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# Enhanced growth and osteogenic differentiation of Induced Pluripotent Stem cells by Extremely Low-Frequency Electromagnetic Field

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**Abstract**

It is accepted that induced pluripotent stem cells (iPSCs) have a great osteogenic potential differentiation, in the present study, we tried to improve this potentials using mechanical and biological stimulation. To achieve this goal, the influence of prolonged pulsed extremely low frequency electromagnetic field (ELF-EMF) (50 Hz and 1.5 mT) was investigated on cultured iPSCs. After evaluation of iPSCs biological behavior under radiation using MTT assay, osteogenic differentiation of stem cells was investigated via common important osteogenic markers such as alkaline phosphatase (ALP) activity, calcium mineral deposition and important bone-related genes. MTT result showed that proliferation rate of iPSCs significantly increased followed by stimulate with ELF-EMF. Osteogenic differentiation characterization demonstrated that potential of stem cells also was significantly increased while these cells cultured under both ELF-EMF and osteogenic medium (OM) in comparison to cultured under ELF-EMF or OM alone. According to the results, concluded that combination of OM and ELF-EMF can be a great supplement for bone differentiation of stem cells and appropriate candidate for use in the treatment of bone defects and osteoporosis patients by accelerating healing process.

***Key words:*** Induced pluripotent stem cell, extremely low ferequency, electromagnetic field, osteoporosis.

## Introduction

Bone defect is one of the major unsolved therapeutic problems in the patients with orthopaedic challenges and osteoporosis. Although, the use of autologous and allogenic bone grafts for patients with bone lesion is reported widely in regenerative approach, their use is limited by restricted availability and significant donor-site morbidity (1, 2). Due to these challenges, tissue engineering has gained much interest from scientists and orthopedic surgeons in 4 last decades (3-5). In this approach, one of the most important tissue engineering components is stem cells and can be isolated from different sources such as inner cell mass or adult tissues, for examples bone marrow, adipose or cord blood, and propagated to reach sufficient population. In 2006, new procedure was introduced to stem cell preparation for the first time via the transduction of murine and human fibroblasts with only four transcription factors including Oct4, Sox2, c-Myc, and Klf4 which generated stem cells named induced pluripotent stem cells (iPSCs) (6, 7). These cells were very similar to embryonic stem cells (ESCs) in characteristics such as morphology, proliferation, gene expression, epigenetic status of pluripotent cell-specific genes and telomerase activity (8). Spectrum of electromagnetic fields (EMF) with frequencies below 300 Hz is called extremely low frequency EMF (ELF-EMF), these frequencies is lower than intermediate frequencies. Over the last four decades, pulsed EMF has been reported that could be applied to promote bone healing. This effectiveness was initially started by Friedenberg and Brighton due to the weak electric current produced by the magnetic field effect on accelerated formation of the bone matrix (9). Several studies demonstrated that likely candidates of Pulsed EMF in bone fractures are the osteoblasts, or their precursors, the mesenchymal stem cells (MSCs) (10, 11). In addition, several studies reported that Pulsed EMF can increase the osteoblast proliferation, differentiation and extracellular matrix (ECM) calcification in *in-vitro and in-vivo* (12-14). One of the most stem cells that their osteogenic differentiation investigated under Pulsed EMF is MSCs, for example Aaron et al. in a study reported that increase in extracellular matrix synthesis and bone maturation is due to Pulsed EMF-enhanced differentiation of MSCs (15). In two another study were showed that pulsed EMF had enhancing role in osteogenesis and proliferation rate of MSCs (16). Furthermore, many studies reported positive effects of Pulsed EMF on the mineralization of osteoblast-like cells on scaffold and tissue culture polystyrene (TCPS) (17-19). As we know that to produce a biological response, electromagnetic signal should be translate into an intelligible biochemical signal. For this reason, some researchers are still challenged by the positive effects of Pulsed EMF on transcriptional level, cell proliferation and differentiation have been reported in osteoblasts (11, 20, 21). In the present study, iPSCs were cultured under radiation pulsed ELF-EMF (50 Hz and 1.5 mT) conditions and after evaluation of their proliferation rate using MTT assay, osteogenic differentiation capacity of stem cells was investigated via valuable osteogenic markers such as alkaline phosphatase (ALP) activity, calcium content assay and osteogenic genes expression.

