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Original Article

Evaluation of the therapeutic potential of infrapatellar fat pad adipose-derived stem cells and their secretome for regenerating knee articular cartilage in a rat model of osteoarthritis

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Abstract

Background: Mesenchymal stem cell (MSC) therapy has ameliorative effects for treating knee osteoarthritis (KOA) disease. Moreover, there is a growing interest in using MSCs-derived secretome (Sec) containing trophic factors secreted by MSCs for KOA treatment. Recently, some studies have suggested that the combination of MSCs and Sec has the potential to treat the diseases. **Aims:** This study aimed to evaluate the ameliorative effects of combined administration of infrapatellar fat pad (IPFP)-derived MSCs, a type of adipose-derived stem cells (ASCs), for treating degenerated cartilage in a rat model of KOA. **Methods:** IPFP-ASCs were isolated from the IPFP of male rats. Sec was obtained from IPFP-ASCs in the fourth passage. Eight weeks after the induction of KOA by collagenase II, the rats were divided into 5 groups (n=5), including a control group with no treatment, and four experimental groups that received sodium hyaluronate (Hyalgan®, Hya), ASCs, Sec, and IPFP-ASCs+Sec, respectively by an infrapatellar injection. To perform the pathological and radiological evaluations, the animals were sacrificed 8 weeks later. **Results:** Our findings indicated that combined administration of the IPFP-ASCs and Sec statistically ($P<0.05$) improved scores of medial tibial and femoral condyles and medial fabella osteophytes. Also, it statistically ($P<0.05$) enhances the cartilage surface, matrix, cell distribution and population viability, and subchondral bone indices. No statistical difference was observed between IPFP-ASCs+Sec and IPFP-ASCs. **Conclusion:** Administration of IPFP-ASCs+Sec has a therapeutic potential to treat KOA in rats. However, there is no difference in the combined administration of IPFP-ASCs and Sec with IPFP-ASCs alone.

Key words: Adipose-derived stem cells, Articular cartilage, Infrapatellar fat pad, Osteoarthritis, Secretome

Introduction

Knee osteoarthritis (KOA) is an inflammatory condition characterized by articular cartilage breakdown, subchondral bone remodeling, and osteophyte formation (Ragni *et al*., 2021). It is the leading cause of pain and disability affecting millions of people, especially older people worldwide (Hunter *et al*., 2014). The worldwide incidence of KOA is about 203 per 10,000 people per year in individuals aged 20 and over (Cui *et al*., 2020).

To date, the management of patients with KOA includes surgical (arthroscopy and osteotomy or joint-replacing procedures) or non-surgical options (use of orthotic devices and pharmacological treatment) to alleviate pain and inflammation (Hussain *et al*., 2016; Hermann *et al*., 2018). However, none of these therapies have been proven to modify disease progression (Grassel and Muschter, 2020), have articular cartilage regenerating potential, or be cost-beneficial (Hussain *et al*., 2016).

Considering all these limitations, recent studies have focused more on stem cell therapy using mesenchymal stem cells (MSCs) and its advantages over conservative methods aimed at ameliorating cartilage structure function in the knee following KOA (Abd-Elsayed, 2018; Shariatzadeh *et al*., 2019; Huang *et al*., 2020). It was shown that MSCs exert their chondroprotective effect through either cell-cell contact, the release of paracrine mediators, or the expression of enzymes (Zhuang *et al*., 2014). Among different sources of MSCs, those derived from adipose tissue are of special interest due to their easy, non-invasive isolation (liposuction) and abundant number (Fraser *et al*., 2006).

