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Original Article

Repair of articular cartilage defects with chondrocyte derived from allogenic bone marrow mesenchymal stem cells (MSCs) in rabbit

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Abstract: To compare the ability of the cartilage precursor cells derived from allogenic and autologous bone marrow mesenchymal stem cells (MSCs) respectively seeded onto "two-phase" allogeneic bone matrix gelatin (BMG) for constructing tissue engineered cartilage in the repair of articular cartilage defects and to observe the repairing efficacy of the two composites for providing the basis for the clinical application of tissue engineered cartilage. After models of articular cartilage defects were made, these rabbits were divided into three groups randomly including 10 in experimental group, 5 in positive control group and 5 in blank group. Rabbits in experimental group were implanted with allogeneic cell-carrier composites, positive control group was implanted with autologous cell-carrier composites and blank group was implanted nothing. Then these rabbits were sacrificed at the 8th and 12th week. Specimens were obtained and underwent general observation, HE staining, immunohistochemistry, and histological score. The defects in experimental group and positive control group were repaired with hyaline-like cartilage tissue while blank group was repaired with fibrous tissues. Histological scores showed that the results of experimental group and positive control group were better than those of blank group at the 8th and 12th week, and the difference was statistically significant ($P<0.05$); There was no significant difference between experimental group and positive control group ($P>0.05$). In conclusion, the composite of allogeneic MSCs-derived cartilage precursor cells and autologous BMG can be used as seed cell to construct tissue engineered cartilage in vivo for the repair of articular cartilage defects without immune rejection, and there is no significant difference in the repairing efficacy of allogenic and autologous MSCs-derived precursor cells. And the effect of the two tissue-engineered cartilage transplantation was better than that of implantation with nothing.

Keywords: Tissue engineering, mesenchymal stem cells (MSCs), bone matrix gelatin (BMG), articular cartilage defects

Introduction

Articular cartilage is a connective tissue with the component of a single cell, which is composed of mono-chondrocyte, fiber and matrix. It has main functions of connecting, supporting, dispersing stress and reducing oscillation. Articular cartilage damage and defects caused by trauma, inflammation, tumor and degeneration are very common in patients with secondary osteoarthritis, which seriously affects the functions of the joint. The regeneration capacity of articular cartilage is very limited, and the effect of the repair of articular cartilage defects is mainly determined by the treatment method. Therefore, the treatment of articular cartilage defects has been one of the key research directions of preclinical and clinical orthopedics [1]. Great progress has been made in the role of MSCs for the basic and applied research of cartilage tissue engineering. Many scholars have showed that MSCs can be used as seed cells to repair articular cartilage defects via conducting plenty of animal experiments [2-4], but the amplification and detection of autologous MSCs require some time, so its clinical application was affected to some extent. In this study, the ability of the cartilage precursor cells derived from autologous and allogenic mesenchymal stem cells (MSCs) seeded onto "two-phase" allogeneic bone matrix gelatin (BMG) for repairing articular cartilage defects were com-

Table 1. Wakitani histological scoring criteria

Score	Cell morphology	Degree of matrix staining	Surface evenness	Cartilage thickness	Binding of the surrounding tissue
0	Hyaline cartilage cells	Normal	Evenness	>2/3	Binding of both sides
1 point	Hyaline cartilage cells in the main	Mildly coloring weakened	Basic evenness	1/3-2/3	Binding of one side
2 points	Fibrocartilage cells in the main	Significantly coloring weakened	Unevenness	<1/3	Not binding
3 points	Non-cartilage cells in the main	Uncolored	Serious unevenness	-	-
4 points	Non-cartilage cells in whole	-	-	-	-

Note: Total score is 14 points; the score of normal articular cartilage is 0.

pared to investigate the feasibility and validity of the composite of allogenic chondrocyte precursor cells and autologous "two-phase" BMG for constructing tissue engineered cartilage.

Materials and methods

Animal grouping and BMG preparation

20 healthy adult Japanese rabbits, male or female, were purchased from the Experimental Animal Center of Kunming Medical College, Kunming, China. They were divided into three groups randomly including 10 in experimental group implanted with allograft cartilage precursor cell-carrier cell-carrier composites, 5 in positive control group implanted with autologous cartilage precursor cell-carrier cell-carrier composites and 5 in blank group implanted nothing as negative control.

