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EFFECTS OF EXTREMELY LOW FREQUENCY MAGNETIC FIELD ON THE PARAMETERS OF OXIDATIVE STRESS IN HEART

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Increasing production of free radicals in organisms is one of the putative mechanisms by which a extremely low frequency magnetic field (ELF-MF) may affect biological systems. The present study was designated to assess if ELF-MF applied in the magnetotherapy, affects generation of reactive oxygen species (ROS) in heart tissue and antioxidant capacity of plasma according to its working time. The experiments were performed on 3 groups of animals: group I - control; group II - exposed to 40 Hz, 7 mT, 30 min/day for 14 days (this field is commonly applied in magnetotherapy); group III - exposed to 40 Hz, 7 mT, 60 min/day for 14 days. Control rats were housed in a separate room without exposure to ELF-MF. Immediately after the last exposure, blood was taken from the tail vein and hearts were removed under anesthesia. The effect of the exposure to ELF-MF on oxidative stress was assessed on the basis of the measurements of thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), total free sulphydryl groups (-SH groups) and reduced glutathione (GSH) concentrations in heart homogenates. The total antioxidant capacity of plasma was measured using ferric reducing ability method (FRAP). Exposure to ELF-MF (40 Hz, 7 mT, 30 min/day for 2 weeks) did not significantly alter tissue TBARS, H_2O_2, total free -SH groups, reduced glutathione (GSH) and total antioxidant capacity of plasma. By contrast, ELF-MF with the same frequency and induction but used for 60 min/day for 14 days caused significant increase in TBARS and H_2O_2 concentration ($P<0.01$) and decrease in the concentration of GSH ($P<0.05$) and total free -SH groups in heart homogenates. Moreover, exposure of rats to ELF-MF (40 Hz, 7 mT, 60 min/day for 2 weeks) resulted in the decrease of plasma antioxidant capacity. Our results indicate that effects of ELF-MF on ROS generation in the heart tissue and antioxidant capacity of plasma depend on its working time.

Key words: *extremely low frequency magnetic field, oxidative stress, heart, lipid peroxidation malondialdehyde, thiobarbituric acid reactive substances*

INTRODUCTION

Low frequency magnetic fields are widely applied in electrical appliances and different equipment such as television sets, computers and kitchen appliances. Recently, extremely low frequency magnetic field (ELF-MF) has been considered to be a therapeutic agent and it has started to be more and more commonly used in medicine. Growing interest of researchers has been noticed in biological effects of ELF-MF on target cells and tissues. Extremely low frequency magnetic field can initiate a number of biochemical and physiological alterations in biological systems of different species (1-5).

Biochemical changes in a cell result from membrane potential or trans-membrane ionic transfer which modifies cell functions. Magnetic field can affect chemical bonds between adjacent atoms with consequent production of free radicals (6). Recent findings suggest that ELF-MF can increase free radical life-span of cell (7, 8). Free radicals, presented as reactive oxygen species and reactive nitrogen species (RNS), are causative factors in the oxidative damage of cellular structures and molecules such as lipids, proteins and nucleic acids. In particular, biological membranes that are rich in unsaturated fatty acids are cellular structures susceptible to free radical attack (9). Free radicals react with unsaturated fatty acids in cell membranes promoting a process called lipid peroxidation. Malondialdehyde (MDA) - the end product of lipid peroxidation is a highly toxic molecule implicated in a range of pathologies by producing oxidative damage in tissues. The heart is more vulnerable to free radical damaging effects than other tissues due to high consumption of oxygen.

A living organism has defense systems against different oxidants. These systems, such as antioxidant vitamins (A, C and E), superoxide dismutase (SOD), and catalase (CAT), glutathione (GSH), and glutathione peroxidase (GSH-Px) protect cells against lipid peroxidation. These antioxidant defense systems can be deteriorated by a magnetic field which leads to oxidative stress (7).

Therefore, the aim of this study was to indicate whether extremely low frequency magnetic field applied in a magnetic field therapy affects ROS generation in the heart tissue and antioxidant capacity of plasma depending on its working time.

