International Journal of Stem Cells Vol. 8, No. 2, 2015 http://dx.doi.org/10.15283/ijsc.2015.8.2.219

ORIGINAL ARTICLE

Fibrin Scaffolds Designing in order to Human Adipose-derived Mesenchymal Stem Cells Differentiation to Chondrocytes in the Presence of TGF-β3

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Background and Objectives: One of the most cellular source used for cartilage tissue engineering are mesenchymal stem cells (MSCs). In present study, human MSCs were used as cellular source. Since scaffold plays an important role in tissue engineering the aim of this study is to assess fibrin scaffold ability in chondrogenic differentiation of adipose-derived mesenchymal stem cells (ADMSCs).
Methods: ADMSCs were isolated and cultured in DMEM medium supplemented with 10% FBS. Also ADMSCs expanded and characterised by flow cytometry. ADMSCs expressed CD44, CD90, CD105 but not CD34. After trypsinization, cells were entered within the fibrin scaffold. Then, chondrogenic medium was added to the scaffold. Seven days after cell culture, cell viability and proliferation were assessed by MTT test. Finally, 14 days after the ending of chondrogenic differentiation, analysis of chondrogenic genes expression was evaluated by RT-PCR and Real time PCR. Also, formation and development of chondrocyte cells was analysed by histological and immunohistochemistry evaluations.
Results: Viability and proliferation as well as chondrogenic genes expression within fibrin scaffold increased significantly compared with control group (cells free scaffold). Also, histological and immunohistochemistry evaluation showed that chondrocyte cells and collagen type II are formed on fibrin scaffold.
Conclusions: Fibrin is a suitable scaffold for chondrogenic differentiation of ADMSCs.

Keywords: Tissue Engineering, Adipose-Derived Mesenchymal Stem Cells (ADMSCs), Chondrogenic Differentiation, Fibrin

Introduction

Cartilage defects repair have been considered extremely difficult due to very slow turnover at the cellular and molecular level and also the low regenerative capacity of articular chondrocytes (1-4). This restricted self-repair capability of the cartilage forced scholars to develop new technologies and appropriate biomaterials to advance tissue integration. In recent years, cartilage regeneration has obtained nice success by method of tissue engineering (5). The tissue engineering has created as a new approach for repairing damaged or disabled tissues/organs such as cartilage, bone and skin (5-7). During tissue engineering the healing capacity of the sick is increased, thereby damaged tissues can be repaired and returned its natural functions (5, 6). An appropriate cell source is a fundamental requirement for a successful tissue engineering application (5, 7). Cellular sources that have been used extensively are chondrocytes and MSCs (6). The select of cell type defines the strategy of cartilage tissue engineering (5). There are obstacles to the chondrocytes-based cell therapy (6). Recent studies have demonstrated that the proliferative capacity

Accepted for publication May 9, 2015, Published online November 30, 2015
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of the terminal differentiated chondrocytes was decreased with in vitro expansion (2). In addition, MSCs have advantages over chondrocytes, since they can be obtained in autologous form in a minimally invasive procedure (4). While the procedure required to harvest autologous cells may initiate joint degeneration (3). Due to this reason, many efforts are being done to use MSCs for cartilage tissue engineering (4). MSCs are multipotent cells which are found in bone marrow, muscle, skin and fat tissue (8). These cells have ability to differentiation into tissues of mesenchymal origin, includin muscle, bone, tandem, adipose tissue and cartilage (9). Lately, research has demonstrated that stem cells derived from human adipose tissue contain multipotent progenitor cells that can differentiate into osteogenic, chondrogenic, myogenic, and neurogenic cells when induced by the suitable biological factors in vitro (10-12). The use of ADMSCs in cartilage tissue engineering requires a lot of research in the field of cell scaffolds (4). The scaffold plays an important role as physical suport and template in tissue engineering by supplying a 3D substrate for cell growth and tissue regeneration (2, 13). The ideal scaffold should have mechanical properties like tissue, display immunologic integrity, and support cell adhesion, migration and differentiation (14). Also, scaffold should not initiate an immunological or 'foreign body' response in the sick (15). Scaffolds can be made of natural materials, of synthetic materials or both (hybrid scaffolds) (16, 17). Natural materials have physiological activities, including cell adhesion and biodegradability (16). Fibrin is one of the natural materials that have high potential for use in tissue engineering (17, 18). It can be isolated from the patient blood and used as an autologous scaffold, without the potential risk of a foreign body reaction or infections (17, 19, 20). In addition, fibrin plays a special role in angiogenesis (21). This is useful for tissue engineering, because one of the main problems in engineered tissues is the limited blood supply (22). During wound healing the fibrin provides an appropriate environment for angiogenesis and thereby recovery of the blood supply (23, 24). Also, fibrin is used in stem cell research (19). Because its cell adhesive properties, biocompatibility and biodegradation (18). Upon contact with fibrin, cells will gradually replace the fibrin scaffold by an adult tissue-specific extracellular matrix (4). According to, these features make fibrin an interesting and widely used protein for tissue engineered scaffolds (17). also, The differentiation medium required to induce chondrogenic differentiation of ADMSCs usually contains a cocktail of growth factors (12). Transforming growth factor-β (TGF-β3) is considered as the most important component (12). TGF-β3 has been regarded as the most powerful chondrogenic growth factor, which induces significant chondrogenic phenotype of ASCs both in vitro and in vivo (4, 12).