Ilia of 15 rabbits in experimental group and positive control group were taken out. After degreased, demineralized and antigen-extracted, these iliac bones were trimmed to 3 mm in height and 3 mm in diameter cylindrical "two-phase structures" with cancellous bone on one side and cortical bone on the other side. Above samples were air-dried with sterilization in ethylene oxide, and stored in -20°C refrigerator for later use. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Affiliated Hospital of Kunming Medical University.

Construction of tissue engineered cell-carrier composites

The cartilage precursor cells, which were induced to express chondrocyte phenotype, were made to cell suspension with cell density of 5×10^6/ml for inoculation. After rewarming, "two-phase" BMG was immersed into above cell suspension. Due to its absorption, BMG absorbed precursor cells uniformly and sank to the bottom of the tube. After incubated for 6 h in incubator, cell-carrier composites were taken out, seeded into 24-well plates and cultured for 3 days. Next the growth of cartilage precursor cells on "two-phase" BMG was observed by scanning electron microscopy on the 3rd day.

Repair of rabbit articular cartilage defects with tissue engineered cartilage

Cell-carrier composites were taken out from the 24-well plate and placed into a centrifugal tube. After the right amount of medium was added, the tube was sealed for later implantation. One rabbit was anesthetized and fixed with supine position on the disinfected operating table in a ventilation environment. Both knee joints of the rabbit were depilated with local disinfection and draping. Medial longitudinal incision was performed on the both knees, and the subcutaneous tissue and fascia were separated; after incision of the joint capsule, the knee joint was exposed. Next the patella was put aside toward lateral flexion knee, thus exposing the femoral condyle. The femoral condyle surface was drilled into about 3 mm until the subchondral bone plate with a drill bit in 3 mm diameter, which was made to bilateral femoral condylar cartilage defect model. The remaining rabbits were made to cartilage defect models according to the method described above. Immediately, above prepared tissue engineered cell-carrier composites were implanted. A total of 20 joints in 10 rabbits were implanted with the composite of allogenic MSCs and autologous BMG in experimental group; a total 10 joints in 5 rabbits were implanted with the composite of autologous MSCs and autologous BMG in positive control

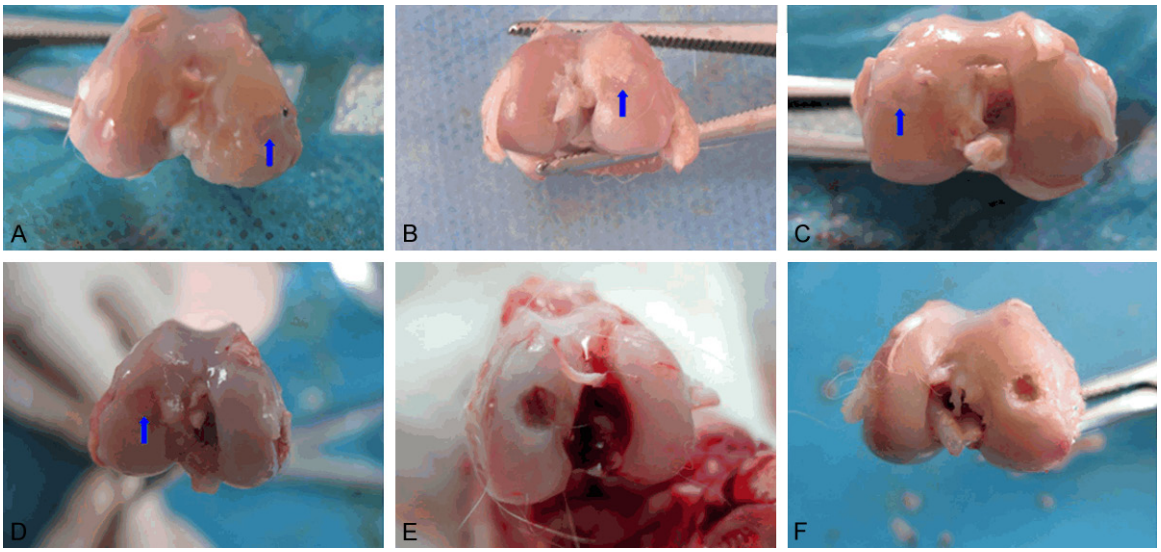

Figure 1. General observation of articular after transplantation in the three groups. A: Experimental group at the 8th week; B: Experimental group at the 12th week; C: Positive control group at the 8th week; D: Positive control group at the 12th week; E: Blank group at the 8th week; F: Blank group at the 12th week.

