JATE JOURNAL APPLIED TISSUE ENGINEERING 2023, Vol9, No1
DOI: https://doi.org/10.22034/JATE.2023.76

# The effect of herbal components, pomegranate and icariin on the chondrogenesis of stem cells in the fibrin- micromass hydrogel system

**Batool Hashemibeni[1], Maryam Aliakbari[1], Mohammad Bakhtiari[1], Mohammad Kazemi[2], Mohsen Setayeshmehr[1*]**

[1]Department of Anatomical Sciences, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
[2]Department of Genetic and Molecular Biology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
[*]Corresponding Author: Mohsen Setayeshmehr, email: m_setayeshmehr@med.mui.ac.ir

**Batool Hashemibeni**
**Associate Professor of Anatomical Sciences**

**Article Type:**
Original Article

Received: 5 August . 2023
Revised: 10 August . 2023
Accepted:16 August 2023
ePublished: 16 August . 2023

**Abstract**

**Introduction:** Tissue engineering has been employed to encourage cartilage renewal using natural or synthetic scaffolds, stem cells, and growth factors. The present study introduces a novel approach based on the chondrogenesis of human adipose-derived mesenchymal stromal cells (ASC)s in the fibrin hydrogel by applying the micro mass culture, using pomegranate and icariin as the herbal components.

**Materials and Methods:** To prepare fibrin hydrogel, a thrombin solution was extracted from human fresh -frozen plasma, and the cryoprecipitate was used as the fibrinogen component following thawing. The ASCs were seeded on the fibrin hydrogel via micro mass culture system. To investigate the chondrogenesis of the ASCs, the cell-seeded hydrogels were cultured in the different culture medias containing TGF-$\beta_3$, pomegranate extract, icariin, and the combined medium containing the Icariin and pomegranate.

**Results:** Gene expression measurements and histological staining confirmed that the ASC-seeded hydrogels cultured in the chondrogenic media containing the herbal components could express cartilage-specific genes and synthesize the cartilage-related macromolecules. However, in the presence of TGF-$\beta_3$, the hydrogels exhibited a significant expression of these markers.

**Conclusion:** These findings suggest that a combination of these two herbal components could significantly stimulate ASCs and increased cellular proliferation, the expression of chondrogenic genes, the enhanced glycosaminoglycan and collagen synthesis, and the reduction of the hypertrophic phenotypes compared to the TGF-$\beta_3$.

***Keywords:*** Cartilage tissue engineering, Fibrin hydrogel, Herbal components, Micromass culture

## Introduction

Articular cartilage has a limited self-repair capacity because of a lack of progenitor cells and vascularity. Traumatic damages and osteoarthritis are among the main reasons for articular cartilage degeneration [1-3]. Typical methods for repairing cartilage damage include micro-fracture, autologous chondrocyte transplantation, and allograft implants. However, none of these attitudes has reached the complete regeneration of the functional cartilage. Therefore, the researchers have recruited tissue engineering (TE) approaches to encourage cartilage regeneration using scaffolds, stem cells, and growth factors [4-6]. Numerous researchers have used fibrin hydrogel for various TE applications to meet precise biological and physical requirements. Furthermore, fibrin hydrogel displays minimal foreign body reaction and inflammation and can absorb through the regeneration and healing progression. Fibrin hydrogel offerings many advantages compared to synthetic biopolymers, such as proper biocompatibility, nontoxic byproducts, and a controllable biodegradation rate.

Likewise, by adjusting the concentration of the precursors and ionic crosslinking can be tuned mechanical stability and the morphology of resulted in fibrin hydrogel. Also, fibrin hydrogel grants uniform cell seeding and distribution, and can enhance cellular interaction. The *in situ* crosslinking and gel formation ability make fibrin appropriate as an injectable hydrogel for developing a compatible biomaterial for a minimally invasive cell delivery method [7,8]. Additionally, autologous fibrin does not involve the risk of allergic reactions and microbial contaminations. Scientists have commonly used fibrin in cartilage tissue engineering (CTE) as a scaffolding biomaterial [9].

A new method called micromass technique has been developed to overcome the problems regarding the needs of scaffolds in TE. Theoretically, in this technique the cells are detached and then cell suspension is aggregated into cellular spheres. The micromass technique allows researchers to monitor tissue development from particular cells to organized tissues in a controlled micro-environment [10,11]. The micromass technique relies to an excessive level on the presence of the proteinaceous extracellular matrix (ECM). The ECM can apply direct or indirect effects on cells to consequently modulate their performance. In addition, these cells adjust the composition of the ECM. This may have resulted in a variety of ways, involving differential expression of specific ECM components in the cells' microenvironment. While most studies regarding micromass technique were done in developmental studies, only limited researches are available concerning its use in TE [10]. The micromass culture system was first used to explore endochondral skeletal development through the embryonic chicken limb bud. This method has been frequently applied to evaluate the induction of the chondrogenesis of mesenchymal stem cells (MSC)s [10,11]. In our previous study, we examined chondrogenic differentiation of human adipose-derived mesenchymal stromal cells (ASC)s cultivated in the micromass culture in the presence of TGF-$\beta_3$. The results exhibited the significant performance of this method in CTE applications [12].

Mesenchymal stem cells are resident in almost all mature tissues and are responsible for beginning reparative cascades in injuries.

The implantation of adult MSCs leads to pain and inflammation decrease, and cartilage renewal in early-phase clinical trials [14]. Human adipose tissue is considered a proper source to isolate ASCs for regenerative medicine purposes because it can be obtained via less invasive protocols in a large number of cells. However, easy isolation procedures and their multipotent differentiation potential are the reason to consider it as a proper source of cells for CTE applications [15-18].

