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Original Article

Cartilage tissue formation from human adipose-derived stem cells via herbal component (Avocado/soybean unsaponifiables) in scaffold-free culture system

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ABSTRACT

Background: The use of stem cells, growth factors, and scaffolds to repair damaged tissues is a new idea in tissue engineering. The aim of the present study is the investigation of Avocado/soybean (A/S) effects on chondrogenic differentiation of human adipose-derived stem cells (hADSCs) in micromass culture to access cartilage tissue with high quality.

Materials and Methods: In this an experimental study After hADSCs characterization, chondrogenic differentiation was induced using transforming growth factor beta 1 (TGF-β1) (10 ng/ml) and different concentrations (5, 10, and 20 μg/ml) of A/S in micromass culture. The efficiency of A/S on specific gene expression (types I, II, and X collagens, SOX9, and aggrecan) was evaluated using quantitative polymerase chain reaction. In addition, histological study was done using hematoxylin and eosin and toluidine blue staining all data were analyzed using one-way analysis of variance (ANOVA) and $P \leq 0.05$ was considered to be statistically significant.

Results: The results of this study indicated that A/S can promote chondrogenic differentiation in a dose-dependent manner. In particular, 5 ng/ml A/S showed the highest expression of type II collagen, SOX9, and aggrecan which are effective and important markers in chondrogenic differentiation. In addition, the expression of types I and X collagens which are hypertrophic and fibrous factors in chondrogenesis is lower in present of 5 ng/ml A/S compared with TGF-β1 group ($P \leq 0.05$). Moreover, the sulfated glycosaminoglycans in the extracellular matrix and the presence of chondrocytes within lacuna were more prominent in 5 ng/ml A/S group than other groups.

Conclusion: It can be concluded that A/S similar to TGF-β1 is able to facilitate the chondrogenic differentiation of hADSCs and do not have adverse effects of TGF-β1. Thus, TGF-β1 can be replaced by A/S in the field of tissue engineering.

Key Words: Adult stem cells, cell culture techniques, tissue engineering

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INTRODUCTION

Tissue engineering using stem cells, growth factors, and scaffolds with aimed to improving the biological

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functions of damaged tissues is a new therapeutic method for tissue regenerative.[1] Due to cartilage tissue have not blood supply and the recovery period of cartilage lesion is long, so tissue engineering can be a suitable method for cartilage repair.

Transforming growth factor beta (TGF-β) family is one the most factors which used for *in vitro* chondrogenic differentiation. It has been reported that TGF-β family members via specific receptors and with intracellular signaling are able to promote cartilage-specific gene expression.[2-4]

In spite of this, our previous study confirms that the expression of type X collagen as a hypertrophic marker is high in cartilage which produced using TGF-β1.[5] On the other hand, a previous study revealed that TGF-β1 can induce type I collagen expression through intracellular signaling effects.[6] Moreover, TGF-β1when used *in vivo* has other side effects such as osteophyte formation and synovial membrane inflammation.[7] Thus, the TGF-β replacing with other agents with fewer side effects is a necessity.

Avocado/soybean (A/S) (ratio 1:2) which is an herbal component used to decrease symptoms of cartilage defects. The main action mechanism of this agent is not clear; nevertheless, several potential pathways have been assumed. Among these mechanisms, increase of TGF-β expression,[8] accumulation of extracellular matrix,[9] and collagenase reduction, fibrinolysis, and matrix metalloproteinase activity inhibition[10] may be significant. It is important to know that A/S is able to alter the cross-linking of collagen fibers and facilitate the wound-healing process.[11] It has been reported that arthrocen (an A/S unsaponifiable) has a potential to reduce joint inflammation and pain associated with end-stage osteoarthritis.[12] In a similar experiment, A/S (300 mg/day) was given to 4822 patients with symptomatic knee osteoarthritis as a routine medication. The results of this study revealed that a large number of patients who received AS for 6 months showed gradual decrease of joint pain, improvement in functional ability, and a considerable diminution in nonsteroidal anti-inflammatory drugs intake.[13]

With respect to the wide beneficial effects A/S, in the present study, we evaluated the effects of several doses of A/S cartilage formation from human adipose-derived stem cells (hADSCs) in micromass culture system.

