



Synergistic effect of pulsed electromagnetic fields and saffron extract on osteogenic differentiation of bone marrow mesenchymal stem cells

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ABSTRACT

Introduction: Pulsed electromagnetic fields (PEMFs) are usually used to treat non-union fractures. Saffron is a medicinal plant with various pharmacologic effects. In the present study, the synergistic effect of PEMF and saffron aqueous extract on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) was evaluated

Methods: BMSCs were isolated from rat femur. After purification, the cells were divided into four groups including control group (did not receive any treatment), saffron alone (800 µg/mL), PEMF alone (50 Hz, 3 times a week, 2 hours for every time, for 2 weeks), and saffron plus PEMF. Cytotoxicity was measured using MTT assay. After calculating the IC50 value for saffron extract (1.5 mg/mL), a lower concentration (800 µg/mL) was combined with PEMF. The differentiation of each cell group was evaluated using alkaline phosphatase (ALP) enzyme activity, alizarin red staining and reverse transcribed polymerase chain reaction (RT-PCR) method.

Results: Saffron aqueous extract decreased dose-dependently the cell viability but the PEMF had not any significant effects on cell viability. The PEMF, saffron extract and their combination increased the ALP activity on day 10 but the increase was significant in synergist group. Alizarin red staining showed that mineralization groups were higher at day 14. RT-PCR results demonstrated that on the day 14 the group treated with both PEMF and saffron expressed osteogenic genes.

Conclusion: Saffron extract combined with PEMFs could promote osteogenesis at the initial stage (the commitment of undifferentiated mesenchymal stem cells into osteoblast), hence, its usage might be beneficial in these patients

Implication for health policy/practice/research/medical education:

Saffron in combination with PEFMs is able to promote osteogenesis at the initial stage in a synergic manner and this combination might be used as a new approach in differentiation of stem cells into bone cells.

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Introduction

Bone defects resulting from resorption trauma, both pathological and physiological, are still a main challenge in orthopedic surgery. The limits of bone regeneration continue to result in failure to fully recover function and quality of life in many people (1). However, in recent decade, the use of stem cells has helped to develop promising cell-based therapies for osseous defects. The use of human embryonic stem cells could be unethical, and hence the use of pluripotent adult stem cells, such

as bone marrow mesenchymal stem cells (BMSCs), in engineering medicine has been considered (2). Recently, autologous cell therapy for bone engineering has been recommended as an interesting choice. The concept of such therapies is based on the especial effect of stem cells to differentiate into osteoblast. BMSCs are most accessible source of stem cells for many tissue engineering and regenerative medicine applications (3). BMSCs can be isolated and easily expanded from a small bone marrow aspirate. In addition, allogeneic transplant of BMSCs is

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possible with no high risk of immune rejection because of their ability to modulate immune responses (4). Growth factors including platelet-derived growth factor, bone morphogenetic proteins (BMPs) and transforming growth factor- β (TGF- β) showed promising effects in differentiation of stem cells into bone cells (5). However, the high cost and rapid degradation of such growth factors have led to limited use of them (6). Therefore, alternative osteogenic agents with lower costs compared to growth factors should be expanded (6).

Low frequency pulsed electromagnetic fields (PEMFs) can be used to treat diseases characterized by pain, inflammation and regeneration (7). PEMFs have a significant potential as a non-invasive physical therapy. PEMFs are effective adjunct to manage non-union long-bone fractures (8). PEMFs are able to stimulate bone tissue to remodel itself. PEMFs have been extensively used in the treatment of skeletal diseases such as osteoporosis and non-union fracture. PEMFs have some physiological effects on cells and tissues, including the up-regulation transcription of collagen type II, preservation of the extracellular matrix (ECM) integrity and increasing prostaglandin E₂, vascular endothelial growth factor and tumor growth factor- β_3 (TGF- β_3) production (9,10).

