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Basic Science

IL-1β promotes disc degeneration and inflammation through direct injection of intervertebral disc in a rat lumbar disc herniation model

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Abstract

BACKGROUND CONTEXT: Lumbar intervertebral disc herniation (LDH) is a common disease that causes low back pain, radiating leg pain, and sensory impairment. Preclinical studies rely heavily upon standardized animal models of human diseases to predict clinical treatment efficacy and to identify and investigate potential adverse events in human subjects. The current method for making the LDH model involves harvesting the nucleus pulposus (NP) from autologous coccygeal discs and applying to the lumbar nerve roots just proximal to the corresponding dorsal root ganglion. However, this surgical method generates a model that exhibits very different characteristics of disc herniation than that observed in human.

PURPOSE: To produce a rat LDH model that better resembles disc herniation in humans and a standardized and uniform LDH model using Interleukin-1 beta (IL-1β).

STUDY DESIGN: Experimental rat LDH model.

METHODS: We exposed the L5–6 disc dorsolaterally on the right side through hemi-laminectomy without nerve compression. Herniation was initiated by puncturing the exposed disc with a 30-gauge needle at a depth of 4 mm. Interleukin-1 beta (IL-1β) was injected simultaneously to heighten the pathological processes of disc degeneration, including inflammatory responses, matrix destruction, and herniation of the NP. We performed histological staining to assess morphological changes, immunohistochemistry to analyze inflammation- and pain-related expression within and around the puncture site of the L5–6 disc, and real-time polymerase chain reaction to examine expression of markers for degenerative processes. In addition, we performed locomotor tests on the rats.

RESULTS: We found that the IL-1β groups showed that the border between the annulus fibrosis and nucleus pulposus was severely interrupted compared to that of the control (puncture only) group. And, the injection of IL-1β leads to accelerated disc degeneration and inflammation in a more consistent manner in LDH model. Functional deficit was consistently induced by puncturing and injection of IL-1β in the exposed disc.

CONCLUSIONS: The method proposed here can be used as an index to control the severity of disc degeneration and inflammation through the injected IL-1β concentration concurrent with surgically induced herniation.

CLINICAL SIGNIFICANCE: Our proposed model may facilitate research in drug development to evaluate the efficacy of potential therapeutic agents for disc herniation and neuropathic pain and may also be used for nonclinical studies to more accurately assess the effectiveness of various treatment strategies according to the severity of disc degeneration.

FDA device/drug status: Not applicable.

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Ethical considerations: All procedures were approved by Jaseng Animal Care and Use Committee (Approval No. JSR-2020-02-002).

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Introduction

Lumbar intervertebral disc herniation (LDH) around the spinal nerve root is a common spinal disease causing radiculopathy, which presents with motor and sensory impairments including paresthesia, sensory loss, and radiating pain [1−3]. Radiating pain has been reported as the most common type of neuropathic pain [4]. In previous studies, autologous transplantation of nucleus pulposus (NP) has been used to create animal models to investigate radiating neuropathic pain caused by LDH around the spinal nerve root [5−12]. When transplanting the NP of an animal to the nerve root, neuropathic pain occurs along with other functional and structural changes of the nerve root. In the study of neuropathic pain with this model, it has been reported that allodynia and hyperalgesia from mechanical stimuli begin 1 day after transplantation and decrease within a few weeks, indicating that these symptoms only last for a short period of time. Conventional animal models induce radiating neuropathic pain using NP harvested from coccygeal discs, but this model does not accurately reproduce clinical cases of radiating pain. In addition, LDH symptoms are not pronounced in these models, and the method of model creation is not standardized. With the exception of LDH cases induced by trauma, degenerative changes of the disc precede NP protrusion, and activities of pro-inflammatory cytokines and chemokines are generally increased in the NP during the degeneration process [13]. Herniation of NP and the resulting sharp increase in inflammatory responses involving immune cells such as macrophages in the epidural space leads to neurotoxicity and pain [14]. Since the induced neuropathy in this model lasts only for a short period of time following NP transplantation, it is not a suitable model of the later stages of increased inflammatory activities and has limited utility for monitoring and assessing changes in long-term patterns of pain.

This study investigates a novel animal model of LDH in which pro-inflammatory cytokine interleukin 1 beta (IL-1β) is injected into discs to rapidly induce NP herniation and inflammatory responses, and subsequently elicit symptoms of nerve compression and pain due to disc herniation. IL-1β is the predominant cytokine involved in the pathological process of intervertebral disc degeneration (IDD). Previous studies showed that IL-1β is the most important inflammatory cytokine for increased matrix degradation, decreased matrix synthesis, and induction of lower back pain [13,15,16]. Compared to the herniated animal models presented in previous studies in which the NP extracted from the rat's coccygeal disc is transplanted between the lumbar spinal cord and the dorsal root ganglion (DRG), the method proposed here induces symptoms of LDH in a considerably shorter time and enables the creation of a standardized animal model. In addition, our model more closely mirrors the pathophysiological environment, leading to clinical pathology mimicking that of a human LDH patient [17]. Therefore, this study ultimately aims to present a model that facilitates long-term follow-up to evaluate the efficacy of therapeutic candidates in neurobehavioral investigations. Moreover, we aim to provide a quick method of creating an accurate and consistent animal model of spinal herniation inducing LDH. This animal model has the potential for application in future research to evaluate the efficacy of various therapeutic substances and elucidate the immune mechanisms underlying LDH.

