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# MAGNETIC FIELD ANTI-INFLAMMATORY EFFECTS IN CROHN'S DISEASE DEPENDS UPON VIABILITY AND CYTOKINE PROFILE OF THE IMMUNE COMPETENT CELLS

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AIM. We investigated effects of pulsating electromagnetic field (PEMF-50 Hz, 45  $\pm$ 5 mT) on viability and cytokine production by human peripheral blood mononuclear cells (PBMC) from healthy donors and from Crohn's disease patients (CD). METHODS. The study was performed after activation of cells with phytohaemaglutinin (PHA) and lipopolisaccharide (LPS). Exposure of PBMC cultures to PEMF from both CD patients and from healthy donors decreased cell's viability of about 10% and 5% (p>0,05) respectively. PEMF influence was most effective after threefold application. Susceptibility of PBMCs to magnetic field exposure differs among the stimulated (PHA, LPS) and not stimulated (NS) cells. Mitogen activated cells during cell division are most susceptible to induction of the cell death as a result of magnetic interaction, contrary PEMF exposure has minimal effect on non-diving PBMCs from CD patients and from controls. Decreased viability of the Crohn derived cells upon magnetic stimulation was accompanied by altered cytokines profile. Exposed and stimulated PBMCs from Crohn patients decreased IFN-y proinflammatory and increased IL-10 anti-inflammatory cytokine production. The electromagnetically induced cell death could be an important step for non-invasive PEMF treatment in chronic inflammatory diseases.

Key words: pulsating electromagnetic field (PEMF), Crohn's disease (CD), cell viability, cytokines, apoptosis

#### INTRODUCTION

An important aspect of medical use of the electromagnetic field (EMF) is the need to explain how it produces a specific biological effects, that has been reported from several studies of cancer and inflammatory disorders (1). Possible harmful EMF effects on human health has led to growing concern and interests in its influence on life processes (2). Effects of EMF are quite heterogeneous with regard to cell type studied, intensity and type of field used, they nonetheless suggest a link between exposition and metabolism of human cells (3). Studies in that topic pointed to altered rate of transcription of c-myc and others genes as well as  $Ca^{+2}$  fluxes, particularly its entry to the cell which is crucial to apoptosis. A breakdown in apoptosis related mechanisms may result in the development of autoimmune disorders (4).

Chronic inflammatory diseases of the gastrointestinal tract are characterized by overractive T-helper (Th)1-mediated response with production of proinflammatory cytokine like IFN- $\gamma$ . Inflammatory process in bowel might result from innate alteration in the IL-10 production pathway as an anti-inflammatory cytokine (5, 6).

Therapies of inflammatory bowel disease are resulting from the exponential advancement in understanding the human intestinal immune system (7). Despite of an array of biological mechanisms (antibodies against proinflammatory cytokines, T-cell antibodies, anti-inflammatory cytokines) involved in gastrointestinal chronic disease with autoimmune pathogenesis like Crohn's disease, current treatment is still difficult and individually inefficient (8). Direct effect of EMF on inflammatory cells viability may offer additional therapeutic opportunities in the management of Crohn's disease. Apoptosis plays a central role in regulating development and homeostasis of lymphocytes. However effects of EMF on apoptosis are inconsistent. Earlier reports published from different laboratories reported no effects of EMF on apoptosis or cytokine production (9, 10, 11).

On the other hand Fanelli *et al* suggested that static EMF exerts strong and reproducible effect of reducing apoptosis in several cell systems via modulation of  $Ca^{+2}$  influx (12). There are also some experimental date showing that stathmin (important T cell signaling phosphoprotein ) expression is reduced after exposure of Jurkat cells to EMF (13).

The aim of our investigations was to evaluate PEMF influence on viability and cytokine production profile of human peripheral blood mononuclear cells (PBMC) involved in pathomechanism of the inflammatory bowel diseases.

#### MATERIAL AND METHODS

#### Patients

PEMF treatment was studied in 8 patients with untreated Crohn's disease (CD) and 8 healthy volunteers paired with the patients and 8 unpaired control with inflammatory processes excluded. All patients suffered from CD (four females and four males) and their characteristics are shown in *Table 1*.

	CD patients	Controls/paired/	Controls/unpaired/
	$(Mean \pm SD)$	$(Mean \pm SD)$	$(Mean \pm SD)$
Number	8	8	8
Age	$(57 \pm 20)$	$(53 \pm 10)$	$(21 \pm 15)$
Sex (F/M)	4 / 4	5 / 3	7/1
Duration of CD	newly diagnosed	-	-

Table 1. Patient's characteristics.

