

Induction of Chondrogenic Differentiation of Human Adipose-Derived Stem Cells with TGF- β 3 in Pellet Culture System

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Abstract

Objective

Adult stem cells which are derived from different tissues, with their unique abilities to self-renew and differentiate into various phenotypes have the potential for cell therapy and tissue engineering. Human adipose tissue is an appropriate source of mesenchymal stem cells with wide differentiation potential for tissue engineering research. In this study isolated stem cells from human subcutaneous adipose tissue were investigated for chondrogenic potential of adipose-derived stem cells (ADSCs) in pellet culture system treated with transforming growth factor- β 3 (TGF- β 3).

Materials and Methods

Human ADSCs were isolated from subcutaneous adipose tissue and digested with collagenase type I. Immunocytochemical method for cell surface antigens was done in order to characterize the cells. The isolated cells were treated with chondrogenic medium, supplemented with TGF- β 3 in pellet culture system and harvested after 21 days. Histological staining was used to evaluate the presence of proteoglycan, with alcian blue. Immunohistochemical method performed for the assessment of cartilage-specific type II collagen and aggrecan. Also, in order to confirm our results, we managed RT-PCR technique.

Results

Chondrogenesis of ADSCs in pellet culture, induced by TGF- β 3 growth factor. Histological and immunohistochemical methods showed deposition of typical cartilage extracellular matrix components in pellets. RT-PCR analysis of cartilage matrix genes, such as type II collagen and aggrecan, also, confirmed the induction of the chondrocytic phenotype in high-density culture upon stimulation with TGF- β 3.

Conclusion

TGF- β 3 promoted chondrogenesis of ADSC in pellet culture system. We suggest that human subcutaneous adipose stem cells could be excellent candidates for the cartilage tissue engineering.

Keywords: Adipose, Chondrogenesis, Stem cell, Tissue engineering, TGF- β 3

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Introduction

Cartilage tissue damage is a rather worldwide problem of many people (1). Osteoarthritis as a result of lesions in the articular cartilage is the most common cause of disability, after trauma or degenerative joint diseases.

Cartilage tissue is an avascular, aneural tissue with limited capacity for self-repair (2). Current treatment methods for cartilage tissue injuries include chondral shaving, subchondral drilling, tissue debridement, microfracture of the subchondral bone, transplantation of autologous and allogeneic osteochondral grafts. The most common clinical treatment for an articular cartilage lesion is to create bleeding microfracture within the subchondral bone in an attempt to stimulate cartilage regeneration (3). While some of these approaches show promise, many lead to formation of fibrous tissue, apoptosis, and further cartilage degeneration (4-6). In addition the repaired tissue in these circumstances lacks the biomechanical properties of normal articular cartilage, leading to inconsistent long-term outcomes (7).

Recently many efforts have been undertaken to repair articular cartilage lesions by transplantation of autologous chondrocytes. A major problem of human chondrocytes in culture is the phenomenon of dedifferentiation. In addition, autologous chondrocyte transplantation has been associated with significant donor-site morbidity and the initiation of osteoarthritic changes in the joint (8). Thus, an alternative cell source is necessary for the repair of cartilage.

Mesenchymal stem cells (MSCs) provide a source for fascinating models of differentiation (9, 10), regenerative medicine (11) and cell therapy (12). There are increasing reports that MSCs can be isolated from various adult mesenchymal tissues such as bone marrow (13), periosteum (14), synovium (15), skeletal muscle (16), adipose tissue (17), dermis (18), blood (19) and trabecular bone (20).

Mesenchymal stem cells with chondrogenic potential are present in many tissues of the body. Those of the adipose tissue are particular interest because, could be harvest easily in large quantities with minimal possibility of

morbidity and discomfort, clinically (21). In addition, the uncomplicated enzyme-based isolation procedures make adipose tissue an attractive source for researchers and clinicians of nearly all medicinal subspecialization (22).

The induction of chondrogenesis of ADSCs appears to require a rounded cell shape through pellet culture or a 3-Dimensional (3-D) hydrogel (23). Pellet culture system that was originally described as a method for preventing the phenotypic modulation of chondrocytes *in vitro* (24) allows cell-cell interactions, analogous to those that occur in precartilage condensation during embryonic development (25).