Materials and methods

In vivo model of disc herniation and local delivery of IL-1β

Adult male Sprague-Dawley (SD) rats (7 weeks old, 230−250 g) were used in this study. All procedures were approved by the Animal Care and Use Committee (Approval No. JSR 2020-02-002-001-A) of our institute. The animals were maintained in individual cages under a 12-hour light/dark cycle with controlled temperature (23°C−25°C) and humidity (45%−50%) with free access to food and water. Briefly, the rats were anesthetized with 2% to 3% isoflurane gas (Forane; BK Pham, Goyang, Korea) and hemi-laminectomy at L5 was performed on the right side using fine rongeurs to expose the L5−6 discs. L5−6 discs were punctured by 30-gauge needles (Sung Shim Medical Co., Ltd., Korea) at depths of 4 mm from the disc surface. IL-1β (PeproTech, Rocky Hill, NJ) was injected simultaneously with disc puncture. The sham animals received a hemi-laminectomy alone on the right side of the L5 level. The cord was then covered with an absorbable hemostat (Surgicel fabric, Johnson and Johnson, Arlington, TX). All rats received an intramuscular injection of 40 mg/kg cefazolin sodium (Cefazolin Inj, Chong Kun Dang pharm, Korea) after suturing. The animals also received oral administration of 10 mg/kg acetaminophen syrup (Tylenol, Janssen Pharmaceutica, Titusville, NJ) after recovery from anesthesia. Rats were divided into five groups (n=18/group): Sham group (hemi-laminectomy without puncture); Control group (puncture only); 1 ng (puncture +IL-1β_1 ng/100 μL); 10 ng (puncture+IL-1β_10 ng/100 μL); 100 ng (puncture+IL-1β_100 ng/100 μL). The rats were sacrificed at 1 or 3 weeks after the operation for histological and molecular analysis.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on rats under isoflurane anesthesia using a 9.4 T animal MRI scanner (Agilent 94/21; Agilent, Santa Clara, CA) at the Korea Basic Science Institute in Ochang. MRI sequence images were acquired in the axial and sagittal planes before surgery and at three time points: 1 day, 1 week, and 2 weeks after disc puncture to analyze changes in NP herniation.

MRI axial images were also acquired 1 week after disc puncture and injection of IL-1β to assess the degree of NP herniation between the groups.

Histological analysis

To reveal the morphological changes and pathophysiological conditions within and around the disc site, the rats were perfused with 0.9% normal saline (Sigma-Aldrich, St. Louis, MO) and 4% paraformaldehyde (Biosesang, Seongnam, Korea) by cardiac perfusion for staining and immunohistochemistry. The lumbar spine containing the L5–6 disc and DRG neurons was extracted, then fixed overnight in 4% paraformaldehyde at 4˚C. Spine specimens were decalcified in decalcification solution (BBC Biochemical, Mount Vernon, WA) until the spine became soft, after which they were rinsed three times with phosphate-buffered saline (PBS) and cryoprotected in 30% sucrose for 3 days. The samples were sectioned in the axial plane into 20-μm thicknesses. Safranin-O/Fast Green staining (Sigma-Aldrich, St. Louis, MO) was performed according to the manufacturer's instructions on the L5–6 disc site to confirm the damage of the punctured disc in each group at 1 week. The stained sections were imaged with an inverted microscope (Eclipse C2 Plus, Nikon, Japan). The grade of damage in intervertebral discs was assessed using a histological grading scale based on 4 categories of degenerative changes with scores ranging from a normal disc with 4 points (1 point in each category) to a severely degenerated disc with 12 points (3 points in each category) [18]. Immunohistochemistry was used to analyze inflammation- and pain-related expression within and around the puncture site of the L5–6 disc. Primary antibodies against monocyte/macrophage CD68 (1:500, Abcam, Cambridge, UK), Aggrecan (1:200, Abcam, Cambridge, UK), MMP3 (1:50, Abcam, Cambridge, UK), TRPV1 (1:200, Alomone, Hadassah Ein Kerem, Israel), and NeuN (1:500, Synaptic Systems, Göttingen, Germany) were incubated overnight at 4˚C. After the sections were washed three times, secondary antibodies (rhodamine goat anti-guinea pig, FITC-goat anti-mouse or anti-rabbit [Jackson ImmunoResearch Laboratories, West Grove, PA]) were diluted to 1: 300 in 2% normal goat serum in PBS. Following 2 hours of incubation, the sections were rinsed three times with PBS. The stained tissue sections were imaged using confocal microscopy (Eclipse C2 Plus, Nikon, Japan). To accurately quantify the fluorescence intensities, confocal images were acquired using identical acquisition settings. Background signals were subtracted using a rolling ball algorithm of ImageJ. The average intensity of CD68, Aggrecan, MMP3, and TRPV1 labeling was measured with ImageJ. The inflammatory response was quantified by manually counting the number of CD68+ cells. TRPV1-positive neurons were also manually counted and the result was expressed as the percentage of neurons expressing TRPV1 to verify the degree of pain in each group.

Real-time polymerase chain reaction

We identified changes in the expression of genes involved in inflammation and disc degeneration using real-time polymerase chain reaction (PCR). RNA was isolated using the Allprep DNA/RNA/protein kit (Qiagen, Hilden, Germany) from L5–6 discs containing the DRG neuron for each group. cDNA was synthesized using random hexamer primers and Accupower RT premix (Bioneer, Daejeon, Korea). All primer pairs were designed using the UCSC Genome Bioinformatics and the NCBI database and are listed in Table. Real-time PCR was performed using SYBR green supermix (Bio-Rad, Hercules, CA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Each real-time PCR was performed as at least a triplicate assay. Expression of each target gene was normalized to GAPDH levels and expressed as the fold change relative to the control group.

