Construction of a tissue engineered intervertebral disc with high biological activity using an allogeneic intervertebral disc supplemented with transfected nucleus pulposus cells expressing exogenous dopamine beta-hydroxylase

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ABSTRACT. This study addressed the *in vitro* construction and biological activity of tissue engineered intervertebral discs with exogenous human dopamine beta-hydroxylase (DBH) nucleus pulposus cells. pSNAV2.0-DBH expression plasmids were utilized to enhance the survival rates of intervertebral disc tissue cells. Various concentrations of transfected nucleus pulposus cells were injected into the discs, and *DBH* mRNA expression was determined using polymerase chain reaction amplification. Polysaccharide content and

total collagen protein content in the engineered disc nucleus pulposus tissue were determined. The visible fluorescence intensities of the 1×10^5 and 1×10^6 groups *vs* the 1×10^4 group were significantly increased ($P < 0.05$); no significant difference was observed between the 1×10^5 and 1×10^6 groups ($P > 0.05$) at 7 days after injection. *DBH* mRNA expression could be detected in the all but the EGFP control group at 14 days culture. No significant difference was observed in the protein content between the 1×10^4 and the control groups at various times, while the protein content was significantly higher in the 1×10^5 *vs* the control and the 1×10^4 groups at 7-, 14-, and 21-day cultures. These results demonstrate that a tissue engineered intervertebral disc with high biological activity can be constructed by utilizing allogeneic intervertebral discs stored in liquid nitrogen and a 1×10^5 transfected nucleus pulposus cell complex with *in vitro* culture for 14 days. This model can be used in animal experiments to study the biological activity of the engineered discs.

Key words: Composite expression; Dopamine beta-hydroxylase; Nucleus pulposus cell; Tissue engineering; Intervertebral disc

INTRODUCTION

Degeneration of the intervertebral discs is the primary cause of pain in the waist and lower extremities and of neck and shoulder pain in adults. With the prolonging of the average life span, the morbidity of spinal degenerative diseases has been rising significantly. The fast pace of life and the high work pressure in modern societies are causing increasing numbers of young adults to suffer from pain in their waists and lower extremities. The intervertebral disc tissue bears the weight of the human head, trunk, and upper limbs. It is more likely to be susceptible to strain from daily life and work than are other tissues. Intervertebral discs are susceptible to degeneration due to a lack of blood supply and a poor intrinsic repair capacity (Lee et al., 2012; Hudson et al., 2013). Intervertebral disc protrusion, spinal canal stenosis, and lumbar spondylolisthesis caused by degeneration of intervertebral discs are frequently occurring diseases seen in clinical orthopedics (Bowles et al., 2012). These diseases have aroused wide concern from clinicians and biomedical researchers in recent decades due to their high morbidities. Standard currently available surgical treatments including removal of the nucleus pulposus (NP) and spinal fusion are not able to solve all of the problems relating to degenerative intervertebral disc diseases. An exploratory goal of research in this field is to be able to remove the diseased intervertebral disc tissue and reconstruct the spinal stability and mobility at the same time.

Animal experiments and preliminary clinical applications with respect to intervertebral disc transplantation have shown that the intervertebral disc is able to survive after transplantation and although it demonstrates degenerative changes in biochemical metabolism and histology, it can still meet the biomechanical demands of physiological activities (Lazebnik et al., 2011). Repair of the degenerative intervertebral disc by using biotechnology has been an important developmental direction in the spinal surgery field in recent years, which aims

to defer the process of intervertebral disc degeneration by influencing cellular metabolism and adjusting the internal disc content of water, protein polysaccharide, and collagen (Silva-Correia et al., 2011; Mehrkens et al., 2012). Direct injection of functioning cells or cytokines into degenerative intervertebral disc tissue is a frequently studied method with demonstrated positive effects upon current usage in patients (Pan et al., 2012).

This study aimed to construct a tissue engineered intervertebral disc *in vitro* using an allogeneic intervertebral disc as support material and NP cells expressing exogenous human dopamine beta-hydroxylase (DBH) as seed cells, and to observe the biological activities and functions thereof.

MATERIAL AND METHODS

Material

Six purebred beagles were provided by the Shanghai Laboratory Animal Centre of The Chinese Academy of Sciences. Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) culture medium and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT, USA); dimethyl sulfoxide (DMSO) was purchased from Beijing Bole Bioscience Development Co., Ltd. (Beijing, China). Ethidium bromide (EB) and fluorescein diacetate (FDA) were purchased from Shanghai Shrek Biotechnology Co., Ltd. (Shanghai, China). Dimethylmethylene blue (DMMB) was purchased from Amresco (Solon, OH, USA) and the protein quantification kit was purchased from Fermentas (Amherst, NY, USA). The lipofectamine liposome transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). The reverse transcription -polymerase chain reaction (RT-PCR) reagents were purchased from Shanghai Meiji Biotechnology Co., Ltd. (Shanghai, China).