However, this cell configuration is not sufficient for the induction of chondrogenesis. The chondrogenic differentiation of the ADSCs required the certain bioactive factors. Many studies have shown that various growth factors such as bone morphogenetic protein-2 and -3 (26, 27) as well as transforming growth factor- β_1 and β_2 (28, 29) can induce mesenchymal stem cells to differentiate into chondrocytes under certain culture conditions. The transforming growth factor- β_3 is the essential growth factor for promoting chondrogenesis *in vivo* (30). This growth factor has significantly increased Sox-9 expression; however, TGF- β_3 cannot alone induces human bone marrow-derived stem cells into completed chondrocyte (31). The effect of TGF- β_3 on chondrogenesis of ADSCs in pellet culture system has not yet been recorded. Therefore, in this study we decided to isolate and culture the subcutaneous adipose derived stem cells and evaluate chondrogenic differentiation potential of ADSCs in pellet culture system, using TGF- β_3 .

Materials and Methods

Isolation of ADSCs

Human subcutaneous adipose tissue was isolated from healthy donors (n=10; age: 24-50) undergoing cesarean surgery.

Adipose tissue was minced into small pieces (1-2 mm³), washed with phosphate-buffered saline (PBS) and digested with 1.5 mg of collagenase type I per mg of fat tissue for 60 min at 37 °C, with continuous shaking. The

floating adipocytes separated from the stromal cells fraction, by multiple centrifugations and washing steps. Red blood cells were removed, using lysis buffer. Stromal cells counted by hemocytometer and plated with 3000 cells/cm² in tissue culture flasks containing DMEM, 10% FBS, 1% Penicillin-streptomycin and incubated at 37 °C, 5% CO₂ conditions. After 24 hrs with changing of medium, nonadherent cells discarded.

Culture media replaced every 2-3 days and the cultures allowed to reach 80% confluence, before trypsinization and replating.

Investigation of cell marker by immunocytochemical method

Although, the isolation of ADSCs was based on the adherence to plastic surface, but in order to characterize these cells further, immunocytochemical study used for detecting of cell surface antigens.

Cell markers of hematopoietic lineage cells CD34, CD45, and mesenchymal cell markers CD44, CD105, receptors of hyaluronan, and TGF-β3 were investigated respectively.

Cells of the passage 4 plated on cover slips and grown until reaching the confluence. They were washed again with PBS two times and fixed with 4% Para formaldehyde for 20 min. Then they washed with PBS, and incubated in acetone and goat serum for 30 min at room temperature. Primary antibodies for CD34, CD44, CD45 and CD105 markers (DAKO Cytomation, Denmark) were applied for 60 min at 37 °C and then washed with PBS. The HRP- labeled secondary antibody applied for 2 hrs at 37 °C, followed by washing with PBS. Diaminobanzidin was used for staining. Counterstaining of cells was done using hematoxylin (Merck, Germany).

Chondrogenic differentiation

ADSCs from the passage 4, harvested with trypsin-EDTA and 2×10⁵ cells/ml centrifuged at 1400 rpm for 8 min in a 15 ml polypropylene conical tube. Then cells were resuspended in chondrogenic medium (CM), consisting of DMEM-high Glucose (Gibco), 1% insulin-transferring-selenium (Sigma), 1.25 mg/ml bovine serum albumin (Sigma), 5 mg/ml linoleic acid (Sigma), 50 mg/ml ascorbate-2-phosphate (Sigma), 1% penicillin-streptomycin (Gibco),

and 10 ng/ml transforming growth factor-β3 (Sigma). After repeating centrifugation at 1400 rpm for 8 min, pellets were cultivated at 37 °C, 5% CO₂ for 21 days. The medium changed every 3 days, and the same medium without TGF-β3 used as the control.

Histological and immunohistochemical analysis

After 21 days of culturing, pellets were harvested, fixed in 10 % formalin for 2 hrs, dehydrated with ethanol, cleared with xylene and finally embedded in paraffin. Then, they sectioned at 5 μm thickness for hematoxylin and eosin (H-E) staining. Sulfated glycosaminoglycans (GAG) were assessed by alcian blue staining.

Immunohistochemical method for evaluation of type II collagen and aggrecan was performed. Briefly, the tissue sections were deparaffinized, treated with trypsin for 30 min to facilitate antibody access. Then, 3% hydrogen peroxide in ethanol for 30 min was added to suppress the endogenous peroxidase activity. Monoclonal antibodies against type II collagen (Serotec) and aggrecan (Serotec) were applied and allowed to react for overnight at 4 °C. Sections incubated with the secondary antibody, rabbit anti-mouse immunoglobulin (DAKO Cytomation, Denmark) for 60 min and the existence of collagen type II plus aggrecan in the extracellular matrix was revealed.