MATERIAL AND METHOD

Chemicals

Thiobarbituric acid (TBA), butylated hydroxytoluene, sodium acetate trihydrate, triethanoloamine hydrochloride (TEA), 5-sulfosalicylic acid hydrate (5-SSA), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), β-NADPH (β-nicotinamide adenine dinucleotide phosphate), glutathione reductase (GR), 2-vinylpyridine, 2,4,6-tripiridyl-s-triazine (TPTZ), ferrous sulphate ($FeSO_4$) and ferric chloride ($FeCl_3$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from POCH (Gliwice, Poland) and were of analytical grade. The TBA solution was prepared by dissolving 0.67 g TBA in 100 ml deionized water and then diluted 1:1 with glacial acetic acid. Phosphate buffers with EDTA (pH 7.4). Sterile, deionized water (resistance >18 MΩ cm, HPLC Water Purification System USF ELGA, England) was used throughout the study.

Animals

Experiments were performed on 21 male Wistar rats weighing 260-280 g, aged 2-3 months. The animals were housed 5 per cage under standard laboratory conditions in 12/12 h light-dark cycle (light on at 7:00 a.m.) at 20±2°C ambient temperature and air humidity of 55±5%. The rat cages were made of non-magnetic material in order to avoid any vibration of heating. All animals received a standard laboratory diet and water ad libitum. All animals were kept for one week in the laboratory for adaptation. The experimental procedures followed the guidelines for the care and use of laboratory animals, and were approved by the Medical University of Lodz Ethics Committee.

Experimental protocol

Animals were randomly divided into three groups as follows:

Group I (n=7) - the control group, animals were housed in the same conditions as the experimental groups II and III except exposure to extremely low frequency magnetic field.

Group II (n=7) - animals were exposed to ELF-MF of the following parameters: rectangular shape, frequency 40 Hz, induction 7 mT, duration of exposure 30 min/day.

Group III (n=7) - animals were exposed to ELF-MF of the following parameters: rectangular shape, frequency 40 Hz, induction 7 mT, duration of exposure 60 min/day.

In group II and III rats were exposed to magnetic field for 14 days in established order always at the same time of the day from 7:00 to 9:00 a.m.

The extremely low frequency magnetic field (40 Hz, 7 mT) was generated by a typical magnetotherapy apparatus MAGNETRONIK MF-10 (Elektronika i Elektromedycyna). The parameters used in our experiments are commonly applied in magnetic field therapy and in experimental studies (10, 11).

Magnetic field in physiotherapy is the most often applied for 2 weeks, but it can be longer than two weeks in special cases *e.g.* in the chronic illnesses of limbs. A magnetotherapy session usually lasts 30 min, but can be lengthened up to 60 min in well-founded cases (clear medical recommendation).

Tissue preparation and biochemical analysis

Blood sampling

Blood samples (0.5 ml/sample) were collected from the tail vein into vials containing lithium heparin after 14-days exposure to the low frequency magnetic field. The samples were used for FRAP measurement.

Tissue preparation

Immediately after the last exposure, the rats were killed with an overdose of i.p. pentobarbital (100 mg/kg). Then, the hearts were excised, rinsed in cold saline to removed excess of blood. After rinsing, approximately 200 mg of organ fragments were cut off and frozen at -80°C for measurement of oxidative stress parameters.