Collection and preservation of intervertebral disc cells

The six beagles were sacrificed under aseptic conditions. The upper sections were detached from the T9-10 intervertebral discs and the lower sections were detached from L7-S1. The surrounding soft tissues, vertebral plates, and the pedicles of the vertebral arches were removed. The capitulum costi were detached. The intervertebral discs were cut with a hacksaw at approximately 3 mm from the upper and lower sides. The intervertebral discs were washed with normal saline while being sawed to prevent the heat generated from damaging the cartilage endplate. A total of 5-7 intervertebral discs were taken from each beagle totaling 32 specimens. Each intervertebral disc was washed repeatedly with normal saline after being taken out completely, immediately placed into a 50-mL centrifuge tube (Constar), and stored in a liquid nitrogen container after addition of DMEM/F12 (1:1) culture medium containing 10% FCS and DMSO (Moss et al., 2011).

Generation of the pSNAV2-DBH construct

*Age*I and *Nhe*I were used to cut the pDC316-DBH-IRES-EGFP plasmid. An approximately 2.2-kb fragment was recovered. *Age*I and *Nhe*I were also used to cut the pSNAV2.0-LacZa plasmid; an approximately 10.3-kb fragment was recovered. The two fragments were

ligated and transformed into *Escherichia coli*. Single clones containing the joined fragments were selected for culture. PCR analysis, and *Age*I and *Nhe*I enzyme digestion were used to confirm construct identity.

Method of constructing tissue engineered intervertebral discs *in vitro*

Methylene blue marking was performed before construction to observe the distribution of the suspension injected within the intervertebral disc. The volume of the cell suspension injected was 20 μL. Then, 5×10^5, 5×10^6, and 5×10^7 cells/mL suspensions were prepared with the transfected NP cells obtained according to a previously reported method (Simard et al., 2015). A 5×10^4 cells/mL cell suspension was prepared for the control group. Cell suspensions at different concentrations were loaded into 1 mL syringes connected to 16G needles for later use. The intervertebral disc cryogenic vials stored in liquid nitrogen were directly soaked in a water bath at 37°C for rewarming, at a rewarming rate of approximately 100°C/min. Each intervertebral disc was washed 3 times with phosphate buffered saline. The 36 intervertebral discs were divided into four groups at random with eight in each group, consisting of: Group A: enhanced green fluorescent protein (EGFP) control group: 20 μL 5×10^6 cells/mL NP-EGFP cell suspension was injected; Group B: 1×10^6 Group: 20 μL 5×10^7 cells/mL NP-hBMP cell suspension was injected; Group C: 1×10^5 Group: 20 μL 5×10^6 cells/mL NP-hBMP cell suspension was injected; Group D: 1×10^4 Group: 20 μL 5×10^5 cells/mL NP-hBMP cell suspension was injected. The injection method consisted of a direct penetration of 5-7 mm into the fibrous ring of the rear middle part of the intervertebral disc. A uniform pressure was applied slowly until 20 μL was injected as indicated by the syringe scale. The syringe needle was pulled back 3 mm and completely extracted after 1 min. The intervertebral discs were placed into 50 mL centrifuge tubes immediately after injection. The centrifuge tubes were vertically placed into a cell incubator for continuous culture after addition of 20 mL complete medium (Clouet et al., 2009).

General observation of the tissue engineered intervertebral discs

At 7, 14, and 21 days after culture, the general appearance of the intervertebral discs in various groups was observed and detected. The observation and detection items included: height and area of intervertebral disc, the relationship between the fibrous ring of the intervertebral disc and the upper and lower endplates, and bone changes between the upper and lower endplates.

Survival of cells injected into the tissue engineered intervertebral disc

At 7, 14, and 21 days after culture, a single intervertebral disc was taken in each group at each time respectively for the generation of frozen sections. The sections were observed immediately with a fluorescence microscope. Images were collected in six visual fields around the needle passage point under the same exposure conditions. The Image Pro Plus6.0 (Media Cybernetics, MD, USA) software was used to analyze the fluorescent brightness, which was in direct proportion to the number of luminant fluorescent cells present in the field. A comparison was made among fluorescence intensities in different groups at different times.

Expression of *DBH* mRNA in tissue engineered intervertebral discs

The NP tissue of the intervertebral discs in each group was divided into 3 fractions. One was subjected to *DBH* mRNA detection; one was subjected to protein polysaccharide detection; and the other was subjected to total collagen detection. *DBH* gene expression was determined in the intervertebral disc tissues of the 1×10^5 group and the EGFP control group at 7, 14, and 21 days after culture by RT-PCR. Using DBH-F: 5'- ATT CCC CTC TAC CGA GAT AG -3' and DBH-R: 5'- TGG TGC TCT GTG GTG TTC -3' as the upstream and downstream primers, the NP tissue containing the *DBH* gene would be expected to support amplification of a 492-bp fragment. Total cellular RNA was extracted in accordance with the regular TRIzol method for extracting tissue RNA. The RNA quality was identified. The RNA concentration and purity were determined. The RT-PCR detection was conducted as per the two-step method (Calderon et al., 2010).

Detection of proteoglycan (PG) content

The PG content within the NP tissue of tissue engineered intervertebral discs in different groups at different times was determined using DMMB staining, described briefly as follows: After removal, the NP tissue was quantified and divided into two equal fractions. One was weighed after being dried and the other was cut into 1 mm^3 pieces with a pair of ophthalmic scissors, digested in an incubator at 60°C for 24 h with papain solution containing 20 mg/mL Ca^{2+} and Mg^{2+}, and centrifuged at 3000 *g* for 5 min. Subsequently, 150 μL supernatant was added to 96-well microplates containing standard S-glycosaminoglycan (GAG) at different concentrations, and 30 mg/L DMMB was added simultaneously with a multi-nozzle sample injector. The absorbance was measured with a 650 nm microplate reader 1 min after color development. A standard curve was plotted and the PG content in the tissue was quantified. Specifically, the PG content in the aliquot was calculated, used to determine the total amount of PG in the tissue fraction, and converted into units in g/mg (dry weight) using the dried weight of the other fraction as a reference.

