Sparstolonin B prevents lumbar intervertebral disc degeneration through toll like receptor 4, NADPH oxidase activation and the protein kinase B signaling pathway

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Abstract. Intervertebral disc degeneration (IVDD) is the most common pathogeny of lumbago. It is the pathological basis for a series of spinal degenerative diseases. For a long time, the diagnosis and treatment of lumbago have rendered difficult, since the pathogeny has not been identified. Therefore, the present study aimed to investigate the protective effect of Sparstolonin B in preventing lumbar intervertebral disc degeneration, and explored its potential mechanism in rats. Firstly, Sparstolonin B effectively reduced the histological score of disc degeneration and increased endplate porosity of L2 superior endplates in a lumbar IVDD rat model. Sparstolonin B significantly inhibited the IVDD-induced inflammatory factors tumor necrosis factor-α, interleukin (IL)-1β and IL-6, oxidative stress factors (malondialdehyde), and superoxide dismutase and caspase-3/9 activities. Treatment with Sparstolonin B significantly suppressed toll-like receptor 4 (TLR4), myeloid differentiation primary response protein 88 (MyD88) and nuclear factor (NF)-κB protein expression, inhibited NAPDH oxidase 2 protein expression and induced phosphoinositide 3-kinase and phosphorylated protein kinase B protein expression in the IVDD rat model. These results demonstrated that Sparstolonin B prevents lumbar IVDD-induced inflammation, oxidative stress and apoptosis through TLR4/MyD88/NF-κB, NADPH oxidase activation and the phosphoinositide 3-kinase/protein kinase B signaling pathway. These results implicate Sparstolonin B for use as a therapeutic agent for IVDD in clinical applications.

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Introduction

Previous research has indicated that intervertebral disc degeneration (IVDD) is associated with biological alterations of the intervertebral disc substrate (1). Research has also determined that a large number of inflammatory substances and enzyme systems exist in degenerated intervertebral disc tissues, primarily consisting of three types: Cytokines, inflammatory medium and protease, as well as its inhibitors (2). The involvement of such materials may lead to relevant inflammatory responses and cause damage of intervertebral disc substrate. There are numerous types of cytokines (CKs), among which, tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 are the most important factors associated with IVDD, and are also the major targets of research (3). TNF-α, IL-1β and IL-6 may affect the normal metabolism of intervertebral disc substrate, which causes internal environment disorder of the intervertebral disc, metabolite accumulation, increased cell apoptosis, inflammatory reaction aggravation, increase of capillary permeability, and intervertebral disc nutritional disorder, which are closely associated with IVDD (4).

Any damage to the intervertebral disc, even minor damage, will cause oxidative stress reaction (5). Nucleus pulposus is the first one affected, which generates a large number of oxygen free radicals (6). Under normal physiological status, the generation and removal of free radicals occurs under a dynamic balancing state (7). External factors, such as multiple-level spinal fracture accompanied by disc intervertebral injuries, may lead to a decrease in the capacity of free radical generation and scavenging (6). The body will be subject to oxidative stress, which leads to free radical accumulation, a rise in body peroxidation levels, cytotoxicity generation and body injury (8).

As age increases, the intervertebral disc exhibits different degrees of aging and degeneration. IVDD mainly presents as reduced numbers of intervertebral disc cells, hypofunction, dehydration of polysaccharide, a decrease in the aggregation of proteoglycans, collagen type and distribution changes, intervertebral disc tension, and pressure weakening or loosing (9,10). The histological alterations eventually lead to changes of intervertebral disc biomechanics (11). It has

been observed that decreasing of intervertebral disc active cells, decreasing of extracellular matrix components and composition change are the pathological bases for IVDD (11). Excessive apoptosis of intervertebral disc cells is the direct cause for intervertebral disc cells decreasing (11).

