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EFFECT OF ELF-EMF ON NUMBER OF APOPTOTIC CELLS; CORRELATION WITH REACTIVE OXYGEN SPECIES AND HSP

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It is by now accepted that extremely low frequency electromagnetic fields ELF-EMF (0–300 Hz) affect biological systems although the mechanism has not been elucidated yet. In this study the effect of ELF-EMF on the number of apoptotic cells of K562 human leukemia cell line induced or not with oxidative stress and the correlation with heat-shock protein 70 (hsp70) levels was investigated. One sample was treated with H_2O_2 while the other was left untreated. ELF-EMF (1 mT, 50 Hz) was applied for 3 hours. ELF-EMF alone caused a decrease in the number of apoptotic cells and a slight increase in viability. However, it increased the number of apoptotic cells. In cells treated with H_2O_2. hsp70 and reactive oxygen species (ROS) levels were increased by ELF-EMF. These results show that the effect of ELF-EMF on biological systems depends on the status of the cell: while in cells not exposed to oxidative stress it is able to decrease the number of apoptotic cells by inducing an increase in hsp levels, it increases the number of apoptotic cells in oxidative stress-induced cells.

Keywords: Extremely low electromagnetic fields (ELF-EMF) – apoptotic cells – heat-shock protein70 – oxidative-stress – K562

INTRODUCTION

Non-ionizing, non-thermal electromagnetic fields produced by electric power systems and electric appliances have been added to the list of environmental agents that are a potential threat to public health [14, 19]. Despite their low energy it has been demonstrated unequivocally that these electromagnetic fields affect living systems at least at the cellular level (for an extensive review see 16 and 25). Although the mechanism of this interaction is still obscure, it has been shown that ELF-EMF can cause changes in cell proliferation, cell differentiation, cell cycle, apoptosis, DNA replication and expression, chromosomal aberrations, changes in Ca^{++} flux and changes in membrane potential [22, 23, 29, 31].

One of the intracellular mechanisms shown to be affected by ELF-EMF is the expression of heatshock proteins that are a family of molecular chaperons induced

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constitutively and the expression of which increases when the cell is subject to stress [19]. Heat-shock proteins are known to play a key role in cellular defense against the effect of stressors and their function in modulating apoptosis is well assessed [5, 8]. Goodman et al. demonstrated that heat-shock protein (hsp) expression was enhanced by exposure to electromagnetic fields [10, 11]. An elaborate study made by Tokalov and Gutzeit showed the effect of ELF-EMF on HS genes (HSP27, HSP60, HSP70 (A, B and C), HSC70, HSP75, HSP78, HSP90). It was demonstrated that even a low dose of ELF-EMF (10 μT) caused an increase in heat-shock proteins especially HSP70 [28]. Tokalov et al. compared the effect of X-ray irradiation, heat shock (41°) and ELF-EMF and found that with respect to HSP gene expression, the three stressors produced similar effects [27].

A plausible mechanism for the interaction of ELF-EMF was given by Brocklehurst and McLauchlan [2] and Simko [25] who proposed that the effect of ELF-EMF on biological systems might be due to the effect of ELF-EMF on free radicals. Free radicals are known to affect various biochemical pathways and reactions including cell proliferation, cellular differentiation and apoptosis [24, 30]. Previous studies showed that ELF-EMF increased the levels of reactive oxygen species [18, 32].

Apoptosis, programmed cell death, is an important physiological mechanism, which is required for embryonic development and to control tissue turnover in adult organisms. Resistance to cell death – particularly apoptotic cell death – is an important aspect of tumorigenesis [4]. Many tumor cells appear to have constitutively elevated levels of heat-shock proteins, which serve to protect them against apoptosis, thus causing chemotherapeutic resistance and increased tumorigenesis [15]. Exposure to ELF-EMF has been linked to increased incidence of leukemia and other tumors [16] and increase of hsp by ELF-EMF may be correlated to these results. On the other hand, increases in free radicals are known to induce apoptosis and ELF-EMF has been shown to increase free radical levels.

This work aimed to determine the effect of ELF-EMF on the number of apoptotic cells of K562 cell line, subjected or not to oxidative stress and correlate it with heat shock protein and reactive oxygen species levels.

