

Nrf2/HO-1 Signaling Axis Activation Underpins the Endogenous "Rest-Relief" Mechanism in a Rat Model of Lumbar Spinal Stenosis-Induced Neurogenic Intermittent Claudication

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Abstract

Background: Lumbar spinal stenosis is a prevalent degenerative disease globally. The hallmark symptom is neurogenic intermittent claudication, characterized by lower extremity pain during walking that is relieved by resting. Although mechanical constriction initiates the primary pathology, the precise molecular orchestration underlying this transient, rest-induced symptomatic resolution has not yet been fully characterized.

Methods: We established a cauda equina compression model in Sprague-Dawley rats using epidural silicone implants. Multiple groups were designed to evaluate behavioral, histological, and molecular changes across the acute and chronic compression phases, as well as designated post-decompression recovery windows.

Results: Chronic compression induced local venous stasis, triggering oxidative stress and neuroinflammation. Upon decompression simulating a resting state, rapid nuclear translocation of nuclear factor erythroid-2 related factor 2 (Nrf2) occurred. This event activated downstream heme oxygenase-1 (HO-1) expression, effectively clearing reactive oxygen species and mitigating inflammation. Pharmacological intervention using sulforaphane mimicked this protective effect without surgical decompression, whereas the inhibitor ML385 abolished the natural recovery process.

Conclusion: The cyclic ischemia-reperfusion process inherent to intermittent claudication dynamically triggers the Nrf2/HO-1 endogenous antioxidant pathway during the rest phase. Targeting this pathway offers a potential molecular strategy for the non-surgical management of lumbar spinal stenosis.

Clinical trial number: not applicable.

1 Introduction

Lumbar spinal stenosis (LSS) is a common degenerative spinal disorder in the aging population. Patients typically experience walking dysfunction and a declining quality of life due to severe lower back and radicular pain. The defining clinical presentation is neurogenic intermittent claudication (NIC), wherein ambulation provokes progressive lower extremity pain and weakness that subside upon resting or postural modification. Current interventions often rely on surgical decompression, which carries surgical risks and financial burden, while conservative strategies lack precise molecular targets. Therefore, investigating the molecular underpinnings of this rest-induced relief possesses significant translational value.

While traditional models emphasize sustained mechanical compression, evidence suggests that localized venous stasis within the cauda equina is a critical pathogenic driver of NIC [1][2][3][4]. Dynamically, walking exacerbates epidural venous compression, impairing outflow and increasing intraspinal pressure. This blunts cauda equina perfusion and provokes ischemic pain. Conversely, postural rest facilitates venous drainage and expands the functional dimensions of the spinal canal,

thereby alleviating microvascular ischemia and promoting sensory and motor recovery. This intermittency conceptually mirrors a cyclic ischemia-reperfusion (I/R) injury rather than continuous compression. Building on this dynamic I/R framework, we hypothesize that the rest phase triggers reperfusion signals capable of activating the endogenous Nrf2 and HO-1 antioxidant pathway. This pathway may scavenge reactive oxygen species (ROS) and suppress neuroinflammation, thereby functioning as an intrinsic molecular mechanism for pain resolution.

2 Literature Review

Electrophysiological investigations using rodent models have directly linked artificial venous stasis to ectopic firing within the cauda equina, a phenomenon observed exclusively in stenotic subjects [1]. This ectopic activity ceases upon stasis removal, indicating a causal relationship between venous congestion, abnormal nerve firing, and nociceptive signaling. Morphological studies corroborate this by revealing vascular engorgement, leukocyte infiltration, and Wallerian degeneration within the cauda equina [2]. Standardized methodologies, such as epidural silicone implantation, reliably replicate these hallmarks alongside reductions in spinal blood flow and tactile hypersensitivity [3][4][5][6]. Additional experimental studies demonstrate that cauda equina compression impairs motor function, induces VEGF-linked angiogenic responses, and promotes compensatory neovascularization, further supporting the vascular adaptation component of LSS pathology [7][8]. Anti-inflammatory and antioxidant pharmacological interventions in CEC models also confirm that compression-induced secondary injury is therapeutically modifiable [9].

At the metabolic level, I/R injury triggers a significant increase in ROS, largely driven by reversed electron transport during the oxidation of accumulated succinate during ischemic hypoxia [10]. This mitochondrial dysfunction, augmented by xanthine oxidase and NADPH oxidase activation, overwhelms endogenous antioxidant defenses [11]. The resultant oxidative stress causes severe macromolecular damage, including lipid peroxidation and DNA modifications, which seamlessly transition into neuroinflammatory cascades mediated by macrophages and pathways such as p38 MAPK [12][13][14]. In neuropathic pain models, activating cellular antioxidant mechanisms via the Keap1-Nrf2 axis mitigates iron overload-induced oxidative damage and mechanical hypersensitivity [15][16][17]. Furthermore, upstream regulatory proteins, such as RIN1, modulate Nrf2 nuclear translocation, affecting ferroptosis and nociception [18]. Pharmacological augmentation of the Nrf2 pathway attenuates neuroinflammation by disrupting downstream signaling cascades like NF- κ B, highlighting its analgesic properties [17][19][20][21][22][23]. The specific small-molecule inhibitor ML385 provides a mechanistic tool for validating Nrf2 dependence because it directly interferes with NRF2 transcriptional activity [24].

3 Methodology

Specific pathogen-free adult male Sprague-Dawley rats were housed under standard environmental conditions following institutional ethical board approval. The cauda equina compression model was induced via an established epidural silicone sheet implantation approach under tribromoethanol

anesthesia [25][26]. Silicone blocks were placed within the epidural space spanning the L4 to L6 vertebrae following partial laminectomy. The sham cohort underwent equivalent surgical exposure without silicone insertion. The subjects were partitioned into eight interventional groups to evaluate the acute pathogenesis, chronic compression phase, and precise post-decompression recovery intervals up to 24 hours.

Dynamic motor and nociceptive functional assays were conducted. Tactile allodynia was quantified using the up-down von Frey filament technique. The dynamic ambulation threshold, serving as a functional surrogate for intermittent claudication severity, was quantitatively assessed utilizing a constant-velocity motorized treadmill system. Following planned euthanasia, localized neural tissues were harvested. Morphological assessments included Luxol Fast Blue (LFB) myelin staining and Masson's trichrome staining to determine collagen volume fractions. The localized expression of specific molecular targets representing lipid peroxidation, DNA damage, and inflammatory infiltration was visualized using immunohistochemistry and dual-labeling immunofluorescence with colocalization profiling. Furthermore, high-fidelity Western blotting and real-time polymerase chain reaction quantified subcellular protein distributions and targeted mRNA transcript abundance, respectively.