RT-PCR analysis

Total cellular RNA isolated by RNX-plus solution (CinnaGen Inc Iran). First strand complementary DNA (cDNA) synthesized from 1 μg total RNA using oligo(dt) as a primer and Revert Aid TM First-strand cDNA synthesis kit (Fermentase), according to the manufacturer's instructions. Subsequent PCR performed as follows: 2.5 μl cDNA, 1X PCR buffer, 200 μM dNTPs, 1 μM of each primer pair (Table 1), and 1 unit / 25 ml reaction Taq DNA Polymerase (Fermentas). Amplification conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 45 sec and extension at 72 °C for 45 sec. The products analyzed on 1% agarose gel and visualized by ethidium bromide staining.

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Table 1. Sequences of different primers were used for RT-PCR.

Name of genes	Sequences of primers	Product size
Aggrecan	F- AGGAGGGCTGGAACAAGTACC R- GGAGGTGGTAATTCCAGGGAACA	350 bp
Collagen II	F- CAGGTCAAGATGGTC R- TTCAGCACCTGTCTCACCA	370 bp
GAPDH	F- GGGCTGCTTTTAACTCTGGT R- GCAGGTTTTTCTAGACGG	680 bp

Results

Cell culture

After 2 days of culturing and at the first changing of medium, spindle bipolar to polygonal fibroblast-like cells, attached to the flask were observed (Figure 1). After 10 to 13 days, the cell culture reached to confluence. Cells in the passage 4 showed uniform monolayer (Figure 2) and needed approximately 1 week to reach 80% confluence. Expanded mesenchymal stem cells displayed a phenotypic single population, which was uniformly negative for hematopoietic antigens such as the early hematopoietic stem cell marker (CD34) (Figure 3), the leukocyte common antigen (CD45) (Figure 4). These MSCs were positive for typical MSC – related antigens, expressing a receptor for TGF- β 3 (endoglin, CD105) (Figure 5), and the hyaluronan receptor (CD44) (Figure 6).

Chondrogenic differentiation

Adipose-derived mesenchymal stem cells in the passage 4 were trypsinized to detach from the surface of flask, cultured for 21 days in high-density system and treated with TGF- β 3. The resulted products, spherical in shape, appeared with more than 1 mm in diameter and 1×10^{-3} g in weight (Figure 7). The chondrogenic potential of ADSCs characterized histologically by H-E and alcian blue staining.

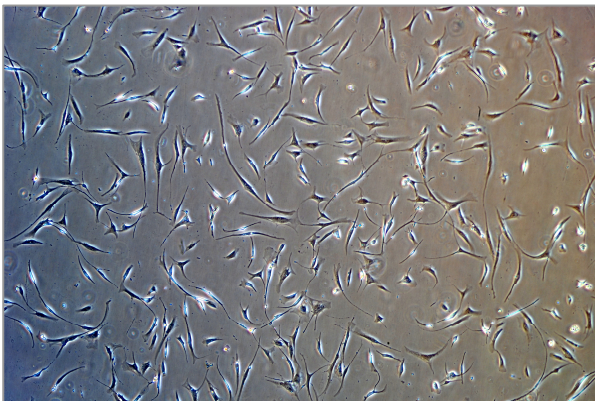


Figure 1. Human subcutaneous adipose-derived stem cells in primary culture after 2 days ($\times 40$).

The histological sections showed a chondrocyte-like cell morphology which located in lacunae (Figure 8). Furthermore, sections of pellets showed the accumulation of extracellular matrix. For showing the existence of proteoglycans in extracellular matrix, metachromatic staining with alcian blue used and the results, indicated matrix of cartilage in sections (Figure 9). To confirm the results, immunohistochemical (IHC) analysis for type II collagen and aggrecan carried out using monoclonal-specific antibody. Positive immunostaining was observed in sections and the existence of type II collagen and aggrecan revealed (Figures 10, 11).

RT-PCR

Total RNA was extracted from pellets on the day 21. Both type II collagen and aggrecan mRNA expression were detected in pellets culture after 21 days (Figure 12). In pellets that were not treated by TGF- β 3, type II collagen and aggrecan mRNA were not depicted. In addition there was no expression of chondrocyte-specific genes in ADSCs from the monolayer culture (Figure 12).

Overall the results showed that, chondrogenesis of subcutaneous adipose stem cells was induced with TGF- β 3 in pellet culture system.