MATERIALS AND METHODS

Cell culture

K562 cells were cultured at 37 °C in suspension in RPMI 1640 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) containing 10% FCS (Gibco-Invitrogen Ltd., Paisley, Scotland) in a humidified 5% CO_2 atmosphere. Cell count was made with trypan blue. Viability was 85–90%. K562 cells were induced to apoptosis with 300 μM H2O2 (Riedel Dehaen D3016).

Exposure system

Electromagnetic field was generated with two solenoids (25 cm diameter and 10 cm height) serially connected each having 400 turns of copper wire. During exposure the cells (control and ELF-EMF applied) were kept in specially designed plastic chambers ($20 \times 15 \times 10$ cm). Water ($37° \pm 0.5$ °C) circulated through these chambers from the same source (M48K water bath, Elektro-Mag, Turkey). Circulation of water was achieved by cavities drilled in the plastic chambers. Desired temperature was reached one hour before field application and was carefully monitored both before, and during exposure to ensure temperature stability. The experimental set-up was located in a secluded corner of the laboratory where field sources from electronic devices had no effect according to measurements. The magnetic field was measured to be 1 mT. Field intensity (measured every 10 minutes) varied for ± 0.05 mT during exposure. For the measurement F. W. Bell Sypris 5100 series was used. A 50 Hz, 1 mT ELF-EMF was applied for three hours.

Number of apoptotic cells, Annexin V staining

After application of ELF-EMF and/or induction with H_2O_2 cells were washed twice with PBS and suspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of approx. 1×10^6 cells/ml. A 5 µl of Annexin V-FITC (Sigma) and 10 µl of propidium iodide was added to both control and induced cell suspension. After incubation at room temperature for 10 minutes the fluorescence of the cells was determined immediately with a flow cytometer (Becton-Dickinson FACS Calibur).

ROS determination

After washing with PBS, cells were plated in 96-well plates (10^4 cells/well). Cells were incubated for 45 min with PBS supplemented with 1 mM p-nitro blue tetrazolium (NBT, Molecular Probes) and 1 mM $CaCl_2$. Measurements were made in the absence or presence of EMF exposure. Reduction of NBT to blue formazan by O^{2-} was measured using a microplate reader (Thermo Multiscan EX) at 550 nm. Intracellular formazan crystals were solubilized in 100 ml DMSO prior to reading.

Heat-shock protein determination

Samples were centrifuged, and washed with PBS. Cell lysates were mixed with sample buffer, subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were first incubated with blocking buffer (5% non-fat milk, 0.1% Tween 20, 20 mM Tris-HCl, pH 7.4, 150 mM

NaCl) for 1 h at room temperature and with anti-Hsp70 antibody (MAB1663, R&B Systems, UK) overnight at 4 °C. After washing with washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) three times the membranes were incubated for 1 h at room temperature with anti-β-actin antibody (A5316, Sigma-Aldrich). Prior to incubation for 1 h at room temperature with anti-mouse IgG Alkaline Phosphatase conjugated secondary antibody (AP308A, Chemicon) the membranes were washed three times with washing buffer. Antibody detection was performed using the BCIP/NBT Color Development Substrate. Band depth was detected with Biodoc Analyzer program.

