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The effect of high frequency electric field on enhancement of chondrogenesis in human adipose-derived stem cells

Ebrahim Esfandiari [1], Shiva Roshankhah [1], Mohammad Mardani [1], Batool Hashemibeni [1*], Erfan Naghsh [2], Mohammad Kazemi [3], Mohammadreza Salahshoor [4]

[1] Department of Anatomical Sciences, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran
[2] Department of Electrical Engineering, Engineering School, Isfahan University, Isfahan, Iran
[3] Department of Molecular Biology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran
[4] Department of Anatomical Sciences, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran

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ABSTRACT

Objective(s): Osteoarthritis (OA) is globally one of the most common diseases from the middle age onwards. Cartilage is an avascular tissue therefore it cannot be repaired in the body. Conservative treatments have failed as a good remedy and cell therapy as a decisive cure is needed. One of the best and easily accessible cell sources for this purpose is adipose-derived stem cells which can be differentiated into chondrocytes by tissue engineering techniques. Chemical and physical inducers have a key role in stem cell – chondrocyte differentiation. We have tried to determine the role of electric fields (EF) in promoting this kind of chondrogenesis process.

Materials and Methods: Human adipose derived stem cells (ADSCs) were extracted from subcutaneous abdominal adipose tissue during cesarean section. A high frequency (60 KHz) EF (20 mv/cm), as a physical inducer for chondrogenesis in a 3D micromass culture system of ADSCs was utilized. Also, MTT, ELISA, flow cytometry, and real-time PCR techniques were used for this study.

Results: We found that using physical electric fields leads to chondrogenesis. Furthermore, results show that using both physical (EF) and chemical (TGFβ3) inducers simultaneously, has best outcomes in chondrogenesis, and expression of SOX_9 and type II collagen genes. It also causes significant decreased expression of type I and X collagen genes in pure EF group compared with control group.

Conclusion: The EF was found as a proper effective inducer in chondrogenic differentiation of human ADSCs micromass culture.

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Introduction

Arthritis is a term used to describe inflammation of the joints. Osteoarthritis (OA), is one of the most common diseases from middle age onwards (1). In osteoarthritis, cartilage undergoes biochemical and biomechanical changes either as a cause or effect of the disease (2). Globally, there are 20.7 million or about %12.1 suffering from this disease (3). Although cartilage has a relatively simple structure compared to other tissues, cartilaginous injuries cannot be cured because chondrocytes cannot proliferate in the body. The limited cartilage blood supply is thought to be responsible for the lack of post injury repair (4). Several procedures, such as microfracture (MFX), osteochondral autograft transfer system, and mosaicplasty are devised to relieve pain, restore function, and delay or halt the progression of focal cartilaginous defects. However, none of the current treatments are definitive, because pain and limitation of joint movement are not completely cured, but are alleviated. In contrast to mentioned treatments, in the last two decades, tissue engineering including autologous chondrocyte implantation (ACI) was presented as a cell therapeutic technique which proved to be a decisive treatment for traumatic acute osteoarthritis and osteoarthritis desiccant. However, treatment of chronic degenerative osteoarthritis, is still problematic and is not cured even with ACI, because of limitations of cell source and also harvested cells in *in vitro* proliferation methods (5–7). The introduction of stem cells in therapeutic domains has opened a new window to chondrocyte transplantation, because it was able to remove limitations in these cell therapy techniques (8). Therefore, current research in this field focuses on producing chondrocytes from stem cell source (9),

*Corresponding author: Batool Hashemibeni, Department of Anatomical Sciences, medical School, HezarJerib Street, University of Medical Sciences, Tel /Fax: +98-311-7922517; 09133651975; email: hashemibeni@med.mui.ac.ir

by differentiation of ADSC (chondrogenesis), which also needs some chemical (e.g. Transforming Growth Factor β) or physical inducers (e.g. electric field or ultrasonic wave). Adipose-derived stem cells are an attractive source of cells for tissue differentiation and clinical applications due to easy access, large amounts of adipose tissue in adults, and convenience as a source for cell proliferation (10, 11). However, many researchers tested many chemical and physical inducers and found that the harvested cartilage tissues are not the same as normal hyaline cartilage, due to having much type I & X collagen and not enough type II collagen (12). Therefore, researchers continue investigating production of a cartilage tissue with better quality. In this study, we use high frequency electric field as a physical inducer for chondrogenesis induction in adipose-derived stem cells, in order to create proper cartilage tissue with more type II collagen and less type I & X collagen.

