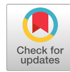
ORIGINAL ARTICLE



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Examination of the effect of xenogeneic mesenchymal stem cells and conditioned medium on cartilage graft viability: a rabbit model

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Background Since cartilage, unlike skin, does not contain vessels, it obtains nutrition by diffusion. This reduces graft viability, resulting in problems such as reductions in size, changes in shape, and resorption of the cartilage graft in the late post-graft period. This study aimed to investigate the effects of adipose-derived mesenchymal cells and conditioned medium (CM) on cartilage graft viability.
Methods Dissections were performed 4 months after the injection of 0.5 mL of CM or 2×10^6 mesenchymal stem cells (MSCs) in 0.5 mL after grafting into a control group and two experimental groups (n=21 rabbits in total). Chondrocyte viability and type II collagen expression in the grafted areas were analyzed by hematoxylin-eosin staining and immunohistochemical methods, respectively.
Results In the MSC and CM groups, chondrocyte proliferation at the graft tissue incision margin (MSC: $P<0.01$, CM: $P<0.0001$), chondrocyte proliferation at the auricular cartilage incision margin (MSC: $P<0.05$, CM: $P<0.0001$), integration of the graft with the surrounding cartilage (MSC: $P<0.001$, CM: $P<0.0001$) and type II collagen expression levels (MSC: $P=0.001$, CM: $P=0.0002$) significantly increased.
Conclusions Xenogenic injection of MSCs and CM contributed to new cartilage production without any tumoral effects or immune reactions. In particular, the cell-free nature of CM strengthened its potential for safe use. Since injections of MSC and CM can preserve cartilage graft viability, interest in this technique is expected to increase as long-term results from clinical studies on the subject become available.

Keywords Cartilage / Adipose-derived mesenchymal stem cells / Graft / Collagen type 2

INTRODUCTION

The annual incidence of cartilage defects is around 415,500 [1]. Alloplastic implants, allografts, and autologous tissues are used to repair cartilage deformities. Autologous cartilage grafts are still viewed as the gold standard among these options. However, donor site morbidity and the resorption of autologous sources are serious problems in today's reconstructive and aesthetic surgery field. The large number of conditions requiring cartilage grafts and the high rate of resorption in cartilage grafts cause undesirable results in the late period after graft placement.

The usage of cartilage grafts is increasing in popularity due to their frequent application in traumatic injuries, tumor resections, congenital anomaly repair, and aesthetic surgery. The donor areas for cartilage grafting are limited. Diffusion provides nutrition to cartilage, unlike the skin and bone tissue. This avascularity reduces the viability of cartilage grafts. Reductions in size, changes in shape, and resorption of cartilage grafts in the late post-graft period are among the main problems [2].

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The ears and nose, which constitute important aesthetic units in the face, are mostly composed of cartilage tissue. The insufficient intrinsic regeneration capacity of the cartilage tissue makes its replacement especially important. In modern medicine, cartilage reconstruction is performed with alloplastic material, allogeneic cartilage grafts, and autogenous grafts. The use of allogeneic cartilage grafts is limited due to the risk of infection and a high degree of resorption [3]. It has also been reported that synthetic alloplastic materials pose a high risk of infection [4]. Today, autologous cartilage grafts are seen as the best reconstruction option. However, donor-site morbidity limits the use of autologous grafts.

With traditional plastic surgery methods, functional disorders and deficiencies of organs are corrected using the principle of "repair like with like." This classical approach causes donor-site morbidity problems. However, regenerative medicine applications using mesenchymal stem cells (MSCs) can prevent donor-site problems by enabling cells, tissues and organs to regain their structure and functions [5].

Several growth factors affect cartilage repair and the differentiation of MSCs into chondrocytes. *In vitro* studies examining growth factors have shown that transforming growth factor-β and fibroblast growth factor-2 induce cell proliferation [6] and promote chondrogenic differentiation of MSCs [7]. In addition, studies have shown that adipose-derived (AD) stem cells promoted successful cartilage production *in vivo* [8].