The aim of this study was to evaluate the ability of fibrin as a natural scaffold for Cell differentiation into cartilage tissue and protect them for growth and expand their in vitro.

Materials and Methods

Materials

Dulbecco's minimum essential medium (DMEM) for cell culture was purchased from Sigma and fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (BRL, Gaithersburg, MD, USA).

Isolation and culture of human ADMSCs

Adipose tissue was harvested from patients by elective surgery (liposuction) and transferred to the laboratory under sterilized condition. Previously, consent was obtained from each patient. Adipose tissue mechanically chopped and washed with PBS (Sigma－Aldrich, St. Louis, MO, USA). Afterward, it was digested with 5 mg/ml collagenase type I (Sigma－Aldrich, St. Louis, MO, USA) at 37°C for 45～60 min. Then, the cell solution was centrifuged at 1800 rpm for 10 min. At the end, the supernatant was removed and the resultant pellet was resuspended in medium contained DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and then cultured in a humidified atmosphere of 95% air, 5% CO2 at 37°C. Medium was replaced every 3 days. When the cells reached 90% confluence at passage 2, ADMSCs were harvested with 0.25% trypsin and used at a density of 1×10^6 cells for any scaffold.

Confirmation of Human Mesenchymal Stem/Stromal Cell Identity by Analysis of MSC Marker Expression

The expression of surface markers of MSCs including CD34, CD44, CD90, and CD105 was tested with flow cytometry technique. Upon reaching 95% confluence in culture, the cells were resuspended in phosphate buffered saline at 1 million/ml. 100 μl of this cell suspension is taken in each Falcon the Fluorescence Activated Cell Sorter (FACS) tubes. Monoclonal antibodies against human CD44 (abcam, Cambridge, USA), CD90 (abcam, Cambridge, USA), CD105 (abcam, Cambridge, USA) and CD34 (abcam, Cambridge, USA), were used. Also, the appropriate amount of conjugated antibody or isotype control is added to each FACS tube. Tubes are incubated at room temperature in the dark for 15～20 min. After the incubation, the cells

are washed with 2 ml of phosphate buffered saline (Ca^{2+} free) and run through a BD FACS Calibur flow cytometer (Becton Dickinson, California, USA). Typically for each tube, 500,000 cells are collected and the data are analyzed using Cell Quest version 3.0.1 software (Becton Dickinson, California, USA). the positive expression was described as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

