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The influence of an electromagnetic field on adipose-derived stem/stromal cells' growth factor secretion: modulation of FGF-2 production by *in vitro* exposure

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Abstract: Adipose-derived stem/stromal cells (ASCs) have tremendous potential for use in regenerative medicine; their secretome is especially important for regenerative processes. We hypothesized that exposure of ASCs to an electromagnetic field (EMF) can influence the proregenerative potential of cells by influencing the secretion of growth factors (GFs) responsible for regenerative properties. We showed that the exposure of ASCs to an EMF (50 Hz; 1.5mT) affected the secretion of GFs as well as the cell cycle process. The most important observation was a statistically significant, 3-fold increase in FGF-2 concentration at 48 h, and a 2-fold decrease at 72 h when compared to the control group. This finding is very important for regenerative medicine, because with precisely adjusted parameters, an EMF can be used to stimulate the production of GFs, mainly of FGF-2, by ASCs, thereby increasing proregenerative properties. The ASC secretome after EMF treatment could be a method for easy, simple and cost-effective stem cell differentiation and therapy facilitation.

Keywords: *ASCs; stem cells; growth factors; electromagnetic field; FGF-2*

INTRODUCTION

The Earth's magnetic field has existed since geological evolution. The first organisms evolved under its influence and have adapted to it. Currently, we live surrounded by modern technologies and sources of artificial electromagnetic fields (EMFs) such as Wi-Fi networks, radio and TV signals, mobile phones, power transmission lines, etc. They are the sources of "electrosmog" that affects living cells and organisms [1,2]. The body of evidence implicating the impact of EMFs on human health is increasing, with the main consequence brain and brain cancer induction [3,4]. Studies have also shown genotoxicity of EMFs at 100 Hz and 5.6 mT, which causes breaks in DNA strands in Vero cells. Presumably, DNA damage is caused by reactive oxygen species (ROS) formed in response to EMF exposure [5-7]. However, an EMF under controlled conditions can have a positive effect. It can be used in medical therapies, as in the treatment of bone diseases [8,9], sources of pain, direct trauma sites or inflammation [10-11], in tumor treating field therapy (TTF), as well as in electric field-based treatment of glioblastoma [12,13], which requires the use of specific EMF parameters [3,14]. This suggests that the effect of EMFs in regenerative medicine depends on its parameters, magnetic flux density and frequency, exposure time and the cell type used for stem cell-based therapies.

Mesenchymal stem cells (MSCs) are multipotential, self-renewing and easily accessible, and can be successfully cultured *in vitro*; they easily differentiate into specialized cell lineages such as myeloid cells, stromal cells, osteocytes, chondrocytes and adipocytes [15,16]. MSCs exposed to an EMF differentiate faster towards osteogenic and chondrogenic lineages but also into other lineages [8,17-19]. Like all living cells, MSCs produce proteins that enable function. Due to their

nature and properties, they secrete factors responsible for differentiation, growth and survival. In this study, adipose-derived stem/stromal cells (ASCs) were used. It has been confirmed that ASCs are superior in the secretion of bioactive factors that stimulate differentiation, proliferation and migration of other cell types, and they also possess protective and supportive factors with antiapoptotic, antifibrotic and antiinflammatory activities when compared to bone marrow MSCs (BM-MSCs) [20,21]. These properties render MSCs a suitable material for therapy in regenerative medicine. However, this therapy might not require the use of whole MSCs but their secretome derivatives, such as conditioned media or exosomes, which provide therapeutic effects [22,23].