In recent years, use of natural products especially medicinal plants has increased. *Crocus sativus* L., commonly known as saffron, is a member of the Iridaceae family. This plant is extensively cultivated in some countries including Iran, India and Greece (11). The main component of saffron is crocetin. Other components of saffron include picrocrocin and safranal which are responsible for its color, flavor and aroma. Anthocyanins, flavonoids, vitamins, amino acids, proteins, starch, minerals and other chemical compounds have been also found in saffron (12). Previous studies have shown that retinoids may have useful effects in prevention of osteoporosis through both enhancing bone formation and suppressing osteoclast activity (13).

Due to the effects of magnetic fields and saffron extract compounds on osteogenic differentiation, in this study the effect of 50 Hz electromagnetic field with 200 g intensity and saffron aquatic extract on osteogenic differentiation of BMSCs from Wistar rats was investigated.

Material and Methods

Design PEMF exposure system

Generator of electromagnetic field was designed and manufactured in the laboratory of developmental biology, Islamic Azad University of Mashhad, Iran. This special circuit included coil, three rheostats, capacitors and ampermeter. To build a coil, around a tube, made of PVC, a suitable amount of copper wire was wrapped. Intensity was calculated according to the equation $B = \mu n I$ (B = the magnetic field intensity by Tesla, $\mu = 4\pi \times 10^{-7}$, n = the number of cycles per unit length, I = amperage). Intensity was controlled by the gauss meter. Culture flasks and plates were placed into solenoid; left and right sides of solenoid were closed and a humidified atmosphere of 5% CO₂ and

95% air at 37°C were established. In this configuration, the magnetic fields were parallel to the flasks and plates.

Preparation of plant extract

Crocus sativa stigmas were collected from Ghaen (Khorasan province, northeast Iran). Stigmas were dried and pulverized. Pulverized stigma of saffron was dissolved in distilled water and soaked for 24 hours at 4°C. The extract was filtered and concentrated by a rotary evaporator and dried by freeze dryer. The extract then was dissolved in deionized water to a final concentration of 100 mg/mL and diluted in culture medium to the working solution prior to use.

Isolation and Expansion of BMSCs

Bone marrow cells were harvested by flushing of femurs bone marrow of 6- to 8-week-old rats with Dulbecco's modified Eagle's medium (DMEM). Then the cells were centrifuged, suspended in DMEM and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. Cells were incubated at 37°C in 5% humidified CO₂ for 24 hours until the medium was replaced to remove non-adherent cells. When cells reached 90% confluency, they were trypsinized with 0.25% trypsin for 5 minutes at 37°C. After centrifugation, the cells were re-suspended and used for other analyses.

Flow cytometry

To confirm that the used cells are stem cells, flow cytometry method agonist stem cell specific markers were done. After cells separation from bottom of the flask, they were centrifuged. The primary antibodies including CD44, CD31 and CD45 were conjugated with isothiocyanate and added to the cells. They were washed with PBS and fixed with formalin. Then, analysis was done by flow cytometer.

In vitro cytotoxicity

Cytotoxicity of saffron extract was evaluated by MTT assay. In this method the cleavage and the conversion of the soluble yellowish MTT into the insoluble purple formazan by active mitochondrial dehydrogenase of living cells is used to develop an assay system for cell proliferation measurement. Briefly, harvested cells were seeded into a 24-well plate (4×10^5 cell/mL) at various amounts of saffron extract (0.5, 1, 1.5 and 2 mg/mL) and exposed to PEMF at 200 g intensity for 2 hours. At the completion of incubations, 100 μ L of MTT solution (5 mg/mL in PBS) was added to each well and left for 4 hours in darkness. Subsequently, the produced insoluble formazan was dissolved with 1 mL of dimethyl sulfoxide and left at room temperature for 20 minutes. Finally, the solution optical density was read by multi well scanning spectrophotometer (Epoch, US) at 570 nm wavelength. The cell viability was calculated using the following equation: Cell viability (%) = $(A_{\text{treated}} / A_{\text{control}}) \times 100$ where A_{treated} and A_{control} are the absorbance of the treated and untreated cells, respectively.

