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Pulsing Electromagnetic Field and Death of Proliferating Peripheral Blood Mononuclear Cells from Patients with Acute Myelogenous Leukemia*

Pulsacyjne pole elektromagnetyczne a śmierć komórkowa proliferujących leukocytów jednojądrzastych pochodzących od pacjentów z ostrą białaczką mieloidalną

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Abstract

Background. A pulsing electromagnetic field (PEMF) influenced the viability of proliferating *in vitro* peripheral blood mononuclear cells isolated from Crohn's disease patients by induction of cell death but did not cause any vital changes in the cells from healthy donors. Experiments with lymphoid cell line U937 have shown a protective effect of PEMF on puromycin-treated cells.

Objectives. The current study aimed to investigate the influence of PEMF on native proliferating leukocytes originating from newly-diagnosed acute myelogenous leukemia (AML) patients.

Material and Methods. The effects of exposure to PEMF were studied in peripheral blood mononuclear cells from 8 patients with AML. Peripheral blood mononuclear cells (PBMCs) were stimulated with three doses of PEMF for 3h each with 24h intervals. After the last stimulation, the cells were double stained with Annexin V and 7-amino-actinomycin D (7-AAD) dye to estimate viability by flow cytometry analysis.

Results. Results indicated an increase of Annexin V-positive as well as double stained- and 7-AAD-positive cells after exposure to threefold PEMF stimulation.

Conclusions. A low-frequency pulsing electromagnetic field induces cell death in native proliferating cells isolated from AML patients. The increased vulnerability of proliferating PBMCs may be potentially applied in the therapy of AML (Adv Clin Exp Med 2011, 20, 6, 721–727).

Key words: pulsing electromagnetic field, acute myelogenous leukemia, apoptosis, necrosis.

Streszczenie

Wprowadzenie. Pulsacyjne pole elektromagnetyczne (PEMF) wpływa na żywotność proliferujących *in vitro* jednojądrzastych leukocytów krwi obwodowej (PBMCs) izolowanych od pacjentów z chorobą Crohna przez wywołanie śmierci komórkowej, a nie powoduje zmian w żywotności komórek od zdrowych dawców. Eksperymenty prowadzone na linii komórkowej U937 wykazały ochronny wpływ PEMF na komórki poddane działaniu puromycyny.

Cel pracy. Bieżące badania mają na celu przetestowanie wpływu PEMF na natywnie proliferujące leukocyty pochodzące od pacjentów z AML.

Materiał i metody. Działanie wywierane przez PEMF było badane na jednojądrzastych leukocytach krwi obwodowej uzyskanej od 8 nowo zdiagnozowanych pacjentów. PBMCs były poddawane 3-krotnie po 3 godz. jednorazowo działaniu PEMF w odstępach 24 godz. Po ostatniej stymulacji wybarwiono komórki aneksyną V i 7-amino-aktynomycyną D do analizy żywotności metodą cytometrii przepływowej.

Wyniki. Uzyskano wzrost liczby komórek pozytywnie barwiących się aneksyną V, 7-amino-aktynomycyną D oraz obydwoma markerami po 3-krotnej ekspozycji PEMF.

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Wnioski. Pulsacyjne pole elektromagnetyczne wywołuje śmierć komórkową w natywnie proliferujących komórkach pochodzących od pacjentów z AML. Zwiększona podatność proliferujących leukocytów jednojądrzastych na działanie PEMF potencjalnie może być wykorzystana do leczenia AML (*Adv Clin Exp Med* 2011, 20, 6, 721–727).

Słowa kluczowe: pulsacyjne pole elektromagnetyczne, ostra białaczka mieloidalna, apoptoza, martwica.

Previous studies have shown that a pulsing electromagnetic field (PEMF) reduces the viability of inflammatory peripheral blood mononuclear cells (PBMCs) originating from patients with Crohn's disease (CD) when stimulated with a mitogen. The effect on viability was doubled in Crohn's patients [1]. Contrarily, lymphoid cell line U937 treated with PEMF and an apoptosis induction agent, puromycin, was protected from cell death [2].

Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), on the other hand, belong to a group of rapidly progressing cancers of the bone marrow and blood with non-inflammatory and multifactor genetic pathogenesis [3]. Acute myeloid leukemia is the most common form of adult acute leukemia, the product of several coexisting genetic alterations and characteristic feature clonal expansion of hematopoietic stem and progenitor cells with blocked differentiation [4, 5].

The genetic changes encompass multiple chromosomal translocations leading to expression of leukemogenic fusion proteins. Analyses of leukemic blasts have revealed heterogeneity with abundant gene mutations and changes in expression of microRNA. Multiple AML-associated lesions target chromatin regulators like histone methyltransferases or histone acetyltransferases, including mixed-lineage leukemia 1 (MLL1) or CREB binding protein/p300 [6]. The pathogenetic mechanism responsible for the AML disorder phenotype plays a pivotal role in clinical efforts to therapeutically exploit the potential reversibility of epigenetic mechanisms and the leukemic state to develop efficient, targeted therapeutic strategies because, for the majority of advanced-age AML patients, the prognosis is poor, 40–50% long-term survival for younger patients [7, 8].

Quite a number of studies have indicated that exposure to extremely low frequency electromagnetic fields could cause DNA damage [9–24] and two studies [25, 26] have reported the effects of EMF fields on DNA repair mechanisms. Since the energy level associated with EMF exposure is not sufficient to cause direct breakage of chemical bonds within molecules, the effects are probably indirect and secondary to other induced biochemical changes in cells. One possibility is DNA damage by free radicals that are formed inside cells and disturb macromolecules like DNA, protein, and membrane lipids. Several reports have indicated

that EMF enhances free radical activity in cells [16–17, 27–30], particularly via the Fenton reaction [17, 31]. Free radicals and interaction with transitional metals (e.g. iron) [15–18, 24] have also been implicated as playing a role in the genotoxic effects observed after exposure to these fields. This data confirmed the genotoxic and cytotoxic influence of PEMF on AML-originating leukocytes in the studies.

The present study was designed to evaluate the influence of low energy PEMF on natively proliferating leukocytes isolated from newly-diagnosed AML patients. The project is directed at considering new procedures, alternatives to highly-toxic and poorly-tolerated standard chemotherapy, especially in older patients.

Material and Methods

Patient Characteristics

For this experiments, the authors obtained blood samples after a hematological classification procedure from newly diagnosed AML patients of the Department of Hematology of the Medical College of Jagiellonian University (Cracow, Poland). Eight newly-diagnosed patients (4 men and 4 women, mean age: 53.4 ± 15.5) were tested (Table 1).

Table 1. Patient characteristics

Tabela 1. Dane pacjentów

AML patients (Chorzy na AML)	
	mean (\pm SD)
Sex – M/F (Płeć)	4/4
FAB classification (Klasyfikacja FAB)	
AML-M1	2/2
M2	1/0
M4	1/1
M5	0/1
Age – years (Wiek – lata)	52 (\pm 12)/49 (\pm 19)
Duration of AML (Czas trwania AML)	newly diagnosed

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from remnant samples of the heparinized blood of 8 patients, taken for medical reasons by a standard Ficoll-Paque (Pharmacia, Sweden) density gradient procedure. The PBMCs were washed with an RPMI medium (Gibco, USA) and adjusted to 10^6 cells/ml in an RPMI culture medium supplemented with L-glutamine + gentamicin (0.2 M and 50 μ g/ml) and 10% human AB serum (both reagents from Sigma-Aldrich, Germany) heat inactivated.

PBMC Cultures

PBMCs (0.2 ml aliquots, cell at three densities – 1×10^6 cells/ml, 0.5×10^6 cells/ml and 0.25×10^6 cells/ml) were seeded in triplicate into 96-well culture plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. The 24 h old cultures of PBMCs were then started on stimulation with a pulsed electromagnetic field for the first time.

Magnetic Stimulation

The generator produced a pulsating field of 50 Hz, 45(\pm 5) mT inside the cell culture incubator. The rationale for choosing such a frequency of the PEMF stemmed from the following reasons: the frequency of magnetic stimulation is higher than the range which directly depolarizes autonomic fibers, and the heating effect is minimal. The 96-well plate with cells was placed in the generator pocket. The field was applied after the 24h culture three times for 3 hours per stimulation (3 \times) with 24 h intervals between stimulations. In the same way, control cultures unstimulated with PEMF were carried out in parallel.

