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Original Work

The assessment of natural scaffolds ability in chondrogenic differentiation of human adipose-derived mesenchymal stem cells

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ABSTRACT: The ability of cartilage to repair damage is limited due to lack of blood vessels and low cell density. Recently, tissue engineering is considerably preferred to other treatments as a way to solve this problem. Regardless of cell sources, one of the crucial factors in tissue engineering is to select an appropriate scaffold, which is essential for healing and renewal procedure of tissues *in vivo* and *in vitro*. Mesenchymal stem cells (MSCs) were isolated from adipose tissue in liposuction surgeries by use of collagenase enzyme. After verification by flow cytometry methods, adipose-derived mesenchymal stem cells (ADMSCs) were embedded into alginate and agarose scaffolds, separately; and then they were cultured in chondrogenic medium for 3 weeks. The ability of alginate and agarose scaffolds was assessed by use of MTT assay and histological analysis. In addition, analysis of chondrogenic genes expression by Real-time PCR was done. The obtained data were analyzed statistically by means of SPSS software. There was a significant difference between alginate and agarose groups in maintaining cells viable but, about chondrogenic differentiation analyzed by use of real-time PCR, statistical analysis has shown a significant difference in expression of aggrecan (as a chondrocyte-specific gene) and collagen II (as an chondrocyte-specific gene) between cell/alginate and cell/agarose and MSCs ($p<0.05$). Chondrocyte differentiation of cells was verified by histological analysis. Alginate scaffold can provide a suitable environment for chondrogenic differentiation of adipose derived mesenchymal stem cells.

KEY WORDS: ***Alginate; Agarose; Chondrogenic differentiation; Mesenchymal stem cell; Tissue engineering***

INTRODUCTION

Cartilage is a specialized connective tissue whose main function is to provide softness and to make joint movements smooth and easy. The ability of cartilage to repair damage is limited due to lack of blood vessels and low cell density[1,2].
Different treatment methods, such as allograft and autograft cartilage transplantation may lead to formation of fibrous tissue, apoptosis and tissue destruction[3,4].

In recent years, transplantation of autologous chondrocyte has been used as an approach to treat joint injuries.
It should be noted that the autologous chondrocyte transplantation method has some limitations and achieving adequate cell numbers is not always possible[5-7]. Thus, many reasons, including damage to healthy cartilage during sampling, restriction of adult chondrocytes in proliferation, decreased number of isolated cells, and reversible phenomenon have necessitated efforts to find other cell sources for cartilage tissue engineering and treating diseases. Beside, transplantation of organs and tissues to treat injuries and illnesses do not always appear to be a viable solution. Appropriate scaffold, regenerative viable cells and physico-

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chemical and growth factors are required for tissue engineering[8-14].

The scaffold surface should be suitable for adhesion, proliferation, migration, and growth of cells, and be able to provide a suitable condition for the formation and replacement of matrix by cells[15] as well as and the ability to sterilize and stabilize in the position of transplantation.

Natural polymers with polypeptide or polysaccharide structures are derived from a living organism and used as a scaffold in tissue engineering[16]. Alginate is a natural biopolymer extracted mainly from brown algae and in lesser amount from bacteria. This substance constitutes 40% of algae by dry weight. In fact, this alginate exists in the extracellular matrix of these algae in combination with calcium, magnesium and sodium. Alginate is a polymer of glucuronic acid and mannuronic acid arranged as chains with opposite pattern of clocks needle[16]. Also, agarose is a polysaccharide extracted from red algae that consists of β-D-galactopyranose and α-D-galactopyranose residues[17].

The family of transforming growth factor β (TGF-β) acts as the chondrogenesis regulator. TGF-β is produced by articular chondrocytes, remains and has an effective role in maintenance of joint chondrocyte and osteoblast proliferation[18]. In addition, it is shown that these proteins have a major determining role in the development of the musculo-skeletal system and are responsible for growth and proliferation of chondrocyte cells[19].

