

Original Article

Treatments for intervertebral discs degeneration by stem cells transplantation: a therapeutic potential comparison between bone marrow stem cells and adipose-derived stem cells

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Abstract: *Objective:* The aim of this study was to compare the therapeutic effects between the transplanted bone mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ADSCs) in rabbits by using a degenerative disc model. *Methods:* Firstly, the intervertebral discs degeneration animal model was constructed by the conventional punctured method. Then, BMSCs and ADSCs were transplanted into the rabbit's model of disc degeneration, respectively. The nucleus pulposus cells (NPCs) transplantation group was set as the positive group, only Dulbecco's modified Eagle's medium (DMEM) injection group was set as the negative group and the health intervertebral disc group was set as the normal control (NC) group. The samples were harvested in 12 weeks after the transplantation. Disc degeneration degree was evaluated by plain radiography as well as the MRI imaging T2-weighted signal intensity, histology, sulfated glycosaminoglycan (sGAG)/DNA, immunochemical staining. Also, the gene expression levels of collagen II and aggrecan were evaluated. *Results:* Twelve weeks after cell-transplantation, the degeneration degree for the DMEM group was significantly higher than the other four groups ($P < 0.05$). Histological analysis indicated no significant difference among NPCs, BMSCs and ADSCs groups, while the other results, including disc height index ratio (%DHI), standard T2-weighted image ratio (%ST2WI), sGAG/DNA assessment and mRNA expression levels of the aggrecan, showed that the therapeutic effects of NPCs and BMSCs groups were much better than that of the ADSCs group. While no significant differences between the NPCs and BMSCs groups were found. *Conclusion:* The results indicated that all types of cells in this study had a positive effect on restraining the discs degeneration. Especially, BMSCs showed a stronger potential in the extracellular matrix synthesis and water content recovery of the discs, similar to that of NPCs. The BMSCs transplantation was more effective in the disc degeneration treatment than ADSCs, which appears to be an ideal substitute for NPCs.

Keywords: Cell therapy, intervertebral disc degeneration, stem cells

Introduction

Low back pain is a common medical and social problem for adults [1, 2]. Intervertebral disc degeneration (IDD), an irreversible phenomenon, is considered as one of the major causes of low back pain. Multiple factors contribute to disc degeneration, including genetic factors, age, immobilization, trauma, tobacco use, diabetes, vascular diseases, and infection [3-7]. The mechanism of disc degeneration is still unknown. IDD is a progressive phenomenon.

Current treatments include cytokine and growth factor induction, gene therapy, tissue engineering and cell transplantation therapy [7, 8].

Among these treatments, cell transplantation is a promising method for treating IDD. Autologous nucleus pulposus cells (NPCs) transplantation is one of the major techniques used to prevent IDD degeneration in animal models [9, 10]. However, the procedure requires many more cells than those that can be harvested from a single disc. The NPCs proliferate slowly *in vitro*.

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Hence, the production of enough NPCs has become a struggle for researchers. Another issue is that cell harvesting from healthy discs may create iatrogenic degeneration in the donor discs. To overcome these problems, alternative cell sources for replacement of NPCs used in IDD therapy have to be explored. In this study, we focused on the investigation of the therapeutic potential of stem cells—bone mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ADSCs) in IDD treatment.

BMSCs are multipotent stem cells that have been found in almost every adult organ. BMSCs can be harvested, isolated and cultured easily *in vitro*. These cells have high plasticity and the capacity to differentiate into a NPCs-like phenotype when appropriately stimulated [11]. In addition, ADSCs are another candidate for stem cell sources, particularly because they are easily obtainable and have multipotent activity. ADSCs can be collected from adipose tissue in human body without significant wound via the minimally invasive procedure [12-14]. Due to these significant advantages, ADSCs can be used as a stem cell source to treat degeneration.

However, to our best knowledge, no studies have focus on the comparison of therapeutic potential between BMSCs and ADSCs in the IDD treatment. Therefore, in this study, we aimed to compare the therapeutic effects between the two types of cells in a degenerative intervertebral disc of rabbits, in order to seek a better substitute for NPCs and provide certain reference both in theory and in the clinical treatment for cell transplantation.

