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PULSED ELECTROMAGNETIC FIELD AFFECTS INTRINSIC AND ENDOPLASMATIC RETICULUM APOPTOSIS INDUCTION PATHWAYS IN MONOMAC6 CELL LINE CULTURE

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Current studies were aimed to elucidate influence of pulsed electromagnetic field stimulation on cell viability and apoptosis induction pathways. For the experimental model we have chosen monocytic cell line MonoMac6 and several apoptosis inducers with different mechanism of death induction like puromycin, colchicine, cyclophosphamide, minocycline and hydrogen peroxide. MonoMac6 cell line was grown at density 1×10^5 cells/well in 96-well culture plates. To induce cell death cell cultures were treated with different apoptosis inducers like puromycin, colchicine, cyclophosphamide, minocycline, hydrogen peroxide and at the same time with pulsed electromagnetic field 50 Hz, 45 ± 5 mT (PEMF) for 4 hour per each stimulation, three times, in 24 hours intervals. Afterwards, cells were harvested for flow cytometry analysis of cell viability measured by annexin V-APC labeled and propidium iodide staining. Expression of apoptosis related genes was evaluated by semi quantitative reverse transcription (RT)-PCR assay. NuPAGE Novex Western blot analysis was carried out for apoptosis inducing factor (AIF) abundance in cytosolic and nuclear extracts of MonoMac6 cells. Puromycin, colchicine and minocycline activated cells and simultaneously treated with PEMF have shown out diminished percentage of annexinV positive (AnV+) cells comparing to controls without PEMF stimulation. MonoMac6 cells puromycin/colchicine and PEMF treated were to a higher extent double stained (AnV+,PI+), which means increased late apoptotic as well as necrotic (PI+) cells, than non-stimulated controls. On the other hand, minocycline activated cells prior to PEMF treatment showed diminished amount of apoptotic and necrotic (annexin V, annexin V and propidium iodide, propidium iodide positive staining) cells. The opposite effect of PEMF on the percentage of annexin V positively stained cells has been achieved after treatment of MonoMac6 culture with cyclophosphamide and hydrogen peroxide. PEMF enhanced early phase of apoptosis induced by both apoptosis inducing agents. The analysis of expression of the apoptosis related genes in MonoMac6 cultures treated with puromycin and exposed to PEMF performed in reverse transcription of polymerase chain reaction (PCR) assay has shown changes in mRNA of genes engaged in intrinsic apoptotic pathway and pathway with AIF abundance. The most influenced was expression of gene belonging to pro-apoptotic family of Bcl-2 and AIF agent. Examination of immunoblots developed with anti-AIF antibody showed that cytosol content of AIF protein was diminished after puromycin and PEMF treatment of MonoMac6 cells. The obtained results indicate that PEMF affects induction of apoptosis in MonoMac6 cells stimulated to death with inducing agents to a different extent. Main finding of the current results is that, PEMF stimulation of MonoMac6 cells simultaneously treated with puromycin caused changes in the Bcl-family genes expression as well as in caspase independent pathway of apoptosis inducing factor (AIF).

Key words: *apoptosis, monocytic cell line MonoMac6, pulsed electromagnetic field, colchicine, cyclophosphamide, hydrogen peroxide, minocycline, puromycin, Bcl-2*

INTRODUCTION

Apoptosis is programmed and regulated cell death playing a key role in tissue and organ development as well as during cell turnover in adult tissues. The ability to induce apoptosis is frequently lost in majority of human tumors. Alternative type of cell death is necrosis, which is uncontrolled process of traumatic cell death caused by acute injury leading to cellular lysis, inflammation and tissue damage. Induction of apoptosis can be triggered by variety of stimuli and activated by three distinct pathways leading to cell death (1, 2).

The extrinsic pathway, often referred to as death receptor signaling pathway, is started by the binding of death activator proteins to cell surface.

The intrinsic pathway, known as mitochondrial mediated, is launched *via* intracellular signals, such as DNA damage, growth factor deprivation, radiation, ischemia and oxidative stress. Under physiological conditions mitochondrial outer membrane displays Bcl-2 molecule that inhibits apoptosis. When internal damage to cell occurs, Bax, a pro-apoptotic molecule migrates to mitochondrial surface and inhibits Bcl-2 protective effect. This interaction results in the releasing of mitochondrial proteins like

cytochrome c, AIF, endonuclease G (ENDO G), Smac/Diablo and HtrA2. Cytochrome c binds Apaf-1 (apoptotic protease activating factor-1) and pro-caspase-9 forming caspase-activating complex directly activating caspase-9, which in turn activates other caspases leading to apoptosis. Despite variance in the types of stimuli that induce apoptosis, both mechanisms turn on caspases to digest cells from within (3-6).

Bcl-2 gene family consists of 'multidomain' death antagonists (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, A1, Bfl-1) and death agonists (Bax, Bad, Bak, Bok/Mtd, Bcl-x_s, Blk, Bim), which function primarily to protect or disrupt the integrity of mitochondrial membranes, respectively (7).

