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Effects of extremely low-frequency electromagnetic field exposure on the skeletal muscle functions in rats

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Abstract

The aim of the present study was to systematically investigate the effects of chronic exposure to extremely low-frequency electromagnetic field (ELF-EMF) on electrophysiological, histological and biochemical properties of the diaphragm muscle in rats. Twenty-nine newly weaned (24 days old, 23–80 g) female ($n = 15$) and male ($n = 14$) Wistar Albino rats were used in this study. The animals were randomly divided into two groups: the control group and the electromagnetic field (EMF) group. The control group was also randomly divided into two groups: the control female group and the control male group. The EMF exposure group was also randomly divided into two groups: the ELF-EMF female group and the ELF-EMF male group. The rats in the ELF-EMF groups were exposed for 4 h daily for up to 7 months to 50 Hz frequency, 1.5 mT magnetic flux density. Under these experimental conditions, electrophysiological parameters (muscle bioelectrical activity parameters: intracellular action potential and resting membrane potential and muscle mechanical activity parameter: force–frequency relationship), biochemical parameters (Na^+, K^+, Cl^- and Ca^{+2} levels in the blood serum of rats; Na^+-K^+ ATP_{ase} enzyme-specific activities in muscle tissue; and free radical metabolism in both muscle tissue and serum) and transmission electron microscopic morphometric parameters of the diaphragm muscle were determined. We found that chronic exposure to ELF-EMF had no significant effect on the histological structure and mechanical activity of the muscle and on the majority of muscle bioelectrical activity parameters, with the exception of some parameters of muscle bioelectrical activity. However, the changes in some bioelectrical activity parameters were relatively small and unlikely to be clinically relevant.

Keywords

Diaphragm muscle, electromagnetic field, intracellular action potential, resting membrane potential, contractile force–frequency relationship

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Introduction

In living organisms, cells and tissues are constantly subject to electromagnetic forces arising from molecular interactions, environmental and externally applied electromagnetic fields (EMFs) (Zhou and Uesaka, 2006). EMFs, especially extremely low-frequency (ELF) ones are one of the most common environmental factors that can influence living systems. EMFs with frequencies between 3 Hz and 300 Hz are generally known as extremely low-frequency electromagnetic fields (ELF-EMFs) (Seyhan, 2010).

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Living organisms are electrochemical systems that use ELF-EMFs from the structure of proteins to cell-to-cell communication and nervous system functions. The widespread use of electricity increases the potential sources of radiation resulting in continuous and/or intermittent exposure of living beings to ELF-EMFs (Boland et al., 2002). Magnetic flux densities of EMFs of the devices offered by the technology, which make our life easier, vary between 1×10^{-4} milli Tesla (mT) and 2.5 mT, and this value, which is more than magnetic flux densities of the EMF of human body (10^{-7} mT) and natural environment (10^{-6} mT) can disrupt the harmony (Seyhan, 2010). Lifetime exposure to EMFs is becoming the subject of significant scientific investigation since it has the potential to cause crucial changes and deleterious effects in biological systems (Kıvrak et al., 2017). Many studies have reported the effects of exposure to ELF-EMFs on various tissues and functions. It is not yet entirely clear, however, whether the effects of ELF-EMFs on the human body is beneficial or harmful. It was found that ELF-EMFs have significant effects on cells and tissues and their functions in the living organisms, such as a marked change in the synaptic plasticity generated in synapses of the dentate gyrus (Komaki et al., 2014); persistent changes in neuronal activity after prolonged exposure to ELF-EMFs (Komaki et al., 2014); dramatic effects from behaviour to physiology and protein expression (Wyszkowska et al., 2016); important alterations of glucose and lipid metabolisms (Hashish et al., 2008); deleterious effect on ultra structure of prostate gland in rat (Khaki et al., 2008); the occurrence of oxidative stress-based nervous system pathologies associated with ageing (Falone et al., 2008); regulation of calcium-related activities in cardiomyocytes (Wei et al., 2015); absence of evidence of persisting unrepaired nuclear DNA single-strand breaks in distinct types of cells in the brain, kidney and liver of adult mice after continuous 8-week 50 Hz magnetic field (MF) exposure with flux density of 0.1 or 1.0 mT (Korr et al., 2014); changes in the synthesis and release of oestradiol-17β (E2) in uterine tissues (Koziorowska et al., 2018); the treatment of cerebral ischaemia by reduction neuronal cell death by low-energy low-frequency pulsed electromagnetic fields (PEMFs) (Gessi et al., 2019); increased bone density, faster recovery, increased formation of new bone, a further opening of the mouth and decreased pain depending on the post-operative PEMF treatment with 1 mT intensity and 40 Hz frequency in mandibular bone fractures (Mohajerani et al., 2019); and the improvement of pain intensity, disability and lumbar range of motion in PEMF exposure with 20 Gauss low intensity, 50 Hz frequency in patients with non-chronic specific low back pain (Elshiwi et al., 2019).

In addition to the above studies, in a recent study, it was shown that both supplemental and ambient MFs modulate myogenesis by stimulating transient receptor potential-C1-mediated calcium entry (Yap et al., 2019). In another study, high-intensity focused electromagnetic field technology has been reported to increase muscle thickness and hypertrophy (Duncan and Dinev, 2019).

However, relatively limited studies have been done on the effects of ELF-EMFs with low intensity on the skeletal muscle (diaphragm-a striated muscle) functions. Our study in this subject is orientated to total body irradiation of laboratory rats by sinusoidal ELF-EMFs (50 Hz frequency, 1.5 mT magnetic flux density). The magnetic flux density used in the present study is in the range of magnetic flux densities emitted from some electrical devices and within the limits contained in occupational and public environment MF exposure guideline standards and it exists in both the public and the occupational environments (Seyhan, 2010; Tsanakas et al., 2006). In our study, we aimed to examine the electrical and mechanical activities of the diaphragm (the main muscle of respiration) in rats exposed to sinusoidal ELF-EMFs from neonatal to adult period (chronic exposure) and so to determine whether homogeneous ELF-EMFs affect diaphragm muscle function. The results of this study may support the idea of whether ELF-EMFs may be a potential factor in the pathogenesis of some muscle diseases, including respiratory disease and skeletal muscle degenerative diseases.

