

Augmented *Cornus officinalis* Levels in Liuwei Dihuang Decoction Inhibits Nucleus Pulposus Cell Pyroptosis to Enhance Therapeutic Efficacy Against Intervertebral Disc Degeneration

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Background: Intervertebral disc (IVD) degeneration (IVDD) is highly prevalent among the elderly population and stands as a leading cause of low back pain. Our prior studies have highlighted the therapeutic potential of Liuwei Dihuang decoction (LWDHD) and its component *Cornus officinalis* (CO)-derived compounds in alleviating IVDD and osteoarthritis, suggesting beneficial effects of CO in treating degenerative osteoarthropathies. However, uncertainty remains regarding the optimal CO dosage within LWDHD and its potential mechanism for effectively treating IVDD.

Objective: To ascertain the optimal dosage of CO within LWDHD for enhancing its therapeutic efficacy in treating IVDD, through a comparison of its effects across varied dosages using a mouse IVDD model.

Methods: Eight-week-old male C57BL/6J mice were subjected to a lumbar spine instability surgery to induce an IVDD model and received a modified LWDHD formulation containing varied dosages of CO (original dose of CO, or 5- or 10-time dose of CO (referred to as 1 × CO, 5 × CO, and 10 × CO)) for 8 weeks. The therapeutic efficacy on IVDD was evaluated through changes in lumbar spine function, histopathological morphology, extracellular matrix metabolism, nucleus pulposus cell viability, sensory nerve ingrowth, and nucleus pulposus (NP) cell pyroptosis.

Results: Augmenting CO levels in LWDHD led to a dose-dependent increase in the levels of CO-sourced active compounds in the plasma of mice. The modified LWDHD formulations, particularly the 5 × CO, exhibited a favorable pharmacological effect on lumbar function, structural integrity, ECM composition, NP cell viability, and sensory nerve ingrowth. Importantly, all 3 formulations notably mitigated NP cell pyroptosis by activating NRF2/KEAP1 pathway, with the 5 × CO formulation exhibiting superior efficacy. Additionally, a comprehensive score analysis indicated that 5 × CO formulation achieved the highest score.

Conclusion: These data underscore that elevating the dosage of CO to a specific threshold can enhance the effectiveness of LWDHD in treating IVDD.

Keywords: intervertebral disc degeneration, liuwei dihuang decoction, nucleus pulposus cell, pyroptosis, *Cornus officinalis*

Introduction

Low back pain (LBP) is a prevalent clinical condition affecting individuals' quality of life and yielding significant social and economic burdens. The prevalence of LBP has escalated from 377.5 million in 1990 to 577 million in 2017.¹ This condition predominantly stems from intervertebral disc (IVD) degeneration (IVDD), impacting 26–42% of LBP

patients.^{2,3} Existing therapeutic approaches encompass pharmaceutical, exercise, and surgical interventions,⁴ focusing on pain alleviation rather than halting the degeneration process, prompting a critical need for effective treatment modalities.

Liuwei Dihuang Decoction (LWDHD), a classic Traditional Chinese Medicine (TCM) prescription derived from the “Jingyue Quanshu”, consisting of 6 herbs, including *Rehmannia glutinosa* Libosch (Shu Di Huang), *Dioscorea opposite Thunb* (Shan Yao), *Cornus officinalis* Sieb. et Zucc (Shan Zhu Yu, CO), *Paeonia suffruticosa* Andr (Dan Pi), *Alisma orientate* (Sam.) Juzep (Ze Xie), *Poria cocos* (Schw.) Wolf (Fu Ling), has showcased significant efficacy in treating various kidney yin deficiency-induced diseases, including LBP.^{5,6} Our recent studies have underscored the therapeutic potential of LWDHD, including its component CO-derived chemical constituent morroniside, in improving lumbar spine instability (LSI) surgery-induced IVDD progression.^{7,8} Further investigations have demonstrated that loganin and morroniside, both chemical constituents sourced from CO, effectively delay joint degeneration by inhibiting chondrocytes pyroptosis,^{9,10} suggesting anti-inflammatory and anti-pyroptosis properties of CO in treating degenerative osteoarthropathies. In parallel, numerous findings from other researchers also demonstrate varied biological activities of CO and its bioactive components, including hypoglycemic, neuroprotective, heart-protective, hepatoprotective, nephroprotective, testis-protective activities, through anti-inflammatory, antioxidant activity, and anti-apoptosis mechanisms.^{11,12} Based on the above findings, we speculate that adjusting the dosage of CO in LWDHD may further enhance the therapeutic impact on IVDD. Nevertheless, uncertainties persist regarding the ideal CO dosage in the original LWDHD formulation and its potential mechanism for effectively treating IVDD.

The mechanisms of IVDD are complex, involving oxidative stress imbalances, reduction of nucleus pulposus (NP) cells, inflammation, and extracellular matrix (ECM) degradation.¹³ Notably, inflammatory elements such as Interleukin-1 β (IL-1 β) have been recognized as pivotal cytokine mediators involved in the pathological alterations of IVDD and LBP, which could intensify the inflammatory response, reduce ECM content by increasing matrix degradase such as matrix metalloproteinase 13 (MMP13), impair cell viability by promoting apoptosis and inhibiting proliferation, and foster neoinnervation.^{14,15}

Pyroptosis is a newly discovered inflammatory programmed cell death process mediated by inflammasomes. Extensive works have highlighted the significance of IL-1 β in IVDD, while the involvement of CASPASE1-mediated inflammasome in promoting various joint compartments has gained considerable attention. Both in vitro and in vivo experiments conducted by our team and other researchers have consistently demonstrated that aberrant oxidative stress-induced dysfunction of nuclear factor E2-related factor 2 (NRF2)/Kelch-like ECH-associated protein 1 (KEAP1) signaling can activate CASPASE1-mediated pyroptosis of NP cell, thereby triggering inflammation and exacerbating IVDD pathogenesis.^{13,16–18} Therefore, targeting NP cell pyroptosis and sensory innervation might offer potential benefits for slowing IVDD progression and relieving LBP.

This study aims to optimize the formulation through a comparative analysis of the therapeutic effects of varying doses of CO within LWDHD (1-, or 5- or 10-time dosage of original LWDHD formulation). To achieve this goal, LSI surgery-induced IVDD mice were treated with the original LWDHD formulation (referred to as 1 \times CO or LWDHD thereafter), or a modified formulation of LWDHD with a 5- or 10-fold increase in the dosage of CO compared to the original formulation (referred to as 5 \times CO and 10 \times CO, respectively). Our findings will illuminate which dosage of CO within LWDHD yields the most optimal therapeutic efficacy for IVDD treatment.

Materials and Methods

Animals

Eight-week-old male C57BL/6J mice were provided by the animal experiments center of Zhejiang Chinese Medical University (Grade SPF, SCXK (Shanghai): 2017-0005). These mice were housed in a specific pathogen-free animal care facility under controlled conditions of 23 \pm 2°C and a 12-h light/dark cycle. All animal experiments were obedient to the ARRIVE guidelines and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 and related guidelines, EU Directive 2010/63/EU for animal experiments.¹⁹ They were given unrestricted access to water and lab chow. All animal experiments followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All procedures involving the mice were approved by the Committee on the Ethics of Animal Experiments of Zhejiang Chinese Medical University (2020-KL-159-02).