Materials and methods

Animal model preparation of degenerative intervertebral disc

Adult New Zealand white rabbits (weight: 2.5-3.0 kg) were provided by the North Sichuan Medical College. Animal experiments were carried out according to the protocols approved by the Animal Experimentation Committee in the Second Clinical Institute of North Sichuan Medical University (NO. 20140702). Rabbits were randomly divided into five groups: the normal control (NC) group, NPCs group, BMSCs group, ADSCs group and DMEM group. And three intervertebral discs of each animal, including L3-4 discs, L4-5 discs and L5-6 discs, were used to identify the sGAG/DNA assessment,

histological analysis and mRNA expression levels of the collagen II and aggrecan, respectively.

The rabbits were anesthetized through the injection of 30 mg/kg of pentobarbital sodium. nucleus pulposus (NP) tissue (5-8 mg wet weight) was aspirated from the rabbits at regions L3-L4, L4-L5 and L5-L6 using an anterolateral approach, with a 21-gauge needle on a 10 ml syringe, as described previously by Sakai D *et al.* [15]. Then rabbits were kept in a controlled environment with access to food and water for 2 weeks.

Isolation, culture and identification of NPCs, BMSCs and ADSCs

Firstly, a nucleus pulposus cell culture was performed according to the method described previously by Feng G *et al.* [16]. NP tissue was harvested from the lumbar discs of 4 two-week-old New Zealand white rabbits that were euthanized by 1% pentobarbital (6 ml/kg) under aseptic conditions. The annulus fibrosus was opened with a #11 scalpel blade, causing a defect through which NP tissue was extruded. Approximate 20 mg of NP tissue was harvested from each disc, and then placed into a 6-well tissue culture plate and incubated at 37°C in 5% CO₂. The basal medium contained DMEM/F12 supplemented with 20% FBS and 50 U/ml penicillin/streptomycin.

Secondly, under pentobarbital anesthesia, the BMSCs were isolated from the femoral bone marrow of 4 two-week-old New Zealand white rabbits. Five milliliter of marrow blood was collected into 2000 U of heparin, as described previously by Sakai D *et al.* [11].

Thirdly, ADSCs were prepared from adult rabbit inguinal fat pads shortly after euthanasia as described previously by Feng G *et al.* [17]. The fat pads were excised, finely minced with scissors and washed three times with PBS containing 100 U/ml penicillin-streptomycin (Gibco, USA) and digested in 0.01% collagenase (Crescent Chemical Co, Inc, USA) at 37°C for 30 min. Then the cells were cultured in high-glucose DMEM (Hyclone) at 37°C with 5% CO₂.

Transplantation of NPCs, BMSCs and ADSCs

Two weeks after the induction of disc degeneration, the two passages of NPCs, BMSCs and ADSCs were gathered for transplantation,

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Table 1. Amplification primers of collagen II, aggrecan and GAPDH

Molecule	Primers	Product size (kb)
Collagen II	ACGCTCAAGTCCCTCAACA (sense)	198
	TGCGAGTTCAGGGAGTTGA (antisense)	
Aggrecan	TCTATCCAGTAGTCACCGCTCT (sense)	223
	AGATAGGTCATCAGTGGCGAGA (antisense)	
GAPDH	TGCTTCTAGGCGGAGTGTTA (sense)	820
	CGTCACATGGCATCTCACGA (antisense)	

alyzeDirect) (%ST2WI = discs T2WI/Water supplement T2WI ×100%).

Biochemical measurement and mRNA expression measurement

After L4-5 discs were isolated, the dissected NP tissues were digested in 200 µL of papain

respectively. The cell suspension was adjusted with DMEM to a final cell density of 1×10^6 cells/mL and used for immediate transplantation. Cell transplantation was performed upon the degeneration-induced discs of the rabbits under 1% pentobarbital anesthesia. Using an insulin microinjector with a 27-gauge needle, 0.03 mL of DMEM containing the NPCs, BMSCs or ADSCs was carefully injected into the degeneration discs, respectively. And 0.03 mL of DMEM without cells was injected in the DMEM group. After injection, histoacryl glue (B. Braun, Melsungen, Germany) was used to seal the injection site and the wounds closed routinely. The samples were harvested at 12 weeks after transplantation for evaluating the degree of degeneration.