With the local inflammatory vascular response stimulated by the fibrous ring damage, the cells at the inflammatory site generate growth factors, which work on intervertebral disc cells isolated by the circulatory system. Through signal transduction, the differentiation and proliferation of intervertebral disc cells and a large amount of extracellular matrix synthesis are promoted (12). It may be the major reason for intervertebral disc fibrosis and degeneration. The majority of research on inflammatory reactions after disc intervertebral injuries focus on the interaction between cytokines (13). However, there are limited studies on the autoimmune response mechanism arising from intervertebral disc tissue. The association between inflammation reactions and immune mechanisms remains to be identified (14). The two may be under a cause-and-effect association, as well as mutual promotion (14). At present, further studies on signal transduction mechanisms underlying inflammation and immunology are undergoing, with the purpose of detecting the specific antigen proteins of nucleus pulposus tissue, to determine the immune foundation for inflammatory cytokines or enzyme reaction during IVDD, and to explore the signal transduction rules of immune response (15). The research is of great significance for studying the immunologic mechanism of fibrous ring of intervertebral disk inflammatory reactions at the molecular level. It can provide a theoretical basis for IVDD prevention (14).

Sparstolonin B is an isocoumarin compound (Fig. 1) and is extracted from the tubers of both *Sparganium stoloniferum* and *Scirpus yagara* (15). Sparstolonin B is a novel toll like receptor 4 (TLR4) antagonist derived from the traditional Chinese medicine 'SanLeng' for the treatment of several inflammatory diseases (16). The present study evaluated the effect of Sparstolonin B in preventing IVDD, and investigated the potential underlying mechanisms in rats.

Materials and methods

Experimental design. Male Sprague-Dawley rats (age, 8-10 weeks; weight, 200-230 g; n=40) were recruited for this study, and were housed at 22-23˚C and 55-60% humidity, 12-h light/dark cycle, free access to food and water. Rats were anesthetized with 1% sodium pentobarbital solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

All animals underwent a midline ventral longitudinal incision to expose the L5/6 intervertebral disc. In experimental rats, ~10 μg Fluoro-Gold neurotracer crystals (Fluorochrome, LLC, Denver, CO, USA) were applied to the surface of the L5/6 intervertebral disc to label the dorsal root ganglion neurons innervating the discs. Rats were randomly divided into four groups (n=10 per group): Sham operation (Sham), IVDD model (model), 100 mg/kg Sparstolonin B (100-Spa B) and 200 mg/kg Sparstolonin B (200-Spa B). The 100-Spa B and 200-Spa B group rats were administered 100 mg/kg/once every three days or 200 mg/kg/once every three days Sparstolonin B (Sigma-Aldrich; Merck KGaA.) for 4 weeks by gavage.

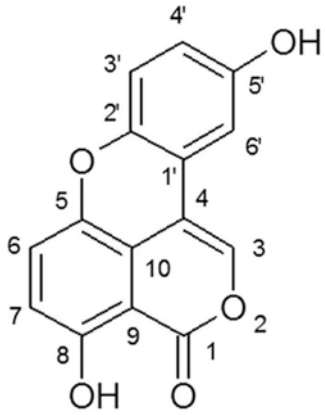

Figure 1. Chemical structure of Sparstolonin B.

The present study was approved by the ethics committee of West China Hospital (Chengdu, China).

Histological evaluation. After Sparstolonin B treatment, the rats were sacrificed using <35 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA), and intervertebral discs or spinal motion segments were harvested as described previously (17). L5-L6 segments were fixed with 10% paraformaldehyde solution for 3-5 days and then fixed with paraformaldehyde solution for 5 days at room temperature. Sections were serial dewaxed, stained with haematoxylin at room temperature for 15 min and rinsed with distilled water. The sections were observed using a Digital Image Analyzer (Ni-E; Nikon Corporation, Tokyo, Japan).

Evaluation of endplate degeneration. L1/2 intervertebral discs were scanned using a Siemens Micro-CT scanning system. Superior endplates were re-established, and volume ratios of marrow contact channels in the endplate and the condition of endplate nutritional supply were evaluated to indicate the state of the endplate.