Chemicals and Reagents

The dosage information of modified LWDHD formulations is presented in Table 1. Each set of medicinal materials was soaked overnight in a large beaker and then decocted in a stainless-steel pot with 2.5 L distilled water. The mixture was boiled down to 1.0 L and subsequently filtered to remove any herb residues. The resulting liquid medicine was then subpacked into 50 mL tubes and stored at -20°C . Prior to use, LWDHD was thawed in the refrigerator at 4°C and allowed to return to room temperature for 1–2 hours before gavage administration.

Primary antibody against AGGRECAN was from Abcam (Cambridge, MA, USA). Primary antibodies against Collagen 2 (COL2), MMP13, Ki-67, Gasdermin D (GSDMD), and IL-1 β were purchased from Ruiying Biological (Jiangsu, China). Primary antibodies against Tyrosine kinase A (TrkA), and NRF2 (S40) were obtained from HuaBio (Hangzhou, China). Primary antibody against CASPASE1 was obtained from ProteinTech Group (Chicago, IL, USA). Primary antibody against KEAP1 was acquired from ImmunoWay Biotechnology Company (Jiangsu, China). Primary antibodies against phospho-I- κ B (p-I- κ B) and phospho-P65 (p-P65) were provided by Cell Signaling Technology (Beverly, MA, USA). Fluorescent secondary antibodies were supplied by Sungene Biotech Co. (Tianjin, China). Unless specified, all chemicals were from Sigma-Aldrich (St. Louis, MO).

Experimental Design

A total of 90 eight-week-old mice were distributed randomly and equally into five groups ($n = 18$): Sham group, Vehicle group, LWDHD group, $5 \times \text{CO}$ group, and $10 \times \text{CO}$ group. All mice, with the exception of those in the Sham group, underwent LSI surgery-induced IVDD modeling, as we previously described.^{8,16}

The mice are anesthetized with isoflurane (3% isoflurane for induction, 2% isoflurane for maintenance). Following shearing and cleaning of the back, the back skin was disinfected with Iodophor and then longitudinally incised along the spine direction, the fascia was passively stripped, the erector spinal muscle of the mice was exposed, and the erector spinal muscles on both sides were passively separated axially (cutting off part of the erector spinal muscle if necessary). Subsequently, the lumbar spinous process of the mice was completely exposed, and the spinous process of L3 ~ L5 segments was completely excised. The supraspinous and interspinous ligaments were removed, followed by disinfection with Iodophor. Subsequently, a check was made for internal bleeding and peritoneum integrity. The muscles, fascia, and skin were sutured layer by layer, and gentamicin hydrochloride was applied to the wound to prevent infection.

Three days after LSI surgery, mice in the LWDHD group, $5 \times \text{CO}$ group, and $10 \times \text{CO}$ group were orally administered with the corresponding LWDHD (0.1 mL/20 g body weight) once a day for 8 consecutive weeks. The other two groups of mice received an equivalent dosage of normal saline. Following 1, 2, and 8 weeks post LSI surgery, 6 mice in each group were euthanized, and the lumbar vertebrae were collected for further analysis.

Table 1 Dosage of Modified LWDHD Formulations

Herb (g) \ Formulation	Original LWDHD	5× CO	10× CO
<i>Rehmannia glutinosa</i> Libosch	160	160	160
<i>Dioscorea opposita</i> Thunb	80	80	80
CO	80	400	800
<i>Paeonia suffruticosa</i> Andr	60	60	60
<i>Alisma orientate</i> (Sam.) Juzep	60	60	60
<i>Poria cocos</i> (Schw.) Wolf	60	60	60

Abbreviations: LWDHD, Liuwei Dihuang Decoction; CO, *Cornus officinalis*. The CO contents in these formulations are highlighted in bold to indicate the differences in amounts across formulations.

HPLC Assay of Morroniside, Loganin, and Paeoniflorin in Mice Plasma

The plasma of IVDD mice was collected following 1 and 8 weeks of LWDHD administration. And 100 μ L of plasma from each mouse was combined with four times the volume of acetonitrile, vortex-mixed for 2 min, and centrifuged at 14000 rpm for 15 min. The resulting supernatant was dried using nitrogen blowing, and the residue was reconstituted with 200 μ L of the initial mobile phase. After vortex-mixing for 30 seconds and centrifuging at 14000 rpm for 20 min, the supernatant was collected and injected for HPLC analysis.

Chromatographic conditions involved utilizing the Waters Xevo TQD triple quadrupole mass spectrometer, Waters Acquity ultrahigh performance liquid chromatograph, and the Waters ACQUITY UPLC C18 column (2.1100 mm, 1.7 m). The mobile phases included (A) 0.1% formic acid in water and (B) acetonitrile. The gradient elution occurred as follows: 0–6 min, 10–15% B; 6–10 min, 15–90% B; 10–10.1 min, 90–10% B; 10.1–12 min, 10% B. A flow rate of 0.3 mL/min, a column temperature of 30°C, and an injection volume of 3 μ L were employed.

For mass spectrometric conditions, an electrospray ionization (ESI) source, negative ion detection, and multiple reaction detection modes (MRM) were used. The capillary voltage was set to 3.4 kV, the ion source temperature to 550°C, the degassing flow rate to 800 L/h, and the cone body flow rate to 50 L/h.

Functional Test

Rearing frequency analysis: The rearing frequency analysis involves placing the mouse in a 1 L beaker. Once the mouse is acclimated to its surroundings, the frequency of upright standing occurrences within one minute is recorded using a camera.

Von-Frey test: Allow the mice to acclimate to the environment for approximately 30 min until they exhibit minimal movement (no longer actively exploring). The middle part of the sole of the hind limb was gently stimulated with a Von-Frey fiber to bend the fiber for several seconds, and the mouse's foot contraction reaction was observed. Rapid foot contraction reaction due to this stimulation is considered a positive response; care should be taken to differentiate this from a reaction caused by physical activity. If there are three positive reactions in five consecutive stimuli, it is marked as "×"; otherwise, it is marked as "O". If a reaction occurs, the pressure is reduced to the previous level; if not, it is increased to the next level, and this operation is repeated (a total of six rounds). The pressure value associated with "×" (the pressure value corresponding to the last used fiber) was recorded for subsequent data analysis.

Open field test: The open field test device comprises an open field reaction box and an automatic data acquisition and processing system. The box measures 30 cm in height and 72 cm in length at the bottom, with the inner walls painted black. A digital camera is situated 2 meters above, with a field of vision covering the entire open field. The experiment is carried out in a quiet environment. Mice are placed in the center of the bottom inside the box, and simultaneous video recording and time tracking are initiated. Observation occurs over a 5-minute period. Following this, the inner walls and bottom of the box are cleaned, mice are replaced, and the test continues. Data are collected on total movement distance, central motion time, and distance relative to the center, for subsequent data processing.