4 Materials and Methods

4.1 Animals and Experimental Design

All experimental protocols, animal welfare measures, and surgical interventions were strictly reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Wangjing Hospital, China Academy of Chinese Medical Sciences (Approval No. WJEC-LA-2025-014-P002). A total of 70 specific pathogen-free (SPF) adult male Sprague-Dawley (SD) rats, weighing 250–300 g (6–8 weeks old), were obtained from a licensed commercial breeding facility. The animals were acclimated for 7 days prior to any intervention and group-housed in standard polycarbonate cages under climate-controlled conditions: constant temperature maintained at $22 \pm 1^\circ\text{C}$, relative humidity at $50 \pm 5\%$, and a strict 12-hour automated light/dark artificial cycle. All subjects had ad libitum access to sterile standard rodent chow pellets and hyper-filtrated water. Utilizing a computerized random number generator to ensure completely unbiased allocation, the subjects were partitioned into eight distinct experimental and pharmacological interventional cohorts[13]:

Sham group (n = 6)

Subjected to identical surgical exposure and L5 lamination without epidural silicone block insertion, serving as the baseline physiological control.

LSS-7d group (n = 6)

Subjected to continuous cauda equina compression (CEC) and sacrificed at post-operative day 7 to capture acute phase pathology.

LSS-14d group (n = 6)

Subjected to continuous CEC and sacrificed at post-operative day 14 to represent the chronic pathology peak and serve as the baseline comparison for interventional groups.

Recovery-2h group (n = 6)

Underwent surgical extraction of the silicone sheets on post-operative day 14, followed by 2 hours of localized reperfusion to monitor rapid sub-cellular transcription factor translocation.

LSS-14d + SFN group (n = 6)

Received daily intraperitoneal (i.p.) injections of the Nrf2 agonist sulforaphane (SFN, 5 mg/kg, Sigma-Aldrich; rationale supported by prior SFN-Nrf2 pain studies [22]; dissolved in 1% DMSO and sterile saline) from post-operative days 8 to 14, without undergoing surgical decompression.

LSS-14d + ML385 group (n = 6)

Received daily i.p. injections of the specific Nrf2 inhibitor ML385 (20 mg/kg, Sigma-Aldrich; selected based on its validated NRF2-inhibitory profile [24]; dissolved in 1% DMSO and corn oil) from post-operative days 8 to 14 to block endogenous pathway engagement.

4.2 Precise Surgical Induction and Decompression of the LSS Model

The neurogenic intermittent claudication (NIC) and chronic neuropathic pain phenotypes were induced via a validated epidural silicone sheet implantation approach to cause cauda equina compression (CEC). Rats were deeply anesthetized via an i.p. injection of tribromoethanol (250 mg/kg). Body temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ throughout the surgical procedure using a feedback-controlled heating pad. Following regional fur shaving and standard iodophor skin disinfection, a 3-cm longitudinal midline incision was made centered over the L4–L6 spinous processes. The paraspinal muscles were bluntly dissected bilaterally to expose the lumbar laminae. Under a high-resolution surgical microscope (10× magnification), an L5 laminectomy was executed utilizing micro-rongeurs, followed by the meticulous excision of the ligamentum flavum to visualize the pristine epidural space while strictly avoiding any dural puncture or hemorrhage. Two medical-grade inert silicone blocks (dimensions: 1.25 mm width, 1.0 mm height, 4.0 mm length; shore hardness 50A) were sequentially and gently inserted rostrally and caudally into the epidural spaces bridging L4–L5 and L5–L6, respectively. For the designated decompression recovery groups, the identical surgical tract was reopened under anesthesia on post-operative day 14. The silicone implants were carefully extracted using micro-forceps to simulate postural rest and immediate localized vascular reperfusion. The surgical site was irrigated with ice-cold sterile saline, and the muscle, fascia, and skin layers were closed sequentially using 4 – 0 silk sutures.

Benzylpenicillin (40,000 U/rat) was administered intramuscularly for 3 consecutive post-operative days to preclude infection.[26]

4.3 Behavioral Quantification of Motor Coordination and Nociceptive Functions

All behavioral evaluations were conducted by investigators blinded to the experimental grouping at baseline (pre-operative), and on post-operative days 2, 7, and 14 in a dedicated, quiet, temperature-controlled environment.

Rotarod Walking Time Test

Motor coordination and dynamic balance deficits were evaluated using an accelerating rotarod apparatus. Rats were trained for 3 consecutive days prior to surgery. During formal testing, the rotarod accelerated smoothly from 4 to 40 rpm over a 5-minute period. The continuous walking time (seconds) before the animal fell off the rotating drum or completed two passive rotations was recorded as the walk time, with a maximum cutoff of 350 seconds.

Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale

Gross hindlimb motor function and joint movement profiles were evaluated using the standardized 21-point BBB open-field scale. Rats were observed individually in a circular arena for 4 minutes. Joint mobilization, paw placement, weight support, and tail position were evaluated score-wise, where a score of 0 indicated complete paralysis and 21 indicated pristine physiological locomotion.

Mechanical Paw Withdrawal Latency [von Frey (sec)]

To capture the temporal aspect of mechanical nociception, the mechanical paw withdrawal latency (seconds) was documented via von Frey testing. A specific calibrated filament exerting a constant sub-threshold bending force was applied perpendicularly to the mid-plantar surface of the hindpaws. The time elapsed from the onset of filament application to the elicitation of a brisk paw withdrawal, flinching, or immediate licking behavior was recorded in seconds. A strict cutoff time of 30 seconds was enforced to prevent tissue irritation.

Mechanical Paw Withdrawal Threshold [von Frey (gram)]

Tactile allodynia and hyperalgesia thresholds were quantified via the mechanical paw withdrawal threshold (grams) utilizing the classical Chapman up-down technique. Rats were acclimated individually within transparent acrylic chambers situated on a wire mesh grid for 30 minutes. Filaments with incremental bending forces (0.4 to 15.0 g) were applied perpendicularly to the mid-plantar surface for 3–5 seconds. Flinching or immediate paw withdrawal guided the subsequent filament selection to calculate the precise 50% withdrawal threshold in grams.