group; A total of 10 joints in 5 rabbits were implanted nothing in blank group. During implantation, cell-rich cancellous bone side of "two-phase" "BMG" was oriented towards the articular cavity while the cortical bone side was close to the subchondral bone plate. Next the knee joint was straightened, the patella was restored and the joint capsule was sutured. After rinsed with iodophor, the fascia and skin were sewn up and the incisions were bandaged. All animals were housed separately with intramuscular injection of antibiotics to prevent infection during postoperative 3 days and dressing change every other day. Meanwhile clinical vital signs of these animals were closely observed.

Observation of the repairing efficacy

5 rabbits in experimental group, 2 in positive control group and 2 in blank group were sacrificed at the 8th week. Femoral condyles at both sides were obtained and underwent general observation, and then these specimens were fixed in 4% paraformaldehyde for 24 h. After decalcification and dehydration, above femoral condyles were paraffin-embedded and made to sections for H&E and Pollak trichrom staining. After type II collagen immunohistochemical staining, the specimens were evaluated with modified Wakitani histological scoring criteria [5] (**Table 1**). The remaining animals were sacrificed at the 12th week and tested according to the method described above.

Statistical analysis

All statistical analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA) and measurement data was shown as the mean ± SD ($\bar{x}$ ± s). Comparison of data among the three groups was analyzed using ANOVA (SNK method) while the data at different time points among the three groups was analyzed using two-sample t test. $P<0.05$ was considered statistically significant.

Results

Comparison of the general observation of articular after transplantation in the three groups

As shown in **Figure 1**, in experimental group, the cartilage defects femoral condyle were repaired with chondroid tissue, and it was white and fragile with slightly rough surface and weak elasticity as well as had the indistinctly visible boundary with adjacent normal tissue at the 8th week; at the 12th week, the repairing tissue was white, delicate and translucent with smooth surface and unclear boundary as well as the elasticity was similar to normal articular cartilage plus the surrounding cartilage was not worn (**Figure 1**). As shown in **Figure 1**, in positive control group, the cartilage defects were repaired with new tissue, which was similar to normal articular hyaline cartilage, and it was fragile with weak elasticity as well as had the

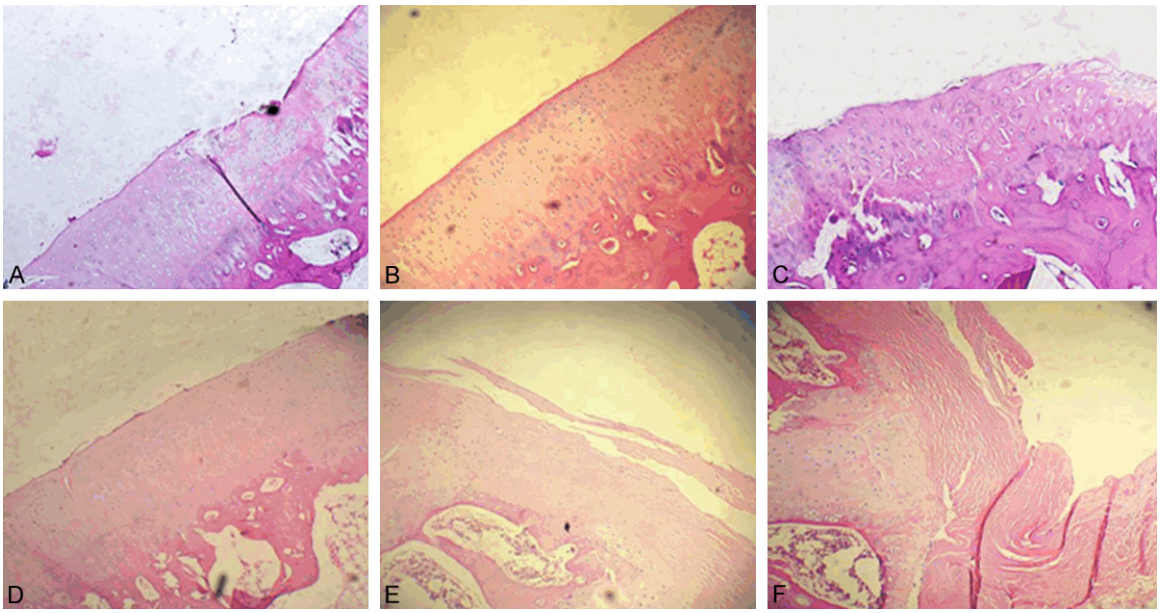

Figure 2. HE staining of repairing tissue (Magnification × 100). A: Experimental group at the 8th week; B: Experimental group at the 12th week; C: Positive control group at the 8th week; D: Positive control group at the 12th week; E: Blank group at the 8th week; F: Blank group at the 12th week.