Methods

Animal preparation and experimental design

Twenty-nine healthy completely weaned female ($n = 15$) and male ($n = 14$) Wistar Albino rats (weanling 24 days, 23–80 g) were used in this study. The rats were obtained from the Experimental Animal Laboratory of Mersin, Turkey. The study was approved by the Research and Ethical Committee of the University of Mersin. The rats were housed in polycarbonate boxes (three or four rats per box) with steel wire tops and rice husk bedding. They were maintained in a controlled atmosphere of 12-h dark/12-h light cycle,

at 22 ± 2°C temperature, and at 50–70% humidity, with free access to pelleted feed and fresh tap water. The animals were supplied with dry food pellets commercially available. The animals were randomly divided into two groups: the control group and the EMF group. The control group was also randomly divided into two groups: the control female group (seven rats) and the control male group (seven rats). The EMF exposure group was also randomly divided into two groups: the ELF-EMF female group (eight rats) and the ELF-EMF male group (seven rats).

ELF-EMF exposure

In our previous study, the homogenous horizontal (field lines horizontal to the bottom plane of the animal's cage) MF was generated by a Helmholtz coil pair in circular configuration (Gunes et al., 2008). A pair of circular coils of 21.5 cm diameter and 22.5 cm distance was constructed by insulated copper wire and made of 160 turns. The resistance of each coil was 2 ohms. MFs were created in the Helmholtz coil systems using a source capable of producing a maximum 10 A current and an output voltage at desired values between 0 V and 250 V. A sinusoidal current of 50 Hz was generated at the output of the circuit (Figure 1). The MF was measured with a Gaussmeter and a special probe (Sypris Test Measurement F.W. Bell 6010 Model Gauss/Tesla meter, Bell Technologies Inc., New Zealand). Fifteen rats in the ELF-EMF groups were housed in the centre of the coils, two per plastic cage, and were exposed to 50 Hz, 1.5 mT magnetic flux density with the exposure period of 4 h/day for 7 months. Fourteen rats in the control groups also underwent same period and same conditions but none received MF in Helmholtz coils.

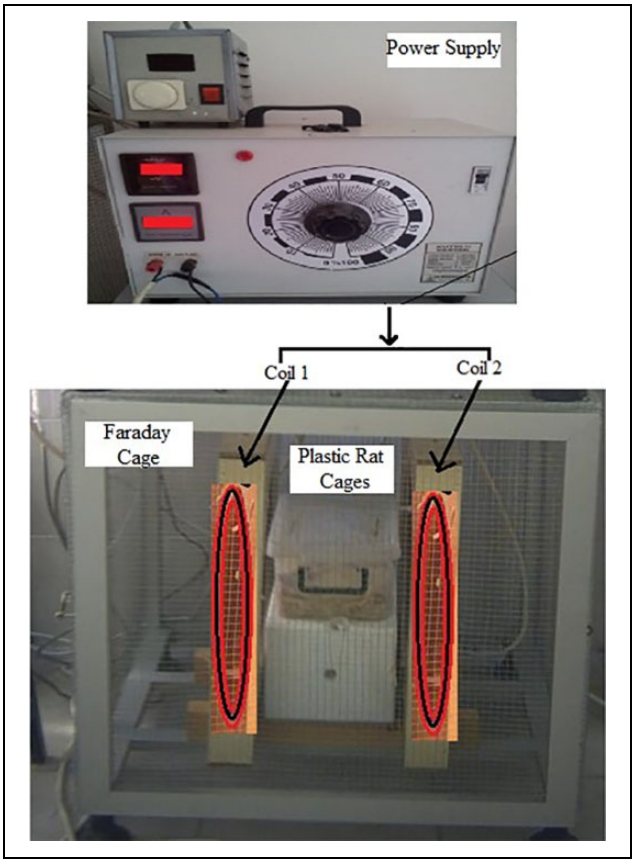

Figure 1. MF exposure setup (Helmholtz coil). The homogenous horizontal (field lines horizontal to the bottom plane of the animal's cage) MF with power frequency of 50 Hz was generated by a Helmholtz coil pair in circular configuration (six). A pair of circular coils of 21.5 cm diameter and 22.5 cm distance was constructed by insulated copper wire and made of 160 turns. The resistance of each coil was 2 ohms. MFs were created in the Helmholtz coil systems by using a power supply producing a maximum 10 A current and an output voltage at desired values between 0 V and 250 V. Eventually, a sinusoidal current of 50 Hz was generated at the output of the circuit. MF: magnetic field.

Preparation of diaphragm muscle samples for biochemical analyses, electron microscopic examination, contractility measurements and electrophysiological recordings

At the end of the chronic ELF-EMF exposure period, the rats in the control and experimental groups were anaesthetized intramuscularly with a mixture of ketamine and xylazine (80 and 10 mg/kg, respectively). Thorax was opened and the blood taken from the heart was placed in glass tubes and the obtained serum samples were stored in deep freezing at −80°C until biochemical analyses were made. The diaphragm was attempted to be isolated very quickly and a strip of the left ventral costal region (4 × 18 mm^2 in size) was removed and placed in a glutaraldehyde solution for use in electron microscopic examination of the muscle. The other few strips obtained from the isolated diaphragm muscle tissues were then placed in the Krebs solution gassed continuously, while the other remaining muscle tissues were placed in glass tubes for use in biochemical assays and freeze-dried at −80°C until analysis.

The diaphragm muscles during contractility measurements and electrophysiological recordings were kept in Krebs solution with the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2,

$NaHCO_3$ 24, KH_2PO_4 1.2, glucose 11 and at pH 7.40. All chemicals were purchased from Merck (Darmstadt, Germany). All the solutions were bubbled with a gas mixture of 5% CO_2 and 95% O_2, and temperature of the bath solution was held constant at 37 $\pm$ 0.5°C for contractility measurements and 23 $\pm$ 1°C for electrophysiological recordings with a heating circulator (Heating Circulator/Model MAY WBC 3044-PR, Ankara, Turkey), which enabled control of the temperature.

Electrophysiological methods

Microelectrode recordings. Each rat diaphragm muscle strip, including the area of the phrenic nerve from the left hemidiaphragm, were placed into tissue chambers (22 ml volume) that were used for the recording of the resting membrane potential and intracellular action potential (AP) (Figure 2). Electrophysiological recordings were performed using conventional microelectrode techniques (Figure 3). Cells were impaled by glass microelectrodes with tip resistances of 8–15 MΩ. The electrical signals were fed through an amplifier (IE-251A, Warner Instruments Corporation, USA) and the data were digitized with a sampling rate of 15,000 samples/s (sampling frequency 15,000 Hz) for intracellular AP and with the sampling rate 200 samples/s (sampling frequency 200 Hz) for resting membrane potential were recorded on a computer. Direct intracellular APs were induced by electrical stimulation of the muscle fibres locally with 2.71 $\pm$ 1.77–7.69 $\pm$ 1.69 mA and 1-ms long pulses.

