

Original Article

Use of Mesenchymal Adult Stem Cell for Cartilage Regeneration by Hydrogel

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ABSTRACT

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Background and Aims: Cartilage is a very specific tissue, which does not have the capacity to heal and renew itself. Although the invention of the method of surgery with autologous chondrocyte transplantation, developed tools to treat the cartilage lesions, it couldn't gain a great success due to problems such as damage to the area of donation. Using the mesenchymal stem cells derived from adipose and culturing and differentiating them on scaffolds was considered appropriate as a successful research and clinical strategy.

Materials and Methods: In the present study, the mesenchymal stem cells were separated from adipose tissue and cultured in two scaffolds of fibrin glue and alginate medium. After 1, 7 and 14 days of cell differentiation, the survival ability of the differentiated cells were analyzed by Chondrogenic MTT. Moreover, type I and II collagen, aggrecan and Sox9 expression were measured via real time-polymerase chain reaction. In addition, cartilage reconstruction on scaffolds was shown by a histological investigation.

Results: Our results showed that the expression of CD90 and CD105 as mesenchymal markers is at a high level whereas the expression of CD34 and CD45 reaches a low level. The LSD test demonstrated that there was no remarkable difference among the chondrogenic MTT, scaffolds groups and control in 7 and 14 days after cell differentiation ($p < 0.05$), although, fibrin glue had the highest expression in chondrogenic gens.

Conclusions: Finding suggests that in order to utilize a new strategy for tissue regeneration utilization of inherent scaffolds such as fibrin glue can act as a protector for mesenchymal stem cells.

Introduction

Over the recent decades, the limited capacity with the self-renewal ability of cartilage tissue has been an obstacle in the way of repairing and regenerating the injuries in this tissue. In recent years, by means of suitable cell source and transplantation systems, new technology such as tissue engineering is being developed for repairing cartilaginous defects [1]. Although autologous chondrocyte transplantation is an approved treatment, this method is rarely used due to the low proliferative capacity of these cells *in vitro* as well as the side effects produced by it in the chondrocytes. However, now the clinical application of cell *in vitro* and the side effects it has in the chondrocytes have made this method to be less employed. Nevertheless, currently, the clinical application of bone marrow-derived mesenchymal stem cells (BM-MSC) has been considered a challenge due to damage to the place of donation and the invasive nature of this method, as well as the pain it imposes on the donor [2]. Adipose-derived mesenchymal stem cells (ADSCs) are becoming increasingly popular as an alternative for BM-MSC in the field of cartilage tissue engineering [3]. These cells can be gained by simple liposuction from subcutaneous fat tissue with the least side effects and the least pain for the donor. The reports suggest that regarding the ability to differentiate into several categories and growth and aging, the MSCs are analogous to mesenchymal stem cells of bone marrow [4, 5]. In addition, morphological analysis and markers expression has been shown that ADSCs are similar to the cells

obtained from bone marrow [6]. It is understood that there is a need for three dimensional culture conditions in order to guide the differentiation of ADSCs into cartilage [7]. Therefore, these cells are cultured on scaffolds *in vitro*. The scaffolds are bio-based materials divided into two categories. First, these materials were used to transfer drug and hormones in the body; however, the next experiments showed that these materials have the ability to provide the best conditions for maintenance and differentiation of cells for designing a scaffold with a suitable physical structure, the possibility of adhesion, movement, expansion, and differentiation of cells, as well as growth and amendment of the new tissue [8]. In tissue engineering, at first, you need a porous material as the extracellular matrix or scaffold for cell growth to be produced. Therefore, the hydrogel scaffolds derived from biological materials that are capable of creating favorable conditions for growth and differentiation of cells can be used to amend soft tissues such as cartilage [9]. Among these biological materials, natural polymers such as alginate and fibrin glue are known to be used in the clinical and experimental study of cartilage. Alginate is a linear polysaccharide with duplicate blocks of Mannuronate and Glucuronate. Due to proper thickness as well as a stem cell delivery system, it is used for recovery of cartilage injuries and the research field related to cartilage tissue engineering [10, 11]. Used for more than 20 years, fibrin is a combination of physiological blood [11]. In recent years, owing to its unique

characteristics, such as compatibility, cell degradation, as well as being autologous fibrin has had different utilizations in the tissue regeneration field. In addition, fibrin glue has been approved by the Food and Drug Administration [12-16].