Infrapatellar fat pad (IPFP) is a yellowish mass of fibrous fatty tissue in the knee structure, which is located intra-capsular and extra-synovial between the femoral condyle, tibial plateau, and patellar tendon (Sun *et al*., 2018). IPFP is considered a unique source of adipose-derived stem cells (ASCs) from knee replacement surgery (Sun *et al*., 2020; Yao *et al*., 2021). IPFP adipose-derived stem cells (IPFP-ASCs), also called IPFP-MSCs, are attracting more and more attention in regenerative medicine, especially for treating KOA due to their high chondrogenic potential (Sun *et al*., 2018; Vahedi *et al*., 2021). It has been reported that IPFP-MSCs have higher clonogenicity and chondrogenic potential in comparison to bone-derived MSCs (BM-MSCs) (Kouroupis *et al*., 2019). The proliferation capability of IPFP-ASCs is higher than synovial fluid-derived MSCs (Garcia *et al*., 2016). The expression of cartilage-related genes, especially SOX9 and COL2A1, are up-regulated in IPFP-ASCs than in the subcutaneous adipose-derived MSCs and Wharton's jelly-derived MSCs (WJ-MSCs) (Ding *et al*., 2015). Also, the IPFP-ASCs can stably maintain their potential for chondrogenic differentiation during proliferation (English *et al*., 2007). In recent years, the potential role of IPFP-ASCs in KOA has been reported in many researches (Zhong *et al*., 2020; Fujii *et al*., 2022; Liao *et al*., 2022). IPFP-ASCs can repair articular cartilage and relieve the pain caused by KOA (Zhong *et al*., 2020; Vahedi *et al*., 2021).

Despite having the capacity for rapid proliferation, differentiation, and self-renewal, some limitations may create doubtful points in MSC transplantation for cartilage repair. The tendency toward osteogenic differentiation could lead to bone formation around the cartilage in the inflammatory area (Kan *et al*., 2017). Hypertrophy during differentiation can make it difficult for differentiated chondrocytes to be stable phenotypically (Loo and Wong, 2021). The quick disappearance of stem cells from the target tissue after application and other considerations (Zhang *et al*., 2019; Loo and Wong, 2021). Also, physiopathological conditions can adversely affect the proliferation capability and differentiation potential of MSCs (Zhong *et al*., 2020).

Secretome (Sec) refers to a group of factors and molecules secreted from stem cells which composed of lipids, proteins, and nucleic acids, affecting numerous cellular processes (Doyle and Wang, 2019; Eleuteri and Fierabracci, 2019; Trzyna and Banaś-Ząbczyk, 2021). Sec provides considerable advantages over stem cells (Harrell *et al*., 2019; Soetjahjo and Utomo, 2022). Therapeutic effects of Sec are enabled to activate anti-apoptotic and pro-survival pathways due to their capacity to deliver genetic material, growth, and immunomodulatory factors to the target cells that lead to tissue repair and regeneration (Harrell *et al*., 2019). Sec can improve the regeneration of the joint cartilage tissue following OA by enhancing chondrocyte proliferation and migration, hindering OA progression, and modulating inflammatory reactions (Soetjahjo and Utomo, 2022).

Recent studies have reported that the combination of MSCs and Sec has the potential to treat diseases (Nabavizadeh *et al*., 2022; Kurniawan *et al*., 2023; Nowzari *et al*., 2023). Hence, in this study, we aimed to conduct a comparative investigation on the effectiveness of the IPFP-ASCs and their Sec for treating KOA-induced rats based on pathological and radiological evaluations.

Materials and Methods

Animals

Fifty adult male Sprague Dawley rats (200 ± 20 g body weight (BW)) were used in the current research. Twenty-five rats were considered as donors of infrapatellar fat pad tissues and the rest were randomly divided into five experimental groups. All animals were kept in standard cages with a 12-h light/dark cycle, 22 ± 2°C temperature, and 55 ± 5% relative humidity. All animals had free access to water and standard food pellets. The experimental procedures were confirmed by the institutional ethical committee for the care and use of animals at the Shiraz University of Medical Science with the approval number of IR.SUMS.REC. 1395.S441. The authors followed all institutional and international guidelines for animal care during this study. The Animal Research Reporting *In vivo* Experiments guidelines (ARRIVE) were also followed up.