Preparation of fibrinogen and thrombin

Fibrinogen and Fresh frozen plasma solutions (FFP) were obtained from Iranian Blood Transfusion Organization. FFP was thawed in water bath at 37°C. Then 15 mL of it was mixed with 10 mL of calcium glucuronate (mid 5:3 Ratio). The obtained solution was incubated for 1 h at 37°C and subsequently centrifuged at 2200 rpm for 10 min. The supernatant was collected as thrombin. Fibrinogen and thrombin solutions were prepared for use as cell culture. To this end, the adipose tissue-derived stem cells at a concentration of 5×10^6 cells per ml were dissolved within thrombin and subsequently fibrinogen was added to them. Then the scaffolds in chondrogenic medium containing DMEM-high glucose along with 50 μg/mL ascorbate 2- phosphate, 1% insulin-transferin-selenium (Sigma－Aldrich, St. Louis, MO, USA), dexamethasone 100 nM (Sigma－Aldrich, St. Louis, MO, USA), 50 mg/mL FBS, 5 μg/mL linoleic acid (Sigma－Aldrich, St. Louis, MO, USA), 1% penicillin/streptomycin and 10 ng/ml transforming growth factor-β_3 (Sigma－Aldrich, St. Louis, MO, USA) was placed in the incubator (37°C, 5% CO2, 99% humidity) for 14 days.

Cell viability measurement

At first, the human adult stem cells were seeded within the scaffolds in chondrogenic medium and then were incubated for 7 days (37°C, 5% CO2, 99% humidity). Cells viability and proliferation were assessed by MTT (3-(4, 5-dimethyl) thiazol-2-yl-2, 5-dimethyl tetrazolium bromide, 5 mg/ml). Compound MTT was added per well after removing chondrogenic medium and incubated at 37°C for 4h. Then, the medium was discarded and intracellular formazan was solubilized by adding 400 μl of dimethyl sulphoxide. The absorbance of each well was read at 570 nm with immunosorbent assay (ELISA) plate reader (Hiperion MPR4, Germany). The ADMSCs without scaffold in MTT assay were also applied as controls and the data was subtracted from measured values. This assay was performed in triplicate.

RT-PCR analysis

Total RNA was isolated from adipose derived mesenchymal stem cells using Trizol® according to the manufacturer's protocol (Invitrogen Life Technologies, Burlington, Ontario, Canada). β-actin was amplified as an control. primers of cartilage-specific genes were designed as follows utilizing primer 3 program that it's primer sequences is shown in Table 1.

After synthesis of single stranded cDNA, RT-PCR was performed. The expression of several cartilage-specific genes (type I and II collagen, SOX9 and aggrecan) was analyzed by RT-PCR.

For this purpose, each reaction was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining and the images were analyzed by the Gel Doc imaging system (Bio-Rad, Hercules, CA, USA).

Expression analysis of chondrogenic genes by Real time PCR

Expression of cartilage-specific markers (type I and II collagen, Sox9 and aggrecan) was evaluated of 14 days af-

Table 1. Primer sequences used for RT-PCR and Real-time PCR

Primer Name	Sequences (3'→5')	Refrence
Collagen type I (COL2A1) Forward	CGTCCAGATGACCTTCCTACG	NM_001844
Colla Collagen type I (COL2A1) Reverse	TGAGCAGGGCCTTCTTGAG	
Collagen type II (COL1A2) Forward	CAGGAAACAGCTATGACC	NM_000089
Collagen type II (COL1A2) Reverse	CTACTCTCAGCCCAGGAGGTCCTG	
Aggrecan (ACAN) Forward	AGGCAGCGTGATCCTTACC	NM_001135
Aggrecan (ACAN) Reverse	GGCCTCTCCAGTCTCATTCTC	
SOX9 Forward	GTACCCGCACTTGCACAAC	NM_000346
SOX9 Reverse	TCTCGCTCTCGTTCAGAAGTC	
GAPDH Forward	CGCTCTCTGCTCCTCCTGTT	NM_001256799
GAPDH Reverse	CCATGGTGTCTGAGCGATGT	
β-Actin Forward	AATCTGGCACCACACCTTCTAC	NM_001101
β-Actin Reverse	CATCTTCCACACCACGGTCTAA	

ter the ending of chondrogenic differentiation. The scaffolds were degraded within liquid nitrogen and then total RNA was extracted of all samples using an AccuZol^{TM} (bioNEER, Daedeok-gu, Daejeon, Korea) in accordance with the manufacturer's protocol. Afterwards, the reverse transcription of RNA was carried to produce a complementary DNA (cDNA) using the AccuPower® RT PreMix (bioNEER, Daedeok-gu, Daejeon, Korea). Real-time PCR was performed using SYBRGreen PCR Master Mix and Rotor-Gene^{TM} 6000 Series Software version 1.7.65 (Corbett Life Science, Australia), and primers of each gene were designed as follows utilizing primer 3 program that it's primer sequences is presented in Table 1:

The reaction was initiated by heating to 95°C for 15 min., followed by 40 cycles of elongation at 59°C for 30 sec and denaturation at 95°C for 15 sec.

