Low-back pain is one of the largest medical and social problems faced in the world today. Intervertebral disc degeneration is considered to be one of the major causes of low-back pain. Once the degenerative process is activated, it is difficult to decelerate. The main causes of degeneration are unknown, but the process is thought to involve the loss of cells and decreased function due to apoptosis. These factors lead to a decreased disc matrix, which is composed of proteoglycan and collagen. Destruction of the IVD follows, with an associated loss of function.

Current treatment options for degenerated disc disease include symptomatic treatment (analgesics, physiotherapy, and cognitive treatment) or surgical treatment (spinal fusion or total disc replacement using an artificial disc); however, none of these methods addresses the underlying problem. In an attempt to find biological treatments to prevent or reverse disc degeneration, cell transplantation therapies have been proposed. To achieve the most effective tissue regeneration, it is desirable to restore the normal cellular constituents of tissues. Therefore, when disc degeneration is induced by the evacuation of NPCs and matrix, a therapy that supplies original NPCs would be appropriate. Autologous NPC transplantation has become one of the major techniques in attempting to achieve the most effective tissue regeneration.

Labortatory investigation

Transplantation of mesenchymal stem cells and nucleus pulposus cells in a degenerative disc model in rabbits: a comparison of 2 cell types as potential candidates for disc regeneration

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Object. The aim of this study was to compare transplanted mesenchymal stem cells (MSCs) with nucleus pulposus cells (NPCs) in a degenerative disc model in rabbits to determine the better candidate for disc cell therapy.

Methods. Mesenchymal stem cells and NPCs were transplanted in a rabbit model of disc degeneration. Changes in disc height, according to plain radiography, T2-weighted signal intensity on MR imaging, histology, sulfated glycosaminoglycan (sGAG)/DNA, and associated gene expression levels, were evaluated among healthy controls without surgery, sham-operated animals in which only disc degeneration was induced, MSC-transplanted animals, and NPC-transplanted animals for a 16-week period.

Results. Sixteen weeks after cell transplantation, in the MSC- and NPC-transplanted groups, the decline in the disc height index was reduced and T2-weighted signal intensity increased compared with the sham-operated group. Safranin O staining showed a high GAG content, which was also supported by sGAG/DNA assessment. Disc regeneration was also confirmed at the gene expression level using real-time polymerase chain reaction. However, no significant differences in expression were found between the NPC- and MSC-transplanted groups.

Conclusions. Study data showed that MSC transplantation is effective for the treatment of disc degeneration and seems to be an ideal substitute for NPCs. (DOI: 10.3171/2010.11.SPINE10285)

Key Words • intervertebral disc degeneration • cell transplantation • mesenchymal stem cell • nucleus pulposus

Abbreviations used in this paper: DHI = disc height index; DMEM = Dulbecco modified Eagle medium; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IVD = intervertebral disc; MSC = mesenchymal stem cell; NP = nucleus pulposus; NPC = nucleus pulposus cell; RT-PCR = real-time polymerase chain reaction; sGAG = sulfated glycosaminoglycan.
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to prevent IVD degeneration in animal models.\textsuperscript{8,11,21–23,31–33} This approach is also the only one currently applied in clinical trials.\textsuperscript{9}

Although the outcome in these studies was mostly promising, harvesting autologous NPCs may not be possible without creating injury to the donor disc. Considering that such cell harvesting is dangerous and can itself initiate degeneration, access to healthy NPCs, especially autologous cells, is very limited in clinical settings. For these reasons, an alternative cell source is adult stem cells, in particular, bone marrow–derived MSCs. Mesenchymal stem cells are multipotent and have the ability to differentiate into an NP-like phenotype when appropriately stimulated.\textsuperscript{17,24–26} Some in vivo experiments have already shown promising results.\textsuperscript{6,12,13,16,28–30,37} However, it is not clear whether MSCs can equal or surpass autologous NPCs in terms of regenerative effects. To our knowledge, an in vivo comparison of the regenerative capacity of NPCs and MSCs in a controlled experimental setting has not been attempted. It is unknown whether a disc height, proteoglycan accumulation, and gene expression similar to those of transplanted NPCs can be achieved from the transplanted MSCs. Therefore, in this study, we examined the regenerative effects of rabbit MSCs and NPCs by performing a comparative study in a classic disc degeneration model in rabbits. Our observations could provide more experimental evidence on choosing a suitable cell source for cell-based therapy in disc regeneration.

