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LOSS OF INTERSTITIAL CELLS OF CAJAL AFTER PULSATING ELECTROMAGNETIC FIELD (PEMF) IN GASTROINTESTINAL TRACT OF THE RATS.

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Exposure to the magnetic field has remarkably increased lately due to fast urbanization and widely available magnetic field in diagnosis and treatment. However, biological effects of the magnetic field are not well recognized. The myoelectric activity recorded from the gastrointestinal and urinary systems is generated by specialized electrically active cells called interstitial cells of Cajal (ICCs). Thus it seems rational that ICC have significant vulnerability to physical factors like an electromagnetic field. The aim of this study was to evaluate the influence of pulsating electromagnetic field (PEMF) (frequency 10 kHz, 30ms, 300 μT burst, with frequency 1Hz) on ICCs density in the rat gastrointestinal tract. Rats were divided into two groups (n=32). The first group was exposed to PEMF continuously for 1, 2, 3, and 4 weeks (n = 16), and the second group (n=16) served as a control. Tissue samples of the rat stomach, duodenum and proximal colon were fixed and paraffin embedded. The tangential sections of 5μm thickness were stained immunohistochemically with anti-c-Kit (sc-168) antibody and visualized finally by DAB as chromogen (brown end product). C-Kit positive branched ICC-like cells were detected under the light microscope, distinguished from the c-kit-negative non-branched smooth muscle cells and from the c-kit positive but non-branched mast cells and quantitatively analyzed by MultiScan computer program. Apoptosis detection was performed with rabbit anti-Bax polyclonal antibody (Calbiochem, Germany) and LSAB ™ 2 visualization system. The surface of c-Kit immunopositive cells decreased after exposure to PEMF in each part of the gastrointestinal tract. Reduced density of ICCs was related to exposure time. The most sensitive to PEMF were ICCs in the fundus of the stomach and in the duodenum, less sensitive were ICCs in the colon and pacemaker areas of the stomach. No marked changes in ICC density in the pyloric part of the stomach were observed. We demonstrate that the PEMF induced apoptosis dependent decrease in ICC expression .

Key words: *interstitial cells of Cajal (ICC), c-Kit immunoreactivity, pulsating electromagnetic field (PEMF), apoptosis*

INTRODUCTION

The electric waves recorded from the gut and urinary system are generated by specialized cells called interstitial cells of Cajal (ICCs) (1,2). ICCs are the key structure of neurotransmission between enteric nervous system and smooth muscles (3, 4). These cells mediate excitatory and/or inhibitory neural inputs to the smooth muscles (3). They are localized mainly in the myenteric plexus of the stomach, small intestine and submuscular plexus of the colon as well as within the circular muscle layer of these parts of gastrointestinal tract (2,5,6). C-Kit-positive intramuscular ICCs appear as long, thin and bipolar cells with 1 or 2 processes seen in the longitudinal and circular muscle layers as well as in the innermost part of it (4,6). Myenteric ICCs are stellate-like cells with large cell bodies and several processes forming a dense network around the myenteric plexus. These electrically very active cells characterized by constant oscillation of membrane potential easily respond to neural and humoral factors (7,8,9). It seems rational that electric nature of ICC causes their significant vulnerability to physical factors like an electromagnetic field (EMF). The fast urbanization and common clinical therapeutic applications has brought almost permanent and high risk of exposure to EMF (10, 11). There are still inadequate data about biological outcome of EMF influence on humans (12).

Thus the aim of our study was the evaluate the influence of long term duration pulsating electromagnetic field on Cajal cells density in the rat gastrointestinal tract.

