

Sinapine thiocyanate alleviates intervertebral disc degeneration by not regulating JAK1/STAT3/NLRP3 signal pathway

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Conflict of interest

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Abstract

Background. Intervertebral disc degeneration (IDD) is a major cause of low back pain. Sinapine thiocyanate (ST) has been reported to have a wide range of biological activities. However, the treatment of IDD with ST has not been studied.

Objectives. To explore the role and mechanism of ST treatment in IDD.

Materials and methods. Nucleus pulposus cells (NPCs) were induced using lipopolysaccharide (LPS), which was used as an in vitro model of IDD. Cell activity, oxidative stress-related indicators and protein expression were detected using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, enzyme-linked immunosorbent assay (ELISA) and western blot. Pyroptosis was evaluated with propidium iodide (PI)/Hoechst double staining and immunofluorescence for NOD-like receptor protein 3 (NLRP3), and pyroptosis-related proteins and inflammatory factors were measured with western blot and ELISA. The pathological changes of IDD were assessed with hematoxylin & eosin (H&E) and safranin-O staining.

Results. Our results showed that ST alleviated LPS-induced degeneration of NPCs, as evidenced by reducing reactive oxygen species (ROS), malondialdehyde (MDA), matrix metalloproteinase-13 (MMP-13), a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), and increasing collagen II and aggrecan expression. Moreover, ST repressed LPS-induced pyroptosis by inhibiting NLRP3, caspase-1 p20, interleukin (IL)-1 β and IL-18. Further studies showed that ST did not restrain the activation of the JAK1/STAT3 signaling pathway induced by colivelin, or of the enhanced pyroptosis induced by polyphyllin VI. Sinapine thiocyanate alleviated IDD in vivo and suppressed NLRP3-mediated pyroptosis and the JAK1/STAT3 signaling pathway.

Conclusions. Sinapine thiocyanate could alleviate IDD, although this did not include a reduction in NLRP3-mediated pyroptosis and inactivation of the JAK1/STAT3 signaling pathway, thus potentially being a candidate drug for IDD treatment.

Key words: intervertebral disc degeneration, pyroptosis, sinapine thiocyanate, JAK1/STAT3 signaling pathway

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Background

Low back pain (LBP) is the main cause of adult disability and labor loss.¹ Intervertebral disc degeneration (IDD) is a common cause of LBP, which causes a huge mental and economic burden to patients and society.² During IDD, the degenerative nucleus pulposus breaks through the fibrous ring and compresses the nerve root, which leads to LBP and affects both work and private life.³ At present, the treatment methods for LBP caused by IDD are relatively limited, mainly including conservative treatment (such as bed rest, traction, physiotherapy, and anti-inflammatory analgesia) and surgical treatments (such as open nucleus pulposus extraction, vertebral fusion, and endoscopic nucleus pulposus extraction). Generally, after 3 months of conservative treatment, if the treatment for patients with IDD is ineffective, surgical treatment is recommended.⁴ Unfortunately, the 2 treatment methods are mainly symptomatic treatment for pain relief, which cannot delay or reverse the pathological process of IDD.^{5,6} The biological mechanism of IDD is complicated, involving mechanical and oxidative stress, DNA damage, inflammatory responses, abnormal signal pathway activation, and abnormal expression of miRNA.^{7–9} Currently, the pathological changes of IDD are mainly characterized by the decrease of nucleus pulposus cells (NPCs) and extracellular matrix (ECM).^{10,11} Hence, exploring the pathological mechanism of NPC death under different stress states is conducive to finding new targets and drugs for the treatment of IDD.

Oxidative inflammatory responses are one of the main causes of abnormal cell function in multiple diseases.^{12–15} So far, researchers found that reactive oxygen species (ROS) were increased in degenerative nucleus pulposus (NP) tissue.¹⁶ Abnormally elevated ROS could downregulate the expression of COL2A1 in human and rat NPCs.¹⁷ Pro-inflammatory cytokines could also repress the synthesis of ECM protein deposition in human and rat NPCs by increasing the expression of matrix-degrading enzymes, namely matrix metalloproteinase (MMP)-3 and MMP-13.¹⁸ Pyroptosis is a form of cell death,¹⁹ the predominant feature of which depends on the activation of caspase-1 mediated by the NLRP3 inflammasome, and which is accompanied by the increase of active inflammatory factors interleukin (IL)-1 β and IL-18.^{20,21} Studies have shown that IDD was accompanied by pyroptosis of NPCs, which mainly presented as the upregulation of NLRP3 and caspase-1 expression.²² Therefore, inhibiting the activation of ROS and NLRP3 is of great significance in delaying the progression of IDD.

The JAK/STAT signaling pathway is involved in many important biological processes, such as immune regulation, inflammatory responses and apoptosis.²³ In pathological conditions, JAK phosphorylation is activated and combined with phosphorylated STAT, which affects the transcription of a series of cytokines and participates in the regulation of the inflammatory response and

oxidative stress.²⁴ Interleukin 21 could stimulate the up-regulation of ADAMTS-7 and MMP-13 by enhancing STAT3 in NPCs.²⁵

Sinapine thiocyanate (ST), whose molecular formula is $C_{17}H_{24}N_2O_5S$, is the main active component of sinapine in semen raphanin.²⁶ Sinapine thiocyanate has a variety of biological activities, including anti-oxidant, anti-inflammatory, anti-radiation, anti-aging, anti-hypertensive, and anti-androgen activity, while also inhibiting angiogenesis.^{27–30} It is reported that ST could also reduce cholesterol and low-density lipoprotein (LDL), significantly improve hypertension symptoms, prevent thrombosis, and attenuate thrombosis caused by inflammatory injury of vascular endothelial cells (VECs).³¹ Sinapine thiocyanate improved vascular endothelial injury in hypertensive rats by inhibiting the activation of NLRP3 and the expression of related inflammatory factors (IL-1 β and IL-18), and also alleviated human umbilical vein endothelial cells (HUVEC) injury induced by the administration of angiotensin II.³² Moreover, ST could inhibit the proliferation and migration of pancreatic cancer cells by upregulating GADD45A.³³ However, the effects and mechanisms of ST in IDD are still unclear. In addition, as a strong inflammatory stimulating factor, lipopolysaccharide (LPS) could lead to gene upregulation and the secretion of diverse pro-inflammatory cytokines and matrix-degrading enzymes, including disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) and MMPs in NP cells, thereby causing a decrease in proteoglycan content and IDD.^{34,35} The current study aimed to explore whether ST could affect ECM degradation and oxidative stress in IDD by regulating NLRP3-mediated pyroptosis via the JAK1/STAT3 signaling pathway. Our study provides new ideas for the research and development of novel methods for IDD treatment.