Target gene was normalized based on glyceraldehyde 3-phosphate dehydrogenase reference gene. The level of expression of each target gene was calculated using $2^{-\triangle\triangle Ct}$.

Histological Examination

14 days after the chondrogenic induction, samples of ADSCs that were seeded on fibrin glue scaffolds and control cultures were rinsed thoroughly with PBS and fixed in 10% formalin for 24 hours. Then, samples after the dehydration with ethanols, were embedded in parafin and sectioned at 5 μm thickness. Finally, samples were stained with hematoxylin/eosin.

Immunohistochemistry

Formaldehyde-fixed tissues were used for the immunohistochemical analysis. Immunohistochemistry was performed using the streptavidin-biotin method, with a SAB-PO kit (Nichirei Co. Ltd., Tokyo, Japan). For immunohistochemical analysis, collagen type II antibody was used as a first antibody. Immunohistochemistry with the collagen type II antibody was performed using the same procedure (streptavidin-biotin method) on freshly frozen tissue sections. Sections 5 mm thick were cut using a cryostat microtome (Bright, Huntingdon, UK), and dried at room temperature for 30 min. After fixation in acetone for 10 min, the sample sections were treated with 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity.

Results

Expression of cell surface markers

As reported in Fig. 2, ASCs identified positive expersion for CD44, CD90, CD105 and negative for CD34, at passage 3 (Fig. 1).

Cell proliferation and viability

Cell viability and proliferation were assessed by MTT

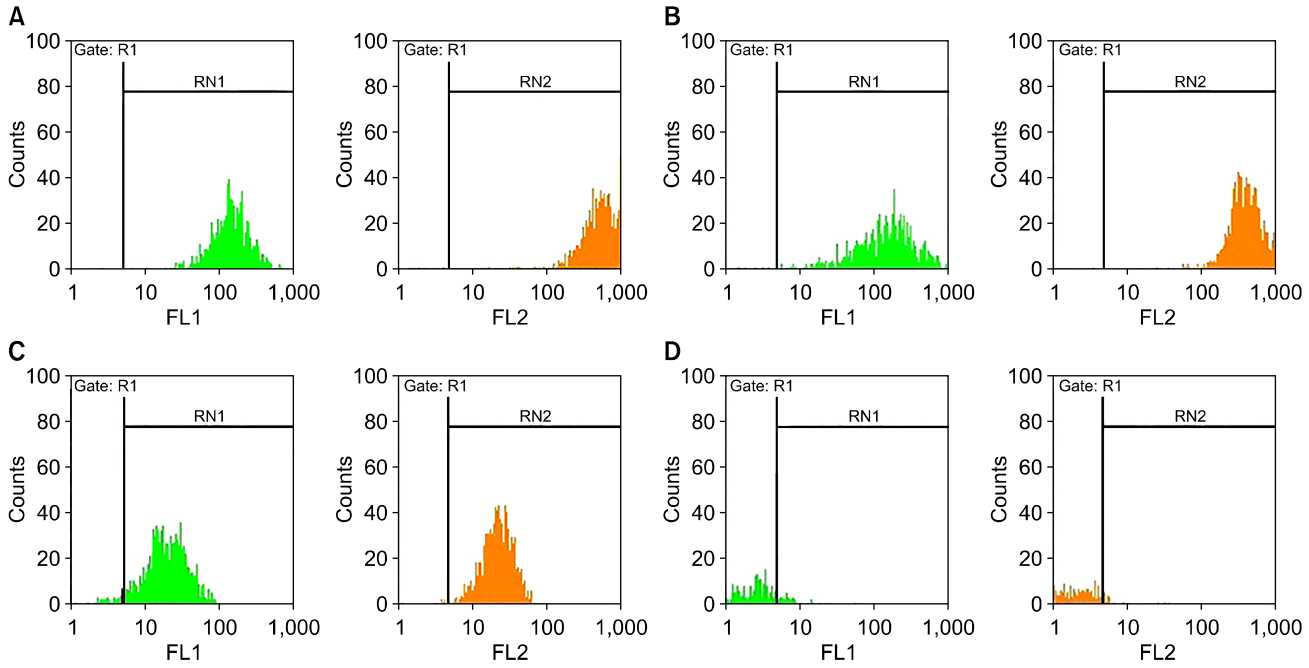

Fig. 1. Flow cytometeric analysis for the determination of the surface markers of MSCs expression. (A) CD44, (B) CD90, (C) CD105 and (D) CD34.

at day 7. Our study demonstrated that the fibrin scaffold significantly increased viability and proliferation cells compared with control group (cells free scaffold) ($p<0.05$) (Fig. 2).