## Materials and methods

### *Cell culture*

Human iPSC lines were gift from Stem Cells Technology Research Center Cell Bank (Tehran, Iran). These

cells were maintained on mitomycin-C (Invitrogen Co. USA) inactivated mouse embryonic fibroblast (MEF) feeder layers in culture plates (Orange Science, Belgium). In brief, iPSCs cultured in DMEM/F12 medium supplemented with 15% knockout serum replacement (KSR) (Invitrogen Co. USA), 0.1 mmol/L non-essential amino acids, 1 mmol/L L-glutamine (all from Invitrogen), 0.1 mmol/L b-mercaptoethanol (Sigma), penicillin/streptomycin (sigma) and 4 ng/ml of human fibroblast growth factor 2 (invitrogen), and about 60% of the medium was replaced every days. For embryoid body (EB) Formation, iPS colonies were deatached with 0.1% Collagenase IV, then transferred onto non-treated six well plates (Jet Biofil, Japan) for 3-5 days in EB medium consisting of human iPS medium without human fibroblast growth factor 2.

***Pulsed extremely low frequency electromagnetic field***

Pulsed extremely low frequency electromagnetic fields (ELF-EMF) generator was placed in a $CO_2$ incubator (5% CO2, 37 8C). After 5 days from EB formation, 50 EBs per $cm^2$ were seeded in six well culture plates (Orange Science, Belgium) using DMEM media supplemented with 10% FBS and after 24 h basal medium was removed and osteogenic induction medium was added. This medium consisted of basal medium supplemented with 3mM β-glycerophosphate (βGP), 50 µg/ml ascorbicacid (AA) and $10^{-9}$ M Dexamethasone. The medium was replaced every two days. Plates were placed in the pulsed ELF-EMF devise and were exposed to pulsed ELF-EMF 8 h per day (a repetition rate of sinusoidal 50 Hz, 1.5mT, pulse-width 350 ns and on and off time of ELF generator was 08:00 and 04:00 respectively) during the period of study.

***MTT assay***

The proliferation rate of iPSCs was evaluated under pulsed ELF-EMF via MTT assay. IPSCs were seeded with an initial cell density of $4 \times 10^3$ cells per $cm^2$ in a 24-well culture plate and incubated at 37°C, 5% $CO_2$. After 1, 2, 3, 4 and 5 days of cell seeding, 50 µl of MTT solution (5 mg/ml in DMEM) was added to each well (n = 3). For conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells, the plate was incubated at 37°C for 3.5 h. For dissolution of the dark-blue intracellular formazan, the supernatant was removed and constant amount of Dimethyl sulfoxide (DMSO) solvent was added. The optical density was read at a wavelength of 570 nm in a micro-plate reader (BioTek Instruments, USA).

***Alizarin red S staining***

Alizarin red staining was carried out to evaluate the mineralized matrix of differentiated stem cells on day 21. The medium was removed, and the cells were washed with PBS and then fixed in cold 4% paraformaldehyde for 20 minutes at 4˚C. Differentiated and control cells were then washed with PBS. The fixed samples were stained with 2% Alizarin red S at pH 7.2 (Sigma). After 10 minutes at room temperature, the cells were washed again with PBS three times and depicted by the inverted light microscope.

***Alkaline Phosphates Activity***

Alkaline phosphatase (ALP) activity was investigated via total protein extraction of stem cells at days 7 and 14 after osteogenic induction using 200 µLof RIPA lysis buffer. The lysate was centrifuged at 15000 RPM at 4 °C for 15 min for sedimentation of cell debris. Then, supernatant was collected and ALP activity was measured using *p*-nitrophenyl phosphate (pNPP) as a phosphatase substrate (ALP Kit, Pars Azmoon Iran). The enzyme activity level (IU) was normalized against the total protein.

***Calcium content assay***

For evaluation of the calcium deposited amount by differentiated stem cells Cresolphthalein Complex one method was used during the period of study. In order to extract the calcium content, the cells were homogenized in 0.6 N HCL (Merck) followed by shaking for 4 h at 4°C. After the addition of reagent (Calcium Content Kit, Pars Azmoon Iran) to calcium solutions, the optical density of samples was measured at 570 nm in a microplate reader (BioTek Instruments, USA). The values of calcium content of samples were obtained from the standard curve of OD versus a serial dilution of calcium concentrations.

