

## Study of chondrogenic potential of stem cells in co-culture with chondrons

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### ABSTRACT

**Objective(s):** Three-dimensional biomimetic scaffolds have widespread applications in biomedical tissue engineering due to similarity of their nanofibrous architecture to native extracellular matrix. Co-culture system has stimulatory effect on chondrogenesis of adult mesenchymal stem cells. This work presents a co-culture strategy using human articular chondrons and adipose-derived stem cells (ASCs) from infrapatellar fat pad (IPFP) for cartilage tissue production.

**Materials and Methods:** Isolated stem cells were characterized by flowcytometry. Electrospun and polycaprolactone (PCL) scaffolds (900 nm fiber diameter) was obtained from Bon Yakhteh (Tehran- Iran) and human infrapatellar fat pad-derived stem cells (IPFP-ASCs) were seeded on them. IPFP-ASCs on scaffolds were co-cultured with articular chondrons using transwell. After 21 day, chondrogenic differentiation of stem cell was evaluated by determining the genes expression of collagen2, aggrecan and Indian hedgehog using real- time RT-PCR.

**Results:** Genes expression of collagen2, aggrecan by IPFP-ASCs did not alter significantly in comparison with control group. However, expression of Indian hedgehog decreased significantly compared to control group ( $P < 0.05$ ).

**Conclusion:** These findings indicate that chondrons obtained from osteoarthritic articular cartilage did not stimulate chondrogenic differentiation of IPFP-ASCs in co-culture.

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### Introduction

Articular cartilage (AC) is an aneural, avascular tissue that covers the diarthrodial joints and due to its avascularity it has limited capacity for regeneration. Therefore, injuries to the AC in adults, do not heal spontaneously and progress towards osteoarthritis (OA) (1, 2).

Tissue engineering represents available choice to repair cartilage and restore joint function (3, 4). This approach requires cell sources, matrix or scaffolds, and appropriate growth factors to promote chondrogenesis (5). The optimum cell source for cartilage tissue engineering is still not being established. Adult mesenchymal stem cells (MSCs) have been investigated

as one of the common cell source for cartilage repair (6, 7). MSCs could be obtained from bone marrow and different other tissues such as adipose tissue, and many other organs (8). Adipose tissue offers an easy available and abundant source for adult stem cells, with minimal donor-site morbidity. Some have used adipose stem cells derived from the infrapatellar fat pad (IPFP), because it has been demonstrated that adipose-derived stem cells (ASCs) from the IPFP encapsulated in fibrin, express collagen type 2 and aggrecan. Additionally, its matrix sulphated glycosaminoglycan (sGAG) content reaches 50% of native cartilage (9).

Scaffolds as a second element in the tissue engineering, provide a 3D environment that are

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desired for the production of cartilaginous tissue. Polycaprolactone (PCL) is a synthetic polymer that has intriguing properties appropriate for cartilage tissue engineering applications, such as good degradability, compatibility and mechanical strength (10). Nevertheless, its low surface wettability due to its rather hydrophobicity affects cell attachment and proliferation. So several surface treatments of polyesters have been attempted to increase their surface hydrophilicity in order to improve the adhesion of cells to PCL (11, 12). Such methods as alkaline hydrolysis and plasma treatment of polymeric scaffolds were proved to improve cell adhesion and enhance cell proliferation and functions (13-15).

The third components of the tissue engineering triad, stimulating factors, have been used to induce, accelerate, and/or enhance cartilage formation. *In vitro* co-culture systems provide a strong tool for cartilage tissue engineering (16) and is used to replace growth factors. The extracellular matrix (ECM) of tissues is a complex reservoir of mediators and growth factors. Therefore, whole tissue chips or ECM components have been used to improve neo-cartilage formation by cultured chondrocytes and chondrocyte-like cells (17, 18).

The chondrocyte and its associated narrow pericellular matrix (PCM) are termed as chondron (19). Previous studies have demonstrated that the PCM of chondron, is primarily defined by the presence of type 6 collagen, but also contains high concentration of proteoglycans including aggrecan (20), hyaluronan, laminins and nidogen-2 (21), decorin and fibronectin (22), and as well as collagen types 2, 9 (23), and collagen type 11 (24), relative to the ECM. In general, the small proteoglycans are thought to have inhibitory functions such as restricting collagen fibrillogenesis, limiting fibronectin adhesion, and binding TGF- $\beta$  modulate matrix synthesis or mitogenic activity (25). The differentiation of chondroprogenitors or MSCs to chondrocyte are characterized by the deposition of cartilage matrix containing collagen 2 and aggrecan. Furthermore, Indian hedgehog (IHH) is one of three hedgehog that specifically expressed by flattened prehypertrophic chondrocytes during development of embryo (26, 27). In the present study the impact PCM of chondrons, in an indirect co-culture model, on chondrogenic potential of IPFP-derived stem cells has been examined. The stem cells were seeded on PCL scaffolds and chondrons were situated on transwell. Chondrogenicity was evaluated by gene expression using Real-time RT-PCR and data were analyzed.