The morphology of adipose derived mesenchymal stem cells

In the study of living and not stained cells using phase contrast microscope, they were determined as small cells with little cytoplasm and elliptic central core. Also, in the primary levels of culture they had a few short cytoplasmic frills. Arriving at the third passage, homogeneity and uniformity in culture of isolated cells was fully determined (Fig. 3).

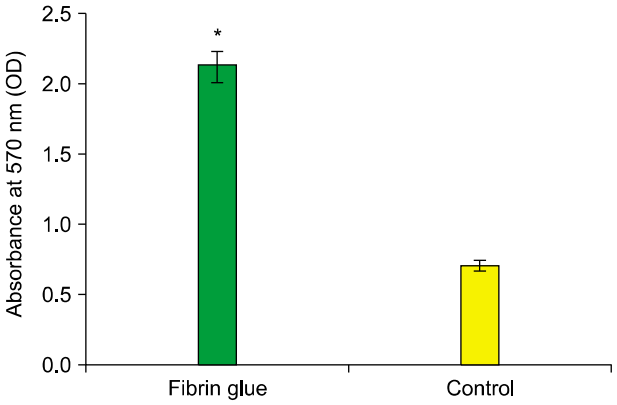

Fig. 2. The comparison of cell viability between MSCs embedded in fibrin scaffold and control group.

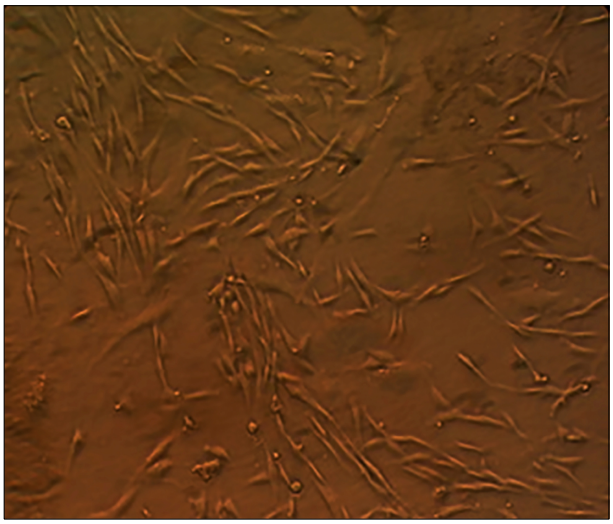

Fig. 3. Image produced by invert microscope of living mesenchymal stem cells isolated from human adipose tissue which spindle cells in the third passage are visible. ×40.

Chondrogenic differentiation

The mRNA expression of collagen I and II, SOX9 and aggrecan was tested to evaluate the differentiated status of ADMSCs on the fibrin scaffold after 14 days of chondrogenic induction. Statistical analysis has shown a significant difference in the expression of all genes (type I and II collagen, SOX9 and aggrecan) between cell/fibrin and ADMSCs ($p<0.05$) (Fig. 4 and 5).

Histological evaluation

With hematoxyline and eosin, the present of chondrocyte in the differentiated cell on the fibrin glue scaffold were determined after 14 days (Fig. 6).

Immunohistochemistry analysis

The finding of this study indicate that the accumulation of type II collagen around the cells, in fibrin gel, gradually appeared over the two weeks after culture and differentiation period (Fig. 7).