Cell isolation and culture

IPFP tissues were collected from 25 rats (about 5 mg) and transferred into a tissue plate. The isolation of ASCs from fat pad tissue was done based on a method previously described by Nowzari *et al*. (2023). To prepare IPFP-ASCs, the tissue sample was transferred into a tissue plate, and washed using phosphate-buffered saline (PBS) solution supplemented with 1% penicillin-streptomycin (PBS-PS; Sigma, USA). Then, to remove connective tissue and arteries, the specimen was digested using 0.1% collagenase type I 37 ± 1°C and shaking for 1 h. Next, an equal volume of Dulbecco's modified Eagle's medium (DMEM; Biovet, Bulgaria) containing 10% fetal bovine serum (FBS; Biovet, Bulgaria) was added to the mixture to stop the digestion. The suspension was then filtered through a 100 µm filter (Falcon, USA) to remove the redundant solid aggregates and centrifuged at 1500 g for 5 min at a temperature of

24 ± 2°C. Using 1 ml of lysis buffer (Promega, Germany), the pellet was re-suspended, incubated (15 min), washed using PBS, and centrifuged at 1500 g for 5 min to clear the suspension from red blood cells. The supernatant was then removed, and the pellet was re-suspended in a complete medium containing DMEM, 20% FBS, and 1 penicillin-streptomycin in a 75 cm^2 culture flask and held in an incubator with a humidified atmosphere of 37°C and 5% CO_2.

Sec preparation

To prepare IPFP-ASCs-derived Sec, MSCs at the third passage were seeded in a T75 tissue culture flask. The confluent cells were supplemented with serum-free medium and cultured for 72 h. To remove cell debris, the medium was collected and centrifuged twice, first at 500 × g for 10 min followed by 3000 × g for 20 min. The supernatant was filtered through a 0.22-mm filter, used as a Sec of ASCs, and stored at -80°C (Tanideh *et al.*, 2021).

Phenotypic characterization

The flow cytometry procedure was done based on (Bahmanpour *et al.*, 2019). The concentration of 1×10^6 cells/ml in the PBS containing 2% FBS, and afterward, a cold PBS containing 10% FBS as a blocking solution was washed for 20 min. Next, the cells were labeled with ready-to-use fluorescein isothiocyanate (FITC)-conjugated anti-CD44 and anti-CD45, phycoerythrin (PE)-conjugated anti-CD34, and PerCP (Peridinin Chlorophyll Protein complex)-conjugated anti-CD90 antibodies (all from Abcam, UK, Cambridge) incubating for 30 min at room temperature. The samples were then analyzed by a flow cytometer (BD FACSCalibur, flow cytometer, USA). Data were then analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

KOA induction

Twenty-five animals were anesthetized with an intraperitoneal injection of ketamine 40 mg/kg (Alfasan, Netherlands) and xylazine 10 mg/kg (Alfasan, Netherlands). Then the rats' knees were shaved. Four mg collagenase II (Sigma-Aldrich, St. Louis, MO, USA) dissolved in a sterile PBS solution and injected in the knee intraarticular space afterward. Treatments were started when KOA was developed after 8 weeks from the time of the injection of collagenase type II (Zare *et al.*, 2020). The development of KOA was confirmed by the radiological evaluations. Radiological images were taken by a mobile X-ray machine (Siemens Mobilett B, Erlangen, Germany) at 55 kV and 6 mA.

Animal grouping

Eight weeks after the injection of collagenase II, animals were randomly divided into the following groups (5 animals in each group):

I. Control: Did not receive any treatment.
II. Hyalgan (Hya): Received 0.1 ml sodium hyaluronate (Hyalgan®, Fidia, Abano Terme, Italy) by an intra-articular injection.
III. IPFP-ASCs: Received 2.5×10^6 ASCs (50 μl) by an intra-articular injection.
IV. Sec: Received an intra-articular injection of Sec (50 μL) by an intra-articular injection.
V. IPFP-ASCs+Sec: Received a combination of IPFP-ASCs (2.5×10^6) and Sec (50 μL) by an intra-articular injection.

