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A novel isolation method for chondrocytes differentiated from synovial fluid-derived mesenchymal stem cell

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Abstract

Mesenchymal stem cells (MSCs) in synovial fluid (SF) actively participate in the regeneration process of healthy joints and have been defined as a good source of chondrocyte cells, which can be obtained by in vitro differentiation of stem cells. This cell population derived from synovial fluid shares cellular characteristics with bone marrow and synovial membrane MSCs with respect to their differentiation potency. Current cell isolation protocols for cartilage therapy mainly focus on the isolation of chondrocytes or MSCs from biological samples. However, the isolation of chondrocytes after the in vitro differentiation from MSCs has not been described in the literature. In this context, we defined a novel method based on Ficoll-Paque density gradient centrifugation for high-throughput isolation of differentiated chondrocytes from human synovial fluid mesenchymal stem cells (hSF-MSCs) without the requirement for cell labeling.

In this study, terminally differentiated chondrocytes were obtained after 21 days of chondrogenic differentiation from hSF-MSCs and were isolated using this novel protocol. The isolated chondrocytes were later analyzed for cell viability and functionality by staining with Alcian Blue and by gene expression analysis for chondrocyte markers.

In conclusion, the novel chondrocyte isolation method described here is capable of achieving low-cost efficiency. According to the minimal process principles, we hope that

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this protocol will find use in both translational research and routine clinical applications involving the differentiation and isolation of chondrocytes in vitro.

Keywords: *Synovial fluid-derived mesenchymal stem cells, chondrocyte, differentiation, isolation*

Sinoviyal sıvı kaynaklı mezenkimal kök hücrelerden farklılaşan kondrositlerin izolasyonu için yeni bir yöntem

Öz

Sinovyal sıvıdaki (SS) mezenkimal kök hücreler (MKH'ler), sağlıklı eklemlerin rejenerasyon sürecine aktif olarak katılır ve in vitro farklılaşma yoluyla elde edilebilen kondrosit hücrelerinin iyi bir kaynağı olarak tanımlanmıştır. SS'dan elde edilen bu hücre popülasyonu, kemik iliği ve sinovyal membran MKH'leriyle benzer şekilde farklılaşma potansiyeli açısından hücresel özellikler paylaşmaktadır. Mevcut hücre izolasyon protokolleri, genellikle biyolojik örneklerden kondrosit veya MKH izolasyonuna odaklanmaktadır. Ancak, MKH'lerden in vitro farklılaştırma yoluyla elde edilen kondrositlerin izolasyonu literatürde henüz tanımlanmamıştır. Bu bağlamda, hücre etiketleme gerektirmeksizin Ficoll-Paque yoğunluk gradyanı santrifüjleme temelli yeni bir yöntem tanımladık. Bu yöntem, insan SS-MKH'lerinden farklılaşmış kondrositlerin yüksek verimli izolasyonunu sağlamaktadır.

Bu çalışmada, iSS-MKH'lerinden 21 günlük kondrojenik farklılaşma sürecinin ardından terminal farklılaşmış kondrositler bu yeni protokol kullanılarak elde edilmiştir. İzole edilen kondrositler, Alcian Blue boyaması ile hücre canlılığı ve fonksiyonelliği açısından incelenmiş ve kondrositlere özgü gen ekspresyonu analizi yapılmıştır.
Sonuç olarak, burada tanımlanan yeni kondrosit izolasyon yöntemi düşük maliyetli ve verimli bir çözüm sunmaktadır. Minimal işlem ilkelerine uygun olarak bu protokolün, hem translasyonel araştırmalar hem de in vitro kondrosit farklılaşması ve izolasyonuna yönelik rutin klinik uygulamalarda kullanılması beklenmektedir.

