Original Article

The Dynamic(s) of Adipose Stem Cell System, Their Survival, and Cessation under the Influence of Electromagnetic Fields

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Abstract

Context: The electromagnetic field (EMF) is one of the external biophysical factors that can influence stem cells' structure and functionality. Depending on its frequency and magnetic flux density, EMF can have both a positive and negative effect on stem cell biology. **Aims:** The aim of the study is to define EMF conditions that support beneficial physiological processes and those that lead to pathophysiological phenomena. Understanding the changes and processes occurring in stem cells after exposure to EMFs of different parameters can be an important factor to be applied in stem cell-based therapies and regenerative medicine. **Materials and Methods:** In this study, using fluorescent microscopy and flow cytometry methods, the influence of EMF on adipose-derived stem cells proliferation, cell cycle, viability, and death were examined. EMF parameters were set in accordance with the ion cyclotron resonance (ICR) theory that influences Ca^{2+} and Mg^{2+} ions influx. Results were statistically developed using the ANOVA and effect size (Cohen's *d*) analyses. **Results:** In this study, the continuous exposure of adipose-derived stem cells to EMF (ICR parameters: 76.6 Hz; 20 μT) causes a statistically significant increase in cell death through the enhancement of apoptotic, necrotic, and autophagic cell numbers. Apart from increased cell deaths after EMF exposure, increased proliferation after 24 h of EMF exposure has been also observed. **Conclusions:** Results presented in this study show that EMF influences stem cell dynamics resulting in a significantly increased cell death, thus altering the stem cell fate. It is important to further establish EMF conditions that support ASCs functioning and beneficial physiological processes for future regenerative medical purposes.

Keywords: Cell death, electromagnetic field, ion cyclotron resonance, stem cell fate, stem cells

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Introduction

Adult stem cells, in their natural state, receive physical and biochemical signals through contact with the cell membrane and soluble proteins in the stem cell niche.[1] These signals may direct stem cells toward various fates, such as differentiation, to maintain regeneration or death as well as cancer stem cell formation in response to improper conditions.[2,3] It has been proven that electromagnetic field (EMF) has an impact on stem cell biology, thus symmetric and asymmetric stem cell division,[4] proliferation, cell cycle, differentiation, cytokine, and growth factors secretion,[2,5-7] therefore, it can be a key factor influencing stem cell fate(s). In this study, it was hypothesized that some EMF parameters may be supportive for stem cell survival and maintenance, while others may direct stem cells to death/cessation or neoplastic transformation/stem cell–cancer stem cell transition(s). In turn, it can help to establish culturing conditions to achieve the beneficial effect and use of adipose stem cells in stem cell-based therapies and regenerative medicine. However, due to the fact that even the simplest biological systems are nonlinear, determining the general mechanism of the effect of EMF conditions on cells is still not obvious. To be precise, in this article, the notion of EMF was used, but the reader should be aware that, in the extremely low-frequency range of EMF, the two components of the field are separated and one has to refer to either electric or magnetic field. Here, only the magnetic component of the

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field was considered but following the literature, the term "EMF" was used.

In this study, to check the biological response of adipose-derived stem cells (ASCs), EMF with specific parameters was applied, which causes altered energy states of Ca^{2+} and Mg^{2+} ions, due to ion cyclotron resonance (ICR) mechanisms. To obtain such parameters, the fact was utilized that energy can be transferred from the EMF to the ions only during ICR resonance.[4,8] In this study, it was hypothesized that EMF, within these specific parameters, will affect the functioning of ASCs and can influence the Ca^{2+} and Mg^{2+} ion influx and therefore change the metabolism of cells. Even though the mechanism *per se* had not been checked, the stem cell response on EMF parameters calculated using this model was monitored. Changes in cell proliferation, survival, and death were observed suggesting the link between EMF parameters and stem cell fate. It is the first work in which the influence of the EMF radiation (with ICR parameters) on adipose stem cells' death types was tested. Findings presented in this study are very important and can help to establish culturing conditions to achieve the beneficial effect and use of adipose stem cells in stem cell-based therapies and regenerative medicine.

