Original Article

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Growth and Chondrogenic Differentiation of Mesenchymal Stem Cells Derived from Human Adipose Tissue on Chitosan Scaffolds

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ABSTRACT

BACKGROUND AND OBJECTIVE: Treatment of cartilage damage for any reason is associated with temporary relief of joint pain. Providing the possibility of differentiating various stem cells into adult tissues can contribute to recovery and treatment of damaged cartilage tissue in skeletal system. In this study, chondrogenic potential of chitosan scaffold, CH-β-GP-HEC, with stem cells derived from human adipose tissue.

METHODS: In this cross-sectional study, adipose tissue-derived stem cells were separated from abdomen of 15 patients who underwent inguinal hernia repair. 6-7×10^5 cells were cultured in plate one-dimensionally and on chitosan scaffold three-dimensionally for 21 days. MTT assay was run to evaluate the toxic effect of scaffold on cell viability. Proliferation and differentiation of cells were studied in the two types of culture after toluidine blue staining. To confirm the formation of cartilage, expression of collagen type II was assessed by immunohistochemistry.

FINDING: In MTT assay, the average OD for cells cultured on scaffold is higher than 0.8 compared with control group, which confirms the nontoxicity of scaffold for culturing stem cells ($p>0.05$). Chondrogenic differentiation of cells on scaffold shows more glycosaminoglycan deposition in the extracellular matrix compared with one-layer culture. Moreover, in group with three-dimensional culture system, cells were spherical and the morphology of nucleus was different from one-layer culture. Regarding immunohistochemistry results, increased synthesis was observed in collagen type II as chondrogenesis markers in three-dimensional culture system compared with one-layer culture.

CONCLUSION: Results of the study revealed that hydrogel scaffold, CH-β-GP-HEC, with porous structure provides a better environment for the growth of mesenchymal stem cells and their differentiation into cartilage tissue.

KEY WORDS: *Chondrogenesis, Adipose Tissue, Mesenchymal stem cells, Chitosan, Scaffold*

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Introduction

Treatment of cartilage damage due to trauma, genetic predispositions or metabolic conditions is usually invasive and is only associated with temporary reduction in joint pain. By providing the possibility of differentiation of stem cells from adult tissue sources, such as cartilage, cell therapy has promising prospects for repair and treatment damage done to cartilage from the skeletal system (1, 2).

Using mesenchymal stem cells (MSC) instead of chondrocytes in cartilage tissue engineering is considered as an alternative source of cells, because they can be harvested with less complications than the cartilage cells from a patient (3, 4). Moreover, proliferation, mass production and maintenance of stem cells for tissue engineering are far easier and more affordable (5, 6).

Stem cells derived from human adipose tissue have the ability to differentiate in different cell lines; similar to bone marrow mesenchymal stem cells (7). Several chemical stimuli have been found that participate in induction of chondrogenic differentiation of MSCs. However, producing a differentiated cell that preserves its function after being transferred to in vivo environment is yet to be discussed (8). In tissue engineering for cell transplantation, cells are cultured on natural and synthetic scaffolds. Several scaffolds were assessed in vivo and in vitro. Hydrogels are types of biomaterials which are very similar to soft tissue in terms of high water content, elastic properties, softness, oxygen permeability and excellent biocompatibility (9).

Among natural hydrogel-based biomaterials, chitosan is presented in tissue engineering as a promising biomaterial (10, 11). Chitosan is a natural amino-polysaccharide, easily degraded in nature and precursor of natural glycosaminoglycan (GAG) (12). Chitosan, as a hydrogel scaffold, can play a key role in cell growth, differentiation and increased cell adhesion by creating a biological and biochemical three-dimensional microenvironment (13-15).

Chitosan-beta glycerol phosphate-hydroxyethyl cellulose (CH-β-GP-HEC) is a chitosan-based scaffold and its biodegradability and biocompatibility effect in liver tissue has been assessed (16). It is also a proper choice as an injectable scaffold for nerve tissue. Assess the ability of the hydrogel for proper support chondrogenic differentiation of stem cells have been studied less. (17). In this study, chitosan-beta glycerol phosphate-hydroxyethyl cellulose was assessed and investigated as a scaffold that supports three-dimensional culture of mesenchymal stem cells derived from human adipose tissue and its effect on chondrogenic potential of these cells.

