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Research article

Cartilage extracellular matrix collagen type II and aggrecan expressions in rabbit cartilage following mesenchymal stem cell implantation

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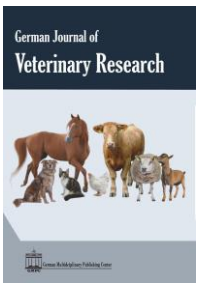

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Abstract

Collagen II and aggrecan are the major components of the cartilage extracellular matrix (ECM) that determine the structure and function of the hyaline articular cartilage. Therefore, their examination should be included in the evaluation of the repair tissue, which is formatted into osteochondral defects (ODs). In this study, the effect of allogeneic-derived stem cells (DSCs), including bone marrow-DSCs (BMDSCs), adipose-DSCs (ADSCs), and synovial membrane-DSCs (SMDSCs), implantation on collagen II and aggrecan production was evaluated. Forty-eight New Zealand rabbits underwent the creation of 3×3mm sized ODs in the femoral patellar groove on both knees and were then randomly assigned to three groups. In all groups, the ODs in the right limbs were filled with fibrin glue (FG), whereas the ODs in the left limbs were filled with ADSCs+FG in group A, with BMDSCs+FG in group B and with SMDSCs+FG in group C. After 12 weeks the repair tissues were immunohistologically assessed for collagen II and aggrecan. Accordingly, FG and ADCSs (p=0.003), BMDSCs (p=0.017), and SMDSCs (p=0.003) implantation resulted in significantly stronger expression of collagen II in the reparative tissue compared with the FG groups. Also, the newly formed tissue in the BMDSCs+FG (p=0.015) and SMDSCs+FG (p=0.003) groups expressed significantly higher aggrecan compared with the FG groups, whereas BMDSCs+FG (p=0.030) and SMDSCs+FG (p=0.002) transplantation promoted significantly higher aggrecan composition compared with ADSCs+FG. These findings indicate the profound impact of BMDSCs and SMDSCs on the ECM quality of the repair tissue. Additionally, the inefficiency of FG to induce a well-developed ECM in the repair area was highlighted.

Keywords: Collagen II, Aggrecan, Extracellular matrix, Cartilage, Rabbit stem cell, Osteochondral defects

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Introduction

Articular cartilage is characterized by its hyaline structure, which facilitates frictionless movements, absolves biomechanical forces, and offers total load support. It is a highly specialized connective tissue composed of a few chondrocytes and a dense extracellular matrix (ECM) containing water (65-80%), collagen fibers (10-15%), proteoglycans (10-15%), and minerals (Horkay, 2012). The organization of collagen fibers determines the mechanical loading of joints, whereas the ability of articular cartilage to resist compression forces is associated with aggrecan concentration (Fox et al., 2009). Several collagen subtypes have been identified in the ECM, supporting cell growth and maintaining cartilage mechanical properties. The main and most prominent collagens are type II, IX, and XI (Alcaide-

Ruggiero et al., 2021). However, collagen II accounts for 90% of the cartilage collagen and is less abundant in the fibrocartilage tissue (Roberts et al., 2009). In addition to the fibrillar collagen network, the ECM contains several proteoglycans, which are important for maintaining the hydration and elasticity of the articular cartilage (Vertel, 1995; Kiani et al., 2020). Aggrecan is the most abundant proteoglycan and plays a critical role in its structure by creating boundaries with hyaluronan and reinforcing the cartilage load-bearing structure (Cederlund and Aspden, 2022). In addition, aggrecan's negative charge attracts water molecules and accordingly participates in the hydration of the ECM (Troeberg and Nagase, 2012).

Osteoarthritis (OA) is the most common joint disease in humans (Yao et al., 2023), horses (Baccarin et al., 2022), and dogs (Anderson et al., 2020), which negatively affects the patient's quality of life. Immunochemical analysis of osteoarthritic cartilage has shown decreased aggrecan and collagen II concentration, particularly at the superficial cartilage layers (Lorenz and Richter, 2006). Also, in the early stages of OA, the loss of aggrecan and the replacement of collagen type II with type I have been presented as indicators of the degeneration process (Krawetz et al., 2022). These alterations in ECM composition during the development of OA progressively interfere with normal cellular activities and eventually result in further cartilage degradation (Maldonado and Nam, 2013). Because of its avascular and hypocellular composition, the articular cartilage has a limited capacity for spontaneous regeneration (Muthu, 2023). Thus, regenerative medicine has proposed many conservative and surgical therapies for cartilage restoration (Cong et al., 2023). In addition, tissue engineering has developed new repair techniques, including mesenchymal stem cell (MSC) implantation.