Anahtar Kelimeler: *Sinovyal sıvı kaynaklı mezenkimal kök hücreler, kondrosit, farklılaşma, izolasyon*

1. Introduction

Mesenchymal stem cells (MSCs) are heterogeneous cell populations with self-renewal and multipotent differentiation capacities that reside in nearly all tissues such as adipose tissue, dental pulp, bone marrow, umbilical cord, placenta, amniotic fluid, and synovial fluids [1]. MSCs display characteristic immunophenotypic profiles ($CD90^+$, $CD44^+$, $CD105^+$, $CD73^+$, and $CD34^-$, $CD14^-$, $CD45^-$ and HLA-DR^-) and multilineage-differentiation potential into osteocytes, adipocytes, and chondrocytes [2]. MSCs play a prominent role in tissue engineering and regenerative medicine along with their immunomodulatory functions and their potential for adipogenic, osteogenic and chondrogenic differentiation. For this reason, these cells are found in many applications for tissue repair and remodeling, and for maintaining homeostasis [3]. Biotechnological

advancements in protocols regarding the induction, cultivation, and straightforward isolation of MSCs have provided convenience in obtaining terminally differentiated cells, such as chondrocytes and osteocytes [4].

For patients with osteoarthritis or synovitis, chondrocyte cells differentiated and isolated from SF-MSCs could be an important source for cell-based therapy of cartilage [5]. In particular, SF-MSCs from the inflammatory microenvironment on intra-articular tissues exhibit improved characteristics for tissue repair and regeneration. Hence, the transplantation of autologous chondrocytes, which were obtained and differentiated from SF-MSCs of osteoarthritic knees, is considered a first-line treatment option for knee osteoarthritis and traumatic cartilage injury [5-7]. However, successful implantation of primary autologous chondrocyte requires healthy cartilage specimens from harvested biopsies.

Cell isolation by density centrifugation is frequently applied to mononuclear cells and leukocytes in blood or bone marrow cells [8, 9]. The principle of this method is to separate cells based on their density differences in in liquid samples. The differences in the densities of these cells and their compartments facilitated separation, depending on the selected medium, into the desired subpopulations. Differential centrifugation is an alternative method to gradient centrifugation for cell separation. The primary difference between these two methods is that density gradient centrifugation focuses on separating cells based on their density, rather than mass or size alone as in differential centrifugation [10, 11]. Therefore, the differential centrifugation is easier to perform, however density gradient centrifugation can sort much smaller particles to increase specificity. Frequently used alternative cell separation methods include, magnetic-activated cell sorting (MACS), buoyancy-activated cell sorting (BACS) and fluorescence-activated cell sorting (FACS) [12-14]. These three methods are highly efficient, but they are not straightforward to perform and are not cost-effective compared to density gradient centrifugation.

In this study, a novel cell isolation method was defined for chondrocytes derived from the differentiated stem cells. Some studies have focused on the isolation of chondrocyte cells from cartilage tissue, but differentiated cells from mesenchymal stem cells *in vitro* might demonstrate differences in size and cellular density, which alters the protocol. The defined protocol for chondrocyte isolation might provide great convenience in autologous transplantation in cell-based therapies.

2. Materials and methods

2.1 Ethical Statement

Ethical approval for this study was granted by the Non-Interventional Clinical Research Ethics Committee of Kocaeli University, under the reference number GOKAEK-2021/22.17, dated December 21, 2021.

2.2 Cell Culture

Human synovial fluid mesenchymal stem cells (hSF-MSCs) were procured from the Center for Stem Cell and Gene Therapies Research and Application at the Kocaeli University. The cells were maintained in DMEM F12 (Gibco, Thermo, Paisley, UK) medium supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo, Paisley, UK), 1 % penicillin-streptomycin (Capricon), and 1% non-essential amino acids (NEAA,

Gibco) at 37°C in a, 5% CO_2 incubator in culture flasks (T75, Corning, NY, USA). Cells extended to 70-80% confluency were detached using 0.25% trypsin-EDTA (Gibco) and collected by centrifuging at 300xg for 5 min. Cell viability was determined by Trypan Blue staining (Gibco) and unstained viable cells were counted using a Thoma counting slide under the microscope.

