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The Effect of Long-Term Extremely Low-Frequency Magnetic Field on Geometric and Biomechanical Properties of Rats' Bone

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Bone is composed of a mineral matrix reinforced by a network of collagen that governs the biomechanical functions of the skeletal system in the body. The purpose of the study was to investigate the possible effect of extremely low-frequency magnetic field (ELF-MF) on geometric and biomechanical properties of rats' bone. In this study, 30 male Sprague-Dawley rats were used. The rats were divided into three groups: two experimental and one control sham. The first and second experimental group ($n = 10$) were exposed to 100 μT and 500 μT-MF during 10 months, 2 h a day, respectively, and the third (sham) ($n = 10$) group was treated like experimental group except ELF-MF exposure in methacrylate boxes. After ELF-MF and sham exposure, geometric and the biomechanical properties of rats' bone, such as cross-sectional area of the femoral shaft, length of the femur, cortical thickness of the femur, ultimate tensile strength (maximum load), displacement, stiffness, energy absorption capacity, elastic modulus, and toughness of bone were determined. The geometric and biomechanical analyses showed that a significant decrease in rats exposed to 100 μT-MF in comparison to sham and 500 μT-MF exposed rats about the values of cross-sectional area of the femoral shaft ($P < 0.05$). Maximum load increased in 100 μT-MF and 500 μT-MF exposed rats when compared to that of the sham rats ($P < 0.05$). The cortical thickness of the femurs of MF-exposed rats (100 μT and 500 μT) were significantly decreased in comparison to that of sham groups' rats ($P < 0.05$ and $P < 0.001$). However, no significant differences were found in the other biomechanical endpoints between each other groups, such as: length of the femur, displacement, stiffness, energy absorption capacity, elastic modulus, and toughness of bone ($P > 0.05$). These experiments demonstrated that 100 μT-MF and 500 μT-MF can affect biomechanical and geometrical properties of rats' bone.

Keywords Bone; Biomechanics; Extremely low-frequency; Electromagnetic fields.

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Introduction

While much of the concern about the hazards of exposure to electromagnetic field (EMF) is related to the characteristics of the magnetic field, in the realm of bone healing it is the induced electric field that is considered to be the exclusive active agent of stimulation (NIEHS, 1998). As described by Bassett (1978), several investigators during the 19th century and the first half of the 20th century reported success in the treatment of bone fractures by electrotherapy. Heightened interest in this area arose during the 1950s and 1960s as the result of several demonstrations of the piezoelectric properties of bone (Tenforde, 1986). It is now well established that exogenous applied EMFs affect bone metabolism, both in vivo and in vitro. Clinical and animal studies have clearly shown that osteogenesis and osteopenia can be regulated electrically in vivo (Chang et al., 2003). Despite the clinical success, negative reports on the in vitro effects of electric stimulation on cellular proliferation, differentiation, and bone formation were reported (Chang et al., 2004a).

