

Chondrogenic activity of two herbal products; pomegranate fruit extract and avocado/soybean unsaponifiable

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Abstract

Background and purpose: Articular cartilage defects aren't repaired by itself. Numerous studies have been conducted in the area of cartilage tissue engineering and some of them considered herbal products. An attempt was made in this study to compare the effects of pomegranate fruit extract (PFE), avocado/soybean unsaponifiable (ASU), and their equal proportional mixture on the chondrogenesis of human adipose-derived stem cells (hADSCs).

Experimental approach: PFE was prepared through the percolation method. ASU powder was dissolved in ethanol at 10 µg/mL concentration and was sterilized. The hADSCs first were isolated, expanded in monolayer culture and identified, and next seeded on fibrin scaffolds. The hADSCs/fibrin scaffolds were divided into 4 groups of control, ASU, PFE, and PFE+ ASU and subjected to *in vitro* induction for 2 weeks. The control group received chondrogenic medium, other groups received chondrogenic medium plus ASU, PFE, or PFE + ASU, respectively. The MTT assay was performed for cell viability evaluation, real-time polymerase chain reaction for expression of cartilage genes, and the toluidine blue, safranin-O, and immunohistochemistry for staining of the constructs.

Findings / Results: Cell viability, cartilage genes expression, matrix staining density, and collagen II protein levels in PFE samples were significantly higher than those of the other groups ($P < 0.05$). Histological assessments revealed more chondrogenic centers ($P < 0.05$) in the PFE group compared to the other groups.

Conclusion and implications: In this study, it was revealed that PFE can be considered as an induction factor for future chondrogenic studies.

Keywords: Avocado; Chondrogenic differentiation; Fibrin hydrogel; Pomegranate; Soybean.

INTRODUCTION

The articular cartilage is an avascular tissue not able to repair its defects effectively (1). There exist some methods like mosaicplasty, autograft, and autologous chondrocyte implantation to cure cartilage lesions (2). One of the disadvantages of these methods is the donor site morbidity that limits their application. Therefore, treatment based on stem cells has been considered (3,4). Applying mesenchymal stem cells (MSCs) with natural scaffolds has resulted in cartilage-like tissue formation *in vitro* (5,6). Fibrin hydrogel is a

natural polymer that retains high water quantity (7). Gel-like nature of fibrin facilitates materials passage and enables cells to be uniformly distributed, therefore fibrin is applied as an optimum scaffold in cartilage tissue engineering (7). Different fibrin formulations have been studied together with human MSCs (hMSCs). The obtained results indicate the high potential of fibrin for inducing chondrogenesis (8).

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Avocado/soybean unsaponifiable (ASU) is a plant-derived product consumed to improve cartilage performance (9). The severity of articular cartilage and bone lesions of the canine model was reduced by ASU treatment (10). Coculturing of osteoarthritic human chondrocytes with osteoblast cells treated with ASU, significantly increased chondrocytes collagen (COL) II mRNA level (11). Researchers revealed that oral uptake of ASU increased transforming growth factor beta (TGF- β s) levels in the joint fluid of experimental dogs (12). *in vitro* studies revealed that ASU is an effective factor to induce chondrogenesis of human adipose-derived stem cells (hADSCs) in hydrogel scaffolds and reduce the cell hypertrophy (13,14).

Pomegranate is cultivated throughout the Mediterranean region and is native to Iran (15). Oral gavage of pomegranate fruit extract (PFE) reduced cartilage destruction and increased proteoglycan stability of the osteoarthritic joint of the mouse (16). The effect of PFE of hADSCs differentiation has been assessed and it has been proved that PFE increases COL II production (17). The count and diameter of cartilage nodules increased significantly by adding PFE to the cultured fetal limb cells (18). The molecular routes through which PFE and ASU perform have not been specified yet.

The objective of this study was to assess the effectiveness of ASU, PFE, and their combination on the chondrogenic induction of hADSCs on fibrin.

