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INFLUENCE OF STATIC AND ALTERNATING MAGNETIC FIELDS ON U937 CELL VIABILITY

Abstract: Presented studies were conducted to assess the influence of alternating (AC) and/or static (DC) magnetic fields (MF) or combined with puromycin (PMC) on U937 cell line viability. The magnitude of DC MF was enclosed in the range (2 ÷ 6) mT. In case of AC MF, four frequencies were set: 12 Hz, 25 Hz, 35 Hz, 50 Hz and magnetic induction value was adjusted to 6.5_{rms} mT. The reaction of samples exposed to MF and/or PMC was presented as cell viability coefficient S defined as a ratio of viable cells in the sample to viable cells in the relevant control sample, analyzed by flow cytometry. For PMC treated sample the percentage of viable cells decreased about factor 3. MF alone did not influence cell viability regardless of the type of exposure while simultaneous action of ACDC MF mode and PMC significantly influenced viability of U937 cells. The viability coefficient S was in the range of (0.13 ± 0.07 ÷ 0.64 ± 0.20) and exerted a non-linear relation with frequency of the AC MF component. The maximal lethal influence (S = 0.13 ± 0.07) was observed for DC MF = 6 mT and frequency of AC MF equal to 35 Hz. Observed bio-effects were confronted with the physical models assuming the Ca^{2+} ions transport disturbance and/or protein complexes dysfunction in cells due to MF action.

Key words: Magnetic Field, U937 Cell Line, Puromycin, Viable Cells.

INTRODUCTION

Technology development, increasing use of electrical appliances in a daily life causes widely distribution of magnetic fields (MF) in our environment. At the same time, epidemiological studies have indicated a rise of different types of human cancer, particularly of hematological malignancies [1, 2]. On the other hand therapeutic role of MF is also presented in the context of magnetotherapy [3]. Therefore, the question about the influence of MF on living organisms remains relevant and still open. Despite many studies [4–13], biophysical mechanism of interaction between MF and biological systems is not fully understood. Attempts to explain observed bio-effects based on developed non-thermal mechanisms have been noted difficult and unclear. Results of experiments are dependent not only on MF parameters, but also on biological material properties [11].

So far, studies have been mainly based on various cell lines due to their potential simplicity as biological objects. Many factors can induce cell death, including electromagnetic irradiation as well as chemical stimulation. Cell death can occur by two distinct mechanisms, apoptosis ("programmed" cell death) and necrosis ("accidental" cell death). These processes differ each other in many observable morphological and biochemical changes in the cells [14] what can be favorable to distinguish between viable and non-viable (damaged) cells using the flow cytometry, for instance.

Presented studies were conducted to assess possible influence of alternating (AC) and/or static (DC) magnetic fields or combined with cytotoxic agent on U937 human lymphoid cell viability. U937 cell line was investigated as an in vitro model for antitumour testing or tumorigenicity. In previous studies three ranges of MF flux densities were most frequently examined (50–100 μT, 15–20 mT and 45–50 mT) [3, 15, 16], hence we decided to complete existing data about DC MF set in the range (2 ÷ 6) mT. Additionally, in case of AC MF component, the amplitude was selected to relate with parameters used in magnetotherapy and to compare obtained data with results of other groups [13].

MATERIALS AND METHODS

CELL CULTURE

U937 cells (human lymphoid cell line; ATCC, Rockville, MD) were cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, USA), 0.2 M L-glutamine and 50 mg/ml gentamicin (Sigma-Aldrich, Germany) at 37°C in humidified atmosphere containing 5% CO_2. U937 cells were passaged every four days and counted with a Fuchs-Rosenthal chamber. The experiments were performed on viable cells assessed by trypan blue exclusion method, in the logarithmic phase of growth. For the experiment U937 cells were seeded into 6-well culture plates at density 2.5×10^5 cells/ml. In order to place cells in MF exposure device, cells were collected to BD Falcon polystyrene test tubes. Cells were harvested and washed with fresh medium after each stimulation. Procedure was verified to obtain the best accuracy and reproducibility.