MATERIAL AND METHODS

In our experiment rats (n = 16) were exposed to PEMF for 1, 2, 3 and 4 weeks respectively, four rats per each exposition duration. The control group consisted of n = 16 rats. At each exposure period four rats were exposed to 24 hours lasting PEMF and simultaneously four animals served as an unexposed control. The magnetic field generated by coil was directed vertically and had a sinusoidal 10kHz frequency course. The 10kHz frequency was located in the audio range and magnetic fields of such frequencies are commonly generated by audio equipment. Rationale for choosing such frequency of PEMF was related to the following reasons: this frequency is higher then the range which directly depolarizes autonomic fibers, heating effect, and the possibility of interference with other sources is minimal. The adjustment of field induction was possible in the range 0 - 323μT. Wave bursts with a duration time of 30ms strength of 300μT and frequency of 1Hz were generated. Inside the cage the magnetic field was homogenous and the value of the amplitude of magnetic induction was within 5% of the nominal value. After each period of exposition tissue samples from both groups were excised from the stomach, duodenum and colon. The tissue sections from both exposed and unexposed rats were immunohistochemically stained with polyclonal anti-

c-Kit antibody to visualize interstitial cells of Cajal. The brown stained c-Kit positive branched ICC-like cells accompanying the Auerbach myenteric ganglia were distinguished from the c-kit-negative non-branched smooth muscle cells and from the c-kit positive but non-branched mast cells (color origins from HRP containing visualization system and DAB as its chromogen) were analyzed under light microscope and quantitatively measured (with MultiScan computer program) as positively stained surface of Cajal cells per 1 mm of tissue.

1. Animals

Male Wistar rats (2 - 3 months old, 250 - 300 g) n = 16 were used in time course exposure to PEMF (groups 1, 2, 3, and 4 weeks) and the control group consisted of n=16, unexposed rats. Rats were housed before an experiment in room where they would be exposed to the magnetic field. The room connotations were maintained on a 12h light-dark cycle and a temperature of 22°C with a relative humidity of 65%. During exposure rats were housed in plastic cages (length 57cm, width 31cm, height 28cm). Each cage was equipped with top ventilation ports. The use of transparent cages and open construction of the coil allowed the animals to be easily viewed. Animals were provided with food and water ad libitum. Jagiellonian University Bioethical Committee approved care and use of the animals.

2. Magnetic field parameters

Rats were exposed to a sinusoidal magnetic field with frequency f=10 kHz and induction B=323μT. The field was switched on for 10ms, every 5s for period of 1, 2, 3, and 4 weeks. Briefly, a computer program was used to drive a coil system located outside around the middle part of the cage. Each coil was made of one set of 63 turns each of 0.5mm^2 copper wire wound in rectangular loops with internal dimensions of 0,57x 0,31m. Epoxy was layered between the loops to glue them together. The coils were wound on dielectric cage. Sinusoidal alternating magnetic field with frequency of 10 kHz generated by the coils was directed vertically. With an exposure level set at 300μT for 30ms, for each second, the heat dissipation from the coil was less than 2W. The heat generated was dissipated due to the large surface area of the coil and good ventilation of the cage and experimental room. Ambient temperature within the cage was kept at the 22° C + 5%. We saw no DC offset during the bursts. The PEMF field was similar to that reported by Frederics et al., however he used 30ms bursts of pulses repeated at a frequency of 1.5 Hz. Similar parameters were used by Sandrey (10). The magnetic field was measured using an induction probe and LeCroy wave runner LT342 500MHz oscilloscope with a valid calibration certificate. The probe has been made as a cylindrical induction coil, made of 200 turns of insulated wire ∅ 0.05mm coiled around a glass pipe with external diameter of 10mm. During measurements the coil's axis was set parallel to the EMF. Control rats for this experiment were simultaneously exposed in the identical wired cages for the same periods of time in a separate room without power source. The ambient magnetic field intensity in our laboratory when the power supply was turned off was 0.12μT.

