Puncture Intervertebral Disc Degeneration Model: A Standard on Rabbit

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Abstract: At present, a large number of people are suffering from low back pain, which becomes an urgent public health concern, intervertebral disc degeneration (IDD) is one of the most significant reasons of this disease. This study is aimed to select an optimal needle size to establish a rabbit IDD model by intervertebral disc puncture and the validity was verified by magnetic resonance imaging, histology and immunohistochemical staining. We divided 12 rabbits into four groups of 3 randomly with number table method, including 1 control group and 3 experimental groups. Then the lumbar 3/4 (L3/4), lumbar 4/5 (L4/5) and lumbar 5/6 (L5/6) discs were punctured by 14G, 16G and 21G needles respectively. According to the MRI and histology, the 16G-needle-punctured disc showed a progressive degeneration, the 14G-needle-punctured disc degenerated acutely one week after the operation while the 21G-needle-punctured disc did not show much degeneration. To sum up, the 16 G needle is the most suitable for making a model of IDD, which can replicate the occurrence, development and outcome of various histopathology of IDD in clinic. Although the 14G needle can cause the acute intervertebral disc injury, it is not an ideal approach to establish an IDD model. 21G needle causes little disc degeneration inversely, and it could be a promising approach to inject kinds of medicine to treat or prevent IDD.

Key words: Animal model, Intervertebral disc degeneration, Low back pain, Optimal choice, Puncture needle, Rabbit model

Introduction

With the increasing aging of population, much more people are suffering from low back pain, which becomes a serious threat to the health of the middle-aged and the elderly. It is said that about 80% people suffer from low back pain in their life, and it was the leading global cause of disability in most countries. From 1996 to 2013, low back pain accounted for the third-third highest personal health care spending in the United States and spending on low back pain increased the most over the 18 years, which makes it an urgent public health concern. Trauma, neoplasia, infection and inflammatory arthritis that affect the spinal structures directly and other biochemical changes or damages on lumbar structure are all contributors to low back pain. Intervertebral disc degeneration (IDD) is one of the most likely causes among them. IDD leads to the abnormal stress distribution in spinal functional units, alters the disc height and then causes the spinal stenosis and segmental instability. At last it results in the nerve root compression and irritation, which manifests as low back pain clinically and even chronically disabled if things go on like this. At present, treatment strategies for such symptoms are very limited, mainly to alleviate pain and postpone the time of operation, whereas the conservative treatments are often unsuccessful and operations are highly invasive and costly. Although different approaches have been proposed from molecular therapy to biomechanical tissue engineering to prevent and treat the degeneration of disc in recent years, and some in vitro experiment have been proved to be effective, the in vivo experiment is still the key to verify the function to treat or prevent IDD. The establishment of animal models, as one of the most basic and commonly used in vitro experimental methods, is a pivotal approach to understand the mechanism of IDD and explore the ideal treatment.

The establishment of a good animal model can replicate the occurrence, development and outcome of various histopathology of IDD to the maximum extent, which is a way to explore this problem efficiently. At present, there are many methods for the establishment of IDD model. For example, disc degeneration is caused by the extraction of some nucleus pulposus (NP) tissue by syringe puncture or by injecting drugs into the intervertebral disc tissue by syringe. What’s more, the removal of the supraspinous ligament and interspinous ligament by surgical method to cause lumbar instability can also lead to IDD. The change of mechanical factors interaction with intervertebral disc is also an approach to establish an IDD model. Afterwards, the feasibility of these methods was verified by imaging and histological examination. However, these methods of modeling are too complicated and there are too many human factors influencing the experimental results. Some researchers are trying to find a simple and effective way of modeling. They used needles to puncture the discs of rabbits to establish a model of IDD. Wang et al. successfully established the L5-6 acupuncture model on the fibrous ring surface of 10 New Zealand white rabbits by using 18G needle. Anderson et al. used 21G needle for puncture, established puncture model and extracted some NP tissues. Sobajima et al. used 16G needle to successfully establish the disc degeneration model, while Kim et al. used 18 and 21G needle to establish the disc degeneration model and mentioned that 21G needle is more advantageous in modeling. These studies were all verified by radiological and histological evidence.

