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The Effect of Low-Frequency Pulsed Electromagnetic Fields on the Differentiation of Permanent Dental Pulp Stem Cells into Odontoblasts

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Introduction: Exposure to pulsed electromagnetic field (PEMF) has been revealed to affect the differentiation and proliferation of human mesenchymal stem cells derived from dental pulp multipotent stromal stem cells (DP-MSCs). This study aimed to investigate the differentiation effect of electromagnetic fields (EMFs) on the DP-MSC. **Materials and Methods**: PEMF was produced by a system comprising a multi-meter autotransformer, solenoid coils, and teslameter. This study included 10 groups of DP-MSCs which underwent different electromagnetic radiation time and beam intensity. Three samples tested for each group. The effect of PEMF with the intensity of 0.5 and 1 mT (mili Tesla) and 50 Hz on the proliferation rate of DP-MSC was evaluated at 20 and 40 minutes per day for seven days. MTT assay was applied to determine the growth and proliferation of DP-MSC. Gene expression of DMP1 for differentiation of DPSCs to odontoblasts was confirmed by Real Time PCR., ANOVA statistical analysis and Kruskal-Wallis test were used to analyze the data. **Results:** The survival in all exposure groups was significantly higher than that in control except in the group of 40 minutes, 1 mT (P<0.05). In 20 minutes, 0.5 mT exposure, the survival intensity is significantly more than others (P<0.05). In general, the intensity of survival was recorded, 20, 0.5 mT≥20, 1 mT≥40, 0.5 mT≥40, 1 mT respectively. Therefore, according to the obtained results, ELF-EMF increases the survival of cells except for one case (40 minutes, 1 mT), even though the effective underlying mechanisms in this process are still unclear. **Conclusions:** The results obtained promise that in the future, by placing an important part of the pulp next to the electromagnetic field, the lost part of the pulp can be reconstructed and the dentin barrier can be created.

Keywords: Bone Marrow Mesenchymal Stem Cells; Dental Pulp; Dental Pulp-derived Mesenchymal Stem Cells; Pulsed Electromagnetic Field

Introduction

Mesenchymal stem cells (MSCs) have become extensively studied over the past decade for their exciting broad-ranging clinical potential, cell biology, and a fundamental building block in the promptly growing field of tissue engineering [1, 2]. MSCs grow readily in the culture dish, have inherent differentiation potentials not found formerly in other cells, and produce abundant beneficial cytokines and growth factors [3, 4]. MSCs are multipotent stem cells found in the bone marrow and play a key role in repairing and making skeletal tissues, such as cartilage, bone, and the fat found in bone marrow [5, 6]. These are not to be confused with hematopoietic stem cells found in the bone marrow and blood [5, 6].The main MSC, containing tissues, such as adipose tissue and bone marrow, are good sources of multipotent cells to reestablish cellular function and replace lost cells [7]. Since the 1960s, the differentiating properties of mesenchymal stem cells into several cell lines have been evaluated and studied by studying different species [8, 9]. Research has shown that these cells can transform into highly differentiated cells such as bone, cartilage, tendons, muscle, adipose tissue, and stroma, supporting hematopoietic stem cells

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in both *in vivo* and *in vitro*. These cells have excellent healing potential in the heart, liver, and bones [8, 10]. Such approaches have been used for the treatment of hematological diseases in recent years [11]. Electromagnetic fields (EMFs) is non-invasive method and have been studied widely as safe approaches [12, 13]. EMFs have been used for many years in both animal and clinical studies to treat different kinds of bone disorders [12, 14]. Acritical study presented that treatment with EMFs helps clinical recovery and bone reestablishment EMFs can improve biomechanical properties and bone mineral density and prevent osteoporosis [15, 16]. Research findings recommended that a low frequency of PEMF pretreatment is a favorable method to enhance the efficacy of cell therapy for peripheral nerve injury repair [17, 18]. Considering the lack of research related to the effect of electromagnetic fields on the differentiation of pulp tissue stem cells into odontoblasts, this study aimed to investigate the impact of electromagnetic fields on the differentiation of dental pulp stem cells. This research demonstrated that placing the vital part of the pulp in the vicinity of EMFs, stimulated and facilitated the reconstruction of the lost part of the pulp and created a dentinal barrier.