Methods

Animal experiments were performed according to a protocol approved by the University Committee on the Use and Care of Animals at Sichuan University. Forty-eight white New Zealand rabbits (mean weight 2.42 ± 0.22 kg) were divided into 4 groups: 12 healthy controls without surgery, 12 sham-operated rabbits that underwent NP aspiration only, 12 NPC–transplanted animals, and 12 MSC–transplanted animals. The rabbits involved in the experiment underwent surgery at regions L3–4, L4–5, and L5–6 and were evaluated at 2, 4, 8, and 16 weeks after transplantation (3 animals at each time point).

Inducing Disc Degeneration in the Rabbit Groups

The surgical procedure performed has been described elsewhere.\textsuperscript{32} Briefly, anesthesia for all surgical procedures was maintained through the inhalation of isoflurane, and the operative field was prepared in a sterile fashion. Nucleus pulposus cell culture was performed according to the method described previously.\textsuperscript{33} Isolation of the Cells and Culture

Nucleus pulposus cell culture was performed according to the method described previously.\textsuperscript{33} Briefly, the aspirated NP was digested with 0.2% Pronase (Sigma Chemical) for 1 hour, followed by 0.025% collagenase (Sigma Chemical) for 8 hours. Nucleus pulposus cells were placed in an incubator (5% CO\textsubscript{2} and 95% room air at 37°C) in DMEM and Ham F-12 medium. The medium was changed every 2 days.

Mesenchymal stem cells were collected via aspiration from the femoral bone using an 18-gauge needle, collecting 10 ml of marrow blood into 1000 U of heparin, as described previously.\textsuperscript{30} The marrow blood was filtered through a cell strainer for excluding any fatty tissues and blood clots, carefully poured over 20 ml of Nycodan Prep 1.077 Animal (Axis-Shield PoC AS), and processed in a centrifuge at 600 G for 30 minutes. Mononucleated cells were recovered from the middle layer, washed 3 times with phosphate-buffered saline, and cultured in 25-cm\textsuperscript{2} flasks in low-glucose DMEM (Gibco) containing 10% fetal bovine serum (Gibco) and antibiotics (penicillin G, 100 U/ml; streptomycin, 0.1 mg/ml; and amphotericin B, 0.25 μg/ml) at 37°C under 5% CO\textsubscript{2}. The MSCs were cultured until 80% confluenve over about 12–15 days to keep better viability for cell transplantation.

Transplantation of NPCs and MSCs

After 2 weeks, NPCs and MSCs were expanded through 1 passage and were gathered for transplantation. As the previous surgeries had been performed from the right lateral side, 0.02 ml of DMEM containing 10\textsuperscript{5} NPCs or MSCs was introduced through the left side of the annulus fibrosus using a 27-gauge needle with a microinjector. To prove the injections were in the proper location, we fashioned a hand-made stopper that was 5 mm in length by cutting a needle cap. The location of the needle tip was detected by sensing an increasing resistance as the needle passed through the annulus and was confirmed with the hand-made stopper to ensure that cells were properly placed into the NP. After injection, all wounds were closed in a routine manner. No secondary operations were performed in the sham operation group.

Radiographic and MR Imaging Analysis

Lateral plain radiographs were obtained while the animals in all groups were under inhalation anesthesia before IVD sample harvest. A previously described procedure was followed to measure the vertebral body and disc heights for calculating the DHI.\textsuperscript{18} Magnetic resonance images were also obtained, as were sagittal images through the lumbar spine (spin echo, TR 3200 seconds, TE 130 seconds, number of excitations 20, field of view 14 cm, slice thickness 3 mm, and no phase wrap). The signal intensity of each disc on T2-weighted imaging was quantified using Analyze 7.0 software (AnalyzeDirect).\textsuperscript{35}

Macroscopic Observations

At 16 weeks after transplantation, 3 rabbits from each group were killed with an excess dose of pentobarbital.
(Euthanasia B solution; Henry Schein, Inc.) and their spines were harvested. The L3–4 discs were isolated with both upper and lower vertebral bodies completely attached and were cut longitudinally at the center of the disc for macroscopic evaluation.