HE Staining, Immunohistochemical (IHC), and Immunofluorescence (IF) Analyses

After fixation in 4% paraformaldehyde for 3 days, the IVD tissues were decalcified with 14% EDTA solution (pH = 7.4) for about 28 days, dehydrated in alcohol, embedded in paraffin, and sliced into 5 μ m sections. The hematoxylin and eosin (HE) staining of the sections was carried out as we previously described.^{8,16} For IHC and IF analysis, the sections underwent thermal repair in sodium citrate buffer, followed by incubation with primary antibodies at specified dilutions (AGGRECAN (1:500 dilution), COL2 (1:300 dilution), MMP13 (1:300 dilution), Ki-67 (1:500 dilution), TrkA (1:500 dilution), CASPASE1 (1:500 dilution), GSDMD (1:500 dilution), IL-1 β (1:500 dilution), NRF2 (S40) (1:500 dilution), KEAP1 (1:500 dilution), p-I- κ B (1:500 dilution), and p-P65 (1:500 dilution)) at 4°C overnight, respectively. Negative control sections were incubated with nonspecific IgG. For IHC staining, the polymer-HRP labeled secondary antibody was added for 30 min the following day. The IHC reaction was visualized using 0.05% diaminobenzidine (ZSGB-BIO, Beijing, China) followed by counterstaining with hematoxylin. For IF analysis, the slides were incubated with fluorescence-conjugated secondary antibody for 30 min in the dark, and then DAPI staining was utilized to estimate the total

cell number. Quantitative histomorphometric analysis was performed in a blinded manner using Image-Pro Plus Software version 6.0 (Media Cybernetics Inc., Rockville, Maryland, USA).

TUNEL Assay

The TUNEL assay to detect DNA breaks was performed using a TUNEL Bright Green Apoptosis Detection Kit (Vazyme Biotech; Nanjing, China), according to the manufacturer's instruction. Negative controls were subjected to incubation in a TdT free-enzyme solution. The number of positive cells was quantified in three randomly selected fields of view using three sections from each sample. DAPI staining was utilized to estimate the total cell number.

Statistical Analysis

All numerical data are presented as mean \pm standard error of mean (SEM). The statistical analysis was performed using GraphPad Prism 8 statistics software (San Diego, CA, USA). The differences between multiple groups were determined by two-way or one-way analysis of variance analysis (ANOVA) with Tukey's multiple comparison test. $p < 0.05$ was considered to be statistically significant.

Results

LWDHD Formulations Improve Lumbar Functional Damage in IVDD Mice

To investigate the effects of these 3 formulations on IVDD progression, mice subjected to LSI surgery received LWDHD, or $5 \times$ CO or $10 \times$ CO via intragastric administration for 8 weeks. The potential bioavailability of the formulations was assessed by determining the plasma concentrations of 3 quality control indexes of LWDHD, including morroniside, loganin, and paeoniflorin (mainly sourced from *Dioscorea opposita Thunb*), using HPLC after 1 and 8 weeks. Compared with original LWDHD, 8 weeks' treatment with $5 \times$ CO or $10 \times$ CO treatment resulted in 2.4 folds and 2.2 folds increase of loganin, with a nonsignificant dose-dependent rise of morroniside. Conversely, no significant differences were observed in the concentrations of paeoniflorin ([Figure S1](#)).

To compare the pharmacological effect of these formulations on the lumbar function of IVDD mice, behavioral assessments, including rearing frequency analysis, Von-Frey test, and open field test, were carried out. IVDD mice exhibited a significant reduction in rearing frequency, while LWDHD and $5 \times$ CO, but not $10 \times$ CO, restored it to a level comparable to that of Sham mice ([Figure 1A](#)). Moreover, the Von-Frey test and open field test Results revealed that $5 \times$ CO and $10 \times$ CO, but not the original LWDHD, significantly reduced that central motion time, and no significant differences were observed in other indexes ([Figure 1B and C](#)), indicating a reduction in anxiety levels in IVDD mice ([Figure 1C](#)).

LWDHD Formulations Attenuate Structure Impairment and ECM Degradation in IVDs of IVDD Mice

To evaluate the impact of these LWDHD formulations on the structure and ECM composition of IVDs of IVDD mice, histomorphological alterations in L4-L5 IVDs were examined using HE staining. IVDD mice exhibited ruptured IVDs with

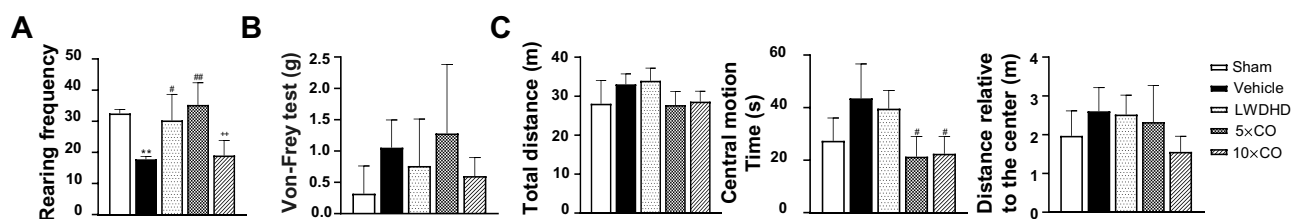


Figure 1 LWDHD formulations enhance lumbar function of IVDD mice. **(A)** Analysis and quantification of rear frequency test performed in IVDD mice after treatment with LWDHD formulation for 8 weeks. **(B)** Quantification of Von-Frey pain test. **(C)** Quantification of total distance, central motion time, and distance relative to the center in open field test of mice. Data are shown as mean \pm SEM ($n = 6$). *Indicates a significant difference compared with the Sham group (** $p < 0.01$), **Means a significant difference compared with the Vehicle group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$), † Denotes a significant difference compared with the LWDHD group ($^{++}p < 0.01$).

Abbreviations: LWDHD, Liuwei Dihuang Decoction; IVDD, Intervertebral disc degeneration.

flattened NP, fissures and folds in annulus fibrosus, and abnormal bone formation in endplates, and LWDHD and 5 × CO significantly reversed these phenotypes (Figure 2A). The degeneration of IVD was further assessed by the histological score system established by Norcross et al,²⁰ and the results showed that administration with LWDHD and 5 × CO, but not 10 × CO, restored histological score (Figure 2B). Subsequently, the expressions of ECM such as AGGRECAN and COL2, and degradase MMP13 were analyzed using IHC analysis. The results showed that all LWDHD formulations significantly alleviate the decrease in AGGRECAN and COL2 and increase in MMP13 in IVDD mice, except for the lack of significant reversal of COL2 in IVDD mice treated with LWDHD (Figures 2C, D–H). Interestingly, 10 × CO treatment caused the most substantial restoration in AGGRECAN, while both the 5 × CO and 10 × CO formulations produced a comparable elevation in COL2. Conversely, the LWDHD led to the most pronounced reduction of MMP13.