2.3 MSCs metabolic and phenotypic characterization

2.3.1 Flow cytometric analysis

For the phenotypic characterization of the MSCs, cells were stained against the antibodies HLA-DR FITC (BD 340688; BD Biosciences, USA), CD34 PE (BD 345802; BD Biosciences, USA), CD73 PE (BD 550257; BD Biosciences, USA), CD90 (555596; BD Biosciences, USA), CD105 PE (BD 560839; BD Biosciences, USA), CD45 FITC (BD 55482; BD Biosciences, USA), analyzed on at flow cytometer (FACS Calibur, BD Biosciences) and evaluated using the Cell Quest software (BD Biosciences, USA) [15]. hSF-MSCs were collected and resuspended in culture medium at a concentration of 1×10^6 cells/ml. Cells (2×10^5) for each marker in the characterization of hSF-MSCs via flow cytometry.

2.3.2 Determination of trilineage potential of MSCs

For the metabolic characterization of osteogenic (osteocytes), adipogenic (adipocytes), and chondrogenic (chondrocytes) differentiation was performed.

2.3.2.1 Analysis of chondrogenic differentiation

First, hSF-MSCs were seeded into T25-flask at a density of $5x10^6$ cell/mL and allow to attach overnight. The complete culture medium was replaced with a chondrogenic differentiation medium cocktail (DMEM-F12 supplemented with 100 nM dexamethasone, 0.05 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 1 % penicillin-streptomycin), and cell culture was maintained. The medium was changed twice a week [16]. Following chondrogenic differentiation, cell-specific staining was performed using Alcian Blue staining (ScienCell Research Laboratories, US). Chondrocyte-specific glycosaminoglycan (GAG) was examined using Alcian Blue staining. Morphological analysis of the cells was performed using an invert microscope and images were captured.

2.3.2.2 Analysis of osteogenic differentiation

To determine osteogenic differentiation, $2x10^5$ hSF-MSCs were seeded into 6 well plate and incubated overnight. The medium was then replaced with an osteogenic differentiation medium cocktail (DMEM-F12 supplemented with 0.05 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 nM dexamethasone, 10 % FBS and 1 % penicillin-streptomycin), and the cell culture was maintained. The medium was changed twice a week [15, 17, 18]. Following osteogenic differentiation, cell-specific staining was performed using Alizarin Red S staining (ScienCell Research Laboratories, US). Calcium deposits were detected using Alizarin Red S staining, morphological analysis of cells was performed using an invert microscope and images were captured.

2.3.2.3 Analysis of adipogenic differentiation

Moreover, on account of analysis of adipogenic differentiation $2x10^5$ hSF-MSCs were seeded into 6 well plate and incubated overnight. Then, the medium was replaced with the adipogenic differentiation medium cocktail (DMEM-F12 supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 50 µM indomethacin, 1 mM dexamethasone, 10 % FBS

and 1 % penicillin-streptomycin), cell culture was maintained. The medium was changed twice a week [15, 16, 18]. Following osteogenic differentiation, cell-specific differential analysis was performed using Oil Red O staining (ScienCell Research Laboratories). Intracellular lipid droplets were detected by Oil Red O staining. Morphological analysis of cells was conducted using an invert microscope and bright-field images were captured concomitantly.

2.4 Isolation of differentiated chondrocytes

After to *in vitro* chondrogenic differentiation of hSF-MSCs, differentiated chondrocytes were washed with phosphate buffered saline (Ca^{2+}/Mg^{2+} free, Dulbecco's PBS). Cells were harvested using trypsin-EDTA and centrifuged at 300xg for 5 min. The supernatant was removed and the cell pellet was resuspended in complete medium and homogenized. To obtain differentiated chondrocytes, the cells were isolated using Ficoll-Paque™ PLUS (GE Healthcare Life Sciences). The cells were gently stirred with Ficoll-Paque at a ratio of 1:1 and centrifuged at 400×g for 30 min, and the mixture was prepared to avoid pipetting. After centrifugation, the cells were carefully collected from the cloudy layer, mixed with 2 ml PBS, and centrifuged at 250×g for 10 min. These processes were repeated twice prior to the cell culture. The isolated chondrocytes were cultured in T25 cm^2 flask. DMEM F12 complete medium (1% penicillin-streptomycin, 1% NEAA, and 10% FBS) was used as the medium.