Radiographic and MRI imaging testing

Prior to harvest, lateral plain radiographs of all groups were taken under 1% pentobarbital anesthesia. Vertebral body heights and disc heights were observed by X-ray and measured using NIH image software. The disc height index (DHI) was described by Lu DS *et al.* [18] (%DHI = postoperative DHI/preoperative DHI ×100%).

Rabbits were tranquilized under 1% pentobarbital anesthesia (3 mL/kg), and placed supine within the MRI imager coil (General Electric Medical Systems, USA). A localizing midsagittal T2-weighted image (TR 2500 msec, TE 100 msec) was used to view the L1-2 through L6-7 intervertebral levels. Next, 5 mm thickness of midsagittal sections was taken using T2-weighted imaging sequences (TR 2800 msec, TE 100 msec) to evaluate signal characteristics within the intervertebral disc. Water supplement capsules on the abdomen of the rabbit were used for the standardized control. The MRI imaging evaluations were performed initially and then postoperatively at 12 weeks and quantified using Analyze 7.0 software (An-

alyzeDirect) (%ST2WI = discs T2WI/Water supplement T2WI ×100%).

digestion buffer (125 mg/mL in sterile PBS, pH 6.0 with 5 mM cysteine hydrochloride) for 18 h at 60°C. Sulfated GAG (sGAG) levels were measured spectrophotometrically after incubation with 1,9-dimethylmethylene blue-chloride (DMMB) dye ($\lambda = 525$ nm) and normalized to total DNA, which measured fluorometrically ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm) using the Hoechst 33258 DNA quantitation kit (Sigma, USA). Total RNA from L5-6 discs was obtained using the E.Z.N.A. Total RNA kit I (OMEGA, USA, R6834-02). Real-time polymerase chain reaction (PCR) analysis was then performed with the Quantitect SYBR Green PCR master mix (Qiagen Valencia, CA, USA. A25742). Standard curves were generated using RT-PCR with a serially diluted cDNA sample mixture. Quantities of the gene expression of aggrecan and Type II collagen were calculated with standard samples and were normalized to GAPDH. Amplification primers are listed in **Table 1**.

Histological observation and immunofluorescence staining observation

After the macroscopic evaluation, L3-4 discs were individually processed for histological studies. Each disc was fixed and embedded in paraffin, sectioned to 5 µm thick using a microtome, and exposed to standard H&E staining. A histological grading system devised by Masuda *et al.* [19] was used. Grade scores were counted by five pathology professors selected randomly and data are presented as the mean ± standard deviation (SD). Samples were sectioned into 5 µm thick and dried in a drying oven for 12 h at 45°C. Aggrecan immunofluorescence was also stained. Sections were visualized with a Nikon TS100 microscope (Nikon, Melville, NY) equipped with a digital camera.

Statistical analysis

Statistical analysis of all the experiment measurement data was performed using SPSS13.0 software. Single-factor analysis of variance was

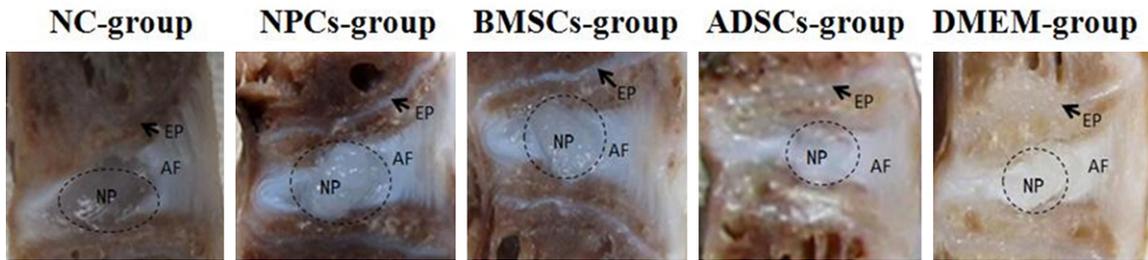


Figure 1. Representative photographs showed sagittal discs in 12 weeks after cell transplantation in each group.