Objectives

Our study aimed to explore whether ST could alleviate IDD in vitro and in vivo.

Materials and methods

Isolated and culture of NPCs

Nucleus pulposus cells were extracted according to a previous study.³⁶ Rats (6–8 weeks, 260–280 g) were euthanized by intraperitoneal injection of sodium pentobarbital (120 mg/kg), sterilized with alcohol, and then the intervertebral disc (L2–L6) tissue was obtained under sterile conditions. After washing, the outer layer of the intervertebral disc annulus fibrosus was cut, and then the nucleus pulposus tissues were stripped and placed in a sterile Petri dish. Nucleus pulposus tissues were disaggregated into

small pieces (~1 mm³), transferred to a 15 mL centrifuge tube, and digested for 20 min with 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) at 30°C. The digestion was terminated using Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, Waltham, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Waltham, USA). The precipitates were collected by centrifugation (1,000 rpm, 5 min), and treated with 0.2% type II collagenase for 2 h; then, the cells were collected and resuspended in DMEM/F12 medium containing 10% FBS. Cells were cultured in an incubator at 37°C and 5% CO₂, and used in subsequent experiments at passages 2 and 3.

MTT assay

Nucleus pulposus cells (5×10³ cells per well) were inoculated in 96-well plates and cultured at 37°C and 5% CO₂ for 24 h. Then, MTT solution (Beyotime Biotechnology, Shanghai, China) was added and cells were cultured for 4 h. Then the culture medium was discarded, and 150 µL dimethyl sulfoxide (DMSO) (100%) was added. The absorbance value at 570 nm was detected using a microplate reader (BioTek, Winooski, USA); 3 independent experiments were performed.

DCFH-DA detection

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay (Beyotime Biotechnology) was made into a working solution with a final concentration of 10 µM using phosphate-buffered saline (PBS). Nucleus pulposus cells (5×10³) were placed into 96-well plates and underwent the indicated treatment. After 24 h, DCFH-DA staining solution was added to each well for 20 min. Next, cells were washed 3 times with serum-free medium, and then the distribution of ROS in cells was observed under fluorescence microscopy (model IX71; Olympus Corp., Tokyo, Japan). Then, the excitation/emission wavelength of 488/525 nm was examined using a microplate reader (Gen5; BioTek, Winooski, USA). At least 3 images were taken per treatment, and relative ROS levels were analyzed by normalizing to the control group. The investigator was blinded to the experimental conditions when taking the images, and 3 independent experiments were performed.

Enzyme-linked immunosorbent assay

Nucleus pulposus cells (4×10⁵ cells per well) were inoculated in 6-well plates containing slides. After 24 h culture, the supernatant was collected by centrifugation (2000 rpm, 20 min), and the expression of IL-1β, IL-18, malondialdehyde (MDA), and superoxide dismutase (SOD) were detected using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three independent experiments were performed.

Western blot

Nucleus pulposus cells (4×10⁵ cells per well) were inoculated in 6-well plates and cultured at 37°C and 5% CO₂. After 24 h, 200 µL radioimmunoprecipitation assay buffer (RIPA) lysate was added to each well, and cells were lysed on ice for 30 min. The supernatant was collected by centrifugation (12,000 rpm, 5 min, 4°C), and the protein content was detected using a bicinchoninic acid (BCA) assay. For each group, 30 µg of protein samples was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then electrically transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1 h with 5% skimmed milk powder solution prepared in Tris-buffered saline + 0.1% Tween-20 (TBST), and then incubated overnight in diluted primary antibody (collagen II (ab34712, 1:1000; Abcam, Cambridge, UK), aggrecan (ab3778, 1:1000; Abcam), MMP-13 (ab39012, 1:1000; Abcam), ADAMTS-5 (ab231595, 1:1000; Abcam), NLRP3 (ab263899, 1:1000; Abcam), ASC (ab151700, 1:1000; Abcam), caspase-1 (ab138483, 1:1000; Abcam), caspase-1 p20 (sc-398715, 1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), JAK1 (ab133666, 1:1000; Abcam), p-JAK1 (ab138005, 1:1000; Abcam), STAT3 (ab68153, 1:1000; Abcam), p-STAT3 (ab109085, 1:1000; Abcam), and GAPDH (ab8245, 1:2000; Abcam)) at 4°C. After washing, the membranes were incubated for 1 h with the diluted second antibody (Goat Anti-Rabbit IgG H&L (1:5000, ab96899; Abcam) or Goat Anti-Mouse IgG H&L (1:5000, ab96879; Abcam)), and then the protein was visualized using an electrochemiluminescence reagent (ECL; Beyotime Biotechnology) in a gel imaging system (Bio-Rad, Hercules, USA). Three independent experiments were performed.

Propidium iodide/Hoechst 33342 double staining

Nucleus pulposus cells (2×10⁴ cells per well) were inoculated in 24-well plates containing slides. After incubating for 24 h, the NPCs were administered Hoechst 33342 and propidium iodide (PI), and stained for 30 min at 4°C. After washing, the cells were observed under a fluorescence microscope (Olympus Corp.). At least 3 images were taken per treatment. The investigator was blinded to the experimental conditions when taking the images. Three independent experiments were performed.

Immunofluorescence

Nucleus pulposus cells (2×10⁴ cells per well) were inoculated in 24-well plates containing slides and cultured at 37°C and 5% CO₂ for 24 h. Next, cells were fixed in 4% paraformaldehyde for 15 min, treated with 0.5% TritonX-100 for 20 min, and then blocked with goat serum for 30 min. The cells were incubated overnight in a wet

box with anti-NLRP3 (ab4207, 1:200; Abcam) at 4°C, and then incubated for 1 h with Alexa 488-conjugated antibody (ab150129, 1:200; Abcam) at room temperature. The nuclei were counterstained for 5 min with DAPI (4',6-diamidino-2-phenylindole) reagent (cat. No. H-1200-10; Vector Laboratories, Burlingame, USA), samples were mounted using an anti-fluorescence quenching agent, and then images were collected using a fluorescent microscope (Olympus Corp.). At least 3 images were taken per treatment. The investigator was blinded to the experimental conditions when taking the images. Three independent experiments were performed.