Determination of lipid peroxidation

The content of lipid peroxidation products in heart homogenates was assayed as TBARS. Briefly, 4 ml 0.25% hydrochloric acid containing the following substances: 0.375% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), 0.015% butylated hydroxytoluene (BHT) were added to 2 ml of the heart homogenates. The samples were boiled for 30 min at 100°C in tightly closed tubes. After cooling them to 10°C, 2.5 ml of butanol was added to each tube and after intensive shaking the tubes were centrifuged for 10 min (3800 r.p.m., 20°C). TBA-reactive substances in the butanol layer were measured spectrofluorometrically using Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT, USA.). Excitation was set and emission was measured at 515 nm and 546 nm, respectively. Readings were converted into µM using regression equation y=0.39, (X-Xo) -1.32, where Y=µmol/l TBARS; X, Xo fluorescence intensity of the sample and control, respectively (arbitral units; AU). The regression equation was prepared from three series of calibration experiments with six increasing concentrations of tetramethoxypropane, used as a standard of TBARS (0.01-50 µM). A mixture of 2 ml of 1.15% potassium chloride, 4 ml of 0.25 M hydrochloric acid was used as a control. Finally, the results were calculated for 50 mg of heart tissue.

Determination of H_2O_2

Briefly, 50 mg of heart tissue fragments were homogenized with 2 ml of 1.15% potassium chloride. Then, 10 µl aliquot of tissue homogenate was mixed with 90 µl of PBS (pH 7.0) and 100 µl of horseradish peroxidase (1 U/ml) containing 400 µmol homovanilic acid (HRP+HVA assay) or with 90 µl of PBS and 100 µl of 1 U/ml horseradish peroxidase only (HRP) assay). Both homogenates were incubated for 60 min at 37°C. Subsequently, 300 µl of PBS and 125 µl of 0.1 M glicyne-NaOH buffer (pH 12.0) with 25 mM EDTA were added to each homogenate sample. Excitation was set at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield UK). Readings were converted into H_20_2 concentration using the regression equation: Y=0.012X-0.007, where Y=H_2O_2 concentration in homogenate (µM); X=intensity of light emission at 420 nm for HRP+HVA assay reduced by HRP assay emission (arbitrary units, AU). The regression equation was prepared from three series of calibration experiments with 10 increasing H_20_2 concentrations (range 10-1000 µM). The lowest H_2O_2 detection was 0.1 nM, with intraassay variability not exceeding 2%.

Measurement of total sulphydryl groups in heart homogenates

Total free -SH groups content in heart homogenates was determined according to the 2.2-dithio-bis-nitrobenzoic acid (DTNB) assay as described by Ellman (12). The absorbance of the samples was measured at 420 nm using a spectrophotometer Pharmacia LKB-Ultrospect III. Readings were converted into sulphydryl group content (µM) using the regression equation Y=-0.2998+241.945X, where Y= free sulfhydryl group content (µM), X= intensity of light emission at 420 nm (arbitrary units, AU). The regression equation was prepared from three repeats of

standard experiments with increasing concentrations of glutathione (range 2-200 µM).

Determination of glutathione levels

Total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in the heart homogenates. Briefly, the hearts were homogenized in cold 5% 5-SSA (1 ml of 5% 5-SSA/100 mg of tissue) using glass homogenizers. Then the homogenates were centrifuged (10000g, 10 min, 4°C) and aliquot samples of the supernatant were frozen at -80°C for a maximum of three weeks. The total GSH determination was performed in 1 ml cuvette containing 0.7 ml of 0.2 mM of NADPH, 0.1 ml of 0.6 mM DTNB, 0.150 ml of H_2O and a 50 µl of sample. The cuvette with the mixture was incubated for 5 min at 37°C and then supplemented with 0.6 U of GR in 100 mM sodium phosphate buffer with EDTA, pH 7.5. The reaction kinetics was followed spectrophotometrically at 412 nm for 5 min by monitoring the increase in absorbance.

GSSG concentration was determined in supernatant aliquots by the same method after opitmalization of pH to 6-7 with 1 M TEA and derivatization of endogenous GSH with 2-vinylpyridine (v:v). Reduced level of supernatant GSH was calculated as the difference between total GSH and GSSG. The increments in absorbance at 412 nm were converted to GSH and GSSG concentrations using a standard curve (3.2-500 µM GSH for total GSH and 0.975-62 GSSG µM for GSSG). GSH and GSSG determinations were repeated three times. The results were expressed in µM.