There are many compounds in the secretome, including growth factors (GFs) responsible for the processes mentioned above. Their presence guarantees the regulation of various processes such as cell proliferation, recovery and differentiation. It has been reported that the presence of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) secreted by ASCs can be significant in wound healing and that these factors promote angiogenic and neurogenic responses [24-26]. In addition, it has been shown on umbilical vein endothelial cells that HGF inhibits the proinflammatory action of VEGF *in vivo* and *in vitro*, which indicates that it has antiinflammatory properties [27]. Interestingly, placental growth factor (PIGF) can bind to the receptor for VEGF and serve as an angiogenesis factor [28]. The platelet-derived growth factor (PDGF) secreted by ASCs also shows antiinflammatory properties by inducing the production of proteins such as TGF-β1 or IL-10, as well as supporting tissue regeneration. The concentration of this factor increases significantly a few days after tissue damage [29]. The stem cell factor (SCF) can also help trigger the migration of MSCs to the site of injury and help tissue regeneration [30]. Similarly, the nerve growth factor (NGF) is also involved in wound healing and cell regeneration and can accelerate these processes and support chondrogenesis [31].

In the process of differentiation towards chondrocytes, adipocytes or osteocytes, not all GFs show similar potency. It has been shown that the epidermal growth factor (EGF) does not possess such properties and may even inhibit osteogenesis, however, it is secreted from platelets and macrophages around wounds and helps with healing [32]. The differentiation of MSCs towards neurogenesis stimulates the brain-derived neurotrophic factor (BDNF), while during differentiation towards chondrogenesis and osteogenesis, fibroblast growth factor 2 (FGF-2) is stimulated [33]. The leukemia inhibitory factor (LIF) is also secreted by MSCs, and by activating the STAT3 signaling pathway it conveys the ability for pluripotency, self-renewal and proliferation [34]. It has been observed that a high level of LIF is present in advanced stages of cancer and is associated with ovarian carcinoma-associated mesenchymal stem cells [35].

Basic fibroblast growth factor (FGF-2 protein) is a GF that increases stem cell proliferation and supports them by maintaining their functions, such as pluripotency, self-renewal and regeneration [33,36-39]. This GF can also increase the differentiation potential of MSCs and affect osteogenesis and chondrogenesis [19,33], and it can stimulate neuropoiesis and angiogenesis [40,41]. Interestingly, FGF-2 protects cells and assists in the maintenance of the integrity of the cell genome under unfavorable conditions that cause damage to DNA strands. Increased production of FGF-2 was noted after exposure of MSCs to γ-rays, which causes DNA damage [42].

An EMF affects many cell functions such as gene expression and thus the production of proteins, including GFs [43]. As we described in our previous work, the EMF can affect the cell cycle, differentiation, proliferation, apoptosis and secretion of trophic factors by MSCs, depending on its parameters, frequency and magnetic flux density [8]. ASCs are characterized by the secretion of high concentrations of GFs and other specific proteins, which makes them a very attractive tool in regenerative medicine [20,21,44]. It has already been shown that the concentration of some proteins secreted by cells (including the MSCs) changed after EMF exposure [43,45-48].

In this study, we focused on GF secretion and on the time-dependent changes in the concentration of GFs in response to EMF exposure. Eleven GFs were examined, including LIF, placental growth factor (PIGF-1), SCF, HGF, vascular endothelial growth factor A (VEGF-A) and D (VEGF-D), beta nerve growth factor (bNGF), EGF, BDNF, FGF-2 and platelet-derived growth factor-BB (PDGF-BB). Additionally, we checked whether an EMF influences the cell cycle. The choice of EMF parameters in this study was based on our previous review [8]. To the best of our knowledge, we are the first to show show

changes in the concentrations of GFs by ASCs after continuous exposure to EMF (50 Hz; 1.5 mT) *in vitro*.

MATERIALS AND METHODS

ASC culture

The Ethics Committee at University of Rzeszow, Rzeszow, Poland (Ethics Committee at University of Rzeszow resolution no. 10/04 /2016) approved the study. Commercially available StemPRO® Human Adipose-Derived Stem Cells, lot no. 1001001 (Invitrogen™, USA) were used. Cells were grown in reduced serum (2%), human mesenchymal stem cell growth-supporting medium MesenPRO RS™ Medium (Gibco™, USA) supplemented by L-glutamine (2 mM; Gibco™), an antibiotic and an antimicotic solution (100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B, respectively; Gibco™). ASCs were cultured at 37°C in the presence of 5% CO_2 (170R CO_2 Incubator, New Brunswick Galaxy®, Eppendorf, Germany). In order to detach adherent ASCs from the culture dishes when passaging, trypsin (TrypLE™ Express Enzyme without phenol red, Gibco™) was used.