Radiographical evaluation

After 8 weeks, the animals were anesthetized and radiological imaging was done in the anterior-posterior (AP) and lateral (LT) positions. All images were taken by the same operator and equipment (Siemens Mobilett B X-ray machine, Erlangen, Germany) at 55 kV and 6 mA. A radiologist blinded to the study evaluated the radiological images at three different times and gave them scores using a radiological grading system (Zare *et al.*, 2020). This system classifies KOA severity based on joint space width and the presence of osteophytes (Table 1).

Histopathological study

Rats were euthanized with CO_2 70% at the end of the 16th week. Specimens from the knee joint were obtained, fixed in 10% buffered formaldehyde, and then transferred into paraffin waxes. Serial sagittal sections were provided and stained with hematoxylin and eosin (H&E) for cellular architecture. All pathological specimens were assessed by a pathologist at three different times which was blinded to the study data. The degree of cartilage repair of each rat was assessed based on 6 indices, including surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization based on the International Cartilage Repair Society (ICRS) scoring system (Mainil-Varlet *et al.*, 2003) with some modifications which was previously mentioned by Zare *et al.* (2020)(Table 2).

Statistical analysis

Statistical analysis and illustration of data were performed using the GraphPad PRISM Software (version 9.5.0, San Diego, CA, USA). The normal distribution of data was assessed by the Shapiro-Wilk test. The

Table 1: The radiological grading system for KOA

Radiographic KOA feature of the medial compartments		Grade 0	Grade 1	Grade 2	Grade 3
Joint space width		Normal	Reduced	Absent	NA
Osteophyte	Medial tibial condyle	Absent	Small	Moderate	Severe
	Medial femoral condyle	Absent	Small	Moderate	Severe
	Medial fabella	Absent	Present		NA

Table 2: Modified International Cartilage Repair Society (ICRS) scoring system, scoring based on histopathological findings

Parameter	Histopathological findings	Score
Surface	Continuity or uniformity	3
	Irregularity	0
Matrix	Hyaline	3
	Hyaline/fibrous cartilage	2
	Fibrous cartilage	1
	Fibrous tissue	0
Cell distribution	Cylindrical	3
	Cylindrical-cluster	2
	Cluster	1
	Irregular/Single cells	0
Cell population viability	Mostly alive	3
	Partially alive	1
	Less than 10% alive	0
Subchondral bone	Normal	3
	Increase reconstruction	2
	Bone necrosis/granular tissue	1
	Separated/broken/callus at the base	0
Cartilage mineralization	Normal	3
	Abnormal/inappropriate location	0

differences between the groups in terms of medial tibial and femoral condyles osteophyte were compared by one-way analysis of variance (ANOVA) test using Tukey's post hoc test. The differences between the groups in terms of joint space width, medial fabella osteophyte, and histopathological parameters were statistically assessed by Kruskal-Wallis's test. The P-value less than 0.05 was considered statistically significant. Data were expressed as mean±standard deviation (SD).

Results

IPFP-ASCs identification

Flow cytometry was used to identify the phenotypes of IPFP-ASCs and assess the MSC surface markers. Cells were stained for CD34, CD45 (hematopoietic cell markers), CD44, and CD90 (MSCs markers). The result showed that the IPFP-ASCs were negative for cell markers CD34 and CD45, while they were positive for MSC markers, CD44 and CD90 (Fig. 1).