Materials and Methods

In this study, for culturing cells, Alginate and DMEM medium (modified eagle's medium of Dulbecco) were acquired from Sigma Company. Fetal bovine serum and penicillin-streptomycin antibiotics were acquired from Gibco Company. This study was approved by the Ethical Committee of the Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran.

Proliferation of the MSCs derived Adipose tissue

In order to isolate MSCs, adipose obtained by liposuction with the patient's consent was transferred from hospital to laboratory in saline containing antibiotic at 4°C, and after several rinses with phosphate-buffered saline and saline, it was divided into smaller pieces. Subsequently, the MSCs were extracted from adipose tissue by digestion with collagenase I enzymes. Then, samples were divided into 3 groups: (Control, Alginate and Fibrin Glue); firstly, 1.5 mg of type I collagenase enzyme per 1 gram of adipose were added and incubated for 45 to 60 min. Then samples was centrifuge at 1400 rpm for 8 min and finally pellet in the culture medium containing penicillin/streptomycin and 10% fasting blood sugar was transferred to cell culture flask. It was then placed in an incubator containing 5% CO₂ and

99% humidity at 37°C. After one day, cells not attached to the bottom of the flask were removed and replaced by fresh medium. The cells were removed every 3 days. Then at passages 3 stage, the cells were prepared to be used [4, 11, 17, 18].

Preparation of chondrogenic medium

In this study, DMEM-High glucose supplemented with insulin- transferrin- 1% selenium, 100 nM dexamethasone, 1% bovine serum albumin, Ascorbate-2-phosphate 50 mg and linoleic acid 50 mg, growth factor transforming growth factor β (TGF- β) (Sigma, Germany) and penicillin- streptomycin 1% were used in order to prepare a medium for the chondrocyte differentiation [4, 5, 11, 18-23].

Fibrinogen and thrombin

Fresh-frozen plasma and fibrinogen were obtained from Qom Organization of Blood Transfusion. Fresh-frozen plasma was added to calcium Gluconate at a ratio of 5 to 3, incubated at 37°C for one hour and centrifuged for 10 min. at 2200 rpm. Next the supernatant was removed and thrombin was isolated. The resulting thrombin and fibrinogen were prepared to be used for culturing the cells. Finally, the ADSCs were first dissolved in thrombin of a concentration of 5×10^6 per one mL; fibrinogen was then, added to them and, after adding the chondrogenic, the medium was put into the incubator at 37°C for 14 days [17, 18].

Preparation of Alginate scaffold

First, Alginate powder (1.2%) was suspended in 0.15 mole/L chloride sodium and then sterilized by filtering. Afterwards, the ADSCs at passage 3 were centrifuged for 8 min. at 1400 rpm after

being plucked off and again suspended in alginate solution. Then, samples were added to chloride calcium 105 Mm solution and placed at room temperature for 15 min. Alginate beads were then rinsed by chloride sodium and DMEM; later, a chondrogenic medium was added followed by addition of 5% CO₂; it was finally put at 37°C for 14 days [10, 11].

Determining cell viability by chondrogenic MTT assay

The viability of cells after 14 days of culturing cells on scaffolds and in the presence of the medium for differentiation to cartilage was assessed and 0.5 mL culture medium of DMEM and 50 mL of MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), 5 mg/ml was added to scaffolds. After 5 hours of incubation, 0.5 mL Dimethyl sulfoxide was added to the solution. After 20 min, the optical density of the cell/scaffold was determined by the microplate reader at a wavelength of 570 nm (Nanodrop, Biotek, USA).

Analysis chondrogenic mRNA expression by real time-Polymerase chain reaction (PCR)

First, scaffolds were destroyed with liquid nitrogen and total RNA was extracted by using AccuZol™ Total RNA extraction solution kit (Bioneer) after 14 days of differentiation of chondrogenic, according to manufacturer's instructions for each scaffold, separately. The reverse transcription from RNA to DNA was fulfilled by means of the AccuPower® CycleScript RT Premix kit (Bioneer) and according to the related guidelines. The primers were designed for each gene using the Primer3 software according to the following sequence (Table 1): the gene was normalized based on the β-actin housekeeping gene; real time-PCR was done by using the SYBR Green PCR master mix (Amplicon) Rotor-Gene 6000 Series (Corbett Life Science, Mortlake, NSW, Australia). [19, 22].

