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Original Article

A porcine model of lumbar disc degeneration induced by superficial layer of annular injury

Jinhui Shi, Zhen Wang, Junjie Niu, Huilin Yang

Department of Orthopedics, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, Jiangsu, China

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Abstract: Purpose: To establish an animal model of lumbar disc degeneration induced by superficial layer of annular injury that closely mimicked human disc degeneration. Methods: Twelve minipigs were used to study lumbar disc degeneration. The animals were separated to two groups; the experimental group includes 9 pigs, while control group includes 3 pigs. Two lumbar discs of each minipig of experimental group were injured by a 3.5 mm diameter trephine, while one adjacent disc was injured by a 4.0 mm diameter trephine. Depth of injury was 3 mm into the annulus fibrosus. The control group was accepted exposed surgery without annular injury. Disc appearance was visualized by magnetic resonance imaging, disc degeneration was evaluated with the Pfirrmann grading scheme. The animals were sacrificed after 1, 2, 3 months after surgery and the harvested lumbar spine submitted to histological examination. Results: At magnetic resonance imaging all injured disc demonstrated markedly degenerative signs. For 3.5 mm subgroup, discs were degenerated to Pfirrmann III and IV, with a moderate disc height decrease. For 4.0 mm subgroup, discs were degenerated to Pfirrmann V, with a severe disc height decrease. Discs in control group showed no degenerative changes. In experimental group, histological analysis showed an undefined border between the nucleus and annulus fibrosus. Reliquus nucleus fragments were detected with fibrous tissue replaced. The central lamellae were distorted and delamination of the outer anterior layers. Conclusions: Superficial layer of annular injury in porcine lumbar discs were found to cause disc degeneration on MRI and histological investigation. The severity of such degenerative changes depends on the severity of annular injury. 3.5 mm diameter trephine injury resulted in moderately disc degeneration, which is more mimicked human disc degeneration in discogenic low back pain patients.

Keywords: Disc degeneration, annular injury, porcine animal model, intervertebral disc

Introduction

Chronic low back pain is a common clinical disease, causing a significant socio-economic problem [1]. In China, a great deal of population is smokers and manual workers, it is much easier for them to suffer low back pain [2]. There is mounting of evidence of relationship between degenerate intervertebral discs (IVD) and low back pain [3, 4].

In order to facilitate systematic investigations of disc degeneration, animal model are often use. There are many different models which have been used in many different species over the years. However, lots of disc degeneration studies were limited using small and/or young animal with immature notochordal discs that generally offer a high regenerative potential, such as rat, mouse and rabbit [5]. These species' discs are very small compare with those in human. Though lots of studies have focus on establishing animal model, in vivo evaluation in large animal models with anatomical and biomechanical properties close to humans is still lacking. In recent years, several innovated therapies have developed to treat degenerative disc, such as mesenchymal stems cells transplantation and injection of hydrogel matrix [6, 7]. Although it is still has a long way to transfer to clinical therapy, biotechnology is tendency of therapy for disc degeneration disease.

The purpose of this study was to find a mimicked, reproducible, and simple animal model for the research of mechanism of human IVD, so as to facilitate new technique to apply in clinical work for disc degenerative disease patients.