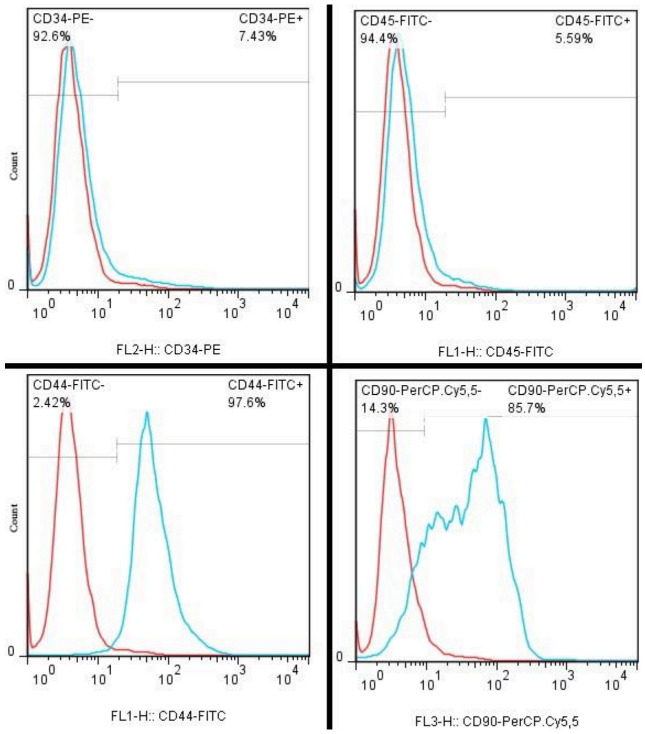

Fig. 1: Flow cytometry study findings indicated that IPFP-ASCs were negative for hematopoietic markers CD34 and CD45 and positive for MSC markers, CD44 and CD90

Radiological findings

The radiological images of the animals' knees and the scores of radiological parameters are illustrated in Figs. 2 and 3, respectively. Regarding joint space width, there was no statistical difference between the groups (Fig. 3A). Scores of both medial tibial and femoral condyles osteophytes were statistically ($P<0.05$) lower in comparison to the Sec, Hya, and control groups (Figs. 3B and C). The medial fabella osteophyte score in the IPFP-ASCs+Sec and IPFP-ASCs groups was statistically ($P<0.05$) decreased compared to the Sec and control groups (Fig. 3D).

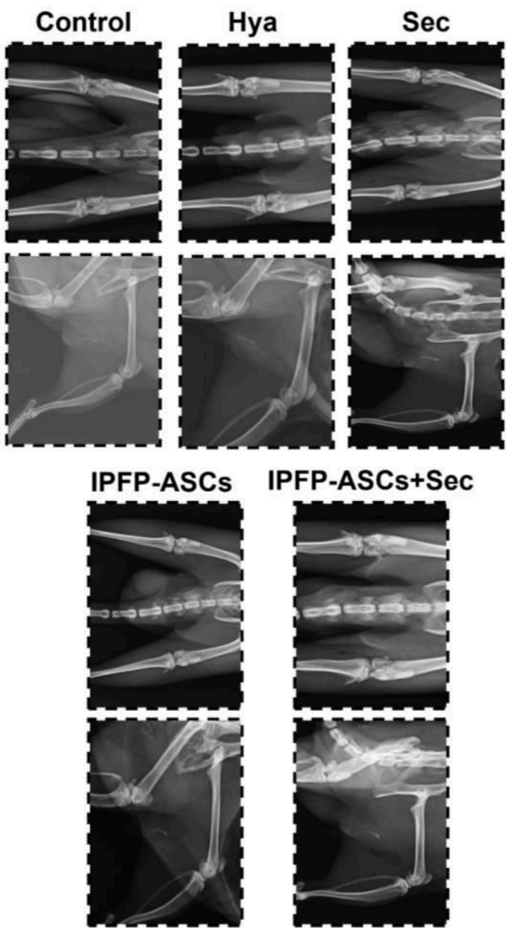

Fig. 2: AP and LT view of knee joints 8 weeks after treatment in KOA-induced rats