Table 1. Primer sequences used for real time-PCR

Primer name	Sequences (5' - 3')	The Genbank (Refseq) accession number
Collagen, type I, alpha 1	F: TGCTAAAGGTGCCAATGGT	XM_011524341.1
	R: ACCAGGTTACCCGCTGTAC	
Aggrecan (ACAN)	F: GAATCAACTGCTGGAGACCA	XM_011521314.1
	R: CCACTGGTAGTCTTGGGCAT	
SRY-box 9 (SOX9)	F: AGTACCCGCACTTGACAAC	NG_012490.1
	R: CGTTCTTCACCGACTTCCTC	
Collagen, type II, alpha 1	F: CTCCTGGAGCATCTGGAGAC	XM_011537935.1
	R: GTCTCACCACGATCACCCCT	
Beta-actin	F: TTCTACAATGAGCTGCGTGTG	NM_001101.3
	R: GGGGTGTTGAAGGTCTCAA	

Flow cytometry

ADSCs in passage 3 were harvested and surface markers of cells measured by using flow cytometry. Antibodies against surface antigens included: CD45, and CD105. Cells were stained via the primary and secondary antibodies (Dako). ADSCs were directly dyed by anti-CD45 and anti-CD105. Cells were analyzed on FACS flow cytometry using Cell Quest Software.

Histological analysis

For histological analysis, the cells grown on scaffolds in 10% volume phosphate buffered formaldehyde were fixed. Then, the concentration sample in different concentrations of ethanol (50%, 70% and 100%) were dehydrated and immersed in paraffin and then cut into pieces of 4 mm thickness. At last, for morphological analysis the pieces were stained by Hematoxylin/Eosin order.

Statistical analysis

In this study, the significance of the differences in the expression level 4 genes between 2 scaffolds was tested using the Least Significant Difference (LSD) test employing Microsoft Excel 2013. The real time-PCR analysis was repeated for each sample 4 times. The statistical analysis was presented by using the SPSS software (version 17). The level of significant analyses was set at a value of $p < 0.05$.

Result

In the first cell culturing, ADSCs grew with the appearance like fibroblast or spindle with the obtained special core (Fig. 1). Freshly AD-MS cells were stained with CD45, CD34, CD90, and

CD105 antibodies. Flow cytometric analysis of cells showed the expression of CD90, CD105 conventional Mesenchymal markers levels were high, and the expression of CD34 and CD45 hematopoietic cell surface markers levels were low (Fig. 2). In order for the differentiation process of chondrocyte to start, at passage 3, 1×10^6 cells per mL were seeded into the scaffolds and differentiation culture media. The differentiation of stem cells into cartilage was examined through investigating the expression of Aggrecan, type I and II Collagen and Sox9 mRNAs in differentiated cells at days 0, 1, 3, 7 and 14. The results demonstrated the expression of ColII and Aggrecan in Fibrin Glue to be more than the Alginate, and expression of ColI lower in Alginate; however, Sox9 increased in Alginate. According to LSD test, regarding the chondrogenic MTT, there was no remarkable difference between the scaffold of fibrin glue and control as well as between the scaffold of fibrin glue and scaffold of alginate in 7 and 14 days after cell differentiation ($p < 0.05$), (Fig. 3 & 4). In addition, the viability and proliferation of ADSCs seeded onto two differentiated scaffolds and adipose-derived stem cells scaffold-free was assessed by MTT at days 0, 7, 14 and 28 after cell differentiation ($p < 0.05$). MTT assay showed a significant difference between the fibrin glue group and the control at days 14 and 28 after cell differentiation. Based on real time PCR analysis and according to LSD test, the expression level of *type I and II Collagen*, *Sox9*, and *Aggrecan* in the presence of fibrin glue with ADSCs (as control) was significantly different in 7 and 14 days after cell differentiation ($p < 0.05$) (Fig. 4).

