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Danshen attenuates cartilage injuries in osteoarthritis *in vivo* and *in vitro* by activating JAK2/STAT3 and AKT pathways

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Abstract: Articular cartilage degradation is a main feature of osteoarthritis (OA). The effects of Danshen, a traditional Chinese herb, in mitigating cartilage damage have been reported before. This study was conducted to investigate the effects of Danshen on cartilage injuries in OA. Rabbit OA models were established by surgical destabilization of the medial meniscus and the anterior and posterior cruciate ligaments in the left knee joint. Injection of Danshen into the articular cavity attenuated OA cartilage destruction *in vivo*. The levels of phosphorylated Janus kinase 2 (JAK2) and phosphorylated signal transducer and activator of transcription 3 (STAT3) were decreased in osteoarthritic cartilage, while they were rescued upon Danshen treatment. Furthermore, chondrocytes isolated from normal rabbit cartilage were exposed to 2 mM sodium nitroprusside (SNP) to establish an OA model *in vitro*. We found that the oxidative stress and chondrocyte apoptosis induced by SNP were suppressed by Danshen. The phosphorylation levels of JAK2 and STAT3 were decreased in response to SNP treatment, whereas they were rescued by Danshen. Additionally, AG490, a specific JAK2 inhibitor, counteracted the anti-apoptotic effect of Danshen. The phosphorylation level of protein kinase B (AKT) was also altered in response to SNP and reversed by Danshen. The anti-apoptotic effect of Danshen was counteracted by AKT pathway inhibitor LY194002. Taken together, Danshen attenuates OA cartilage destruction by regulating the JAK2/STAT3 and AKT signaling pathways.

Key words: AKT, apoptosis, articular cartilage degradation, Danshen, JAK2/STAT3

Introduction

Osteoarthritis (OA) is the most common arthritis disease representing joint damage during daily activities. This disease is a major cause of activity limitation,

physical disability, and increased health cost [11]. It will be the fourth highest cause of disability worldwide by the year 2020 [26]. Therefore, the therapies for OA have gained more attention. OA mainly affects the knees, hips, hands, and spine joints, and the knee joints are most

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Supplementary Figures: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/exanim>



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commonly involved. There are some pharmacologic therapies for knee OA, such as NSAIDs [3, 4, 6], associated with adverse effects. Hence, safe and effective therapies for OA should be investigated. OA is characterized by articular cartilage degeneration [25]. Articular cartilage mainly consists of chondrocytes and extracellular matrix (ECM). Chondrocytes promote ECM formation or degradation by producing anabolic or catabolic factors [8]. Hence, chondrocytes perform a crucial role in articular cartilage degeneration and the pathological process of OA [5]. Inhibition of chondrocyte apoptosis may be a novel therapeutic target for the management of OA.

Natural plant products have been used as flavors, fragrances and medicines for thousands of years, and attract more attention with respect to their potential uses in various diseases, with rare reports showing their adverse effects. Among these plant products, various kinds have been identified as having specific medical usages including *Salvia miltiorrhiza* (Danshen). Danshen, as one of the most important herbs, has been traditionally used to treat cardiovascular diseases for centuries [24], and it has been reported to have medical usage for various liver diseases [35] and acquired immunodeficiency syndrome [1]. Rare adverse effects have been reported in trials. Recently, Danshen has been reported to prevent articular cartilage degeneration in rabbits with OA by inhibiting oxidative stress [3]. Our previous study showed that Danshen attenuates osteoarthritis through inhibition of nuclear factor- κ B (NF- κ B) signaling [28]. However, the therapeutic effects of Danshen on OA and underlying mechanisms still need to be further unraveled.

The Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and protein kinase B (AKT) pathways play important roles in inflammation. The JAK2/STAT3 pathway is also implicated in OA. Activation of the JAK2/STAT3 pathway is regulated by inflammatory cytokines stimulation during the progression of OA [16]. Inhibition of the JAK2/STAT3 pathway can ameliorate apoptosis in damaged chondrocytes [34]. The AKT pathway plays an important role in cartilage anabolic and catabolic processes in response to inflammatory cytokines [10]. The AKT pathway is also involved in OA. Inhibition of the AKT pathway promotes autophagy in articular chondrocytes and attenuates inflammatory responses in rats with OA [29]. On the other hand, whether the JAK2/STAT3 and AKT pathways

were involved in the effect of Danshen on OA remains unclear.