Functional assessments

Locomotor function was evaluated using three assessments after herniation induction: Von Frey test, the Basso, Beattie, and Bresnahan (BBB) scale, and the horizontal ladder test. The Von Frey test is a method of evaluating behavior in response to pain, once a week after surgery. Briefly, rats were maintained for adaptation in the testing environment 15 minutes prior to measurement. The latency for the paw withdrawal response was measured upon applying mechanical stimulation to the center of both hind paws using the Von Frey filament (Ugo Basile, Varese, Italy). A positive avoidance response was indicated by lifting, whipping, licking, or running of the paw during stimulation, and the average value of three or more measurements was used. The BBB scale was performed once a week after surgery and was expressed as a score from 0 to 21 points (no hindlimb movement is 0, and normal hindlimb movement is 21) [19]. Two independent observers analyzed the motion of the hindlimbs for 4 minutes in an open field (cylindrical acrylic box; 90 cm diameter, 15 cm height) and the average value was used as the representative score. The ladder walking test was performed to assess the balancing ability of the rats [20]. All rats walked on the metal runway

Table
Primer sequences used for real-time PCR analysis

Gene	5′-3′	Primer sequence
IL-1β	Forward	TTGCTTCCAAGCCCTTGACT
	Reverse	GGTCGTCATCATCCCACGAG
IL-10	Forward	TAACTGCACCCACTTCCCAG
	Reverse	AGGCTTGGCAACCCAAGTAA
Aggrecan	Forward	GCCTCTCAAGCCCTTGTCTG
	Reverse	GATCTCACACAGGTCCCCTC
MMP-3	Forward	ATGATGAACGATGGACAGATGA
	Reverse	CATTGGCTGAGTGAAAGAGACC
GAPDH	Forward	CCCCCAATGTATCCGTTGTG
	Reverse	TAGCCCAGGATGCCCTTTAGT

(2.5 cm between grids) from left to right three times and their movements were captured with a digital camcorder. The ladder score was calculated as below.

$$\text{Ladder score} = \text{erroneous steps of hind limb} / \text{total steps of hind limb} \times 100\,(\%)$$

The locomotor test of each group was examined every 7 days until sacrifice. All locomotor tests were analyzed by two observers who were blinded to the identity of the rats.

Statistical analysis

All results are statistically confirmed by Prism software (GraphPad, San Diego, CA) as the means±standard error of the mean [21]. Multiple comparisons among five groups were analyzed via one-way analysis of variance with Tukey's post hoc test. Differences were considered statistically significant if $^{\#}p<.05$, $^{\#\#}p<.01$, $^{\#\#\#}p<.001$ vs sham group and $^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$ vs control group.

Results

Experimental design and surgical procedure of the LDH model

The L5–6 discs of rats were punctured dorsolaterally on the right side and were simultaneously injected with IL-1β to create an LDH model that more accurately depicts human disc herniation. Fig. 1A is a schematic diagram illustrating the process. The neurological symptoms of disc herniation in this model were controlled by the concentration of injected IL-1β. Rats in the sham group underwent only a right-sided hemi-laminectomy without nerve compression and disc damage. The inset of the surgical images illustrates the procedures performed. The first image from the right shows that the lumbar 5 vertebrae was positioned in the center site before removing the spine (Fig. 1B). Hemi-laminectomy was performed until the L5–6 disc was exposed without nerve compression (Fig. 1C). After exposing the L5–6 disc, the needle was inserted obliquely on the side of spinal cord and within the disc through the space between the spinal cord and the DRG (Fig. 1D).

Histological analysis of LDH model injected with varying concentrations of IL-1β

We performed MRI analysis to determine the time points for NP herniation after the disc puncture. The same rat was tracked every week for 2 weeks following surgery. The T2-weighted axial plane MRI image showed that the amount of NP within the disc decreased dramatically in the first week after surgery and noticeably at 2 weeks compared to a normal disc (Fig. 2A). Furthermore, images were also acquired

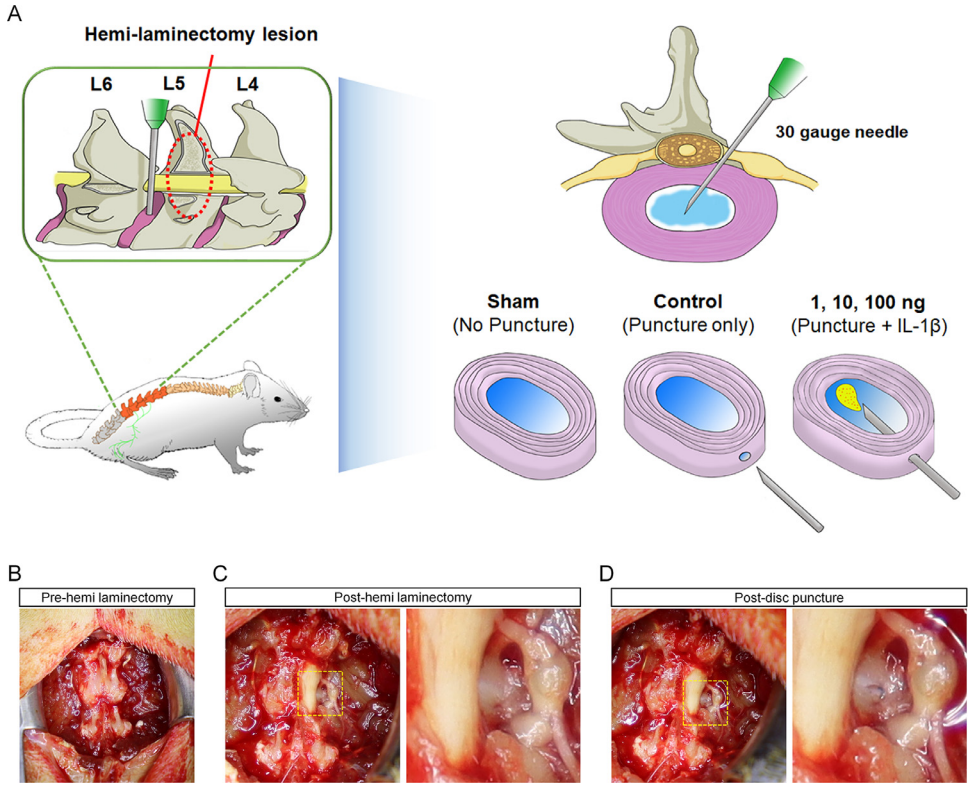

Fig. 1. Scheme of the lumbar disc herniation (LDH) experimental design and sequence of surgical procedure. (A) An experimental scheme for induction of disc herniation by using IL-1β injection. (B) The process of exposing the right-hand half of the lumbar spinal cord and dorsal root ganglion (DRG) for disc herniation, through hemi-laminectomy. Also, (C) and (D) demonstrate the disc dorsolaterally before and after needle puncture. Note the visible disc before and after needle puncture (in yellow dashed boxes).