3. Tissue preparations for immunochemistry

The stomach, duodenum, and proximal colon were removed from the rats immediately after they had been killed by lethal injection of pentobarbital (40 - 60 mg/kg Vetbutal, Biowet, Pulawy - Poland). After excision, specimens were cleaned of digestive material with saline and cut into 2cm long segments in the case of the duodenum and colon. From the stomach samples: the fundus, pylorus and pacemaker area were taken out. All the specimens were fixed in 4% paraformaldehyde in 0,1M phosphate-buffered saline pH 7,4; for 6hrs at 4°C. After fixation tissues were dehydrated using graded alcohol, cleared with xylene and infiltrated with paraffin wax. Paraffin-embedded

tissues were cut into longitudinal sections (5μm thick), collected on poly-L-lysine coated slides, and dehydrated in an oven at 37°C for a few hours. Prior to staining, tissue slides were deparaffinized to remove embedding media and rehydrated with xylene and graded alcohol.

4. Immunohistochemistry

Heat induced epitope retrieval of tissue slides was carried out by using Target Retrieval Solution (DAKOCytomation, Denmark) to unmask antigen determinants. Immunostaining procedure was performed with rabbit polyclonal anti-c-Kit antibody, dilution 1:200 (sc-168, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and LSAB™ 2 (DAKOCytomation, Denmark) visualization system (HRP labeled) using diaminobenzidine - DAB - (DAKOCytomation, Denmark) as a chromogen. The cells containing brown deposits of DAB (result of the immunohistochemical reaction) in the region of the muscular zone were considered as positive and morphometrically assessed. The counterstaining was done with Mayer's hematoxylin (DAKO, Danemark), after a thorough rinse with distilled water, the slides were mounted in DPX mounting medium (Fluka, Biochemika). Omitting the primary antibody negative controls were performed.

Apoptosis detection was performed with rabbit anti-Bax polyclonal antibody (Calbiochem, Germany) and LSAB ™ 2 visualization system (13). Some positive background staining occurring in specimens resulted from the presence of erythrocytes, tissue mast cells and possibly not completely blocked excessive endogenous peroxidase.

5. Computerized image analysis

The immunoreaction products were observed under a light Zeiss Axiophot microscope linked with CCD Color Camera and analyzed using MultiScan (CSS) software. The brown stained ICC (color origins from used HRP containing visualization system) were quantitatively assessed by image analysis (MultiScan software, CSS Warszawa, Poland). Data were expressed as positively stained surface of Cajal cells per 1 mm of length of the analyzed tissue section. At least 5 serial slides for every part of gastrointestinal tract examined in each animal were analyzed.

6. Data and statistical analysis

Data were expressed as mean and standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA). This was followed by post-hoc LSD tests. Statistical significance was set at $p < 0.05$. The statistical analysis was performed using a Statistica 5.5 software package (StatSoft, Tulsa, OK, USA).

RESULTS

Duodenum

C-Kit expression on ICC in rats exposed to PEMF was diminished after 1-week of exposure, and decreased up to 4th week achieving plateau after two weeks (*Fig. 1*). In duodenum control tissue specimens ICCs appeared mainly as abundant c-Kit positive cells surrounding the myenteric plexus. The first two weeks of rat's exposure to PEMF caused the highest reduction in c-Kit immunoreactivity within duodenal ICCs. The longer duration of PEMF exposure caused the more significant changes in c-Kit immunoreactivity.

Colon

Expression of c-Kit antigen obtained in colon tissues of rats exposed to PEMF were analogous to duodenal. The amount of immunopositive cells was reduced in rats exposed to PEMF. Especially significant differences can be seen between first and second week of exposure (*Fig.2*). During the next two weeks of exposure the density of immunopositive surface of ICCs remained on the same level, reaching plateau (*Fig. 2*).

