JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2012, 63, 4, 397-401
www.jpp.krakow.pl

K. JUSZCZAK[1,2], J. KASZUBA-ZWOINSKA[1], P.J. THOR[1]

PULSATING ELECTROMAGNETIC FIELD STIMULATION OF UROTHELIAL CELLS INDUCES APOPTOSIS AND DIMINISHES NECROSIS: NEW INSIGHT TO MAGNETIC THERAPY IN UROLOGY

[1]Department of Pathophysiology, Jagiellonian University, Medical College, Cracow, Poland;
[2]Department of Urology, Rydygier Memorial Hospital, Cracow, Poland

The evidence of electromagnetic therapy (EMT) efficacy in stress and/or urge urinary incontinence, as well as in detrusor overactivity is generally lacking in the literature. The potential EMT action of neuromuscular tissue depolarization has been described. Because there is no data on the influence of pulsating electromagnetic fields (PEMF) on the urothelium, we evaluated the effect of PEMF stimulation on rat urothelial cultured cells (RUCC). In our study 15 Wistar rats were used for RUCC preparation. RUCC were exposed to PEMF (50 Hz, 45±5 mT) three times for 4 hours each with 24-hour intervals. The unexposed RUCC was in the same incubator, but in a distance of 35 cm from the PEMF generator. Annexin V-APC (AnV+) labelled was used to determine the percentage of apoptotic cells and propidium iodide (PI+), as standard flow cytometric viability probe to distinguish necrotic cells from viable ones. The results are presented in percentage values. The flow cytometric analysis was carried out on a FACS calibur flow cytometer using Cell-Quest software. In PEMF-unstimulated RUCC, the percentage of AnV+, PI+, and AnV+PI+ positive cells were 1.24±0.34%, 11.03±1.55%, and 12.43±1.96%, respectively. The percentages of AnV+, PI+, and AnV+PI+ positive cells obtained after PEMF stimulation were 1.45±0.16% ($p=0.027$), 7.03±1.76% ($p<0.001$), and 9.48±3.40% ($p=0.003$), respectively. The PEMF stimulation of RUCC induces apoptosis (increase of AnV+ cells) and inhibits necrosis (decrease of PI+ cells) of urothelial cells. This leads us to the conclusion that a low-frequency pulsating electromagnetic field stimulation induces apoptosis and diminishes necrosis of rat urothelial cells in culture.

Key words: *apoptosis, necrosis, overactive bladder, magnetic therapy, pulsating electromagnetic field, urothelial cells*

INTRODUCTION

Electromagnetic field (EMF) induces cell death, therefore it seems to be an option for non-invasive treatment with low side-effect risks and without problems of drug interactions in chronic inflammatory diseases (1). Pulsating electromagnetic field (PEMF) stimulation showed anti-inflammatory effects in Crohn's disease patients by induction of peripheral blood mononuclear cells apoptosis and changes in cytokine profile (2). In the last decade, extracorporeal EMF stimulation of pelvic muscle floor has been introduced for treating urge and/or stress urinary incontinence, and also overactive bladder (OAB) (3, 4).

The urothelium as an "active barrier" plays an important role in storage and voiding *via* detrusor muscle motor activity modulation, as well as seeming to contribute to OAB and LUT (lower urinary tract) symptoms (5). Neurogenic and myogenic mechanisms, and also changes in the urothelium underlying overactive bladder (OAB) have been described (6). In response to different stimuli, urothelial cells can release many substances including prostaglandins (PG), adenosine triphosphate (ATP), nitric oxide (NO), acetylcholine (Ach), *etc*. which affect urinary bladder compartments (smooth muscle, afferent and efferent nerve endings, interstitial and immune cells). Afferent nerve fibres stimulation (*via* vanilloid TRPV1-6 and ankyrin TRPA1 receptors) may release substance P (SP), calcitonin gene-related peptide (CGRP) and interleukins generating blood vessels, mastocytes and lymphocytes response, and, in a consequence, alter the urinary bladder sensory and/or motor activity due to neurogenic inflammation (7-9). Overstimulation of afferent unmyelinated C-fibres and its local effector function (*via* neurogenic inflammation) by substances released from urothelium may induce detrusor overactivity leading to OAB symptoms and/or urge incontinence (10, 11). Diminishing the release of the urothelial cells' mediators to reduce afferent nerve overstimulation seems to be crucial in urinary bladder sensory and motor activity control. Moreover, there is still no evidence on the influence of PEMF on the urothelium.

