Original Article

Optimizing a novel method for low intensity ultrasound in chondrogenesis induction

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Abstract

Background: Hyaline cartilage tissue of joints is susceptible to injuries due to avascularity. Mesenchymal stem cells (MSCs) are used for cartilage tissue engineering. Among MSCs, adipose stem cells (ASCs) are attractive because of accessibility, their large number, and rapid growth. Common *in vitro* protocols successfully induce chondrogenic differentiation by expression of multiple cartilage-specific molecules. However, transforming growth factor β (TGFβ) promotes chondrogenesis to terminal stages. Despite much attention being given to the influences of biochemical factors on chondrogenesis of MSCs, few studies have examined the chondrogenic effect of mechanical factors such as ultrasound as a feasible tool.

Materials and Methods: In this study, we focused on inducing chondrogenesis in the early stages of differentiation by using low-intensity ultrasound (LIUS). Four groups of ASC pellets (control, ultrasound, TGFβ, and ultrasound/TGF) were cultured under chondrogenic (10 ng/ml of TGFβ3) and ultrasound conditions (200 mW/cm^2, 10 min/day). After 2 weeks, differentiation was evaluated by histology, quantitative gene expression analysis, and immunohistochemistry.

Results: Our data demonstrated that ultrasound differentiated pellets showed increased expression of early chondrogenesis marker, *Col2A*, than those in TGFβ groups ($P < 0.001$), and *Col2B* and *Col10* expression were more prominent in TGFβ groups. Immunostaining of sections showed Col2 fibrils around lacuna in LIUS and TGFβ treated groups.

Conclusion: Using LIUS resulted in early chondrogenesis in comparison with terminally differentiated chondrocytes by TGFβ. Therefore, LIUS might provide an applicable, safe, efficient, and cheap tool for chondrogenic differentiation of ASCs in cartilage tissue engineering.

Key Words: Adipose stem cell, cartilage tissue engineering, chondrocyte, chondrogenesis, low intensity ultrasound

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INTRODUCTION

Articular cartilage (AC) damage and degeneration are among the most common disorders due to avascularity that results in limited capacity for self-repair. On the other hand, still there is no efficient treatment for this kind of diseases.[1] However, tissue engineering approaches by using mesenchymal stem cells (MSCs) are promising and adipose tissue has been described as an alternative source for autologous adult MSCs.[2] Zuk *et al*. showed that these cells can differentiate toward the adipogenic, chondrogenic, neurogenic, osteogenic, and myogenic lineages.[3]

Chondrogenesis of MSCs requires inducers such as transforming growth factor β (TGFβ).[4-6] But the end result of this differentiation often is terminal differentiation with transient cartilage properties.[7,8] Previous studies have found other types of chondrogenic stimulants such as mechanical loadings. Numerous publications have shown the positive effects of biomechanical conditioning such as mechanical compression and hydrostatic pressure on the chondrogenic differentiation of MSCs. Angele *et al*. showed that cyclic hydrostatic pressure stimulated chondrogenic differentiation of bone marrow derived MSCs (BM-MSCs) in pellet culture.[9] Studies of transplanted MSCs reported that application of mechanical stimuli improved cartilage healing in the rabbit knee joint. Huang *et al*. suggested that cyclic compressive loading could promote chondrogenesis of rabbit BM-MSCs by inducing endogenous TGFβ.[9-12] However, among the mechanical loading types, ultrasound is a noninvasive, cheap, and easy to apply tool. Biological effects of ultrasound on chondrocytes and cartilage metabolism have also presented some promising results.[13] Ultrasound has also been found to be effective on chondrogenesis of bone marrow MSCs. These studies have used ultrasound transducer directly on cells like chondrocytes or MSCs in order to induce chondrogenesis differentiation.[14,15] While for *in vivo* conditions or future clinical applications, it is necessary to consider the distance between transducer of ultrasound waves and target cells. Thus, we designed a novel method for calculating this distance to induce chondrogenesis on adipose stem cells (ASCs). The goal of this study was to optimize the conditions for chondrogenesis of ASCs by low intensity ultrasound (LIUS) and to compare chondrogenic stage of chondrocytes that were differentiated by ultrasound and TGFβ3.

