The effect of 900 MHz microwave GSM signal on the proliferation of endothelial cells *in vitro*

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Abstract

Pulsed electromagnetic fields (PEMF) have been shown to be clinically beneficial, effective for bone and wound repair, but their mechanism of action is not fully elucidated. Angiogenesis plays an important role in injured tissue healing and during the wound repair many potential angiogenic factors are released. It was reported that PEMF may increase angiogenesis through endothelial release of basic fibroblast growth factor (bFGF). Moreover, an increase of protein synthesis by cells exposed to a 1,800-MHz radiofrequency mobile phone PEMF was detected by proteome profiling.

We previously reported that immune activity of lymphocytes and monocytes in culture can be intensified by 900 MHz microwaves. In the present study, we performed experiments with murine endothelial cell line HECa10, exposed to the influence of pulse modulated 900 MHz microwave electromagnetic field. Fifteen minutes exposure resulted in significant stimulation of endothelial cells proliferation rate, what we evaluated by two independent methods (Alamar Blue reduction and ^3H thymidine incorporation).

Key words: *microwave GSM, endothelial cells, proliferation in vitro.*

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Introduction

Angiogenesis, the growth of new vessels from existing microvasculature, has an obvious and essential role in wound healing [1]. Wound healing is a complex biological process, comprised of a series of sequential events aiming to repair injured tissue. Immune system participates in this process not only through elimination of infective agents at the site of injury, but also by contribution to the process of healing [2].

A lot of molecules and cells of the immune system participate in wound healing process. Growth factors secreted by different cells (macrophages, neutrophils, lymphocytes, fibroblasts, platelets) induce cells to migrate, divide or produce other factors influenced on wound healing. These factors bind to target cells via specific cell surface receptors and exert inhibitory or stimulatory response. The first phase of the wound healing process (cellular migration and inflammation) lasts several days and is initiated by the process of blood clotting and platelet degranulation. Granules of platelets release various growth factors (PDGF, IGF1, EGF, TGF-β, TSP1) which initiate sequences of events leading to healing.

A pleiotropic cytokine macrophage migration inhibitory factor (MIF) appears early in wound and exerts both positive and negative effects on the healing process. Macrophages have many functions in wounds, including host defense, the promotion of inflammation, the removal of apoptotic cells, and the influence on cell proliferation and tissue repair.

Neutrophils and macrophages enter the wound and begin to synthetise and secrete other important growth factors, among them interleukin 1 (IL-1) and proangiogenic cytokines vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF, or FGF-2). These factors stimulate migration of fibroblasts, epithelial cells and vascular endothelial cells into wound [3-10].

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The second phase of wound healing (proliferative and repair) lasts from 2 to 4 weeks. The fibroblasts, endothelial cells, keratinocytes continue to produce growth factors (keratinocyte-derived autocrine factor KAF, TGF-β, bFGF, PDGF, VEGF), which cause proliferation, synthesis of extracellular matrix proteins and new blood vessels formation.

The third phase of wound healing-remodeling of the scar, lasts from 1 month to 1 year. During this time a balance is reached between synthesis of extracellular matrix proteins and their degradation by proteases [3, 5, 11-13].

Pulsed electromagnetic fields (PEMF) have been shown to be clinically beneficial, effective for bone and wound repair and pain and edema reduction, but their mechanism of action is not fully elucidated.

Studies performed in diabetic rats by Goudarzi *et al.* revealed that exposition to extremely low frequency pulsed electromagnetic fields (ELF PEMFs, 20 Hz, 4 ms, 8 mT) for 1 h per day, accelerated diabetic wound healing [14].

During the wound repair many potential angiogenic factors are released.

The study performed in mice by Callaghan *et al.* demonstrates that pulsed electromagnetic fields are able to accelerate wound healing under diabetic and normal conditions by up-regulation of FGF-2-mediated angiogenesis. Cultured medium from human umbilical vein endothelial cells in pulsed electromagnetic fields exhibited a three-fold increase in FGF-2 [15]. This supported the earlier study of Tepper *et al*., where PEMFs, consisted of asymmetric 4.5 ms pulses repeated at 15 Hz, with a magnetic flux density rising from 0 to 12 G in 200 μs and returning to 0 G in 25 μs, increased *in vitro* and *in vivo* angiogenesis through endothelial release of FGF-2 [16].

Recent development of low energy and high frequency EMF emitters (mobile phones, radar and microwave broadcast stations) increased the interest on the risk of their possible harmful influence, and on the other hand, on the potential of their therapeutic application.

It was shown by Dąbrowski *et al.* that T cell immune competence and monocyte immunogenicity in the population of mononuclear cells isolated from the human blood (PBMC) can be modulated *in vitro* by exposition of the cells to the influence of pulse modulated 1300 MHz microwave electromagnetic field. The same authors reported that immune activity of lymphocytes and monocytes in culture can be intensified by 900 MHz microwaves [17, 18].