Recent studies have shown that MSCs exert their protective effects mainly through the factors they secrete [9]. Thus, the therapeutic benefits of MSCs are based on the release of biologically active factors. Therefore, a new therapeutic approach related to the application of MSCs has been introduced. This method includes the use of medium containing biologically active factors and extracellular vesicles, generally called MSC-conditioned medium (MSC-CM) [10]. Proteomic analyses with MSC-CM have identified more than 100 proteins (including cytokines, chemokines, and growth factors) in CM that exert anti-inflammatory, anti-apoptotic, anti-fibrotic, and regenerative effects [11]. MSC-CM has been applied in different disease models, and the results showed that its functions are similar to those of MSCs, including neuroprotection, immunosuppression, tissue repair, and anti-inflammatory effects [12].

Although AD-MSCs are widely used in plastic, reconstructive, and aesthetic surgery, the use of AD-MSC-CM is generally prominent in wound healing and hair regeneration studies [13]. Very little research has investigated their use for cartilage tissue healing and regeneration. However, it is thought that growth factors and exosomes in the CM trigger chondrocytes in the medium, increase cartilage production, and increase cartilage graft viability [14].

In this study, we aimed to investigate the effect of xenogeneic AD-MSC and CM injections on cartilage graft viability in a rabbit model. The effects of xenogeneic AD-MSC and CM injections on the healing of cartilage grafts were demonstrated using histological and immunohistochemical methods in a comparative manner.

METHODS

Animals and experimental groups

The study was carried out by Kobay DHL I.C. (Ankara, Turkey), and animal experiments were carried out with the approval of the ethics committee (protocol number: 523). In total, 21 New Zealand male white rabbits weighing between 2,500 and 3,000 g were used. Ad libitum nutrition was provided with pellet feed and water. All procedures were performed under general anesthesia induced by intraperitoneal injection using ketamine hydrochloride (30 mg/kg; Ketalar, Eczacibasi) and xylazine hydrochloride (5 mg/kg; Rompun, Bayer).

The animals were randomly divided into three groups, with seven rabbits in each group. The procedures were performed in both (right and left) ears of the animals. In group 1 (control group; $n=7$), only cartilage grafting was performed on both ears. In group 2 (the MSC group; $n=7$), a single dose of 2×10^6 MSCs in 0.5 mL of medium was injected subcutaneously into both ears in four quadrants, in line with the cartilage graft, immediately after the grafting procedure. In group 3 (the CM group; $n=7$), a single dose of 0.5 mL of cell-free CM was injected subcutaneously into both ears in four regions, in line with the cartilage graft, immediately after the grafting procedure.

Preparation of AD-MSCs and CMs for injection processes

Canine-derived AD-MSCs (VetStem, D009/40) were purchased from Tekkgen Health Services Company. The cells were characterized using the flow cytometric method by the manufacturer (Supplementary Tables 1, 2). For the culture of canine-derived AD-MSCs, low-glucose Dulbecco serum supplemented with 15% fetal bovine serum (F7524; Sigma Aldrich), 2% L-glutamine (G6392; Sigma Aldrich) and 1% antibiotic-antimycotic solution (A5955; Sigma Aldrich) modified Eagle's medium (L0060; Biowest) medium was used. Frozen cell vials were heated at 37 °C for 1 to 2 minutes. Liquid suspensions of the cells were then transferred to a sterile tube and brought to a final volume of 5 mL with the appropriate medium. Next, the cell suspensions were centrifuged at 1,500 rpm for 5 minutes, and the supernatant was removed. The cell pellet was suspended in 1 mL of medium before being transferred to a T25 flask containing 4 mL of medium. The cells in the flask were placed in a 37 °C incubator under an atmosphere of 5% CO_2 for culturing. After cells reaching 80% confluence were removed by trypsinization, they were counted with a Scepter 3.0 Handheld Automated Cell Counter (Merck, PHCC340KIT), followed by passage to 6-well cell culture plates at 10^6 cells/well.

To obtain MSC-CM, AD-MSCs were cultured until they reached 70% confluence. The medium was replaced with medium containing less FBS (7%, 5%, 2%, 0%) every 2 to 3 days. In this way, the cells

sequentially adapted to the serum-free environment, preventing the development of oxidative stress-related changes, toxins, and unwanted proteins [15]. Just before injections into the third group, the medium from the cells was collected and filtered using 0.22-µm filters. Insulin syringes containing 0.5 mL of CM were prepared. Simultaneously, cells in the wells were removed by trypsinization for injections into group 2. After counting, insulin injectors containing 2×10^6 MSC in 0.5 mL of serum-free medium were prepared.