Determination of biological factors. The T12/L1 and L1/2 intervertebral discs were immediately lysed using lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) on ice for 30-60 min and the protein concentration was quantified by an Enhanced Bicinchoninic Acid (BCA) Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Total proteins (5 μg) were used to measure TNF-α (cat. no. PT516), IL-1β (cat. no. PI303), IL-6 (cat. no. PI328), malondialdehyde (cat. no. S0131), and superoxide dismutase (SOD; cat. no. S0101) using ELISA assay kits (Beyotime Institute of Biotechnology) at 450 nm. Total proteins (5 μg) were used to measure caspase-3/9 activities using caspase-3/9 activities kits (cat. no. C115; Beyotime Institute of Biotechnology) at 405 nm.

Western blot analysis. The T12/L1 and L1/2 intervertebral discs were immediately lysed using lysis buffer on ice for 30-60 min and the protein concentration was determined using an Enhanced BCA Protein Assay kit. Total proteins (50 μg) were separated by 8-10% SDS-PAGE at 100 V for

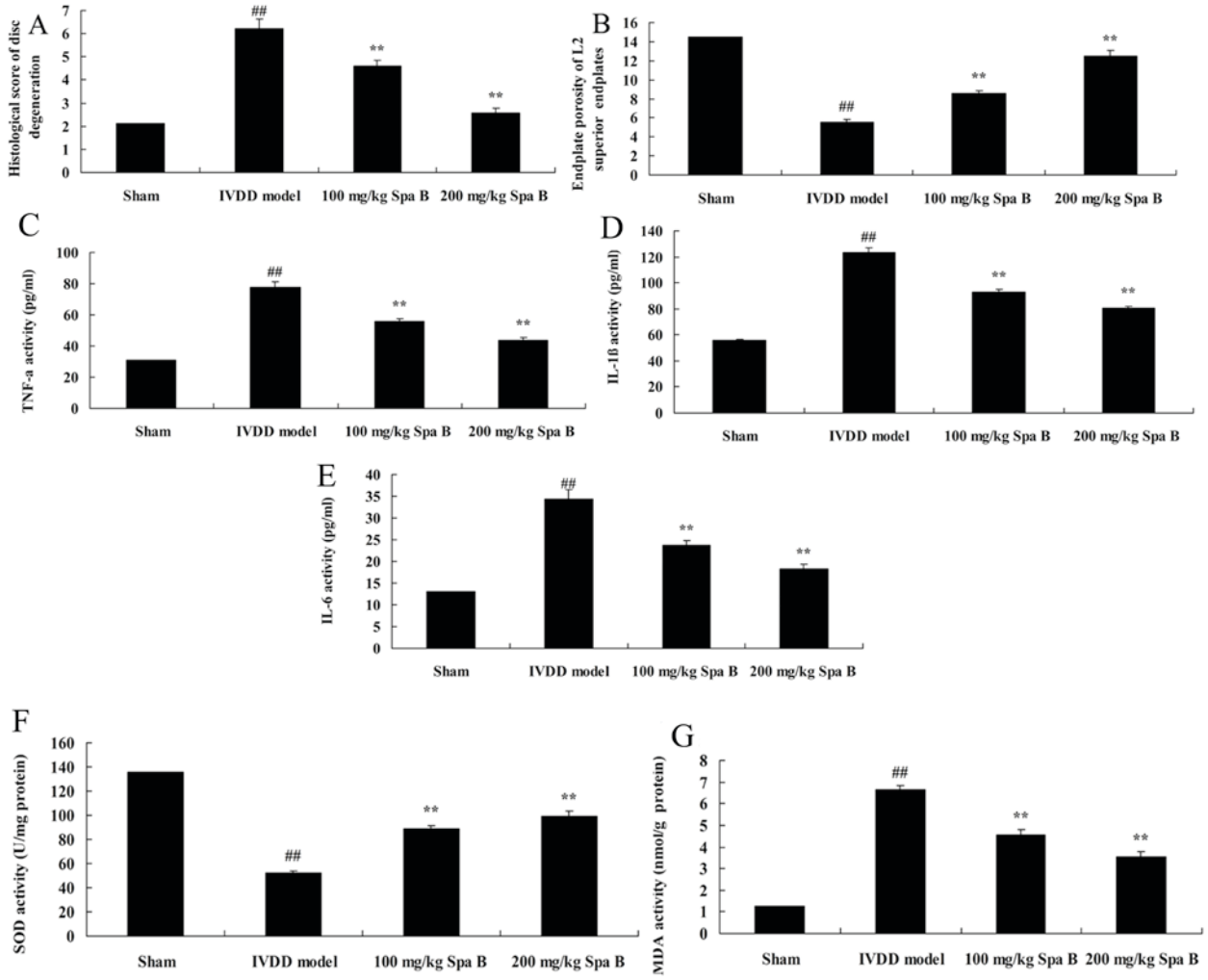

Figure 2. Sparstolonin B effects on disc degeneration, endplate porosity of L2 superior endplates, inflammation and oxidative stress in IVDD. (A) Histological score of disc degeneration. (B) Increased endplate porosity of L2 superior endplates. (C) TNF-α, (D) IL-1β, (E) IL-6, (F) SOD and (G) MDA activities levels in an IVDD model. Data are presented as the mean ± standard error. ##P<0.01 vs. sham group; **P<0.01 vs. IVDD model group. Spa B, Sparstolonin B; IVDD, intervertebral disc degeneration; IL, interleukin; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; MDA, malondialdehyde.