In addition to the mitochondrial mediated pathway and death receptor signaling pathway third mechanism is present and it functions without caspases. The third pathway is associated with endoplasmic reticulum (ER) where are assembled polypeptide chains destined for secretion or routing into various subcellular compartments. It regulates cellular responses to stress and intracellular Ca²⁺ levels. A variety of toxic stimuli results in ER stress that leads to apoptosis. Apoptosis-inducing factor (AIF) is one of the main participants of this pathway. It is localized in the mitochondrial membrane and released from the mitochondria, translocates to the nucleus and triggers apoptosis when a cell receives a signal. AIF acts in a caspase-independent manner. However, in response to certain apoptotic stimuli, AIF cooperates with, or functionally substitutes for, the mitochondrial pathway, whereas in others, AIF constitutes a not crucial death signal (8-10).

Previously obtained data have shown, that pulsed electromagnetic field interaction (50 Hz, 45 mT) caused apoptosis of the peripheral blood mononuclear cells (PBMC) originated from patients with Crohn's disease and changed secretion of cytokines (11). Experiments with U937 lymphoid cell line cultures treated with PEMF revealed that PEMF decreased the apoptosis of cells (12). In order to discriminate and clarify mechanisms responsible for cell death after PEMF, cell culture was stimulated with death inducer - puromycin. Puromycin treatment combined with simultaneous single PEMF exposition reduced the death cells rate. Three fold PEMF exposition increased viability by about 25%. Our data was in agreement with Grassi *et al.* (13). He found that 50 Hz EMF exposure enhanced proliferation and inhibited puromycin induced death in human neuroblastoma and rat neuroendocrine cells (13).

Current studies are aimed to elucidate influence of the pulsed electromagnetic field stimulation on cell viability and find changes in apoptosis induction pathways gene expression. Experimental model is based on monocytic cell line-MonoMac6 and several apoptosis inducers (with different death induction mechanisms) like puromycin, colchicine, cyclophosphamide, minocycline and hydrogen peroxide, which act in the same time as PEMF interacts on cell cultures.

MATERIALS AND METHODS

Cell culture

Human monocytic MonoMac6 cell line was obtained from Ziegler-Heitbrock German Collection of Microorganisms and Cell Cultures.

Cells were cultured in RPMI 1640 medium (Gibco-BRL, USA), supplemented with 10% (v/v) fetal calf serum (Gibco-BRL, USA) heat inactivated, L-glutamine 0.2 M and gentamicin 50 mg/ml (Sigma-Aldrich, Germany) at 37°C in a 5%CO₂ incubator of 90% humidity. Cells viability was monitored by trypan blue exclusion method and counted with a haemocytometer (Fuchs-Rosenthal chamber). The experiments

were performed on cells in the logarithmic phase of growth under condition of ≥98% viability, as assessed by trypan blue exclusion.

For experiments MonoMac6 cells were seeded into 96-well (Nunck, Denmark) culture plates and grown at density 1×10⁵ cells/well in threefold repetition per each sample. After four days of experiment cells were harvested by centrifugation at 280 g for 10 min and used for further analysis. Each density point has always been done as threefold repetition and each value represented a mean from the pulled density experimental points.

Magnetic stimulation

The generator produced pulsed electromagnetic field 50 Hz at a flux density 45±5 mT (built and provided by the Institute of Electron Technology, Cracow, Poland) inside the cell culture incubator. The choice of such frequency of PEMF was related to the following reasons: frequency of magnetic stimulation is higher than the range, which directly depolarizes autonomic fibers, heating effect minimal and all power devices generate EMF with such frequency. The 96-well plate with cells was placed in the generator pocket. The field was applied for 4h per each stimulation with 24 hours intervals between stimulations if more than one has been used. The control samples were in the same incubator but in a distance of 35 cm from the generator.

Cell death induction and electromagnetic stimulation

To induce cell death several cell proliferation inhibitors and death inducing agents were chosen for experiments: a) puromycin, b) colchicine, c) cyclophosphamide monohydrate, e) minocycline hydrochloride, f) hydrogen peroxide solution. All reagents were purchased from Sigma-Aldrich, Germany.

MonoMac6 cells were seeded at density 1×10⁵ cells/well into 96-well plates in triplicates per each experimental point. After 24 h of incubation cells were stimulated independently with one of death inducing agent and simultaneously exposed to PEMF (50 Hz, 45±5 mT) for 4 hours. Final concentration of puromycin, colchicine, cyclophosphamide, minocycline used for cell death induction was 100 µg/ml and 0.4 mM for hydrogen peroxide solution. Then cells were harvested, washed three times with fresh medium, resuspended at initial volume and cultivated for 24 hours, afterwards death induction and PEMF exposure were repeated in 24 hours intervals to obtain threefold death induction and PEMF stimulation. Following third death induction of puromycin, colchicine, cyclophosphamide, minocycline and PEMF dose cells were harvested for flow cytometric analysis. Cells without death inducing agent treatment were non stimulated (NS), cells which have not undergone PEMF constituted a control group (C). Each assay was repeated at least three times. Cells after puromycin and PEMF treatment were collected for extraction of RNA and reverse transcription-PCR and Western blot analysis.