indistinctly visible boundary with adjacent normal tissue at the 8th week; at the 12th week, the repairing tissue was white, delicate and translucent with smooth surface and faint boundary as well as it was well-integrated with adjacent tissue plus the elasticity was similar to normal articular cartilage (**Figure 1**). As shown in **Figure 1**, in blank group, the defects were only partially repaired and manifested as cavity, which was filled with fibrous tissue. It was pale white, opaque and the surrounding cartilage was worn at the 8th week; at the 12th week, there was more fibrous tissue growth, but it was not integrated with adjacent tissue (**Figure 1**).

Comparison of the results of H&E staining

As shown in **Figure 2**, in experimental group, the defects were repaired with chondroid tissue with smooth surface, and the repairing tissue contained a large number of cells, most of which were mainly circular or oval hyaline cartilage-like cells in disordered arrangement at the 8th week; at the 12th week, cartilage-like tissue was smooth and well-integrated with adjacent tissue without clear boundary. In addition, the cell arrangement tended to be normal and the subchondral bone was remodeled as well as the surrounding cartilage had no obvious degeneration and there was no residual structure of BMG in repairing tissue. HE staining showed that there were no obvious lymphocyte aggregation and no dead cartilage cells with positive eosinophilic staining of matrix and no staining of nuclear around the graft (**Figure 2**).

As shown in **Figure 2**, in positive control group, there were several layers of circular and oblong cells on the repairing surface, which was parallel to the cartilage surface and the lower radiation area had a large number of immature chondrocytes as well as subchondral bone gradually formed and there was a firm connection around repairing tissue at the 8th week; at the 12th week, the repairing surface was smooth with formation of typical hyaline cartilage cells and there was chondrocellular mitosis in radiation zone as well as cartilage pouch was obvious and subchondral bone formed well. In contrast, the defects were repaired with fibrous tissue at the 8th and 12th week in blank group (**Figure 2**).

Comparison of the results of Pollak trichrom staining

As shown in **Figure 3**, normal cartilage and repairing area were dyed blue, repairing cartilage layer arranged in good order and repairing tissue was closely connected with adjacent tissue at the 8th and 12th week in experimental group and positive control group. In blank group, the defects were repaired with fibrous