Statistical Analysis

ANOVA was used to analysis of viability and chondrogenic differentiation of cells in fibrin glue scaffolds. Data is presented as means plus or minus one standard error

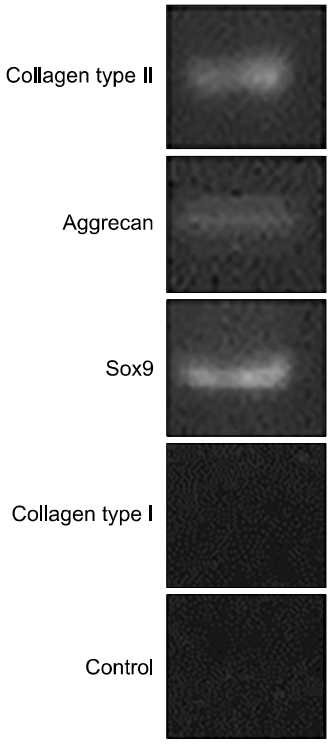

Fig. 4. The analysis of collagen Type II, Aggrecan and SOX9 genes expression in cell/fibrin using RT-PCR method.

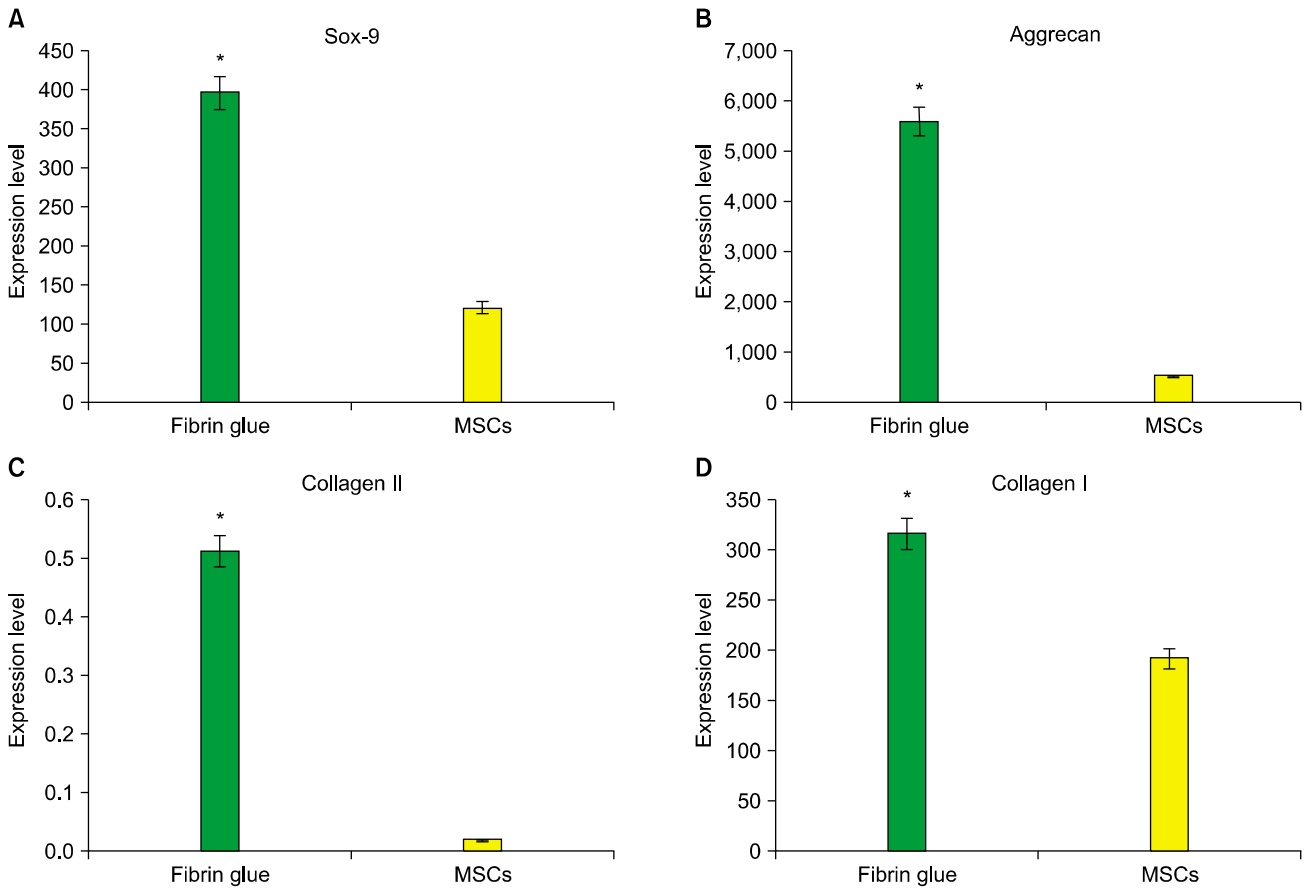

Fig. 5. The expression of collagen I, Aggrecan, SOX9 and Collagen II in cell/fibrin vs. MSCs.