The control group consisted of 8 patients (5 females and 3 males paired with age and sex) and 8 unpaired controls that underwent routine blood examination prior to various elective medical procedures. All patients and control subjects gave informed consent before the examination.

### Isolation of PBMCs

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

### PBMC cultures

PBMCs (0.2 ml aliquots,  $1x10^6$  cells/ml) were seeded in triplicate into 96-well culture plates and incubated at 37°C in a humified atmosphere containing 5% CO<sub>2</sub> in the presence of mitogens: 1 µg/ml of phytohemaglutynin (PHA; Sigma-Aldrich, Germany) or 1 µg/ml of lipopolysaccharide (LPS; Sigma-Aldrich, Germany) for 48 h. Parallel were cultured PBMCs unstimulated. The 48 h old cultures o PBMCs were started to stimulate with magnetic field for the first time. Intervals between stimulations were 24 h long.

### Cytokine production

To determine cytokine production in PBMC cultures from the controls and CD patients supernatants were collected always 24 h after exposure to electromagnetic field. At the same time were collected supernatants from unexposed to PEMF cultures.

IFN- $\gamma$  and IL-10 were measured using commercially available ELISA kits (BD PharMingen, San Diego, USA) according to the manufacturer's procedure.

#### Magnetic stimulation

The generator produced pulsating field 50 Hz,  $45 \pm 5$  mT inside the cell culture incubator. Rationale for choosing such frequency of PEMF was related to the following reasons: frequency of magnetic stimulation is higher then the range, which directly depolarizes autonomic fibers and heating effect, is minimal. The 96-well plate with cells was placed in the generator pocket. The field was applied after 48 h of culture three times for 3h per each stimulation (3x) with 24 h intervals between stimulations. The same way unstimulated with PEMF control cultures were parallel carried out.

#### Vital staining AO/EB

Vital stains were used for morphological assessment of PBMCs viability.

At the end of the culture period (after third magnetic simulation) cells of each sample were combined and centrifuged (1000 x g, 10 min). The pellets were resuspended in PBS (500  $\mu$ l),(Sigma-Aldrich, Germany) and supplemented with a dye mixture (10  $\mu$ l; acridine orange [100  $\mu$ g/ml] and ethidium bromide [100  $\mu$ g/ml] (both Sigma-Aldrich, Germany). Aliquots (20  $\mu$ l) were assessed by fluorescent microscopy.

Live cells showed normal nuclear characteristics (bright green chromatin), while late apoptotic had condensed and/or fragmented nuclei (bright orange chromatin), and necrotic exhibited a bright orange nuclear stain but no clear condensation. The apoptotic and necrotic cells were counted together as died cells and the number of viable cells in each sample was expressed as a percentage of the total cell number (minimum of 200 cells per count; in duplicate).

#### Statistical analysis

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

#### RESULTS

## Effect of PEMF dose (unpaired control).

Magnetic stimulation exerts its significant effect only after threefold application (3x) what has been found out for PBMCs originated from the controls. In our PBMC control cultures without stimulation (NS) viability of cells achieved  $84 \pm 3\%$  and in PHA and LPS stimulated cells viability mounted  $89 \pm 4\%$ . First and second dose of PEMF applied on non stimulated PBMCs did not change cell's viability (data not shown). Third dose of PEMF caused reduction in control PBMC viability to  $83 \pm 4\%$  after PHA to  $78 \pm 4\%$  in LPS stimulated cells (p<0.05, *Fig. 1*).

## Effects of PEMF (CD vs. paired control).

Viability of the stimulated cells in control group without PEMF were about 89  $\pm$  4%, after PEMF (3x) their viability decreases only about 5% (p<0.05, *Fig. 2*). Sensitivity of PHA and LPS stimulated cells to PEMF was similar.

In PBMCs stimulated cultures isolated from CD patient's viability was lower achieving about  $81 \pm 3\%$  without PEMF. In PBMC cultures obtained from CD patients application of PEMF(3x), decreased cell's viability about 10% ( p<0.05, *Fig.* 2). However susceptibility of PBMC to PEMF differs between cells stimulated with PHA and LPS achieving  $75 \pm 5\%$  and  $71 \pm 5\%$  level of viability respectively. Mitogen activated cells from CD patients during cell division undergo apoptosis and necrosis as a result of magnetic interaction, contrary PEMF exposure has minimal effect on non stimulated PBMCs and cells from healthy donors (*Fig.* 2 and *Fig.* 3). However, for PBMCs from CD patients there is no effect exerted by mitogens on cell viability comparing to the control PBMCs where viability after PHA and LPS stimulation rised, p<0.05 (*Fig.* 2 and *Fig.* 3).