Several species of animals have been selected to make models of IDD. Rats, rabbits, sheep, dogs, pigs and other mammals were...
included, and they all have their own advantages and disadvantages, so it is necessary to select through comparison to find a most suitable model. An ideal model of IDD always include the following conditions\cite{21,27}: 1) Being similar and comparable to the process of human IDD; 2) Being easy to operate and economical; 3) Being repeatable. Pigs, sheep and dogs are hard to use in large quantities due to their high cost, complicated operation, poor repeatability and long experimental course. At present, the model of IDD established by rats and rabbits are most widely applied because they are easy to achieve and their MRI results of degenerated discs are in line with that of human discs\cite{23,20}, and they also cost less than other animals. In our previous experiment\cite{27}, we successfully established an acupuncture rat caudal IDD model and compared the effects of different specifications of needles on the model. Anatomically, rabbits’ disc has a high degree of homology to human disc due to the presence of facet joints, paraspinous muscles, and ligaments\cite{29}, which makes it an excellent object to establish an IDD model. In this study, we established rabbit models of IDD by puncture needles likewise.

The technique of establishing an IDD model of rabbit is various but not mature enough, one of the most important reasons is there has not been enough research to show how to select a puncture needle of the appropriate specification to improve the success of model establishing. In terms of this, we selected different commonly used specifications of puncture needle to make rabbit IDD models in order to find the most suitable specification.

**Materials and Methods**

**Materials**

The animal center of Soochow University provided the New Zealand white rabbits used in this experiment (weighing 3.0-3.2kg, n=12). The rabbits were examined by MRI and X-ray in advance to eliminate congenital spine deformity and degenerative disc disease. Thereafter, we divided the 12 standard New Zealand white rabbits into four groups of 3 randomly with number table method, including 1 control group and 3 experimental groups. Each rabbit was free to food and water and kept in a dedicated room with constant temperature and humidity.

**Establishment of a rabbit intervertebral disc degeneration model**

For the establishment of the intervertebral disc puncture model, the puncture method used is roughly the same, but the specifications of needle used in each study are different, the impact of needle specifications on the model is certainly exist. Therefore, we tried to establish the acupuncture model with needles of different specifications, including 14G, 16G and 21G needles, and the lumbar 3/4 (L3/4), lumbar 4/5 (L4/5) and lumbar 5/6 (L5/6) discs were punctured by 14G, 16G and 21G needles respectively.

The experiment was approved by the animal committee of The First Affiliated Hospital of Soochow University (approval number 323/2019). Each rabbit was weighed and underwent segment position at the L3/4, L4/5, L5/6 by X-ray before the operation. After anesthesia of etamine and chlorpromazine by intramuscular injection, the rabbits were placed in a prone position. The whole operation was carried out in a sterile environment. The puncture position was disinfected with an iodine for environment. The puncture position was disinfected with an iodine for

**Magnetic resonance examination**

Three rabbits were randomly selected at 1 week, 2 weeks and 4 weeks after operation from the experimental group. After anesthesia, the rabbits were obtained on a 1.5 T MRI scanner (Philips Medical Systems, Andover, Massachusetts, USA), using the parameters of T2-weighted sagittal plane: repetition time/ echo time: 3500/102 ms, field of view: 15.0, thickness: 3 mm, interval: 0 mm. Thereafter, a 5-grade modified Pfirrmann system\cite{21} was used to evaluate the degree of disc degeneration: Grade I: The structure of disc is homogeneous, bright white and with expression of hyperintense; Grade II: The structure of disc is inhomogeneous with or without horizontal bands, with expression of hyperintense; Grade III: The structure of disc is inhomogeneous and gray, with intermediate to high-intensity signal; Grade IV: The structure of disc is inhomogeneous and gray to black, with a intermediate signal; Grade V: The structure of disc is inhomogeneous and black, with a low-intensity signal. All MRI results would be graded separately by two experienced radiologists. Grade I is marked as 1, Grade II as 2, and so on.