AP properties were characterized as follows: amplitude (difference between resting membrane potential and the peak positive voltage), overshoot (amount by which voltage exceeded 0 mV at the peak of the AP), area (the integral of membrane potential during the AP measured relative to resting membrane potential), depolarization time (duration) (time required for the AP to depolarize of the way up from resting membrane potential) and half-repolarization time (time required for the AP to repolarize 50% of the way back to resting membrane potential).

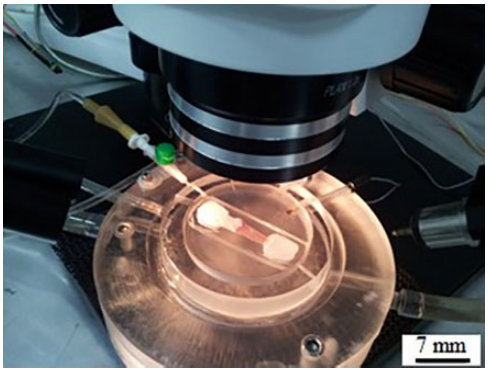

Figure 2. Photograph of rat diaphragm muscle strip (approximately 4–5 mm wide and 17–20 mm long) taken by a binocular stereo microscope in the Krebs solution fixed on paraffin at the base of the tissue chamber from the rib and tendon sections for recording of the resting membrane potential and intracellular AP. AP: action potential.

Measurement of diaphragm muscle contractility. Muscles were mounted vertically into the isolated organ (Isolated Organ Bath Stand Set – MAY-IOB S99, Ankara, Turkey) baths between an isometric force transducer (FDT-05 Force Displacement Transducer, BIOPAC

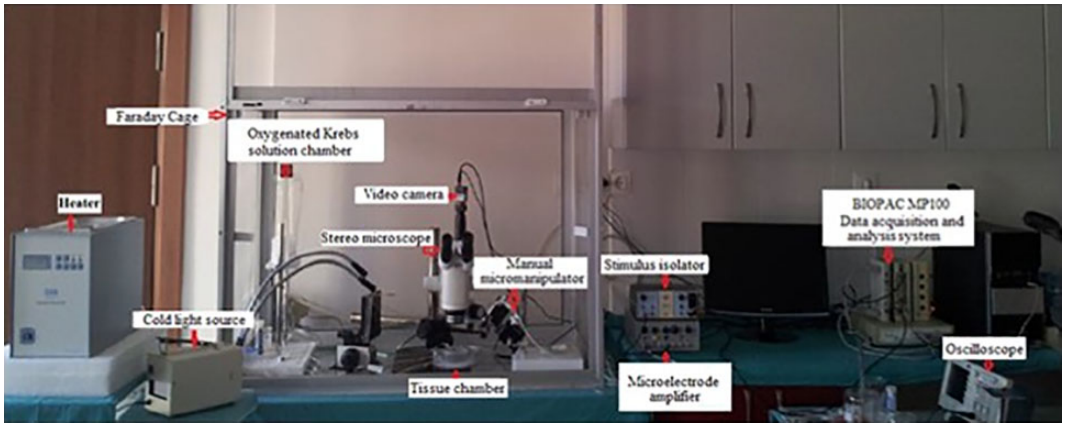

Figure 3. Schematic of the entire experimental system used to record resting membrane potential and intracellular AP. AP: action potential.

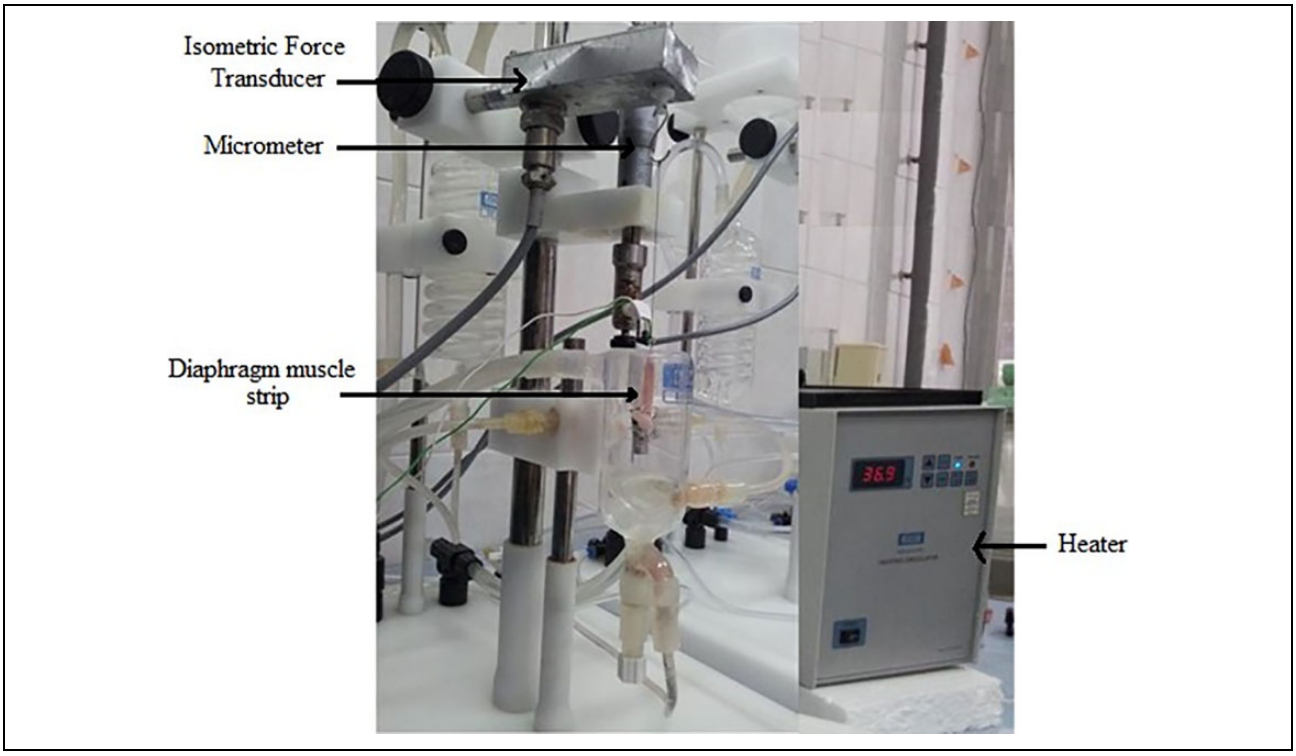

Figure 4. The isolated organ bath system for accurate recording of diaphragm muscle strip isometric contraction/release.

