to electromagnetic field 12 hours a day), arrowhead; strong immunoreactivity; (**D**) Group 4 (exposed to electromagnetic field 24 hours a day), arrowhead; strong immunoreactivity.

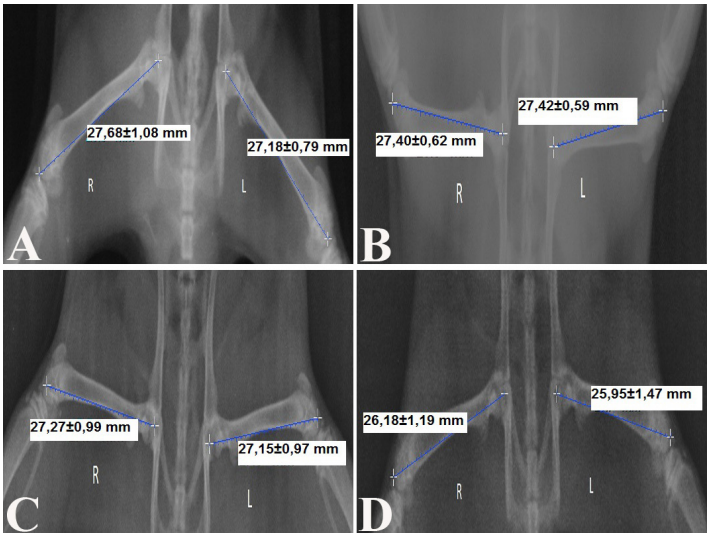

FIGURE 5 - Femur bone measurements of the all groups by radiologically. (**A**) Group 1 (not exposed to electromagnetic field); (**B**) Group 2 (exposed to electromagnetic field 6 hours a day); (**C**) Group 3 (exposed to electromagnetic field 12 hours a day); (**D**) Group 4 (exposed to electromagnetic field 24 hours a day).

TABLE 3 - The evaluation of bone tissue morphology of femur, tibia and ulna.

Parameters	**Group1** (Means± S.D)	**Group2** (Means± S.D)	**Group3** (Means± S.D)	**Group4** (Means± S.D)
Weight	152.83±8.90	144.90±7.82[a]	148.89±16.29[a]	124.32±14.65[a]
Femur R	27.68±1.08[b]	27.40±0.62[b]	27.27±0.99[b]	26.18±1.49
Femur L	27.18±0.79[b]	27.42±0.59[b]	27.15±0.97[b]	25.95±1.47
FBD R	2.49±0.15[C]	2.48±0.13[C]	2.56±0.13[C]	2.25±0.17
FMD R	1.50±0.10[C]	2.18±0.45[C,d]	2.54±0.10[C,d]	2.29±0.15
FBD L	2.53±0.11	1.53±0.08[e,f]	1.54±0.09[e,f]	1.32±0.11[e]
FMD L	1.53±0.06[g]	1.55±0.06[g]	1.54±0.05[g]	1.36±0.11
Femur head R	3.02±0.14[h]	3.24±0.09	3.12±0.11	3.07±0.14[h]
Femur head L	2.97±0.19[h]	3.24±0.09	3.10±0.10	2.99±0.28[h]
Tibia length R	29.83±1.07	29.68±1.11	29.87±1.01	29.09±1.63
Tibia length L	29.89±1.32	30.65±3.31	29.84±1.25	28.97±1.52
Ulna length R	27.26±72.61	25.13±0.66	25.03±0.69	23.70±0.99
Ulna length L	27.82±72.45	24.29±0.57	24.92±0.69	23.68±0.94

FBD, Femur bicortical distance; FMD, Femur medullary distance.
[a,d]p<0.05 *vs* group I; [h]<0.05 *vs* goup II; [b,e]p<0.05 *vs* group IV