MATERIALS AND METHODS

Adipose stem cells isolation and culture

Informed consent and local ethical committee approval were obtained for the use of adipose tissue specimens for this research. ASCs were isolated from subcutaneous adipose tissue that was harvested from patients undergoing elective surgical procedures. Cells were isolated from adipose tissue using methods previously described with minor modifications.[16] Briefly, the obtained tissue was washed with phosphate-buffered saline (PBS) to remove red blood cells, chopped into small pieces of about 25-50 mm^3, and the extracellular matrix was digested for 60 min at 37°C with collagenase I (0.5 mg) for each gram of adipose tissue, St. Louis, MO, USAin PBS. The ASC-containing cell suspension was centrifuged at 600 g and the pellet was resuspended in culture medium, which was composed of Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 500 μg/ml streptomycin sulfate (Sigma), 600 μg/ml penicillin (Sigma), and 10% placental human serum.[17] Cultures were washed with PBS buffer after 24 h plating to remove unattached cells and erythrocytes, and then re-fed with fresh medium.[17,18] Cultures were maintained at 37°C with 5% CO2 and fed two times per week.

For pellet culture, ASCs were differentiated in 15-ml Falcon tubes. ASC pellets were formed by centrifuging 2×10^5 cells at 500 g in serum-free basal chondrogenic medium consisting of high glucose DMEM (DMEM-HG; Gibco, Paisley, Renfrewshire, UK), 10^{-7} M dexamethasone (Sigma, St. Louis, MO, USA), 200 μM ascorbic acid 2-phosphate (Sigma), 1% bovine serum albumin (BSA; Sigma), Insulin-Transferrin-Selenium (ITS; Gibco, Paisley, Renfrewshire, UK), and 1% streptomycin sulfate (Sigma)/penicillin (Sigma). Four groups of (control, ultrasound, TGFβ, and ultrasound/TGFβ) pellets were cultured in basal chondrogenic medium for 14 days. In this study, we added TGFβ3 (10 ng/ml) to basal chondrogenic medium for TGFβ containing groups.

Low-intensity ultrasound stimulation

With the LIUS device (Novin, Tehran, Iran), continuous wave at 1 MHz and intensity of 200 mW/cm^2 was applied for 10 min/day in ultrasound groups. The distance between transducer and cultures was determined by the Sarvazyan method [Figure 1a].[19-21] A chamber was designed based on the distance determined [Figure 1b and c] and was covered with sound absorbent material for reducing reflected beams [Figure 1d]. Ultrasound treatment was carried out in this water-filled chamber that was placed in an incubator at 37°C. Our instrument consists of an ultrasound generator and transducer coupled tank in incubator. All experiments were performed on three sets of samples.