## Materials and Methods

### Cell isolation, culture and doubling time

IPFP was obtained from patients (aged 24, 25 and 46 years; n= 3) undergoing anterior cruciate ligament (ACL) surgery. Before surgery the purpose

of study was explained to the patients and a written consent was obtained from each patient. Briefly the tissue was washed 3 times with phosphate buffered saline (PBS, Sigma, USA), and diced finely and then digested with 0.1% collagenase 1 (Gibco, USA) for 50-55 min at 37 °C. Enzymatic activity was neutralized by Dulbecco's modified Eagle's medium (DMEM-low glucose, Gibco, UK), containing 10% fetal bovine serum (FBS, Gibco, E.U. Approved (South American)) and centrifuged at 1400 rpm for 10 min. Then, the pellet was resuspended, washed 2 times with medium, and seeded on culture flask. Medium of culture flask containing DMEM, 10% FBS, 1% penicillin-streptomycin (Sigma-Aldrich, USA) and maintained in incubator at 37°C, 5% CO<sub>2</sub> and 97% humidity (28). At the time of passage, cell viability was determined by trypan blue staining. For freezing, after culturing through passage 1 or 2, the cells were suspended in a cryopreservation medium containing 90% FBS and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich, USA).

Doubling time from passage 0 to 1 was calculated using the algorithm:

$$\text{Doubling Time} = T [\log 2 / \log (N2 / N1)]$$

T= days of expansion; N1= number of plating cells; N2 = number of harvested cells at the end

### Enzymatic isolations of chondrons

Articular cartilage was obtained from patients (aged 46, 55, and 62 years; n = 3) who underwent total hip or knee arthroplasty due to osteoarthritis. Only macroscopically normal-looking cartilage was diced for chondrons isolation. Chondron isolation was accorded on a previously published protocol (29) with slight modification. The cartilage pieces were treated with 0.3% dispase (Gibco, USA) and 0.2% collagenase 2 (Gibco, USA) in PBS for 5 hr. Enzymes activity were neutralized with DMEM, containing 10% FBS and centrifuged at 1400 rpm for 10 min. The cells were washed and seeded on culture flasks in medium containing DMEM, 10% FBS, 1% penicillin- streptomycin, and 25  $\mu$ g/ml ascorbic acid (Sigma-Aldrich, USA). For freezing, after 24 hr, floating cells were washed and suspended in a cryopreservation medium containing 90% FBS and 10% DMSO.

### Scaffold characterization

PCL scaffold was obtained from a Stem Cell Technology Company (Bon Yakhteh-Tehran, Iran). According to company's instruction the nanofibrous PCL sheet was plasma treated. Before using, scanning electron microscope (MIRA3 FEG-SEM) was used for the observation of the structural morphology of scaffold. The samples of scaffold were cut from the nanofibrous sheet using a 7 mm dermal punch and coated with gold by a sputter-coater. Diameter of fibers in the electrospun scaffold is mean of 10

nanofibers which were measured on scanning electron micrographs. The average fiber diameter was determined from measurements taken perpendicular to the long axis of the fibers within representative microscopic fields (30).

#### **Cell surface epitope characterization and flowcytometry**

IPFP-ASCs from passage 2 were characterized for mesenchymal stem cell surface protein expression by flowcytometry. The monoclonal antibodies used were PE labeled anti- CD90 and FITS labeled anti-CD44 (BD Bioscience, USA), PerCP labeled anti-CD31 (R&D systems) and CD45 (Abcam, UK). The cells ( $3 \times 10^5$  cells) were washed and incubated with antibodies for 25 min at 4 °C temperature in dark environment. At least 10,000 events were acquired on BD caliber (BD ebioscience), and the data were analyzed using flowing software (PerttuTerho, Version: 2.5.1).

