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Bioelectrochemistry 57 (2002) 107–111

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Effect of 7 mT static magnetic field and iron ions on rat lymphocytes: apoptosis, necrosis and free radical processes

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Received 17 April 2001; received in revised form 19 November 2001; accepted 28 December 2001

Abstract

Simultaneous exposure of rat lymphocytes to 7 mT static magnetic field (SMF) and iron ions caused an increase in the number of cells with DNA damage. The mechanism by which MF induces DNA damage and the possible cytotoxic consequences are not known. However, we suppose that free radicals are involved. Potentially, the deterioration of DNA molecules by simultaneous exposure to 7 mT SMF and iron ions may lead to cell death: apoptosis or necrosis. The possible prooxidative properties of these two agents may result in an induction of the lipid peroxidation process as a marker of free radical mechanism in the cells. Experiments were performed on rat blood lymphocytes incubated for 3 h in Helmholtz coils at SMF of flux density 7 mT. During SMF exposure, some samples were treated with ferrous chloride (10 μg/ml), the rest serving as controls. We used the dye exclusion method with the DNA-fluorochromes: ethidium bromide and acridine orange. No significant differences were observed between unexposed lymphocytes incubated with medium alone and lymphocytes exposed to 7 mT SMF. Three-hour incubation with $FeCl_2$ (10 μg/ml) did not affect cell viability. However, when lymphocytes were exposed to 7 mT SMF and simultaneously treated with $FeCl_2$, there was a significant increase in the percentage of apoptotic and necrotic cells accompanied by significant alterations in cell viability. As compared to lipid peroxidation, there is a significant increase in the amount of lipid peroxidation end products MDA + 4 HNE in rat lymphocytes after simultaneous exposure to 7 mT SMF and $FeCl_2$ (vs. to the control samples and those exposed to SMF alone). This suggests that 7 mT static magnetic field in the presence of Fe^{2+} ions can increase the concentration of oxygen free radicals and thus may lead to cell death.

Keywords: Static magnetic field; Apoptosis; Necrosis; Lipid peroxidation; Lymphocytes

1. Introduction

Weak static or extremely low-frequency magnetic fields may interact with biological systems. One of the potential mechanisms by which static magnetic fields (SMFs) may interact with the living organism is through electronic interactions, i.e. radical pairs mechanism. Magnetic fields (MFs) influence the kinetics of reactions with radical pair intermediates [1,2]. External MF can increase the concentration of free radicals in living cells. Transition metals, e.g. iron or copper, are among the most important agents that can cause damage of DNA, RNA and other macromolecules through the production of oxygen free radicals (ROS), by Fenton reactions or by interaction with cellular thiols [3]. When ROS react with nonradicals, new free radicals can be formed, which leads to chain reactions, i.e. lipid peroxidation. In our earlier study, we demonstrated that 5 mT static magnetic field and iron ions increased lipid peroxidation in isolated rat liver microsomes [4].

At the cellular level: lipids, proteins, carbohydrates and nucleic acids may be damaged by reactions with ROS. Moreover, the increase of the concentration of ROS may give rise to functional and morphological disturbances in the cell through the oxidative stress leading to reversible or irreversible tissue injury, e.g. DNA damage [3,5]. Data by Singh and Lai [6,7] support the hypothesis that exposure to a power frequency (60 Hz) magnetic field at flux densities of 0.1, 0.25 and 0.5 mT cause DNA damage in the cells (rat brain cells) with the involvement of oxygen free radicals processes. Similarly, exposure to 7 mT magnetic field, static or 50 Hz, can induce DNA damage in rat lymphocytes if the cells were simultaneously treated with $FeCl_2$. The mechanism of this effect remains unknown. Potentially, the level of ROS induced by Fe^{2+} ions could be additionally

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1567-5394/02/$ - see front matter
PII: S1567-5394(02)00059-2

enhanced by the exposure to SMF as a result of the effect of magnetic fields due to radical pairs mechanism [8].