Histological and immunocytochemical analysis

Pellets were harvested and fixed in 10% buffered

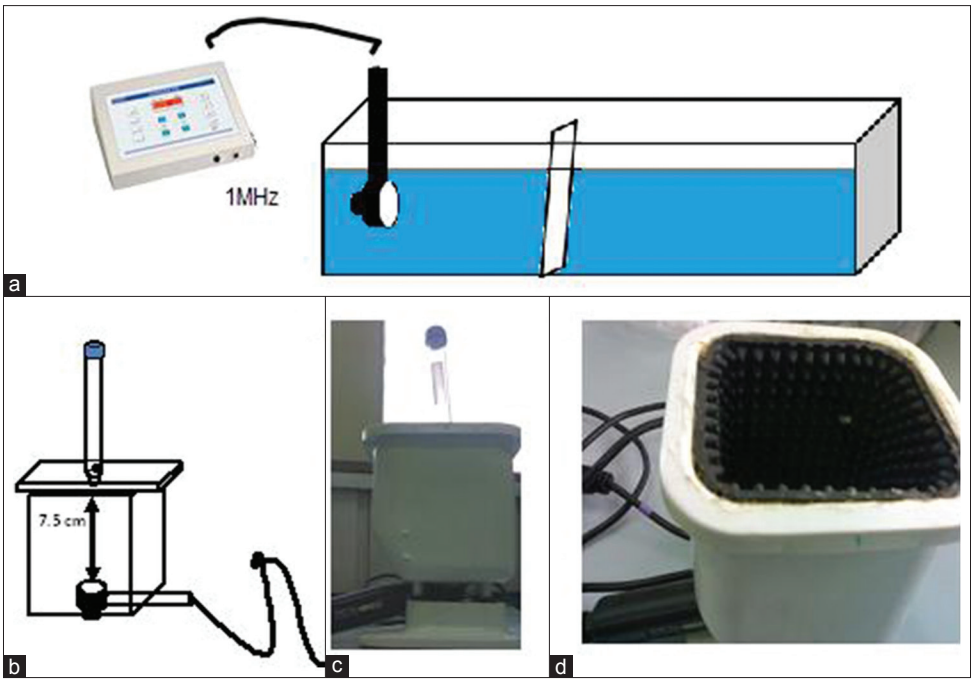

Figure 1: Instruments used for LIUS calibration and stimulations. (a) Determining optimal distance, (b) Schematic view (b) The chamber for LIUS exposure to ASCs pelltes. (d) Inside of chamber for LIUS exposure

formalin for 2 h at room temperature. The fixed pellets, after tissue possessing, were embedded in paraffin and routinely stained. Also, proteoglycans were visualized by staining with toluidine blue for 5 min at 60°C. For immunocytochemical assessment, sections post fixation with acetone for 5 min were washed in PBS (pH 7.5) for 30 min at 37°C and then peroxidase activity was blocked by a 30-min incubation in 0.3% H2O2 in ethanol. After two 5-min rinses with PBS, the pellets were pretreated with 1 mg pepsin (Sigma) (1 ml in 0.5 M acetic acid) for 40 min at 37°C for optimal antigen retrieval and then were washed two times for 5 min with PBS. The procedure was followed by overnight incubation at room temperature with the indicated antibodies in PBS containing 0.1% BSA. Primary antibodies to collagen type II and aggrecan (1-100) were obtained from Serotec, Oxford, UK. All incubations were performed in a humidified chamber. After extensive washing with PBS to remove residual primary antibody, reactivity was detected using secondary antibody (Horse radish peroxidise; DAKO cytomation, cyan, Ely, UK) for 60 min at room temperature. Peroxidase activity was visualized using diaminobenzidine (DAB) as the substrate (DAKO cytomation). The sections were incubated for 10 min. The reaction was stopped by rinsing with water and the sections were counterstained with Hematoxylin (Merck; Darmstadt, Germany). The sections were dehydrated through graded alcohols, cleared with xylene, and permanently mounted.

Gene expression

RNA isolation was performed using Trizol (Invitrogen, Eugene, OR, USA). After cDNA synthesis (Ferementase), real-time polymerase chain reaction (PCR) was performed in two separate wells for each of our samples (ABI SYBR Green kit). Undiluted cDNA (2 μl) was used in 20 μl PCR mix. Relative gene expression of aggrecan (AGG), collagen types 2A and B and collagen type 10 of the treatment groups was determined to housekeeping gene expression (18s) and then normalized to untreated (day 0) ASCs.

Statistical analysis

Data were obtained from three independent donors. For statistical analysis, the data were presented as means ± SEM. Significances were tested by one-way analysis of variance (ANOVA) in Graph Pad Prism program. Differences were considered significant if $P < 0.05$.

RESULTS

After 2 weeks of chondrogenic differentiation using LIUS and TGFβ, the gene expression results of this study show that LIUS induces chondrogenesis in ASCs. The expression of *Col2A* increased significantly in the LIUS group versus control and TGFβ groups [Figure 2a]. However, *Col2B* and aggrecan expressions were very high in the TGFβ group ($P < 0.001$) [Figure 2b]. LIUS stimulated *Col2B* and aggrecan in comparison to the control group, but was not