In many studies, extremely low-frequency (ELF) magnetic fields (MF) were used to treat fresh fracture healing, prevent and reverse of osteoporosis, and heal ununited congenital pseudoarthosis of the tibia and surgically resistant nonunions in adults (Grace et al., 1998; Tabrah et al., 1990, 1998; Chang and Chang, 2003). In addition to ELF-MF, some non ionizing electromagnetic waves were also used as alternative treatment method (Moazezi et al., 2008). Some investigators reported that ELF-MF may affect the behaviors of osteoblast-like cells, stimulate the osteoblasts in the early stages of culture, accelerate cellular proliferation of osteoblasts, and stimulate increasing of total bone mineral content (Chang et al., 2004a; Jonathan et al., 2000; Diniz et al., 2002; Akpolat, 2008). Diniz et al. (2002) suggested that the stimulatory effect of pulsed electromagnetic field (PEMF) was most likely associated with enhancement of the cellular differentiation, but not with the increase in the number of cells. However, Chang et al. (2004a) reported that the effect of PEMF stimulation on the bone tissue formation was most likely associated with the increase in the number of cells, but not with the enhancement of the osteoblasts' differentiation. The results obtained by Hanay et al. (2005) indicate that a 15 Hz PEMF stimulus on monolayers of an osteoblast-like cell line led to a depression in proliferation with a concomitant increase in alkaline phosphatase production. Chang et al. suggested that ELF-PEMF can both enhance and suppress the formation of osteoclast-like cells in bone marrow culture, and that osteoclastogenesis can be inhibited by PEMF stimulation, putatively due to a concomitant decrease in local factor production depending on the induced electric field intensity (Chang et al., 2003, 2004b). In some studies, ELF-MF were also used for the treatment of arthritic patients (Jacobson et al., 2001; Poornapria et al., 1998). Vera et al. (1999) investigated possible mass and bone density alterations in second-generation OF1 mice exposed chronically since birth to a magnetic field of 50 Hz and 15 μT. In this study, no significant differences were observed in relation to densitometric parameters such as total mass, total density, and mechanical parameters like periosteal and endosteal circumferences, etc. Chang and Chang (2003) demonstrated that extremely low-intensity, low-frequency, single PEMFs significantly suppressed the trabecular bone loss and restored the trabecular bone structure in bilateral ovariectomized rats. Zhang et al. (2006) showed that many bone indexes are significantly elevated after rotary non uniform magnetic field (RMF) exposure compared to the control ovariectomized (OVX) group and confirmed mechanistic evidence that strong magnetic field exposure could

effectively increase bone density and might be used to treat osteoporosis. They suggested that synergy of daily RMF exposure (30 min a day for 30 days using an 8 Hz rotary 0.4 T MF) with calcium supplement tended to increase the indexes of thigh bone density, energy absorption, maximum load, maximum flexibility, and elastic deformation as compared to those of untreated OVX control group. However, Tabrah et al. (1998) suggested that the enhancing effect of PEMFs in bone density should be further studied, alone and in combination with exercise and pharmacologic agents such as the bisphosphonates and hormones, as prophylaxis in the osteoporosis-prone postmenopausal woman and as a possible block to the demineralization effect of microgravity.

There are many studies in relation to the effect of ELF-MF on bone fractures, osteoporosis, osteoarthritis, and osteoblast and osteoclast cells. However, a few studies were reported in the literature considering the effects of ELF-MF on biomechanical properties of rat bone. Bone strength is an important parameter, measured by a biomechanical test of the bone specimen, as other properties of bone. Strength (maximum load), stiffness, energy absorption capacity, elastic modulus (young modulus), and toughness are evaluated by biomechanical tests and all of these parameters affect bone fragility. In the present report, it was designed to determine whether any changes occur in the biomechanical properties and histologic structure of bone when rats are exposed to ELF-MF for long term. To the best our knowledge, no biomechanical measurements based upon similar experiments were made earlier.

Materials and Methods

Subjects and Animal Care

The experiments were performed on 30 male Sprague-Dawley rats with initial weights of 304–380 g obtained from Medical Science Application and Research Center of Dicle University, aged 4 months at the beginning of the study, and fed with standard pelleted food (TAVAS Inc., Adana, Turkey). The rats were divided into three groups ($n = 10$): two experimental and one sham-exposed control. The animals were kept in 14/10 h light/dark environment at constant temperature of $22 \pm 3^{\circ}$C, $45 \pm 10\%$ humidity. This protocol was approved by the local ethics committee.

