Lumbar Intervertebral Disc Puncture under C-arm Fluoroscopy: a New Rat Model of Lumbar Intervertebral Disc Degeneration

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Abstract: To establish a minimally invasive rat model of lumbar intervertebral disc degeneration (IDD) to better understand the pathophysiology of the human condition. The annulus fibrosus of lumbar level 4–5 (L4-5) and L5-6 discs were punctured by 27-gauge needles using the posterior approach under C-arm fluoroscopic guidance. Magnetic resonance imaging (MRI), histological examination by hematoxylin and eosin (H&E) staining, and reverse transcription polymerase chain reaction (RT-PCR) were performed at baseline and 2, 4, and 8 weeks after disc puncture surgery to determine the degree of degeneration. All sixty discs (thirty rats) were punctured successfully. Only two of thirty rats subjected to the procedure exhibited immediate neurological symptoms. The MRI results indicated a gradual increase in Pfirrmann grade from 4 to 8 weeks post-surgery (P<0.05), and H&E staining demonstrated a parallel increase in histological grade (P<0.05). Expression levels of aggrecan, type II collagen (Col2), and Sox9 mRNAs, which encode disc components, decreased gradually post-surgery. In contrast, mRNA expression of type I collagen (Col1), an indicator of fibrosis, increased (P<0.05). The procedure of annular puncture using a 27-gauge needle under C-arm fluoroscopic guidance had a high success rate. Histological, MRI, and RT-PCR results revealed that the rat model of disc degeneration is a progressive pathological process that is similar to human IDD.

Key words: animal model, intervertebral disc degeneration, magnetic resonance imaging, nucleus pulposus

Introduction

Low back pain is one of the most common orthopaedic conditions, afflicting up to 75% of adults at some period in their lives [3, 9, 10]. The major contributor to low back pain is lumbar intervertebral disc degeneration (IDD) [1, 4, 8], a chronic disease involving progressive changes in disc composition and structure, leading ultimately to chronic pain and dysfunction [23, 24]. The precise pathogenesis and pathophysiology of IDD are unknown, necessitating the development of animal models to simulate human disc degeneration for studies on pathology, biochemistry and treatment.

Several animal models of IDD have been described [19, 22], each with its own advantages and disadvantages for studying pathogenesis, pathophysiology, and treatment of IDD [15, 23]. These models can be divided into two categories: spontaneous IDD models and experimentally induced IDD models [22]. Spontaneous IDD models, such as the sand rat [7] and Chinese ham-
ster [21], can simulate the slow pathological process of human disc degeneration. However, the low frequency and inconsistency of degeneration limit the use of these models [17]. Experimentally induced IDD models involve the induction of structural damage by a blade [16], needle [12, 17], or drill [11]. Annular needle puncture can cause a slow, cumulative degeneration with pathological and biochemical changes similar to those observed in human IDD. This simple operation, which has good reproducibility and reliability, is commonly used to establish animal models of IDD. However, use of this model is limited because damage or irritation of the vertebral body periosteum may accelerate IDD [17]. Furthermore, the surgery may cause severe trauma and requires long surgical exposure time, increasing the risk of infection and mortality.

In the current study, we developed and characterized an IDD rat model in which a 27-gauge needle was used to puncture the annulus fibrosus by the posterior approach under C-arm fluoroscopic guidance, a procedure that caused minimal trauma. The reliability of this model was assessed by magnetic resonance imaging (MRI), histological staining, and reverse transcription polymerase chain reaction (RT-PCR) analysis of gene expression changes.

Fig. 1. Intraoperative fluoroscopy images from a representative rat
Both the anteroposterior radiograph (a) and lateral radiograph (b) showed the tips of the needles were located in the center of the discs. An enlarged image (c) from 1b indicated needles locations. The numbers (1–6) indicated the lumbar vertebrae.

