Cell Biochem Biophys (2011) 59:71–77
DOI 10.1007/s12013-010-9113-0

ORIGINAL RESEARCH

Oxidative Stress and Apoptosis in Relation to Exposure to Magnetic Field

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Published online: 8 September 2010

Abstract We investigated the effect of extremely low-frequency electromagnetic field (ELF-EMF) with pulse trains exposure on lipid peroxidation, and, hence, oxidative stress in the rat liver tissue. The parameters that we measured were the levels of plasma alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase as well as plasma albumin, bilirubin, and total protein levels in 30 adult male Wistar rats exposed to ELF. We also determined the percentage of apoptotic and necrotic cells of the kidney extracts from the animals by flow cytometry method. Apoptotic cell death was further characterized by monitoring DNA degradation using gel electrophoresis. The results showed an increase in the levels of oxidative stress indicators, and the flow cytometric data suggested a possible relationship between the exposure to magnetic field and the cell death. We showed significantly lower necrotic cell percentages in experimental animals compared to either unexposed or sham control groups. However, DNA ladder analyses did not differentiate between the groups. Our results were discussed in relation to the response of biological systems to EMF.

Keywords Electromagnetic field · Lipid peroxidation · Antioxidant enzymes · Apoptosis · DNA fragmentation

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Introduction

Biological systems respond to a wide range of electromagnetic field (EMF); however, the mechanism of the EMF's influence on biological structures remains to be clarified. Most of the effects reported so far indicated that the majority of EMF was tolerable by the living organisms without detectable detrimental effects [1]. On the other hand, a number of epidemiological studies have suggested an increased risk for developing cancers, particularly leukemia, brain, and breast cancer upon residential or occupational exposure to EMFs [2, 3]. Biochemical studies showed a considerable disruption in the metabolism of carbohydrates, lipids, and proteins as reflected by altered blood glucose levels as well as accelerated glycolysis and glycogenolysis [4]. EMFs were reported to influence enzyme action, signal transduction, protein synthesis, and gene expression, which all have important roles in regulating cell growth [5]. Exposure to extremely low-frequency pulsed electromagnetic fields (ELF-PEMFs) caused an increased oxidative stress in chick embryos [6], mammalian cultured cells [7], and human erythrocytes [8]. The increased oxidative stress involves oxidative DNA damage and lipid peroxidation [9] with an ultimate effect of on a number of systemic disturbances and cell death [10, 11].

Cell viability of unicellular organisms in response to EMF was investigated by a number of researchers [1, 12]. However, a report, on the systematic comparison of different forms of cell death: necrosis, apoptosis, and autophagy, does not exist. Although these three forms of cell death share common molecular effectors and signaling routes, apoptotic cell death differs from necrosis and autophagy by a number of morphological and biochemical characteristics [13]. Necrosis is accompanied by swelling and lysis with an ultimate consequence of inflamantation. Apoptosis, on the other hand, is a highly conserved and tightly regulated inducible cell response that reveals chromatin condensation without a change in cellular integrity [14]. Apoptotic bodies formed at the surface of apoptotic cells are recognized by fagocytic cells.

In this study, we investigated whether EMF with pulse trains exposure causes lipid peroxidation and, hence, oxidative stress in the rat liver tissue. We measured the levels of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities as well as plasma albumin, bilirubin, and total protein levels. We also determined the percentage of apoptotic and necrotic cells of the kidney extracts from female Wistar rats by flow cytometry method. Apoptotic cell death was further characterized by monitoring DNA degradation using gel electrophoresis.

Materials and Methods

Reagents

All reagents used were of analytic grade and were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents were purchased from Merck (México).

Animals

Male Wistar-Albino rats, weighing from 190 to 220 g were randomly divided into control (C; $n = 10$) and EMF-exposed (EMF; $n = 10$) groups. A sham exposure was also performed under the same environmental conditions using another apparatus including only the pair of Helmholtz coil, in a Faraday cage. The animals in this group were not exposed to electrical transients when the field was turned on. The animals were maintained in normoxic conditions and were housed in groups of four in plexyglass cages at 23 ± 2°C under a 12–12 h light–dark cycle with lights on from 8 a.m. to 8 p.m. with free access to basal diets and water. All the animals received care according to the criteria outlined in the "Guide for Care and Use of Laboratory Animals" prepared by the Natural Academy of Science, also adopted and promulgated by Cukurova University.