One of the most important biological consequences of the macromolecules damage by ROS could be cell death. ROS may directly or indirectly participate in the initiation of apoptotic or necrotic cell death. Cell death induced by either physiological or nonphysiological agents, including chemical (drugs, environmental toxicants) and physical (ionising radiation) factors, could be related to ROS production [9–12].

It has been found that an extremely low-frequency MF can induce apoptosis mainly in transformed cells, in vitro [13–15]. Recently, Blumenthal et al. [16] reported that low-frequency magnetic field (0.25 mT DC) could initiate apoptosis in rat tendon fibroblast cell cultures.

In the present study, we show that the deterioration of DNA molecules by simultaneous exposure to 7 mT SMF and Fe^{2+} ions may result in cell death: apoptosis or necrosis. The possible prooxidative properties of these two agents may lead to an induction of lipid peroxidation process, marking free radical mechanism in the cells.

2. Materials and methods

2.1. Isolation of lymphocytes

The experiments were performed on male albino Wistar rats (outbreed stock Imp: DAK), aged 3–4 months, weighing 260–280 g. The animals were fed on laboratory chow (MURIGRAN, Biowet, Poland) and given tap water ad libitum. Blood samples were collected by femoral vein puncture into heparinized tubes for cell death evaluation or into vacutainer tubes containing K_3EDTA for lipid peroxidation assay, pooled from a few (6–10) rats and immediately processed. Lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by layering 5 ml of whole blood onto 4 ml of Histopaque gradients (Sigma, St Louis, MO) and centrifuged at 2000 rpm for 30 min at room temperature. Lymphocytes were aspirated from the gradient–plasma interfaces and washed twice in phosphate-buffered saline (PBS). The final cell pellets were resuspended in RPMI 1640 medium with L-glutamine. Viability of cells was checked by supravital staining with 0.1% trypan blue and only the samples containing at least 95% viable lymphocytes were accepted for the experiments.

2.2. Magnetic field exposure

Exposure to MF was performed inside a pair of Helmholtz coils (35 cm in diameter), which provided a highly homogenous field ($\pm$ 5%). For measurements of flux density (magnitude and distribution), a gaussmeter, model 9500 A, with STF 99-0404 probe (F.W. Bell, USA) was applied; the range of measurements was 3 μT–30 T, accuracy $\pm$ 0.1% for static field.

As described earlier in detail [10], lymphocyte suspensions were exposed to MFs in a small water bath (with no metal parts) at 37.0 $\pm$ 0.2 °C, which was placed inside the coils. An identical water bath with control samples was placed in the natural static magnetic field (about 50 μT). Both baths were coupled with a thermostat to form a closed system of water circulation and temperature control.

2.3. Cell treatments

Two milliliters of suspension of lymphocytes (each containing 10^6 lymphocytes) and 3 ml of RPMI 1640 medium with L-glutamine were added to each tube. Part of the samples was treated with ferrous chloride (final concentration: 10 or 20 μg/ml), while the rest served as controls. The exposed samples were placed in the bath inside the coils and the control samples were kept in water bath outside the coils. The 3-h incubation and/or exposure to SMF at 7 mT flux density was initiated.

After 3-h exposure, the cells were washed twice in RPMI 1640 medium, resuspended in the same medium and immediately used for cell death evaluation or lipid peroxidation assay.

For cell death evaluation, two samples were prepared for each experimental point and the experiment was repeated twice. For lipid peroxidation assessment, the experiment was repeated three times and the final results were represented as the mean values from the two samples for each experimental endpoint. Statistical analysis of the experimental data was performed with Student's *t*-test for comparison of controls (medium) and static magnetic field or ferrous ions. Results with levels of $p < 0.05$ were considered as statistically significant.