Materials and Methods

Experimental animals
Forty domestic male Sprague-Dawley rats (weight, 300–400 g; age, 4–6 months) were used in this study. Adults were selected because younger rats are too small for the surgery, while older rats have natural disc degeneration. Ten rats were sacrificed prior to surgery to provide normal disc specimens for histology and RT-PCR. Surgery was performed on the remaining thirty rats. This study was approved by the Laboratory Animal Care Committee of Jiangsu University, and the animal experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

Surgical procedure
Animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g) (HuaYi Biotechnology, Shanghai, China). The animal was laid prone on the fluoroscopy tube of the C-arm system (MM-M17AB, Philips, Eindhoven, Netherlands). The animal’s back was shaved, and the operative field was sterilized with betadine (LiKang Pharma, Shanghai, China). A 20–30 mm longitudinal posterior midline incision was made from the lumbar level 3 (L3) process to the L6 process. The right facet joints of lumbar level 4–5 (L4–5) and L5–6 were exposed by blunt dissection of paraspinal muscles. The L4–5 and L5–6 discs were then punctured.
with 27-gauge needles (HuaYi Bio-technology, Shanghai, China) along the outside of the facet joints under C-arm fluoroscopic guidance until the needle tips reached the disc center (Fig. 1). After puncture, the deep fascia, superficial fascia, and skin were closed in layers with 3–0 sutures (Ethibond®, Johnson & Johnson, Somerville, NJ, USA). Perioperative data, including operative time, blood loss, and complications, were recorded for each animal.

MRI

Eight rats were chosen randomly for MRI scans before surgery and eight each at 2, 4, and 8 weeks after surgery. Rats were placed prone inside the 3.0-T MRI scanner (Magnetom TrioTim, Siemens, Erlangen, Germany) with the lumbar region centered over a 127 mm diameter circular surface coil. Sagittal T2-weighted images were obtained using the following settings: fast spin echo sequence, TR=4,070 milliseconds, TE=241 milliseconds, field of view=160 mm, and section thickness=1.5 mm. The Pfirrmann classification [20], with grades ranging from I (normal) to V (advanced degeneration), was used to assess the degree of disc degeneration.

Histological assessment

Two rats (four discs) were chosen randomly before surgery and two each at 2, 4, and 8 weeks after surgery for histology (8 rats in total). The intact specimens, including the annulus fibrosus, the nucleus pulposus, both endplates, and the adjacent vertebral body bone, were fixed in 4% paraformaldehyde (Haide Bio-technology, Beijing, China) for 24 h, decalcified in 10% dilute nitric acid solution (Haide Bio-technology, Beijing, China) for 24 h, and washed with running tap water for 30 mins (min). The fixed specimens were embedded in paraffin and cut into 4-µm thick serial mid-sagittal sections for hematoxylin and eosin (H&E) staining. The degree of IDD was assessed by a histological grading scale [17], with scores ranging from grade 4 (normal) to grade 12 (severely degenerated). This grading scale is based on degenerative changes in 4 regions: the annulus fibrosus, the border between the annulus fibrosus and the nucleus pulposus, the parenchyma of the nucleus pulposus, and the matrix of the nucleus pulposus. Each region can be graded 1 (normal) to 3 (severely degenerated).

RT-PCR

Eight rats (sixteen discs) were randomly chosen before surgery and eight each at 2, 4, and 8 weeks after surgery for RT-PCR. Total RNA was extracted from the nucleus pulposus using Trizol reagent (Invitrogen, Carlsbad, CA, USA). A 1-µg sample of total RNA was reverse transcribed into cDNA using the Thermo OneStep RT-PCR Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer’s protocol. A 1-µl volume of cDNA template was used for each RT-PCR reaction, and sequences were amplified using Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) and primers (Table 1) designed and synthesized by Sangon Biotech (Shanghai, China). The PCR conditions used were specific to each target transcript. Aggrecan: denaturation for 3 min at 94°C, followed by 36 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 6 min; type II collagen (Col2): denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min; Sox9: denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min; type I collagen (Col1): denaturation for 3 min at