MATERIALS AND METHODS

The chemicals for this study were from Sigma-Aldrich (St. Luis, MO, USA), unless stated otherwise.

Avocado/soybean unsaponifiable preparation

ASU powder was purchased from Expanscience Laboratories (Courbevoie, France). ASU powder (0.1 mg) was dissolved in 10 mL of pure ethanol to obtain a solution at 10 μ g/mL concentration.

Pomegranate fruit extract preparation

The pomegranate was collected from Najaf Abad city (Isfahan, I.R. Iran) and after being verified by Pharmacognosy Department of School of Pharmacy and Pharmaceutical

Sciences (Isfahan University of Medical Sciences, I.R. Iran). They were peeled, their arils were collected and dried. The husks were dried, powdered, and their extract was obtained by applying the percolation method (19). The extract was concentrated at 45 °C by applying a rotary evaporator, and first, freeze-dried then stored at -20 °C. The product was dissolved in phosphate-buffered saline (PBS; Bioidea Co., I.R.Iran) before use (20).

Thrombin and fibrinogen preparation

One bag of fresh frozen plasma and one bag of cryoprecipitate (Cryo) bag were provided from the blood transfusion center of Isfahan, I.R. Iran. The products were melted at 37 °C. Ten mL calcium gluconate (Sina Daru Lab, I.R. Iran) was added to 16 mL of fresh frozen plasma in a sterile 50 mL tube and incubated for 60-90 min at 37 °C. The clear supernatant containing thrombin was removed after centrifugation at 581 g for 10 min (21). The Cryo was consumed as a rich source of fibrinogen (15 mg/mL). Cryo also contains other ingredients including von Willebrand and factor VIII, factor XIII, and fibronectin (22).

Separation and proliferation of hADSCs

Subcutaneous fat pieces (~120 g) were removed from three young volunteers of liposuction surgery in Al-Zahra hospital (Isfahan, I.R. Iran) after informed consent. The pieces were rinsed by PBS containing 1% antibiotics (Gibco, Germany). Subsequently, tissues were minced by a knife subjected to the sterile condition and were digested with 0.075% collagenase I solution (Gibco, Germany) at 37 °C for 1 h. After adding the neutralizing medium and centrifugation at 403 g for 10 min, the supernatant was separated. The pellet containing hADSCs was cultured in the T-75 flask containing medium (DMEM + 10% FBS, and 1% antibiotics) in an incubator (set at 5% CO₂ and 37 °C). The initial medium was changed 24 h after the introduction and then in every 3-day periods. After 80% confluency, the cells were isolated by applying 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Bioidea Co., I.R. Iran) and sub-cultured for three consecutive passages.

All of the protocols involving human tissue were approved by the Ethics Committee of Isfahan University of Medical Sciences, I.R. Iran (Ethic No. IR.mui.rec.1396.3.124).

Identification of stem cells using flow cytometry

The positive and negative markers were used to characterize hADSCs. A cluster of differentiation (CD) 14 and CD45 as negative markers and CD44 as a positive one was evaluated according to the protocol (23,24).

Chondrogenic induction of seeded hADSCs on fibrin scaffold

The hADSCs from passage 3 were harvested through trypsin-EDTA and centrifuged at 459 g for 8 min. The pellet was suspended in a fresh Cryo solution. A volume of 1×10^6 cells/500 μ L Cryo and an equal volume of thrombin solution was poured into the required wells of a 24-well culture plate simultaneously. After 10 min incubation and random division of the scaffolds, the chondrogenic medium (1 mL) was added to each well with respect to the groups of control, ASU, PFE, and PFE/ASU. Chondrogenic differentiation medium composed of DMEM-high glucose supplemented with 1% antibiotics, 100 nM dexamethasone, 50 μ g/mL ascorbate-2-phosphate, 50 mg/mL ITS Premix (Becton Dickinson, USA), 6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenious acid, 40 μ g/mL proline, 100 μ g/mL sodium pyruvate, 5.35 mg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin (BSA) was added to the control wells. ASU (10 μ g/mL), PFE (5 μ g/mL) + ASU (5 μ g/mL) and PFE (10 μ g/mL) were dissolved in the medium consumed for ASU, PFE, and PFE/ ASU groups, respectively. The medium changed every 2-3 days, and the differentiation time lasted for 14 days in favorable conditions (37 °C, 5% CO₂, 99% humidity) (25).

