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EFFECTS OF EXTREMELY LOW FREQUENCY MAGNETIC FIELD ON OXIDATIVE BALANCE IN BRAIN OF RATS.

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Extremely low frequency magnetic field (ELF-MF) may result in oxidative DNA damage and lipid peroxidation with an ultimate effect on a number of systemic disturbances and cell death. The aim of the study is to assess the effect of ELF-MF parameters most frequently used in magnetotherapy on reactive oxygen species generation (ROS) in brain tissue of experimental animals depending on the time of exposure to this field. The research material included adult male Sprague-Dawley rats, aged 3-4 months. The animals were divided into 3 groups: I - control (shame) group; II - exposed to the following parameters of the magnetic field: 7 mT, 40 Hz, 30 min/day, 10 days; III - exposed to the ELF-MF parameters of 7 mT, 40 Hz, 60 min/day, 10 days. The selected parameters of oxidative stress: thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), total free sulphydryl groups (-SH groups) and protein in brain homogenates were measured after the exposure of rats to the magnetic field. ELF-MF parameters of 7 mT, 40 Hz, 30 min/day for 10 days caused a significant increase in lipid peroxidation and insignificant increase in H_2O_2 and free -SH groups. The same ELF-MF parameters but applied for 60 min/day caused a significant increase in free -SH groups and protein concentration in the brain homogenates indicating the adaptive mechanism. The study has shown that ELF-MF applied for 30 min/day for 10 days can affect free radical generation in the brain. Prolongation of the exposure to ELF-MF (60/min/day) caused adaptation to this field. The effect of ELF-MF irradiation on oxidative stress parameters depends on the time of animal exposure to magnetic field.

Key words: *brain, extremely low frequency magnetic field, oxidative stress, reactive oxygen species, hydrogen peroxide, superoxide dismutase, lipid peroxidation*

INTRODUCTION

Magnetic field is inseparably connected with the surrounding environment, both natural and resulting from technological progress. It is produced not only as the side effect of the activity of many appliances but it is also used in medicine for diagnostic and therapeutic purposes. Magnetotherapy and magnetostimulation are commonly used in physical medicine. Magnetic fields applied in magnetotherapy, according to generally accepted physical medicine criteria, have frequency lower than 100 Hz and magnetic induction ranging from 0.1 mT to 20 mT. The induction is 2-3 times higher than magnetic induction of the earth field which is 30 to 70 μT. Recent studies have shown that extremely low frequency magnetic field (ELF-MF) can change cell behavior and activation by affecting the biochemical and/or biophysical processes. Physical processes at the atomic level are the basis of reactions between biomolecules in ELF-MF, since the field can magnetically affect chemical bonds between adjacent atoms and alter energy levels and spin orientation of electrons. This contributes to an increase in the activity, concentration, and lifetime of free radicals (1, 2). Although oxygen is required for many important aerobic cellular reactions, it may undergo electron transfer reactions, which generate highly reactive membrane-toxic intermediates such as superoxide, hydrogen peroxide or the hydroxyl radical. Excess oxygen free radicals induce lipid peroxidation, especially in brain, which is very vulnerable to free radical insults because it contains high concentrations of easily peroxidizable fatty acids (3). Therefore, overproduction of reactive oxygen species (ROS) can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA (4). Of many biological targets of oxidative stress, lipids are the most involved class of biomolecules. A biomarker for lipid peroxidation is malondialdehyde (MDA), it is a highly toxic molecule and it has been implicated in a range of disease pathologies by producing oxidative damage in tissues (5). Many studies reveal the ELF-MF effects on lipid peroxidation (6, 7).

The living organism has defensive systems against free radicals, such as the production of antioxidant enzymes: catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) and antioxidant vitamins: A, C and E (8). Among these antioxidant enzymes, superoxide dismutase (SOD) is critically important for the brain. These antioxidant defense systems can be deteriorated by the magnetic field which leads to oxidative stress (9).