Therefore, the objective of our study was to examine the effect of pulsating electromagnetic field (PEMF) stimulation on rat urothelial cultured cells (RUCC).

MATERIAL AND METHODS

Animals

Urothelial cells were isolated from 15 adult female Wistar rats (weight: 200-250 g). Rats were housed individually per

cage. The animal room was maintained at a constant temperature of 23°C, humidity and a 12:12 h alternating light-dark cycle. They were fed with animal food (Labofeed; Kcynia, Poland) without water restraint. The study has been approved by the Regional Animals Ethical Committee.

Urothelial cells isolation and culture

Rat urothelial cell cultures were prepared according to the procedure described by Birder *et al.* (12), with slight modification of the experimental protocol. 15 female Wistar rats were anesthetized with intraperitoneal injection of 1.2 g/kg urethane (Sigma-Aldrich, St. Louis, USA), and the urinary bladders were removed and placed in cold Dulbecco's phosphate buffered saline (Sigma, Germany) containing penicillin/streptomycin/fungizone (PSF; 1%; Sigma, Germany). The bladder was cut open to expose the urothelium and incubated in dispase (2.5 mg/ml; Sigma, Germany) overnight at 4°C. Urothelial cells were gently scraped from the underlying tissue, placed in trypsin - EDTA solution (0.25% wt/vol; Sigma, Germany) for 10-15 min. at 37°C, and dissociated by trituration. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma, Germany) containing 10% fetal bovine serum - FBS (GibcoBRL; Invitrogen, Grand Island, NY) to arrest the trypsin reaction and centrifuged at 416 g for 10 min. The supernatant was removed and cells were suspended in keratinocyte basal medium (Invitrogen) with 1% PSF, counted with a hemacytometer and seeded on 96-well plate at density 0.5×10^6 cells/ml in fourfold repetition separately each bladder cells and cultured at 37°C in a 5% CO_2 incubator of 90% humidity. The medium was changed every day. Cells were used for experiments after 48 hours of culture.

Urothelial cell culture exposure to pulsating electromagnetic field

The generator which produces low energy pulsating electromagnetic field (PEMF) of 50 Hz and 45±5 mT peak was designed to generate such PEMF inside the cell culture incubator. The rationale for choosing such frequency of PEMF was as follows: firstly, frequency of magnetic stimulation is higher than the range, which directly depolarizes autonomic fibers; and secondly, the heating effect is minimal (13). The 96-well plate with urothelial cells was placed in a pocket of the generator. The PEMF was applied three times, for 4 hours each stimulation per day, with 24-hour intervals between stimulations. The control RUCC was in the same incubator, but in a distance of 35 cm from the PEMF generator to avoid the influence of magnetic field on the control culture.

In vitro animal model of rat urothelial cell culture (control group)

The control urothelial cultures (seeded in fourfold repetitions per animal) at density 0.5×10^6 cells/ml/well were in the same incubator, but in a distance of 35 cm from the PMS generator.