Materials and Methods

Isolation and proliferation of adipose derived stem cells

Human ADSCs were extracted from subcutaneous abdominal adipose tissue taken from women who underwent caesarean section (30–40 years). Adipose tissue was mechanically minced and washed with PBS (Sigma) and was then digested with collagenase IA (1 mg/1g). The cell solution was centrifuged at 1500 rpm for 10 min. The pellet was resuspended in culture medium containing DMEM-LG supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco) and was then cultured and kept at 37˚C, 5% CO_2 conditions. In order to examine the morphology of the cells, photographs were taken by an invert microscope, at different time intervals (13).

Flow cytometry

The percentage of cell markers was quantified by flow cytometry. Cells were released with trypsin-EDTA, rinsed, and suspended in PBS. Cell suspension was split into aliquots (100 µl), an unstained group, 5 µl mouse antibody IgG 1,2 (negative control), 5 µl mouse anti-human monoclonal CD105(Abcam) and mouse anti-human monoclonal CD 44 (DAKO Cytomation), 5 µl mouse anti-human monoclonal CD 14,45 (IQ Product). Next, samples were incubated for 30 min in the dark at 4˚C. The cells were washed and centrifuged at 1500 rpm for 10 min. The supernatant was removed, the labeled cell pellet was resuspended in 200 µl PBS, and the FACS analysis was done (14).

In vitro chondrogenic differentiation

Chondrogenic differentiation media contained

DMEM-HG (High Glucose) (Gibco), penicillin and streptomycin 1% (Gibco), dexamethasone 10^{-7}M (Sigma), ascorbate-2-phosphate 50 µg/ml(Sigma), bovine serum albumin 0.5 mg/ml (Sigma), linoleic acid 5 µg/ml (Sigma), 10 mg/ml insulin, 5.5 mg/l transferring, 5 µg/l selenium (ITS) (Sigma), with and without adding transforming growth factor-β 3 (TGFβ3) 10 ng/ml (Sigma) (14). In this study, a 3D micromass culture system of ADSCs was used. Briefly, for passage two, the cells were trypsinized and resuspended in growth media at a density of 250,000 cells in 12.5 µl medium. The microliter droplets were seeded in a 24-well plate and allowed to form cell aggregates and were incubated at 37˚C for two and a half hrs. Chondrogenic differentiation media was carefully added around the cell aggregates after this time (15).

Capacitively coupled electrical stimulation

Capacitively electric fields (20 mv/cm, 60 KHz) were delivered to the cultured cells. The electric field was designed by the microcontroller (ATMEGA16). In this way, first pulse wave was generated by the microcontroller. Then, the electric field intensity was amplified by the push-pull electronic circuit and applied to the cells which were cultured in modified cooper tissue culture plate (16).

Experimental design

The study setup included four groups; a control group consisting of ADSCs in chondrogenic media without TGF-β, and 3 experimental groups including a subgroup of cells in chondrogenic media with TGF-β, one subgroup of cells in chondrogenic media without TGF-β and applied EF stimulation, and another one of cells in chondrogenic media with TGF-β and applied EF stimulation. The applied signal was a pulsed wave applied for 20 min daily for 7 days.

MTT assay (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide)

The viability of ADSCs with EF exposure was assessed by the MTT assay on 7th day. At first, the medium of each well was removed, rinsed with PBS, and replaced with 400 µl serum free medium and a 40 µl MTT solution. Next, it was incubated at 37˚C, 5% CO_2 for 4 hr. The medium was discarded and 400 µl DMSO (Sigma) was added to each well, and was incubated in dark for 2 hr. Next, 100 µl of the solution was transferred to a 96-well plate and absorbance of each well was read at 570 nm with ELISA reader (Hiperion MPR4). The assays were performed in triplicate (17, 18).