| Gene            | Primer Sequence                        |
|-----------------|----------------------------------------|
| Aggrecan        | Forward: 5’-CTCTGGGATCTATCGGTGTA -3’   |
|                 | Reverse: 5’-TCGGTCAAGAGTCCAGTGTG- 3’   |
| Type II collagen| Forward: 5’-TCAGGTGTTGGTGAGCAAATA- 3’ |
|                 | Reverse: 5’-CCGGACTGTGAAGTTAGGATG- 3’ |
| Sox-9           | Forward: 5’-GGCTCTACCTACCATGCTICA- 3’ |
|                 | Reverse: 5’-AATGCGTACATGGATGTTA -3’   |
| Type I collagen | Forward: 5’-GGGGAAGACAGTCGAAATA- 3’   |
|                 | Reverse: 5’-GATTGGACAGGAGGGATT -3’    |
| β-actin         | Forward: 5’-TGAGTGAGCACCAAGAGGG-3’    |
|                 | Reverse: 5’-CTGGAAGGTGACGAGGAGG-3’    |
94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min; β-actin (internal standard): denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. The PCR products were separated on 1.5% agarose gels (Gene, Hong Kong, China) and visualized on a gel doc system (GBOX/CHEMI-XT16, Syngene, Cambridge, UK). The RT-PCR experiments for quantitative analysis of changes in aggrecan, Col2, Sox9, and Coll expression levels were replicated 4 times, and band density (grey scale) normalized to that of β-actin.

**Statistical analyses**

Statistical analyses were conducted using the Statistical Package for Social Sciences System Version 14.0 (SPSS, Chicago, IL, USA). Nonparametric data (Pfirrmann grading and histological grading) were analyzed by the Kruskal-Wallis H test. Gene expression changes at different observed times were analyzed by One-way ANOVA test. A P-value<0.05 was considered statistically significant.

| Grade | Pre-op (n=16) | Post-op 2 w | Post-op 4 w | Post-op 8 w |
|-------|---------------|-------------|-------------|-------------|
| I     | 15            | 12          | 1           | 0           |
| II    | 1             | 3           | 9           | 3           |
| III   | 0             | 1           | 5           | 4           |
| IV    | 0             | 0           | 1           | 7           |
| V     | 0             | 0           | 0           | 2           |

*The degree of disc degeneration was assessed in MRI images pre-operation (pre-op; n=16) and 2 (post-op 2 w; n=16), 4 (post-op 4 w; n=16), and 8 (post-op 8 w; n=16) weeks post-operation using the Pfirrmann classification [20]. Classifications range from I (normal) to V (advanced degeneration). **p<0.01; ***p<0.001

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**Table 2. Results of Pfirrmann classification***

**Fig. 2.** Sagittal T2-weighted images by MRI at 4 observed time points

Pre-operative image (a) showed the homogeneous structures of the L4–5 and L5–6 discs. A hyperintense signal was still seen at 2 weeks post-operation (b), but the area of high intensity decreased in L4–5 disc (arrow). At 4 weeks post-operation (c), T2-weighted image by MRI showed an intermediate gray signal in the L4–5 and L5–6 discs (arrows). At 8 weeks post-operation (d), signal intensity from both L4–5 and L5–6 discs was significantly reduced. A ‘black disc’ (L4–5, arrow) was observed in several T2-weighted images at this time. The numbers (1–6) indicated the lumbar vertebrae.