Cell death evaluation by flow cytometric analysis

Twenty four hours after last PEMF stimulation, the urothelial cells were harvested from culture plates by trypsinization and washed three times with cold PBS (Sigma, Germany) then stained according to the manufacturer's procedure for FACS analysis. APC-conjugated annexin V (BD, Pharmingen™, USA) was used to determine the percentage of cells within the population that were undergoing apoptosis. Propidium iodide (PI) (BD, Pharmingen™, USA) was used as standard flow cytometric viability probe to distinguish necrotic cells from viable ones. Annexin V-APC positive cells were analysed as apoptotic, Annexin V-APC and PI positive were either in the end stage of apoptosis or undergoing necrosis and analysed as already dead, and PI positive cells were necrotic. In apoptosis the cell membrane alterations consist of the translocation of phosphatidylserine (PS) from the inner side of the membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Therefore the measurement of annexin V binding to the cell membrane surface is indicative for apoptosis cells (14). PI accumulation inside necrotic cells distinguishes between apoptotic and necrotic cells. Apoptotic cells can be distinguished from AnV- living cells by using flow cytometric procedure. PI allows a further distinction of necrotic (AnV+/PI+), apoptotic (AnV+/PI-) cells. Furthermore, when the cells are incubated with annexin V prior to harvesting, the former cell populations can be separated from cells damaged during isolation (AnV-/PI+).

For staining, urothelial cells were washed twice with cold PBS and resuspended in 1× binding buffer (BD, Pharmingen™, USA) at a concentration 1×10^6 cells/ml. Then 100 µl of solution was transferred to a 5 ml culture tube and 5 µl of annexin V-APC and 5 µl of PI were added. Cells were gently vortexed and incubated in dark for 15 minutes at RT. Prior to flow cytometric analysis 400 µl of 1× binding buffer was added and cells were analyzed on a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest software. The Cell-Quest software provided the percentage calculation of the cell types in RUCC. Controls to set up compensation and quadrants included unstained cells, cells stained with annexin V-APC alone (for FL-4 fluorescence) and cells stained with PI alone (detected in FL-3). A minimum 10,000 events were collected on each sample.

Statistical analysis

All data was expressed as mean and (±) standard deviation (SD) and compared using the Student *t*-test considering $p<0.05$ defined as significantly different.

RESULTS

In vitro rat urothelial cells culture (control RUCC)

The density of RUCC was 0.5×10^6 cells/ml. The RUCC characterized high urothelial cells viability (about >75% of viable urothelial cells in the culture). The rat urothelial cell culture was characterized by 1.24% ±0.34% of apoptotic cells (AnV+ cells) and 11.03% ±1.55% of necrotic cells (PI+ cells). The percentage of AnV+PI+ positive cells was 12.43% ±1.96% (*Table 1; Fig. 1*).

Effect of pulsating electromagnetic field stimulation on rat urothelial cells culture

The flow cytometry analysis of rat urothelial cell culture was performed after the last pulsating electromagnetic field (PEMF) stimulation of the cells culture. PEMF stimulation significantly induced the apoptosis (an increase of 16.9%, as compared to PEMF-unstimulated culture), and inhibited the necrosis (a decrease of 36.3%, as compared to PEMF-unstimulated culture) of the urothelial cells in rat urothelial cells culture (RUCC) (*Fig. 2*). The percentages of apoptotic (AnV+) and necrotic (PI+) urothelial cells obtained after PEMF stimulation were 1.45% ±0.16% ($p=0.027$) and 7.03% ±1.76% ($p<0.001$), respectively. Additionally, AnV+PI+ positive urothelial cells measured as a percentage of double stained cells - annexin V and propidium

Table 1. The percentages of rat urothelial cells types (AnV+, PI+, AnV+PI+) in rat urothelial cells culture (RUCC) unstimulated and stimulated with pulsating electromagnetic field (PEMF).

Percentage of rat urothelial cells in RUCC	Control rat urothelial cells culture (RUCC) – PEMF unstimulated RUCC	RUCC after PEMF stimulation	p
Annexin V positive cells (**AnV+**) [%]	1.24 ± 0.34	1.45 ± 0.16*	*0.027
Annexin V and PI positive cells (**AnV+PI+**) [%]	12.43 ± 1.96	9.48 ± 3.40*	*0.003
PI positive cells (**PI+**) [%]	11.03 ± 1.55	7.03 ± 1.76*	*<0.001

* *vs*. control RUCC ($p<0.05$); *Abbreviations:* PEMF - pulsating electromagnetic field; RUCC - rat urothelial cells culture

