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Original Article

Repair of the rabbit inverterbral disc with transplantation of autologous BMSCs-"two-phase" BMG complex

Jun Hu[1], Jun Shu[2], Xiaofeng Yuan[1], Bangxu Nie[1], Rong Qin[3], Shuguang Zhou[4], Weiqiang Li[2], Bo Pu[2]

[1]Department of Orthopedics, The First People's Hospital of Kunming, Kunming 650011, China; [2]Department of Orthopedics, The Second Affiliated Hospital of Kunming Medical University, Kunming 650101, China; [3]Department of Gastroenterology, Yanan Hospital of Kunming, Kunming 650041, China; [4]Department of Orthopedics, The Affiliated Hospital of Hunan University of Medicine and The Third People's Hospital of Huaihua, Huaihua 418000, China

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Abstract: Objective: To explore transplanting autologous bone marrow mesenchymal stem cells (MSCs) compounded with "two-phase" autologous bone matrix gelatin (BMG) into the defects after surgical removal of rabbit intervertebral disc and the feasibility and efficacy of repairing disc. Methods: 48 experimental Japanese rabbits were randomly divided into control group (n=24) and experimental group (n=24). After rabbit models of total removing intervertebral disc were made, experimental group was implanted with autogeneic cell-carrier complex, while control group was only implanted with autologous BMG. Then these rabbits were sacrificed at the 8th and 12th week. Specimens were obtained and underwent general observation and HE staining. Sulfuric acid-carbazole method was used to assay aggrecan, while immunohistochemistry and RT-PCR were performed to detect Collagen II. Results: Intervertebral discs of rabbits in experimental group were repaired with the growth of cartilage endplate and fibrocartilage tissue, while repairing discs in control group was not as good as that in experimental group. Moreover, levels of aggrecan and Collagen II of cartilage endplate and fibrocartilage tissue in experimental group were significantly increased when compared with that in control group ($P<0.05$) and the difference was statistically significant. Conclusion: MSCs can repair the rabbit inverterbral disc with the growth of cartilage endplate and fibrocartilage tissue.

Keywords: Bone marrow mesenchymal cells, inverterbral disc, inverterbral disc cells, collagen II

Introduction

Degenerative disc disease refers to a group of clinical syndromes, mainly manifested as neck, shoulder, waist, back and limb pain, which is caused by intervertebral disc degeneration (IDD). The occurrence and development of IDD are slow and complex, and the specific pathophysiological mechanisms are not yet fully understood at present [1-3], therefore, the treatment of degenerative disc disease is only limited to traditional methods [4], which can relieve some clinical symptoms caused by disc degeneration to a certain extent but cannot completely solve the problem and even has the risk of complications.

In recent years, with the emergence of tissue engineering and cell therapy, animal autologous BMSCs are transplanted into the degenerative intervertebral disc tissue, and the results show that BMSCs can differentiate into spindle shaped cells which are similar to intervertebral disc cells and can survive for a long time, meanwhile, loss of nucleus pulposus cells and disordered fibrous ring arrangement can be improved to a certain degree [5, 6]. However, there is no similar report in the study of orthotopic implantation of induced BMSCs into the defects after surgical removal of intervertebral disc. Therefore, this study was conducted to observe the effect of orthotopic implantation of BMSCs induced in vitro and seeded onto "two-phase" autologous BMG into the defects after surgical removal of intervertebral disc via simulating the upper and lower disc cartilage endplate structures, which was based on BMSCs as seed cells and "two-phase" autologous BMG

as cell carriers. This study aimed to provide an experimental basis for intervertebral disc transplantation.

Materials and methods

Animal grouping and BMSCs obtaining

24 healthy adult Japanese rabbits, about 2.2 kg in weigh, male or female, were purchased from the Experimental Animal Center of Kunming Medical College. All these rabbits were anesthetized with 3% sodium pentobarbital via ear vine, and about 3-5 ml marrow were extracted, as well as BMSCs was isolated from the bone marrow blood for purification, induction and identification, which provided adequate seed cells for the repair of intervertebral disc. 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), 8th 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.