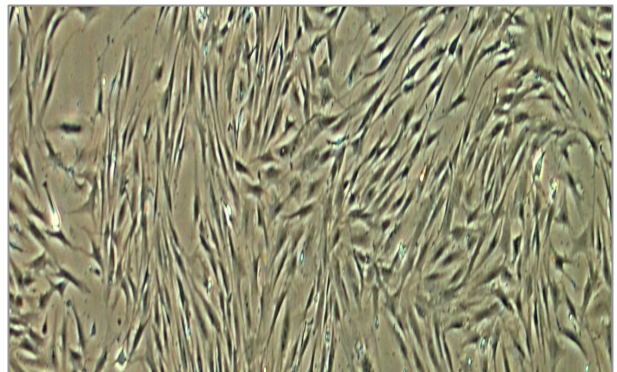


Figure 2. Adipose-derived stem cells have reached 80% confluence in the passage 4. Cells show uniform shape in monolayer culture ($\times 40$).

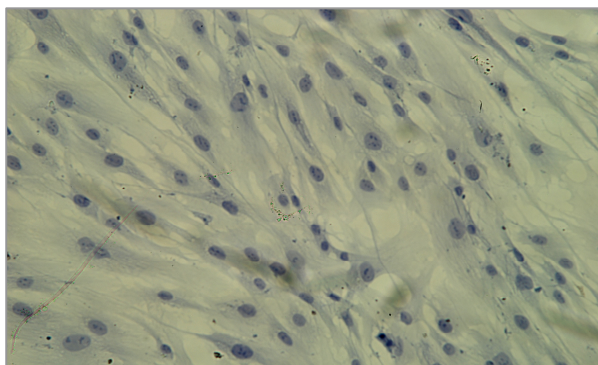


Figure 3. Immunocytochemical method shown that ADSCs were not expressed CD34, a hematopoietic marker, thus cells stained with hematoxylin ($\times 400$).

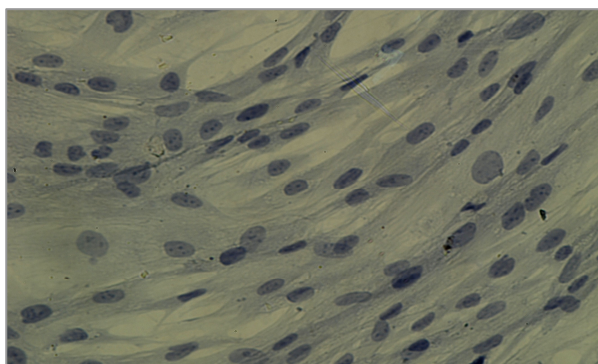


Figure 4. Immunocytochemical method shown that ADSCs were not expressed CD45, a leukocyte common antigen, thus cells stained with hematoxylin ($\times 400$).

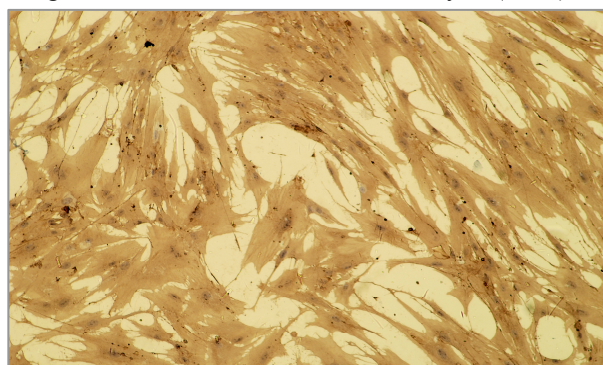


Figure 5. Immunocytochemical method shown ADSCs express CD105 marker, transforming growth factor- $\beta 3$ receptor ($\times 400$).

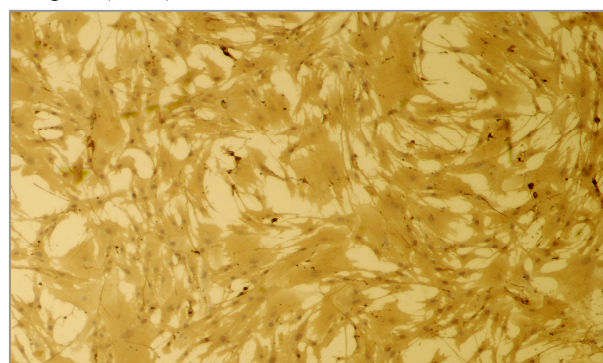


Figure 6. Immunocytochemical method shown ADSCs express CD44 marker, hyaluronan receptor ($\times 400$).