Some members of the Transforming growth factor-β (TGF-β) superfamily, such as TGF-$\beta_1$, TGF-$\beta_3$, and $BMP_6$, are vital factors in the chondrogenesis of MSCs, extracellular matrix (ECM) synthesis, and cartilage development [19]. However, according to some previous studies, the overexpression of TGF-$\beta_3$ in murine joints could lead to inflammation, osteophyte stimulation, and synovial fluid increase [20,21]. Furthermore, these growth factors (GF)s not only are expensive but also have a short half-time and rapid degradation. Consequently, researchers are looking for other GFs with higher efficacy and lesser hypertrophic effects for CTE applications [17].

In southeast Asian countries, the *Herba Epimedii* (HEP) is famous as a traditional Chinese herb for treating osteoporosis [22]. The key active factor of HEP, icariin (ICA; C33H40O15), has several pharmacological effects such as promoting cerebral blood flow and blood circulation, regulating immunity, and antitumor activities. In addition, icariin can stimulate the proliferation and development of osteoblasts, anti-lipid peroxidation, enhance estrogenic activity, protect neurons from ischemia/reperfusion damage, and prevent and treat atherosclerosis [23]. Furthermore, many researchers have endorsed icariin as a potential promotor for cartilage healing and an alternative to the chondrogenic GFs [24]. Some research studies have demonstrated that icariin is a nontoxic and strong chondrocyte anabolic mediator that can stimulate the proliferation of chondrocytes and decrease the degradation of the cartilaginous ECM [25], and enhance cartilage-specific gene expression, including the SRY-type high mobility group box 9 (SOX9), collagen II and aggrecan *in vitro* [26].

Pomegranate (*Punica granatum L.*), a fruit belonging to the family Punicaceae, contains crude fiber, sugars, pectin, anthocyanin, several tannins, and flavonoids in their juice and seed oil. Pomegranate peel polysaccharides have significant antioxidant potential in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, and superoxide anion radical scavenging *in vitro* [27, 28].

Furthermore, pomegranate has significant antioxidant, anti-inflammatory, anti-carcinogenic, anti-mutagenic, and anti-microbial effects on chondrocytes [29-32]. These unique characteristics have made pomegranates more and more popular worldwide [33]. Recently published studies have demonstrated that pomegranate extract can prevent the destruction of the ECM of knee joints and cartilage damage in the mouse's osteoarthritis model [34,35]. In the previous work, our group described the positive effects of the pomegranate seed extract on the chondrogenesis of ASCs; the results showed the overexpression of type II collagen in the differentiated cells [36].

In this study, we investigated the effect of the herbal components of pomegranate and icariin and their combination on the chondrogenesis of ASCs in the fibrin hydrogel through the micromass culture. To prepare fibrin hydrogel, a thrombin solution was extracted from FFP, and the cryoprecipitate was used as the fibrinogen component following thawing. Then, the ASCs/fibrin suspension was seeded on the bottom of the culture plate in the form of a droplet, accordingly. Following gelation, the seeded droplets were cultured in the chondrogenic media in the micromass culture system without/with TGF-$\beta_3$, pomegranate, icariin, and their combination to evaluate the chondrogenic potential of these herbal components via gene expression and histology methods.

## Materials and Methods

### Isolation and Culture of ASCs

The ASCs were isolated from the subcutaneous adipose tissue following liposuction from 4 female donors (Al Zahra Hospital, Isfahan) with the body–mass index in the range of 25-30 and between 25-45 years old. The Institutional Ethics Committee of Isfahan University of Medical Sciences approved the informed permission of the study. According to the previous works to digest the adipose tissue, collagenase type IA was used at 37 °C for 30 min [11,16].

The cells were cultured on 75-$cm^2$ flasks at a starting density of 6000 cells/$cm^2$ through a culture media containing the Dulbecco's modified Eagle's medium (DMEM, Bioidea, Iran), 10% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (pen/strep, Bioidea, Iran), and 10 ng/mL basic fibroblast growth factor (b-FGF, Royan, Iran). Consequently, the cells were incubated at 37°C in 5% $CO_2$, and the culture media was changed every 48 hours. After reaching 90% confluence, the cells were harvested using 0.05% trypsin- EDTA (Bioidea, Iran) and seeded on the scaffolds following counting [37,39].

The cryoprecipitate and human fresh-frozen plasma (FFP) were prepared from the Blood Donation Center (Isfahan, Iran) as the raw materials. Thrombin solution was extracted via a previously established method. In short, 400 mM $CaCl_2$ was added to FFP (1:9 vol/vol); then, the clots were incubated for 90 min at 37 °C, then centrifuged at 2200 rpm at 4°C for 10 min to obtain the thrombin concentrate. Following thawing, the cryoprecipitate was used as the fibrinogen component with any purification [16,17,40].

**Preparation of the fibrin hydrogel**

The cryoprecipitate and human fresh-frozen plasma (FFP) were prepared from the Blood Donation Center (Isfahan, Iran) as the raw materials. Thrombin solution was extracted via a previously established method. In short, 400 mM $CaCl_2$ was added to FFP (1:9 vol/vol); then, the clots were incubated for 90 min at 37 °C, then centrifuged at 2200 rpm at 4°C for 10 min to obtain the thrombin concentrate. Following thawing, the cryoprecipitate was used as the fibrinogen component with any purification [16,17,40].

