308

Available online at www.sciencedirect.com

SCIENCE DIRECT

ELSEVIER

JNMU

Journal of Nanjing Medical University, 2008, 22(5):308–312

www.elsevier.com/locate/jnmu

Research Paper

An animal model to create intervertebral disc degeneration characterized by radiography and molecular biology☆

Zhengming Sun, Miao Liu, Yingang Zhang*

Department of Orthopaedics, the First Affiliated Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China

Received 18 July, 2008

Abstract

Objectives: To develop a rabbit model of intervertebral disc degeneration that more exactly simulates the pathological changes of human intervertebral disc degeneration. **Methods:** Twelve New Zealand white rabbits were utilized to establish three different disc injury models according to the following protocol; group A: anulus punctures were done with a 18-gauge needle at L2-L3 and L5-L6; Group B: intradiscal injection of interleukin-1 IL-1 β with a 23-gauge needle at L3-L4; and Group C: intradiscal injection of phosphate buffer saline(PBS) with a 23-gauge needle at L4-L5. The L1-L2 level was used as a control. Rabbits were killed after 24 weeks. The intervertebral disc height was measured by lateral plain radiographs. After the radiographic measurements were obtained, the intervertebral discs were removed and analyzed for DNA, sulfated glycosaminoglycan(s-GAG) and water contents of nucleus pulposus. **Results:** The intervertebral disc height, s-GAG, and water contents in anulus needle punctures were significantly decreased in Group A, but the DNA content in the nucleus pulposus was significantly increased when compared to the control. The significant decrease of disc height and water contents were demonstrated, only the s-GAG and DNA contents did not show a significant difference in Group B when compared to the control. The significant decrease of disc height, s-GAG, water, and DNA contents did not show in Group C when compared to the control. **Conclusion:** The 18-gauge puncture models produced the most consistent disc degeneration in the rabbit lumbar spine.

Key words: intervertebral disc degeneration; animal model; rabbit

INTRODUCTION

Animal models are powerful tools used to study the pathways of degenerative change and uncover cause-and-effect relationships in a complex environment. Lumbar disc disease(LDD) caused by degeneration of intervertebral discs(IVD) of the lumbar spine, is one of the most common disorders seen in orthopaedic practices. Despite the prevalence of degenerative IVD disease, thus far, treatment has been limited to addressing the symptoms rather than to the application of repair methodologies. Many of the methods used to mitigate symptoms are actually destructive to disc integrity[1]. However, with the development of molecular biology and biomaterials, many novel therapeutic interventions to repair disc have been proposed, such as growth factor injection[2,3], cell transplantation[4-8], tissue engineering[9] and gene therapy[10]. Therefore it is necessary to develop appropriate experimental animal models, which mimic the degenerative process often occurring in human IVD breakdown and can be used for pharmacological studies or the development of new biological interventions.

The overall purpose of the present study was to establish a simple, reproducible rabbit model of disc degeneration with 18-gauge needles for annulus puncture, 23-gauge needle for injection of interleukin-1β and phosphate buffer saline respectively. To achieve this aim, radiologic and molecular biology studies were performed to monitor the progress of disc degeneration.

☆ This study was supported by National Natural Science Foundation of China(30400163)

*Corresponding author

E-mail address: zyingang@mail.xjtu.edu.cn

MATERIALS AND METHODS

Animals and tissue harvest

Twelve New Zealand white rabbits were offered by Animal Experimentation Center of Xi'an Jiaotong University(all female, 7 months old, 4.6~5.1 kg body weight), and were used to test 4 different disc degeneration models(***Table 1***). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Xi'an Jiaotong University. The right anterior surface of the lumbar intervertebral discs was exposed through retroperitoneal approach. Five disc spaces of each rabbit(L1-L2 to L5-L6) were used for the experiment, and each disc space was treated according to the following protocol:①L1-L2 was used as the control;②Group A:L2-L3 and L5-L6 received 1 anulus punctures into the nucleus with a 18-gauge needle; ③ Group B: L3-L4 was injected with interleukin-1β 10 μl(Sigma Chemical, USA) using a 23-gauge needle (Fisher Company, USA); ④ Group C:L4-L5 received 10 μl phosphate buffer saline(PBS) using a 23-gauge needle(***Table 1***). In the anulus punctures, each needle tip was inserted to the center of the nucleus.

