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Original Article

Comparison of Poly(Lactic-Co-Glycolic Acid) and Poly(Lactic-Co-Glycolic Acid)/Hyaluronic Acid Based Scaffolds for Chondrogenesis of Human Adipose-derived Stem Cells by Avocado/Soybean Unsaponifiables

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ABSTRACT

Background: Corporation of Hyaluronic acid (HA) with PLGA is an effective way to potentially enhance chondrogenesis. The aim of this study was to use HA macroporous biodegradable poly(lactic acid-co-glycolic acid) [PLGA] scaffold to enhance the attachment, proliferation and differentiation of chondrocytes for cartilage tissue engineering and articular cartilage regeneration of human adipose derived stem cells (hADSCs) in the presence of avocado/soybean unsaponifible (ASU).

Methods: The PLGA and PLGA/HA scaffolds were prepared and hADSCs were cultured separately on the scaffolds and 14 days after differentiation, chondrogenic genes in each scaffold evaluated using real time PCR and cell viability examined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: The viability and proliferation of cells in-group of PLGA significantly decreased in comparison with the control (P=0.002) and PLGA/HA (P=0.013) groups. The expression of (SOX9), Aggrecan (AGG), and Collagen type II (Col II) genes was significantly higher in the PLGA and PLGA/HA groups compared to the control group (P≥0.05). The gene expression of SOX9 (P=0.003) and AGG (P=0.009) was significantly higher in the PLGA/HA groups compared to the PLGA group. The results of real time PCR showed that collagen type X (Col X) gene expression in the PLGA group, was significantly higher than the control and PLGA/HA groups (P=0.000).

Conclusion: The corporation of HA with PLGA is an effective way to potentially enhance chondrogenesis and articular cartilage regeneration of hADSCs in the presence of avocado/soybean unsaponifiables (ASU).

Keywords: Hyaluronic Acid, Poly (Lactic-Co-Glycolic) Acid, Chondrogenesis, Human Adipose-derived Stem Cell, Scaffold

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Introduction

The number of reconstruction methods for tissue defects arising from various reasons have been expanded, however, there are many problems to dominate. Cartilage is a unique tissue without any blood vessels in parenchymal tissue, and its regeneration hardly occurs after being injured. Reconstruction methods for cartilage defects applied are autologous or homologous cartilage grafts, and the use a synthetic prosthetic device (1). Among them, autologous tissue grafts are the most common idea. However, autologous cartilage grafts have also some disadvantages such as donor incidence, adversity in grafting and trimming for the eligible shape, and distinct qualities between the donor and recipient (1,2). To overcome these short approaches, studies of the cartilage organization using tissue engineering have been carried out since the late 1980s. Some studies have shown the actual eventuality of cartilage tissue rehabilitation using tissue engineering by successfully forming the tissue in a desired shape by grafting chondrocytes on three-dimensional (3-D), high molecular scaffolds (3,4). Three significant factors of tissue engineering are the biological characteristics of cells, selection of biocompatible scaffolds, and assorting to in vivo environments. In contrast with other organs, it is especially difficult to use clinically the cartilage tissue organized by tissue engineering due to the problems of resorption and deformation (5). Selecting biocompatible scaffolds is one of the important points for solving serious problems, and thus, many studies are concentrating on this issue.