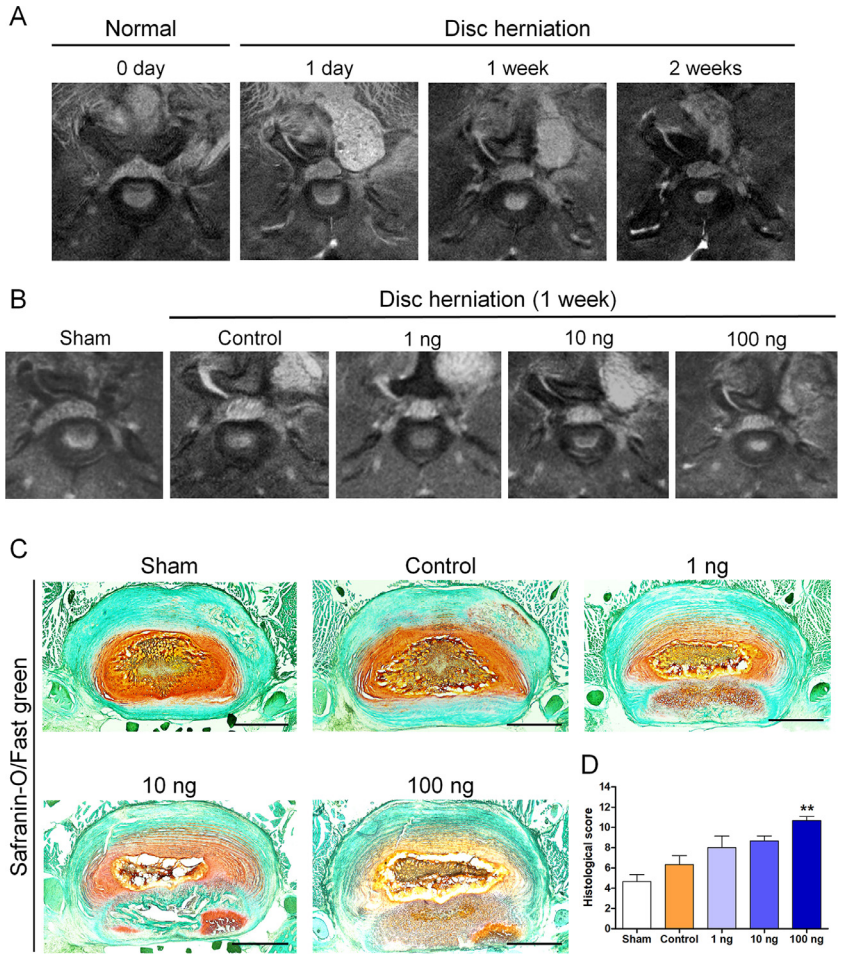

Fig. 2. Histological analysis of the LDH model injected with IL-1β of varying concentration. (A) MRI of the lumbar spine after needle puncture in L5–6 disc. (B) Representative MRI axial images at 1 week in each group. (C) Representative axial Safranin O/Fast green stained images of the L5–6 disc. Scale bar=400 μm. (D) Histological score of discs at 1 week after the puncture and injection of IL-1β. Data are expressed as the means±standard error of the mean. Significant differences indicated as **p<.01 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

in the sagittal plane before surgery and at three time points: 1 day, 1 week, and 2 weeks after disc puncture (Supplementary Fig. S1). Although MRI sagittal images showed a tendency toward reduced and herniated NP, disc protrusion was not observed following the disc puncture. In addition, we scanned the punctured disc axially to compare the degree of NP herniation between the groups after disc puncture and simultaneous injection of IL-1β. The results showed that NP herniation occurred much faster in the IL-1β group than in the control group. The remaining NP within the disc was reduced in a dose-dependent manner. Therefore, we confirmed that NP gradually escaped through the hole of the ruptured disc and that IL-1β can promote NP herniation.

Next, we evaluated the morphological change of the disc following puncture and injection of IL-1β by Safranin-O/Fast Green staining (Fig. 2C). The shape of the disc remained intact and the annulus fibrosis (AF) had a normal pattern of fibrocartilage lamellas in sham-operated rats, whereas in LDH model rats, the disc was ruptured and the border between the AF and NP was interrupted. In particular, the IL-1β-injected groups showed that the border between the annulus fibrosis and nucleus pulposus was severely interrupted compared to that of the control group. These characteristics were quantified using a histologic grading scale ranging from 4 to 12 (Fig. 2D). The sham group scored 4 and showed characteristics of a normal disc: intact AF with a normal pattern of fibrocartilage lamellas and well-defined border between the AF and NP. In contrast, the score of the IL-1β group gradually increased in a dose-dependent manner, with significantly higher scores compared with the control group. These findings confirmed that the concentration of IL-1β can be used as an index to control the severity of the degenerative disc model.

In vivo inflammation in LDH model injected with varying concentrations of IL-1β

We next quantitatively evaluated the degree of inflammation by immunohistochemistry. CD68 was used as an

inflammatory marker associated with the recruitment of monocytes/macrophages. We determined the number of $CD68^+$ cells and quantified the intensity of CD68 expression within the disc, nerve root, and DRG neuron of an adjacent lesion site in each group. The sham group did not exhibit any inflammatory responses within the disc, nerve root, or DRG neuron (Fig. 3A). In contrast, the control group exhibited slightly more inflammatory cell infiltration at the margin of the disc. However, no inflammatory cells were detected in the DRG neuron and other peripheral nerves around the puncture site. In the IL-1β groups, inflammatory cells were localized within the disc, nerve root, and DRG neuron. Furthermore, quantification results revealed that the relative number and intensity of CD68 positive cells significantly increased in the IL-1β groups (Fig. 3B, C). In particular, the IL-1β group injected with 100 ng of the cytokine experienced the greatest increase in the number of $CD68^+$ cells and intensity of CD68 expression. These results suggest that IL-1β induces a significant increase in inflammation. In addition, the degree of inflammation is highly correlated with an increased concentration of IL-1β. In the process of producing a standardized and replicable LDH model, inflammation is an important driver of disc disease pathology. Therefore, IL-1β may be used to induce inflammation in a more consistent manner in LDH model.