Creation of a cartilage graft model and injections

Both ears were shaved. The rabbits were placed in the supine position, and both ears were approached posteriorly. Antisepsis was achieved with povidone-iodine (Batticon, Adeka). After the skin incision with a no. 15 scalpel, the skin flap was elevated so that it would not come into contact with the cartilage graft incision line (Fig. 1A and B). The cartilage boundaries were determined with a 10-mm-diameter punch (Fig. 1C). A 10×10 mm cartilage graft was obtained using a fine-tipped elevator (Fig. 1D and F). The anterior perichondrium was preserved (Fig. 1E). The graft was placed back into the donor area (Fig. 1G) [16].

The cartilage graft was fixed with two round 5.0 propylene sutures (Dogsan), and the skin was closed and dressed in such a way that the skin was permanently sealed (Fig. 1H and I). No procedure was applied to group 1, which was the control group, after suturing. In the other groups, after suturing, MSC was injected into four quadrants in line with the cartilage graft in group 2, while CM was injected into the corresponding regions in group 3 (Fig. 1J). The animals were followed for 120 days. On the 120th day, the ears of the rabbits were shaved under general anesthesia. After providing antisepsis with povidone-iodine (Batticon, Adeka), cartilage grafts were accessed by entering through the old incision borders (Fig. 2A). The cartilage was removed with 5 mm of intact cartilage around the grafts (Fig. 2B). The animals were then sacrificed by ether inhalation. All samples were placed in 10% neutral buffered formalin (HT501320; Sigma Aldrich) solution for fixation to be used in histopathological examinations.

Histological examinations

Tissues fixed in 10% neutral buffered formalin were dehydrated by

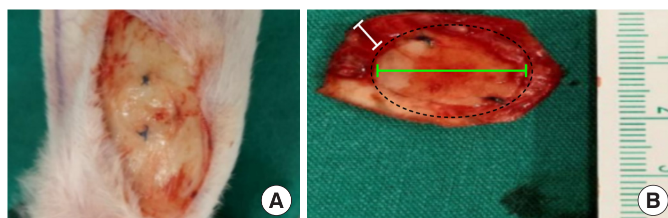

Fig. 2. Exploration of cartilage grafts by entering through the old incision (A). The area bounded by the green line is the graft tissue incision margin: the area bounded by the white line is the margin of the ear cartilage incision, dashed black lines denote the integration of the graft with the surrounding cartilage (B).