#### **Seeding of IPFP- ASCs into PCL scaffolds and co-culture**

The scaffolds were sterilized under ultraviolet (UV) light for 2 hr on each side. The sterilized 7 mm scaffold discs were placed into 24-well culture plate. Each scaffold was seeded with IPFP-ASCs at concentration of  $5 \times 10^5$  cells/specimen and then incubated for 2 hr to allow the cells to attach. Then 1 ml medium [DMEM, 1% FBS, 1% penicillin-streptomycin, and 25 µg/ml ascorbic acid] was added to each well. For co-culturing transwells (0.4 µm pore size, polyester membrane, Greiner, Germany) were placed into the 24-wells plates that had IPFP-ASCs /scaffold. The chondrons were suspended in chondrogenic medium [DMEM, 1% FBS, 1% penicillin-streptomycin, and 25 µg/ml ascorbic acid] at a concentration of  $5 \times 10^5$  cell/300 µl. Cell suspension was added to the transwell. The 24-well culture plate was incubated at 37°C in 5% CO<sub>2</sub> for 21 days. Hundred µl medium were removed from the lower chambers and equal volume fresh medium were added to upper chambers every 2 days.

#### **Real-Time RT-PCR**

After 21 days, each IPFP-ASCs seeded scaffold was homogenized under liquid nitrogen using a mortar. RNA was isolated from the samples by using RNX- Plus (Sinaclon, IRAN) according to the manufacturer's instructions. The concentration of RNA was estimated spectrophotometrically with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA) at A<sub>260/280</sub>. The RNA samples were reverse transcribed into first-strand cDNA using the AccuPower® RT PreMix (Bioneer). Real Time-PCR

reactions were performed using the SYBRGreen PCR Mastermix (Applied Biosystems, USA), according to the manufacturer's instructions. These gene primers were purchased from (Bioneer). The cartilage-specific oligonucleotide primers which were used, were aggrecan (forward 5'-AGGGCGAGTGGGAATGATGTT -3'; reverse 5'-GGTGGCTGTGCCCTTTTAC -3'), and collagen type 2a1 (forward 5'-ATGCCACACTCAAGTCCCTCAA-3'; reverse 5'-CGCAAGTCTCGCCAGTCTCC-3'), (IHH) (forward 5'-CACTTCTGCC-TGGTCCTGTT-3'; reverse 5'-GGGCTGAAGTCTGTAGG-3'), housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-CAAGATCATCAGCAATGCCTCC-3'; reverse, 5'-GCCA-TCACGCCACAGTTTCC -3'). All experiments were performed in triplicate for each sample. Interpretation of the results was performed using the Pfaffle method and the CT values were normalized with respect to GAPDH expression.

#### **Statistical analysis**

Results are presented as mean±standard deviation. T-test method was used for statistical analysis.  $P < 0.05$  were considered statistically significant.

## **Results**

#### **Cell morphology and doubling time**

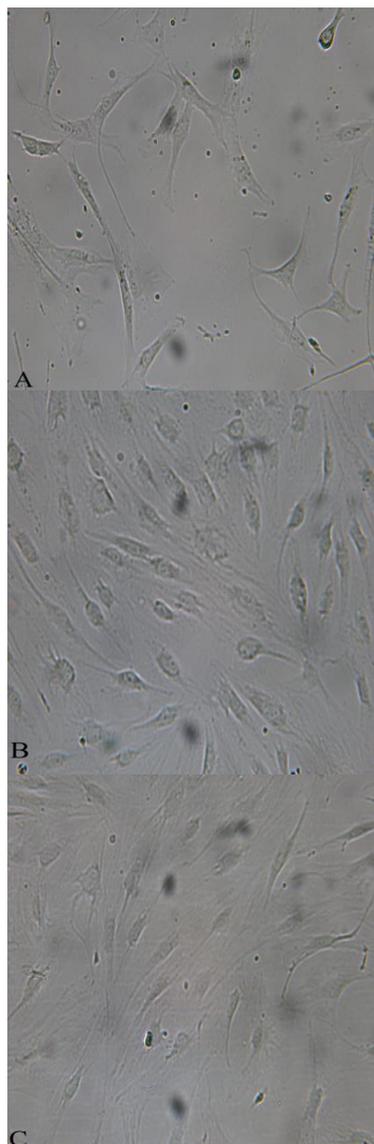
IPFP-ASCs were able to adhere to tissue culture flasks where as non-adherent cells such as red blood cells, were removed by media change. Cells proliferated rapidly and doubling time was  $1.99 \pm 0.36$  days. The initial adherent cells grew into spindle, or triangular-shaped cells (Figure 1A). At passage 1 (P1), the shape of cells shifted towards a fibroblast-like form (Figure 1B). At the second passage, IPFP-ASCs appeared to adopt a more uniform fibroblast-like shape (Figure 1C).