Materials and Methods

Adipose-derived stem cells culturing

StemPRO human adipose-derived stem cells (lot number 1001002, Invitrogen, Van Allen Way Carlsbad, CA) were seeded in density 5000 viable cells/cm^2 and grown in DMEM/F12 medium (Life Technologies, Paisley, UK), supplemented by 10% FBS (Biological Industries, Cromwell, CT) with an antibiotic and antimycotic mixed solution (Life Technologies, Paisley, UK, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B). Passaging adherent ASCs, trypsin (TrypLE Express Enzyme without Phenol Red Life Technologies, Paisley, UK) was used. ASCs were cultured at 37°C in the presence of 5% CO_2 and 95% humidity (New Brunswick Galaxy 170R CO_2 Incubator, Eppendorf, Hamburg, Germany). ASCs from the third passage were used for each experiment.

Electromagnetic field parameters calculation

The EMF parameters (frequency and amplitude) were estimated based on the ICR theory.[9-11] According to this theory, the physiological ionic activity of some ions can be altered by the biological action of the EMF in the extremely low-frequency region. To calculate the parameters, the well-established equation $f = \frac{1}{2\pi}\frac{q}{m}B_e$ was used where f is the frequency of applied external magnetic field, B_e its amplitude, and q and m are charge and mass of an ion, respectively. The equation provides linear dependence $f(B_e)$ of frequency as a function of the external field and allows one to calculate the resonant frequency for a given external field (B_e) and chosen ion (defined by $\frac{q}{m}$ ratio). According to this equation, one can calculate ratios $\frac{f}{B_e} = \frac{1}{2\pi}\frac{q}{m}$ for chosen ions. These ratios for Ca^{2+} and Mg^{2+} ions are equal 0.766 Hz/μT and 1.263 Hz/μT, respectively. However, if consider the fundamental 5th harmonic for $f_2 = (2 \times 2 + 1)f$ Ca^{2+} and 3rd harmonic $f_1 = (2 \times 1 + 1)f$ for Mg^{2+}, then above ratios become almost equal (the 5th harmonic of Ca^{2+} is slightly more than 1% greater than the 3rd harmonic for Mg^{2+}) and there are $\frac{f_2}{B_e} = 3.832\ Hz/\mu T$ and $\frac{f_1}{B_e} = 3.788\frac{Hz}{\mu T}$ respectively. From the other side $\frac{76.6\ (Hz)}{20\ (\mu T)} = 3.83\ Hz/\mu T$, what may suggest that EMF with a frequency equal to 76.6 Hz and an amplitude of 20 μT can simultaneously affect two different ions (Ca^{2+} and Mg^{2+}). Therefore, to investigate the proliferation, survival, and death of ASCs sinusoidal EMF with parameters, the frequency equal to 76.6 Hz and the amplitude of 20 μT tuned to the fundamental 5th and 3rd cyclotron resonance harmonics for Ca^{2+} and Mg^{2+} ions have been chosen.

Electromagnetic field exposure system and electromagnetic field exposure of adipose-derived stem cells

ASCs were placed in the EMF-generating device consisting of a cylindrical magnetic field applicator and a dedicated electric signal generator (COMEF, Katowice, Poland) [Figure 1a]. The source of the sinusoidal EMF was solenoid with 280 turns/coil [Figure 1b] (resistance of the coil: 3.6 Ω; inductance: 2.5 mH) with a length of 19.9 cm and a diameter of 10.5 cm placed in an open Teflon tube to create an applicator of magnetic field with final dimensions of length 24.5 cm and a diameter of 9.5/14 cm (inside/outside). The solenoid was located in the cell culture incubator. In each experiment (n = 3) cell samples, seeded on 6 cm culture plate dishes (10^4 cells/sample), were placed in an EMF generator system [Figure 1a] for continuous EMF exposure (for 12 and 24 h). To ensure that experiments were performed in identical conditions, 3 samples were exposed to EMF (76.6 Hz; 20 μT) at once (in the middle of the solenoid where the same homogeneous condition is maintained). Similarly, control samples (not exposed to EMF) were prepared and cultured for the same time in different cell incubators, with maintained same cell culture condition. Appropriate cell culture conditions