LWDHD Formulations Mitigate NP Cell Viability and Invasion of Sensory Nerves in IVDD Mice

To assess whether modified formulations of LWDHD could affect NP cell viability and innervation in IVDD mice, the apoptosis and proliferation status of NP cells were determined using TUNEL assay and IF assay of Ki-67, a specific marker for proliferation. TUNEL results showed that 1 × CO and 10 × CO, but not 5 × CO, resulted in a significant reduction in TUNEL-positive cells in IVDs of IVDD mice (Figure 3A and B). Consistent with TUNEL results, IF results

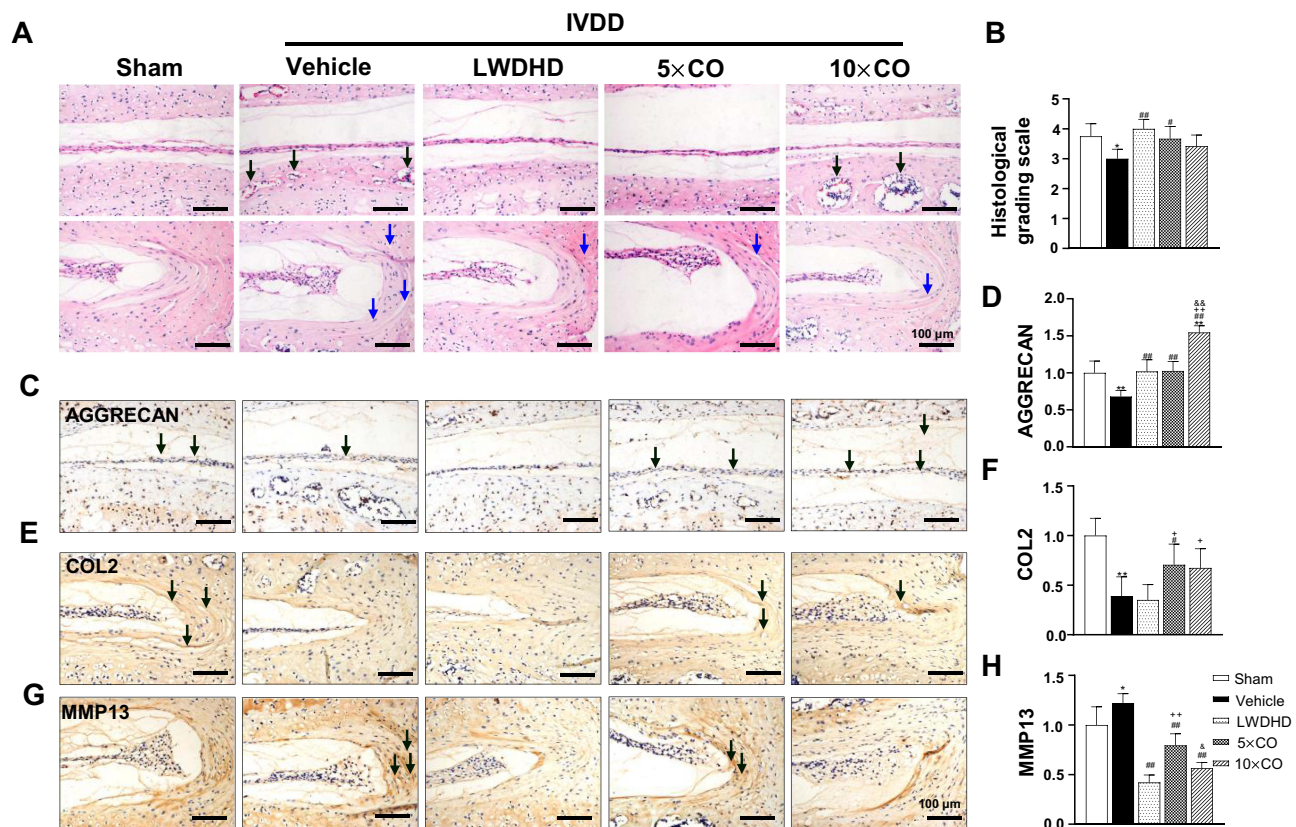


Figure 2 LWDHD formulations alleviate IVDD progression in LSI surgery-induced IVDD mice. **(A)** HE staining of the IVDs of IVDD mice after treatment with LWDHD formulation for 8 weeks. Black arrows indicate ectopic bone formation. Blue arrows indicate tearing in annulus fibrosus. **(B)** Histological grading scores of IVD tissues in **(A)**. **(C and D)** IHC analysis and corresponding quantification of AGGRECAN in IVDs of IVDD mice. Black arrows indicate high expression of AGGRECAN. **(E–H)** IHC analysis and corresponding quantification of COL2 **(E and F)** and MMP13 **(G and H)** in IVDs of mice. Black arrows indicate high expression of COL2 and MMP13. Data are shown as mean \pm SEM (n = 6). *Denotes a significant difference compared with the Sham group (* p < 0.05, ** p < 0.01), #Means a significant difference compared with the Vehicle group (### p < 0.01), *Indicates a significant difference compared with the LWDHD group (* p < 0.05, ** p < 0.01), &Signifies a significant difference compared with the 5 × CO group (& p < 0.05, && p < 0.01).

Abbreviations: LWDHD, Liuwei Dihuang Decoction; IVDD, Intervertebral disc degeneration; LSI, Lumbar surgery instability; IVD, Intervertebral disc; IHC, Immunohistochemical; COL2, Collagen 2; MMP13, Matrix metalloproteinase 13; CO, *Cornus officinalis*; 5 × CO, a modified formulation of LWDHD with a 5-fold increase in the dosage of CO.