Methods

Preparing chitosan-based hydrogel: Chitosan powder with molecular weight of 1000 kDa was sterilized by autoclave and exposed to dry air for two hours. 0.225 g of the powder was dissolved in 9 ml hydrochloric acid (0.1 M) by shaking. On the other hand, 2.25 g β-Glycerophosphate (β-GP) (Sigma) was dissolved in 3.5 ml distilled water and was sterilized by a 0.2 µ filter. To prevent gelation, both products were chilled on ice for 15 minutes. Then, cold β-GP solution was added to chitosan solution drop by drop while stirring continuously to form a clear solution. After that, 0.125 g hydroxyethyl cellulose (HEC) (Sigma) was dissolved in 10 ml DMEM medium and was added to CH-β-GP solution. All solutions were prepared at 4°C.

Isolation and culture of stem cells: In this cross-sectional study, adipose tissue samples were obtained from inguinal region of 15 patients aged 20-35 were referred for inguinal hernia surgery based on guidelines of ethics committee of Mazandaran university of medical sciences and informed consent of patients admitted to Sari Imam Khomeini Hospital. Then, adipose tissue samples in falcon tubes containing PBS with antibiotics were transferred to cell culture laboratory, department of anatomy. Enzymatic digestion method was used for isolation of stem cells derived from adipose tissue (18).

After washing adipose tissue samples in PBS buffer for three times and cleansing the blood and excess adipose tissue, they were divided into few-millimeter-sized pieces. For enzymatic digestion, they were incubated with collagenase enzyme type I (sigma) and 5% CO2 at 37°C.

Then, the enzyme was neutralized using DMEM (Bio Idea) containing 10% FBS. Cell suspension was centrifuged and the supernatant was removed. DMEM culture containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) was added to cellular pellet and cell suspension was transferred to cell culture flask (T-25). The third or fourth passage cells were used for chondrogenic differentiation.

Flow cytometry: Flow cytometry was used to confirm the stem cell type and to identify cell surface markers. For this purpose, two specific surface markers of mesenchymal stem/stromal cells (MSCs) including CD105 and CD90 and two specific surface markers of hematopoietic stem cells (HSCs) including CD31 and CD45 were examined (19).

After counting cells passage 4 with hemocytometer, about 2×105 cells were transferred to each Falcon control tube and test tube. Then, they were centrifuged at 2500 rpm for 5 minutes and after removing supernatant, cell pellet was dissolved in 3% BSA and

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was incubated on ice for 30 minutes. After that, CD90, CD45, CD31 and CD105 antibodies conjugated with phycoerythrin (PE) were added to test tubes. The isotypes antibody anti IgG1 conjugated PE were added to negative control tube. Samples were incubated for one hour at room temperature in darkness. Then, PBS was added to tubes and were centrifuged at 2500 rpm for 5 minutes. Supernatant was removed and labeled cells were dissolved in 2cc of PBS buffer and were analyzed by flow cytometry.

Culture and chondrogenic differentiation in monolayer technique: In monolayer culture, supernatant is removed after cell trypsinization and centrifusion. Cellular pellet was suspended in chondrogenic differentiation medium (Invitrogen). 6-7×10^5 cells were cultivated in a 24 well plate and chondrogenic differentiation medium was replaced every two days. Cells were cultured with differentiation medium for 21 days. DMEM medium was used for control group.

Culture and chondrogenic differentiation on chitosan-beta glycerol phosphate-hydroxyethyl cellulose scaffold: Prepared scaffolds were first sterilized with 70% alcohol and UV rays. Each piece of scaffold was placed in a 24 well plate and 6-7×10^5 cells were cultured in the center of each scaffold. The cell-containing plate was incubated for 2 hours. Then, 1cc of chondrogenic differentiation medium was added to each well. DMEM medium was used for control group. Culture medium was replaced every two days. Cells were cultured on scaffold for 21 days.

MTT assay: MTT assay was run to assess the toxicity of chitosan-beta glycerol phosphate-hydroxyethyl cellulose scaffold. Similar to "culture and differentiation" section, ADSCs cells were cultured in 24 well plates. 24 hours after incubation, 10 µl hydrogel was added to each well.