ELF-PEMF Exposure System and Field Characteristics

ELF-PEMF was generated in a pair of Helmholtz coil of 60 cm diameter and 30 cm distance [15]. The coils were placed in a Faraday cage (90 cm × 90 cm × 50 cm) with grounded shielding to prevent environmental electromagnetic interaction (Fig. 1a). Helmholtz coils were connected to a power supply and a microprocessor-controlled frequency pulse generator (ILFA Electronic, Adana, Turkey). The intensity of the magnetic field was measured by a Hall-effect probe of a Gauss meter (FW Bell model 6010, Sypris/Tesla meter). The time-varying PEMF consisted of quasi-triangular waveform, a rise time of 0.5 ms, and a fall time of 9.5 ms [15]. The wave form of induced electric field, measured by a search coil probe (50 turns of 30 gauge soft copper wire) with an internal diameter of 50 mm was placed in the core-center axis of the Helmholtz coils. The corresponding induced electric field was a unipolar rectangular wave form having peak electric fields of 0.6 V/m in plexyglass cage located in between the Helmholtz coils. The shape of the induced voltage wave forms was measured with a search coil by transferring to osilloscope. The distribution of the magnetic density was measured using a Gauss Meter, and the density was homogeneous within 5% difference in the exposure area (plexyglass cage: 30 cm long, 30 cm wide, and 20 cm high). Shapes of observed induced potential and current pulses outgoing from generator were found to be identical.

The animals were put into a 30 × 30 × 20 cm^3 plexyglass cage and housed in the core-center of the Helmholtz coils. The cage with perforation to permit air passage for breathing was used. Each animal was placed in a plexyglass cage located between the Helmholtz coils, and was subjected to a whole-body exposure to PEMF for 1 h every day. Pulsed square-wave magnetic field was applied with consecutive four pulse trains of 1-, 10-, 20-, and 40-Hz frequency at an intensity of 1.5 mT. Each frequency train was given 4-min and 1-min intervals between each frequency (Fig. 1b). PEMF-exposed rats were exposed to a vertical and pulsed square-wave magnetic field for 1 h/day for 30 days, whereas the control rats were treated in the same way without exposure to the magnetic field. Following 10-day acclimatization to laboratory conditions, PEMF was carried out in a silent room with a temperature of 21–25°C. After 30 days of PEMF exposure, the rats were killed under Xylazine (Rompun®; Bayer, Leverkusen, Germany) and Ketamine (Ketalar®, Eczacibasi, İstanbul, Turkey) anesthesia, and the tissue samples of liver

