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Randomized, Experimental Trial

Impact of Needle Size on the Onset and the Progression of Disc Degeneration in Rats

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Background: Numerous animal models of intervertebral disc (IVD) degeneration have been proposed in the literature. The rat caudal disc has been used in disc degeneration studies because of its low cost and simplicity. However, no consensus on the size of the needle to be used during this process has been reached, yet.

Objectives: This study aims to select an optimal needle size to establish a reproducible IVD degeneration model.

Study Design: This is a randomized, experimental trial.

Setting: Cell therapy and experimental surgery of musculoskeletal system LR18SP1 Lab, The Faculty of Medicine of Sfax, Tunisia.

Methods: The validity was verified by magnetic resonance imaging (MRI), histological, and immunohistochemical examinations.

Results: The MRI, histological, and immunohistochemical examinations showed that a disc that is perforated with a 21G needle degenerated acutely one week after the surgery, while a 29G needle puncture failed to develop disc degeneration. A 25G needle induced progressive degeneration in the IVD.

Limitations: This study was not very long (6 weeks).

Conclusions: We conclude that the size of the needle affects the onset and the progression of disc degeneration;, a larger needle size leads to a more extended histological and radiographic degeneration within the IVD and in a relatively short time. Therefore, a 21G needle is an optimal choice to induce rapid degeneration in rats' caudal discs. However, the use of a 29G needle failed to establish a degenerative IVD model, which makes it ideal for IVD injection of drugs, plasmids, and growth factors. A 25G needle may be used to induce gradual degeneration.

Key words: Degenerative intervertebral disc, different needle sizes, caudal spine, animal model, optimal choice

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Intervertebral disc (IVD) degeneration is one of the most common public health problems and one of the most important reasons for low back pain and sciatica (1). The causes of IVD degeneration are thought to be multifactorial (age, hereditary factors, altered mechanical loading, etc.) (2). The current treatment options include conservative management (rest, anti-inflammatory/analgesic drugs, or bioactive agents) and aggressive surgical interventions: dynamic stabilization, spinal fusion, and disc arthroplasty (3). All these techniques are costly and correlated with significant complications. Many therapies are currently being

investigated as promising treatment methods, such as molecular therapy, biomechanical tissue engineering (4), and ozone therapy (5).

To test novel therapeutic strategies and explain the physiopathological mechanisms of disc degeneration, the establishment of a reproducible and progressive animal model that can consistently reproduce disc degeneration and mimic the degenerative process occurring in humans has become essential (6). Numerous animal models of disc degeneration have been proposed in the literature based on mechanical loading (7), nicotine inhalation (8), structural injury (9), spontaneous (10), chemical induction (11), genetic modification (12), and models. The above-mentioned methods are costly, sophisticated, and can demand special equipment. Given the complicated nature of disc degeneration, there is still no consensus on the "perfect" animal model that mimics the process of human disc degeneration – although several categories of animal models have been developed. An animal model must be ethically sound, reproducible, uncomplicated, cost-effective, and clinically relevant to the human situation (6).

In recent years, it has been shown that disc degeneration can be induced by introducing needles of defined sizes into the caudal disc in rats. This model of needle puncture has gained popularity principally because of its reproducibility, and the short time required to induce the desired degenerative effect similar to many of those observed in human disc degeneration (13). This model was first used in the lumbar spine of rabbits (14), and because of its benefits, it was then used in rodents like Sprague-Dawley rats (15,16). Later, the rat tail disc was proposed as a platform for the puncture model of inducing disc degeneration (17). The rat caudal disc is easy to manipulate, easily accessible to interventions, and does not require skin opening or direct exposure of the disc during manipulation. Thus, this model does not involve a risk of damage to surrounding structures (18).

However, there is still no consensus on the size of the needle to be used during this process. Therefore, the purpose of this study was to determine the optimal size of the needle that is required to establish a simple, reliable, and less invasive model of disc degeneration in the rat caudal spine. To achieve this goal, we punctured the tail discs of 18 Sprague-Dawley rats with 3 different size needles for each one. Magnetic resonance imaging (MRI), histological, and immunohistochemical examinations were used to evaluate the progression of disc degeneration.