Determination of collagen content

The content of collagen in the NP tissue was determined using the hydroxyproline detection method. Specific procedures were in accordance with the instructions for the hydroxyproline detection kit. Briefly, these were as follows: a quantified amount of NP tissue was cut into 1-mm^3 pieces, hydrolyzed for 30 min after addition of alkaline hydrolysate in a water bath filled with boiling water, and centrifuged at 3500 *g* for 20 min after the pH was adjusted to 6-6.8 and following addition of activated carbon. The supernatant was removed and detection liquid was added and well mixed with the pellet. The sample was placed in a water bath at 60°C for 30 min, centrifuged at 3000 *g* for 15 min, and subjected to optical path colorimetric detection at 550 nm and 1 cm after the supernatant was removed. The absorbance was measured after distilled water was used to calibrate the colorimeter to zero. The computational formula used to determine collagen content was as follows: hydroxyproline content (μg/mL) $= (A_{determination\ tube} - A_{blank\ tube}) / (A_{standard\ tube} - A_{blank\ tube})$ x content in the standard tube (5 μg/mL) x total volume of hydrolysate (mL) / sampling volume (mL) collagen (μg/mL).

Statistical analysis

The SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Variance analysis was used for comparisons among groups and the q test was conducted for pairwise comparisons among groups.

RESULTS

Construction and identification of pSNAV2.0-DBH plasmid

The *Age*I and *Nhe*I restriction enzymes were used to digest the pDC316-DBH-IRES-EGFP and pSNAV2.0-LacZa plasmid. T4 DNA ligase was used to join the 13.6-kb vector and 3.4-kb *DBH* fragments. A positive *DBH* product band could be observed in the successfully constructed plasmid using PCR (Figure 1).
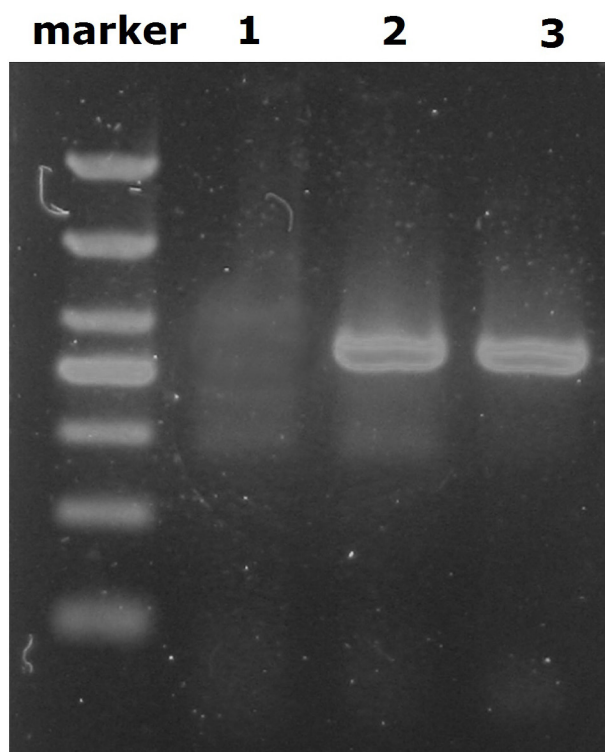

Figure 1. PCR identification of the pSNAV2.0-DBM plasmid. *Lane 1* = Negative control; *lane 2* = positive control (PCR product with the original BMP7 plasmid as the template); *lane 3* = pSNAV2.0-DBM plasmid; this PCR product was consistent with that of the positive control. Marker = DL2000. PCR, polymerase chain reaction.

The pSNAV2.0-DBH plasmid was cleaved by *Age*I and *Nhe*I to identify the target fragments. It was observed that there were two bands, 13.6 and 3.4 kb, as expected. The electrophoresis result is shown in Figure 2.
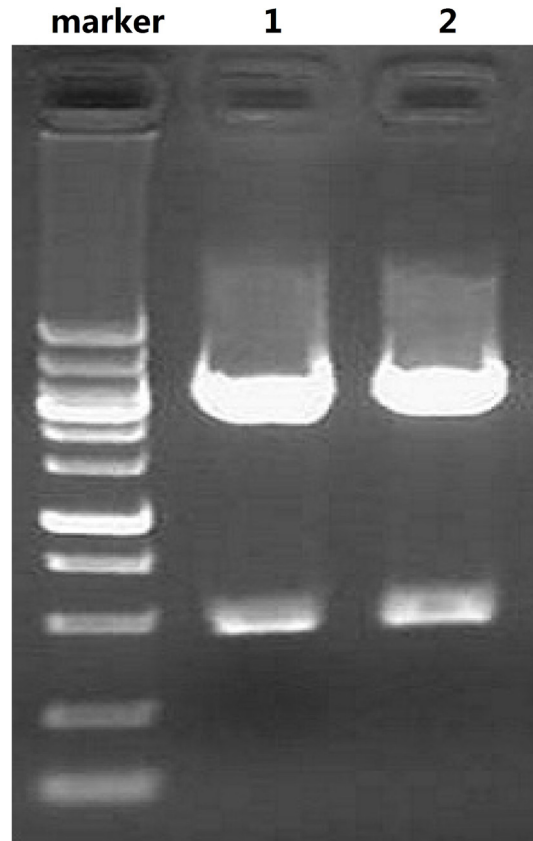
Figure 2. Electropherogram of the PCR products from the pSNAV2.0-DBM plasmid following double digestion with *Eco*RI + *Sal*I (*lanes 1* and *2*). Marker = DL2000. PCR, polymerase chain reaction.