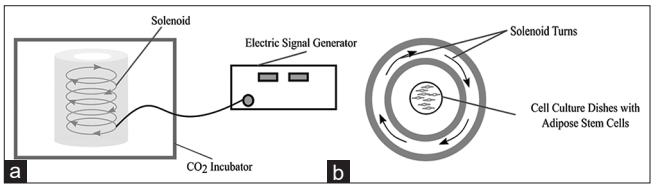

Figure 1: The electromagnetic field exposure system. (a) Electromagnetic field applicator placed in CO_2 Incubator New Brunswick Galaxy® 170R connected with electromagnetic field generator; (b) coil cross-section

were provided (37°C, 5% CO_2, 95% humidity) in both incubators for the control and experimental samples. The only difference was the absence of a magnetic field in the control system. It is worth mentioning that the EMF did not cause any additional vibration or heat in ASCs if magnetic flux density is below 2 mT in the used exposure system. Background external DC (earth's) and AC fields were measured during the experiments and were at the same level in two separate incubators.

Adipose-derived stem cells viability

To check ASCs viability after EMF exposure, the Zeiss Axio Observer Z1-Inverted Fluorescence Microscope (magnification × 40) was used. Analysis based on fluorescent staining and manual counting was used with fluorescein diacetate (FDA, Serva, Heidelberg, Germany) and propidium iodide (PI, Cayman CHEMICAL, Ann Arbor, MI). To each cell culture dish, 2 µl/ml of FDA was added and incubated for 15 min at 37°C, in the dark. In the next step, 20 µl/ml of PI was added and incubated for 1 min at 37°C, in the dark. Both dead and live cells were visualized and scored under a fluorescence microscope (1000 cells from randomly selected fields of view). Each experiment was performed in triplicate.

Adipose-derived stem cells apoptosis and necrosis

To assess the extent of ASCs apoptosis and necrosis after EMF exposure and visualize the changes, a Zeiss Axio Observer Z1-Inverted Fluorescence Microscope (magnification × 40) and Muse Cell Analyzer were used.

For the identification of apoptotic and necrotic cells, based on a staining method with a mixture of fluorescent dyes 0.4 mg/ml Hoechst 33342 (Invitrogen, Van Allen Way Carlsbad, CA) and 0.5 mg/ml PI (Cayman CHEMICAL, Ann Arbor, MI), a necessary amount of staining solution was added to the cell culture dishes and incubated for 5 min at 37°C, in the dark. Apoptotic and necrotic cells were visualized and scored under a fluorescent microscope (1000 cells from randomly selected fields of view; Hoechst 33342: 350 nm/460 nm excitation/emission; PI: 535 nm/617 nm excitation/emission).

To determine the level of advancement of the apoptosis process, a Muse Cell Analyzer and Muse Annexin V and Dead Cell Kit (Luminex, Commercial Ave, Northbrook, IL) were used. Analysis was performed using a dead cell marker and calcium-dependent phospholipid-binding protein Annexin V and 7-AAD according to the manufacturer's instructions (Muse Annexin V and Dead Cell Kit Catalog No. MCH100105). Briefly, after each experiment, cells were trypsinized and 100 µL of cells suspensions containing 1% FBS were prepared for analysis. Next, 100 µL of Annexin V and dead cell reagent was added to each sample and mixed. Samples were stained for 20 min in the dark and then analyzed with Muse Cell Analyzer. Each experiment was performed in triplicate.

