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Synovium-Derived Mesenchymal Stem Cells in Combination with Low Molecular Weight Hyaluronic Acid for Cartilage Repair

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Abstract

Regeneration of damaged articular cartilage remains one of the most complex and unresolved problems in traumatology and orthopedics. In this study, we investigated whether intra-articular injection of synovium-derived mesenchymal stem cells (SD-MSCs) with low molecular weight hyaluronic acid (LMWHA) could promote the regeneration of damaged cartilage in rabbits. To answer this question, rabbits' SD-MSCs were harvested, expanded in culture, and characterized by CFU assay and a multilineage differentiation test. For in vivo study, we created a defect within the cartilage layer without destroying subchondral bone. Two weeks after the cartilage defect, SD-MSCs (2×10^6 cells) were suspended in 0.5% LMWHA and injected into the left knee, and hyaluronic acid (HA) solution alone was placed into the right knee. Cartilage regeneration in experimental and control groups was evaluated macroscopically and histologically at Days 30, 60, and 90. The results of the study showed an early process of cartilage regeneration in the defect area on Day 30 after intra-articular MSCs-HA injection. Histological studies revealed that cartilage defect was covered by a thin layer of spindle-shaped undifferentiated cells and proliferated chondroblasts, in contrast to a single HA injection, which did not induce cartilage regeneration. On Day 60, we observed that the size of the cartilage defect after MSCs-HA injection significantly decreased, compared to one after HA injection. On Day 90, the cartilage defect in a knee treated with MSCs-HA was fully regenerated and was similar to intact cartilage. Thus, the combined application of the MSCs, HA, and chondroinductive proteins have a high therapeutic effect on cartilage defect regeneration in rabbits.(**International Journal of Biomedicine. 2022;12(4):548-553.**).

Keywords: mesenchymal stem cells • hyaluronic acid • growth factors • cartilage defect • regeneration • cell therapy

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Abbreviations

BMP, bone morphogenic protein; **CFU**, colony-forming unit; **CCM**, complete culture medium; **CT**, cartilage tissue; **GFs**, growth factors; **HA**, hyaluronic acid; **LMWHA**, low molecular weight HA; **PBS**, phosphate-buffered saline; **SM**, synovial membrane; **SD-MSCs**, synovium-derived mesenchymal stem cells; **TGF-β**, transforming growth factor-beta.

Introduction

Regeneration of damaged articular cartilage remains one of the most complex and unresolved problems in traumatology and orthopedics.(1) The absence of its own perichondrium results in poor cellular regeneration. Only in peripheral injuries with the areas adjacent to the synovial membrane is the process of histotypical restoration of hyaline cartilage tissue (CT) observed. In deep injuries communicating with the bone marrow canal, migration of mesenchymal stem cells (MSCs) from the bone marrow to the defect area is ensured. This occurrence can serve as a

cellular source for regeneration.[2] However, even if damaged hyaline cartilage is restored in this manner, the cartilage is formed with fibrous tissue, which differs significantly in architectonics, the biochemical composition of the matrix, and mechanical properties.

The current non-surgical methods, including physiotherapy and intra-articular injections, as well as surgical procedures, such as multiple microperforations of the articular surface, abrasion, and microfractures, are aimed at stimulating the CT regeneration and are not able to provide a complete and sustainable cure without complication.[3]

Advanced options for cellular arthroplasty involve a combination of cellular technology with a complex surgical technique, among these methods - transplantation of autologous chondrocyte under a periosteal patch or a resorbable collagen membrane.[4,5] This technology is called autologous chondrocyte implantation. Even though this method is able to improve cartilage defect regeneration to some extent, it has certain limitations. The major of these are trauma, in cases when a transplant is taken from an adjacent healthy area of cartilage, difficulties in obtaining a sufficient number of chondrocytes, and the expansion of chondrocytes in culture. Additionally, another disadvantage is incomplete recovery, which is explained by the formation of fibrous cartilage but not functional hyaline tissue.[6,7] Moreover, there is a question of the optimal source of cells, their acceptable carrier to the damaged area, and immobilization for the complete and efficient recovery of damaged cartilage.

