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Original Article

SINUSOIDAL ELECTROMAGNETIC FIELD DECREASES OSTEOGENIC DIFFERENTIATION OF RAT BONE MARROW MESENCHYMAL STEM CELLS

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ABSTRACT

Objective: The widespread use of household electrical appliances generating electric and magnetic fields was a significant focus of WHO attention because of its serious threat to human health, especially osteogenesis. This research investigated the effect of 50 Hz frequency (1 mT intensity) sinusoidal EMF (SEMF) on the osteogenic differentiation of rat bone marrow stem cells (rBMSCs) *in vitro*.

Methods: Experimental groups were: positive control (cells cultured in osteogenic medium supplemented with 7-10 M Dexamethasone, negative control (cells cultured in α-MEM/10% FBS, 10 mmol Beta-Glycerol-Phosphate, 15% FBS, 50 ug/ml Ascorbic Acid bi-Phosphate, 100 unit/ml Penicillin) and for the EMF group, cells exposed to SEMF (50 Hz, 1 mT, 30 min/day) for 14 and 21 d. Alizarin red staining, Alkaline phosphatase activity, and QRT-PCR were performed.

Results: The EMF group exhibited weaker positive stains for ALP and Alizarin red than the positive control group. The *Runx2* and *Ocn* gene expression levels were significantly decreased compared to negative control at 14 and 21 d of EMF exposure, respectively. After 14 and 21 d of exposure, *Runx2* and *Ocn* gene expression were much lower in the EMF group than in the positive control group.

Conclusion: SEMF (1 mT, 50 Hz, 30 min/day) could retarded osteogenesis and reduce the osteogenic differentiation of rBMSCs.

Keywords: Sinusoidal electromagnetic field, Bone marrow stem cells, *Runx2* and *Ocn* genes

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INTRODUCTION

The spectrum of electromagnetic waves with extensive frequency range includes extremely low frequency (ELF), radio frequency, ultraviolet (U. V.), infrared, gamma, and X-rays. Bone marrow mesenchymal stem cells (BMSCs), known as bone marrow stromal cells (BMSCs) [1], increase rapidly *in vitro*, migrate, and differentiate into different tissues [2]. These cells can differentiate into the osteoblast lineage *in vitro* [3, 4]. Horwitz *et al.* showed that allogeneic bone marrow transplantation in kids increases bone mineral density with osteogenesis imperfect [2]. They are also a promising and abundant source for treating inherited diseases and repairing tissues such as cartilage, bone, and myocardium. In animal models, bone marrow cells were transplanted to skeletal and cardiac muscle, vascular endothelium, liver, lung, intestine, nerve tissue, and skin epithelium [5]. The function and Differentiation of MSCs depend on environmental factors and biophysical stimuli [6, 7]. As a biophysical agent, the pulsed electromagnetic field (PEMF) released Ca^{2+}ions from the smooth endoplasmic reticulum [6]. Calcium entry and membrane depolarization lead to the expression of Ca^{2+}-binding proteins, including calmodulin, in MSCs [8]. The rise in cytosolic Ca2+content initiates the wnt/β-catenin signaling pathway, which is essential in osteogenesis and stimulates bone-related genes such as *Runx2* and *Ocn*, this improved MSC osteogenic differentiation [6, 8]. Bassett *et al.* (1982) devised a magnetic field across the broken site with Helmholtz coils to induce osteogenesis [9]. They suggested that applied external electrical energy could change the behavior of bone cells [10]. Gap junction intercellular communication was inhibited in Chinese lung cells and mouse fibroblasts by 24 h exposure to 50Hz EMF at 0.2-0.8 mT [11, 12].

It could be affected by EMF on plasma membrane gap junction proteins. During the proliferation phase of osteoblasts, ELF inhibited gap junction communication. However, ELF was relatively effective in preventing gap junction communication during the differentiation phase. ELF may only affect pre-osteoblasts or osteoblasts that are not fully developed [13].