Histopathological findings

Histopathological images and indices scores from the

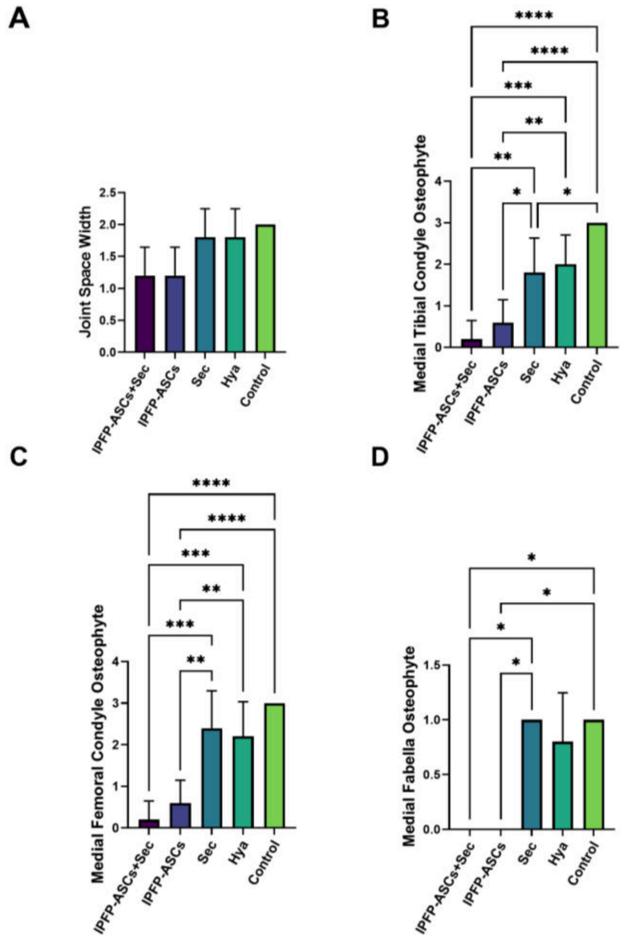

Fig. 3: Radiological scores of knee joints in the different groups. IPFP-ASCs+Sec: Infrapatellar fat pad adipose-derived stem cells+Secretome, IPFP-ASCs: Infrapatellar fat pad adipose-derived stem cells, Sec: Secretome, and Hya: Hyalgan®. Data are shown as mean±SD (n=5). * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001, respectively

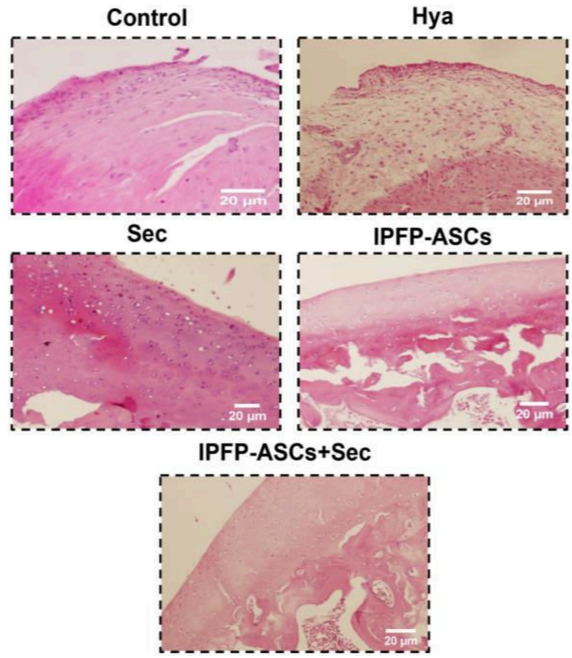

Fig. 4: H&E-stained sections from the surface of articular cartilage of all groups. IPFP-ASCs+Sec: Infrapatellar fat pad adipose-derived stem cells+Secretome, IPFP-ASCs: Infrapatellar fat pad adipose-derived stem cells, Sec: Secretome, and Hya: Hyalgan®. Severely irregular articular surfaces comprise the mostly fibrous cartilaginous matrix (control). Mild articular surface irregularity with hyaline cartilage matrix foci (Hya). Cluster cell distribution and a mixture of the hyaline-fibrous matrix (IPFP-ASCs). A mixture of cylindrical-cluster arrangement of chondrocytes with moderate irregularity of articular surface (Sec). Smooth continuous articular surface with the cylindrical distribution of chondrocytes (IPFP-ASCs+Sec)

right knee of rats from the different groups are represented in Figs. 4 and 5A-F, respectively. Regarding cartilage surface and subchondral bone, the groups that had been treated with IPFP-ASCs+Sec and IPFP-ASCs showed a statistical (P<0.05) increase compared to the control group (Figs. 5A and E).