performed to evaluate statistical significance among many groups, While SNK were Used to Evaluate statistical significance between two groups. The data are expressed as mean \pm SD. The $\alpha = 0.05$ was considered to be inspection level and p -value of less than 0.05 was considered to be statistically significant.

Results

Macroscopic findings

Twelve weeks after cell transplantation, the discs from the NC group showed an intact NP without a narrowing of the disc space. In the DMEM group, the discs showed obvious degeneration. The structure of the annulus fibrosus appeared to be disturbed, and an area of the nucleus pulposus was further reduced, interrupting the boundary between the nucleus pulposus and annulus fibrosus (**Figure 1**). The three cell-transplanted groups showed the height of disc was undisturbed. Also, the white translucent gel organization appeared in an area of the nucleus pulposus with a clear border between the NP and the annulus fibrosus, although whose shape appeared not as perfect as the NC group discs (**Figure 1**).

Evaluation of disc height and MRI T2-weighted images signal

Compared with the NC group, the %DHI in the NPCS, BMSCs, ADSCs and DMEM groups all decreased in 12 weeks after cell transplantation. Among the three cell groups, the %DHI in the NPCS and BMSCs groups were obviously higher than that in the ADSCs group ($P < 0.05$). The %DHI had no significant difference between the NPCS and BMSCs groups. The %DHI in the ADSCs group was remarkably higher than that in the DMEM group ($P < 0.05$). These results demonstrated that the cell transplantation could alleviate the height loss of the degenerated disc (**Figure 2A**).

In 12 weeks after cell transplantation, the intervertebral disc T2-weighted images in NC group exhibited high signal. The %ST2WI in the other groups was significantly lower than that in the NC group ($P < 0.05$). However, no significant differences were noted between the NPCS and BMSCs groups. The %ST2WI of the NPCS, BMSCs group was significantly higher than that of the ADSCs group ($P < 0.05$, **Figure 2C**). Therefore, it was speculated that the three kinds of cells in this study all maintained their extracellular matrix and water content in nucleus pulposus, but the NPCS and BMSCs displayed more effectively than ADSCs in IDD regeneration. However, the %ST2WI images signal of the L3-4, L4-5, and L5-6 discs in the DMEM group showed the lowest signal, suggesting significant degeneration for the DMEM group (**Figure 2B**).

Histological analysis and immunohistochemical analysis

In 12 weeks after transplantation, the NC group discs indicated of an oval-shaped NP and intact annulus fibrosis. The NPCS, BMSCs and ADSCs groups also showed abundant extracellular matrix in nucleus pulposus region, indicating regeneration of the nucleus pulposus. However, in the DMEM group, the border between the annulus fibrosus and the nucleus pulposus region was unclear. Extracellular matrix partial deleted and accompanied the collapse of the annulus fibrosus. The discs degeneration was obviously observed in the DMEM group (**Figure 3**).

For aggrecan immunofluorescence, the similar tendency appeared among the five groups, which means that immunofluorescence staining of the NPCS, BMSCs, ADSCs and NC groups all showed positive results while that of the DMEM group showed a negative result (**Figure 3**). Based on the above results, it was concluded

