Original Article

Induction of chondrogenic differentiation of human adipose-derived stem cells by low frequency electric field

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Abstract

Background: Since when the cartilage damage (e.g., with the osteoarthritis) it could not be repaired in the body, hence for its reconstruction needs cell therapy. For this purpose, adipose-derived stem cells (ADSCs) is one of the best cell sources because by the tissue engineering techniques it can be differentiated into chondrocytes. Chemical and physical inducers is required order to stem cells to chondrocytes differentiating. We have decided to define the role of electric field (EF) in inducing chondrogenesis process.

Materials and Methods: A low frequency EF applied the ADSCs as a physical inducer for chondrogenesis in a 3D micromass culture system which ADSCs were extracted from subcutaneous abdominal adipose tissue. Also enzyme-linked immunosorbent assay, methyl thiazolyl tetrazolium, real time polymerase chain reaction and flowcytometry techniques were used for this study.

Results: We found that the 20 minutes application of 1 kHz, 20 mv/cm EF leads to chondrogenesis in ADSCs. Although our results suggest that application of physical (EF) and chemical (transforming growth factor-β_3) inducers at the same time, have best results in expression of collagen type II and SOX_9 genes. It is also seen EF makes significant decreased expression of collagens type I and X genes.

Conclusion: The low frequency EF can be a good motivator to promote chondrogenic differentiation of human ADSCs.

Key Words: Low frequency electric field, chondrogenesis, Adipose derived stem cells

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INTRODUCTION

Osteoarthritis which affecting most often the hips, knees, and spine is the one of the world's most common diseases.[1] In osteoarthritis, articular cartilage is degenerated and disappears so bones under the cartilage rub together and cause pain, swelling, soft-tissue inflammation and reduced range of motion in the joints.[2] It is estimated that 2% of the world population under the age of 45 suffers from osteoarthritis.[3] Yet, cartilaginous injuries cannot be cured in the body because cartilage is avascular tissue.[4] Therefore, cell therapy, a technique come

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out from tissue engineering, is a promising treatment method. In last two decades autologous chondrocyte transplantation (ACT) was demonstrated to be one of the best treatment for traumatic acute osteoarthritis and osteoarthritis desiccant but because of cells harvested limitation from the body, treatment of the chronic degenerative osteoarthritis is still a problematic issue.[5-7] Since stem cells have the unique capacity to self renew and differentiate into the various cell types[8] so researchers focus on producing chondrocyte from stem cell source[9] such as adipose derived stem cell (ADSC) which also needs some chemical (e.g., transforming growth factor-β [TGF-β]) or physical inducers (e.g., electric field [EF] or ultrasonic wave). Because of easy access, large amounts of adipose tissue in adults and convenient source for cell proliferation, ADSCs are an interesting cells which used in tissue differentiation and clinical applications.[10,11] As many investigators found that the cartilage harvested in the laboratories are not the same as normal hyaline cartilage, because of having much type I and X collagen and not enough type II collagen.[12] Therefore researches continued their efforts to better quality cartilage tissue production. Electric stimulation is one of physical inducers that cells may feel it. External ES has been shown to induce various cellular and molecular responses including microfilament reorganization, cell surface receptor redistribution, changes in intercellular calcium dynamic, enhanced differentiation, proliferation, angiogenesis and protein biosynthesis.[13] Hence we use low frequency EF in order to get access to achieve proper cartilage tissue with more collagen type II and less collagen type I and X as a physical inducer which induction of adipose-derived stem cells to chondrocyte.

MATERIALS AND METHODS

Cells isolation and study setup

Human ADSCs are extracted from subcutaneous abdominal adipose tissue and then breake up mechanically and washed with phosphate buffered saline (PBS) (Sigma) and next it is enzematic digested. The cell solution is centrifuged and pellet is resuspended in culture medium contained Dulbecco's modified eagle medium (DMEM) –low glucose supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (Gibco) and then is cultured and kept at 37°C, 5% CO_2 conditions. Then photographs are taken by an invert microscope due to examine the morphology of the cells, at different time intervals.[14] The study setup includes 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 1KHz EF stimulation and another one consisting of cells in chondrogenic media with both of TGF-β and 1KHz EF stimulation. The signal was appleid for 20 min daily for 7 days.