EMF system and exposure of ASCs

ASCs were exposed to sinusoidal EMF supplied by an electromagnetic field device consisting of a cylindrical in shape magnetic field applicator and a dedicated precision electric signal generator (COMEF, Poland). A solenoid with 280 turns per coil (resistance of the coil is 3.6 Ω and inductance is 2.5 mH) with a length of 19.9 cm and a diameter of 10.5 cm, was placed in an a magnetic Teflon tube to create an applicator of the magnetic field with the following dimensions: length 24.5, diameter 9.5/14 cm (inside/outside). This system was located in the cell culture incubator where all conditions for cell growth, such as temperature, atmosphere composition and humidity, were constant and controlled (Supplementary Fig. S1). The EMF did not cause any additional heat or vortex motions. After three passages of culture, the cells were plated in separate cell culture dishes, placed in the coil with a homogeneous EMF area with a frequency of 50 Hz and a flux density of 1.5 mT for 24, 48 and 72 h. Both experimental and control samples (which were placed in a separate incubator without the EMF effect) were performed in triplicate (n=3). To determine the protein concentration, the collected medium was used after each experiment. After morphological analyses, the cells were used for cell cycle analysis.

Morphological analysis of ASCs

Changes in cell morphology were monitored by inverted microscopy (magnification: 20x, ZEISS Primovert, Germany). Images for each control and experimental sample after 24, 48 and 72 h were collected.

Identification of growth factors by the Luminex® FLEXMAP 3D® System

After three different exposure times (24, 48 and 72 h), growth factors, including LIF, PIGF-1, SCF, HGF, VEGF-A, VEGF-D, bNGF, EGF, BDNF, FGF-2, PDGF-BB, were detected in the collected cell medium (with a reduced serum content of 2% instead of 10%). The Growth Factor 11-Plex Human ProcartaPlex™ Panel Invitrogen™, USA, was used according to the manufacturer's instructions (19 October 2015 Rev.21).

Antigen standards by dilution of the antigen standard vial with cell culture medium were prepared. To prepare the plates for analysis, a magnetic beads mix was added to each well. Standards, the blank and samples (cell culture supernatant) were applied. The plate was sealed and shaken in the dark at room temperature and then kept at 4°C and incubated overnight. The next day, the detection antibody mixture was added to each well and incubated. Streptavidin-Phycoerythrin (PE) was added to every well, and the plate was sealed and shaken in the dark, followed by the addition of Reading Buffer. The plate was analyzed using a Luminex® FLEXMAP 3D® System, USA.

Cell cycle analysis

The cell cycle was analyzed using a Muse™ Cell Analyzer and Muse® Cell Cycle Kit (Luminex, USA). The analysis was performed according to the manufacturer's instructions. Two hundred µL of cells from every exposure time was transferred to tubes, centrifuged at 300 × g for 5 min and washed once with 1× phosphate-buffered saline (PBS). Subsequently, 200 µL of ice-cold

70% ethanol was added slowly and incubated for 3 h at -20°C. To the new tubes, 200 µL of fixed cells were added and the cells were centrifuged again and washed with PBS. In the next step, 200 µL of Muse™ Cell Cycle reagent was added and incubated for 30 min at room temperature in the dark. The cells were transferred to a Muse™ Cell Analyzer.

Statistical analysis

The obtained results were analyzed using *Statistica* 13.1 software (StatSoft, Poland). ANOVA with post-hoc Tukey's test was used to determine the differences between and within the control and experimental groups in time. Results with $p<0.05$ were considered statistically significant.