Culture medium was replaced every two days, similar to previous section. After 21 days, 100 µl MTT solution was added to each well and they were incubated at 37°C for 4 hours. To dissolve formazan crystals, DMSO solvent was added to each well at the same volume and was placed in the dark for 15 minutes. Finally, light absorption of the resulting solution was read at wavelength of 570 nm.

Histological assessment: After 21 days of culturing cells in 24 well plates, samples after two times washing with PBS buffer were fixed in 4% paraformaldehyde for 20 minutes. After being washed with distilled water, toluidine blue stain was added. Samples were then examined with an optical microscope.

After 21 days culturing cells on scaffold, all samples were then washing, fixation, dehydration and clarification, paraffin blocks were obtained from scaffolds. Samples sectioned at 5µ thickness were stained with toluidine blue. Samples were then examined with an optical microscope.

Immunohistochemical assessment: To perform an immunohistochemical assessment of cartilage formation, specimens were fixed in acetone after preparing slides with 5µ thickness, clarification and dehydration. Slides were retrieved in microwave for 20 minutes. Samples were washed in TBS (Tris-buffered saline) buffer. Hydrogen peroxide activity of tissues with 3% H_2O_2 solution was suppressed in 70% ethanol for 5 minutes at room temperature.

After being washed in TBS buffer, samples were incubated with Antibody Anti-Collagen II at 4°C overnight. After washing the slides in TBS buffer, they were incubated with Goat Anti-Rabbit IgG H&L (HRP) at room temperature for 2 hours. After being washed in TBS buffer, samples were incubated with DAB substrate for 10 minutes and after being washed with running water for 5 minutes, they were examined with an optical microscope.

Statistical analysis: Statistical analysis was done using SPSS 17 software. MTT assay data were expressed by one-way ANOVA and $p<0.05$ was considered significant.

Results

Morphology of cultured cells: After 2 days of culturing and replacing the first culture medium, fusiform fibroblast-like cells with cytoplasmic appendages attached to the flask were observed. After 10 days, cultured cells reached 80% confluence and they were then passaged.

Results of analyzing surface marker expression by flow cytometry: Analyzing mesenchymal stem cell markers by flow cytometry technique revealed that cells passage 4 attached to the bottom of the flask expressed on average 81.1% of cells CD105 marker and 99.2% of cells CD90 marker, which is one of the specific markers of mesenchymal stem/stromal cells (Fig 1). Nevertheless, the same cells expressed less than 7% of hematopoietic cell markers such as CD31 and CD45 (Fig 2).

MTT assay results: MTT assay was run to assess the effect of CH-β-GP-HEC hydrogel on cell viability in vitro. Cells were cultured on hydrogel in wells decreased OD a low level. Average OD read at wavelength of 570 nm was >0.8 for control group and ≥0.8 for the cultured on hydrogel group, which does not indicate a significant statistical difference between these two groups and confirms nontoxicity of scaffold for stem cells culture (Fig 3).

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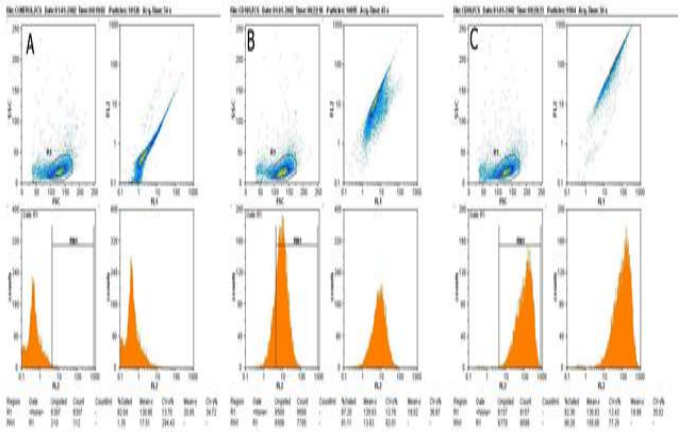


Figure 1. Flow cytometry assay. Negative stromal surface markers in CD105 (B) and CD90 (C) groups compared with control group (A)