MATERIALS AND METHODS

Human adipose-derived stem cells isolation and culture

This is an experimental study which was done at Isfahan University of Medical Sciences. All materials (except those specified) which used in this study were purchased from Sigma-Aldrich. Moreover, all procedures were approved by the Ethics Committee of Isfahan University of Medical Sciences. To hADSCs isolation, all following steps were done according to the previous study.[14] Human abdominal fat was obtained from lipoaspirate samples; the samples were washed with phosphate-buffer saline (PBS) and then treated with 0.075% type I collagenase. Enzyme activity was neutralized after 30 min, the samples were centrifuged at 1400 rpm for 10 min, and finally, the cellular pellet was resuspended in Dulbecco's modified Eagle medium (DMEM/low glucose) solution and was cultured in standard condition.

Cell surface marker characterization

To identify specific markers of stem cell, hADSCs (within third passages) incubated with respective fluorochrome-conjugated antibodies against CD14, CD44, CD105, and CD90 which were conjugated with FITC (BioLegend Company) for 30 min. After incubation, the cells were washed with PBS and stained with DAPI. Finally, the mean percent of fluorescent cells were analyzed by a fluorescence microscope (Olympus BX51, Tokyo City, Japan).

Micromass culture system and chondrogenic differentiation

The induction of hADSCs into chondrocyte was carried out according to the previous study.[15] Briefly, hADSCs within the third passages at a density of 2.5×10^5 cells in 12.5 μl medium were placed in a 24-well plate in a 37°C humidified incubator with a 5% CO_2 environment for ½ h to form cell aggregates. In the following, chondrogenic differentiation media (1 ml) consisting of DMEM-high glucose (Gibco), penicillin and streptomycin 1% (Gibco), dexamethasone 10-7M, ascorbate-2-phosphate 50 μg/ml, bovine serum albumin 0.5 mg/ml, linoleic acid 5 μg/ml, 10 μg/ml insulin, 5.5 μg/l transferring, 5 μg/l selenium (ITS), with adding TGF-β1 10 ng/ml (Group 1), A/S 5 μg/ml (Group 2),[16] A/S 10 μg/ml (Group 3),[16] A/S 20 μg/ml (Group 4),[16] TGF-β1/A/S 10 μg/ml (Group 5), and without any growth factor (group control) were carefully added around the cell aggregates. The medium was substituted

every 3–4 days for 14 days. Meanwhile, the mean expression of cartilage-specific genes in stem cells was evaluated before induction of chondrogenesis and was considered as stem group.

Histological study

Three samples in each group were fixed with formaldehyde and then dehydrated in ascending ethanol. In the following, they were clarified with xylol, embedded in paraffin, sectioned into 4-μm thickness, and finally stained using hematoxylin and eosin and toluidine blue. The comparison of glycosaminoglycans secretion in the extracellular matrix and the morphology of chondrocytes within lacuna were studied using a light microscope (Nikon, Japan).

Real-time polymerase chain reaction

To determine the cartilage-specific genes expression, RNA extraction (three samples per group) was done using TRIzol reagents (Invitrogen). In the following, RNA was reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit (Fermentase) with oligo (dT) primers, and then real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR Master Mix (Fermentase) and the Step One Plus™ quantitative real-time PCR detection system (Applied Biosystems). Primers were designed for each gene using the Allele ID software (Primer Biosoft), which generated the following sequences: Collagen II (Forward: CTGGTGATGATGGTGAAG, Reverse: CCTGGATAACCTCTGTGA), collagen X (Forward: AGAATCCATCTGAGAATATGC, Reverse: CCTCTTACTGCTATACCTTTAC), SOX9 (Forward: TTCAGCAGCCAATAAGTG, Reverse: GTGGAATGTCTTGAAGGTTA), aggrecan (Forward: GTGGGACTGAAGTTCTTG, Reverse: GTTGTCATGGTCTGAAGTT). In addition, GAPDH primer was used as an internal control within these sequences: Forward: AAGCTCATTTCCTGGTATG, Reverse: CTTCTTCTTGTGCTCTTG.