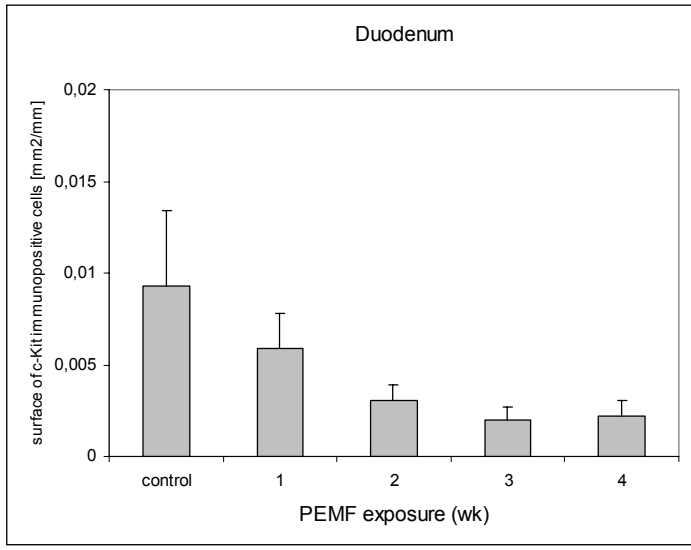

Fig.1. The effect of 1, 2, 3, and 4week duration PEMF exposures on ICC c-Kit expression (±S.D.) in duodenum of rats (n=16).

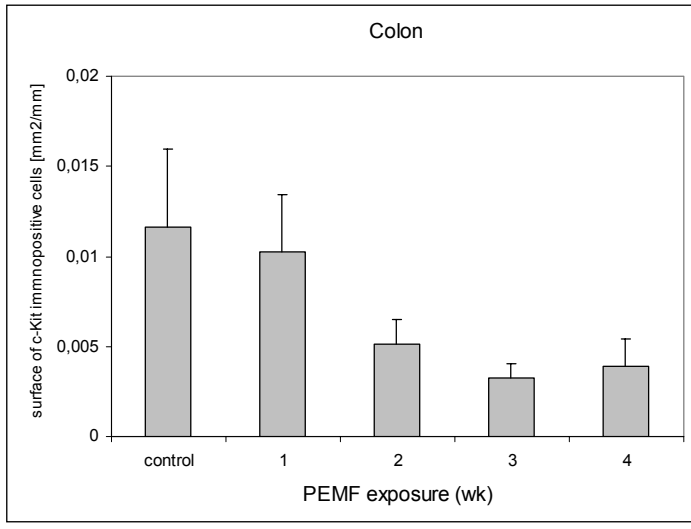

Fig.2. The effect of 1, 2, 3, and 4 weeks of PEMF exposures on ICC c-Kit expression (±S.D.) in colon of rats (n=16).

Stomach

Analysis of the ICCs of the stomach were distinguished and we separately studied three physiologically important gastric parts: fundus, pylorus and pacemaker areas (2/3 of the bigger curvature). Gastric ICCs were mostly present as myenteric type cells localized in circular or longitudinal muscle layers and in the innermost part of the circular muscles.

In the fundal and pacemaker areas of the stomach rising time of exposure from one to four weeks of PEMF triggered proportional decrease in ICCs density. However the pyloric part of the stomach in terms of amount of c-Kit immunopositive cells has no clear trend to be reduced. ICCs presence in pylorus area were rather resistant to month lasting PEMF exposure, and after this period, expression of c-Kit was still near control value (*Fig.3*). Percentage loss of the ICC densities throughout the gastrointestinal tract is presented in *Table 1*.