Methods

Behavioral Tests

To avoid the pain caused by needle puncture, a feasibility test was carried out 15 days before the implementation of our study. Twelve rats were divided into 3 groups of 4: sham group, punctured group (where the rats were punctured in the same injection site and using the same needle gauges that will be used in our study thereafter), and a treated group, where rats were punctured and received an intramuscular injection of tramadol HCl ampule 100 mg (Teriak) as an analgesic agent, in a dose of 3 mg/kg for 3 days postpuncture. The rats were videotaped by a fixed video camera and behavioral analysis was performed on days one and 14.

Animals

A total of 18 male Sprague-Dawley rats, aged 12-14 weeks and weighing 300-350g, were studied. The animals were obtained from the Central Pharmacy (SIPHAT), and kept in the central animal house. All rats were housed in groups of 3 rats per cage. The rodents had free access to a commercial pellet diet (Sicco) and water as desired. They were kept under climate-controlled conditions. The room temperature was kept at 23°C ± 1°C and the humidity at 45% - 50% with a 12-hour light-dark cycle.

At one, 3, and 6 weeks postpuncture, 6 rats were randomly selected. Three of them were explored by MRI under anesthesia; then, the 6 were euthanized by injection of an overdose of ketamine, and their tails were sent for histological and immunohistochemical study. All experimental procedures were approved by the Tunisian ethical committee for the care and use of laboratory animals, following internationally recognized principles and judged commendable by the Committee of Animal Ethics (Protocol no. 94–1939).

Model Establishment

All the rats were anesthetized with a mixture of ketamine (90 mg/kg, Panpharma) and xylazine (10 mg/kg, Unimed) intramuscularly. The animals were then placed into a prone position. The coccygeal intervertebral discs Co6/7, Co7/8, and Co8/9 were selected for this study. They were identified by digital palpation and confirmed by radiography. These discs were noted by making a ring using a permanent marker on the skin corresponding to the levels to be punctured. The Co6/7, Co7/8, and Co8/9 discs were punctured by 21G, 25G, and 29G needles, respectively. The unpunctured disc (Co9/10) was left intact as a control.

In the manipulated discs, the tip of the needle was carefully inserted into the center of the nucleus pulposus (NP) through the annulus fibrosus (AF) at a controlled depth of penetration of exactly 5 mm, rotated 360° twice, and held for 30 seconds before extraction. To avoid exceeding the depth of 5 mm, all the needles used were marked. To prevent infection, the skin of the tail was sterilized before and after manipulation and each needle was used for puncturing only one disc. After the procedure, the rats returned to normal cage activity and were monitored daily for one, 3, or 6 weeks (i.e., the number of weeks they would be kept after the puncture), with no signs of pain or distress.

MRI Examination

MRI examinations were performed on 9 anesthetized rats (3 rats chosen randomly at each term: one, 3, and 6 weeks). The signal and structural changes of the caudal disc were evaluated by sagittal T2-weighted MRI slices. Images were acquired with a 7T MR system (Bruker BioSpin). The tail was introduced into a tube containing a 0.1 M CuSO4 solution to increase the contrast in the image and reduce the effects of susceptibility. We used a T2-weighted sagittal plane with the following parameters: repetition time/echo time, 2000/70 milliseconds; the field of view, 50 mm; the number of averages, 2. The slide thickness was one mm; interval, 0 mm; matrix, 256. A 2-cm-diameter surface receive coil and a 60 mm volume resonator were used. To grade the severity of IVD degeneration a 4-grade modified Pfirrmann system was used (19). The interpretation of the MRIs was accomplished by 3 spine surgeons in a blinded fashion to follow-up time and needle size. The quantitative data were presented as the mean of the 3 evaluations.