CHEMICAL STIMULATION

Cells were supplemented with PMC, a known unselective cell death inducer acting via inhibition of translation in protein synthesis process [17, 18], at concentration of 100 μg/ml (Sigma-Aldrich, Germany). Puromycin was applied simultaneously

with MF exposition to stimulated samples and separately added to PMC control sample. After particular treatment cells were harvested and washed with fresh medium, resuspended at initial volume and finally analyzed for viable/damaged cells contribution. Chemical stimulation was performed in a form of single (2.5 h) or triple (3 × 2.5 h) repetition with 24 h intervals.

EXPOSURE SYSTEM

Measurements were performed in a few steps. Cells were exposed to homogenous DC MF alone or combined with perpendicularly oriented sinusoidal component. The magnitude of DC MF component ranged from 2 mT to 6 mT. In case of correspondent AC MF, four different frequencies were set: 12 Hz, 25 Hz, 35 Hz, 50 Hz and magnetic induction value was adjusted to 6.5_{rms} mT. The exposition was repeated (1 ÷ 3) times (by 2.5 h each one) with 24 h intervals.

The MF in user defined mode (AC MF, DC MF or ACDC MF) were produced by specially designed Helmholtz coils (HC). Tubes containing U937 cells were positioned in the center of the coils. The coils with exposed samples as well as control ones were maintained in an incubator. During exposure to MF, temperature inside the coils was kept at 37 ± 0.1°C.

The homogeneity of the MF in the sample volume was tested and ensured with the accuracy of a few% by using the Gaussmeter equipped with Hall probe. Control samples were set in area out of MFs produced by HC (the background magnetic field for static and alternating components was as follows: < 0.05 ± 0.01 mT and < 0.03 ± 0.01 mT, respectively). In the first step, the frequency (50 Hz) of AC MF was chosen in a view of wide abundance of 50 Hz MF sources. Another three values of frequencies were set to establish potential resonant behaviour of the exposed samples.

DETECTION SYSTEM

Cell viability was estimated with a BD FACSCalibur (San Jose, CA) flow cytometer equipped with a 15-mW 488-nm blue argon ion laser and with a 10-mW 635-nm red diode laser. U937 cells were stained with Annexin V-APC labeled (BD Biosciences, USA) and with 7-amino-actinomycin D (7-AAD; BD Biosciences, USA) in order to distinguish between viable cells and cells affected by cell death processes (damaged), by analyzing the fluorescence intensity of these dyes. Annexin V-APC and double-positive Annexin V-APC and 7-AAD cells were analyzed as damaged ones. Viable population was registered as Annexin V-APC and 7-AAD negative. Staining was performed on cells cultivated for 24 h, after last puromycin and MF stimulation. CellQuest Pro software (BD, San Jose, CA) was used to

analyze the percentage of viable/non-viable cells. Number of events collected for each sample was no less than 10^4 to obtain reliable statistics. The details of the applied experimental procedure are given elsewhere [19].

STATISTICS

Results are presented as a viability coefficient S, defined as a ratio of viable cells in the sample exposed to MF alone or in combination with PMC (MF + PMC), to viable cells in the relevant control sample (sham one or only PMC treated). In case of experiments with the use of cytotoxic agent alone, the percentage of viable cells in the sample was used to assess results. Viability coefficient S was estimated as the mean of no less than six experiments (± standard deviation). Statistical analysis was performed by U-Mann Whitney and Kruskal-Wallis test considering $P < 0.05$ as significant.

RESULTS

Data analysis has shown that magnetic fields alone did not influence the U937 cell line viability. Even AC+DC components combination did not change this effect considerably, regardless of used DC MF magnitude (2 mT or 6 mT) and frequency selection (12 Hz, 25 Hz, 35 Hz, 50 Hz) in case of 6.5_{rms} mT AC MF, as well as number of exposure repetitions (1 ÷ 3). Viability coefficient S was enclosed in the range (0.98 ± 0.02 ÷ 1.01 ± 0.02) for various modes of the magnetic field exposure.

Application of PMC influenced the U937 cell viability. Effects depended on dosage, time of stimulation, time of detection and sample processing, as established by test measurements. The percentage of viable cells in the samples varied in the range (0.20 ± 0.1 ÷ 93.5 ± 0.5)%. For further studies the parameters of PMC application causing decrease of the viable cells percentage in the sample to about 30% were chosen. It was related to 100 μg/ml as a dose applied in triple repetition (3 × 2.5 h) with 24 h interval between applications.

