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Original Article

Spinal manipulative therapy promotes nucleus pulposus resorption and alleviates pain in a rat model of lumbar disc herniation via JNK-dependent regulation of inflammation and tissue remodeling

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Abstract: Objective: Lumbar disc herniation (LDH) often resolves spontaneously through inflammation-mediated resorption of the extruded nucleus pulposus (NP). While spinal manipulative therapy (SMT) is clinically used to alleviate LDH-related pain, its role in modulating NP resorption and the underlying molecular mechanisms remain unclear. This study aimed to determine whether SMT promotes NP clearance and to elucidate its signaling pathways. Methods: A rat model of LDH was established by autologous NP implantation onto the L5 nerve root. Animals received either SMT (every other day), epidural betamethasone (SHD, weekly), or sham treatment. Mechanical allodynia was assessed over 28 days. Histology, immunohistochemistry, ELISA, RT-qPCR, and Western blotting were performed on NP tissues. To confirm pathway specificity, experiments were replicated in JNK2-knockout (JNK-KO) mice. Results: SMT significantly attenuated mechanical allodynia from day 3 onward and promoted near-complete NP resorption by day 28-outperforming SHD in both analgesia and tissue restoration. Unlike SHD's broad anti-inflammatory suppression, SMT maintained a balanced microenvironment: moderately elevating TNF-α, IL-1β, VEGF, and MMP-3 to support macrophage recruitment, neovascularization, and matrix remodeling, while reducing NP cell apoptosis via Bax/Bcl-2 regulation. These effects were accompanied by selective inhibition of NF-κB and MAPK (p38, ERK, JNK) phosphorylation. Crucially, all benefits of SMT were abolished in JNK-KO mice, confirming JNK as an essential upstream mediator. Conclusion: SMT promotes pain relief and nucleus pulposus resorption in LDH by inhibiting inflammation and accelerating tissue remodeling through a JNK-dependent mechanism.

Keywords: Spinal manipulation, lumbar disc herniation, JNK, inflammation, apoptosis

Introduction

Lumbar disc herniation (LDH) is a leading cause of chronic low back pain and sciatica, imposing a substantial burden on patients' quality of life and contributing significantly to rising healthcare expenditures [1, 2]. Clinical imaging studies have consistently demonstrated that extruded or sequestered nucleus pulposus (NP) tissue can undergo spontaneous resorption over time [3, 4], a process now recognized as central to natural recovery. This resorption is not passive but actively driven by a localized inflammatory response, which facilitates macrophage infiltration, neovascularization, and extracellular matrix degradation [5, 6]. Key molecular mediators in this cascade include pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), angiogenic factors like vascular endothelial growth factor (VEGF), and matrix-degrading enzymes including matrix metalloproteinase-3 (MMP-3) [7, 8].

While conventional anti-inflammatory therapies - particularly glucocorticoids - are effective in alleviating acute pain, they carry a critical drawback: the potential suppression of essential cytokine signaling pathways which are required for NP clearance and tissue repair [9, 10]. This

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unintended inhibition may delay or impair the body's intrinsic healing mechanisms, highlighting a fundamental limitation of purely symptomatic treatment strategies. In contrast, spinal manipulative therapy (SMT) has long been employed as a conservative, non-surgical intervention for LDH in clinical practice [11, 12]. Emerging evidence suggests that SMT not only reduces pain but may also preserve-or even enhance the inflammatory microenvironment necessary for NP resorption [13, 14]. However, the precise molecular mechanisms underlying these dual effects remain poorly defined.

Beyond secreted cytokines, intracellular signaling networks are increasingly recognized as pivotal regulators of inflammation and tissue remodeling in LDH. Among these, the nuclear factor κB (NF-κB) pathway and mitogen-activated protein kinases (MAPKs) - including p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) - orchestrate the expression of inflammatory mediators and matrix-degrading enzymes in response to disc injury [15, 16]. Notably, JNK signaling has been implicated in macrophage activation and MMP production, positioning it as a potential mechanistic link between manual therapy and biological resorption. Yet, whether SMT exerts its therapeutic effects through selective modulation of these pathways - particularly without inducing the broad immunosuppression associated with glucocorticoids - has not been systematically investigated.

To address this gap, we utilized a well-established rat model of lumbar disc herniation to evaluate whether SMT can simultaneously alleviate pain and foster an inflammatory milieu conducive to NP resorption. By directly comparing SMT with glucocorticoid treatment (simulated by SHD), our study aims to determine if SMT promotes the body's natural repair response while avoiding the excessive immune suppression commonly induced by steroids. This work thus seeks to provide a mechanistic rationale for SMT as a biologically informed, non-pharmacological strategy in LDH management - one that aligns symptom relief with active tissue resolution.

Materials and methods

Animals and grouping

The animal experiment followed a 28-day timeline after surgery. Sixty male Sprague Dawley rats (300-350 g; Shandong Keshuda Biotechnology Co., Ltd., Jinan, China) were randomly assigned to four groups (n=15 per group): the control group underwent sham surgery with laminectomy but no nucleus pulposus (NP) implantation; the model group received autologous NP implanted extradurally at the left L5 nerve root; the SHD group received the same NP implantation followed by weekly epidural injections of betamethasone starting on postoperative day 2; and the SMT group underwent NP implantation and then received simulated spinal manipulation every other day for a total of 14 sessions over four weeks. All procedures complied with the "Guidelines for the Care and Use of Experimental Animals" and were approved by the Animal Ethics Committee of Shandong First Medical University Neck-Shoulder and Lumbocrural Pain Hospital. To investigate the role of the JNK signaling pathway, parallel experiments were conducted using homozygous JNK2$^{-/-}$ C57BL/6 mice (Saige Bio, Guangzhou, China), with genotypes confirmed by PCR and baseline physiological and locomotor functions verified as comparable to wild-type littermates; these mice were allocated into corresponding groups with n=12 per group to account for transgenic variability. On postoperative day 28, rats were subdivided for terminal analyses: all 15 animals per group were used for behavioral testing, while 6 rats per group were dedicated to histology and immunohistochemistry, and another 6 independent rats per group were used for molecular assays including Western blotting, ELISA, and RT-qPCR; the mouse cohorts followed an analogous subsampling strategy based on their group size. All animals were humanely euthanized at designated time points under deep anesthesia induced by intraperitoneal injection of pentobarbital sodium (150 mg/kg). Death was confirmed by the absence of respiration, loss of corneal reflex, and cessation of heartbeat. This method is consistent with the AVMA Guidelines for the Euthanasia of Animals (2020 Edition) and was approved by the Institutional Animal Care and Use Committee of Shandong First Medical University Neck-Shoulder and Lumbocrural Pain Hospital.