In the present study, the effects of Danshen on articular cartilage degeneration were evaluated in rabbits with surgery-induced OA *in vivo* and isolated chondrocytes treated with sodium nitroprusside (SNP) to establish a cell model of OA. We explored the associated molecular mechanisms against cartilage damage. This study was beneficial to the utilization of Danshen in clinical applications for OA patients.

Materials and Methods

Experimental OA and treatments

Thirty healthy mature New Zealand white rabbits (6 months old, male, weighing 3.0 ± 0.5 kg) were purchased from Tianjing Yuda Experimental Animal Breeding Co., Ltd. (Tianjing, China). Danshen injection was purchased from Chiatai Qingchunbao Pharmaceutical Co., Ltd. (Hangzhou, Zhejiang, China), sodium hyaluronate (SH) injection was obtained from Aladdin Industrial Corporation (Shanghai, China). Experimental OA models were established by surgically destabilizing the left knee joint through transection of the medial collateral ligament and anterior and posterior cruciate ligaments and excision of the medial meniscus [13]. After the surgery, the incisions were sutured but not fixed and were exposed to penicillin (2×10^4 U/kg/day) for 7 days. In this experiment, the rabbits were divided into a control group, Danshen group, OA model group, OA model+SH group, and OA model+Danshen group. Normal rabbits in the Danshen group were administered with Danshen (1.05 g/day, intra-articular injection). The surgery-induced OA rabbits in the OA group were administered normal saline (0.7 ml/day). OA rabbits in the OA+SH group were administered 1% SH in 0.4 ml/day (intra-articular injection). OA rabbits in the OA+Danshen group were administered Danshen (1.05 g/day, intra-articular injection). All treatments were administered for 5 weeks, and then rabbit articular cartilage was collected for subsequent experiments. In this study, the animal treatment was in accordance with the guidelines for animal experiments of Heilongjiang University of Chinese Medicine, and the experiments were approved by the Animal Care and Use Committee of Heilongjiang University of Chinese Medicine.

Histopathological analysis

The samples were decalcified with 10% EDTA. After complete decalcification, the samples were dehydrated in ethanol, permeabilized in xylene, embedded in paraffin, and then cut into 5 μm sections. The sections were deparaffinized, rehydrated, and then stained with hematoxylin (Solarbio, Beijing, China) and eosin (H&E; Sinopharm, Shanghai, China). The slides were examined by light microscopy (Olympus, Tokyo, Japan).

Chondrocyte isolation, culture, and identification

Normal articular cartilage was obtained from the rabbit knee joint and cut into small pieces in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Hyclone, Cramlington, England, UK). The pieces were digested with 0.1% type II collagenase and 0.2% trypsin (Beyotime, Beijing, China) at 37°C for 2.5 h. The released cells were cultured at 37°C in 5% CO₂ humidified environment. Then the cells were treated with Red Blood Cell Lysis Buffer (Solarbio) for 3 min. Cell suspension was centrifuged at 1500 r/min for 5 min. After washing with PBS, the cells were resuspended with DMEM and counted with Trypan blue. The cell density was set to $1 \times 10^5/\text{ml}$, and the cells were seeded into T25 culture flasks. After 24 h, the cells were washed with PBS and cultured with DMEM containing with 10% FBS. Then the cells were subcultured for subsequent experiments.

Chondrocyte identification was performed by toluidine blue staining. The cells were washed with distilled water and stained with 0.5% toluidine blue (URchem, Shanghai, China) for 10 min. Then they were treated with a graded series of ethanol. The cells were imaged using a microscope (Olympus, Tokyo, Japan).

Cell treatment

Chondrocytes were cultured in DMEM at 37°C in 5% CO₂ humidified environment and treated at 80% confluence. For analysis of the inhibitory effects on apoptosis and oxidant stress of Danshen and SH, the chondrocytes were randomized into five groups: the control group, Danshen group, SNP group, SH+SNP group, and Danshen+SNP group (75 mg/ml Danshen for 24 h or 100 mg/ml SH for 24 h and then 2 mM SNP for 24 h). For analysis of the underlying mechanism of Danshen, the chondrocytes were randomized into five groups: the control group, SNP group, Danshen+SNP group, AG490+Danshen+SNP group, and

LY294002+Danshen+SNP group, (75 mg/ml Danshen for 24 h, 40 μM AG490 for 24 h, and/or 25 mM LY294002 for 1 h, and then 2 mM SNP for 24 h).