Assessment of cell viability after chondrogenic induction of hADSCs

Cell viability was assessed by MTT assay after the 14th day of differentiation. The chondrogenic medium was drained out of the

wells to be replaced by MTT solution at 0.5 mg/mL and incubated for 2 h at 37 °C until the formazan purple color appears under the microscope. Then, the constructs were incubated with 400 μ L of dimethyl sulphoxide (DMSO) for 30 min at room temperature. The optical density (OD) was measured at 570 nm wavelength.

RNA extraction and real-time polymerase chain reaction

Expression of specific cartilage genes including *COL2A1*, *aggrecan (ACAN)*, *SOX9*, *SMAD3*, and hypertrophic factor *COL10A1* was assessed using real-time polymerase chain reaction (RT-PCR) (24,25). Constructs were rinsed with PBS. Total RNA was extracted from constructs by Yekta Tajhiz Kit (Tehran, I.R. Iran) according to the manufacturer's instructions. Isolated RNA quantity and quality were determined by measuring absorbance at 260 and 280 nm with a nanodrop 2000 (Thermo scientific, USA). Five mg of total RNA was used to synthesize cDNA using Biofact 2X Onestep RT-PCR Master Mix kit (Biofact, Korea) and oligo dT primers. The primers for all assayed genes were used according to previous researches (Table 1). The RT-PCR was performed using Biofact 2X RT-PCR Master Mix (high ROX) containing SYBR Green (Biofact, Korea) and the Step One Plus™ RT-PCR detection system (Applied Biosystems, USA). The PCR amplification conditions consisted of 15 min at 95 °C followed by 40 cycles of denaturation step at 95 °C for 20 sec, annealing and extension for 1 min at 60 °C. Melting curve analysis was used to determine the melting temperature of specific amplification products and primer. These experiments were carried out in triplicate and independently repeated at least three times. *GAPDH* was used as an endogenous control. The expression level of each target gene was calculated as $2^{-\Delta\Delta C_t}$, as previously described.

Table 1. The primer sequences of genes used for the real-time polymerase chain reaction.

Genes	Forward primers (5' to 3')	Reverse primers(5' to 3')
<i>COL2A1</i>	CTGGTGATGATGGTGAAG	CCTGGATAACCTCTGTGA
<i>ACAN</i>	GTGGGACTGAAGTTCTTG	GTTGTCATGGTCTGAAGTT
<i>SOX9</i>	TTCAGCAGCCAATAAGTG	GTGGAATGTCTTGAAGGTTA
<i>COL10A1</i>	AGAATCCATCTGAGAATATGC	CCTCTTACTGCTATACCTTTA
<i>SMAD3</i>	AGCGGAGTACAGGAGACA	ACACTGGAACAGCGGATG
<i>GAPDH</i>	TTGAGGTCAATGAAGGGGTC	GAAGGTGAAGGTCGGAGTCA

Histological assessment of the constructs

Histology of constructs at the 14th day of chondrogenic differentiation was assessed. The constructs were fixed in formalin 10% solution for 18 h, dehydrated, infiltrated, and blocked in paraffin. Afterward, 5 μ m sections were prepared. Hematoxylin and eosin (H&E) staining was carried out to review the overall evaluation. Safranin-O staining was performed for glycosaminoglycans content of the sections in the following steps: staining with Weigert's iron hematoxylin solution for 10 min, fast green for 5 min, next rinsed in 1% acetic acid and then stained with 0.1% safranin-O solution for 10 min. The sections were dehydrated, cleared, and mounted. For toluidine blue staining, the sections were immersed in the toluidine blue, and the additional colors were removed by filter paper.