Magnetic Field Generation and Exposure Procedure

The MF was generated in a device designed by us that had one pair of Helmholtz coils of 25 cm in diameter in a Faraday cage ($130 \times 65 \times 80$ cm) that earthed shielding against the electric component. This magnet was constructed by winding 225 turns of insulated soft copper wire with a diameter of 1.0 mm. Coils were placed horizontally as facing one another and the distance between coils was 25 cm. An AC current produced by an AC power supply (DAYM, Turkey) was passed through the device. The current in the wires of the energized exposure coils was 0.12 A for 100 μT-MF and 0.50 A for 500 μT-MF, which resulted 50 Hz MF. The MF intensities were measured once per week as 100 μT and 500 μT-MF in different 15 points of methacrylate cage by using digital teslameter (Phywe, 209101074, Göttingen, Germany) to ensure homogeneity of the field during the course of the experiment by a person who is not involved in the animal experiment. MF measurements

showed that, at the conditions of the experiment, the MF exposure system produced a stable flux density of 100 μT-MF, 500μT-MF, and stable frequency of 50 Hz with neglible harmonics and no transients. The 50 Hz stray fields in the sham-exposure system were 0.1 μT. The static earth magnetic field was measured with a Bell 7030 Gauss/Teslameter (F.W. Bell, Inc., Orlando, FL). The component parallel to the exposure field was 14 μT and the component perpendicular to the exposed field was 34 μT. All field measurements were performed by persons not involved in the animal experiments. Observers were not aware of which group of rats was ELF-MF-or sham-exposed, i.e., the whole study was done blind. No temperature differences were observed between exposure and sham coils during the exposure. The first and second experimental groups were exposed to 100 μT and 500 μT-MF during 10 months, 2 h a day, respectively. The third group was sham that were treated like experimental group except ELF-MF exposure (corresponding to first and second groups, respectively) in methacrylate boxes. The rats were free in methacrylate cage inside the coils. After ten months of MF exposure, the study was terminated. Immediately after the last exposure, blood of the animals was collected by cardiac puncture under ketamine anesthesia (100 mg/kg, intramuscularly) to kill rats. At termination, the left and right femur of each animal was harvested. The left femur was stored at $-20^{\circ}C$ until mechanical testing. The length of the femurs was measured with a digital clipper for the elastic modulus and toughness calculation. Cross-sectional area of the femoral shaft was measured by computerized tomography (ARSTAR 40; Erlangen, Germany) and calculated by Point Counting Method (sensitivity with 1.5 mm).

Biomechanical Test

Biomechanical measurements were performed at the mid-diaphysis of the left femur. Tensile test was performed to measure the ultimate tensile strength, displacement, stiffness, energy absorption capacity, elastic modulus, and toughness of bone. After thawing within isotonic NaCl at room temperature, samples were tested using biomaterial testing machine (MAY 03; USA). For the tensile test, the femur bone was mounted horizontally in the machine by using colacryl. Distance between the two ends was 3 mm. The tensile loading speed in all tests was 1 mm/s. Data were transferred to the computers translating the numerical signals by 16-bit A/D converter for off line analysis. The sampling rate chosen was 200 sample/s. During mounting and testing of the specimens, normal Ringer's solution was regularly applied for the prevention of drying. Load-displacement data were recorded using BIOPAC MP 100 Acquisition System Version 3.5.7 (Santa Barbara, CA). Ultimate tensile strength, displacement, stiffness, and energy absorption capacity were determined from this curve. Ultimate tensile strength is the maximal load in tension that a material can sustain before failure (N). Displacement is the transverse displacement at the point of loading (mm). Stiffness was defined by the slope of the linear portion of the load-displacement curve (N/mm). The area under the load-displacement curve was defined as energy absorption capacity, which is the energy stated the effect of mineral content of the femoral shaft on bone biomechanic (mJ). The load-displacement recordings were normalized by cross-sectional area and this curve was converted to a stress-strain curve. Stress-strain curves for each specimen were generated. The elastic modulus and toughness were determined. Elastic modulus was defined by the slope of the linear portion of the stress-strain curve

(GPa). The area under the stress-strain curve was defined as toughness, which is the energy required to cause breaking of the femoral shaft (J/m^3) (Gurgul et al., 2008).