Despite the extensive research in cartilage defects and MSC implantation techniques, the acquired data from previous studies are limited regarding the immunochemistry analysis of the reparative tissue formatted into animal ODs. The majority of previous studies have almost exclusively focussed on the presentation of histological and macroscopic findings of the repair tissue (Brittberg et al., 1994; Chen et al., 2005; Ishii et al., 2007; Koga et al., 2008; Im and Lee, 2010; Kim et al., 2012; Lee et al., 2012; Shimomura et al., 2014; Ye et al., 2018; Hsu et al., 2018; Jia et al., 2018; Yin et al., 2018; Duan et al., 2019; Khanmohammadi et al., 2019; Mahmoud et al., 2019; You et al., 2020; Anatolitou et al., 2023). However, Alcaide-Ruggiero et al. (2021) revealed that the immunohistological assessment of the ECM should also be included in the histopathological evaluation of the repaired tissue to estimate the tissue's quality and structure. In addition, the zonal organization of ECM is a unique feature that characterizes the hyaline articular cartilage and can further distinguish it from other types of cartilage (Alcaide-Ruggiero et al., 2023). Nevertheless, only a few studies have investigated the presence and arrangement of the ECM in the repair tissue and presented findings regarding some collagen types. Additionally, the distribution of aggrecan in the reparative tissue has not been widely investigated (Chen et al., 2005; Xie et al., 2010; Lee et al., 2012; Lee et al., 2013; Ye et al., 2018; Jia et al., 2018; Yin et al., 2018; Duan et al., 2019; Khanmohammadi et al., 2019; Oshima et al., 2019; You et al., 2020; Yang et al., 2021). Another limitation of the existing literature is that most of these results have been presented as qualitative data without scoring systems.

The goal of this randomized, controlled, double-blind study was to perform an immunohistochemical analysis of collagen II and aggrecan in-depth to obtain greater evidence on the quality of the reparative tissue after allogeneic bone marrow- (BMDSCs), adipose- (ADSCs), and synovial membrane- (SMDSCs) derived stem cell implantation into rabbit ODs. Our conclusions are presented quantitatively and may provide additional information about the nature of the newly formed tissue in rabbit's ODs.

Material and methods

All procedures were performed according to the international guidelines and with the approval of the Veterinary Service of the Region of Attica, Hellenic Republic (1155/13-03-2018). Isolation of allogeneic BMDSCs, ADCSs, and SMDSCs was performed as described in a previous study using female New Zealand white rabbits. In addition, the *in vitro* capacity of MSCs to form colonies and exhibit chondrogenesis has been

described previously (Bami et al., 2020). In this study, passage 3 cells were used.

Isolation of BMDSCs

After anesthesia induction by intramuscular injection of xylazine (Xylapan, Vetoquinol, Towcester, UK) 5 mg/kg and ketamine (Imalgene, Merial, Lyon, France) 35 mg/kg, 5mL of blood was aspirated from the bone marrow of rabbits through penetration of the cortex of the iliac spine using a 15G gauge needle (Kimal, Worcester, UK) and treated accordingly to induce hemolysis. The blood sample was centrifuged at 500g for 10 minutes, and the isolated cell pellets were resuspended in low glucose DMEM with 15% MSC qualified fetal bovine serum (FBS), 2mM L-glutamine, 1×antibiotic-antimycotic and 1×non-essential amino acids (Life Technologies, Carlsbad, USA). Then, the cells were seeded in well plates, and after 48 hrs, the detached cells were washed with phosphate-buffered saline (PBS). Cultivation was continued until 90% confluence, by changing the culture medium every three days.