In order to develop an effective cell preparation, it was necessary to choose the optimal source of MSCs. An effective solution might be the use of synovial MSCs obtained from the same individual.[8-11] SD-MSCs are more effectively involved in the activation of chondrogenesis and have a higher proliferative and chondrogenic potential than MSCs derived from bone marrow or adipose tissue.[12-14] Other advantages of using SD-MSCs in cellular therapy for cartilage defects are the ease of isolation, less traumatic nature of material sampling using arthroscopy, and obtaining a sufficient amount of MSCs from a small fragment of synovial tissue, which can completely self-repair in a short period of time.[15]

As a biocompatible and biodegradable agent, we chose low molecular weight hyaluronic acid (LMWHA). HA plays the role of a lubricant and shock absorber, an energy-accumulating agent between opposite cartilage, and a semi-permeable barrier that regulates metabolic processes between cartilage and synovial fluid.[16] Thus, the use of this biopolymer will not only reduce pain in the joint, but also increase the therapeutic efficacy of restoring damaged CT.

Additionally, in the development of the drug, we propose the addition of chondroinductive growth factors such as TGF-β1 and BMP-4 in a certain combination and optimal concentration, which will increase the regeneration of cartilage defects and other damage to the knee joint.[17]

In this study, we obtained and characterized primary cultures of SD-MSCs from the knee joints of experimental rabbits. In addition, we studied the effect of MSCs and HA on cartilage defect regeneration in rabbits. In vivo data showed that SD-MSCs after intra-articular injection were distributed mainly in the area of the defect, suggesting that the cells have a tropism for damaged areas of CT. It was also revealed that the intra-articular injection of MSCs with HA leads to a complete repair of the cartilage defect within 90 days, compared with the individual use of HA, which did not have an effect on cartilage regeneration. In contrast, the combined administration of SD-MSCs with HA and growth factors resulted in a significant acceleration of the regeneration process in cartilage defects with a complete restoration of hyaline-like cartilage within 30 days.

Thus, the results of this study demonstrate that SD-MSCs and growth factors (TGF-β1 and BMP-4) play a crucial role in CT repair. The combined application of the MSCs, HA, and chondroinductive proteins have a high therapeutic effect on cartilage defect regeneration in rabbits. It is assumed that the results of this work might serve as a basis for the application in orthopedics, namely in cell-based therapy for cartilage defects.

Materials and Methods

Animals

Skeletally mature male grey Giant rabbits were purchased from the "KletkaMaster" company (Saint Petersburg, Russian Federation). Rabbits were held in large cages at a temperature of 23°C and relative humidity of 60%. The access to food and water for all experimental animals was ad libitum. All procedures from this study were approved by the local Ethical Committee and the Institutional Review Board of the National Center for Biotechnology (IRB 00013497). All experimental procedures were performed following the guidelines for the care and use of laboratory animals.

Isolation and cultivation of rabbit SD-MSCs

The isolation of the synovial membrane from the knee joints of rabbits was performed under general calypsol anesthesia (5 mg/kg intramuscularly). The synovium was rinsed with a mixture of antimycotic-antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B) in phosphate-buffered saline, minced into 1-2mm^3 pieces, and processed with 0.3% collagenase type II solution for 16 hours at 37°C. The resulting cell suspension was filtered through a 70-μm cell strainer (BD Biosciences, USA) to remove the remaining tissue fragments. Following that, the cells were resuspended in α-MEM complete culture medium, counted in a hemocytometer and cultured in a T75 cell culture flask (Corning, USA) at 37°C and 5% CO_2. After 2 days, the cells unattached to the plastic were removed, and the fraction of adherent cells was cultivated until cells reached 80-90% confluence. Passaging of the MSCs was performed with TrypLE™ Express (Thermo Fisher Scientific, USA) with an interval of 5-7 days. The medium in the cell culture was changed every 2 days.