Histologic Analysis

After euthanasia, right femurs were harvested and fixed in 10% neutral buffered formalin (NBF) for 12 h at room temperature. The femurs were decalcified in 5% formic acid for 48 h. The femurs were placed in 10% NBF for transport to the histotechnology facility, where they were dehydrated in a series of alcohols, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin-eosin for measurement of cortical bone thickness using light microscopy. Diaphysial cortical bone thickness was measured with ocular micrometer (Olympus CH-2). Ten random areas were selected and average thickness was calculated for each femur.

Statistical Analysis

Descriptive values of data were represented as means ± standard error of the mean (SEM). After checking for normal distribution with Shapiro-Wilks test, data were analyzed by Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks and post-hoc tests using Dunn procedure. P value less than 0.05 was considered significant. Statistical analysis was performed by using SPSS 11.5 software (Lead Technologies, Inc., Chicago, IL). Graphs were drawn by use of Statistica 6.1 program (StatSoft Inc., Tulsa, OK).

Results

In this study, it was determined some geometric and biomechanical parameters of rats' bone to investigate the effect of ELF-MF on bone. These parameters are cross-sectional area of the femoral shaft, length of the femur, cortical thickness of the femur, ultimate tensile strength (maximum load), displacement, stiffness, energy absorption capacity, elastic modulus, and toughness of bone. The geometric and biomechanical parameters and their statistical analysis were shown in Tables 1 and 2. The cortical

Table 1
Geometric properties of sham and experimental groups (values were expressed as means ± SEM)

Groups	Cross sectional area (mm^2)	Length (mm)	Cortical thickness (mm)
Sham	11.49 ± 0.56	37.68 ± 0.26	0.445 ± 0.0163
100 μT-MF	8.06 ± 0.44[a]	37.27 ± 0.28	0.393 ± 0.0108[a]
500 μT-MF	13.48 ± 1.03[b]	37.43 ± 0.43	0.376 ± 0,00905[a]

MF: Magnetic Field.

[a] $P < 0.05$ as compare to sham group by nonparametric Kruskal–Wallis one-way analysis of variance followed by the Dunn post-hoc test.

[b] $P < 0.001$ as compare to 100 μT-MF group by nonparametric Kruskal–Wallis one-way analysis of variance followed by the Dunn post-hoc test.

Table 2
Biomechanical parameters of sham and experimental groups (values were expressed as means ± SEM)

Groups	Displacement (mm)	Maximum load (N)	Stiffness (N/mm)	Energy absorption capacity (mJ)	Toughness (J/m^3)	Elastic modulus (GPa)
Sham ($n = 10$)	1.44 ± 0.19	297.46 ± 32.26	405.75 ± 124.63	225.89 ± 43.21	0.00299 ± 0.0008	1.202 ± 0.40
100 μT-MF ($n = 10$)	1.63 ± 0.60	376.64 ± 34.09[a]	499.73 ± 146.30	318.48 ± 136.71	0.00385 ± 0.0012	2.177 ± 0.57
500 μT-MF ($n = 10$)	1.13 ± 0.37	418.91 ± 29.35[a]	930.32 ± 194.33	240.98 ± 77.05	0.00140 ± 0.0003	2.791 ± 0.75

MF: Magnetic Field.
[a] $P < 0.05$ as compare to sham group by nonparametric Kruskal–Wallis one-way analysis of variance followed by the Dunn post-hoc test.
[b] $P < 0.001$ as compare to 100 μT-MF group by nonparametric Kruskal–Wallis one-way analysis of variance followed by the Dunn post-hoc test.