Isolation of ADSCs

Adipose tissue was aseptically excised from the inguinal area of rabbits after euthanasia with intramuscular pentobarbital (Thiopental, IFET, Athens, Greece) 150 mg/kg injection. The tissue was washed with sterile PBS, cut into small pieces, and digested in collagenase type I. Enzyme stop medium was added to neutralize collagenase activity, and the cells were then sedimented by centrifugation at 400 ×g for 5 minutes and resuspended in red blood cell lysis buffer. After filtration and centrifugation, the cell pellets were resuspended in low-glucose DMEM, 10% MSC qualified FBS, 2mM L-glutamine, 1× Antibiotic-Antimycotic and 1× non-essential amino acids (Life Technologies, Carlsbad, USA) and plated on tissue culture dishes. Cultivation continued until confluence.

Isolation of SMDSCs

The rabbits were euthanized by intramuscular pentobarbital (Thiopental, IFET, Athens, Greece) 150 mg/kg injection, and the synovial membrane was harvested aseptically. The synovial tissues were washed with sterile PBS 3% penicillin/streptomycin, minced, and enzymatically digested with 0.02% type I collagenase in PBS at 37°C for 6 hours. After filtration, the cells were resuspended in low glucose Dulbecco's modified Eagle's medium (DMEM), with 10% MSC qualified FBS, 2 mML-glutamine, 1× antibiotic-antimycotic and 1× non-essential amino acids (Life Technologies, Carlsbad, USA), washed with PBS, and cultured until confluence was reached.

Experimental design and surgical procedure

Forty-eight skeletally mature (10-12 months old) intact female New Zealand rabbits weighing approximately 4.5 kilograms were included in this study. Before the procedures, the rabbits were acclimatized for 1 week.

General anesthesia induction was completed with an intramuscular injection of xylazine (Xylapan, Vetoquinol, Towcester, UK) 5 mg/kg and ketamine (Imalgene, Merial, Lyon, France) 35 mg/kg. With the standard aseptic technique, both knee joints were operated via a lateral parapatellar incision. Using a surgical drill, a 3 mm depth and 3 mm diameter defect was made centrally in the patellar groove. The depth of the defect was premeasured and achieved using a drill stopper (Veterinary Instrumentation, Sheffield, UK). Finally, the surgical field was lavaged with water for injection (Water for injection, Fresenius Kabi Hellas, Athens, Greece) before implantation.

MSC and fibrin glue administration

The FG contained in a double syringe system of fibrinogen and thrombin was prepared according to the manufacturer's recommendations for sterile thawing (Tisseel, Baxter, Deerfield, USA). All rabbits were randomly divided into three groups (Table 1): After the attachment of the FG to the adjacent cartilage, the surgical site was lavaged with water for injection (Water for injection, Fresenius Kabi Hellas, Athens, Greece), and the patellar was relocated. The joint capsule was sutured using 4/0 Monocryl sutures (Ethicon, Johnson and Johnson Medical, Somerville, NJ), and the surgical incision was closed in three layers. Postoperatively, meloxicam (Meloxidyl, Ceva, Libourne, France) 0.6mg/kg and enrofloxacin (Enrotron, aniMedica GmbH, Frankfurt, Germany)10 mg/kg were administered subcutaneously for 7 days. The animals were allowed to move freely and evaluated for signs of discomfort, pain, or infection. However, most of them could bear weight on both limbs after surgery.