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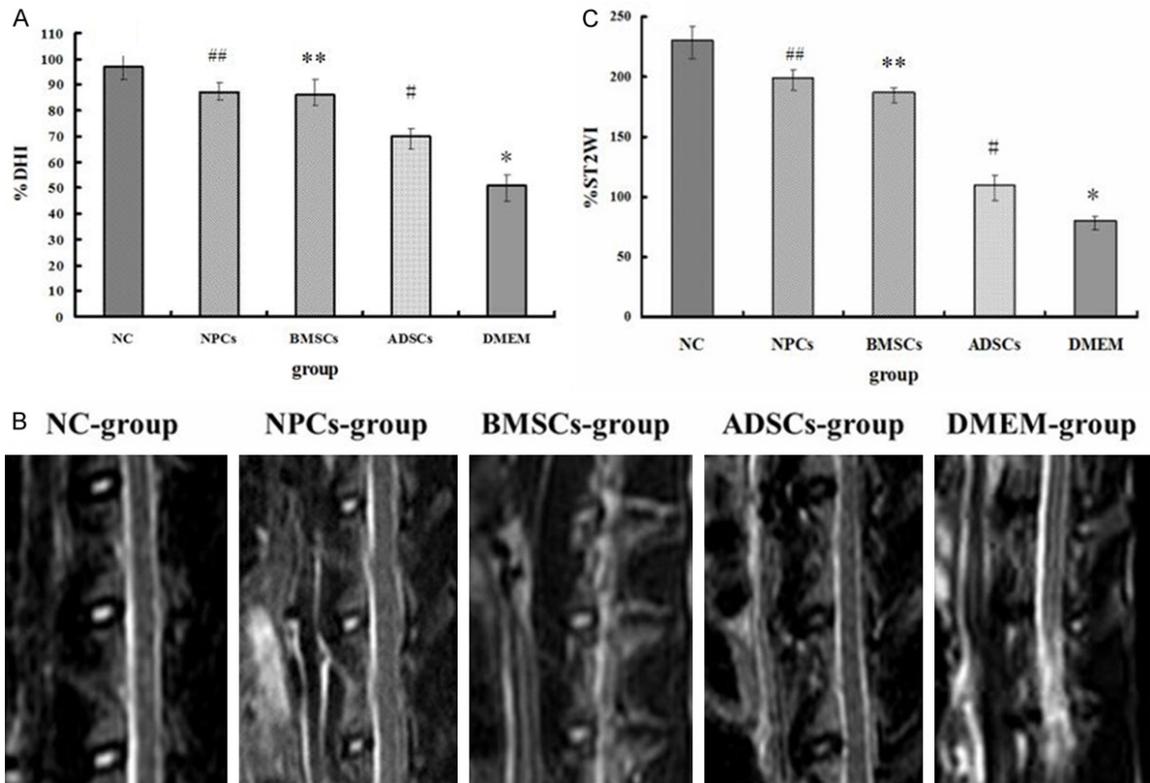


Figure 2. A: Bar graph demonstrating the %DHI obtained in 12 weeks after cell transplantation. * $P < 0.05$, compared with the NC group, NPCs group, BMSCs group and ADSCs group; # $P < 0.05$, compared with the NC group, NPCs group and BMSCs group. ** $P < 0.05$, compared with the NC group; ### $P < 0.05$, compared with the NC group. B: Representative MRI images in each group in 12 weeks after cell transplantation. C: Bar graph demonstrating the %ST2WI obtained in 12 weeks after cell transplantation. * $P < 0.05$, compared with the NC group, NPCs group, BMSCs group and ADSCs group; # $P < 0.05$, compared with the NC group, NPCs group and BMSCs group; ** $P < 0.05$, compared with the NC group; ### $P < 0.05$, compared with the NC group.