Disk degeneration in LDH model injected with varying concentrations of IL-1β

Proteolytic matrix destruction is a common feature of disc herniation. Aggrecan is the major proteoglycan composing the disc and has a key role in maintaining water content inside the disc [22]. We examined the expression of aggrecan by immunohistochemistry (Fig. 4A). The nucleus pulposus of the sham group was strongly immunolabeled for aggrecan. In contrast, the distribution of aggrecan in the nucleus pulposus decreased in a dose-dependent manner in

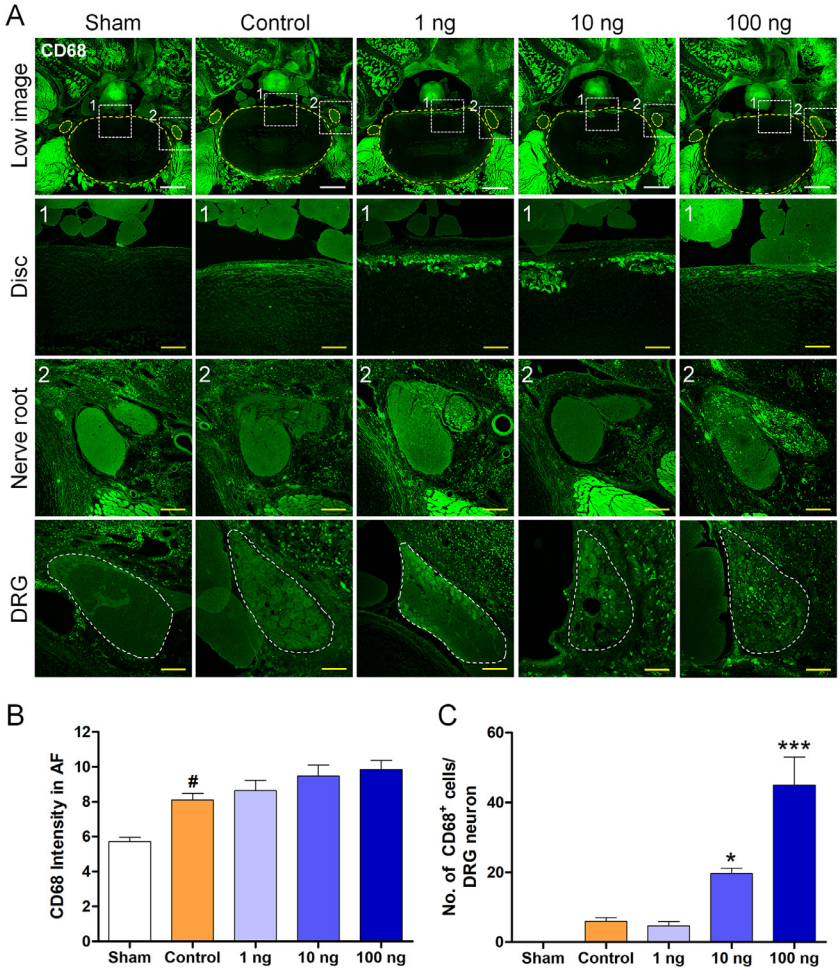

Fig. 3. In Vivo inflammation in LDH model injected with varying concentrations of IL-1β. (A) Representative images of immunohistochemical staining for inflammatory cells in disc, nerve root and DRG neuron site of LDH model at 1 week. White scale bar=1 mm, yellow scale bar=200 μm. (B, C) Quantification of (B) CD68 intensity and (C) number within the annulus fibrosis (AF) and DRG neuron. Data are expressed as the means±standard error of the mean. Significant differences indicated as $^{\#}p<.05$ vs the sham group; *p<.05 and ***p<.001 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