Cell death evaluation-annexin V-PI assays for apoptosis

Twenty four hours after third death inducing agent and PEMF stimulation of MonoMac6 culture, cells were harvested, washed twice with cold PBS (Sigma-Aldrich, Germany) and resuspended in 1× binding buffer (1.0 mmol/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH=7.4, 140 mmol/L NaOH, 2.5 mmol/L CaCl₂), (BD Biosciences, USA) and stained with annexin V - propidium iodide (PI) assays and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol. Briefly, cells at density 1×10⁶ cells/ml were stained with 5 µl annexinV-allophycocyanin conjugate (APC) and 10 µl PI (5 µg/ml) in 1× binding buffer, gently

vortexed and incubated for 20 min at room temperature in the dark (BD Biosciences, USA). Prior to flow cytometric analysis 400 µl of 1x binding buffer was added and cells were analyzed on a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest software.

Annexin V-APC was used to quantitatively determine the percentage of cells within population that were undergoing apoptosis. Propidium iodide as standard flow cytometric viability probe was used to distinguish viable from non-viable cells. Annexin V-APC positive cells were analyzed as apoptotic. Annexin V-APC and PI staining cells were either in the end stage of apoptosis or undergoing necrosis and analyzed as already dead. PI stained cells were necrotic.

Suggested controls to set up compensation and quadrants encompassed unstained cells, cells stained with annexinV-APC alone (for FL-4 fluorescence) and with PI alone (detected in FL-3). A minimum 10,000 events were collected on each sample.

Extraction of RNA and reverse transcription polymerase chain reaction RT-PCR

As described before, MonoMac6 cells were seeded at density 1×10^5 cells/well into 96-well plates in triplicates per each experimental point. After 24 hours of incubation cells were treated with puromycin as one of death inducing agents and simultaneously exposed to PEMF (50 Hz, 45 ± 5 mT) for 4 hours. Then cells were harvested, washed three times with fresh medium, resuspended at initial volume and cultivated for 24 hours, afterwards death induction and PEMF exposure were repeated in 24 hours intervals to obtain threefold death induction and PEMF stimulation. At 24 hours following third stimulation the cells were harvested and total cellular RNA was isolated using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's recommendation (35). After precipitation, RNA was resuspended in RNase free water (HyClone Laboratories, Inc., USA) and its concentration was estimated by absorbance at 260 nm wavelength with NanoDrop1000 (Thermo Fisher Scientific Inc.USA) The RNA A_{260}/A_{280} ratios were between 1.6 and 1.8.

Semiquantitative reverse transcription (RT)-PCR was performed using 2 µg of total RNA and One-Step RT-PCR kit (Qiagen, Germany). Sequence of specific primers (Sigma-Aldrich, Germany), is collected in *Table 1*.

The RT-PCR applied was 30 min at 50°C for reverse transcription, 15 min at 95°C for HotStarTaq DNA polymerase activation. Omniscript and Sensiscript reverse transcriptases inactivation and cDNA template denaturation followed by 40 cycles consisting of 1 min denaturation at 94°C, 1 min of annealing at 50–68°C dependently on T_m of the used primer and 2 min of extension at 72°C. The program was terminated by a final extension of 10 min at 72°C. The RT-PCR reaction was performed in Professional Basic Gradient thermal cyclor

(Biometra, Berlin, Germany). Amplified products were detected by electrophoretic analysis on 2% agarose gels (Bio-Rad, Hercules, CA, USA) stained with ethidium bromide (Bio-Rad, Hercules, California, USA) 0.5 µg/ml followed by examination under UV light using Transluminator (Vilber Lourmat, France) and analysed by PhotoCapt software (Vilber Lourmat, France). Location of a predicted PCR products was confirmed using O'Gene Ruler 50 bp DNA Ladder (Fermentas Life Sciences, San Francisco, CA, USA) as a standard size marker.

Cytosol and nuclear protein isolation from MonoMac6 cultures puromycin and pulsed electromagnetic field stimulated

Cytosol extracts of cells were isolated using the following procedure: Mono Mac6 cells 24 hours after last simultaneous cell treatment with puromycin 100 µg/ml and PEMF were harvested, washed in ice-cold PBS and resuspended in buffer A (10 mM HEPES pH 7.8; 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF). After 15 min. incubation, nonidet (NP-40) was added. The samples were vortexed shortly and centrifuged 14,000 rpm/min for 15 min. Supernatants containing cytosol proteins were collected and validated for protein concentration. Pellets were resuspended in buffer C (50 mM HEPES pH 7.8; 50 mM KCl, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol). The samples were incubated for 20 min, next centrifuged 14,000 rpm/min for 5 min and the supernatants containing nuclear proteins were collected and validated for protein concentration. After centrifugation supernatants were assayed for protein content using spectrophotometric measurement of absorbance at 280 nm (NanoDrop1000) and bovine serum albumin as standard (14). All reagents for cytosol and nuclear protein isolation were purchased from Sigma-Aldrich, Germany.

NuPAGE Novex Western blot for apoptosis inducing factor abundance in cytosolic and nuclear extracts of MonoMac6 cells

Equal amount of cytosol, nuclear proteins (20 µg per each sample) and molecular weight Novex® Sharp Protein Standard (Invitrogen, CA, USA), were separated in 4-12%Bis-Tris Gel NuPAGE® electrophoresis using MES SDS Running Buffer (Invitrogen, CA, USA). Separated proteins were dry blotted onto nitrocellulose iBlot®gel transfer stacks in iBlot Gel transfer device for 7 min. The nitrocellulose membrane was blocked with Western Dot™ blocking Buffet overnight at 4°C. Blocking procedure was followed with 1 hour exposure to rabbit anti-human polyclonal primary antibody against AIF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Santa Cruz, CA, USA) as a control of immunoblotting, both diluted 1:500 at final concentration. Straightly, according to manufacturer's procedure detection step was developed with

Table 1. Reverse transcription-PCR primer sequences.