Establishment of intervertebral disc herniation model

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Sinopharm, Shanghai, China), and ~5 mg autologous caudal NP mixed

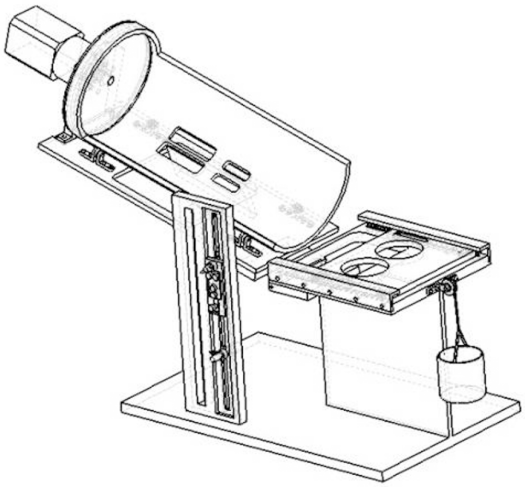

Figure 1. Customized simulation device for spinal manipulation in rats.

with 0.1 mL sterile saline (HyClone, Logan, UT, USA) was implanted extradurally at the left L5 nerve root; controls underwent sham surgery. JNK-KO mice were anesthetized with 2% isoflurane (RWD Life Science, Shenzhen, China) in oxygen (Praxair, Danbury, CT, USA), and received ~2 mg donor NP in 0.05 mL saline via Hamilton microsyringe (Reno, NV, USA). All animals were housed in SPF conditions with daily monitoring for complications.

SHD group treatment

From postoperative day 2, SHD rats and mice received weekly epidural injections of betamethasone (Aspen Pharma, Dublin, Ireland; 0.5 mg/mL, 0.1 mL for rats; 0.3 mg/mL, 0.05 mL for mice) diluted in sterile saline (HyClone) using a 30G Hamilton syringe, guided stereotactically near L5.

SMT grouping processing

SMT was delivered every other day for 4 weeks using a custom device (**Figure 1**): rats were prone-fixed on an acrylic base (Tianjin Yiheng Plastic, Tianjin, China) with foam padding (Dow Corning, Midland, MI, USA); a spring-loaded robotic arm tipped with a 3 mm silicone probe (Dow Corning) applied force via a Futek pressure sensor (Irvine, CA, USA), controlled by a Mitutoyo micrometer (Kawasaki, Japan), with real-time readout on a National Instruments screen (Austin, TX, USA). The system was calibrated weekly with Ohaus standard weights (Parsippany, NJ, USA).

SMT operation protocol

Under light 2% isoflurane anesthesia (RWD Life Science), the L5 spinous process was marked and targeted. A preload of 0.5 N was applied, followed by a rapid thrust of 2.0±0.1 N over 0.5 s, mimicking clinical impulse. Rats recovered in a 28°C Thermo Fisher cage (Waltham, MA, USA) with no adverse events observed.

Organizational examination

On day 28, rats (n=4/group) were euthanized and perfused with heparinized saline (Jianfeng Pharma, Shandong, China) and 4% paraformaldehyde (Solarbio, Beijing, China). L5 spinal cords and NP tissues were post-fixed, cryoprotected in 30% sucrose (Aladdin, Shanghai, China), and sectioned (40 μm) on a Leica CM1950 cryostat (Wetzlar, Germany). HE staining used reagents from Solarbio, and sections were imaged on an Olympus BX53 microscope (Tokyo, Japan). NP weight was measured pre- and post-implantation using a Mettler Toledo balance (Columbus, OH, USA). To enable objective assessment, histopathological changes in HE-stained sections - including inflammatory cell infiltration, fibrosis, neovascularization, and structural disorganization - were evaluated using a semi-quantitative scoring system adapted from prior disc degeneration studies [17]. Each parameter was graded on a scale of 0 (none) to 4 (severe), and a total histological score was calculated per animal by summing subscores across five non-overlapping fields (×200 magnification).

Immunohistochemistry

Adjacent sections underwent antigen retrieval in citrate buffer (Beyotime, Shanghai, China), blocked with 5% BSA (Beyotime), and incubated overnight with primary antibodies against SP, IL-1β, TNF-α, VEGF, and MMP-3 (1:500; R&D Systems, Minneapolis, MN, USA). Detection used HRP-secondary antibody (Wako, Osaka, Japan) and DAB kit (ZSGB-BIO, Beijing, China). Staining intensity (IOD) was quantified in five 400× fields per section using ImageJ (NIH, Bethesda, MD, USA).

Protein imprinting

NP tissues (n=6/group) were homogenized in RIPA buffer (Beyotime) with protease/phosphatase inhibitors (MedChemExpress, Monmouth

Junction, NJ, USA). Proteins (50 μg) were separated on 10% SDS-PAGE (Epizyme, Shanghai, China), transferred to PVDF membranes (Millipore, Burlington, MA, USA), and probed with antibodies against p/t-NF-κB, p38, ERK, JNK (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Santa Cruz, Dallas, TX, USA). Bands were visualized with ECL (Millipore) and quantified via ImageJ.

ELISA for detecting the protein levels of TNF-α, IL-1β, and IL-6

NP homogenates (n=6/group) were prepared in PBS (HyClone) and centrifuged. Cytokine levels were measured using rat-specific ELISA kits (R&D Systems) per protocol, with absorbance read at 450 nm on a Thermo Varioskan LUX plate reader (Waltham, MA, USA). Results were normalized to total protein (pg/mg).

RT qPCR detection of TNF-α, IL-1β, and IL-6 mRNA levels

Total RNA was extracted from NP tissue (n=6/group) using TRIzol (Invitrogen, Carlsbad, CA, USA), reverse-transcribed with TaKaRa PrimeScript™ RT kit (Kusatsu, Japan), and analyzed by qPCR on a StepOnePlus system (Thermo Fisher) using TB Green® Premix (TaKaRa) and primers from Sangon Biotech (Shanghai, China). Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as reference.

Statistical analysis

Data are presented as mean ± SD. Data normality was assessed using the Shapiro-Wilk test. For non-normally distributed data (which included all primary outcomes), group comparisons at a single time point (e.g., Day 28) across four independent groups were analyzed using the Kruskal-Wallis H test, followed by Dunn's post hoc test with Bonferroni correction. Longitudinal behavioral data (repeated measures over 4 weeks) were analyzed using aligned ranks transformation (ART) ANOVA to account for both group and time effects under non-parametric conditions, with post hoc pairwise comparisons adjusted by the Benjamini-Hochberg FDR procedure. Sample size (n=6-15/group) was determined using G*Power 3.1 to achieve ≥80% power (α=0.05, two-sided). All analyses were performed in SPSS v17.0 and R (v4.3.1, ARTool package), and reviewed by a certified biostatistician.