Fibroblastic colony forming unit test

Cells isolated from the rabbit synovial tissue were seeded into Petri dishes at a rate of 1cell/cm^2 and cultured in complete culture medium for 14 days at 37°C and 5% CO_2. At the end of the cultivation period, the cells were washed with phosphate-buffered saline and stained with 0.5% crystal violet solution for 5 min at room temperature. After washing twice with phosphate-buffered saline, the formed colonies were dried and counted using an SZ61 stereomicroscope (Olympus, Germany).

Multilineage differentiation test

For differentiation into chondrocytes, at passage 4 MSCs were resuspended in a differentiation medium consisting of high glucose DMEM medium, 1% ITS+Premix (BD Biosciences, USA), 100μM ascorbate-2-phosphate (Sigma, USA), 10^{-7}M dexamethasone (Sigma, USA), and 10ng/mL TGFβ1 (Sigma, USA) at a concentration of 1.25×10^6 cells/mL. To create chondrogenic cell pellets, each well of a 96-well polypropylene plate was loaded with 2.5×10^5 cells, centrifuged at 400g, and transferred to a CO_2 incubator at 37°C, and 5% CO_2. The medium was changed 3 times a week. On day 21 of differentiation, cell pellets were collected and fixed in 10% neutral buffered formalin. Samples were placed into paraffin, cut on a microtome, and processed for staining with hematoxylin-eosin (H&E).

For osteogenic differentiation of MSCs, at passage 4 we used an induction medium containing 10^{-7}M dexamethasone, 10mM β-glycerol-phosphate, and 50 μM ascorbate-2-phosphate. After 3 weeks of cultivation, the cells were stained with Alizarin Red.

MSCs were differentiated into adipocytes by culturing them in an induction medium containing 10^{-6}M dexamethasone, 0.5μM 3-isobutyl-1-methylxanthine, and 10ng/mL insulin for 3 weeks at the same passage as chondrocytes and osteoblasts and stained with Oil Red O.

Cartilage defect model and intra-articular injection of SD-MSCs

Surgical intervention was performed under ketamine anesthesia at the concentration of 5mg/kg of the rabbit body weight. After anesthesia was achieved, the experimental animals were fixed on the operating table. A 4 mm diameter cartilage defect was formed in the intercondylar area of the thigh, in the area of the femoral-patellar joint. To unify the modeled defect, we used the COR kit for mosaic chondroplasty of the femoral condyles (Johnson&Johnson, USA). To exclude the reparative function of the bone marrow, the defect was performed within the cartilage without destroying the subchondral bone. The joint cavity was washed with sterile phosphate-buffered saline, the wound was sutured in layers. Three days post-operation gentamicin was used to prevent purulent complications.

Two weeks after the cartilage defect, to evaluate the effect of HA in combination with SD-MSCs, the cells at the concentration of 2×10^6/100μL of DMEM media were suspended in 0.5% of LMWHA (*OSTENIL®*, TRB CHEMEDICA AG, Haar/Munich, Germany) and injected into the left knee. HA solution alone in the same concentration was used as a control, which was administered into the right joint of the same rabbits. The procedure was performed 3 times at an interval of 7 days. The determination of cartilage defect regeneration in the intermuscular region of the knee joint was evaluated by macroscopic and histological analyses at different time points on Days 30, 60, and 90.

To study the possibility of enhancing the regeneration of a cartilage defect, the following experimental groups were used: Group 1: 0.5% HA, TGF-β1 (100 ng/mL) and BMP-4 (500 ng/mL); Group 2: 0.5% HA, 5×10^6 of MSCs, and TGF-β1 (100 ng/mL) and BMP-4 (500 ng/mL). A 0.5% HA was used as a control.