Combination of DC MF and PMC did not provoke cell viability changes in the samples. Viability coefficient S was equal to 1.11 ± 0.05. Similar results were observed for the most abundant 50 Hz AC MF (S = 1.04 ± 0.05).

However, simultaneous action of ACDC MF and PMC significantly influenced the U937 cell viability in the samples (Fig. 1). The viability coefficient S was enclosed in the range (0.13 ± 0.07 ÷ 0.64 ± 0.20) and showed a non-linear relation with used frequencies of the AC MF component. The maximal lethal influence (S = 0.13 ± 0.07) was observed for the following parameters of magnetic fields: DC MF = 6 mT, AC MF = 6.5_{rms} mT and f = 35 Hz. It indicates that alternating

and static magnetic field combination could amplify changes caused by cytotoxic agent, leading to cell death more effectively. For most abundant frequency (50 Hz), amplification of cell death processes was about 4 times less compared to the minimum (Fig. 1).

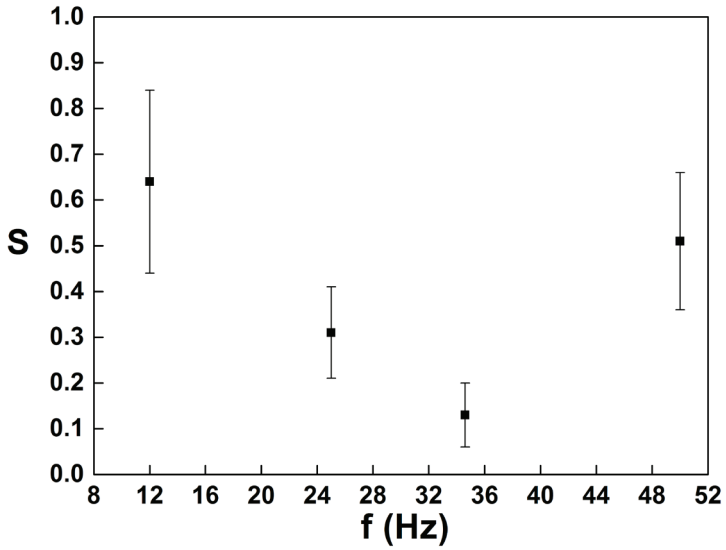

Fig. 1. Changes of viability coefficient S in accordance with frequency f of alternating magnetic field component (DC MF = 6 mT and AC MF = 6.5_{rms} mT). Data expressed as mean ± SD (n ≥ 6).

DISCUSSION

Obtained results showed that magnetic fields alone did not significantly influence U937 cell viability, regardless of used magnetic flux densities, frequencies, as well as chosen modes of the MF exposure. Foregoing findings are consistent with conclusions of some other groups [5, 11, 12], although the other studies suggest growth inhibition of selected human tumor cell lines exposed to MF [10, 20]. Existing controversies revealed dependence between caused effects and type of cells [7, 9, 11, 21].

Chemical stimulation with puromycin induces cell death through disturbance of protein synthesis process. Effectiveness of its action varies with applied dosage, time-course of application and type of cells [22]. In the present work, threefold application (3 × 2.5 h) with 24 h interval between applications of PMC in high dose (100 μg/ml) dramatically changed percentage of U937 viable cells in the sample compared to sham one.

Combination of MF with action of different chemical agents, PMC for instance, can also influence cell viability in a different ways. As an example, in [13] authors showed no changes in the number of rat lymphocytes with DNA damage exposed only to 7 mT DC MF. However, simultaneous exposure of cells to $FeCl_2$ and 7 mT DC MF or 50 Hz 7_{rms} mT AC MF caused significant increase of the damaged cells population due to possible radical pairs mechanism.