Material and Methods

This was an *in vitro* study on 10 groups of human dental pulp-derived mesenchymal stem cells, three samples were tested in each group (n=3).

Differentiation of dental pulp-derived mesenchymal stem cells (DPSCs) into odontoblasts

Human DPSCs were purchased from Tabriz University of Medical Sciences, then DPSCs was cultured in α-MEM containing (MEM Alpha Gibco™ Fisher Scientific) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, America) and 1% penicillin and streptomycin (Gibco, Life Technologies) was transferred to a culture T25 flask and incubated 37° C containing 5% CO_2 and 95% humidity. After 24 h, the floating cells with the medium are removed from the T25 flask. 2×10^5 DPSCs were transferred to 24 well plates to differentiate into odontoblasts. For differentiation into odontoblasts, pulp stem cells were exposed to this differentiating medium containing "Minimum Essential Medium" (MEM Alpha; Gibco™ Fisher Scientific, Waltham, MA, USA) containing10% fetal bovine serum (FBS; Gibco, Life Technologies, NY, USA), Dexamethasone, 10 mL sodium beta glycerophosphate, 50 mg/mL vitamin C, 5 ng /mL TGF-β1 for three weeks.

Table 1. Characteristics of the ten groups of dental pulp-derived mesenchymal stem cells which underwent different electromagnetic radiation time and beam intensity [mT (mili Tesla)]

Group	Radiation Time	Intensity	With/without differentiating cell groups (DCG)
1	40 min	1 mT	With DCG
2	40 min	0.5 mT	With DCG
3	40 min	1 mT	Without DCG
4	40 min	0.5 mT	Without DCG
5	20 min	0.5 mT	With DCG
6	20 min	1 mT	With DCG
7	20 min	0.5 mT	Without DCG
8	20 min	1 mT	Without DCG
9	_	_	With DCG
10	_	_	Without DCG

Ionizing radiation on cells

The study groups are as follows: 1-Tooth pulp mesenchymal stem cells differentiating factors (containing 10% FBS, dexamethasone, 10 mL sodium beta glycerophosphate, 50 mg/mL vitamin C, 5 ng/mL TGF-β1) 2-Tooth pulp mesenchymal stem cells+differentiating factors (containing 10% FBS, dexamethasone, 10 mm sodium beta glycerophosphate, 50 mg/mL vitamin C, 5 ng/ml TGF-β1). Minutes a day. 3-Tooth pulp mesenchymal stem cells+electromagnetic radiation with a frequency of half and 1 millisec for 20 min and 40 min a day. In addition, 3 samples were evaluated in each group. This study included 10 cell groups: 1) DP-MSCs+TGF-β1, 2) DP-MSCs+TGF-β1, 40 min, 1 mT (mili Tesla), 3) DP-MSCs+TGF-β1, 40 min, 0.5 mT, 4) DP-MSCs+TGF-β1, 20 min, 1 mT, 5) DP-MSCs+TGF-β1, 20 min, 0.5 mT 6) DP-MSCs+40 min, 1 mT, 7) P-MSCs+40 min, 0.5 mT, 8) P-MSCs+20 min, 1 mT, 9) P-MSCs+20 min, 0.5 mT, 10) DP-MSCs+without TGF-β1. More details of the groups are summarized in Table 1.