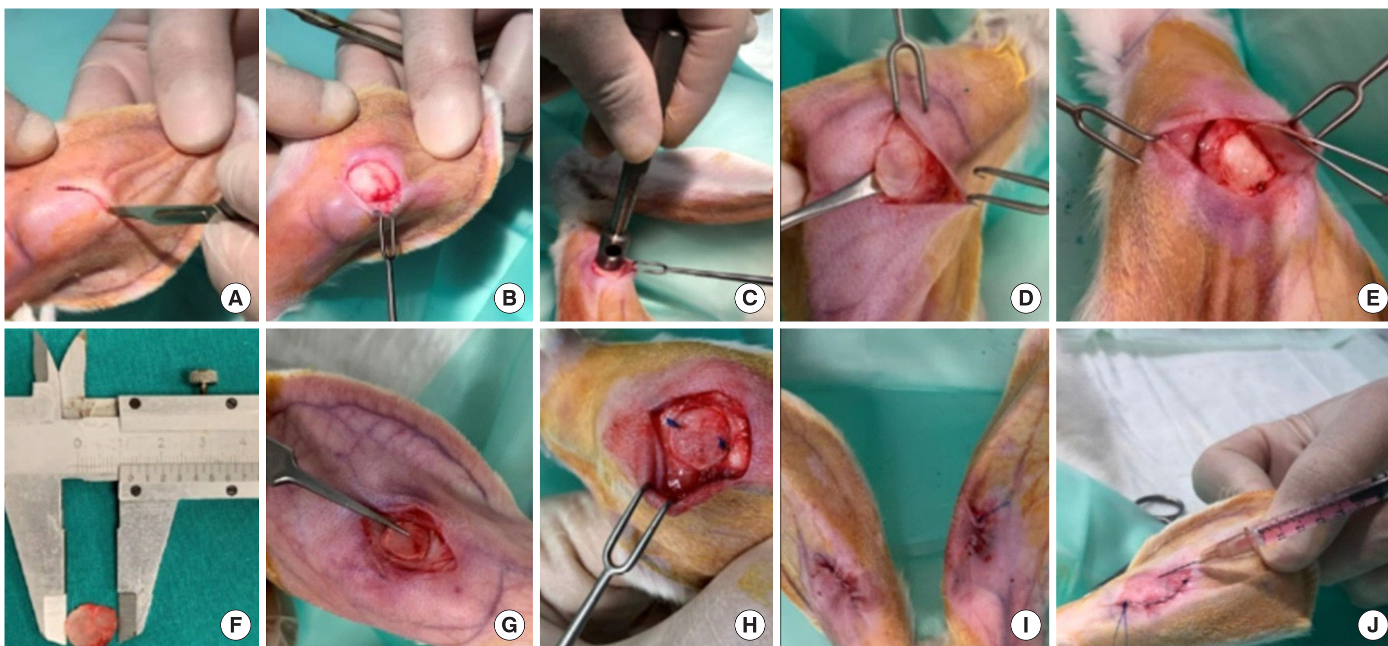

Fig. 1. Surgical stages of cartilage graft model creation. Elevation of the skin flap after the skin incision so that it does not come into contact with the cartilage graft incision line (A, B). Determination of cartilage boundaries with a 10 mm diameter punch (C), 10×10 mm cartilage graft removal using a fine-tipped elevator (D, F), demonstration of the anterior perichondrium (E). Placing the graft back into the donor area (G), fixation of the cartilage graft and permanent closure of the skin (H, I), conditioned medium and mesenchymal stem cell injections (J).

passing through a series of ascending grade ethanol (70%, 90%, 96%, 100%) (100986; Merck). Tissues were then cleared in xylene (108298; Sigma Aldrich) for 1 hour, kept overnight in liquid paraffin (107164; Sigma Aldrich) in an oven at 56 °C, and embedded in paraffin blocks. Next, 4-µm sections were taken from the tissues using a microtome. Hematoxylin-eosin (H&E), Masson trichrome, and type II collagen immunohistochemical stains were applied to sections taken from each tissue.

H&E staining

Sections were deparaffinized by xylene and rehydrated through a descending alcohol series (100%, 96%, 90%, 70%). Sections were kept in hematoxylin (ab220365; Abcam) for 15 minutes to stain the nuclei. Then, sections stained with eosin (ab246823; Abcam) for 1 minute were washed with distilled water and passed through a series of ascending ethanol concentrations (70%, 90%, 96%, 100%). After the sections were embedded in xylene and kept for 10 minutes, they were covered with mounting medium (05-BMHM100; Bio Optica) using a cover glass.

Immunohistochemical staining

For immunohistochemical analysis, transverse sections were incubated with a type II collagen rabbit polyclonal antibody (28459-1-AP; Proteintech) at 4 °C overnight. Then, after washing three times with phosphate-buffered saline (PBS), biotinylated secondary antibody solution (TL-125-HL; Thermo Fisher Scientific) was applied for 10 minutes. Streptavidin peroxidase solution (TL-125-HL; Thermo Fisher Scientific) was applied to the sections, which were washed again in PBS for 10 minutes. Next, AEC chromogen solution (TA-125HA; Thermo Fisher Scientific) was applied for 10 minutes. After washing the sections with distilled water, they were kept in hematoxylin for 2 minutes and counterstained. The sections were covered with mounting medium using a cover glass.

Table 1. Evaluation criteria for the ear cartilage incision margin

Score	Assessment criteria
0	There is no proliferation of chondrocytes.
1	There are very few proliferating chondrocytes.
2	There are few proliferating chondrocytes.
3	There is marked chondrocyte proliferation.
4	There is a considerably increased, abundant chondrocyte proliferation.