Results presented in our work revealed that combination of DC MF or AC MF (50 Hz) and PMC did not influence the U937 cell viability in the samples. In case of DC MF, its protective role against PMC — induced cell death according to some authors [8, 11, 23] was not confirmed. Mentioned discrepancy is probably due to some differences in experimental conditions (density of cells, cytotoxic agent dosage, time of chemical stimulation, etc.). Unobservable effect for AC MF + PMC stays in contrast with our previous work [19], where simultaneous action of 50 Hz pulsating electromagnetic field (PEMF) with PMC on the same cell line viability was examined. However, described protective role of PEMF against PMC — induced cell death was obtained for the much higher (45 ± 5 mT) amplitude of used MF compared to present paper and for different signal waveform.

In turn, simultaneous action of ACDC MF mode and PMC increased number of damaged U937 cells. The viability coefficient S changed in non-linear way with used frequencies of the AC MF component, obtaining minimum for 35 Hz. Whereas, tests with three times lower magnitude of DC MF component were ineffective, no changes in cell viability were noticed. It seems to indicate also B_{AC}/B_{DC} ratio dependency of the observed bio-effects [24, 25] and requires further studies.

To explain differences between data obtained by various scientific groups one has to note that simple comparison is not trivial or maybe even not possible for many reasons. Firstly, observed effects are strictly dependent on applied MF parameters such as amplitude, frequency, signal waveform, as well as dose and type of used chemical drug. Secondly, sensitivity to external stimulation varies for different types of cells. Another crucial point is matched time of chemical and/or physical stimulation and interval between exposition and further detection. Finally, chosen detection method and way of samples processing may also influence the results [26].

Our findings indicate a range of MF parameters and details of cytotoxic agent application appropriate to influence U937 cell viability due to simultaneous action of physical and chemical factors. Synergy of MF and PMC determines changes in studied system, which finally could provoke biochemical signal cascades development. Energy of used MF is not enough to cause lethal lesions in the cell in direct way [4, 25, 27]. Thus, cytotoxic agent is needed firstly to decrease the “natural cellular defense mechanisms”. Puromycin, accomplished this role through disturbance of protein synthesis process, what finally can lead to deregulation of intracellular signal pathways.

In cellular signaling not only peptides are involved but also key biological ions (e.g., Ca^{2+}) [28]. The Ca^{2+} ions play an important role as signaling molecules in cells, what corresponds to their intracellular concentration maintained through different mechanisms (pump, channels, proteins) [29]. For instance, action of some signaling proteins depends on Ca^{2+} ions binding, the same time maintaining low concentration of Ca^{2+} ions in the cytoplasm (0.1 ÷ 0.2 μM) [30, 31]. Thus, deregulation of protein formation process can influence Ca^{2+} homeostasis and Ca^{2+}-dependent signaling pathways in the cell [32–40]. Initiated perturbations at the level of biochemical signaling in the cell might lead to more frequent and easier cell death upon exposure to external MF, for instance.

Basic problem is related to indicate the biophysical mechanism(-s) responsible for observed changes in cell viability caused by MF action on "chemically weaken" cell. Concerning physiological role of Ca^{2+} ions, the findings are mainly focused on physical models assuming mechanisms generating the ionic transport disturbance and/or ion-protein complexes dysfunction in cells due to MF. Three physical models: ion cyclotron resonance (ICR), free ions' forced-vibration mechanism (FIFVM), and mechanism of magnetosomes motion in magnetic fields were used to confront presented results.

ION CYCLOTRON RESONANCE

The ICR model was used to interpret bioactivity of low-frequency MF combined with low-magnitude DC MF [41–44]. The interpretation of ICR model for bio-systems is based on the assumption that ion-protein complex in the cell proclaims harmonic oscillator with many vibrational energy levels. Applied DC MF with magnitude B_{DC} causes Zeeman splitting of energy level E into two sublevels (E_1, E_2) with energy difference equal to $h(\frac{qB_{DC}}{2\pi m}) = hf_c$ [45]. Additional exposure to AC MF can influence the probability of ion-protein complex transitions between energy states that depends on Bessel function argument [46]. Bioactivity of the ion-protein complex depends on its spatial configuration related to energy state of the complex. Thus, applied ACDC MF can finally lead to changes in cell signaling. Calmodulin with bound Ca^{2+} ions sets an example of bioactive "ion-protein" complex in the cells [31].