**Histological examination**

After MRI examination, the rabbits were euthanized and post-operation disc specimens were taken for histological examination. During HE examination, the rabbits were dissected into the following seven sections: I. **Annulus Fibrosus (AF).**

| Grade for morphology | Morphology Change Under Optical Microscope | Grade |
|----------------------|--------------------------------------------|-------|
| I                    | Normal texture and free of damage and distortion | 1     |
|                      | The damaged and distortion area is less than 30% | 2     |
|                      | The damaged and distortion area is more than 30% | 3     |
| II                   | Normal | 1     |
|                      | Micro disrupted | 2     |
|                      | Medium or severe disrupted | 3     |
| III                  | Normal cells with large amounts of vacuoles | 1     |
|                      | Cells and vacuoles decreased slightly | 2     |
|                      | Cells decreased moderately or severely without vacuoles | 3     |
| IV                   | Normal gel appearance | 1     |
|                      | Slightly congealed | 2     |
|                      | Morderate or severe condensation | 3     |
staining, paraffin sections were dewaxed by toluene and successively washed by xylene (I), (II) for 5 minutes, 100% ethanol for 2 minutes, 95% ethanol for 1 min, and then 80% and 75% ethanol for 1 min respectively. Afterwards, the sections were washed with distilled water for 2 minutes. Then they were stained in hematoxylin for 5 minutes and washed with floating water for 5 minutes, soaked in 75% hydrochloric acid ethanol for 30 seconds, and in water for 15 minutes, then dipped dyeing in 0.5% eosin solution for 2 minutes. Subsequently, we soaked the section in 95% ethanol and anhydrous ethanol for 3 min respectively for dehydration, and then soaked in xylene (I) and (II) for 3 min respectively. Finally, Paraffin sections were sealed with neutral resin. The results of HE would be graded according to the grade for morphology (Table 1).

Statistical Analysis
The data analysis was conducted by SPSS 21.0 software (SPSS Inc., Chicago, IL), with the process of the nonparametric Kruskal-Wallis test and Mann-Whitney U test on MRI and histological grades, and P<0.05 was considered significant.

Results
None of the 12 rabbits died during the operation, the incision healed well and there was no infection. After operation, it was observed that all the rabbits had a good diet and normal activities.

MRI
One week post-operatively, the L3/4 disc punctured by 14G needle turned out to be dimmer than pre-operation, which means the decrease of high-signal areas. Meanwhile, the L4/5 and L5/6 discs punctured by

Figure 1. MRI results of IDD. a) The first week post-operation, signal of L3/4 (14G) decreased obviously. Signals of L4/5 (16G) and L5/6 (21G) were dimmer than before, L5/6 (21G) signal decreased a little and remained hyperintense like normal disc. b) The second week after operation, signal from L3/4 (14G) tend to become hypointense. Signal from L4/5 (16G) decreased sequentially. c) Four weeks later, the MRI signal of L3/4 (14G) turned out to be hypointense and the disc space collapsed, signal of L4/5 (16G) decreased further, while the L5/6 (21G) signal remained hypointense.

Figure 2. Pfirrmann scores of the MRI results. Scores of 14G group were significantly higher than other groups. Pfirrmann scores of the 16G group increased obviously (P < 0.05). Pfirrmann scores of the 21G group increased slightly in 4 weeks (P > 0.05).
16G and 21G needles were almost similar to the normal disc, presenting high signal areas on the magnetic resonance image (Fig. 1a). Two weeks after operation, the L3/4 disc manifested as further decreased high-signal areas and intervertebral disc height while signals from the L4/5 disc punctured by 16G needle began to decrease and signals from L5/6 disc punctured by 21G needle were still unchanged (Fig. 1b). Eventually, the L3/4 disc punctured by 14G needle showed no obvious high-signal areas at all four weeks post-operatively and the height of intervertebral disc decreased markedly. Meanwhile, the high signal areas and intervertebral disc height from L4/5 discs punctured by 16G needle were further decreased. However, the magnetic resonance image of L5/6 disc punctured by 21G needle remained unchanged (Fig. 1c). According to the radiologists, the Pfirrman scores of L3/4 disc were higher than that of normal disc at one week post-operatively (normal disc Pfirrman Score = 1; P < 0.05). At the fourth week, the Pfirrman scores of L5/6 disc were a little more than that at the first week and turned out to be no statistic differences compared to the normal disc (P>0.05, Fig. 2). In addition, the Pfirrman scores of L4/5 disc showed a significant increase compared with the first week (P< 0.05, Fig. 2).