**Histological Analysis**

After macroscopic evaluation, L3–4 and L4–5 discs were individually processed for histological studies. Each disc was fixed in 10% neutral-buffered formalin for 1 week, decalcified in 22.5% formic acid and 10% sodium citrate for approximately 2–3 days, and processed for paraffin embedding and sectioning into sagittal sections (10 μm thick) using a microtome. Sections were stained with H & E and Safranin O for evaluation. A histological grading system devised by Masuda et al. was used for evaluation of the degenerative changes. Briefly, the slides were graded based on the histological appearance of 4 parameters: the annulus fibrosus, the border between the annulus fibrosus and the NP, the cellularity of the NP, and the matrix of the NP. Each of the 4 parameters was given a grade of 1, 2, or 3. The sum of the grades for each parameter yielded a total grade. Total grades ranged from 4 to 12, with 12 representing severe degeneration.

**Quantitative RT-PCR**

The L5–6 discs were harvested mainly to extract total RNA for RT-PCR and biochemical analyses. Total RNA was isolated from the NP by using the Trizol reagent (Invitrogen) followed by the RNasey mini kit (Qiagen), and DNA was digested with a RNase-free DNase set (Qiagen) according to the manufacturer’s protocol. Reverse transcription was performed with TaqMan reverse transcription reagents (Applied Biosystems). Type II collagen, aggrecan, and GAPDH gene expression were quantified with RT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems). A positive standard curve for each primer was obtained using RT-PCR with a serially diluted cDNA sample mixture. Quantities of gene expression of aggrecan and Type II collagens were calculated with standard samples and were normalized with GAPDH.

**Biochemical Analyses**

After discs were isolated from each level, the dissected NP was digested with papain solution (280 μg/ml in 50 mM sodium phosphate, pH 6.5, containing 5 mM N-acetylcysteine and 50 mM EDTA) for 24 hours at 65°C. The proteoglycan in the digested solution, mainly sGAG, was assayed using the dimethylmethylene blue method. Total sGAG in the disc for each group was normalized according to the tested DNA amount, and then the ratio of sGAG/DNA was compared and reported.

**Statistical Analysis**

Results are presented as the means ± SDs. The significance of differences among the 4 groups was determined using the t-test. Differences were regarded as significant when p was < 0.05.
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Histological Analysis

Histological analysis at 16 weeks after cell transplantation also showed the significant regenerative effects of cell transplantation (Fig. 4). Control group discs showed oval-shaped NP, without collapse of the inner annular structure, which constitutes at least one-half of the disc area at the midsagittal cross-section. The annulus fibrosis was intact, and the border between the annulus fibrosis and the NP was clearly defined. In the sham-operated group, the NP area in the 21-gauge needle–aspirated disc decreased and became irregular, accompanying the collapse of the inner annulus morphology from 4 weeks. Fibrosis in the nucleus due to cell invasion from the surrounding region was also found. Conversely, discs in the NPC-treated group still displayed features of disc degeneration to a certain extent at 16 weeks posttreatment but were more even and regular than those in the sham-operated groups. Mesenchymal stem cell–transplanted discs showed a relatively preserved inner annulus structure with minimal fibrosis in the nucleus region. The positively stained areas in the sham-operated and NPC-treated groups were smaller and weaker than those in the MSC-treated group.

Semiquantitative histological scores were 4 ± 0 for healthy controls, 7.4 ± 0.9 for the MSC-transplanted group, 6.2 ± 0.7 for the NPC-transplanted group, and 10.4 ± 1.4 for the sham group. Statistical analysis showed that the histological scores of the 3 groups after degeneration induction significantly increased compared with the control group (p < 0.05). The score of the sham group was significantly higher than in NPC and MSC groups (p < 0.05). However, there was no significant difference between the NPC and MSC groups.