Results

SMT alleviates mechanical allodynia and promotes nucleus pulposus resorption in a rat model of intervertebral disc herniation

Following epidural autologous NP implantation, all rats in the Model, SHD, and SMT groups developed significant ipsilateral mechanical allodynia by day 1, which persisted throughout the 28-day observation period. In the Model group (NP implantation only), the paw withdrawal threshold (PWT) dropped by approximately 50% post-surgery and showed no recovery, confirming successful establishment of an LDH model with L5 radiculopathy. Compared to the sham Control group, both SHD (weekly epidural betamethasone) and SMT (every-other-day spinal manipulation) partially reversed this hypersensitivity. The SHD group exhibited transient pain relief on days 1, 3, 7, and 14, but its effect dissipated by day 28. In contrast, SMT began to show efficacy from day 3 onward, and its analgesic effect was sustained through day 28, at which point it was significantly superior to SHD (**Figure 2A**). Histological analysis of NP tissue at day 28 (n=6 per group) further revealed that while the Model group displayed severe structural disruption, inflammatory cell infiltration, and fibrosis, the SMT group maintained near-normal NP architecture with uniform cell distribution, minimal inflammation, and markedly fewer residual NP cells indicative of enhanced spontaneous resorption. The SHD group showed partial improvement but retained mild fibrosis and disorganized matrix (**Figure 2B**, **2C**). Together, these findings demonstrate that SMT not only provides longer-lasting pain relief than steroid therapy but also more effectively supports tissue restoration and NP clearance.

SMT regulates inflammation and remodeling pathways to promote spontaneous NP absorption

To explore the molecular basis of NP resorption, we examined key mediators of inflammation and tissue remodeling via immunohistochemistry. As expected, NP implantation significantly upregulated IL-1β, TNF-α, VEGF, and MMP-3 in the Model group compared to Control. SHD treatment strongly suppressed all

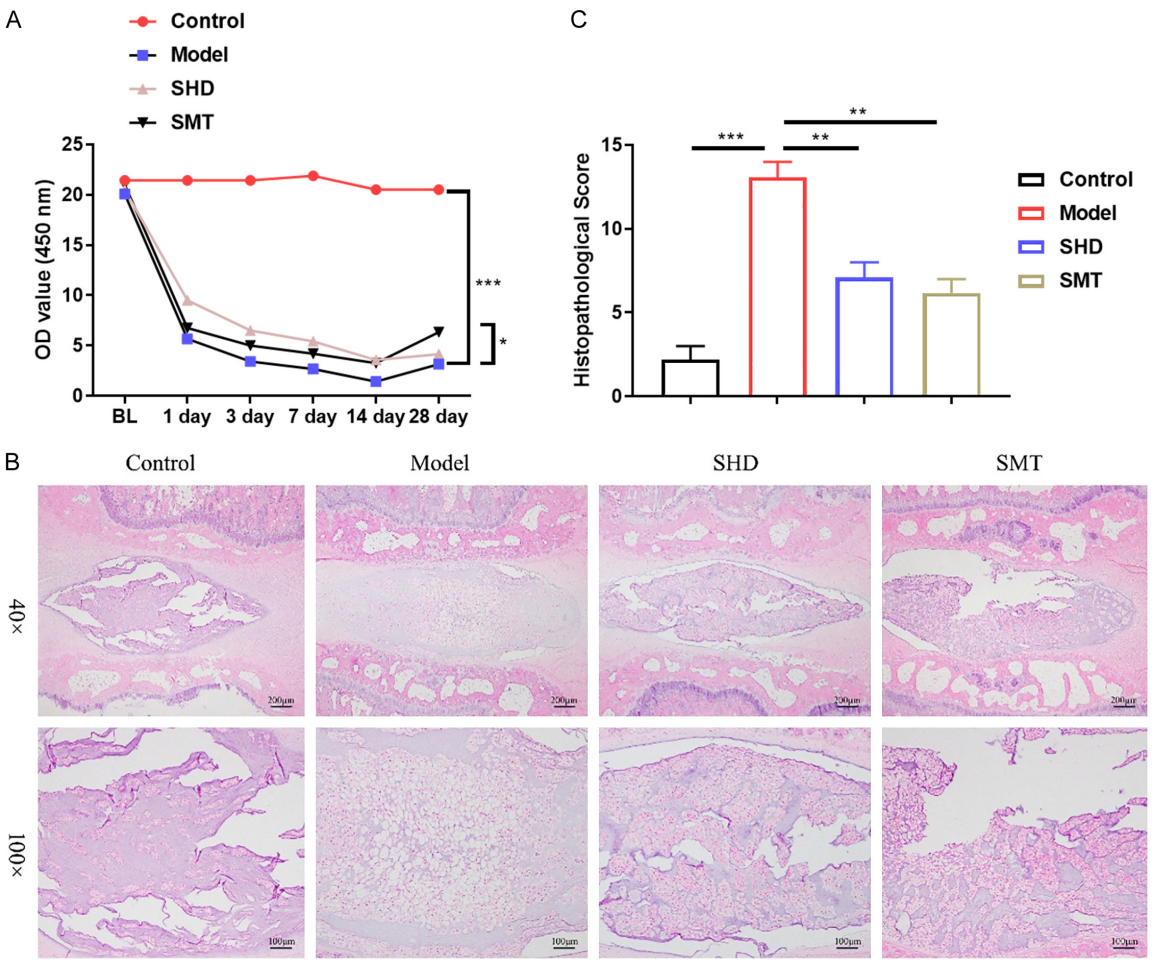

Figure 2. SMT alleviates mechanical allodynia and helps NP tissue recover. A. SMT continuously relieved pain from day 3 to day 28. On the 28th day, the 50% claw reduction threshold (PWT) of the SMT group was significantly higher than that of the SHD group. *P<0.05, ***P<0.001. B. H&E staining showed severe damage to the model group structure, partial recovery in the SHD group, and almost normal tissue structure in the SMT group. C. Quantification of histopathological changes using a semi-quantitative scoring system (0-16 total score; see Methods). Scale bar: 100 μm. Data are presented as mean ± SD; n=6 per group. *P<0.05, **P<0.01, ***P<0.001.

four markers by day 28 (P<0.05 vs. Model), consistent with its broad anti-inflammatory action. Strikingly, SMT induced a distinct regulatory pattern: while it reduced these factors relative to the Model group, their expression remained moderately higher than that in the SHD group (**Figure 3A**). This balanced profile aligns with the biological requirements for effective resorption - moderate levels of IL-1β and TNF-α facilitate macrophage recruitment, VEGF supports neovascularization, and MMP-3 enables controlled matrix degradation. These observations were corroborated by ELISA and RT-qPCR, which confirmed elevated pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in the Model group and greater suppression by SMT than SHD (**Figure 3B**). Thus, unlike the blanket immunosuppression caused by steroids, SMT appears to fine-tune the inflammatory microenvironment to simultaneously alleviate pain and promote reparative processes.