**Table 1.** Primers used in real-time RT-PCR

| Gene | Primer sequence(F,R, 5´-> 3´) | Product length (bp) |
|---|---|---|
| **Beta Actin** | GTCCTCTCCCAAGTCCACGGG<br>AGACCAAAAGCCTTCAT | 198 |
| **Runx2** | GCCTTCAAGGTGGTAGCCC<br>CGTTACCCGCCATGACAGTA | 67 |
| **Osteocalcin** | GCAAAGGTGCAGCCTTTGTG<br>GGCTCCCAGCCATTGATACAG | 80 |
| **Osteonectin** | AGGTATCTGTGGGAGCTAATC<br>ATTGCTGCACACCTTCTC | 121 |
| **Collagen type 1 (Col1)** | TGG AGCAAGAGGCGAGAG<br>CACCAGCATCACCCTTAGC | 121 |
| **ALP** | CCTAAAAGGGCAGAAGAAGGAC<br>TCCACCTAGGATCACATCAATG | 444 |
| **osteoprotegerin** | AAGATCATCCAAGATATTGACC<br>TGCTCCCACTTTCTTTCC | 123 |
| **Matrix metaloproteinase 1 (MMP1)** | GTCTCTGAGGGTCAAGCAG<br>CACGATGTAAGTTGTACTCTCTG | 190 |
| **Matrix metaloproteinase 3 (MMP3)** | GTTTTCTCCTGCCTGTGC<br>GACAAGGTGCAAGCTAAGC | 129 |

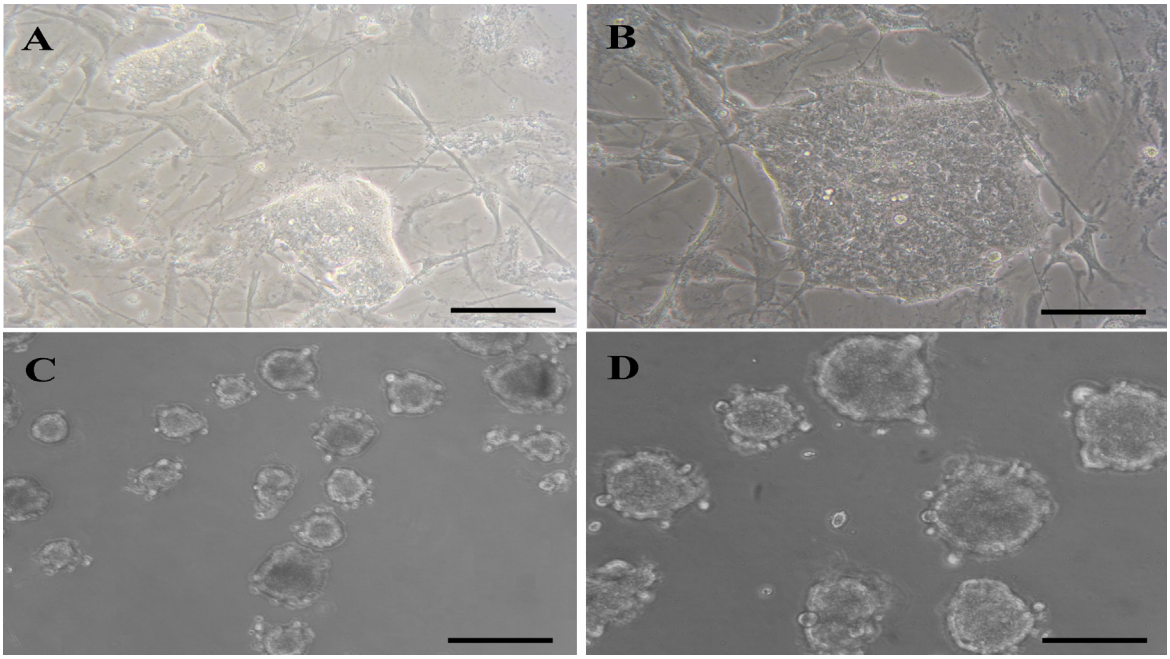


**Figure 1.** Optical micrographs of iPS colonies (a and b) and EBs after 5 days (C and d), with two magnifications (10 × and 40 ×).