Moreover, the expression level of *Aggrecan*, *Sox9* and *Collagen type I* was statistically significant in Alginate with control at 14 days after cell differentiation ($p < 0.05$). Furthermore, the real time-PCR evaluation revealed the difference between *type I Collagen* gene expression, *Alginate* and control at 7 days after cell differentiation. The existence and development of chondrocytes on the scaffolds

were approved by histological analysis. Hematoxylin and eosin staining has showed no detected type II Collagen and Aggrecan in control samples (Fig. 5). Light positive staining of type II collagen and Aggrecan was detected in ADSCs seeded in the fibrin glue and Alginate scaffold within the differentiation medium culture.

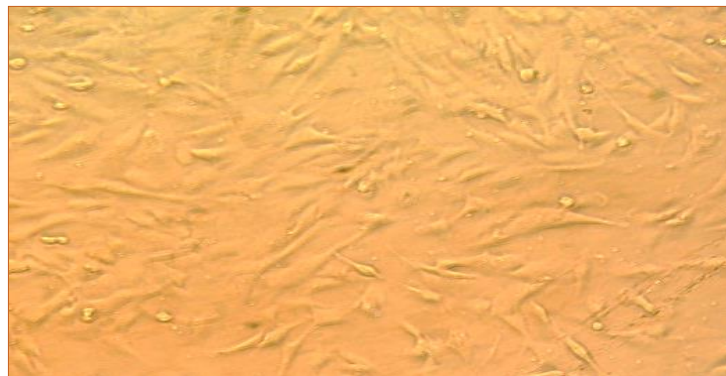


Fig. 1. Adipose-derived mesenchymal stem cells morphology under an Olympus OLY28-10-CB inverted Microscope

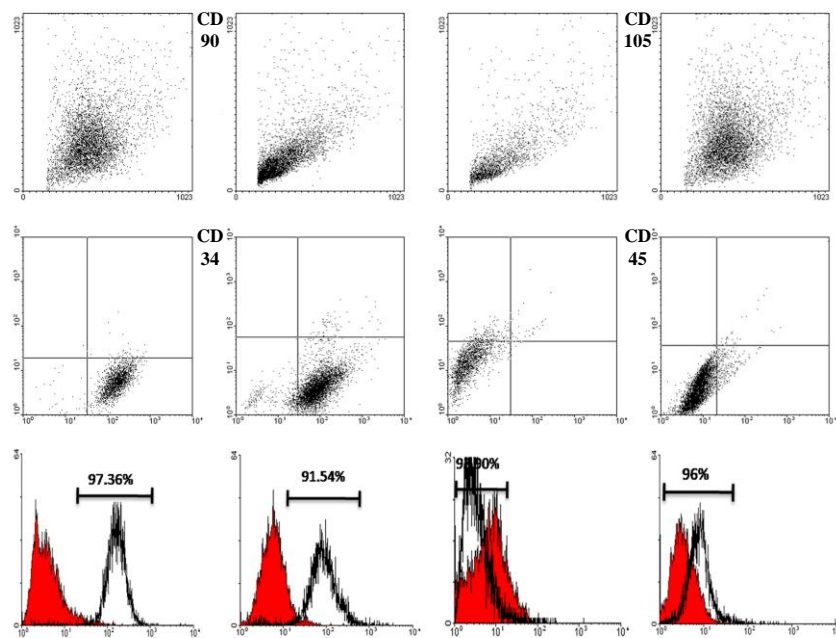


Fig. 2. Flow cytometry analysis. Characterization of AD-MSCs: CD90, CD105 as mesenchymal markers and CD45, CD34 as hematopoietic cell surface markers have shown the high expression of CD90, CD105 and low expression of CD34, CD45.