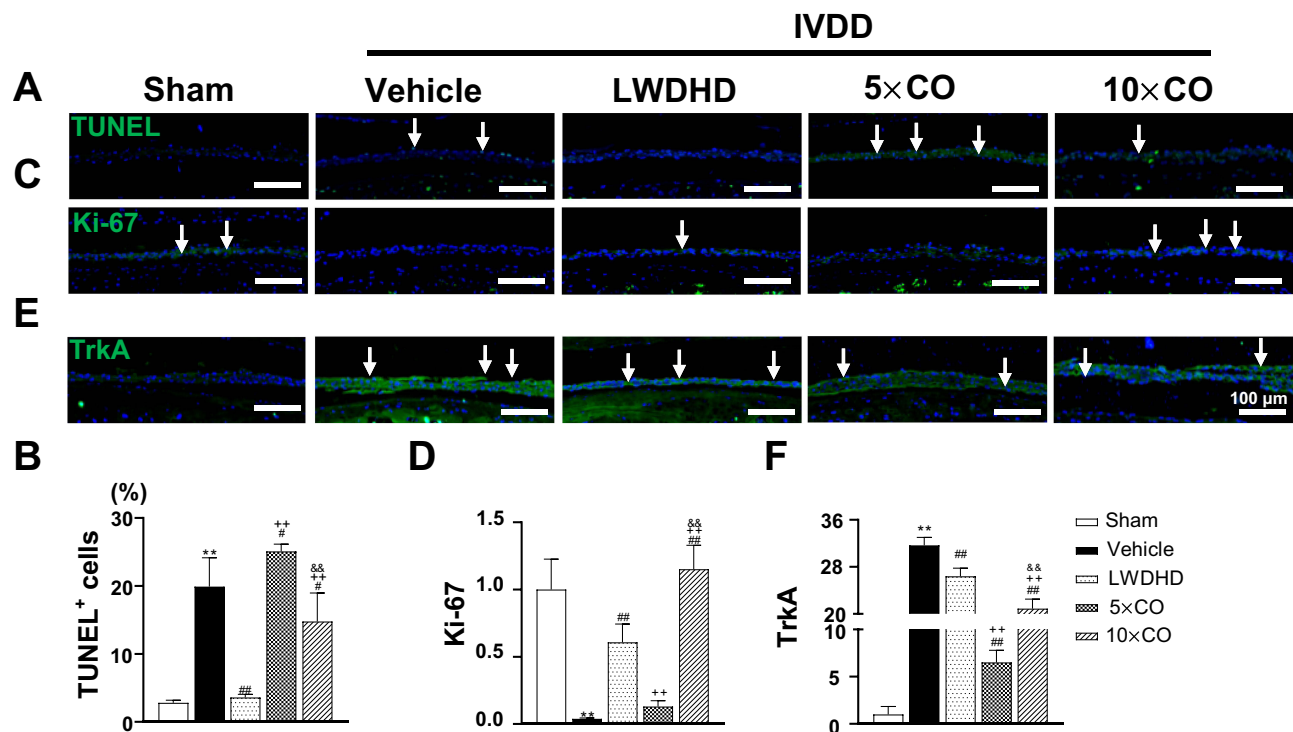


Figure 3 LWDHD formulations mitigate NP cell viability and innervation in IVDs of IVDD mice. **(A and B)** TUNEL assay of the IVD section **(A)** and corresponding qualification **(B)** of the rate of positive cells in NP tissues of IVDD mice after treatment with LWDHD formulation for 8 weeks. DAPI stains nuclei (Blue). White arrows indicate high expression in the IVDs. **(C–F)** IF staining for Ki-67 **(C and D)** and TrkA expression **(E and F)** in IVDs of IVDD mice after 8 weeks of treatment with LWDHD formulation. White arrows indicate high expression in the IVDs. Data are shown as mean \pm SEM (n = 6). **Denotes a significant difference compared with the Sham group (** $p < 0.01$), ##Means a significant difference compared with the Vehicle group (* $p < 0.05$, ### $p < 0.01$), *Indicates a significant difference compared with the LWDHD group (* $p < 0.01$), &Means a significant difference compared with the 5 \times CO group (& $p < 0.01$).

Abbreviations: LWDHD, Liuwei Dihuang Decoction; NP, nucleus pulposus; IVD, Intervertebral disc; IVDD, Intervertebral disc degeneration; TrkA, Tyrosine kinase A; CO, *Cornus officinalis*; 5 \times CO, a modified formulation of LWDHD with a 5-fold increase in the dosage of CO.

of Ki-67 showed that 1 \times CO and 10 \times CO, but not 5 \times CO, led to a significant upregulation in Ki-67 expression of IVDs of IVDD mice (Figure 3C and D).

It has been well established that sensory nerve ingrowth into IVD was triggered by nerve growth factor/high-affinity TrkA signaling.²¹ To identify whether modified formulations of LWDHD could affect sensory nerve ingrowth, the expression of TrkA was assessed using IF analysis. The results showed that a noticeable increase in positive TrkA expression was observed in the NP of IVDD mice, and all 3 LWDHD formulations treatment decreased the increased expression of TrkA in IVDs of IVDD mice, with the 5 \times CO-treated mice demonstrating the most pronounced inhibitory effect (Figure 3E and F). All these findings suggest that the efficacy of LWDHD with different dosages of CO in treating IVDD varies slightly.

LWDHD Formulations Inhibit NP Pyroptosis in IVDD Mice

In a previous study, we demonstrated that activated pyroptosis of NP cells is involved in the progression of IVDD.¹⁶ To analyze whether 3 LWDHD formulations could affect NP pyroptosis, the expression of key proteins in pyroptosis, including CASPASE1, GSDMD, and IL-1 β was determined. Consistent with our previous results, we found a marked elevation in CASPASE1, GSDMD, and IL-1 β expression within the NP tissues of IVDD mice, and all these formulations significantly reduced these elevations compared with IVDD model mice, except for a higher expression of GSDMD was noted in 10 \times CO group compared to all other groups. Notably, among the LWDHD formulations, the 5 \times CO treatment demonstrated superior efficacy in reducing the expression of CASPASE1, GSDMD, and IL-1 β . Moreover, when compared with the 5 \times CO group, 10 \times CO treatment led to a slight increase in CASPASE1 and IL-1 β , indicating a potential counterproductive effect of CO overdose (Figure 4A–F). These data suggest that the 5 \times CO treatment is more effective in inhibiting NP pyroptosis.