General observations of the tissue engineered intervertebral discs

At 7 and 14 days after culture *in vitro*, no significant change was observed in the height and area of the intervertebral disc compared with those before culture, the fibrous ring of intervertebral disc did not protrude from the end-plate edge, and the bone connected with the end-plate was compact without decalcification. At 21 days after culture *in vitro*, no significant change was observed in the height and area of the intervertebral disc, and the fibrous ring of intervertebral disc did not protrude from the end-plate edge; however, significant decalcification were observed to have occurred to the bone connected with the end-plate, and the spatium interosseum increased.

Survival of cells injected into the intervertebral disc

It was observed that the visible fluorescence intensity of the 1×10^5 and the 1×10^6 groups significantly increased compared with that of the 1×10^4 group ($P < 0.05$) while there was no significant difference in fluorescence intensity between the 1×10^5 and the 1×10^6 groups ($P > 0.05$)

at 7 days after quantifying the fluorescence intensity of the tissue engineered intervertebral disc at different times in different groups. At 14 days, the fluorescence intensity of the 1×10^5 group was significantly higher than those of the 1×10^6 and the 1×10^4 groups. At 21 days, there was no significant difference in the cell distribution rates between the 1×10^5 and the 1×10^4 groups and those at 7 days ($P > 0.05$); the cell distribution rate of the 1×10^6 group decreased partially and the cell distribution rate of the 1×10^5 group was significantly higher than those of the other two groups (Figure 3).
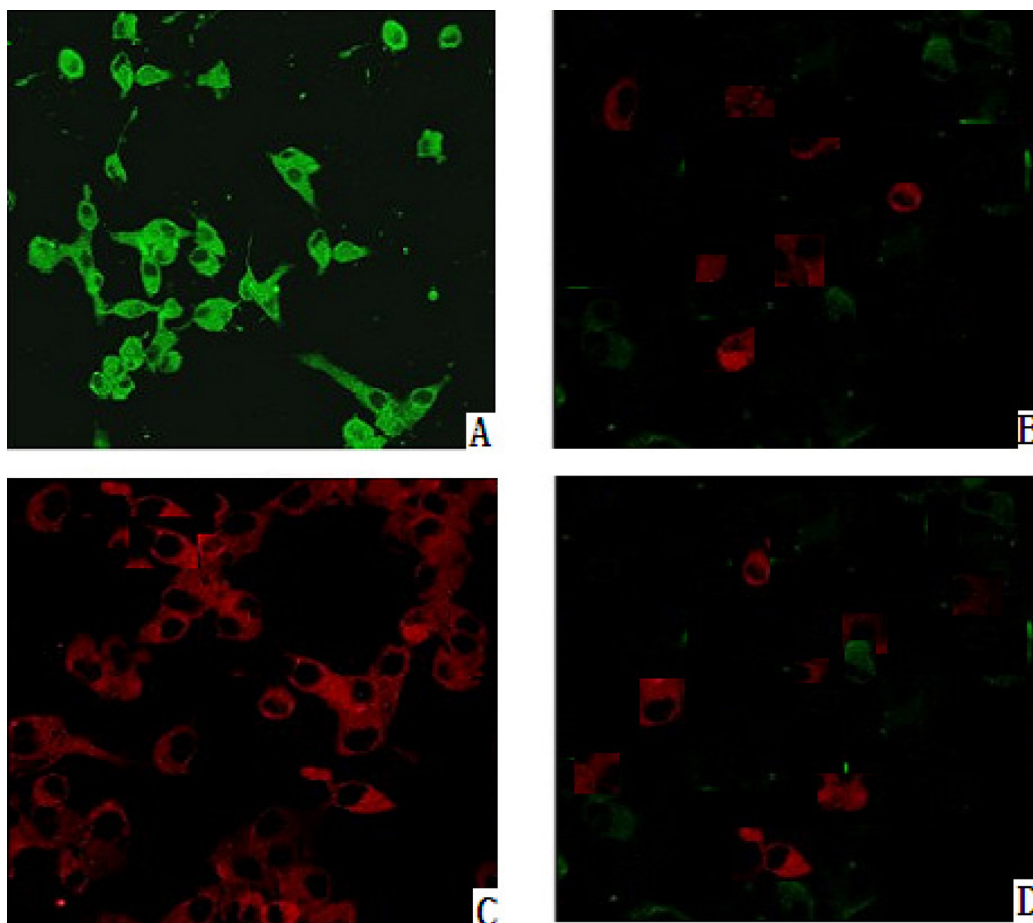

Figure 3. Fluorescence microscopic observation of the different groups following injection with NP cells. **A.** EGFP control group. It was observed that the NP cells expressing EGFP were widely distributed in the NP tissue. **B.**, **C.** and **D.** represent the 1×10^6, 1×10^5, and 1×10^4 groups. The injected NP cells were widely distributed in the NP tissue after transfection and the fluorescence intensity of the 1×10^5 group increased significantly compared with those of the other two groups. NP cells, nucleus pulposus cells; EGFP, enhanced green fluorescent protein. (Red fluorescence: DyLight 549, Cytoplasm staining).