2.4. Fluorescence microscopy analysis of cell death (apoptosis, necrosis)

For studying cell death and morphological changes in the nucleus, we used dye exclusion method in which viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be visualised by fluorescence microscopy after staining with the DNA-fluorochromes (fluorescent DNA-binding dyes): ethidium bromide and acridine orange. Ethidium bromide does not penetrate the plasma membrane in viable cells and only stains the nonviable cells, but acridine orange penetrates the plasma membrane without permeabilisation and stains viable and nonviable cells. Apoptotic cells were identified by morphological features, such as nuclear fragmentation and chromatin condensation. Morphological characteristics of necrosis include swelling of the cytoplasm and organelles, which are associated with membrane lysis. Fluorescence microscopy with differential uptake of fluorescent DNA binding dyes is a method of choice for its simplicity, rapidity and accuracy [17].

Immediately after the 3 h exposure to a 7 mT static magnetic field, ethidium bromide (1 μg/ml) and acridine

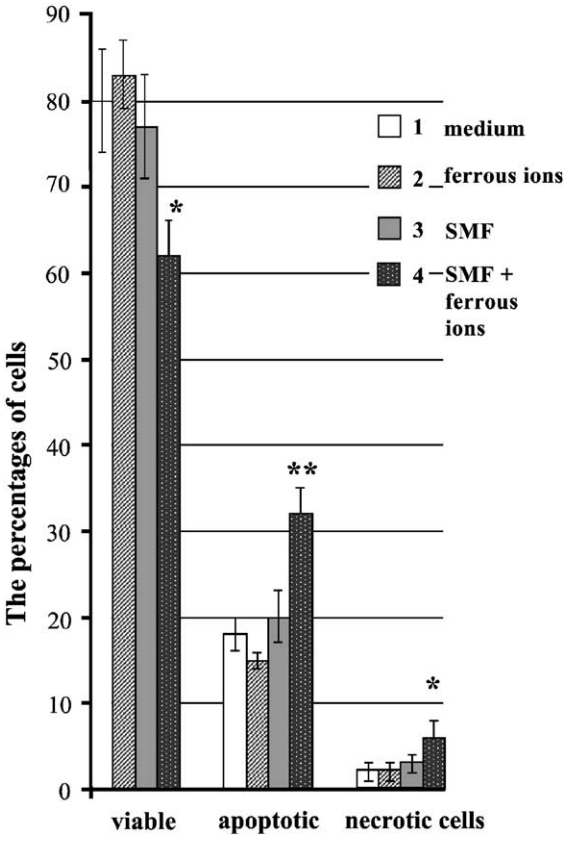

Fig. 1. Detection of apoptosis and necrosis (percent of cells) of rat lymphocytes after a 3-h in vitro exposure to 7 mT static magnetic field and/or to ferrous chloride (10 μg/ml). Data (the means ± S.D. from two independent experiments) are shown for: control (medium) (1), ferrous chloride (2), 7 mT static magnetic field (3), 7 mT static magnetic field and ferrous chloride (4). Statistically significant effects with $p \leq 0.05$ are indicated in the figures by one asterisk, highly statistically significant effects with $p \leq 0.001$ are indicated by two asterisks, as compared to the control (medium). Statistical analysis was carried out using Student's *t*-test.

orange (1 μg/ml), were added to cell suspension in medium (2×10^6 cells/ml) and incubated at room temperature for 5 min [18]. In all samples, 100 cells were scored and analysed by fluorescence microscopy (Olympus BX 40, UV 410). Viable cells fluoresced green, whereas nonviable cells had orange nuclei.

2.5. Lipid peroxidation assay

Immediately after the 3-h incubation and/or exposure to a 7 mT static magnetic field, the cell suspension in medium (containing 6×10^6 cells) was washed twice with PBS and lysed three times by freezing and thawing in 20 mM Tris–HCl, pH 7.4. The level of lipid peroxidation was expressed as the amount of malondialdehyde (MDA) + 4-hydroxynonenal (4-HNE) (in nM/mg protein), as major lipid peroxidation end products. The amounts of MDA + 4-HNE were determined with BIOXYTECH LPO-586 Assay kit (OXIS Int.), exactly as described by the manufacturer. This assay is based on the reaction of a chromogenic reagent *N*-methyl-2-phenylindole with MDA and 4-hydroxyalkenals at 45 °C to yield a stable chromophore with maximal absorbance at 586 nm. The concentration of MDA + 4-HNE was measured spectrophotometrically with 4-hydroxynonenal as standard. In the assay, we added 5 mM (final concentration) butylated hydroxytoluene (BHT) to prevent sample autooxidation.