**Proliferation and differentiation of the ASCs on the scaffolds**

Following isolation, ASCs were re-suspended in thrombin (cell density, $10\times10^6$ cells/ml), then fibrinogen was added to the mixture and mixed by pipetting. ASCs/fibrin suspension was seeded on the bottom of the 24-well culture plate in the form of a droplet. ASCs/fibrin droplets were allowed to form a gel and attached to the culture plate for 20 min at 37°C before adding the culture media. ASCs/fibrin droplets were further incubated in the medium at 37°C in an atmosphere of 5% $CO_2$ and 95% humidity; the medium was changed every 48 hours. According to the previous works, on days 1 and 14 of culture, cell viability and proliferation within the scaffolds were evaluated via the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) assay [16].

The cell/fibrin hydrogels seeded on the 24 well plates were cultured in the well-known micromass system. Fibrin scaffolds were cultured for 14 and 21 days in 1 ml of the culture media (control medium, CRL) composed of DMEM high-glucose (Bioidea, Iran), 1% pen/strep, L-ascorbic acid 2-phosphate (37.5 mg/ml; Sigma, A8960), 1% ITS + Premix (Gibco, 41400-45), and 10 nM dexamethasone (Sigma, D4902). In addition, the scaffolds were cultivated for 14 and 21 days in the culture media containing 10 ng/ml TGF-$\beta_3$ (TGF) medium (Sigma, T-9705), pomegranate (pom) medium containing the 100 µl/ml pomegranate extract (Shahreza, Iran), icariin (Ica) medium containing the $1\times10^{-5}$ Molar ($10^{-5}$ M) icariin (Iran), and the combination (P/I) medium containing the $10^{-5}$ M icariin and 100 µl/ml pomegranate to evaluate the chondrogenesis of the ASCs [11]. The fibrin hydrogels were subsequently incubated in the $CO_2$ incubator at 37°C; the media were changed every 2 days, and the cultures were discharged on days 14 and 21 for further evaluation [15-17].

**Real-time polymerase chain reaction (RT-PCR)**

The RT-PCT was carried on according to the previously established method. In short, after 14 days of culture, the scaffolds were discharged and three constructs per each group were moved to the 1.5 ml plastic vials and crushed using a homogenizer following snap-frozen in liquid Nitrogen, then lysed using the super RNA Extraction Kit for Tissue and Cells (YT9080, Iran). After that, the purification system was used to extract total RNA based on the manufacturer's optional procedure. The high -capacity cDNA Synthesis Kit (YT4500, Iran) was used to synthesize the single-stranded cDNA following RNA extraction. Real-time PCR experiment was performed using a Step One Plus real-time PCR system (Applied Biosystem). The primers were designed using Allele ID 7.6 software according to the previously published works, to compare the transcript levels for different genes: aggrecan (ACAN), types II collagen (COLIIA1), types X collagen (COLX), and SOX9 (Table 1). To analyze relative gene expression, 2X Real-Time PCR Master Mix, High ROX SYBR® Green1 (BioFACT) was used. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer was used as the internal control. To calculate relative gene expression, comparative Ct (ΔΔCt) was applied [16,41].

**Histological analyses**

After 21 days of culture, the scaffolds were fixed for 24 hours at 4°C in 10% buffered formalin in the PBS solution. Then, the scaffolds were processed using increasing the ethanol solutions before being cleared by the xylene series. After that, the samples were impregnated and embedded in paraffin and then cut into 5 µm slices. Xylene-cleared sections were stained using general histological staining, Hematoxylin, and Eosin (H&E). For the staining of glycosaminoglycans (GAG)s, Toluidine blue staining (0.125%, 20 sec) was performed. In addition, in another method for GAGs staining, xylene-cleared sections were treated with hematoxylin for 5 min, 0.02% fast green for 1 min, and 1% aqueous Safranin-O solution for 30 min; then, were rinsed in distilled water, dehydrated, cleared and mounted on the microscope slides [37,42].

**Table1:** Gene sequences of the primers

| Gene | Forward and reverse primer sequences |
|---|---|
| **Collagen II-F** | CTGGTGATGATGGTGAAG |
| **Collagen II-R** | CCTGGATAAC-CTCTGTGA |
| **Sox9-F** | TTCAGCAGCCAA-TAAGTG |
| **Sox9-R** | TTCAGCAGCCAA-TAAGTG |
| **Collagen X-F** | AGAATCCATCTGAGAA-TATGC |
| **Collagen -R** | CCTCTTACTGCTATAC-CTTTAC |
| **Aggrecan-F** | CCTT-GGAGGTCGTGGTGAAAGG |
| **Aggrecan-R** | AG-GTGAACTTCTCTGGCGACGT |
| **GAPDH-F** | AAGCTCATTTCCTGG-TATG |
| **GAPDH-R** | CTTCCTCTTGTGCTCTTG |

**Statistical analysis**

For the statistical analysis, the data were evaluated using the Kolmogorov-Simonov test for the normal distribution analysis of variables. In addition, to determine the significance between different groups and different time points ($\alpha = 0.05$) of the MTT and RT-PCR results, one-way analysis of variance with the LSD *post-hoc* test was employed.

## Results

### MTT assay

The results of the MTT assay on days 1 and 14 displayed the viability of the ASCs in the fibrin hydrogels (ACSs/Fibrin) in the presence of the herbal components compared to the control group. (Fig. 1). The analysis showed the significant proliferation of ASCs on the cultured fibrin scaffolds on day 14 compared to day 1. Furthermore, ASCs metabolic activity in hydrogels was higher than that of 2D cell cultures at 14 days. However, treatment of the ASCs in the ACSs/Fibrin constructs using icariin and TGF-$B_3$ led to significant increases in cell viability on day 14 of culture (145.80±0.22% and 138.20±0.04%, respectively), compared to the control group (132.87±0.14%). Nerveless, treatment of the ASCs using Pom and Pom/Ica supplements couldn't lead to significant increases in cell viability on day 14 of culture (131.71±0.06% and 133.25±0.17%, respectively), compared to a control group (Fig. 1).