Histological Analysis

Joints with cartilage defects were fixed in 4% paraformaldehyde solution (pH=7.2). After washing in phosphate-buffered saline, the samples were decalcified, then dehydrated consistently in 70%, 95%, 95%, 100%, and 100% ethanol and immersed in xylene. Then, the samples were infiltrated with paraffin, embedded into paraffin blocks, and cut into 5μm sections. Before staining, sections were treated with xylene and sequentially rehydrated in 100%, 100%, 95%, 95%, and 70% ethyl alcohol and distilled water in order to remove paraffin. The sections from each defect were stained with modified Mayer's H&E, sequentially dehydrated and cleared with ethyl alcohol and xylene, and mounted in histological medium Bio Mount HM (Bio-Optica, Italy). The stained samples were analyzed using a light microscope (Carl Zeiss, Germany). The cartilage defect regeneration area was measured using the AnalySIS® program (Olympus, Germany).

Statistical analysis was performed using The GraphPad Prism 8 software. Baseline characteristics were summarized as frequencies and percentages for categorical variables and as mean±SD for continuous variables. Inter-group comparisons were performed using Student's t-test. A probability value of $P<0.05$ was considered statistically significant.

Results

At the first stage of this study, MSCs were isolated from the synovium of the knee joints of 12 mature rabbits and further characterized. The isolated cells had a fibroblast-like morphology (Figure 1A), the capacity to adhere to culture plastic, and a high ability to proliferate and form cell colonies (Figure 1B-C). Moreover, it has been found that the cells also were able to differentiate into adipocytes, osteoblasts, and chondrocytes when cultured in selective differentiation media (Figure 1D-F). MSCs differentiated into adipocyte-like cells and formed lipid vacuoles in the cytoplasm, which were stained by Oil Red O. Osteoblast-like cells accumulated calcium deposits by Alizarin Red staining. Moreover, chondrogenic differentiation resulted in the formation of chondrogenic pellets with the characteristic hyaline-like morphology shown by H&E staining.

At the same stage of the synovial membrane isolation, we created a massive cartilage defect 4 mm in diameter in the intermuscular region of the knee joint. In order to avoid the release of bone marrow progenitor cells into the defect area, the defect was performed up to the border of the subchondral bone. After the synovial membrane isolation, MSCs were cultured for 14 days to produce an appropriate cell mass. The macroscopic and histological analyses after using the HA in combination with MSCs showed interesting results (Figure 2).

As shown in Figure 2G, 30 days after MSC administration, the area of cartilage defect was markedly reduced, compared to the control. On Day 60, the macroscopic analysis demonstrated that the cartilage defect was substantially repaired and looked almost like native articular cartilage (Figure 2H). On Day 90 after MSC transplantation, the area of the cartilage defect was completely recovered (Figure 2I). These results were confirmed by the histologic analysis. Histology showed that on Day 30, the formation of the fibrocartilaginous layer containing both undifferentiated cells and chondrocytes was observed (Figure 2J). On Day 60, histology showed that the formation of hyaline-like cartilage occurred. As can be seen in Figure 2K, the emerging

hyaline-like cartilage consists of three conditional layers: 1) the upper fibrous layer, consisting of undifferentiated cells surrounded by collagen fibers; 2) a layer containing chondroblasts, and 3) a layer of hyaline-like cartilage containing clusters of chondrocytes. On Day 90, the analysis showed the formation of hyaline cartilage with almost fully repaired cartilage (Figure 2L).