In the present study, we performed pilot experiments with murine endothelial cell line HECa10, exposed to the influence of pulse modulated 900 MHz microwave electromagnetic field. Fifteen minutes exposure resulted in significant stimulation of endothelial cells proliferation rate, what we evaluated by two independent methods (Alamar Blue reduction and ^{3}H thymidine incorporation).

Material and methods

Cell cultures

HECa10 cells (mouse endothelial cell line) were maintained in 75 cm^2 (BD Bioscience) culture flasks under standard culture conditions of 5% CO_2 in air at 37°C with medium renewal every 2–3 days. The culture media used was DMEM (4.5 g/l glucose, PAA) containing 200 mM glutamine (PAA), 1/100 penicillin – streptomycin solution and 1/1000 gentamicin solution supplemented with 10% FBS (PAA). When subconfluent (~80%), cells were split 1 : 3. Cells passaged for 24 times, were used for the experiments. HECa10 cells were trypsinized from subconfluent cultures, suspended in culture medium with 5% FBS, and then seeded into wells of a 96-well plate (0.2 ml per well) at concentrations of 5 × 10^4 cells/ml and next incubated at standard culture conditions of 5% CO_2 in air at 37°C.

Exposure conditions to microwaves

Exposure was performed according to the method described previously [18]. Briefly, 15 min, 1 hour and 3 hours after beginning of the experiment, the cultures were placed in the anechoic chamber and were exposed to microwaves (MW) (900 MHz, 20 V/m, SAR 0.024 W/kg) for 15 min. Control cultures were not exposed to MW. The miniature anechoic chamber (MAC) was a cube of 40 × 40 × 40 cm of external dimensions located inside the ASSAB incubator. The internal dimensions of the chamber were 23 × 23 × 23 cm. This special anechoic chamber was constructed and technically tested in the Department of Microwave Safety, Military Institute of Hygiene and Epidemiology in Warsaw, Poland. The chamber, containing the microplate with cultured cells and MW-emitting antenna, was installed inside the ASSAB CO_2 incubator so the HECa10 cells could be exposed to MW at choosing period of culturing without removing them from the incubator.

An identical plate of control cultures was set up and placed in the ASSAB incubator beyond the chamber.

Alamar Blue assay for cell proliferation

After a 20-h culture, media were discarded and 0.2 ml of fresh DMEM medium (supplemented with 5% FBS, without Fenol Red) with Alamar Blue (AB) (1 : 10 Alamar Blue in medium) was added directly to the wells. Cells were incubated for 5 h at 37°C and 5% CO_2. After this time, an absorbance was measured (FLUOstar Omega, BMG Labtech) at 578 nm and 630 nm.

The results were calculated (after correction for background values of samples containing medium without cells) as a mean % of AB reduction ±SD for 24 non-irradiated cultures and for 16 cultures irradiated in various times after beginning of the experiment (total number of cultures 72), according to the general protocol described in [19] with own modifications.

^{3}H thymidine assay for cell proliferation

Cells were incubated at standard culture conditions of 5% CO_2 in air at 37°C for 24 hours. For the last 18 h of incubation, ^{3}H thymidine (^{3}HTdR, Amersham, UK, spec. act. 5 Ci/mM) was added into the cultures in a dose of 0.4 μCi/culture. At 24 h the cultures were harvested and incorporation of ^{3}HTdR was measured in Packard Tri carb 2100 TR scintillation counter. The results were calculated as a mean value of dpm ±SEM for six non-irradiated cultures and for six cultures irradiated in various times after beginning of the experiment (total number of cultures 24).

Statistical evaluation of the results

Statistical analysis was done by 1-way analysis of variance (ANOVA) and the significance of differences between the groups was verified by Tukey-Kramer Multiple Comparisons Test and unpaired *t* test (GraphPad Prism software).

Results

The results of the test performed with Alamar Blue and evaluated after 25 h of culture are presented on Figure 1. and in Table 1. Microwave radiation highly significantly stimulated Alamar Blue reduction by growing endothelial cells independently of the time-point of exposure.

The results of the test performed with ^{3}H thymidine and evaluated after 24 h of culture are presented on the Figure 2 and in Table 2. In all time-points exposure to microwaves significantly stimulated ^{3}H thymidine incorporation by endothelial cells. However, the best results were obtained when exposure to microwaves was performed 15 minutes after establishing of the culture.