Immunofluorescence imaging

Constructs were first, fixed with 4% paraformaldehyde overnight on the 14th day. Next, rinsed with PBS, dehydrated, and then embedded in paraffin. The samples were sectioned at 4 μ m, deparaffinized, and rehydrated. To retrieve antigen, the sections were incubated in 0.1% pepsin for 15 min at 37 °C, and then blocking was carried out with 3% BSA. Polyclonal goat anti-human COL2A1 antibody (24128; Abcam) was consumed overnight at 4 °C, which was followed by incubation with 10 μ g/mL fluorescein isothiocyanate (FITC) rabbit anti-goat

secondary antibody (31509; Invitrogen, USA) for 1 h at room temperature. The DNA was labeled with 4', 6-diamidino-2- phenylindole (DAPI). Negative controls without primary antibodies were prepared. Images were taken using an Axiovert 200M fluorescence microscope (Carl Zeiss Light Microscopy, Germany). The positive cells were counted to determine COL II level.

Statistical analysis

One-way ANOVA and Kruskal-Wallis followed by Tukey post-hoc test were used to compare the cell viability and gene expression results between groups using SPSS software, $P < 0.05$ considered significant. Histological data were reported as mean \pm SEM. Kruskal-Wallis used for comparison of the chondrogenic center results.

RESULTS

Pre-induction assessment of hADSCs

HADSCs harvested in passage 3 appeared in spindle shape. The cells reached 80% confluence and were distributed evenly (Fig. 1A). The content of culture flasks was observed through a high magnification inverted microscope that revealed no contamination, dead or detached cells (Fig. 1A). Analysis of the CD markers on the hADSCs demonstrated that 1.14% of cells were positive for CD14/45 and 96.3% were CD44⁺ (Fig. 1B).