MTT assay

Cells were seeded in a 96-well plate (3×10^3 per well). Then the cells were treated at 80% confluence and cultured for 24 h. After culture, 5 mg/ml MTT (Sigma, St. Louis, MO, USA) was added to the cells, and the cells were incubated for 4 h. Then 200 μl dimethyl sulphoxide was added to the cells. The absorbance at 490 nm was measured using a microplate reader (Biotek, Winooski, VT, USA). Five replicates were performed for each sample.

Flow cytometry assay

Flow cytometry was performed to analyze the apoptosis of chondrocytes. After treatment, cells were collected and washed with cold PBS. The cells were resuspended with 500 μl binding buffer and incubated with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) in the dark for 15 min. Cells were detected using flow cytometer (BD, Franklin Lakes, NJ, USA).

Hoechst 33342 fluorescence staining

Hoechst 33342 staining was performed with a Hoechst Staining Kit (Beyotime) to detect the apoptotic cells in normal and treated chondrocytes. In brief, the cells were seeded in a 12-well plate and treated at 80% confluence. After treatments, the cells were fixed and stained with Hoechst staining solution. The cells were observed using a fluorescence microscope (Olympus).

Western blot

Primary antibodies against JAK2 (1:500; rabbit polyclonal antibody, bs-23004R, Bioss, Beijing, China), phosphorylated JAK2 (p-JAK2) (1:500; rabbit polyclonal antibody, bs-2485R, Bioss), STAT3 (1:400; rabbit polyclonal antibody, bs-20382R, Bioss), p-STAT3 (1:500; rabbit polyclonal antibody, bs-3429R, Bioss), B-cell lymphoma 2 (Bcl-2) (1:500; rabbit polyclonal antibody, bs-0032R, Bioss), Bcl-2-associated X protein (Bax) (1:500; rabbit polyclonal antibody, bs-4564R, Bioss), cleaved caspase-3 (1:500; rabbit polyclonal antibody, WL01992, Wanleibio, Shenyang, China) and cleaved poly (ADP-ribose) polymerase (PARP) (1:500; rabbit polyclonal antibody, WL01932, Wanleibio), AKT (1:500; rabbit polyclonal antibody, bs-6951R, Bioss), or p-AKT

(1:1,000; mouse monoclonal antibody, bsm-33281M, Bioss) were diluted in 5% skim milk.

Protein samples were extracted by RIPA (Beyotime) with 1% phenylmethanesulfonyl fluoride (PMSF, Beyotime). The protein concentration was measured using a BCA Protein Assay Kit (Beyotime) according to the manufacturer's instructions. Same amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies overnight at 4°C. After four washes, the membranes were incubated with horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG (H+L) (A0208, Beyotime) or HRP-labeled Goat Anti-Mouse IgG (H+L) (A0216, Beyotime) diluted in 1:5000. The membranes were developed with an ECL Kit (7sea Biotech, Shanghai, China), and the blots were imaged by a gel imaging system (Liuyi, Beijing, China). The protein levels were normalized with β -actin. Each experiment was performed in triplicate.

Detection of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT)

The levels of GSH, SOD, and CAT in chondrocytes were measured using the corresponding GSH assay kit, SOD assay kit, and CAT assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions.

Statistical analysis

Data were analyzed with Graphpad Prism 6. Comparisons between groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison. All data are presented as the mean \pm SD, and $P < 0.05$ was considered statistically significant.

Results

Danshen attenuates OA cartilage destruction and induces JAK2/STAT3 activation in vivo

The results from H&E staining showed that cartilage tissues in the control group showed a normal structure. In the OA group, the cartilage surface layer was destroyed, with reduced and irregularly clustered chondrocytes. However, Danshen or SH treatment obviously attenuated cartilage destruction and recuperated the

structure of the cartilage surface layer (Fig. 1A). Western blot assay showed that the phosphorylation levels of JAK2 and STAT3 were decreased significantly in the OA group as compared with the control group. Danshen or SH treatment significantly increased p-JAK2 and p-STAT3 levels in rabbits with surgery-induced OA (Fig. 1B). Our data illustrate that Danshen treatment attenuates OA cartilage destruction *in vivo*, maybe partly through the JAK2/STAT3 signaling pathway.