1.5 h and then transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with 5% non-fat dry milk at room temperature and incubated with primary antibodies against TLR4 (cat. no. 14358; 1:2,000), myeloid differentiation primary response protein 88 (MyD88; cat. no. 4283; 1:2,000), nuclear factor (NF)-κB (cat. no. 8242; 1:2,000), NAPDH oxidase 2 (NOX2), phosphoinositide 3-kinase (PI3K; cat. no. 4249; 1:2,000), phosphorylated-protein kinase B (Akt; cat. no. 9614; 1:2,000) and GAPDH (cat. no. 5174; 1:5,000; all from Cell Signaling Technology, Inc.) overnight at 4˚C. After washing with PBS with 0.1% Tween 20, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Proteins were detected with an Enhanced Chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified by Image J version 3.0 software (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Data are expressed as the mean ± standard error using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance by Tukey's post hoc test was used for multiple group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Sparstolonin B effects on IVDD. The present study used an IVDD model *in vivo* to determine the histological score of disc degeneration and endplate porosity of L2 superior endplates in lumbar IVDD after treatment with Sparstolonin B. As presented in Fig. 2A, a significant increase of histological score of disc degeneration was observed in the IVDD model group compared with the sham group. Meanwhile, the inhibition of endplate porosity of L2 superior endplates in lumbar IVDD was markedly observed compared with the sham group (Fig. 2B). Treatment with Sparstolonin B (100 and 200 mg/kg) significantly reversed these changes in IVDD rats, compared with the IVDD model group (Fig. 2A and B).

Sparstolonin B effects on inflammation in IVDD. To investigate the protective effect of Sparstolonin B on inflammation in IVDD, TNF-α, IL-1β and IL-6 levels were measured by