After 12 weeks, the rabbits were anesthetized with an intramuscular injection of xylazine (Xylapan, Vetoquinol UK) 5 mg/kg and ketamine (Imalgene, Merial, France) 35 mg/kg. All animals were euthanized with pentobarbital (Thiopental, IFET, Greece) 150 mg/kg intraperitoneally, and the distal femur condyles were collected (Figure 1). Sections 4-6 μm thick were cut from paraffin-embedded tissue and mounted on positively charged glass slides (Superfrost Plus, Thermo Scientific, Copenhagen, Denmark). The deparaffinization process was performed on a LEICA BOND automated immunohistochemistry staining system. Antigen retrieval was performed with pepsin for 10 minutes at 99°C, citrate buffer 0,01M ph=6 (Bond epitope retrieval solution 1), and EDTA ph=8 (Bond epitope retrieval solution 2) for 30 minutes. To investigate the expression of collagen II and aggrecan, immunohistochemical staining was performed by incubation with monoclonal mouse primary antibodies to collagen II (1:100, II anti-collagen II antibody [2B1.5] ab 185430, Abcam, Cambridge, UK) and aggrecan (1:100, recombinant anti-aggrecan antibody [6-B-4] ab 3778, Abcam, Cambridge, UK). First, the sections were treated with peroxidase block for 5 minutes, washed 3 times with wash buffer, and incubated with the markers for 30 minutes. The sections were then washed out thrice, covered with the post-primary block for 20 minutes, and washed three times before polymer application for 20 minutes. Subsequently, the slides were rinsed three times, and the DAB chromogen substrate was applied for 10 minutes. Before being counterstained with hematoxylin for 5 minutes, the slides were rewashed three times. This was followed by dehydration of the sections in an ascending series of alcohols, clarification in xylene baths, and finally, coverslipping with a mounting medium. Semiquantitative expression of type II collagen and aggrecan in the repair site was blindly graded based on a previously reported scoring system Grevenstein et al. (2020) (Table 2).

Statistical analysis

All analyses were performed using SPSS vr 21.00 (IBM Corporation, Somers, NY, USA). The variables were expressed as the median and intra-quadratic range (IQR) because of the ordinal data. Comparisons of the immunohistochemical expression of collagen II and aggrecan were completed using the non-parametric Kruskal-Wallis test and Dunn test adjusted by the Benjamini-Hochberg FDR method for pairwise comparisons. Comparisons between the right and left limbs for the immunohistochemical expression of collagen II and aggrecan were performed using the non-parametric Wilcoxon test. All tests were two-sided, and statistical significance was set at $p<0.05$.

Table 1: Experimental design.

Group	Number	Treatment
A	16	FG was transplanted into the defect of the right limb (50μL/defect), whereas the defect in the left limb was implanted with ADSCs (2×10^7cells/mL) and sealed with FG (50μL/defect).
B	16	FG was transplanted into the defect of the right limb (50μL/defect), whereas the defect in the left limb was implanted with BMDSCs (2×10^7cells/mL) and sealed with FG (50μL/defect).
C	16	FG was transplanted into the defect of the right limb (50μL/defect), whereas the defect in the left limb was implanted with SMDSCs (2×10^7cells/mL) and sealed with FG (50μL/defect).

FG: Fibrin glue, ADSCs: Adipose-derived stem cells, BMDSCs: Bone marrow-derived stem cells, SMDSCs: Synovial membrane-derived stem cells