Gene	Forward primer	Revers primer
GAPDH	GGCCAAGATCATCCATGACAAC	ACCAGGACATGAGCTTGACAAAGT
AIF	GGGAGGACTACGGCAAAGGT	CTTCCTTGCTATTGGCATTTCG
Bcl-2	GGGTACGATAACCGGGAGAT	ACAGTTCCACAAAGGCATCC
Bax	TTTGCTTCAGGGTTTCATCC	GGAGGAAGTCCAATGTCCAG
Caspase-9	TGTCTACTACTTCCCAGGTTTT	GTGAGCCCACTGCTCAAAGAT
c-Myc	TCGGGGCTTTATCTAACTCG	GCTGCTATGGGCAAAGTTTC
ENDO G	GTACCAGGTCATCGGCAAGAAA	CGTAGGTGCGGAGCTCAATT
p21	AGAACC GGCTGGGGATGT	AGGGCTTCCTCTTGAGAAA
Smac	GGAGCCAGAGCTGAGATGAC	CCAGCTTGTTTCTGCTTTC

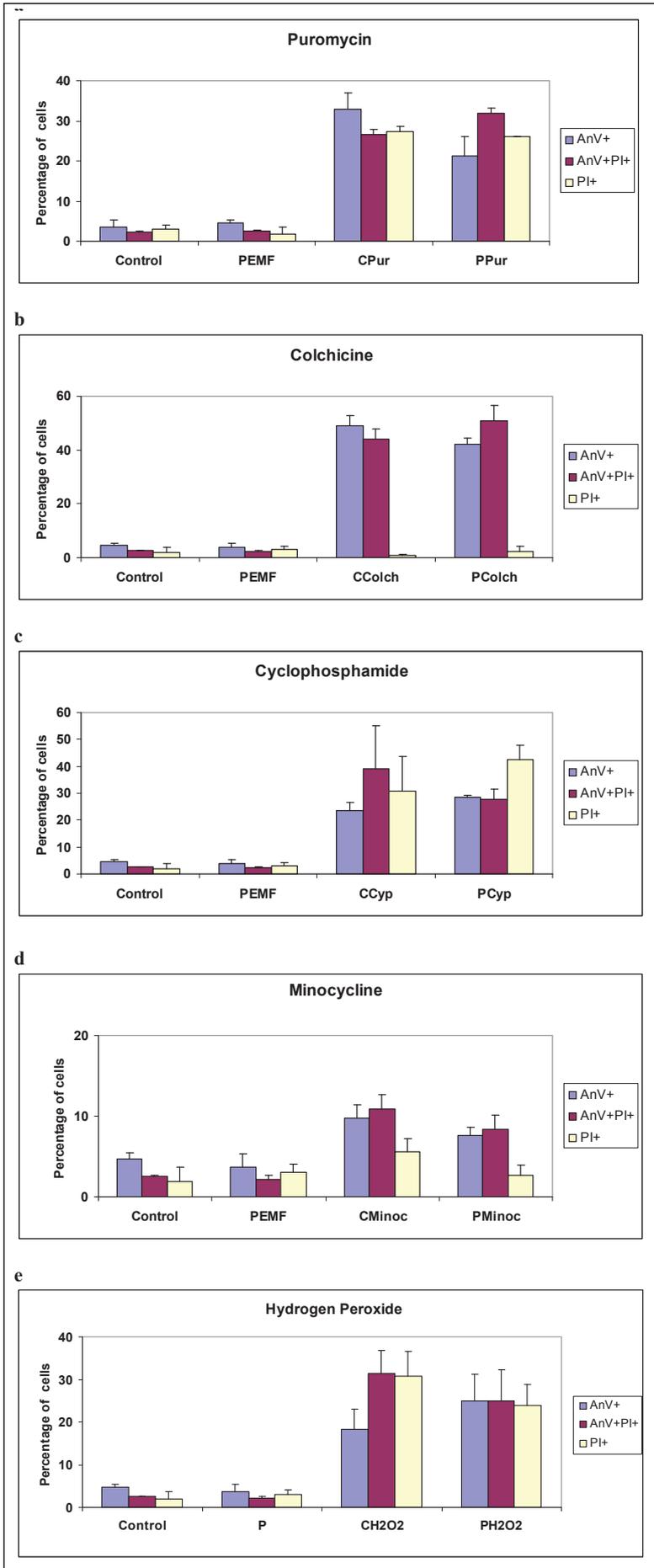


Fig. 1. Apoptosis (AnV+), late apoptosis (AnV+,PI) and necrosis (PI+) of MonoMac6 cell cultures upon **a)** puromycin (Pur), **b)** colchicine (Colch), **c)** cyclophosphamide (Cyp), **d)** minocycline (Minoc), **e)** hydrogen peroxide (H₂O₂) treatment and simultaneous PEMF stimulation three times for 4 hours each one (PPur, PColch, PCyp, PMinoc, PH₂O₂); control cultures without PEMF exposure treated with puromycin (Pur), colchicine (Colch), cyclophosphamide (Cyp), minocycline (Minoc), hydrogen peroxide (H₂O₂) (CPur, CColch, CCyp, CMinoc, CH₂O₂) by flow cytometry analysis. The data are expressed as mean (±S.D.) of 3 experiments performed in threefold repetition; statistical significance was determined by Student *t*-test analysis as P<0.05.