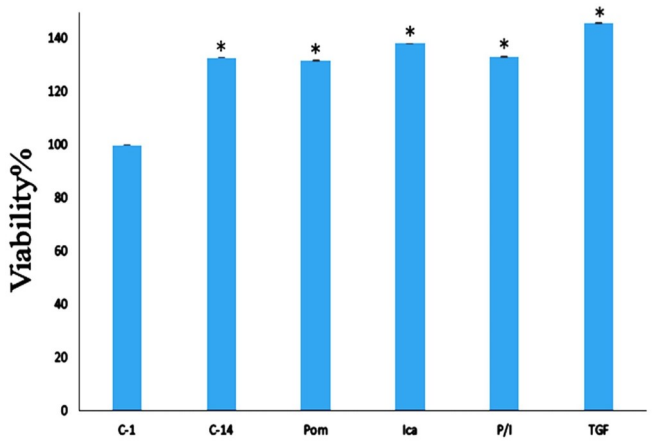


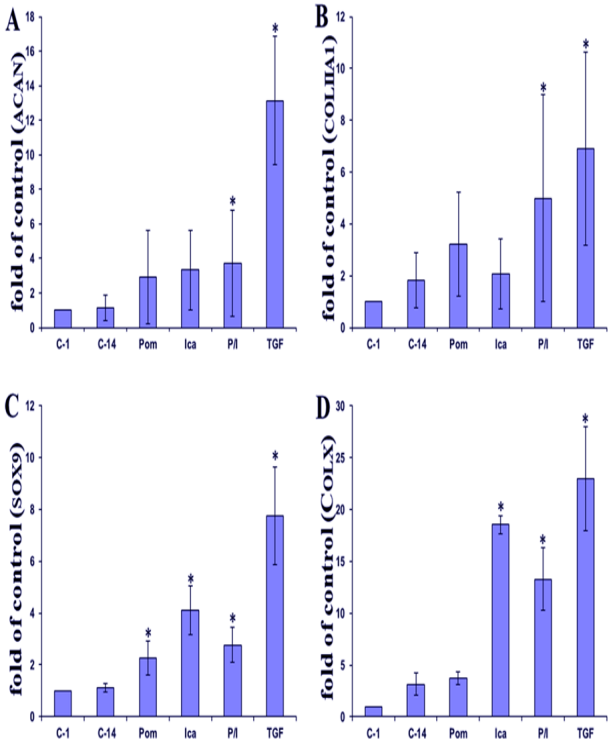

**Figure 1:** Comparison of the MTT assay results, showing the viability of ASCs in the (ASCs/Fibrin) hydrogels compared to the control group. (Data presented as mean ± SD, *p < 0.05, as compared to the day 1; n=3).

### Gene Expression

The capacity of the cell-seeded hydrogels and supplemented with different media to enhance chondrogenic differentiation of ASCs, which is a main concern for cartilage regeneration, were assessed by RT-PCR. The three chondrogenesis gene markers, SOX9, ACAN, COLIIA1, and a hypertrophic gene marker, COLX, were analyzed in the control, pomegranate extract (Pom), icariin (Ica), pomegranate/icariin (P/I) and TGF-$\beta_3$ (TGF) groups on the day 14 (Fig. 2). In Pom/Ic medium, the three positive chondrogenic markers were significantly upregulated by the ASCs/Fibrin hydrogels. COLIIA1 was significantly increased relative to day 1, which exhibited an approximately 5-fold increase upon day 14. ACAN and SOX9 transcript values were also significantly upregulated by 3.7-fold and 3-fold on day 14, respectively. Likewise, the significant upregulation of COLX was observed. By day 14, COLX levels had been increased nearly by 13 folds, relative to day 1. However, in the TGF medium, the three positive chondrogenic markers were significantly upregulated by the fibrin hydrogel, as compared to the P/I group. In addition, COLIIA1 was significantly increased relative to day 1, which exhibited an approximately 6.9-fold increase on day 14. ACAN transcript values were also significantly upregulated by 13.1-folds on day 14 of culture. Furthermore, a slight trend representing an increase in SOX9 production was noted through day 14 in the P/I and TGF groups, which showed 2.8 and 7.8-fold upregulation, respectively. Nevertheless, the significant upregulation of COLX was observed in the TGF group, as compared to the P/I group. By day 14, COLX levels had been increased approximately by 23 fold, relative to day 1(Figure 2).

**Figure 2:** Expression of the cartilaginous transcripts for the fibrin-micro mass scaffolds on day 14

## Histology




To confirm chondrogenesis and new matrix synthesis and approve RT-PCR results, histological studies involving hematoxylin and eosin staining to show cell infiltration within the hydrogels, and Toluidine blue and Safranin-O/fast green (S-O) staining to confirm chondrogenesis and GAGs formation, were performed. In the control medium, the fibrin hydrogel in the micromass system, after 21 days of culture, established minimal matrix production, as compared to day 1 (Fig. 3). In the fibrin scaffolds, supplementation with TGF-$\beta_3$ and Pom/Ic media significantly improved the synthesis of the new matrix that was stained strongly for GAGs, while the TGF group exhibited the most synthesized matrix, as compared to the Pom/Ic group. Furthermore, in the hydrogels, cell distribution, and new matrix deposition were uniform within the scaffolds (Fig. 3).