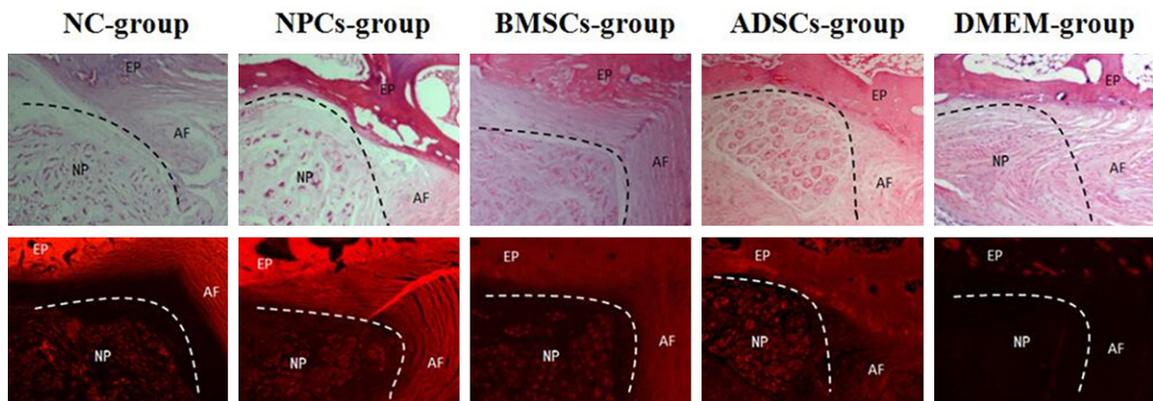


Figure 3. Representative photomicrographs showing H&E and aggrecan immunofluorescence stainings in each group in 12 weeks after transplantation (Scale Bar = 150 μm).

ed that the transplantations of the NPCs, BMSCs and ADSCs were favorable for the expressions of aggrecan, and further promoted the secretion of extra cellular matrix.

Biochemical analyses

The production ratios of sGAG/DNA levels in the NPCs, BMSCs, ADSCs and DMEM groups

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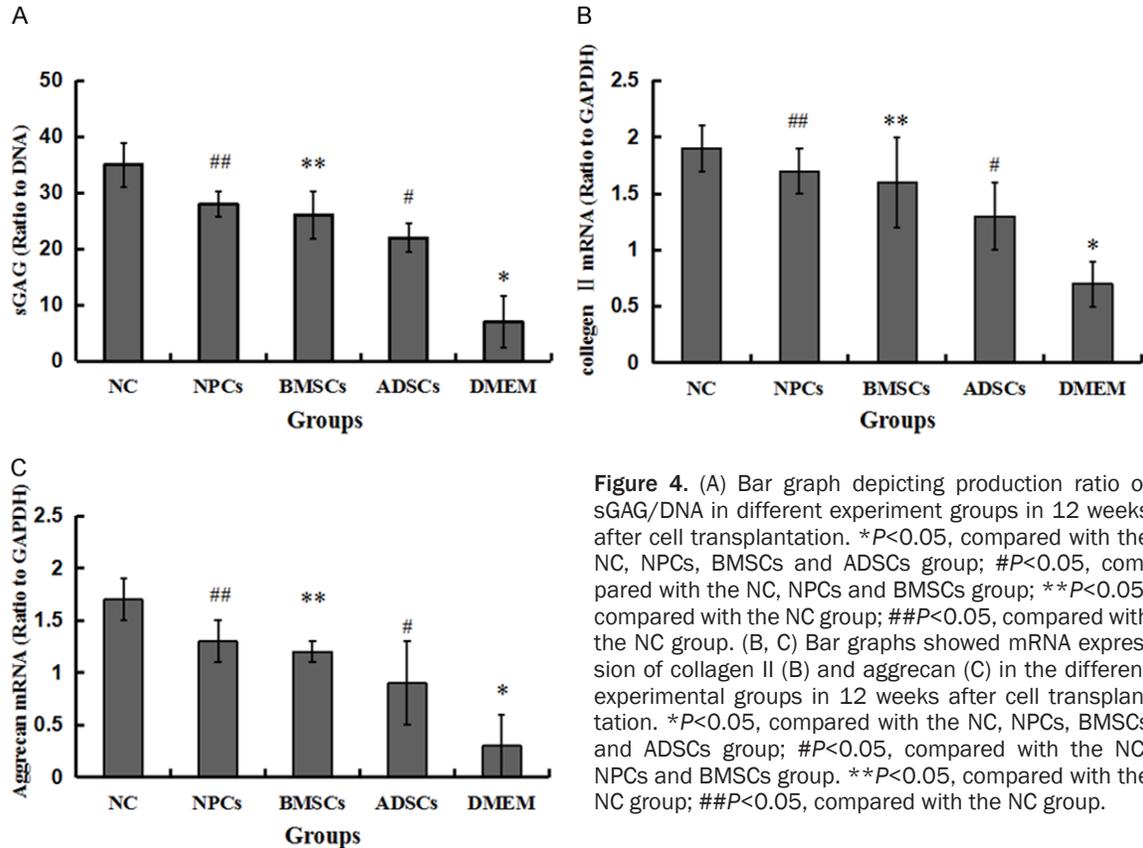