Table 1 **The three disc models**

Level of intervertebral space	Groups
L1-L2	control
L2-L3	1 anulus puncture with an 18 gauge needle(Group A)
L3-L4	IL-1β :10 μl of 1 mmol/L with a 23-gauge needle(Group B)
L4-L5	PBS: 10 μl with a 23-gauge needle(Group C)
L5-L6	1 anulus puncture with an 18 gauge needle(Group A)

Twenty-four weeks after surgery, lateral plain radiographs were obtained keeping all rabbits under general anesthesia using ketamine hydrochloride(25 mg/kg) when they were sacrificed. Immediately after death, portions of the nucleus pulposus were carefully removed from the lumbar intervertebral discs and collected into preweighted 1.5 ml tubes. The water, DNA and sulfated-glycosaminoglycan(s-GAG) content of each nucleus pulposus were measured.

The nucleus

The nucleus tissues were weighed before and after lyophilizing the tissue in a -60℃ vacuum chamber. Water content of the nucleus was expressed as a percentage (%) of wet weight:(wet weight-dry weight)/wet weight × 100%. Results at the experimental level was compared to that at the control level(L1-L2) and expressed as a ratio.

1,9-Dimethylmethylene blue(DMMB) staining for s-GAG was used to measure proteoglycan content in the nucleus pulposus[11,12]. Briefly, 40 U of papain dissolved in 250 μl of phosphate buffer with 10 mmol/L EDTA, and 10 mmol/L cysteine was added into each lyophilized sample. The tissue was digested overnight at 60℃. After complete digestion, the tissue s-GAG was determined by mixing 20 μl of a 1:1 000 sample with 200 μl of DMMB solution to measure the absorbance at a wavelength of 515 nm wavelength. A standard curve was made with known concentrations of chondroitin sulfate. Standardized s-GAG content was expressed as a percentage(%) of dry weight:s-GAG weight/dry weight of nucleus × 100%. Results at the experimental level were compared to that at the control level(L1-L2) and expressed as a ratio.

DNA content was evaluated by fluorescence method using Hoechst dye 33258[13]. The fluorescence of the samples was read using a spectroflurometer with an emission of 458 nm and excitation of 356 nm. A standard curve was prepared from known concentrations of calf thymus DNA. Results at the experimental level was compared to that at the control level(L1-L2) and expressed as a ratio.

Plain radiograph documentation of rabbit disc degeneration

A plain lateral radiograph was taken to measure the change in disc height, the three-center line method being used[14]. On plain lateral film, four landmarks were chosen at the uppermost or lowermost corners of the vertebral bodies. Two straight lines were drawn using four landmarks. Three additional lines-A, B, and C-were drawn by dividing evenly the distance between the first two lines(***Fig. 1***). Each disc height was measured with computer software(Grabit version 1.51, Datatrend Software, Raleigh, NC) by averaging the distance at three different sites:(D+E+F)/3(***Fig. 1***). The disc height at the experimental level was compared to that at the control level(L1-L2) and expressed as a ratio.

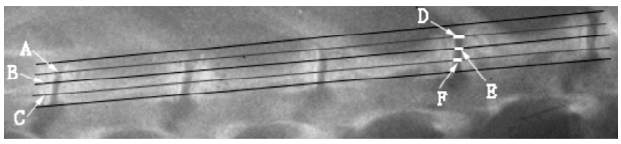


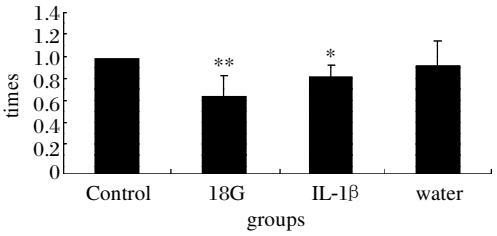
Fig. 1 On plain lateral film, four landmarks were chosen at the uppermost or lowermost corners of the vertebral bodies. Two straight lines were drawn using four landmarks. Three additional lines-A, B, and C-were drawn by dividing evenly the distance between the first two lines. Each disc height was measured by averaging the distance at three different sites:(D+E+F)/3.