**Fig. 3.** Changes in the Pfirrmann classification grade after annular puncture

The degree of disc degeneration was assessed from T2-weighted images acquired before surgery (pre-op; n=16) and at 2 (post-op 2 w; n=16), 4 (post-op 4 w; n=16), and 8 (post-op 8 w; n=16) weeks post-operation using the Pfirrmann classification [20]. Grade ranged from I (normal) to V (advanced degeneration). **p<0.01; ***p<0.001

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Statistical analyses were conducted using the Statistical Package for Social Sciences System Version 14.0 (SPSS, Chicago, IL, USA). Nonparametric data (Pfirrmann grading and histological grading) were analyzed by the Kruskal-Wallis H test. Gene expression changes at different observed times were analyzed by One-way ANOVA test. A P-value<0.05 was considered statistically significant.
Results

Surgery

All sixty discs (thirty rats) were punctured successfully confirmed by intra-operative fluoroscopy. The mean operative time was 17.4 ± 4.3 min and the average blood loss was 0.3 ± 0.1 ml. Only two of thirty rats exhibited immediately observable neurologic symptoms after surgery, a limp in the right hind limb. Otherwise, there were no immediate post-surgical deficits.

MRI assessment

The Pfirrmann classification results at the 4 time points are shown in Table 2. At 2 weeks after surgery, the signal intensities on T2-weighted images of most punctured discs were similar, with only a few discs exhibiting a slight decrease (Fig. 2). At 4 weeks after surgery, the degree of degeneration in the punctured discs was mainly grade II to III according to the Pfirrmann classification. At 8 weeks after surgery, T2-weighted images revealed severe decrease in signal intensity, and ‘black disc’ changes were found in several cases. The MRI results showed a gradual increase in Pfirrmann grade after needle puncture (Fig. 3, P<0.05).

Histological assessment

Histological (H&E) staining of sagittal sections indicated that the severity of IDD increased gradually post-surgery. Normal discs sections (Figs. 4a and b) showed intact annulus fibrosus, a well-defined border between the annulus fibrosus and nucleus pulposus, and a nucleus pulposus consisting of numerous chondrocyte-like cells. There were no differences in histological characteristics between control discs and discs examined 2 weeks after surgery (Figs. 4c and d). At 4 weeks after surgery, however, the border between the annulus fibrosus and nucleus pulposus was less distinct than in the pre-operative normal disc and there was a loss of nucleus pulposus accompanied by a reduction in the number of chondrocyte-like cells (Figs. 4e and f). At 8 weeks after surgery, cracks and ruptures of collagen fibers could be seen in the annulus fibrosus, most of the contents of the nucleus pulposus was lost, and there were very few chondrocyte-like cells (Figs. 4g and h).

RT-PCR Results

Expression levels of aggrecan, Col2, and Sox9 mRNAs decreased over time post-surgery (P<0.05), while expression of Col1 mRNA increased (Fig. 5; P<0.05).

Discussion

We established an IDD model in rats by annular puncture from the posterior approach using 27-gauge needles under C-arm fluoroscopic guidance. Rats exhibited gradually increasing IDD-like changes as determined by both Pfirrmann classification and histological grading. Additionally, RT-PCR results showed a decrease in the mRNA expression levels of aggrecan, Col2, and Sox9, three major components of lumbar discs [13]. In contrast, expression of Col1, an indicator of fibrosis, increased. These findings were consistent with the matrix breakdown observed in human IDD associated with decreased aggrecan and Col2 expression and fibrosis associated with up-regulation of Col1 [6]. The punctured discs exhibited slow progressive degeneration as revealed by MRI, histology, and RT-PCR starting at 2 weeks and continuing for at least 8 weeks post-surgery (although there were no significant differences in Pfirrmann and histological grades between baseline and 2 weeks post-surgery). Thus, this model demonstrated progressive degeneration resembling human IDD [5, 6].

It was reported that rats reach approximately 90% of skeletal maturity 12 weeks after birth [27], and the nu-
cleus pulposus start to degenerate after the first 12 months of life [25]. Thus, we chose mature rats aged 4–6 months as subjects in order to avoid impacts of age on disc degeneration.