SMT inhibits abnormal NF-κB and MAPK signaling without interfering with basal pathway activity

Given the central role of NF-κB and MAPK pathways in neuroinflammation, we assessed their activation status in NP tissue at day 28 via Western blot. SMT significantly reduced phosphorylation of NF-κB p65, p38, ERK, and JNK compared to the Model group, while total protein levels remained unchanged across groups (**Figure 4**). This indicates that SMT selectively

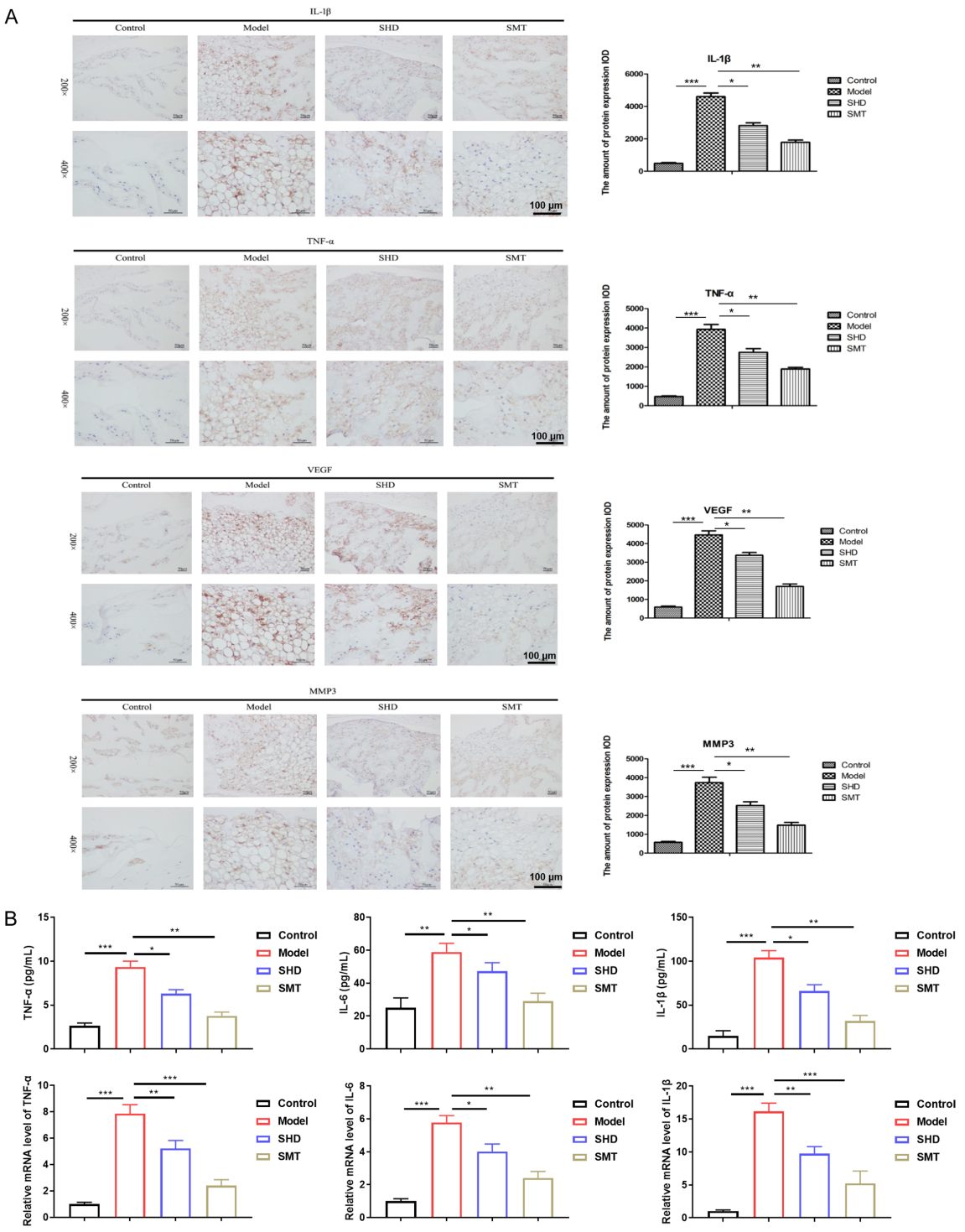

Figure 3. SMT regulates inflammation and remodeling reactions. A. The IHC results showed that SMT maintained IL-1β, TNF-α, VEGF, and MMP-3 at moderate levels, lower than the model group but higher than the SHD group (*P<0.05, n=6). Scale bar: 50 μm. B. ELISA and RT qPCR detection confirmed that the cytokines (TNF-α, IL-1β, IL-6) produced by SMT are moderately expressed, which helps create a microenvironment that promotes reabsorption. Data are presented as mean ± SD; n=6 per group. *P<0.05, **P<0.01, ***P<0.001.

dampens pathological overactivation of these signaling cascades without disrupting their baseline expression, which is essential for cellular homeostasis and tissue repair. Such targeted inhibition likely underlies SMT's ability to mitigate inflammation-driven damage while

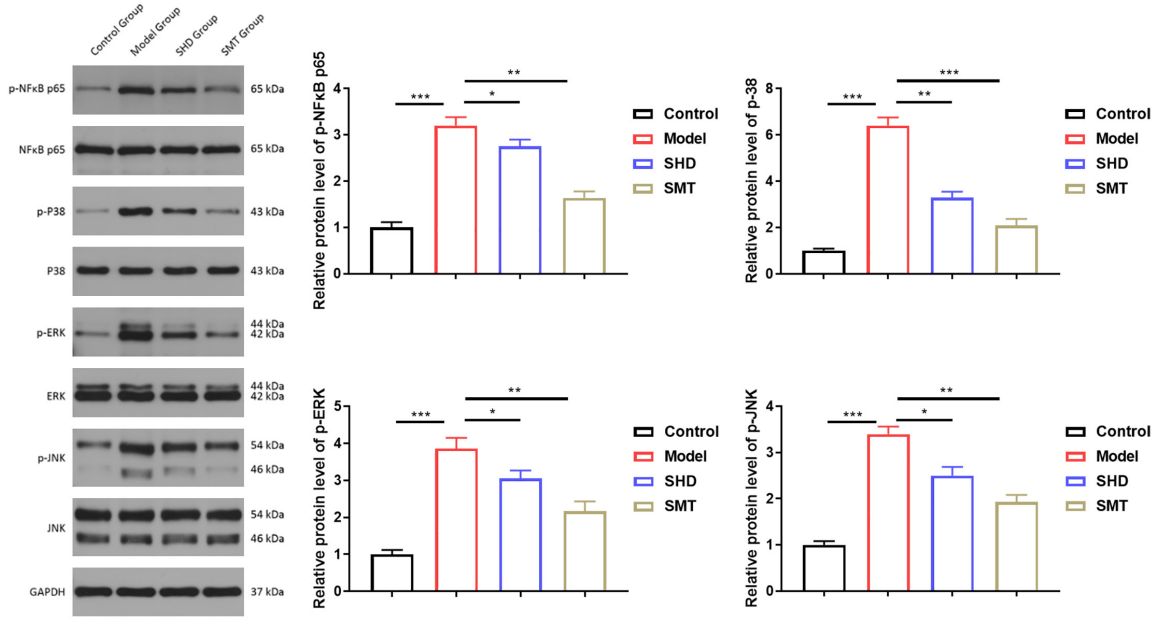

Figure 4. SMT inhibits the activation of pathological NF-κB and MAPK. Western blot showed that compared with the model group, SMT reduced the phosphorylation of NF-κB p65, p38, ERK, and JNK (*P<0.05), but did not change the total protein level (n=6). Data are presented as mean ± SD; n=6 per group. *P<0.05, **P<0.01, ***P<0.001.

preserving physiological functions necessary for NP resorption.