### *Real-Time RT-PCR*

Relative Quantification of Collagen type 1 (Col1), Runt-related transcription factor 2 (Runx2), Osteocalcin (OC), Osteonectin (OCN), ALP, osteoprotegerin (OSP), Matrix metaloproteinase 1 (MMP1) and Matrix metaloproteinase 3 (MMP3) genes expression were carried out on differentiated cells compare to controls at 7 and 14 days during the period of study. Total RNA was extracted using Qiazol reagent (Qiagen) according to manufacturer's protocol. The first-strand cDNA was synthesized using random hexamers (Vivantis, USA) in a total reaction volume of 25 µl using M-MLV Reverse Transcriptase (Vivantis Cat No: RTPL12). The PCR parameters included denaturation at 95°C for 3 min, then 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. For PCR amplification and real- time RT-PCR, primer sequences are illustrated in Table 1. Real-time RT-PCR was performed using Takara SYBR Premix Ex Taq Master. The relative quantification model was applied to calculate the expression of the target gene in comparison to β-actin used as an endogenous control. Gene expression levels were quantified by Rotor Gene 6000 (Corbett, Concorde, NSW, and Australia).

## Results

### *Stem cell culture*

Human iPSCs were seeded on mitomycin-C inactivated MEF cells and microscopic photograph of iPSCs colonies was shown in Fig 1(A and B). EB formation was carried out after deatache of iPS colonies and then was transferred into non-treated six well plates and depicted during 5 days (Fig 1C and D). Result of MTT assay showed that on day 3 only proliferation rate of iPSCs treated with both osteogenic medium and ELF-EMF increased significantly in comparison to control (Fig. 2) and on days 5 and 7 no significant change observed between cells treated with osteogenic medium and ELF-EMF. However, highest proliferation rate of iPSCs detected in cells cultured under combination of osteogenic medium and ELF-EMF during the period of study.

### *Assessment of osteogenic markers*

*Alizarin Red staining, ALP activity and calcium content*

IPSCs morphology was depicted after 14 days culture under osteogenic induction medium (Fig 3A),

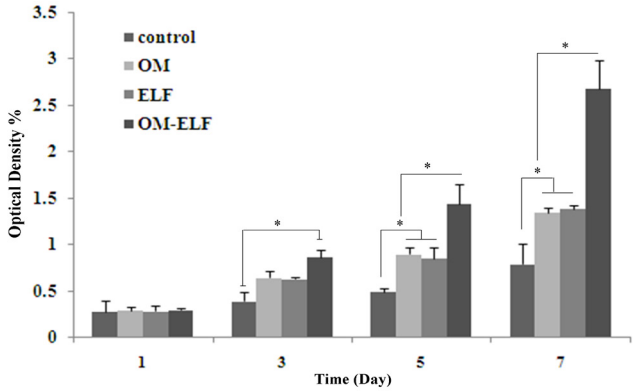


**Figure 2.** Proliferation rate of iPSCs cultured under osteogenic medium (OM), ELF-EMF and both OM and EMF-ELF during 1, 3, 5 and 7 days, the significant difference ($P < 0.05$) has been shown between the groups indicated by asterisks.

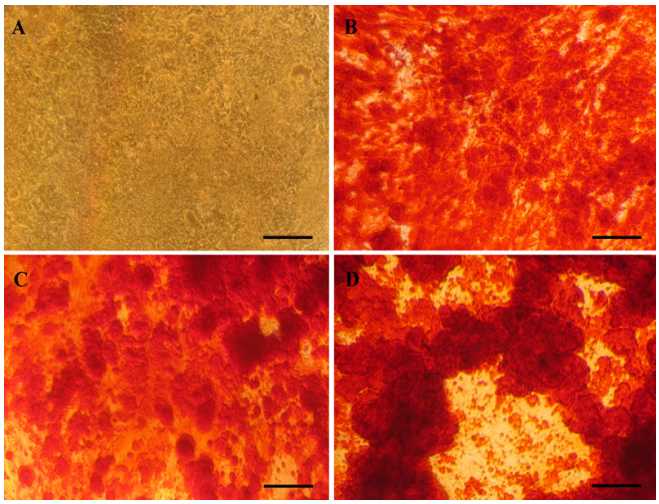


**Figure 3.** Optical micrographs of osteogenic differentiation of iPSCs at day 14 (a), Alizarin Red S staining of iPSCs after 14 days culture under osteogenic medium (OM) (b), ELF-EMF (c) and both OM and ELF-EMF (d), with 40 × magnification.