Systems Inc., Santa Barbara, California, USA) and a holder attached to a micrometer that allowed adjustment of the preparation to optimal length (Figure 4). Isometric force transducer output was connected to the amplifier module (MAY-GTA-200, Ankara, Turkey) on the BIOPAC MP100 system (BIOPAC MP100 Systems Inc., Santa Barbara, California). The diaphragm muscle strip was placed in between two platinum wire electrodes to allow direct contact of the electrodes to the tissue. The Krebs solution where the diaphragm muscle was placed was replaced every 15 min, and the muscle was incubated in this medium for 30 min to balance it by adapting the bathroom environment. After the equilibration period in the Krebs solution, preload values that bring each diaphragm muscle to the optimal length (L_0) were measured before isometric force responses formed by each diaphragm muscle for stimuli at different frequencies (5, 10, 20, 40, 60, 80, 100 and 150 Hz) were recorded. Moreover, appropriate preload values that brought the muscle preparation to its optimal length were adjusted by means of a micrometer attached to one end of the muscle before each alert protocol was applied. The maximum alert output of the stimulator on the BIOPAC MP100 was ± 5 V. This value was found not to be a suitable range for supramaximal stimulus voltage required to induce muscles. Therefore, a stimulator module (STM100A, BIOPAC Systems Inc.) on the BIOPAC MP100 system was connected to a stimulus isolator (MAY-ISO150-A Serial No: 200,001–1 Stimulus Isolated Power Supply, Ankara, Turkey), which can be adjusted up to the output voltage of 150 V, and then preliminary trials were performed; it was observed that the diaphragm muscle reached the supramaximal amplitude with about 40 V. Thus, the duration and the amplitude of the stimulus voltage were used respectively, as 1 ms and 40 V to obtain the records of mechanical activity at the supramaximal amplitude in the diaphragm muscles during the study. Stimuli at different frequencies (5, 10, 20, 40, 60, 80, 100 and 150 Hz) were given with an interval of 5 min for the diaphragm muscles which were brought to the optimal length (L_0), and the mechanical responses of the muscle were recorded on the BIOPAC MP100 data acquisition and analysis system with AcqKnowledge Software (BIOPAC MP100 System Inc.), which transfers the responses transmitted through an isometric force transducer to the computer via a difference amplifier. The maximum contraction force was expressed in grams (g) and normalized to be divided by the cross-sectional area (CSA). Following the removal of non-muscle tissue, muscle strips were

blotted and weighed, and force per CSA was calculated according to the following equation (Close, 1972):

$$\frac{\text{Maximum contraction force (g)}}{\text{CSA (cm}^2\text{)}} = \frac{\text{g}}{\text{Muscle mass (g)}/L_0\text{(cm)} \times 1.056\ \left(\text{g/cm}^3\right)}$$

Biochemical assays

Blood samples taken from the rats through the heart of the experimental and control groups were left in the room for 20 min and then centrifuged at 4000 r/min for 5 min. Na^+, K^+, Cl^- and Ca^{+2} levels were measured on the same day from centrifuged serum samples by Roche Diagnostics Cobas Integra 800 Instrument (Midland, Ontario, Canada) at Mersin University, Medical Faculty Hospital, Medical Biochemistry Laboratory. Na^+, K^+ and Cl^- levels were measured by the ion-selective electrode principle and the Ca^{+2} ion levels were measured by the colorimetric method. At the end of the study, the Na^+-K^+ ATPase enzyme-specific activities in muscle tissue of ELF-EMF were determined and also the effects of total oxidant status (TOS) and total antioxidant status (TAS) on free radical metabolism in both tissue and serum were determined.

Electron microscopic examination

Sample preparation for electron microscopy. For transmission electron microscopic (TEM) evaluation of the diaphragm muscle, tissue samples were fixed with 2.5% glutaraldehyde (Cat #16400,450 ml, EMS), postfixed with 1% osmium tetroxide (Cat #19110, 10 × 1 g, EMS), dehydrated in graded alcohol series, cleared with propylene oxide (Serva Lot 100109, 500 ml) and embedded in epon. Thin sections (50–70 nm) were cut by an ultramicrotome (UCT-125; Leica Microsystems GmbH, Wien, Austria) and contrasted with uranyl acetate and lead citrate. Sections were examined with TEM (JEM-1011; JEOL Ltd, Tokyo, Japan) and photographed with a microscope-attached digital camera (Megaview III, Olympus GmbH, Germany). Electron micrographs were taken at 12,000× magnification randomly from diaphragm muscle samples for all groups.

Statistical analysis

The data were expressed as median and mean ± standard deviation. MedCalc 12.3.0 (MedCalc Software Ltd., Belgium) and SPSS 11.5.0 (SPSS Software IBM Ltd., USA) software package was used for analysis. After the normal distribution of measures was confirmed with the Kolmogorov–Smirnov test, the data were analysed by analysis of variance and differences in parameters between groups were analysed with the least significant difference post hoc test. The $p < 0.05$ value was defined as being statistically significant.

Results

Electrophysiological evaluations

The cumulative effect has been evaluated based on gender by considering the electric and mechanical activity changes that occur in the skeletal muscle as a result of ionic movements in the cell under long-term ELF-EMF exposure.

Intracellular APs and resting membrane potential recording in the isolated diaphragm muscle strips

Intracellular AP and resting membrane potential were recorded by ealuating at least 45 cells in the each diaphragm muscle strip isolated from all groups.