Danshen restores the antioxidant system decreased by SNP

Chondrocytes were first isolated from rabbit cartilage tissues and identified with toluidine blue staining by their spindle-shaped morphology (Supplementary Fig. 1). The levels of antioxidants GSH, SOD, and CAT were assessed in our study. As shown in Fig. 2, in the SNP-induced OA model *in vitro*, the level of GSH and activities of SOD and CAT were decreased. However, after treatment with Danshen or SH, the level of GSH and activities of SOD and CAT were restored (Fig. 2). These results demonstrate that Danshen restores the antioxidant system in the OA model *in vitro*.

Danshen inhibits apoptosis and promotes proliferation and JAK2/STAT3 activation in chondrocytes in vitro

Apoptosis and proliferation of chondrocytes were detected. The results from flow cytometry showed that Danshen or SH treatment significantly decreased SNP-induced apoptosis of chondrocytes (Fig. 3A). Hoechst 33342 fluorescence staining also showed more chromatin pyknosis in the SNP group than in the control group, and these increased chromatin pyknosis induced by SNP was reversed after Danshen or SH treatment (Supplementary Fig. 2A). Thus, we concluded that Danshen inhibited chondrocyte apoptosis induced by SNP. This conclusion was also confirmed by increased level of anti-apoptotic factor Bcl-2 and decreased levels of proapoptotic Bax, cleaved caspase-3, and cleaved PARP in SNP-treated chondrocytes upon Danshen treatment (Supplementary Fig. 2B). The MTT assay showed that Danshen or SH treatment significantly increased SNP-reduced proliferation of chondrocytes (Fig. 3B). The levels of phosphorylated JAK2 and STAT3 in chondrocytes were assessed by western blot. Danshen or SH treatment significantly increased p-JAK2 and p-STAT3 levels, which were reduced by SNP in chondrocytes (Fig. 4). The above results demonstrate that Danshen inhibits