Tissue engineering has been proved to be one of the most promising alternative therapies for articular cartilage defects. In this strategy, a biodegradable 3-D porous scaffold is required to incorporate the transplanted cells as a supportive matrix and to guide the organization of new tissue. Multiple scaffolds have been prepared from biodegradable synthetic polymers and naturally derived polymers for the tissue engineering of cartilage. Naturally, derived polymers such as collagen and hyaluronic acid have hydrophilic surfaces and specific cell interaction peptides, which are excellent for cell growth, but their weak mechanical properties, make it very difficult to withstand compression when implanted into the cartilage defect (6). On the other hand, the most famous artificial polymers for cartilage regeneration constructs are poly lactic acid (PLA), which is present in both L and D forms, poly-glycolic acid (PGA), and their copolymer poly (lactic-co-glycolic) acid (PLGA). PLGA shows high biocompatibility, the potential to break down into safe monomer units, a beneficial range of mechanical characteristics, and governable degradation time depending on the copolymer ratio, but their hydrophobic surface is not favorable for cell seeding. The ideal scaffold for cartilage tissue engineering should have good cell affinity and enough mechanical strength to serve as an initial support (7). Due to the importance of scaffolds in cartilage tissue engineering, the aim of this study was to compare two PLGA and PLGA/HA scaffolds for chondrogenesis of hADSCs by ASU.

The cell source for tissue engineering can be obtained from several tissues or organs, one of which is adipose tissue. Adipose-derived mesenchymal stem cells (ADSCs) are mesenchymal stem cells (MSC) obtained from perivascular white adipose tissue, including subcutaneous adipose tissue. The isolation of ADSC is relatively easy and produces a higher yield of cells compared to other adult stem cell source tissues (8). In addition to using different types of stem cells, various growth factors are also used in tissue engineering. Growth factors play a key role in the proliferation, apoptosis, and differentiation of stem cells. Transforming growth factor-β (TGF-βs) family is widely used in the cartilage tissue engineering. TGF-βs induce the expression of some genes, like Col II and glycan, and also, facilitate the construction of glycosaminoglycan (GAGs) (9,10). Although TGF-βs have an inducing impact on chondrogenesis of bone marrow mesenchymal stem cells (BM-MSCs) and ADSCs, they have some limitations that need to be taken into account when using them. Their high price and short half-life are two of their disadvantages. Besides, these growth factors contribute to the hypertrophy of chondrocytes (9,10).

ASU are natural botanical extracts of avocado and soybean oils, consisting of the remaining fraction that cannot be made into soap after saponification. ASU is formed of two-thirds soybean unsaponifiables and one-third avocado. The strenuous components remain unknown. ASU is a member of the symptomatic slow-acting drugs for OA, which are impressive in mitigating OA symptoms in patients. The

application of ASU on knee osteoarthritis has been proven to be secure and effective in many clinical studies (11). The purpose of this study was to compare biodegradable PLGA and HA scaffold in promoting chondrogenesis of hADSCs in the presence of ASU.

Materials and Methods

Fabrication of the PLGA and PLGA/HA hybrid scaffold

The PLGA scaffold was prepared via solvent casting/progeny leaching (SC/PL) penetrating method using methylene chloride, as previously described with few modifications (12). Briefly, polymer/solvent solution (8% w/v concentration of PLGA in methylene chloride) was added in a cylindrical silicone cast (7 mm in diameter and 3 mm in height), which was filled with sodium chloride salt particles (NaCl) (approximately 180 μm particle sizes) as progeny. Then, the scaffolds were dried at room temperature for 24 hours. In order to leach out the NaCl particles, the samples were saturated in deionized water for 2 days. During this period, water was renewed three times. Then, the samples were freeze-dried at -80°C for 48 h in a freeze-dryer (Christ Alpha2_4Ld Plus, Germany) to produce highly porous construct with no solvent remaining in their structures. Composite scaffolds were prepared by saturation of PLGA scaffolds in 2% of solution of HA for 24 hours (13). Finally, the prepared porous hybrid scaffolds were sterilized with ultraviolet irradiation and 70% ethanol.

All chemicals were purchased from Sigma Aldrich Company (St Louis, MO, USA), unless stated otherwise. ASU was purchased from Perarian Pars Company, Iran.