2.5 qRT-PCR

The gene expression levels of chondrogenic cell-specific markers in the samples were determined by qRT-PCR analysis. Collagen type 2 (COL2), SRY-Box Transcription Factor 9 (SOX9), Prolactin, and Cartilage Oligomeric Matrix Protein (COMP) were selected for analysis explore both chondrogenic differentiation and maturation. First, total RNA was isolated from cell samples in RNase free microcentrifuge tube using the GeneJET RNA Isolation kit (Thermo Scientific), according to the kit's protocol. cDNA was synthesized using a Revert Aid First Standard cDNA Synthesis Kit (Thermo Scientific). qRT-PCR was performed on a Roche Light Cycler II 480 RT-PCR system (Mannheim, Germany) using 2X qPCR SYBR-Green Master Mix (A.B.T.™ without ROX) [19]. Actin-β was used as the housekeeping gene in the analysis. The Cp values were automatically calculated using LC480 SW1.5 software. The primer sequences are listed in Table I.

Table I. Sequences of forward and reverse primers for qRT-PCR analysis.

Genes	**Primer Sequences (5'-3')**
Actin-β forward	5' AGA GAA GCT GTG CTA TGT TG 3'
Actin-β reverse	5' GTA CTC CTG CTT GCT GAT CC 3'
Sox9 forward	5' TGA AGA AGG AGA GCG AGG AA 3'
Sox9 reverse	5' GGG GCT GGT ACT TGT AAT CG 3'
Collagen type 2α1 forward	5' ACG GCG AGA AGG GAG AAG TTG 3'
Collagen type 2α1 reverse	5' GGG GGT CCA GGG TTG CCA TTG 3'
Prolactin reverse	5' ATG AAC ATC AAA GGA TCG C 3'
Prolactin forward	5' TTA GCA TGG TTT GTT GTG 3'
COMP reverse	5' CCG ACA GCA ACG TGG TCT T 3'
COMP forward	5' CAG GTT GGC CCA GAT GAT G'

2.6 Statistical Analysis

Samples from groups without chondrogenic differentiation (control group) and groups with differentiation were analyzed in triplicate. The results were analyzed using the Livak method [20]. Standard deviation was calculated using the Minitab 14 software (one way ANOVA) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) [21].

3. Result

hSF-MSCs were cultured successfully in DMEM F12 medium with 10% FBS, 1% penicillin-streptomycin and 1% NEAA. The cells showed a spindle-like elongated mesenchymal cell morphology. Flow cytometric analysis of CD73, CD90, CD105, CD45, HLA-DR and CD34 antibodies revealed typical markers for MSCs. The assessment for CD73 (Fig 1A-i), CD90 (Fig 1A-ii) and CD105 (Fig 1A-iii) showed that 98.06%, 94.97% and 98.38% of the cell population were positive, respectively. In contrast, it was confirmed that the cells in culture were negative for HLA-DR (0.59%) (Fig 1A-iv), CD45 (0.61%) (Fig 1A-v), and CD34 (9.34%) (Fig 1A-vi).