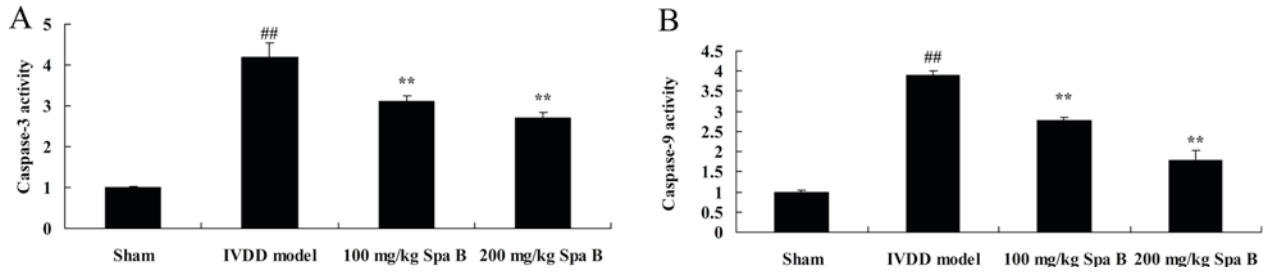

Figure 3. Sparstolonin B effects on caspase-3/9 activities in IVDD. (A) Caspase-3 and (B) caspase-9 activity in an IVDD model. Data are presented as the mean ± standard error. ##P<0.01 vs. sham group; **P<0.01 vs. IVDD model group. Spa B, Sparstolonin B; IVDD, intervertebral disc degeneration.

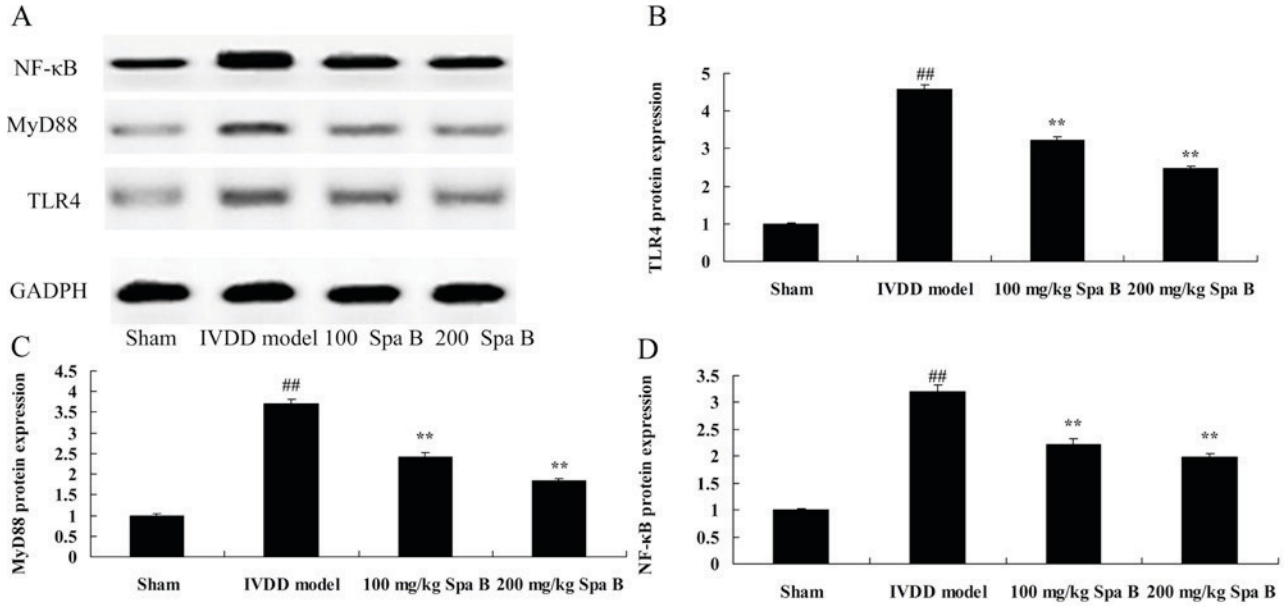

Figure 4. Sparstolonin B effects on TLR4, MyD88 and NF-κB protein expression in IVDD. (A) Representative western blot images of TLR4, MyD88 and NF-κB protein expression levels. Quantification of (B) TLR4, (C) MyD88 and (D) NF-κB protein expression levels. GAPDH served as a loading control. Data are presented as the mean ± standard error. ##P<0.01 vs. sham group; **P<0.01 vs. IVDD model group. Spa B, Sparstolonin B; IVDD, intervertebral disc degeneration; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB; MyD88, myeloid differentiation primary response protein 88.