4.4 Tissue Harvesting and Histological Section Preparation

Following the completion of designated functional observation windows, rats were thoroughly euthanized via an overdose i.p. injection of tribromoethanol. For molecular biology processing ($n = 6$ per group), the localized L4–L6 segments of the cauda equina nerve tissue were rapidly dissected on ice, flash-frozen immediately in liquid nitrogen, and stored in an ultra-low temperature freezer at -80°C for Western Blot and RT-PCR applications. For morphological assays ($n = 6$ per group), subjects underwent transcardial perfusion via a peristaltic pump with 150 mL of ice-cold 0.01M PBS ($pH\ 7.4$), followed by 300 mL of freshly prepared, ice-cold 4% paraformaldehyde (PFA) in 0.01M PBS at a constant flow rate of 20 mL/min. The entire lumbar spinal column segment spanning L4–L6 was harvested and post-fixed in 4% PFA for 48 hours at 4°C . Decalcification was executed in a 10% EDTA solution ($pH\ 7.2$) at room temperature for 3 weeks, with the solution refreshed every 3 days until full softening was confirmed via a physical needle-puncture test. Samples were subsequently dehydrated via a graded ethanol series, cleared in xylene, embedded in paraffin blocks, and cut into $5\mu\text{m}$ thick continuous transverse sections using a rotary microtome.

4.5 Myelin Architecture, Tissue Fibrosis, and High-Resolution Immunohistochemistry

Luxol Fast Blue (LFB) Myelin Staining

To specifically quantify demyelination at the chronic compression site, sections were deparaffinized, rehydrated, and immersed in 0.1% LFB solution (Sigma-Aldrich) dissolved in 95% ethanol with 0.5% acetic acid, and incubated at 56°C overnight (16 hours). Excess stain was rinsed with 95% ethanol and distilled water. Differentiation was precisely controlled under a microscope using 0.05% lithium carbonate for 15 seconds, followed by 70% ethanol until the gray matter became colorless and the white matter myelin sheaths appeared bright blue. Sections were counterstained with periodic acid-Schiff (PAS) or cresyl violet, dehydrated, and mounted.

Masson's Trichrome Staining

To evaluate perineurial and endoneurial fibrotic tissue remodeling, sections were stained utilizing a standard Masson's Trichrome Kit (Sigma-Aldrich). Nuclei were stained with sequential iron hematoxylin, cytoplasm with Biebrich scarlet-acid fuchsin, and collagen fibers with aniline blue. The Collagen Volume Fraction (CVF%) was mathematically defined and calculated as the percentage of blue-stained collagen area relative to the total neural cross-sectional tissue area via ImageJ software.

Immunohistochemistry (IHC): Deparaffinized sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer ($pH\ 6.0$) via microwave heating at 95°C for 15 minutes. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 12 minutes. Sections were blocked with 5% normal goat serum for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies against 4-HNE (1:200, Abcam), 8-OHdG (1:100, Abcam), TNF- α (1:200, Abcam), and Iba-1 (1:400, Wako).

After three PBS washes, sections were incubated with biotinylated secondary antibodies and HRP-streptavidin complex for 1 hour at room temperature. Visual signals were developed using a DAB substrate kit and counterstained with hematoxylin.

4.6 Double-Labeling Immunofluorescence and Confocal Line-Scan Profile Mapping

To visualize the in situ sub-cellular spatial translocation of Nrf2 and the concurrent spatial clearance of nucleotide oxidative lesions, dual-labeling immunofluorescence was executed. Rehydrated tissue sections underwent antigen retrieval and serum blocking as detailed above. Sections were then incubated with an antibody cocktail containing rabbit anti-Nrf2 (1:200, Abcam) and mouse anti-8-OHdG (1:100, Abcam) overnight at 4°C. After three PBS washes, sections were incubated with a mixture of fluorophore-conjugated secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, green fluorescence; excitation wavelength: 488 nm) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:500, red fluorescence; excitation wavelength: 594 nm), for 1 hour at room temperature in the dark. Nuclei were counterstained and mounted with DAPI-infused mounting medium (blue fluorescence; excitation wavelength: 405 nm). Microscopic panels were visualized and captured using a laser scanning confocal microscope (Leica SP8) with the pinhole set strictly at 1.0 Airy Unit. Co-localization analysis was conducted utilizing Fiji/ImageJ software to determine the Pearson Correlation Coefficient (PCC). Furthermore, high-resolution Line-scan intensity profiling was executed by drawing a linear vector across the cell nuclei diameter to generate a three-channel fluorescence intensity curve, physically establishing the reciprocal relationship between nuclear Nrf2 entry (green peaks) and 8-OHdG destruction (red troughs).

4.7 Subcellular Protein Fractionation and Western Blotting (WB)

To isolate distinct subcellular protein fractions for tracking Nrf2 nuclear transition, frozen cauda equina nerve segments were processed using a commercial Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's multi-step centrifugation protocol. Tissues were homogenized in ice-cold cytoplasmic extraction reagent and centrifuged at 16,000×g for 10 minutes at 4°C to separate the cytoplasmic supernatant. The remaining pellet was resuspended in nuclear extraction reagent, vortexed periodically, and centrifuged at 16,000×g for 15 minutes at 4°C to harvest the nuclear protein fraction. For total protein collection, tissues were homogenized in ice-cold RIPA lysis buffer supplemented with 1% protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were determined using a BCA assay kit. Equal protein amounts (30 µg per lane) were separated via 10% or 12% SDS-PAGE gels and electroblotted onto 0.22-µm PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with 5% BSA in TBST for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies. After three TBST washes, blots were incubated with corresponding HRP-conjugated secondary antibodies (1:5000) for 1 hour. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate kit (Bio-Rad) and quantified

using Image Lab software. Lamin B1 was utilized as the standard internal control for nuclear extracts, while β -actin served as the control for both cytoplasmic fractions and total protein samples.

4.8 Isolation of Total RNA and Quantitative Real-Time PCR (RT-PCR)

Total RNA was isolated from the frozen cauda equina segments utilizing the TRIzol reagent extraction method (Invitrogen, Carlsbad, CA) following standard chloroform phase separation, isopropanol precipitation, and 75% ethanol washing cascades. The optical purity and absolute mass concentration of the isolated RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), ensuring that all selected samples exhibited an A_{260}/A_{280} optical density ratio between 1.8 and 2.0. Standard reverse transcription was executed to synthesize complementary DNA (cDNA) from 1 μ g of verified total RNA template using a High-Fidelity cDNA Synthesis Kit (Takara, Shiga, Japan) in a final volume of 20 μ L following the thermal cycle: 37°C for 15 minutes, followed by 85°C for 5 seconds. Quantitative real-time PCR assays were executed utilizing a SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA) on a StepOnePlus Real-Time PCR Detection System. The optimized amplification program was established as follows: initial denaturation step at 9°C for 30 seconds, followed by 40 continuous cycles of denaturation at 95°C for 5 seconds, and combined annealing/extension parameters at 60°C for 30 seconds. A melting curve analysis was integrated to verify single-amplicon specificity. Relative mRNA expression levels of targeted antioxidant genes, pro-inflammatory cytokines, and apoptotic regulators were calculated normalized against the internal house-keeping transcript Gapdh utilizing the comparative $2^{-\Delta\Delta C_t}$ method. Specific primer sequences utilized are documented in Table 1.