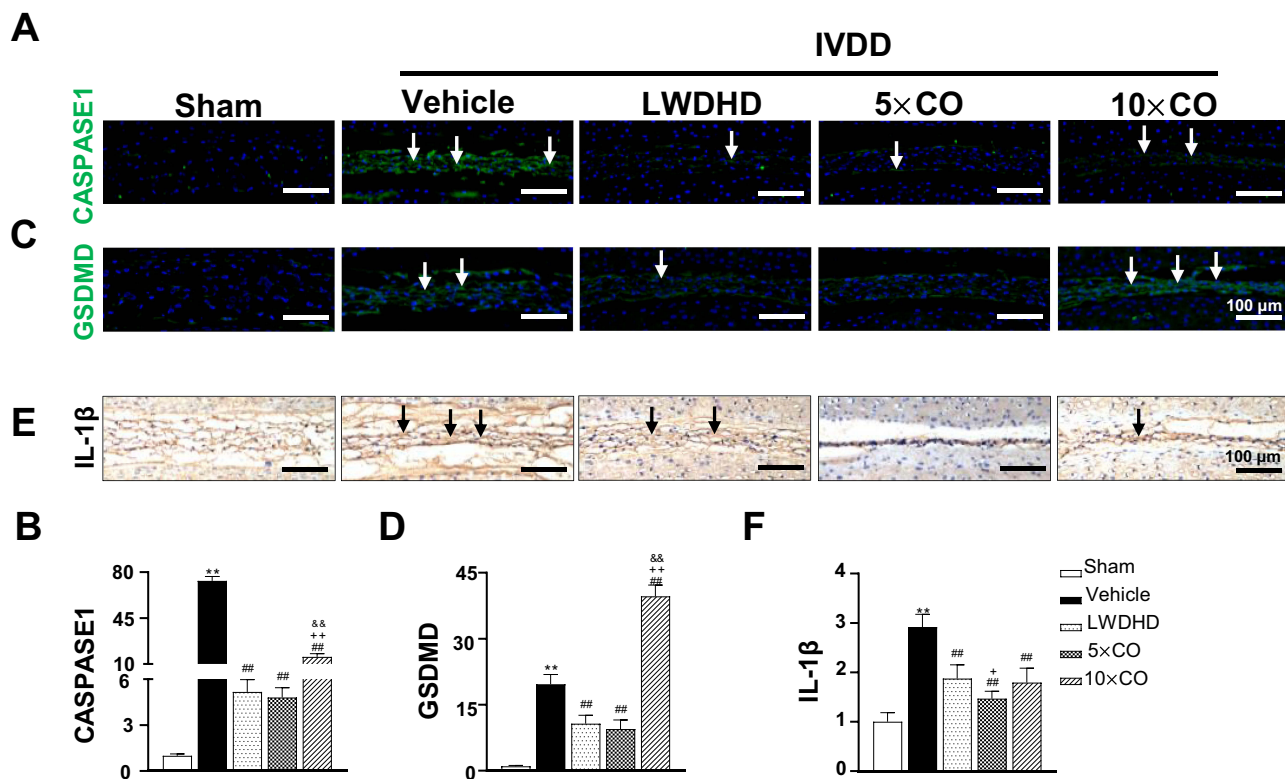


Figure 4 LWDHD formulations inhibit NP cell pyroptosis in IVDD mice. (A–D) IF staining and corresponding qualification of CASPASE1 (A and B), GSDMD (C and D) in IVDs of IVDD mice after treatment with LWDHD formulation for 2 weeks. White arrows indicate high expression of CASPASE1 and GSDMD in NP tissues. (E and F) IHC staining and corresponding qualification of IL-1 β in IVDs of IVDD mice. Black arrows indicate high expression of IL-1 β in NP tissues. Data are shown as mean \pm SEM (n = 6). *Denotes a significant difference compared with the Sham group (** $p < 0.01$), #Means a significant difference compared with the Vehicle group (### $p < 0.01$), +Indicates a significant difference compared with the LWDHD group (* $p < 0.05$, ** $p < 0.01$), &#Means a significant difference compared with the 5 \times CO group (&# $p < 0.01$). **Abbreviations:** LWDHD, Liuwei Dihuang Decoction; NP, nucleus pulposus; IVDD, Intervertebral disc degeneration; GSDMD, Gasdermin D; IVD, Intervertebral disc; IHC, Immunohistochemical; IL-1 β , Interleukin-1 β ; CO, *Cornus officinalis*; 5 \times CO, a modified formulation of LWDHD with a 5-fold increase in the dosage of CO.

LWDHD Formulations Activate NRF2/KEAP1 Pathway in NP Tissues

To elucidate the possible upstream mechanism through which LWDHD delays the progression of IVDD, we examined the activity of NRF2/KEAP1 pathway and downstream NF- κ B pathway by analyzing the expression of antioxidant response protein NRF2 and its negative feedback protein KEAP1, as well as the p-I- κ B and p-P65 using IF analysis. As shown in Figure 5, we found that LSI surgery resulted in almost no expression of NRF2 (S40) in IVDD mice, and 1.4-fold, 3.4-fold, and 8.7-fold increases of KEAP1, p-I- κ B, and p-p65. Notably, 3 LWDHD formulation significantly reversed the decrease of NRF2 (S40) in a dose-dependent manner, while 1 \times CO and 5 \times CO significantly reduced the increased expression of KEAP1 to the level of Sham mice, except that 10 \times CO treatment resulted in a higher KEAP1 than all other groups (2.3-fold of Sham mice). Intriguingly, all 3 LWDHD formulations could significantly reduce the elevated expression of p-P65, with 1 \times CO and 10 \times CO demonstrating significantly more inhibitory effects on the increase of p-P65 expression in IVDD mice than 5 \times CO treatments (Figure 5G and H). Additionally, 1 \times CO and 10 \times CO but not 5 \times CO remarkably suppressed the increase of p-I- κ B in IVDD mice (Figure 5E and F).

5 \times CO as an Optimal Level for Delaying IVDD Progression

To facilitate a more intuitive and comprehensive comparison of the therapeutic efficacy of 3 LWDHD formulations, we devised a scoring standard to assess their combined efficacy within IVD tissues (refer to Table S1). After computing the total score for each LWDHD formulation, and the results revealed that 5 \times CO obtained the highest score (Table 2), implying that LWDHD containing this particular dosage may represent the most effective option for delaying the progression of IVDD.