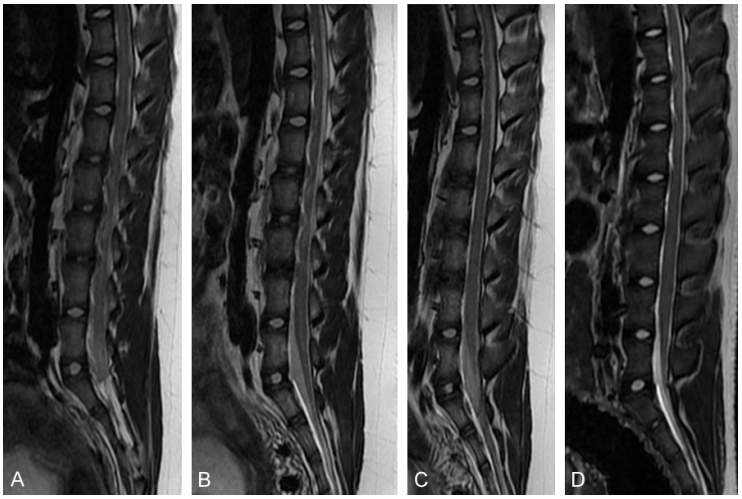

Figure 1. A-D. T2-weighted sigittal MRI scans, showing changes of lumbar disc degeneration after superfical annular injury. A. One month after injury: L1-2, L2-3, L3-4 discs are inhomogeneous with an intermediate gray signal intensity. The distinction between the nucleus and annulus is unclear, and the disc height is slightly decreased. B. Two months after injury: L1-2, L2-3 discs showed no obvious aggressive changes compared with one month, while the structure of L3-4 disc was inhomogeneous with a hypointense black signal intensity. C. Three months after injury: the structure of L1-2, L2-3 discs is inhomogeneous with a hypointense dark gray signal intensity. The distinction between the nucleus and annulus is lost, and the disc height is moderately decreased. L3-4 disc was black signal intersity, and disc height was significantly decreased. D. Control group at three month: the structure of the disc is homogeneous with a bright, hyperintense white signal intensity and a normal disc height.

In this model, superficial layer of annular injury induced by trephine were made in porcine's lumbar disc. The disc morphologic and histological changes were investigated by Magnetic Resonance Imaging (MRI) and histology.

Materials and methods

Animals

Twelve minipigs, initially 12 months of age and weighing 40 kg, were used in the study. The Animal Research Committee at the Soochow University approved the surgical protocol prior to the performance of animal experiments. The animals were kept in housing units that met the recommended weight-space specification, and were provided with water and nutritionally balanced feed.

Surgical procedure

Each animal was sedated by an intramuscular injection of ketamine (20 mg/kg body weight) and tranquilize (10 mg/kg body weight), and then anaesthetized by intravenous injections of 3% Pentobarbital Sodium (1 ml/kg body weight). The intervertebral discs of L1-2, L2-3, L3-4 were exposed through a left retroperitoneal approach. In experimental group, a 3.5 mm diameter trephine was used to create a lesion on lateral annular fibrous of L1-2 and L2-3, the depth of lesion was controlled as 3 mm. The fragment of annular fibrous from lesion was removed. A 4.0 mm diameter trephine was used to create a lesion on lateral annular fibrous of L3-4, the depth of lesion was same. Make sure the inner aunlar fibrous was intact, without nucleus pulposus outflow. In control group, only exposure of lumbar discs was done, without damage to disc structure. The discs were successfully lesioned in all experimental animals. Postoperatively, the animals recuperated in a facility for a period of 3 months, where they were monitored daily. MRI and histologic evaluation was investigated at 1, 2, 3 month. Each investigation included 2 experimental minipigs and 1 control minipig.

Degeneration grading and disc height index (DHI) evaluated by magnetic resonance imaging

Animals were placed supine position in a GE 3.0-T HDXT MR unit. After anesthesia, sagittal T1- and T2-weigh images were obtained using 4-mm slices. A presaturation band was placed anterior to the spine to cancel out abdominal motion. Axial T2-weighted imaging of disc spaces was performed. Image data were transferred to DICOM format. Pfirrmann grading scheme [8] were used to evaluate disc degeneration. The discs were assigned 1 of 5 grades according to MRI findings. The discs were graded by 2 spine surgeons blinded to the study. Disc Height Index (DHI) calculations were performed as described by Yoon and Masuda [9, 10]. The anterior, middle, and posterior portion of the IVD were measured to calculate the average value, then dividing them by the average of adjacent vertebral body heights.

Table 1. DHI values of L1-2 and L2-3 in 3.5 mm trephine subgroup and control group

Evaluate time point	DHI (L1-2, L2-3)		
	3.5 mm	Control	*P* value
Pre-surgery	0.33±0.02	0.34±0.03	0.15
1 month	0.30±0.04	0.34±0.03	0.04
2 month	0.29±0.02	0.33±0.04	0.02
3 month	0.27±0.03	0.34±0.04	0.00

Table 2. DHI values of L3-4 in 4.0 mm trephine subgroup and control group

Evaluate time point	DHI (L3-4)		
	4.0 mm	Control	*P* value
Pre-surgery	0.28±0.02	0.29±0.02	0.16
1 month	0.17±0.04	0.28±0.03	0.01
2 month	0.15±0.02	0.29±0.03	0.00
3 month	0.15±0.03	0.28±0.02	0.00

All values are given as means ± standard deviation. DHI = disc height index.