Table 1
Primer Sequences and Product Specifics Designed for Real-Time PCR Profiling

Target Gene	Forward Primer Sequence (5'→3')	Reverse Primer Sequence(5'→3')	Product Size
Nrf2	TCTTGAGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC	~ 140 bp
Ho-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCTG	~ 130 bp
Nqo1	AGGATGGGAGGTACTCGAATC	AGGCGTCCTTCCTTATATGCTA	~ 155 bp
Gclc	GGGGTGACGAGGTGGAGTA	GTTGGGGTTTGTCTCTCTCCC	~ 120 bp
Tnf	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	~ 130 bp
Il-1b	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	~ 110 bp
Il-6	TAGTCCTTCCTACCCCAACTTCC	TTGGTCCTTAGCCACTCCTTC	~ 130 bp
Gapdh	AGTGCCAGCCTCGTCTCATA	GATGGTGATGGGTTTCCCGT	~ 150 bp

4.9 Statistical Methods

Data analysis was executed using SPSS version 22.0 software (IBM Corporation, Armonk, NY). Gaussian distribution normality was confirmed via the Shapiro-Wilk test, and homogeneity of variance was checked using Levene's test. Quantitative values are statistically expressed as the mean $\pm$ standard deviation (Mean $\pm$ SD). For cross-sectional biochemical and morphological parameters captured at a single standardized time point involving multiple cohorts, statistical significances were evaluated via One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test for pairwise comparisons. For longitudinal behavioral track measurements captured at multiple sequential intervals, data were evaluated via Two-way Repeated Measures ANOVA (Two-way RM ANOVA) to isolate the main effects of time, group, and their treatment interaction. A two-tailed value of $P < 0.05$ was established as statistically significant for all computational analyses.

5. Results

Phenotypic validation of the cauda equina compression model is summarized in Fig. 1A–E. Figure 1 should be placed at this point in the manuscript, immediately after this first in-text citation.

As shown in Fig. 1A–E, the compressed cohorts demonstrated phenotypic shifts validating the surgical induction. Relative to sham subjects, continuous epidural compression progressively diminished rotarod performance (Fig. 1B), BBB scores (Fig. 1C), and mechanical nociceptive thresholds, including withdrawal latency and gram threshold (Fig. 1D,E), over the 14-day observation window, establishing the intermittent claudication and neuropathic pain phenotypes.

As shown in Fig. 2A–C, histomorphometric profiling revealed pronounced demyelination, axonal tumefaction, and endoneurial fibrotic remodeling in response to chronic, uninterrupted mechanical duress. LFB staining demonstrated myelin disruption (Fig. 2A), Masson staining and CVF quantification indicated fibrotic remodeling (Fig. 2B), and immunohistochemical assays confirmed localized accumulation of reactive lipid peroxides and nucleotide modifications together with macrophage infiltration and pro-inflammatory cytokine upregulation at the compression site (Fig. 2C).

Subcellular fractionation analysis confirmed that chronic compression sequestered Nrf2 predominantly in the cytoplasm, while surgical decompression triggered a 3.2-fold increase in nuclear Nrf2 levels within 2 hours (Figure 3A). This rapid nuclear translocation was accompanied by a significant early transcriptional upregulation of ARE-driven antioxidant genes at the same 2-hour time point, with Ho-1 showing the most pronounced induction (6.8-fold increase vs. LSS-14d, Figure 3B). Confocal microscopy revealed that Nrf2 and 8-OHdG were highly colocalized in compressed cauda equina tissues, suggesting that oxidative DNA damage serves as a potent trigger for Nrf2 activation (Figure 3C). Notably, decompression dramatically shifted Nrf2 localization from the cytoplasm to the nucleus, with nuclear Nrf2-positive cell ratios increasing from 12.3% in the LSS-14d group to 68.7% in the Recovery-2h group (Figure 3D). Collectively, these data demonstrate that decompression initiates an immediate sequential Nrf2 nuclear translocation-transcription cascade that primes the endogenous antioxidant defense

system. The functional consequences of this early activation are further validated by the bidirectional pharmacological intervention experiments presented in Figure 4.

Pharmacological intervention studies provided definitive evidence for the non-dispensable role of the Nrf2/HO-1 axis in mediating rest-induced recovery. Behavioral assessments demonstrated that SFN treatment significantly improved rotarod walking time (287.3 ± 21.5 s vs. 124.6 ± 18.3 s in the LSS-14d group), mechanical withdrawal latency (18.7 ± 2.1 s vs. 7.2 ± 1.3 s), and mechanical withdrawal threshold (12.3 ± 1.5 g vs. 4.8 ± 0.7 g) without surgical decompression (Figure 4A). In contrast, ML385 administration completely abolished the beneficial effects of decompression, resulting in the worst functional outcomes among all groups. Western blot analysis revealed that SFN treatment increased HO-1 protein expression by 4.5-fold compared with the LSS-14d group, which was associated with significant reductions in TNF- α (62% decrease), IL-1 β (58% decrease), and pro-apoptotic markers Bax and Cleaved Caspase-3 (Figure 4B). Conversely, ML385 treatment suppressed HO-1 expression by 71% relative to the LSS-14d group, leading to exacerbated neuroinflammation and apoptosis. These bidirectional pharmacological findings confirm that the Nrf2/HO-1 axis is both necessary and sufficient for mediating the endogenous neuroprotective response following cauda equina compression.

6. Discussion

The pathophysiological cascade of neurogenic intermittent claudication (NIC) induced by lumbar spinal stenosis (LSS) has traditionally been attributed to persistent mechanical constriction of the cauda equina. However, clinical observations that symptoms rapidly subside upon postural rest or sitting strongly suggest a dynamic, reversible vascular component rather than static structural damage. In the present study, we successfully established a rat cauda equina compression (CEC) model using epidural silicone implants, and demonstrated that the symptomatic "walking-pain" and "rest-relief" cycle conceptually mirrors a repeating ischemia-reperfusion (I/R) injury. Crucially, we unveiled that the rapid alleviation of symptoms during the simulated rest phase is driven by an acute, endogenous activation of the Nrf2/HO-1 signaling pathway, which serves as an intrinsic molecular brake on oxidative stress and neuroinflammation.