**Fig. 2.** Left: Representative lateral radiographs showing lumbar levels in healthy control (NC), sham-operated (SO), NPC-transplanted (NP), and MSC-transplanted (MSC) groups at 16 weeks after transplantation. The NP and MSC groups showed a lower decrease in the DHI compared with the SO group. Furthermore, sham animals showed ventral osteophyte formation indicating progression of the degenerative process. Right: Graph demonstrating the DHI obtained at 0, 2, 4, 6, and 16 weeks after cell transplantation. Restoration of the DHI began 4 weeks after cell transplantation, whereas discs from sham animals showed a constant decrease. *p < 0.05, compared with the SO group; #p < 0.05, compared with the NC group.

**Fig. 3.** Representative MR images (left) and quantitative analysis (right) of T2-weighted signal intensity of the different experimental groups at 16 weeks after cell transplantation. Significant recovery of T2-weighted signal intensity was seen in the L3–4, L4–5, and L5–6 discs in the MSC and NP groups compared with the SO group. *p < 0.05, in a comparison between the MSC and SO group; **p < 0.05, in a comparison between the NP and SO group.
Real-Time PCR

Figure 5 illustrates that aggrecan and Type II collagen gene expressions in the sham-operated, NPC, and MSC groups were significantly lower than in healthy controls (p < 0.05). A significant upregulation of gene expression was found in the NPC and MSC groups compared with the sham group (p < 0.05 for Type II collagen gene expression and p < 0.01 for aggrecan gene expression). The MSC group showed a higher mRNA expression of aggrecan than did the NPC group, although the increase was not significant (p > 0.05). The mRNA level of Type II collagen was not significantly different (p > 0.05) between the NPC and MSC groups.

Biochemical Analyses

The production ratio of sGAG/DNA levels in the NPC and MSC groups was lower than in the healthy controls (p < 0.05; Fig. 6). Both the discs with NPC and MSC transplantation showed significant increases in the sGAG/DNA ratio compared with the sham-operated group (p < 0.05). However, the difference between the NPC and MSC groups was not statistically significant (p > 0.05).

Discussion

Intervertebral disc degeneration is mostly characterized by changes in disc morphology and the composition of the extracellular matrix, as well as by the loss of disc cells and water content. Cell therapy provides a potential approach for treating degenerative disc disease. Authors of previous animal studies have reported the use of various cell types, such as NPCs, chondrocytes, and MSCs. To our knowledge, this study represents the first systematic comparison of MSCs and NPCs with respect to their regeneration capacity in a rabbit disc degeneration model.

Since Nishimura and Mochida demonstrated that the reinsertion of autologous NP tissue slowed degeneration in the rat IVD, the regeneration of disc tissue is likely to be achieved by transplanting cells that possess many characteristics of the native cells. Although transplant-
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ed NPCs have proven to be effective in clinical trials, the efficiency of MSC transplantation is still worthy of further investigation. Our findings indicated that transplanted MSCs have abilities comparable with NPCs: 1) to preserve disc structure and maintain disc height with minimal degeneration at all time points; 2) to maintain the gene expression levels of proteoglycan and collagen Type II; and 3) to produce almost the same amount of proteoglycan. Therefore, our data support the idea that transplanted MSCs and NPCs have essentially similar effects in inhibiting disc degeneration.

Based on the present data, it appears that both MSCs and NPCs possess the appropriate characteristics for cell-based therapy in disc regeneration. For clinical applications, however, the choice of cell type is influenced by various considerations. The density of NPCs in IVDs is low, and harvest may create iatrogenic degeneration in the donor disc. Moreover, expansion of the NPCs is difficult because they easily lose their phenotype characteristics in monolayer growth. In contrast, MSCs are easy to harvest, isolate, and grow, with minimum involvement of in vitro techniques. Adult MSCs are multipotent stem cells that have been found in almost every organ in adulthood. These cells have high plasticity and the capacity to differentiate into an NP-like phenotype when appropriately stimulated. In addition, MSCs are considered suitable for autologous and allogeneic transplantation, as they lack the expression of human leukocyte antigen class II antigens. Therefore, MSCs might be of greater value than NPCs in a clinical situation.