Radiation system; extremely low-frequency electromagnetic fields (ELF-EMFs)

The device producing a sinusoidal magnetic field was a coil (solenoid) with a cylindrical acrylic core with an inner diameter of 20 cm and a length of 24 cm. 720 turns of coated copper wire with a diameter of 1 mm wrapped around the solenoid core. The solenoid will be placed horizontally in the laboratory and connected in series to the auto transfer on a voltage percentage scale. The autotransformer was connected to the electricity with a frequency of 50 Hz and a voltage of 220 volts. By feeding the solenoid with a current of 1.202 mA and 395 mA and voltage of 64.4 and 19.9 V, a sinusoidal magnetic field with intensities of half and 1 mA Tesla was produced. The solenoid was connected to an oscilloscope and a multimeter to check the input current and waveform. The system was calibrated and the resulting magnetic

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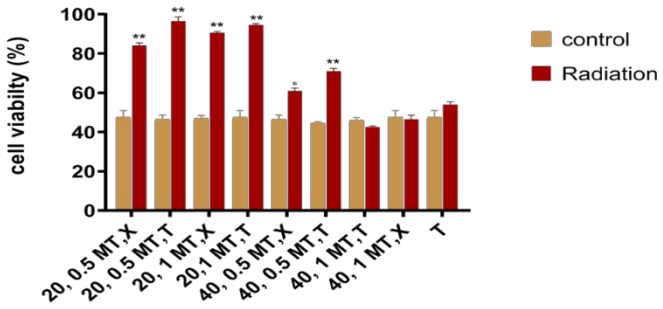

Figure.1. Viability of DP-MSCs with MTT assay. Significant difference in Viability cell in DP-MSCs in (20 min, 0.5 mT, X), (20 min, 0.5 mT, T), (20 min, 1 mT, X), (20 min, 1 mT, T), (40 min, 0.5 mT, X), (40 min, 0.5 mT, T) with respect to control observed (X; without differentiating cell groups, T; with differentiating cell groups). **P*<0.05; ***P*<0.01

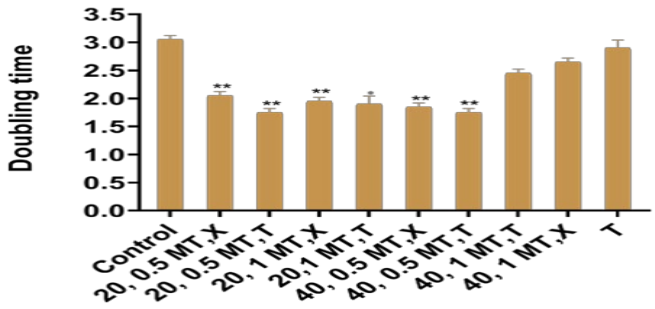

Figure.2. Evaluation of doubling time in cell groups, significant difference in doubling time in DP-MSCs in (20 min, 0.5 mT , X), (20 min, 0.5 mT , T), (20 min, 1 mT , X), (20 min, 1 mT , T) , (40 min, 0.5 mT , X), (40 min, 0.5 mT , T) with respect to control observed (X; Without differentiating cell groups, T; With differentiating cell groups), **P*<0.05; ***P*<0.01

field was measured using a teslameter (GMBH 51662; Leybold Didactic, Huerth, Germany). The uniformity of the magnetic field at the center of the coil was 1%. In all experiments, the platelets were irradiated in the center of the solenoid (with maximum uniformity). Intra-selenoid temperature was measured during the experiment with a thermometer.

MTT assay

For this purpose, DPSCs were seeded into 96-well plates with 80000 cells in passage 3 and incubated for 24 h with α-MEM. Well plates are removed from the incubator and drained using a pasteurizer pipette. Each well was rinsed once with 100 µl of PBS solution. Then 100 µL of FBS-free culture medium and 10 µl of MTT solution (5 mg/mL concentration Life Technologies, England) are added to each well, and the plates are incubatedin a 37˚ for 4 h. After this time, the plates are removed from the incubator and 100 µL of isopropyl alcohol was added in order to dissolve the formazan crystals to each of well. Then the contents of each well are transferred from the plate to another well and light absorption was quantified by measuring wavelength 570 nm by using The ELISA reader (Multiskan spectrum, 51118650; Thermo scientific, MA, USA) and percentage of cell life was quantified.