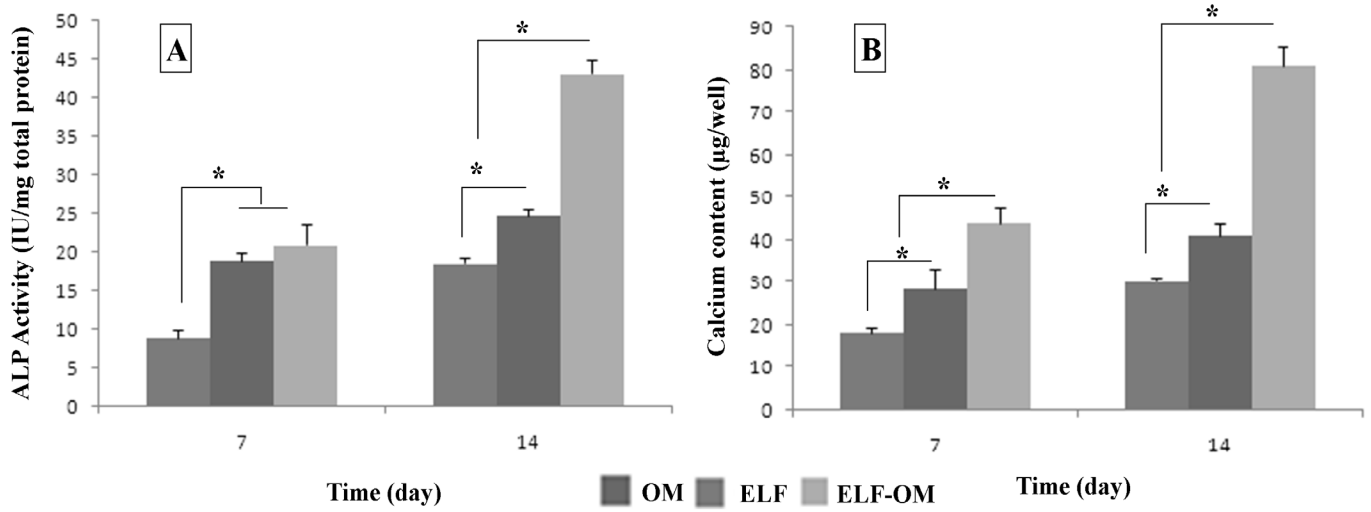


**Figure 4.** ALP activity (a) and calcium content (b) of iPSCs cultured under osteogenic medium (OM), ELF-EMF and both OM and EMF-ELF during 7 and 14 days, the significant difference ($P < 0.05$) has been shown between the groups indicated by asterisks.

the biomineralization and secreted extracellular matrix were visualized on TCPS using Alizarin Red staining on day 14. As showed in Fig 3B, iPSCs cultured simultaneously under osteogenic medium (OM) and exposed ELF (Fig 3D) obviously secreted more extracellular matrix and biomineralization than either alone qualitatively (Fig 3B and C). The pattern of ALP activity during osteogenic differentiation of iPSCs cultured under OM, ELF and OM-ELF was similar but exhibited significant different values ($P < 0.05$). Significantly increase in ALP activity was detected on day 7 in iPSCs cultured under ELF and OM-ELF compared to OM and also highest ALP activity observed in iPSCs cultured under OM-ELF on day 14 (Fig. 4A). Mineralization is one of the most important late markers of osteogenic differentiation which was also investigated for iPSCs during the period of study. As showed in Fig. 4B, the same increasing trend of calcium deposition was observed in all groups during the period of study ($P < 0.05$). In addition, iPSCs cultured under OM-ELF demonstrated significantly highest amount of induced mineral precipitation in comparison to that cultured under OM and ELF on days 7 and 14.