WesternDot™ 625 Western blot kit containing biotinylated goat anti-rabbit secondary antibody followed by a Odot® 625 streptavidin conjugate. The obtained fluorescence signal from protein detection was examined under UV light using Transluminator (Vilber Lourmat, France) and analysed by PhotoCapt software (Vilber Lourmat, France).

Statistical analysis

The data were expressed as mean and (\pm) standard deviation (S.D.) and compared using the Student t-test considering $P < 0.05$ defined as significantly different.

RESULTS

Cell death induction and pulsed electromagnetic field stimulation in annexinV-PI assays

To examine the MonoMac6 cells viability changes upon PEMF treatment different apoptosis inducers and proliferation inhibitors were chosen for experiments: puromycin, colchicine, cyclophosphamide, minocycline and hydrogen peroxide. Puromycin, colchicine, minocycline induced cells and simultaneously treated with PEMF have shown out diminished percentage of annexin V positive (AnV+) cells when compared to controls without PEMF stimulation (Fig. 1a, 1b, 1c., Table 2).

MonoMac6 cells puromycin/colchicine and PEMF treated were to a higher extent of double stained (AnV+, PI+), which

means late apoptotic as well as necrotic (PI+) when compared to the controls non stimulated with PEMF. Minocycline induced cells prior to PEMF treatment exhibited diminished apoptotic and necrotic (annexin V, annexin V and propidium iodide, propidium iodide positive staining) cells. The opposite effect of PEMF on the percentage of annexin V positively stained cells occurred after treatment of MonoMac6 culture with cyclophosphamide or hydrogen peroxide.

Cyclophosphamide activated - PEMF exposed cells indicated higher rate of early apoptotic cells - annexin V stained, but diminished percentage of the late apoptotic and necrotic cells and higher rate of necrotic cells (Fig. 1d, 1e; Table 2).

As a death inducing agent was used hydrogen peroxide, in MonoMac6 culture hydrogen peroxide - PEMF treated, cells showed increased percentage of annexin V positive cells and decreased annexin V and propidium iodide, and propidium iodide, comparing to PEMF unexposed ones (Fig. 1e, Table 2).

Puromycin and pulsed electromagnetic field treatment influence change in apoptosis related genes expression and apoptosis inducing factor protein

MonoMac6 cells were seeded at density 1×10^5 cells/well into 96-well plates were treated with puromycin and simultaneously exposed to PEMF (50 Hz, 45 ± 5 mT) 3 times, 4 h per each stimulation in 24 h intervals. Following third death induction of puromycin and PEMF dose cells were harvested for total RNA isolation, cytosol and nuclear cellular extracts preparing. The analysis of expression of the apoptosis related genes in RT-PCR

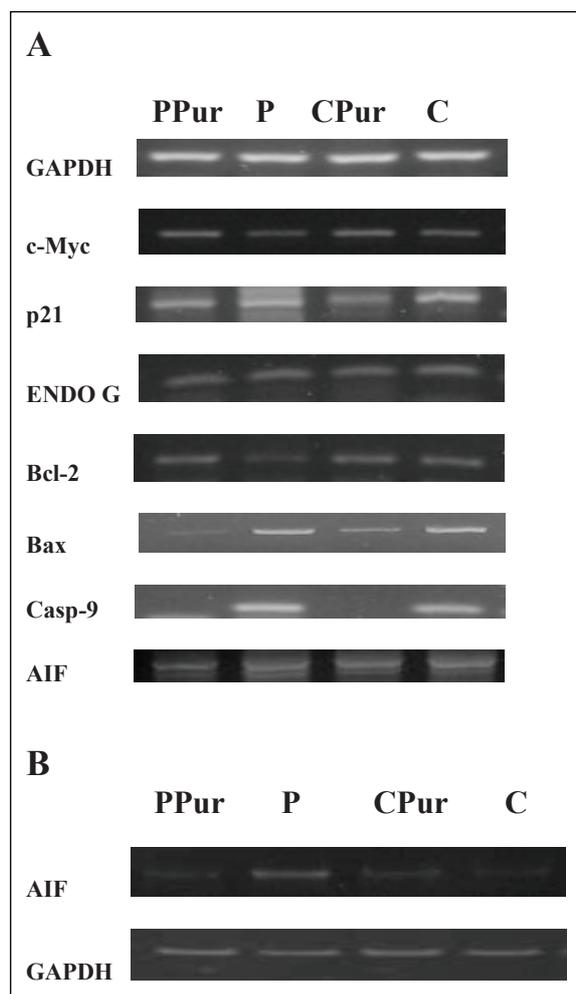


Fig. 2. Expression of puromycin-PEMF sensitive and insensitive genes in MonoMac6 culture treated with puromycin and PEMF stimulated (described under Material and Methods). **A**). RT-PCR products on agarose gels stained with ethidium bromide: C-control MonoMac6 culture, CPur - MonoMac6 cells treated with puromycin, P - PEMF exposed MonoMac6 cells, PPur - puromycin treated and PEMF exposed MonoMac6 culture. **B**) Cytosol extract proteins detected by immunoblots detected with WesternDot™ 625 Western Blot Kit: C-control MonoMac6 culture, CPur - MonoMac6 cells treated with puromycin, P - PEMF exposed MonoMac6 cells, PPur - puromycin treated and PEMF exposed MonoMac6 culture.