Histological Examination

After MRI, the rats were euthanized and IVDs Co6/7, Co7/8, and Co8/9 with adjacent vertebral bodies were harvested for histological analysis to determine the grade of degeneration. All harvested discs were fixed in 10% neutral-buffered formalin for 24 hours at room temperature, then decalcified in 10% ethylenedinitrilotetraacetic acid for 20 days to attain complete decalcification. The specimens were embedded in paraffin, dehydrated in graded ethanol (100%, 2 minutes; 95%, one minute; 80%, one minute; 75%, one minute), washed in xylene twice for 2 minutes, divided into sagittal sections (5 µm thick) by using a microtome (Leica) and stained with hematoxylin and eosin (H&E) for cellular constituents.

The histological sections were analyzed qualitatively under a light microscope (Olympus), then graded by a pathologist according to the grading established by Han et al (16). The histological scale consisting of 5 categories was used to assess the cellularity and morphology of the AF and NP, as well as the boundary between the 2 structures. Each category was scored from one, corresponding to a normal disc, to 3, corresponding to a completely degenerated disc. Thus, the score varies from 5, for a normal disc, to 15 for a severely degenerated disc. The sum of each score per category was presented for each animal and results were determined as a mean score per group.

Picrosirius Red Staining

This coloring aims to identify the appearance of a collagen fiber (collagen type I and type III). Tissue sections were deparaffinized in xylene and rehydrated by a graded series of ethanol. These sections were incubated in Weigert's Iron hematoxylin for 10 minutes and then washed in tap water. Sections were then stained with Picrosiruis (0.5 g Sirius red and 500 mL saturated aqueous solution of picric acid) for one hour and then washed in water and ethanol. After air drying, the slides were mounted on DPX (VWR) and analyzed in a Zeiss Axiovert 200 inverted microscope (Zeiss). Three sections per slide were assigned a score for the overall staining, where + = weak, ++ = moderate, and +++ = strong.

Immunohistochemical (IHC) Staining of Type II Collagen

Immunohistochemistry was performed for collagen type II. Briefly, 2 sections were deparaffinized with xylene and rehydrated through graded ethanol. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 minutes at 37°C. The slides were rinsed twice in phosphate-buffered saline solution for 5 minutes and boiled in 0.01 M citric acid buffer for antigen retrieval at 95°C for 15-20 minutes, followed by incubation with mouse monoclonal antibody to collagen Type II (Abcam). All sections were then incubated overnight at 4°C. The signal was treated with diaminobenzidine substrate (DAKO). The slides were counterstained with H&E and mounted. The slides were then dehydrated, washed, mounted, and imaged using a DXM 1200 C (Nikon) CCD camera.

Statistical Analysis

The Kruskal-Wallis and Mann-Whitney U tests were used to analyze the nonparametric data (histologic grading and MRI observations). All the data were expressed as mean ± standard deviation. All statistical measurements were performed using IBM SPSS Statistics 20.0 (IBM Corporation). The significance level was defined was as $P < 0.05$.

Results

Behavioral Tests

On the first day postpuncture, there were no differences in immobilization among the 3 groups. There was reduced locomotion in the punctured group; we also noticed leg lifting in 2 rats.

At 14 days, the punctured group displayed notably reduced locomotion and increased immobility but leg lifting was no longer present. However, no differences were observed between the sham and treated groups. From this perspective, treatment with tramadol HCl remarkably reduced this behavior. Therefore, the rats involved in the groups in our study received one injection of tramadol for 3 days postpuncture.

MRI

One week after the operation, the signal of the Co6/7 disc punctured by a 21G needle decreased considerably. Meanwhile, discs Co7/8 and Co8/9 punctured by 25G and 29G needles, respectively, presented high signals like the ones seen in the control disc (Co9/10) (Fig.1A).

Three weeks postoperatively, the Co6/7 disc manifested as a further decrease in IVD height. The signals from the Co7/8 disc punctured by a 25G needle began to decrease whereas signals from the Co8/9 disc punctured by a 29G needle remained unchanged (Fig.1B).