Autologous ilium obtaining and BMG preparing

Ilium of anesthetized rabbit was taken out and the soft tissue on bone was removed and cleaned. After degreased, demineralized and antigen-extracted, these iliac bones were trimmed to a sponge-like, soft and easy plastic "two-phase" structure with cancellous bone on the one side and cortical bone on the other side.

Preparation of BMSCs/BMG complex

After rewarming, "two-phase" BMG of experimental group was immersed into corresponding autologous BMSCs cell suspension with sterile ophthalmic tweezers. Due to its absorption, BMG absorbed cell suspension uniformly and sank to the bottom of the tube within few minutes. The tube was placed in the culture incubator for 6 hours until the cells grew initial attachment. Next cell-carrier composites were taken out, seeded into 24-well plates and cultured for 3 days. And the sells grew with full attachment to the carrier for modeling use. Rewarmed "two-phase" BMG of control group was only seeded into 24-well plates, which only concluded culture medium.

Rabbit disc model construction and BMSCs/BMG complex implantation

48 experimental Japanese rabbits were randomly divided into two groups. Control group (n=24) was only implanted with autologous BMG while experimental group (n=24) was implanted with autogeneic cell-carrier complex. After the rabbits were anesthetized, the L3-4 was exposed. The fenestration was at the upper and the lower edge of the left side of the disc next to the vertebra, and then the intervertebral disc was completely removed, which included the upper and lower endplate. The cell-carrier complex was implanted in experimental group while BMG was implanted in control group. All animals were housed separately with intramuscular injection of antibiotics to prevent infection during postoperative 3 days. Meanwhile clinical vital signs of these animals were closely observed.

Sampling and effect analysis

12 rabbits in experimental group and 12 rabbits in control group randomly selected were sacrificed by air embolism, and L3-4 discs together with L3, L4 vertebrae were removed at the postoperative 8th and 12th week respectively. 6 discs of them each in experimental group and in control group underwent general observation and were cut into two halves at the middle of the intervertebral disc: one half was performed for determination the level of proteoglycan with sulfuric acid-carbazole method, while the other was immediately placed in liquid nitrogen to detect the expression of aggrecan and Collagen II mRNA via RT-PCR. The left 6 discs of them each in experimental group and in control group underwent general observation, and were fixed in 4% paraformaldehyde for 24 h. After decalcification and dehydration, above discs were paraffin-embedded and made to sections for HE staining and collagen II immunohistochemical staining.

Immunohistochemical determination of collagen II

The disc tissue samples were paraffin-embedded. After desodium and rehydration, 3% hydrogen peroxide solution was added, and the sections were incubated at room temperature to block endogenous peroxidase activity. 50 μl primary antibody Mouse anti-rabbit Col II Antibody (1:75) was added in each section at

Table 1. Primer sequences and amplification conditions

Gene	Primer sequence	Annealing temperature	Amplification length	Cycle times
Aggrecan	F 5'-TTGGAGGTCGTGGTGAAAGG-3' R 5'-TCTCACGCCAGGGAACTCAT-3'	60°C	258 bp	38
Collagen II	F 5'-CTGGCAAAGATGGTGAGACAGGTG-3' R 5'-GACCATCAGTGCCAGGAGTGC-3'	60°C	294 bp	38
β-actin	F 5'-GTGGGGCGCCCCAGGCACCA-3' R 5'-CTTCCTTAATGTCACGCACGATTTC-3'	56°C	540 bp	30