ELISA. There were significant increases of TNF-α (Fig. 2C), IL-1β (Fig. 2D) and IL-6 (Fig. 2E) levels in the IVDD model group, compared with the sham group. However, treatment with Sparstolonin B (100 and 200 mg/kg) significantly reduced TNF-α, IL-1β and IL-6 content levels in IVDD rats, compared with IVDD model rats (Fig. 2C-E).

Sparstolonin B effects on oxidative stress in IVDD. To clarify the protective effect of Sparstolonin B on oxidative stress in IVDD, MDA and SOD content levels were measured by ELISA. Inhibition of SOD content (Fig. 2F) and induction of MDA content (Fig. 2G) were markedly observed compared with the sham group. Sparstolonin B treatment (100 and 200 mg/kg) significantly reversed the inhibition of SOD content and induction of MDA content in IVDD rats, compared with the IVDD model (Fig. 2F and G).

Sparstolonin B effects on caspase-3/9 activities in IVDD. To determine the protective effect of Sparstolonin B on apoptosis in IVDD, caspase-3/9 activities were assessed by ELISA. As presented in Fig. 3, the caspase-3/9 activities of IVDD model rats were markedly higher compared with the sham group. After treatment with 100 and 200 mg/kg Sparstolonin B, the induction of caspase-3/9 activities were significantly inhibited, compared with the IVDD model (Fig. 3).

Sparstolonin B effects on TLR4, MyD88 and NF-κB protein expression in IVDD. The present study examined whether TLR4, MyD88 and NF-κB were involved in the protection effect of Sparstolonin B on IVDD. TLR4, MyD88 and NF-κB protein expression levels were measured using western blot analysis. Western blot analysis demonstrated that TLR4, MyD88 and NF-κB protein expression levels in the IVDD model group were significantly increased, compared with sham group (Fig. 4). Sparstolonin B (100 and 200 mg/kg) significantly suppressed TLR4, MyD88 and NF-κB protein expression levels in IVDD rats, compared with IVDD model (Fig. 4).

Sparstolonin B effects on NOX2, PI3K and p-Akt protein expression in IVDD. The effect of Sparstolonin B on NOX2, PI3K and p-Akt protein expression levels were examined (Fig. 5). Compared with sham operation group, NOX2 protein expression in the IVDD model group was increased (Fig. 5A and B).