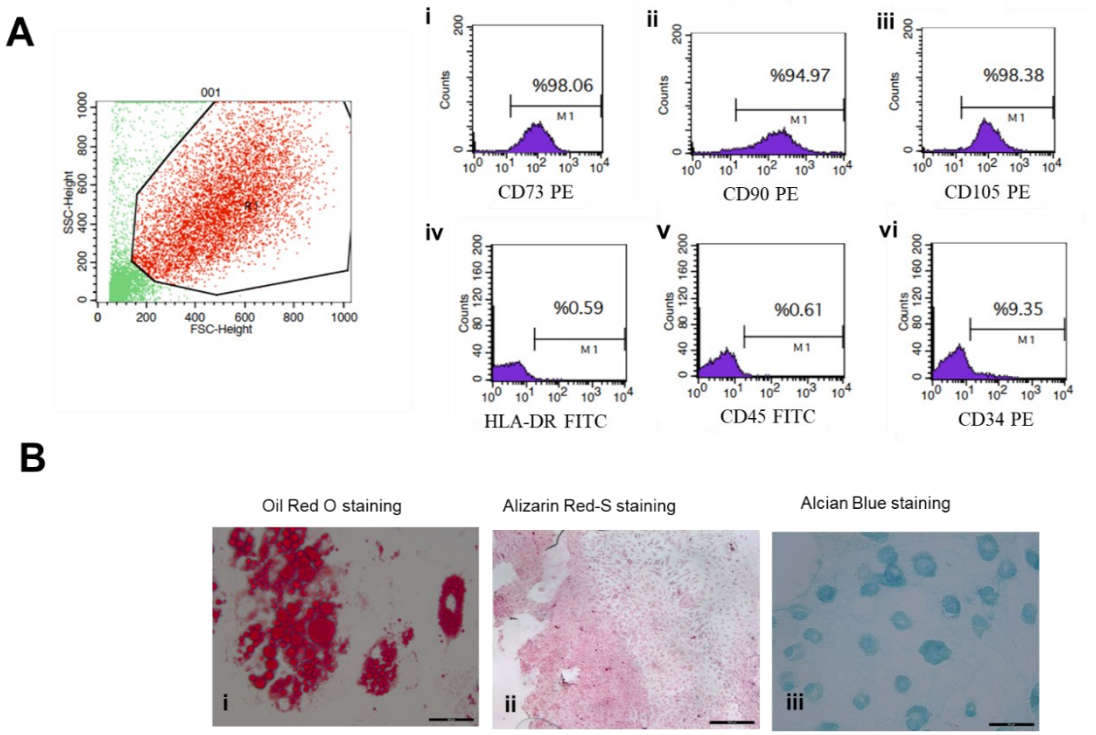

Figure 1. MSCs were from synovial fluid were characterized after the culture according to the MSC-specific markers. The characterization was performed by flow cytometer (A) according to their cell surface markers and by cell differentiation capacity (B). Scale bar 50 µm.

The ratio of CD34-positive cells was slightly elevated. However, this weak population of CD34-positive cells is commonly observed in hSF-MSCs [22]. Adipogenic differentiation supplements were added to the medium containing hSF-MSCs were given in order to induce adipogenic differentiation. Oil Red O was used to dye the lipid droplets red at the end of the third week (Fig 1B-i). Media containing osteogenic differentiation

supplements was administered to hSF-MSCs to promote osteogenic differentiation. Calcium nodules were stained red with Alizarin Red S at the end of the third week (Fig 1B-ii). For chondrogenic differentiation, hSF-MSCs were administered a medium containing chondrogenic differentiation supplement. At the end of the third week, the chondrogenic extracellular matrix was stained red with Alcian Blue (Fig 1B-iii).