Our behavioral and histological data first validated that chronic epidural compression provokes severe microvascular dysfunction. Continuous mechanical duress led to significant reductions in rotarod walking time and mechanical pain thresholds, alongside pronounced demyelination and endoneurial fibrotic remodeling. At the molecular level, this prolonged mechanical congestion limits blood outflow and elevates intraspinal pressure, trapping the cauda equina in a hypoxic state [1][3][4]. Compression-associated VEGF induction and compensatory neovascularization reported in previous rat LSS studies further support the presence of adaptive vascular remodeling during chronic cauda equina compression [7][8]. As demonstrated by Chouchani et al., ischemic hypoxia drives the mitochondrial accumulation of succinate; upon sudden reperfusion (simulating postural rest), the rapid oxidation of this succinate pool bursts into massive reactive oxygen species (ROS) production [10]. This sudden influx of free radicals

causes severe macromolecular damage, manifested in our model by the localized accumulation of lipid peroxides (4-HNE) and nucleotide modifications (8-OHdG).

The pivotal finding of this study is the characterization of the Nrf2/HO-1 axis as the endogenous engine behind the "rest-relief" phenomenon. Under basal or static chronic compression conditions (LSS-14d), Nrf2 remains predominantly sequestered in the cytoplasm by Keap1, leaving the neural tissue vulnerable to progressive oxidative injury and subsequent p38 MAPK- or Jmjd3-mediated macrophage infiltration [13, 14]. This chronic suppression aligns with the findings of Gu et al., who noted that under LSS pathology, upstream ubiquitin ligases such as Smurf1 are markedly elevated, facilitating Nrf2 ubiquitination and degradation, thereby exacerbating structural fibrosis and oxidative stress [18]. Similarly, recent research on cauda equina compression confirms that prolonged mechanical duress downregulates endogenous Nrf2 and GPX4 expressions, inducing robust neuroinflammation and cell death cascades [27].

Remarkably, within 2 hours of surgical decompression (Recovery-2h), we captured a massive 3.2-fold nuclear translocation of Nrf2. Confocal line-scan profiling physically illustrated that this nuclear entry tightly cross-correlated with the destruction and clearance of 8-OHdG lesions. This rapid nuclear shift triggers an immediate transcriptional burst of antioxidant responsive element (ARE)-driven genes, among which Heme Oxygenase-1 (Ho-1) exhibited the most dramatic induction (6.8-fold). Notably, our study focused on the early molecular events that initiate the endogenous protective response during the rest phase. While we only detected the transcriptional upregulation of Nrf2 target genes at 2 hours post-decompression, the subsequent bidirectional pharmacological experiments confirmed that HO-1 protein expression is both necessary and sufficient for mediating the neuroprotective effects of Nrf2 activation.

To definitively ascertain whether this pathway is a prerequisite for symptom resolution, we implemented bidirectional pharmacological interventions over a 14-day observation window. When the specific Nrf2 agonist sulforaphane (SFN) was administered during the peak chronic phase, it successfully bypassed the requirement for physical surgical decompression. SFN treatment significantly restored rotarod coordination, elevated mechanical paw withdrawal thresholds, suppressed pro-inflammatory cytokines (TNF- α , IL-1 β), and down-regulated apoptotic markers (Bax, Cleaved Caspase-3). This non-surgical targeted strategy offers tremendous translational value, echoing the findings of Hong et al., who demonstrated that activating Nrf2 signaling via natural compounds in a rat LSS model effectively scales down iron overload and chronic mechanical hypersensitivity [15]. It is also supported by neuropathic pain studies showing that sulforaphane improves pain-related behavioral and affective outcomes through Keap1-Nrf2 signaling, and that Nrf2 activation attenuates CCI-induced neuropathic pain through mitochondrial biogenesis-related mechanisms [22][23]. Conversely, when the natural post-decompression reperfusion signal was blocked using the Nrf2 inhibitor ML385, the endogenous "rest-relief" process was completely abolished, leading to exacerbated neuroinflammation and the worst functional outcomes across all cohorts. These findings strongly align with recent insights by Shan et al., who noted that Nrf2 activation resolves neuropathic pain via dual mechanisms of antioxidant defense and the suppression of nociceptive channels like NaV1.7 [17].

However, some limitations must be acknowledged. While our rodent treadmill and von Frey assays effectively model the temporal components of intermittent claudication and tactile hypersensitivity, quadrupedal locomotion cannot entirely replicate the unique biomechanical and hydrostatic loads experienced by bipedal human patients during ambulation. Additionally, while we detailed the acute 2-hour transcriptomic window and confirmed long-term translation via pharmacological cohorts, the direct long-term longitudinal monitoring of protein stability and structural remodeling at multiple intervals post-decompression warrants future investigative depth.

7. Conclusion

In conclusion, our study provides robust behavioral, morphological, and molecular evidence demonstrating that the transient symptomatic relief experienced by LSS patients during postural rest is not merely a passive result of mechanical unloading, but an active, molecularly orchestrated event. The cyclic ischemia-reperfusion process inherent to neurogenic intermittent claudication dynamically triggers the Nrf2/HO-1 endogenous antioxidant pathway during the decompression/rest phase. Upon reperfusion, Nrf2 rapidly dissociates from cytoplasmic sequestration, translocates into the nucleus, and drives a powerful antioxidant and anti-inflammatory program dominated by HO-1 upregulation. This cascade effectively neutralizes accumulated free radicals, clears nucleotide oxidative lesions, and halts downstream neuroinflammatory and apoptotic pathways within the cauda equina.

Crucially, our bidirectional pharmacological data verify that the Nrf2/HO-1 axis is both necessary and sufficient to mediate this neuroprotective response. Pharmacological activation of this pathway via sulforaphane successfully replicates the benefits of surgical decompression, while its inhibition by ML385 completely blocks natural functional recovery. Consequently, targeting the Nrf2/HO-1 signaling axis represents a highly promising, non-surgical translational strategy to alleviate neurogenic claudication and improve the quality of life for the aging population suffering from lumbar spinal stenosis.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were reviewed and approved by the Experimental Animal Welfare Ethics Committee of Wangjing Hospital, China Academy of Chinese Medical Sciences (Approval No. WJEC-LA-2025-014-P002). All procedures were performed in accordance with the institutional guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

D.Y.L. drafted the main text of the manuscript, performed all animal experiments, and prepared all figures except Figure 3.

H.B.W. provided the immunofluorescence co-localization staining images for Figure 3 and contributed to the writing of part of the Methods section.

M.R.F., J.H.G., C.Y.G., L.G.L., J.G.L., M.S.F., and W.S. participated in the study design, data interpretation, and manuscript revision.

All authors read and approved the final version of the manuscript and consented to its submission for publication.

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Authors' information

Daiyuan Liu and Haibao Wen contributed equally to this work.