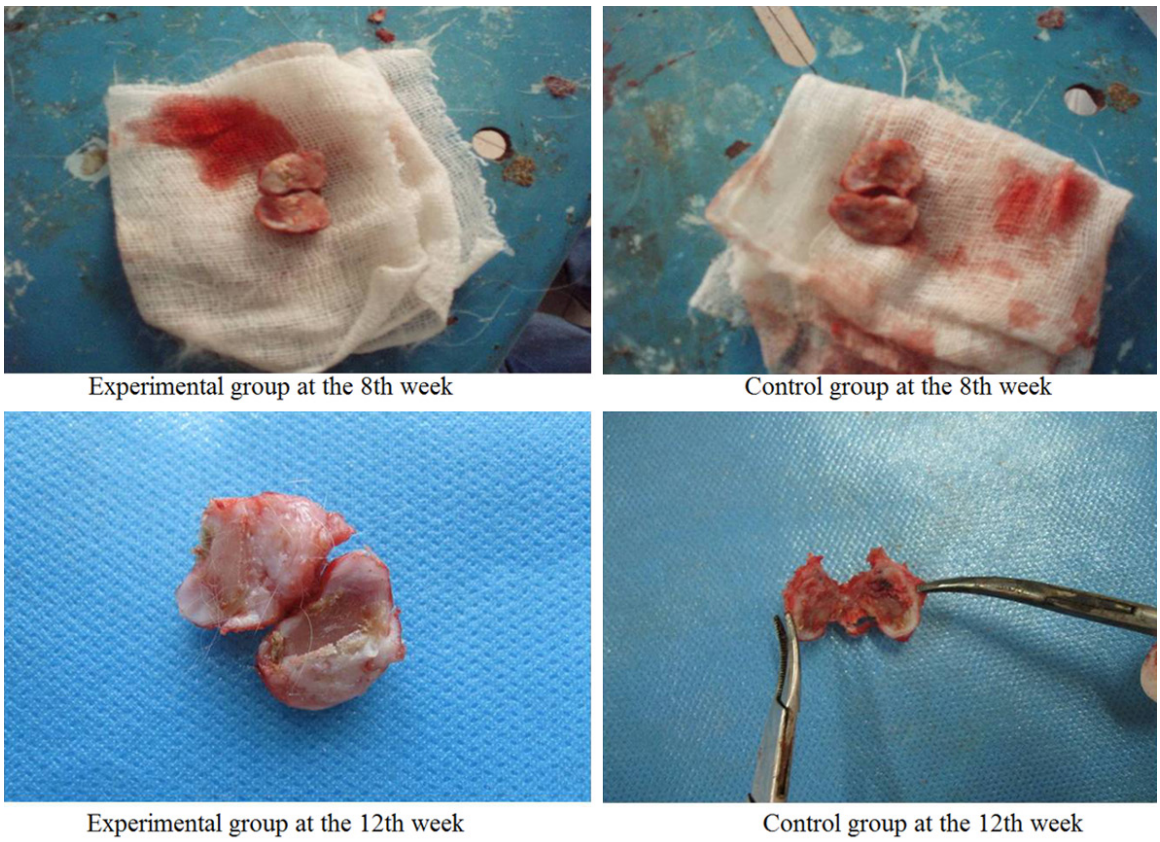

Figure 1. General observation of interveterbral disc.

4°C overnight, and 50 ul of polymer reinforcing agent (reagent A) and enzyme-labeled anti-mouse/rabbit polymer (reagent B) were added. Next diaminobenzidine (DAB) was used as the chromogen; after thoroughly rinsed with distilled water, sections were counterstained with hematoxylin and gradient dehydrated in ethanol with xylene as clearing medium and mounted by neutral resin. Above sections was observed with microscope. HPIAS-1000 high resolution pathological image analysis system was applied to measure 6 random high power fields of per disc sample for quantitative evaluation of the gray value of the chordrocytal matrix and fibrocartilage which were in positive immunostaining (0~255 grade, 0 is the deepest, and 255 is the lowest).

Determination of proteoglycan level with sulfuric acid-carbazole

20 mg disc was degreased with acetone and ether. After dried for 4 h at constant temperature, it was placed in 10 ml test tube with papaya enzyme added for digestion. Next above tube was centrifuged in 20% trichloroacetic acid and the supernatant was extracted. After 5 ml distilled water was added, 500 μl mixtures were taken out. As 3 ml concentrated H_2SO_4 was added and it was placed in an ice water