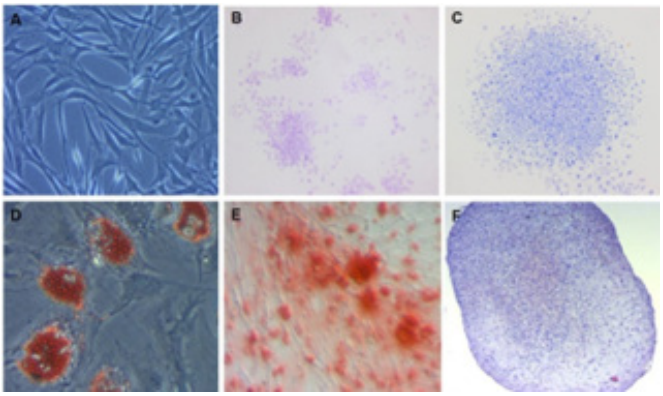

Fig. 1. *Rabbit SD-MSC characterization. A) Phase-contrast image of a live cell culture with fibroblast-like morphology. B) CFU assay. SD-MSCs are able to proliferate and form colonies rapidly. C) Enlarged image of CFU assay. D-F) Multilineage differentiation test. D) Differentiation of MSCs into adipocytes. Lipid vacuoles stained with Oil Red O are visible. E) Differentiation of MSCs into osteoblasts. Calcium deposits are visible in the cells stained with Alizarin Red. E) Differentiation of MSCs into chondrocytes. H&E staining.*

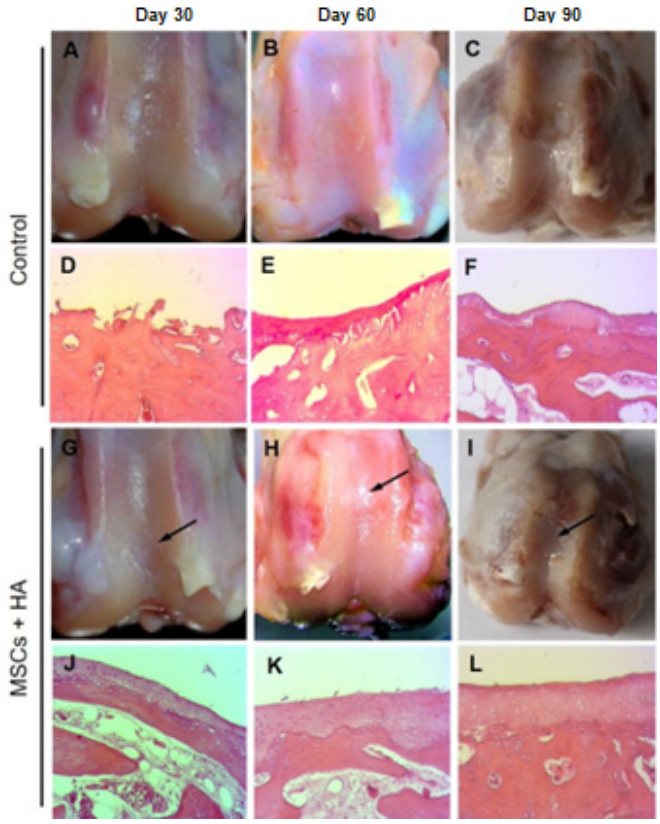

Fig. 2. *Macroscopic (A-C, G-I) and histological (D-F, J-L) analyses of cartilage defect regeneration after intra-articular injection of rabbit synovial MSCs at the indicated time points. The black arrow shows a regenerating cartilage defect. HA only was used as a control.*

Macroscopic and histological analyses of articular cartilage regeneration after the application of HA with growth factors (TGF-β1 and BMP-4) showed that on Day 30, there was a marginal repair of the defect by about 40%-50% (Figures 3B and 3E). Apparently, the addition of TGF-β1 and BMP-4 leads to the activation of the proliferation of endogenous chondroblasts, resulting in the regeneration of the cartilage defect.

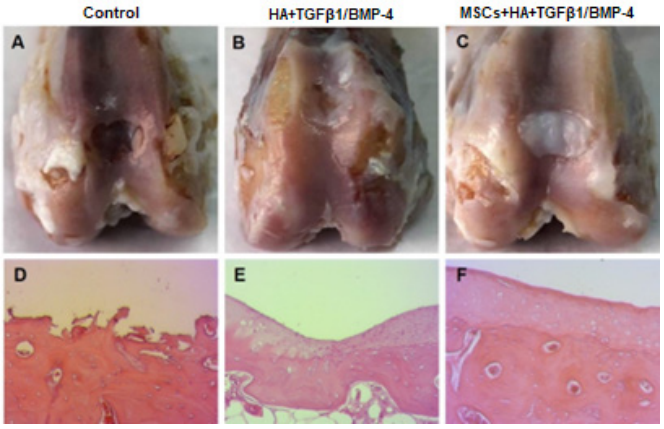

Fig. 3. *Macroscopic (A-C) and histological (D-F) analyses of cartilage defect regeneration after intra-articular injection of rabbit SD-MSCs on Day 0 and Day 30.*