Table 2. Percentage of MonoMac6 cell in control culture (C), in PEMF stimulated (PEMF), in cultures stimulated with apoptosis inducers: puromycin (Pur), colchicine (Colch), cyclophosphamide (Cyp), minocycline (Minoc), hydrogen peroxide (H₂O₂) in control cultures (CPur, CColch, CCyp, CMinoc, CH₂O₂) and PEMF stimulated (PPur, PColch, PCyp, PMinoc, PH₂O₂) cultures.

MM6 cells	Annexin V positive cells (AnV+)% (±SD)	Annexin V&PI positive cells (AnV+PI+)% (±SD)	PI positive cells (PI)% (±SD)	P
C	3.67 (±1.63)	2.21 (±0.40)	3.0 (±1.75)	*0.050
PEMF	4.64 (±0.76)	2.48 (±0.19)	1.86 (±1.05)	**0.012 ***0.029
CPur	32.81 (±4.25)	26.69 (±1.1)	27.43 (±1.28)	*0.014 **0.003
PPur	21.35 (±4.82)	31.79 (±1.3)	26.03 (±0.01)	**0.050
CH ₂ O ₂	18.37 (±4.61)	31.31 (±5.4)	30.86 (±5.75)	*0.049 **0.050
PH ₂ O ₂	24.91 (±6.2)	24.84 (±7.4)	23.96 (±4.77)	***0.050
CCyp	23.6 (±2.98)	39.13 (±15.88)	30.87 (±12.86)	*0.002 **0.034
PCyp	28.32 (±1.07)	27.83 (±3.6)	42.36 (±5.3)	***0.050
CMinoc	9.75 (±1.61)	10.93 (±1.76)	5.62 (±1.56)	*0.019 **0.042
PMinoc	7.58 (±0.99)	8.34 (±1.79)	2.68 (±1.27)	***0.028
CColch	49 (±3.68)	44 (±3.67)	0.84 (±0.48)	*0.005
PColch	42 (±2.6)	50.86 (±5.7)	2.45 (±1.89)	**0.029 ***0.025

* statistically significant differences between Annexin V positive cells in Control (C) with and PEMF (P) cultures

** statistically significant differences between Annexin V and PI positive cells in Control and PEMF cultures

*** statistically significant differences between PI positive cells in Control and PEMF cultures

assay has shown changes in mRNA of genes engaged in intrinsic apoptotic pathway and pathway with AIF abundance upon PEMF stimulation of puromycin treated cells. The most influenced was expression of Bax gene- pro-apoptotic member of Bcl-2 family, and AIF gene. In cultures stimulated with puromycin and PEMF, Bax gene mRNA was down regulated, caspase-9 mRNA expression was completely blocked but Bcl-2 mRNA was up regulated. AIF mRNA seemed to be also slightly down regulated. ENDO G mRNA and mRNAs of proliferatory and cell cycle regulatory genes like c-Myc or p21 were detected with the same intensity of bands in all experimental points without visible differences in their expression upon puromycin and PEMF treatment.

Bcl-2 family protein products were not found in MonoMac6 protein extracts in spite of the presence of pro-apoptotic regulation of expression within their mRNA transcripts, but examination of immunoblots developed with anti-AIF antibody showed that cytosol content of AIF protein was diminished upon puromycin and PEMF treatment of MonoMac6 cells (Fig. 2b). Nuclear extract protein analysis has not revealed presence of any one of them by immunoblots examination.

DISCUSSION

The main finding of this study is that PEMF treatment of puromycin activated MonoMac6 cells affects intrinsic and endoplasmatic reticulum dependent apoptotic pathways. In current research, we have compared early apoptosis, late apoptosis, and necrosis in MonoMac6 cell line culture upon cell death induction by several apoptosis inducers treatment combined with PEMF stimulation and analysed changes in

apoptosis related gene expression. Our studies were aimed to elucidate mechanism responsible for viability changes. In order to address the problem, we utilized besides puromycin which is potent inhibitor of telomerase activity in leukemic cell lines and inducer of apoptosis, the other apoptosis inducers like colchicine, cyclophosphamide, minocycline and hydrogen peroxide (15-20).

Puromycin treated and PEMF stimulated MonoMac6 cultures have shown lower percentage of annexin V (34.9%), annexin V and propidium iodide (19.1%) and propidium iodide (0.5%) positively stained cells comparing to cultures not exposed to PEMF, respectively (Fig. 1a, Table 2). Currently obtained results are consistent with previous finding that puromycin and PEMF stimulated lymphoid U937 cells were less susceptible to apoptosis induction than cultures not stimulated with PEMF (12).