The therapeutic effect of JNK signal on SMT and SHD in LDH is crucial

To determine whether JNK is functionally required for the observed effects, we replicated the LDH model in *JNK2*$^{-/-}$ (JNK2 knockout) mice. In these animals, neither SMT nor SHD significantly altered the expression of VEGF or MMP-3 compared to the Model group (**Figure 5A**, **5B**), and both interventions failed to modulate pro-inflammatory cytokines at the protein or mRNA levels (**Figure 5C**, **5D**). In wild-type mice, SMT induced moderate cytokine elevation conducive to resorption, while SHD strongly suppressed them. However, this differential regulation was completely lost in JNK2$^{-/-}$ mice. These results demonstrate that JNK2 serves as a non-redundant signaling hub essential for both therapies to exert their downstream effects on inflammation, angiogenesis, and matrix remodeling.

SMT exerts anti apoptotic effects on Bax/Bcl-2 in NP tissue through JNK dependent regulation

We next investigated whether SMT influences apoptosis during NP remodeling. TUNEL staining showed that NP implantation increased apoptosis in wild-type mice, which was further exacerbated by SHD but significantly attenuated by SMT (P<0.05 vs. Model). However, this protective effect disappeared in JNK2$^{-/-}$ mice, where no differences in apoptosis index were observed among groups (**Figure 6A**). At the molecular level, SMT in wild-type mice downregulated Bax, upregulated Bcl-2, and lowered the Bax/Bcl-2 ratio, whereas these changes were absent in JNK2$^{-/-}$ mice. Moreover, the Bax/Bcl-2 ratio in the SMT-treated JNK2$^{-/-}$ group remained significantly higher than that in the wild-type SMT group (**Figure 6B**, **6C**). These data confirm that SMT's anti-apoptotic action, which is critical for preserving functional NP cells during resorption, is entirely dependent on intact JNK signaling.

SMT fails to inhibit NF-κB and MAPK signaling in JNK-KO mice

Finally, to test whether JNK acts upstream of other inflammatory pathways, we examined NF-κB and MAPK activation in JNK2$^{-/-}$ mice. In wild-type animals, SMT robustly suppressed phosphorylation of NF-κB p65, p38, and ERK (**Figure 7**). In contrast, this inhibitory effect was completely abolished for NF-κB p65 and markedly weakened for p38 and ERK in JNK2$^{-/-}$ mice, despite residual statistical significance for the latter two. These findings provide genetic evi-

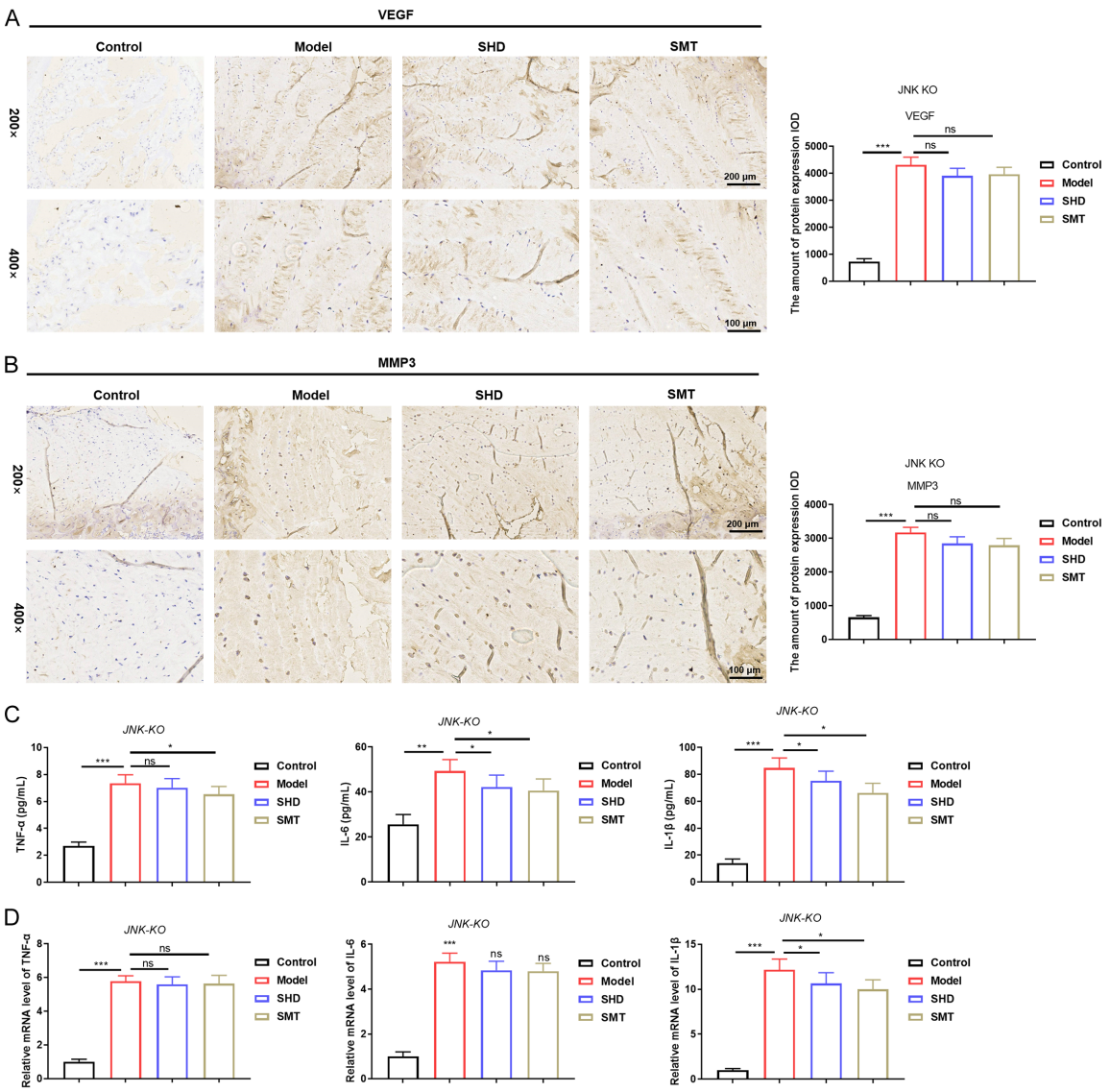

Figure 5. The efficacy of JNK in SMT and SHD is crucial. A, B. Immunohistochemical staining was performed to detect the expression of VEGF and MMP-3 in nucleus pulposus (NP) tissues of JNK-KO mice and wild-type mice 28 days after surgery. Integrated optical density (IOD) values were used for quantification (n=6 per group). C, D. ELISA and RT-qPCR were performed to detect the protein and mRNA expression levels of TNF-α, IL-1β and IL-6 in NP tissues of JNK-KO mice and wild-type mice 28 days after surgery. Data are presented as mean ± SD; n=6 per group. Ns: P>0.05, *P<0.05, **P<0.01, ***P<0.001.

dence that JNK is a critical upstream regulator through which SMT modulates broader inflammatory signaling networks. Without functional JNK, SMT loses its capacity to coordinate the multi-pathway suppression necessary for therapeutic efficacy in LDH.