RESULTS

In order to detect if an EMF influences GF secretion and cell cycle distribution, samples from 4 independent experiments were analyzed. The results are presented on Figs. 1 and 2. The data set with mean values and standard deviation is also provided on the Supplementary Table S1.

Morphological analysis of ASCs

Cell morphology was monitored during the experiment. No morphological changes between the control and experimental samples were observed (Supplementary Fig. S2).

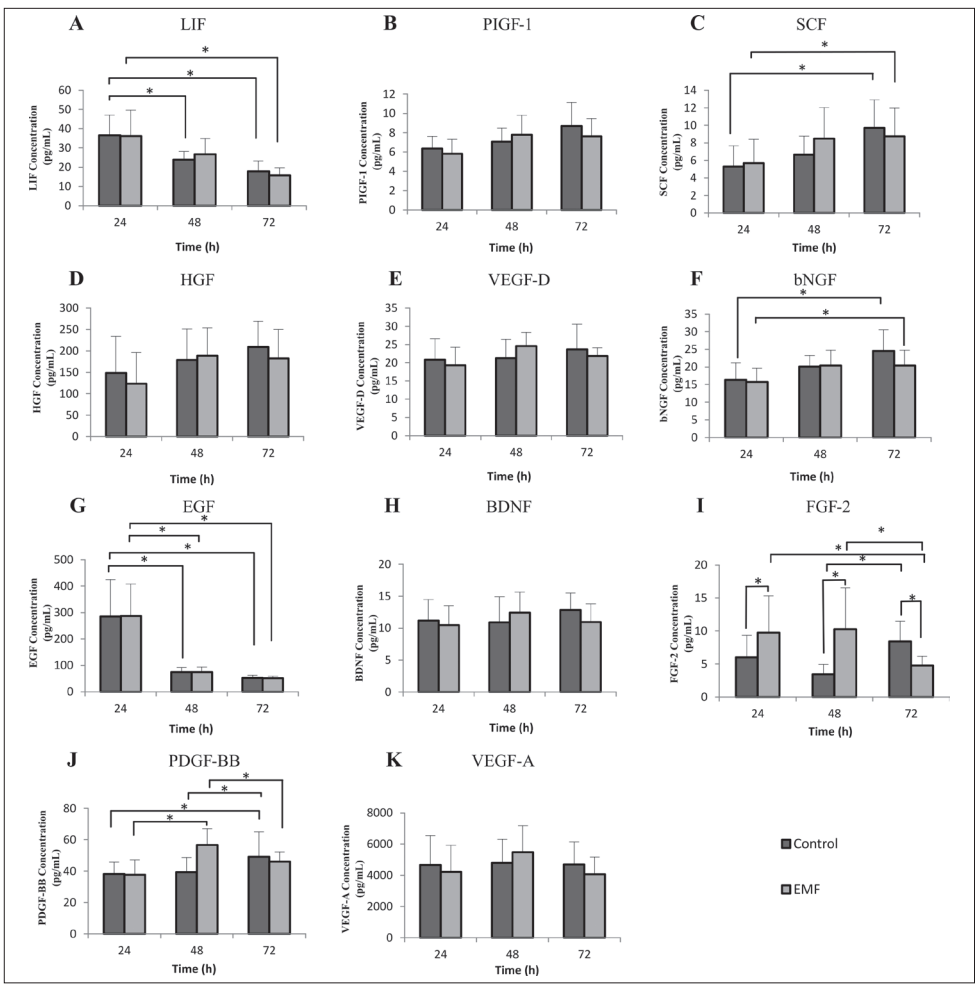

Fig. 1. Mean concentration of secreted GFs by ASCs: leukemia inhibitory factor LIF (**A**); placental growth factor 1 PIGF-1 (**B**); stem cell factor SCF (**C**); hepatocyte growth factor HGF (**D**); vascular endothelial growth factor D VEGF-D (**E**); beta-nerve growth factor bNGF (**F**); epidermal growth factor EGF (**G**); brain-derived neurotrophic factor BDNF (**H**); basic fibroblast growth factor FGF-2 (**I**); platelet-derived growth factor-BB PDGF-BB (**J**); vascular endothelial growth factor A VEGF-A (**K**), after electromagnetic field treatment EMF and in the control group. * indicates $p<0.05$.