Determination of FRAP of plasma

The automated FRAP assay was carried out on an Ultraspect (Pharmacia LKB) spectrophotometer using the method described by Benzie and Strain (13) with modifications. In this method, Fe^{3+} ion is reduced to Fe^{2+} ion at low pH causing formation of a colored ferrous-TPTZ complex, which results in an increase in absorbance at 593 nm. This test measured total antioxidant capacity determined by nonenzymatic antioxidants; the main contributors in this test are ascorbic acid and uric acid, whereas plasma proteins and low molecular weight -SH groups containing compounds, such as glutathione, have very low activity in this method.

In brief, 50 µl of plasma were mixed with 50 µl of deionized water and then added to 900 µl of FRAP reagent (prewarmed to 37°C FRAP reagent (10 volumes of 300-mmol/l acetate buffer, pH 3.6+1 vol of 10 mmol/l TPTZ in 40 mmol/l HCl+1 vol of 20 mmol/l $FeCl_3$). Sample absorbance at 593 nm was continuously measured for 10 min at 37°C. 100 µl of water was added to the control (blank) samples. Calibration curve was performed for aqueous solutions of ferrous sulphate ($FeSO_4$) (50-1200 µM). Results were expressed in concentration of Fe^{3+} ions reduced to Fe^{2+} ions according to the formula:

Y (µM)=1235.5X+33.662 (where Y is concentration of Fe^{3+} reduced ions and X is sample absorbance at 593 nm).

Statistical analysis

The data are presented as mean±SE. The statistical analysis was performed by ANOVA followed by Duncan's multiple range tests as post-hoc. P value lower than 0.05 was considered significant.

RESULTS

Measurement of oxidative stress parameters

TBARS, H_2O_2 and -SH groups values in the heart tissue are shown in *Table 1*. With regard to the TBARS and H_2O_2 concentration we found that extremely low frequency magnetic field 40 Hz, 7 mT, 30 min/day for 2 week caused slight increase when compared to the control, however, the differences between groups were not significant (P>0.05). No statistically significant changes in the content of total free -SH groups in the heart were observed after extremely low frequency magnetic field (40 Hz, 7 mT, 30 min/day for 2 weeks) exposure when compared to the control. Determination of heart GSH (*Table 2*) and ferric reducing ability of plasma (*Fig. 1*) showed a slight decrease in antioxidant defense though the statistical analysis were insignificant (P>0.05).

Table 1. Effect of extremely low frequency magnetic field exposure to 40 Hz, 7 mT, 30 min/day or 60 min/day on some parameters of oxidative stress in heart homogenates in rats. Data are mean±SEM of seven experiments. The data were statistically evaluated by one-way ANOVA.

Parameters	Control (without magnetic field)	30 min/day for 2 weeks	60 min/day for 2 weeks
TBARS (µM)	3.98 ± 0.32	4.51 ± 0.23 ††	11.34 ± 1.38 ##
H_2O_2 (µM)	6.21 ± 0.44	7.08 ± 0.43 †††	10.91 ± 1.02 ##
Total free -SH groups (µM)	14.23 ± 1.13	14.62 ± 2.13	10.69 ± 0.911 #

TBARS- thiobarbituric acid reactive substances; H_2O_2- hydrogen peroxide; -SH groups- total free sulphydryl groups.
# P<0.05; ## P<0.01 *vs*. control, †† P<0.01; ††† P<0.02 *vs*. 60 min/day for 2 weeks

Table 2. Changes in heart glutathione metabolism in control (rats without ELF-MF) and after ELF-MF exposure to 40 Hz, 7 mT, 30 min/day or 60 min/day for 2 weeks. Data are mean±SEM of seven experiments. The data were statistically evaluated by one-way ANOVA.