Discussion

We used a new epidural NP implantation model in mice that replicated the pathological features of LDH in patients with L5 radiculopathy. Our research has found that spinal massage therapy has a dual therapeutic effect: promoting postganglionic fiber absorption and alleviating mechanical hyperalgesia. Mechanical hyperalgesia is a key clinical symptom of lumbar disc herniation, which directly affects the quality of life of patients. It is worth noting that mechanical hyperalgesia, manifested by a decrease in pain threshold, is the main symptom of nerve root stimulation triggered by nerve implantation stimulation. When evaluating the therapeutic effect of lumbar disc herniation,

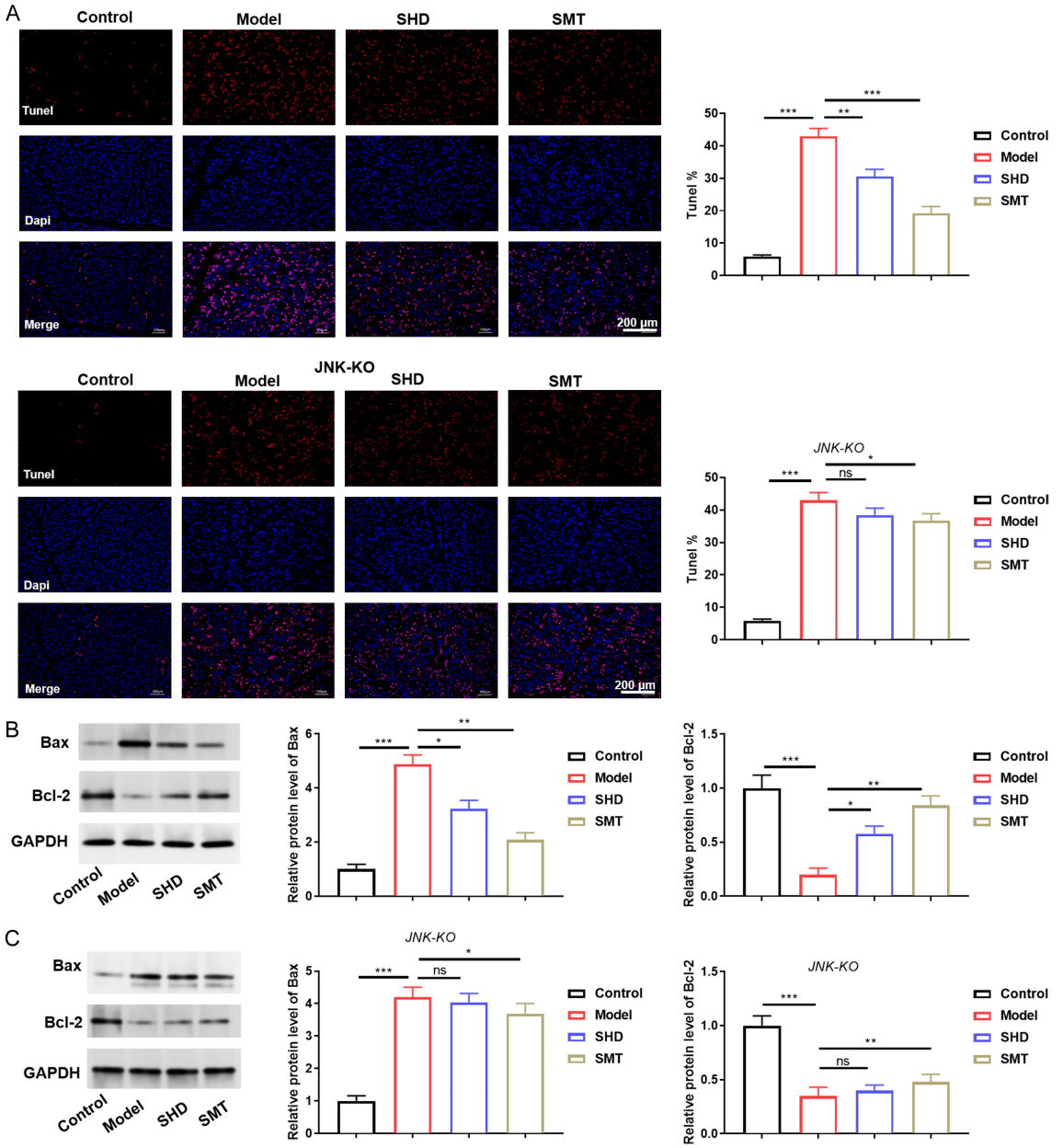

Figure 6. SMT reduces NP cell apoptosis through JNK dependent Bax/Bcl-2 regulation. A. TUNEL staining was used to detect apoptotic cells in NP tissues of WT and JNK-KO mice. Scale bar: 50 μm. B, C. Western blot or immunohistochemistry (consistent with experimental methods) was used to detect the expression of Bax and Bcl-2 in NP tissues of WT and JNK-KO mice, and the Bax/Bcl-2 ratio was calculated. The data are presented as mean ± SD (n=6). Ns: P>0.05, *P<0.05, **P<0.01, ***P<0.001.

relieving this symptom is equally important as tissue repair [18].