Chondrogenic differentiation

A 3D micromass culture system of ADSCs is used. Chondrogenic differentiation media containing DMEM- high glucose (Gibco), penicillin and streptomycin 1% (Gibco), dexamethasone 10^{-7} M (Sigma), ascorbat-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 (insulin-transferrin-selenium) (Sigma), with and without adding TGF-β_3 10 ng/ml (Sigma).[15] Briefly the cells with 250,000 cells/12.5 μl density in passage two are trypsinized and resuspended in growth media. The droplets are seeded in 24-well plate and after 2½ hour at 37°C incubation, cell aggregates. after this time chondrogenic differentiation media is carefully added around the cells.[16]

Electric stimulation design

Electric field (1 kHz, 20 mv/cm) was delivered to the cultured cells, based on our previous experiment. The EF is designed by the microcontroller (ATMEGA16) [Figure 1]. In this way, first pulse waves generated by the microcontroller. Then, the EF intensity is amplified by the push-pull electronic circuit and applied to the cells which are cultured in modified cooper tissue culture plate. One modality to restore this important signaling system is the provision of a capacitively coupled EF. The term "capacitive" is used since the arrangement of conductive plates and targeted cells/tissue resembles a capacitor (an electrical element used to temporarily store a charge) with the tissue/cells in their dielectric medium placed between a pair of conductive plates. Stimulation occurs by a transfer

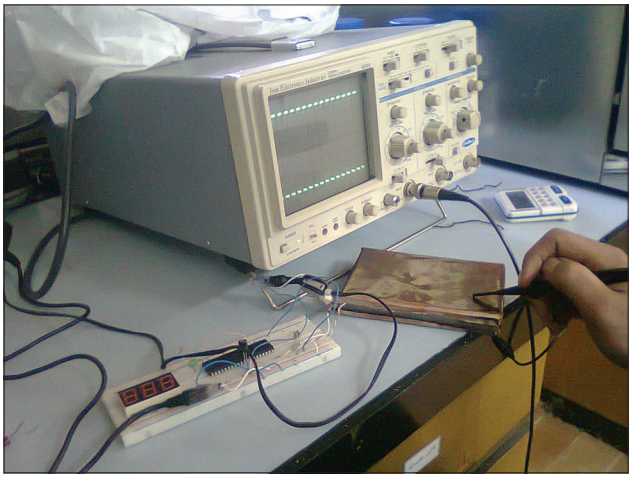

Figure 1: Experimental design used for electrical stimulation. Electric fields (20 mv/cm, 1 kHz) are delivered to the cultured cells

of electrical energy from the capacitor to the targeted tissue (coupling) via the induced EF.[17]

Flow cytometry technique

Flow cytometric analysis of human ADSCs was perform using specific FITC and PE coupled antibodies against surface markers. Cells are released with trypsin-EDTA, rinsed and suspended in PBS. Cell suspension is split into 5 groups (100 µl); an unstained group (negative control), 5 µl mouse antibody IG1, 2, 5 µl mouse anti-human monocolonal CD 90 (Abcam) and mouse anti-human monocolonal CD 44 (DAKO Cytomation), 5 µl mouse anti-human monocolonal CD 14 and 45 (IQ Product). Next, samples were incubated in the dark at 4°C for 30 minutes. The cells centrifuged at 1500 rpm. After removing of supernatant, the labeled cell pellet resuspended in 200 µl PBS and to fluorescence activated cell sorter analysis is done.[18]

Methyl thiazolyl tetrazolium (MTT) assay

The viability of ADSCs which exposure with low frequency EF were examined by the MTT assay on 7th day. Briefly, the medium of wells was removed then rinsed with PBS and replaced with 40 µl MTT solutions and 400 µl serum free medium. Next, they were incubated at 37°C, 5% CO_2 for 4 h. The content of wells was discarded and 400 µl DMSO (Sigma) is added to each well and was incubated in dark for 2 h. Next 100 µl of the each well's solution was transferred to 96-well plate and absorbance of each well was read at 570 nm with enzyme-linked immunosorbent assay (ELISA) reader (Hiperion MPR4). It should be noted that the assays were performed in triplicate.[19]