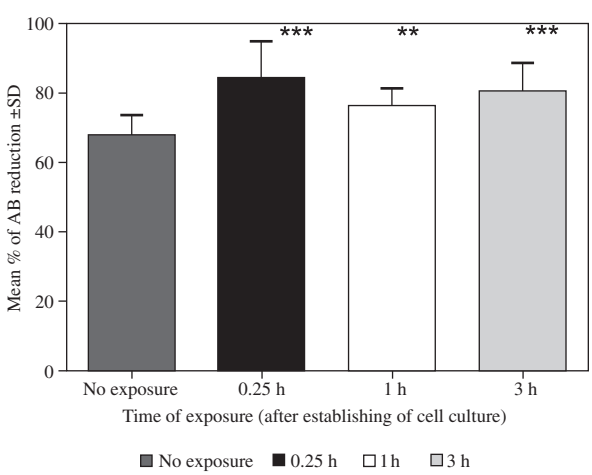
Discussion

Alamar Blue is an indicator dye which incorporates an oxidation-reduction (REDOX) indicator that changes color in response to the chemical reduction of growth medium, resulting from cell growth. This assay is designed to quantitatively measure the proliferation of various cell lines. The assay is simple to perform since the indicator is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays [20].

Alamar blue assay has been shown to be an alternative to the ^{3}H-thymidine incorporation assay [21]. In the present study we obtained comparable results by these two methods. Endothelial cells proliferation, independently of the time-point of cells cultures exposure to 900 MHz

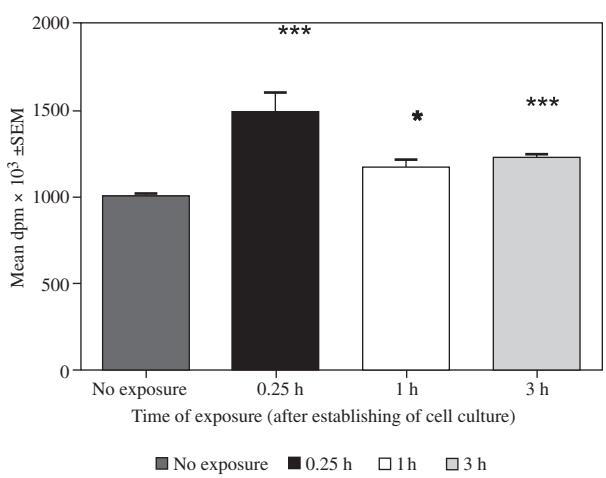
Table 1. One-way analysis of variance of the results obtained by Alamar Blue test

P value		< 0.0001
P value summary		****
Are means signif. different? (*P* < 0.05)		Yes
Number of groups		4
F		19.22
R square		0.4070
Tukey's Multiple Comparison Test		q
0 h vs. 0.25 h	* * *	10.50
0 h vs. 1 h	* *	4.59
0 h vs. 3 h	* * *	6.81



***p < 0.01; ***p < 0.001*

Fig. 1. The results of the experiments evaluated by Alamar Blue test



**p < 0.05; ***p < 0.001*

Fig. 2. The results of the experiments performed with ^{3}H thymidine

Table 2. Evaluation of the results obtained with ^{3}H thymidine by one-way analysis of variance and unpaired *t* test

P value	0.0004
P value summary	***
Are means signif. different? (*P* < 0.05)	yes
Number of groups	4
F	9.703
R square	0.5927
Tukey's Multiple Comparison Test	
No exposure vs. 0.25 h	
Significant * * *	
Unpaired t test (no exposure vs. 1 h)	
P value	0.0121
P value summary	*
Are means signif. different? (*P* < 0.05)	Yes
One- or two-tailed *P* value?	Two-tailed
t, df	t = 3.060 df = 10
Unpaired t test (no exposure vs. 3 h)	
P value	< 0.0001
P value summary	***
Are means signif. different? (*P* < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 10.26 df = 10

microwave radiation, was significantly enhanced in comparison to the non-exposed controls.

Endothelial cells proliferation is one of the main links in the angiogenesis chain. Enhancing vascularization in healing wound is essential for successful tissue repair. A number of approaches to improve angiogenesis in tissue – engineered skin injuries were reported [22, 23], among them the influence of pulsed electromagnetic fields [24]. There are some attempts to elucidate the molecular mechanism of this phenomenon. Nylund and Leszczynski reported the results of proteomics analysis of human endothelial cell line after exposure to GSM 900 radiation. Such exposure significantly altered the expression of many proteins, and two of them were determined to be isoforms of cytoskeletal vimentin [25]. In the study of O'Connor *et al.* exposure to GSM RF fields did not affect calcium homeostasis in human endothelial cells [26]. Using four different cell kinds Gerner *et al.* obtained increased protein synthesis by exposure to a 1,800-MHz radio-frequency mobile phone electromagnetic field, detected by proteome profiling. Authors postulate that it reflects an increased rate of protein turnover resulting from protein folding problems caused by the interference of radio-frequency electromagnetic fields with hydrogen bonds [27].

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