Adipose-derived stem cells autophagy

Identification of autophagy was performed using an acridine orange staining method (Life Technologies, Paisley, UK). To every cell culture dish, 5 µg/ml of acridine orange was added and incubated at 37°C for 15 min in the dark. After this time, autophagic cells were visualized and scored under a Zeiss Axio Observer Z1-Inverted Fluorescence Microscope (magnification ×40; 1000 cells from randomly selected fields of view; 546 nm/640 nm excitation/emission). Each experiment was performed in triplicate.

To support data from manual counting, the western blot analysis for LC3 protein was performed. After 12 and 24 h of EMF treatment, ASCs from control ($n = 3$) and EMF exposed group ($n = 3$) were lysed in a hot SDS-loading buffer (125 mM Tris-HCl pH 6.8; 100 mM DTT; 4% SDS; 10% glycerol). Next, boiled in water bath for 10 min and centrifuged for 10 min (10,000 × g). Supernatants were collected and analyzed. The concentration of protein was estimated with the Bradford method. Protein samples (80 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto an immobilon-P membrane (Sigma Aldrich, USA). Next, the membrane was blocked with 3% low-fat milk in PBS for 1 h and incubated overnight with anit-LC3 diluted 1:1000 (Sigma, USA). Membranes were washed for 10 min with 0.05% Triton X-100 in PBS (Sigma Aldrich, USA), each membrane was three times. Next, membranes were incubated with alkaline phosphatase-conjugated antimouse IgG or antirabbit IgG in 1:30,000 dilution for 2 h (Sigma Aldrich, USA). Membranes visualization was performed with an alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium) in color development DMF buffer (Sigma Aldrich, USA). The data were normalized relative to β-actin diluted 1:2000 (Sigma Aldrich, USA).

Adipose-derived stem cells proliferation

To check ASCs ability for proliferation after EMF exposure, a Muse Cell Analyzer and Muse Ki67 Proliferation Kit (Luminex, Commercial Ave Northbrook, IL, USA) were used. The analysis basing on Ki67 expression was performed according to the manufacturer's protocol (Muse Ki67 Proliferation Kit, Catalog No. MCH100114). Briefly, after each experiment cells were trypsinized from cell culture dishes and then transferred to separated tubes. Next, washed with PBS (Life Technologies, Paisley, UK) and centrifuged (5 min, 300 ×g). Each sample of cell sediment was fixated with fixation solution for 15 min at room temperature. Next, washed with 150 µL of assay buffer and treated with 100 µL of permeabilization buffer at room temperature. After 15 min, each sample was washed once again with assay buffer and incubated for 15 min at room temperature. Next, Muse® Hu Ki-67-PE was added to each sample, mixed, and incubated for 30 min in the dark. After incubation, 150 µL of assay buffer was added to each sample and analyzed on the Muse® Cell Analyzer. Each experiment was performed in triplicate.

Cell cycle analysis

The cell cycle was analyzed using a Muse Cell Cycle Kit and Muse Cell Analyzer (Luminex, Northbrook, IL, USA). The analysis basis for using nuclear DNA intercalating stain PI and RNAse A was performed according to the manufacturer's

instructions (Muse Cell Cycle Kit User's Guide Catalog No. MCH100106). Briefly, after each experiment, ASCs were trypsynized and suspended in 200 µL of culture media in separated tubes. Next, cells were fixated with 200 µL of 70% ice ethanol and incubated for 3 h at − 20°C. Following, cells were centrifuged (5 min, 300 ×g) and washed with PBS (Life Technologies, Paisley, UK). To each sample, 200 µL of Muse™ Cell Cycle Reagent was added and incubated for 30 min in the dark at room temperature. Next, cells were analyzed with Muse® Cell Analyzer. Each experiment was performed in triplicate.

Statistical analysis

The obtained results were analyzed using Statistica 13.1 software (StatSoft, Krakow, Poland). The one-way analysis of variance and effect size (Cohen's *d*) were calculated to determine if differences for the control and experimental groups can be considered as statistically significant ($P < 0.01$).