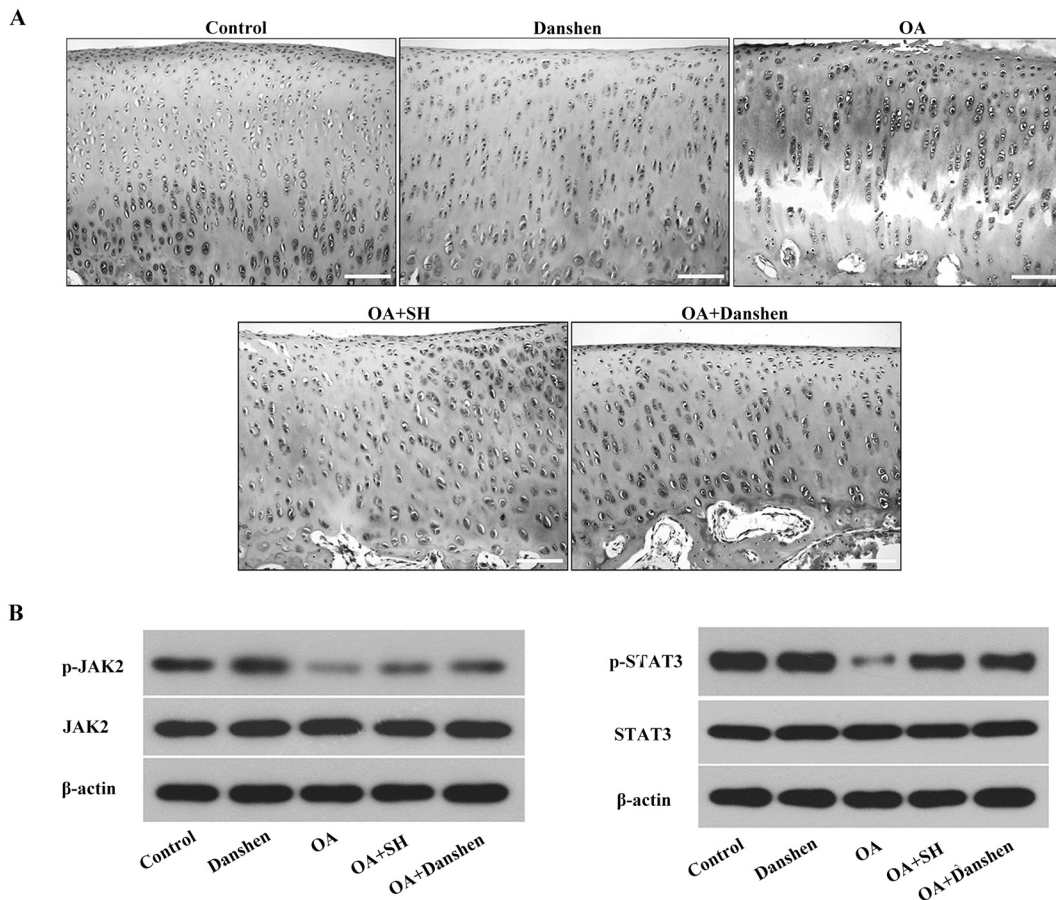


Fig. 1. Danshen attenuated articular cartilage degradation *in vivo* and activated the JAK2/STAT3 pathway. (A) Histological examination (H&E staining) of articular cartilage in Danshen- or SH-treated OA models. Scale bar=100 μ m. (B) Western blot was performed to assess the levels of phosphorylated and total JAK2 and STAT3 in articular cartilage.

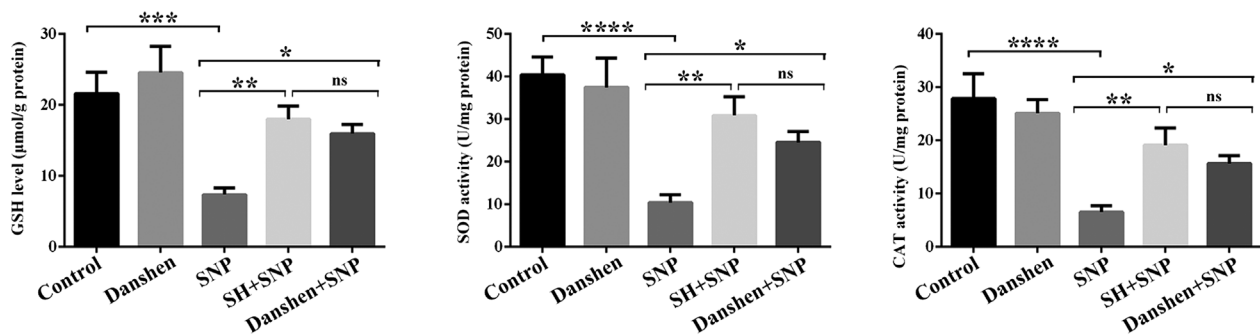


Fig. 2. Danshen rescued the antioxidant system decreased by SNP. The chondrocytes were randomized into five groups: the control group, Danshen group, SNP group, SH+SNP group, and Danshen+SNP group. The levels of GSH, SOD, and CAT in each group were measured with corresponding kits. Error bars indicate the standard deviation. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001.

apoptosis and promotes proliferation and JAK2/STAT3 activation in SNP-treated chondrocytes.

Danshen inhibits SNP-induced cartilage degeneration via the JAK2-STAT3 signaling pathway

The effect of AG490, an inhibitor of the JAK signaling pathway, on p-JAK2 and JAK2 was determined by west-