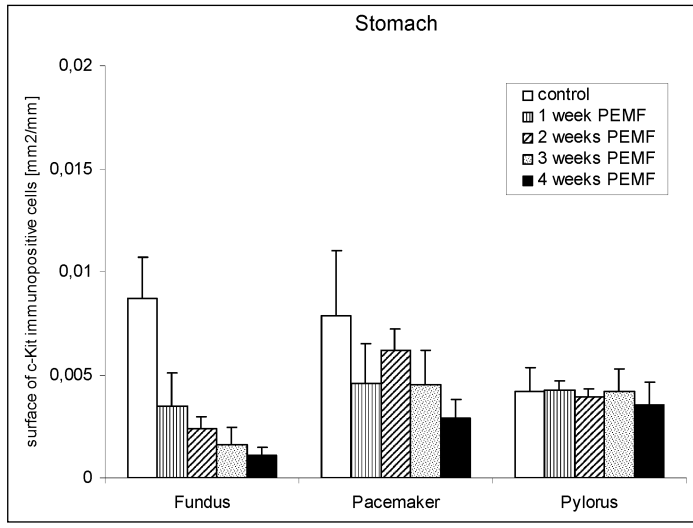

Fig.3. The effect of 1, 2, 3, and 4 weeks of PEMF exposures on ICC c-Kit expression (±S.D.) in the fundus, pylorus, and pacemaker stomach area in rats (n=16).

Table 1. Percentage [%] of ICCs present in the rat's gastrointestinal tract upon different periods of exposure to PEMF in comparison to control group. Results expressed as mean (SD).

PEMF exposure	1 week	2 weeks	3 weeks	4 weeks
Duodenum	63.0 (20.9) *	32.8 (9.4) *	21.1 (7.6) *	23.4 (9.5) *
Proximal colon	88.3 (27.1)	44.1 (12.1) *	33.7 (6.4) *	33.7 (12.8) *
Fundus	40.1 (22.7) *	27.0 (18.0) *	18.1 (6.6.) *	12.2 (9.8) *
Pylorus	100.0 (10.8)	93.3 (10.0)	99.1 (26.9)	83.3 (32.4)
Pacemaker	58.1 (27.8)	78.7 (16.9)	57.5 (26.8)	36.7 (20.5)