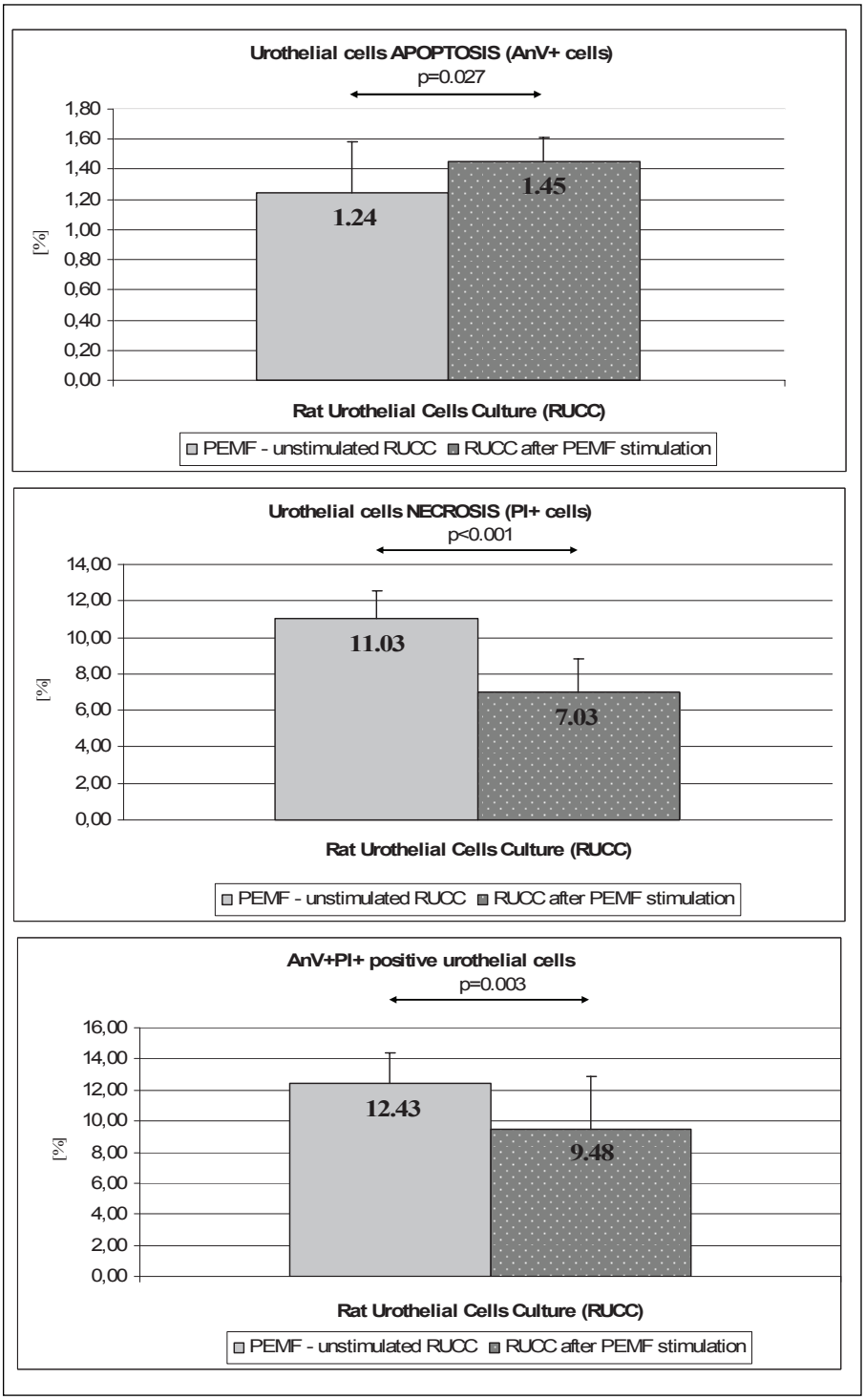

Fig. 1. The percentage of apoptotic (AnV+ cells), necrotic (PI+ cells), as well as AnV+PI+ positive urothelial cells in rat urothelial cells culture (RUCC) unstimulated and stimulated with pulsating electromagnetic field (PEMF).