In summary, the results of studies showed that EMF with different intensities and frequencies positively affects proliferation without affecting or reducing proliferation and increasing differentiation [14]. Following PEMF treatment, osteoblast proliferation, differentiation, and bone tissue-like formation increased [10]. Aaron *et al.* (1996) indicated that PEMF stimulates MSC differentiation, increasing bone maturation in the extracellular matrix [15]. Jansen *et al.* (2010) observed a high level of some signs of bone growth, like BMP2 (3-5 fold), osteoprotegerin (1-7 fold), and osteocalcin (2 fold) in BMSC post-induced by EMF [16]. However, different studies disagree on how PEMF stimulates the development of osteoblasts and the growth of cell lines *in vitro* [17], which depends on the waveform, frequency, intensity, duration of exposure, type, and cell age [16, 18]. Conflicting reports have been reported, making it difficult to draw any clear conclusion.

Because of the widespread use of electromagnetic devices nowadays, some gaps in knowledge about the bio-environmental effects of EMF have motivated many studies. Although different species are exposed to SEMF, there is no consensus about the effects of EMF on osteogenesis.

This study aimed to determine the effect of SEMF with an intensity of 1 mT and frequency of 50 Hz on the osteogenic differentiation of rat BMSCs.

MATERIALS AND METHODS

Magnetic field generator

This study investigated the effect of a magnetic field with an intensity of 1 mT and a frequency of 50 Hz on cell samples. The magnetic field must be the same over the entire sample space, and the magnetic flux lines must stay parallel. The device was designed according to a recent study [19].

rBMSCs isolation and culture

Femur and tibia bones were collected under sterile conditions from Wistar rats (6-8 w old) and situated in a culture dish that had been

sterilized and was under a laminar hood. Research ethics at Damghan University was approved for all tests (IR. DU. REC.1400.014), and this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. (NIH Publication No. 23-80, revised 1996). Bone marrow was flushed into a Falcon tube and centrifuged at 1200 rpm for 5 min. After removing the supernatant, the cell pellets were grown in α-MEM (α-minimal essential medium; Gibco/BRL; cat. No. 52100-0.3) with 10% FBS (Gibco, cat. No. 12203C) added and left to grow. Every three days, the medium was changed. The cells were passaged at 70% to 80% confluency [20]. The stem cells were previously confirmed by Ghorbanian *et al.* (2012) [21, 22].

Experimental groups

rBMSCs at passage four were examined in three groups: positive control (cultured cells in osteogenic medium containing 7-10 M Dexamethasone, negative control (cultured cells in α-MEM containing 10% FBS), 50 ug/ml Ascorbic Acid bi-Phosphate, 10 mmol Beta-Glycerol-Phosphate, 15% FBS, 100 unit/ml Penicillin) and EMF (cells exposed to SEMF at a frequency of 50 Hz, intensity 1 mT, half an hour daily [23, 24].

Alizarin red staining

On day 14, the cells were stained with Alizarin Red (069K1639, Sigma-Aldrich). After removing the medium, the cells were washed twice with cold PBS. Each sample was set in 4% paraformaldehyde (Merck, Germany) that was kept out for 10 min at 4 °C. Following removing the fixative, the cells underwent two washes with PBS and were subsequently stained with 400 µl of Alizarin Red solution at a pH of 7.2 for 30 min. Later, the cells underwent two rounds of PBS rinsing and were subsequently examined under a microscope [25].

Alkaline phosphatase activity

On day 14 after induction, the cells' total protein was lysed using 30 µl of Triton X-100 lysis solution to assess the activity of alkaline phosphatase (ALP). While the solution was spinning at 2000 rpm and 4 °C for 10 min, the ALP activity of the supernatant was measured at 405 nm with a microplate reader from BioTek Instruments (USA). The phosphatase substrate used was para nitrophenyl phosphate (pNPP). The enzyme activity (I. U.) level was then compared to the amount of protein in the sample [26].