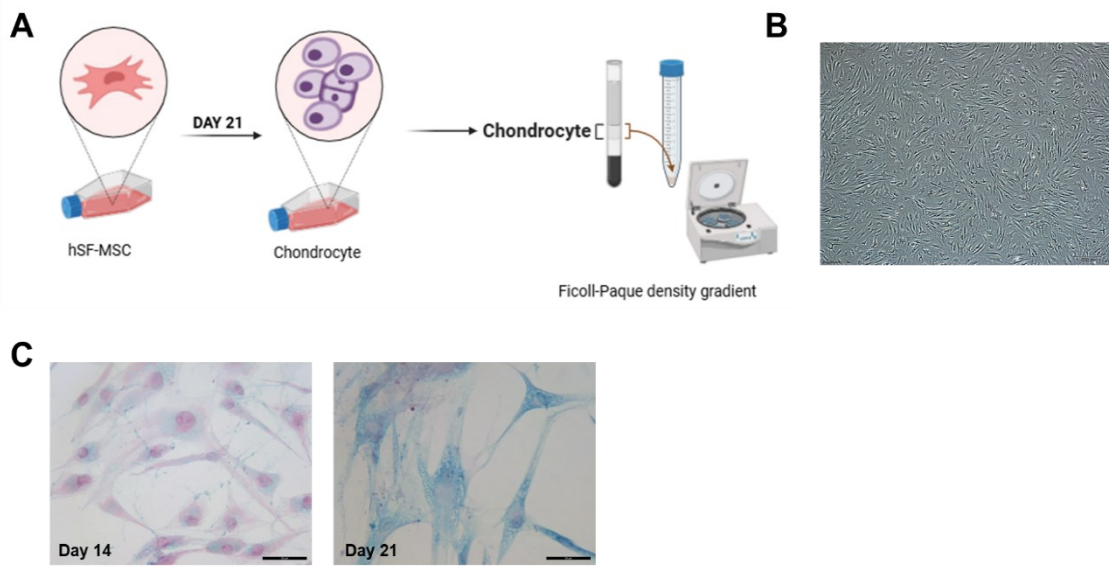

Figure 2. hSF-MSCs, cells were isolated and characterized after the chondrogenic differentiation (A). Chondrocyte (B) scale bar 200 µm. Cells were stained with Alcian Blue dye at Day 14 and Day 21. At the end of 21 days of differentiation for the induction into chondrocytes, cells were differentiated (C). Scale bar 50 µm.

The cells were chemically induced for chondrogenic differentiation and isolation *in vitro* (Fig 2A). Differentiation occurred after approximately 21 days (Fig 2B). During this time, MSCs altered their characteristics, elongated cell morphology and contracted. At the end of 21 days, the cells were fixed and stained for glycosaminoglycan (GAG) using Alcian Blue. Intense Alcian Blue staining confirmed that the cells were differentiated into mature chondrocytes. Following chondrocyte differentiation, cells were isolated. Ficoll-paque isolation is the preferred method for cell separation. The procedure did not involve cell labelling to track or to bind cells to isolate. The cells in the buffy-coat layer were collected using a Pasteur pipette and recovered by centrifugation. Gene expression analysis of cell pellets was performed to confirm that the cells expressed chondrocyte marker genes. According to the results of the analysis, there was a 2-fold increase in COL2 gene expression in chondrocyte cells compared to that in undifferentiated control cells (Fig 3A). This elevated level of significant gene expression indicated successful differentiation, as MSCs do not usually express COL2. COMP gene expression also increased by 1.1-fold compared to that in the control (Fig 3B). Although the increase was weak, statistical analysis showed that this change in the expression was statistically significant. Remarkably, the early differentiation marker for chondrocytes, SOX9, decreased 10-fold in chondrocyte differentiated cells compared to undifferentiated control cells (Fig 3C). This alteration in SOX9 expression indicated the formation of mature chondrocytes, which was also supported by GAG expression (Fig 2C). Unexpectedly, there was a 3-fold decrease in prolactin gene expression (Fig 3D).