Finally, all data were analyzed using one-way analysis of variance (ANOVA). In addition, Data are presented as mean ± standard error of the mean and $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Human adipose-derived stem cells characterization

As shown in Figure 1a and b, hADSCS before micromass formation exhibited fibroblast-like morphology; however, after micromass formation, these cells were aggregated and numerous cellular mass was formed. Moreover, immunocytochemistry results indicated that the high percent of hADSCs was CD105 and CD90 positive (98.27 ± 0.17 and 99.2 ± 0.11, respectively) but was CD14 and CD45 negative [Figure 1c-f].

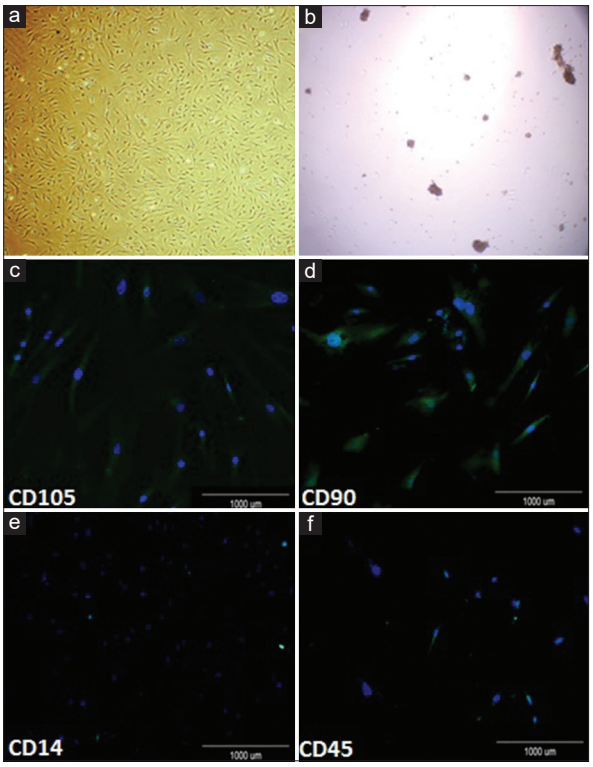

Figure 1: Phase contrast images of cell morphology. Human adipose-derived stem cells within third passage (a), human adipose-derived stem cells micromass formation before chondrogenesis (b) and CD marker expression in human adipose-derived stem cells (c-f) (a and b, ×40).

Histological analysis of samples

Morphological study of the samples revealed that in 5 μg A/S group, the sulfated glycosaminoglycans in the extracellular matrix were higher than other groups [Figure 2]. In addition, the presence of chondrocytes within lacuna was more prominent in this group [Figure 3].

Real-time polymerase chain reaction analysis

The results of real-time PCR indicated that A/S administered for 2 weeks significantly increased cartilage-specific genes expression of hADSCs in a dose-dependent manner. As seen in Figure 4, in 5 ng/ml A/S administered group, the expression of aggrecan and collagen II genes was upregulated in comparison with TGF-β, stem, and control groups ($P \leq 0.05$), but the expression of collagen I and

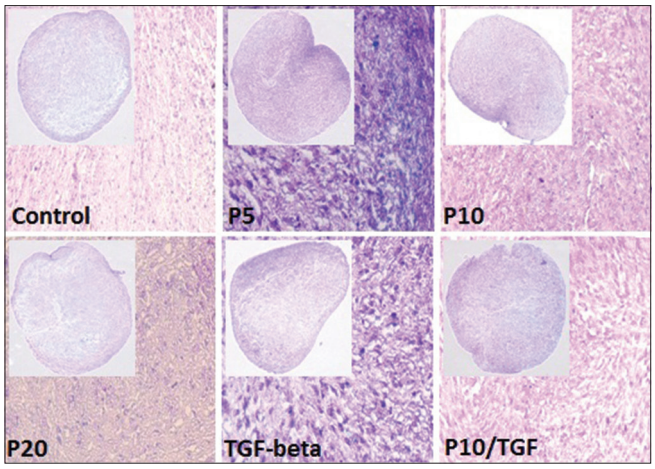

Figure 2: Toluidine blue staining of the samples in end stage of chondrogenesis. The presence of sulfated glycosaminoglycans in the extracellular matrix was higher in 5 μg Avocado/soybean group than other groups (×40 and ×100).