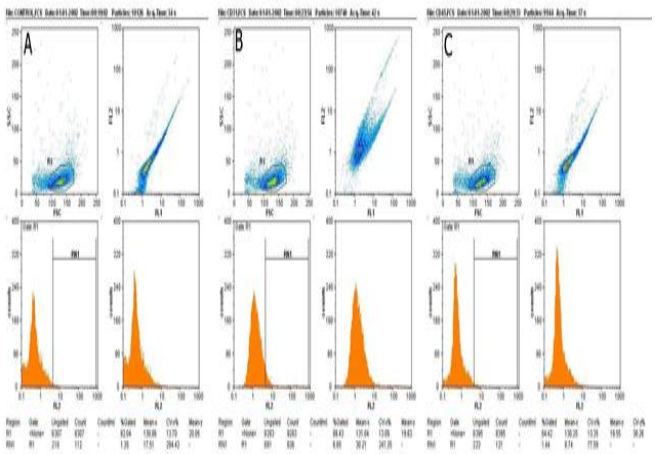


Figure 2 Flow cytometry assay. Negative hematopoietic surface markers in CD31 (B) and CD45 (C) groups compared with control group (A)

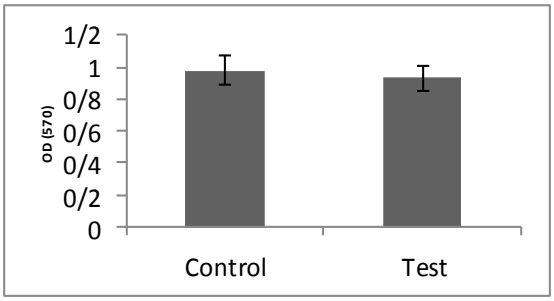


Figure 3 MTT method shown viability of hADSCs cultured on hydrogel on day 3 (p>0.05)

Results of histological and immunohistological assessment of cells cultured in differentiation medium: 21 days after differentiation, chondrogenesis capacity of mesenchymal cells in both monolayer and three-dimensional (scaffold) methods was revealed histologically using toluidine blue staining. Spherical cells with basophilic nucleus were identified in three-dimensional culture group that had different morphology compared to monolayer culture. In addition, basophilic extracellular matrix was abundantly observed around cells, but cell differentiation and secretion of extracellular matrix in monolayer method was minimal. Toluidine blue staining was used to show the presence of proteoglycan in extracellular matrix. Blue color indicated existence of methachromatic and the presence of cartilaginous matrix (Fig 4). In order to confirm the results, immunohistochemical analysis was done for collagen type 2 using monoclonal antibody. The difference in color of cells bound to DAB indicated that cells in scaffold secreted more matrix compared with monolayer culture cells and scaffold without differentiation medium (Fig 5).

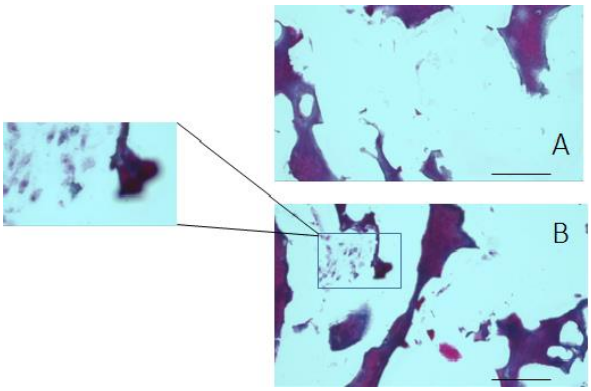


Figure 4. Colony formation in cartilage cells as a result of differentiation of stem cells derived adipose tissue in chitosan scaffold on day 21 (B) and its comparison with control group (A). Toluidine blue staining. Scale bar=20µ×40

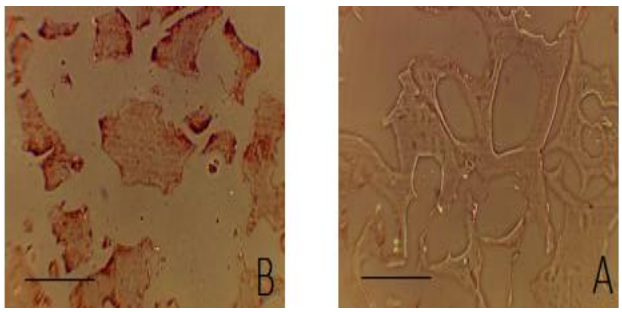


Figure 5. Immunohistochemical staining. Brown color indicates the existence of collagen type II in extracellular matrix, showing higher color density in three-dimentional culture (scaffold) (B) compared with monolayer culture (A). Scale bar=20µ×40