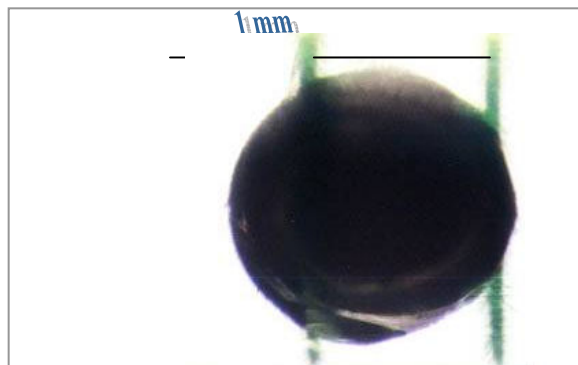


Figure 7. Gross appearance of construct after 21 days treatment with TGF- $\beta 3$ in pellet culture system ($\times 40$).

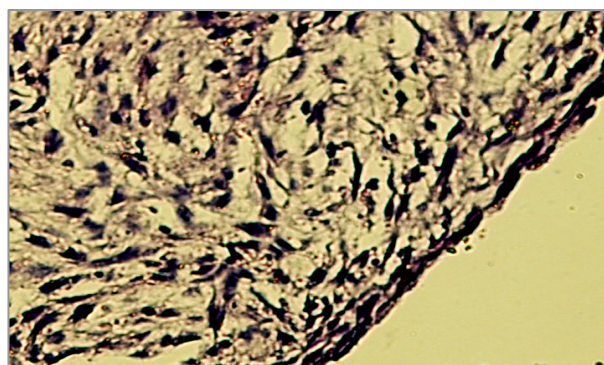


Figure 8. The section of the resulted construct. Chondrocytes are located in lacuna (H-E) ($\times 400$).

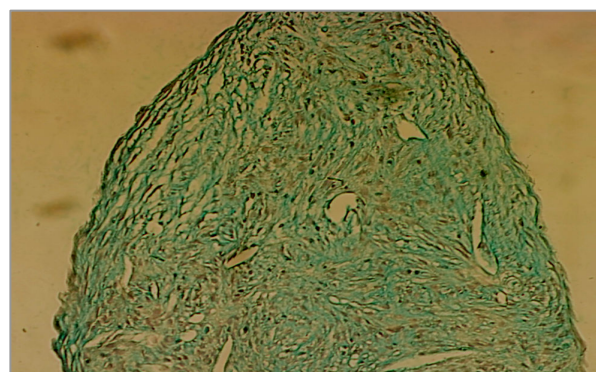


Figure 9. Alcian blue staining of resulted tissue. Existence of methachromatic extracellular matrix in tissue was revealed ($\times 200$).

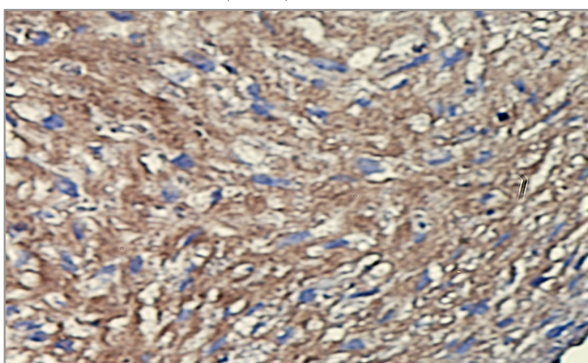


Figure 10. Immunohistochemical method revealed existence of aggrecan in extracellular matrix after chondrogenic induction ($\times 400$).

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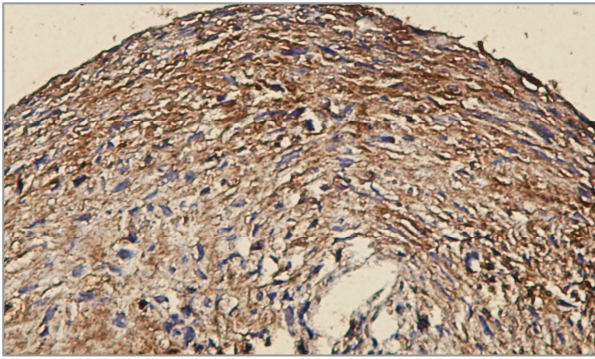


Figure 11. Immunohistochemical method revealed existence of type II collagen in extracellular matrix after chondrogenic induction ($\times 300$).