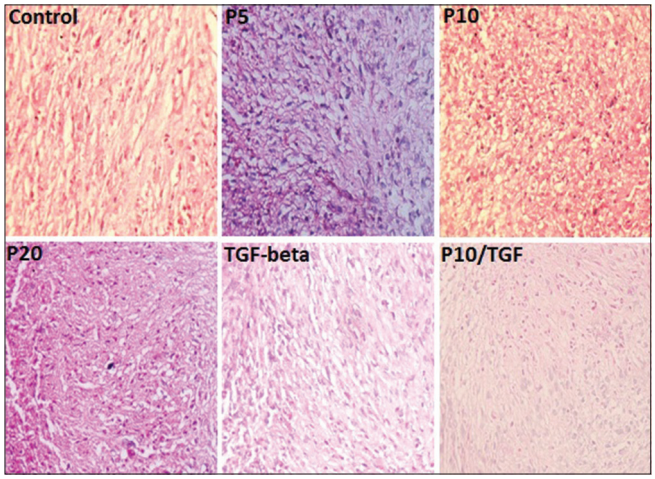

Figure 3: The samples in all groups. The presence of chondrocytes within lacuna was more prominentin 5 μg Avocado/soybean group than other groups (H and E, ×100).

collagen X was lower than TGF-β group ($P \geq 0.05$). Therefore, 5 ng/ml A/S applied for 2 weeks was determined to be the optimal treatment for highest cartilage-specific genes expression.

DISCUSSION

Age-related disorders including inflammatory, chronic, and acute joints lesions are principal cause of disability in the elderly. Of these, arthritis is the most common type of degenerative disorder which can lead to severe disability.[17] It has been shown, for example, that stem cell transplantation is able to inhibit cartilage degeneration progression and capable to promote cartilage regeneration via paracrine effects including antioxidative and anti-inflammatory effects as well as chondroprotective effects.[18-21] It is important to know that stem cell transplants to cartilage repair may be to create teratomas;[22,23] thus, *in vitro* predifferentiation of these cells by the use of specific agent such as TGF-β family is suggested. It has been reported that TGF-β family members are able to promote chondrogenic differentiation and endochondral ossification.[24,25] Although these multifunctional growth factors by intracellular signaling through specific receptors involved in several biological and pathological processes of the body, the exact roles of them in maintaining stem cells and tissue homeostasis have not been well documented.[26]

According to our current published data, TGF-β3 is able to promote chondrogenic differentiation of hADSCs via increased chondrogenic genes expression in these cells.[27] On the other hand, in similar experiment, TGF-β3 affect significantly on cellular proliferation, but TGF-β1 promotes chondrocyte differentiation.[28] Of interest, our recent study showed that TGF-β1is able to express a high level of collagen type X which is a hypertrophic marker during chondrogenic differentiation.[5] Unfortunately, due to the high number of side effects of TGF-β isoforms, the use of other effective agent on chondrogenesis is recommended.

A/S which is a plant extract has several effects on cell differentiation, cell viability, and proliferation. In addition, A/S also is able to reduce reactive oxygen species via inhibits cholesterol absorption and biosynthesis. Meanwhile, this agent in clinical level can reduce pain and stiffness and so is able to improve joint function.[10] Overall, A/S is able to modify osteoarthritis pathogenesis through inhibiting some molecules and pathways, prohibit cartilage degeneration via inhibiting the produce and function of matrix metalloproteinases, inhibit fibrinolysis through stimulating the expression of plasminogen activator inhibitor, promote cartilage repair via stimulating collagen and aggrecan synthesis, decrease cholesterol absorption and endogenous cholesterol biosynthesis, and diminish pain and joint stiffness.[29]

Here, in the presence of different dosage of A/S, we have differentiated hADECs into chondrocytes, and then, the expression of cartilage-specific genes was evaluated.