Six weeks postoperatively, the Co6/7 disc punctured by a 21G needle became thinner and showed a reduced high-signal area. Also, the height of the IVD decreased considerably. Meanwhile, the Co7/8 disc punctured by a 25G needle size decreased further and the MRI signal of the IVD Co8/9 punctured by a 29G needle remained unchanged (Fig. 1C).

The Pfirrmann scores of the Co6/7 discs were significantly higher than the nonpunctured discs at one week postpuncture ($P < 0.05$) (normal disc Pfirrman Score = one; $P < 0.05$). Six weeks postpuncture, the Pfirrmann scores of the Co7/8 discs increased significantly compared to those of the first week ($P > 0.05$). The Pfirrmann scores of the Co8/9 discs at the sixth week presented no statistical changes when compared with the normal disc ($P > 0.05$) (Fig.2).

Histology

One week postpuncture, the NP of the Co6/7 disc punctured by a 21G needle was irregularly contoured and its size decreased. We also observed damage to the AF. Meanwhile, the limit between the AF and NP was progressively disrupted. These processes became more severe in the sixth week, and most of the NP cells were gradually replaced by fibrous tissue (Figs. 3A, 3D, and 3G).

One week postpuncture the NP of disc Co7/8 punctured by a 25G needle had a rounded form and an intact limit dividing it from the surrounding AF. Three weeks postpuncture, the NP was shrunken, the AF was twisted, and the border between the NP and AF became less obvious. At 6 weeks, the NP was even smaller and the AF was disrupted (Figs. 3B, 3E, and 3H) while the Co8/9 IVD punctured by a 29G needle was just a little damaged (Figs. 3C, 3F, and 3I). Depending on the histological scoring system, the scores of the discs punctured by 21G and 25G needles increased significantly in a time-dependent manner ($P < 0.05$) while no significant differences in the scores were observed in

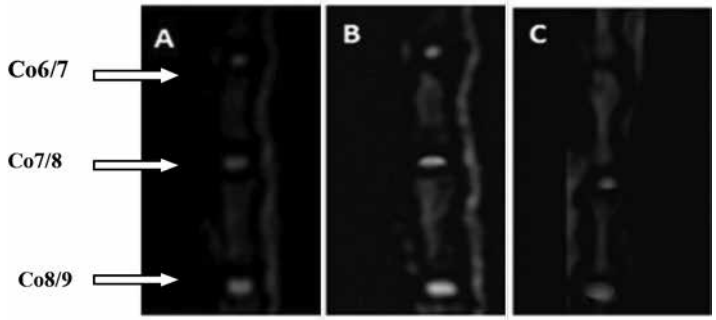

Fig. 1. A. *One week after puncture, the signal from IVD Co6/7 (21G) decreased obviously. Discs Co7/8 (25G) and Co8/9 (29G) exhibited high signals like a nonpunctured disc.* B. *Three weeks after puncture, signal from IVD Co6/7 (21G) further decreased. Co7/8 (25G) began to decrease. Co8/9 (29G) is still unchanged.* C. *Six weeks later, the signal of IVD Co7/8 (25G) decreased further, whereas the signal from IVD Co8/9 (29G) is still unchanged.*

the disc punctured by a 29G needle ($P > 0.05$, Fig. 4).

Collagen Quantification

For the quantification of the collagen, we performed picrosirius red staining that identifies collagen type I (Col I) and type III (Col III) in the AF. The immunohistochemistry method was used against collagen type II (Col II) to identify it in the NP (20). The picrosirius red staining allows the identification under polarized light of large collagen type I fibers by their orange/red birefringence, and thin collagen type III fibers by their green birefringence..

Collagen types I, II, and III expressions in discs punctured by a 21G needle decreased over time starting at one week postoperative. Expressions of collagen I and II of discs punctured by a 25G needle decreased from the third week postpuncture. At 6 weeks postpuncture, the levels of Col I, II, and III decreased further (Figs. 4F, 6F). However, the levels of Col I, II, and III of the discs punctured by a 29G needle turned out to be similar to the control disc (Figs. 5B, 5E, 6G, 6H, 6I). These results show that the expression of Col types I, II, and III can be reduced by puncture with a 21G or 25G needle, but not with a 29G needle.