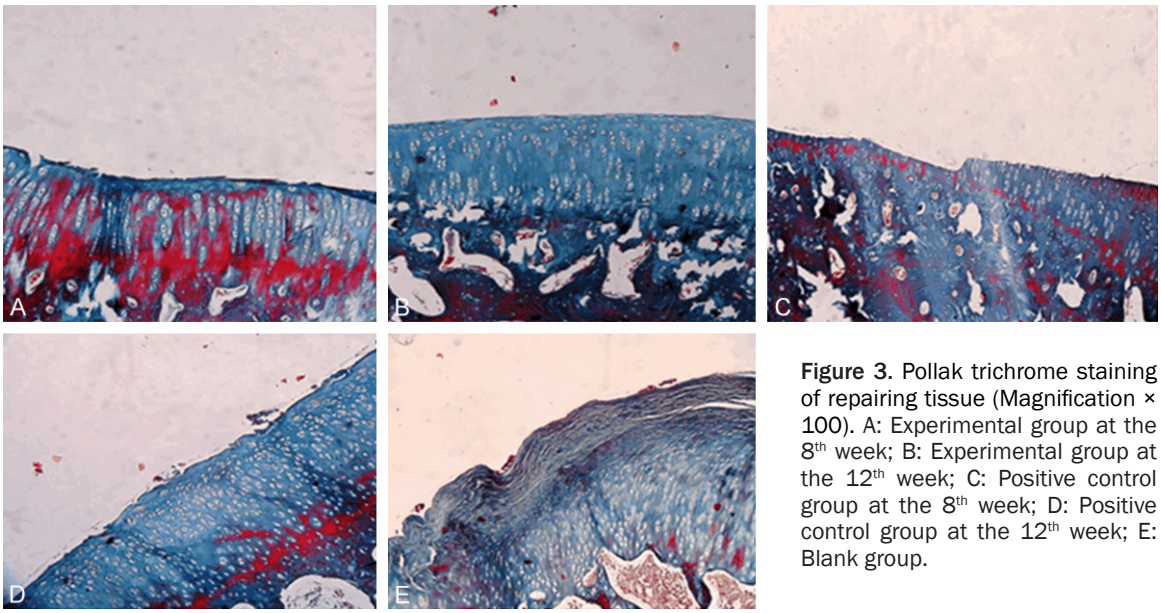

Figure 3. Pollak trichrome staining of repairing tissue (Magnification × 100). A: Experimental group at the 8th week; B: Experimental group at the 12th week; C: Positive control group at the 8th week; D: Positive control group at the 12th week; E: Blank group.

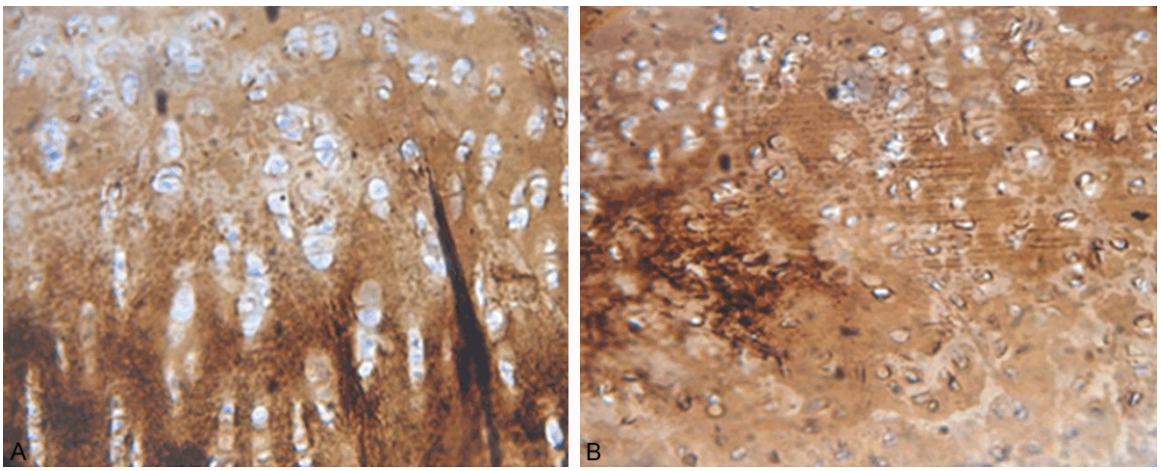

Figure 4. Type II collagen immunohistochemical staining of cells and matrix (Magnification × 200). A: Experimental group at the 8th week; B: Experimental group at the 12th week.

tissue, so only normal cartilage was stained blue (**Figure 3**).

Results of type II collagen immunohistochemical staining

As shown in **Figure 4**, positive staining was seen in cytoplasm and matrix at the 8th and 12th week in experimental group and positive control group, especially, strongly positive was seen at the 12th week.

Results of Wakitani histological score

As shown in **Table 2**, the results showed that every score of experimental group and positive control group was better than that of blank group at the 8th and 12th week, and the difference was statistically significant ($P<0.05$); There was no significant difference between experimental group and positive control group at different time points ($P>0.05$). The difference in pairwise comparisons at different time points were significantly significance in the three groups ($P<0.05$).

Discussion

Loss of cartilage caused by osteoarthritis or trauma is a major cause of joint pain and disability in the elderly. Articular cartilage has very