#### **Scaffold characterization**

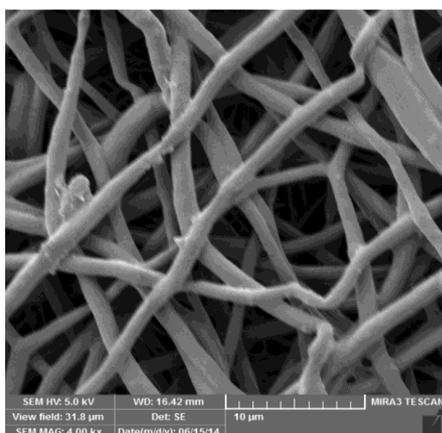
SEM imaging was first performed to observe the structural morphology of fibers in the electrospun PCL scaffold. The surface of plasma treated scaffold was smooth. The average diameter of nanofibers was approximately 900 nm. The orientation of the fibers were random (Figure 2).

#### **Cell surface epitope characterization and flowcytometry**

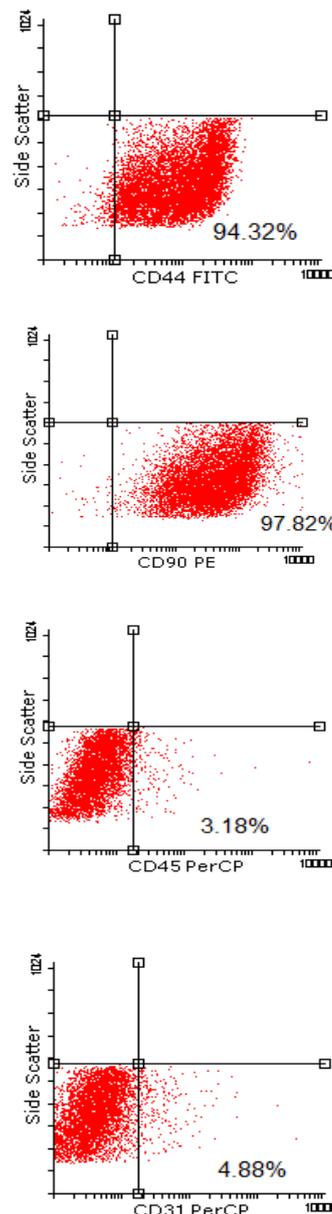
IPFP-ASCs expressed the mesenchymal stem cell markers such as CD44 (94.32%), CD90 (97.82%). However, few cells expressed hematopoietic marker such as CD45 (3.18%), or the endothelial marker CD31 (4.88%). Figure 3 shows positive and negative mesenchymal cell surface markers at passage 2.



**Figure 1.** Morphology of IPFP-ASCs in culture observed with an inverted phase-contrast microscope at different passages. The shape of cells changed during passages: p0 (A), p1 (B) and passage2 (C), 20X



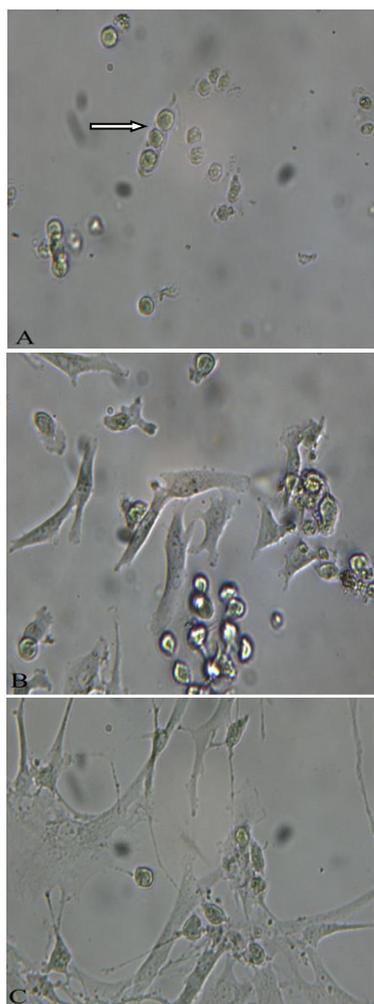
**Figure 2.** Architecture of electrospun PCL nanofiber scaffold as seen with a scanning electron microscope at 4.00KX