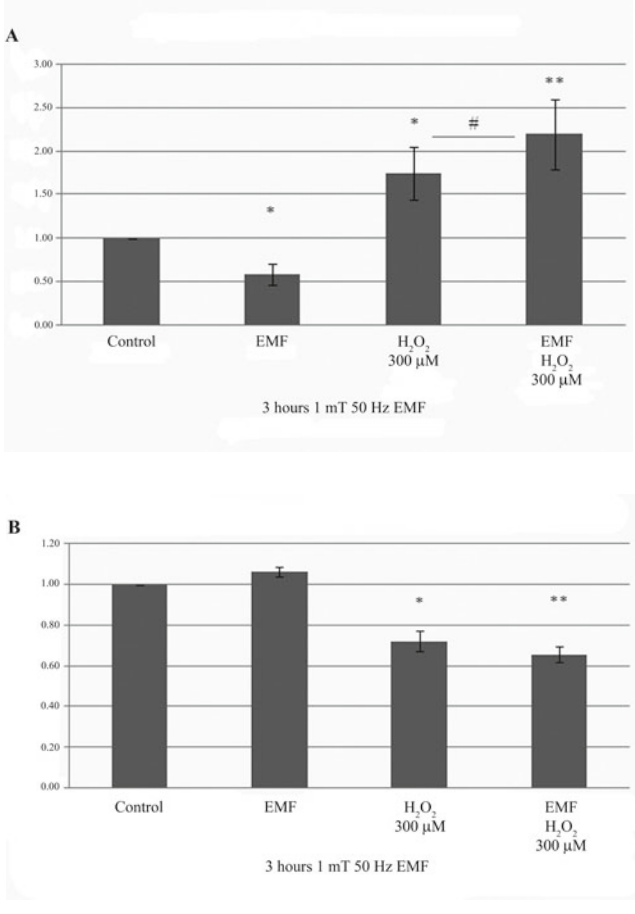

Fig. 1. Effect of ELF-EMF on the number of apoptotic cells of K562 cell line. Apoptosis was induced with H_2O_2. Both H_2O_2 treated and untreated cells were subjected to ELF-EMF (50 Hz, 1 mT) for three hours. Detection of the number of apoptotic cells was made with Annexin V and results were normalized to control values. (A) number of apoptotic cells; (B) viability of K562 cells induced or not with hydrogen peroxide. ELF-EMF significantly increased number of apoptotic cells subjected to oxidative stress (A) and decreased viability (B). Bars represent Standard Deviation of four independent experiments made in triplicate (*$p < 0.05$, **$p < 0.01$ with respect to control, #$p < 0.05$)

Statistical analysis

Each measurement was made in triplicate and four independent experiments were made. Results are mean of four independent experiments. Statistical analysis was made using Student *t*-test where indicated.

RESULTS

K562 cells were treated with H_2O_2 or left untreated and subjected to ELF-EMF (50 Hz, 1 mT) for three hours. Hydrogen peroxide was added at the onset of ELF application. Control cells were kept in similar conditions except for H_2O_2 treatment and ELF-EMF application. Apoptotic cell ratio was 3.3% in control cells similar to the ratio found by Liu et al. [17]. Hydrogen peroxide as an exogenous source of oxidative stress is known to induce apoptosis at mild concentrations [30]. When K562 cells were treated with hydrogen peroxide (300 μM) the ratio of apoptotic cells increased by about 70% (Fig. 1A) and viability decreased as expected (Fig. 1B). A further increase of about 40% in the number of apoptotic cells was observed upon ELF-EMF application to H_2O_2 treated cells (Fig. 1A) indicating that ELF-EMF acted as a co-stressor enhancing the apoptotic effect of H_2O_2. Correspondingly ELF-EMF application caused a slight decrease in viability of cells treated with hydrogen peroxide (Fig. 1B).

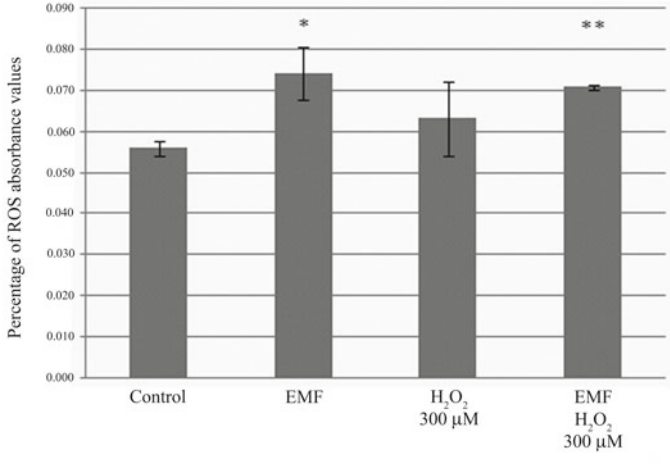

Fig. 2. Effect of ELF-EMF on ROS. Cells were treated with H_2O_2 or left untreated and subjected to ELF-EMF (50 Hz, 1 mT) for three hours. ROS levels were determined with NBT assay. The increase in ROS levels in cells subjected to ELF-EMF is evident. Bars represent Standard Deviation of four independent experiments made in triplicate (*$p<0.05$, **$p<0.01$ with respect to control)