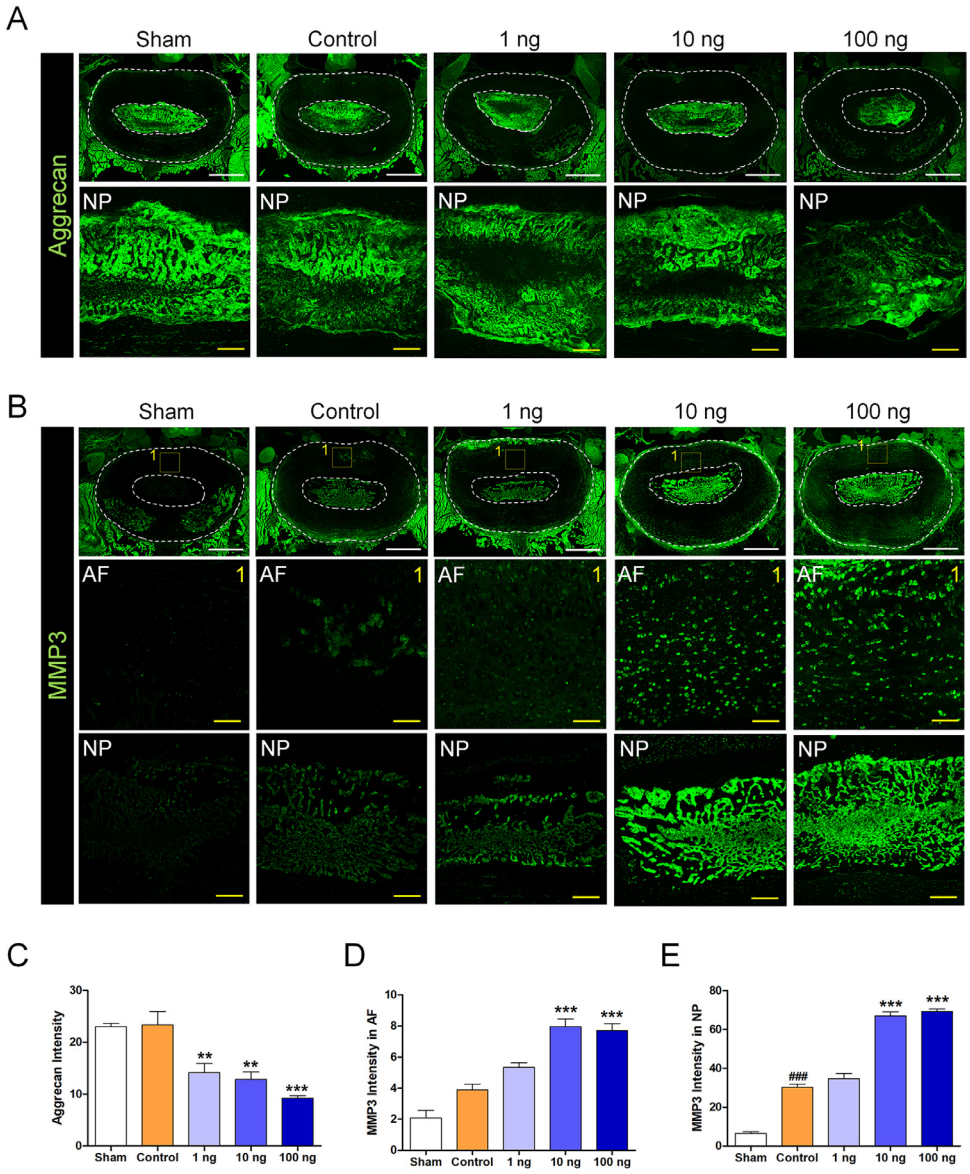

Fig. 4. Disc degeneration in LDH model injected with varying concentrations of IL-1β. (A) Representative images of immunohistochemical staining for aggrecan and matrix metalloproteinase-3 (MMP-3) in L5–6 disc. White scale bar=1mm, yellow scale bar=200 μm. (C–E) Quantification of fluorescence intensities of (C) aggrecan, (D) MMP3 in AF, and (E) MMP3 in NP. Significant differences indicated as $^{###}$p<.001 vs the sham group; **p<.01, and ***p<.001 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

IL-1β groups. However, the control group did not exhibit reduced expression of aggrecan compared with the sham group. We quantified the intensity of aggrecan immunofluorescence in the nucleus pulposus for each group (Fig. 4C). IL-1β injection led to a dose-dependent moderate and significant decrease in the disc.

The intervertebral disc is primarily composed of water, collagen, and proteoglycan. Matrix metalloproteinase 3 (MMP3) is an enzyme that facilitates the destruction and degradation of proteoglycans, collagen, gelatin, laminin, and other extracellular matrix components [23]. We observed a dramatic increase in MMP3 expression in both the AF and NP of IL-1β injected groups (Fig. 4B). Quantification of the intensity of MMP3 expression in AF and NP sites supported this observation (Fig. 4D, E), confirming that the injection of IL-1β leads to accelerated disc degeneration and may provide a more accurate animal model of disc herniation.

Characterization of neuropathic pain in LDH model injected with varying concentrations of IL-1β

The activity of transient receptor potential vanilloid subtype 1 (TRPV1) has been known to play a crucial role in neuropathic pain [24]. TRPV1 expression is involved in the regulation of the pain pathway in afferent sensory neurons.

We analyzed TRPV1 expression in DRG sensory neuron by immunohistochemical staining (Fig. 5A). Interestingly, IL-1β induces dramatic enhancement of expression of TRPV1 in DRG neurons. Quantitatively, the percentage of TRPV1$^+$ neuron in NeuN$^+$ neuron in DRG was 16.7±4.0% in control rats, 16.9±2.3% in 1 ng-injected rats, 22.1±1.8% in 10 ng-injected rats, and 26.1±4.8% in 100 ng-injected rats, respectively. This increase in TRPV1-positive neuron was significant in 100 ng-injected rats (Fig. 5B). The quantified intensity of TRPV1 in each group was also significantly increased in IL-1β rats injected with 10 ng and 100 ng compared with control rats (Fig. 5C). Therefore, TRPV1 activity can be enhanced by injecting the IL-1β into the disc and is involved in neuropathic pain.

Gene expression associated with inflammation and pain in the LDH model

We confirmed the mRNA expression in disc and DRG neuron for pro-, anti-inflammatory, and disc degeneration-related genes in the LDH model injected with IL-1β at 1 week. Results demonstrated that the expression of pro-inflammatory gene (Table) including *IL-1β* was upregulated significantly in IL-1β treated groups compared to control in a dose-dependent manner (Fig. 6A). The *IL-10* gene, an anti-inflammatory cytokine, was downregulated at 1 week in the IL-1β groups and was significantly decreased in the IL-1β groups injected with 10 and 100 ng (Fig. 6B). We further analyzed the expression of disc degeneration-related genes in each group at 1 week after disc herniation. The mRNA level of *aggrecan* was downregulated at 1 week in IL-1β group but was not dose-dependent. The group receiving 100 ng of IL-1β was only just statistically significant compared to the control group (Fig. 6C). In addition, the *MMP3* gene was also dose-dependently upregulated in the IL-1β groups (Fig. 6D). Although, this gene analysis does not clarify whether IL-1β was more effective in inducing the LDH model, these data support the concept that IL-1β mediates disc degeneration through aggrecan and MMP3 expression.