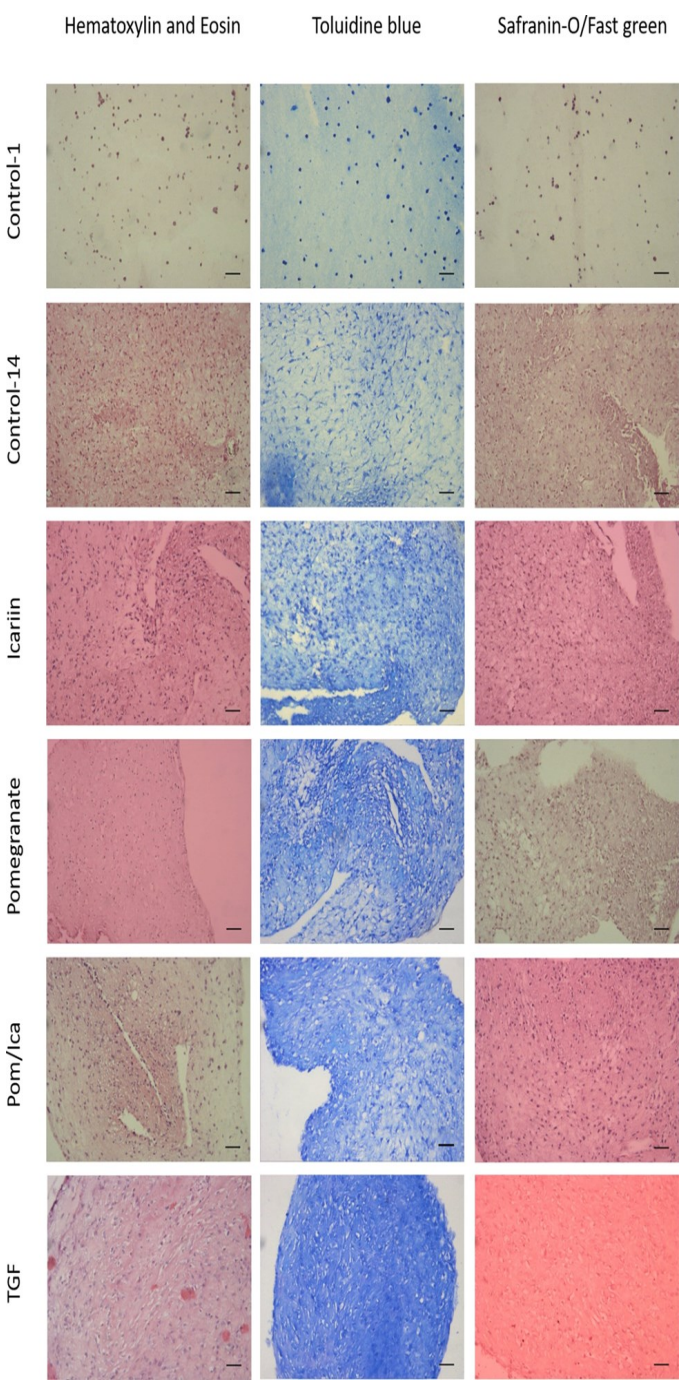



**Figure 3:** Chondrogenesis in the ASCs/Fibrin-micromass culture. Cartilaginous constructs formed by ASCs cultured under control, Pomegranate extract (Pom), icariin (Ica), Pomegranate/icariin (P/I), and TGF-$\beta_3$ (TGF) media. Hematoxylin and eosin, Toluidine blue and Safranin-O/fast green staining, after 21 days of culture. General morphology, indicating a central core surrounded by a basophil transition zone stained with H&E, metachromatic staining with TB, and red staining with S-O, typical of the cartilage phenotype.

The rich deposited neo-matrix was stained and presented in both TGF and combination groups, compared to the control medium (scale bar = 50 µm).on day 14 in the control medium (C-14) and in the presence of the Pomegranate extract (Pom), icariin (Ica), Pomegranate/icariin (P/I), and TGF-$\beta_3$ (TGF). Relative quantification of **A**: ACAN, **B**: COLIIA1, **C**: SOX9, and **D**: COLX gene expression normalized to day 1 (C-1) transcript values exposed upregulation of the genes. (Data presented as mean ±SD; *$p < 0.05$, as compared to day 1; n = 3).

## Discussion

The results of the MTT assay showed an increase in cell viability on day 14 for the Pomegranate extract (Pom), icariin (Ica), and Pomegranate/icariin (P/I) media, as compared to the control medium. This, therefore, suggested that these herbal components were cell compatible, consistent with the previous reports. Previous works [36] have also reported the cell compatibility of the Pom extract. Furthermore, ASCs seeded on the fibrin hydrogel displayed comparable proliferative capability concerning the control 2D culture. Some other researchers have also demonstrated that icariin is a nontoxic agent promoting the proliferation of chondrocytes [25]. The results of the study carried out by our group in 2020 showed that the cell viability of the differentiated cells in fibrin/icariin nanoparticles was significantly increased, as compared to the fibrin control group [43].