Statistical analysis

The statistical significance of the differences was evaluated using GraphPad Prism version 6.0 (GraphPad LLC, San Diego, CA) using the two-tailed Student t-test. A P-value less than 0.05 was considered statistically significant. The error bars in all graphs are standard deviations.

RESULTS

H&E staining

All specimens stained with H&E were evaluated in terms of chondrocyte proliferation at the incision border of the graft tissue, chondrocyte proliferation at the incision border of the ear cartilage, and the surrounding cartilage integration of the graft. The scoring criteria of Serel et al. [16] were modified and used to evaluate the specimens. Chondrocyte proliferation at the incision border of the graft tissue and chondrocyte proliferation at the incision border of the ear cartilage were evaluated according to the criteria in Table 1, and the graft's surrounding cartilage integration was assessed using the criteria in Table 2.

Chondrocyte proliferation at the graft tissue incision border, chondrocyte proliferation at the auricular cartilage incision border, and the integration of the graft cartilage tissue with the surrounding cartilage tissue increased in the CM group (group 3) compared to the control group (group 1) (Fig. 3).

The statistical analysis showed that chondrocyte proliferation at the graft tissue incision margin was significantly greater in both the CM group (group 3) and in the MSC group (group 2) than in the control group (group 1) ($P < 0.0001$ and $P < 0.01$, respectively). Furthermore, significantly greater chondrocyte proliferation at the graft tissue incision margin was found in the CM group (group 3) than in the MSC group (group 2) ($P < 0.01$) (Fig. 4A).

Chondrocyte proliferation at the margin of the ear cartilage incision was likewise significantly greater in both the CM group (group 3) and the MSC group (group 2) than in the control group (group 1) ($P < 0.0001$ and $P < 0.05$, respectively), and the CM group (group 3) showed significantly greater chondrocyte proliferation at the

Table 2. Evaluation criteria for the integration of the graft with the surrounding cartilage

Score	Assessment criteria
0	There is no integration. It is filled with connective tissue between the ear cartilage and the graft.
1	The space between the ear cartilage and the graft is partially filled with fibroblasts.
2	The area between the ear cartilage and the graft is completely filled with fibroblasts.
3	The area between the ear cartilage and the graft is filled with chondroblasts.
4	Full integration available. The area between the ear cartilage and the graft is completely filled with chondrocytes.

margin of the ear cartilage incision than the MSC group (group 2) (P < 0.01) (Fig. 4B).

The integration of the graft with the surrounding cartilage was significantly greater in both the CM group (group 3) and the MSC group (group 2) than in the control group (group 1) (P < 0.0001 and P < 0.001, respectively) (Fig. 4C).

Immunohistochemical staining

All samples subjected to immunohistochemical staining were visualized with a light microscope, and density analysis was performed with the ImageJ program (National Institutes of Health) in order to semi-quantitatively measure the expression levels of type II collagen (Fig. 5). When type II collagen expression levels were compared in the control (group 1), MSC (group 2), and CM (group 3) groups,

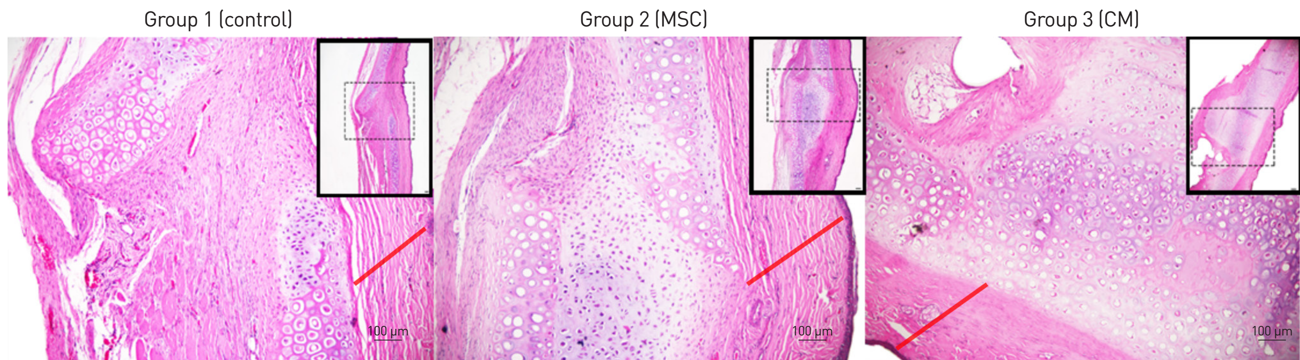

Fig. 3. Images of hematoxylin and eosin staining in different groups (× 10, insert: × 4). The areas bounded by the red line represent the perichondrium region. MSC, mesenchymal stem cell; CM, conditioned medium.