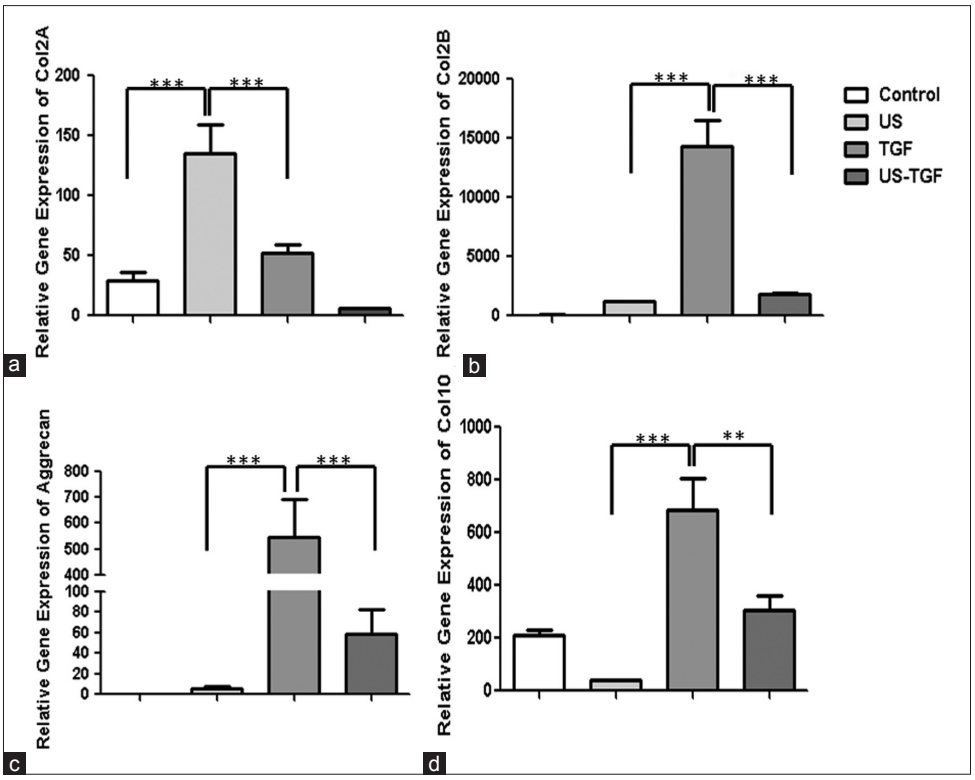

Figure 2: Effect of LIUS stimulation on the gene expression of cartilage differentiation markers. The cells were cultured for up to 14 days with or without LIPUS and TGFβ stimulation for 10 min/day. The mRNA expression of Col2A, Col2B, aggrecan and Col 10 were determined using real-time PCR. The data shown are the mean ± SEM of three separate experiments. ** means is P<0.01 and *** means is P<0.001

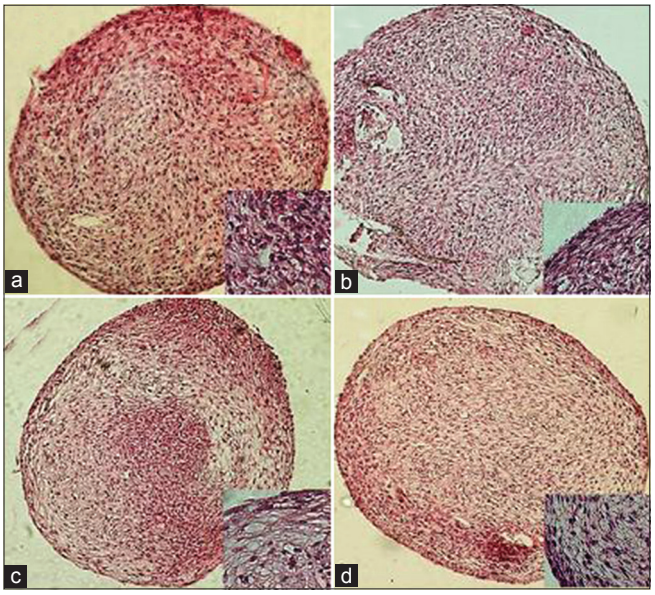

Figure 3: Micrographs of H and E staining of four experimental groups of ASC pellets, ×60 (inset, ×150); (a) Control; (b) LIUS; (c) TGF; and (d) LIUS–TGF