ELISA assay

The aggrecan (AGC) amount in the culture media was measured on 7th day, according to protocol of Human Aggrecan Direct ELSA kit (Invitrogen). At first, 100 µl supernatant media were added to ELISA plate so AGC molecules bound to antibodies which coated in each well. Next, the secondary enzyme conjugated antibodies which links to antigens was added and formed antigen-antibody sandwiches. Finally, it's substrate is added and the absorbance of them was measured at a wavelength of 450 nm by spectrophotometer.[20]

Real-time polymerase chain reaction

Real-time quantitative RT-PCR is performed to quantitatively estimate the messenger ribonucleic acid (mRNA) expression of types I, II, X collagens and SOX_9 genes in ADSCs at all groups. Total RNA was isolated by RNeasy mini kit (Qiagen) and in order to eliminate the genomic deoxyribonucleic acid (DNA) treated by RNase-free DNase set (Qiagen). The RNA concentration using by biophotometer (Eppendorf) was examined. Total RNA (100 ng) using by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) was reverse-transcribed to complementary DNA (cDNA) according to the manufacturer's instructions. In this step the Maxima SYBR Green RoxqPCR master mix kit (Fermentas) was used. Primer sequences which used in this study were CTGGTGATGATGGTGAAG for collagen II (forward) and CCTGGATAACCTCTGTGA for collagen II (reverse), TTTCCAAGACACAAACATGA for SOX_9 (forward) and AAAGTCCAGTTTCTCGTTGA for SOX_9 (reverse), AGAATCCATCTGAGAATATGC for collagen x (forward) and CCTCTTACTGCTATACCTTTAC for collagen x (reverse), CCTCCAGGGCTCCAACGAG for collagen I (forward) and TCAATCACTGTCTTGCCCCA for collagen I (reverse). Real-time PCR reactions are performed using step analyzed using the comparative Ct (ΔΔCt) method. The relative expression level of genes is calculated by determining a ratio between the amount of genes and that of endogenous control (GAPDH: AAGCTCATTTCCTGGTATG [forward] CTTCCTCTTGTGCTCTTG [reverse]). Melting curve in order to measuring of the melting temperature of specific amplification products and primer dimmers was produced. It should be noted that these experiments were done in triplicate and were repeated at least 3 times.[21]

Statistical tests

The Kolmogorov-Smirnov test was used for evaluating normal distribution of variables. In order to comparison of results in different groups one-way analysis of variance with the least significant difference *post hoc* test was done.

RESULTS

Morphology of ADSCs after EF exposure

The morphology of cells at EF exposure and control groups are almost the same so that the period of 7 days all were spindle-shaped [Figure 2].

Micromass formation

Micromass droplets aggregate spherically in all groups just 24 hour. Also micromasses at low frequency EF exposed groups same as control group condense on the 4th day [Figure 3].

Flow cytometry

This analysis confirms stemness of the extracted cells from adipose tissue. The results show that they are negative for CD 14, 45 (0.14%) but express CD 44, 90 (89.69%), at high level [Figure 4].

MTT

Following MTT solution treatment, the dark blue formazan crystals are seen in ADSCs which indicates

their metabolic activity. Comparison of MTT assay results between control and exposed groups is done and shows that however, the applying low frequency EF, improves viability and proliferation of ADSCs, but they don't have significant differences ($P > 0.05$). These results show that low frequency EF is not fatal to cells in through 7 days [Figure 5].

ELISA

The results show that the content of AGC which produced by cells that exposed to low frequency EF are more than control group on the 7th day, but they had not significant differences ($P > 0.05$).The addition of TGF-β3 to chondrogenic media caused to AGC production more than the amount produced by control group and group that just applied EF, but they don't have significant differences ($P > 0.05$). On the other hand, in a group having a growth factor and low frequency EF applied produced more AGC than control group ($P < 0.001$) [Figure 6].