Cell Death Evaluation

Annexin V-APC labeled (BD Biosciences, USA) was used to quantitatively determine the percentage of cells within the population that were undergoing apoptosis.

7-amino-actinomycin D (7-AAD, BD Biosciences, USA) as a standard flow cytometric viability probe was used to distinguish viable from non-viable cells. Annexin V-APC positive cells were analyzed as apoptotic and both Annexin V-APC and 7-AAD were either in the end stage of apoptosis or undergoing necrosis analyzed as already dead.

For staining, U 937 cells were washed twice with cold PBS and resuspended in a $1 \times$ binding

buffer (BD Biosciences, USA) at concentration 1×10^6 cells/ml. Then, 100 μ l of the solution was transferred to a 5 ml culture tube and 5 μ l of Annexin V-APC and 5 μ l of 7-AAD were added. The cells were gently vortexed and incubated in darkness for 15 min at RT. Prior to flow cytometric analysis, 400 μ l of the $1 \times$ binding buffer was added and the cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest software.

Suggested controls to set up compensation and quadrants encompassed unstained cells, cells stained with Annexin V-APC alone (for FL-4 fluorescence) and cells stained with 7-AAD alone (detected in FL-3). A minimum 10,000 events were collected on each sample.

Statistical Analysis

Data were expressed as mean and (\pm) standard deviation (SD) and compared using the Student's *t*-test, with $P < 0.05$ considered as significantly different.

Results

PBMCs isolated from the blood of AML patients were cultivated at different ranges of culture densities – 1×10^6 cells/ml, 0.5×10^6 cells/ml and 0.25×10^6 cells/ml, for 4 days. Starting from the second day of culture, the cells were stimulated with low energy PEMF (50 Hz, 45 \pm 5mT peak) three times for 3 h per a day in 24 hour intervals. After the last stimulation, the cells were stained with Annexin V and 7-AAD dye for flow cytometry analysis.

The percentages of apoptotic cells obtained after PEMF exposure were 34.83 (\pm 14.38)%, 33.1 (\pm 6.84)% and 57.51 (\pm 13.28)% for density 1×10^6 cells/ml, 0.5×10^6 cells/ml and 0.25×10^6 cells/ml, respectively. PBMCs not stimulated with PEMF achieved the level of Annexin V-positive cells 20.12 (\pm 7.46)%, 17.6 (\pm 5.63)% and 35.11 (\pm 13.73)% at their respective densities (Fig. 1).

PEMF induced the strongest apoptosis at 0.5×10^6 cells/ml density of cultivated PBMCs.

As regards necrotic cells measured by 7-AAD binding, similar PEMF-induced effects were obtained as with apoptosis, but to a much smaller extent.

The most significant change in the amount of necrotic cells in the PBMC population was found at 1×10^6 cells/ml density and equaled 6.08 (\pm 1.27)% after threefold PEMF stimulation, contrary to unstimulated cells, where necrosis was 3.31 (\pm 2.82)% (Fig. 2).

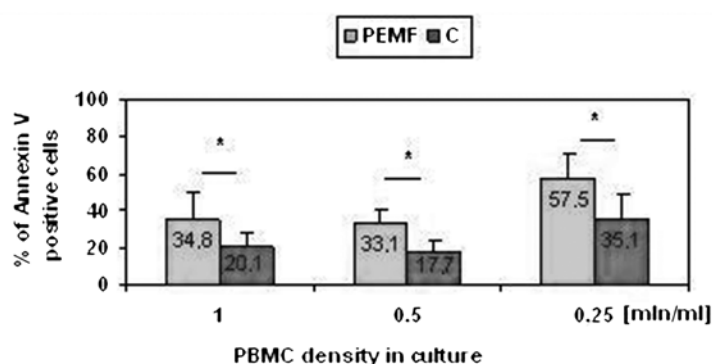


Fig. 1. Apoptosis of AML-originated PBMCs cultivated *in vitro* at three different densities during a 4 day culture and simultaneously threefold stimulated with PEMF 3h per day with 24 h intervals; C – PBMCs not treated with PEMF; PEMF – PBMCs exposed to PEMF. Data is expressed as mean (\pm SD); statistical significance was determined by the Student *t*-test analysis as $*P < 0.05$