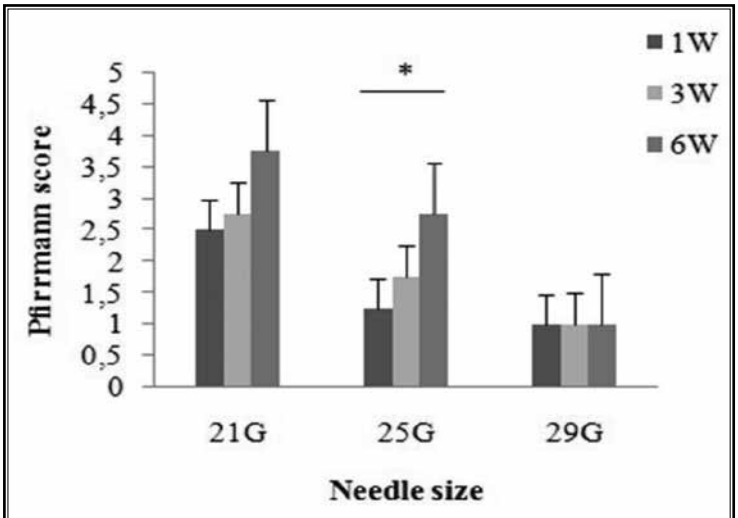

Fig. 2. *Pfirrmann scores of the 21G group were significantly higher than the other groups. The scores of the 25G group at 6 weeks were significantly increased compared to the first week ($P < 0.05$). The 29G group increased slightly in 6 weeks ($P > 0.05$).*

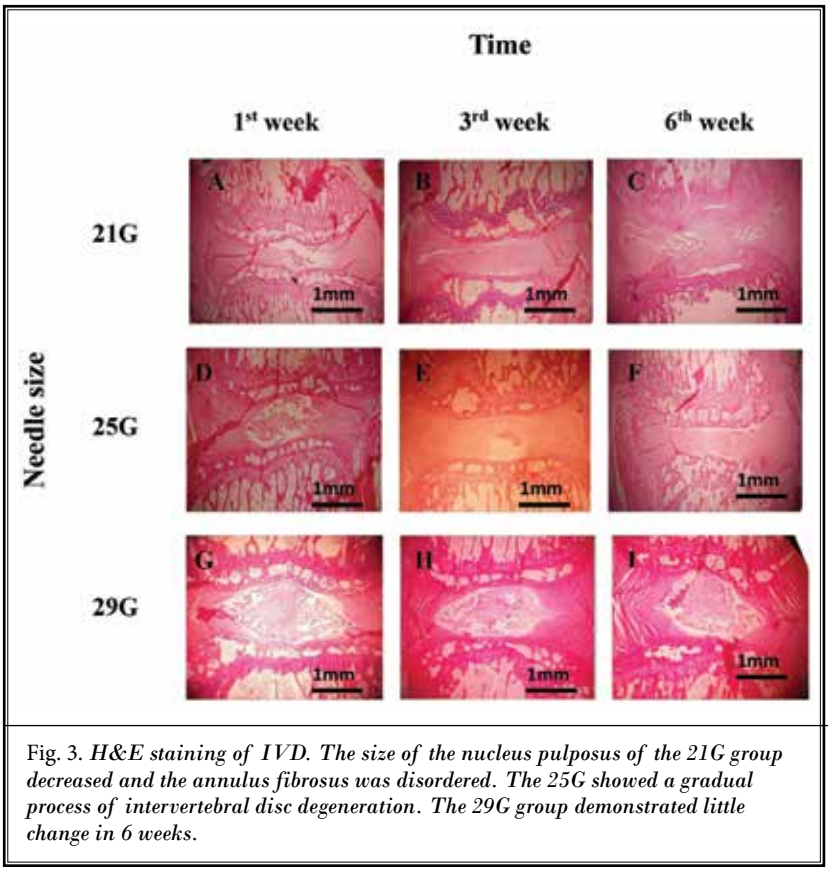

Fig. 3. *H&E staining of IVD. The size of the nucleus pulposus of the 21G group decreased and the annulus fibrosus was disordered. The 25G showed a gradual process of intervertebral disc degeneration. The 29G group demonstrated little change in 6 weeks.*