Real time PCR

The results of real-time PCR indicated that expression of type II collagen and SOX_9 genes in the group which have both of TGF-β and low frequency EF applied are higher than control group, significantly ($P < 0.001$). Expression of type II collagen and SOX_9 genes in group that just applied low frequency EF are more than group that just TGF-β was used, but they are not significantly different ($P > 0.05$) [Figure 7].

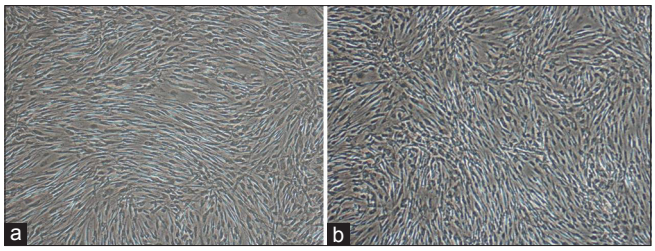

Figure 2: Representative invert microscopy images of adipose derived stem cells before and after treatment with electric field (EF) at passage 0 on the 7th day. (a) Control group.(b) the group was applied EF. Magnification ×20

Also the results of real-time PCR show that type X collagen (as hypertrophic marker) genes expression in group that just applied EF are significantly lower than ($P < 0.001$) control group. Although expression of type I collagen genes in this group are lower than other groups, but they had not significant differences ($P > 0.05$).

DISCUSSION

Although, the information about the mechanism of electrical stimulation on cells differentiation are still unknown, but some mechanisms has been raised to a clearer horizon. For example, it is demonstrated that the electric stimulation on bone cells cultured caused an increase in cytosolic Ca^{2+} via voltage-gated calcium channels leading to activated calmodulin and an increase in TGF-β mRNA,[22-25] we have designed our study is based on the same idea but we doubt whether the same mechanism(s) is operating in ADSCs. Different electrical stimulation techniques have been demonstrated that they are effective in therapeutic managements and also in tissue culture improvement, including application of changing magnetic fields and capacitative coupling with metal plates placed

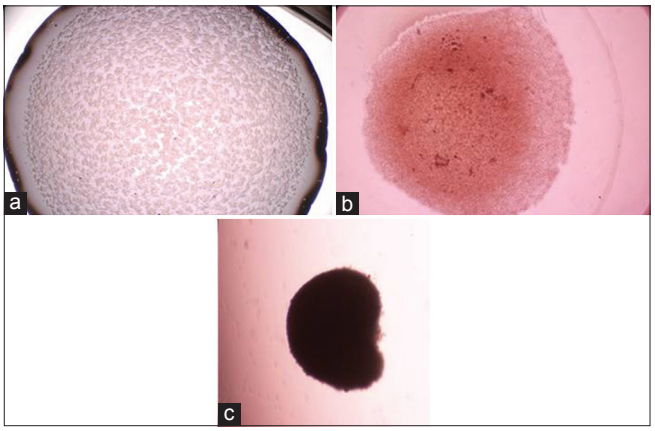

Figure 3: Representative invert microscopy images of micromass droplet in electric field applied group. (a) 1st day. (b) 2nd day. (c) 3rd day. Magnification ×20