Figure 4. (A) Bar graph depicting production ratio of sGAG/DNA in different experiment groups in 12 weeks after cell transplantation. * $P < 0.05$, compared with the NC, NPCs, BMSCs and ADSCs group; # $P < 0.05$, compared with the NC, NPCs and BMSCs group; ** $P < 0.05$, compared with the NC group; ## $P < 0.05$, compared with the NC group. (B, C) Bar graphs showed mRNA expression of collagen II (B) and aggrecan (C) in the different experimental groups in 12 weeks after cell transplantation. * $P < 0.05$, compared with the NC, NPCs, BMSCs and ADSCs group; # $P < 0.05$, compared with the NC, NPCs and BMSCs group. ** $P < 0.05$, compared with the NC group; ## $P < 0.05$, compared with the NC group.

were lower than that in the NC group ($P < 0.05$). However, the sGAG/DNA ratios in the NPCs, BMSCs and ADSCs group were significantly higher than that in the DMEM group ($P < 0.05$). And the ratios in the NPCs and BMSCs were obviously higher than that in the ADSCs transplantation group ($P < 0.05$). But the difference between the NPCs and BMSCs groups was not statistically significant ($P > 0.05$, **Figure 4A**).

Real-time PCR

The collagen II and aggrecan gene expression levels in the NPCs, BMSCs, ADSCs and DMEM groups were significantly lower than those in the NC groups ($P < 0.05$) in 12 weeks after cell transplantation. However, a significant enhancement of gene expression was found in the NPCs, BMSCs and ADSCs groups when compared with the DMEM groups ($P < 0.05$). Collagen II and Aggrecan gene expressions in the NPCs and BMSCs groups were significantly higher than those in the ADSCs groups. The gene expression levels of the collagen II and Aggrecan were not significantly different ($P >$

0.05) between the NPCs and BMSCs groups (**Figure 4B, 4C**).

Discussion

Several researchers have utilized cell-transplantation therapy for disc regeneration. Autologous disc cell and stem cell therapies using animal models have been attempted to decelerate disc degeneration. NPCs, as the existing cells in a natural intervertebral disc, have been widely used in the treatment of intervertebral disc degeneration [9]. However, the isolation of IVD cells from a healthy IVD is unrealistic as it could result in damage to the disc [20]. Therefore, NPCs are not ideal seed cells for intervertebral disc degeneration treatment. Instead, stem cells can be differentiated to a NPCs-like phenotype in special condition. These characteristics make BMSCs recognized as the best option for cell therapy because of their accessibility and proliferative capability. In this study, we deemed the NPCs group as a positive control, the DMEM group as a negative control and the NC group as a normal control.

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Based on these groups, we represented systematic comparison of ADSCs and BMSCs with respect to their restorability in a rabbit disc degeneration model.

In 12 weeks after cells transplantation, the restoration effects of the degenerated disc were evaluated by X-ray, the T2-weighted signal intensity of MRI. In clinical testing, the MRI was the first and most sensitive evaluation index to intervertebral disc degenerated. Based on these parameters, disc regeneration was achieved successfully with water and proteoglycan restoration in NPCs, BMSCs and ADSCs groups. The height and MRI signal changes in this study were consistent with previous researches [15]. The nucleus pulposus signal from MRI decreased in the NPCs, BMSCs and ADSCs groups, but the morphological structure remained relatively intact. However, MRI observations revealed that the DMEM group showed a typical low signal sample in 12 weeks after cell transplantation, which demonstrated that the water content of nucleus pulposus decreased significantly and the intervertebral disc degeneration was serious. These data demonstrated that the injection of the DMEM could not cause a similar repair effect to the cell transplantation groups.