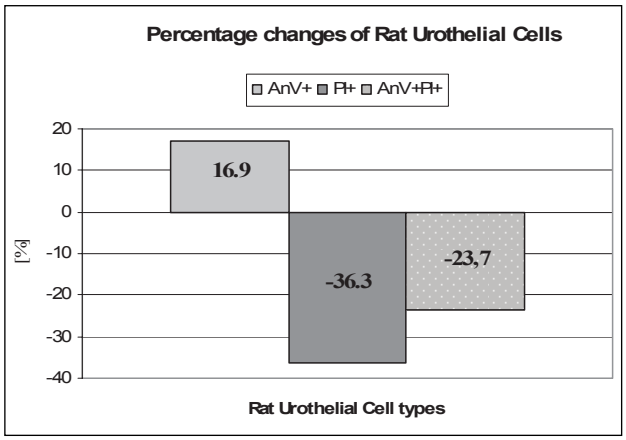

Fig. 2. The percentage changes of rat urothelial cells in RUCC (AnV+, PI+, AnV+PI+) after pulsating electromagnetic field (PEMF) stimulation, as compared to unstimulated RUCC (control). The percentage of increment (positive values) or decrement (negative values) of the amount of different types of rat urothelial cells were compared to the values of normal RUCC (set at 100%). For example, in RUCC after PEMF stimulation there is increased amount of AnV+ cells, as compared to control (PEMF unstimulated) RUCC. The increase is about 16.9% of the mean value of PEMF-unexposed RUCC.

iodide positive in RUCC, upon PEMF exposure, achieved lower level (9.48% ±3.40%, p=0.003), as compared to control unstimulated RUCC (a decrease of 23.7%, as compared to PEMF-unstimulated culture) (*Table 1, Fig. 1, 2*).

DISCUSSION

The potential mechanisms of electromagnetic field (EMF) action on lower urinary tracts, especially urinary bladder are poorly described in the literature. Irrespective of this, the influence of EMF on urothelium is still unknown. Therefore, we explored the effect of pulsating EMF stimulation on rat urothelial cell culture (RUCC). Our main findings revealed that low-frequency PEMF stimulation (50Hz, 45±5 mT) induces apoptosis (increase of AnV+ cells) and inhibits necrosis (decrease of PI+ cells) of urothelial cells in culture.

Apoptosis (programmed process) and necrosis (incidental process, induced by extracellular conditions) are two different ways of cell death (15). Independent molecular pathways regulate apoptosis, such a [1] extrinsic *via* Fas-R or TNF-R death receptor, and [2] intrinsic or mitochondrial: the stress mediated by pro-apoptotic members of Bcl-2 family *e.g. via* drugs, PEMF, *etc*. (16-18). However, the potential influence of PEMF on apoptosis and necrosis of urothelial cells is still unknown. PEMF affects cell proliferation *in vitro*, which depends on the cell's type, the magnetic field's physical parameters (frequency, strength, waveform), and also time exposure. Additionally, DNA damage, signal transduction pathway alterations, and changes in immune system cells (*e.g.* lymphocytes) function after PEMF exposure was described (19, 20). Apoptotic cells are phagocytosed and digested by macrophages, preventing the release of intracellular substances to the extracellular space. Apoptosis of the urothelium is at a low rate in naive conditions (21). Contrary to apoptosis, urothelium necrosis distributes an extensive release of substances from dead cells, which activates afferent nerve fibres and induces neurogenic inflammation leading to detrusor overactivity or exacerbation of chronic urinary bladder illness.