Histology of intervertebral discs

After euthanasia at predetermined time points, the animals by intravenous injection of an over dose of anaesthetics, spines were excised and fixed in 4% paraformaldehyde for 48 h, and transferred to the PBS solution. IVD were partially decalcified by using a rapid decalcifying formic acid/hydrochloric acid mixture. The IVD area was cut in cross-section with a razor blade and embedded in paraffin wax. Sections (5 μm) of the cross-section were stained with H&E and Masson's trichrome stain and imaged by using bright-field microscopy (Zeiss Axio Imager M1).

Statistical anyalysis

Statistical analyses included the comparison with experimental group and control group in MR imaging. DHI were analyzed using the paired Student t-test. Results were considered significant at probability values <0.05. Statistical comparisons were analyzed with SPSS 13.0 (SPSS Inc.).

Results

All of the animals successfully accepted the surgery. There were not any signs of infection or neurovascular injury. In experimental group, no nucleus pulposus leakage happened after annular lesion was made by trephine. The 12 minipigs had an uneventful postoperative course, including resumption of standing from the first postoperative day and increase of weight from the third week after surgery.

MRI analysis

Degeneration grading of disc was evaluated by Pfirrmann grading scheme. After superficial annular lesions were made, the discs had a significantly degeneration changes. At one months, L1-2, L2-3 and L3-4 disc demonstrated Grade III. At two months, L1-2, L2-3 discs showed a Grade III, while L3-4 discs showed a Grade V. At three month, L1-2, L2-3 discs showed a Grade IV, while L3-4 discs still showed a Grade V (**Figure 1A-D**).

Disc height index

Disc height decreased after annular injury in experimental group since one month evaluation. Disc height in control group showed no decrease at each time point. The DHI in experimental group was found to have a lower value than in the control group (P<0.01). In experimental group, 4.0 mm trephine subgroup showed a significant decrease compare with 3.5 mm subgroup in each time point (P<0.05) (**Tables 1**, **2**).

Histopathological results

Gross observations: The disc in experimental group showed an obvious disc height decrease. The annular lesion was filled with granulation tissue. Cross section observations showed significant nucleus pulposus collapse after injury comparing with no injury group. The area of nucleus pulposus decreased and replaced with fibrous tissue. The ability of hydrophilia also weakens because of gel content significantly decreased (**Figure 2A**, **2B**).

HE and masson stains: Histologic sections of the discs were examined using HE and Masson stains at 1, 2, and 3 months. In experimental group, all of the lesioned discs were significantly degenerated at three time points. Of Pfirrmann Grade III and IV, loss of nuclear material was demonstrated, accompanied with fibrous tissue taken place. Nuclear clefts were isolated inside of fibrous. Cells of nuclear were reduced. Annular fibrous showed a disordered structure without layers. The distinct of annular and nuclear was vanished. Of Grade V, most of the contents of the nucleus pulposus have

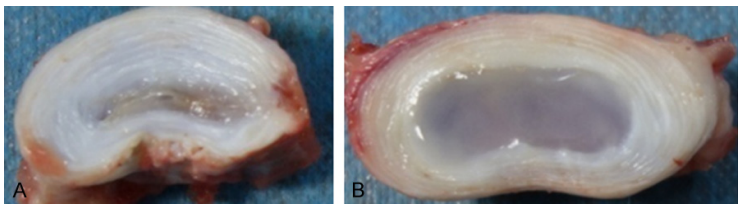

Figure 2. Picture of gross findings at 3 months. A. Lesioned disc showed a significant decrease of gel content of nucleus pulposus. The distinction between the nucleus and annulus was indistinguishable. Lesion on the right side of the superficial annular for injury was filled with granulation tissue. B. Unlesioned disc had a distinct border between nucleus pulposus and annulus fibrosus. Nucleus pulposus had a plenty of gel content so as to maintain water and proteoglycans.