**Figure 3.** Cells were tested against human antigens CD31, CD44, CD45, CD90. All experiments were conducted at passage 2

**Cell culture and co-culture**

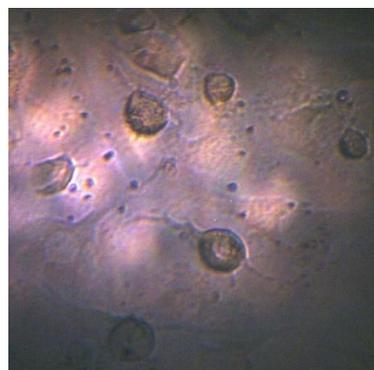
Before using the chondrons for co-culturing, they were cultured in DMEM, containing 10% FBS, 1% penicillin–streptomycin, and 25 μg/ml ascorbic acid. After 15 days of culture in flask, PCM of the chondrocytes disappeared and chondrocytes attached on floor of flask. These events happened gradually. In the 1<sup>st</sup> day, all the chondrons had PCM and were floating. Figure 4A shows the cells at the 1<sup>st</sup> day of culture, the white arrows indicate a group of chondrons consisting of a few cells which are in connection with adjacent group by a tail-like segment that ensure linear continuity between adjacent groups. But on the 8<sup>th</sup> day, some of them lost PCM and



**Figure 4.** Morphology of cultured chondrons as seen with an inverted phase-contrast microscope during 15 day culture, 1<sup>st</sup> day (A), 8<sup>th</sup> day (B), 15<sup>th</sup> day (C), 10X

transformed to flat form cells (Figure 4B). On the 15<sup>th</sup> day all of chondrons lost their PCM and the cells adhered to culture flasks (Figure 4C).

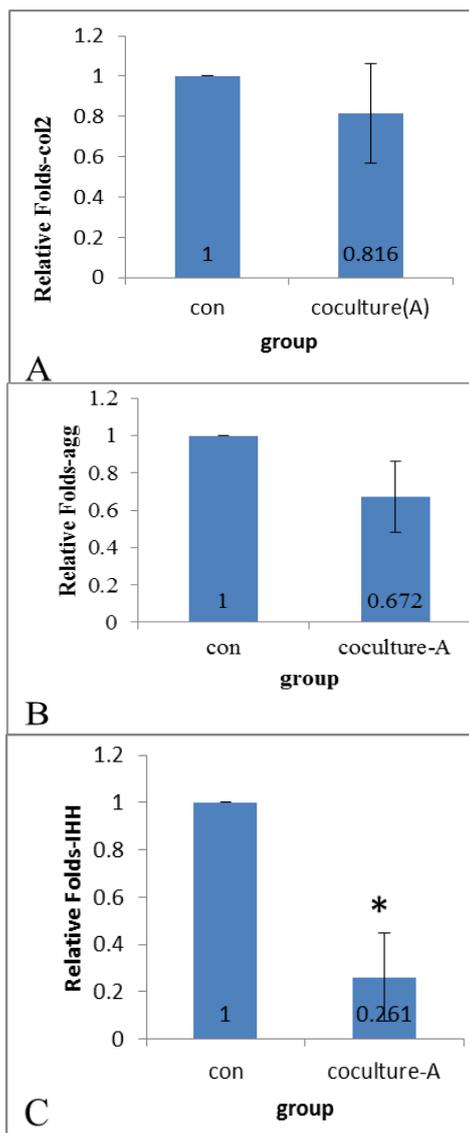
Figure 5 shows the IPFP-ASCs seeded on PCL scaffold. As the Figure 5 shows, the cultured cells on PCL, have roundish shape and have a few processes after 21 days.



**Figure 5.** IPFP-ASCs on PCL scaffold after 21 days, observed with inverted phase contrast microscope, 20X

**Real-Time RT-PCR**

To determine the effects of chondron co-culture on IPFP-ASCs chondrogenesis, we examined the mRNA levels of collagen type 2 (a1), aggrecan, and IHH in IPFP-ASCs/scaffold by quantitative RT-PCR. For the co-culture group, IPFP-ASCs/scaffold showed slight decrease in collagen type 2 (a1) and aggrecan (Figure 6A, 6B) but the changes were not significant. However the mRNA levels of IHH had severe reduction (Figure 6C) and it was statistically significant ( $P < 0.05$ ).