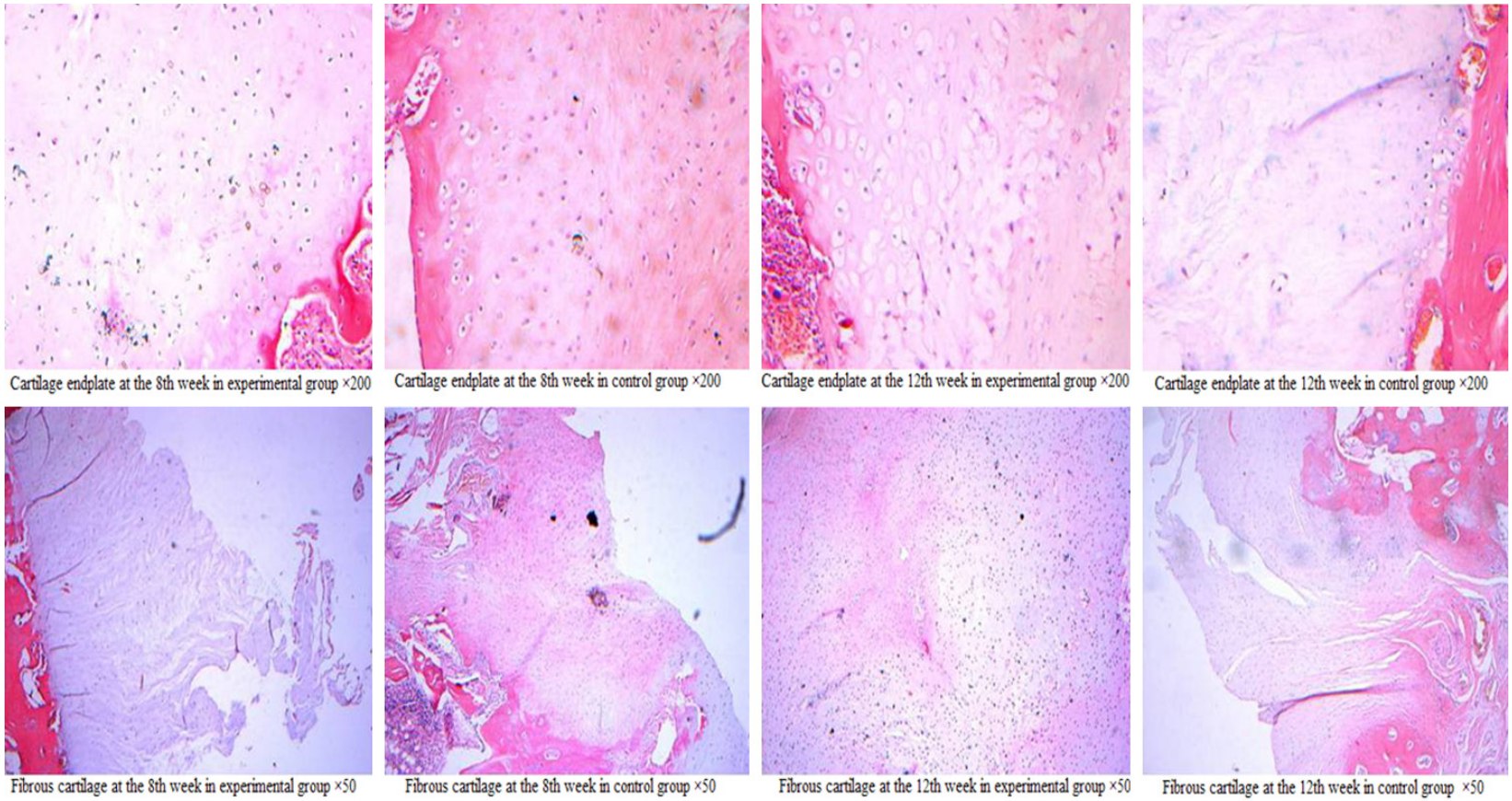

Figure 2. HE staining results of cartilage endplate and fibrous cartilage.

Table 2. Comparison of the thickness of cartilage endplate and fibrous cartilage in the two groups at the 8th week and the 12th week (um, $\bar{x}\pm s$)

Group	Time (week)	Case	Cartilage endplate	Fibrous cartilage
Experimental group	8	6	156.7±11.5*	829.6±78.6*
	12	6	189.5±12.7*	1193.9±201.7*
Control group	8	6	92.4±7.5	580.3±78.2
	12	6	124.1±15.7	796.8±73.5

Note: Compared with control group at corresponding time, *P<0.05.

bath for 20 min at 100°C to measure the absorbance at 535 nm in 100 μl carbazole [7, 8]. At last, the content of uronic acid was calculated.