RESULTS

Change of intervertebral disc height

The disc height ratio to control was measured using the 3 center lines method(***Fig. 1***). The disc height decreased significantly in the 18 gauge group($P < 0.01$) and in the IL1β-injection group($P < 0.05$). The PBS

injection model showed no difference when compared to the control(***Fig. 2***).

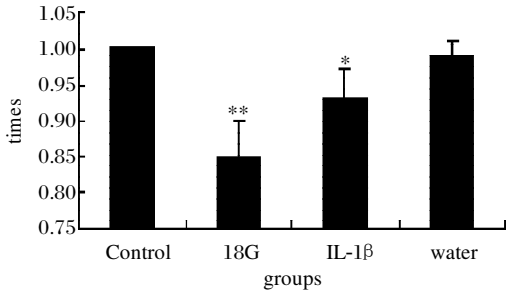


Compared with the control, $^{**}P < 0.01$,$^{*}P < 0.05$.

Fig. 2 Intervertebral disc hight ratio to the control

Water content of nucleus

The water content ratio to control of the nucleus was measured and expressed as a percentage of wet disc weight(***Table 2***). Compared to the control, the water content in 1-puncture(18-gauge) and that in injection (IL-1β significantly decreased(former:$P < 0.01$; latter: $P < 0.05$). The injection(PBS) model showed no difference when compared to the control(***Fig. 3***). The pattern of water content was similar to the pattern of disc height change of the nucleus.

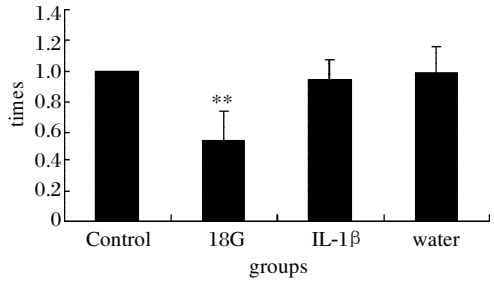


Compared with the control, $^{**}P < 0.01$,$^{*}P < 0.05$.

Fig. 3 Water content ratio to the control

Sulfated-Glycosaminoglycan content of nucleus

The s-GAG content ratio to control of the nucleus was measured and expressed as a percentage of dry disc weight(***Table 2***). Only the result for 1-puncture(18-gauge) model showed a statistically significant difference when compared to control($P < 0.01$, ***Fig. 4***).

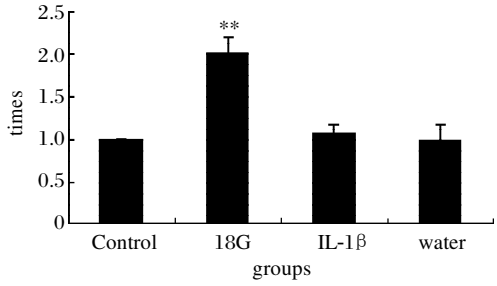


Compared with the control, $^{**}P < 0.01$.

Fig. 4 The s-GAG content ratio to the control of the nucleus

DNA content of nucleus

The DNA content ratio to control of the nucleus was measured and expressed as a percentage of dry disc weight(***Table 2***). Only the result for 1-puncture(18-gauge) model showed a statistically significant difference as compared to control ($P < 0.01$, ***Fig. 5***).



Compared to the control, $^{**}P < 0.01$.