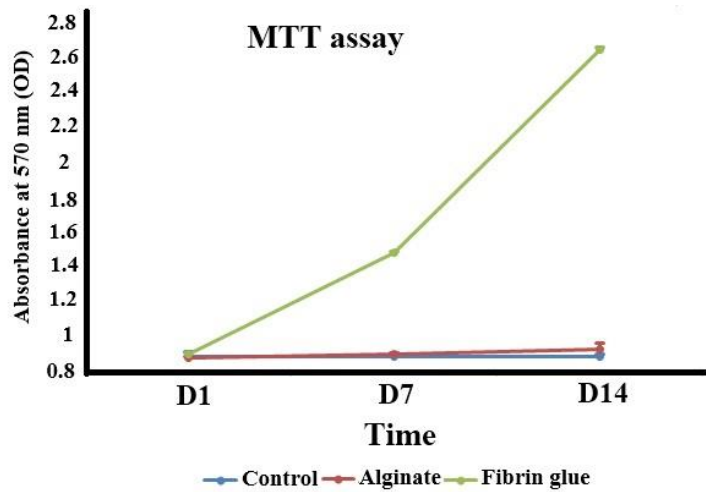


Fig. 3. The MTT analysis of the differentiated ADSCs into cartilage in fibrin glue and Alginate scaffolds. Data showed that the percentage of survival cells increase in fibrin glue compared with Alginate and control groups. D= Day

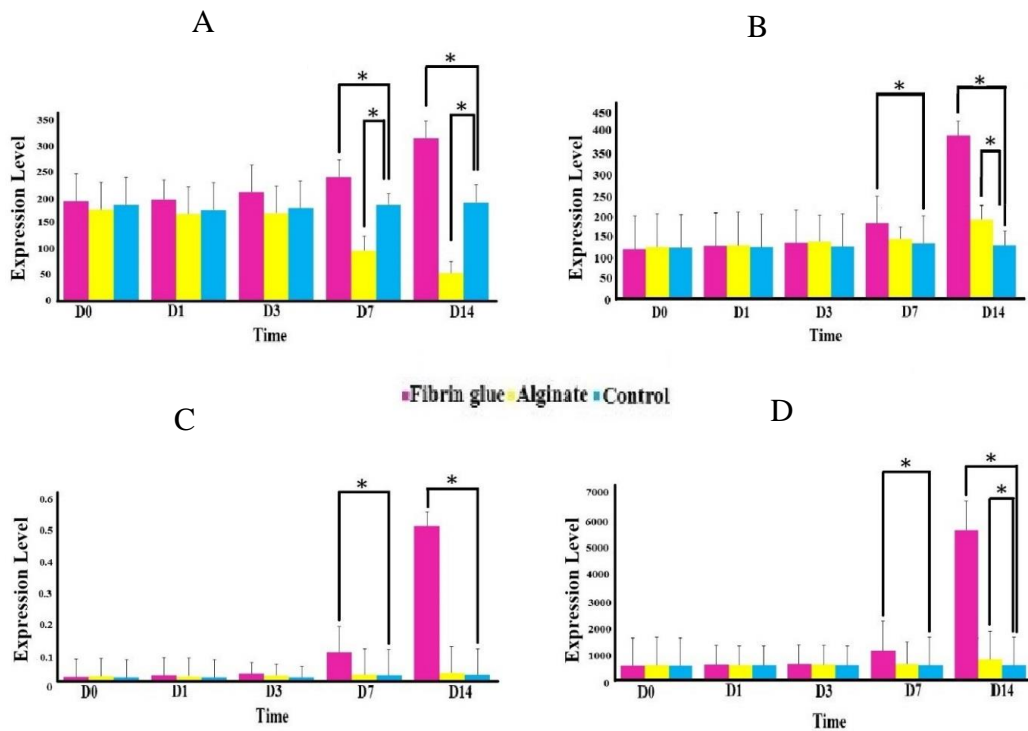


Fig. 4. The expression of *type I Collagen* (A), *SOX9* (B), *type II Collagen* (C) *Aggrecan* (D) genes in differentiated cells on 2 different scaffold and control at days 0, 7, 14 and 28 after cell differentiation. The results showed that the expression of *CollIII* and *Aggrecan* in Fibrin Glue is more than Alginate and expression of *CollI* was lower in Alginate but *Sox9* increased in Alginate, The value was normalized with respect to the level of β -actin mRNA. The results represented means \pm SD from duplicate determinations, representative of 3 independent experiments compared with the control. The statistical difference in comparison with the control group was analyzed by LSD test and differences were statistically significant at $p \leq 0.05$; D= Day