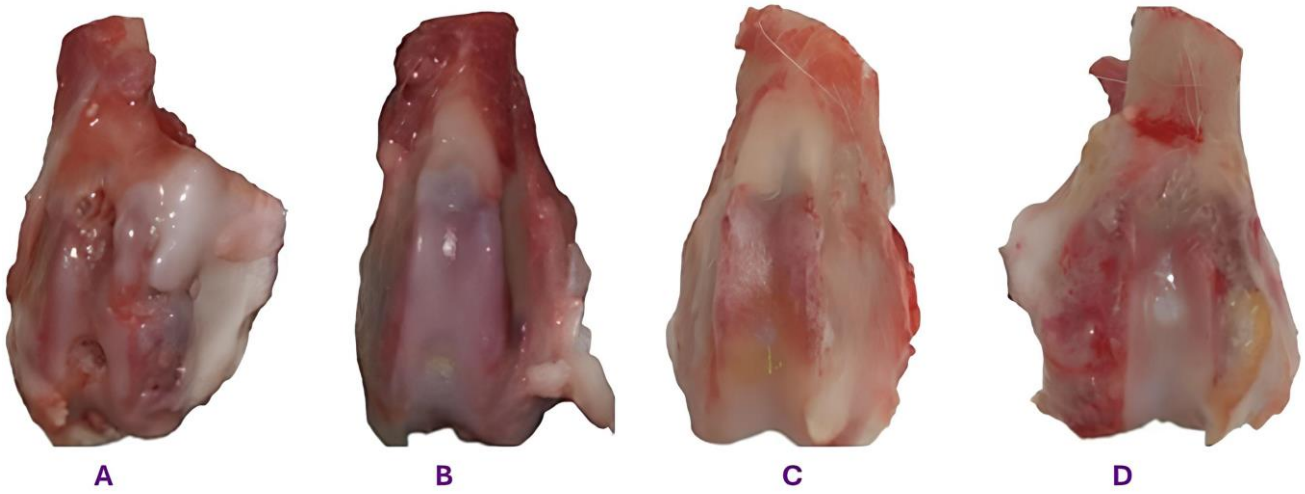

Figure 1: Macroscopical features of osteochondral defects (ODs) in different groups. (A) The reparative tissue that formed into ODs, filled with fibrin glue (FG), exhibited partial growth, poor integration with the native cartilage, and some fissuring. (B) The defects in the bone marrow-derived stem cells (BMDSCs + FG group were completely covered with newly formed tissue that had well-defined margins and a fibrillated surface. (C) In the BMDSCs + FG group, the reparative tissue filled the defect completely, with indistinct demarcating borders and a fibrillated appearance. (D) The defects in the synovial membrane-derived stem cells (SMDSCs) + FG group were fully covered with smooth, glossy, and well-integrated newly formed tissue.

Table 2: Scoring system from 0 to 3 for immunohistochemical staining intensity (Grevenstein et al., 2020)

Collagen II staining	**Scores**
Intense	3
Moderate	2
Weak	1
None	0
Aggrecan staining	
Intense	3
Moderate	2
Weak	1
None	0
Total immunohistochemistry score	0-6

Human lung tissue and human cartilage were used as positive controls for anti-collagen II and anti-aggrecan antibodies. Like negative controls, samples of human liver incubated with PBS were used. A light microscope fitted with a digital camera recorded the images of the stained specimens. All samples were examined independently by one blinded observer to sample characteristics.

Results

Type II collagen

As assessed immunohistochemically, no statistical difference was detected between the expression of type II collagen in the newly formed cartilage tissue among the different groups either in the right (p=0.626) or in the left limbs (p=0.121).

However, the newly formed cartilage in the left limbs showed significantly stronger staining for collagen II in groups A (p=0.003), B (p=0.017), and C (p=0.003) compared to the right limbs (Table 3). The defects in the right limbs were filled with tissue that exhibited lower staining intensity for type II collagen than that in the normal cartilage (score 1). In the left limbs, the cartilage showed positive staining in most areas for groups A, B (score 2), and C (score 3) (Table 3).

Aggrecan

Aggrecan immunostaining revealed no statistical difference (p=0.144) among the different groups for the right limbs. Nevertheless, a significantly higher expression level of aggrecan was found for groups B (p=0.030) and C (p=0.002) compared with group A (p=0.007) (Table 3). The intensity of staining in the repaired areas in

groups B and C was similar to normal cartilage (score 3), whereas aggrecan expression was average in group A (score 2) (Figure 3).

Lastly, the immunohistochemistry staining for aggrecan was significantly stronger for the left limbs only in groups B (p=0.015) and C (p=0.003) (score 3) (Table 3). whereas it was very light in the right limbs (score 1 and 1.5, respectively) (Figure 3).

Table 3: Comparison of collagen type II expression in the repair tissue between the right and the left limbs. And the comparison of aggrecan expression in the repair tissue between the groups of the left limbs and between the right and the left limbs.

	Group	Mean± SD	Median	IQR
Aggrecan left	A (ADSCs+FG)	1.73±0.80	2.00	1.00
	B (BMDSCs+FG)	2.33±0.90	3.00	1.00
	C (SMDSCs+FG)	2.64±0.63	3.00	1.00
Aggrecan right	A (FG)	1.27±0.80	1.00	1.00
	B (FG)	1.27±0.70	1.00	1.00
	C (FG)	1.43±0.85	1.50	1.00
Collagen II left	A (ADSCs+FG)	1.80±0.86	2.00	1.00
	B (BMDSCs+FG)	2.20±0.94	2.00	1.00
	C (SMDSCs+FG)	2.43±0.76	3.00	1.00
Collagen II right	A (FG)	0.93±0.59	1.00	0.00
	B (FG)	1.20±0.68	1.00	0.00
	C (FG)	1.07±0.73	1.00	1.25

SD: Standard deviation; IQR: intra-quadratic range; FG: Fibrin glue; ADSCs: Adipose-derived stem cells; BMDSCs: Bone marrow-derived stem cells; SMDSCs: Synovial membrane-derived stem cells.