*Osteogenic-related Gene's expression analysis*

The relative expression of eight important osteogenic-related genes was investigated in iPSCs cultured under OM, ELF and OM-ELF (Fig 5). An increasing trend of all gene expression observed during the osteogenic differentiation process. The expression level of Col-1 mRNA in iPSCs treated with ELF-EMF and OM-ELF-EMF significantly up-regulated in comparison to cell treated with OM on day 7, while highest it expression was detected in cells treated with OM-ELF-EMF on day 14. Runx2 expression was showed decreasing trend and this down-regulation more seen in cell cultured under OM-ELF-EMF during the period of study. The expression of osteonectin on day 7 none significantly changed between different groups. But higher expression of this gene observed on cells treated with ELF-EMF in comparison to cells treated with OM and OM-ELF-EMF, although iPSCs cultured under OM-ELF-EMF showed more expression compared with OM. Osteocalcin showed similar trend to col-1 and highest expression of it observed in cells cultured under OM-ELF-EMF. More significantly expression of ALP detected on cells cultured under OM-ELF-EMF on day 14, while on day 7 ALP expression in cells treated with ELF-EMF and OM-ELF-EMF was similar. None significant osteoprotegerin (OSP) expression observed on day 7 between groups, but it expression on day 14 significantly more

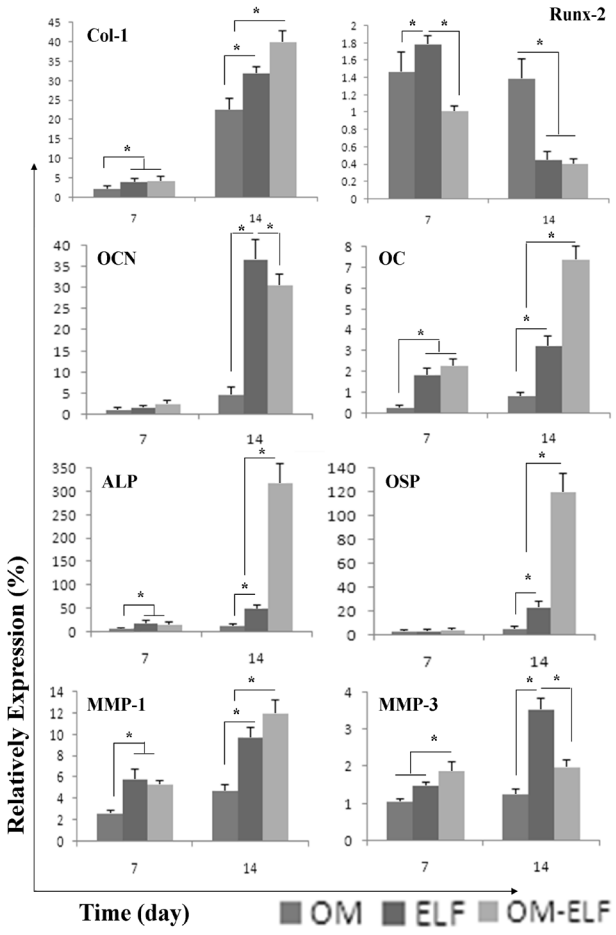


**Figure 5.** Relative expression of Collagen type 1 (Col1), Runt-related transcription factor 2 (Runx2), Osteocalcin (OC), Osteonectin (OCN), ALP, osteoprotegerin (OSP), Matrix metaloproteinase 1 (MMP1) and Matrix metaloproteinase 3 (MMP3) on days 7 and 14 in iPSCs cultured under osteogenic medium (OM), ELF-EMF and both OM and EMF-ELF during 7 and 14 days during osteogenic process, the significant difference ($P < 0.05$) has been shown between the groups indicated by asterisks.

up-regulated in cells treated with OM-ELF-EMF compared with other groups. The different expression of MMP-1 on day 7 between cells treated with ELF-EMF and OM-ELF-EMF not significant while in both groups it expression was significantly enhanced in comparison to OM group. In cells cultured under OM-ELF-EMF, MMP-3 expression was significantly higher than other groups on day 7 and significant up-regulated it expression observed in cells treated with ELF-EMF compared with OM and OM-ELF-EMF on day 14.