Isolation, proliferation, and culture of stem cells in scaffolds

hADSCs were extracted from subcutaneous abdominal adipose tissue taken from women who underwent caesarean section (30-40 years). Adipose tissue was mechanically minced and washed with PBS, and then, was designed with collagenase IA (1 mg/1g). The cell solution was centrifuged at 1500 rpm for 10 min. The pellet was resuspended in the culture medium containing DMEM-LG supplemented with 10% FBS, 1% penicillin, and streptomycin (Gibco), and then, was cultured and kept at 37°C, 5% CO2 conditions. In order to examine the morphology of the cells, photographs were taken by an invert microscope, at different time intervals (Figure 1) (14). After the isolation of hADSCs and three passages, the cells were seeded in the PLGA and PLGA/HA scaffolds. Triplicate of each subgroup was prepared and cultured for 14 days. After the end of the treatment period, the cells were isolated from the PLGA and PLGA/HA scaffolds. Cell proliferation was assessed by MTT assay and cartilage specific gene expression was evaluated by the real-time PCR.

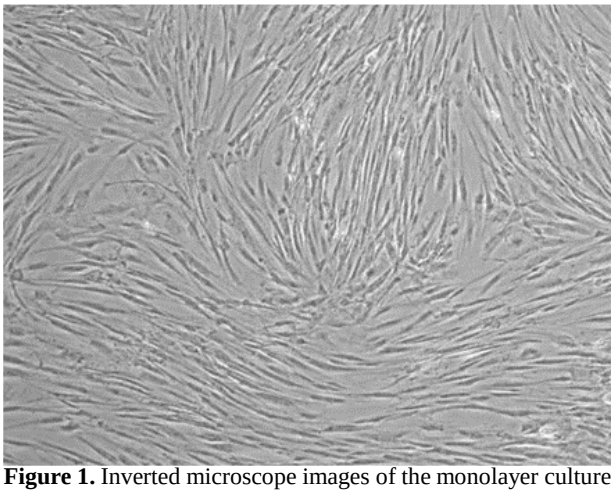

Figure 1. Inverted microscope images of the monolayer culture of ADSCs in the third passage (magnification x 40).

In vitro chondrogenic differentiation

The hADSCs cultured from passage 3, were resuspended in chondrogenic medium (high glucose Dulbecco's modified Eagle medium, supplemented with 100 µg sodium pyruvate, 10 µg /ml ASU, 100 nM dexamethasone, 1% ITS + Premix, 40 µg/ml proline, 50 µg/ml ascorbate-2-phosphate, and 1% penicillin-streptomycin, 0.5 mg/ml bovine serum albumin, 5 µg/ml linoleic acid) (15). For loading the cells on the PLGA and PLGA/HA scaffolds, a 24-well plate containing 2×10^6 in 200 ml of medium was loaded on each scaffold, then, the plate was incubated at 37°C and %5 CO_2 for 2 hours. Then, 500 µml of chondrogenic medium was added to each well. The half amount of medium was replaced every 3 days.

MTT assay

Viability tests were applied using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) colorimetric assay. On day 14, the medium was removed and replaced with 400 µl DMEM high glucose and 40 µl of MTT solution (5 mg/ml in PBS). Then, it was incubated at 37°C, 5% CO_2 for 4 hours. The medium was then discarded and 400 µl dimethyl sulfoxide (DMSO) was added to each well and incubated for 2 hours at room temperature in the dark. DMSO dissolved the formazan crystals and created a purple color. Finally, 100 µl of each well was transferred to the 96-well plates and the amount of light absorption or optical density (OD) was measured at a wavelength of 570 nm with an ELISA Reader (Hyperion MPR4). All measurements were done in triplicates (16,17).