Different studies on chondrogenesis of stem cells have shown various and even conflicting results in three-dimensional culture system. Also the effects of various growth factors are differently reported. So, in this study we decided to isolate mesenchymal stem cells from subcutaneous human adipose tissue and after proliferating, they were cultured on the alginate and agarose scaffolds, by adding TGF-β3 as growth factor, for 28 days to produce cartilage tissue. The purpose of this study was to investigate the ability of alginate and agarose scaffolds to maintain cell viability and also to determine chondrogenic genes expression in mesenchymal stem cells embedded in two scaffolds, separately.

METHODOLOGY

Cell isolation and expansion of human ADMSCs

After obtaining written consent from patients, adipose-derived mesenchymal stem cells (ADMSCs) were isolated from adipose tissue derived from liposuction surgeries. Briefly, the adipose tissue was weighed and cut into few millimeters pieces under sterile conditions. In order to digest tissue, 1.5 mg collagenase I (sigma) per gram of adipose tissue was applied at 37°C for 45-60 min. After enzymatic digestion of tissue, DMEM (sigma) containing 10% FBS and 100 U/ml penicillin/ streptomycin, in equal volume with applied enzyme, was added to the cell suspension to neutralize it. Then, the suspension was centrifuged for 10 minutes in 1400 rpm. The obtained cell deposit was seeded in the cell culture flask in DMEM culture medium supplemented with 10% FBS and 100 U/ml penicillin/ streptomycin at 37˚C, 5% CO_2 and 95% air.

Flow Cytometry

Flow cytometry was performed according to our previous study[10].

Chondrogenic differentiation on alginate scaffold

Alginate solution (1.2%) was prepared by dissolving alginate powder in 5 ml of 0.9% NaCl. Then it was passed through 0.22µm filter. To culture cells on the alginate scaffold and induce chondrogenic differentiation, after trypsinization of mesenchymal stem cells in passage 2, they were washed by PBS and centrifuged at 1400 rpm for 10 minutes. After counting cells, about 2×10^5 cell/ml was centrifuged again and 0.4 ml alginate solution was added to the cell deposit. Then, cell-alginate mixture was dropped into 105 mM CaCl2 solution. Within 15 min alginate beads containing cells formed. Then they were washed with NaCl solution 0.9% and DMEM–high glucose. Finally, chondrogenic medium containing 100 U/ml penicillin/ streptomycin, 1% transferring-selenious-insulin (sigma), 10-7 mole dexamethasone (sigma), 1% bovine serum albumin (sigma), 50 µg/ml ascorbate-2 phosphate, 5 µg/ml linoleic acid (sigma) and 10 ng/ml transforming growth factor-β3 (sigma) was added to alginate beads. Ultimately they were placed in an incubator (37°C, 5% CO_2, 99% humidity and PH 7.4).

Chondrogenic differentiation on agarose scaffold

Similarly, cells were suspended in 2% (w/v) low-melting point agarose (Type VII, Sigma) at a concentration of 10^6 cells/ml. The agarose molds were allowed to gel at 4°C for 10 min. At last chondrogenic medium containing 100 U/ml penicillin/ streptomycin, 1% transferring-selenious-insulin (sigma), 10-7 mole dexamethason (sigma), 1% bovine serum albumin (sigma), 50 µg/ml ascorbate-2 phosphate, 5 µg/ml linoleic acid (sigma) and 10 ng/ml transforming growth factor-β3 (sigma) was added to alginate beads. Ultimately they were placed in an incubator (37°C, 5% CO_2, 99% humidity and PH 7.4).

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Assessment of Cell viability

After 7 days, MTT (3-(4, 5-dimethyl) thiazol-2-yl-2, 5-dimethyl tetrazolium bromide) assay was used to assay cell viability and proliferation. MTT solution (5mg/ml) was added to scaffolds/cells for 4hr and then intra cellular formazan was solubilized by adding 1ml dimethyl sulfoxide for 20 min. Absorbance at 570 nm was measured on a microplate reader.