## Discussion

In the previous study, we were showed increased osteogenic differentiation potential of iPSCs cultured on tissue culture plate and nanofibrous scaffolds in comparison to the TCPS (22, 23). In another study we also compared osteogenic potential of iPSCs to adipose tissue derived MSCs and enhanced osteogenic potential was detected in iPSCs compared to the MSCs (24). This study designed to evaluation of osteo-stimolatory effects of ELF-EMF radiation to iPSCs, because of this we were carried out this study in three groups such as iPSCs cultured under osteogenic medium as control group, iPSCs cultured under ELF-EMF irradiation and iPSCs cultured under osteogenic medium plus ELF-EMF irradiation. Common osteogenic markers such as ALP activity, calcium mineral deposition and eight important bone-related genes were investigated during the period of study. There are multiple and conflicting reports about the ELF-EMF irradiation effects on the proliferation rates of cells. Sakai et al. reported that proliferation rate and glycosaminoglycan synthesis were increased in rabbit costal growth cartilage cells and human articular cartilage cells via cultured under 6.4 Hz irradiation (25). Sun et al. also showed that pulsed ELF-EMF exposure could increase proliferation rate of bone marrow (BM) MSCs. They were cultured BM-MSCs under ELF-EMF (15 Hz) 8 h irradiation per day (26). In addition to proliferation rate assay, multi-lineage differentiation potential also investigated and result demonstrated irradiated cells have similar differentiation capacity to the control group. In another study, rat primary osteoblastic cells were treated with pulsed EMF alone or together with bone morphogenetic protein 2 (BMP-2) and results showed that the cell proliferation and the osteogenic differentiation of these cells increased in comparison to the control greoup (27). However, a few studies also reported that pulsed EMF exposure has a depressing effect on cell growth. For example, McLeod et al. demonstrated proliferation rate of osteoblasts was decreased when cultured under specific EMF, while ALP activity of them was increased (28). In three other studies were observed that pulsed EMF treatment on the MG-63, ROS 17/2.8, and MLO-Y4 cell lines has no effect or has a negative effect on the cell proliferation rate (29-31). Our results were showed significantly increased iPSCs proliferation rate after treated with pulsed ELF-EMF and osteogenic medium plus ELF-EMF. Several studies have been reported osteo-stimulatory and osteo-conductiviry effects of pulsed EMF. Matziolis et al. was investigated 0.05 Hz effect on the osteogenic predifferentiation of human BM-MSCs and enhanced osteopontin (OPN), integrin-β1, transforming growth factor-β-receptor 1 (TGF-β-R1) and SMAD5 expression detected in comparison to the control group (32). Jansen et al. reported significant differentiation of BM-MSCs stimulated with 8 h exposure by 15 Hz EMF (33). It was showed that increased mineralization without altering alkaline phosphatase activity in the irradiated stem cells, and also increased mRNA levels of BMP-2, transforming growth factor-beta 1 (TNF-β-1), osteoprotegerin, matrix metalloproteinase-1(MMP-1) and -3 (MMP-3), osteocalcin, and bone sialoprotein observed in the exposure cells comparison to control. As cell contact and communications play critical role in the osteogenesis process, studies showed that EMF can affect and increase these communications via increased electrical conductivity of gap junctions. Sreedharan and Zhang also demonstrated that EMF could stimulate gap junctions which are one of the specialized intercellular channels for ions and small molecules exchange between adjacent cells (34). In this study higher ALP activity and calcium content assay observed in irradiated iPSCs than cells cultured under osteogenic medium and also highest measure of these factors detected in cells treated with both osteogenic medium and ELF-EMF. Moreover, highest significantly increased expression of eight bone-related genes in iPSCs treated with both osteogenic medium and ELF-EMF indicate that combination of ELF-EMF and osteogenic medium could have more suitable for osteogenic differentiation induction of iPSCs.

Despite contradictory reports about the effects of ELF-EMF exposure on stem cells, the present study showed that ELF-EMF had prominent osteogenic induction properties. In addition applying ELF-EMF in combination with osteogenic medium can be a great supplement for bone differentiation of stem cells and could also be an appropriate candidate for use in the treatment of bone defects.

## Acknowledgments

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