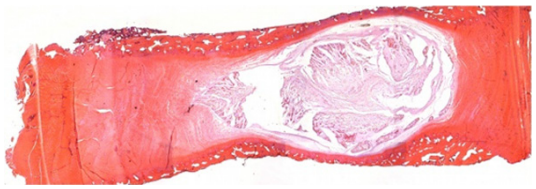

Figure 3. The normal interverbral disc of control group, showed a regular arrangement layers of annular fibrosus. The structure of nucleus pulposus was intact, had a clear boundary with inner annular fibrosus. (HE stains, ×25).

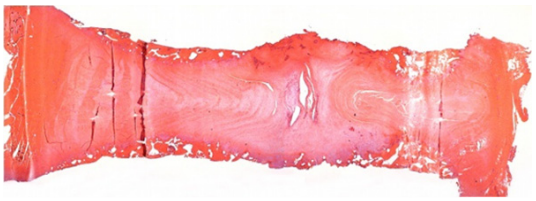

Figure 4. The residual annulus fibrosus was deformed and distorted, displaying an irregular and indistinct boundary with residual nucleus pulposus. Most of the nucleus pulposus had vanished and remnants were visible scattered throughout the central part of the intervertebral disc. (HE staining, ×25).

been vanished, and the annulus fibrosus broken, lost the arrangement of wavy fibrocartilage lamellas, fibrous tissue full filled in the area of nucleus pulposus. No changes were observed in the discs in the control group and adjacent to the lesion discs (**Figures 3**-**6**).

Discussion

Degenerate intervertebral disc (IVD) is a major contributor to low back pain. It is still lack of effective therapy to treat low back pain caused by disc degeneration. Therefore, to reveal the relationship between IVD and pain generation must be studied [11-14]. Because of the difficulty of obtaining human disc tissue, there have been many researches of animal models to mimic human lumbar disc degeneration, so as to analyze the mechanism of disc degeneration and find new treatment options [15, 16].

Injury models are common used because the exact timing of the insult can be precisely controlled, especially lesion or puncture to annular [3, 17]. While annular injury is believed to play a critical role in human IVD degeneration. Rabbit annular puncture models were first used to study DDD [10], however, their discs are very small compared with those in humans, so little intervene ways could be used to treat disc degeneration. Therefore, researchers tried to find large animal models to study the pathogenesis and treatment of DDD that may be more relevant to the human disc. Yoon et al [9] reported to use a 3.2-mm-diameter trephine to a 5-mm depth into annular fibrosus in porcine lumbar disc. The results showed a reliable model of sequential disc degeneration in miniature pigs. Significant disc degeneration was observed in the early stages of 5 weeks and no improvement by the final time point of 39 weeks. Osti et al [3] analyzed the effects of a surgical incision limited to the external annulus in sheep discs. The results showed this injury induces progressive degeneration in the inner anulus and nucleus pulpous, including loss of distinction between anulus and nucleus, marked degeneration of the nucleus, nuclear material was replaced with chondroid tissue, severe clefting extending to the peripheral portion of the disc, sclerosis, and ossification of vertebral endplates. Cinotti and Holm et al [18, 19] studied porcine disc degeneration model induced by endplate injury. They found endplate injury lead to degenerative changes in the disc tissue on MRI, histologic, and biochemical investigations. The severity of such degenerative changes was related to the severity of endplate injuries.

The model in this study has several advantages as following. First, the approach is simply and

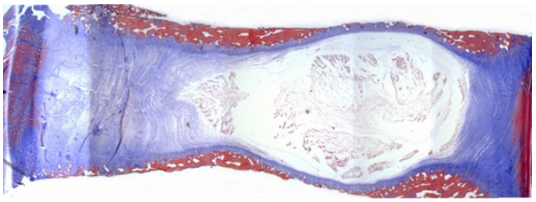

Figure 5. In the control groups,the annulus fibrosus arranges dense and neat. The nucleus pulposus forms regularly, it has many nucleus pulposus cells. A clear boundaries was showed between nucleus pulposus and annulus fibrosus. (Masson staining, ×25).