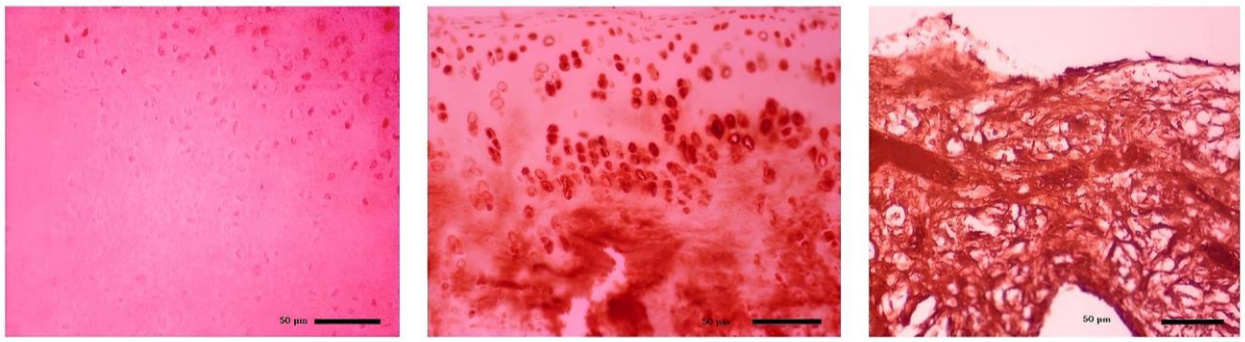

Figure 2: Immunohistochemistry staining for collagen type II in the newly formed cartilage tissue among the different groups (a) The reparative tissue that formed in ODs filled with FG exhibited weak staining intensity for collagen type II (score 1, bar: 50 µm). (b) The defects in the ADSCs + FG and BMDSCs + FG groups showed moderate staining in most areas (score 2, bar: 50 µm), while (c) the SMDSCs + FG group demonstrated intense staining in the repaired regions (score 3, bar: 50 µm).

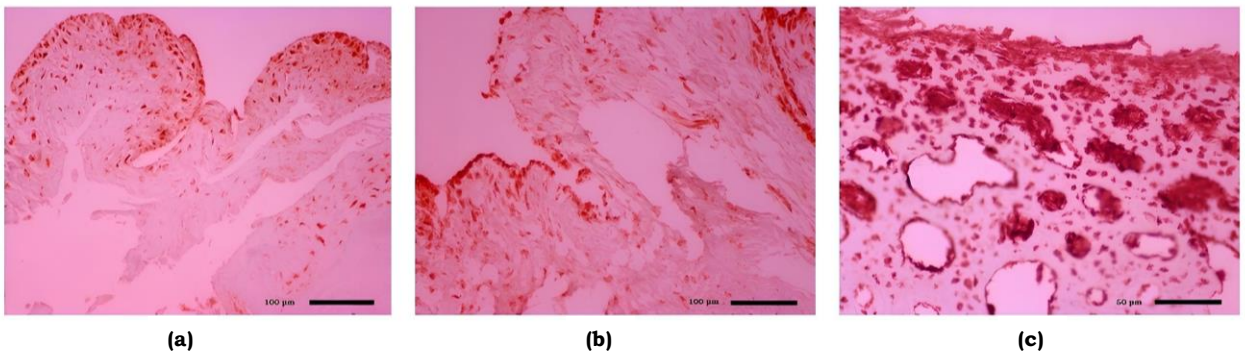

Figure 3: Immunohistochemistry staining for aggrecan in the newly formed cartilage tissue among the different groups. (a) The reparative tissue that formed in ODs filled with FG exhibited weak staining intensity for aggrecan (score 1, bar: 100 µm). (b) The defects in the ADSCs + FG group showed moderate staining for aggrecan (score 2, bar: 100 µm), while (c) the SMDSCs + FG and BMDSCs + FG groups demonstrated intense staining in the repaired regions (score 3, bar: 50 µm).