Puromycin is known potent inhibitor of telomerase activity and independently of its differentiation - apoptosis inducer in HL-60, U937 and K562 human leukemic lines, also commonly used chemotherapeutic agent (16). Flow cytometry analysis of cell cycle of the drug treated cells of these leukemic lines showed that puromycin unselectively induced apoptosis.

Our data is in agreement with Grassi *et al.* (13). He found that 50 Hz EMF exposure enhanced proliferation and inhibited puromycin induced death in human neuroblastoma and rat neuroendocrine cells, caused by increased expression of voltage-gated Ca²⁺ channels on plasma membrane of the exposed cells and consequent increase in Ca²⁺ influx responsible for the electromagnetic modulation of cell proliferation and apoptosis (13, 21).

The expression study of a broad spectrum of pro-apoptotic and anti-apoptotic genes in puromycin-PEMF treated

MonoMac6 cultures has shown that cellular levels of their transcriptional products were affected by PEMF stimulation. Important variations were revealed in content of Bcl-2 family gene members Bcl-2, Bax and caspase-9 as well as in gene not belonging to Bcl-2 family but engaged in apoptosis regulation in caspase independent manner - AIF gene. Our analysis of Bcl-2 mRNA expression has proved that PEMF interaction elevated Bcl-2 mRNA in puromycin treated cells but diminished mRNA of pro-apoptotic member - Bax gene.

The obtained results are partially confirmed by the investigation of the gene expression of Bcl-2, Bax, p53 and hsp70 in freshly isolated and in culture-aged human lymphocytes that indicates the modulation of these genes by static magnetic field (SMF) exposure in the used experimental conditions, in a gene-, age- and time-dependent manner. The exposure of isolated lymphocytes to 6 mT SMF for up to 24 hours modulated increased Bax and p53 and decreased hsp70, and Bcl-2. The amount of increment and/or decrement of the proteins varied for each gene examined and was independent of the apoptotic inducers (22).

The caspase-independent pathway of apoptosis was also confirmed by the observation of the decrease of AIF mRNA expression and correlated with AIF protein level in puromycin and PEMF treated MonoMac6 cells. The obtained data are partially consistent with investigations carried out with human gastric adenocarcinoma AGS and human bladder carcinoma J82 cells activated with transglutaminase-2 and photodynamic therapy that resulted in caspase-dependent and caspase-independent apoptotic cell death with AIF releasing (23). In studies with cyclohexyl compounds against HL-60, REH, MOLT-4, KG-1, JVM-2, and K-562 leukemic cell lines authors have found that cell death was mediated by caspase-independent apoptosis associated with mitochondrial dysfunction, and AIF translocation. These results supports our hypothesis (24). The apoptosis process is also regulated and influenced by p53 - protein is known as tumor suppressor protein controls expression of p21 protein which functions as a regulator of cell cycle progression at S phase, and c-Myc gene expression, which is very strong protooncogene, playing an important role in regulation of proliferation, growth, differentiation and apoptosis (25). Analysis of p53, p21 and c-Myc mRNAs has not revealed significant changes in these apoptosis regulatory gene expression in PEMF stimulated-puromycin induced cells, although protein 53 is an important protein for apoptosis, it is capable of affecting both the intrinsic and extrinsic pathways, but p53 mutations exist in 50% of tumor cells (26). The p53 gene is frequently inactivated in half of lung human adenocarcinomas (AdC) however, it is not genetically altered in the other half of lung AdCs (27). Therefore, it is possible that p53 is inactivated by other mechanisms like HPV infections in lung AdCs (28). There are also some p53 wild type tumor cell lines, such as HCT116 and U2OS, which are sensitive to inhibition of serine/threonine-protein kinase (Chk1) due to attenuated p21 (waf1) induction upon DNA damage. A novel mechanism for disruption of the p53-p21 (waf1) pathway as currently known involves either mutation of p53 or reduction of p53 protein levels. In consequence, this attenuated p21 (waf1) expression may render some p53 wild type tumors sensitive to a combination of DNA damage plus checkpoint inhibition. Our findings of gene-dependent modulation in long-term exposure system are in agreement with literature data (29, 30).

Colchicine-PEMF stimulation effect exerted on MonoMac6 cells was similar to the result obtained in the case of puromycin treated cells as it regards early apoptosis analysis. Colchicine and PEMF treated cells have shown 14.2% lower annexin V staining, but amounts of late apoptotic and necrotic

cells were 15.5% and 52.1% higher comparing to cultures not exposed to PEMF.

Apoptosis induction in MonoMac6 culture carried out also with colchicine an alkaloid, the microtubule disruptor that has been widely used to treat gout and cancer. The effect of colchicine on apoptosis and the apoptosis-associated signaling pathways studied in human normal liver cells L-02 revealed that cell apoptosis was mediated by acting *via* the intrinsic apoptotic pathway - the activation of caspase-3 and -9, up-regulation of Bax and down-regulation of Bcl-2 genes (17).

Our flow cytometry cell viability analysis confirmed, that PEMF inhibits early apoptotic stage of cell death, but increases level of late apoptosis and necrosis in colchicine treated MonoMac6 cells.