Also, the histological and immunofluorescence staining methods were used to observe the regenerative intervertebral disc. HE staining (**Figure 3**) displayed that the regeneration of the degenerated disc in the cells transplanted groups was better than the DMEM group. In addition, as the aggrecan was the specific extracellular matrix of the nucleus pulposus cells. The immunofluorescence staining was adopted to identify the expression of the extracellular matrix. In the cells transplanted groups, these immunofluorescence results suggested that the extracellular matrix expression was universally positive in nucleus pulposus region, as shown in **Figure 3**. The increased collagen may provide tensile strength and anchor the tissue to the bone, and aggrecan may contribute to high osmotic pressure for the absorption of water. But the extracellular matrix expression in the DMEM group was negative in 12 weeks after transplantation, which supported the disc degenerative procedure. Thus, the increased extracellular matrix may effectively assist in restoring the biological and mechanical functions of the degenerated discs. Based on the histological and immunofluorescence

staining results, delaying the degeneration and maintaining the function of the intervertebral disc can be achieved through stem cell transplantation.

Moreover, the ratio of the sGAG/DNA level and the real-time PCR confirmed that the repair effect of the BMSCs was better than the ADSCs, although degeneration scores have no significant difference among NPCs, BMSCs and ADSCs groups. It is noteworthy that stem cells, as a promising therapeutic strategy, have also been widely investigated in IDD repair and regeneration [21]. However, the BMSCs or ADSCs cannot synthesis the extracellular matrix the same as nucleus pulposus cells, although their amplification ability is stronger than nucleus pulposus cells [22]. But several studies [23-25] suggested that the residual NP cells within the discs may interact with the injected BMSCs or ADSCs to repair the degenerated discs. Growth factors released from the degenerate NPCs may effectively stimulate stem cells to produce type II collagen and aggrecan [26]. Strassburg *et al.* [27] suggested that cellular interactions between BMSCs or ADSCs and degenerate NP cells stimulate the endogenous degenerated NP cell population to regain a non-degenerate phenotype and consequently enhance matrix synthesis for self-repair. In addition, BMSCs exert a trophic effect on degenerate NP cells. When BMSCs was co-culture with either non-degenerate or degenerate NP cells, the mRNA expressions of growth factors including CDMP-1, TGF- β 1, IGF-1 and CTGF of BMSCs were enhanced. Also, BMSCs significantly increased mRNA expression for CDMP-1 and TGF- β 1 in degenerate NP cells following direct co-culture [21]. The increased growth factor gene expression might be the mechanism responsible for the observed BMSCs or ADSCs repair of intervertebral disc degeneration.

For another, in comparison with BMSCs, ADSCs were obtained more easily, but showed to be a little weaker in terms of the treatment effect. Some researchers have found that the ADSCs have an inferior differentiation capability to bone or cartilage compared to BMSCs when equal amounts of bioactive factors are present [14]. That may be the reason why the treatment effect of the ADSCs group was not as good as the BMSCs group. However, the ADSCs are conveniently abundant, which provides the stem cells a great advantage over its peers.

Despite the growing number of research data on cell-based experimental therapy for IDD, it is noteworthy that cell leak is inevitable during the injection process. Nowadays, much attention has been paid on the inhibition of cell leak. Frith et al. [28] developed an injectable hydrogel incorporating mesenchymal cells for intervertebral disc regeneration and proved that such a system could overcome the deficiency of cell leak. Therefore, the incorporation between cell injection and microspheres will be used in the intervertebral disc regeneration in our following experiments.

In this study, the results indicated that BMSCs showed a stronger potential in the extracellular matrix synthesis and water content recovery of the discs than ADSCs. Furthermore, the disc degeneration treatment effect of the BMSCs transplantation was similar to that of NPCs, which appears to be an ideal substitute for NPCs.

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Disclosure of conflict of interest

None.

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