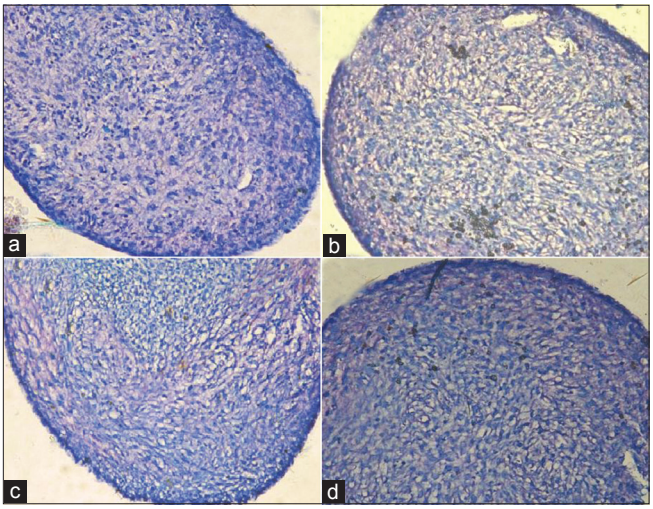

Figure 4: Toluidine blue staining of four experimental groups of ASCs, ×100: (a) Control; (b) LIUS; (c) TGF; and (d) LIUS–TGF

statistically significant [Figure 2b and c]. Also, collagen 10 expression was dramatically prominent in the TGFβ group versus LIUS containing groups.

The routine histologic results of sections showed cartilage-like appearance in induced ASCs [Figure 3 c-d] and larger lacunas in TGFβ containing groups [Figure 3c and d, inset]. Toluidine blue staining revealed metachromasia in experimental groups because of proteoglycan [Figure 4].

Collagen type 2 is an essential component of hyaline cartilage. Our results demonstrated the expression following LIUS and TGFβ treatment, suggesting that

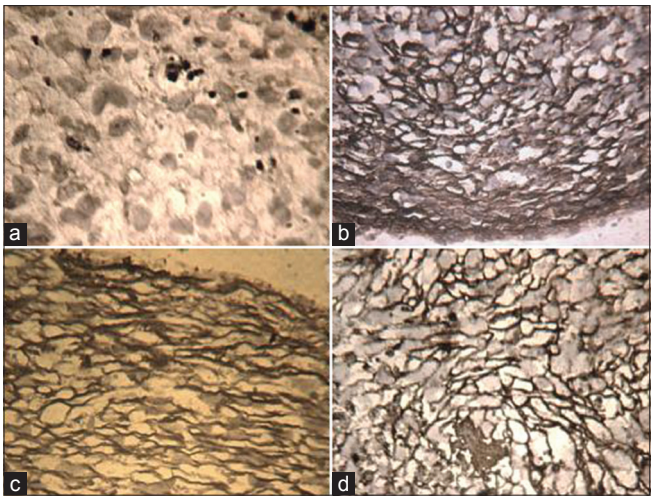

Figure 5: Effect of LIUS and TGF; on the chondrogenic differentiation of ASCs. Immunostaining of collagen in sections of experimental pellets: (a) Control; (b) LIUS; (c) TGF; and (d) LIUS–TGF

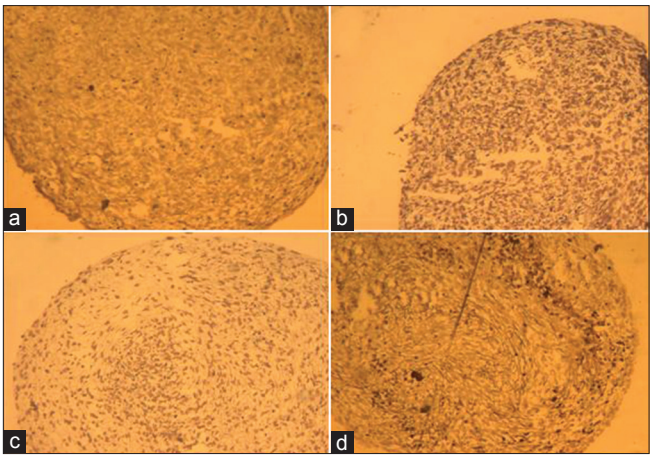

Figure 6: Effect of LIUS and TGF; on the chondrogenic differentiation of ASCs. Immunostaining of aggrecan in sections of experimental pellets: (a) Control; (b) LIUS; (c) TGF; and (d) LIUS–TGF