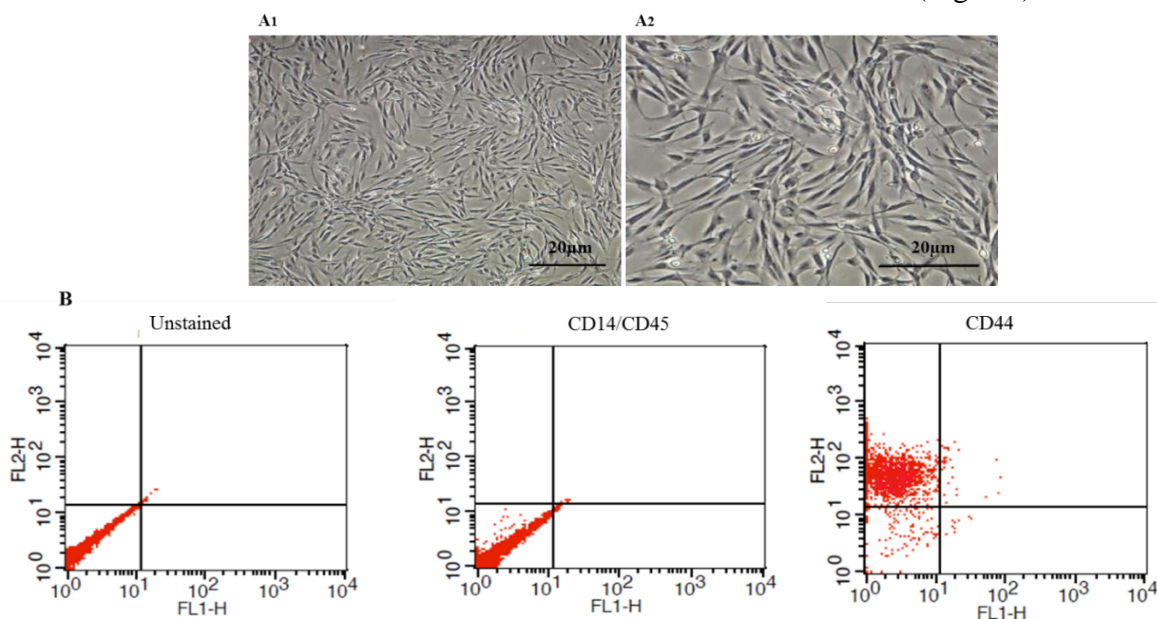


Fig. 1. Pre-differentiation examination of passage 3 hADSCs. Inverted microscopic images of hADSCs at (A₁) 20 \times and (A₂) 40 \times magnification. (B) Flow cytometry analysis of hADSCs; CD14 and CD45 as negative and CD44 as a positive stem cell markers were analyzed. CD, Cluster of differentiation.

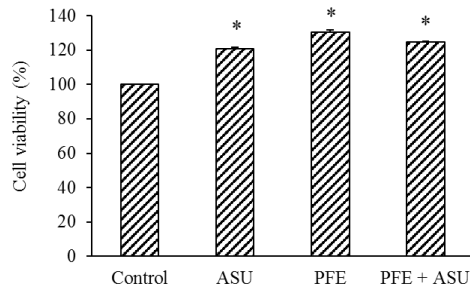


Fig. 2. MTT assay results at the 14th day of differentiation. * $P < 0.05$ indicates a significant difference in comparison to the control group. ASU, avocado/soybean unsaponifiable; PFE, pomegranate fruit extract.

Evaluation of cell viability after chondrogenic induction of hADSCs

The mean values of OD in each group were calculated and the viability percentage was determined in comparison with the control group (Fig. 2). MTT assay indicated significant differences between treated groups and the control ($P < 0.05$). The highest cell viability was observed in the PFE group.

Evaluation of genes' expression using RT-PCR

The quantitative analyses of cartilage-specific genes' expression including *COLII*,

SMAD3, *SOX9* and *ACAN* at the 14th days of hADSCs differentiation was conducted (Fig. 3). Results indicated a significant difference between all groups regarding all genes' expression ($P < 0.05$). Higher expression of the examined cartilage-specific genes was observed in PFE than the other groups (Fig. 3). There were significant differences among all samples in the *COLX* gene expression ($P < 0.01$) except between ASU and PFE+ASU groups ($P > 0.01$).

Histological findings

H&E staining exhibited the general differentiation status of the specimens (Fig. 4A), and was applied to evaluate the average of chondrogenesis centers count in successive sections. Chondrogenic centers were defined by the high density of round or polygonal-shaped cells with a chondrocyte appearance. This parameter was different between treated groups and controls (Fig. 4B, $P < 0.05$). More chondrogenic centers were observed in the PFE samples. The intensity of safranin-O and toluidine blue staining was related to the number of glycosaminoglycans. The control sections were displayed low or negative staining.