Consistent with the pathological features of degenerative diseases, the model group (only transplanted nucleus pulposus tissue) showed two typical changes: significant degeneration of nucleus pulposus tissue (visible through HE staining) and persistent mechanical hyperalgesia. Histological observations showed that the nucleus pulposus structure in the model group was severely damaged and the number of cells decreased, accompanied by inflammatory cell infiltration and fibrosis - consistent with previous research results on degenerative disease models [19, 20]. In terms of behavior, the pain threshold (PWT) of the affected hind limb in the model group decreased sharply the day after

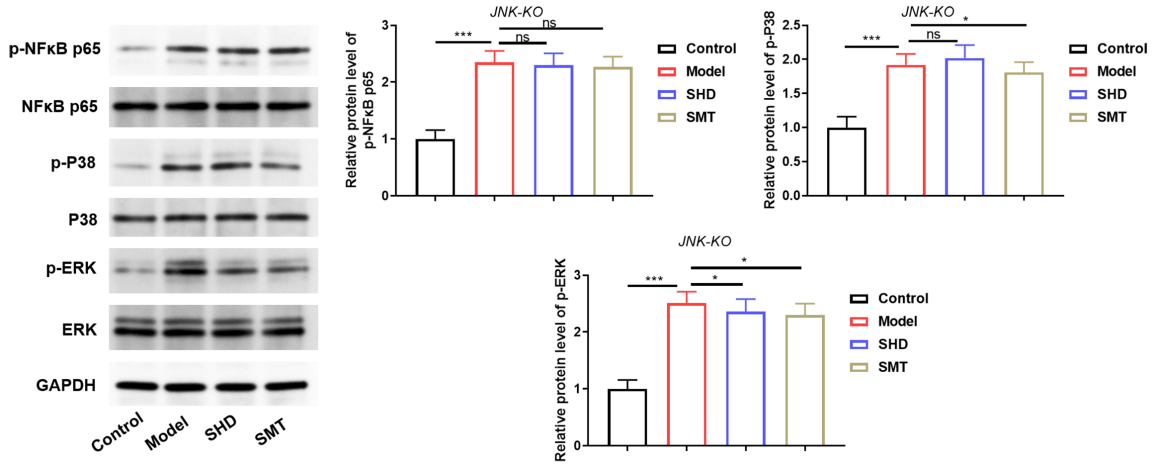

Figure 7. Effect of SMT on the expression levels of p-NF-κB p65, p-p38, and p-ERK in JNK-KO mice. Representative Western blot images showing the protein expression levels of phosphorylated NF-κB p65 (p-NF-κB p65), phosphorylated p38 (p-p38), phosphorylated ERK (p-ERK), total NF-κB p65 (NF-κB p65), total p38 (p38), total ERK (ERK), and GAPDH as a loading control in WT and JNK-KO mice under different treatment conditions: Control, Model, SHD, and SMT groups. Data are presented as mean ± SD; n=6 per group. Ns: P>0.05, *P<0.05, **P<0.01, ***P<0.001.

surgery and did not recover within 28 days. These pathological and behavioral abnormalities demonstrate the effectiveness of our LDH model. It replicates the tissue damage and pain symptoms of clinical LDH, providing a reliable platform for testing intervention measures. Our model construction method - implanting autologous coccyx NP in the epidural space near the L5 nerve root without damaging the posterior joint - follows the classic LDH model protocol [21, 22], so it is reproducible and comparable to early studies.

A key finding of our research is the different analgesic effects of SMT and steroid hormone drugs (SHD, betamethasone). The SHD group alleviated the reduction of PWT in the early to mid postoperative period (days 1, 3, 7, and 14), which is consistent with the known short-term anti-inflammatory effects of glucocorticoids. Steroids can rapidly inhibit pro-inflammatory cytokines (such as IL-1β, TNF-α) to reduce nerve root inflammation and temporarily alleviate pain [23, 24]. But this pain relief did not last: by the 28th day, the PWT reduction in the SHD group was no different from that in the model group. In contrast, the pain relief of SMT was slower but lasted longer. On the third day, it first reduced the decrease in PWT, and this effect persisted until the 28th day, even statistically superior to the SHD group on the 28th day. The long-term pain relief advantage of SMT is consistent with early evidence that SMT alleviates LDH related pain while maintaining beneficial biological processes [25, 26], and is supported by studies demonstrating the role of SMT in regulating pain signaling.

Compared with SHD's broad immunosuppression, SMT achieves a more nuanced "inflammation-balancing" effect by sufficiently dampening pathological signaling to relieve pain while preserving cytokine activity necessary for tissue clearance and repair. This distinction is clinically meaningful: whereas steroids may delay spontaneous resorption of herniated NP by over-suppressing macrophage-recruiting signals (e.g., TNF-α, IL-1β), SMT fosters a reparative microenvironment that addresses both symptoms and underlying compression [27]. Such a balanced immunomodulatory profile may confer broader applicability beyond LDH. For instance, in other spinal disorders driven by dysregulated inflammation - such as cervical radiculopathy, ankylosing spondylitis, or post-surgical fibrosis - where complete suppression of inflammation could impair healing or immune surveillance, SMT's ability to fine-tune rather than abolish inflammatory responses might offer a safer and more sustainable therapeutic strategy.

The persistence of SMT pain relief comes from its coordinated regulation of tissue repair and inflammation balance, which is different from SHD, which only masks pain without address-

ing the fundamental cause of NP compression. Histologically, the NP structure of the SMT group was almost normal, with minimal inflammation and fewer NP cells (indicating faster SNP absorption). This smaller NP volume directly alleviates the mechanical compression of the L5 nerve root, which is the main cause of nerve root pain. Meanwhile, IHC results showed that SMT slightly increased the levels of IL-1β, TNF-α, VEGF, and MMP-3 compared to SHD. These cytokines are not only pro-inflammatory: moderate levels of IL-1β and TNF-α help recruit macrophages to engulf degenerated NP tissue [28, 29]. VEGF promotes the growth of new blood vessels and provides nutrients for tissue repair (a key component of spontaneous NP reabsorption [30, 31]), while MMP-3 helps to break down the matrix in a controlled manner to aid SNP reabsorption [32]. In contrast, SHD strongly suppressed these cytokines even in the late stage, which may explain the reason for its pain relief and regression. By excessively inhibiting cytokines that are crucial for NP reabsorption, SHD cannot solve NP compression, leading to sustained nerve stimulation and pain recurrence. This is consistent with clinical observations that long-term use of steroids may hinder spontaneous NP absorption [33], further confirming our findings.