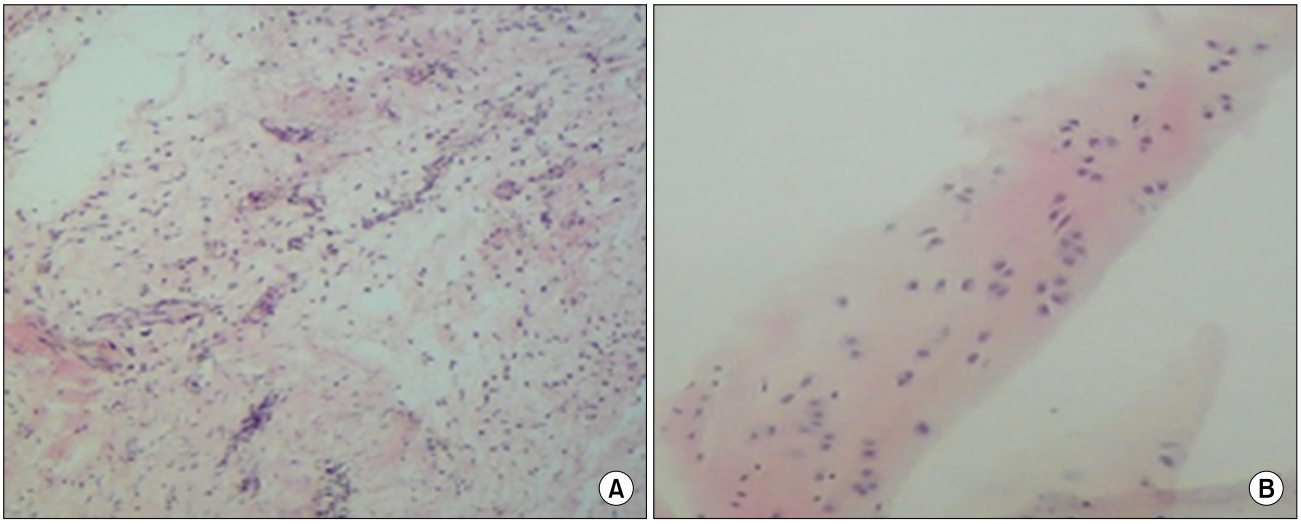

Fig. 6. Histological examination of the ADMSCs using haematoxylin and eosin staining. After 4 weeks there was significant cartilage formation with high cell density in ADMSCs seeded on the Fibrin glue scaffold (original magnification ×10) (A). Staining of native cartilage used as control with hematoxylin/eosin is demonstrated in (B) (×50 magnification).

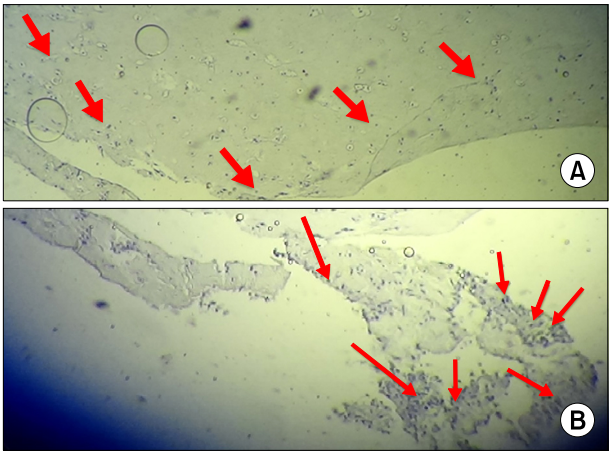

Fig. 7. The analysis of collagen type II antibody (arrowheads) in cell/fibrin (A) and control group (B) using immunohistochemistry evaluation.

of the mean throughout. Significance is defined as $p<0.05$.