Enzyme-linked immunosorbent assay

The amount of aggrecan (AGC) was quantified in supernatant culture media on the 7th day, according to protocol of Human Aggrecan Direct ELSA kit (Invitrogen). Briefly, supernatant media as antigens were added to ELISA plate and AGC molecules bound to coated antibodies. Next, the enzyme conjugated antibodies were added which linked to

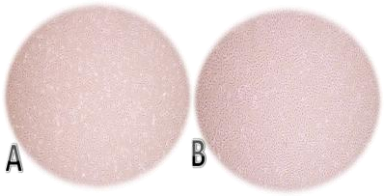

Figure 1. Invert microscopic images (×40), of ADSCs before (A) and after (B) treatment with EF at passage 0 on the seventh day

antigens and formed antigen-antibody sandwiches. Finally, enzyme`s substrate was added and the absorbance of the mixture was measured at a wavelength of 450 nm by spectrophotometer (19).

RNA isolation and real-time polymerase chain reaction (PCR)

Real-time quantitative RT-PCR was performed to quantitatively estimate the mRNA expression of type I, II, X collagens, and SOX9 genes in ADSCs at different groups. Total RNA was isolated by RNeasy mini kit (Qiagen), treated by RNase-free DNase set (Qiagen) to eliminate the genomic DNA. The RNA concentration was determined using a biophotometer (Eppendorf). Total RNA (100 ng) was reverse-transcribed to cDNA by using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The Maxima SYBR Green Rox qPCR master mix kit (Fermentas) was used for real-time RT-PCR. Primer sequences are shown in Table 1. Real-time PCR reactions were performed by using the Comparative Ct (ΔΔCt) method. The relative expression level of genes was computed by calculating the ratio of the amount of genes to that of endogenous control (GAPDH). Melting curve to determine the melting temperature of specific amplification was produced. These experiments were carried out in triplicate and were independently repeated at least 3 times (20).

Statistical tests

The Kolmogorov-Smirnov test was used for assessing normal distribution of variables and ANOVA (one-way analysis of variance) with the LSD *post hoc* test for the comparison of results in different groups.

Results

Morphology of ADSCs after EF exposure

The morphology of EF exposure and control groups were almost the same and all of them were uniform and spindle-shaped for the duration of the 7 days.

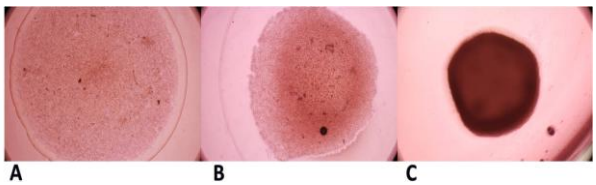

Figure 2. Invert microscopic images of micromass droplet in EF applied group. A: first day, B: second day, C: third day. Mag. ×40

Table 1. Gene sequence of primers

Gene	Primer sequences
Collagen II-F	CTGGTGATGATGGTGAAG
Collagen II -R	CCTGGATAACCTCTGTGA
Sox_9 -F	TTCAGCAGCCAATAAGTG
Sox_9 -R	TTCAGCAGCCAATAAGTG
Collagen x -F	AGAATCCATCTGAGAATATGC
Collagen x - R	CCTCTTACTGCTATACCTTTAC
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTTCCTCTTGTGCTCTTG
Collagen I-F	CCTCCAGGGCTCCAACGAG
Collagen I -R	TCAATCACTGTCTTGCCCCA

Micromass formation

Micromass droplets aggregate spherically in 24 hr in all groups; while cell droplets in EF exposed groups condense and the mass form appears in day 3, but in control group this process is performed on the 4th day.

Flow cytometry

This analysis confirms stemness of the isolated cells. The results show that they are negative for CD 14 and 45(0.14%) but expressed CD 44 and CD 90 (89.69%) at high level.

MTT

Followed by treatment with MTT solution, the dark blue formazan crystals are seen in ADSCs which indicates their metabolic activity. However, applying EF improves viability and proliferation of ADSCs, but the comparison of results shows that they don't have significant differences (P>0.05). These results show that EF is not fatal to the cells during the 7 days.