Intervertebral disc degeneration rat model

Sprague Dawley (SD) rats (female, $n = 24$) were randomly divided into 4 groups: sham operation group (sham, $n = 6$), intervertebral disc degeneration group (IDD, $n = 6$), intervertebral disc degeneration + 4 mg/kg ST group (IDD + 4 mg/kg ST, $n = 6$), and intervertebral disc degeneration + 8 mg/kg ST group (IDD + 8 mg/kg ST, $n = 6$). The concentrations of ST were used based on previous investigations.³² Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.6 mL/kg), the C5/6 intervertebral disc was marked, and the local skin was disinfected with alcohol. The C5/6 intervertebral discs in the IDD group and ST treatment group were punctured with a 30 G sterile needle from the dorsal side along the horizontal direction of the cone end plate with a penetration depth of 5 mm, and the needle was rotated 360° and retained for 30 s. The sham group was sutured after skin incision only without the needle penetration. Following the operation, 100,000 units of penicillin were injected intraperitoneally for 3 days to prevent infection. After the operation, the ST treatment group was administrated 4 mg/kg and 8 mg/kg per day by gavage, and the sham group and IDD group were given the same amount of normal saline by gavage. The treatment continued for 4 weeks, at which time rats were euthanized by intraperitoneal injection of sodium pentobarbital (120 mg/kg). All animal experiments were approved by the animal ethics committee of the Suzhou Hospital of Traditional Chinese Medicine, affiliated with Nanjing University of Chinese Medicine. Three independent experiments were performed.

Hematoxylin & eosin and safranin-O staining

Nucleus pulposus tissues from rat discs were fixed in 40 g/L paraformaldehyde and then decalcified in 10% EDTA for 15 days. After preparation and dehydration, IDD tissues were embedded in paraffin and cut into 5 µm continuous sections. According to previous studies, we detected IDD by safranin-O staining and hematoxylin & eosin (H&E) staining. The slices were placed in the oven (70°C) for 1 h, soaked in xylene for 30 min, rehydrated with a gradient concentration of absolute ethanol (100%,

95%, 85%, 75%), stained with hematoxylin for 3 min, and then treated with 2% acetic acid for 1 min and ammonia for 1 min. For H&E staining, the sections were treated for 5 s with 95% absolute ethanol and then stained for 2 min with an eosin staining solution. For safranin-O staining, the sections were stained with fast green for 6 min, differentiated with 1% acetic acid for 15 s, and then stained with safranin-O for 6 min. Subsequently, the slices were dehydrated using gradient concentrations of absolute ethanol (75%, 85%, 95%, 100%), treated for 6 min with xylene solution, and then sealed with neutral resin. The results of 6 random fields were observed and photographed in an optical microscope (model IX-71; Olympus Corp.). At least 3 images were taken per treatment. The investigator was blinded to the experimental conditions when taking the images. Three independent experiments were performed.

Statistical analyses

IBM SPSS v. 22.0 software (IBM Corp., Armonk, USA) was used to analyze the experimental data, and data were expressed as mean \pm standard deviation ($M \pm SD$) for all data points. Multiple comparisons were performed using non-parametric analysis of variance (ANOVA) (Kruskal–Wallis test) followed by Dunn's post hoc test without any correction. A p -value of <0.05 was considered statistically significant.

Results

Sinapine thiocyanate attenuated LPS-induced decrease in the activity of NPCs

First, we evaluated the effect of ST on the activity of NPCs. The chemical structure of ST is shown in Fig. 1A. Sinapine thiocyanate at concentrations of 0, 10, 25, 50, 75, and 100 mg/L was added into NP cells for 24 h, and MTT assay showed that ST (0–75 mg/L) had no toxicity to NPCs, but ST at 100 mg/L significantly weakened their activity to 83.76% (Fig. 1B). In addition, LPS (10 µg/mL) was used to maintain the degenerative status of NP cells. Nucleus pulposus cells were stimulated with 10 µg/mL LPS, 10 µg/mL LPS + 25 mg/L ST, 10 µg/mL LPS + 50 mg/L ST, or 10 µg/mL LPS + 75 mg/L ST for 24 h. The results showed that LPS could reduce the activity of NPCs, which is reversed by ST (75 mg/L) treatment in a concentration-dependent manner (Fig. 1C).

Sinapine thiocyanate restrained LPS-induced degeneration of NPCs

Next, the effect of ST on LPS-induced degeneration of NPCs was explored. Lipopolysaccharide facilitated increased ROS in NPCs, which was inhibited by ST

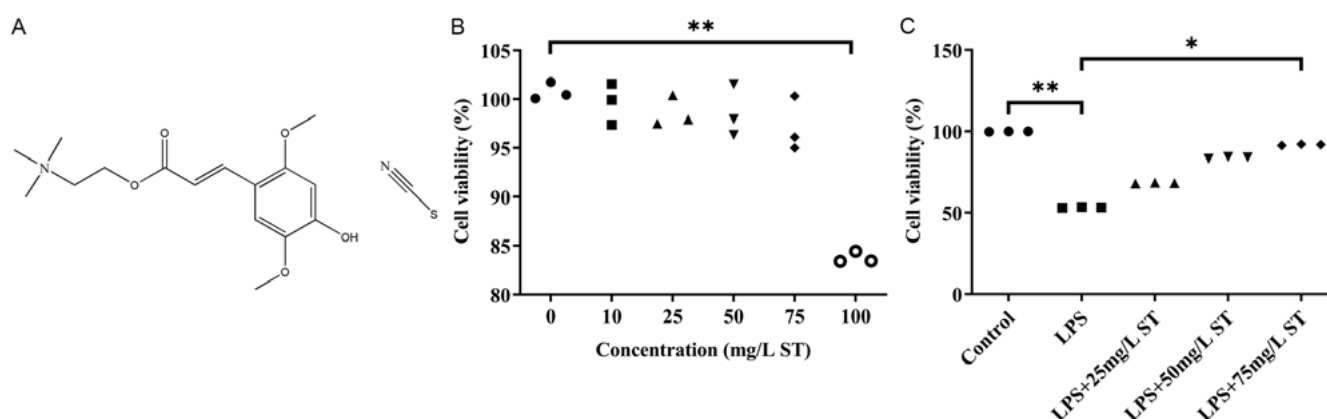


Fig. 1. Effect of sinapine thiocyanate on the activity of nucleus pulposus cells. A. The chemical structural formula of sinapine thiocyanate; B,C. The viability of nucleus pulposus cells was measured using MTT assay

** $p < 0.01$; * $p < 0.05$.

in a concentration-dependent manner (Fig. 2A,B). Oxidative stress-related indexes MDA and SOD were also measured, showing that ST could block the increase of MDA and decrease of SOD induced by LPS in NPCs (Fig. 2C,D). Moreover, LPS significantly reduced the expression of collagen II and aggrecan, and increased the expression of MMP13 and ADAMTS-5. However, ST inhibited the loss of collagen II and aggrecan and repressed the expression of MMP13 and ADAMTS-5 in LPS-induced NPCs (Fig. 2E–I). These results suggested that ST effectively alleviated the degeneration of NPCs induced by LPS.