Treatment of BMSCs with PEMF and saffron

BMSCs were seeded in 24-well plates (5×10^4). When over 80% confluence was reached, osteogenesis was induced by 50 Hz PEMF three times a week (2 hours for every time) for 2 weeks. In some groups 800 $\mu\text{g/mL}$ saffron alongside with PEMF was added. The medium was replaced once every three days and treated with saffron again. On day 10, cells were collected for measuring alkaline phosphatase (ALP) activity and on day 14, reverse transcribed polymerase chain reaction (RT-PCR) analysis and alizarin red staining were done. Control group was put in off generator in a condition same as experimental groups.

Alizarin red staining

Cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. After washing with distilled water once, 1 mL alizarin red solution (2%, pH 4.2) was added to each well in a 24-well plate. The staining solution was removed after 10 minutes. Each well was washed with distilled water 4 to 5 times. Finally it was observed by an inverted microscope (Biomed, Korea).

Measurement of alkaline phosphatase activity

BMSCs were cultured in 6-well plate dishes for 24 hours as described previously and then were treated with 50 Hz 200 g intensity three days a week for 10 days. To determine ALP activity level, total cell protein was extracted by 200 μL NP40 buffer (Sigma, UK). The lysate was then centrifuged at $14000 \times g$ at 4°C for 15 minutes. The protein concentrations were determined by the border of Ford assay reagent. ALP activity was measured by ALP assay kit (Parsazmun, Iran) using p-nitrophenyl phosphate as

substrate and ALP provided in the kit as standard.

Semi-quantification RT-PCR analysis

Osteocalcin mRNA expression was analyzed by RT-PCR. For this propose RNA was extracted from approximately $10^{10} \times 6$ BMSCs treated with PEMF, saffron and a combination of both using RNEasy per the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using advantage RT-for-PCR per the manufacturer's instructions. After initial denaturation at 94°C for 5 minutes, cDNA was amplified using a termocycler (Perkin Elmer Applied Biosystems, Boston, MA) at 94°C for 40 seconds, 56°C for 50 seconds and 72°C for 60 seconds for 35 cycles. Primers used for amplification are forward primers shown in Table 1. The PCR products were analyzed by the electrophoresis of samples in 1.5% agarose gels stained with ethidium bromide. Results were analyzed by Image J software for measurement of band density.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). Statistical analyses were done by analysis of variance (ANOVA) test. $P < 0.05$ was considered as significant level.

Results

Flow cytometric results confirmed presence of BMSCs. Cells were positive for CD44 but negative for CD45 and CD31 (Figure 1).

Cytotoxicity study results

Saffron extract led to time and dose-dependent inhibition of viability at 24 hours and 48 hours. Inhibitory