Analysis of chondrogenic genes expression by Real time PCR

Expression rate of collagen type I and II, Sox9 and aggrecan was evaluated 14 days after chondrogenic differentiation. The scaffolds were separately degraded within liquid nitrogen and then total RNA was extracted from all samples using an Accuzol™ (BioNEER) according to the manufacturer's protocol. Reverse transcription of RNA was carried out to produce a complementary DNA (cDNA) using the Accupower® RTpreM1X (BioNEER). Real-time PCR was performed using SYBRGreen PCR Master Mix and Rotor-Gene™ 6000 Series Software version 1.7.65 (Corbett Life Science). Also, primers of each gene were designed as follows utilizing primer 3 program with primer sequences as mentioned in **Table 1**.

Table 1: Real-time PCR primer sequences

Primer Name	Sequences (5' -> 3')
Collagen I	F-TTGTACAGACATGACAAGAGGC R-CTCTACCTGGGTACTACCCA
Aggrecan	F-CAGAGTGAAATCCACCAAGT R-TGTCCGTGGACAAACAGGTA
Sox9	F-TACGACTACACGCACCACCA R-TTAGGATCATCTGCGCCATC
Collagen II	F-ACACAGCGCCTTGAGAAGAG R-TTCTACGGTCTCCCCAGAGA

Histological Examination:

At the 28th day, samples of ADMSC, which were seeded on alginate and agarose scaffolds, were histologically analyzed. Cartilage tissue was considered as the control in this evaluation: after fixation with 10% neutral-buffered formalin for at least 24 hr, specimens (N = 2) were embedded within paraffin and sectioned at 5µm thickness. For histological evaluation, sections were deparaffinized, rehydrated through a series of graded ethanol, and stained with hematoxylin/eosin counterstaining.

RESULT AND DISCUSSION

In the study of living and unstained cells using phase contrast microscopy, 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 were fully determined (**Figure 1**).

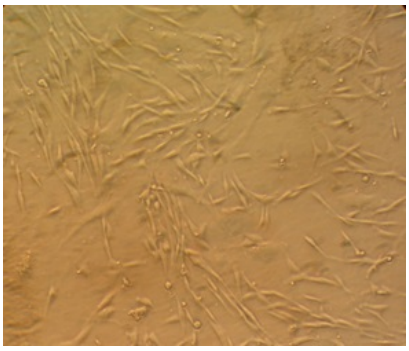

Figure 1: Image produced by invert microscope of living mesenchymal stem cells isolated from human adipose tissue in which spindle cells in the third passage are visible. ×40

In this study, flow cytometry showed that the majority of human adipose derived stem cells (hASCs) are of mesodermal or mesenchymal origin. The isolated cells expressed transcripts for markers CD44, CD90 and CD105, but failed to express the hematopoietic marker CD45 (**Figure 2**).

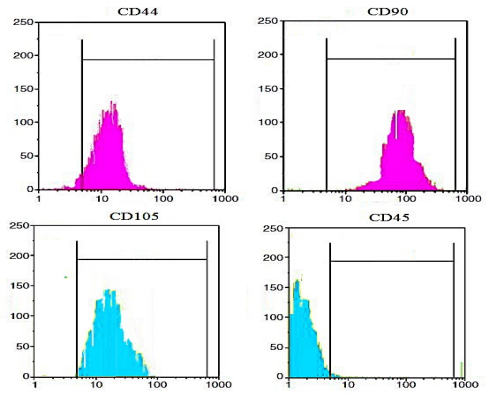

Figure 2: The comparison of cell viability between MSCs embedded in two scaffolds and control

To assess alginate and agarose scaffolds as suitable environments for maintaining viability of cells and providing them appropriate conditions to proliferate, MTT assay was applied. Although a greater viability of mesenchymal stem cells in the two scaffolds was observed, statistically significant

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difference was detected between alginate and the other groups (**Figure 3**).