The aim of the study was to investigate whether extremely low frequency magnetic field parameters, commonly used in

magnetotherapy, of 40 Hz, 7 mT, 30 or 60 min/day for 10 days induce oxidative stress in rat brain in relation to the exposure time.

MATERIAL AND METHODS

Animals

Thirty male Sprague-Dawley rats, aged 3-4 months, body weight 300-350 g, bred in animal quarters of the Medical Faculty of the Medical University of Lodz, were used in the experiments. The animals were kept in standard conditions *i.e.* at room temperature (20±2°C), artificial lighting for 12 hours and 12 hours of darkness, in metal cages, 5 rats in each. The animals were fed with LSM Murigran granulated fodder for rodents and water *ad libitum*. The study was approved by the Local Ethics Committee for Experiments on Animals of the medical University of Lodz, No. 40/LB 368/2007.

The animals (n=30) were randomly divided into 3 experimental groups and exposed to the changing magnetic field of the following parameters:

- group I (n=10) the control (sham) group, animals were housed in the same conditions as the experimental groups II and III except the exposure to extremely low frequency magnetic field;
- group II (n=10) exposed to rectangular field, frequency of 40 Hz, induction 7 mT, exposure time 30 min/day for two weeks;
- group III (n=10) exposed to rectangular field, frequency of 40 Hz, induction 7 mT, exposure time 60 min/day for two weeks.

The magnetic field was generated by a typical apparatus used in magnetotherapy - MAGNETRONIK MF-10 (Electronics and Electromedicine Otwock). The animals were exposed to low frequency magnetic field for the period of two weeks without Saturday-Sunday break as it usually happens in the case of magnetotherapy procedures. The exposure took place every day, for 10 days for each group, preserving the order, at the same time of the day between 7.00 and 10.00 a.m. The animals in plastic containers that did not limit their movement were placed inside an applicator being part of the set for magnetotherapy. The magnetic field used in magnetotherapy is most frequently applied for the period of 2 weeks. Longer time of exposure can be applied in special cases *e.g.* fractures or in wound and burn therapy. The selection of the magnetic field frequency parameters, 40 Hz, 7 mT, resulted from the analysis of referrals of patients to magnetotherapy and from the analysis of literature (10-12). The time of procedures is usually 30 min; it can be prolonged to 60 min. depending on indications (10).

Organ preparation

After completion of the exposure to magnetic field (after the period of 10 days) the animals were decapitated with an overdose of i.p. pentobarbital (100 mg/kg) (13). Then the brain tissues were excised, rinsed with cooled 0.9% NaCl solution to remove the excess of blood, dried and frozen at -80°C for measurements of oxidative stress parameters.

Determination of lipid peroxidation

The lipid peroxidation product content in the lung homogenates was assayed for TBARS, previously described by Yagi *et al.* (14) with some modifications. Briefly, brain tissue section (50 mg) was prepared and then homogenized in 2 ml of 1.15% KCl. 1 ml of the homogenate was moved to glass tubes into which 2 ml of reaction mixture containing 0.25 M of hydrochloric acid, 15% TCA, 0.375% TBA and 0.015% BHT were added. 1 ml of 1.15% KCl was used as control. The samples were mixed and incubated for 20 min. at 100°C. After cooling with cold water, 2.5 ml of L-butanol was added to each tube and after intensive shaking the tubes were centrifuged (10 min, 3800 rpm, 25°C). A Perkin Elmer Luminescence Spectrometer LS-50B was used to measure the supernatant fluorescence at emission wavelength of 546 nm and after excitation with 515 nm wave. Readings were converted into µM using regression equation $Y=0.39\ (x-x_o) - 1.32$, where Y=µmol/l TBARS; x, x_o 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 and 4 ml of 0.25 M hydrochloric acid was used as a control. Finally, the results were calculated for 50 mg of the brain tissue.