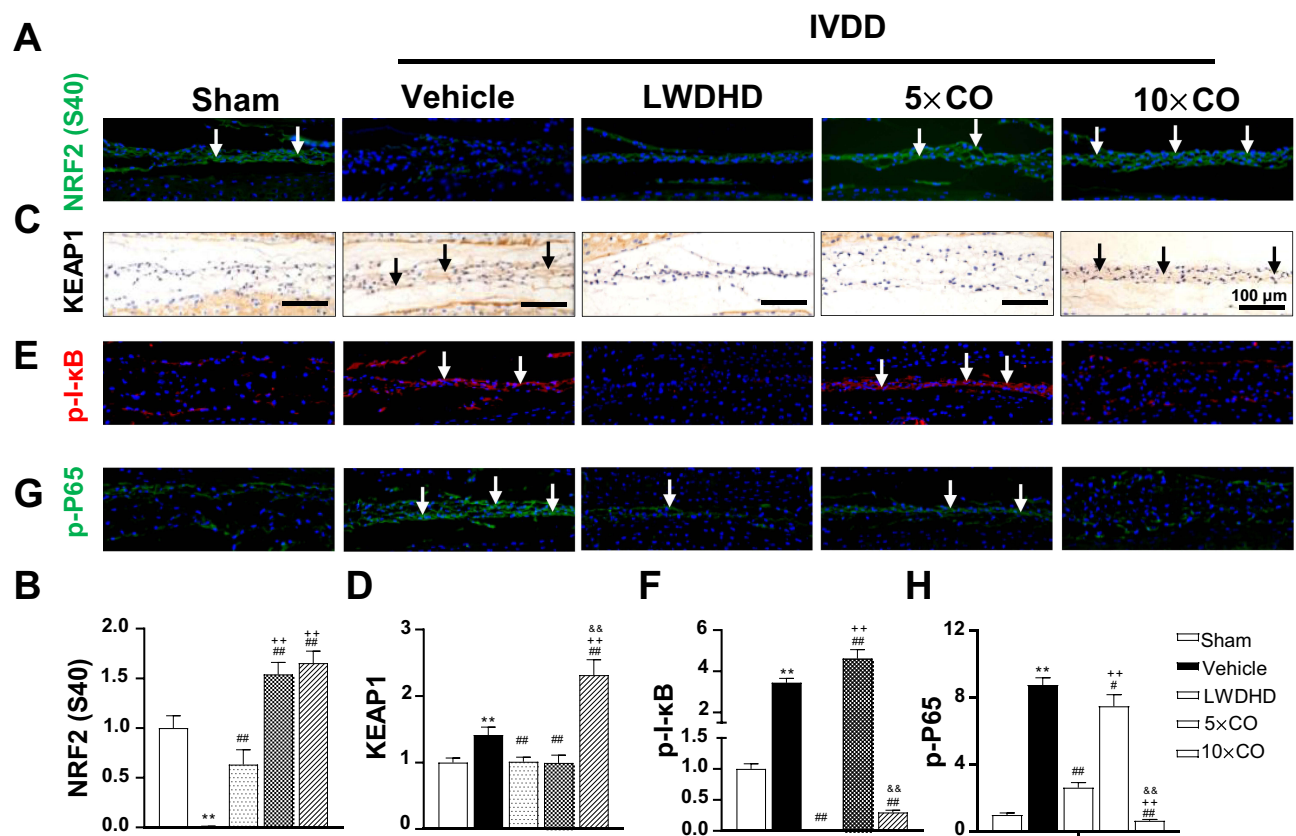


Figure 5 LWLWHD formulations activate the NRF2 antioxidant pathway in IVDs of IVDD mice. (**A** and **B**) IF staining and corresponding qualification of NRF2 (S40) in IVDs of IVDD mice after treatment with LWLWHD formulation for 1 week. White arrows indicate high expression of NRF2 (S40) in NP tissues. (**C** and **D**) IHC staining and corresponding qualification of KEAP1 in IVDs of IVDD mice. Black arrows indicate high expression of KEAP1 in NP tissues. (**E–H**) IF staining and corresponding qualification of p-I-kB (**E** and **F**) and p-P65 (**G** and **H**) in IVDs of IVDD mice. Data are shown as mean \pm SEM (n = 6). *Denotes a significant difference compared with the Sham group (** $p < 0.01$), #Means a significant difference compared with the Vehicle group ([#] $p < 0.05$, ^{##} $p < 0.01$), *Indicates a significant difference compared with the LWLWHD group (*^{*} $p < 0.01$), &#amp;#Means a significant difference compared with the 5 \times CO group (&#amp;# $p < 0.01$).

Abbreviations: LWLWHD, Liuwei Dihuang Decoction; NRF2, NF-E2-related factor 2; IVD, Intervertebral disc; IVDD, Intervertebral disc degeneration; IF, Immunofluorescent; KEAP1, Kelch-like ECH-associated protein 1; p-I-kB; phospho-I-kB; p-P65, phospho-P65; CO, *Cornus officinalis*; 5 \times CO, a modified formulation of LWLWHD with a 5-fold increase in the dosage of CO.

Discussion

IVDD is a clinical common problem characterized by impaired structural integrity with reduced NP cell viability, diminished ECM matrix content, inflammation, and nerve ingrowth,²² lacking effective disease-modifying therapies. LWLWHD has historically been a prominent prescription in TCM, historically utilized for various kidney yin deficiency-induced conditions, including LBP. Our earlier studies have highlighted the potential of LWLWHD, including its component CO-derived compound mornoniside, for the treatment of IVDD disorder by protecting NP cells from injury,⁸ and suggesting loganin and mornoniside, two CO's active components, in mitigating knee joint degeneration by targeting chondrocyte pyroptosis.^{9,10} These findings collectively suggest anti-inflammatory and anti-pyroptosis properties of CO in treating degenerative osteoarthropathies. However, uncertainty remains regarding the original LWLWHD formulation's optimal CO dosage and its potential mechanism for effectively treating IVDD. In the present study, we compared the therapeutic efficacy of 2 modified formulations of LWLWHD containing varying doses of CO (5- or 10-time original dosage) (referred to as 5 \times CO and 10 \times CO, respectively) against the original LWLWHD formulation using LSI surgery-induced IVDD mice. Our present results demonstrated that the administration of modified LWLWHD formulations, particularly with 5 \times CO, exhibited notable improvements in lumbar function, structural integrity, ECM composition, NP cell viability, and sensory nerve ingrowth in IVDs of IVDD mice. Importantly, 5 \times CO formulation particularly suppressed NP pyroptosis by activating NRF2/KEAP1 pathway, offering promising evidence for its potential

Table 2 Combined Score of Modified Formulations of LWDHD in Delaying IVDD

Group \ Item	Sham	Vehicle	LWDHD	5 × CO	10 × CO
Rearing frequency analysis	2	0	2	3	0
Von-Frey test	0	2	1	3	1
Total distance	2	0	0	3	2
Central motion time	1	3	2	1	1
Distance relative to the center	2	0	0	1	2
Histological grading scale	3	1	3	3	2
AGGRECAN	1	1	2	2	3
COL2	3	1	1	2	2
MMP13	1	0	2	2	2
TUNEL	3	0	3	0	3
Ki-67	3	0	2	0	3
TrkA	3	0	1	3	1
CASPASE1	3	0	2	3	0
GSDMD	3	0	1	2	0
IL-1 β	3	0	1	2	1
NRF2 (S40)	1	0	1	3	3
KEAP1	3	2	2	3	0
p-I- κ B	2	0	3	0	3
p-P65	3	0	2	0	3
Total	42	10	31	36	32

Abbreviations: LWDHD, Liuwei Dihuang Decoction; IVDD, Intervertebral disc degeneration; CO, *Cornus officinalis*; COL2, Collagen 2; MMP13, matrix metalloproteinase 13; TrkA, Tyrosine kinase A; GSDMD, Gasdermin D; IL-1 β , Interleukin-1 β ; NRF2, NF-E2-related factor 2; KEAP1, Kelch-like ECH-associated protein 1; p-I- κ B; phospho-I- κ B; p-P65, phospho-P65.

as an effective therapeutic approach to mitigate IVDD progression (Figure 6). This presents a robust rationale for considering LWDHD, specifically with the 5 × CO formulation, as a potential intervention strategy for IVDD.

Morroniside, one of the main iridoid glycosides in CO, has been shown in previous research to significantly improve the anti-oxidation and anti-apoptosis capabilities of mice liver when administered at doses of 20 mg/kg and 100 mg/kg body weight per day for 8 weeks, with the 100 mg/kg dose outperforming the 20 mg/kg dose.²³ Based on these findings, we hypothesized that a similar dose-dependent effect might be observed in IVD tissue if the dosage of CO is increased. Therefore, we decided to test a dosage that is 5 times higher than the original prescription to evaluate this potential effect. Additionally, to further investigate the dose-dependency of CO's therapeutic effects, we included a group with a dosage that is 10 times the original prescription. This experimental design allows us to comprehensively assess the efficacy of CO at varying dosages in the modified LWDHD formulations.

Pyroptosis, a form of programmed cell death that is facilitated by CASPASE1, stimulates cells to release inflammatory factors, including IL-1 β , thereby initiating pro-inflammatory reactions in adjacent cells.²⁴ Differing from other types of cell death, such as apoptosis, necrosis, and autophagy, its distinctive features encompass pore formation on the plasma membrane, cellular swelling and rupture, followed by the release of inflammatory cytokines (eg, IL-1 β and IL-18) and intracellular