Real Time-PCR

First, total RNA of DPSCs was extracted by RNX-plus, and finally, cDNA is synthesized by H Minus First Strand cDNA Synthesis Kit (Takara Perfect Real Time) using 0.2 Random Hexamer and 1 µg total RNA. The technical program includes: an initial temperature and humidity of 95˚ C for 10 min and then 40 cycles with 95˚ C for 10 sec, 60˚ C for 30 sec, and 72˚ C for sec, respectively. RT-PCR is performed to quantify the number of copies of the desired genes. Therefore, for this purpose, 1 µL of each primer, 10 µL of SYBR Green Master Mix (SYBER® Premix EX Taq TM II) and 10 µL of water (dH_2O) are added and 2 µL of cDNA sample is added to the above set. The real-time PCR reaction is performed with the help of real-time PCR system (Applied bio systems life technologies; ABi Step one plus) and for each sample in duplicate with triplicate repetition, and finally, data analysis is performed using Perl Primer software.

Statistical analysis

The results are shown as mean and standard deviation. The Kruskal-Wallis test was used to compare gene expression in the target groups after 20 and 40 min. Statistical ANOVA analysis is used to analyze the data. In this study, *P*-values less than 0.05 were considered statistically significant ($P \leq 0.05$).

Result

Viability assay (MTT)

The effect of extremely low-frequency electromagnetic fields on survival of human DPSCs. The survival in all exposure groups was significantly higher than that in the control except in the group of 40 min, 1 mT (**P*<0.05). In 20 min, 0.5 mT exposure, the survival intensity is significantly more than others (**P*<0.05) (X). In general, the intensity of survival was recorded, 20, 0.5 mT≥20, 1 mT ≥ 40, 0.5 mT ≥ 40, 1 mT respectively. Therefore, according to the obtained results, ELF-EMF increases the survival of cells except for one case (40 min, 1 mT) (Figure 1).

Proliferation effect assessment

In all of groups doubling time was significantly less than control group except for 3 groups such as 40 min, 1 mT, X group, 40 min, 1 mT group and differentiation group T) (*P*<0.05).In other words, ELF-EMF reduces the doubling time of cells. This result showed that in groups (20 min, 0.5 mT, X group, 20 min, 0.5 mT, T group, 20 min, 1 mT, T group, 20 min, 1 mT, X group, 40 min,

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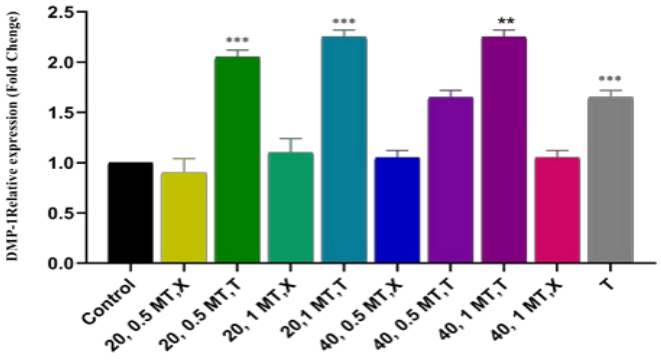

Figure 3. The expression of DP-MSC in different cell group. Result showed significant difference in DMP-relative expression in DP-MSCs (20 MIN, 0.5 mT, T), (20 MIN, 1 mT, T), (40 MIN, 1 mT, T) and DP-MSCs+TGFβ1 with respect to control (X; without differentiating cell groups, T; with differentiating cell groups). **P*<0.05; ***P*<0.01

0.5 mT, X group and 40 min, 0.5 mT, T group) was significant difference compare to control group (*P*<0.01). Significant difference observed in 20 min, 1 mT, T group compared to control group (*P*<0.05). Therefore, based on the obtained results, the ELF-MEF increases cell growth. Means and standard errors of the optical density (OD) of six replicates were calculated. (Percentage of life=OD control/OD sample) (Figure 2).