Discussion

In our study, we found that increasing the exposure duration to EMF during the prenatal period not only decreased the levels of resting cartilage, but also increased the loss of proteoglycan and the number of apoptotic chondrocytes. There were no obvious changes in the levels of proliferative and hypertrophic cartilages and epiphyseal plates in relation to EMF exposure duration. By increasing the duration of EMF exposure, there was a significant increase in the degeneration of myocytes, myocyte swelling, and in the number of apoptotic myocytes. We also determined that an increase in the duration of EMF exposure reduced CN activities in both bone and muscle tissues. Even though it was mildly statistically significant, EMF exposure duration, especially with 24 hours of exposure, negatively affected the development of the femur, tibia, and ulna. However, we found no reduction in chondrocyte development. While there are a few EMF studies that have found positive effects of low dosage EMF on the development of bones, other studies have claimed otherwise. On the other hand, some authors have argued that high dosages of EMF have a positive effect on bone development[16-18]. Two studies that investigated EMF exposure on *ovariectomized* rats with osteoporosis found that EMF positively affected bone development and obviously decreased bone loss[16,17]. EMF has been reported to stimulate the differentiation of cartilage cells and to have positive effects on bone tissue by increasing the level of bone alkaline phosphate[19]. However, van der Jagt *et al*.[10] investigated the effects of EMF on osteoporotic and nonosteoporotic rats; they found no positive effects of EMF on the development of cancellous and trabecular bone in osteoporotic rats. Atay *et al*.[4] did not find an important effect of 1800 MHz radiation on bone mineral density levels of the spine and femur. Conversely, Yildiz *et al*.[18] reported that bone mineral density levels of the spine and femur were decreased due to exposure to 900 MHz and 1800 MHz radiation.

Our study is different from other studies since we investigated the effect of EMF on bone and muscle tissues during the prenatal period since tissues are more susceptible to EMF during this time. This may be the reason we found a deceleration in bone development and intensive damage in myocytes. EMF is thought to act on Ca^{2+} mechanisms by an electric current inducer[20]. An increase in intracellular Ca^{2+} may stimulate CN activity. However, CN activity may not always increase; unfortunately, there is not enough information in this regard.

CN stimulates cellular and muscular development. CN also increases osteoblast activation and CN inhibitors leads to osteoporosis[21]. While CN leads to apoptosis of osteoclasts by stimulating TGF-β, it also leads to bone development by increasing osteoblast activity and leads to chondrocyte development by stimulating TGF-β[22]. Low CN levels have also been shown to decrease osteoblast activity. However, there are studies that have reported that CN is not essential for chondrocyte development and intracellular calcium directly stimulates TGF-β[23]. CN overexpression harms chondrocytes and collagen by stimulating metalloprotease[24]. In the literature, there is only one study that has investigated the effect of EMF on CN[25]. This study reported increases in CN activity in hippocampal tissue due to EMF exposure[25]. We found CN activity to be decreased in both bone and muscle tissues and bone development was decelerated due to EMF exposure during the prenatal period. In our study, high dosages of EMF exposure during the prenatal period and the investigation of CN activity in bone and muscle tissues may explain the different results between this study and the study of Manikonda *et al*.[25]

Exposure to high dosages of EMF during the fetal period may suppress CN activity, however, further studies are needed to clarify this relationship. It has been reported that EMF exposure during the prenatal period may lead to teratogenic effects, cranial and thoracic skeletal anomalies, DNA[1] and chromosome anomalies, and negative effects on male and female reproductive systems. Many studies have reported that low frequency EMF may have healing effects on bone tissue and stimulatory effects on chondrocytes[26,27]. However, the positive or negative effects of both the exposure to high frequency and the duration of this exposure on bone and muscle tissues during the prenatal period have not been investigated. In contrast to previous studies, in our study, which investigated bone and muscle tissue damage by both the exposure and duration to high frequencies during the prenatal period, there was intensive damage to myocytes and an obvious increase in apoptosis. EMF increases the development of chondrocytes by stimulating the release of TGF-β, then decreases the release of cytokines like interleukin-1β, and inhibits matrix metalloproteinase[28,29]. In our study, even though CN levels were low, chondrocyte development was similar to the control group due to the direct effects of EMF. However, an increase in chondrocyte cell apoptosis might suggest that the exposure to EMF during the prenatal period had harmful effects, rather than benefits, on chondrocyte cells. CN stimulates apoptosis through various pathways. However, in our study, while chondrocyte apoptosis was increased, CN levels were low. EMF leads to oxidative stress and DNA damage. EMF increases intracellular calcium, which

stimulates apoptosis through a direct effect[30]. Therefore, we may have found an increase in apoptosis due to a toxic dosage of EMF during the prenatal period. EMF stimulates the development of chondrocytes and conversely, it stimulates chondrocyte apoptosis and tissue damage by its toxic effects. In contrast to the adult stage, in the prenatal period, EMF may decrease osteoblastic activity and suppressed the development of myocytes by suppressing CN in bone tissues.