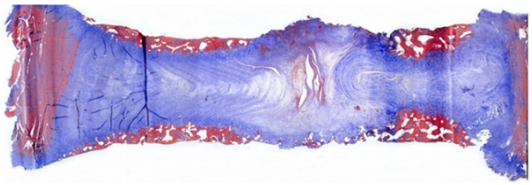

Figure 6. In the experimental groups, the annulus fibrosus layered disorder, distorted and broken. The nucleus pulposus turned fibrosis, cells were decreasing, the collagen arranges disorder. (Masson staining, ×25).

safe. In lateral position, it was easy to touch transverse process, which could be apparent marker for exposure. After dissection of the attachment points of paraspinal muscle by lance, it was easy to push away it from lateral lumbar spine. The approach could provide a clear field of vision from L1 to L5 lumbar spine, and easy to identify sequence of vertebrae by ribs. Second, the depth of lesion was strict limited on 3 mm, so as to confirm only outer annular layers were lesioned. In some studies [17, 20], a peripheral injury (made with a stab) resembling a radial tear was extended through the inner anulus, even into the nucleus pulpous, which induces nucleus pulpous avulsion and disc degeneration develops relatively quickly. However, acute prolapse of nucleus pulpous is thought to be a rare pathomechanism of human disc degeneration. Therefore, it may be the model of choice for studying disc regeneration and the effect of therapy such as growth factors [10, 21]. Third, the severity of degeneration of disc could be set, in 3.5 mm subgroup, the grade of disc degeneration kept as Grade III in first two months, there were still part of nucleus residual along with some nuclear cells. The moderate disc degeneration was similar with young adult low back pain patients according to MRI appearance. We concluded that process could mimic human discogenic low back pain. And residual nucleus provided timing to innovate technique for treating degeneration. Degeneration of lesion disc was aggressive. No regeneration of discs appeared in all experimental group.

To evaluate the progress of disc degeneration, this study used MRI and histological examinations. The signal loss of discs on T2-weighted MRI correlates with progressive degenerative changes of intervertebral discs [22]. The brightness of the nucleus has also been shown to correlate directly with proteoglycan concentration, which indicated the hydrophilicity of nucleus pulpous. In experimental group, 3.5 mm lesion group showed a Grade III degeneration, 4.0 mm lesion group showed a Grade IV degeneration. The degree of disc degeneration had a positive correlation with lesion. We conclude using different diameter trephine could precise control disc degeneration. That is very important for explore mechanism of degeneration, and also a window phase of biotherapeutics and gene therapy intervene.

The histological assessment of disc degeneration in this study was evaluated by HE and Masson stains. HE staining can clearly demonstrate a variety of different tissue structures of disc. In experimental group, investigated section of the nucleus pulposus was no longer demonstrable as an intact structure with a clear boundary, but replaced by newly formed hyaline cartilage and scar tissue among the residing fibrocartilage. Areas with remnants of nucleus pulposus were enclosed by a fibrous capsule. Masson staining showed no obvious inflammation inside of annular and nucleus was observed in all lesion discs. The disc was avascular except at its periphery. No vessel penetrated inside of inner annular and neucleus, so normal disc was immuno-privilege. Our models avoid lesion at inner annular, so maintain inner annular and neucleus structure intact. No inflammation occurred inside of disc. The lesion of superficial annular lesion healed with scar fulfilled. No neucleus pulpous leakage happened from lesion. At three months, there was not any recognizable necrotic tissue, except for scattered encapsulated fragments of the nucleus pulposus.

The lamellae in the external annulus were often found to be interrupted. Fragmentation and infold bulging of the inner annular lamellae were commonly seen.

In conclusions, degenerative changes occurred at annular and nucleus pulposus after a superficial annular injury. The severity of degeneration depends on the size of annular lesion. The superficial annular lesion can induce a simply, reproducible, reliable lumbar disc degenerative model of minipig, this model can be used in both study of mechanism and innovative therapy of IVD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huilin Yang, Department of Orthopedics, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, Jiangsu, China. Tel: +86 512 6778 0101; Fax: +86 512 6522 8072; E-mail: suzhouyang-8101@126.com

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