Discussion

Since the cartilage defects due to lack of blood vessels and nerves cannot repair spontaneously, tissue engineering can provide the possibility of achieving to cartilage (25). Adult mesenchymal stem cells have the potential for growth and differentiation to the chondrocyte (12). The previous studies have clearly shown that MSC inhibit T-cell responses induced by mitogens and alloantigens (26). Therefore, they are the ideal candidates for cartilage tissue regeneration (12). MSC isolated from several types of tissues have the potential to differentiate into mesoderm cell lineages, especially chondrocytes (26-28). In a recent study, several types of MSCs, derived from adipose tissue, bone marrow, were encapsulated in fibrin hydrogel with TGF-β3 and then evaluated for their differentiation capacity (19). The results showed that of all three types of MSCs, cartilage cells were produced (19, 28). ADMSCs in comparison with bone marrow MSCs have several advantages including feasibility of harvesting in a large amount with a simple, repeatable, and minimally invasive method, the high frequency of MSCs, fast and easy expansion in culture, and higher passage cells still retaining stem cell phenotypes and pluripotency (29-31). In addition, the main benefits of ADMSCs compared with bone marrow mesenchymal stem cells are less effect of age or morbidity of patients on quality (32-34). The several experimental studies suggest that ADMSCs reduce hypertrophy and dedifferentiation of chondrocytes, protect against joint destruction, and decrease the development and progress of osteoarthritis (35-38). Our results are also consistent with previous research and show that ADMSCs are an appealing source for the treatment of osteoarthritis. Therefore, considering the numerous benefits of using ADMSCs, in the present study, they were used as the cell source. In this study, we corroborated the previous findings that ADMSCs cells can differentiate into cartilage under defined culture conditions. The researches have shown that the cell's response to chondrogenic mediators may depend on the physical and biological properties of scaffold. Considering the previous studies, the subject is quite apparent that the scaffold selection influences the growth and differentiation of adult stem cells (39). Fibrin is a natural scaffold which can be applied autologously and has an important role in angiogenesis as well as having biodegradability and biocompatibility. In addition, it can achieve high seeding efficiency and uniform cell distribution. Due to the hydrophilic nature of fibrin and high amounts of water, it can be used as appropriate extracellular material for providing cell growth (19). Therefore in this study, the ability of fibrin to maintain cell viability was assessed to detect a suitable environment for inducing chondrogenic differentiation in ADMSCs. The results of MTT assay showed that the survival ability of cells in fibrin scaffold compared to controls increased significantly (Fig. 2). The following results are supporting for our review:

In a study Pelaez et al. (39) the capability of fibrin hydrogels to support chondrogenesis of bone marrow mesenchymal stem cells was evaluated. This study confirmed the suitability of fibrin hydrogel for supporting chondrogenesis and improved viability, proliferation and chondrogenic differentiation. Also, in a study conducted by Ho et al. (40) fibrin was introduced as an optimized environment promoting chondrogenesis of ADMSCs in vitro (40). Various methods are used to prove the existence of cartilage in obtained structures resulted of chondrogenic differentiation induction (41). As it has been stated in many studies, evidence suggests that the chondrogenesis, by increasing the expression and accumulation of collagen type II and aggrecan is known and TGF-β can also be effective on Chondrogenesis (42-44). Previous studies demonstrated that TGF-β3 led to significantly higher collagen type II expression of ADMSCs (4, 12).

In this research, using real time PCR technique was found that increased the expression of all genes within fibrin scaffolds was found compared to the control group ($p<0.05$). However, as seen in Fig. 5B and C, ADMSCs encapsulated in fibrin a highly the enhancement in expression of collagen type II and aggrecan genes (as the

chondrogenesis indicator) compared to the control group ($p<0.05$). These results with the results of Ho et al. and Ahmed et al. were consistent (40, 43). These observations indicate that chondrogenesis induction using fibrin scaffold was successful.

Conclusion

According to the results of our study, the fibrin is an appropriate scaffold for chondrogenic differentiation of ADMSCs and to protect of these cells for growth and expand their in vitro.

Acknowledgements

This work was funded by grants from The Academic Center for Education, Culture and Research, Qom Branch, Qom, Iran.

Potential conflict of interest

authors have no conflicting financial interest.

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