Sinapine thiocyanate suppressed LPS-induced pyroptosis of NPCs

The death of NPCs is key to IDD,³⁷ and we assessed whether ST could induce NPCs pyroptosis. Hoechst and PI double staining were performed, highlighting that LPS boosted nuclear PI and increased the red fluorescence compared with the control group, while this was attenuated by the addition of ST (Fig. 3A). Moreover, the expression of NLRP3 was detected using immunofluorescence. The results showed that the fluorescence signal of NLRP3 was increased in LPS-treated NPCs, and this could be gradually weakened by the addition of ST (Fig. 3B). Furthermore, pyroptosis-associated proteins were assessed with western blot, which showed that LPS-induced facilitation of NLRP3, ASC and caspase-1 p20 was inhibited by ST (Fig. 4A–D). Expression of IL-1 β and IL-18 downstream of NLRP3 was significantly diminished by ST in LPS-induced NPCs (Fig. 4E,F). In addition, the effect of ST on the JAK1/STAT3 signaling pathway was analyzed. The results showed that ST could decrease the activation of the JAK1/STAT3 signaling pathway induced by LPS in NPCs (Fig. 4G–I). These results indicated that ST restrained NLRP3-mediated pyroptosis and JAK1/STAT3 signaling pathway in LPS-induced NPCs.

Sinapine thiocyanate alleviated NPCs degeneration, but not through JAK1/STAT3/NLRP3 signaling

The role of NLRP3-mediated pyroptosis and JAK1/STAT3 signaling in the protective effect of ST on NPCs were then explored. Polyphyllin VI has been reported to activate pyroptosis by increasing NLRP3.³⁸ Our findings showed that polyphyllin VI enhanced LPS-induced upregulation of NLRP3 (Fig. 5A,B). Moreover, ST reversed the effect of polyphyllin VI on collagen II and aggrecan in LPS-induced NPCs (Fig. 5C,D). In addition, the increase of IL-1 β and IL-18 induced by polyphyllin VI was not significantly attenuated by ST in LPS-induced NPCs (Fig. 5E,F). We used colivelin as an activator of STAT3,³⁹ which further induced ROS production in LPS-treated NPCs, which was not prevented by the addition of ST (Fig. 6A,B). Colivelin significantly increased the ratio of p-STAT3/STAT3, further increased NLRP3, and did not significantly reduce the expression of collagen II and aggrecan in LPS-induced NPCs, which were not significantly restored by ST (Fig. 6C–G). Finally, ST treatment did not significantly block colivelin-induced increases in IL-1 β and IL-18 (Fig. 6H,I). These results suggest that ST may resist LPS-induced degeneration of NPCs, but not by regulating the JAK/STAT3/NLRP3 signaling pathway.

Sinapine thiocyanate repressed IDD in vivo

The improvement of ST on IDD was then verified in vivo. The IDD model was constructed through annulus fibrosus puncture, which caused a reduction of NPCs and the loss of ECM. The results of H&E and safranin-O staining confirmed the successful construction of the model. Compared to the control group, the LPS group showed various degenerative changes, including a reduction in NP size and dense ECM, and the addition of ST effectively

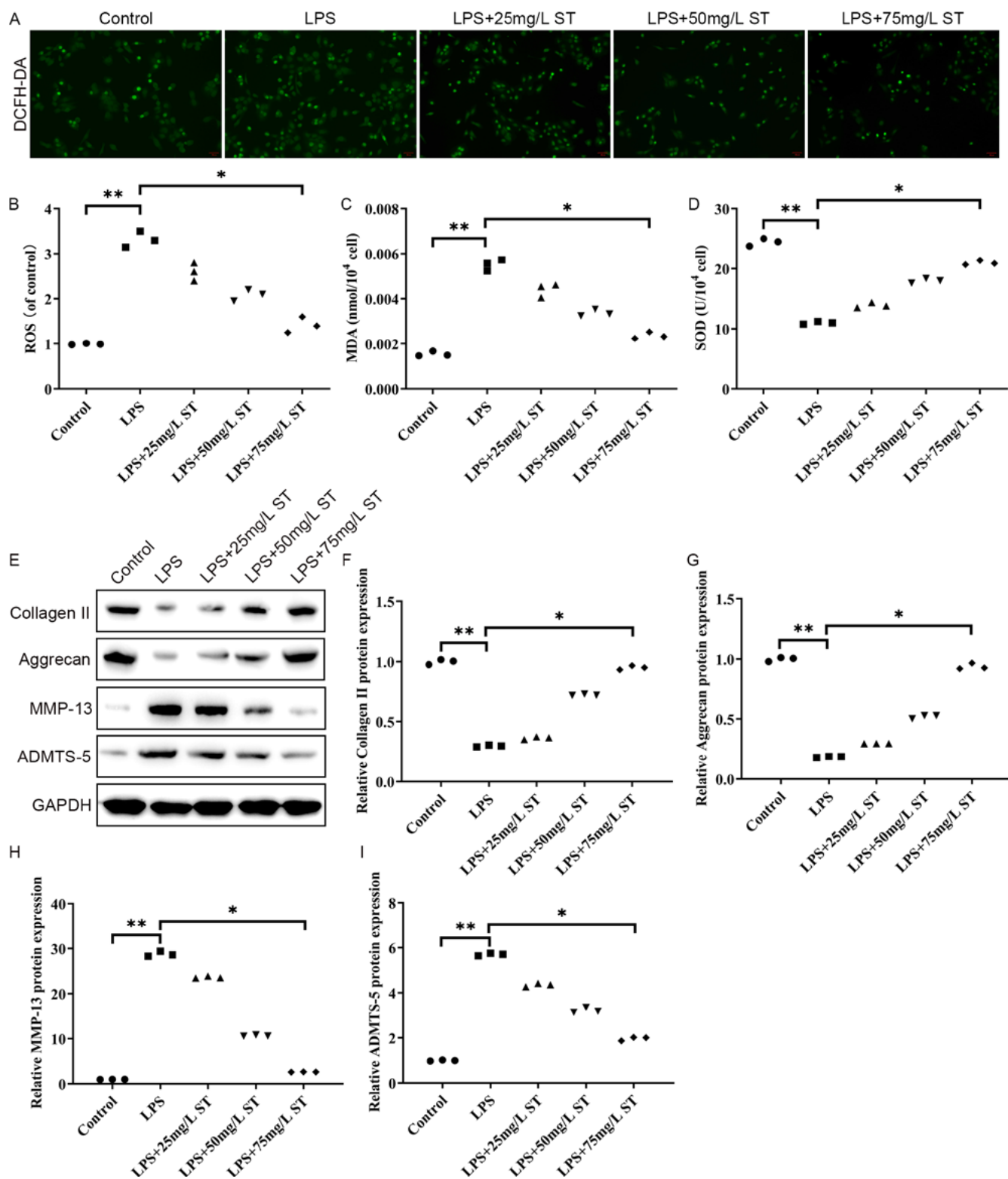


Fig. 2. Effect of sinapine thiocyanate on the degeneration of nucleus pulposus cells. A,B. Reactive oxygen species (ROS) levels were assessed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay method; C,D. The content of malondialdehyde (MDA) and superoxide dismutase (SOD) was evaluated with enzyme-linked immunosorbent assay (ELISA); E-I. The expression of collagen II, aggrecan, matrix metalloproteinase (MMP)-13, and ADMTS-5 was detected with western blot