**Histology examination**

One week post-operatively, the AF from L3/4 disc punctured by 14G needle were damaged with the absence of NP cells. Meanwhile, the boundary between AF and NP were also disrupted. These processes became more severe until the fourth week, and most of the NP cells were lost or replaced by much fibrosis. On the contrary, the L5/6 disc punctured by 21G needle turned out to be little damaged over the four weeks, including the absence of only a little NP cells and most of the integrity of AF (Fig. 3a, d, g, h and i). As for the L4/5 disc punctured by 16G needle, the NP cells lost as time went by especially at the second week. Meanwhile, the AF was twisted and the boundary between NP cells and AF was disrupted. At the fourth week, there was obviously a void of NP cells and the boundary seemed to be blurry partly (Fig. 3d, e and f). In line with the histological scoring system, the scores of disc punctured by 14G and 16G needles are significant and increase over time (P<0.05), while the scores of 21G-needle-punctured disc present slightly increasing in 4 weeks and are not significant (P > 0.05, Fig. 4).

**Immunohistochemical staining**

As the immunohistochemical staining interpreted, the expression of collagen II and aggrecan from L3/4 disc punctured by 14G needle decreased over time from the first week post-operation while the expression of collagen X increased (Fig. 5, 6, 7. a, b and c). Meanwhile, with a lighter degree than that from L3/4 disc, the expression of collagen II and aggrecan from L4/5 disc punctured by 16G needle began to decrease and the collagen X expression began to increase from the second week.
Figure 5. Immunohistochemical staining of collagen II. Collagen II expression turned out to be decreased gradually in the 4 weeks. The 14G group expressed the least collagen II while the 21G group expressed the most.

Figure 6. Immunohistochemical staining of aggrecan. Aggrecan expression turned out to be decreased gradually in the 4 weeks. The 14G group expressed the least aggrecan and decreased faster than others while the 21G group decreased slightly.
The level of collagen II, X and aggrecan from L5/6 disc punctured by 21G needle turned out to be similar to that from normal disc. It can be seen from the above results that 14G and 16G puncture needle can result in the less expression of collagen II and aggrecan and the higher expression of collagen X.

Discussion

In this study, we successfully established an intervertebral disc degeneration model through disc puncture with different specifications of needle. This study followed the 3R principle strictly because the choice of rabbit to establish an IDD model was thoughtful, the number of selected rabbits was reasonable and the welfare of rabbits is guaranteed before and after operation. The annulus fibrous puncture method, as a more mature technology, can stably induce intervertebral disc degeneration, and its mechanism may be that the damage of AF reduces its water retention capacity, resulting in chronic dehydration and degeneration of NP tissue, and the time of degeneration is usually several weeks. Masuda et al. firstly proposed to puncture rabbit intervertebral disc with 16G, 18G and 21G needles for 5 mm, and compared with traditional disc injury model. Finally, all the models of intervertebral disc puncture caused intervertebral disc degeneration, and the degeneration of intervertebral disc was slower than that of AF model. Obviously, the degree of intervertebral disc injury caused by different specifications of puncture needle is different, so it is necessary to choose the most suitable specification of puncture needle to make the model more successful.

A healthy disc sits between two adjacent vertebrae, connecting the two adjacent vertebrae to maintain spinal flexibility and provide a cushion against the weight of the spine. Intervertebral disc is composed of AF and NP. The AF anchors to the cartilaginous endplates connecting to the vertebral bodies and keeps the NP in the center position. The main changes of IDD are the decrease of extracellular matrix, the decrease of chondroid cells in NP, the destruction of intervertebral disc structural integrity and angiogenesis.

MRI has an unparalleled advantage in tissue imaging, and it can clearly demonstrate the degeneration of the intervertebral disc. When the content of proteoglycan and collagen II in the intervertebral disc decreases, it directly leads to the loss of water in the NP. On MRI, it shows decreased signal intensity of the intervertebral disc and stenosis of the intervertebral disc height. Histological examination is a classic method to verify disc generation for its matrix expression of NP cells, AF and other tissues. The damaged and distortion area of AF, the decreased cells without vacuoles and the disruption of boundary between AF and NP can help assess the degree of disc degeneration. What’s more, the change of intervertebral disc height can also be observed through HE. The less expression of collagen II and aggrecan with the higher expression of collagen X indicates the more severity of disc degeneration. Collagen II is the main component of NP and has high water content, so that it can withstand various compressive stresses and shear forces on the disc, absorb concussion, and protect the stability of other structures and functions, such as the vertebral body and the intervertebral disc. The expression of collagen X marks the appearance of hypertrophic chondrocytes and the appearance of matrix calcification while the decrease of aggrecan may cause the disc to be unable to resist mechanical stress, thus affecting its repair process. These factors above can be tested by immunohistochemical staining.