Discussion

Needle puncture is one of the techniques used to establish an animal model that mimics human disc degeneration (16,17). Many species of animals – including rabbits, pigs, sheep, dogs, etc. – have been used in the creation of animal models (9,14,16). However, the use of primates is restricted due to high costs and ethical limitations. The use of mammals is also limited because of the difficulty they cause in the establishment of the model, the long experimental course, the high cost, and the model's low reproducibility. Additionally, the use of rabbits is lim-

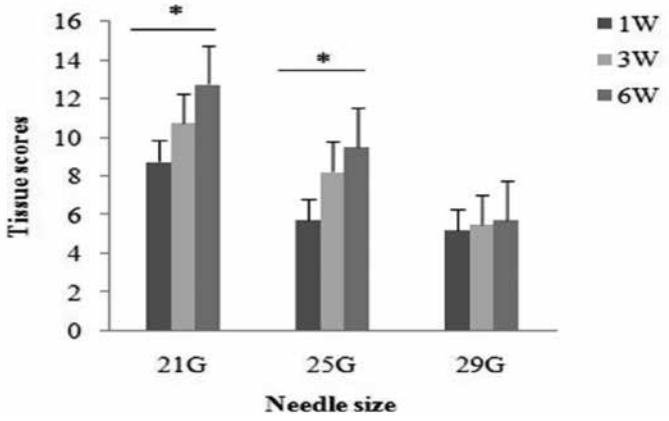

Fig. 4. *Histological scores of the 21G group were significantly higher than other groups. Histological scores of the 21G and 25G groups at the 6th week were significantly increased compared to the first week ($P < 0.05$). The 29G group presented slight increases in 6 weeks ($P > 0.05$).*

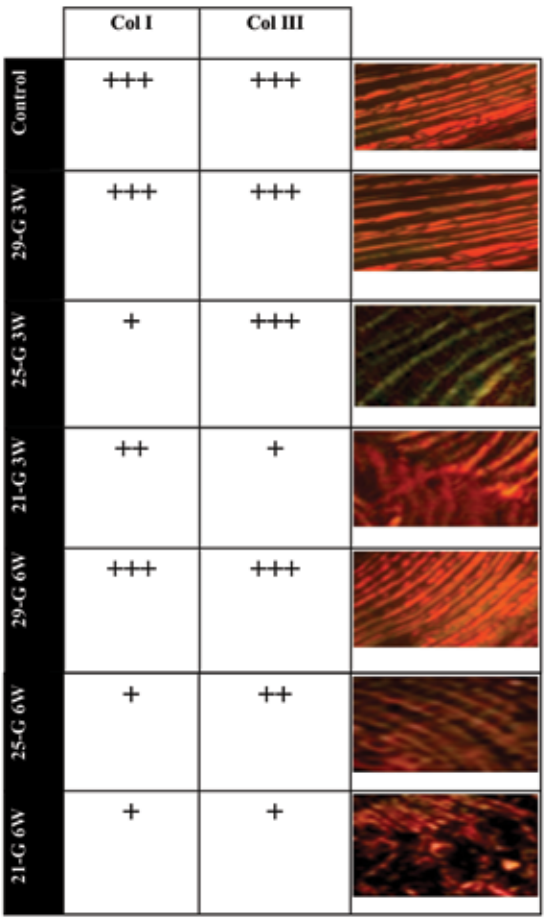

Fig. 5. *Picrosirius red staining for collagen type I and collagen type III in the annulus fibrosus only. The collagen type I fibers are shown in red/orange (the larger fibers), and the collagen type III fibers are shown in green (the thinner fibers), where + = weak, ++ = moderate, and +++ = strong, under polarized light.*
Scale bars : 200μm

ited due to the high infection rate, the model-creation complexity, and the lack of availability of relevant antibodies for the research. Hence, rats are better than the other animals because of the availability of relevant antibodies and low cost (21).