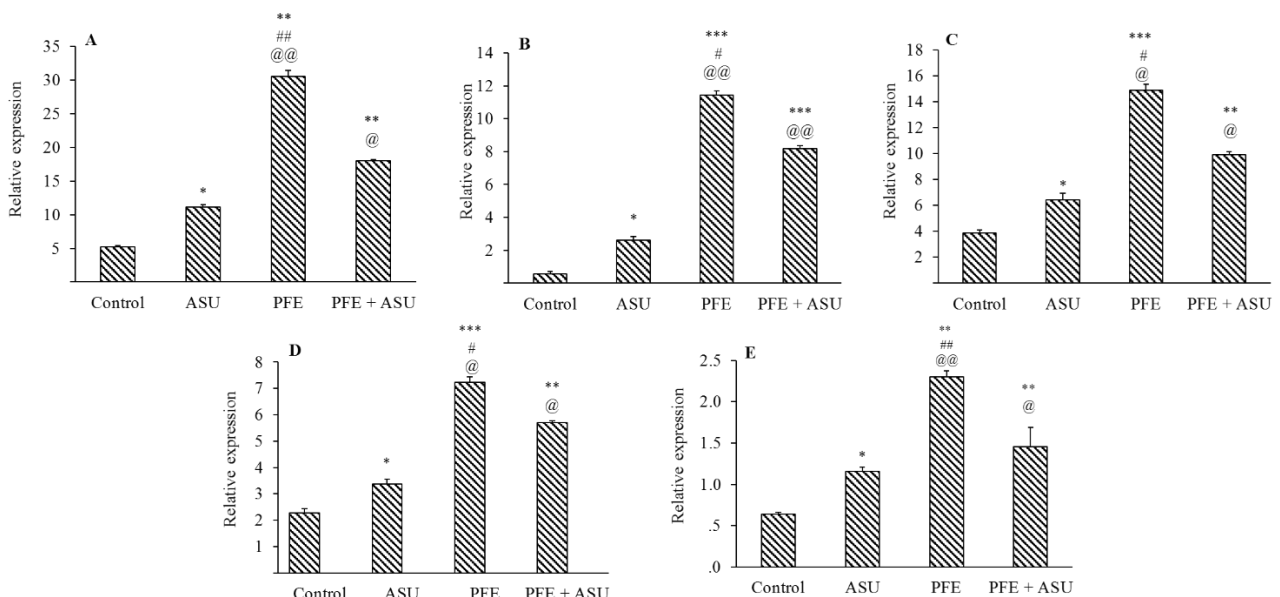


Fig. 3. Real-time polymerase chain reaction results of *COL II*, *ACAN*, *SMAD3*, *SOX9*, and *COLX* genes' expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant differences compared to the control; @ $P < 0.05$ and @@ $P < 0.01$ indicate significant differences vs ASU; and the # $P < 0.05$ and ## $P < 0.01$ considered significantly different from PFE + ASU group. ASU, avocado/soybean unsaponifiable; PFE, pomegranate fruit extract.

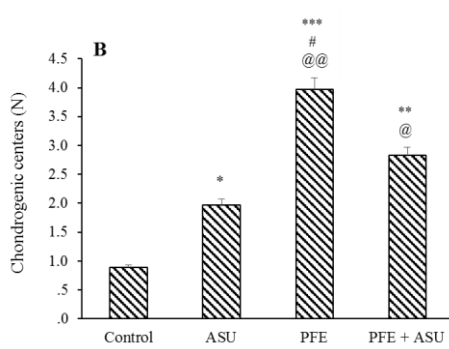


Fig. 4. Histological results of hADSCs differentiation on fibrin scaffold on the 14th day. (A) Microscopic presentation of control, ASU, PFE, and PFE + ASU groups. Red and blue of safranin-O and toluidine blue staining indicating the matrix synthesis due to chondrogenesis (magnification 40×). (B) The average count of chondrogenic centers. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared to the control; @ $P < 0.05$ and @@ $P < 0.01$

indicate significant differences vs ASU; and # $P < 0.05$ considered significantly different from PFE + ASU. ASU, avocado/soybean unsaponifiable; PFE, pomegranate fruit extract; H&E, hematoxylin and eosin.

Immunofluorescence evaluation of COL II production

The level of COL II protein was evaluated applying the immunostaining method on the 14th day of induction. Green color appearance in the cytoplasm is indicative of COL II production due to the chondrogenic induction of hADSCs (Fig. 5A). Appropriate fluorescent images were selected in terms of quality and 300 cells counted in each group manually. The percentage of COL II positive cells was obtained from each group (Fig. 5B). There were significant differences among all groups ($P < 0.05$).