Microelectrode impedance, and resting membrane potential, stimulating current intensity, peak latency, amplitude, total duration, area, depolarization time and half-repolarization time and overshoot of APs in all four groups (control female, control male, ELF-EMF female and ELF-EMF male) were measured (Table 1). When the stimulating current intensity applied to the diaphragm muscle of the female rats was compared between the control groups and the ELF-EMF groups, it was determined that exposure of female diaphragm muscles to ELF-EMF can produce a response with statistically significant a lower-stimulating current intensity ($p < 0.05$). In male rats, it was found that ELF-EMF exposure increased the peak latency (ms) ($p < 0.05$) and depolarization duration (ms) ($p < 0.001$) at a statistically significant level. In addition, when the ELF-EMF male and ELF-EMF female groups were compared, it was observed that the peak latency (ms) and the depolarization time (ms) increased in male rats at a significant level ($p < 0.05$). It was determined that being exposed to ELF-EMF increased the AP area (mV·ms) in female rats when compared with the control female rats at a statistically significant level ($p < 0.05$).

Table 1. Descriptive statistics for microelectrode impedance, membrane potential, current intensity and intracellular APs parameters that were recorded from diaphragm muscle samples for all groups.[a]

	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
Microelectrode impedance (MΩ)	10.70 ± 1.60	11.59 ± 2.62	9.21 ± 1.11	9.00 ± 1.34
Resting membrane potential (mV)	−68.73 ± 4.33	−72.61 ± 3.05	−69.35 ± 2.03	−69.71 ± 2.92
Current intensity (mA)	7.69 ± 1.69	4.83 ± 2.97	2.71 ± 1.77[b]	2.89 ± 1.82
Peak latency (ms)	2.49 ± 0.57	1.90 ± 0.28	2.12 ± 0.34	2.61 ± 0.46[c,d]
Amplitude (mV)	80.47 ± 7.61	88.64 ± 4.48	82.60 ± 6.04	85.29 ± 2.76
Total time (ms)	6.95 ± 1.17	7.22 ± 1.09	7.87 ± 0.78	7.72 ± 1.00
Area (mV·ms)	0.112 ± 0.02	0.119 ± 0.01	0.128 ± 0.01[b]	0.129 ± 0.01
Depolarization time (ms)	0.97 ± 0.38	0.68 ± 0.14	0.88 ± 0.22	1.29 ± 0.32[d,e]
Half-repolarization time (ms)	2.96 ± 0.59	3.28 ± 0.48	3.48 ± 0.35	3.23 ± 0.53
Overshoot (mV)	11.93 ± 4.67	15.08 ± 2.72	12.06 ± 3.66	14.95 ± 2.61

AP: action potential; ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure.
[a]Values are means + standard deviation.[b]$p < 0.05$, significantly different from control female.
[c]$p < 0.05$, significantly different from control male.
[d]$p < 0.05$, significantly different from ELF-EMF female.
[e]$p < 0.001$, significantly different from control male.

Table 2. Descriptive statistics for pre-tension (g) and the maximum contractile force values (g/cm^2) that were recorded from the diaphragm muscles for stimuli protocols at different frequencies (one single square pulse stimulus − T_w, 5, 10, 20, 40, 60, 80, 100 and 150 Hz).[a]

	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
Pre-tension values (g)	1.85 ± 0.29	2.59 ± 0.29	1.71 ± 0.27	1.96 ± 0.29
T_W	192.19 ± 93.71	232.99 ± 147.4	172.79 ± 82.59	166.52 ± 131.3
5 Hz	202.63 ± 102.8	227.03 ± 134.9	182.70 ± 57.24	166.27 ± 128.2
10 Hz	254.19 ± 148.5	280.39 ± 180,6	200.59 ± 98.63	195.10 ± 162.6
20 Hz	337.30 ± 172.9	340.21 ± 222.8	234.54 ± 117.9	219.55 ± 184.8
40 Hz	500.82 ± 241.5	558.65 ± 365.2	373.37 ± 191.9	354.62 ± 300.4
60 Hz	566.19 ± 326.3	718.92 ± 459.41	489.19 ± 249.6	441.09 ± 381.4
80 Hz	609.37 ± 405.8	769.08 ± 511.9	524.52 ± 283.4	470.83 ± 410.0
100 Hz	577.80 ± 382.2	767.58 ± 507.6	529.31 ± 297.2	475.84 ± 418.7
150 Hz	558.29 ± 409.4	791.45 ± 523.7	555.57 ± 308.7	480.59 ± 424.8

ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure; T_W: twitch.
[a]The duration and the amplitude of the one single square pulse stimulus were used respectively, as 1 ms and 40 V to obtain the records of mechanical activity at the supramaximal amplitude in the diaphragm muscles during the study. Values are means + standard deviation. No statistically significant differences in the pre-tension and maximum contractile force values between any groups ($p > 0.05$).

Mechanical activity data of diaphragm muscles

The force–frequency relationship. To create maximum contraction force in the diaphragm muscle, the pre-tensions that brought the muscle to the optimum length were applied. When the pre-tension values that brought the diaphragm muscle to the optimum length to obtain maximum contraction force were compared among the groups, no statistically significant differences were detected (Table 2). This result shows that the records are obtained in the same standard for each experimental group. After the pre-tensions that were proper to bring each diaphragm muscle to its own optimum length were applied to the muscle, single stimulant (twitch) (T_W) and 5, 10, 20, 40, 60, 80, 100 and 150 Hz frequency stimuli were sent to the muscle with 5-min intervals, and the maximum contraction force responses for each stimulus protocol were recorded (Table 2). When values of maximum contraction force were compared among the groups, no statistically significant differences were detected ($p > 0.05$).

The contraction times of the recorded pulse curves applied as one single square pulse (1 ms time and

Table 3. Values for the duration (ms) to reach the maximum pulse forces as a result of stimuli at different frequencies in the diaphragm muscle mechanical activity.[c]

	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
5 Hz	302.53 ± 14.18	283.44 ± 31.72	281.30 ± 26.27	261.33 ± 42.06
10 Hz	297.83 ± 36.87	351.43 ± 142.42	241.44 ± 134.5	153.13 ± 67.94[b]
20 Hz	301.17 ± 111.2	195.29 ± 77.67	180.75 ± 105.78[a]	142.14 ± 73.51
40 Hz	352.37 ± 188.3	369.37 ± 165.6	119.54 ± 21.96[a]	223.44 ± 201.6
60 Hz	478.48 ± 53.41	414.06 ± 157.6	327.68 ± 172.8	260.36 ± 200.6
80Hz	473.13 ± 68.58	417.88 ± 139.0	312.96 ± 177.0	273.57 ± 210.1
100 Hz	366.83 ± 70.82	312.43 ± 85.78	266.00 ± 135.58	226.14 ± 160.21
150 Hz	439.67 ± 121.9	336.86 ± 144.77	301.50 ± 128.58	254.29 ± 206.25

ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure.
[a]$p < 0.05$, significantly different from control female.
[b]$p < 0.001$, significantly different from control male.
[c]Values are means + standard deviation.