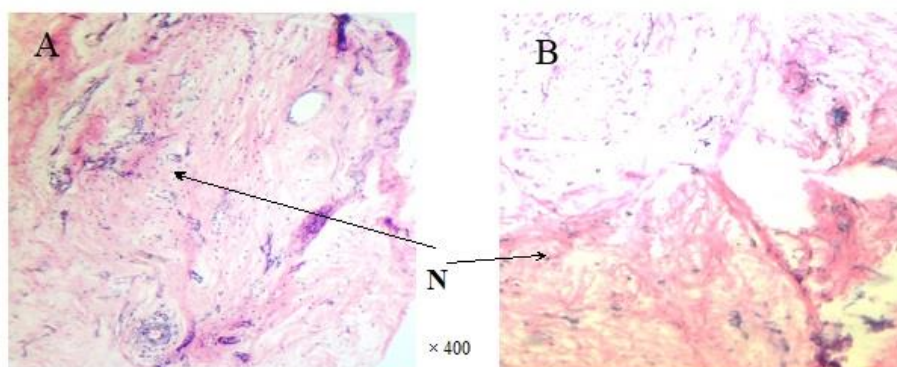


Fig. 5. The histological analysis of the differentiated cells (cartilage) on the fibrin glue (A) and Alginate (B) scaffolds in 14 days after cell differentiation. (H and E stain). N=Nucleus

Discussion

Tissue engineering is a field integrating different branches of science and engineering, trying to create tissues and organs with natural normal functions in order to treat human diseases and repair injuries [12, 13]. Tissue engineering includes culturing cells and establishing interconnections among them via suitable scaffolds in order to provide the conditions favorable to the growth, migration and differentiation of cells into a special tissue [14, 17, 30]. Selecting a biological material as an extracellular matrix depends on the kind and characteristics of the tissue in question; and the used scaffolds in tissue engineering must have special characteristics essential for the cell regeneration and should be able to provide the required mechanical strength [13]. For example, hydrogels are a group of biopolymers with advantages like a high level of water (usually more than 30% of its weight), efficient material and the ability to encapsulate the cells uniformly [9]. These materials contain a hydrophilic polymer chain

and consist of natural hydrogels such as Alginate and fibrin, thus offering structural similarities to human tissues. From a macromolecular point of view, they are biocompatible, relatively inexpensive, having low toxicity and widespread availability and acceptance. Fibrin glue has been widely used to repair and regenerate the cartilage tissue in tissue engineering [9, 13, 14, 24]. Accepted as a suitable biological environment for cell transplantation due to its biocompatibility, degradation, and capacity of cell connections, fibrin glue is a mixture of fibrinogen and thrombin [17, 18, 25- 27]. In addition, immunologically not toxic, alginate is another hydrogel that has wide applicability in tissue engineering. Alginate is widely used in culture cartilage tissue [31]. The Alginate has no special interaction with mammalian cells and this is one of its problems [10, 11]. Generally, growth factors, cell, and the scaffold are the most effective issues in the tissue culture field. The MSCs have many advantages compared to

chondrocytes in such a way that they can be produced with the lowest level of invasion and injuries, in the autologous form by a very simple method [5]. In the 1970s, multipotential MSCs were introduced for the first time as bone stromal cells [22]. These cells can be produced in a lot of body fissures because of their availability, ability in self-renewal and differentiation into different lineages such as cartilage, so they have been turned into a very important means and tools in regenerative medicine [4]. The ADSCs are counted as a suitable candidate in cell therapy and injuries of cartilage tissue [4]. In cell therapy strategies, transplantation requires many cells while the cartilage tissue samples contain few chondrocytes and have no suitable angiogenesis to repair and amend this tissue. That is why the chondrocytes taken from the cartilage tissue do not have the required potential to repair and rebuild the injuries of the tissue [17]. The MSCs are a suitable alternative for these cells. Recently, due to their invasive nature and the pain and injuries they have for the donor as well as their high risk for the donor in the process of isolation, the MSCs taken from bone marrow is less used. Instead, the ADSCs are used due to their advantages such as lack of damage to the donor, simple isolation procedure, loss of gaining cells, availability and the ability to change into different cell levels [5,11,32]. Besides, the results from different studies reveal that the ADSCs are similar to the (BM- MSC) regarding the growth capability and differentiate into various cell lineages [5]. Some studies had demonstrated the fibrin glue