Discussion

This study demonstrated the inferior capacity of FG to induce the production of ECM with sufficient amounts of collagen II and aggrecan. Moreover, the feasibility of allogeneic MSCs in promoting collagen type II expression in the repair tissue was recognized. In addition, the superior efficacy of SMDSCs and BMDSCs in inducing higher aggrecan concentrations into the newly formed tissue compared with ADSCs was highlighted.

Biological scaffolds such as FG could be a potential option for MSC transplantation because of their adhesive and biodegradable properties. However, the effects of FG on cell migration and proliferation remain controversial. According to Homminga and coworkers, the cocultivation of FG and rabbit chondrocytes enhanced cell proliferation and ECM production *in vitro* (Homminga et al., 1993). Nevertheless, in more recent *in vitro* studies, FG was proved to be not the most suitable choice for supporting tissue engineering. The migration and proliferation of human chondrocytes in collagenic membranes were weak after the use of FG over the resorbable membrane, indicating impairment of cartilage regeneration (Migliorini et al., 2022). In addition, FG could not support chondrocyte adhesion and growth in cell cultures, while the production of cartilaginous matrix lacked the normal levels of collagen II and aggrecan (Wang et al., 2016).

Moreover, minimal information is available regarding the efficacy of FG in inducing the production of normal ECM *in vivo*. According to these findings, FG has a low capacity to produce adequate levels of collagen type II. Brittberg et al. (1994) claimed no chondrocyte migration inside the implanted FG into rabbit ODs, while the repair tissue was overall assessed as histologically inferior compared with the untreated defects. Also, in previous reports, immunohistological analysis of the reparative tissue formed inside rabbit ODs after FG implantation revealed light to moderate intensity of collagen type II expression (Ishii et al., 2007; Yin et al., 2018; Khanmohammadi et al., 2019; You et al., 2020). Besides, as evaluated by Sirius red staining, the collagen fibers in the regenerated tissue had a random and irregular orientation (Yin et al., 2018).

Additionally, the use of porcine fibrin sealant in a rabbit model for cartilage regeneration resulted in the formation of tissue with inconsistent and heterogeneous staining of collagen type II (Yang et al., 2021). Similarly, in our study, FG did not promote the production of cartilaginous ECM, whereas the expression of collagen II and aggrecan in the repair tissue remained weak. To the authors' knowledge, this is the first study to demonstrate *in vivo* findings about the impact of FG on ECM composition regarding aggrecan. The low aggrecan expression found in this study reinforces the presence of a non-well-developed ECM in the repair area, which is significant for tissue resistance to mechanical stress. These results support evidence from our previous conclusions about the inferior efficacy of FG in treating rabbit ODs due to the poor histological features of the reparative tissue (Anatolitou et al., 2023).

Interestingly, the accumulation of collagen type II was augmented after the addition of allogeneic MSCs in FG. Previous *in vivo* studies that examined the impact of human-derived MSC implantation on collagen expression in the repair tissue presented that collagen type II was produced from the 3rd month postoperatively (Khanmohammadi et al., 2019; You et al., 2020). Accordingly, in another study, after 6 months, the expression of collagen type II in the reparative tissue was similar to that of the healthy cartilage (Khanmohammadi et al., 2019). In addition, the implantation of allogeneic ADSCs into rabbit ODs promoted at a higher rate the formation of collagen type II compared with FG, while the collagen fibers organization was found to be similar to normal cartilage (Yin et al., 2018).