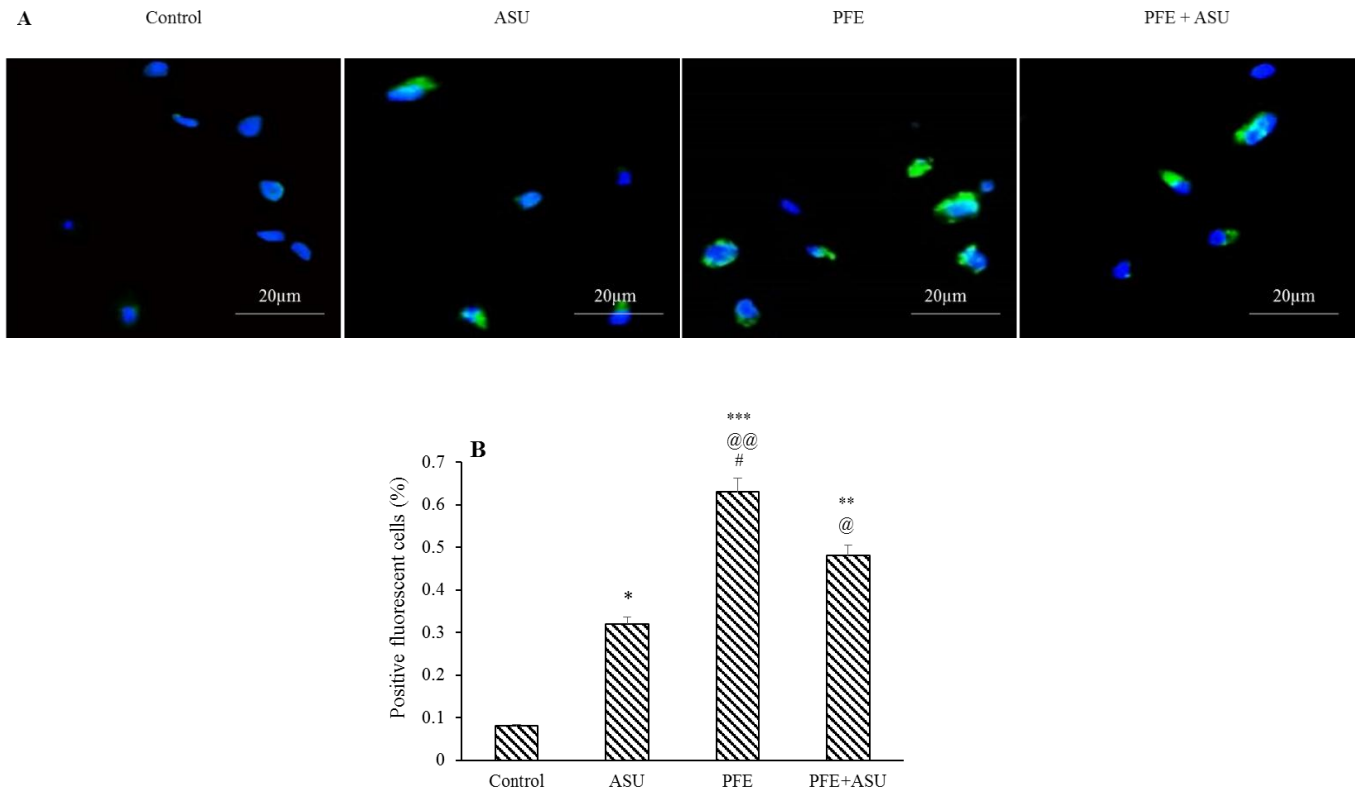


Fig. 5. Immunofluorescence results of fibrin / hADSCs constructs on the 14th day of chondrogenic induction. (A) Production of COL II protein indicated by green color in the cytoplasm of cells. Nuclei stained with DAPI stain (blue). Staining was more prominent in the PFE group (magnification 40×). (B) The percentage of COL II positive cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared to the control; @ $P < 0.05$ and @@ $P < 0.01$ indicate significant differences vs ASU; and the # $P < 0.05$ considered significantly different from PFE + ASU. ASU, avocado/soybean unsaponifiable; PFE, pomegranate fruit extract; DAPI, 6-diamidino-2- phenylindole; COL, collagen.