Discussion

In the present study, CH-β-GP-HEC scaffold created a proper three-dimensional structure for growth and chondrogenic differentiation of mesenchymal stem cells derived human adipose tissue in vitro. The

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number of mesenchymal stem cells decreases in the process of chondrogenesis in vitro and in vivo due to apoptosis (20-22). In addition, chondrogenic potential of stem cells requires high cell density in vitro and in vivo (23). According to the above-mentioned statements and high demand for cell in cell therapy, using mesenchymal stem cells is more appropriate than using chondrocytes. Many studies have demonstrated that ADSCs have high proliferative potential and can be differentiated into various cell lines including chondrocytes. This type of stem cells can be abundantly obtained from adipose tissue with minimum damage (24).

Although some studies have shown that BMSCs are more appropriate for chondrogenesis than ADSCs (1), many studies have shown than ADSCs benefit from appropriate chondrogenic potential (8, 25-27). For repairing cartilage defects with cell therapy, cell suspension is associated with certain restrictions. Methods in which cells attach to cartilage defect locally are less invasive and are more attractive for clinical use (28). However, when mesenchymal cells are injected intra-articularly, they attach to synovial membrane and only a small amount of them can be observed at the location of cartilage defect. When more cells are injected, more cells attach to the location of cartilage defect, yet more cells attach to synovial membrane, increasing the risk of proliferation of the synovial membrane (28). Therefore, using scaffolds for cell transfer to the location of defects will be more appropriate. Natural polymers such as chitosan are biodegradable and biocompatible materials (29). The level of degradation depends on the degree of deacetylation (30). In previous study demonstrated that after being embedded within liver tissue for 180 days, the scaffold shrank while its morphology did not change. Gelatinization period of CH-β-GP-HEC scaffold solution is about 30 minutes which is enough for cell therapy and drug delivery into neural tissue.

Adding cells to the gel decreases gelatinization period (31). Naderi et al. have reported that the same period for CH-β-GP-HEC scaffold is 20 minutes (32). In hydrogels, crosslinks increase the viscosity of solution and subsequently decrease diffusion. In alginate hydrogels, viscosity increases with cell density. According to their result, although high cell density is appropriate for cell therapy, increasing viscosity and mechanical force may damage the cells (33). Hydrogel viscosity may influence water diffusion, cell growth, cell differentiation and drug release (34). When CH-β-GP-HEC scaffold was embedded within liver tissue, migration and proliferation of host tissue cells into the scaffold was reported and no toxicity was observed (16).

According to MTT assay results, high viability rate of cells cultured on CH-β-GP-HEC scaffold, with or without differentiation medium, indicates high biocompatibility of scaffolds in contact with ADSCs. Optical microscopy was used to demonstrate the penetration and expansion of cells within the scaffold. 21 days after the beginning of culture and differentiation, histological images illustrated high penetration and colonization inside scaffold pores. GAG is one of the cartilaginous matrix molecules that for studying cartilaginous differentiation of MSC. 21 days after the beginning of differentiation, GAG increase was observed compared with monolayer culture. According to histology results, differentiated cells were spherical similar to chondrocyte cells and formed a cellular accumulation. This shows that cells are healthy, they move toward each other and they have interact with each other. It is well known that cell density takes place in the course of joint development in vitro (6).

Therefore, cell-cell interaction may be significant in chondrogenic differentiation of MSCs in this system. Increased secretion of collagen type II was comparable with monolayer culture system; 21 days after the beginning of cell culture in the presence of differentiation medium in three-dimensional culture system (scaffold). Secretion of collagen type II and glycosaminoglycans (GAGs) indicates that CH-β-GP-HEC scaffold has a high potential for inducing chondrogenic differentiation, particularly when compared with monolayer culture.

Results of the present study demonstrated that similar compounds to body tissues that were used in scaffold structure along with differential factors create a desirable condition for chondrogenic differentiation of ADSCs compared with monolayer culture. Thus, using biometrical scaffolds with differential factors is a powerful tool for enhancing the potentials of chondrogenesis and creates a better and more enhanced environment for chondrogenic differentiation of mesenchymal stem cells.

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