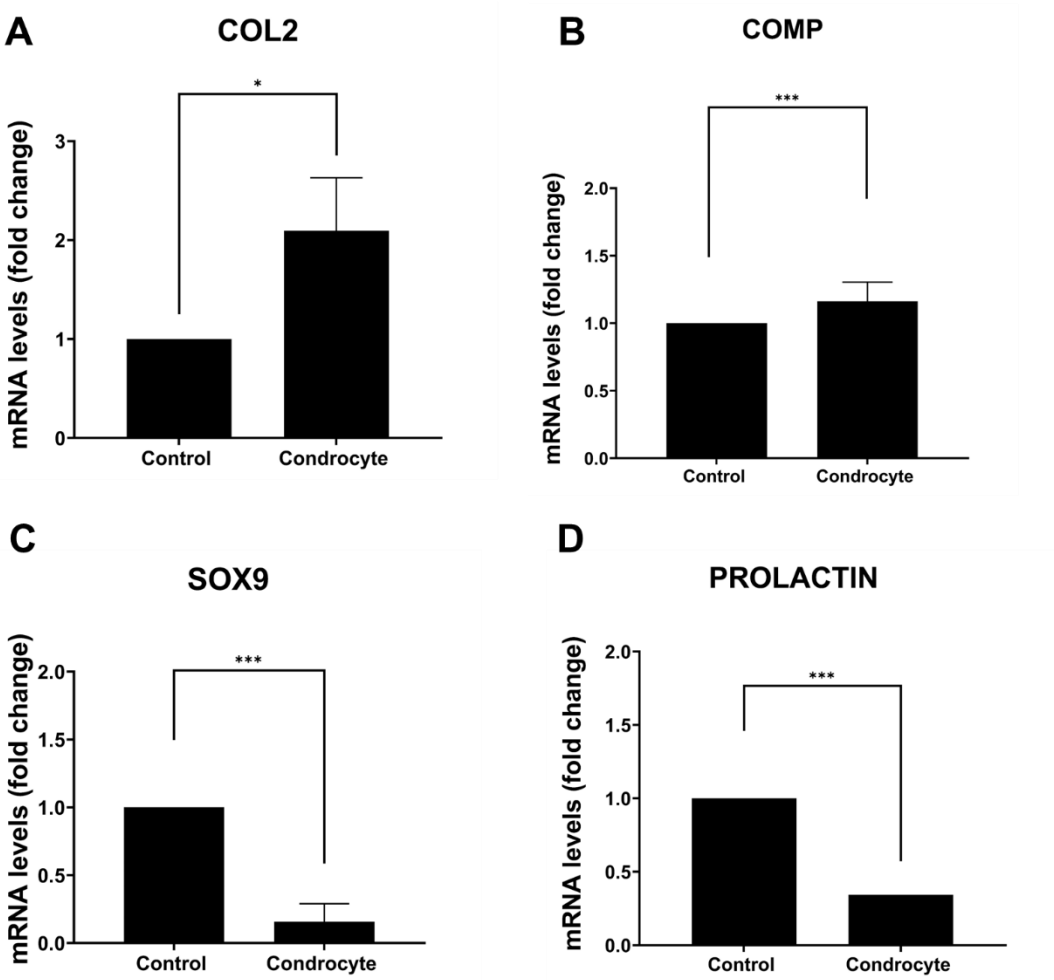

Figure 3. COL2 (A), COMP (B), SOX9 (C), Prolactin (D) gene expression profile of chondrocytes differentiated form hSF-MSCs using qRT-PCR analysis. Chondrocytes were differentiated using undifferentiated SF MSCs as controls. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered.

4. Discussion

Osteoarthritis (OA) causes cartilage degeneration, which leads to patient suffering and has a significant effect on society. Surgical approaches for effective cartilage repair were not recently available, until new techniques emerged. This was made possible by the autologous chondrocyte transplantation technique, which was first introduced in 1994 [23]. Hyaline cartilage is the most abundant type of cartilage in the body, covers the surface of the joints, and cannot repair itself owing to a lack of vascularization. Owing to the chondrocyte differentiation ability and immunomodulatory properties of MSCs, they have been widely investigated as therapeutic agents for the treatments of osteoarthritis. MSCs are a powerful solution for cell-based therapy because of their role in cartilage regeneration and restoration of joint health. Current treatments for OA are required to support their unmet medical needs through advances in biomedical technology to improve efficiency [6]. Preclinical studies have demonstrated the benefits of intra-articular MSC therapy in improving pain relief and tissue function [24-26]. Early clinical studies have shown that MSC-therapy may be effective in treating knee OA and might be helpful in reducing pain. Although some trials have observed changes in the disease, this has not

been consistent [27-30]. The use of cultured adipose tissue-derived MSCs (ADMSCs) in autologous transplantation was shown in an early phase (I/II) study, which provided radiological evidence for increased cartilage volume and histological confirmation of hyaline-like cartilage [29]. Similarly, the use of allogenic bone marrow-derived MSCs in OA was shown to improve pain and function, enhance cartilage quality, and stabilize disease within 12 months compared with the control group [30].

Cartilage is a tissue composed solely of chondrocytes, surrounded by a collagen-rich extracellular matrix organized by chondrocytes. Chondrocytes can have variable cell morphology in tissue or culture, which is adjusted by the type of collagen surrounding the cells. When chondrocytes are isolated from their tissue microenvironment, they exhibit differentiation features by producing type I and III collagens. With the onset of osteoarthritis, the proliferation, viability, and secretion profile of chondrocytes undergo significant alterations [31]. Current Osteoarthritis Research Society International (OARSI) guidelines for the treatment of knee and hip OA focus primarily on pharmacological treatment, including analgesics and anti-inflammatory drugs, which are known to have long-term side effects [32]. Prosthetic surgery is currently the final treatment for OA. Further research is ongoing to develop new drugs that can slow disease progression. Various tips aimed at maintaining chondrogenic phenotypes have been investigated [31].