In vivo functional assessment of the LDH model

The locomotor function of LDH rats was assessed using three methods (BBB, ladder, and Von Frey tests) to determine whether IL-1β injection was more effective than controls. The BBB score of the control group decreased significantly after 1 week compared with that of the sham group. The control group had an average score of 18 points and displayed consistent plantar stepping with consistent

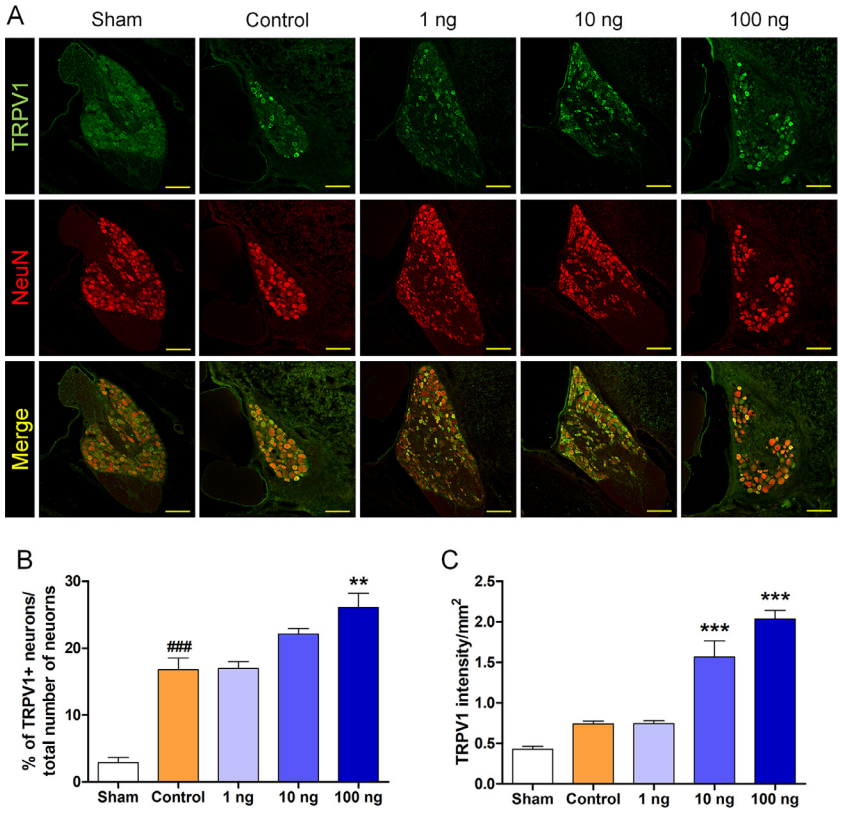

Fig. 5. Characterization of neuropathic pain in LDH model injected with varying concentrations of IL-1β. (A) Representative images of immunohistochemical staining for transient receptor potential vanilloid1 (TRPV1) in DRG neuron. Scale bar=200 μm. Quantification of (B) the percentage of TRPV1$^+$ neurons and (C) intensity within DRG neuron. Significant differences indicated as $^{###}$p<.001 vs the sham group; **p<.01, and ***p<.001 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

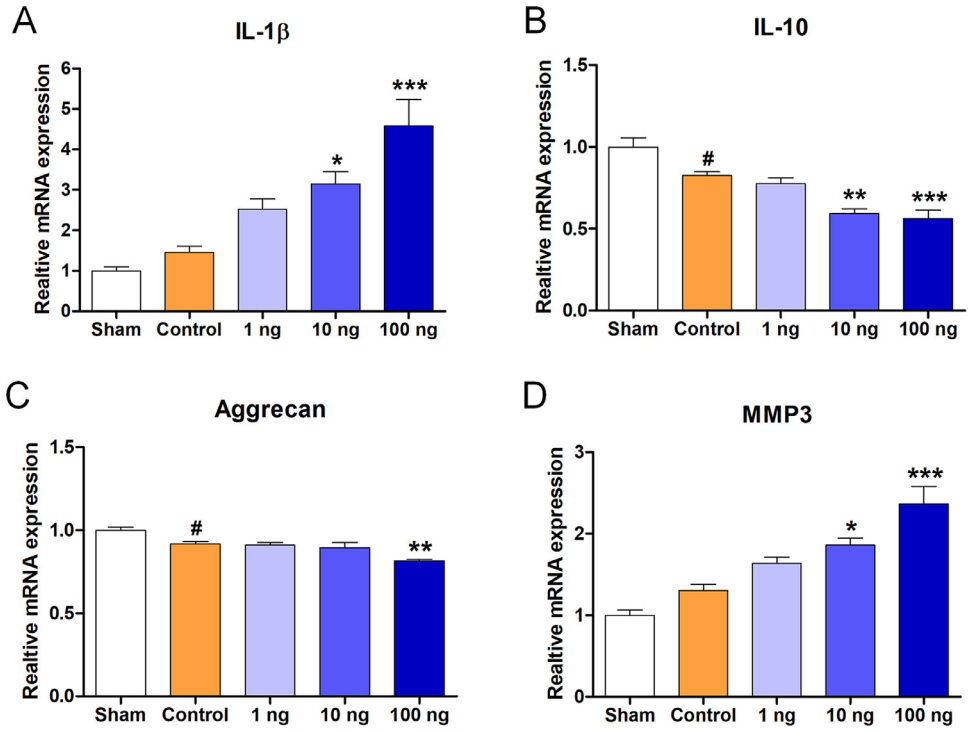

Fig. 6. Gene expression associated with pro-, anti-inflammation, and disc degeneration in the LDH model injected with varying concentrations of IL-1β; (A) IL-1β, (B) IL-10, (C) aggrecan, and (D) MMP3. Significant differences indicated as $^{\#}$p<.05 vs the sham group; *p<.05, **p<.01, and ***p<.001 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

coordination; toe clearance was also consistently observed in beyond 90% of cases. The IL-1β rats had an average BBB score of 16 points in the first week, which is approximately two points lower than the average score of the control group. A significant difference in the scores between the control and IL-1β groups persisted until 3 weeks following surgery. However, there were no significant differences among the groups injected with varying concentrations of IL-1β (Fig. 7A). Compared with the results of the BBB test, the ladder test more rapidly revealed markedly pronounced behavioral differences. In particular, compared to the control group, the IL-1β groups showed lower frequencies of stepping through forelimb-hindlimb coordination. The sham group had a foot fault rate of approximately 10% in week 1 but showed gradual improvement and had a foot fault rate of approximately 5% in the final assessment at 3 weeks. The IL-1β groups injected with varying concentrations showed a significantly increased foot fault frequency compared to the control group for up to 3 weeks (Fig. 7B). In the Von Frey test, the control group showed a withdrawal latency of 4 to 5 seconds in the first week. There was a significant difference between the sham and control groups in week 1. Following surgery to induce the LDH model, the rats showed increased sensitivity of the nerves in their legs, resulting in shorter withdrawal latencies. Of note, rats injected with 100 ng of IL-1β showed faster withdrawal