Ryc. 1. Apoptoza PBMCs pochodzących od pacjentów z AML hodowanych *in vitro* w 3 różnych gęstościach przez 4 dni i jednocześnie stymulowanych 3-krotnie PEMF przez 3 godz. w odstępach 24 godz.; C – PBMC niestymulowane PEMF; PEMF – PBMCs eksponowane na PEMF. Dane są wyrażone jako średnia (\pm SD); istotność statystyczną określono testem *t*-Studenta jako $*P < 0,05$

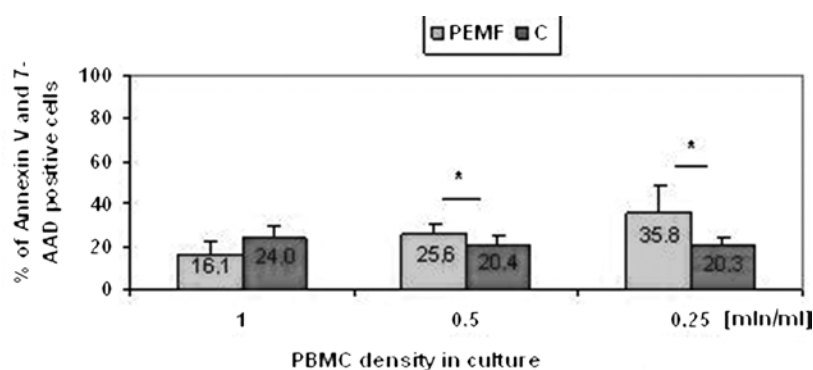


Fig. 2. Necrosis of PBMCs originating from AML patients, cultivated *in vitro* at three different densities during a 4 day culture and simultaneously threefold stimulated with PEMF 3 h per day with 24 h intervals; C – PBMCs not treated with PEMF; PEMF – PBMCs exposed to PEMF. Data is expressed as mean (\pm SD); statistical significance was determined by the Student *t*-test analysis as $*P < 0.05$

Ryc. 2. Martwica PBMCs pochodzących od pacjentów z AML hodowanych *in vitro* w 3 różnych gęstościach przez 4 dni i jednocześnie stymulowanych 3-krotnie PEMF przez 3 godz. w odstępach 24 godz.; C – PBMC niestymulowane PEMF; PEMF – PBMCs eksponowane na PEMF. Dane są wyrażone jako średnia (\pm SD); istotność statystyczną określono testem *t*-Studenta jako $*P < 0,05$

Late apoptotic and necrotic cells measured as a percentage of double stained cells – Annexin V and 7-AAD positive, upon PEMF exposure, achieved levels 76% higher at the middle density of the culture than in the case of the (PEMF-unexposed) control group (Fig. 3).

Discussion

Previously, the authors have described that PEMF caused apoptosis of the peripheral blood mononuclear cells (PBMC) originating from patients with Crohn's disease and the changed secretion of cytokines, but not triggered cell death induction in leukocytes from healthy donors.

Leukocytes treated *in vitro* with mitogens under PEMF influence were more susceptible to apoptosis than non-dividing cells and cell death depends upon the dose of PEMF [1]. The authors have also tested the U937 lymphoid cell line to prove PEMF-induced cellular interaction. In order to elucidate the mechanisms responsible for cell death after PEMF, they stimulated U937 cells with an apoptosis inducing agent, puromycin, prior to PEMF exposure. Puromycin treatment combined with simultaneous PEMF exposure reduced the rate of cell death. Threefold PEMF exposure increased cell viability about 25%. The results obtained [2] have shown the protective effect of PEMF on death induction in the case of puromycin-treated cells.