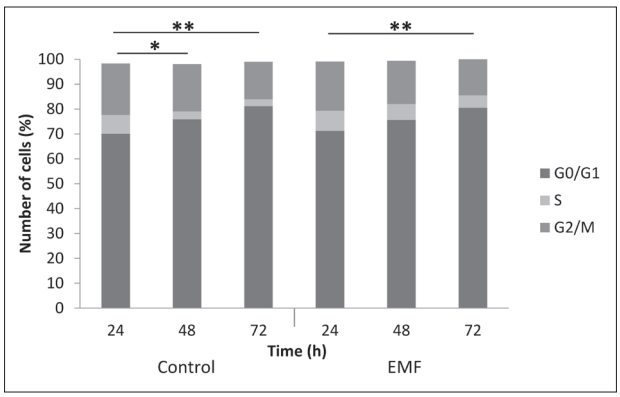

Fig. 2. Cell cycle distribution analysis of ASCs after exposure to an EMF and in the control group. * indicates p<0.05 for 24-48 h (G0/G1- and S-phases); ** indicates p<0.05 for 24-72 h (G0/G1-, S- and G2/M-phases).

Growth factor identification

As can be seen on Fig. 1, each analyzed GF in the cell culture with time (24C, 48C, 72C) exhibited a tendency to either increase (SCF, bNGF, PDGF-BB), decrease (LIF, EGF) or to remain more less at the same level (without statistical significance: PIFG-1, HGF, VEGF-D, BDNF, VEGF-A). This means that even without any external stimulation, the production of GFs and their release is subject to regulatory processes. After EMF exposure (24E, 48E, 72E), the concentrations of SCF, bNGF and PDGF-BB increased, exhibiting a trend that was similar to the control samples; likewise, the concentrations of LIF and EGF decreased. Analysis of the abovementioned GFs, however, did not reveal statistically significant changes after EMF exposure between control samples and samples exposed to EMF. The concentration of FGF-2 was statistically significantly increased after 24 and 48 h of EMF exposure and decreased at 72 h.

Only the concentration of FGF-2 after EMF exposure (Fig. 1) showed statistically significant differences compared to the control at every examined time. An opposite tendency in the production and secretion during EMF exposure (24E, 48E, 72E) versus control samples (24C, 48C, 72C) can be seen, where in the control group, the concentration of FGF-2 decreased after 48 h and increased after 72 h. This sudden, statistically significant decrease in FGF-2 concentration after 72 h of EMF exposure to a lower level when compared to other GFs is of note. It suggests a strong influence of EMF exposure, leading the regulation of FGF-2 production and secretion. This phenomenon may be due to a change in culture conditions and cell adaptation.

Another interesting observation is that after 48 h of EMF exposure, increased levels of LIF, PIGF-1, bNGF, SCF, HGFVEGF-A, VEGF-D, BDNF, FGF-2 and PDGF-BB were observed. These values were not maintained when exposure lasted for 72 h. This led us to conclude that EMF exposure brings into play processes in the cells that lead to the production and secretion of GFs, as manifested by the levels of GFs in the culture media after 48 h. Differences in changes in concentration of GFs in the experimental group as compared to the control group suggest that exposure to EMF exerted an influence at the level of secretion.