Detection of the expression of aggrecan and collagen II mRNA with RT-PCR

The total RNA was extracted from the tissue with the kits, and the cDNA was generated by reverse transcription. The Premier Primer 5 software was used to design primers (**Table 1**), and aggrecan and collagen II mRNA were amplified by PCR. The reaction condition was as follow: predenaturation for 2 min at 94°C, denaturation for 40 s at 94°C; annealing for 50 s, extension for 1 min at 72°C (cycle parameter was shown in **Table 1**); termination for 10 min at 72°C and preservation at 4°C. The amplified products were identified 1.5% agarose gel electrophoresis with β-actin as a control sample volume, and electrophoretic bands were performed for quantitative analysis with Bio-Rad quantity one.

Statistical analysis

All data were performed using SPSS version 11.5 software and measurement data was shown as the mean ± SD ($\bar{x}\pm s$). Comparison of averages was analyzed using t-test between the two groups and *P* value was calculated. P<0.05 was considered statistically significant.

Results

General observation of intervertebral disc

At the 8th week, intervertebral discs in experimental group were repaired with cartilage tissue without obvious normal fibrous ring-like structure, and the position of original nucleus pulposus was filled with cartilage tissue, but its color was slightly lighter than that of repaired fibrous ring. Intervertebral discs in control group were partly repaired with cartilage tissue without fibrous ring-like structure, and the position of original nucleus pulposus was partly filled with cartilage tissue as well as its color was slightly lighter than that of experimental group (**Figure 1**).

At the 12th week, the whole intervertebral discs in experimental group were fully repaired with cartilage tissue without obvious normal fibrous ring-like structure, which was more complete and smooth than that at the 8th week, and the repair of original nucleus pulposus was more adequate than that at the 8th week. A small part of intervertebral discs in control group were repaired with cartilage tissue without fibrous ring-like structure, and original nucleus position was filled with much fibrous tissue as well little cartilage tissue (**Figure 1**).

HE staining

Cartilage endplate: At the 8th week, HE staining showed that a large number of transparent cartilages was seen in experimental group, and the thickness was more than that of control group, while a thin layer of cartilage endplate was seen in control group. At the 12th week, HE staining showed that the thickness of the endplate in experimental group increased obviously while a thinner layer of cartilage endplate was seen in control group (**Figure 2**).

Fibrous cartilage: At the 8th week, HE staining showed that much fibrocartilage was seen in experimental group, and the thickness was more than that of control group, while a thin layer of fibrocartilage was seen in control group. At the 12th week, HE staining showed that a large number of fibrocartilage was seen in experimental group, of which the thickness was more than that at the 8th week, while a thinner layer of fibrocartilage was seen in control group (**Figure 2**).

In the two groups, the thickness of cartilage endplate and fibrocartilage was compared, and the results showed both of them in experimental group was significantly thicker than those in