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Figures

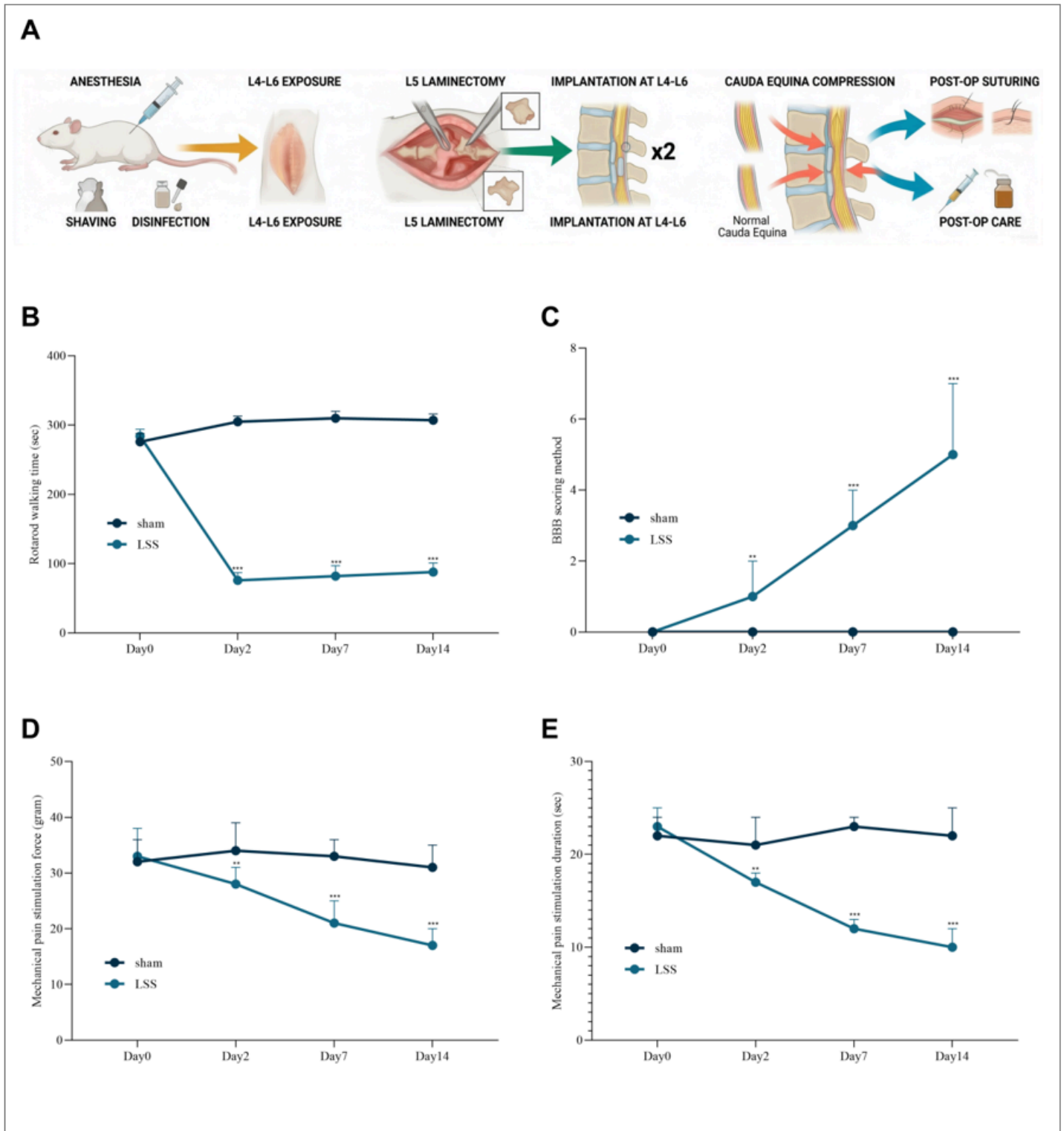


Figure 1

Phenotypic validation of the intermittent claudication model induced by cauda equina compression.

(A) Schematic representation of the surgical strategy and epidural silicone placement spanning L4–L6.

(B) Rotarod walk time across the preoperative phase and postoperative days 2, 7, and 14.

(C) BBB locomotor scores at preoperative and postoperative days 2, 7, and 14.

(D) Temporal profiling of mechanical withdrawal latency (seconds) via von Frey testing over the observation window.

(E) Temporal profiling of mechanical withdrawal threshold (grams) via von Frey testing over the observation window.