Results

Adipose-derived stem cells viability

Here, a significant, two-fold increase ($P < 0.01$) of dead cells in ASCs population after 12 and 24 h of EMF exposed (5%–13%) compared to nonexposed (11%–21%) cells is reported [Figure 2a]. It has been also revealed that the cell death changed with time ($P < 0.01$) within two tested groups: control (5%–11%) and EMF exposed (13%–21%). These changes were enhanced in the EMF exposed group. To validate cell viability, a fluorescein diacetate and PI double staining method and fluorescence microscope visualization were used [Figure 2e].

Adipose-derived stem cells apoptosis, necrosis, and autophagy

Following that, new samples have been set, like those described above, to investigate types of cell death occurring under EMF influence. Near two-fold increase of apoptotic, necrotic, and autophagic cells, compared to the control group, after 12 and 24 h of EMF exposure is reported [Figure 2c and d]. The level of apoptosis increased from 9.8% to 20.7% and 13.9% to 24,9%, necrosis from 1% to 2.1% and 1.1% to 2.6%, and autophagy from 8.6% to 18.9% and 10.7% to 20.7%, after 12 and 24 h of EMF exposure, respectively. It shows that the autophagic cells (10.7%) appeared in higher number after 12 h than apoptotic cells (9.8%). Changes can also be observed in apoptosis stages revealed in our study, where after 24 h of EMF exposure, the number of early apoptotic cells increase from 5.6% to 8.18%, as well as late apoptotic cells increase from 1.95% to 3.22%, respectively, compared to the control groups [Figure 2d]. Investigation of changes in cell death was based on fluorescent staining methods to detect characteristic morphological features for each cell death type such as apoptosis (cell shrinkage, membrane blebbing, apoptotic bodies), necrosis (loss of plasma membrane integrity, increase in cell volume) [Figure 2f] as well as autophagy (acidic vesicular organelles) [Figure 2g]. Moreover, to confirm differences in autophagic cell number in the control and EMF exposed group,