Determination of protein concentration

Lowry's method (15) was used to determine protein in the brain homogenates with the use of a Pharmacia LKB Ultraspec III UV/VISIBLE spectrophotometer. The values for absorbance at 750 nm were converted into protein concentration using a standard curve for 10 increasing bovine serum albumin concentrations (5-250 µg/ml).

Determination of the concentration of free-SH groups

Ellman's colorimetric method (16) was used for the determination of the tissue concentration of free-SH groups. It lies in the measurement of absorption of reduced thiol groups combined with DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The organ segments (50 mg) were homogenized in 20 volumes of 6% TCA at 0°C, then the homogenate was centrifuged at 10,000 g for 20 min at 4°C. The free-SH groups were determined at 420 nm with a spectrophotometer (Pharmacia LKB Ultrospec III UV Spectrophotometer). Readings were converted into sulphydryl group content (µM) using the regression equation Y= -0.299+241.954 x, where: Y - concentration of free-SH groups in µM, x - intensity of light emission at 420 nm (arbitral units, AU). The regression equation was prepared from three repeats of standard experiments with increasing concentrations of glutathione (range 2-200 µM).

Determination of H_2O_2 concentration

The hydrogen peroxide concentration in the brain homogenates was determined according to the Ruch *et al.* method (17). The test was performed with the use of a detection method, which consists in following the reaction of H_2O_2 with appropriate substrate (*e.g.* homovanilic acid HVA) with horseradish peroxidase as a catalyzer: 50 mg segments were homogenized in 2 ml of 1.15% solution of KCl. Then, 10 µl of the homogenate was mixed with 90 µl of PBS (phosphate-buffered saline; pH 7.4) and 100 µl of horseradish peroxidase (HRP) solution (1 u/ml) containing 400 µM of homovanilic acid (HVA). In the second experiment 10 µl of homogenate was mixed with 90 µl of PBS (pH 7.4) and 100 µl of HRP (1 µ/ml). Both samples were incubated for 60 min. at 37°C. Then, 300 µl of PBS (pH=7.4) and 125 µl of glycine buffer (pH=12) with EDTA (25 mM) were added to each sample. The emission intensity of wavelength 420 nm after excitation set at 312 nm was measured with a spectrofluorimeter LS-50 (Perkin Elmer-Norwalk, CT). The concentration of H_2O_2 was calculated according to the regression equation: Y=0.012 x - 0.007, where: Y=H_2O_2 concentration in µM converted into 50 mg of the tested organ, x - emission of 420 nm in arbitral units. The lowest detection was 0.1 nM with variability not exceeding 2% for standard solution of H_2O_2 in the range from 0.1 to 0.5 µM.

Statistical analysis

The results were analyzed with Statistica 8.0 software. ANOVA followed by Tukey's tests as post-hoc was applied. Data are presented as means ±S.E.M. $p \leq 0.05$ is regarded as being statistically significant.

RESULTS

The test results of the oxidative stress parameters of TBARS, -SH groups, H_2O_2 and protein in brain homogenates of animals exposed to ELF-MF, are showed in *Table 1*.

It results from the *Table* that the concentration values of the products of lipid peroxidation process increased in brain homogenates and this increase was statistically significant when the exposure time was 30 min/day as compared to the control group (2.7±0.14 *vs.* 1.3±0.02; $p<0.0001$). However, prolongation of the time of animal exposure to magnetic field up to 60 min/day resulted in decreasing tendency in the quantity of lipid peroxidation products in relation to group II, where the time of exposure to ELF-MF was 30 min/day (2.5±0.42 *vs*. 2.7±0.14; $p>0.05$). In the case of H_2O_2 concentration, more increase in the value of this parameter was observed when the exposure time was 30 min/day than 60 min/day in comparison to group I but these changes are not statistically significant. The concentration of free -SH groups in the investigated organ insignificantly increased at the exposure time of 30 min/day ($p>0.05$) and significantly increased at the exposure of animals to ELF-MF 60 min/day for 10 days in comparison to control and group I ($p<0.01$; $p<0.0001$, respectively). The concentration of total protein slightly decreased after the exposure to ELF-MF 30 min/day and significantly increased after the exposure to 60 min/day for 2 weeks ($p<0.05$) in comparison to group I (30 min/day).