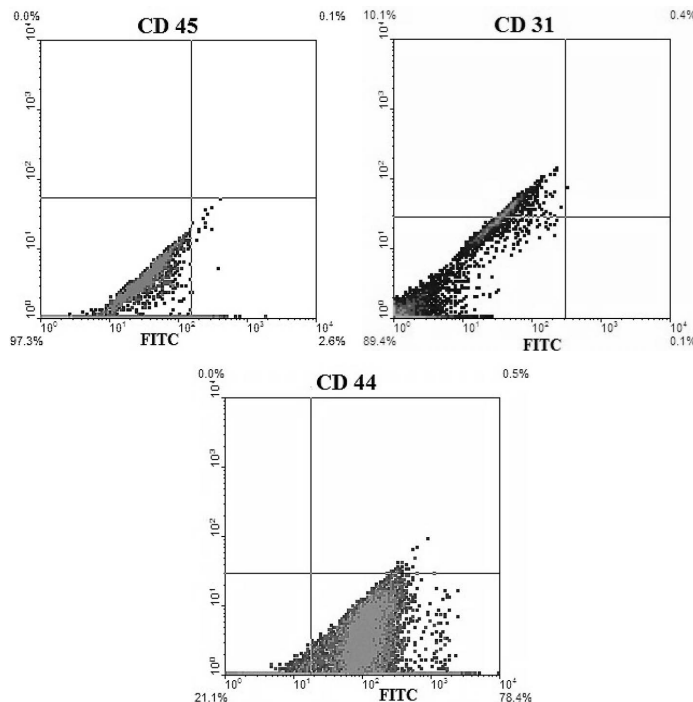


Figure 1. Flow cytometric analysis of isolated BMSCs.

BMSCs were expression specific surface marker CD44 but they were not hematopoietic marker (CD45); endothelial marker (CD31).

Table 1. Sequencing of used primers

| Gene | Forward | Reverse |
|------------|---------------------------|------------------------------|
| Beta Actin | 5'CCCGCCGCCAGCTCACCATGG3' | 5'AAGGTCTCAAACATGATCTGGGTC3' |
| OCN | 5'GTGCAGAGTCCAGCAAAGGT3' | 5'CGATAGGCCTCTGAAAGC3' |
| OPN | 5'ACAGCCAGGACTCCATTGAC3' | 5'ACACTATCACCTCGGCCATC3' |

concentration (IC₅₀) that decreased half of the cells viability was about 1.5 mg/mL (Figure 2). The lower IC₅₀ value (800 µg/mL) was used to induce osteoblast differentiation. The BMSCs viability values were around 80% when treated with 800 µg/mL of saffron extract. The viability of cells that exposed to PEMF did not show significant change compared to the control groups.

Alizarin red staining results

Matrix mineralization showed that PEMF and saffron extract had positive effects on osteogenic differentiation of BMSCs. However the greatest difference was observed in the PEMF+saffron extract groups compared to the control, PEMF and saffron extract groups (Figure 3).

ALP activity measurement results

In the present study ALP activity was detected using Naphthol AS-MX Phosphate stain. The results showed that ALP activity increased at day 10 compared to the control. However in PEMF+saffron extract group, increase was significant compared to saffron extract and PEMF groups (Figure 4).

Semi-quantification RT-PCR results

Significant up regulation of osteocalcin expression was seen in the groups treated with saffron extract and PEMF+saffron. However in the groups treated with PEMF alone, osteopontin gene (OPN) expression did not show any significant changes. However osteocalcin gene (OCN) expression in all the experimental groups increased but this increase was not statistically significant compared to the control group (Figure 5).

Discussion

Bone cells and mineralized matrix comprise two main components of bone. Osteoblasts are main bone cells. They

produce organic bone's matter and lead mineralization of matrix (14,15). The biological activity and proliferation rate of the osteoblasts have been directly correlated with the bone formation rate. However, for bone repair and osteoporosis treatment osteoblast proliferation is main factor. BMSCs are the source of bone-forming osteoblasts (2). Hence it is necessary to identify factors that enhance the differentiated stem cell into osteoblast. Stimulatory effects of the PEMF on hard tissues such as bone and soft tissue such as muscles, tendons and nerves, have been demonstrated. Saffron is a medical plant with different pharmacological effects. In the present