Expression of *DBH* mRNA in the tissue engineered intervertebral discs

The results of RT-PCR detection of the expression of *DBH* in NP tissues among differ-

ent groups showed that the expression of *DBH* mRNA could be detected in the remaining three groups with the exception of the control group at 14 days after culture. Based on a comparison between the gray value and the internal control gray value of the target gene, the content of *DBH* in the 1×10^5 group was found to be relatively high (Figure 4).
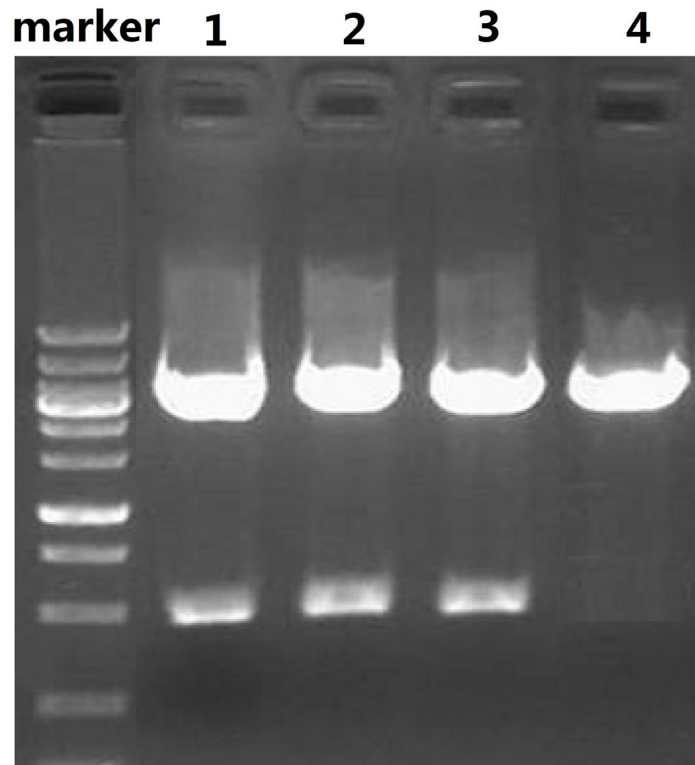

Figure 4. Electropherogram showing the results of PCR amplification determination of *hBMP7* mRNA expression in the nucleus pulposus tissues. *Lane 1* = 1×10^4 group; *lane 2* = 1×10^6 group; *lane 3* = 1×10^5 group; and *lane 4* = EGFP group. PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein.

PG content in the NP tissue of the tissue engineered intervertebral discs

Detection of the PG content in the NP tissues among different groups using the DMMB method showed that: at 7, 14, and 21 days, there were no significant differences in PG content between the 1×10^4 group and the EGFP control group at different times whereas the content of PG in the 1×10^5 group was significantly higher than those in the control and the 1×10^4 groups. In addition, the content at 7 days was significantly higher than those at 14 and 21 days whereas there were no significant differences between the PG contents at 14 and 21 days, and the PG content in the 1×10^6 group decreased significantly with time and increased slightly at 7 days compared with that in the 1×10^5 group while the contents of PG at 14 and 21 days were significantly lower than that in the 1×10^5 group. The specific PG contents are shown in Figure 5.
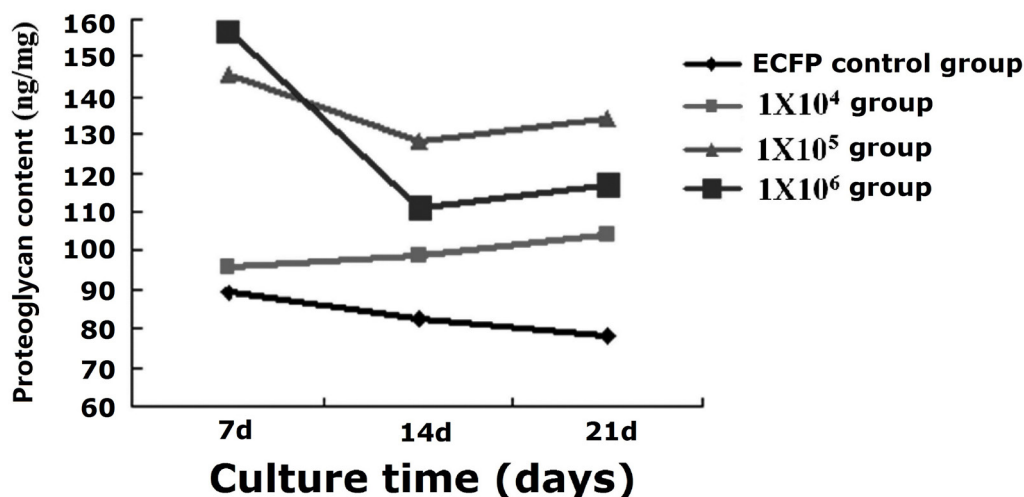
Figure 5. Proteoglycan content of the different nucleus pulposus tissues.