* - results statistically significant

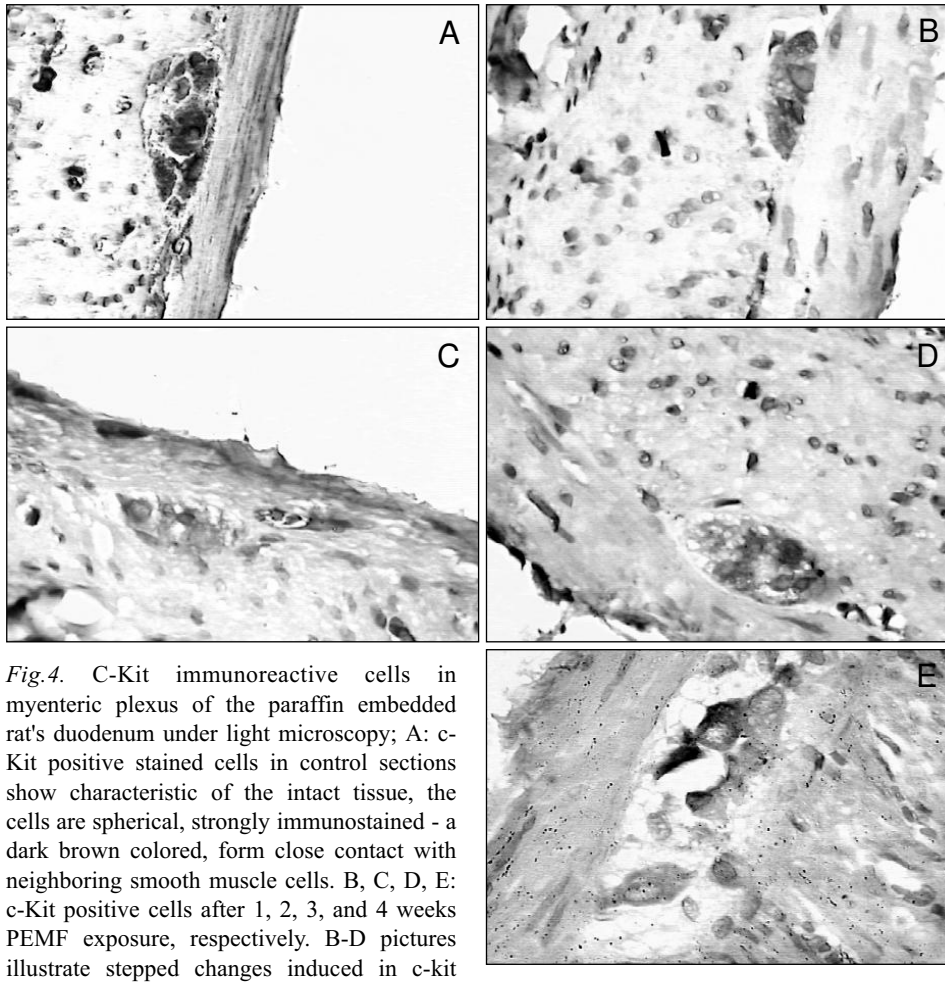

Fig.4. C-Kit immunoreactive cells in myenteric plexus of the paraffin embedded rat's duodenum under light microscopy; A: c-Kit positive stained cells in control sections show characteristic of the intact tissue, the cells are spherical, strongly immunostained - a dark brown colored, form close contact with neighboring smooth muscle cells. B, C, D, E: c-Kit positive cells after 1, 2, 3, and 4 weeks PEMF exposure, respectively. B-D pictures illustrate stepped changes induced in c-kit positive cells and surrounding smooth muscle tissues. A distinctive feature of c-Kit stained cells is shrinkage of the cytoplasm and nucleus, the cells manifest a lesser extent of immunohistochemical reactivity (a weak brown color stained cells). Magnification = x 400.

Apoptosis

The one of possible PEMF induced changes in the gastrointestinal tract is triggering of apoptosis in c-Kit positive cells. PEMF belongs to group of stressful agents which mediate stress-induced apoptotic pathway regulated by members of Bcl-2 protein family containing pro- and anti-apoptotic proteins. We have shown that PEMF causes expression of pro-apoptotic Bax protein in c-Kit immunoreactive myenteric cells, *Fig.5*.

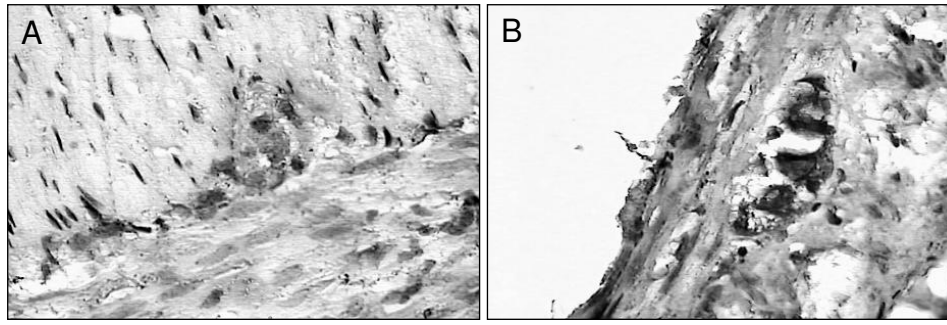

Fig.5. Apoptosis detected in c-Kit immunopositive cells of the rat duodenum with a-Bax antibody: A - rat not exposed to PEMF; B - rat exposed to PEMF for one week.

DISCUSSION

Beneficial effects of selected electromagnetic fields on bones, joints, and neurologic disorders, as well as wound healing determined their therapeutical application (14,15,16). Mostly anti-inflammatory aspects of electromagnetic field exposures in electromagnetic therapy were reported (11). Pulsating electromagnetic field (PEMF) effects on the nervous system were well recognized. PEMF is able to affect membrane potential of neurons as reported by several authors (17,18) and may influence autonomic nervous system activity (19, 20). Neurons and cells with intrinsic property of spontaneous cyclic depolarization are especially sensitive to physical stimuli affecting membrane potential (21,18). Except therapeutic use, generally long exposure to EMF is recognized as harmful. Biological effects of low and high frequency EMF exposures are subjected to investigations to settle risk-assessment guidelines for humans (22). Some negative associations between EMF and cancer has been considered (23,24). Mechanisms responsible for EMF bioeffects are still not fully clarified and may be related to functional and/or structural changes within enteric nervous system and smooth muscles itself.