** $p < 0.01$; * $p < 0.05$.

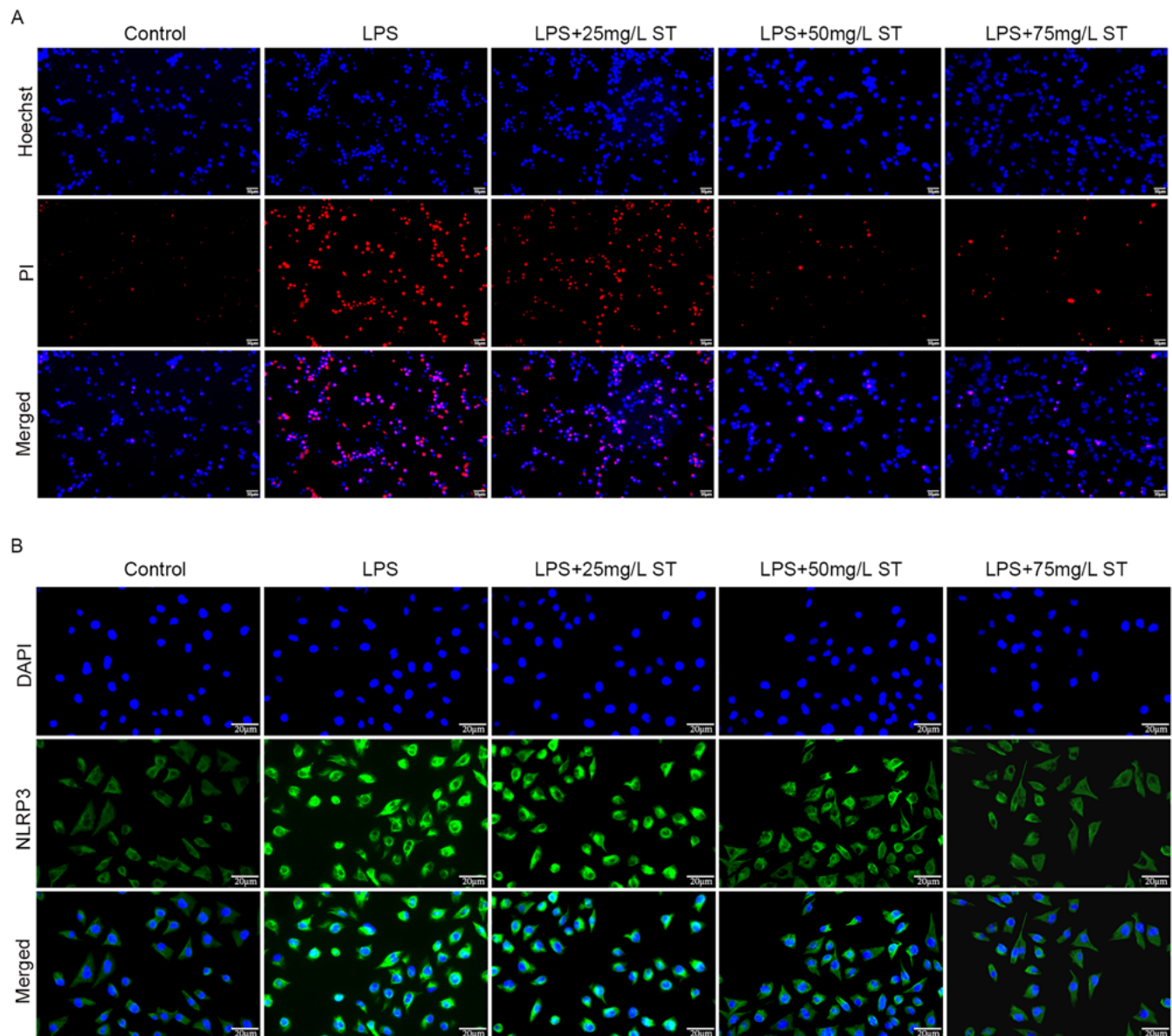


Fig. 3. Effect of sinapine thiocyanate on pyroptosis. A. Cell death of nucleus pulposus cells was measured with propidium iodide/Hoechst double staining; B. NLRP3 expression was assessed with immunofluorescence

alleviated these symptoms in IDD (Fig. 7A, 7B). Moreover, ST significantly inhibited the activation of JAK1/STAT3, reduced the expression of NLRP3, and promoted the expression of collagen II and aggrecan in IDD tissue (Fig. 7C–H). Finally, the high levels of IL-1 β and IL-18 in our IDD model were reduced following treatment with ST (Fig. 7I,J). These results demonstrated that ST-attenuated IDD reduced the activation of JAK1/STAT3 and restrained NLRP3-mediated pyroptosis *in vivo*.

Discussion

In this study, LPS was used to maintain the degenerative status of NPCs *in vitro* because LPS stimulates the decrease of ECM and increase of NLRP3 inflammasome

in NPCs.^{40–42} Our study is the first to report the effect of ST on IDD, and we demonstrated that ST improved the activity of NPCs, reduced ROS and MDA, increased SOD, expedited collagen and aggrecan expression, and reduced MMP-13 and ADAMTS-5 expression in LPS-treated NPCs. These results indicate that ST alleviated the degeneration of NPCs.