increased secretion of collagen 2 had occurred in the LIUS group similar to TGFβ group. Following Col2 antibody staining, the secreted Col2 around lacuna was strongly positive in both the LIUS group and TGFβ group. There were also no apparent differences in Col2 fibril positivity between the three experimental groups and no thick fibrils in the control group [Figure 5]. Also, we showed that there was more aggrecan protein in TGF pellets than in those treated with LIUS. These results were parallel with our gene expression observation [Figure 6].

DISCUSSION

Based on the current knowledge of tissue engineering technology and adipose tissue stem cell technology,[2] we have formulated an innovative physical approach for chondrogenic differentiation. In this method, LIUS produced functional chondrocytes in contrast to TGFβ differentiated chondrocytes, which were almost useless because of unwanted production of collagen type 10. Both induction methods can differentiate ASCs to chondrocytes which can express collagen type 2 protein, but gene expression results revealed LIUS produces *Col2A* more than *Col2B*. Type IIA collagen is the splice variant of type II collagen that has been found in prechondrocytes and immature chondrocytes. Johnstone *et al.*'s study showed that when chondrogenesis was achieved, the morphology of the aggregate changed from the appearance of a mesenchymal cell condensation to that of cellular cartilage, as seen in embryonic limb formation.[23] Furthermore, at day 7 post-aggregation, the presence of type IIA collagen mRNA was detected by reverse transcription (RT)-PCR.[5]

Optimization of chondrogenesis protocol to generate stable cartilage is necessary for clinical use. Permanent cartilage development has not been elucidated; however, it is obvious that physical activity of fetus play important role in joint development.[22,23] Our focus is to obtain early stage of chondrocytes from ASCs that are suitable for transplantation in articular cartilage defects. A pervious study has found dynamic compressive loading in the early stage of BM-MSC chondrogenesis.[11] This finding confirmed the results of this study by LIUS. Maintenance of the chondrocyte phenotype is critical for the formation of cartilaginous matrices cell therapies using autologous chondrocytes for repairing cartilage injuries. Tein showed that low-intensity pulsed ultrasound (LIPUS) stimulates aggrecan and type II collagen synthesis in chondrocytes.[24]

LIPUS showed similar stimulatory effects on aggrecan and type II collagen synthesis of human chondrocytes; the effects on synthesis of aggrecan appeared earlier than that of type II collagen. Several studies have found that exposure to PLIUS can significantly modulate the chondrocyte functions.[25, 30-32]

Some investigations used LIUS of pulsed wave at 30 mW/cm^2 and showed ultrasound maintains chondrocyte properties *in vitro* and *in vivo*,[1,15] Park *et al*. used directly continuous wave at 200 mW/cm^2 to induce chondrogenic differentiation,[33] but we applied this mode of waves and the same intensity at 7.5 cm distance. There are major differences between studies of this kind, therefore discrepancy of results could be caused by differences in the cell source, with or without scaffold, and LIUS stimulation mode, particularly transducer–cell distance. It is possible that the LIUS stimulation mode could be the most critical factor and LIUS activity could be dependent on TGFβ.[31] Findings suggest that there is a complex network for the control

of MSC chondrogenesis. On the other hand, the exact mechanism of the effect of mechanical loadings such as ultrasound on MSCs has not been elucidated.

CONCLUSION

Our new method of ultrasound application can induce chondrogenesis in ASCs. LIUS interestingly produces chondrocytes in early stages instead of TGFβ that it is desirable for cartilage tissue engineering purposes. Thus, LIUS can be used as an efficient tool for future clinical applications.

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