Icariin has been described to exhibit multiple effects on bone marrow-derived MSCs (BMSCs), including its proliferation, osteogenic and chondrogenic differentiation. Lio et al., in 2020, reported that Ica protected the rabbit BMSCs from the damage induced by oxygen, glucose, and serum deprivation induced apoptosis; via inhibitory regulation of the endoplasmic reticulum stress -mediated autophagy related to the MAPK signaling pathway. These results have delivered visions for a potential therapeutic strategy in OA [44]. In one other study, Wang et al., 2020, revealed that Ica treatment could support chondrocyte vitality by promoting HIF-1α expression and anaerobic glycolysis. Therefore, they concluded that Ica could be considered a novel clinical treatment for OA [45]. A research group, in 2020, also determined the synergistic effects of a formula containing the dried pomegranate concentrate powder, *Eucommiae Cortex*, and *Achyranthis Radix* (PCP:EC: AR). They informed that the oral administration of the PCP:EC:AR displayed potent osteoarthritis (OA) protective effects in an OA rabbit model induced by surgery [46]. In the present study, we evaluated the effect of the herbal components of Pom and Ica and their synergic effects on the chondrogenesis of ASCs in the fibrin hydrogel through the micromass culture. According to the results, gene transcript levels on day 14 for SOX9, ACAN, COLII A1, and COLX were upregulated in the fibrin hydrogels in both P/I and TGF groups compared to the ASCs on day 1. Nevertheless, the significant upregulation of SOX9 and COLX in the Pom and Ica groups was observed compared to the control group. On the other hand, the upregulation of ACAN and COLII A1 in the Pom and Ica groups was not significant compared to the control group. Therefore, the observed results suggested that the combination of Pomegranate extract and icariin could induce the chondrogenic potential and provide support for the chondrogenesis ability of the ASCs within the fibrin hydrogel in the micromass culture. Consequently, the combination of these active biomolecules might have synergic effects on the chondrogenic differentiation of the ASCs.

. However, the previous work [36] has shown the overexpression of type II collagen in differentiated cells in the presence of the Pom extract. The other study, confirmed that icariin could enhance cartilage-specific gene expression, including SOX9 and collagen II, aggrecan *in vitro* [26]. Furthermore, in a recent study performed by our group in 2020, the effect of fibrin/icariin nanoparticles on the chondrogenesis of the stem cells was evaluated; the quantitative RT-PCR analysis demonstrated that Ica upregulated the cartilaginous-specific gene expression [43]. As shown by the histological images, the distribution of the cells within all experimental groups was form as well as the control group. uniform as well as the control group. Histological sections in all TGF-$\beta_3$ and P/I treated samples showed the expression of GAGs macromolecules. General morphology demonstrated a central core surrounded by a basophil transition zone staining with H&E, metachromatic staining with TB, and red staining with S-O, representative of the cartilage phenotype. The abundant synthesized neo-matrix was observed in the TGF and combination groups compared to the control group. Fibrin hydrogels treated with TGF-$\beta_3$ showed a higher synthesis of GAGs matrices, as compared to the combination group. However, it should be mentioned that the TGF-$\beta_3$ treated ASC-fibrin group produced a hypertrophic phenotype, as shown by histological staining (Fig. 3), that was according to COLX gene upregulation. Since ASC-fibrin treated by P/I induced the synthesis of GAGs and minimized a hypertrophic phenotype, ASCs responded to signals generated from these herbal components to differentiate towards a more hyaline chondrocyte phenotype. However, according to previous reports, the proper stimuli to induce rapid tissue remodeling are necessary to induce collagen biosynthesis [47]. As observed and evaluated in this study via RT-PCR and histology studies, by proper ASCs stimulation through these herbal components, GAGs, and COLII biosynthesis was significantly induced during the 21-day culture period.

## Conclusion

In the present study, the effect of pomegranate, icariin, and their combination as herbal components were investigated on the chondrogenesis of ASCs in the fibrin hydrogel through the micromass culture. the ASCs/fibrin hydrogels were seeded on the culture plate in the form of a droplet. The ASCs/fibrin hydrogels then were cultured in the chondrogenic media without/with TGF-$\beta_3$, pomegranate, icariin, and their combination to investigate the chondrogenic potential of the herbal components compared to the TGF-$\beta_3$ in the micromass culture system. The results of the MTT assay presented an increase in the cell viability on day 14 for the treated groups compared to the 2D control, thereby suggesting that these herbal components were cell compatible and nontoxic. The RT-PCR and histological results indicated that fibrin scaffolds treated with TGF and P/I could stimulate ASCs chondrogenesis. Furthermore, the combination of these two herbal components could significantly stimulate ASCs and produce the most required products in terms of increased cellular proliferation, the expression of chondrogenic genes, enhanced GAG, and collagen synthesis. In addition, the combination group (P/I) presented a significant reduction of the hypertrophic phenotypes and expression of the COLX, as compared to the TGF group.

**Acknowledgments**

This work was supported by grants received from the Isfahan University of Medical Sciences, Isfahan, Iran.

**Conflict of Interests**

The author declares any conflict of interest.