We selected the caudal IVD of the rat since it has the advantage of being easier to locate and operate on than the lumbar IVD, which requires a complete exposure of the peritoneal cavity of the animal and a surgical procedure that can be associated with increased morbidity (22). In this model, the needle was introduced via a very small incision without disturbing the surrounding ligamentous tissues and without interference with normal physiological function. Moreover, excessive dissection of the surrounding tissues can induce the formation of osteophytes that interfere in the evaluation of IVD degeneration (9).

As a recent animal model, using rats has been accepted by an increasing number of scientists. The caudal IVDs of rats were advantageous to be used in this study because of their similarity to human discs in their physiological features, anatomic structure, and biochemical components (22). Furthermore, this handling technique is very simple, reproducible, inexpensive, efficient, and significantly less invasive than alternative methods.

In this puncture model, the effect of the size of the needle, the number of rotations, and the depth of puncture have been proposed as important factors influencing the grade of disc degeneration. It has also been noted that the rotation of the needle leads to a more rapid degeneration (9,16), which was also proven by our study. Similarly, Aoki et al (23) showed that the depth of puncture had an effect on the degree of IVD degeneration. Many studies of this model in the rat caudal disc have been accepted but, to date, there has been no standardization in terms of the effect of the

initial injury severity. The standardization of this model is essential to evaluate the effect of therapeutic interventions. Our study analyzed a steadily triggered degenerative cascade using various needle gauges and showed stepwise improvements before achieving extreme degeneration.

MRI is the most direct method to grade the severity of IVD degeneration. The measurement of MRI assessment showed that the discs punctured with a 21G needle had significantly more severe degeneration when compared with the control group. The differences were observed in the discs punctured with a 25G needle only at the third week postpuncture. The 29G needle failed to cause IVD degeneration since no significant difference was detected.

For histological H&E analysis, significant differences were seen in the punctured discs (especially by 21G and 25G needles) when compared with control discs. The normal AF is composed of several layers of fibrocartilage made up of fibroblast in the outer layer and chondrocyte-like cells in the inner layer, while the NP consists mainly of chondrocyte-like cells and notochordal cells. The degenerated discs showed a shrunken NP, annular layer disorganization, and increased cell death. In addition, the degeneration of the IVD leads to annular tears and the inner annulus bulges inward. It was also noted that the osteophyte formation in the cartilage and formation of chondrocyte-like cells at the junction of the NP and AF resembled the typical appearance of human degenerative discs and animal models previously reported (24).