Fig. 5 DNA content ratio to the control

Table 2 **The results of ratio of water content, sulfated glycosaminoglycan content, DNA and intervertebral disc height**

	control	Group A	Group B	Group C
IVD height ratio to control	1	$0.67 \pm 0.18^{**}$	$0.84 \pm 0.11^{*}$	0.94 ± 0.22
Water content ratio to control	1	$0.85 \pm 0.05^{**}$	$0.93 \pm 0.04^{*}$	0.99 ± 0.02
s-GAG content ratio to control	1	$0.53 \pm 0.20^{**}$	0.94 ± 0.13	0.99 ± 0.17
DNA content ratio to control	1	$2.01 \pm 0.19^{**}$	1.06 ± 0.10	0.97 ± 0.20

Compared with the control, $^{**}P < 0.01$, $^{*}P < 0.05$; IVD = intervertebral disc; s-GAG=sulfated glycosaminoglycan.

DISCUSSION

In the present study, through measuring lateral "stab" of rabbit lumbar discs with an 18-gauge needle, the intervertebral disc height, s-GAG, and water contents over 24 weeks, showed a significant decrease compared with what was seen in the control. The significant decrease of disc height and water contents were demonstrated, whereas only the s-GAG contents did not show a significant difference in injection of interleukin-1β as compared to the control. A significant decrease of disc height, s-GAG, and water contents were not shown with injection of phosphate buffer saline, as com-

pared to the control.

Normally the nucleus pulposus of intervertebral disc, which has no blood supply, is the location of immune escape. In this case a series of auto-immune responses will be activated, leading the nucleus to apoptosis, induced by disc degeneration after the fibrous ring broken, and nucleus pulposus is exposed to the serum[15]. The present study using 18-gauge puncture needle successfully established a model of disc degeneration similar to that found in humans[16]. However, no obvious disc degeneration was observed in the model of injection of sterile PBS 10 μl with 23-gauge puncture needle. Lotz[17] considered that it was associated with the size of the crack of anulus fibrosus. But some researchers found that obvious disc degeneration was observed to puncture anulus fibrosus with 16-gauge, 18-gauge or 21-gauge needle[18-20]. Korecki *et al.*[21] considered that small gauge needle(25G) puncture had immediate and progressive mechanical and biologic consequences with important implications. In our study, some degree of disc degeneration can be caused by the injection of IL-1β with a 23-gauge needle. Nevertheless, the water and s-GAG content showed no significant difference in the simple injection of the IL-1 β group compared with the PBS. Maitre *et al.*[22] believed that IL-1β can induce the formation of a variety of cytokines and activation of MMP, causing the decomposition of disc matrixa and inducing disc degeneration. Therefore, we believe that the mechanical injury plays a dominant position in the model of disc degeneration.

In addition, the DNA content showed no significant difference with the injection of IL-1β and phosphate buffer saline, when compared to the control, whereas only anulus punctures with an 18-gauge needle obtained a statistical significance. We thought, that as there is some formation of connective tissue,(which can occur with damage of the nucleus pulposus and reduction of chordoma) concentrations of certain cells in the nucleus pulposus become higher. Nomura *et al*[23] also found some connective tissue was formed with degeneration of the intervertebral disc.

We have successfully established a model of disc degeneration. But the extent of disc degeneration was not suitable for the study of treatment of disc degeneration. In subsequent studies, therefore, in order to find the best level of disc degeneration and carry out the genetic or biological treatment, the level of disc degeneration should be detected at different time.

References

[1] Peng Chen, Guoxing Zhu. Analysis of palindromic lubar intervertebral disc protrusion after surgery. *JNMU* 2006; 20:992-4.

[2] Takegami K, Thonar EJ, An HS, Kamada H, Masuda K. Osteogenic protein-1 enhances matrix replenishment by intervertebral disc cells previously exposed to interleukin-1. *Spine* 2002; 27:1318-25.

[3] An HS, Takegami K, Kamada H, Nguyen CM, Thonar EJ, Singh K, *et al.* Intradiscal administration of osteogenic protein-1 increases intervertebral disc height and proteoglycan content in the nucleus pulposus in normal adolescent rabbits. *Spine* 2005; 30:25-31; discussion 31-2.

[4] Meisel HJ, Siodla V, Ganey T, Minkus Y, Hutton WC, Alasevic OJ. Clinical experience in cell-based therapeutics: disc chondrocyte transplantation A treatment for degenerated or damaged intervertebral disc. *Biomol Eng* 2007; 24: 5-21.