Fig. 2. Pulsating magnetic field effects PBMCs (PBMCs from CD - Crohn'disease patients and Ccontrol volunteers) viability measured by AO/EB staining. NS - nonstimulated PBMC cultures; PHA, LPS – PBMC cultures stimulated with PHA or LPS respectively; NS-PEMF, PHA-PEMF, LPS-PEMF – parallel cultures stimulated with threefold dose of 50 Hz,  $45 \pm 5$  mT magnetic field, three hours each; statistical significance of PEMF induced viability determined by Student t-test analysis (\* p<0.05, \*\*p<0.01).

PHA

LPS

60

NS

Cytokine IFN- $\gamma$ . Production of IFN- $\gamma$  by unexposed PBMC from healthy volunteers (C) oscillates at the negligible levels. Control PBMCs stimulated by

LPS



*Fig. 3.* Comparison of the viability of PBMC cultures originated from Crohn'disease patients – CD and control volunteers - C) measured by AO/EB staining. NS – nonstimulated PBMC cultures; PHA, LPS – PBMC cultures stimulated with PHA or LPS respectively; NS-PEMF, PHA-PEMF, LPS-PEMF – parallel cultures stimulated with threefold dose of 50 Hz,  $45 \pm 5$  mT magnetic field, four hours each; statistical significance was determined by Student *t*-test analysis (\* p<0.05, \*\*\*p<0.001).



*Fig. 4.* IFN- $\gamma$  production of PBMC cultures from Crohn's disease patients (CD) and control group (C). PBMC stimulation was performed with LPS or PHA, both 1 µg/ml final concentration. PEMF exposures were in 24 h intervals. Cytokine level in cell culture supernatants 24 h after each PEMF exposure.

Data represent the mean value of eight donors, from duplicates (\* p<0.05, by Student *t*-test analysis).

PHA exposed to one dose PEMF (1x) show decrease of IFN- $\gamma$  from 264 ± 179 to 168 ± 69 pg/ml, and in LPS stimulated diminish IFN- $\gamma$  release from 363 ± 264 to 306 ± 205 pg/ml. After second PEMF (2x) IFN- $\gamma$  level decreases from 252 ± 115 to 174 ± 61 pg/ml, and after third dose from 246 ± 81 to 193 ± 11 pg/ml respectively, p<0.05 (*Fig. 4*).

The effects of PEMF on IFN- $\gamma$  production was seen mostly in PBMCs stimulated with PHA and LPS from CD patients. PHA stimulated PBMCs after PEMF (3x) decreased IFN- $\gamma$  release from 100 ± 58 pg/ml to 53 ± 28 pg/ml (*Fig.* 4). In LPS stimulated PBMCs the strongest effect was obtained after second PEMF (2x) application. IFN- $\gamma$  level falls from 189 ± 126 pg/ml to 128 ± 75 pg/ml (data not shown, p<0.05).

*Cytokine IL-10.* A contrary results were obtained with the IL-10 release. PEMF increased IL-10 production by PBMC from CD patients and healthy donors. LPS



*Fig. 5.* IL-10 production of PBMC cultures from Crohn's disease patients (CD) and control group (C). PBMC stimulation was performed with LPS or PHA, both 1  $\mu$ g/ml final concentration. PEMF exposures were in 24 h intervals. Cytokine level in cell culture supernatants, 24 h after each PEMF exposure.

Data represent the mean value of eight donors, from duplicates (\* p < 0.05, by Student *t*-test analysis).

stimulated PBMCs from control group increased IL-10 release after PEMF (1x) from  $688 \pm 388$  pg/ml to  $761 \pm 473$  pg/ml (data not shown) and after PEMF (3x) from  $227 \pm 58$  pg/ml to  $306 \pm 22$  pg/ml, p<0.05 (*Fig. 5*). PHA stimulated PBMC cultures from control group increased production of IL-10 PEMF(1x) from  $328 \pm 180$  pg/ml to  $372 \pm 240$  pg/ml (data not shown). In LPS stimulated cells from CD patients highest increase in IL-10 production was achieved after third dose of PEMF from  $135 \pm 3$  pg/ml to  $220 \pm 139$  pg/ml (*Fig. 5*).

All data represent the mean value of eight donors + SD, measurements always were done in duplicates.

#### DISCUSSION

A number of in vitro studies have revealed that magnetic field exposure affects cellular processes of proliferation which depends upon cell type, line and PEMF range of radiofrequency, strength and waveform of the magnetic field and time of exposure (14 - 16). Among cellular processes, proliferation has been studied the most broadly and revealed strong inhibitory effects of electromagnetic stimulation on cell division.