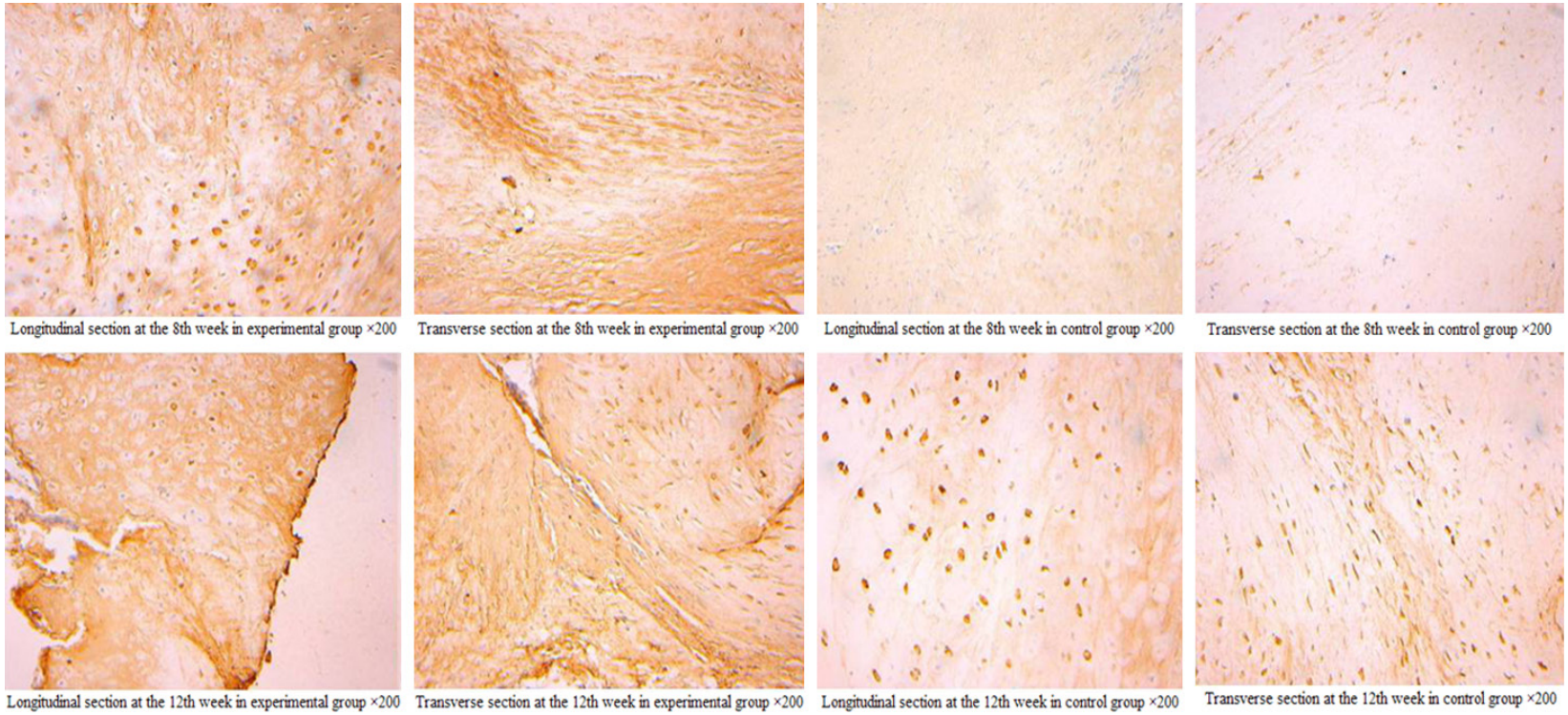

Figure 3. Collagen II immunohistochemical staining results.

Table 3. Comparison of the gray value of cartilage endplate and fibrous cartilage in positive immunohistochemical staining in the two groups at the 8th week and the 12th week ($\bar{x}$±s)

Group	Time (week)	Case	Cartilage endplate	Fibrous cartilage
Experimental group	8	6	187.5±11.9*	166.6±7.7*
	12	6	168.0±7.6*	154.4±9.0*
Control group	8	6	245.0±13.7	247.1±14.4
	12	6	201.6±9.0	215.9±10.8

Note: Compared with control group at corresponding time, *P<0.05.

Table 4. Comparison of the level of aggrecan in cartilage endplate and fibrous cartilage in the two groups at the 8th week and the 12th week (mg/100 mg, $\bar{x}$±s)

Group	Time (week)	Case	Cartilage endplate	Fibrous cartilage
Experimental group	8	6	4.374±0.116*	3.607±0.118*
	12	6	7.222±0.234*	6.978±0.146*
Control group	8	6	1.758±0.109	1.159±0.137
	12	6	3.353±0.153	2.369±0.190

Note: Compared with control group at corresponding time, *P<0.05.

control group, and the difference was statistically significant (**Table 2**, P<0.05).

Collagen II immunohistochemical staining

At the 8th week and the 12th week, Collagen II immunohistochemical staining on matrix around cartilage endplate and fibrous cartilage in both groups was positive, which presented pale yellow or brown little particles. Samples in control group was weak positive, presenting pale yellow particles while experimental group was strong positive presenting brown small particles (**Figure 3**).

The gray value of chondrocyte matrix and fibrocartilage which were in positive immunostaining: the average gray values of experimental group at each time point were significantly decreased when compared with control group, the difference was statistically significant (**Table 3**, P<0.05).

Comparison of aggrecan level

Aggrecan levels of cartilage endplate and fibrous cartilage in experimental group at the 8th week and the 12th week were significantly increased when compared with control group (P<0.05, **Table 4**).