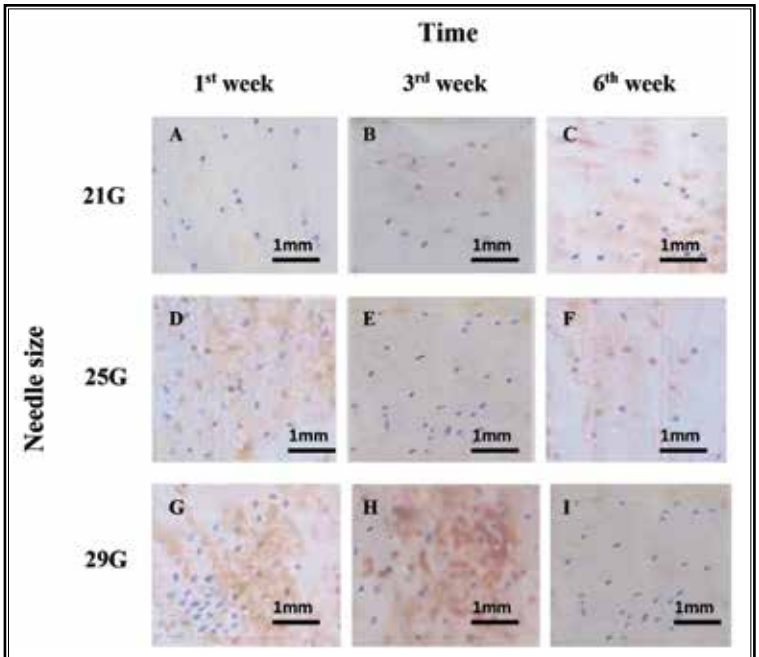

Fig. 6. *Immunohistochemical staining of collagen type II. Collagen II level decreased gradually in the 6 weeks. The 29G needle puncture expressed the most collagen II while the 29G needle puncture expressed the least.*

Immunohistochemistry is also an important method to evaluate disc degeneration. Therefore, the expression of collagen type II is one of the indicators used to evaluate the degree of disc degeneration. The collagen type II content decreases with the size of the needle used. Thus, the thicker the needle puncture, the less collagen type II expression. Previous studies showed that, in the case of NP degeneration, there is an increase in collagen type I and a reduction in collagen type II (25).

In the study by Cunha et al (26), 21G and 25G needles can induce a disc herniation. However, in our model, we did not find obvious disc herniation in the punctured site. At 6 weeks we also did not observe spontaneous regression. This can be attributed to the different approaches to establish IVD injury.

Even though this study was limited to 6 weeks of follow-up, we were able to conclude that the establishment of the IVD degeneration model is directly correlated to the needle gauge used. The larger needle size (21G) created more degenerative changes with time, particularly when compared with the smaller needle gauge (29G). The difference of degeneration was significant in almost all parameters when 21G and 29G were compared at a 6-week time point. The possible hypothesis is that the larger needle gauge produced more biomechanical and biochemical deterioration in the intervertebral disc which later accelerated the degeneration. Zhang et al (17) demonstrated that a 21G needle puncture into a rat tail disc induces a progressive disc degeneration process without spontaneous recovery. Kim et al (27) also mentioned that the 21G needle is more advantageous for establishing disc degeneration in their rat model based on 3 IVD punctures at once. These studies were all verified by radiological and histological evidence.

Our results were consistent with those of previous studies. Indeed, the 21G and 25G needles were found to be optimal for inducing a model of disc degeneration but the 29G needle could not induce it, suggesting that the latter should be used to inject plasmids, growth factors, or other pharmacological reagents or

cells to avoid damage. This may support the concept that establishing a proper disc degeneration model may need the use of a large size needle track to induce continuously progressive degeneration changes (28). Elliott et al (29) indicated that the size of a needle (ratio of the needle diameter to the height) must be over 40% to induce significant IVD degeneration. Also, Hu et al (30) demonstrated that the 21G (ratio 0.63) needle would cause rapid and severe degeneration in rat caudal discs (height 1.3 mm) while a 25G (ratio 0.39) needle can induce a significant but less severe disc degeneration. The degree and rate of degeneration were different with each needle size. Masuda et al (9) used a surgical blade to establish IVD degeneration in a rabbit that was relatively larger than 40% of disc height. The results showed that stabbing was less controllable than needle puncture for producing disc degeneration.

As any animal model has limitations, this model has the disadvantage that rat tail discs are different from the human lumbar spine in terms of mechanical loading, anatomy, dimensions, composition, and metabolism (31).

Conclusions

In our present study, a simple disc degeneration model was created using percutaneous needle puncture in rat tail discs. This is a minimally invasive, cost-effective, qualified, and reproducible method for the analysis of the pathophysiological features of disc degeneration. A 21G needle is an optimal selection to establish a rapid degeneration model whereas a 29G needle can be used to inject plasmids, growth factors, or drugs. A 25G needle may be used to induce a gradual degeneration in rat caudal discs.

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