Protein was determined by the Bio-Rad assay kit (BIO-RAD Lab.), with bovine serum albumin as a standard. This kit, based on the Bradford dye-binding procedure, is a simple colorimetric assay for measuring total protein concentration, e.g. in cell suspension.

3. Results

The percentages of viable and nonviable: apoptotic or necrotic, rat lymphocytes after 3-h in vitro exposure to a static magnetic field (7 mT) and/or ferrous chloride (10 μg/ml) are presented in Fig. 1. We did not find any statistically significant differences between unexposed lymphocytes incubated for 3 h with medium alone and lymphocytes exposed to 7 mT SMF for 3 h. Incubation of lymphocytes for 3 h with $FeCl_2$ (10 μg/ml) did not affect cell viability. At this concentration, ferrous chloride alone did not induce any noxious effect on rat lymphocytes, in line with previous results [19]. Similarly, exposure of lymphocytes suspended in medium to a 7 mT SMF for 3 h did not affect cell viability. However, when lymphocytes exposed for 3 h to a 7 mT SMF were simultaneously treated with $FeCl_2$ (10 μg/ml), there was a significant increase in the percentage of apoptotic and necrotic cells, accompanied by significant alterations in viability.

Table 1 demonstrates the changes on the level of lipid peroxidation (expressed as amount of MDA + 4-HNE) in rat blood lymphocytes after in vitro exposure (3 h) to Fe^{2+} ions and/or 7 mT SMF. We did not find any statistically signifi-

Table 1
Lipid peroxidation (expressed as amount of MDA + 4-HNE in nmol/mg protein) in rat blood lymphocytes exposed in vitro (3 h) to the 7 mT static magnetic field (SMF) and/or $FeCl_2$ (mean ± SD)

	Unexposed cells (nM MDA + 4-HNE/mg protein)	Cells exposed to SMF, 7 mT (nM MDA + 4-HNE/mg protein)
Control group (medium RPMI 1640)	10.21 ± 0.52	10.41 ± 0.57
$FeCl_2$ (10 μg/ml)	18.22 ± 0.53[a]	19.02 ± 1.11[a]
$FeCl_2$ (20 μg/ml)	25.77 ± 0.52[a,b]	31.70 ± 1.67[a,b,c]

MDA = malondialdehyde, lipid peroxidation product.
4-HNE = 4-hydroxynonenal, lipid peroxidation product.
[a] Statistically significant compared to cells exposed or unexposed to SMF and incubated with RPMI 1640, $P < 0.05$.
[b] Statistically significant compared to cells exposed or unexposed to SMF and incubated with RPMI 1640 and $FeCl_2$ (10 μg/ml), $P < 0.05$.
[c] Statistically significant compared to cells incubated with RPMI 1640 and iron ions ($FeCl_2$, 20 μg/ml), $P < 0.05$.

cant differences between the amount of lipid peroxidation products (MDA + 4-HNE) in the samples incubated for 3 h with medium alone and the samples exposed to 7 mT SMF. This assay, as had been expected, showed the induction of lipid peroxidation by Fe^{2+} ions. The amount of MDA + 4-HNE in the samples exposed to $FeCl_2$ was about two times higher than in controls. Moreover, in cells exposed to 7 mT SMF and $FeCl_2$ at the concentration of 10 μg/ml, no statistically significant changes in the level of lipid peroxidation were observed, as compared to the samples unexposed to SMF. When we added $FeCl_2$ at the concentration of 20 μg/ml, significant changes occurred. No changes in protein content were observed for rat lymphocytes exposed to the 7 mT static magnetic field and/or iron ions, compared with the control samples (data not shown).