Discussion

At the present study, we confirmed the chondrogenic capacity of ADSCs in the presence of TGF-β3. ADSCs were isolated from the human adipose tissue that obtained from cesarean surgery. Previous studies characterized ADSCs and revealed that ADSCs have multi – lineage potential, but, in those studies fat tissue isolated in the liposuction surgery (17, 31, 32).

Characterization of MSCs has been performed using the expression of cell – specific proteins and CD markers (27, 33, 34). We showed that like MSCs, ADSC cells express CD44, CD105 markers but, do not express the CD34 and CD45 antigens. Our results demonstrated that surgery procedure had no effect on the surface markers.

Many researches have shown that the essential conditions for chondrogenesis from human MSCs are a 3-D culture or high density cell culture, and the growth factors (17, 27, 31, 32).

Pellet or micromass culture system was originally described as a method for preventing the phenotypic modulation of chondrocytes (24). Since the introduction of micromass culture system by Johnston *et al* (27), scientists have utilized this system for different purposes. Some researchers have used this culture system to evaluate the chondrogenic potential of the cells isolated by different methods (35, 36). Others used it to study the role of macromolecules such as TGF-β family in promoting chondrogenic differentiation (37, 38).

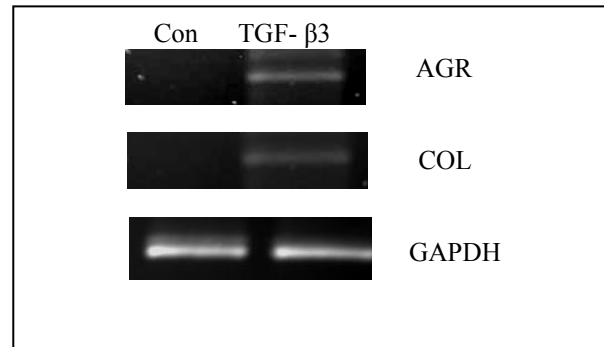


Figure 12. RT-PCR results showed expression of cartilage specific molecules, aggrecan (AGR) and type II collagen (COL) in TGF-β3 treated group. GAPDH used as housekeeping gene.

Our investigation showed that the pellet having been cultured in chondrogenic medium supplemented with TGF-β3 for 21 days, differentiated into tissue composed of chondrocyte-like cells and a plenty of matrix accumulated among them.

Since the cartilage-specific molecules were produced in pellets as evidenced by RT-PCR and immunohistochemical methods, we concluded that the tissue could be cartilage.

Zuk *et al* isolated processed lipoaspirate cells (PLA) from human adipose tissue and induced them into adipogenic, osteogenic, chondrogenic and neurogenic lineage cells. Also, they used TGF-β1 for chondrogenic differentiation in micromass culture, during 2 weeks (17), while in the present study we used TGF-β3 growth factor, in order to do chondrogenic induction for the first time in pellet culture, during 3 weeks.

Some researchers that investigated chondrogenic potential of human adipose-derived stem cells, used growth factor of TGF-β1 in pellet culture system or various scaffolds (23, 39, 40).

Estes and colleagues studied chondrogenesis of ADSCs influenced with TGF-β3 and other growth factors on alginate scaffold, during 1 week (32), however, we did not used any scaffold in our study.

Jin *et al* in 2007 demonstrated that human ADSC could be induced into chondrocyte-like phenotype in pellet culture system using human TGF-β2 treatment *in vitro* (31). They used TGF-β2 while we used TGF-β3 growth factor. In addition Jin *et al.* showed the

expression of the chondrocyte marker genes at 7 and 14 days of the induction but, in our study the expression of cartilage specific-genes was revealed after 21 days of induction.

To our knowledge, this is the first report that TGF- β 3 can induce differentiation of ADSCs into the chondrocyte in pellet culture system.

Conclusion

In summary, our study demonstrated the chondrogenesis of human subcutaneous adipose-derived stem cells, induced by TGF- β 3 in the pellet culture, for the first time

in Iran. Therefore, ADSCs, induced with appropriate growth factors, may serve as a valuable source of easily accessible mesenchymal stem cells, for producing stem cell derived cartilage.

Acknowledgements

The authors would like to express their gratitude to the Vice-Chancellor for Research of Isfahan University of Medical Sciences, to Dr Peyman Adibi, Dr. Haghighat, Obstetrics and Gynecologist, also, to Miss Sadeghi, MSc and Mrs Amuzegar, histological laboratory technician for their kind collaboration.

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