Table 4. Values for P_i/P_0 rates, which are among diaphragm muscle mechanical activity parameters.[a]

P_i/P_0	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
P_{TW}/P_0	0.44 ± 0.24	0.49 ± 0.54	0.45 ± 0.30	0.49 ± 0.21
$P_{5\ Hz}/P_0$	0.48 ± 0.23	0.46 ± 0.45	0.42 ± 0.26	0.50 ± 0.22
$P_{10\ Hz}/P_0$	0.59 ± 0.29	0.50 ± 0.39	0.49 ± 0.27	0.54 ± 0.20
$P_{20\ Hz}/P_0$	0.75 ± 0.39	0.54 ± 0.30	0.54 ± 0.24	0.58 ± 0.17
$P_{40\ Hz}/P_0$	1.02 ± 0.27	0.83 ± 0.41	0.79 ± 0.22	0.86 ± 0.16
$P_{60\ Hz}/P_0$	1.10 ± 0.19	1.02 ± 0.39	0.99 ± 0.17	1.01 ± 0.11
$P_{80\ Hz}/P_0$	1.11 ± 0.19	0.98 ± 0.16	1.03 ± 0.14	1.05 ± 0.09
$P_{100\ Hz}/P_0$	1.08 ± 0.17	0.97 ± 0.11	1.03 ± 0.13	1.05 ± 0.10
$P_{150\ Hz}/P_0$	1.02 ± 0.06	1.00 ± 0.06	1.05 ± 0.05	1.05 ± 0.09

i: T_w, 5, 10, 20, 40, 60, 80, 100 and 150; T_w: The pulse that occurs with one single stimulant; P_i: the force that is created to the stimulant at different frequencies (T_w, 5, 10, 20, 40, 60, 80, 100 and 150 Hz); P_0: the force that is created to the stimulant at 150 Hz; ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure.
[a]Values are means + standard deviation.

supramaximal amplitude) were measured (Table 3). In male rats, it was found that low-frequency MF exposure reduced the maximum contraction force time obtained as a result of 10 Hz stimulant at a statistically significant level when compared with the control male group ($p < 0.001$). Meanwhile, it was also determined that the maximum contraction force time obtained as a result of 20 and 40 Hz stimuli applied in female rats was reduced at a statistically significant level as a result of EMF exposure when compared with the control group ($p < 0.05$). The maximum contraction force values (P_i) obtained for each frequency applied were normalized by dividing by the maximum contraction force value obtained by giving 150 Hz (P_0) (Table 4). No statistically significant significance was detected between the groups for the parameters aside from the maximum contraction time (ms), which is among the parameters that were evaluated to determine the mechanical activity characteristics.

Biochemical evaluation

The effects of the ELF-EMF on Na^+, K^+, Cl^- and Ca^{+2} levels in the blood serum of rats. It was determined in female rats that long-term ELF-EMF exposure increased the K^+ and Ca^{+2} levels at a statistically significant level ($p < 0.01$). In male rats, on the other hand, it was determined that long-term ELF-EMF exposure decreased the Ca^{+2} levels at a statistically significant level when compared with the rats in the control group ($p < 0.01$). As a result of ELF-EMF, when the change in Ca^{+2} levels are compared as based on the gender variable, it was found to be at a lower level in male rats at a statistically significant level ($p < 0.001$) (Table 5).

Table 5. The mean and standard deviation values of the groups for Na^+, K^+, Cl^- and Ca^{+2} measured in the serum.[d]

	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
Serum Na^+(mEq/l)	139.00 ± 2.89	140.00 ± 3.70	138.37 ± 2.72	139.29 ± 1.38
Serum K^+(mEq/l)	4.34 ± 0.33	5.55 ± 0.47	5.09 ± 0.48[a]	5.29 ± 0.45
Serum Ca^{+2}(mEq/l)	5.32 ± 0.15	5.51 ± 0.31	5.78 ± 0.15[a]	5.16 ± 0.15[b,c]
Serum Cl^- (mEq/l)	103.00 ± 3.37	101.00 ± 4.08	99.38 ± 2.39	100.14 ± 1.68

ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure.
[a]$p < 0.01$, significantly different from control female.
[b]$p < 0.01$, significantly different from control male.
[c]$p < 0.001$, significantly different from ELF-EMF female.
[d]Values are means + standard deviation.

Table 6. The values of the TOS and TAS both in the diaphragm muscle tissue and in the blood serum for all groups.[c]

	Control female ($n = 7$)	Control male ($n = 7$)	ELF-EMF female ($n = 8$)	ELF-EMF male ($n = 7$)
TISSUE_TAS (mmol Trolox Eq/l)	0.49 ± 0.17	1.08 ± 0.17	0.68 ± 0.16	0.56 ± 0.17[a]
SERUM_TAS (mmol Trolox Eq/l)	1.11 ± 0.05	0.91 ± 0.05	0.92 ± 0.05[b]	1.02 ± 0.05
TISSUE_TOS (mmol H_2O_2 Eq/l)	4.96 ± 0.82	4.95 ± 0.82	4.38 ± 0.77	6.92 ± 0.82
SERUM_TOS (mmol H_2O_2 Eq/l)	3.10 ± 1.50	5.07 ± 1.50	5.28 ± 1.41	4.51 ± 1.50

ELF-EMF: 50 Hz extremely low-frequency electromagnetic field exposure; TOS: total oxidant status; TAS: total antioxidant status.
[a]$p < 0.05$, significantly different from control male.
[b]$p < 0.01$, significantly different from control female.
[c]Values are means + standard deviation.

TOS and TAS both in the diaphragm muscles and in the blood serum of the rats. No statistically significant differences were determined between all the experimental groups when the total oxidant activity, which was measured both in the tissue and in the serum were compared among the groups. It was found that the total antioxidant value measured in the diaphragm muscle in the male rats reduced at a statistically significant level as a result of being exposed to ELF-EMF ($p < 0.05$). In female rats, it was observed that the exposure to ELF-EMF reduced the total antioxidant values in serum at a statistically significant level ($p < 0.01$) (Table 6). In addition, the Na-K ATPase activity levels measured in the diaphragm muscles of the rats did not show any statistically significant differences when measured among the groups.