Conclusions

Fetal chondrocytes of bone tissue and myocytes of muscle tissue are more susceptible to electromagnetic field (EMF) exposure during the prenatal period. EMF may lead to the formation of oxygen radicals by a direct toxic effect, and increase apoptosis and excessive osteoblast inhibition by suppressing CN. EMF exposure during the prenatal period may lead to obvious damage to muscle, bone, and cartilage tissues.

References

1. Pourlis AF. Reproductive and developmental effects of EMF in vertebrate animal models. Pathophysiology. 2009;16(2-3):179-89. doi: 10.1016/j.pathophys.2009.01.010.
2. Nisbet HO, Nisbet C, Akar A, Cevik M, Karayigit MO .Effects of exposure to electromagnetic field (1.8/0.9 GHz) on testicular function and structure in growing rats. Res Vet Sci. 2012;93(2):1001-5. doi: 10.1016/j.rvsc.2011.10.023.
3. Bedir R, Tumkaya L, Sehitoglu I, Kalkan Y, Yilmaz A, Sahin OZ. The effect of exposure of rats during prenatal period to radiation spreading from mobile phones on renal development. Ren Fail. 2015;37(2):305-9. doi: 10.3109/0886022X.2014.985995.
4. Atay T, Aslan A, Heybeli N, Hurriyet Aydogan N, Baydar M, Ermol C, Yildiz M. Effects of 1800 MHz electromagnetic field emitted from cellular, phones on bone tissue. Balkan Med J. 2009;26:292-6.
5. Ferreira AR, Knakievicz T, Pasquali MA, Gelain DP, Dal-Pizzol F, Fernandez CE, de Salles AA, Ferreira HB, Moreira JC. Ultra high frequency- electromagnetic field irradiation during pregnancy leads to an increase in erythrocytes micronuclei incidence in rat offspring. Life Sci. 2006 Dec 3;80(1):43-50. PMID: 16978664.
6. Sehitoglu I, Tumkaya L, Kalkan Y, Bedir R, Cure MC, Zorba OU, Cure E, Yuce S. Biochemical and histopathological effects on the rat testis after exposure to electromagnetic field during fetal period. Arch Esp Urol. 2015;68(6):562-8. PMID: 26179793.
7. Trosic, I, Busljeta, I, Modlic, B. Investigation of the genotoxic effect of microwave irradiation in rat bone marrow cells: in vivo exposure. Mutagenesis. 2004;19(5):361–4. PMID: 15388808.
8. Libshitz HI. Radiation changes in bone- Sem Roentgenol.1994;29(1):15-37.
9. Trock DH. Electromagnetic fields and magnets. Investigational treatment for musculoskeletal disorders. Rheum Dis Clin North Am. 2000 Feb;26(1):51-62. PMID: 10680193.
10. van der Jagt OP, van der Linden JC, Waarsing JH, Verhaar JA, Weinans H. Electromagnetic fields do not affect bone micro-architecture in osteoporotic rats. Bone Joint Res. 2014 Jul;3(7):230-5. doi: 10.1302/2046-3758.37.2000221.
11. Kalkan Y, Tumkaya L, Bostan H, Tomak Y, Yilmaz A. Effects of sugammadex on immunoreactivity of calcineurin in rat testes cells after neuromuscular block: a pilot study. J Mol Histol. 2012;43(2):235-41. doi: 10.1007/s10735-011-9384-9.
12. Rusnak F, Mertz P. Calcineurin: form and function. Physiol Rev. 2000;80(4):1483-521. PMID: 11015619.
13. Sun L, Blair HC, Peng Y, Zaidi N, Adebanjo OA, Wu XB, Wu XY, Iqbal J, Epstein S, Abe E, Moonga BS, Zaidi M. Calcineurin regulates bone formation bythe osteoblast. Proc Natl Acad Sci U S A. 2005;102(47):17130-5. PMID: 16286645.
14. Schulz RA, Yutzey KE.Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. Dev Biol. 2004;266(1):1-16. PMID: 14729474.
15. Ochiogu IS, Uchendu CN, Ihedioha JI. A new and simple method of confirmatory detection of mating in albıno rats (*Rattus norvegicus*) An Res Int.2006;3(3): 527-30.
16. Chang K, Chang WH. Pulsed electromagnetic fields prevent osteoporosis in an ovariectomized female rat model: a prostaglandin E2-associated process. Bioelectromagnetics. 2003;24(3):189-98. PMID: 12669302.
17. Sert C, Mustafa D, Duz MZ, Aksen F, Kaya A. The preventive effect on bone loss of 50-Hz, 1-mT electromagnetic field in ovariectomized rats. J Bone Miner Metab. 2002;20(6):345-9. PMID: 12434162.
18. Yildiz M, Cicek E, Cerci SS, Cerci C, Oral B, Koyu A. Influence of electromagnetic fields and protective effect of CAPE on bone mineral density in rats. Arch Med Res. 2006;37(7):818-21. PMID: 16971219.
19. Ardeshirylajimi A, Soleimani M. Enhanced growth and osteogenic differentiation of induced pluripotent stem cells by extremely low-frequency electromagnetic field. Cell Mol Biol (Noisy-le-grand). 2015;61(1):36-41. PMID: 25817344.
20. Otter MW, McLeod KJ, Rubin CT. Effects of electromagnetic fields in experimental fracture repair. Clin Orthop Relat Res. 1998;(355 Suppl):S90-104. PMID: 9917630.
21. Li H1, He JW, Fu BS, Wang K, Jiang N, Wang GY, Zhang J, Wang GS, Yang Y, Chen GH. Immunosuppressant-related hip pain after orthotopic liver transplant. Exp Clin Transplant. 2013;11(1):32-8. doi: 10.6002/ect.2012.0026.
22. Yoo SA, Park BH, Yoon HJ, Lee JY, Song JH, Kim HA, Cho CS, Kim WU. Calcineurin modulates the catabolic and anabolic activity of chondrocytes and participates in the progression of experimental osteoarthritis. Arthritis Rheum. 2007;56(7):2299-311. PMID: 17599750.
23. van der Windt AE, Jahr H, Farrell E, Verhaar JA, Weinans H, van Osch GJ. Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGFbeta1 production. Tissue Eng Part A. 2010;16(1):1-10. doi: 10.1089/ten.TEA.2009.0082.
24. Yoo SA, Park BH, Park GS, Koh HS, Lee MS, Ryu SH, Miyazawa K, Park SH, Cho CS,Kim WU. Calcineurin is expressed and plays a critical role in inflammatory arthritis. J Immunol. 2006;177(4):2681-90. PMID: 16888030.
25. Manikonda PK, Rajendra P, Devendranath D, Gunasekaran B, Channakeshava, Aradhya SR, Sashidhar RB, Subramanyam C. Extremely low frequency magnetic fields induce oxidative stress in rat brain. Gen Physiol Biophys. 2014;33(1):81-90. doi: 10.4149/gpb_2013059.
26. Lin HY, Lu KH. Repairing large bone fractures with low frequency electromagnetic fields. J Orthop Res. 2010 Feb;28(2):265-70. doi: 10.1002/jor.20964.
27. Chang SH, Hsiao YW, Lin HY. Low-frequency electromagnetic field exposure accelerates chondrocytic phenotype expression on chitosan substrate. Orthopedics. 2011;34(1):20. doi: 10.3928/01477447-20101123-10.