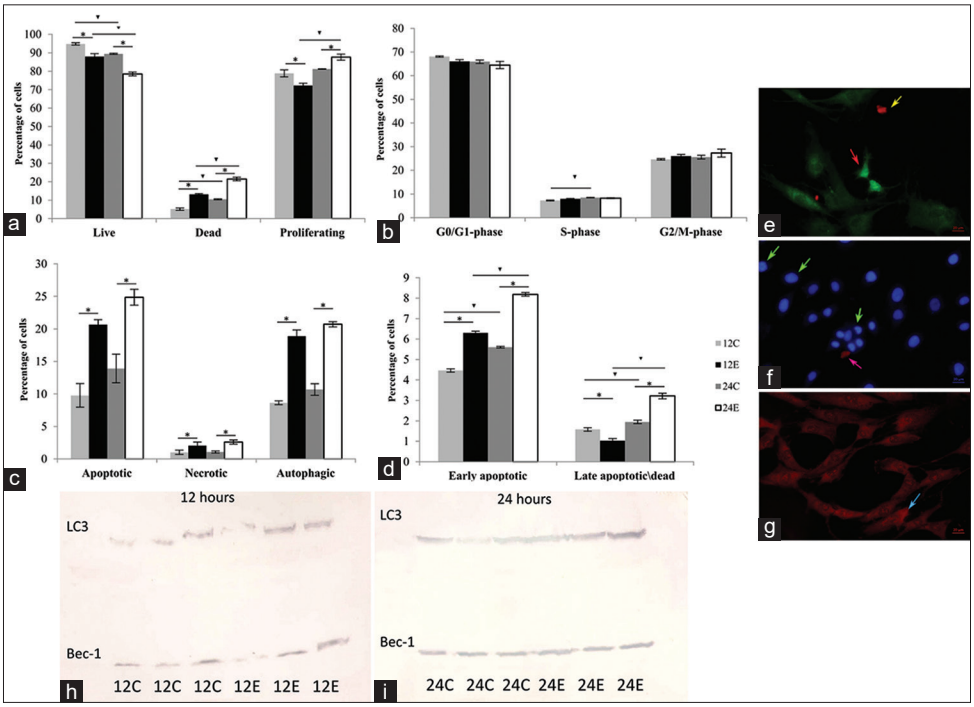

Figure 2: The effect of electromagnetic field on adipose-derived stem cells after 12 and 24 h (a) viability, proliferation; (b) cell cycle; (c) apoptosis, necrosis, autophagy; (d) apoptosis stages; cells visualization (fluorescence microscope, magnification, ×40); (e) viability; (f) apoptosis and necrosis; (g) autophagy; (h) western blot analysis for LC3 after 12 hours and (i) 24 hours. Arrows: Red-live cells; yellow-dead cells; green-apoptosis; pink-necrosis; blue-autophagy. *: Changes statistically significant for experimental (e) to control (c) groups and ▼: Indicates changes statistically significant within one of these groups ($P < 0.01$)

western blot analysis for LC3 was performed [Figure 2h, 2i]. The results show that autophagy marker protein LC3 shows increased concentration after EMF exposure, mostly after 24 h of EMF treatment comparing to the control samples [Figure 2h].

Adipose-derived stem cells proliferation

To investigate the proliferation changes, new samples were prepared with the same conditions as before. Subsequently, using a Muse Cell Analyzer and a dedicated kit based on determining Ki-67 (+) and Ki-67 (−) cells, proliferation changes after EMF exposure were defined. Comparing results to nonexposed cells, proliferation decreased after 12 h (79%–72%), to be increased (81%–88%) after 24 h ($P < 0.01$) with EMF stimulation [Figure 2a]. Within the control group, proliferation remained at a similar level after 12 and 24 h of the experiment (79% and 81%) but has changed in the EMF exposed group in time increasing from 72% to 88% [Figure 2a].

Cell cycle analysis

When investigating the cell cycle distribution, and its changes after EMF treatment, Muse Cell Analyzer and dedicated kit for all cell cycle analyses were used. A slight increase (7.27%–7.97%) in the percentage of cells at the S-phase after 12 h [Figure 2b] when DNA synthesis occurred was observed. On the other hand, also significant changes ($P < 0.01$) in S-phase cells numbers in the control group at 12–24 h occurred (7.27%–8.5%) but not in samples exposed to EMF where the number of S-phase cells changed increased from 7.97% to 8.23% [Figure 2b]. Nevertheless, statistically significant changes in the cell cycle compared to EMF exposed and nonexposed samples were not observed.

Discussion

In this study, EMF parameters were calculated to influence Ca^{2+} and Mg^{2+} ions which are essential in signal transduction pathways (such as cell division and growth), cell metabolism, and cell death.[11-13] Ca^{2+} ions act as a mediator changing for example the transmembrane potential and may act as a mediator in the interaction with the EMF. The parameters based on ICR can tune the frequency to that characteristic for individual ions, thereby selectively changing ion concentration in the cell.[4,14] These changes may lead to pathophysiological cell responses, such as increased cell death, reported in this study [Figure 2a].

Apoptosis pathways may be induced by microenvironmental perturbations, such as oxidative stress, reactive oxygen spices (ROS) overload, or DNA damage[12,15-17] controlled by intracellular Ca^{2+} ions[13]. Their increase might have been affected by disturbed calcium homeostasis in ASCs, which cause early oxidative and stress responses, leading to pathophysiological processes like increased cell death.[13,16,18] Interestingly, autophagy processes can protect cells from the negative impact of oxidative stress and apoptosis by activating adenosine monophosphate-activated protein kinase/mammalian target of rapamycin signaling pathway, thus reducing the accumulation of DNA damage in cells.[19,20] Instead, necrosis as a passive process occurs as a consequence of harmful conditions that cause loss of plasma membrane integrity.[15] This may occur because of a ROS or Ca^{2+} overload[12] caused by membrane surface depolarization and Ca^{2+} sensitive channel activation after EMF exposure.[4] Perhaps, the parameters of the EMF used in this study have a significant importance concerning adipose tissue stem cell necrosis. Increased cell death involving the disintegration of the cell membrane in this work is consistent with the approach and influence of EMF parameters selected based on ICR theory and Ca^{2+} transport through the cell membrane.[11,21]