While chondrocytes play a very important role in the treatment of osteoarthritis, the fact that long-term treatment results have not yet been obtained with autologous or allogeneic transplantation reveals the need for further studies on chondrocytes. Autologous transplantation cannot be considered effective because of the low number of chondrocyte cells that can be isolated from individuals with existing diseases [33]. To release chondrocytes, a combination of enzymatic and mechanical digestion of cartilage tissues is required for chondrocyte isolation to release cells from the tissue matrix. Primary chondrocytes are extracted and proliferated in monolayer culture by adhering to standard tissue culture plastics for several days, usually in small clusters [34]. A study was conducted to develop an improved protocol for isolation chondrocytes by enzymatic digestion using collagenase II. Chondrocyte isolation was achieved by performing two additional digestions with 5- and 3-hour intervals between them, instead of using existing methods of digesting fragmented cartilage for extended periods of time (usually 14-16 hours). The results showed that compared to any conventional isolation protocol, this multiple digestion method was able to increase the total number of cells by more than 5-fold. High expressions of collagen type II (Col II), aggrecan, and COMP genes has been observed [35]. Studies have also been conducted to enhance the chondrocyte cell efficiency by optimizing the amount of Col II [36]. Additionally, research has been conducted to improve the efficiency of chondrocyte isolation through enzymatic reactions created using various combinations of animal tissues studies [37-43]. The methods for isolating chondrocytes are currently inconsistent, highlighting the need for further development in this area. No tangible information is available when searching the literature on *in vitro* chondrocyte isolation.

This study presents a Ficoll-Paque method for isolating chondrocytes efficiently and cost-effectively. *In vitro* isolation is a practical option because of the low quantity and viability of chondrocytes isolated from tissues used for autologous or allogeneic transplantation. Flow cytometry confirmed the mesenchymal characterization of the chondrocytes obtained from hSF-MSCs. Subsequently, the cells were differentiated in a cytokine cocktail for 21 days. Chondrocytes that had undergone differentiation and changed their

morphology were centrifuged with Ficoll at a 1:1 ratio to obtain a buffy-coat layer. The cells were collected from this layer and washed. Upon examining the genes expressed in these chondrocytes, it was found that they exhibited an expression profile for collagen type-2, Sox-9, prolactin, and COMP consistent with the literature, confirming that these cells were intact mature chondrocytes, which was also supported by the staining of GAG with Alcian Blue.

In a recent study by Shen et al. (2022), chondrocytes were filtered through a 70 μm cell strainer following enzymatic digestion, and cell viability and size were assessed by flow cytometry. RNA-seq was performed to determine the expression of 13 chondrocyte-marker genes including COL2A1, ACAN, COMP and SOX9 [36]. A similar method was applied to isolate chondrocytes by Mumammad et al. (2021), but different from the previous paper in this study, the cells were allowed to expand in culture. Importantly the passaged chondrocytes in P2 and P3 exhibited a fibroblast-like phenotype with increased expression of collagen type 1 and decreased expression of collagen type 2 and aggrecan [44]. This loss of chondrocyte-cell characteristics is explained by the dedifferentiation or degeneration of the cells. However, this alteration could also be explained by the contamination of other cells, such as MSCs, which might become dominant in the culture during cell expansion. In another study by Naranda et al. (2017), isolated primary human adult articular chondrocytes from waste cartilage tissue. These studies focused on cell viability and isolation efficiency, but proper characterization following the isolation of any non-chondrocyte is cells lacking [45]. In contrast to these studies, in this study we focused on cell separation using Ficoll to get highly pure chondrocytes that are safely applicable to cell transplantations for OA treatment.

Isolation of chondrocytes requires the combined use of enzymatic and mechanical digestion of cartilage tissues. In most cases, mixed cell cultures are performed directly to expand the cells. However, the presence of non-chondrocyte cells may induce immune rejection following transplantation. In this study, we propose an applicable separation method for chondrocytes that does not adversely affect their viability and function.

Conclusion

Our method is particularly useful for all researchers who intend to better explore the potential of chondrocytes. The proposed cell separation method is a non-toxic, economical, practical and applicable approach at the laboratory scale that can support the cell manufacturing required for chondrocyte-based therapies.

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