Real-time PCR

The qPCR technique evaluated genes and *Runx2* in treated rBMSCs [27]. According to the manufacturer's instructions, The RNX-plus solution (Sinaclon, Iran) was used to extract RNA. The purity of the extracted RNA was examined using a spectrophotometer (Eppendorf Biop Hotometer). Samples with 260/230 ≥ 1.4 adsorbents and 260/280 ≥ 1.8 were analyzed. PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan) synthesized cDNA. Rotor-Gene 6000 PCR machine with RealQ Plus Master Mix Green (Amplicon, Denmark) was used for real-time PCR. Primer sequences (table 1) were made with AlleleID software version 7.5 (Premierbiosoft, USA). In this study, the housekeeping gene *Tbp* was used as internal control, and the expression level of each gene was compared with *Tbp.* The relative expression of genes in the treatment groups compared to the control group was calculated using-$2^{\Delta\Delta ct}$, and the reaction solution volume was tenµl in the end. Finally, the RT-qPCR reaction with the amplicon amplification program includes initial denaturation: 95 °C, 15 min, denaturation: 95 °C, 15 seconds, annealing and extension: 60 °C, 45 seconds, melt: 65-94 °C was performed [28].

Statistical analysis

SPSS software version 16 was used for statistical analysis. A one-way ANOVA test and a Tukey post-hoc test were used to see if there were significant differences between the test groups. The Independent-sample T-test was used to assess the significance of treated cells on days 14 and 21. $P<0.05$ was considered significant, and each experiment was conducted thrice.

RESULTS

Morphologies of cultured rat bone marrow-derived stem cells

Phase contrast images of rBMSCs are shown in fig. 1. The primary cultured rBMSCs were triangular or polyhedral in shape (A) and then exhibited spindle-shaped morphology. As the passage number increased, the blood cell density decreased, and the rBMSCs gradually changed into flat morphology (B).

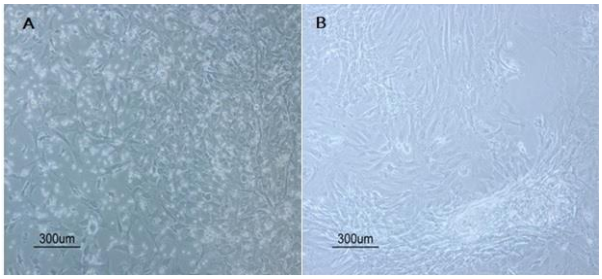

Fig. 1: Phase contrast images of rBMSCs at P0 (A) and P3 (B) were shown (×100). The cells appeared flat morphology, and the blood cells were removed with increasing passage number

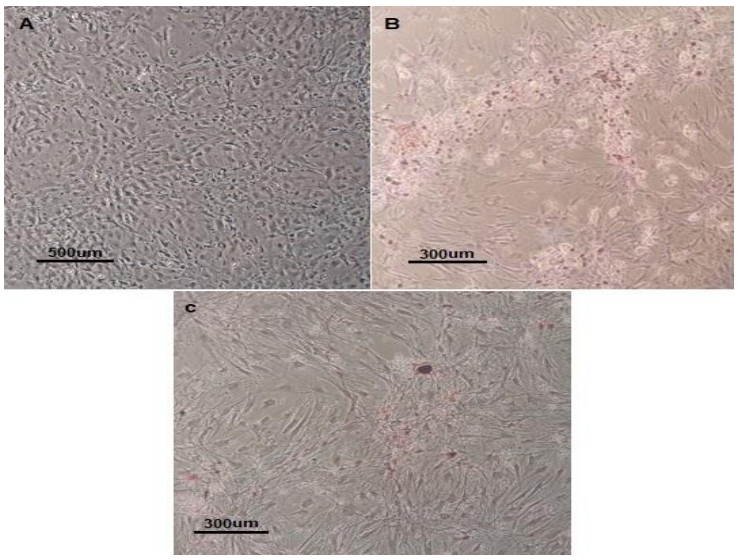

Fig. 2: Alizarin red staining after 14 d of treatment in experimental groups. A: Undifferentiated cells of the negative control group; B: Positive control group; C: EMF group

Alizarin red staining

Alizarin red staining was used to see how well rBMSCs turned into osteoblasts after 14 d of treatment. According to fig. 2, undifferentiated cells of the negative control group were observed (A). The positively stained regions were more in the positive control (B) than the EMF group (C).

Alkaline phosphatase activity

The results shown in fig. 3 (A-C) indicate higher mineralization in the positive control than in the EMF group.