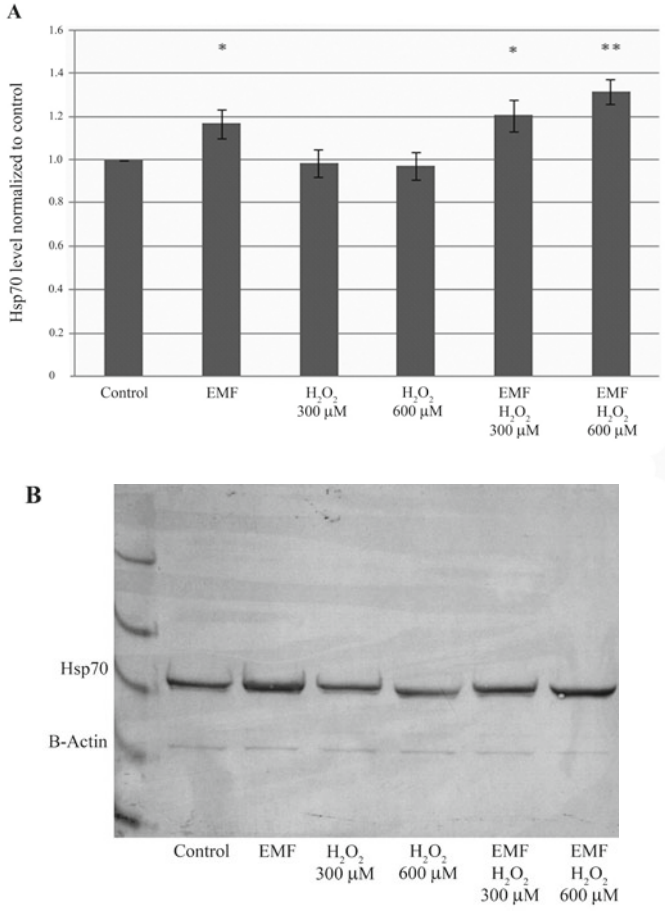

Fig. 3. Effect of ELF-EMF on heat shock protein 70 levels. ELF-EMF (50 Hz, 1 mT) was applied for three hours to cells treated with H_2O_2 or to untreated cells. hsp70 levels were determined with Western blot. (A) hsp level normalized to control values, (B) representative Western blot. ELF-EMF caused a slight increase in hsp70 level in oxidative stress induced cells. Bars represent Standard Deviation of four independent experiments made in triplicate (*$p < 0.05$ with respect to control)

ROS levels were determined after three hours of exposure to ELF-EMF. The effect of ELF-EMF on ROS level was seen to be pronounced (Fig. 2). A 30% increase in ROS levels in ELF-EMF applied cells was observed. Interestingly, this increase was larger than the increase induced by H_2O_2 alone. ELF-EMF application caused a slight increase in ROS levels of H_2O_2 treated cells suggesting the free radical stabilizing effect of ELF-EMF. However this increase did not give rise to a larger increase in ROS levels than the one caused by ELF-EMF application alone indicating that ELF-EMF did not act synergistically with H_2O_2.

Hydrogen peroxide was used as a positive control and also to determine the effect of ELF-EMF on heat shock protein expression as a second stressor. Cells were exposed to 300 μM H_2O_2 or 600 M H_2O_2 and ELF-EMF was applied immediately after H_2O_2 addition for three hours. Western blot was made to determine hsp70 level and heat shock protein levels were normalized to beta-actin. A significant increase (20% to 25% for 300 μM H_2O_2 and 600 M H_2O_2, respectively) in hsp70 expression was observed upon ELF-EMF application with respect to H_2O_2 treated cells (Fig. 3) indicating the crucial effect of ELF-EMF. Although statistically not significant, hsp levels were seen to be slightly higher for hydrogen peroxide treated cells subjected to ELF-EMF than for cells subjected to ELF-EMF application alone.

DISCUSSION

In this study we investigated the effect of ELF-EMF on the number of apoptotic of cells in the presence or absence of H_2O_2 and correlated the results with altered ROS and hsp levels.