Parameters	Control (without magnetic field)	30 min/day for 2 weeks	60 min/day for 2 weeks
Total GSH (µM)	73.193 ± 12.15	55.59 ± 9.8	36.78 ± 5.99 #
GSSG (µM)	22.19 ± 1.31	26.43 ± 2.75	28.33 ± 5.13
GSH (µM)	51.39 ± 11.35	34.67 ± 8.01	16.47 ± 4.86 #

Total GSH - total glutathione; GSH - reduced glutathione; GSSG - oxidised glutathione;
# P<0.05 *vs*. control.ELF-MF extremely low frequency magnetic field

On the contrary, the extremely low frequency magnetic field at the same frequency and induction but applied for 60 min/day for 2 weeks caused a marked modification of all parameters of the oxidative stress (*Table 1*). In particular, the concentration of TBARS, as an indicator of oxidative damage of lipids, and H_2O_2 were significantly higher in these rats when compared to the control (P<0.01 and P<0.05, respectively). Total free -SH group and antioxidant capacity of plasma (FRAP) were significantly lower in rats exposed to the low frequency magnetic field when compared to the control (P<0.02 and P<0.01, respectively) (*Fig. 2*). Myocardial status of glutathione metabolism was also changed in the group of rats exposed to magnetic field 40 Hz, 7 mT, 60 min/day for 2 weeks. There was a 32% and 50% decrease in the reduced (P<0.01) and total GSH (P<0.05), respectively, in animals exposed to magnetic field as compared to the controls. Redox status (GSH/GSSG ratio), an oxidative stress indicator was found to be significantly reduced when compared to the control in rats exposed to the extremely low frequency magnetic field 40 Hz, 7 mT, 60 min/day for 2 weeks (P<0.05) (*Table 2*).

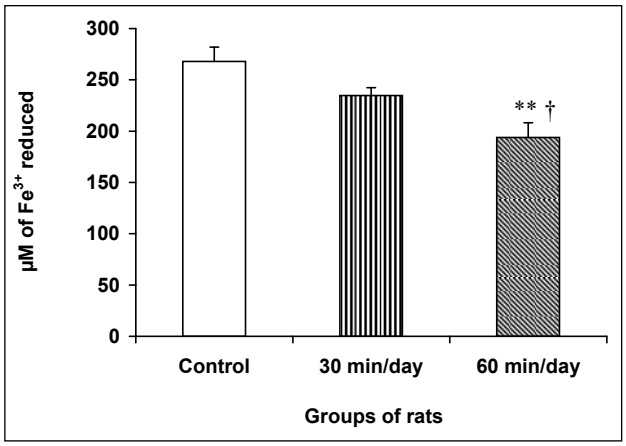

Fig. 1. Ferric reducing ability of plasma in control (rats without magnetic field) and after extremely low frequency magnetic field exposure of rats to 40 Hz, 7 mT, 30 min/day or 60 min/day for 2 weeks (mean±SEM, n=7). The data were statistically evaluated by one-way ANOVA. ** P<0.01 *vs*. control; † P< 0.05 *vs*. 30 min/day for 2 weeks.

DISCUSSION

The current study was designed to establish whether working time of extremely low frequency magnetic field used in the magnetic field therapy has influence on production of free radicals in a heart tissue. In this investigation we demonstrated that the exposure of rats to the extremely low frequency magnetic field 40 Hz, 7 mT, and 30 min/day for 2 weeks slightly increased the lipid peroxidative process and H_2O_2 concentration. Furthermore, concentration of GSH in heart homogenates and ferric reducing ability of plasma were also slightly diminished. This finding suggests that ELF-MF 30 min/day for 2 weeks do not markedly affect ROS generation and antioxidant defense in heart tissue. These results conflict with our earlier findings where rats exposure to ELF-FM for 2 weeks, 30 min/day significantly increased TBARS concentration in plasma (14). This may suggest that changes in the oxidative markers in blood do not always reflect changes in tissues as it was shown previously by Arguelles *et al*. (15). Moreover, several studies indicate that low frequency magnetic field has no effect on ROS generation in various tissues. For instance, Singh *et al*. (16) reported that lipid peroxidation was inhibited in mice exposed to a 2 mT magnetic field. Akdag *et al*. (17) showed that the exposure of male rats to 50-Hz magnetic field (2 hours/day for 2 months) had no effect on MDA level in the liver, kidney and in the brain. Exposure of neural culture to magnetic field of 50 Hz, 1 mT for 7 days was also demonstrated to have no significant effects on the main antioxidant defense (18). Some other studies reported that extremely low frequency electromagnetic field 50 Hz, 1 mT applied at different length time can reduce inflammatory process by inhibiting NF-κB signaling pathway (19). Similarly, exposure of human peripheral blood mononuclear cells to pulsating magnetic field (50 Hz, 45±5 mT) three times for 3 hours per each stimulation with 24 intervals between stimulations, increases the production of anti-inflammatory cytokine IL-10 (20).