Alkaline phosphatase activity in experimental groups was evaluated after 14 d of treatment. It can be seen in fig. 4 that the positive control group had more ALP activity than the negative control group ($P<0.05$). Not so with the EMF group; compared to the positive control, they showed a significant decrease ($P<0.05$).

Expression of osteogenesis-related genes, including *Runx2* and *Ocn*

After 14 d of treatment, the *Runx2* gene expression was analyzed using real-time PCR (fig. 5). The gene expression was significantly decreased in the EMF group compared with the negative and positive controls ($P<0.05$).

After 21 d of treatment, the positive control group showed a significant increase in gene expression compared to the negative control, and the EMF group showed a significant decrease in that compared to the positive control ($P<0.05$).

Finally, the expression levels of the *Runx2* gene after 14 d were compared with those after 21 d. The *Runx2* mRNA level of positive control on day 21 significantly increased compared to the same group on day 14 ($P<0.05$).

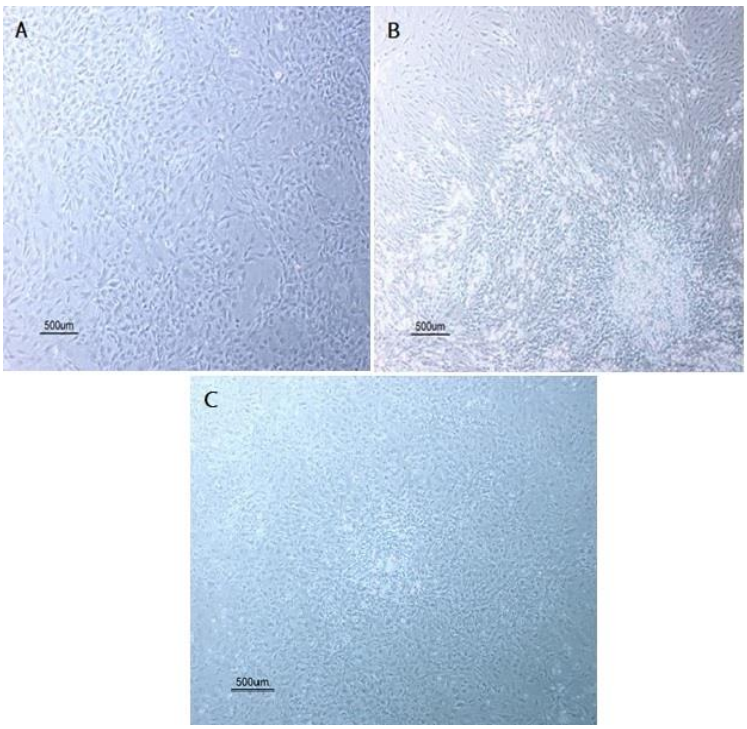

Fig. 3: Mineralization activity of experimental groups. A: Negative control (×400); B: Positive control (×400); C: EMF group (×400). The small calcified particles appeared as white dots

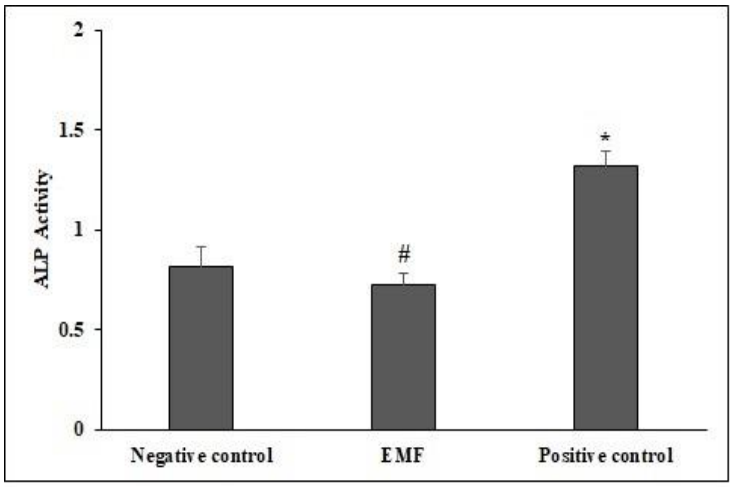

Fig. 4: Alkaline phosphatase activity in negative control, EMF, and positive control groups after 14 d of treatment. *significant increase versus negative control, # significant decrease versus positive control. Values are mean±SD n=3