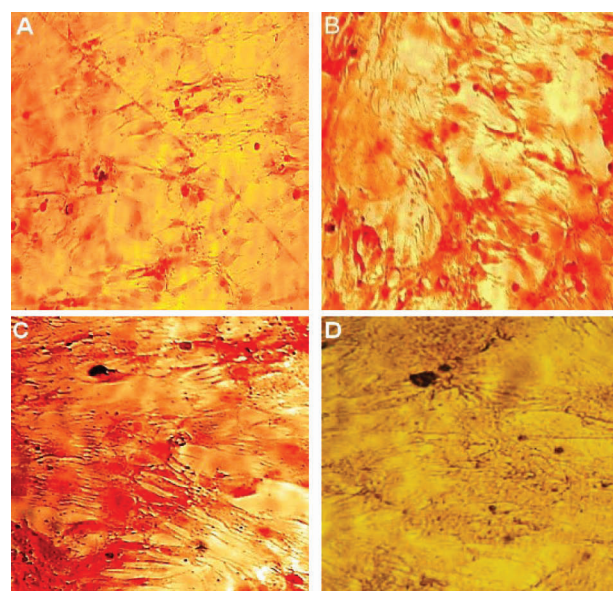


Figure 3. Alizarin red staining of BMSCs. Exposure to PEMF (A), saffron extract (800 µg/mL) (B) and saffron extract with PEMF after 14 days (C) comparing with untreated group (D). Synergic use of saffron extract and PEMF induced osteogenic differentiation more efficiency than each one.

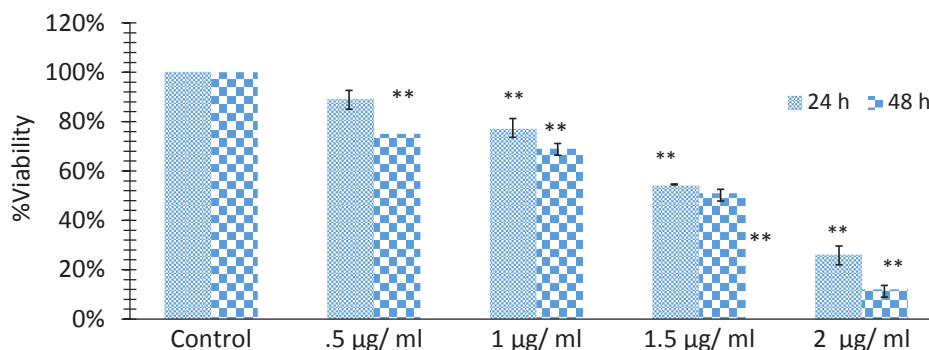


Figure 2. Cytotoxicity of saffron extract.

Saffron extract decreased cell viability dose dependently. Data are indicated as Mean ± SEM; * $P < 0.05$, ** $P < 0.01$.

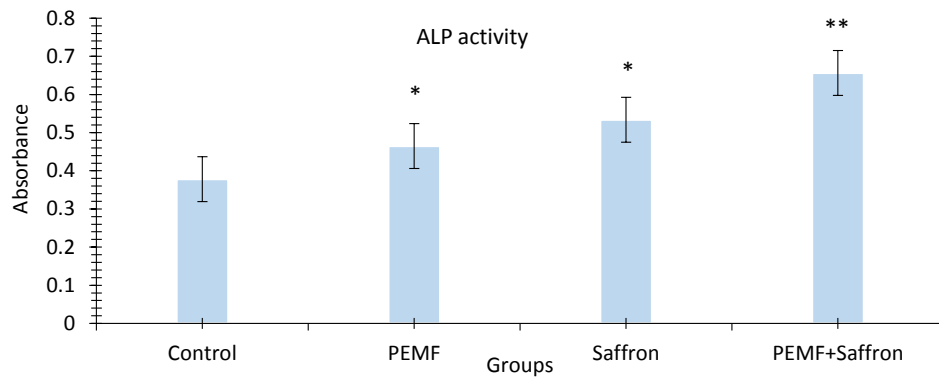


Figure 4. Diagram of the measurement alkaline phosphatase activity in different groups at 10th days, compared to control groups.