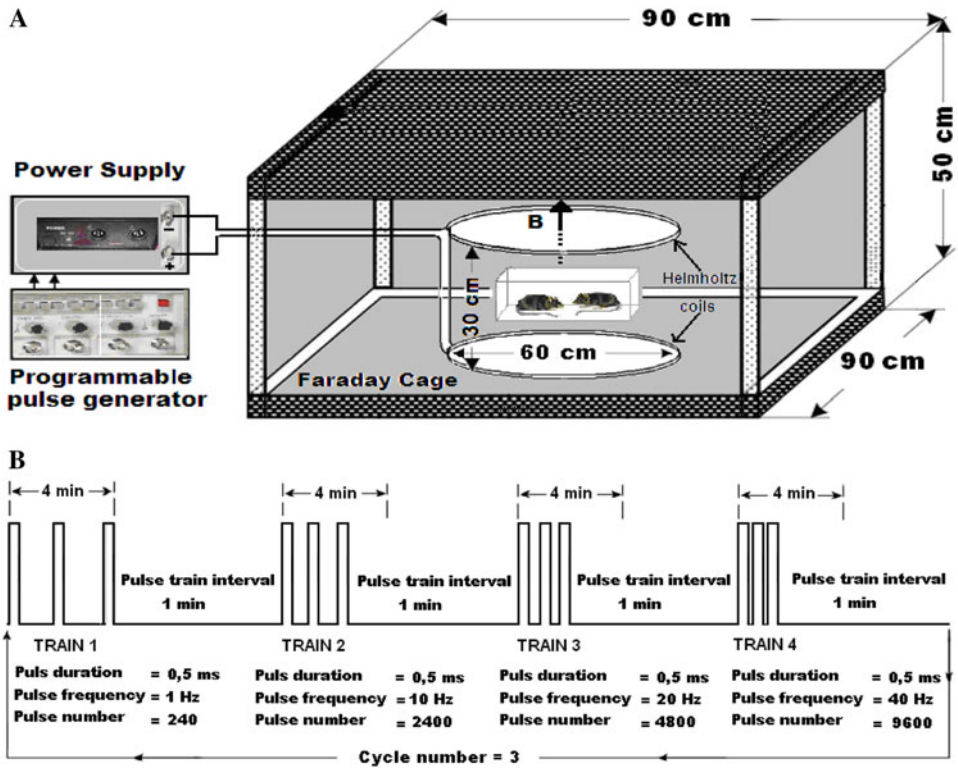

Fig. 1 Extremely low-frequency square pulsed electromagnetic field exposure system and pulse protocol for the rats. **a** ELF-PEMF was generated in a pair of Helmholtz coils, 60 cm in diameter, with 100 turns of 18 gauge (1.3 mm in diameter) insulated soft copper wire each in a Faraday cage. A pulse generator produced magnetic field amplitude of 1.5 mT in Helmholtz coils, and a digital timing device, programmable pulse generator, controlled the timing. **b** Schematized pulsed electromagnetic fields application protocol

and kidney of each animal were harvested and stored at −70°C.

Blood Sampling Protocol

Blood samples (~1 ml) were collected by cardiac puncture using sterile needle and syringe to be used in the liver function tests for measuring ALT, AST, and ALP, albumin, bilirubin, and total protein levels. Serum was prepared by transferring the blood sample into a clean, sterile centrifuge tube. The sample was centrifuged at 3,000×*g* for 5 min, and the supernatant serum sample removed by aspiration using a Pastuer pipette. The sample was then transferred into a sterile container and stored at −4°C until further analysis. All the samples were analyzed by a Cobas Integra 800 biochemical automatic analyzer (Roche Diagnostics, GmbH, Mannheim, Germany).

Preparation of Liver Homogenates for Enzymatic and Protein Assays

The liver tissues were rapidly excised and placed into ice-cold homogenization medium (HM). The tissues were weighed, finely minced, washed with HM rinsed with ice-cold deionized water, and then dried using filter paper. Fractions of tissues (~500 mg) were gently homogenized (20% w:v) in buffer (Tris–HCl 10 mmol/l, EDTA 1 mmol/l, PMSF 1 mmol/l; pH 7.5) using a glass Potter–Elvehjem homogenizer set. The homogenates were centrifuged at 1,000×*g* for 10 min and recentrifuged at 13,000×*g* for 20 min at +4°C [16].

Determination of Oxidative Stress Parameters

Tissue specimens of 50 mg was homogenized in 0.15 mol/l KCI for malondialdehyde (MDA) assignation. After the homogenate was centrifuged at 3,000 rpm, the MDA levels in 50 µl of tissue homogenate were determined by thiobarbituric acid (TBA) reaction. The measurements of MDA concentration and superoxide dismutase (SOD) activities were carried out as described [17, 18]. Protein concentration was determined according to Lowry method [19].

Homogenization of Kidney Extracts and Flow Cytometric Analyses

The kidney tissue samples were minced into pieces of approximately 1 mm^3 in phosphate buffered saline (PBS). Blood samples were removed with rinsing in the homogenization buffer. Detection of apoptotic cells was performed by annexin/propidium iodide (annexin/PI) staining. Cells (5×10^6) were reacted with fluorescein isothiocyanate (FITC)—labeled Annexin V (Immunotech, Marseille, France) in the presence of PI and analyzed by flow cytometry according to the manufacturer's instructions. Apoptotic cells were monitored as Annexin-V positive,

whereas the cells stained with propidium iodide (PI) were characterized as necrotic cells. The ratios of living cells, apoptotic cells, and necrotic cells were determined.

DNA Fragmentation Assay

Apoptotic degradation of DNA was analyzed by agarose gel electrophoresis using Apoptotic DNA Ladder Extraction Kit (BioVision, Bethesda, MD, USA). Isolated DNA samples were run on 1. 2% agarose gel containing 0. 5 μg/ml Ethidium bromide (Etd-Br) (5 V/cm) [20]. Electrophoresis was conducted until the bromophenol blue dye had migrated 75% down the gel. DNA was visualized by trans-illumination under UV light (Vilber Lourmat) and photographed. To check the reproducibility, all the reactions were repeated at least once more.