[5] Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K, *et al.* Transplantation of mesenchymal stem cells in a canine disc degeneration model. *J Orthop Res* 2008; 26:589-600.

[6] Jintang Wang, Hong Zhang, Yingang Zhang, Xiaowei Zhang, Meng Li. The study of migration of bone mesenchymal stem cells transplanted in intervertebral discs of rabbits and expression of exogenous gene. *JNMU* 2006, 20:287-91.

[7] Bertram H, Kroeber M, Wang H, Unglaub F, Guehring T, Carstens C, *et al.* Matrix-assisted cell transfer for intervertebral disc cell therapy. *Biochem Biophys Res Commun* 2005; 331: 1185-92.

[8] Zhang YG, Guo X, Xu P, Kang LL, Li J. Bone mesenchymal stem cells transplanted into rabbit intervertebral discs can increase proteoglycans. *Clin Orthop Relat Res* 2005; 430:219-26.

[9] Sato M, Asazuma T, Ishihara M, Ishihara M, Kikuchi T, Kikuchi M, *et al.* An experimental study of the regeneration of the intervertebral disc with an allograft of cultured annulus fibrosus cells using a tissue-engineering method. *Spine* 2003; 28:548-53.

[10] Cui M, Wan Y, Anderson DG, Shen FH, Leo BM, Laurencin CT, *et al.* Mouse growth and differentiation factor-5 protein and DNA therapy potentiates intervertebral disc cell aggregation and chondrogenic gene expression. *Spine* 2008; 8:287-95.

[11] Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986; 883:173-7.

[12] Goldberg RL, Kolibas LM. An improved method for determining proteoglycans synthesized by chondrocytes in culture. *Connect Tissue Res* 1990; 24:265-75.

[13] West DC, Sattar A, Kumar S. A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. *Anal Biochem* 1985; 147:289-95.

[14] Kim KS, Yoon ST, Li J, Park JS, Hutton WC. Disc degeneration in the rabbit: a biochemical and radiological comparison between four disc injury models. *Spine* 2005; 30:33-7.

[15] Satoh K, Konno S, Nishiyama K, Olmarker K, Kikuchi S. Presence and distribution of antigen-antibody complexes in the herniated nucleus pulposus. *Spine* 1999; 24:1980-4.

[16] Yingang Zhang, Guo xiong. Biological progress of mechanism and restoration of disc degeneration. *Chinese Journal of Orthopaedic* 2004(in Chinese); 24:119-21.

[17] Lotz JC. Animal models of intervertebral disc degeneration: lessons learned. *Spine* 2004; 29:2742-50.

[18] Sobajima S, Shimer AL, Chadderdon RC, Kompel JF, Kim JS, Gilbertson LG, *et al.* Quantitative analysis of gene expression in a rabbit model of intervertebral disc degeneration by real-time polymerase chain reaction. *Spine J* 2005; 30:14-23.

[19] Masuda K, Aota Y, Muehleman C, Imai Y, Okuma M, Thonar EJ,

et al. A novel rabbit model of mild, reproducible disc degeneration by an anulus needle puncture: correlation between the degree of disc injury and radiological and histological appearances of disc degeneration. *Spine* 2005; 30: 5-14.

[20] Sobajima S, Kompel JF, Kim JS, Wallach CJ, Robertson DD, Vogt MT, *et al.* A slowly progressive and reproducible animal model of intervertebral disc degeneration characterized by MRI, X-ray, and histology. *Spine* 2005; 30:15-24.

[21] Korecki CL, Costi JJ, Iatridis JC. Needle puncture injury affects intervertebral *disc* mechanics and biology in an organ culture model. *Spine* 2008; 33:235-41.

[22] Le Maitre CL, Freemont AJ. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res Ther* 2005; 7:R732-45.

[23] Nomura T, Mochida J, Okuma, Nishimura K, Sakabe K. Nucles pulposus allograft retards intervertebral disc degeneration. *Clin Orthop Res* 2001; 389:94-101.