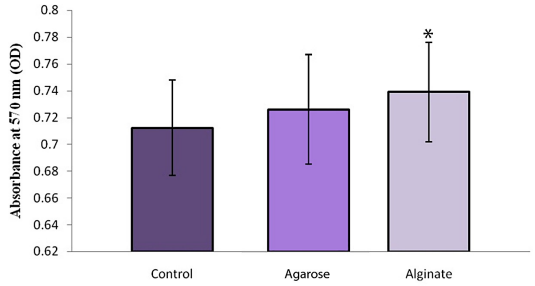

Figure 3: The comparison of cell viability between MSCs embedded in two scaffolds and control

However, regarding chondrogenic differentiation, which was assessed by the use of real-time PCR, the results were supportive to select both alginate and agarose as two scaffolds for cartilage tissue engineering. Statistical analysis has shown a significant difference in expression of aggrecan (as a chondrocyte-specific gene), collagen type II (as an chondrocyte-specific gene) and sox9 (as an chondrocyte-specific gene) between cell/alginate, cell/agarose and ADMSCs as control ($p<0.05$). But collagen I as a osteogenic gene was not expressed differently (**Figure 4**).

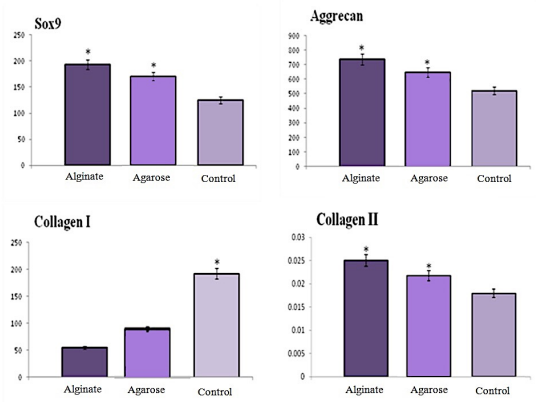

Figure 4: The expression of Sox9, Aggrecan, Collagen type I and collagen type II in cell/alginate and cell/agarose versus control

The accumulation and presence of chondrocytes were examined by hematoxylin/eosin staining at the 4th week (**Figure 5**). Histological examination revealed that pores were filled with chondrocytes, and the cells were uniformly distributed in the cell-alginate constructs at 4 weeks. The histological response for agarose scaffold was weak.

While cartilage has no blood vessels and nerves and damage of this tissue is not repairable, tissue engineering can provide the possibility of achieving this for cartilage.

In the past, chondrocytes encapsulated in the different scaffolds were used as the cell source in for *in vitro* chondrogenesis. But chondrocytes could not keep their biochemical and morphological properties and, because obtaining chondrocytes requires surgery on the patient with some risk of injuring cartilage, researchers tried to find another cell source, which has cartilage differential ability.

Research has shown that to design cartilage tissue, high density of cells and suitable interaction between cells is necessary which can be provided through Pellet Culture System and culture on three-dimensional scaffolds to induce chondrogenesis[17,18,20,21].

It should be noted that for chondrogenic differentiation various scaffolds have been used in extensive research, and that each of them has some advantages and disadvantages. Although synthetic polymers have suitable mechanical properties in tissue engineering, they do not have good biocompatibility as well as inability to provide appropriate cell adhesion. Moreover, their preparation is complex in laboratory conditions[22,23]. There are also disadvantages in different types of natural scaffolds, including low stability, lack of good mechanical properties and fast degradation[24,25].

Alginate as a natural polysaccharide easily changes to gel state without the need for organic solvents and its preparation as a scaffold does not require alteration of pH or utilization of heat or toxic activators. In addition, in the gel state alginate has porosity and facilitates the release of macromolecules[25,26]. Due to the hydrophilic nature of alginate and high amounts of water, it can be used as an extracellular material suitable for providing cell growth. Thus in the present study alginate was selected as scaffold.

In this study, the ability of alginate to retain cell viability and to provide a suitable environment for inducing chondrogenic differentiation in adipose-derived mesenchymal stem cells was assessed.