Data Presentation and Statistical Analysis

Data were reported as the mean ± SD. Statistical significance of the differences between means was assessed using the ANOVA test followed by the Bonferroni post-hoc test and paired Student's *t*-test. The levels of significance were set at $P < 0.001$ and $P < 0.05$. The data were analyzed using SPSS, statistical Package (Version 12).

Results

This study was designed to investigate whether ELF with pulse trains EMF exposure causes lipid peroxidation and, hence, oxidative stress in the rat liver tissue. The results of liver function tests covering plasma ALT, ASP, and ALP levels in three animal groups are shown in Fig. 2. As seen in Fig. 2, the levels of the three enzymes in the MF group were significantly higher than in either C or SH groups ($P < 0.001$), whereas these parameters were comparable within C and SH groups. The activity of ALT increased from 44.8 U/l (the control value) to 70.1 U/l upon exposure to PEMF. Likewise, ALP and AST levels increased by 25% and 35%, respectively, with the applied field intensity of 1.5 mT (Fig. 2).

Figure 3 shows serum albumin, bilirubin, and total protein levels in C, SH, and MF groups. Considering the C and SH groups, there was a significant increase in the levels of serum albumin, bilirubin, and total protein by 24, 44,

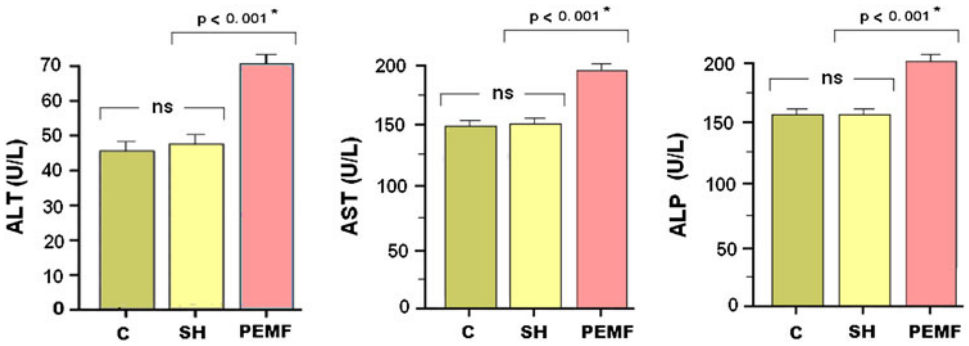

Fig. 2 Plasma ALT, AST, and ALP levels in control group, sham (non-exposed PEMF) control, and pulse trains PEMF-exposed groups ($n = 10$, $N = 2$). Data were presented as the mean ± SD. Statistical significance of the differences between means was assessed using the ANOVA test followed by the Bonferroni post-hoc test. The level of significance was set at * $P < 0.001$; $n = 10$, $N = 2$. *n* number of animals, *N* number of replication. *ns* not significant

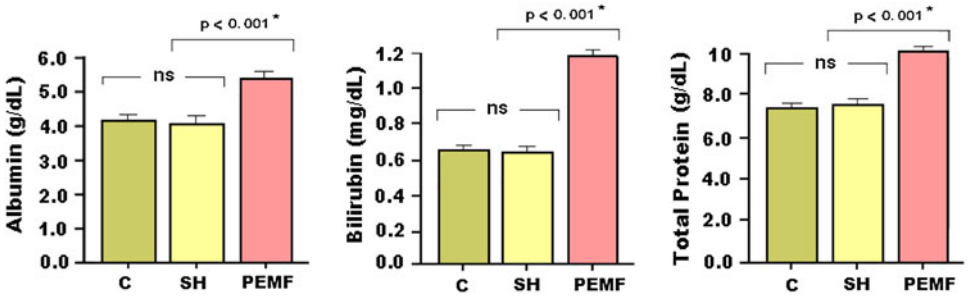

Fig. 3 Plasma albumin, bilirubin, and total protein levels in control, sham, and PEMF-exposed group rats. Data were presented as the mean ± SD. Statistical significance of the differences between means was assessed using the ANOVA test followed by the Bonferroni post-hoc test. The level of significance was set at *$P < 0.001$; $n = 10$, $N = 2$. *n* number of animals, *N* number of replication. *ns* not significant