**Figure 6.** Relative genes expression of chondrogenic cell/PCL and cell/PCL co-culture. Co-culture conditions decreased expression collagen2a1 (A), aggrecan (B) and IHH (C)  $\ast = P < 0.05$

**Discussion**

In this study we investigated whether chondrons are able for chondrogenic induction of ASCs from the IPFP on the nanofibrous PCL scaffold.

The study showed plastic-adherent property of ASCs in culture, 94.32% of ASCs expressed the MSCs positive CD markers (CD44) and 97.82% of ASCs expressed the CD90. While about 2% of ASCs expressed negative cell surface marker (CD31: platelet endothelial cell adhesion molecule and CD45: leukocyte common antigen), at second passage. So the cell surface epitope characterization and flowcytometry of IPFP population showed a similar staining pattern to that of bone marrow-derived stem cells. Our result showed the percentage of negative markers is high, but it seems to decrease with increasing passage number because cells became more homogeneous. Taken together, the results suggest that the ASCs from the IPFP is a relatively homogenous population of mesenchymal cells with low contamination by endothelial and leukocyte cells.

Two properties have been established in the present study. First it is shown that ASCs isolated from the IPFP were more homogenous fibroblast-like shape in passage 2. Second, we found that the contents of pericellular matrix of chondrons released into the culture medium by 15 days. Based on these findings, we used chondrons for co-culture in transwell to evaluate the effect of PCM on ASCs. In an study it is demonstrated that chondrocytes on nanofibrous scaffolds had higher rates of proliferation and maintained a rounded morphology, which is characteristic of the chondrocyte phenotype (31). Consistent with this result, the seeded IPFP-ASCs on scaffold, in our study, had a round morphology. We also found that 21 day after culture, the attached IPFP-ASCs on nanofibrous PCL scaffold still had roundish shape and grew a few branched processes.

Previous study showed IPFP-ASCs can successfully undergo chondrogenesis using TGF $\beta$ 3 and BMP6 and the cartilage-like tissue (32). Release of growth factors such as transforming growth factor beta 1 (TGF $\beta$ 1), insulin-like growth factor 1 (IGF-1), bone morphogenetic proteins 2 (BMP2) were demonstrated in the supernatants of co-cultured bone marrow stem cells (BMSC) and articular chondrocytes (33). In our study, probably releasing of similar factors were involved in cell differentiation.

Some studies showed that presence of the PCM in chondrons had a profound effect on chondrocyte gene expression (34-36). Up-regulation of the heat shock protein 70, may contribute to the robustness and active matrix production of chondrons (34). In this study, we expected that the release of factors from PCM of chondrocytes into the microenvironment could be absorbed by the nanofibrous PCL and stimulate IPFP-ASCs. The nanofibrous constructs were found to selectively enhance the adsorption of specific proteins, such as fibronectin and vitronectin (37). Since previous studies have reported that

fibronectin binds to growth factors (38), so we assumed that growth factors may also attach to factors released from chondrons. However, the results of real-time RT-PCR in this study suggest that co-culture conditions had deleterious effect on chondrogenesis. From this point, our findings are consistent with a previous study showing that conditioned medium from primary osteoarthritic (P0) chondrocytes could not induce chondrogenic differentiation of MSCs (39).

Previous studies have also demonstrated that injured and osteoarthritic joints show significantly higher levels of pro-inflammatory cytokines. For example interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF- $\alpha$ ), as well as pro-catabolic enzymes and mediators such as metalloproteinases (MMPs), aggrecanases, prostaglandins, and nitric oxide are overexpressed in these joints which could reason tissue degradation, pain, and inflammation (40-42). Other studies showed the deleterious effects of IL-1 on the chondrogenesis of ASCs (43) and MSCs (44-47). Based on these studies, it is probable that in the present study, the presence of similar substances in the microenvironment during the initial 21-day culture period inhibited gene expression. In addition to these substances in the microenvironment, there may be other factors overshadowed.

On the other side, it is known that IHH is secreted from mature cells, it seems that co-culture condition in our study, inhibited cell maturation and therefore secretion of IHH was reduced.

There are limitations related to the design of the current study. First, it was not possible to use chondrons from healthy normal cartilage of young adults, so we used sample of OA patients which would have yielded different, perhaps more dramatic, results. Second, the conditioned medium is expected to contain various growth factors and cytokines secreted from chondrons but it was not checked in the present study.

## Conclusion

In summary, our findings show that nanofibrous scaffold is ideal for IPFP-ASCs attachment. The differential effects of co-culture with chondrons from OA patients did not improve chondrogenesis of IPFP-ASCs.

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