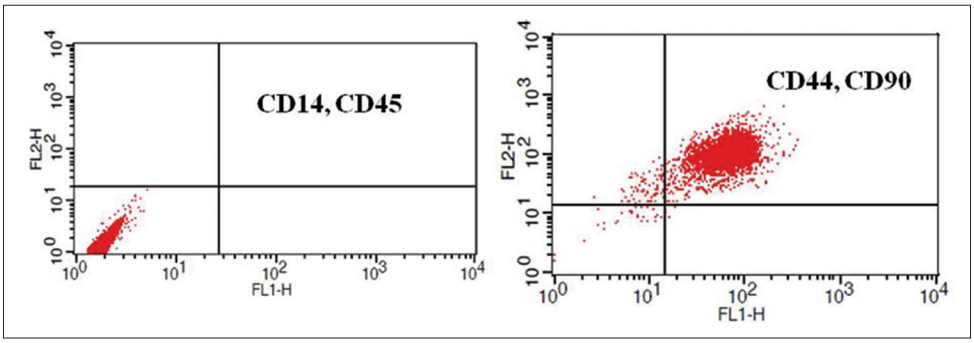

Figure 4: Flow cytometry dot plots of cell markers in adipose derived stem cells. CD90 (a receptor for transforming growth factor-β), CD44 (hyaluronic acid receptor), CD14, 45 (hematopoietic markers). CD14, 45 (0.14%), CD44, CD90 (89.69%)

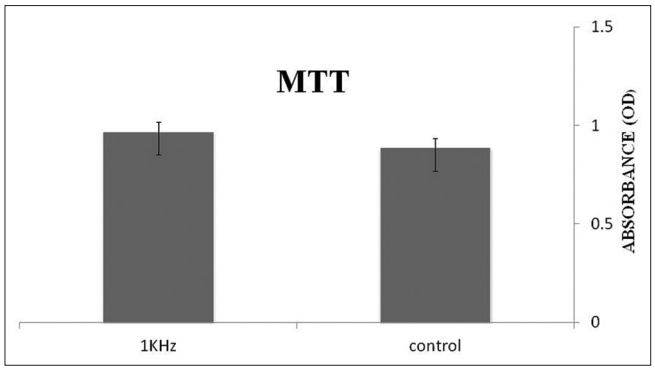

Figure 5: Comparison of MTT assay results between two groups. They have not significant differences ($P > 0.05$). C: Control group. 1 kHz: The group has been affected by 1 kHz electric field

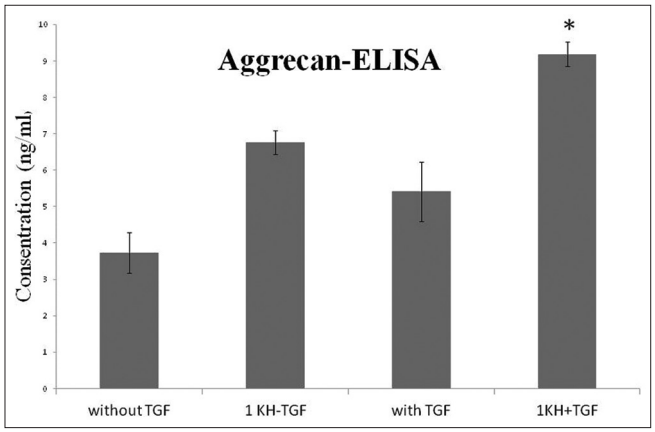

Figure 6: The results of enzyme-linked immunosorbent assay analysis for aggrecan in supernatant media of groups at 7th day. Values are the mean ± standard error of triplicate experiments. *$P < 0.05$ versus control group. C- transforming growth factor (TGF): Control group without transforming growth factor. 1 kHz-TGF: The group has been affected by 1 kHz electric field (EF) without TGF-β3. TGF: The group has been affected by TGF-β3. 1 kHz + TGF: The group has been affected by 1 kHz EF and TGF-β3

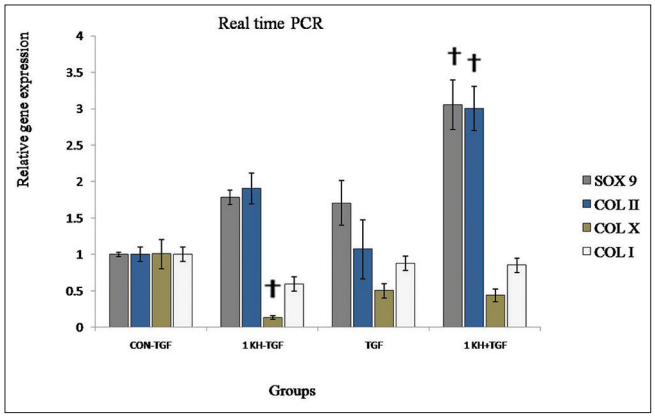

Figure 7: Results of real time polymerase chain reaction for type I, II and X collagen and SOX_9 genes in all groups. Values are the mean ± standard error of triplicate experiments. †$P < 0.001$ versus control group. C- transforming growth factor (TGF): control group without TGF-β3. 1 KH-TGF: the group has been affected by 1 kHz EF without TGF-β3. TGF: the group has been affected by TGF. 1 KH + TGF: the group has been affected by 1 kHz electric field and TGF-β3