Pyroptosis is an “inflammatory death”, which is mainly characterized by the activation of caspase-1 or caspase-11 with the activity of inflammatory factors IL-1 β and IL-18.⁴³ NOD-like receptor protein 3 has also been shown to recruit and activate caspase-1 to induce pyroptosis.⁴³ At present, studies have confirmed that NLRP3 and IL-1 β play key roles in the pathogenesis of IDD.^{44,45} Comparing 45 degenerative intervertebral discs with 7 normal intervertebral discs, Chen et al. found that NLRP3 and

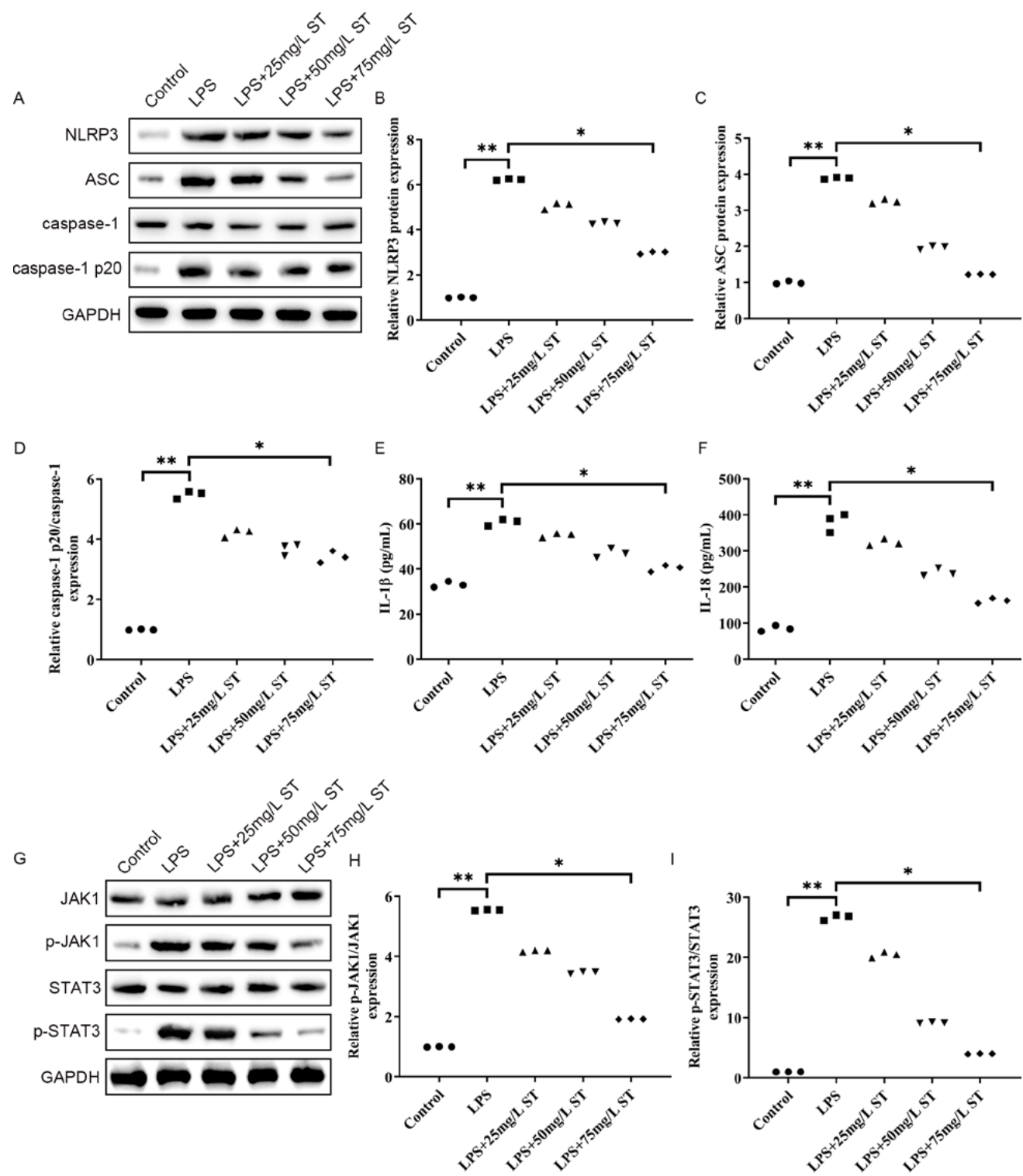


Fig. 4. Sinapine thiocyanate repressed NLRP3-mediated pyroptosis and JAK1/STAT3 signaling pathway. A–D. The expression of NLRP3, ASC, caspase-1, and caspase-1 p20 was detected with western blot; E, F. The content of interleukin (IL)-1β and IL-18 was evaluated using enzyme-linked immunosorbent assay (ELISA); G–I. The expression of JAK, p-JAK, STAT3, and p-STAT3 was measured with western blot

**p < 0.01; *p < 0.05.

its downstream targets, caspase-1 and IL-1β, were significantly upregulated in the degenerative group.⁴⁶ Zhang et al. established a mouse IDD model using the annulus fibrosus puncture method, and compared with the sham operation group, the expression levels of NLRP3, caspase-1,

p20 and gasdermin D (GSDMD) in the IDD model group were significantly increased.⁴⁷ Studies have shown that knocking down NLRP3 or inhibiting caspase-1 expression could weaken the inflammatory response and degeneration of NPCs.⁴⁸ Sinapine thiocyanate has been reported