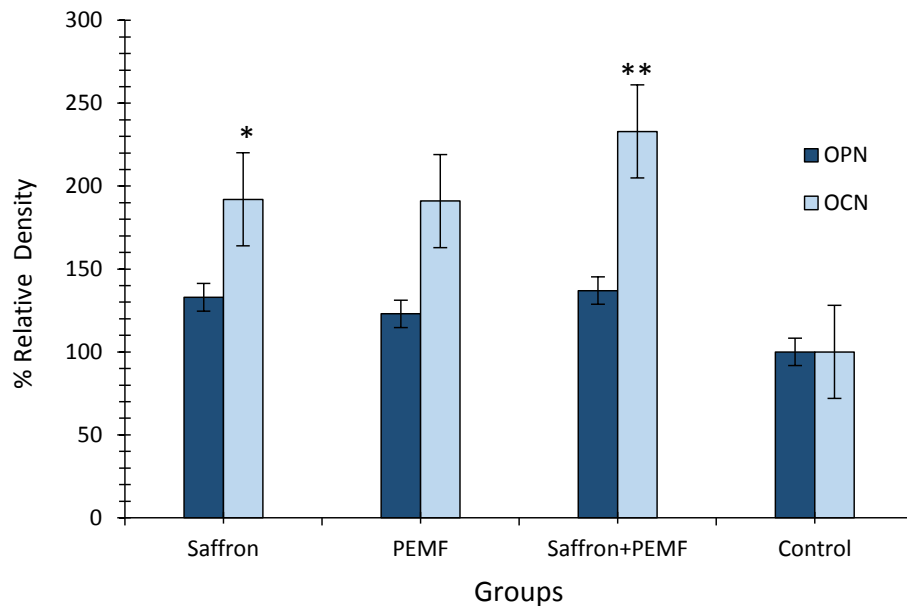


Figure 5. Comparison of osteogenic genes (OPN and OCN) expression on BMSCs treated with saffron extract, PEMF and PEMF plus saffron. The expression of OPN and OCN in the group treated with PEMF and saffron together increased significantly. Data are indicated as Mean ± SEM; * $P < 0.05$, ** $P < 0.01$.

study, differentiation effects of non-invasion PEMF and aqueous extract of saffron on BMSCs was investigated. Differentiation of stem cells was assessed by alizarin red staining, ALP activity and semi quantification RT-PCR. Alizarin red is widely used to detect MSCs differentiation into osteoblast. At the end of the day 14, alizarin red staining was evaluated to detect stem cells differentiation. In another research it was reported that sinusoidal EMF of 15 Hz, 1 mT in promoted osteogenic differentiation of the stem cells (16). Also, in this study, we demonstrated that saffron extract could differentiate BMSCs into osteoblast, however the greatest difference was observed in the groups treated with both saffron extract and PEMF compared to the control group. Once osteoblasts are active, they begin to produce huge amounts of ALP enzyme (14); hence, in this study ALP activity was measured to evaluate osteoblast differentiation and PEMF and saffron extract increased ALP activity. Saffron extract also could stimulate calcium deposition with BMSCs, which was higher in synergic group than the groups treated with either saffron extract or

PEMF. Another study reported that PEMF increased ALP activity and mineralized nodule formation, and stimulated osteoblast-specific mRNA expression of RUNX2, ALP, BMP-2, DLX and bone sialoprotein genes (16). These data are consistent with our findings. Osteogenesis involving the mesenchymal cells differentiation happens throughout a multi-step molecular pathway which is regulated by various transcription factors and signaling proteins. The mineralized ECM is mainly composed of type I collagen and considerable amounts of matrix protein, osteopontin, bone sialoprotein, BMPs, TGF- β and the inorganic mineral hydroxylapatite (17). Analysis of bone cell-specific markers like OCN and OPN detection is vastly used to detect osteoblasts differentiation. In this study OCN and OPN genes expression were assessed by comparative semi-quantification RT-PCR. Densitometry analysis revealed that expression of OCN and OPN increased in the treatment groups compared to the control groups. Expression level of OPN was higher than OCN due to the fact that expression of the gene started in earlier

stage of osteoblast differentiation. However, considerable up-regulation of OPN gene expression was seen after 14 days of treatment.