Total collagen content in the NP tissue of the tissue engineered intervertebral discs

Detection of total collagen in the NP tissues of the tissue engineered intervertebral discs among different groups using the hydroxyproline method demonstrated that: at 7, 14, and 21 days, there were no significant differences in the total collagen contents between the 1 x 10^4 and the control groups at different times while the content of total collagen in the 1 x 10^5 group was significantly higher than those in the control and the 1 x 10^4 groups. In addition, the content at 7 days was significantly higher than those at 14 and 21 days while there were no significant differences between the total collagen contents at 14 and 21 days. The total collagen content in the 1 x 10^6 group decreased significantly with time and increased slightly at 7 days compared with that in the 1 x 10^5 group while the contents of total collagen at 14 and 21 days were significantly lower than that in the 1 x 10^5 group. The specific total collagen contents are shown in Figure 6.

DISCUSSION

As a treatment method for degenerative diseases of intervertebral discs, the tissue engineered intervertebral disc is at the preliminary study stage. Currently, many research institutes are studying the construction of tissue engineered intervertebral discs and their biological functions (Nerurkar et al., 2010). To date, no method has been able to construct a mature tissue engineered intervertebral disc. Research on support materials is still at the preliminary stage. At present, both artificially synthesized and natural support materials have the problem of incompatibility with disc tissue. It is difficult to enable artificially synthesized support material to have a structure and function similar to that of the natural intervertebral disc. The support material is not expected to further exhibit a significant role in repairing the degenerative intervertebral disc in animals or in clinical experiments (Miyazaki et al., 2009; Richardson et

al., 2010). At present, *in vivo* and *in vitro* experimental research on tissue engineered intervertebral disc is at the preliminary stage and many basic scientific problems remain to be solved. Theoretically, the construction of a tissue engineered intervertebral disc with a fine structure and complex physiological functions requires more stringent requirements and presents a higher difficulty compared with construction of skin and skeleton with their relatively-simple anatomical structures and physiological functions (Mäenpää et al., 2010). Based on current research, the difficulties in constructing tissue engineered intervertebral discs lie in the construction of the support material and construction of an NP that functions in the intervertebral disc (Kandel et al., 2008).
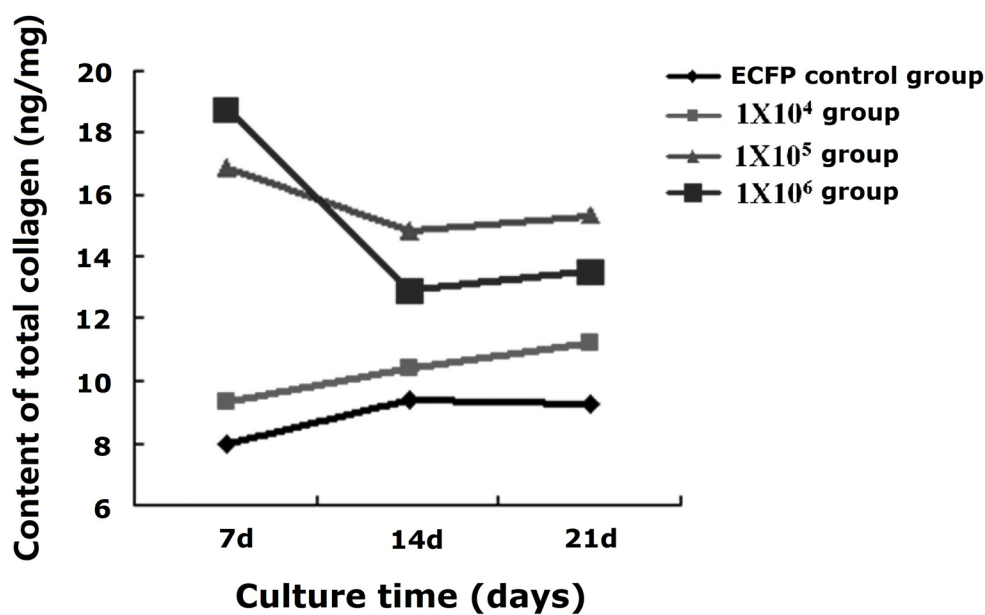

Figure 6. Total collagen content of the different nucleus pulposus tissues.

An ideal support of the tissue engineered intervertebral disc should meet the following requirements: 1) good biocompatibility, 2) the fibrous ring tissue constructed should be sufficiently connected to the upper and lower end-plates and should be well sealed, and 3) the fibrous ring tissue should have sufficient compressive strength. However, the existing available artificial material is not able to meet the requirements for tissue engineered intervertebral discs in either biocompatibility or strength (Lumpkins et al., 2008; Nesti et al., 2008; Lumpkins and McFetridge, 2009). The problems of connection force and sealability have not yet been solved in the techniques currently used for connecting the artificial fibrous ring and the artificial upper and lower end-plates (Saldanha et al., 2008; Foss et al., 2013). In contrast, the allogeneic frozen intervertebral disc used in the present experiment is able to completely meet the above three conditions as the support material for the tissue engineered intervertebral disc.