The real-time PCR results demonstrated that during chondrogenic differentiation, A/S a dose-dependent

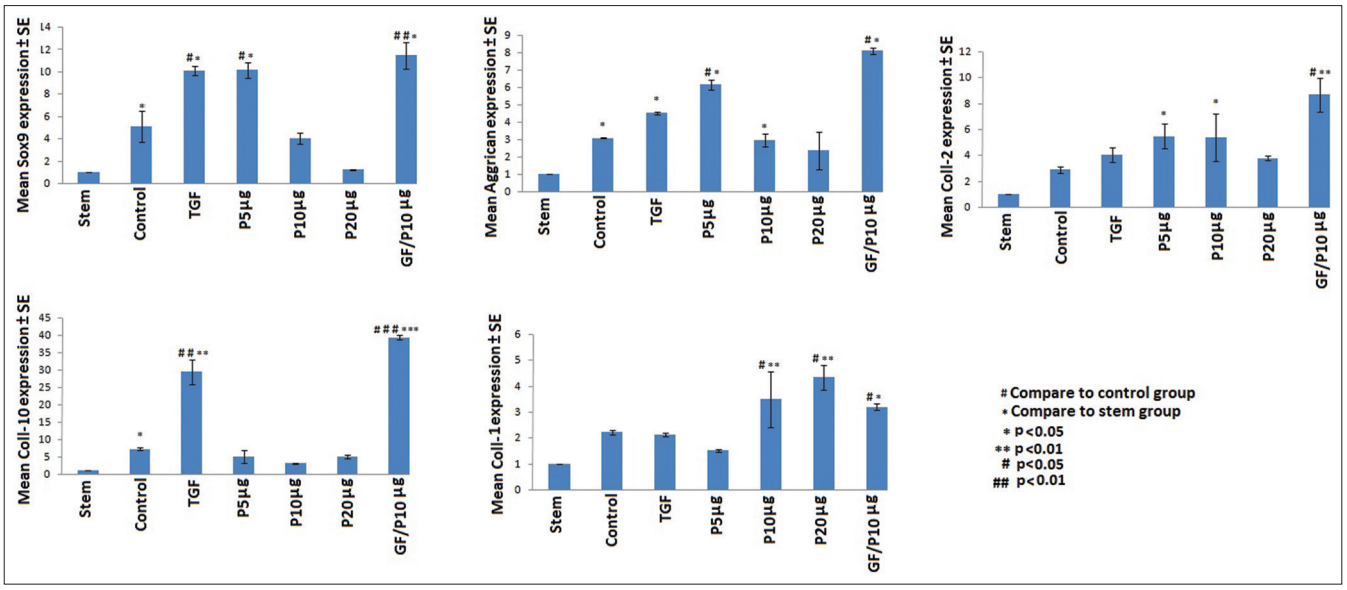

Figure 4: The results of real-time polymerase chain reaction for cartilage-specific genes expression. The expression of aggrecan, collagen II, and SOX9 genes was similar in transforming growth factor beta and avocado/soybean 5 ng/ml treated group and was increased significantly compared to stem and control groups ($P < 0.05$). In addition, the expression of collagen I and collagen X was lower in avocado/soybean 5 ng/ml treated group than transforming growth factor beta group.

manner has potentially beneficial effects on cartilage-specific genes expression. As seen in Figure 2, 5 ng/ml A/S has the highest effect on aggrecan, collagen II, and SOX9 genes expression. In addition, the expression of collagen I and collagen X genes is at low level which is another beneficial effect. These results is consistent with the recent study by others, which have also shown that, AS is able to reduces the expression of type X collagen better than other agents such as kartogenin in the stem cells.[30]

Although, the TGF-β1/(A/S) administration can increase cartilage-specific gene expression, this composition is not able to reduce the expression of hypertrophic (collagen X) and fibrocartilage (collagen I) markers. Therefore, to *in vitro* chondrogenic differentiation, 5 ng/ml A/S is an effective and beneficial agent compared to other dosage.

CONCLUSION

It can be concluded that A/S is able to promote chondrogenic differentiation of hADSCs by the effect on gene expression via intracellular signaling pathway. Thus, this agent with 5 ng/ml dosage may be an ideal agent for *in vitro* chondrogenic differentiation of stem cells to application in cartilage lesions.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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