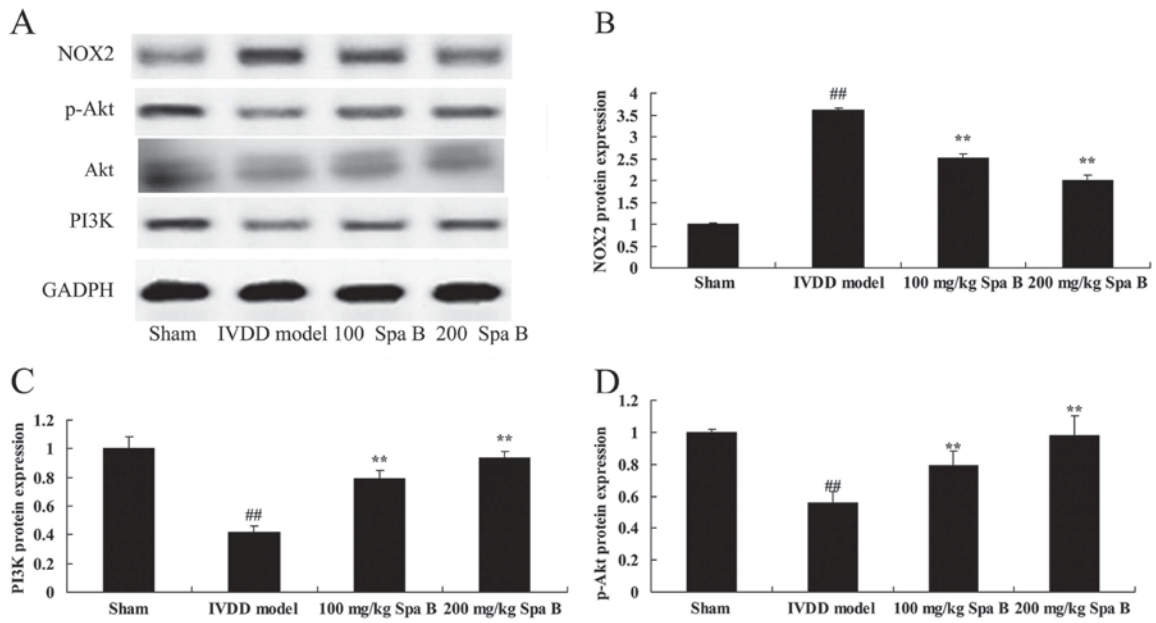

Figure 5. Sparstolonin B effects on NOX2, PI3K and p-Akt protein expression in IVDD. (A) Representative western blot images of NOX2, PI3K and p-Akt protein expression levels. Quantification of (B) NOX2, (C) PI3K and (D) p-Akt protein expression in an IVDD model. GAPDH served as a loading control. Data are presented as the mean ± standard error. $^{\#\#}$P<0.01 vs. sham group; **P<0.01 vs. IVDD model group. Spa B, Sparstolonin B; IVDD, intervertebral disc degeneration; Akt, protein kinase B; p-, phosphorylated; PI3K, phosphoinositide 3-kinase; NOX2, NAPDH oxidase 2.

Sparstolonin B (100 and 200 mg/kg) significantly suppressed NOX2 protein expression in IVDD rat, compared IVDD model group (Fig. 5A and B). Conversely, protein expression levels of PI3K (Fig. 5C) and p-Akt (Fig. 5D) were decreased in the IVDD model group compared with the sham-operated group; however, both doses of Sparstolonin B significantly ameliorated this effect.

Discussion

IVDD is a syndrome presenting intervertebral disc deformation, accompanied by progressive fibrosis, which causes corresponding lesions of adjacent joints and ligament, spinal instability, or even compression of spinal cord, nerve root and spinal artery, and other corresponding clinical symptoms and physical signs (18). It is the premise and basic pathological process for a series of spinal degenerative diseases (19). Generally, the intervertebral disc of humans will start degenerating from 20 years old. It has been hypothesized that IVDD is induced by a variety of factors, including aging, nutrition, immune and trauma (19). In case of multiple segmental spinal fracture and after spinal internal fixation surgery, the degeneration rate of intervertebral disc will increase. IVDD deteriorates gradually as age increases (20). However, its pathogenic mechanism is still not clear. The present study demonstrated that Sparstolonin B (100 and 200 mg/kg) significantly reversed these changes in IVDD rats.

The activation of toll receptor signaling pathways participates in the destruction of articular cartilage and synovial membrane process; TLR4 is a member of toll receptor family (21). It is mainly expressed in various immune cells. A previous study has identified that TLR4 is highly expressed in the articular cartilage and synovial membrane of IVDD (21). The TLR4 signaling pathway is closely associated with the pathogenetic mechanism of IVDD (22). NF-κB is an essential signal transduction molecule located in the downstream signaling pathway of toll receptors (22). Many *in vivo* cellular processes, such as inflammation, immune response, cell apoptosis, tumor occurrence and metastasis, are regulated by NF-κB. These results suggested that Sparstolonin B (100 and 200 mg/kg) significantly reduced TNF-α, IL-1β and IL-6 content levels in IVDD rats through suppression of the TLR4/NF-κB signaling pathway. It has been demonstrated that Sparstolonin B protects mice against endotoxin shock by inhibiting the TLR2/4 signaling pathway (23).