Detection of mRNA expression by RT-PCR

To comparison of the genes expression of odontoblast cells (DMP1) before and after exposure to 0.5 mm and 1mm Tesla for 20 and 40 minutes per day (frequency 50 Hz, 220 V, 1CM2), Real time PCR technique was used appropriately. Accordingly, the amount of expressed gene in odontoblast cells in irradiated and non-irradiated groups were evaluated successfully. The results of Real-time PCR showed that the expression of exposed cells is higher than the control group. As shown in Figure 3, the highest expression was recorded in 20 min,1 mT (T) (*P*<0.001), 40 minutes 1 mT (T) (*P*<0.01), and 20 min 0.5 mT (T) (*P*<0.001) compared to control group, while the lowest expression was recorded in 20 min, 0.5 mT (X), 20 min 1 mT (X), 40 min, 0.5 mT (X), 40 min 1 mT (X), and control group cells respectively. Therefore, extremely low-frequency electromagnetic fields can effectively affect gene expression in DP-MSC (Figure 3).

Morphology of DPSCs exposed to ELF-EMF

Alizarin red staining was done to show the differentiation potential of dental pulp stem cells. Morphology of Cells exposed to ELF-EMF (20 min-0.5–1 mT) has been exposure were illustrated. As shown in Figure 4, no significant morphological changes were observed in different cell groups.

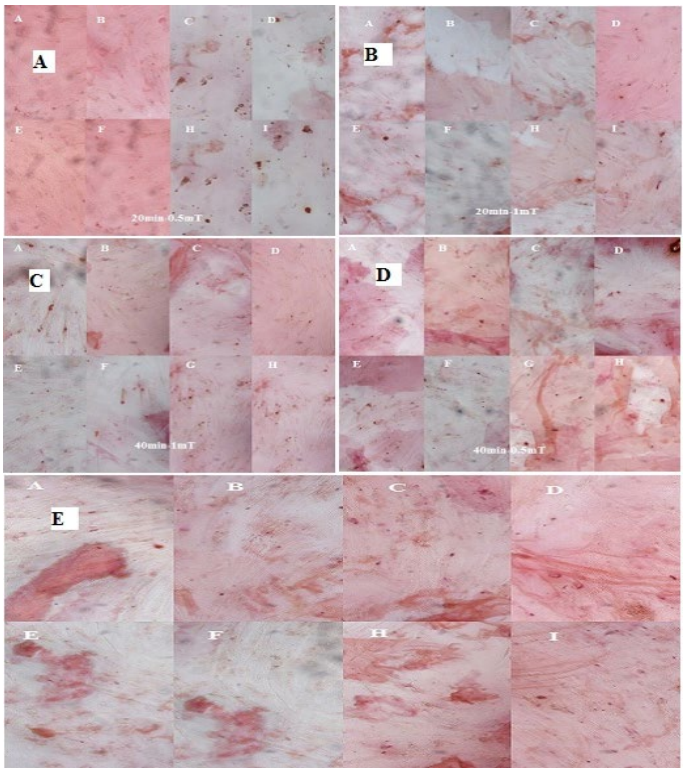

Figure 4. *A*) Morphology of Cells exposed to ELF-EMF (20 minutes, 0.5mT); *B*) Morphology of Cells exposed to ELF-EMF (20 minutes-1 mT); *C*) Morphology of Cells exposed to ELF-EMF (40 minutes 1 mT); *D*) Morphology of Cells exposed to ELF-EMF (40 minutes 0.5 mT); *E*) Morphology of Cells exposed to ELF-EMF (control group)

Discussion

The present study results indicated that short duration exposure to ELF-EMF can significantly improve of dental pulp stem cells (DP-MSC). In this research, two intensities (0.5 and 1 mT) and duration (20 and 40 min) of ELF-EMF were nominated to assess the effect of different intensities and duration of magnetic field on the survival of DP-MSC. For this purpose, MTT assay, doubling time, and real-time PCR were applied appropriately. The survival and PE in exposure groups were more than those in control groups except in 1 mT and 40 min. Findings revealed that ELF-EMF has maximum effect in the dose of 1 mT, 20 min. This research evaluates the effect of duration (20, 40 min) and intensity (0.5, 1 mT) on DP-MSC survival. There are nomajor differences between groups of 0.5 mT with 20 and 40 mins' exposure of ELF-EMF. However, the survival of cells in 1 mT at 40 mins' group was less than that in a group of 20 mins' exposure. Accordingly, by reducing the period of exposure and radiation dose, the survival is improved at 1 mT intensity. Consequently, the survival of DP-MSC has no direct association with exposure doses. As a result, at 40 min of exposure, the cell