Regarding cartilage matrix as an aspect of hyaline or fibrosis tissue, treated groups, including Sec, ASCs, and IPFP-ASCs+Sec showed statistically higher scores than the control group (P<0.05 for IPFP-ASCs and Sec and P<0.001 for IPFP+Sec, respectively) (Fig. 5).

Both cell distribution and population viability had a statistically (P<0.05) increase in the rats treated with IPFP-ASCs+Sec and IPFP-ASCs in comparison to the control animals and those treated with Hya (Figs. 5C and D). Except for the cartilage matrix score, the Sec group had no statistical difference compared to the other groups. No statistical difference was observed between groups in terms of cartilage mineralization (Fig. 5F).

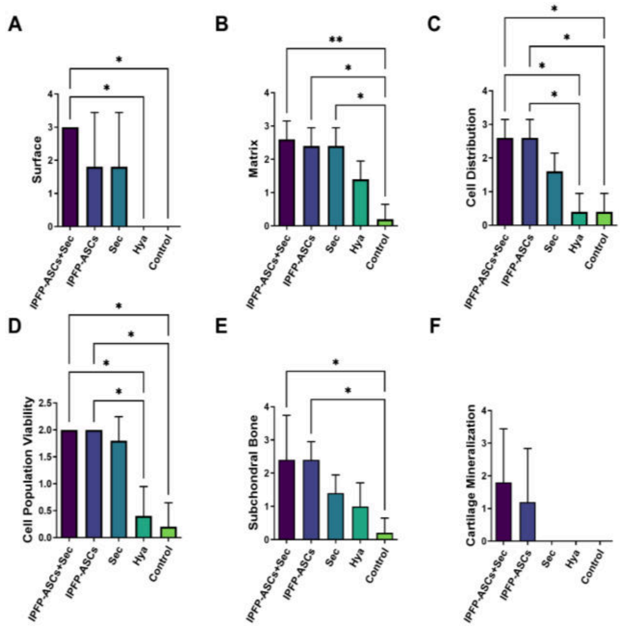

Fig. 5: Histological scores of KOA in rat models. IPFP-ASCs+Sec: Infrapatellar fat pad adipose-derived stem cells+Secretome, IPFP-ASCs: Infrapatellar fat pad adipose-derived stem cells, Sec: Secretome, and Hya: Hyalgan®. Data are shown as mean±SD (n=5). * P<0.05 and ** P<0.01, respectively

Discussion

In this study, for the first time, we have attempted to evaluate and compare the efficacy of treating KOA by Sec, IPFP-ASCs, and a combination of IPFP-ASCs and Sec. Findings from this study revealed that combined administration of the IPFP-ASCs and Sec statistically (P<0.05) improved scores of medial tibial and femoral condyles and medial fabella osteophytes. Also, it statistically (P<0.05) enhanced the cartilage surface, matrix, cell distribution and population viability, and subchondral bone indices.

KOA causes progressive and detectable changes in the tissue metabolism and architecture of the articular-related tissues (Primorac *et al*., 2020). Deterioration of subchondral bone, articular sclerosis, endochondral ossification, modification of osteoblastic and osteoclastic activity, and alterations in cartilage tissue metabolites following KOA result in the formation of osteophytes (Chen *et al*., 2017; Xu *et al*., 2017) which was observable in the radiological image of KOA induced rats. However, the combination of IPFP-ASCs and Sec reduced the score of articular osteophytes. Similarly, Nowzari *et al*. (2023) have reported combined intraarticular injection of dental pulp-derived MSCs (DP-MSCs) and their Sec improves the score of articular osteophytes. Considering these findings, it seems combination of MSCs and their Sec can prevent or down-regulated mechanisms that are involved in the formation of osteophytes. However, molecular and molecular investigations should be conducted to confirm our speculation.