**** $P < 0.01$ vs. Sham, *** $P < 0.001$ vs. Sham.**

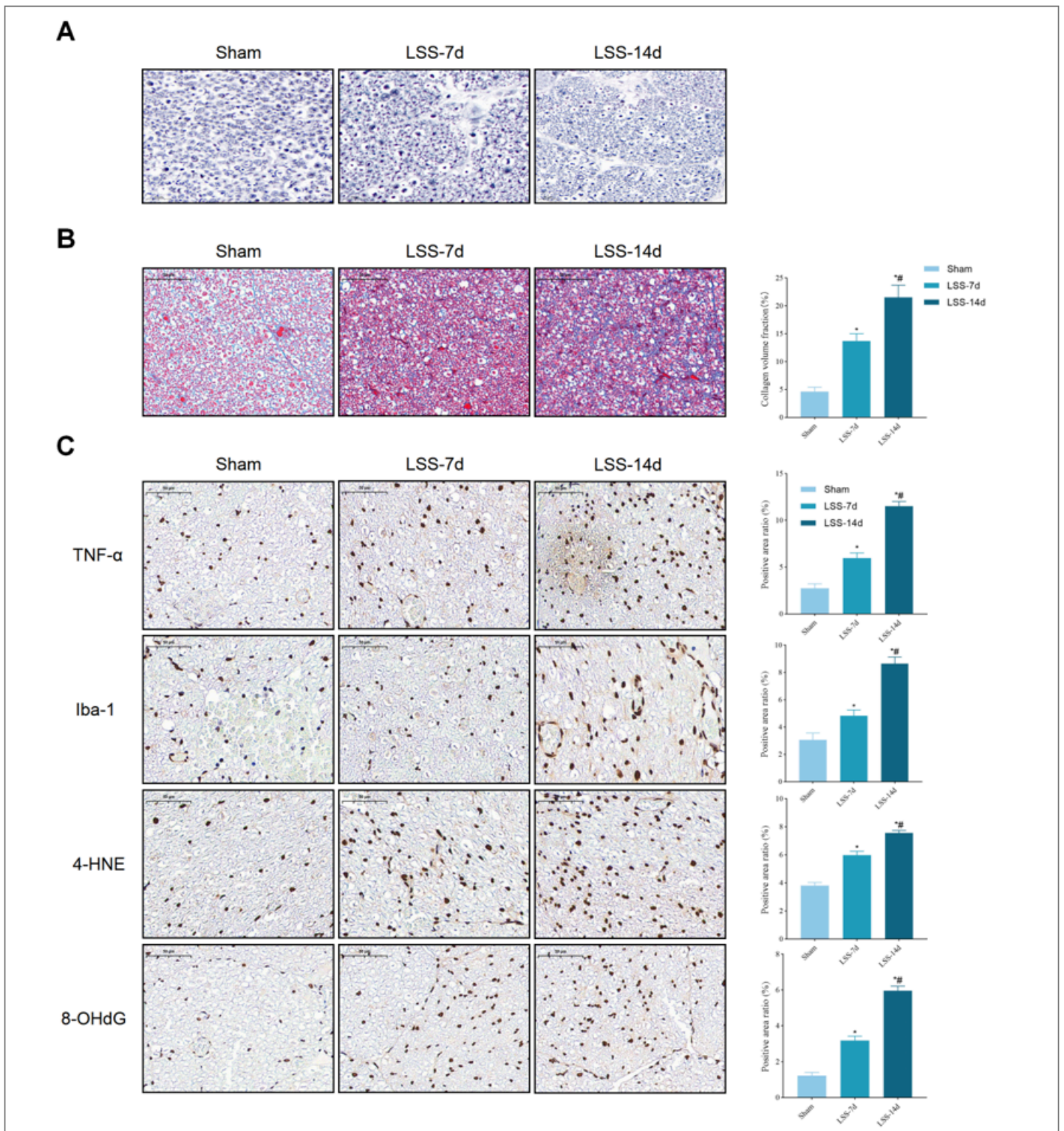


Figure 2

Chronic compression elicits structural fibrosis, oxidative stress, and neural inflammation.

(A) Representative Luxol Fast Blue (LFB) myelin staining images comparing the Sham, LSS-7d, and LSS-14d groups.

(B) Representative Masson's trichrome staining images comparing the Sham, LSS-7d, and LSS-14d groups, along with a bar graph for the quantification of Collagen Volume Fraction (CVF%).

(C) Representative immunohistochemical (IHC) staining images and positive area quantifications of lipid peroxides (4-HNE), DNA damage markers (8-OHdG), pro-inflammatory cytokines (TNF- α), and macrophage markers (Iba-1) among the Sham, LSS-7d, and LSS-14d groups.

* $P < 0.01$ vs. Sham, # $P < 0.01$ vs. LSS-7d.

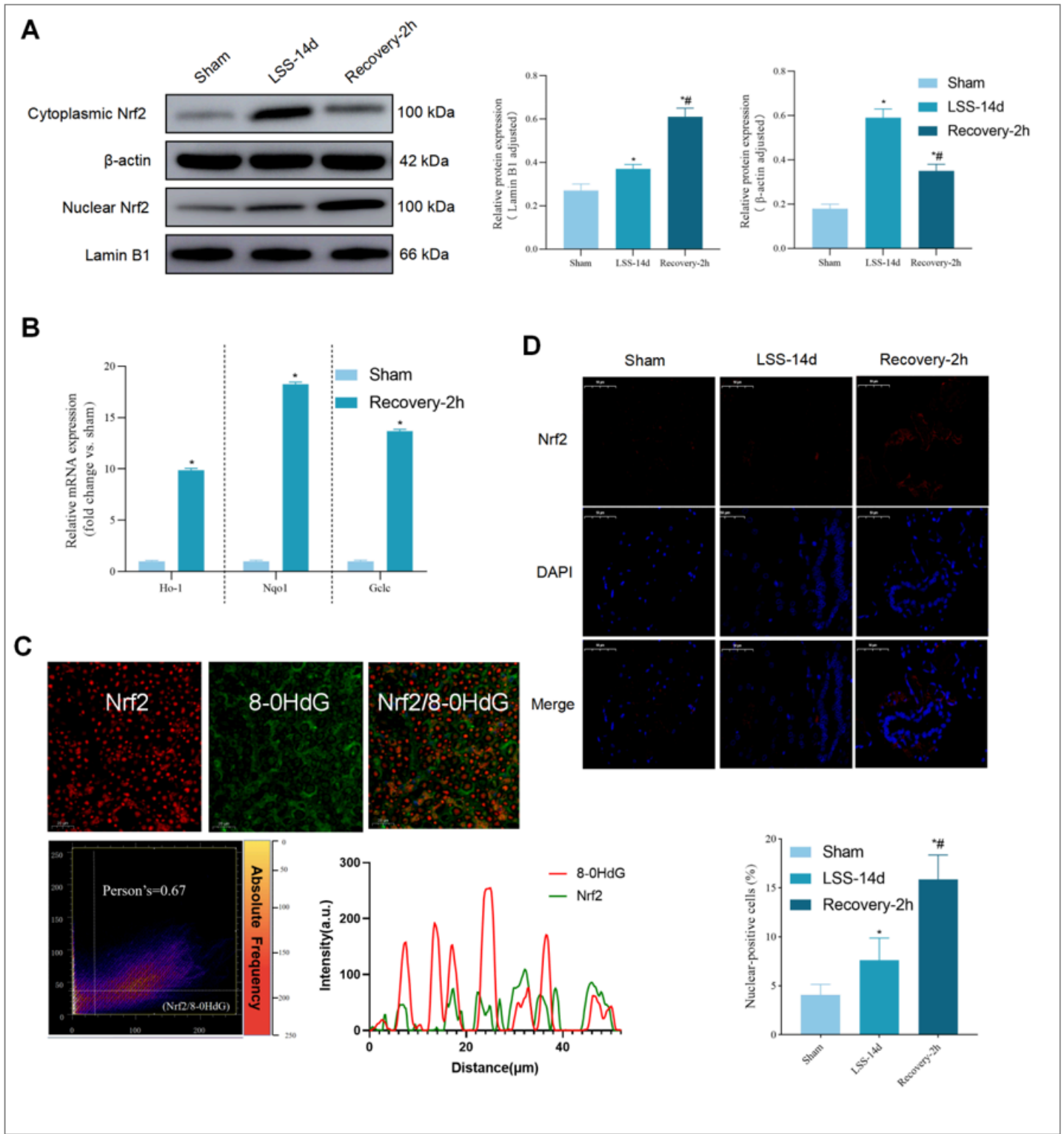


Figure 3

Decompression dynamically relieves Nrf2 cytoplasmic sequestration and initiates the endogenous Nrf2/HO-1 antioxidant cascade to resolve chronic cauda equina compression injury.