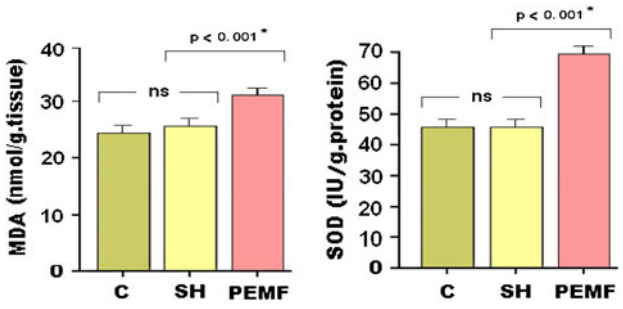

Fig. 4 Effects of pulse trains PEMF exposure on MDA concentration and SOD activity in the liver samples ($n = 10$, $N = 2$). Statistical evaluation was carried out as indicated in Fig. 2 legend (*n* number of animals, *N* number of replication. *ns* not significant)

and 25%, respectively, following a PEMF exposure at the same intensity ($P < 0.001$) (Fig. 3).

We also investigated whether ELF with pulse trains PEMF exposure causes lipid peroxidation and, hence, oxidative stress in the rat liver tissue. For this purpose, we addressed possible effects of the long-term exposure to ELF with square pulse trains PEMF on MDA and SOD in the liver homogenates and liver enzyme levels. The results showed that the lipid peroxidation increased from 24.1 ± 2. 2 nmol/g fresh tissue MDA (the control value) to 30. 3 ± 3.4 nmol/g fresh tissue MDA (Fig. 4). On the other hand, the concentration of SOD increased from 44. 5 ± 7.1 IU/g protein (the control value) to 68.6 ± 8.4 IU/g protein following the magnetic field intensity of 1.5 mT (Fig. 4). Augmentation of lipid peroxidation in liver tissue was observed after 1.5 mT PEMF exposure period of 1 h/day for 30 days. These alterations for SOD levels in PEMF-exposed group was statistically significant ($p < 0.001$). Significant difference was not found between the control and the sham-exposed control groups in lipid peroxidation level and antioxidant enzyme activity ($p > 0.001$).

We next aimed to determine the percent ratios of apoptotic and necrotic cells among the three groups (Table 1) in a selected number of tissue samples. As seen in Table 1, there was not any statistically significant difference among viable cell percentages of the groups. Likewise, apoptotic cell percentages of the control, sham, and experimental animals were comparable to each other. However, necrotic cell percentage of the group exposed to magnetic field was significantly lower than those of the other groups ($P < 0.05$).

Because the DNA ladder formation is a characteristic property of apoptosis, we then monitored DNA fragmentation from the isolated DNA samples [14]. Among the ladder-detecting procedures, visualization of DNA fragmentation on agarose gel is an easy and rapid way of monitoring DNA ladders. The method we employed for DNA isolation specifically extracted laddered DNA, not genomic DNA. A representative assay result is illustrated

Table 1 The average percent ratios of viable, apoptotic, and necrotic cells obtained in control ($n = 6$, $N = 2$), and PEMF-exposed ($n = 6$, $N = 2$) groups

Cell status (%)	Control group ($n = 6$, $N = 2$)	PEMF-exposed group ($n = 6$, $N = 2$)
Viable	97.02 ± 1.24	98.73 ± 1.45
Apoptotic	0.10 ± 0.00	0.21 ± 0.01*
Necrotic	0.60 ± 0.11	0.42 ± 0.12*
P		<0.05

Data were reported as the percent ratios. All values were expressed as % means ± % SD. Statistical analysis was performed using the paired Student's *t*-test. The level of significance was set at $P < 0.05$; $n = 10$, $N = 2$

n number of animals, *N* number of replication, *PEMF* pulsed electromagnetic field

* $P < 0.05$ vs. control group

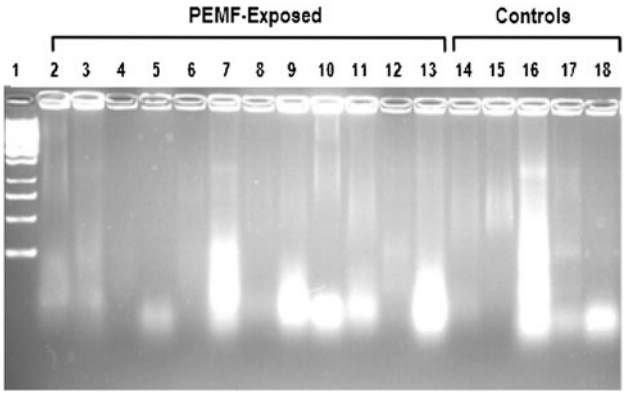

Fig. 5 A representative agarose gel analysis of DNA fragmentation. *Lane 1* 1 kb DNA ladder. Representative DNA samples from the selected PEMF-exposed animals and unexposed controls were loaded onto the wells of 2–13 and 14–18, respectively. 1 kb. DNA ladder was loaded onto the *lane* 1