A number of animal models of IVD degeneration have been described in the literature, each with its own advantages and disadvantages for the purposes of testing novel therapies. Although the optimum model for disc degeneration would be a nonhuman primate model, there is a degree of validation in the use of a small animal, such as the rabbit, when considering the cost of developing therapeutic approaches. In smaller animals, such as mice or rats, IVDs are very small, and thus measuring disc heights on simple radiographs or MR images is very difficult, although it is possible to measure the proteoglycan production, disc cell proliferation, and mRNA levels. Therefore, we chose the rabbit for our experimental model. This model has been used in many cell transplantation studies. It mimics nucleotomy, a popular surgical procedure used in the treatment of herniated lumbar discs. Nucleus pulposus tissue is evacuated in this procedure, and it causes partial degeneration shown as NPC loss, resulting in a decrease in proteoglycan content and disc height. In our study, it is obvious from the results of histological, radiographic, and MR imaging analysis in the sham-operated group that degeneration was successfully induced.

The approach to delivering cells into the degenerated discs of rabbits was established by Okuma et al. In that study, decelerating disc degeneration was successful by reinserting NPCs that were activated by coculture with annulus fibrosus cells. Sakai et al. used biodegradable carriers, such as atelocollagen gel, for the delivery of cells. The benefit of using a gel carrier lies in mimicking the natural environment and increasing possible survival for the injected cells. However, in the present study, we did not use a coculture method or carriers because it is difficult to maintain the same baseline for 2 cell types after external manipulation. The result raises the possibility that the native IVD cells in vivo could induce MSC differentiation without the need for external manipulation. However, the number of surviving cells or the percentage of cells that differentiate toward chondrocyte-like cells remains to be investigated in future studies.

In a previous study, a second DMEM injection or a needle puncture was performed in the sham group. Almost the same extent of degenerative change was found between the 2 groups. Similar results were also reported in other studies. These data demonstrated that the injection of DMEM or saline did not cause a regenerative effect similar to cell transplantation. In the present study, we mainly focused on comparing the regenerative capacity of MSCs and NPCs in the same animal model; therefore, we did not perform a second operation in the sham rabbits.

Although the current study demonstrated successful results in retarding disc degeneration, several issues remain for future resolution. The first issue is the need for an animal model more closely mimicking human anatomy and physiology. One reason for this is that the IVD cells of rabbits consist mainly of notochordal NPCs, as opposed to the phenotype of adult human NPCs. Another reason is that animal models usually have a better IVD nutrition status compared with that of the degenerating human IVD. However, because the process of IVD degeneration is not uniform, it is almost impossible to establish an animal model for naturally occurring human disc degeneration. A second issue in this study is that the optimal number of MSCs is unknown. Although the transplantation of approximately 1 million cells per IVD effectively led to the regeneration of IVDs in a rabbit model, this number is not necessarily optimal for achieving similar data in humans. Therefore, further investigation is essential. A third issue is the need for a long-term study utilizing radiological, histological, and biochemical analyses to more definitively show that the transplantation of MSCs would be a useful treatment for degenerating IVDs. Due to problems of breeding and research facility expenses, we could only follow up the animals for
16 weeks after transplantation. For clinical applications, longer-term data to look for problems, such as ossification and spine fusion, subsequent to MSCs transplantation is needed.

Conclusions

In summary, transplanted MSCs and NPCs had comparable effects in inhibiting disc degeneration. Because it is easier to obtain MSCs than NPCs, there is a good chance that the former may become an alternative source of cells for broad clinical applications.

Disclosure

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Author contributions to the study and manuscript preparation include the following: Conception and design: H Liu. Acquisition of data: Feng, X Zhao, Shi, X Liu, X Zhao. Analysis and interpretation of data: Feng, X Zhao. Drafting the article: H Liu, H Zhang. Review and approval of the manuscript and approved it for submission: all authors. Statistical analysis: Feng, X Zhao, Chen. Administrative/technical/material support: Feng, W Zhang, Wang. Study supervision: H Liu.

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