Electron microscopic evaluation

When the diaphragm tissues of the rats were examined under the electron microscope, it was determined that the muscle cells had normal morphological characteristics. It was seen that the myofibrils in the sarcoplasm showed a regular order, the sarcomere structure was preserved, the mitochondria among the myofibrils, the cisterna of the sarcoplasmic reticulum (SR) and other organelles were in normal structure (Figure 5).

Discussion

It is well known that an ELF-EMF can cause substantial changes at the cellular level. For this purpose, in this study, it was recorded that the resting membrane potential and intracellular muscle AP of diaphragmatic muscle cells exposed to ELF-EMF using microelectrode recording method. However, the sinusoidal ELF-EMF (50 Hz frequency and 1.5 mT magnetic flux density 5 days in each week during the 7 months) applied to diaphragm muscle isolated from rats in our study did not create a statistically significant change in the resting membrane potential of the male and the female MF groups according to their own control groups. In a study, the resting membrane potential of the diaphragm muscle isolated from rats chronically exposed to static MF of 20 mT magnetic flux density was measured as -72.8 ± 0.4 mV and compared to the control group (-76.5 ± 0.6 mV), this value was an increase in the depolarizing direction and statistically significant (Itegin et al., 1995). In

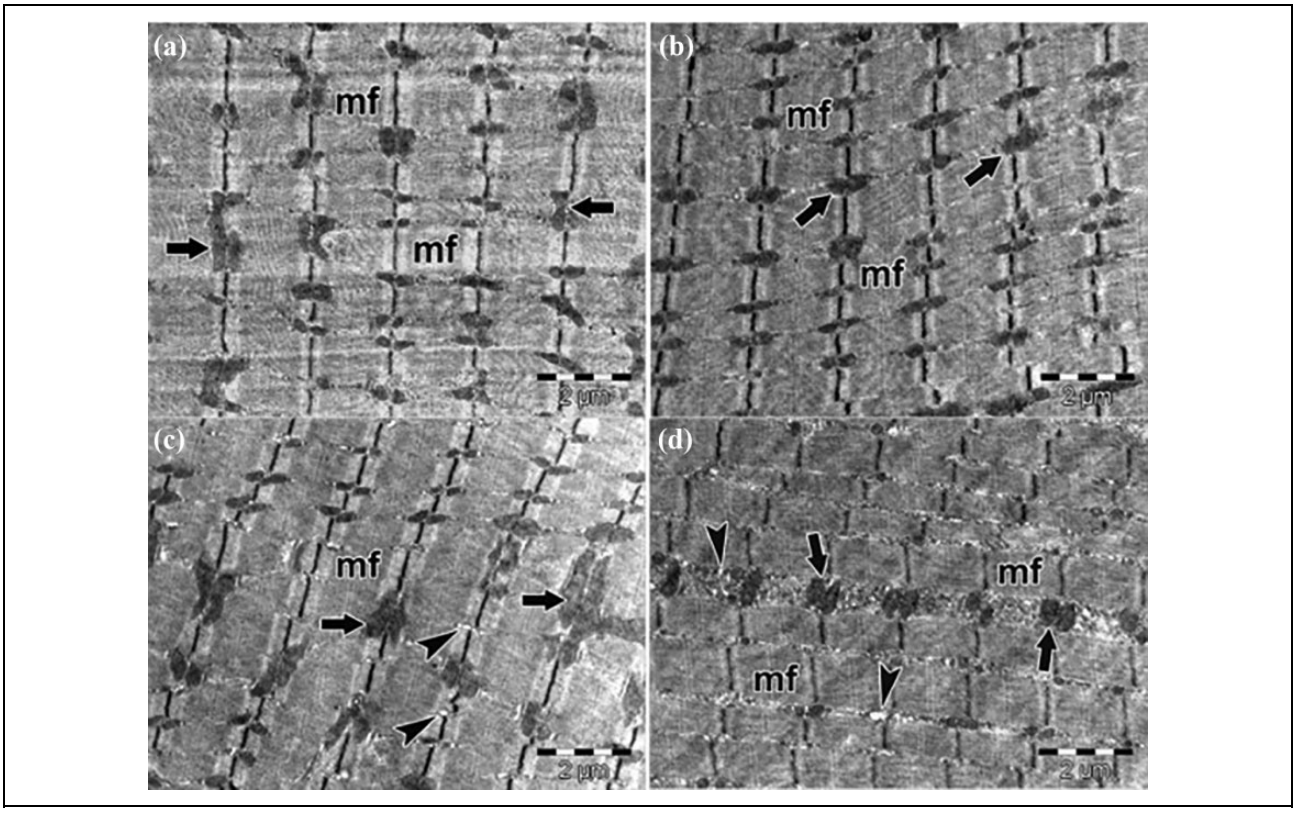

Figure 5. It is seen that the muscle cells have normal morphological characteristics and the mf have a regular order. The mitochondria (the arrow) and SR cisterna (arrow-head). ×12,000. (a) Control female, (b) control male, (c) ELF-EMF female and (d) ELF-EMF male. mf: myofibrils; SR: sarcoplasmic reticulum.

another study in which modulated MF (50 Hz frequency, 5 mT magnetic flux density) was applied for 4 weeks to diaphragm muscle isolated from rats, it has been reported that the resting membrane potential of the group treated with ELF-EMF was depolarized according to the control group (Demirkazık, 2005). But no significant difference in the amplitude of AP has been determined. Therefore, it has been suggested that exposure to modulated ELF-EMF may have affected the Na^+-K^+ pump and/or K^+ channel kinetics, thereby reducing resting membrane potential (membrane depolarization) in the mentioned studies. In our study, resting membrane potential was not depolarized significantly by ELF-EMF exposure, in contrast to other studies with high magnetic flux density. In addition, in our study, the Na-K ATPase activity levels measured in the diaphragm muscles of the rats did not show any statistically significant difference when measured among the groups. These results may suggest that K^+ channel kinetics and/or Na^+-K^+ pump can be affected only by EMF exposure with high magnetic flux density.

Skeletal muscles contain a large number of K^+ channels, each of which plays a different role in the regulation of cellular electrophysiological function and whose activation processes are regulated by voltage, calcium or adenosine triphosphate (ATP) (van Lunteren et al., 2001). Therefore, K^+ channels play key roles in regulating skeletal muscle contractility, including that of the diaphragm. They act in concert with Cl^- channels to determine resting membrane potential, thereby controlling membrane excitability and hence the propensity for muscle activation. In addition, they act together with Na^+ channels to determine the height and duration of intracellular APs, thereby controlling the extent and time course of Ca^{2+} release from the SR and hence force generation (van Lunteren et al., 2001).