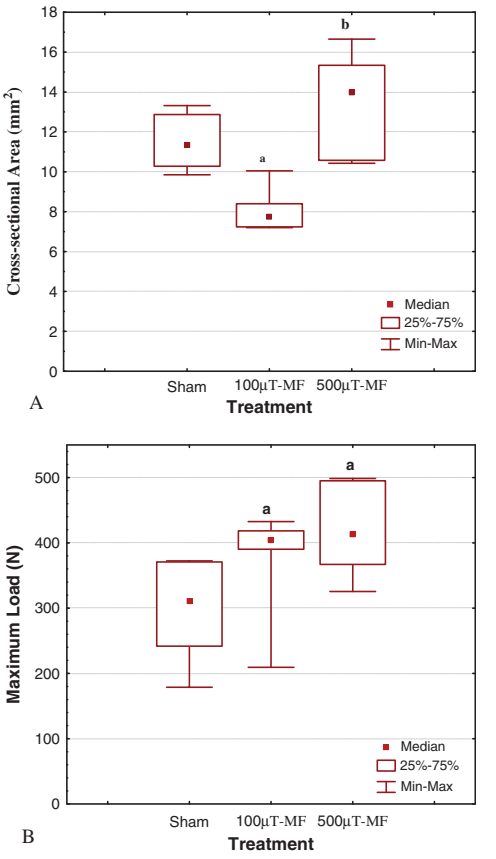

Figure 1. Cross-sectional area and maximum load parameters of rats' femurs. MF: rats which were exposed to magnetic field. a: $P < 0.05$ as compare to sham group; b: $P < 0.001$ as compare to 100 μT-MF group. The results were compared by using the Kruskal–Wallis one-way analysis of variance followed by the Dunn post-hoc test.

thickness of the femurs of MF-exposed rats (100 μT and 500 μT) were significantly decreased in comparison to that of sham groups' rats ($P < 0.05$) (Table 1). It was determined a significant decrease in rats exposed to 100 μT-MF in comparison to sham and 500 μT-MF-exposed rats about the values of cross-sectional area of the femoral shaft ($P < 0.05$ and $P < 0.001$, respectively; Figure 1A; Table 1). With respect to maximum load, it was found to be increased in 100 μT-MF- and 500 μT-MF-exposed rats when compared to the sham rats ($P < 0.05$; Figure 1B). However, no significant differences were found in the other biomechanical endpoints between each other groups, such as: length of the femur, displacement, stiffness, energy absorption capacity, elastic modulus, and toughness ($P > 0.05$) (Table 2).

Discussion

In this study, we investigated the effects of ELF-MF at the flux intensities of 100 μT and 500 μT exposure for 2 h a day up to 10 months on biomechanical properties of rat bone. For the evaluation, load-displacement data were recorded and ultimate tensile

strength (maximum load), displacement, stiffness, and energy absorption capacity were determined from this curve. The load-displacement recordings were normalized by cross-sectional area and this curve was converted to a stress-strain curve. The elastic modulus and toughness parameters of bone were determined from this curve.

Maximum load is a widely used parameter for biomechanical evaluation and represents the maximum tensile force important for fracture. Displacement, stiffness, and energy absorption capacity are also important parameters for biomechanical test of bone (Peng et al., 1994; Reddy et al., 2001). These parameters demonstrate the extrinsic properties (structural properties) of bone tissue (Burr, 2002). In the present study, maximum load increased in 100 μT-MF- and 500 μT-MF-exposed rats when compared to the sham rats. No statistically significant differences were found between the experimental groups' rats and the sham rats in the values of displacement and energy absorption capacity (Table 1). In addition, it was determined that the values of bone stiffness were higher in 100 μT- and 500 μT-MF- exposed rats than that of sham rats. This increase that is directly proportional with MF strength but not significant can stem from ELF-EMF stimulation. Hence, it can be suggested from these results that ELF-MF at the flux intensities of 100 μT and 500 μT-MF may have an effect on the mineralization of bone. A lot of studies suggest this stimulation with their results both in vivo and in vitro experiments (Chang and Chang, 2003; Chang et al., 2004a; Grace et al., 1998; Tabrah et al., 1990, 1998; Jonathan et al., 2000; Diniz et al., 2002; Zhang et al., 2006; Gurgul et al., 2008).