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viability of the 0.5 mT group was significantly higher than that of one of the 1 mT groups. Even with 20 minutes of ELFEMF exposure, there was no clear difference in DPMSC survival at 1 mT and 0.5 mT. Cells show similar responses to various intensities of ELFEMF with short exposure times. Subsequently, the expression of DP-MSC cells in all exposure groups was considerably higher than that in control groups except in group of 1 mT, 40 min. Accordingly, it is revealed the 60 Hz, 2 mT sinusoidal EMF treatment stimulated the growth of cortical neuronal and fetal osteoblasts cells at 7 and 14 days incubation [19]. Some research showed EMFs accelerated or induced the osteogenic differentiation of BMSCs directly. For the example, it is revealed that 75 Hz, 1.5 mT EMFs as a bioactive factor improved the osteogenesis of adipose-derived stem cells[20]. Disagreed with our results findings indicated the pulsed electromagnetic field increases the proliferation and differentiation of osteoblast cells and the expression of related genes such as insulin growth factor, alkaline phosphatase, runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN). Intracellular calcium is not present in osteoblasts when irradiated. Pulse electromagnetic field does not affect osteoblast proliferation and differentiation and can increase cell response rate and amplitude of intracellular calcium induced by extracellular calcium stimulation[21]. In agreement with our results, it is investigated 50 Hz sinusoidal EMFs considerably promoted the mineralization potentials and differentiation of osteoblasts in an intensity-dependent manner[22]. Consistent with our results, it is revealed that pulsed electromagnetic field (PEMF) affects osteoblast differentiation and proliferation through improved intracellular calcium transients, which delivered a clue to treat bone-related diseases with PEMF[21]. Finding discovered the effects of freezing on the survival rate of dental pulp stem cells. Similarly our results it is investigated that PEMF irradiation increases the survival rate and generally improves the freezing process, which may be due to the stability of the membrane to resist damage caused by ice crystals during freezing [23]. Research that agreed with our work showed that 15 Hz, 7 mT PEMF had a stimulatory effect on the osteoblasts. This stimulatory effect was most likely associated with improvement of cellular differentiation[24]. The result of the research documented that 15 Hz, 1 mT sinusoidal EMFs directly regulated the differentiation direction of BMSCs, and EMFs promoted osteogenic differentiation even though they reserved adi pogenic differentiation of BMSCs[25]. Similarly, research presented that the EMF treatment might inhibit adipogenic differentiation and promote osteogenic differentiation of BMSCs under the environment of dexamethasone [26]. Consistent with our study it has been found that EMFs of 7.5 Hz, 0.4 T inhibited the adipogenic differentiation of MSCs via JNK-dependent Wnt signaling pathway [27].Our findings were consistent with the results of a study by Shahbazi *et al.*; it was concluded that ELF-EMF increases the survival and proliferation of mesenchymal stem cells with intensities of 0.5 and 1 ms Tesla [28]. Consistent with the present study results, it was found that the proliferation of neuronal stem cells under magnetic field irradiation increased by 1 mT and 50 Hz for 6 to 24 h[29]. Also, in a similar study, a low-frequency electromagnetic field with an intensity of 3 to 3.6 milliseconds for 30 minutes increased the proliferation of stem cells, consistent with our study's results [30]. However, these findings are inconsistent with the results reported by Yan *et al.*, who stated that low-frequency electromagnetic field radiation inhibits the growth of mesenchymal stem cells and has a negative effect on cell growth and proliferation [31, 32]. The results obtained in this study are almost in agreement with previous studies, although more comprehensive conclusions require extensive research. Additional studies were proposed to evaluate the effect of PEMF with further intensities and duration on these cells.

Conclusion

Therefore, according to the obtained results, ELF-EMF increases the survival of cells except for one case (40 min, 1 mT). However, the mechanisms involved in this process are still mysterious and require further research in the future.

Conflict of Interest: 'None declared'.

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