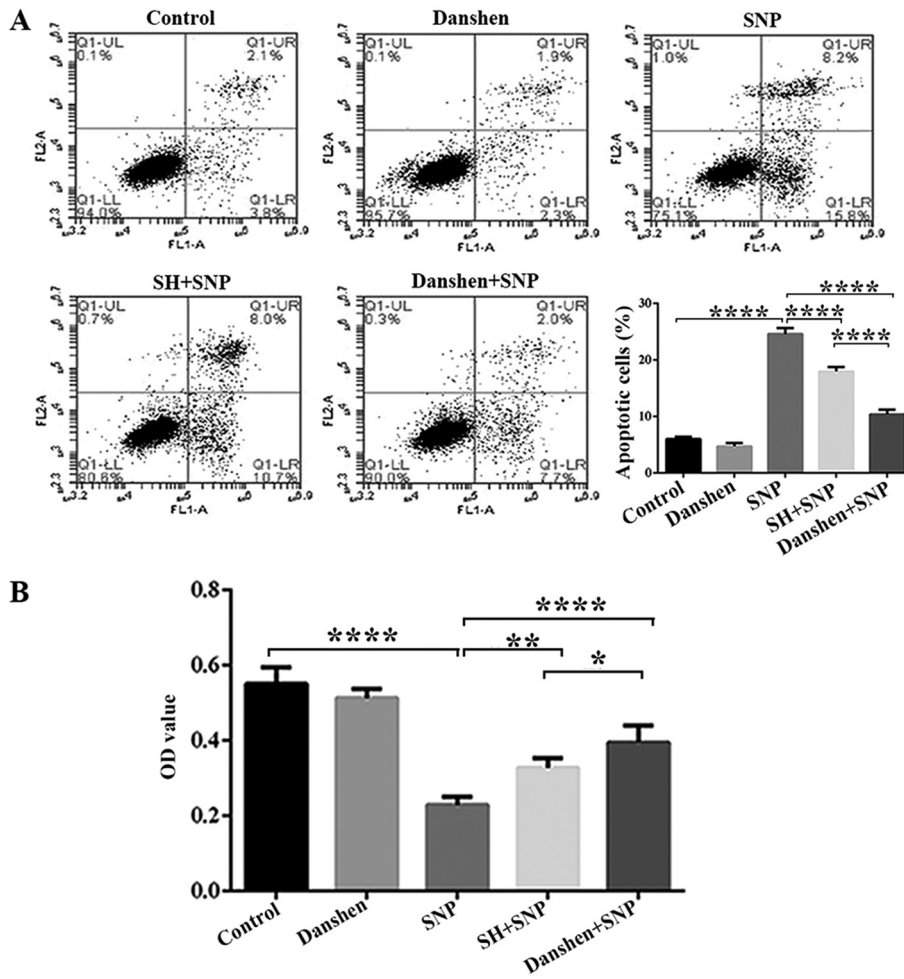


Fig. 3. Danshen ameliorated SNP-induced chondrocyte apoptosis and enhanced SNP-reduced proliferation of chondrocytes. (A) Chondrocyte apoptosis was determined by flow cytometry assay. FL1 corresponds to Annexin-FITC and FL2 corresponds to PI. (B) Proliferation of chondrocytes was detected by MTT assay. Error bars indicate the standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

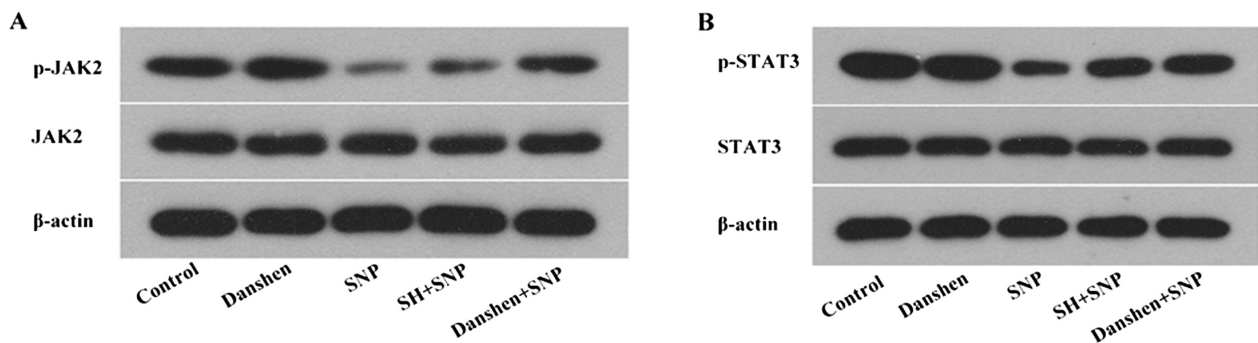


Fig. 4. Danshen activated the JAK2/ STAT3 pathway. JAK and p-JAK2 (A) and STAT3 and p-STAT3 (B) were detected by western blot. The levels of p-JAK and p-STAT3 were upregulated by Danshen in SNP-treated chondrocytes.