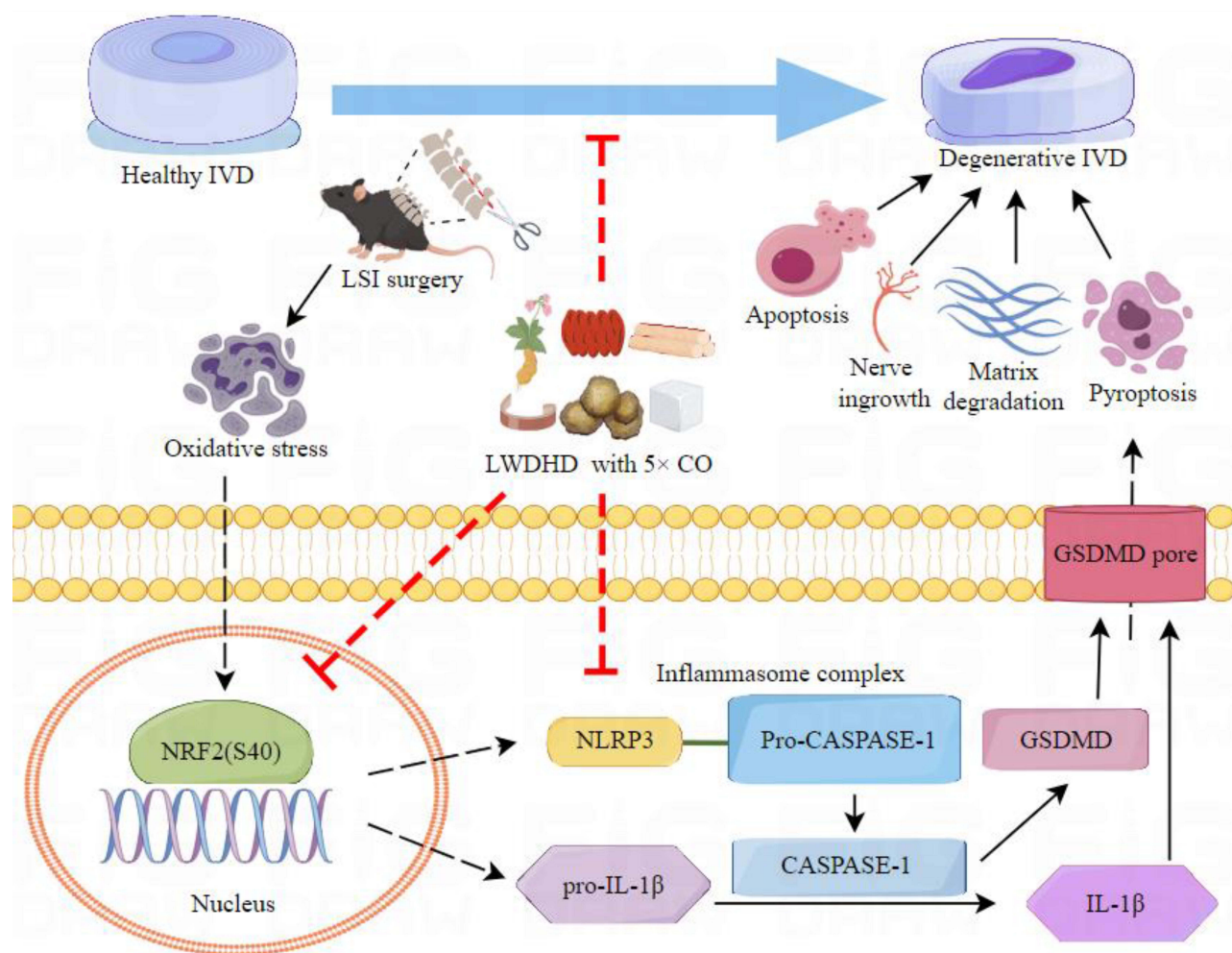


Figure 6 Schematic illustration of the mechanism by which LWDHD formulation, containing varying dosages of CO, protects against IVDD progression through the inhibition of NP pyroptosis by activating the NRF2 pathway. The schematic illustration is drawn by Figdraw.

Abbreviations: LWDHD, Liuwei Dihuang Decoction; CO, *Cornus officinalis*; IVDD, Intervertebral disc degeneration; NP, nucleus pulposus; NRF2, NF-E2-related factor 2.

materials.²⁴ Previous studies have revealed that the heightened levels of IL-1 β resulting from pyroptosis have been linked to impediments in ECM synthesis, which in turn facilitates the degradation of ECM and induces apoptosis in IVD cells.¹⁶ However, it remains uncertain whether the inhibition of pyroptosis can be effective against IVDD. Our findings discovered that the utilization of the original formulation of LWDHD has the potential to mitigate pyroptosis, and increasing the dosage of CO by a factor of 5 could further improve the anti-pyroptosis effect by further lowering the level of IL-1 β . Notably, the contradiction arises from the lack of significant differences in the levels of CASPASE1 and GSDMD between 1 \times CO-treated mice and 5 \times CO-treated mice, suggesting that the superior improvement of IL-1 β by 5 \times CO may be attributed to the targeting of other pyroptosis-related molecules, such as NOD-like receptor thermal protein domain associated protein 3 (NLRP3). Subsequently, we aim to conduct further experiments to confirm this.

Pyroptosis can be induced by various stimuli, including oxidative stress.²⁵ The nuclear factor NRF2/KEAP1 pathway plays a crucial role in regulating the body's antioxidant status. The latest findings from our work and others have revealed a strong association between NRF2/KEAP1 pathway dysfunction and the development of various spinal-related diseases including IVDD and osteoporosis, and thyroid toxicity.^{17,18,26,27} Consistent with these findings, our results demonstrated that LWDHD containing varying CO significantly upregulated the decreased NRF2 (S40) but lowered the increased KEAP1 expression in NP tissues, except for a higher upregulation in KEAP1 in 10 \times CO-treated mice. These results suggest that moderately increasing the CO dose in LWDHD can achieve better anti-oxidant and anti-pyroptosis effects on NP cells.

While our findings are groundbreaking, it is important to acknowledge several limitations. First, the relatively short study duration of 8 weeks did not allow for the assessment of long-term treatment effects and potential side effects. To address these issues, we intend to further investigate the efficacy and potential side effects of IVDD disorder after 12 and 24 weeks of treatment with LWDHD formulations. Moreover, in our previous study, a total of 71 active ingredients of LWDHD were initially selected based on specific screening criteria, including oral bioavailability (OB) $\geq 30\%$ and drug-likeness (DL) ≥ 0.18 , after retrieving the TCM Systems Pharmacology Database and Analysis Platform (<http://tcmspw.com/tcmspw.php>).²⁷ However, it remains uncertain whether the overall effectiveness of TCM formulation is inferior or superior to the combination of active ingredients in this formulation. Consequently, in future experiments, we intend to conduct further comparative investigations on the efficacy of formulation with the combination of active ingredients in this formulation. Furthermore, the detailed molecular mechanism explaining this therapeutic effect of modified LWDHD formulation is not fully elaborated. We will further confirm the specific mechanism of NRF2-mediated NP cell apoptosis in LWDHD intervention for the treatment of IVDD using transgenic animal and in vitro NP cell models. The implementation of all these aspects will significantly enhance our understanding of the therapeutic efficiency of LWDHD for IVDD disorder.

Conclusions

In summary, our findings provide valuable considerations for optimizing the formulation of LWDHD in the management of IVDD. Comprehensive therapeutic efficacy score analysis suggests that the 5 × CO is a superior modified formulation for treating IVDD, demonstrating better improvement in NP cell pyroptosis, reduction in sensory innervation, and enhancement of overall lumbar spine function.

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Disclosure

The authors report no conflicts of interest in this work.

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