28. Ongaro A, Pellati A, Setti S, Masieri FF, Aquila G, Fini M, Caruso A, De Mattei M. Electromagnetic fields counteract IL-1β activity during chondrogenesis of bovine mesenchymal stem cells. J Tissue Eng Regen Med. 2015 Dec;9(12):E229-38. doi: 10.1002/term.1671.
29. Zhu H, Cai X, Fan X. Effect of puerarin on matrix metalloproteinase-2 in human Fetal sclera fibroblasts treated with low frequency electromagnetic fields. J Tradit Chin Med. 2013;33(5):664-8. PMID: 24660593.
30. Tumkaya L, Kalkan Y, Bas O, Yilmaz A. Mobile phone radiation during pubertal development has no effect on testicular histology in rats. Toxicol Ind Health. 2013 Oct 9. [Epub ahead of print]. PMID: 24097363.

Correspondence:
Adem Erkut
Department of Orthopedic Surgery, Recep Tayyip Erdogan University
Islampasa mh, Rize 53100 Turkey
ademerkut2006@ttmail.com

Received: Oct 16, 2015
Review: Dec 17, 2015
Accepted: Jan 19, 2016
Conflict of interest: none
Financial source: none

[1]Research performed at Histology and Embryology Laboratory, Department of Surgery, Faculty of Medicine, Recep Tayyip Erdogan University, Turkey.