In our study, we randomly divided 12 New Zealand white rabbits
into three groups and used 14G, 16G and 21G needle to puncture intervertebral discs at the same segment respectively. This method to establish an IDD model can avoid individual’s disc variations. Many scholars have already carried out similar experiments. Kim et al.21 tested different approaches to cause IDD on rabbits’ 4 lumbar discs. Camptothecin injection to L2/3 disc, L3/4 nucleus aspiration, L4/5 and L5/6 AF puncture by two specifications of needles were conducted and eventually they found that AF puncture was a relatively better way to establish a rabbit IDD model. In recent years, Ishikawa et al.22 used needles to puncture different times on L2/3, L3/4 and L4/5 to compare the degeneration of intervertebral disc in rabbits. The height of intervertebral disc is an essential factor to be considered when establishing an IDD model. Elliott et al.30 found that a puncture needle may directly alter mechanical properties via NP depressurization and/or AF damage, depending on the relative needle size. The height of rabbit lumbar disc is greater than that of rat coccygeal disc, so in this study, we selected the larger specification of puncture needle relatively to guarantee the validity and rationality of the establishment of IDD model. The results of MRI examination, HE staining and immunohistochemical staining of collagen II, X and aggrecan showed that the 14G-needle-punctured disc degenerated acutely one week after the operation. This may be due to the higher ratio of diameter to the height of puncture needle was more likely to cause larger trauma and lead to more severe disc degeneration as time went by38, 39. The 21G-needle-punctured disc did not show much degeneration over the four weeks and the 16G-needle-punctured disc showed a progressive degeneration which is similar to the process of human IDD. Compared with the acute intervertebral disc injury model caused by 14G needle, the animal model of progressive degeneration can better simulate the nature of human intervertebral disc degeneration and evaluate the effect of biotherapy.

Modeling methods for IDD are various, which are lacking in uniform standard, including induced by intervene of biomechanical mechanism, surgery, chemicals and needles. The models established by needle puncture is more convenient comparing with the former methods. In addition, this modeling method causes little damage to animals, which has a low incidence of infection and a short operation time. Furthermore, this method has good repetitiveness, lower prices, convenience in feeding and applicability in large-scale experiments. However, each model has its own advantage and disadvantage which is unavoidable. There remain some problems in this modeling method, which is also the cause of controversy. First, the establishment of puncture model is of poor stability, and there is lack in guiding basis in the process of puncture. As a result, the puncture is often not conducted in an ideal position, and the NP of the intervertebral disc is not punctured, which may not cause the degeneration of the intervertebral disc. In other words, the lack of operational guidance can lead to the failure of the disc degeneration model. At present, the C-arm can be a flexible assistance to provide guidance but the radiation exposure could be a problem need to be solved urgently. Second, New Zealand white rabbits intervertebral disc surface is small, comparing with its volume, which is not conducive to nutrition and metabolic infiltration, is not suitable for long-term in vitro culture. Third, compared with other methods for the establishment of intervertebral disc degeneration model, the degenerative effect caused by puncture model is generally light in imaging and histological manifestations, which may not meet the expectation of researchers. In addition, most degenerative changes occur slowly, and it takes a long time to cause obvious degenerative manifestations. These are the current problems of rabbit IDD models, and we are committed to improving them. Oppositely, this study to establish a rabbit IDD model can prove us with more precise approaches to simulate the human IDD process and investigate the effect of different kinds of drugs by injecting to the intervertebral disc.

According to our study, the 16 G needle is the most suitable for making a model of IDD, which induces the IDD process much more similarly to that of human IDD compared with the 14G and 21G needles, so it is an optimal choice to replicate the occurrence, development and outcome of various histopathology of IDD in clinic and can be applied as a standard model of IDD for further experiment. The 14G needle caused the acute intervertebral disc injury, and it is not an ideal approach to establish an IDD model. On the contrary, 21G needle causes little disc degeneration, and it could be a promising approach to puncture the disc to achieve tissue with minimally invasive for test or to inject kinds of medicine to treat or prevent IDD.

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Conflict of Interest

The authors have declared that no COI exists.

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