DISCUSSION

The present study was designated to establish whether ELF-MF applied in the magnetic field therapy has an influence on free radical generation in the brain tissue in relation to the exposure time. In this study, we found that the exposure of rats to the low frequency magnetic field of 40 Hz, 7 mT and 30 min/day for 10 days significantly increased TBARS concentration and insignificantly increased H_2O_2 and free -SH group concentrations. TBARS is formed through the peroxidation of unsaturated fatty acids, nucleic acid and proteins, and thiobarbituric acid reacts with the products of this peroxidation (18). Therefore, TBARS are widely used as an index of biogenic macromolecules, particularly lipid peroxidation. The brain is rich in lipids and especially in polyunsaturated fatty acids, which are very sensitive to peroxidation (19). Our present results are in line with our earlier findings where rats exposure to ELF-MF for 2 weeks 30 min/day increased TBARS concentration in plasma and in heart (20). The results of the present study on lipid peroxidation in the brain homogenates are also consistent with the findings of other studies. For instance, Akdag *et al.* (21) showed that ELF-MF caused increased concentration of MDA in brain rats. Bediz *et al.* (22) revealed that a long-term exposure to ELF-MF increases lipid peroxidation in the brain, which may be ameliorated by zinc supplementation.

The increase in lipid peroxidation in our study is a result of increased ROS production, which in turn leads to excessive peroxidation of polyunsaturated fatty acids, causing neuron damage (23, 24).

It was previously reported that ELF- MF as a potent activator of macrophages (25) produces an abundance ROS which are directly involved in oxidative damage to cellular macromolecules, such as lipids, proteins, and nucleic acids in the tissues. The increased formation of ROS in our study was indicated by the increased level of H_2O_2 in the brains of rats exposed to ELF-MF for 30 min/day for 10 days. H_2O_2 is produced also from superoxide anion by the action of superoxide dismutase and is one of the most stable toxic oxygen metabolites. H_2O_2 is a volatile compound and easily crosses cell membranes by simple diffusion promoting radical reactions at a great distance from its origin. In normal conditions H_2O_2 is scavenged by CAT or GPx to water and oxygen. In the brain, CAT activity is low and the antioxidant enzyme, superoxide dismutase, is localized mainly in neurons (26), while GPx and GSH are mainly found in astrocytes (27). A deficit of these enzymes and their localization in the brain suggest that the neural tissue may be especially susceptible to hydroxyl radicals (28). In the case of an inadequate level of enzymes able to degrade H_2O_2, more H_2O_2 may be converted into toxic hydroxyl radicals that could then contribute to oxidative stress (8). In our study, ELF-MF 40 Hz, 7 mT applied for 60 min/day for 10 days resulted in a slight decrease in TBARS and H_2O_2 concentration as compared to animals exposed to magnetic field for 30 min/day indicating the diminished ROS generation and lipid peroxidation.

The results of the present study also demonstrated that magnetic field (40 Hz, 7 mT) applied for 30 min/day for 10 days caused a slight decrease in the total protein concentration

Table 1. The effect of low frequency magnetic field (ELF-MF) parameters of 7 mT, 40Hz, 30 min/day or 60 min/day for the period of 10 days on TBARS, H_2O_2, free -SH groups and protein concentration in rat brain. Means ±S.E.M. are given, n=10 in each group. The data were statistically evaluated by one-way ANOVA.
TBARS- thiobarbituric acid reactive substances; H_2O_2 - hydrogen peroxide; -SH groups - total free sulphydryl groups; # denotes $p<0.01$ *vs*. control; * denotes $p<0.0001$ *vs*. control; ** denotes $p<0.0001$ *vs*. 30 min/day for 10 days; ## denotes $p<0.05$ *vs*. 30 min/day for 10 days.