Real time-polymerase chain reaction (Real-time PCR) analysis

Total RNA was extracted using RNeasy Mini columns (Qiagen), according to the manufacturer's instructions. The RNA samples were subjected to reverse transcription-PCR (RT-PCR) analysis in a single-step procedure using the Titan One Tube RT-PCR System (Roche). Reverse transcription was carried out at 50°C with specific primers, followed by hot-start PCR in the same tube. Primers used in this study were:

GAPDH: 5′-AAGCTCATTTCCTGGTATG-3′ forward and 5′-CTTCCTCTTGTGCTCTTG-3′ reverse; SOX9: 5′-TTCAGCAGCCAATAAGTG-3′ forward and 5′-TTCAGCAGCCAATAAGTG-3′ reverse; AGG: 5′-GTGGGACTGAAGTTCTTG-3′ forward and 5′-GTTGTCATGGTCTGAAGTT-3′ Col II: 5′-CTGGTGATGATGGTGAAG-3′ forward and 5′-CCTGGATAACCTCTGTGA-3′ reverse; Col X: 5′-AGAATCCATCTGAGAATATGC-3′ forward and 5′-CCTCTTACTGCTATACCTTTAC-3′ reverse

Statistical analysis

One-way ANOVA test was used to evaluate the differences between groups and the Tukey's test in SPSS software was operated to determine the differences between each two groups. Statistically significant level was considered at $P\leq0.05$.

Results

Cell viability assay

The MTT assay results are exhibited in Figure 2. The viability and proliferation of cells in the group of PLGA significantly decreased compared to the control ($P=0.002$) and PLGA/HA ($P=0.013$) groups. In addition, viability in the control group, was higher than that in the PLGA/HA group but it was not significant ($P\geq0.05$).

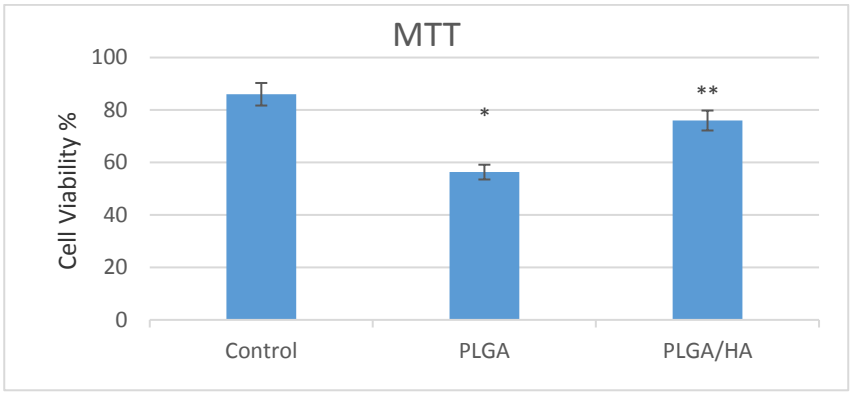

Figure 2. The MTT assay results 14 days after the culture of hADSCs in the chondrogenic medium supplemented with ASU in the PLGA and PLGA/HA scaffolds. P≤0.05 was considered significant.
*Significance compared to the control group.
**Significance compared to the PLGA group.

Gene expression analysis

On day 14, the expression of SOX9, AGG, and Col II genes was significantly higher in the PLGA and PLGA/HA groups compared to the control group (P≥0.05) (Figure 3A-C). The gene expression of SOX9 (P=0.003) and AGG (P=0.009) was significantly higher in the PLGA/HA groups compared to the PLGA group (Figure 2). Also, the gene expression of Col II in the PLGA/HA group, was higher than that in the PLGA group but it was not significant (P≥0.05) (Figure 3C).

The results of real-time PCR showed that the gene expression of Coll X (as hypertrophic marker) in the PLGA group, was significantly higher than that in the control and PLGA/HA groups (P=0.000). However, the gene expression of Coll X had no significant differences between the control and PLGA/HA groups (Figure 3 D).