The evaluation of the articular cartilage defect regeneration after the use of MSCs in combination with HA and growth factors demonstrated a significant acceleration of the regeneration in the defective area. As shown on macroscopic and histological images (Figures 3C and 3F), 30 days after the intra-articular injection, complete closure of the defect area with hyaline-like CT was observed. Spontaneous recovery was not observed in the control samples. Additionally, the macroscopic results were quantitatively evaluated. As shown in Figure 4, the regeneration area of the defect after HA in combination with growth factor administration was at the level of 2 mm and was significantly higher than the control (P<0.05). In contrast to the treatment with hyaluronic acid + growth factors, the combination of hyaluronic acid with growth factors and MSCs had a more pronounced effect with a regeneration area of 4 mm (P<0.05).

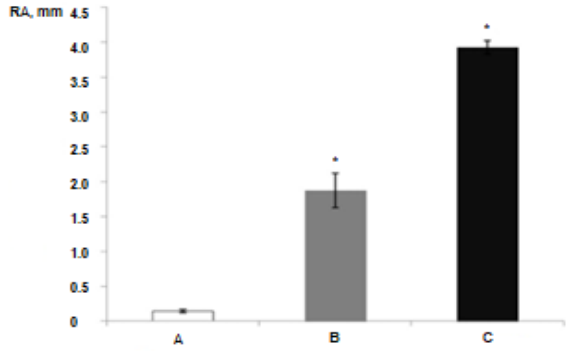

Fig. 4. *Quantitative measurements for the macroscopic analysis of cartilage defect regeneration after intra-articular administration of rabbit SD-MSCs and growth factors.* ***RA*** *- Regeneration area (mm).* ***A.*** *Control: 0.14±0.02 mm;* ***B.*** *HA+TGFβ1/BMP-4: 1.87±0.25 mm;* ***C.*** *MSCs+HA+TGFβ1/BMP-4: 3.9±0.09 mm. *- Significant difference from control, P<0.05.*

Discussion

The development and implementation of cell-based preparations for the treatment of degenerative diseases of joints remain one of the promising directions in cellular therapy and tissue engineering. In this regard, biotechnological methods for obtaining cellular preparations encounter the need to address several issues: 1) the optimal cell source; 2) a method for their delivery to the defect area; 3) an adequate biocompatible matrix.

In this study, as an optimal source of MSCs, we chose the synovial membrane, which can be easily isolated from a patient during arthroscopic procedures. It is justified by several studies, which showed that MSCs isolated from the synovial membrane have a higher proliferative potential and a higher potential than MSCs to differentiate into chondrocytes from bone marrow or adipose tissue.[15,18] At the same time, the functional activity of synovial MSCs remains at a high level, regardless of the person's age. The procedure for isolating MSCs from the synovial membrane of a human or animal is relatively simple to perform, which includes the following steps: 1) sampling of the synovial membrane from the knee joint, 2) washing in a sterile buffer with antibiotics, 3) mincing the tissue into small pieces, 4) processing with collagenase, 5) filtering through a special nylon filter, and 6) counting seeding the cells in a culture dish, followed by cultivation in a CO_2 incubator. Using this technique, we managed to obtain primary cultures of rabbit SD-MSCs. The resulting cultures of MSCs had high adhesiveness to the culture plastic and the ability to form fibroblastic CFU. Moreover, they differentiated into adipocytes, chondrocytes, and osteoblasts.