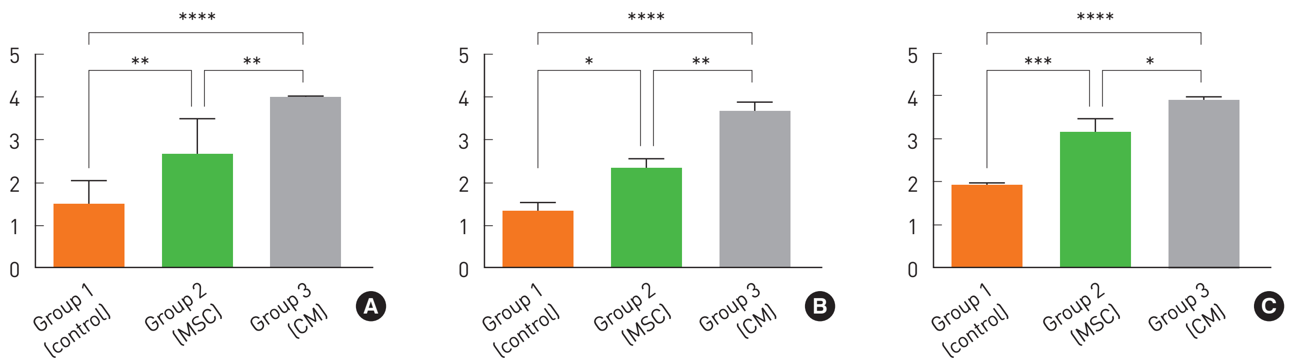

Fig. 4. Statistical comparison of chondrocyte proliferation at the border of the graft tissue incision (A), chondrocyte proliferation at the border of the auricular cartilage incision (B), and the integration of the graft with the surrounding cartilage (C) between the groups. MSC, mesenchymal stem cell; CM, conditioned medium. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

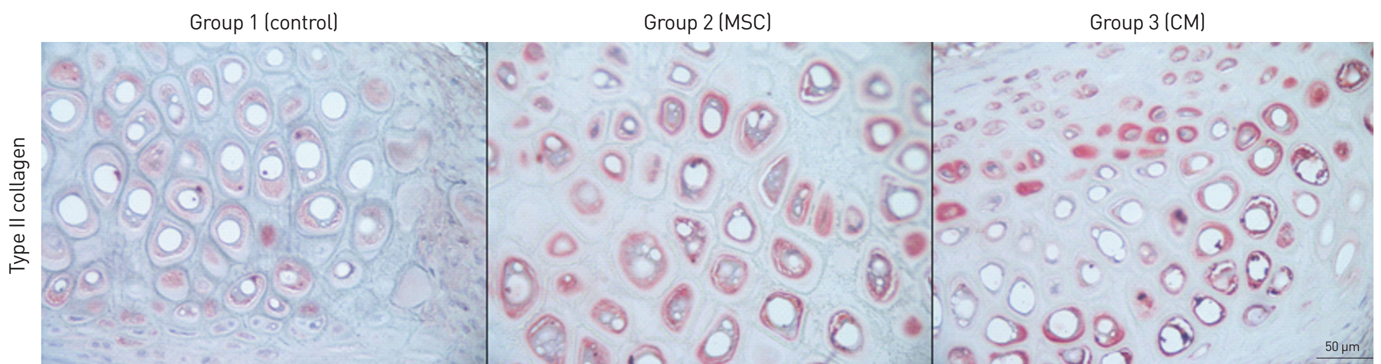

Fig. 5. Images of type II collagen expression, shown by immunohistochemical staining, in different groups (× 20). MSC, mesenchymal stem cell; CM, conditioned medium.