Expression of aggrecan and collagen II mRNA

RT-PCR analysis showed that the expression of aggrecan and Collagen II mRNA was detected in experimental and control group at the 8th week and the 12th week. The expression of aggrecan and Collagen II mRNA in the experimental group was significantly enhanced when compared with control group (P<0.05, **Figure 4**; **Table 5**).

Discussion

Mesenchymal stem cells (MSCs), mainly in the bone marrow as well as in extraskeletal tissue derived from mesenchyma over embryonic period, are non hematopoietic stem cells. It has many characteristics of easily sampling, small damage to the body, strong ability of proliferation and immune tolerance. Moreover, in different conditions, it has the potential to differentiate into osteoblasts, chondroblasts, adipocytes, fibroblasts, nerve cells [9-11]. It is this property that can repair the intervertebral disc. In the appropriate treatment condition, mesenchymal stem cells can differentiate into disc-like cells, which results in a large number of Collagen II and aggregation, thus achieving the purpose of repair. As a result, MSCs, as seed cells, has received more and more attention on the phenotypic differentiation into the intervertebral disc cells [12, 13]. Due to lack of cell carrier, cells are lost easily, and can not form an effective cell concentration at the implantation site during the period of the transplantation of BMSCs. The extracellular matrix (ECM) has the functions of maintaining the growth of cells, regulating the formation of the cell and affecting cell growth, migration, proliferation and function [14, 15]. Therefore, the preparation and selection of ECM, which benefits to the attachment, proliferation and differentiation of seed cells, are important in intervertebral disc tissue engineering. On the basis of demineralization, BMG is prepared with the method of continuous chemical treatment, which is designed as a "two-phase" carrier structure with one side of cancellous bone and the other side of the bone cortex. The bone cor-

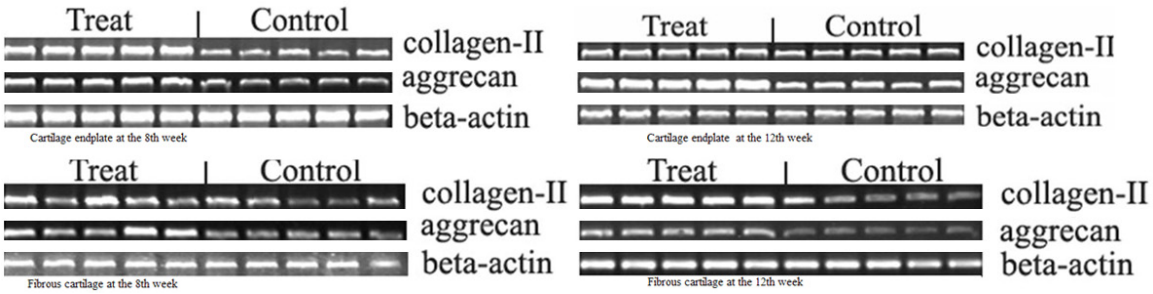

Figure 4. Expression of aggrecan and Collagen II mRNA.

Table 5. Comparison of the expression of aggrecan and collagen II mRNA in cartilage endplate and fibrous cartilage in the two groups at the 8th week and the 12th week ($\bar{x}\pm s$)

Group	Time (week)	Case	Cartilage endplate aggrecan	Collagen II	Fibrous cartilage aggrecan	Collagen II
Experimental group	8	5	108.94±2.59*	142.15±5.46*	67.30±9.67*	74.45±15.96*
	12	5	172.27±4.80*	174.77±4.65*	104.79±24.79*	91.58±6.91*
Control group	8	5	79.28±4.23	113.15±1.22	44.62±1.86	54.14±8.91
	12	5	128.91±8.06	130.87±5.39	65.37±7.55	72.94±11.26

Note: Compared with control group at corresponding time, *P<0.05.

tex side of the "two-phase" structure is similar to the morphology of the upper and the lower cartilage endplate of intervertebral disc with big internal space in loose part, which can cover more cells. Cartilage precursor cells can interact with each other in such a three-dimensional culture after inoculation.