**References**

1. Sutherland AJ, Beck EC, Dennis SC, Converse GL, Hopkins RA, Berkland CJ, et al. Decellularized cartilage may be a chondroinductive material for osteochondral tissue engineering. PloS one. 2015;10(5):e0121966.
2. 2.Athanasiou KA, Darling EM, Hu JC, DuRaine GD, Reddi AH. Articular cartilage: CRC Press; 2013.
3. Huey DJ, Hu JC, Athanasiou KA. Unlike bone, cartilage regeneration remains elusive. Science. 2012;338(6109):917-21.
4. Liao I, Moutos FT, Estes BT, Zhao X, Guilak F. Composite three-dimensional woven scaffolds with interpenetrating network hydrogels to create functional synthetic articular cartilage. Advanced functional materials. 2013;23 (47):5833-9.
5. Blum MM, Ovaert TC. Low friction hydrogel for articular cartilage repair: evaluation of mechanical and tribological properties in comparison with natural cartilage tissue. Materials Science and Engineering: C. 2013;33(7):4377-83.
6. Setayeshmehr M, Esfandiari E, Rafieinia M, Hashemibeni B, Taheri-Kafrani A, Samadikuchaksaraei A, et al. Hybrid and composite scaffolds based on extracellular matrices for cartilage tissue engineering. Tissue Engineering Part B: Reviews. 2019;25(3):202-24.
7. Li Y, Meng H, Liu Y, Lee BP. Fibrin gel as an injectable biodegradable scaffold and cell carrier for tissue engineering. The Scientific World Journal. 2015; 2015: 1-10.
8. Wang W, Li B, Yang J, Xin L, Li Y, Yin H, et al. The restoration of full-thickness cartilage defects with BMSCs and TGF-beta 1 loaded PLGA/fibrin gel constructs. Biomaterials. 2010;31(34):8964-73.
9. Hashemibeni B, Pourentezari M, Valiani A, Zamani M, Mardani M. Effect of icariin on the chondrogenesis of human adipose derived stem cells on poly (lactic-co-glycolic) acid/fibrin composite scaffold. International Journal of Advanced Biotechnology and Research. 2017;8 (2):595-605.
10. Handschel JG, Depprich RA, Kübler NR, Wiesmann H-P, Ommerborn M, Meyer U. Prospects of micromass culture technology in tissue engineering. Head & face medicine. 2007;3 (1):1-4.
11. Teimouri M, Hashemibeni B, Mardani M. Comparison of aggrecan gene expression in chondrogenesis of adipose-derived stem cells in pellet and micromass culture systems. Tehran University Medical Journal TUMS Publications. 2018;76(2):90-5.
12. Setayeshmehr M, Esfandiari E, Hashemibeni B, Tavakoli AH, Rafienia M, Samadikuchaksaraei A, et al. Chondrogenesis of human adipose-derived mesenchymal stromal cells on the [devitalized costal cartilage matrix/poly (vinyl alcohol)/fibrin] hybrid scaffolds. European Polymer Journal. 2019;118:528-41.
13. Davis A, Guldberg RE, Samsonraj RM. Osteoimmunological Implications in Regenerative Medicine: Cross-Talk Between Mesenchymal Stem Cells and Immune Cells. Journal of Musculoskeletal Research. 2020.
14. Hsiao Y-m, Hu C-C, Chang C-H, Chen M-F, Chang Y. Adult mesenchymal stem cell-based approaches for osteoarthritis: current perspectives and challenges. Journal of Musculoskele-

Musculoskeletal Research. 2020.

15 .Hashemibeni B, Razavi S, Esfandiary E, Karbasi S, Mardani M, Nasresfahani M. Induction of chondrogenic differentiation of human adipose-derived stem cells with TGF-$\beta_3$ in pellet culture system. Iranian Journal of Basic Medical Sciences. 2008;11(1):10-7..

16.Hashemibeni B, Valiani A, Esmaeli M, Kazemi M, Aliakbari M, Iranpour FG. Comparison of the efficacy of piascledine and transforming growth factor β1 on chondrogenic differentiation of human adipose-derived stem cells in fibrin and fibrin-alginate scaffolds. Iranian journal of basic medical sciences. 2018;21(2):212.

17.Valiani A, Izadi M, Bahramian H, Esfandiari E, Hashemibeni B. Comparison between the effect of kartogenin and TGFβ3 on chondrogenesis of human adipose-derived stem cells in fibrin scaffold. Bratislavske lekarske listy. 2017;118(10):591-7.

18.Esfandiari E, Roshankhah S, Mardani M, Hashemibeni B, Naghsh E, Kazemi M, et al. The effect of high frequency electric field on enhancement of chondrogenesis in human adipose-derived stem cells. Iranian Journal of Basic Medical Sciences. 2014;17(8):571-6.

19.Kawamura K, Chu CR, Sobajima S, Robbins PD, Fu FH, Izzo NJ, et al. Adenoviral-mediated transfer of TGF-β1 but not IGF-1 induces chondrogenic differentiation of human mesenchymal stem cells in pellet cultures. Experimental hematology. 2005;33(8):865-72.

20.Newfeld SJ, Wisotzkey RG, Kumar S. Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-β family ligands, receptors and Smad signal transducers. Genetics. 1999;152(2):783-95.

21.Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development. 1995;121(6):1845-54.

22.Buhrmann C, Honarvar A, Setayeshmehr M, Karbasi S, Shakibaei M, Valiani A. Herbal Remedies as Potential in Cartilage Tissue Engineering: An Overview of New Therapeutic Approaches and Strategies. Molecules. 2020;25(13):3075.

23.LIU K-p, WANG L-f, Yang L, Bin Y, Chao D, Yang W. Preparation, pharmacokinetics, and tissue distribution properties of icariin-loaded stealth solid lipid nanoparticles in mice. Chinese Herbal Medicines. 2012;4(2):170-4.

24.Wang ZC, Sun HJ, Li KH, Fu C, Liu MZ. Icariin promotes directed chondrogenic differentiation of bone marrow mesenchymal stem cells but not hypertrophy in vitro. Experimental and therapeutic medicine. 2014;8(5):1528-34.

25.Li D, Yuan T, Zhang X, Xiao Y, Wang R, Fan Y. Icariin: a potential promoting compound for cartilage tissue engineering. Osteoarthritis and cartilage. 2012;20(12):1647-56.

26.Wang Z, Li K, Sun H, Wang J, Fu Z, Liu M. Icariin promotes stable chondrogenic differentiation of bone marrow mesenchymal stem cells in self-assembling peptide nanofiber hydrogel scaffolds. Molecular medicine reports. 2018;17(6):8237-43.

27.Li Y, Zhu C-p, Zhai X-c, Zhang Y, Duan Z, Sun J-r. Optimization of enzyme assisted extraction of polysaccharides from pomegranate peel by response surface methodology and their antioxidant potential. Chinese Herbal Medicines. 2018;10(4):416-23.