In our study we also indicated that ELF-MF at the same frequency (40 Hz) and induction (7 mT) but applied for 60 min per day for 2 weeks is able to influence oxidative-antioxidative balance in the heart. Levels of TBARS and H_2O_2 in the heart tissue of rats exposed to the extremely low frequency magnetic field (40 Hz, 7 mT, 60 min/day for 2 weeks) were significantly higher in comparison to the control rats. This ELF-MF caused also significant increase in TBARS in plasma (14). These finding indicate that ELF-MF applied for 60 min/day caused

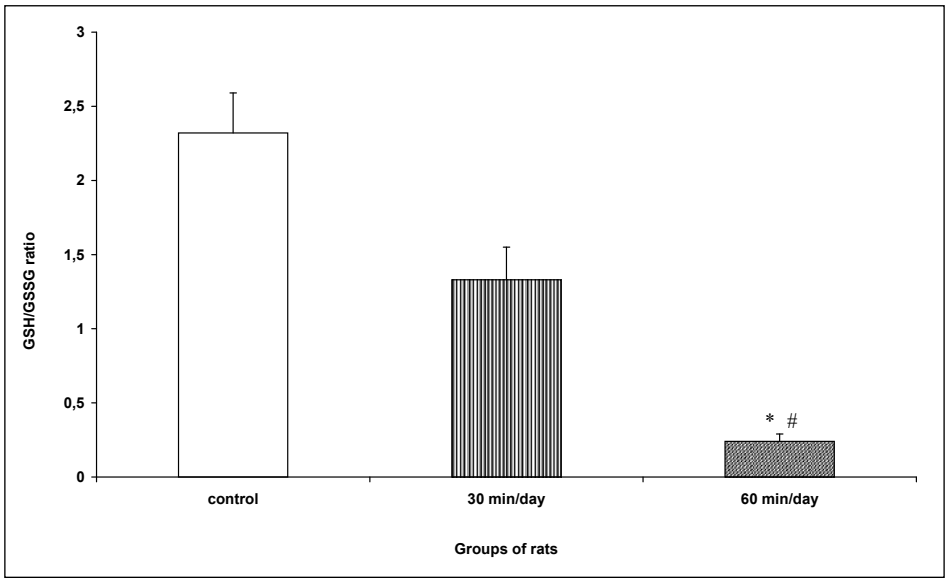

Fig. 2. Glutathione redox ratio GSH/GSSG in heart homogenates in control (without magnetic field) and after extremely low frequency magnetic field exposure of rats to 40 Hz, 7 mT, 30 min/day or 60 min/day for 2 weeks (mean±SEM, n=7). The data were statistically evaluated by one-way ANOVA. GSH-reduced glutathione; GSSG-oxidized glutathione. # P<0.001 *vs*. control; * P<0.01 *vs*. 30 min/day for 2 week.

ROS overproduction in a rat's heart and plasma. ROS and RNS, if not removed or neutralized, react with lipids, proteins, and nucleic acids, damaging the cellular functions and eventually causing death (21-23). Lipid peroxidation is commonly used as an index for measuring the damage that occurs in cell membranes as a result of free radical insult. Biological membranes are cellular structures rich in unsaturated fatty acids and therefore, susceptible to free radical attack (24). This study is consistent with other reports showing that exposure to ELF-MF increased free radical generation in the liver (4), brain (25), kidneys (1), lymphocytes and erythrocytes (26).