ELISA

The general results show that the amount of produced AGC from cells exposed to EF are more than that of control group, on the 7th day (P<0.05). The addition of TGFβ3 to chondrogenic media leads to enhanced AGC production compared to the control group, but the difference is not significant (P>0.05). On the other hand, the group with a growth factor and exposed to EF produced more AGC than control group (P<0.001) and other groups (P<0.05). Findings indicate, although the presence of TGFβ3 can increase AGC production, EF is more effective (P<0.05).

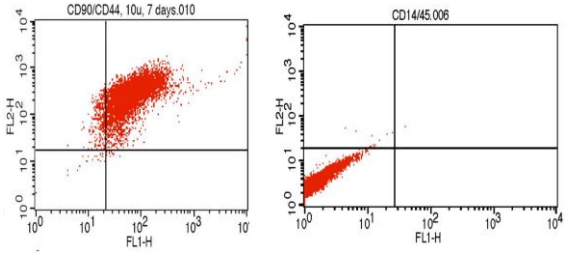

Figure 3. Flow cytometry dot plots of cell markers in ADSCs. CD90, 44- CD14,45. CD14, 45 (0.14%), CD44, CD90 (89.69%)

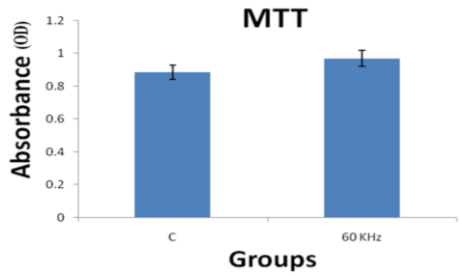

Figure 4. Comparison of MTT assay results between two groups They have no significant differences (P>0.05). C: control group
60 KHz: the group has been affected by 60 KHz electric field

Real-time PCR

The results of real-time PCR indicate that type II collagen and SOX_9 gene expression in the group having TGFβ and exposed to EF are significantly higher (P<0.001) than the control group and other groups. Expression of type II collagen and SOX_9 genes in the group with applied EF only, is more than in the group affected just by TGFβ, but they are not significantly different (P>0.05).

The results of real-time PCR show that type X (as hypertrophic marker) and I (as fibro cartilage marker) collagen gene expression in the group affected by EF only, is significantly lower than control group (P<0.001). Although expression of type I and X collagen genes in this group is lower than the group just affected by TGFβ and the group affected by both TGFβ and exposure to EF, differences are not significant (P>0.05).

Discussion

We found that using both physical (EF) and chemical (TGFβ3) inducers simultaneously, leads to the best results in chondrogenesis, and specifically expression of type II collagen and SOX_9 genes. A variety of techniques of electrical stimulation proved

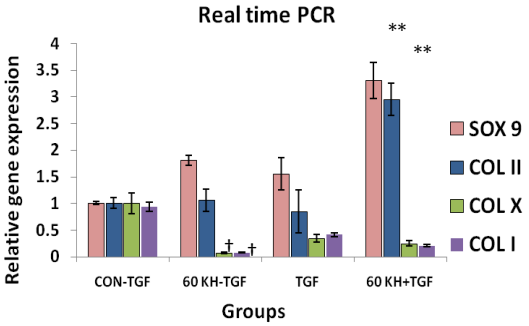

Figure 6. Results of real-time PCR for type I, II & X collagens and SOX_9 genes in all groups. Values are Means ±SE of triplicate experiments
**: P<0.001 vs. other groups
†: P<0.001 vs. control group
C-TGFβ3: control group without transforming growth factor.
60- TGFβ3: the group has been affected by 60 KHz EF without TGFβ3
TGFβ3: the group has been affected by TGF
60+ TGFβ3: the group has been affected by 60 KHz EF and TGFβ3