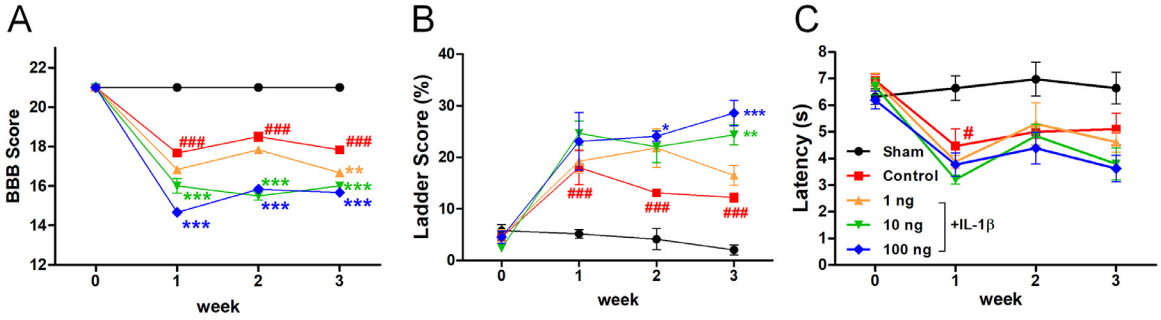

Fig. 7. In vivo functional assessment of the LDH model injected with varying concentrations of IL-1β until 3 weeks. (A) BBB score, (B) Ladder score, and (C) Von Frey test. Significant differences indicated as $^{\#}$p<.05, $^{\#\#\#}$p<.001 vs the sham group; *p<.05, **p<.01, and ***p<.001 vs the control group were analyzed via one-way analysis of variance with Tukey's post hoc test.

latencies than the control group (Fig. 7C). Therefore, simultaneously administered IL-1β induced reduction of locomotor activity in our LDH model.

Discussion

Animal models for preclinical studies serve as essential means to elucidate the mechanisms of human diseases and explore their prevention, diagnosis, and treatment methods. Thus, it is crucial that an animal disease model exhibits clinically comparable pathological findings and pathophysiological similarities to human cases. Numerous studies have proposed creating an animal model of lumbar disc herniation by harvesting the NP from the coccygeal discs of the animal and transplanting the autologous NP between the DRG and the spinal cord. We also attempted to make a lumbar disc herniation model using this method (data not shown). However, due to the lack of standardization of the protocol regarding the amount of autologous NP to be transplanted onto the nerve root, it was difficult to fix the transplanted NP as some was lost during transfer. We performed behavioral tests weekly after transplantation to examine changes in locomotor function, and we found that the changes in behavior were very small when compared to the sham-operated group.

Here, we proposed a method for creating an animal model that exhibits pathophysiology that is comparable to clinical cases of disc herniation. In this method, the exposed disc is artificially punctured so that the NP can protrude from the AF, inducing the herniation of NP. The herniated NP dehydrates and hardens, and irritates the nerves to cause low back pain and degeneration of the disc. However, as shown in the results of the control group in this study, the effect of puncturing the disc alone was not sufficient to induce these changes in the disc, and the spontaneous recovery rate was too high to facilitate long-term follow-up and accurate evaluation of the efficacy of potential therapeutic candidates for disc herniation. Therefore, we explored a more accurate and replicable disc herniation model by puncturing the disc and injecting a given concentration of IL-1β at the time of disc puncture, inspired by the idea of using drugs affecting IL-1β to treat the NP in current in vitro models of disc herniation [25–29]. The present study thus proposes a standardized method of creating a disc herniation model that allows control of disease severity by assessing histological and molecular changes corresponding to the concentration of injected IL-1β.

In current clinical practice, LDH patients are classified by severity before appropriate responsive measures and treatment strategies are applied [30]. To evaluate the exact effects of the various treatment strategies based on severity, systematic comparisons may be conducted to evaluate the reproducibility of clinical symptoms, degree of damage, and treatment outcomes by creating LDH model of differential severity using our proposed method. To date, studies investigating the treatment of LDH have been conducted using various approaches. Although a complete cure of patients has not been achieved so far, diverse treatment methods based on the pathophysiology of this condition have been proposed, and several studies are still underway. An animal model that perfectly recapitulates the symptoms and abnormal findings in the recovery process of disc herniation in humans is not yet available, and continuous research is needed to assess the advantages and disadvantages of the options currently available to select the optimal animal model for these purposes. Moreover, continuous efforts should be made for the development of new animal models that will more clearly represent the mechanism and pathophysiology of disc herniation.

In conclusion, compared to the conventional method of creating a disc herniation model of SD rats with autologous NP transplantation, this study proposes a new method of animal model creation that follows a route of clinical pathology similar to that of clinical cases of disc herniation; this is a model in which uniform neuropathic pain and inflammatory responses were induced with the injection of IL-1β. In particular, the degrees of disc degeneration and damage were differentially evaluated according to the concentration of injected IL-1β, and using the findings of this study, an index was developed to modulate the severity of disc degeneration based on the concentration of IL-1β. In the future, our proposed model may be used for nonclinical research studies to develop and test strategies to alleviate disc degeneration.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.spinee.2021.01.014.

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