The influence of PEMF on minocycline MonoMac6 cultures resulted in diminished amount of all death markers measured by annexin V (22.2%), annexin V plus propidium iodide (23.6%) and propidium iodide staining (52.3%), whereas only minocycline treated cells showed increased percentage of early and late apoptotic population and necrotic ones comparing to the controls. The obtained data indicate, that PEMF inhibits both types of cell death apoptosis and necrosis upon minocycline induction of monoMac6 cell line. Our results are confirmed by investigations carried out with minocycline as a broad-spectrum tetracycline antibiotic which exhibits anti-inflammatory properties independent of its antibiotic activity. The anti-inflammatory effects of minocycline in human monocytes revealed that minocycline inhibits points of convergence of distinct and interacting signaling pathways mediating multiple inflammatory signals which may influence monocyte activation, traffic and recruitment into the brain (31). Minocycline effectively crosses the blood-brain barrier and has demonstrated neuroprotective qualities in experimental models of CNS trauma, stroke, spinal cord injury, and neurodegenerative diseases including amyotrophic lateral sclerosis, Huntington disease, Parkinson disease, and multiple sclerosis (32-35).

Effects of PEMF on MonoMac6 cultures stimulated for death with hydrogen peroxide as an apoptosis inducer exhibited increase by about 35.5% of early apoptotic population measured by annexin V staining of cells and decrease of late apoptotic (19.8 %) and necrotic population (22.35%) of cells in comparison to PEMF not stimulated cells. Hydrogen peroxide is known to induce apoptosis *via* production of reactive oxygen species (ROS) that includes hydroxyl radical (HO[•]), superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and organic peroxide radicals. ROS are known important mediators of both types of cell death - apoptosis and necrosis. H₂O₂ represents a particularly important molecule because it is generated under nearly all oxidative stress conditions and it can participate in several fundamental intracellular processes. It triggers the intrinsic pathway (such as oxidative or genotoxic stress) mainly transduced to mitochondria which then undergoes a series of biochemical events resulting in mitochondria membranes permeabilization and release of pro-apoptotic molecules from mitochondria, such as cytochrome c (36-39).

Our studies confirmed that cell death induced by hydrogen peroxide treatment triggers in general intrinsic pathways but PEMF enhances early phase of apoptosis but diminishes late apoptosis and necrosis in cells treated with hydrogen peroxide. The obtained effect has strictly anti-inflammatory meaning and might be useful as an additional component in therapy of chronic inflammatory diseases.

In turn, cyclophosphamide treatment of MonoMac6 cultures combined with PEMF stimulation increased of early apoptosis and necrosis rate of about 20% and 37.2%, respectively, but decreased level of late apoptotic cells of 28.8% when compared to controls.

Cyclophosphamide slows or stops cell growth and is mainly used with other chemotherapy agents in the treatment of lymphomas, some forms of brain cancer, leukemia and solid tumors. At higher doses, it is associated with increased cytotoxicity and immunosuppression (40, 41). The influence of cyclophosphamide (CYP) on the rat urothelial cultured cells (RUCC) resulted in induction of apoptotic and necrotic population of RUCC (42). The investigations with fucosyltransferase IV (FUT4), which is an essential enzyme that catalyzes the synthesis of difucosylated oligosaccharide found out that FUT4 inhibited cell apoptosis induced by cyclophosphamide through decreasing the expression of apoptotic proteins Bax, caspase 3, and PARP and increasing the expression of anti-apoptotic protein Bcl-2 via the ERK/MAPK and PI3K/Akt signaling pathways in A431 cells (43). Our previous findings have confirmed previous results showing that threefold stimulation of PBMCs from AML patients with PEMF increased significantly apoptotic cells number (44).

In conclusion, our results indicate that PEMF affects apoptosis induction pathways in MonoMac6 cells stimulated with death inducers. PEMF stimulation with puromycin treatment caused changes in expression of Bcl-2 family members increasing content of anti-apoptotic Bcl-2 gene and gene of caspase independent pathway - AIF. Opposite effect was achieved as far as it regards pro-apoptotic Bcl-2 family members genes - Bax and Bcl-xL.

Experiments with human lymphoidal cells (Jurkat) and ciprofloxacin as a inhibitor of the mitochondrial topoisomerase II showed that at 25 µg/ml concentration ciprofloxacin inhibited proliferation of cells with induction of chromosomal instability without any symptoms of cell death, while if used at much higher concentration exceeding 80 µg/ml induced apoptosis (45).

Photophoresis therapy that involved extracorporeal exposure of isolated human peripheral blood leukocytes to UVA irradiation in the presence of different photosensitizers revealed high increase of apoptosis of the photochemically treated lymphocytes in dose and time dependent manner, regardless of the photosensitizer used in phototreatment (46).

Studies focused on a potential role of macrophages in the proliferation of total human peripheral blood mononuclear leukocytes in the milieu of *H. pylori* and *E. coli* LPS found significant proportion of human lymphocytes exhibiting the features of apoptosis in cultures treated with *H. pylori* LPS, suggesting a role of apoptotic changes in LPS-dependent antiproliferative activity towards non-adherent lymphocytes (47).

Action of PEMF to apoptosis and necrosis inducers may be related to the expression of ATP-binding cassette transporter proteins like P-glycoprotein, product of MDR1 or MRP genes, which are actively effluxing a wide range of structurally and functionally diverse compounds from target cells and the same provide resistance do death induction (48). Obtained data revealed that PEMF-puromycin tandem effect could be beneficial in anti-cancer therapy to protect healthy tissues.

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