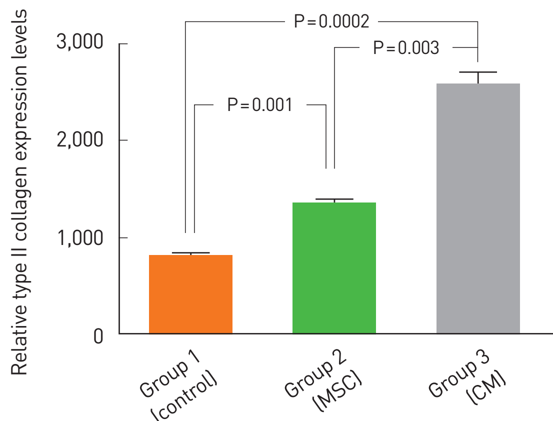

Fig. 6. Statistical comparison of type II collagen expression levels between groups.

the expression of type II collagen was found to be significantly greater in the CM group ($P=0.0002$) and the MSC group ($P=0.001$) than in the control group. The expression of type II collagen was also significantly greater in the CM group (group 3) than in the MSC group (group 2) ($P=0.003$) (Fig. 6).

DISCUSSION

MSCs are multipotent stem cells that can be isolated from a variety of sources. AD-MSCs are preferred over MSCs obtained from other regions because they are available from more sources, are easier to isolate, have high proliferation and low immunogenicity, and are able to produce high levels of trophic factors, including neuroprotective, angiogenic, antioxidant, and anti-inflammatory factors [17]. Studies investigating their regeneration potential have also shown that MSCs can differentiate into chondrocytes under the right conditions [18].

In a previous study, MSCs obtained from the ear, MSCs derived from bone marrow, and MSCs derived from adipose tissue were compared regarding the production of new cartilage, and it was shown that cartilage production increased with all types of MSCs [19]. In our *in vivo* study, in accordance with the literature, the stem cell injection group showed more favorable results in terms of cartilage viability and new cartilage formation than the control group.

MSC-CM has the potential to be used as a biological drug to replace live cell application. In addition, MSC-CM treatment is reproducible over time because it does not cause immunological reactions [20].

Oh et al. [21] compared AD stem cells and their nutrient secretomes in rabbit ear cartilage defects. They showed that the stem cells caused a regenerative increase in ear cartilage and significantly increased insulin-like growth factor-1, transforming growth factor-β1, and type II collagen levels compared to the medium secretome group. Although the expression of type II collagen was significantly higher in the MSC group in our study than in the control group, the CM group also showed significantly better results compared to the stem cell group. An explanation for the difference between these study results may be that we performed an extra cartilage graft in addition to the CM injection to the defect area.

Allogeneic cartilage grafts pose problems due to their high resorption rate (>70%) and risk of disease transmission [22]. For this reason, an autologous cartilage graft was used in our study, but xenogeneic MSC and CM injections were performed. Although allogeneic MSC injection is seen as a more suitable alternative, studies have shown that there is no significant difference between xenogeneic implantation results. For example, a previous study found that both allogeneic and xenogeneic MSCs repaired cisplatin-induced renal dysfunction, ameliorated tissue damage, and improved regenerative scores [23]. No significant immune reaction or deterioration of vital signs was reported in the xenogeneic MSC-injected groups. Similarly, no immune reaction was observed in our study.

It is thought that the growth factors in CM trigger chondrocytes and increase cartilage production by activating the cells in the environment. In the production of cartilage, there is a high amount of type II collagen in the environment. Since it is not known exactly in which direction MSCs will differentiate, activating the chondrogenic pathway in the medium is seen as a major advantage of CM compared to stem cells. We observed that the chondrogenic pathway was activated, new chondrocytes were formed, and an extracellular matrix rich in type II collagen was generated in group 3, in which only CM was given without stem cells. We suggest that xenogeneic CM injections can be used safely in clinical practice, since it prevents cartilage graft resorption and contributes to new cartilage production without any tumoral effect or immune reaction.

NOTES

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

The study was carried out by Kobay DHL I.C. (Ankara, Turkey), and animal experiments were carried out with the approval of the ethics committee (protocol number: 523).

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Supplemental material

Supplementary materials can be found via https://doi.org/10.14730/aaps.2022.00773

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