Seven days after culturing stem cells, MTT assay was performed and it was demonstrated that the ability of cells to survive in alginate scaffold was more in comparison with agarose scaffold and the control group (**Figure 3**). This result is supported by a study, which reported enhanced viability and metabolism of chondrocytes in alginate scaffold[27].

To prove the existence of cartilage in obtained structures resulting from chondrogenic differentiation induction, different methods are used. As has been stated in other studies, the existence of collagen type II and aggrecan are considered cartilage indicators[28]. After fourteen days real-time PCR was done and it was found that the expression of sox9, collagen type II and aggrecan were significantly enhanced by differentiated cells in alginate and agarose scaffolds ($p<0.05$). However, there was no statistically significant difference in the expression of collagen type I (**Figure 4**). Similarly, Munirah *et al*

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confirmed chondrogenic properties by the expression of genes encoding collagen type II, aggrecan proteogelican and sox9[29].
On the other hand, expression of collagen type I has been known as the osteogenesis indicator. Our results showed that differentiated cells in alginate scaffold have a significantly lowered amount of collagen type I expression ($p<0.05$). While mesenchymal stem cells (MSCs) within their native environment of the stem cell niche receive biochemical stimuli from surrounding cells which induce osteogenesis in them and as a result they have innate ability to differentiate into bone, decreased expression of collagen type I in alginate and agarose lead us to conclude that alginate is an appropriate scaffold to be used in cartilage tissue engineering.

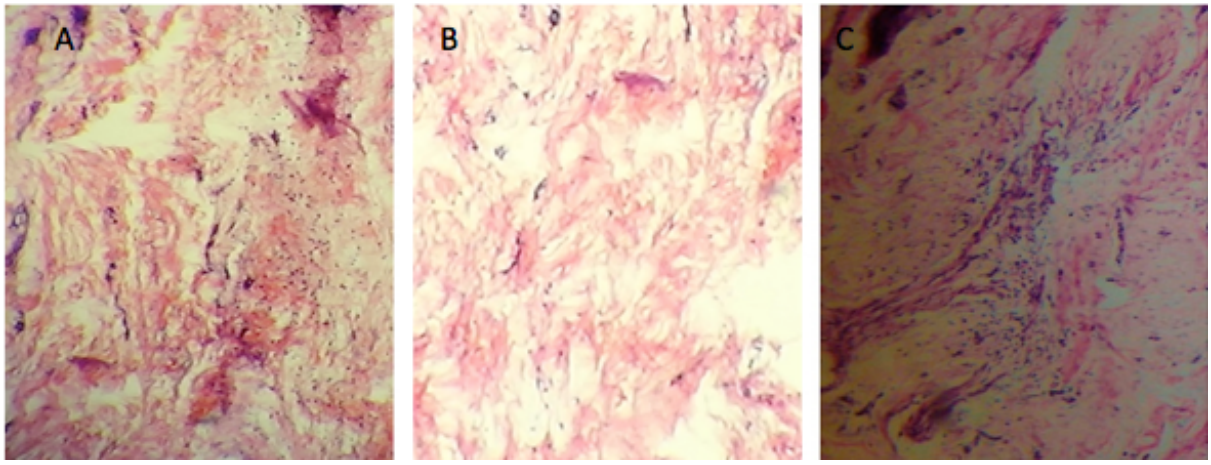

Figure 5: Histological evaluation of cells cultured on alginate and agarose scaffolds at fourth week. Alginate scaffold (A), agarose scaffold (B) and staining of native cartilage used as control with hematoxylin/eosin is demonstrated in Figure (C) (×10 magnification)

CONCLUSION

We showed that alginate scaffold could provide a suitable environment for chondrogenic differentiation of human adipose derived mesenchymal stem cells. Also, alginate scaffold can enhance the growth and proliferation of human adipose derived mesenchymal stem cells.
This study recommends that suitable biomaterials support the chondrogenic differentiation of human adipose derived mesenchymal stem cells, and that selection of appropriate scaffolds could have significant effects on tissue engineering approaches.

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