Conclusion

The results of this study indicate that saffron extract in combination with PMEF can promote osteogenesis at the initial stage (the commitment of undifferentiated MSCs into osteoblast). This method can be as a new approach in differentiation of stem cells into bone cells.

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Authors' contributions

JB designed the study, TR and MM did the laboratory works. MAS analyzed the results. All authors read and confirmed the manuscript for publication.

Conflict of interests

The authors declare no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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References

1. Shekkeris AS, Jaiswal PK, Khan WK. Clinical applications of mesenchymal stem cells in the treatment of fracture non-union and bone defects. *Curr Stem Cell Res Ther*. 2012;7(2):127-33.
2. Potu BK, Bhat KM, Rao MS, et al. Petroleum ether extract of *Cissus quadrangularis* (Linn.) enhances bone marrow mesenchymal stem cell proliferation and facilitates osteoblastogenesis. *Clinics (Sao Paulo)*. 2009;64(10):993-8.
3. Arinzeh TL. Mesenchymal stem cells for bone repair: preclinical studies and potential orthopedic applications. *Foot Ankle Clin*. 2005;10(4):651-65.
4. Undale AH, Westendorf JJ, Yaszemski MJ, Khosla S. Mesenchymal stem cells for bone repair and metabolic bone diseases. *Mayo Clin Proc*. 2009;84(10):893-902.
5. Kumar A, Salimath BP, Stark GB, Finkenzeller G. Platelet-derived growth factor receptor signaling is not involved in osteogenic differentiation of human mesenchymal stem cells. *Tissue Engineering Part A*. 2010;16(3):983-93.
6. Fan JJ, Cao LG, Wu T, et al. The dose-effect of icariin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cells. *Molecules*. 2011;16(12):10123-33.
7. Ganesan K, Gengadharan AC, Balachandran C, Manohar BM, Puvanakrishnan R. Low frequency pulsed electromagnetic field--a viable alternative therapy for arthritis. *Indian J Exp Biol*. 2009;47(12):939-48.
8. Qu C, Qin QH, Kang Y. A hypothetical mechanism of bone remodeling and modeling under electromagnetic loads. *Biomaterials*. 2006;27(21):4050-7.
9. De Mattei M, Gagliano N, Moscheni C, et al. Changes in polyamines, c-myc and c-fos gene expression in osteoblast-like cells exposed to pulsed electromagnetic fields. *Bioelectromagnetics*. 2005;26(3):207-14.
10. Mayer-Wagner S, Passberger A, Sievers B, Aigner J, Summer B, Schiergens TS, et al. Effects of low frequency electromagnetic fields on the chondrogenic differentiation of human mesenchymal stem cells. *Bioelectromagnetics*. 2011;32(4):283-90.
11. Hosseinzadeh H, Modaghegh MH, Saffari Z. *Crocus sativus* L. (Saffron) extract and its active constituents (crocin and safranal) on ischemia-reperfusion in rat skeletal muscle. *Evid Based Complement Alternat Med*. 2009;6(3):343-50.
12. Bhargava V. Medicinal uses and pharmacological properties of *Crocus sativus* Linn (Saffron). *Int J Pharm Pharm Sci*. 2011;3(3):22-6.
13. Liu H, Xue WJ, Xie YF, Feng XS, Huo FQ. Tea polyphenol-induced neuron-like differentiation of mouse mesenchymal stem cells. *Chin J Physiol*. 2011;54(2):111-7.
14. Kalfas IH. Principles of bone healing. *Neurosurg Focus*. 2001;10(4):E1.
15. Cool SM, Nurcombe V. Substrate induction of osteogenesis from marrow-derived mesenchymal precursors. *Stem Cells Dev*. 2005;14(6):632-42.
16. Yang Y, Tao C, Zhao D, Li F, Zhao W, Wu H. EMF acts on rat bone marrow mesenchymal stem cells to promote differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Bioelectromagnetics*. 2010;31(4):277-85.
17. Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev*. 2000;21(4):393-411.