Table 2. Wakitani score of tissue sections at each time point in three groups

Group	Time (week)	Joint (number)	Score of cell morphology	Score of matrix staining	Score of surface evenness	Score of cartilage thickness	Score of binding of the surrounding tissue	Total point
Experimental group	8	10	1.1±0.3	1.1±0.6	1.4±0.5	1.2±0.6	1.1±0.6	5.9±2.0
	12	10	0.3±0.5	0.4±0.5	0.6±0.7	0.4±0.5	0.4±0.5	2.1±2.1
Positive control group	8	4	1.0±0.0	1.5±0.6	1.3±0.5	1.0±0.0	1.3±0.5	6.0±1.4
	12	6	0.2±0.4	0.3±0.5	0.2±0.4	0.2±0.4	0.3±0.5	1.2±1.5
Blank group	8	4	3.3±0.5	2.8±0.5	2.8±0.5	2.0±0.0	2.0±0.0	12.8±1.0
	12	6	2.2±0.4	1.5±0.5	1.7±0.5	1.2±0.4	1.3±0.5	7.8±1.9

limited capacity to repair itself, therefore, articular cartilage defects often lead to more severe articular cartilage degeneration [6]. The rising tissue engineering provides a new choice for the repair of articular cartilage defects, and the acquisition of seed cell is the basis for the construction of tissue engineered cartilage. Mesenchymal stem cells, or MSCs, are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, adipocytes, bone marrow stromal cells, nerve cells and epithelial cells, all of which were derived from the mesoderm [7, 8]. What's more, isolation of MSCs is convenient and the process has little effect on human body, so the role of MSCs as seed cells is especially prominent in tissue engineering. However, isolation and cultivation of MSCs in vitro have a great risk, and its cultivation and proliferation to a sufficient quantity need at least 1-2 months. In addition, the number of MSCs cells decreases with age, specially its differentiation potential also decreases in the elderly. In some cases, the capacity of MSCs amplification in vitro is limited by the reduction of bone marrow cells in patients with bone marrow damage, bone marrow fibrosis or chemotherapy [9]. In this regard, allogeneic MSCs shows obvious advantages that it can be preamplified in vitro and cryopreserved in time, which can save time and reduce the pain of patients.

Chondrocyte is immunogenic with tissue-specific antigen on its surface, and has a weak response to collagen and protein [10]. However, MSCs is a king of "immune amnesty" cells, because it does not express major histocompatibility complex (MHC) II molecules and co-stimulatory molecules (B7 and CD40), which are necessary to activate T cells in immune rejection, and it also can inhibit T cell proliferation induced by allogeneic cells [11, 12]. Le et al. [13] found that osteoblasts, chondrocytes and adipocytes derived from MSCs did not cause a reaction of allogeneic T cells, and differentiated MSCs was still capable of inhibiting mixed lymphocyte reaction. A conclusion was drawn that undifferentiated and differentiated MSCs didn't induce allogeneic T cell proliferation but can regulate immune response [14, 15], which provided a reliable theoretical basis for allogeneic MSCs transplantation.

Cartilage tissue engineering requires not only a sufficient quantity of seed cells but also perfect cell-carrier scaffolds, which is a three-dimensional scaffold for the survival and adherence of seed cells prior to tissue formation and provides space for proliferation, differentiation, nutrient exchange, metabolism and extracellular matrix secretion and other physiological activities in cells. Because BMG is considered to have good inductive osteogenesis potential, it can be used as a suitable alternative to autologous bone and an ideal graft material for repairing bone defects. Therefore, BMG has been widely used and promoted in clinical at home and abroad recently [16].

In this study, the composite of autologous BMG and cartilage precursor cells derived from allogeneic MSCs in vitro was implanted into articular cartilage defects without inflammatory cell infiltration at the 8th and 12th week and obvious lymphocyte aggregation around the grafts as well as obvious immune rejection, which indicated that the method of the cells derived from allogeneic MSCs for the repair of articular cartilage defects was feasible. The results showed that, in experimental group, the cartilage defects in femoral condyle were repaired with chondroid tissue at the 8th week and rich hyaline-like cartilage tissue without obvious degeneration in new cartilage at the 12th week, and there was no statistic difference between

experimental group and positive control group, which suggested that implantation of cartilage precursor cells derived from allogeneic MSCs was capable of repairing articular cartilage defects.

The comparative study on the capabilities of cartilage precursor cells derived from autologous and allogenic MSCs respectively seeded onto "two-phase" allogeneic BMG for constructing tissue engineered cartilage in the repair of articular cartilage defects was conducted through drawing material and evaluating the repairing efficacy at the 8th and 12th week, and the satisfactory result was achieved, which proved that the composite of "two-phase" allogeneic BMG and cartilage precursor cells derived from autologous MSCs could effectively repair articular cartilage defects. Meanwhile, this laid a foundation for the clinical application of tissue engineering in the repair of cartilage defects.

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Disclosure of conflict of interest

None.

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