Cell cycle analysis

The results of cell cycle analysis that were obtained using Muse™ Cell Analyzer and Muse® Cell Cycle Kit (Luminex, USA. These findings are summarized in Fig. 2. Most of the cells, both in the control and treated samples, were arrested at low activity and resting G0/G1-phases (70.05-81.1% of cells). A low percentage of cells was in the S-phase during DNA synthesis (2.7-8.1% of cells). The number of cells in cell division G2/M-phases was in the range of 14.50-20.75% cells. Statistically significant changes were observed for control and experimental cells with regard to the G0/G1-, S- and G2/M-phases after 72 h when compared to 24 h. In the controls for G0/G1- and S-phases, there were statistically significant differences after 48 h of culturing. In the experimental and control samples, the percentages of S- and G2/M-phases gradually decreased over time; however, in treated samples, a higher percentage of cells in S-phase vs the control, and a lower percentage of cells in G2/M-phases were detected. Perhaps the exposure to EMF affected DNA synthesis, hence the observed increase in the percentage of S-phase cells in samples exposed to EMF.

DISCUSSION

The main purpose of this study was to examine the effect of the EMF on the secretion of GFs by ASCs *in vitro*. The most significant discovery of the present

study was that EMF influences the statistically significantly production and secretion of FGF-2 to the medium of an *in vitro* culture. It should be noted that the medium contained a reduced serum content (2% instead of the usual 10%), to eliminate the effect of GFs from the serum.

ASCs can naturally secrete high amounts of GFs [8,20,21]. Their presence guarantees the regulation of various processes, such as cell proliferation, healing and differentiation. It was revealed that FGF-2, like SCF and PDGF-BB, are not the GFs that are secreted by cells at high concentrations, while VEGF is – without medium changes (as also shown in our study), and without EMF stimulation [44]. Our study revealed no significant increase in the secretion of VEGF in the experimental group when compared to the control, but it is notable that its concentration increased after 48 h, like SCF and PDGF-BB. However, our results showed a significant increase in FGF-2 concentration after EMF exposure, which indicates that EMF affected the ASCs, which in turn affected the secretion of GFs. Another study also showed that the level of VEGF-A is not significantly increased after the exposure of retinal pigment epithelial cells to EMF (50 Hz; 1 mT) [47], which indicates that our assumption is correct. This suggests that specific changes occur in cells depending on the prevailing conditions, and also in response to them.

FGF-2 is a GF that helps in maintaining the integrity of the stem cell genome. It is secreted and activated after DNA damage caused by the stress factor (γ-rays) in epithelial stem cells [42]. The increased secretion of FGF-2 can also be induced by DNA strand breaks in response to EMF exposure (50 Hz; 1.5 mT) [5,6]. An extremely low frequency electromagnetic field (ELF-EMF) (0-200 Hz) can increase ROS formation that can damage DNA as well as lipids, proteins and sugars, and activate signaling pathways for gene expression, proliferation, apoptosis and other processes [7]. Studies have shown that the level of H_2O_2 is increased after ELF-EMF exposure in BM-MSCs and phosphorylation of the EGF receptor, revealing the induction of differentiation-linked processes [7,17,49]. Changes in ROS levels can be observed after 90 min of exposure to EMF with similar parameters (50 Hz; 1 mT) [17]. An increased number of S-phase cells has also been observed; the Vero cell line after EMF exposure (100 Hz; 5.6 mT) showed an increase of S-phase and high damaged DNA cells [5]. DNA damage can occur via the homologous recombination pathway occurring only in the S- and G2-phases [42]. The observed increase in the number of G0/G1-phase cells points to DNA repair in MSCs (which was also observed after exposure of MSCs to γ-rays) in which double-strand DNA breaks are repaired by the non-homologous end-joining (NHEJ) pathway [42]. In this study, no statistically significant changes in G2/M-phases were observed.