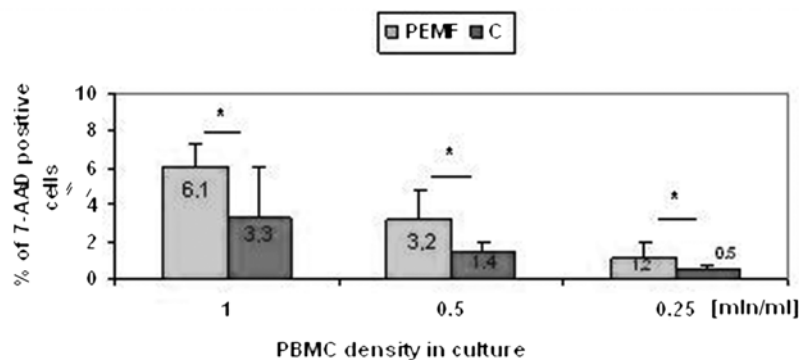


Fig. 3. Percentage of the dead double-stained (late apoptotic and necrotic) PBMCs isolated from AML patients after a 4-day *in vitro* culture at different densities and threefold PEMF stimulation, 3 h per day with 24h intervals; C – PBMCs not stimulated with PEMF; PEMF – PBMCs stimulated with PEMF. Data is expressed as mean (\pm SD); statistical significance was determined by the Student t-test analysis as *P < 0.05

Ryc. 3. Liczba komórek apoptotycznych i martwiczych wyrażona jako procent podwójnie barwiących się komórek (aneksyną V i 7-AAD) po 4-dniowej hodowli *in vitro* prowadzonej przy różnych gęstościach początkowych i 3-krotnej stymulacji PEMF po 3 godz. na dzień w odstępach 24 godz. C – PBMC niestymulowane PEMF; PEMF – PBMCs eksponowane na PEMF. Dane są wyrażone jako średnia (\pm SD); istotność statystyczną określono testem *t*-Studenta jako *P < 0,05

Thus authors current investigations were aimed at researching the effect of 50 Hz, 45 (\pm 5) mT PEMF interactions exerted on natively-proliferating leukocytes isolated from patients with acute myeloid leukemia.

AML is the result of the clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations with the presence of more than 20% of undifferentiated cells [3, 4]. Leukemic blasts from patients with acute myeloid leukemia (AML) have revealed a marked heterogeneity with regard to the presence of acquired gene mutations and changes in gene and microRNA expression [8]. Chromatin-modulating mechanisms are also mediating the transforming activity of key drivers of leukemogenesis by aberrant recruitment of corepressors [5, 32]. Many of the identified genetic alterations not only represent independent prognosticators, but also may constitute targets for specific therapeutic intervention like electromagnetic stimulation.

The main finding is that the damaging PEMF effect on the AML cell profile depends upon cell density, apoptosis is strongest at the middle but necrosis at the highest density of cultivated PBMCs. Threefold stimulation of PBMCs with PEMF increased apoptotic cell numbers up to 87% at middle density and the number of necrotic cells at the highest density reached 83% compared to unexposed cells.

Differences in the extent of the PEMF-induced effects on PBMCs from AML patients may depend upon the heterogeneity of the isolated population of leukocytes from the blood (including blasts forms) as well as arrays of molecular changes in the genome responsible for pathomechanisms. [3–8, 32–34]. The results obtained strongly support the hypothesis that PEMF activates the death pathways in proliferating cells. Gluck et al. [40] confirmed our results on cell lines by showing that 50 Hz; 3,5 mT pulsed electromagnetic field with fourfold stimulation of the U937 line inhibited the proliferation of cells about 85% compared to their initial at a 1.5×10^5 cells/ml concentration. The parameters of the field and density of the culture were both similar conditions.

The death induction effect by PEMF exposure of the native proliferating PBMCs isolated from AML patients has been achieved for all densities of the cultured leukocytes but the strongest increase in the percentage of Annexin V and 7-AAD positive cells was obtained at the middle density.

Considering the numerous side effects of conventional chemotherapy, the age of patients suffering from AML (the majority are elderly) and the complicated pathogenesis, electromagnetic exposure could have clinical implications as an alternative/additional, non toxic, noninvasive future prospective therapy of these proliferative diseases [7, 44].

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