Based on research on the survival and functions of a fresh intervertebral disc in a

rhesus monkey, it has been observed that an autologous intervertebral disc retains partial function after transplantation. Further analysis of the survival and functions of frozen fresh allogeneic intervertebral discs transplanted into the body of a rhesus monkey has shown that the storage temperature and preserving fluid have a significant effect on the subsequent functions of the intervertebral disc, and that the cryopreserved intervertebral disc has an immunoprotective effect (Strange and Oyen, 2012; Wiltsey et al., 2013). Mietsch et al. (2013) have found that the immunogenicity of fresh-transplanted cartilage tissue is higher than that of frozen tissue. Hegewald et al. (2011) have also studied the transplantation of allogeneic intervertebral discs using a canine model and found that the damage to intervertebral disc cells stored at -196°C is less severe than that to those stored at -80°C, with no evidence being observed of immunological rejection. Li et al. (2010) have used transplantation of allogeneic intervertebral discs to treat degenerative intervertebral discs and have found that the allogeneic intervertebral disc is able to survive after transplantation and to meet the demand for physiological activities in biomechanics in spite of degenerative changes in biochemical metabolism and histology. Therefore, the allogeneic intervertebral disc preserved in liquid nitrogen is the most ideal support material for tissue engineered intervertebral discs at present (Huang et al., 2010).

The experimental research described in this study demonstrated that when the constructed intervertebral disc was cultured *in vitro* no significant changes were observed in the general structure of the intervertebral disc at the first 7 and 14 days after culture whereas the bone connected with the intervertebral disc and the upper and lower end-plates decalcified significantly at 21 days after culture, which significantly impacted the healing of the transplanted intervertebral disc. Healing between the allogeneic intervertebral disc and the autologous vertebral body primarily consists of the osseous healing of the cancellous bone of the autologous vertebral body and the cancellous bone at both ends of the allogeneic intervertebral disc. The cancellous bone of the intervertebral disc became significantly decalcified and osteoporosis occurred at 21 days after culture *in vitro*, which undoubtedly and significantly affected the healing of the tissue engineered intervertebral disc within the body and thus influenced the effect of treatment using this method.

The intervertebral disc is a tissue without its own blood supply and its nutrient substance permeates from the gap between the upper and lower end-plates. A thin layer of cancellous bone is retained in the upper and lower end-plates of the allogeneic intervertebral disc for permeation of nutrient solution and exudation of metabolites (Yang and Li, 2009; Leckie et al., 2013). The intervertebral disc is a site with relatively insufficient nutrition and oxygen. In this study, transfected NP cells cultured *in vitro* were injected into the intervertebral disc after being concentrated, altering their living environment. Following injection, the NP cells might have been subject to apoptosis, decreased proliferation capacity, and slow growth during their adaptation to the new environment (Jin et al., 2013; Morimoto et al., 2013).

Our study incorporated an injection of 20 μL NP cell suspension containing 1×10^5 cells and a canine intervertebral disc preserved in liquid nitrogen was used as the support material. After being cultured *in vitro* for 7 days, many cells were found to have survived and the PG content and total collagen in the NP tissue increased significantly. These results suggest that this system represents an ideal tissue engineered intervertebral disc, laying a foundation for further *in vivo* studies on tissue engineered intervertebral discs.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Bowles RD, Gebhard HH, Dyke JP, Ballon DJ, et al. (2012). Image-based tissue engineering of a total intervertebral disc implant for restoration of function to the rat lumbar spine. *NMR Biomed.* 25: 443-451.

Calderon L, Collin E, Velasco-Bayon D, Murphy M, et al. (2010). Type II collagen-hyaluronan hydrogel-a step towards a scaffold for intervertebral disc tissue engineering. *Eur. Cell Mater.* 20: 134-148.

Clouet J, Vinatier C, Merceron C, Pot-Voucel M, et al. (2009). The intervertebral disc: from pathophysiology to tissue engineering. *Joint Bone Spine* 76: 614-618.

Foss BL, Maxwell TW and Deng Y (2013). Chondroprotective supplementation promotes the mechanical properties of injectable scaffold for human nucleus pulposus tissue engineering. *J. Mech. Behav. Biomed. Mater.* 29: 56-67.

Hegewald AA, Enz A, Endres M, Sittinger M, et al. (2011). Engineering of polymer-based grafts with cells derived from human nucleus pulposus tissue of the lumbar spine. *J. Tissue Eng. Regen. Med.* 5: 275-282.

Huang B, Li CQ, Zhou Y, Luo G, et al. (2010). Collagen II/hyaluronan/chondroitin-6-sulfate tri-copolymer scaffold for nucleus pulposus tissue engineering. *J. Biomed. Mater. Res. B Appl. Biomater.* 92: 322-331.

Hudson KD, Alimi M, Grunert P, Härtl R, et al. (2013). Recent advances in biological therapies for disc degeneration: tissue engineering of the annulus fibrosus, nucleus pulposus and whole intervertebral discs. *Curr. Opin. Biotechnol.* 24: 872-879.

Jin ES, Min J, Jeon SR, Choi KH, et al. (2013). Analysis of molecular expression in adipose tissue-derived mesenchymal stem cells: prospects for use in the treatment of intervertebral disc degeneration. *J. Korean Neurosurg. Soc.* 53: 207-212.

Kandel R, Roberts S and Urban JP (2008). Tissue engineering and the intervertebral disc: the challenges. *Eur. Spine J.* 17: 480-491.