outside the culture, direct probe placement within bone tissue culture, which induce an alternating EF within the tissue.[22,26] In this regard, we tested effects of selective and specific capacitively coupled electrical signals in chondrogenesis in ADSCs.We found in our study that handling low frequency EF and TGF-β_3 at the same time lead to induce ADSCs to chondrocyte so that these cells express type II collagen and SOX_9 genes (special genes in chondrogenesis), Although, as it was mentioned earlier, these effects were fortified by using of TGF-β 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 and the method of culture and also the features of our EF, are different from other researches. Clearly, our investigation is a novel study in which effects of low frequency EF on chondrogenesis has been examined and it was in the following of our previous study.[17] However, the effect of EF on various cells have been carried out in the past studies. For example, Szasz N. use of 25 mA/cm^2 1 kHz for 20 h found that the 20 h application of a 1 kHz EF increased DNA synthesis by 35% on days 6-9 of culture and statistically significant increase in total cell count for all samples and culture periods.[23,27] In a study done by MacGinitie *et al*. use of 1 kHz 10-30 mA/cm^2 increase protein synthesis in articular cartilage explants.[26,28] Hronik-Tupaj *et al*. differentiated Mesenchymal Stem Cell human to osteogenesis. They demonstrated that use of 20 mv/cm 60 kHz EF 40 min/day can increase in alkaline phosphatase (ALP) mRNA and collagen I mRNA.[27,29] Hammerick *et al*. in differentiated ASCm to Osteogenesis by pulsed 6 V/cm 50 hz EF 6 h/day. They found increase in cytoskeletal tension, ALP, osteopontin, runt-related transcription factor 2, COL I, cytosolic free calcium and no significant change in cell proliferation. Hammerick *et al*. use of 1V/cm electric field 4 h/day differentiated Adipose Derived Stem Cell Human to osteogenesis. They found increase in mineral deposition, increase of calcium within cells.[28,30] Brighton *et al*. use of 20 mv/cm 60 kHz EF, 6 h/day found a significant increase in proliferation of cells but no change in ALP activity.[29,31] Wang *et al*. use of 20 mv/cm 60 kHz, 1 h/day and found up-regulation of AGC, COL II genes expression in chondrocytes.[30,32] Brighton *et al*. use of 20 mv/cm 60 kHz, 1 h/day and found proliferation of bovine articular cartilage chondrocyte and up-regulation of AGC, COL II genes expression.[31,33] In the mentioned studies, it has been reported that 1 kHz frequency is the effective frequency on cartilage proliferation and biosynthesis improvement. Also some researches were shown biologic effects of (0-1 kHz) frequency interval is so near to the physiologic frequency of body cells.[24,26,28,32] Therefore, in this paper we chose this frequency to examine its effect on chondrogenesis for the first time. Researchers have

been concluded that the MSCs chondrogenesis in *in-vitro* furthermore expression of type II collagen and SOX_9 genes also express type I and X collagens genes and therefore, leading into fibrocartilage and terminal hypertrophy (initiation of ossification) respectively.[25,33] In our present study it became clear that low frequency EF promoted chondrogenesis and partly inhibited the expression of type I and X collagens.

CONCLUSION AND SUGGESTION

The low frequency EF was found as a safe and cheap inducer in chondrogenic differentiation of human ADSCs micromass cultures. Next studies should be designed about how can get access to significant chondrogenesis just with electric stimulator without any chemical inducers.

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Conflicts of interest

There are no conflicts of interest.

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