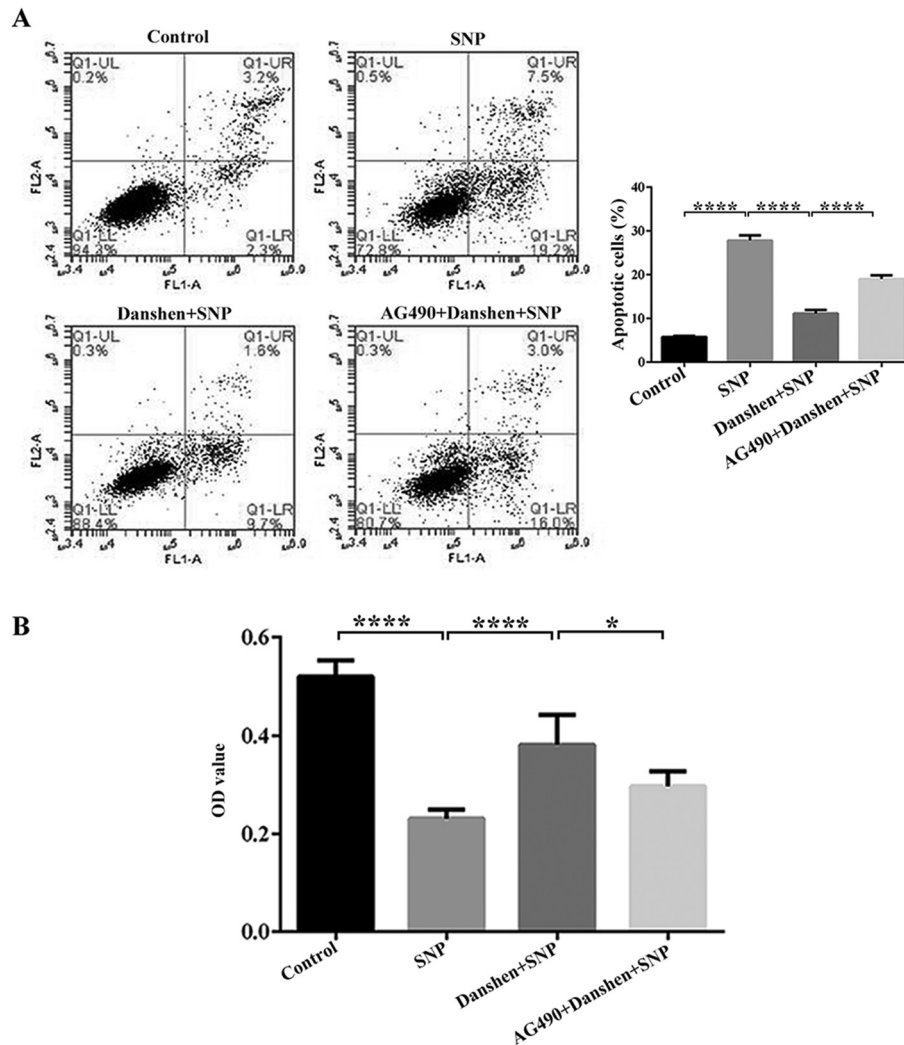


Fig. 5. Danshen attenuated SNP-induced chondrocyte apoptosis via the JAK2/STAT3 signaling pathway. (A) Flow cytometry showed that Danshen's inhibitory effects on SNP-induced apoptosis of chondrocytes were abrogated by AG490 treatment. FL1 corresponds to Annexin-FITC and FL2 corresponds to PI. (B) An MTT assay showed that Danshen's effects on proliferation of chondrocytes were inhibited by AG490 treatment. Error bars indicate the standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

ern blot. The level of p-JAK2 was not affected obviously by AG490 at 5 μM or 10 μM , but was slightly inhibited at 20 μM and significantly inhibited at 40 μM and 80 μM (Supplementary Fig. 3). The expression of JAK2 was not affected by AG490 treatment (Supplementary Fig. 3). AG490 at 40 μM was chosen for the subsequent experiments.

To determine whether Danshen inhibits the apoptosis of chondrocytes via the JAK2/STAT3 signaling pathway, AG490 was used to treat chondrocytes. Danshen treatment inhibited SNP-induced apoptosis and enhanced proliferation of chondrocytes. However, these effects

were significantly abrogated by AG490 treatment (Fig. 5 and Supplementary Fig. 4A). Additionally, Danshen treatment also reversed alterations in apoptosis-related proteins, and this effect of Danshen was abrogated by AG490 (Supplementary Fig. 4B). Therefore, we conclude that Danshen inhibits cartilage degeneration through the JAK2/STAT3 signaling pathway.