Lazebnik M, Singh M, Glatt P, Friis LA, et al. (2011). Biomimetic method for combining the nucleus pulposus and annulus fibrosus for intervertebral disc tissue engineering. *J. Tissue Eng. Regen. Med.* 5: e179-e187.

Leckie SK, Sowa GA, Bechara BP, Hartman RA, et al. (2013). Injection of human umbilical tissue-derived cells into the nucleus pulposus alters the course of intervertebral disc degeneration *in vivo*. *Spine J.* 13: 263-272.

Lee KI, Moon SH, Kim H, Kwon UH, et al. (2012). Tissue engineering of the intervertebral disc with cultured nucleus pulposus cells using atelocollagen scaffold and growth factors. *Spine* 37: 452-458.

Li CQ, Huang B, Luo G, Zhang CZ, et al. (2010). Construction of collagen II/hyaluronate/chondroitin-6-sulfate tri-copolymer scaffold for nucleus pulposus tissue engineering and preliminary analysis of its physico-chemical properties and biocompatibility. *J. Mater. Sci. Mater. Med.* 21: 741-751.

Lumpkins SB and McFetridge PS (2009). Regional variations in the viscoelastic compressive properties of the temporomandibular joint disc and implications toward tissue engineering. *J. Biomed. Mater. Res. A* 90: 784-791.

Lumpkins SB, Pierre N and McFetridge PS (2008). A mechanical evaluation of three decellularization methods in the design of a xenogeneic scaffold for tissue engineering the temporomandibular joint disc. *Acta Biomater.* 4: 808-816.

Mäenpää K, Ellä V, Mauno J, Kellomäki M, et al. (2010). Use of adipose stem cells and polylactide discs for tissue engineering of the temporomandibular joint disc. *J. R. Soc. Interface* 7: 177-188.

Mehrkens A, Müller AM, Valderrabano V, Schären S, et al. (2012). Tissue engineering approaches to degenerative disc disease-a meta-analysis of controlled animal trials. *Osteoarthritis Cartilage* 20: 1316-1325.

Mietsch A, Neidlinger-Wilke C, Schrezenmeier H, Mauer UM, et al. (2013). Evaluation of platelet-rich plasma and hydrostatic pressure regarding cell differentiation in nucleus pulposus tissue engineering. *J. Tissue Eng. Regen. Med.* 7: 244-252.

Miyazaki T, Kobayashi S, Takeno K, Meir A, et al. (2009). A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? *Tissue Eng.* 15: 3835-3846.

Morimoto R, Akeda K, Iida R, Nishimura A, et al. (2013). Tissue renin-angiotensin system in the intervertebral disc. *Spine* 38: E129-136.

Moss IL, Gordon L, Woodhouse KA, Whyne CM, et al. (2011). A novel thiol-modified hyaluronan and elastin-like polypetide composite material for tissue engineering of the nucleus pulposus of the intervertebral disc. *Spine* 36: 1022-1029.

Nerurkar NL, Elliott DM and Mauck RL (2010). Mechanical design criteria for intervertebral disc tissue engineering. *J. Biomech.* 43: 1017-1030.

Nesti LJ, Li WJ, Shanti RM, Jiang YJ, et al. (2008). Intervertebral disc tissue engineering using a novel hyaluronic acid-nanofibrous scaffold (HANFS) amalgam. *Tissue Eng.* 14: 1527-1537.

Pan Y, Chu T, Dong S, Hao Y, et al. (2012). Cells scaffold complex for intervertebral disc anulus fibrosus tissue engineering: *in vitro* culture and product analysis. *Mol. Biol. Rep.* 39: 8581-8594.

Richardson SM, Hoyland JA, Mobasheri R, Csaki C, et al. (2010). Mesenchymal stem cells in regenerative medicine: opportunities and challenges for articular cartilage and intervertebral disc tissue engineering. *J. Cell Physiol.* 222: 23-32.

Saldanha KJ, Piper SL, Ainslie KM, Kim HT, et al. (2008). Magnetic resonance imaging of iron oxide labelled stem cells: applications to tissue engineering based regeneration of the intervertebral disc. *Eur. Cell Mater.* 16: 17-25.

Silva-Correia J, Oliveira JM, Caridade SG, Oliveira JT, et al. (2011). Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications. *J. Tissue Eng. Regen. Med.* 5: e97-e107.

Simard JC, Vallieres F, de Liz R, Lavastre V, et al. (2015). Silver nanoparticles induce degradation of the endoplasmic reticulum stress sensor activating transcription factor-6 leading to activation of the NLRP-3 inflammasome. *J. Biol. Chem.* 290: 5926-5939.

Strange DG and Oyen ML (2012). Composite hydrogels for nucleus pulposus tissue engineering. *J. Mech. Behav. Biomed. Mater.* 11: 16-26.

Wiltsey C, Kubinski P, Christiani T, Toomer K, et al. (2013). Characterization of injectable hydrogels based on poly(N-isopropylacrylamide)-g-chondroitin sulfate with adhesive properties for nucleus pulposus tissue engineering. *J. Mater. Sci. Mater. Med.* 24: 837-847.

Yang X and Li X (2009). Nucleus pulposus tissue engineering: a brief review. *Eur. Spine J.* 18: 1564-1572.-