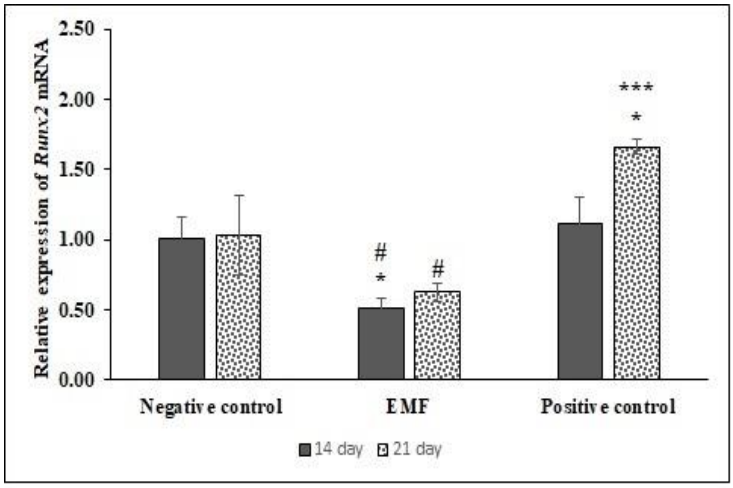

Fig. 5: Relative expression of *Runx2* mRNA levels in experimental groups. *P<0.05 versus negative control and #significant decrease versus positive control at the same time. ***significant difference compared to the same group on day 14. Values are mean±SD n= 3

According to fig. 6, after 14 d of treatment, *Ocn* gene expression compared to the negative control group increased significantly in the positive control group. It decreased significantly in the EMF group compared to the positive control (P<0.05).

After 21 d of treatment, the positive control group showed a significant increase in *Ocn* gene expression compared to the negative control, and the EMF group showed a significant decrease in that compared to the negative and positive controls (P<0.05).

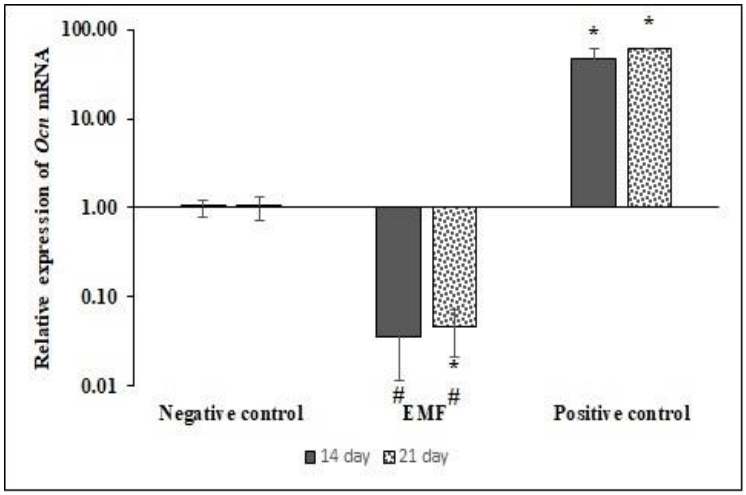

Fig. 6: Relative expression of *Ocn* mRNA levels in experimental groups. *P<0.05 versus negative control and #significant decrease versus positive control at the same time. Values are mean±SD n= 3; finally, we examined how much the Ocn gene was expressed after 14 and 21 d. There wasn't a big difference between the groups in the amount of Ocn mRNA on day 21 compared to day 14 (P>0.05)

DISCUSSION

Devices generating extremely low-frequency fields have found wide applications in daily life. This highlights the need for further research to investigate critical mechanisms involved in osteogenesis. The findings suggest suitable solutions to deal with the destructive effects of EMF on bone formation. Osteogenesis is a complex set of events involving the differentiation of MSCs for new bone production [17, 29]. Lim *et al.* (2013) showed that extremely low frequency-PEMFs (ELF-PEMFs) at 50 and 100 Hz increased osteogenesis in human alveolar bone mesenchymal stem cells [29]. Also, Zhong *et al.* (2012) reported that EMF with a frequency of 50 Hz and an intensity of 0.5 mT accelerates cell proliferation and increases cell differentiation [30]. However, there have been adverse reports of the effects of electrical stimuli on cell differentiation proliferation and bone formation *in vitro* [9].