Considering ICR model, molecular masses of hypothetical elements of a biochemical complex which could interact with magnetic fields were estimated. Assuming B_{DC} = 6 mT, f_c = 35 Hz and q = (1 ÷ 10) e, calculated molecular masses are enclosed in the range (2.7 ÷ 27) kDa. Many proteins playing a crucial physiological role in the cell have similar masses. At this stage of studies it was impossible to determine a particular complex existing in the U937 cell.

FREE ION'S FORCED-VIBRATION MECHANISM

Proposed mechanism [47] was based on the assumption that observed bio-effects in the cells exposed to external oscillating electromagnetic field (EMF) can be explained through ion's forced-vibration, caused by applied field. Once amplitude of the ion's forced-vibration exceeds some critical value, the oscillating electric charge can influence an electro-sensitive ion channels gating, thus disrupting cellular electrochemical equilibrium and cell function.

To confront discussed model with results obtained for AC MF and ACDC MF action on U937 cells only magnetic fields will be further considered. Under assumption of certain physical properties of the cell, Panagopoulos D.J. *et al.* presented relation between ion oscillating amplitude and displacement Δr_j necessary to disturb electro-sensitive ion channel gating:

$$\frac{B_1 uq}{\lambda\omega} \geq \Delta r_j \tag{1}$$

where λ — attenuation coefficient for the ion movement;
B_1 — AC MF amplitude;
u — ion velocity;
q — ion charge;
ω — angular frequency of the AC MF.

Estimated values of Δr_j are equal to $4 \cdot 10^{-12}$ m and $2 \cdot 10^{-12}$ m for single- and double-valence cations inside the ion channel, respectively. Results obtained for AC MF (6.5_{rms} mT; f = 50 Hz) were confronted with FIFVM model for key biological Ca^{2+} ions. Made calculations assuming $\lambda \cong 10^{-12}$ kg/s and u = 0.25 m/s indicated 2.3×10^{-12} m as an oscillating amplitude for Ca^{2+} ion. It meant that relation (1) was hardly fulfilled for used AC MF, hence the possibility of ion channel gating modulation and further changes in U937 cell viability were doubtful. Above calculations stay in accordance with our observations, no bio-effects were noticed.

Considering results obtained for used static (with magnitude B_{DC}) and alternating magnetic field combination (ACDC MF), relation (1) modifies to:

$$\frac{\sqrt{B_1^{\,2} + B_{DC}^{\,2}}\, uq}{\lambda\omega} \geq \Delta r_j \tag{2}$$

The dependence between ion oscillation amplitude and frequencies from the range (10 ÷ 100) Hz is presented in Fig. 2 for Ca^{2+} ion. It is well seen that relation (2) for 6 mT DC MF combined with 6.5_{rms} mT AC MF was fulfilled in a full range of tested frequencies (12 ÷ 50) Hz. Thus, performed estimations indicated possibility of the ion channel gating modulation through Ca^{2+} ion forced-vibration. It stays in agreement with results presented in Fig. 1, where changes of the viability coefficient S upon ACDC MF action, were experimentally proved.

In case of applied ACDC MF, the ion movement is more complex due to superposition of two movements forced by DC MF and AC MF, respectively. Thus, the involvement of strong enough DC MF might also influence the electro-sensitive ion channels gating. For three times lower magnitude of DC MF and 50 Hz AC MF an oscillating amplitude was comparable with the one obtained for AC MF acting alone and was not enough to cause any changes in U937 cells.