As previously described, HA is a natural biopolymer that provides the viscoelastic properties of the synovial fluid. In the synovial fluid, HA acts as a lubricant and shock absorber, an energy-accumulating agent between opposite cartilage, and regulates metabolic processes between cartilage and synovial fluid.[16,19] Moreover, HA has been shown to have an anti-inflammatory, anabolic, and analgesic effect when injected into an injured joint.[20] Thus, given its unique properties, we have chosen this biopolymer for use in the development of our cell preparation. In our study, we used a commercial Ostenil drug based on 1% LMWHA, which is commonly used for local therapy of osteoarthritis.

In addition, after observing the positive impact of HA in combination with SD-MSCs administration, we questioned how to enhance the therapeutic effect of the cellular preparation. According to the literature, there are several key GFs that increase MSC proliferation and their differentiation into chondrocytes, among which is TGF-β1, which plays a central role in chondrogenesis.[21] This factor stimulates the synthesizing activity of chondrocytes and acts against the catabolic activity of the inflammatory mediator, IL-1, and also increases the proliferation and chondrogenic differentiation of bone marrow MSCs. Moreover, the cultivation of MSCs with the addition of TGF-β1 led to the suppression of the expression of the collagen I gene, and at the same time, activated the expression of collagen II, which is synthesized during the formation of hyaline cartilage.[22]

Other important factors that participate in chondrogenesis and osteogenesis are BMPs, which are homodimeric molecules belonging to the TGF-β superfamily. There are 13 types of BMPs (from BMP-2 to BMP-14) that are involved in the regeneration of cartilage and bone tissue.[23] The most studied of them are BMP-2 and BMP-7, which are already used in clinical practice to repair nonunion fractures. In the previous study, it was found that TGF-β1 in combination with BMP-4, has a significant effect on both chondrogenic differentiation and the synthesis of extracellular matrix and glycosaminoglycans in chondrogenic micropellets.[17] Apparently, BMP-4 acts synergistically with TGF-β1, stimulating chondrogenesis in synovial MSCs and chondroprogenitor cells. Similar results were obtained by other researchers who showed that the combination of TGF-β3 and BMP-4 is necessary to stimulate chondrogenesis, while chondoprogenitor cells can differentiate into chondrocytes in the presence of BMP-4 alone.

At the next stage of the study, we evaluated the regenerative potential of SD-MSCs in rabbits with a massive defect in the cartilage of the knee joint. In order to exclude the reparative function of the bone marrow, the defect was performed within the cartilage, without destroying the subchondral bone. In our study, we used SD-MSCs in combination with HA, which served not only as a scaffold for cell delivery, but also as an anti-inflammatory and analgesic agent. The macroscopic and histological analyses for the evaluation of cartilage defect regeneration at different time points showed that intra-articular injection of synovial MSCs with HA significantly accelerates the process of regeneration of damaged CT in rabbits. In subsequent periods of observation, it was shown that a significant acceleration in the recovery of a cartilage defect led to the formation of hyaline-like cartilage on Day 90 after the introduction of MSCs with HA.

Animal studies showed that the combination of TGF-β1 and BMP-4 with SD-MSCs and HA significantly accelerated the process of cartilage defect repair in experimental rabbits. Already on Day 30 after the injection of HA and MSCs with GFs, we observed a complete closure of the defect by hyaline-like cartilage. In contrast, the intra-articular injection of HA with MSCs only resulted in the restoration of damaged CT on Day 90. Apparently, the addition of TGF-β and BMP-4 leads to stimulation of the proliferation and differentiation of MSCs and endogenous chondroblasts, resulting in the regeneration of the cartilage defect.

In conclusion, based on the obtained data, it can be said that the combined intra-articular application of SD-MSCs with HA and TGF-β+BMP-4 significantly accelerates the process of regeneration of damaged CT, compared to their separate use.

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effectiveness of diagnosis, treatment of injures, consequences of injures, diseases of the limbs, spine and pelvis."

Competing Interests

The authors declare that they have no competing interests.

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