FGF-2 belongs to a family of proteins that promote the self-renewal of cells and maintains their stemness [33,36] by stimulating proliferation via the PI3K/AKT-MDM2 pathway [48], and inhibiting cellular aging *in vitro*. There is evidence on the regenerative effect of FGF-2 supplementation on ASC proliferation from healthy and diabetic donors, where in the second group the regeneration of cells was slower [37]. It was shown that in human periodontal ligament stem cells, supplementation of FGF-2 into the culture medium causes a significant increase in proliferation as well as the expression of a marker determining the stemness of the cells and a decrease in apoptosis after 5-7 days. Similarly, for BM-MSCs, FGF-2 supplementation helps maintain pluripotency [38].

The ELF-EMF (0-100 Hz) increases the differentiation potential of MSCs and has an impact on ion dynamics and signaling molecules as well as gene expression that leads to stimulation or inhibition of the cell pathway responsible for biological processes [43].

The pathway in which FGF-2 affects different types of differentiation of stem cells is unclear. The increased amount of FGF-2 increases stem cell chondrogenic and osteogenic potential [19,33,39], and may increase the secretion of collagen, glycosaminoglycan and other chondrogenic markers typical for this tissue [19,39,50]. On the other hand, FGF-2 has an inhibitory effect on early and late chondrogenesis [50,51] in which FGF-9 and FGF-18 are involved [52]. Increased amounts of type II collagen are characteristic of hypertrophy classified as the late stage of post-mitotic chondrogenesis [51]. The addition of FGF-2 reduced chondral growth induced by TGF-β1/BMP-2 by involving the Erk pathway and inhibited the phosphorylation of Smad1 and Smad2 proteins by blocking the signaling

pathway for chondrogenic markers such as TGF-β1/BMP-2 [53]. This suggests that increased secretion of FGF-2 in ASCs at 24 h and 48 h was a first response to EMF exposure, with cells attempting to maintain their stemness and proliferation, and not proceed into chondrogenesis or osteogenesis. FGF-2 also stimulates neuropoiesis and angiogenesis and is essential for the differentiation and function of the central nervous system [40,41], and it can help increase the level of neuronal precursors [36]. BDNF, which also supports the stimulation of neurogenesis [54], exhibited an increase in concentration after 48 h and a decrease after 72 h. A similar relationship was reported after exposure of pheochromocytoma rat cells to an EMF (75 Hz) under hypoxic conditions, where BDNF acted as a protective factor [46].

FGF-2 also has antiapoptotic, proangiogenic and regenerative properties [20,40]. Therefore, it plays a significant role in the growth and differentiation of cells under normal and conditions and in cancer [55]. In our study, we observed an increase in the number of number S-phase cells, similarly to another study performed on human epidermal stem cells (50 Hz; 5 mT) [56].

It seems that the stimulation of cells with EMF interferes with processes related to DNA preservation and replication, resulting in an increase in S-phase cells after EMF exposure. It may also stimulate the secretion of GFs and affect the regenerative capability of the cells. The beneficial effects of pulsed EMF on wound healing and the significantly increased level of FGF-2 secretion and the level of mRNA encoding for this GF in human umbilical vein endothelial cells has been demonstrated earlier [57-59].

CONCLUSIONS

In conclusion, ASCs cultured *in vitro* secrete high concentrations of growth factors into the medium in a time-dependent manner. The EMF (50 Hz; 1.5 mT) impacted the production of GFs, which is very important with respect to proregenerative properties. Our study revealed that the most significant change was a 3-fold increase in FGF-2 concentration after 48 h post EMF exposure, and a rapid, 5-fold decrease after 72 h. The parameters of the EMF used in our study also affected the cell cycle. In EMF-exposed cells, the numbers of cells in the G0/G1-, S- and G2/M-phases were statistically significantly changed over time and were characterized by an increase in the number of cells in the S-phase. We intend to verify whether exposure to an EMF with different parameters will also produce such a significant change in the concentrations of FGF-2 and other GFs. It would be worthwhile testing how such alterations in FGF-2 concentration after EMF exposure influence the expression of chondrogenic and osteogenic markers as well as the process of differentiation.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Trzyna%20et%20al_5199_Supplementary%20Material.pdf