use as a scaffold to grow and differentiate the MSCs. The results of these studies suggest that when the MSCs are put in three-dimensional culturing condition, they can differentiate into cartilage cell [18]. Some studies have shown human ADMSCs can differentiate into cartilage cells provided that they are cultured on a three-dimensional matrix and in a medium containing special regulator materials (dexamethasone, TGF- β) capable of solving [8]. In a study conducted by Ahmed et al. in 2011, it became clear that the encapsulation of MSCs on the fibrin glue scaffold can cause an augment in the expression of the *type II Collagen* gene [17]. In this study, after encapsulation of producing cartilage, a large number of cartilage chondrocyte-shaped cells were observed in fibrin glue scaffolds [12,17,33]. These results were approved by histological analyses. Furthermore, it was shown that when mixed with chondrocytes, the fibrin glue can be used as a promising method to repair tissue in cartilage tissue engineering and cause collagen accumulation, especially under the culture condition of low oxygen [14]. In the study of Ho et al., they showed that the MSCs derived from bone marrow have higher ability for differentiation into cartilage on fibrin glue scaffolds and in a medium containing TGF- β 1 factor, compared to the scaffold hybrid fibrin glue/Alginate, and produce more type II collagen as well [23]. Li et al. (2009) demonstrated that polyurethane scaffold can induce the production of cartilage by MSCs [20, 21]. Jung et al. proved that by a hydrogel fibrin scaffold and in a medium containing TGF- β 3 factor respectively, we can

differentiate ADSCs into cartilage, express cartilage-specific genes and proteins; the results of our study also proved this [15, 18]. Im et al. (2005) compared the ADSCs differentiation potency into cartilage as BM-MSC [5]. In another study, Yang et al. used a hydrogel fibrin scaffold containing genes like *SOX5*, *SOX6*, and *SOX9*, that could differentiate the ADSCs into mature chondrocytes and show the expression of special cartilage genes and production of the proteins resulting from these genes in the differentiated cells [19]. Proulx et al. reported that the MSCs can grow in a kind of scaffold derived from fibrin, keep and protect their growth and their multipotent on these scaffolds [29]. The results from other studies suggest that TGF- β 3 can increase the expression of *type II collagen* gene in encapsulated MSCs in Alginate scaffold; nevertheless, Estes et al. demonstrated that TGF- β 3 is not able to change the gene expression and production of cartilage proteins in ADSCs cultured in alginate scaffold [4, 25, 28]. However, the increased production of cartilage markers in MSCs in the presence of TGF- β 3 has been proven. Some studies have indicated that type II collagen in ADSCs is similar to natural chondrocytes in hyaluronic acid scaffolds [28]. In some studies, the alginate was referred to as a cell carrier in order to support the process of cartilage production by cells and cartilage genes expression [10] in such a way that when the MSCs are put into Alginate, they gain the ability to express high levels of *type II*

collagen compared to plate. Some previous studies showed their ability to grow quickly, hold their phenotype and have the potential to be differentiated into different categories. In Bauge and et al studies, the expression of two genes including *Aggrecan* and *type II Collagen* in both of the scaffolds highly increased [2]. However, the expression level in the three-dimensional hydrogel of fibrin glue was higher than that in alginate, which is pursuant to the results of ours. In this study, it was revealed that in comparison with Alginate, fibrin glue is able to provide suitable conditions for the ADSCs to grow and differentiate into cartilage category [22]. To sum up, ADSCs have shown high capacity in producing and developing the cartilage. This study was in line with the previous investigations.

Conclusion

The results of our study suggests that the selection of scaffold cells affects the growth and differentiation of stem cells. Natural hydrogels like fibrin have shown unique characteristics that enable them to provide a suitable background for growth the MSCs and differentiate into cartilage in order to be used as a promising and essential factor in tissue engineering.

Conflicts of interest

The authors declare that they have no conflict of interest.

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