Histopathological scoring remains the gold standard for quantifying OA progression in animal models (Haubruck *et al*., 2023). Administration of IPFP-ASCs+Sec improved the histopathologic score of subchondral bone. Subchondral bone remodeling has a key role in OA pathophysiology (Rezuş *et al*., 2021; Zhu *et al*., 2021). As mentioned above, the deterioration of subchondral bone is related to the formation of osteophytes (Chen *et al*., 2017). Hence, it may be another reason for the prevention of osteophyte formation following the administration of IPFP-MSCs accompanied by their Sec. The present results are in line with previous reports (Tanideh *et al*., 2021; Nowzari *et al*., 2023).

The articular cartilage matrix is composed of components, including collagens (mainly collagen type II), proteoglycans, glycosaminoglycans, and glycoproteins that are produced by chondrocytes (Chen *et al*., 2017; Primorac *et al*., 2020). They can be degraded by enzymes, such as matrix metalloproteinases (MMPs) and aggrecanases (Primorac *et al*., 2020; Rezuş *et al*., 2021). There is a balance between the production of chondral matrix components and their degradations by those enzymes. KOA can disturb this balance by increasing chondrocyte death and over-expression of degrading enzymes (Primorac *et al*., 2020). Administration of IPFP-ASCs+Sec in this study improved the cartilage matrix score. MSCs and their Sec can prevent chondrocyte apoptosis, up-regulate the production of matrix components, and modulate the degrading enzyme production and activity (Ferreira *et al*., 2018; Harrell *et al*., 2019).

Cell distribution and their viability scores were remarkably increased in the rats treated with IPFP-ASCs and IPFP-ASCs+Sec. As mentioned above, MSCs and their Sec can inhibit chondrocytes apoptosis (D'Arrigo *et al*., 2019; Zhang *et al*., 2019; Kangari *et al*., 2020; Vahedi *et al*., 2021). Also, it may be due to the increase in cell proliferation and chondrogenic differentiation of MSCs and Sec (D'Arrigo *et al*., 2019; Harrell *et al*., 2019; Vahedi *et al*., 2021; Kouroupis *et al*., 2022; Liao *et al*., 2022). Moreover, elevation in the score of the articular surface can be due to enhancement of cell viability and proliferation as well as matrix component production.

Hya is an FDA-approved local treatment for KOA (Householder *et al*., 2023). Our results showed that treating with both IPFP-ASCs and IPFP-ASCs+Sec significantly improved radiological and histopathological parameters of the knee when compared to Hya, which is in line with previous studies (Nabavizadeh *et al*., 2022; Nowzari *et al*., 2023). These findings indicate that stem cell therapy has better advantages than Hya to treat KOA.

However, no statistical difference was observed between IPFP-ASCs+Sec and IPFP-ASCs in this study. Similar to our findings Nowzari *et al*. (2023) have reported there was no significant difference between animals treated with DP-MSCs and those who received the combination of DP-MSCs and their Sec. Also, Tanideh *et al*. (2021) observed similar findings when they applied synovial-derived stem cells and their Sec in a rat model of KOA. This may be due to insufficient concentration of administrated Sec or the short-term period of the present study. Hence, we suggest future studies apply higher concentrations of Sec and expand the period of study. In contrast to our result, some previous studies have reported better efficacy following the combined administration of stem cells and Sec. Nabavizadeh *et al*. (2022) have reported combination of the synovial membrane-derived MSCs, Sec, and PRP improves radiological and histopathological indices in the KOA-induced rats better than their alone administration.

On the other hand, we observed that alone administration of Sec just improved the scores of medial femoral condyle osteophyte and cartilage matrix. These results showed that the therapeutic potential of a single administration of Sec against KOA is weak, which is similar to findings from prior reports (Oh *et al*., 2020; Nabavizadeh *et al*., 2022; Nowzari *et al*., 2023).

The present study had some limitations, including the conduction of some special staining, such as alcian-blue, Masson's trichrome, and Safranin-O. Also, gene and protein expression evaluations should be performed to shed light on changes at the molecular and biochemical levels.

In conclusion, the current study demonstrated that while both ASCs and Sec have the chondroprotective effect when used together, they had no better effect compared to ASCs alone based on histopathological and radiological evaluations. However, more experimental studies need to be conducted regarding the combined administration of IPFP and Sec for KOA treatment.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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