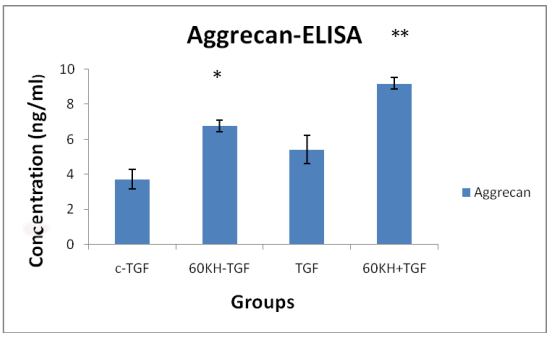

Figure 5. ELISA analysis for aggrecan in supernatant media of groups at seventh day. Values are Means±SE of triplicate experiments
*: P<0.05 vs. control group. **: P<0.001 vs. control group
C-TGFβ3: control group without transforming growth factor
60- TGFβ3: the group has been affected by 60 KHz electric field without TGFβ3
TGFβ3: the group has been affected by TGF
60+ TGFβ3: the group has been affected by 60 KHz electric field and TGFβ3

to be effective in therapeutic management and also in tissue culture improvement, including direct probe placement within bone tissue culture, application of changing magnetic fields, and capacitative coupling with metal plates placed outside the culture, which induce an alternating electric field within the tissue (21). In this regard, we applied just the latter of the above mentioned physical inducers in our study. We tested effects of selective and specific capacitively coupled electrical signals in enhancement of chondrogenesis in ADSCs, although, as it was mentioned earlier, these effects were fortified by using TGFβ simultaneously. It should be emphasized that it is difficult to compare our *in vitro* results with those of others, because all elements of our study, such as the source of cells, the culture method, and the features of our electric field are different from other research. Clearly, our investigation is the only study in which effects of EF on chondrogenesis are examined. However, several studies have shown the impact of EF on osteogenic differentiation *in vitro*. For example, Hronik-Tupaj *et al* (22), Hammerick *et al* (23), and also Seth D *et al* (21), were all able to differentiate stem cells into osteoblasts through EF with different features; 20 mv/cm 60 kHz frequency, 6V/cm 50 Hz, and 1V/cm 50 Hz, respectively. In *in vitro* MSCs chondrogenesis, there is usually a tendency to gene expression of type I and X collagens, therefore, fibrocartilage, and terminal hypertrophy (initiation of ossification) result respectively (24). Researchers have been investigating a technique in which they can get rid of these two unwanted collagens. Our results clearly meet this serious need. Here, electric field not only promoted chondrogenesis, but also inhibited the expression of type I and X collagens, significantly. Although, the knowledge about the mechanism of electrical stimulation on cell differentiation is still

unknown, in some studies some mechanisms are raised to a clearer horizon. For example, it is demonstrated that the capacitively coupled electric stimulation of cultured bone cells causes an increase in cytosolic Ca^{2+} via voltage-gated calcium channels leading to activated calmodulin and an increase in TGFβ mRNA (25–27). We doubt whether the same mechanism(s) is operating in ADSCs.

Conclusion

The coupled-capacity electric field was found as a proper effective inducer in chondrogenic differentiation of human ADSCs micromass cultures. Future studies may work on accessing significant chondrogenesis with just EF stimulator, as a safe, easy, and cheap inducer.