Generally, it is believed that oxidative stress will occur when the balance between the generation and scavenging of oxygen free radicals is destroyed (21). The resulting damage is the primary cause for cell aging. The cells will protect themselves through a series of antioxidant system against free radicals (24). The extracellular antioxidant system will participate in resistance and alleviating of oxidative damage (25). Aging is a process affected by multiple factors (25). Oxidative stress is closely associated with aging, and aging is an essential factor for IVDD (25). Our previous study demonstrated that Sparstolonin B significantly reversed the inhibition of superoxide dismutase content and induction of malondialdehyde content in IVDD rats. It has been demonstrated that Sparstolonin B attenuates early liver inflammation via NADPH oxidase activation (16).

NADPH oxidase is detectable in neutrophile granulocytes. Neutrophile granulocytes generate millimole levels of O_2 during phagocytosis. It also serves important effects on host non-specific defense. The genovariation of the important subunits of enzyme may lead to chronic guanulomatous disease (characterized by recurrent episodes of lethal infection) (26). Among them, gp91phox is the basic component of NADPH oxidase. Additionally, p47phox is the key subunit for activity of NADPH oxidase. Under normal circumstances, NADPH

oxidase is under a dormant state in neutrophile granulocyte. If appropriately stimulated, it will be activated rapidly (27). The cytoplasmic component p47phox is then subject to phosphorylation and p67phox displacement (26). Eventually, it will be activated due to the accumulation with cell membrane components, gp91phox and p22phox. NADPH serves as the electron donor to catalyze oxygen to O_2 and further generate a reactive oxygen species (26). The results of the present study suggested that Sparstolonin B significantly suppressed NOX2 protein expression in IVDD rats. Furthermore, it also has been detected that cell apoptosis may participate in pathophysiological changes of intervertebral disc tissue degeneration (28). It has been indicated that cell apoptosis serves important effects on IVDD process (29). Cell excessive apoptosis will lead to a decrease of intervertebral disc activity cells (29). The subsequent decreasing of extracellular matrix synthesis and composition change are the pathology bases for IVDD (29). Previous research has indicated that the oxidative stress arising from reactive oxygen is an essential link causing cell apoptosis (30,31).

The PI3K/Akt signaling pathway is involved and activated substrates after acidification include serine or threonine residues (such as Bcl-2-associated death promoter, NF-KB and caspase-9) have biological functions including resistance to contabescence and growth promotion (32). Studies have verified that the PI3K/Akt transduction pathway is closely associated with cartilage cell apoptosis (33). It has been demonstrated that after adding PI3K inhibitor into rat bone chondrocytes, the growth and differentiation velocity are decreased significantly, and that the apoptotic cell ratio increases. The difference in the number of apoptotic cells arising from biomechanical changes is also increased through this pathway (33). Consequently, the PI3K/Akt transduction pathway is significant for the apoptosis of chondrocytes (33). In the present study, it was demonstrated that 100 and 200 mg/kg Sparstolonin B significantly induced the PI3K/Akt signaling pathway in IVDD rats. Liang *et al* (15) reported that Sparstolonin B suppresses endothelial cell inflammation through extracellular signal-regulated kinase 1/2 and the Akt signaling pathway.

In conclusion, the results of the present study demonstrated that Sparstolonin B prevents IVDD, and inhibits IVDD-induced inflammation, oxidative stress and apoptosis through TLR4/MyD88/NF-κB, NADPH oxidase activation and the PI3K/Akt signaling pathway. Sparstolonin B may affect autophagy or other mechanisms underlying IVDD, which require further study. Therefore, Sparstolonin B has the potential to be used as a therapeutic agent for IVDD in clinical applications.

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