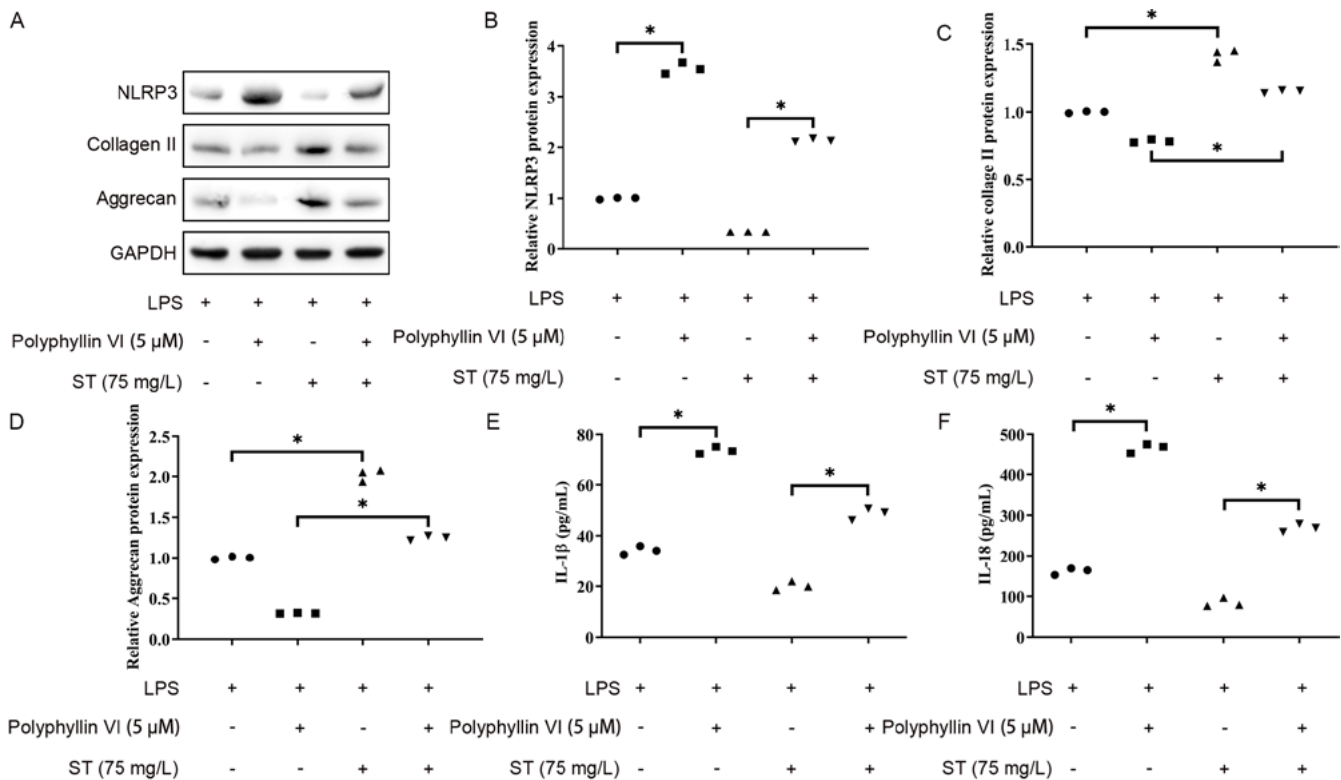


Fig. 5. Sinapine thiocyanate restrained degeneration of nucleus pulposus cells by reducing NLRP3-mediated pyroptosis. A–D. The expression of NLRP3, collagen II and aggrecan was measured with western blot; E, F. The content of interleukin (IL)-1 β and IL-18 was evaluated with enzyme-linked immunosorbent assay (ELISA)

* $p < 0.05$.

to reduce the dysfunction of VECs caused by hypertension by inhibiting NLRP3-mediated pyroptosis.³² Similarly, our study found that ST could reduce pyroptosis of LPS-treated NPCs by inhibiting NLRP3, caspase-1, p20, IL-1 β , and IL-18. The activation of pyroptosis was also induced by polyphyllin VI, which was confirmed by the change of NLRP3, IL-1 β and IL-18. However, ST treatment did not significantly reverse the activation of pyroptosis induced by polyphyllin VI. These results suggest that ST might protect NPCs, but not by regulating NLRP3-mediated pyroptosis. Moreover, polyphyllin VI might modulate the effect of ST on IDD.

Our study found that ST could also inhibit the activation of the JAK1/STAT3 signaling pathway. JAK/STAT signaling is a common pathway for a variety of cytokines and growth factors to transmit signals into target cells, which mediates a variety of biological reactions, including cell proliferation, differentiation, migration, apoptosis, and inflammation.⁴⁹ The activation of JAK/STAT signaling promotes the occurrence and development of various diseases, including IDD.^{23,50} Resveratrol reversed the degeneration of NPCs by increasing ECM production (collagen II and aggrecan), and repressing the activation of JAK1/STAT3 and the secretion of IL-6.⁵¹ Meanwhile, STAT3 could combine with NLRP3 to promote cell death.⁵² In our study, colivelin, the activator of STAT3, could upregulate ROS and NLRP3-mediated pyroptosis and not significantly enhance the degeneration of NPCs, while the addition of ST did not

significantly restore these effects. In addition, ST could only partially restore NPC function following colivelin treatment. Our rat IDD model was used to further verify the role of ST in IDD. Sinapine thiocyanate effectively alleviated IDD, reduced NLRP3-mediated pyroptosis and restrained the activation of the JAK1/STAT3 signaling pathway.

Limitations

The question whether ST alleviated IDD in vivo by regulating JAK1/STAT3/NLRP3 needs further verification. In addition, ST might regulate pyroptosis in IDD through a variety of signaling pathways, and our study lacked the required exploration. Furthermore, our experimental sample size seriously affects the conclusions we can draw. Increased sample sizes are required to further verify our conclusion.

Conclusions

These findings provide a new perspective on the treatment of IDD. Our data demonstrated that ST suppressed LPS-induced degeneration of NPCs, but not through regulating JAK1/STAT3/NLRP3 signaling. Sinapine thiocyanate may, therefore, become a candidate drug for IDD treatment.