The retroperitoneal approach often used for annular puncture can expose the anterolateral surface of the discs. This approach does facilitate accurate puncture, as depth and trajectory are easily controlled, but also prolongs surgical time, results in more severe trauma, and increases the risks of infection and mortality. To circumvent these problems, we used a posterior approach to puncture L4–5 and L5–6 discs. Although the discs were not exposed in this posterior approach, puncture could still be performed quickly (17.4 ± 4.3 min) with minimal blood loss (0.3 ± 0.1 ml) and did not require extensive dissection, reducing irritation of prevertebral structures. Moreover, the depth and trajectory could be precisely controlled by C-arm fluoroscopic guidance, ensuring that the needle reached the center of the nucleus pulposus. However, this procedure did require skill.

**Fig. 4.** Histological changes after annular puncture
H&E-stained sagittal sections before (a & b) and 2 weeks (c & d) after surgery showed intact annulus fibrosus and cell-enriched nucleus pulposus. From 4 (e & f) to 8 weeks (g & h) post-surgery, the number of chondrocyte-like cells in nucleus pulposus was reduced gradually, and cracks (arrows) among the layers of collagen fibers of the annulus fibrosus appeared.

**Fig. 5.** Changing mRNA expression levels of aggrecan, type II collagen (Col2), Sox9, type I collagen (Col1), and β-actin
RT-PCR was used to examine the expression of genes related to disc degeneration before surgery (A) and 2 (B), 4 (C), and 8 weeks (D) post-operation. Gene expression was normalized to β-actin.
to avoid excessive damage to discs and accelerated degeneration caused by multiple punctures. In addition, a long residence time and needle rotation should be avoided after puncture to prevent acceleration of IDD [17].

The extent of injury to the annulus pulposus affects the subsequent progression of IDD. A previous report showed that the #11 scalpel blade used in the classic ‘stab’ model [16] led to development of IDD within 2 weeks. Alternatively, a 16 or 18-gauge needle resulted in a slow, progressive, and reproducible rabbit IDD, similar to the changes seen in human IDD [17, 23]. Another study suggested that a 27-gauge needle could be used to introduce pharmacological reagents or cells in rabbit discs while minimizing damage [2]. However, the size of the needle should be based on the animal’s body type. For IDD rat models, a 27-gauge needle is usually used to puncture the discs [18]. In addition, the rate of IDD progression can be modified by the number of punctures. For example, multiple punctures with a 21-gauge needle led to greater damage to rabbit discs compared to a single puncture using an 18-gauge needle [14].

In this study, we used MRI, not radiography to assess the degree of IDD. Although radiography offers a favorable measurement of lumbar disc height, rat disc height is too small to be measured accurately by radiography, is easily affected by posture and degree of muscle relaxation, and correlates significantly with the changes in the annulus fibrosus rather than the nucleus pulposus [17]. In addition, the signal intensity on T2-weighted images can reflect biochemical as well as physical changes during IDD [26]. Indeed, the signal reduction observed was consistent with human IDD. The 3.0-T MRI scanner used in the present study also helped in Pfirrmann grading because the T2-weighted images clearly showed both the boundary of the annulus fibrosus and nucleus pulposus and the signal intensity from the nucleus pulposus.

Limitations of this study include no comparative analysis with other established animal models of IDD, such as sand rats. In addition, quantitative analysis of MRI was not performed, and the number of discs for histological analysis was small. Nonetheless, we demonstrate a new model with high success rate for inducing slowly progressive IDD.

In summary, lumbar intervertebral disc puncture from the posterior approach using 27-gauge needles under C-arm fluoroscopic guidance reliably induced IDD, was minimally invasive, caused little bleeding, and was easily controlled. Moreover, the progressive changes in MRI, histology, and gene expression mirrored changes observed in human IDD. This rat model of IDD may be suitable for studying the pathogenesis of IDD and testing the effectiveness of growth factors or cell-based therapies for promoting disc regeneration.

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