Elastic modulus (Young modulus) and toughness have an effect on bone strength, and are also important parameters for bone biomechanical evaluation. These parameters demonstrate the intrinsic properties (material properties) of bone structure and combinational changes of these parameters indicate different situations (Gurgul et al., 2008; Burr, 2002). For example, if the elastic moduli of bone increase while the toughness decrease, we can say that the bone which fits this situation is highly mineralized (Burr, 2002). In the present study, the values of elastic modulus and toughness in 100 μT-MF-exposed rats increased when compared to sham rats. The elastic modulus of bone in 500 μT-MF-exposed rats increased in comparison to the sham rats, in contrast its toughness values were reduced.

At increasing levels of stiffness, the tissue can become brittle, reducing the energy required for fracture and toughness contribute to bone collagen integrity (Gurgul et al., 2008; Burr, 2002). The stiffness values of 500 μT-MF-exposed rats were higher than the sham and 100 μT-MF rats, in contrast its toughness values were lower.

With regard to the results obtained from the overall biomechanical parameters, it can be indicated that ELF-MF seems to be demonstrating a strength-dependent effect on the mechanic properties of bone. It can be also suggested that ELF-MF has effect not only on the mineralization of bone, but also on the collagen integrity. Our previous study indicated that ELF-MF (50 Hz, 1 mT, 4 h/d for 45 d) can affect bone mineralization and collagen integrity and this flux intensity caused a deteriorative effect on bone structure (Gurgul et al., 2008).

Bone biomechanical parameters are related with bone material properties (matrix calcification, the composition and spatial arrangement of crystals, collagen fibers, and lamellae) and geometric properties (trabecular network, macrostructure of the cortex, and cortical shell; Gurgul et al., 2008; Ferretti et al., 2001; Martin et al., 1993). The width of cortical bone and cortical area is an important parameter in the evaluation of cortical bone quality and bone strength. A significant reduction was found in 100 μT-MF-exposed rats compared to sham and 500 μT-MF-exposed

rats in relation to cross-sectional area of the femoral shaft. The cross-sectional area of the femoral shaft in 500 μT-MF-exposed rats were higher than sham. However, this increase was not statistically significant. It was also found that femoral cortical thickness was significantly decreased in MF-exposed rats than in sham. The results of the cortical thickness are in good agreement with the results of Gurgul et al. (2008). According to the results of cross-sectional area and femoral cortical thickness, it is suggested that long-term ELF MF exposure can affect cortical bone quality and bone strength. The results of the cross-sectional area are not in agreement with dose-response characteristic. Several cellular activities may be influenced by weak EMFs, showing unusual dose-response characteristic (Goodman et al., 1995) since the mechanisms of the modified biochemical and physiological processes is directly linked to the frequency (Blank, 1995). Taken together, EMF effects on cells depend on a number of factors including the frequency, the contribution of electrical field components generated by the coil (McLeod et al., 1996), level of magnetic induction, cell density, and cell type and assay system used. Zhang et al. (2006) reported that rats, receiving RMF exposure for 30 min per day plus calcium supplement, had a higher maximum load, elastic deformation, energy absorption, and elastic energy absorption than that OVX models, indicating that the joint treatment of RMF and calcium had a favorable influence on repairing or maintaining the structure of the capitellum (Fitzsimmons et al., 1989). The results of the study carried out by Zhang et al. (2006) are consistent with the results of the present study in relation to energy absorption capacity and elastic modulus compared to sham.

In conclusion, it is suggested that low-intensity ELF-MF can affect the biomechanical properties of rat bones, especially cortical bone quality and bone strength. Its effect can be on the bone mineralization, and therefore, it is also suggested that ELF-MF cannot affect the intrinsic properties of bone structure. However, further detailed studies should be done to explore the interaction of ELF-MF and bone cell.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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