Our Western blot results reveal a novel and critical molecular mechanism behind the dual efficacy of SMT. Contrary to the initial hypothesis of broad pathway inhibition, we found that SMT specifically activated the JNK signaling pathway (increasing p-JNK levels), while reducing the phosphorylation levels of NF-κB p65, p38, and ERK. This indicates that SMT does not specifically inhibit inflammation, but rather exerts an "upstream" regulatory effect by inhibiting JNK. De-activated JNK plays a central role in regulating the overall inflammatory response, thereby inhibiting the excessive activation of harmful pathways such as NF-κB, p38, and ERK, which are closely related to excessive inflammation and nerve root damage in LDH [15, 34]. The key is that this JNK dependent regulation enables SMT to construct a balanced microenvironment - while inhibiting pathological signals, maintaining the basic activity required for homeostasis and repair. Our genetic experiments ultimately confirmed the indispensable role of JNK: in JNK-KO mice, all therapeutic effects of SMT completely disappeared. The upregulation of VEGF and MMP-3, balanced cytokine profile, inhibition of NF-κB/p38/ERK pathway, and anti apoptotic effect all disappeared, which irrefutably established JNK's position as the core signaling hub of SMT pleiotropy. Although our initial Western blot analysis demonstrated that SMT modulated the phosphorylation of multiple signaling molecules - including NF-κB p65, p38, ERK, and JNK - we specifically selected the JNK2 isoform (encoded by *Mapk9*) for functional validation based on compelling biological and practical considerations. First, accumulating evidence indicates that JNK2, rather than JNK1, plays a dominant role in stress-induced inflammation, macrophage activation, and extracellular matrix remodeling - processes central to nucleus pulposus resorption in lumbar disc herniation. Second, prior studies in disc degeneration and radiculopathy models have linked JNK2 activation to the upregulation of key mediators observed in our study, including TNF-α, IL-1β, MMP-3, and VEGF. Third, *Mapk9*$^{-/-}$ C57BL/6 mice are viable, fertile, and exhibit no overt developmental abnormalities, making them suitable for mechanistic interrogation without confounding systemic effects. Given the functional redundancy among MAPK family members, targeting JNK2 allowed us to dissect its specific contribution to SMT's therapeutic effects while preserving basal JNK1 activity. This focused approach enabled a more precise evaluation of the pathway dependency underlying SMT-mediated pain relief and tissue repair.

We need to recognize the limitations of this study, especially when interpreting behavioral data. A follow-up period of 28 days is sufficient to observe the analgesic and absorption effects in the short to medium term, but may not capture the long-term effects on ganglion absorption and related pathophysiological processes, including whether the analgesic effect of spinal manipulation therapy lasts for more than 28 days. Future research with longer follow-up periods is needed to determine whether the analgesic effect of SMT persists or enhances over time. Secondly, although the sample size of this study (15 rats per group for behavioral testing and 6 rats per group for histology/immunohistochemistry/Western blotting) meets the minimum requirements of 1-2

level journals and has the basic statistical ability to detect the main intervention effects, there are still limitations. For example, it may not be able to detect clinically significant rare or subtle effects. Future studies on larger groups (possibly 20-30 per group) can further confirm whether the impact of SMT on NP reabsorption is reproducible. A larger sample size allows for more detailed subgroup analysis, taking into account confounding factors such as genetic differences or baseline physiological changes in animal, which are known to affect the risk and progression of LDH. In addition, there are inherent differences between rat models and human LDH [35], which may limit direct translation into clinical environments. Human LDH is influenced by complex factors such as work-related exposure [35], repeated minor injuries [36], and genetic susceptibility [37] - these factors have not been fully replicated in our animal model.

More and more evidence suggests that mechanical stress and inflammation interact in intervertebral disc herniation [35], and genetic factors [37], work exposure [35], and repeated minor injuries affect risk. Imaging studies have shown that spontaneous degeneration of intervertebral discs [38, 39] is associated with local cytokine driven neovascularization [40, 41]. Balancing pro-inflammatory and anti-inflammatory pathways is crucial [42]: excessive inflammation can cause pain and tissue damage, while insufficient inflammation can hinder absorption. Our research results confirm this balance mechanism: spinal massage therapy does not eliminate inflammation, but regulates inflammation to promote repair. This is consistent with the theory that mechanical intervention can finely regulate proteolytic enzymes to assist in matrix remodeling. From a clinical perspective, our findings support a shift toward personalized SMT protocols. Given individual variability in pain sensitivity, herniation size, and inflammatory status, the frequency, intensity, and duration of SMT should be tailored rather than standardized. For example, patients with larger extrusions or elevated inflammatory biomarkers (TNF-α, IL-6) might benefit from more frequent initial sessions to modulate JNK2-mediated signaling, followed by tapering as resorption progresses. Moreover, combining SMT with structured rehabilitation - such as core stabilization exercises, neural mobilization, or aerobic conditioning - may synergistically enhance outcomes. Exercise itself can modulate MAPK/NF-κB pathways and promote macrophage polarization toward a reparative phenotype, potentially amplifying SMT's "balanced inflammation" effect. Future clinical trials should therefore test integrated protocols that pair precisely dosed SMT with stage-specific rehabilitation to maximize both symptom relief and structural recovery. In addition, rehabilitation training can improve clinical efficacy, indicating that there may be a synergistic effect between SMT and exercise, which requires further research. Biological scaffolds and immunomodulators are other means of promoting intervertebral disc healing, and combining them with SMT may enhance the therapeutic effect.

In short, our research confirms the unique value of SMT as a conservative treatment option for lumbar disc herniation: it not only provides long-lasting pain relief (which is superior to steroids in the long term), but also promotes intervertebral disc tissue absorption by maintaining a controlled inflammatory microenvironment. By inhibiting the JNK pathway, spinal manipulation therapy coordinates a balanced inflammatory response, inhibits excessive activation of the NF-κB/MAPK (p38/ERK) signaling pathway, and promotes intervertebral disc tissue clearance and injury repair. This mechanism targets both the symptoms (pain) and causes (nerve root compression) of lumbar disc herniation. These data indicate that spinal manipulation therapy is a highly promising conservative treatment for lumbar disc herniation, and more clinical studies are needed to validate its efficacy in human patients.

Despite its mechanistic insights, this study has several important limitations. First, the widely used rat model of autologous tail NP implantation primarily recapitulates the inflammatory component of LDH but does not fully mimic the natural course of human disease, which involves progressive disc degeneration, annulus fibrosus rupture, sustained mechanical compression of nerve roots, and dynamic interactions between biomechanical stress and biochemical inflammation. Our model, relying on extradural NP placement, induces robust radiculopathy through inflammation but largely lacks chronic compression, potentially limiting the

translational relevance of therapeutic responses, especially for interventions targeting mechanical pathophysiology. Second, while SMT in rats provides proof-of-concept evidence for non-pharmacological modulation of JNK2-mediated neuroinflammation, it cannot fully replicate the complexity of clinical manual therapy in humans. Third, although our findings implicate JNK2 as a key mediator of SMT's effects, the potential involvement of other JNK isoforms - particularly JNK1 - was not evaluated. Future studies using isoform-specific inhibitors, double-knockout models, or phospho-isoform profiling will be needed to fully dissect the contributions of individual JNK family members. Nevertheless, our findings support SMT as a promising anti-inflammatory strategy. Future studies should validate these results in large-animal models that incorporate both compression and degeneration, explore optimal SMT parameters (force, frequency, duration), analyze human herniated disc tissues for JNK2 activation, and ultimately translate these insights into biomarker-guided clinical trials.

Conclusion

In the LDH rat model, SMT can alleviate excessive inflammation activation while maintaining favorable conditions for intervertebral disc tissue resorption. By partially inhibiting the overactivation of the NF-κB/MAPK pathway, SMT achieves a balanced regulation of the inflammatory environment, thereby promoting intervertebral disc clearance and tissue repair. These data indicate that SMT is a potential conservative therapy for treating LDH and deserves further clinical exploration.

Disclosure of conflict of interest

None.

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