Brain	Control (without magnetic field)	ELFMF 30 min/day for 10 days	ELFMF 60 min/day for 10 days
TBARS (µmol/l)	1.31±0.02	2.7±0.1*↑	2.5±0.4
H_2O_2 (µmol/l)	0.038±0.005	0.054±0.001	0.05±0.01
–SH groups (µmol/l)	5.3±0.3	8.7±1.0	44.22±1.78**#↑
Total protein (µg/ml)	250.3±21.8	221.72±19,72	305.25±17.88##↑

and a slight increase in the content of -SH groups in the rat brain homogenates. The decrease in the total protein concentration may be related to the decrease of their synthesis and may indicate a diminished antioxidant protection. The results of this study are in agreement with studies of other authors. Thus Falone *et al.*(13) indicated a decrease in copper-zinc superoxide dismutase in rat cortices after rat exposure to ELF-MF (50 Hz, 0.1 mT, 10 days). Zwirska-Korczala (29) also observed a decrease in Mn SOD and Cu/Zn SOD activity after exposure of cell culture to ELF-MF 185-195 Hz, 120 μT for 24 and 48 h. Hashish *et al.* (30) reported a decrease in protein concentration in liver of mice exposed to ELF-MF (50 Hz, 1.4 MT, 30 days).

In our study exposure of rats to ELF-MF 60 min/day for 10 days caused a significant increase in the content of -SH groups and protein in the brain homogenates. The Trx (thioredoxin) and Grx (glutaredoxin) systems control cellular redox potential, keeping a reducing thiol-rich intracellular state, which on generation of reactive oxygen species signals through thiol redox control mechanisms (31, 32).

The levels of ROS in mammals are partially controlled by endogenous antioxidants mainly GSH (33, 34). GSH concentrations in plasma and tissue are inversely related to the level of oxidative stress (35) preventing cellular damage against toxic insults including free radicals (36, 37). An increase in total thiol groups signifies that ROS and oxidative stress are decreasing what probably protects the tissues and organs against oxidative stress. The increase in total protein concentration in brain homogenates after the exposure of rat to ELF-MF for 60 min/day is in accordance with the results of Di Loreto (31) who found a significant mRNA and protein expression of both neurotrophic factor (BDNF) and receptor TrkB (tyrosine kinase B receptor) in 1 mT ELF-MF-exposed neurons. It has been established that BDNF plays a role in the adaptative responses to oxidative stress and can prevent ROS-mediated neuronal cell death (38-40). Moreover, ELF-MF resulted in an increase in interleukin concentration (IL-1β) (31). In addition, some researchers observed an increase in total protein, alanine and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) concentration after the exposure of rats to ELF-MF of parameters 40 Hz, 1.5 mT, 1 h/day for 30 days (32). A significant increase in sulphydryl groups and protein concentration can indicate an adaptive mechanism to magnetic field due to activation of the system controlling the body's oxidative balance.

In summary, ELF-MF with parameters commonly applied in magnetic field therapy 40 Hz, 7 mT, 30 min/day for 10 days significantly increased lipid peroxidation indicating that these animals might be under high level of oxidative stress. By contrast, ELF-MF (40 Hz, 7 mT) applied for 60 min/day for 10 days induced an increase of free -SH groups and protein concentration indicating an adaptive mechanism to magnetic field. Effect of ELF-MF on ROS generation depends on the time of exposure to this field.

However, the presented above results of our study and the reports of other authors indicate that it is difficult to unequivocally determine the effect of magnetic field on the course of redox processes due to the parameters used in the performed ELF-MF experiments such as field induction, its frequency, as well as an impulse shape and time of exposure. Thus, studies in this range should be continued to obtain as many conclusions as possible.

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