Intervertebral disc structure, a jelly-like organ without the core and blood vessels, is rather special and has a complete package, which is mainly composed of a central nucleus pulposus, the surrounding fibrous ring and the upper and the lower cartilage endplate. The cells located in the outer and inner layer of the intervertebral disc annulus are originated from the mesenchymal cells, and the inner layer is a kind of chondromimetic cells. The endplate not only has the function of barrier, but also plays a nutritional intermediary role. The nutrients in blood vessels of the vertebral body can diffuse into the intervertebral disc through marrow cavity-sinus-cartilage interface for nurturing the nucleus pulposus and annulus inner layer [16]. Nucleus pulposus cells in adults are mostly chondromimetic cells, which are different from that in articular cartilage and fibrous ring. The number of cells in the intervertebral disc is small (accounting for about 1%). These cells have limited intrinsic self-renewal ability and are rich in extracellular matrix, which constitutes a scaffold for cell distribution, including water, proteoglycan, collagen and non-collagen protein. The water absorption of proteoglycan makes the disc keep a high hydrostatic pressure to maintain disc height; moreover, proteoglycan concentration determines the disc permeability, which influences the pathway of nutrition, chemical medium and cell metabolites. Collagen is mainly against the tension load, which the intervertebral disc must bear; Collagen I and Collagen II account for about 80%, while Collagen III, V, VI, IX and XI is little, which involve in the composition of collagen fibers.

As time goes by, the nucleus pulposus of intervertebral disc undergo a transformation. In the early time, it occupies nearly half of the intervertebral disc and is mainly composed of notochordal tissue containing many vacuoles, known as notochordal nucleus pulposus, which consists of the multi-core cords and notochord cell clusters surrounded by a soft gelatinous matrix. After birth, as the number of notochord cells decreases gradually, chondromimetic cells appears in the nucleus pulposus, and more and more collagen fiber also emerges, and then notochordal nucleus pulposus will be gradually replaced by fibrocartilaginous nucleus pulposus which contains chondromimetic cells. What's more, this conversion process is

often completed in about 20 years old. In the transformation of nucleus form, the chondromimetic cells play a central role and eventually become the main cell types of mature or adult nucleus pulposus.

In this study, the nucleus pulposus and annulus structure of the intervertebral disc were repaired with fibrocartilage containing chondromimetic cells, and a large number of aggrecan and Collagen II were detected in fibrous cartilage tissue, which suggested that induced BMSCs could differentiate into chondromimetic cells in vivo and eventually became the nucleus-like phenotype fibrocartilage tissue. However, because of the lack of specific surface markers of nucleus pulposus cells and fibrous ring cells, chondromimetic-like phenotype could be expressed, and its identification mainly depended on Collagen II and aggrecan, which were the specific markers of cartilage phenotype. This experiment could detect Collagen II and aggrecan in different parts of the regenerated disc, so it is difficult to determine whether grafted BMSCs expressed nucleus pulposus specific phenotype or annulus phenotype. As a result, identification of the characteristics of the cell phenotype becomes the focus in the future.

In addition, the results of HE staining showed that cartilage endplate and fibrous cartilage tissue of experimental group were significantly thicker than that of control group, and immunohistochemical staining and RT-PCR assay showed that the expression of Collagen II of cartilage endplate and fibrous cartilage tissue in experimental group was significantly enhanced than that in control group. With biochemical methods, aggrecan and Collagen II were detected in the interveterbral discs of experimental group and control group, and experimental group was significantly higher than control group with significant difference. This explained that the orthotopic transplantation of BMSCs into rabbit's interveterbral disc could differentiate into disc cells, increase the level of aggrecan and Collagen II, and repair cartilage endplate and fibrous cartilage with the intervention of TGF-β1. It was said that the complete regeneration of the intervertebral disc became a reality.

This study explored the possibility of the complete regeneration of intervertebral disc via the transplantation of BMSCs to provide an experimental basis for future studies. Tissue engineering technology brings hope for the regeneration of disc tissue, but it is not thorough, so pre-clinical studies are needed in the future.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Shu, Department of Orthopedics, The Second Affiliated Hospital of Kunming Medical University, 374 Dianmian Road, Kunming 650101, Yunnan Province, China. E-mail: junshucn@126.com

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