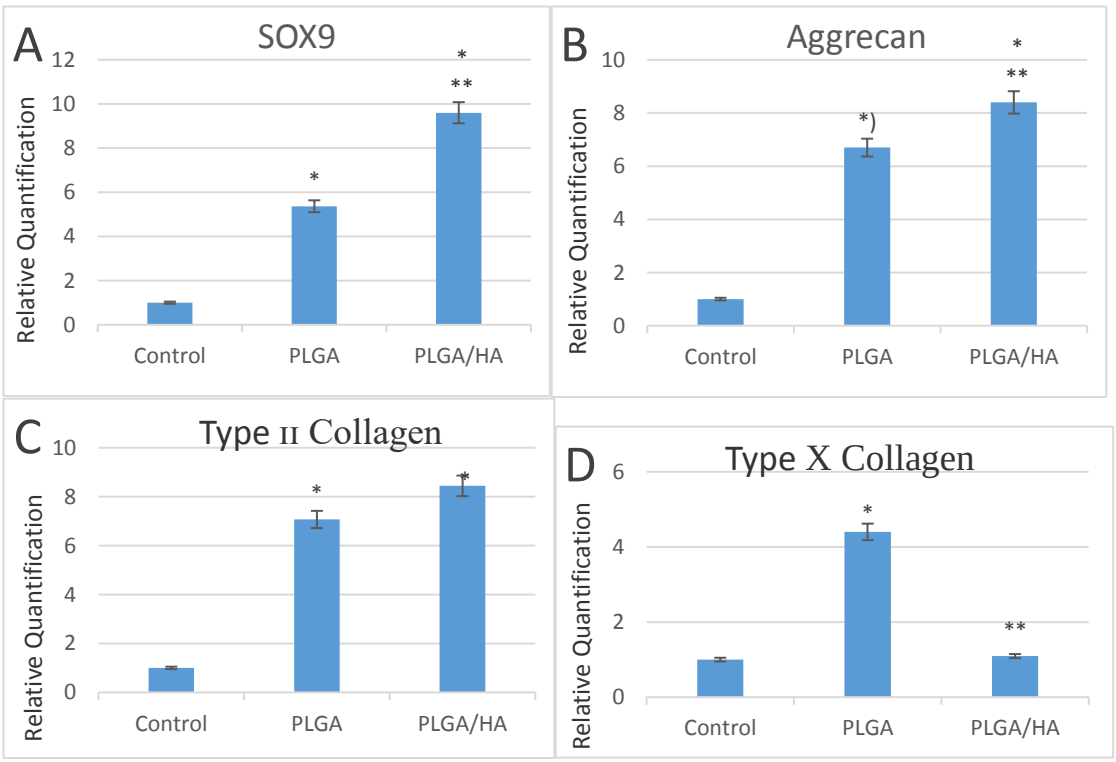

Figure 3. The results of SOX9 (A), AGG (B), Col II (C) and Coll X (D) genes expression in different groups 14 days after the culture of hADSCs. P≤0.05 was considered significant.
*Significance compared to the control group.
**Significance compared to the PLGA group.

Discussion

This study demonstrated that the corporation of HA with PLGA is an effective way to potentially enhance chondrogenesis and articular cartilage regeneration of hADSCs in the presence of ASU. Human articular cartilage is an avascular structure, which any damage to it, poses significant hurdles to repair strategies (18).

Tissue engineering is the in vivo regeneration and remodeling of tissue for the purpose of repairing, replacing, maintaining, or enhancing organ function, and the in vtro engineering and growing of functional tissue substitutes for in vivo implantation as a biological substitute for damaged tissues and organs (19). Successful tissue engineering relies on four specific criteria: cells, growth factors, scaffolds, and the mechanical environment (18).

MSCs have shown much promise with respect to their use in cartilage tissue engineering. MSCs can be obtained from many different tissue sources. Among these, adipose tissue can provide an abundant source of adipose-derived mesenchymal stem cells (ADMSCs) (20). Previous studies showed that ADSCs also have multipotential differentiation ability, including chondrogenesis (14,16).

In previous studies, several researchers have studied chondrogenesis of ADSCs in different scaffolds and various growth factors. However, there is no information about chondrogenesis of hADSCs in the PLGA and PLGA/HA scaffolds via ASU. The results of the present study showed that ASU can increase the extracellular matrix (ECM) and up-regulate the expression of chondrogenesis-related genes in hADSCs.