At the proliferation phase, the proliferation of rBMSCs was enhanced, while their differentiation was inhibited under exposure to EMF. But at the differentiation phase, it was vice versa [31].

This study investigated the effect of a sinusoidal electromagnetic field on extracellular matrix mineralization, alkaline phosphatase activity, and expression of *Runx2* and *Ocn* osteogenic genes in rBMSCs.

The regions that responded positively to Alizarin red staining were lower in the EMF group than in the positive control group. These results contradict the results of Yang *et al.* (2010), who showed that SEMF with a frequency of 15 Hz and an intensity of 1 mT increased the formation of mineral nodules in rBMSCs [32]. Chang *et al.* (2004) showed that PEMF with a frequency of 15 Hz and an intensity of 0.1 mT for 14 d did not affect the mineral nodes of rat calvarial osteoblasts [9]. The effects of EMF on the proliferation and the Differentiation of MSCs are very different and sometimes inconsistent [14]. These effects depend on the waveform, frequency, intensity, exposure duration, type, and age of the cell [16, 18]. Our results showed alkaline phosphatase activity in the EMF group was significantly reduced compared to the positive control. This is similar to the results of Yang *et al.* (2008), who reported that PEMF with a frequency of 48 Hz and an intensity of 1.5 mT for 24 h

significantly reduced alkaline phosphatase activity [33]. Schwarts *et al.* (2007) showed that PEMF at 15 Hz for 24 d did not affect ALP activity in BMSCs [34]. In contrast, Zhou *et al.* (2014) showed that SEMF with a frequency of 50 Hz, intensities of 1.8 and 3.6 mT, 30 min/day for 15 d increases matrix mineralization and alkaline phosphatase activity in rat osteoblasts [18]. The contrasting effects may be due to the use of different intensities and types of cells.

Runx2 expression level dropped considerably in the EMF group after 14 d of treatment versus the negative and positive controls and after 21 d of treatment compared to the positive control. This is pretty close to the outcomes of Tsai *et al.* (2009), who reported that PEMF with a frequency of 7.5 Hz, intensity of 0.13 mT, 2 h a day for 10 d leads to a significant reduction of *Runx2* in MSCs [35]. In contrast, Yang *et al.* (2010) reported that SEMF with a frequency of 15 Hz and an intensity of 1 mT increased the expression of the *Runx2* gene in rBMSCs [32]. The contrast between our results and the results of Yang *et al.* is probably due to the use of different frequencies.

Ocn gene expression reduced dramatically in the EMF group after 14 d of treatment compared to the positive control following 21 d of treatment compared to the negative and positive controls. These results are consistent with the results of Junsen *et al.* (2010), who reported that exposure to BMSCs at a frequency of 15 Hz, the intensity of 0.1 mT for 14 d, reduces the expression of *Runx2* and *Ocn* genes [15] and Liu *et al.* (2014) stated decreased *Runx2* and *Ocn* genes expression in BMSCs exposed to EMF with a frequency of 50 Hz and an intensity of 1 mT for 2 h/day for up to 14 d [36].

CONCLUSION

However, we could not identify the significance of the disparity between our result and the other findings. The discrepancies in results represent unidentified variations in laboratory techniques and materials. This study used sinusoidal EMF with a frequency of 50 Hz, intensity of 1 mT, and half an hour daily as a stimulus for osteogenic differentiation. SEMF with the mentioned features reduced the osteogenic differentiation of rBMSCs.

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Nil

AUTHORS CONTRIBUTIONS

All authors contributed equally to this work regarding the scope of the subject, the literature review and analysis, drafting, revision, editing, and final approval of the manuscript.

CONFLICT OF INTERESTS

The authors have no conflicts of interest.

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