However, studies that used different scaffold materials reached similar conclusions about the efficacy of MSC in producing cartilaginous ECM. Immunochemistry analysis of the tissue formed into rabbit ODs showed that the addition of ADCSs (Oshima et al., 2019) and BMDSCs (Chen et al., 2005; Xie et al., 2010; Duan et al., 2019) inside different biomaterials advanced the expression of collagen type II. Moreover, the transplantation of autologous SMDSCs with COL/HA/FG-44 (Koga et al., 2008) and PRP (Sakaguchi et al., 2005) into ODs enhanced the accumulation of collagen type II fibers. Also, according to Jia et al. (2018), similar results were presented after the intra-articular injection

of autologous SF-MSCs into rabbits' knees regarding the collagen type II concentration in the newly formed tissue inside ODs. Only one study claimed that collagen type II concentration did not differ between the MSC and scaffold groups (Yin et al., 2018). According to a histomorphometric analysis of the repair tissue, the authors concluded that the implantation of an acellular dermal matrix and ADSCs promoted tissue production with similar ECM features. However, these types of scaffolds have superior chondrogenic properties due to their unique compound, which may have interfered with the results (Sutherland et al., 2015).

Even though all studies highlighted MSC's potential to produce a healthy ECM, no comparisons could be easily made because of the high protocols' variability. In our study, we compared the impact of three different MSCs on cartilaginous matrix composition. According to our results, although no significant difference was detected between the three groups, the expression of collagen II in the SMDSCs+FG group was higher and similar to native cartilage. Also, the expression of aggrecan was proved to be more intense in the BMDSCs+FG and SMDSCs+FG groups. The *in vitro* features of BMDSCs and SMDSCs have been previously investigated. According to Bami et al. (2020), ADSCs expressed aggrecan and collagen type II much lower than BMDSCs and SMDSCs. In addition, their chondrogenic superiority was indicated due to their larger pellet size in the culture system. Another *in vitro* chondrogenesis assay in rats claimed that the pellets derived from SMDSCs were larger than those from ADSCs and BMDSCs because of greater ECM production (Lee et al., 2013). Similar features were presented for human BMDSCs and SMDSCs regarding pellet size and the amount of formed ECM (Sakaguchi et al., 2005). Besides, human BMDSCs were shown to express aggrecan *in vitro* constitutively (Mwale et al., 2006), whereas SMDSCs exhibited aggrecan secretion both *in vitro* and *in vivo* (Krawetz et al., 2022). Also, collagen type II and aggrecan expression were markedly higher in SMDSCs cell pellets than in BMDSCs (Ogata et al., 2015).

However, the results from *in vivo* studies remained controversial. The implantation of equine SMDSCs in rats' ODs induced a higher rate of collagen type II production than BMDSCs (Zayed et al., 2018). On the contrary, the intra-articular implantation of BMDSCs and cocultured MSCs in rabbits resulted in excessive collagen type II expression in the reparative tissue, in contrast to SMDSCs (Mahmoud et al., 2019). However, these results were not statistically confirmed. In the present study, we demonstrate that BMDSCs and SMDSCs could promote the production of reparative tissue with superior immunohistological features regarding aggrecan, while SMDSCs possess great potential to induce higher concentrations of collagen II, too. To the authors' knowledge, this is the first study to present *in vivo* findings about aggrecan after MSCs' implantation. These findings complement those of our previous study, which depicted the superior osteochondrogenic efficacy of SMDSCs regarding the macroscopic and histological features of the repair tissue (Anatolitou et al., 2023).

Nevertheless, this study has several limitations. The immunohistochemistry results' quantification system was simplified. A simple scoring system was decided, but more subcategories might be more suitable for detecting differences in stain distribution among the cartilage layers. In addition, aggrecan and collagen type II have been selected as the major components of the articular ECM. However, other collagen types could also be estimated to allow a more extensive evaluation of the ECM composition and an accurate determination of the quality of the repair tissue. Finally, the follow-up was twelve weeks, which may interfere with long-term effects. Future long-term studies, which should also compare the imaging features of the reparative tissue, should be conducted.

Conclusions

From an immunohistochemical point of view, we demonstrated that FG implantation into rabbit ODs resulted in the production of ECM with insufficient amounts of collagen II and aggrecan. Moreover, we mentioned that the addition of allogeneic MSCs into FG improved the expression of collagen type II. Lastly, we revealed that SMDSCs and BMDSCs are the most superior sources of MSCs in inducing the production of cartilaginous ECM.

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