PEMF anti-inflammatory influence was described in Crohn's disease by induction of peripheral blood mononuclear cells apoptosis and changes in cytokine profile (2). Anti-inflammatory PEMF backgrounds may be due to free radicals profile by the radical pair mechanisms. EMF inhibits reactive oxygen production by neutrophils in inflammatory process in mice (22). Contrarily, a rise in the formation of reactive oxygen species by macrophages derived from mouse bone marrow, indicate the induction of an oxidative burst (23). The formation of reactive oxygen species in different pathological conditions (*e.g.* inflammation) may affect urinary bladder function leading to OAB (24). Christmas (25) and Harrington *et al*. (26) studies revealed different T cells sub-populations within urinary bladder mucosa, as follows: $CD8^+$ T cells are sparsely scattered in the urothelium and present in lamina propria, while $CD4^+$ T cells are within the urothelium to a lower extent than $CD8^+$ T cells. Moreover, Liu *et al*. (27) indicted that urothelial Ag-specific $CD4^+$ T cells can function as direct effector cells to induce bladder autoimmune inflammation independent of CD8+ T cells. Apoptosis plays a main regulatory role in lymphocyte development and homeostasis. However, the molecular mechanisms of PEMF-induced apoptosis are inconsistent, EMF may reduce apoptosis *via* Ca^{2+} influx modulation and/or alteration of c-myc transcription rate (28). Moreover, EMF induces free radical production and changes the redox homeostasis, and consequently may lead to DNA-damage and apoptosis *via* direct interaction between reactive oxygen species (29). Also, Lai *et al*. (30) revealed that the DNA strand breaks after EMF in rat brain cells, and that effect was diminished by melatonin (a radical scavenger).

Several probable mechanisms of PEMF action affect urinary bladder activity, as follows: [1] changes in urothelium apoptosis/necrosis balance, and secondarily in cellular substance release regulating the nerves, interstitial cells, and immune cells (lymphocytes, *etc*.), [2] direct effect on nerve fibres (depolarization) and sub-population of lymphocytes in urinary bladder mucosa, [3] changes in the formation of reactive oxygen species.

Recently, magnetic therapy (MT) has become an alternative option for the treatment of urge and/or stress urinary incontinence, and overactive bladder (especially in the case of neurogenic detrusor overactivity). The advantages of such treatment are the completely non-invasive nature, excellent safety and tolerability (31). However, the evidence based on randomized controlled trials for evaluation of the efficacy of MT in urinary incontinence and OAB treatment is generally poor (32). Morris *et al*. (4) observed that MT in women with idiopathic detrusor overactivity diminishes detrusor contractility in the acute term of MT, and also reduces urge episodes in prolonged mode. The potential mechanisms of electromagnetic field action on lower urinary tracts (LUT) are only partially described, especially due to neuromuscular compartments. It is believed that nerves (afferent, efferent, and autonomic fibres) are particularly sensitive to the effects of PEMF-induced eddy currents causing its depolarization, and, in a consequence, may regulate local blood flow and other factors (still unknown). PEMF stimulation activates efferent nerves and motor end plates of pelvic-floor muscle, providing better muscle strength and endurance. On the other hand, PEMF may affect the somatic nerve firing rate responsible for pelvic muscle and sphincter tone (3). Moreover, the imbalance between apoptosis and necrosis of the urothelium affects the detrusor muscle activity (11, 33). The 'pro-apoptotic' and 'anti-necrotic' action of PEMF on urothelial cells may be potentially applied in the therapy of functional disorders of urinary bladder (*e.g.* overactive bladder, urge incontinence, *etc*.). Nevertheless, further experimental and clinical studies are strongly required for the evaluation of the potential role of PEMF actions on urinary bladder.

A low-frequency pulsating electromagnetic field (PEMF) induces apoptosis and diminishes necrosis of urothelial cells. However, further evaluation is strongly required to explain the potential molecular mechanism of pulsating electromagnetic field application on urothelium.

Conflict of interests: None declared.

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Received: March 22, 2012
Accepted: June 12, 2012

Author's address: Dr. Kajetan Juszczak Department of Pathophysiology, Jagiellonian University Medical College, 18 Czysta Street, 31-121 Cracow, Poland.
E-mail: kajus13@poczta.onet.pl