Also, in our study, when intracellular muscle AP curves recorded from diaphragmatic muscle cells exposed to ELF-EMF using the microelectrode recording method were analysed, it was found that there was no difference between the control and the experimental groups in terms of amplitude and overshoot parameters of AP. However, in our study, the

prolongation in the peak latency and duration of depolarization in male rats exposed to ELF-EMF according to the control group suggests that Na^+ channel kinetics may be affected by ELF-EMF. This means that the mean open time of Na^+ channels was increased and the ensemble average showed a prolonged inward Na^+ current. This prolonged inward Na^+ current could cause repetitive APs and the clinical syndrome. Voltage-gated Na^+ channels are responsible for the depolarization phase of the AP as well as from the duration of the AP and hence the release of Ca^{2+} from the SR, thus controlling the formation of force (van Lunteren et al., 2001; van Lunterenand Manubay, 2001). In our study, the total antioxidant values of male and female rats in diaphragm muscle or serum, respectively, were found to be decreased by exposure to ELF-EMF. This result may be interpreted that antioxidants are used to improve a partial change in bioelectric activity (peak latency, depolarization duration, current intensity and area) of muscle cells caused by EMF and thus muscle mechanical activity was not affected in any group.

It has also been reported that as latency increases, amplitude and overshoot values of muscle AP decrease statistically significantly in the study performed on the diaphragm exposing to 20 mT (200 Gauss) magnitude of MF mentioned above (Itegin et al., 1995). The prolongation in the peak latency in male rats exposed to EMF in our study is similar to the above study.

In our study, the effects of chronic exposure to ELF-EMF on the mechanical activity (contraction and relaxation) of rat diaphragm muscle were also investigated, and it was observed that muscle mechanical activity was not affected in any group. As a result of the literature review, it was found that only a few studies examined the effects of ELF-EMF on the mechanical activity of rat diaphragm muscle. In a study examining the effects of chronically applied static MF (200 Gauss) on biomechanical responses of the isolated rat diaphragm muscle, force of muscle twitch was found to decrease significantly in the MF-exposed group. This finding is attributed to the enhancing effect of the MF on Ca^{2+}-ATPase activity (Itegin et al., 1995). The calcium pump (also known as, Ca^{2+}-ATPase or Sarcoendoplasmic reticulum calcium transport ATPase (SERCA) is a membrane transport protein ubiquitously found in the endoplasmic reticulum of all eukaryotic cells. As a calcium transporter, SERCA maintains the low cytosolic calcium level that enables a vast array of signalling pathways and physiological processes (e.g. synaptic transmission, muscle contraction and fertilization). In muscle cells, SERCA promotes relaxation by pumping calcium ions from the cytosol into the lumen of the SR, the main storage compartment for intracellular calcium (Primeau et al., 2018). In another study that investigated the effect of MF on the isometric contraction properties of soleus and extensor digitorum longus muscles in rats, it has been reported that MF increases both muscle contraction forces compared to control values (Pelit et al., 2008). Also, MF decreased the contraction time of the two muscles of rats. In addition, the isometric contraction forces obtained by different stimulating frequencies (10, 20, 50 and 100 Hz) showed a significant linear increase in the tetanic contraction. On the other hand, Stefl et al. (2006) investigated a potential effect of homogeneous sinusoidal MF (50 Hz, 10 mT) on bioenergetics (ATP, creatine phosphate, creatine, lactate, pyruvate and inorganic phosphate) of rat skeletal muscle and they reported that neither repeated exposure nor the acute exposure of rats to the sinusoidal MF has any important influence on the level of important energy metabolites in rat skeletal muscle.

In our study, it was observed that muscle mechanical activity was not affected in any group. But it was found that ELF-EMF exposure has decreased the duration (ms) to reach the maximum pulse forces as a result of stimuli at 20 and 40 Hz frequencies in the female rat muscle mechanical activity. Also, ELF-EMF exposure has decreased the duration (ms) to reach the maximum pulse forces at only 10 Hz frequency stimuli in the male rat muscle mechanical activity. In addition, in our study, as a result of histological examinations, diaphragmatic muscle cells in experimental groups had normal morphological features. The mitochondria among the myofibrils, the cisterna of the SR and other organelles were detected as normal. This result may be due to the use of lower intense MF in our study compared to studies in which muscle mechanical activity after MF exposure is affected. Moreover, many studies demonstrated different biological effects of low-frequency MFs during different exposure times. This indicates that exposure information (induction, frequency and exposure time) should be similar for comparison and assessment of the possible effect of MF. Different study groups have used different experimental settings of EMFs, including different frequencies, field intensities, total exposure times and waveforms. In our study, the total exposure time to the sinusoidal ELF-EMF of a rat

began immediately after birth and continued until the seventh month (adulthood). There has been no study of applying such a long exposure time.

Conclusion

In this study, changes in electrical and mechanical activity in skeletal muscle as a result of ionic movements in cells under the cumulative effect of long-term ELF-EMF exposure were evaluated in rats according to gender. While chronic exposure to ELF-EMF does not have an effect on the histological structure and mechanical activity of the diaphragm muscle, it can be said that it causes a partial change in some bioelectrical activity parameters. It was found that MF has a different effect on some bioelectric parameters according to gender. In the light of the findings of this study, magnetic flux density and duration of exposure to ELF-EMF should be probably increased in the next studies. Thus, perhaps the possible pathological reflections of the partial change in muscle bioelectric activity of ELF-EMF are likely to be seen in the histological structure and mechanical activity of the muscle. In this way, the idea of whether ELF-EMFs may be a potential factor in the pathogenesis of certain muscle diseases, including respiratory disease and skeletal muscle degenerative diseases, can be discussed.

Author contributions

SG and BB contributed to study design. SG and SY conducted the surgical procedures. SG, BB, SY and CHT performed mechanogram recordings and *in vitro* bioelectrical activity recordings by microelectrode in diaphragm muscle of rats and analysed that results. EB and GB performed ultrastructural analysis with electron microscopy and participated in the interpretation of the ultrastructural analysis results. BC performed biochemical analysis and participated in the interpretation of the biochemical analysis results. DO performed the statistical analysis. SG (head author), BB (corresponding author) and CHT wrote the manuscript. All authors read and approved the final manuscript.

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