In this study, a statistically significant increase of proliferation after 24 h of continuous exposure to EMF was observed. A decrease in proliferation after 12 h might be due to a superoxide anion formation induced by disturbing Ca^{2+} influx through EMF stimulation, whereby a proliferation increase after 24 h manifests when cells achieve a redox balance [Figure 2a].[16-18] Increased proliferation may also be due to higher concentrations of pro-proliferative FGF-2 protein, secreted by ASCs. Previous study has already demonstrated that after 24 h of EMF (50 Hz) exposure, ASCs secrete an increased amount of FGF-2.[7]

The ASCs, in response to new environmental conditions like EMF stimulation, might enter into different programs/stages (e.g. differentiation, death/cessation) and adaptation processes to compensate for negative effects such as an increased number of dead cells (e.g. increased proliferation).[4] In this study, the stronger impact of EMF on cell death after 12 h was observed, as opposed to 24 h [Figure 2a]. This might be due to increased proliferation and therefore a higher amount of new cells after 24 h. It is important to note here that stem cells are characterized by a low proliferation ability,[22] and an increased proliferation may be a signal of losing stemness and entering into one of the stem cell fate programs, e.g. differentiation or stem cell-cancer stem cell transition.[3,23,24] The stem cell fate may be affected by EMF stimulation, especially in terms of ICR-like mechanism(s) influencing Ca^{2+} ion concentration, thus changing cell division, microtubule dynamic instability, and division axis, leading to asymmetric stem cell division.[4,23,24]

Other researchers in the reviewed literature have already confirmed a dependence of time and EMF frequency (1–50 Hz) on stem cell survival, as well as proliferation. Two dependencies have been shown after 7 days of periodic EMF stimulation: the shorter the time of exposure, the higher the rate of proliferation and the lower the frequency, the lower the increase of proliferating cell number(s).[25,26] However, in this study, even though increased proliferation after 24 h of EMF exposure was observed, a significant change in dividing cells under G2/M-phases [Figure 2b] was not. However, changes in the S-phase under the influence of the EMF were observed. A slight increase of the percentage of S-phase cells in this study may have been caused by DNA damage, ROS formation, and single-strand breaks generated by increased intracellular calcium concentration.[13,27]

Conclusions

In summary, ASCs exposed *in vitro* to EMF (with ICR parameters supposed to influenced Ca^{2+} and Mg^{2+} ions) changed stem cells' fate toward increased cell death/cessation. The statistical level of apoptosis, necrosis, and autophagy significantly increased after EMF (ICR parameters: 76.6 Hz; 20 μT) exposure for 12 and 24. EMF exposure resulted in a greater than two-fold increase in the percentage of dead cells. This confirms that EMF can have an impact on ASCs and can direct them toward specific programs such as cell death and determine their fate and "destiny." Therefore, it seems extremely important to establish EMF conditions that support ASCs functioning, maintenance of stemness, and the occurrence of beneficial physiological features for future regenerative medical purposes and likewise to identify triggers that stimulate the pathological processes of stem cells. It would be worth checking if ASCs will show increased ROS levels after EMF exposure and how the redox balance will change, including Ca^{2+} transport dynamics. This collective and cumulative approach will eventually deliver a scope of knowledge that may improve the usage of EMF in stem cell maintenance, differentiation as well as regulation, direction, and therefore successful therapies.

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Conflicts of interest

There are no conflicts of interest.

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