4. Discussion

The 3-h exposure to the 7 mT static magnetic field in the presence of ferrous ions ($FeCl_2$, 10 μg/ml) significantly enhances lymphocytes death, both apoptotic and necrotic. This effect was not caused by either 7 mT SMF or ferrous ions alone, respectively. The mechanism by which 7 mT SMF could affect cell death is not known. However, we suppose that these effects may involve free radicals, similar to the increase in the number of cells with DNA damage after simultaneous exposure of lymphocytes to $FeCl_2$ and 7 mT SMF or $FeCl_2$ and 50 Hz MF (7 mT rms) [8]. $FeCl_2$ at the concentration tested (10 μg/ml) or 7 mT SMF did not induce any noxious effect on the cells. Therefore, the level of ROS induced by iron ions could be additionally enhanced by the exposure to SMF as a result of the magnetic fields effect due to radical pairs mechanism. The involvement of oxygen free radicals in the MF effects is also suggested for 60 Hz magnetic field-induced DNA strand breaks, blocked by free radical scavengers [20]. EMF exposure leads to crosslink formation (DNA–protein and DNA–DNA) in brain cells of the rat [7].

The biological effect of SMF on the basis of determination of lipid peroxidation level in the cells is suggested as a specific process inducing toxic effect through the free radical formation. In the membrane, polyunsaturated fatty acid can generate the aldehydes upon decompositions via the free radical reaction: malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA and 4-HNE has been used as an indicator of lipid peroxidation. For the initiation of the lipid peroxidation process, we used $FeCl_2$. The Fe^{2+} ions is well known to be one of the key factors of free radical processes as well as one of the initiators lipid peroxidation in membrane [3,21]. Recently, Schafer et al. [22] have shown that Fe^{2+}–dioxygen chemistry mechanism is even more important in initiating lipid peroxidation in cell membrane than the OH°-dependent reaction.

The results indicate that exposure to 7 mT SMF for 3 h combined with simultaneous treatment of cells with Fe^{2+} ions stimulates lipid peroxidation in rat blood lymphocytes. Significant changes in the level of lipid peroxidation were observed in cells exposed to 7 mT SMF and $FeCl_2$ at the concentration of 20 μg/ml; a discrete tendency, without significant changes, were detected in the samples exposed to 7 mT SMF and treated with $FeCl_2$ at the concentration of 10 μg/ml, as compared to the samples unexposed to SMF. The oxygen free radical production by Fe^{2+} ions appears further enhanced by MF, when lipid peroxidation is stimulated by 10 μg/ml $FeCl_2$ and SMF. When we measured with very reactive aldehydes, as end-products of this process, we were not able to detect changes. Exposures of rat lymphocytes to the 7 mT 50 Hz MF and 10 or 20 μg/ml of $FeCl_2$ gave similar results. The number of cells with DNA damage was the same, i.e. no significant differences between 10 μg/ml of $FeCl_2$ and MF or 20 μg/ml of $FeCl_2$ and MF were observed (Zmyślony et al., unpublished data).

Previously, we demonstrated that 5 mT static magnetic field under iron ions stimulation increased lipid peroxidation in isolated rat liver microsomes [4]. Aristarkhov et al. [23] demonstrated a 9% increase of lipid peroxidation level in liposomes (1,2-dioleophosphatidylcholine) exposed to a 8 mT SMF. SMF can, under in vitro chemical stimulation, influence lipid peroxidation kinetics in liposomes and can enhance the production of oxygen free radicals in human red blood cells [24,25].

In summary, we conclude that exposure of lymphocytes to a 7 mT SMF and iron ions may simulate free radical reactions involving ROS, e.g. lipid peroxidation, leading to increased cell death. Therefore, it is suggested that 7 mT static magnetic field in the presence of iron ions can increase the concentration of oxygen free radicals. The data support the hypothesis that lipid peroxidation plays an important role in cell death. The product of lipid hydroperoxide degradation are responsible for damaging the DNA in the cell [5]. Fe^{2+} ions at a higher concentration catalyse the Fenton reaction and OH° radicals production, crosslinks in DNA leading to cell death [26].

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