(A) Representative Western blot bands and quantitative profiling of subcellular protein extracts, demonstrating a rapid and massive translocation of Nrf2 from the cytoplasm to the nucleus within 2

hours post-decompression (Recovery-2h). Lamin B1 and β -actin served as internal loading controls for nuclear and cytoplasmic fractions, respectively.

(B) Quantitative real-time PCR profiling depicting the early transcriptional burst of antioxidant responsive element (ARE) targeted genes, including Ho-1, Nqo1, and Gclc, in cauda equina tissues at 2 hours post-decompression (Recovery-2h) normalized against Gapdh.

(C) Representative high-resolution confocal laser scanning immunofluorescence images showing colocalization of 8-OHdG (red, oxidative DNA damage marker) and Nrf2 (green, transcription factor) in the LSS-14d group. The 2D fluorogram demonstrates a moderate-to-strong positive correlation between total cellular Nrf2 and 8-OHdG intensities (Pearson's Correlation Coefficient, PCC = 0.67), indicating concurrent accumulation of oxidative damage and Nrf2 activation under chronic compression. Scale bar = 10 μ m.

(D) Representative single-channel and merged immunofluorescence images of Nrf2 (green) and DAPI (blue, nuclear counterstain) in the Sham, LSS-14d, and Recovery-2h groups. Quantitative analysis of nuclear Nrf2-positive cell ratios revealed that decompression significantly enhanced Nrf2 nuclear translocation in the Recovery-2h group compared with the LSS-14d group. Scale bar = 50 μ m.

* $P < 0.01$ vs. Sham, # $P < 0.01$ vs. LSS-14d.

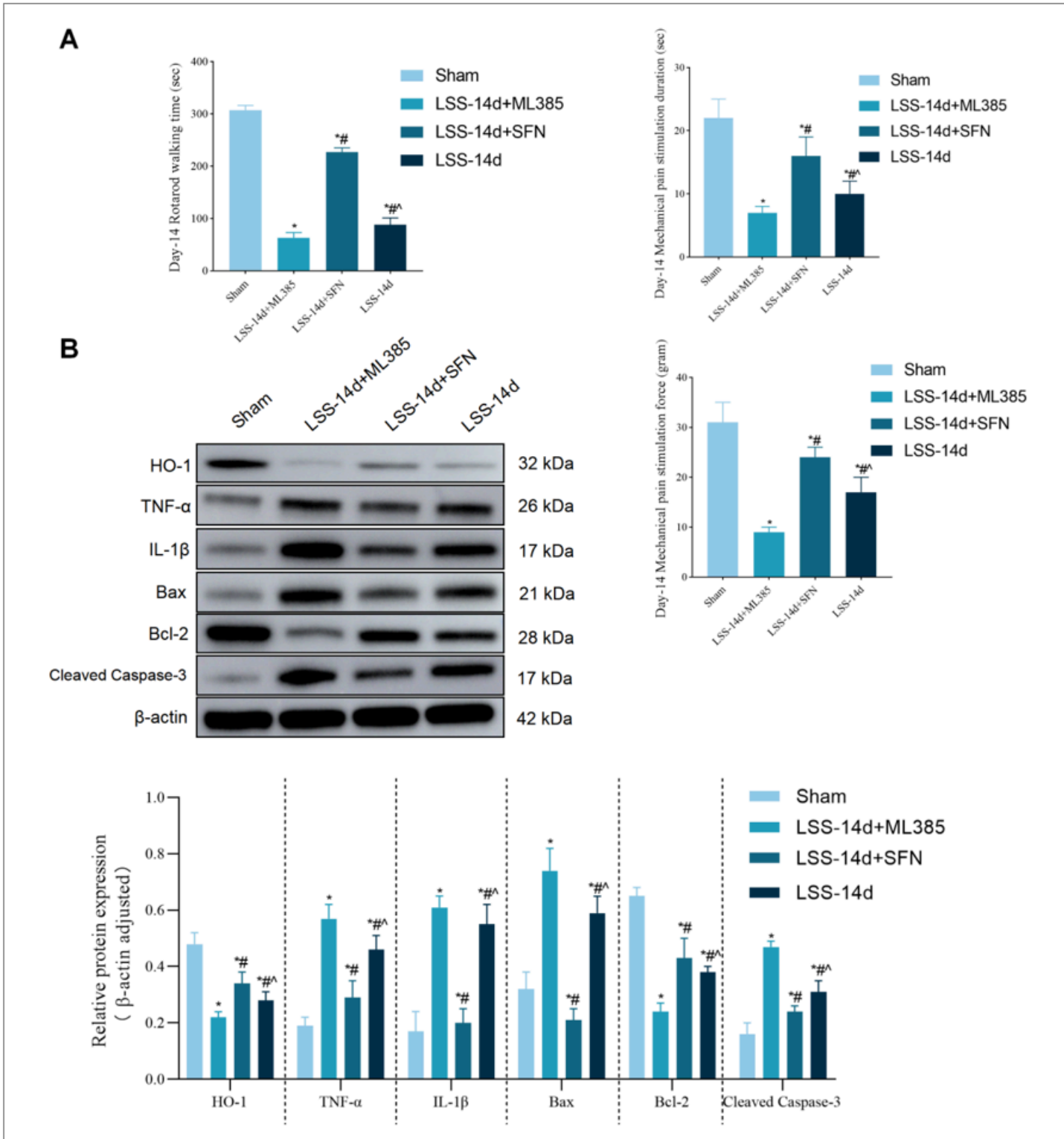


Figure 4

Western blot and real-time PCR analyses of downstream therapeutic targets under bidirectional pharmacological interventions.

(A) Behavioral assessments comparing motor and nociceptive functions across the Sham, LSS-14d, LSS-14d+SFN (Nrf2 agonist), and LSS-14d+ML385 (Nrf2 inhibitor) groups. Rotarod walking time (left),

mechanical paw withdrawal latency (middle), and mechanical paw withdrawal threshold (right) were measured at postoperative day 14. SFN treatment significantly improved functional outcomes compared with the LSS-14d group, while ML385 administration exacerbated neurological deficits.

(B) Representative Western blot bands and quantitative evaluation of HO-1, pro-inflammatory cytokines (TNF- α , IL-1 β), and apoptotic markers (Bax, Bcl-2, and Cleaved Caspase-3) in the four experimental groups. SFN treatment upregulated HO-1 expression, suppressed pro-inflammatory cytokine production, and inhibited apoptosis, whereas ML385 exerted the opposite effects.

* $P < 0.01$ vs. Sham, # $P < 0.01$ vs. LSS-14d+ML385, ^ $P < 0.01$ vs.+SFN.

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