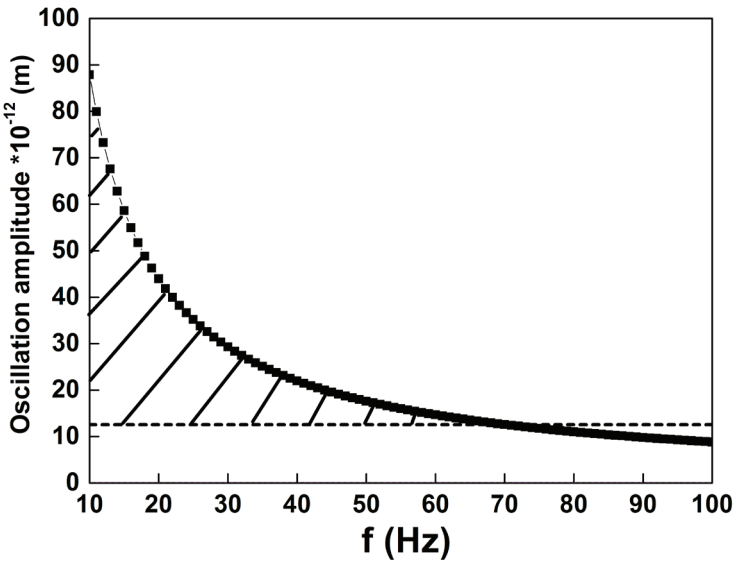

Fig. 2. Oscillation amplitude for Ca^{2+} ion vs. frequency of alternating magnetic field component. B_1 = 9.2 mT and B_{DC} = 6 mT; $\lambda \cong 10^{-12}$ kg/s; u = 0.25 m/s. In the hatched region oscillation amplitudes for Ca^{2+} ion follow the relation (2). Dashed line corresponds to $\Delta r_j = 4\pi \cdot 10^{-12}$ m.

The FIFVM model does not determine a full interpretation of MF influence on U937 cell line viability, particularly in the context of S(f) non-linearity (Fig. 1). It mainly arises from difficulties to define appropriate values of parameters used in the ion movement equation. Unknown value of ion oscillation circular self-frequency ω_0 and assumption that $\lambda >> \omega_0$ exclude analysis of resonant effects.

Nevertheless, basing on the FIFVM model, the calcium ions likely influence on the ion channel gating this way triggering biochemical signal cascades leading to changes of U937 cell line viability.

MAGNETIC FIELDS ACTION ON MAGNETOSOMES

Bioactivity of magnetic fields was also interpreted in a frame of MF action on particles with non-zero magnetic moment (magnetosomes) [48]. This type of structures,

built up from magnetite crystals, were found in a variety of human tissues and some leukemic cell line [49]. Magnetosome with magnetic moment $\boldsymbol{\mu}$ placed in the static magnetic field B_{DC}, will tend to align with the direction of this field. If additional alternating magnetic field $B_1 \sin\omega t$ is applied, the magnetic moment will be reoriented vs. B_{DC} direction (at an angle θ). Inasmuch the magnetic moment reorientation takes a place in the viscous medium, there are three torques on the magnetosome, which limit its movement [27].

Nevertheless, the magnetosome rotational motion alone does not explain the cell reaction to MF action. Some authors suggested the possible linkage between rotation of magnetosome and gating of mechanically sensitive ion channels. The proposed mechanism involves the role of the cell cytoskeleton [50].

Considering parameters of used ACDC MF and according to formulas for rotational viscous drag coefficient and for magnetic moment of a single-domain magnetosome [51–53], the radius of a potential magnetosome necessary to produce an effective magnetosome-induced cytoskeletal tension leading to the ion channel activity was estimated as ≥ 11 nm.

Nevertheless, the value of magnetosome rotation angle (an important variable of quotation used in the model) varied little with the used range ($12 \div 50$) Hz of MF frequency. This did not correspond with the frequency-related results (Fig. 1) obtained in the experiments. Thus, S(f) dependence obtained for U937 cell line cannot be explained by mechanism involves magnetosomes, even such particles would be found in the cells.

CONCLUSIONS

The results exhibited that ACDC MF may influence U937 cell viability in the presence of puromycin, in contrast to action of magnetic fields alone. Basing on made estimations in a frame of tested models, we conclude that observed bio-effects are probably caused by perturbations of calcium ions transport through ion channels, what further could trigger changes of Ca^{2+} ions concentration in the cell compartments. Puromycin as used chemical agent could also influence Ca^{2+} ions intracellular distribution, but on the chemical way [28]. Therefore, we suppose that simultaneous action of ACDC MF and PMC led to cell death through deregulation of calcium dependent cell signaling pathways.

The results confirmed that further studies on static and low-frequency magnetic fields influence on U937 cells or other suchlike cell lines would be required to find biophysical mechanism(-s) explaining particular effects.

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