Data obtained in our experiments indicate that exposure to PEMF causes the partial loss of c-Kit immunoreactivity of ICCs in the stomach and bowel. Decrease of ICC surface in duodenum and colon was near a half at one week and one-fourth after month lasting exposures to PEMF. Although PEMF influence on the gastric ICCs expression were less convincing and depend upon their localization within the stomach but corresponded with other findings in duodenum and colon. Our current study correlates with previous observation that long term stimulation of vagal nerves with a small current supplied by microchip dramatically decreases ICC expression (25). In analogy to that currents generated by EMF in the enteric nervous system could be responsible for damaging effects on ICCs. To prevent possible direct PEMF external depolarization of the

gastrointestinal neural net, high frequency of PEMF was chosen however, despite of this, direct effects on membrane potentials cannot be completely excluded. This confirms Nordenstrom's hypothesis of biologic closed electric circuits (26). These results indicated on high sensitivity of ICC to any external direct and indirect electrical manipulation.

As was depicted by Panagopoulos et al. PEMF seems to be biologically more active than continuous one and is able to irregularly gate electrosensitive channels on the plasma membrane causing disruption of the cell's electrochemical balance and function (27). This may explain our findings of remarkable ICC depression after rat exposure to EMF. Cells reaction to EMF may vary depending on different cells sensitivity. This may explain irregular changes of ICC expression in studied gastrointestinal regions in response to EMF in our study. For example, the pyloric ICCs seem not to be so sensitive to PEMF, their c-Kit immunoreactivity remained almost unchanged after one month of exposition. The possible explanation of resistance pyloric ICCs to PEMF could be related to two observations: first the pylorus is quite abundant in ICCs and secondly two types of ICCs are present, different in morphology, distribution, functions and presumably sensitivity to PEMF (3, 28, 29). We could also presume, that both types of ICCs are equally resistant to PEMF but the phenotype of the cell has been changed loosing ability to express c-Kit antigen or the antigen itself has been structurally modified. Additional studies are necessary to explain this phenomenon.

It has been clearly shown that a high-intensity magnetic field also affected the morphology of smooth muscle cell assemblies and changed their structure and orientation (30,31). Additionally, channels and membrane deformation due to EMF has been recently reported (32). These correspond well with hypothesis that EMF cause a reorientation of diamagnetic molecular domains within the membrane (33). One can speculate that migration of smooth muscles induced by long term use of EMF may affect their relationship with ICC leading to loss of c-kit expression. Reported cell's structure and function changes following EMF exposure activate the common execution mechanisms of apoptosis (34). Although we applied low-intensity EMF it is of interest that a stronger magnetic field strength does not always induces stronger biological effect (35).

Another probable elucidation of our observations could be the possibility that PEMF induces only change in the ICC phenotype. The phenotype of ICC expressed as c-Kit positive immunoreactivity is necessary for maintenance the morphology and function of ICCs. Loss of the immunoreactivity due to structural changes results not only in lack of c-Kit expression but also apoptotic regulatory proteins induction (28, 36, 37). It is well known that EMF affects myoblast differentiation and this could affect c-kit expression on ICCs (38). Clinical observation confirmed our results, EMF is effective in the treatment of functional pelvic floor disorders (39).

In conclusion, the main finding of our studies is evidence for the impairing effect of high frequency, low intensity PEMF on ICCs in the gastrointestinal tract.

Future prospects are aimed to investigate the molecular changes responsible for the resistance of the gastric ICCs to PEMF. On the other hand there is an open possibility that PEMF could be a tool in the new non invasive treatment of the myogenic type dysmotility of the gastrointestinal tract as well as the other body organs pacemaker cells.

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