DISCUSSION

The potential effects of PFE, ASU, and their combination on the chondrogenic process was assessed in this study. The MTT assay results indicated the highest viability of hADSCs induced by medium contained PFE at the 14th day of differentiation. According to the obtained results, the potential of PFE for induction of chondrogenesis has been identified. The other two groups revealed significant chondrogenesis compared to the control.

The PFE used in this study included pomegranate juice and the oil extracted from its seeds. Several *in vitro* and *in vivo* studies have proved significant clinical effects of pomegranate (26). Pomegranate juice is rich in tannins and possesses many therapeutic benefits (27-29). Pomegranate seed oil, which is composed primarily of punicalic acid and sterols, exhibit pharmacological functions (30). PFE has strong antioxidant properties due to its soluble polyphenols like anthocyanins, hydrolyzable tannins, and punicalagin (31,32). Prodelphinidin, a tannin that can be found in the pomegranate, increased the synthesis of cartilage matrix proteins, proteoglycans, and COL II in human chondrocytes (33). Applying PFE on chondrocytes isolated from human osteoarthritic cartilage samples, significantly inhibited cartilage degradation effects of interleukin 1- β (32). Shukla *et al.* revealed that oral administration of PFE in the osteoarthritic mice model significantly reduced the inflammatory cytokine, interleukin 6, and severity of arthritis (34). They concluded that metabolites of PFE could be effective against nitric oxide and prostaglandin E2 production in chondrocytes.

ASU is a natural product of avocado/soybean consumed at the clinical level to reduce joint stiffness and pain (35). Previous studies have proved the inhibitory effect of ASU on interleukin 1- β . In addition to the anti-inflammatory contribution, ASU has anabolic effects and can stimulate the synthesis of COL II in chondrocytes (36). Treatment with ASU caused an increase in TGF- β 1 and TGF- β 2, the most well-known chondrogenic inducers, levels in the canine joint fluid (12).

ASU enhanced the proliferation and differentiation of ADSCs in fibrin scaffolds (37).

Relative expression of cartilage-specific genes and synthesis of cartilage matrix in constructs induced by PFE was notable (Fig. 3). The PCR results of the hyaline cartilage-specific genes are IN consistent with the previous study indicated a significant increase of COL II in presence of PFE-contained medium (17).

Here it is found that the expression of *SMAD3* and *SOX9* by PFE-contained medium was significantly higher than that of the control (Fig. 3). *SMAD3* together with *SMAD4* and *SOX9* form a transcriptional complex and are placed in the enhancer region of *COL2A1* and activate its expression (38). *SMAD3* pathway inhibits the chondrocyte maturation and hypertrophy activated by some other SMADs like *SMAD6* (39). Overexpression of *SMAD3* and *SOX9* and low level of *COLX* genes expression in this study is probably because of the positive contribution of PFE in the non-hypertrophic pathway of chondrogenesis.

The relationship between the chondrogenesis of hADSCs and PFE/ASU mixture was first investigated in this study. Also, a part of the possible molecular pathways in which the herbal products induce chondrogenesis was investigated.

This study is subject to no research constraint. In this study, the potential of two herbal preparations in chondrogenesis of hADSCs was assessed. Further research is requiring to characterize the role of their constituents and molecular pathways activate therein.

CONCLUSION

Here it is revealed that PFE promoted the proliferation and differentiation of hADSCs, enhanced extracellular matrix synthesis, and increased the expression levels of cartilage-specific genes. PFE had a strong effect on increasing the chondrogenic markers, suggesting that PFE may be a potential promoting compound in cartilage tissue engineering and may be applied in experimental chondrogenesis studies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that no conflict of interest in this study.

AUTHORS' CONTRIBUTION

All authors conceived and planned the experiments. A. Teimourinejad carried out the experiments. H. Bahramian and B. Hashemibeni supervised the project. M. Kazemi participated in preparing facilities and conducting RT-PCR experiments. All authors contributed to the interpretation of the results. A. Teimourinejad wrote the manuscript. All authors contributed to the final version of the manuscript.

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