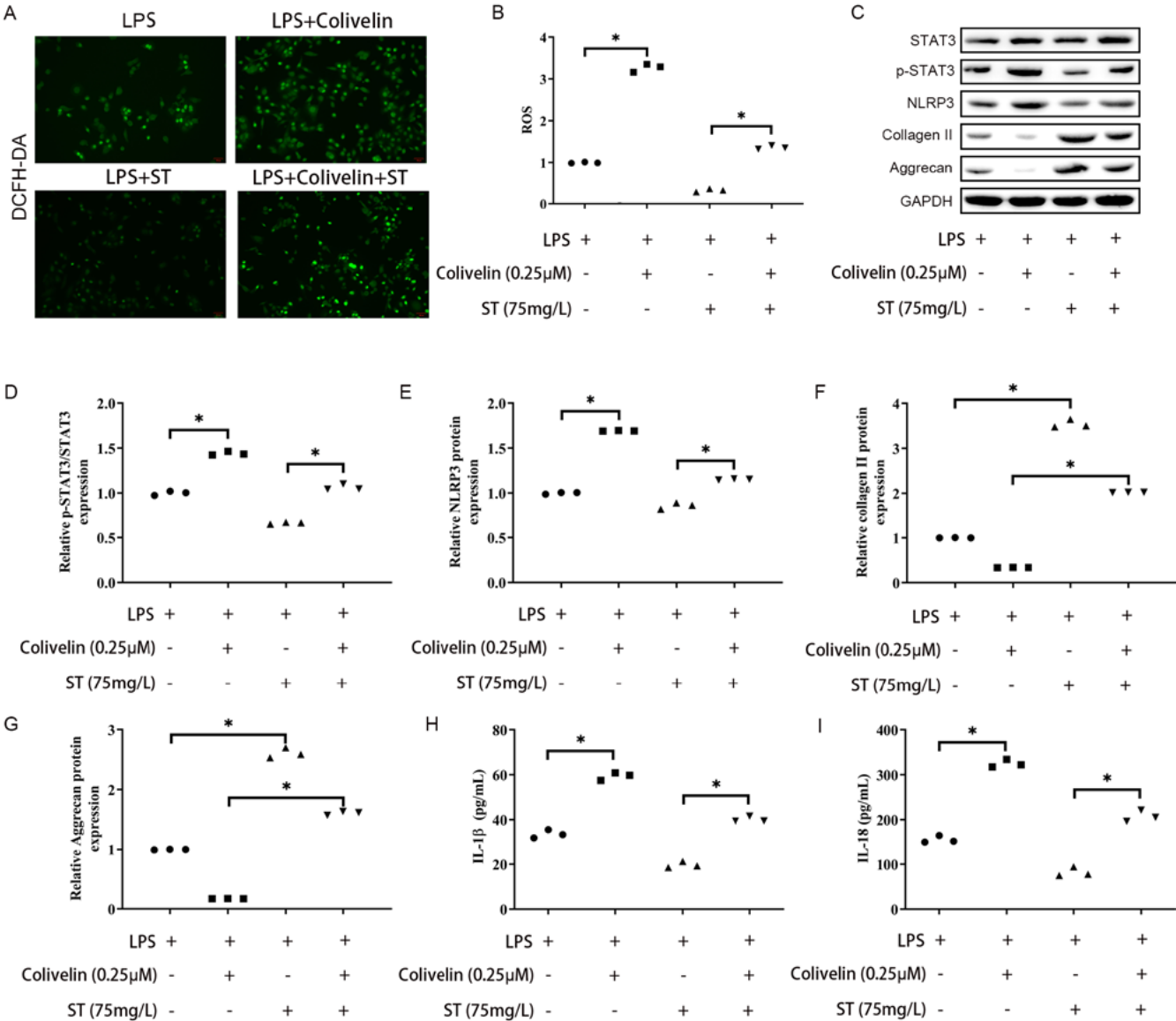


Fig. 6. Sinapine thiocyanate restrained degeneration of nucleus pulposus cells by JAK1/STAT3/NLRP3 signaling pathway. A–B. Reactive oxygen species (ROS) levels were assessed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay method; C–G. The expression of STAT3, p-STAT3, NLRP3, collagen II, and aggrecan was measured with western blot. H–I. The content of interleukin (IL)-1β and IL-18 was evaluated using enzyme-linked immunosorbent assay (ELISA)

*p < 0.05.

Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.10222233>. The package includes the following files:

Supplementary Table 1. The analysis results of data from Fig. 1 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test without any correction.

Supplementary Table 2. The analysis results of data from Fig. 2 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test without any correction.

Supplementary Table 3. The analysis results of data from Fig. 3 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test without any correction.

Supplementary Table 4. The analysis results of data from Fig. 4 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test without any correction.

Supplementary Table 5. The analysis results of data from Fig. 5 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test without any correction.

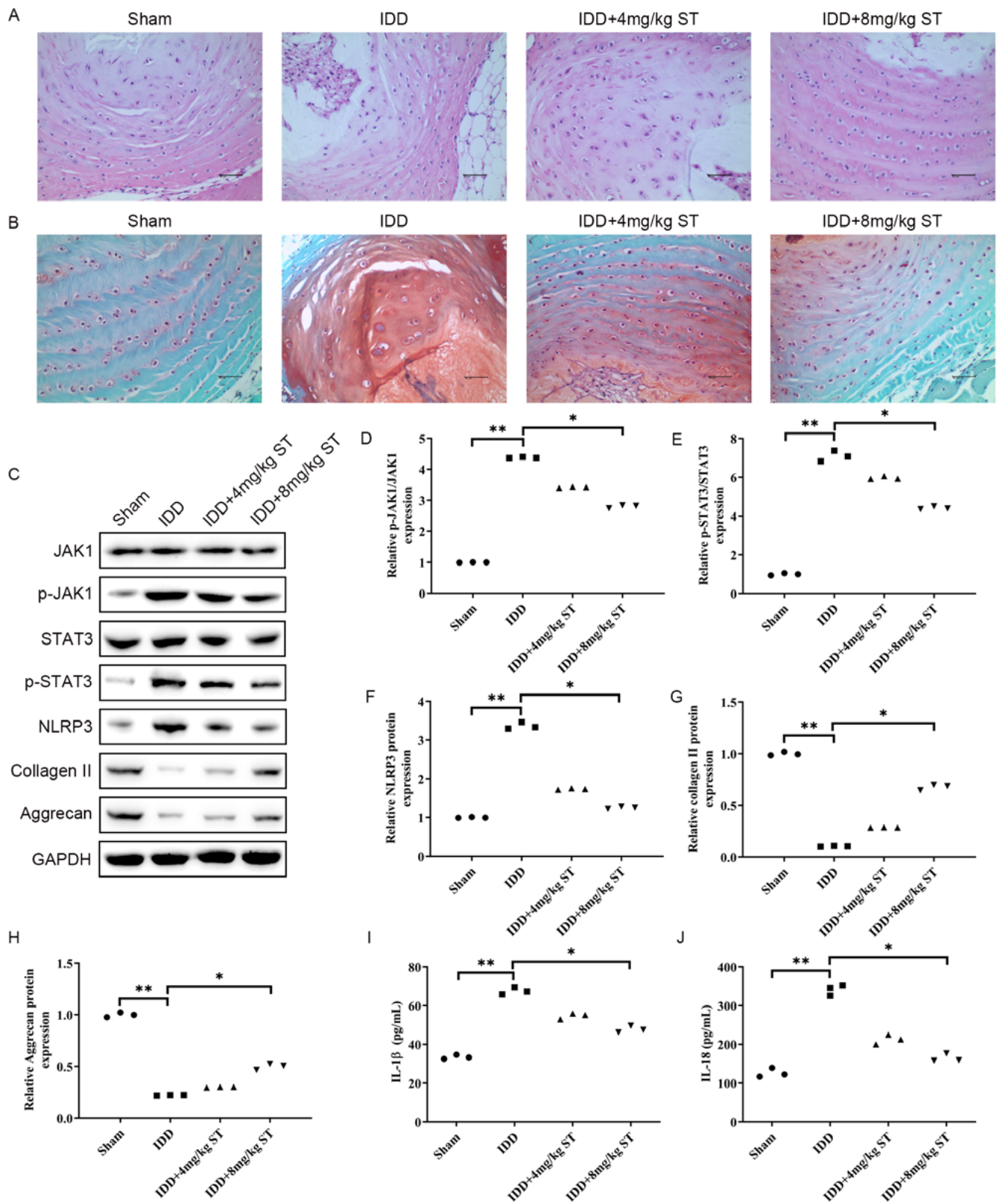


Fig. 7. Sinapine thiocyanate improved intervertebral disc degeneration (IDD) in vivo. A, B. Pathological change of the intervertebral disc was assessed with hematoxylin & eosin (H&E) and safranin-O staining; C–H. The expression of JAK, p-JAK, STAT3, p-STAT3, NLRP3, collagen II, and aggrecan was detected using western blot; I–J. The content of interleukin (IL)-1 β and IL-18 was evaluated with enzyme-linked immunosorbent assay (ELISA)

**p < 0.01; *p < 0.05.

Supplementary Table 6. The analysis results of data from Fig. 6 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test without any correction.

Supplementary Table 7. The analysis results of data from Fig. 7 through non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test without any correction.

Data availability

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

Consent for publication

Not applicable.

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