in Fig. 5. As seen in Fig. 5, we detected a partial DNA fragmentation both in a selected number of experimental (Fig. 5, lanes 2–11) and non-exposed groups (Fig. 5, lanes 12–18). There was an overload DNA onto agarose in lanes 7, 9–11, 13, and 19 when the lanes were compared next to each other. Quantitative analyses of DNA band intensities using densitometer did not give rise to a significant difference in banding pattern among the animals exposed or unexposed groups (data not shown).

Discussion

In this study, we investigated the influence of PEMF on liver function tests, lipid peroxidation, and antioxidant enzyme in rats as well as viable, apoptotic, and necrotic cell percentages. The major findings were that the PEMF

exposure induced MDA, ALT, AST, ALP, SOD, and plasma antioxidant protein (albumin and bilirubin) levels. These are specific liver enzymes that increase in hepatic diseases and toxic damage of liver cells [21]. The elevation in the levels of plasma albumin, bilirubin, and total protein, observed in this study, may result from the damaged cells which leak into circulation after exposure to alternative magnetic field [22]. We also demonstrated that the PEMF exposure is associated with a high level of bilirubin and MDA compared to control rats. Elevated MDA is an indicator of lipid peroxidation in liver.

Moreover, increased SOD activity, detected in liver samples of EMF-exposed rats, can be interpreted as a compensation to the increased anion formation. Increased plasma bilirubin level in PEMF-exposed group could also be a compensatory/retaliatory phenomenon in response to cellular peroxidative changes. This is because bilirubin functions as a powerful antioxidant, anti-mutagen, and an endogenous tissue protector in vivo [23]. Thus, there is a positive correlation between MDA content and plasma bilirubin level, which supports the study findings of Pratibha et al. [23]. On the contrary, another group of authors did not observe any significant modification in plasma bilirubin level in rats under the effect of long-term PEMF. This discrepancy may be explained by the intensity, duration, and the shape of the pulse/configuration of the exposure [24]. We also showed that a significant increase in ALP activity after exposure to PEMF ($P < 0.001$), which were in accordance with the findings of Kula et al. [25] showing an elevation of AST and ALT concentrations in steelworkers exposed to EMF. Similarly, other researchers have reported that the EMF induced significant elevations in the levels of plasma AST and ALT concentrations.

ELF-PEMF can also affect the physiological processes and enhance the production of free radicals [26]. These effects can induce changes in chemical signals, and unwanted effects may be produced at the molecular and biochemical levels in cells [27]. This observation supports the hypothesis that the physiological equilibrium state of a biological system is important to its response to a potentially effective EMF [28]. Our results in PEMF-exposed rats demonstrated that antioxidant status was compromised with important component of the antioxidant defense mechanism being significantly increased (35%). In comparison to the sham-exposed rats, antioxidative defense was sufficient to protect the liver structures against lipid peroxidation exposed to 1.5 mT PEMF.

Although the relationship between magnetic field and apoptosis is rather limiting, this issue has been probed by a number of investigators [1, 29, 30]. Some investigators reported a diminished or delayed apoptosis upon exposure to magnetic field [2, 4]. On the other hand, an elevated apoptosis was reported in thymic cells while neither kidney nor liver cells responded to the applied magnetic field as thymic cells did [3, 5]. In this study, flow cytometry results showed a significantly lower necrotic cell percentages in experimental animals compared to either unexposed or sham control groups. On the other hand, DNA ladder analyses did not differentiate between the groups. Although the effect of magnetic field on apoptosis is not proven to be a clearly established issue, the release of extracellular calcium ion has been proposed as a critical step in the process [5, 6].

Taken together, our results clearly showed an increase in the levels of oxidative stress indicators. The flow cytometric data suggest a possible relationship between the exposure to magnetic field and the cell death, and significantly lower necrotic cell percentages in experimental animals compared to either unexposed or sham control groups were demonstrated in this study. However, DNA ladder analyses did not differentiate between the groups.

Acknowledgment The authors acknowledge with thanks the support provided by the Biochemistry department of Cukurova Medical School by way of valuable contributions in the determination of oxidative stress parameters.

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