The exogenous use of growth factors such as transforming growth factors-β (TGF-βs) and BMPs were tested to induce chondrogenic differentiation from stem cells (21), but these exogenous recombinant growth factors have a short half-time and are expensive (22), and especially, TGF-βs induce apoptosis in terminal chondrogenic differentiation, Yoo et al. (1998) showed that the use of TGF-β1 as a growth factor in chondrogenic process induced the expression of Coll II in the 4^{th} day, while in the 11^{th} day, stimulated the expression of Coll X, which is a marker of hypertrophy (23). Indrawattana et al. (2004) showed that BMP-6 alone could not stimulate chondrogenesis (24). Due to the problems related to recombinant growth factor and safety and efficiency of recombinant growth factor, there is a restriction for the exogenous use of growth factor in clinical treatment. Previous studies showed that ASU has a chondroprotective effect on chondrocytes. Moreover, ASUs has anabolic effects and is capable of stimulating endogenous production of TGF-βs in chondrocytes (25).

In the present study, ASU was used in chondrogenic media and the findings revealed that there is an up-regulation in the gene expression profile of SOX9, Coll II, and AGG. Henrotin et al. (2003) reported that ASU could stimulate the expression of Coll II in chondrocytes during culture in a monolayer environment (26). Altinel et al. (2007) showed that ASU treatment caused an increase in the levels of TGF-β1 and TGF-β2 in the joint fluid compared to controls (27). This finding suggests that ASU is probably capable of inducing endogenous production of TGF-βs in hADSCs, and as a result, chondrogenesis induction can occur in them.

Previous study reported that high density of cells and cellular interaction is essential to produce cartilage tissue in the process of chondrogenesis, and through 3-D scaffold, high density of cell and cellular interaction can be achieved (28-30). Structure of scaffolds can promote biological functions of cells and extracellular matrix (ECM) synthesis (31). It should be noted that many studies have been done on the induction of chondrogenesis using a variety of scaffold that each has their own advantages and disadvantages. Natural scaffolds are also a number of disadvantages such as low mechanical properties and high rate enzymatic host degradation (5,32). Previous studies showed that PLGA porous scaffolds are very popular synthetic scaffold due to their good mechanical properties and degradation behavior (33). However, they are not bioactive to support stem cells growth and differentiation and maintain the chondrocyte phenotype; with surface modification by a high bioactive agent, such natural polymers can greatly enhance their bioactivity and biocompatibility (34,35). In the present study, the PLGA also compared to control increased the expression of specific cartilage genes, on the other hand, it caused a significant increase in the gene expression associated with hypertrophy (Coll X). By impregnating the scaffolds with HA, they can better mimic ECM for cells, consequently, adhesion property and chondrogenesis and cartilage formation are enhanced (36,37). Also,

Dowthwaite et al. (1998) reported that HA has a biological role in the cartilage tissue to regulate and associate with many cellular responses, such as cellular adhesion, proliferation, and differentiation (38). Consistent with the results of the present study, Song et al. (2013) reported that incorporation of HA in the PLGA scaffold can increase the production of AGG and Col II (13).

Furthermore, the expression of collagen X, a hypertrophic factor, was considered as a limitation during differentiation of hADSCs to chondrocytes (39). In this study, it was found that Coll X gene expression had no significant differences between the control and PLGA/HA groups, whereas the expression level of this gene in the PLGA group, was significantly higher than that in the control and PLGA/HA groups. So, the PLGA/HA scaffold caused less chondrocyte hypertrophy.

Conclusion

According to the results of this study, the PLGA/HA scaffold is an effective way to potentially enhance chondrogenesis and articular cartilage regeneration of hADSCs in the presence of ASU. This can be due to the increase of special markers of hyaline cartilage and reduction of hypertrophic marker compared to the PLGA scaffold.

Acknowledgments

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Conflict of interests

The authors declare that they have no conflict of interests.

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