In the heart, toxic oxygen metabolites can be generated by endothelium, fibroblasts, and cardiomiocytes. Their activation results in production of superoxide anion, which is dismutated to H_2O_2. H_2O_2 is able to react with membrane lipids and causes their peroxidation. In addition, cooperating with transition metal ions, it converts to hydroxyl radicals, which are considered the most reactive and potentially dangerous compounds (27). Moreover, H_2O_2 causes activation of $I_{Ca\text{-}L}$ channels in ventricular myocytes, increases mitochondrial uptake of calcium and increases superoxide anion production by mitochondria (28). In normal conditions H_2O_2 is scavenged by catalase (CAT) or glutathione peroxidase (GPx) to water and oxygen. Destruction of H_2O_2 by these enzymes has inhibitory effect on hydroxyl radical formation.

The results of the present study also demonstrated that plasma ferric reducing ability in rats exposed to the extremely low frequency magnetic field (40 Hz, 7 mT, 60 min/day for 2 weeks) was significantly lower than in the control group. Such decrease could be a result of the diminished defense mechanism against free radicals -induced oxidative stress in the low frequency magnetic field. Although, FRAP assay does not qualify the values of a specific antioxidant, it is considered to be one of the reliable parameters to monitor the overall antioxidative status (13). The obtained results are consistent with the previous reports which suggested that low frequency magnetic field reduces antioxidant defense of an organism (1) and that the formation of free radicals and behavior of antioxidant enzymes depend on the working time of magnetic field (29).

For further investigations of the antioxidant capacity in rats exposed to the extremely low frequency magnetic field we measured total free sulfhydryl groups and glutathione in heart homogenates. We indicated that the heart tissue of rats exposed to magnetic field 40 Hz, 7 mT, 60 min had always lower levels of total free sulfhydryl groups and reduced glutathione in comparison to the control ($P<0.05$). Lowering of total free sulfhydryl groups' concentration leads to abnormal oxidation of SH-containing proteins. This may contribute to the reduced antioxidant capacity of plasma factors as thiols play a fundamental antioxidant role in protection of cellular and extracellular function against oxidative stress (30).

GSH is considered to be the first-line defense against oxidative damage and radical generation where GSH functions as a scavenger and cofactor in metabolic detoxification of ROS (30, 31). In particular, tissue GSH plays a major role in the antioxidant defense by direct detoxification of ROS or in a GPx-catalized mechanism. Myocardial status of glutathione metabolism during oxidative stress in rats may be improved by lipoic acid, which is a potent thiol antioxidant (32). It was also demonstrated that platelet activating factor (PAF) possesses cardioprotective effect which depends on opening of mitochondrial K_{ATP} channels and on a redox-sensible signal in the pre-ischemic phase of heart (33).

In this study, the decrease in reduced GSH and total free-SH group's content induced by the extremely low frequency magnetic field may result from the oxidative damages. In addition, a significant decrease in the redox ratio GSH/GSSG by the magnetic field of 40 Hz, 7 mT, 60 min/day for 14 days is inversely related to the oxidative stress during which ROS are produced and heart membranes are damaged.

The magnetic field is an efficient factor increasing blood flow, favouring bone formation, reducing production of proinflamatory molecules (19). Recent study also indicated that magnetically induced vagus nerve stimulation suppressed food intake (34).

CONCLUSION

Extremely low frequency magnetic field of parameters commonly applied in magnetic field therapy: 40 Hz, 7 mT, 30 min/day for 2 weeks, slightly influences ROS generation in the heart tissue as well as the antioxidant defense of organism. On the contrary, extremely low frequency magnetic field of the same parameters but working time of 60 min/day for 2 weeks results in markedly increased ROS generation and decreased total antioxidant capacity, which indicate the existence of profound oxidative stress in the heart. This findings suggest that the effect of ELF-MF irradiation on oxidative stress parameters depend on working time of this field.

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Conflict of interests: None declared.

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