In studies population of PBMCs is composed mostly from lymphocytes 90%, where lymphocytes Th are abundant in 70 - 80%. Subpopulation of Th lymphocytes, subset Th1 is engaged in pathogenetic mechanism of a great number of the inflammatory chronic diseases of the gastrointestinal tract like Crohn's or ulcerative colitis by producing proinflammatory cytokines (17). Inflammatory bowel diseases are characterized by overactive T-helper (Th) 1-mediated response associated with high expression of IL-12 towards resident bacterial flora in genetically susceptible individuals. Some studies carried on knockout mice with deficiency of IL-10 have shown that chronic enterocolitis was developed (5, 18, 19).

In mouse model important role of IL-10 production has been proved by showing that IL-10 crambene-induced protection in acute pancreatitis occured via pancreatic acinar cell apoptosis and anti-inflammatory pathways (20).

Our interest was pointed to investigate magnetic field influences on viability of lymphocytes originating from CD patients. Since is known, that magnetic field exerts its effect mainly on proliferating cells (14, 15, 21, 22), we isolated PBMCs and stimulated T-cell with well known mitogens PHA and LPS. We confirmed these data by showing that PBMCs from healthy donors are not sensitive to PEMF. After 48 h duration of cultures cells were treated with pulsating magnetic field 50 Hz with a flux density 45 mT for three hours/day with 24 h intervals.

*In vitro* T-cell exposure to EMFs also affects production of cytokines. We continued these observation finding that exposure to magnetic field causes change in production profile pro- and anti-inflammatory cytokines. Effects were most pronounced in PBMC cultures stimulated with mitogens.

Decrease of IFN- $\gamma$  after three doses of PEMF in stimulated with PHA cultures from Crohn's disease patients was lower than in unstimulated cultures. Maximal increase in IL-10 production was obtained also in cultures from Crohn's patients stimulated with LPS after PEMF. Thus PEMF exerts the strongest effect on cytokine production either pro-inflammatory like IFN- $\gamma$  or anti-inflammatory like IL-10 in cells isolated from patients with CD.

The strongest inhibitory effect of the magnetic field interacting with proliferating cells was visible in PBMCs from CD patients (*Fig. 3*). Contrary to our results Scarfi et al. studying human lymphocytes grown 72 hr exposed to 50Hz saw toothed field at 0.025 T did not observe significant effects (23). These differences could be attributed to lower magnetic field flux dentisity in his experiments. On the other hand Fioro *et al.* using hamster V79 cells exposed for 10 days to 50Hz sinusoidal magnetic field at 200  $\mu$ T confirmed our data by observation that beside chromosomal alteration cell viability decreased to 50% after 10 days of exposition (24).

Main finding of our studies is that electromagnetic field therapy may be used in inflammatory bowel diseases treatment, currently dominated by pharmaceutical and surgical interventions. Anti-inflammatory effects of EMF comes from hypothesis that its affects the number of free radicals by the radical pair mechanisms. Lushnikov *et al.* showed that low-intensity ultrahigh frequency electromagnetic radiation also reduces the severity of inflammation by inhibition production of reactive oxygen intermediates by neutrophils in inflammatory process in mice (25). The investigations of the influence of EMF on phagocytic activity of mouse bone marrow derived macrophages showed a rise in formation of reactive oxygen species, indicating the induction of an oxidative burst (26).

Methods of vital staining with acridine orange/ethidium bromide has shown, that decreased viability of proliferating cells was due induction of apoptosis and necrosis in the exposed to PEMF proliferating cells. We have also carried out preliminary experiments assessment of apoptotic marker like annexin V binding and propidium iodide staining for necrosis by flow cytometry analysis (FACS). The data obtained from FACS confirmed microscopic results. The magnetic field exposure increases the death of PHA or LPS stimulated lymphocytes by apoptosis and necrosis. These results suggest that a magnetic field was effective mostly on immune cells during cell division, having no influence on non dividing cells in used doses.

Results obtained from our previous experiments with exposure of rats to long lasting magnetic stimulation are in agreement with current data. They showed that exposure of rats to PEMF has caused loss of c-Kit immunoreactivity in the gastrointestinal tract of animals due to magnetic field induced apoptosis in c-Kit positive cells (27).

Thus electromagnetically induced cell death could be a step for non-invasive application with low risk side effects and without problem of drug interactions in chronic inflammatory diseases (28). Future prospects are aimed to explain molecular mechanism of the effects exerted by PEMF application, responsible for generation of cellular changes in PBMC viability and protein expression pattern.

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