.28Gorji M, Zargar A, Setayeshmehr M, Ghasemi N, Soleimani M, Kazemi M, et al. Releasing and structural/mechanical properties of nanoparticle/Punica granatum (Pomegranate) in poly(lactic-co-glycolic) acid/fibrin as nano-composite scaffold.

28.Gorji M, Zargar A, Setayeshmehr M, Ghasemi N, Soleimani M, Kazemi M, et al. Releasing and structural/mechanical properties of nanoparticle/ Punica granatum (Pomegranate) in poly(lactic-co-glycolic) acid/fibrin as nano-composite scaffold. Bratislavske lekarske listy. 2021;122(1):54-64.

29. Gullon B, Pintado ME, Pérez-Álvarez JA, Viuda-Martos M. Assessment of polyphenolic profile and antibacterial activity of pomegranate peel (Punica granatum) flour obtained from co-product of juice extraction. Food Control. 2016;59:94-8.

30.Mansour E, Ben Khaled A, Lachiheb B, Abid M, Bachar K, Ferchichi A. Phenolic compounds, antioxidant, and antibacterial activities of peel extract from Tunisian pomegranate. J Agr Sci Tech. 2013;15:1393-403.

31.Sadeghi N, Jannat B, Oveisi M, Hajimahmoodi M, Photovat M. Antioxidant activity of Iranian pomegranate (Punica granatum L.) seed extracts. J Agr Sci Tech. 2009;11:633-8.

32.Yasoubi P, Barzegar M, Sahari M, Azizi M. Total phenolic contents and antioxidant activity of pomegranate (Punica granatum L.) peel extracts. J Agric Sci Technol. 2007;9:35-42.

33. Choi B-R, Ku S-K, Kang S-J, Park H-R, Sung M-S, Lee Y-J, et al. Anti-osteoarthritic effects of a combination of pomegranate concentrate powder, Eucommiae cortex and Achyranthis radix in rats. Journal of Korean Medicine. 2018;39(4):86-113.

34.Qu W, Breksa III AP, Pan Z, Ma H. Quantitative determination of major polyphenol constituents in pomegranate products. Food Chemistry. 2012;132(3):1585-91.

35. Akhavan H, Barzegar M, Weidlich H, Zimmermann BF. Phenolic compounds and antioxidant activity of juices from ten Iranian pomegranate cultivars depend on extraction. Journal of Chemistry. 2015;2015.

36.Katani M, Zolfaghari B, Soleimani M, Valiani A, Hashemibeni B. The effect of pomegranate extract on producing type ii collagen in differentiation of adipose-derived stem cells into chondrocytes.

37.Cheng N-C, Estes BT, Young T-H, Guilak F. Genipin-crosslinked cartilage-derived matrix as a scaffold for human adipose-derived stem cell chondrogenesis. Tissue engineering Part A. 2012;19(3-4):484-96.

38.Kabiri A, Esfandiari E, Hashemibeni B, Kazemi M, Mardani M, Esmaeili A. Effects of FGF-2 on human adipose tissue derived adult stem cells morphology and chondrogenesis enhancement in Transwell culture. Biochemical and biophysical research communications. 2012;424(2):234-8.

39.Sarveazad A, Babahajian A, Bakhtiari M, Soleimani M, Behnam B, Yari A, et al. The combined application of human adipose derived stem cells and Chondroitinase ABC in treatment of a spinal cord injury model. Neuropeptides. 2017;61:39-47.

40.Yang SH, Wu CC, Shih TTF, Chen PQ, Lin FH. Three-dimensional Culture of Human Nucleus Pulposus Cells in Fibrin Clot: Comparisons on Cellular Proliferation and Matrix Synthesis With Cells in Alginate. Artificial organs. 2008;32(1):70-3.

41.Garrigues NW, Little D, Sanchez-Adams J, Ruch DS, Guilak F. Electrospun cartilage-derived matrix scaffolds for cartilage tissue engineering. Journal of biomedical materials research Part A. 2014;102(11):3998-4008.

42.Gong YY, Xue JX, Zhang WJ, Zhou GD, Liu W, Cao Y. A sandwich model for engineering cartilage with acellular cartilage sheets and chondrocytes. Biomaterials. 2011;32(9):2265-73.

43.Gorji M, Ghasemi N, Setayeshmehr M, Zargar A, Kazemi M, Soleimani M, et al. The Effects of Fibrin-icariin Nanoparticle Loaded in Poly (lactic-co-glycolic) Acid Scaffold as a Localized Delivery

System on Chondrogenesis of Human Adipose-derived Stem Cells. Adv Biomed Res. 2020;9:6-.

44. Liu D, Tang W, Zhang H, Huang H, Zhang Z, Tang D, et al. Icariin protects rabbit BMSCs against OGD-induced apoptosis by inhibiting ERs-mediated autophagy via MAPK signaling pathway. Life Sciences. 2020;253:117730.

44.Wang P, Xiong X, Zhang J, Qin S, Wang W, Liu Z. Icariin increases chondrocyte vitality by promoting hypoxia-inducible factor-1α expression and anaerobic glycolysis. The Knee. 2020;27(1):18-25.

46.Choi B-R, Kang S-J, Kim J-L, Lee Y-J, Ku S-K. Anti-Osteoarthritic Effects of a Mixture of Dried Pomegranate Concentrate Powder, Eucommiae Cortex, and Achyranthis Radix 5: 4: 1 (g/g) in a Surgically Induced Osteoarthritic Rabbit Model. Nutrients. 2020;12(3):852.

47. Ng K, DeFrancis J, Kugler L, Kelly T-A, Ho M, O'Conor C, et al. Amino acids supply in culture media is not a limiting factor in the matrix synthesis of engineered cartilage tissue. Amino acids. 2008;35(2):433-8.