ELF-EMF increased the number of apoptotic cells in cell populations induced with H_2O_2 while it caused a decrease in non-induced cell populations (Fig. 1A). Our results showing a decrease in the number of apoptotic cells and an increase in viability are in good agreement with the results of Falone et al. [7] who showed that ELF-EMF increased viability of SH-SY5Y cells. These authors suggested that the shift towards a more reduced intracellular environment deduced by measuring glutathione S-transferase, glutathione peroxidase and glutamylcysteine synthetase, affected the signal transduction pathway, thus increasing the expression of anti-apoptotic and repair-related proteins Bcl-2 and p53. We suggest that in addition to these findings, the decrease in the number of apoptotic cells and the increase in viability can be attributed to an increase in heat-shock protein hsp70 levels induced by ELF-EMF (Fig. 3A). The increase in hsp70 levels (Fig. 3A) under the effect of ELF-EMF is in accordance with the results of Goodman et al. [10] and Tokalov and Gutzeit [28]. Guo et al. [12] showed that hsp70 significantly increased activities of glutathione peroxidase and glutathione reductase. They suggested that this might provide a new insight into the cytoprotection induced by hsp70. In addition the anti-apoptotic role of hsp70 by interfering with the apoptotic cascade [9] must also be taken into account with the above consideration for the decrease in the number of apoptotic cells. Overall, it can be hypothesized that an increase in hsp70 induced by ELF-EMF would cause reduction in the number of apoptotic cells by triggering a shift towards a more reduced environment thus causing expression of anti-apoptotic proteins, and also by directly influencing the apoptotic cascade. On the other hand ELF-EMF increased the number of apoptotic cells in oxidative stress induced cell populations. This can be attributed in part to the slight increase in ROS levels in H_2O_2 induced cells exposed to ELF-EMF. However it is evident that further studies are required to decipher the mechanism that caused the increase in the number of apoptotic cells.

We have determined a significant increase in ROS levels (Fig. 2) in cells exposed to ELF-EMF. Our results are in agreement with those of Wolf et al. [32], Lupke et al. [18] and De Nicola et al. [6]. However despite the increase in ROS levels caused by ELF-EMF application alone, the decrease in the number of apoptotic cells suggests that the increase in heat shock protein level enhanced by ELF-EMF suppressed the anticipated increase in the number of apoptotic cells that would be triggered by increased ROS levels.

As stated previously ELF-EMF are considered to be promoters rather than initiators of cancer and are therefore listed as potential environmental pollutants. The increase that ELF-EMF induces in heat shock protein and ROS can be considered to be some of the causes, the others being their interference with signal transduction mechanism of the cell [7, 21].

It is known that many pollutants exert their deleterious effects by inducing oxidative stress [26]. ELF-EMF, are considered to be weak stressors [13] which alone can or cannot generate physiological responses. However ELF-EMF as co-stressor can augment or dampen the effect of another weak stressor thus causing a significant outcome. Thus ELF-EMF may cause increased adverse effects on biological systems in more polluted areas acting as a co-stressor in oxidative stress production. In addition an increase in heat-shock proteins and the consequent decrease in the number of apoptotic cells may not be beneficial under malignant conditions. Therefore there is no doubt that they may have adverse health effects. On the other hand as stated previously the cytoprotective effect of increased heat shock protein levels induced by ELF-EMF can be used as an alternative to hyperthermia in medical treatments [3]. Thus either in risk assessment or for probable medical application they must be considered accordingly.

As a whole, the effect of ELF-EMF on biological systems must be considered to be dependent on the status of the system. These results demonstrate that ELF-EMF decreases the number of apoptotic cells of an erythroleukemia cell line, by increasing heat-shock protein levels albeit an increase in ROS. However as a co-stressor it augments the effect of oxidative stress on the number of apoptotic cells one reason being the stabilizing effect on free radicals. The increase in heat-shock protein levels induced by ELF-EMF is not able to reverse the increase in the number of apoptotic cells caused by oxidative stress. Further studies concerning the effect of ELF-EMF taking into account the cell type and different stressors are required. Nevertheless we think that these findings can be promising for beneficial uses if carefully controlled settings can be achieved.

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