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References

1. Marlovits P, Zeller P, Singer C, Resinger V. Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol 2006; 57:24–31.
2. Kuo K, LiWan J, Mauck L, Tuan S. Cartilage tissue engineering: it's potential and uses. Curr Opin Rheumatol 2006; 18:64-73.
3. Kurtz S, Ong K, Lau E, Mowat F, Halpern M. Projections of primary and revision hip and knee arthroplasty in the United States from 2005 to 2030. J Bone Joint Surg Am 2007; 89:780-785.
4. Ahmed N, Stanford WL, Kandel RA. Mesenchymal stem and progenitor cells for cartilage repair. Skeletal Radiol 2007;36:909-12.
5. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331:889-895.
6. Esfandiari E, Nazem Kh, Safdarian A, Fesharaki M, Moulavi F, Shakibaei M, *et al* . Treatment of full thickness cartilage defects in human knees with Autologous Chondrocyte Transplantation. Res Med Sci 2011; 16:855–861.
7. Esfandiary E, Shakibaei M, Amirpour N, Razavi Sh, Nasresfahani M, Moulavi F, *et al*. Study of human chondrocyte redifferntiation capacity in three-dimensional hydrogel culture. Iran J Basic Med Sci 2008; 11:152-158.
8. Raghunath J, Salacinski J, Sales M, Butler E, Seifalian M. Advancing cartilage tissue engineering: the application of stem cell technology. Curr Opin Biotechnol 2005; 16:503-509.
9. Song H, Chang W, Song BW, Hwang KC. Specific differentiation of mesenchymal stem cells by small molecules. Am J Stem Cells 2012; 1:22-30.
10. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al* . Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13:4279-4295.
11. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al* . Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001; 7:211-28.
12. Chung C, Burdick JA. Engineering cartilage tissue. Adv Drug Deliv Rev. 2008; 60: 243-62.
13. Hashemibeni B, Razavi S, Esfandiary E, Karbasi S, Mardani M, Nasresfahani M. Induction of chondrogenic differentiation of human adipose-derived stem cells with TGF-β3 in pellet culture system. Iran J Basic Med Sci 2008; 11:10-17.
14. Ansar M, Esfandiariy E, Mardani M, Hashemibeni B, Zarkesh H, Hatef M, *et al*. A comparative study of aggrecan synthesis between natural articular chondrocytes and differentiated chondrocytes from adipose derived stem cells in 3D culture. Adv Biomed Res 2012; 1:30-37.
15. Yue X, Balooch G, Chio M, Bekerman E, Ritchie R, Longaker M. Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells using an in i*n vitro* three dimentional micromass culture system. Biochem Biophys Res Commun 2007; 359:311-316.
16. Xu J, Wang W, Clark C, Brighton T. Signal transduction in electrically stimulated articular chondrocytes involves translocation of extracellular calcium through voltage-gated channels. Osteoarthritis Cartilage 2009; 17:397-405.
17. Esfandiary E, Valiani A, Hashemibeni B, Moradi I, Narimani M. The evaluation of toxicity of carbon nanotubes on the human adipose-derived-stem cells *in vitro*. Adv Biomed Res 2013; 4:17-25.
18. Yan J, Dong L, Zhang B, Qi N. Effects of extremely low-frequency magnetic field on growth and differentiation of human mesenchymal stem cells. Electromagn Biol Med 2010; 29:165-176.
19.Mardani M, Hashemibeni B, Ansar M, Zarkesh Esfahani H, Kazemi M, Goharian V, *et al* . Comparison between chondrogenic markers of differentiated chondrocytes from adipose derived stem cells and articular chondrocytes *In vitro*. Iran J Basic Med Sci 2013; 16:763-771.
20. Creecy C, Neill C, Arulanandam B, Sylvia V, Navara C, Bizios R. Mesenchymal stem cell osteodifferentiation in response to alternating electric current. Tissue Eng 2012; 19:10-17.
21. Seth M, McQuilling J, Grossfeld R, Lubischer J, Clarke L, Loboa E. Application of low-frequency alternating current electric fields Via interdigitated electrodes: effects on cellular viability, cytoplasmic calcium, and osteogenic differentiation of human adipose-derived stem cells. Tissue eng 2010; 16:1377-1366.
22. Hronik-Tupaj M, Rice W, Cronin-Golomb M, Kaplan D, Georgakoudi I. Osteoblastic differentiation and stress response of human mesenchymal stem cells exposed to alternating current electric fields. Biomed Eng 2011; 10:9-15.
23. Hammerick K, James A, Huang Z, Prinz F, Longaker F. Pulsed direct current electric fields enhance osteogenesis in adipose-derived stromal cells. Tissue eng 2010; 16:917-931.
24. Mayer-Wagner S, Passberger A, Sievers B, Aigner J, Summer B, Schiergens T, *et al* . Effects of low frequency electromagnetic fields on the chondrogenic differentiationof human mesenchymal stem cells. Bioelectromagnetics 2011; 32:283-290.

25. Wang W, Wang Z, Zhang G, Clark C, Brighton C. Up-regulation of chondrocytes matrix genes and products by electric fields. Clin Orthop Relat Res 2004; 427:5163-5173.
26. Gan J, Fredericks D, Glazer P. Direct current and capacitive coupling electrical stimulation upregulates osteopromotive factors for spinal fusions. Spine 2005; 15:15-22.
27. Goodwin C, Brighton C, Guyer R. A double blind study of capacitively coupled electrical stimulation as an adjunct to lumbar spinal fusions. Spine 1999; 24:1349-1357.