Danshen inhibits SNP-induced cartilage degeneration via the AKT signaling pathway

The role of the AKT signaling pathway was also explored in our study. The level of AKT showed no obvious

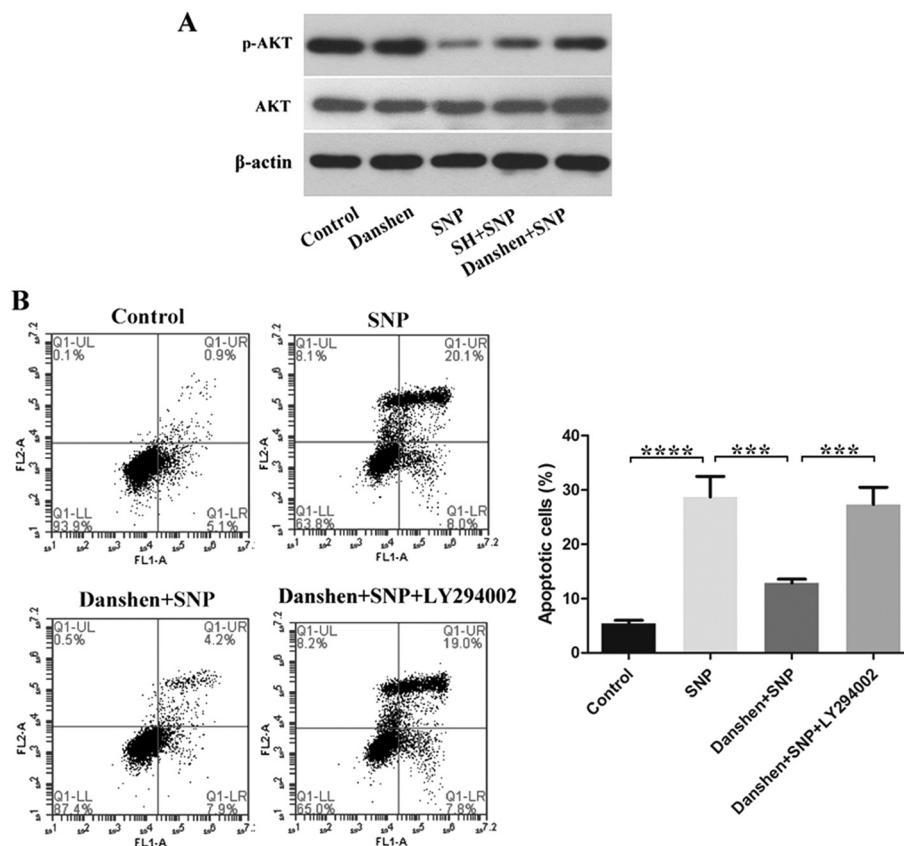


Fig. 6. Danshen ameliorated SNP-induced chondrocyte apoptosis via the AKT signaling pathway. (A) Western blot was performed to assess AKT and p-AKT levels. The level of p-AKT was increased by Danshen in SNP-treated chondrocytes. (B) Flow cytometry showed that Danshen's inhibitory effects on SNP-induced apoptosis of chondrocytes were abrogated by LY294002. FL1 corresponds to Annexin-FITC and FL2 corresponds to PI. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

changes (Fig. 6A). In the SNP-induced OA model *in vitro*, the level of p-AKT was decreased. This change was reversed upon treatment with Danshen or SH (Fig. 6A). This information indicates that the AKT signaling pathway may be involved in the effects of Danshen on OA. Moreover, LY294002, an inhibitor of the AKT signaling pathway, was used to treat chondrocytes. Danshen treatment inhibited SNP-induced apoptosis, but this effect was abolished by LY294002 (Fig. 6B). Thus, we conclude that Danshen inhibits cartilage degeneration through the AKT signaling pathway.

Discussion

In the present study, we explored the effects of Danshen on OA. The results of our study showed that Danshen attenuated OA cartilage destruction *in vivo* and

reduced oxidative stress and apoptosis of chondrocytes in an OA model *in vitro*. Further study revealed that the JAK2/STAT3 and AKT pathways were involved in the effects of Danshen on OA. Our study indicates that Danshen may become a promising drug for OA treatment.

Articular cartilage degeneration is involved in the progression of OA, and it is suggested as a novel therapeutic target for OA. Recently, in the reports of Bai *et al* and our previous study, Danshen was reported to prevent articular cartilage degeneration in OA rabbits by inhibiting oxidative stress or the NF- κ B signaling pathway [2, 28]. Our present study also showed that Danshen prevented articular cartilage degeneration and inhibited chondrocyte apoptosis, which is consistent with previous reports. Our previous study also showed that Danshen can reduce proteoglycan loss in cartilage tissues [28]. Thus, Danshen has the potential to become a ther-

apeutic target for OA.

Oxidative stress plays a crucial role in multiple physiological and pathological processes. Various intracellular signaling pathways are activated by oxidative stress, leading to cell apoptosis or overgrowth [20]. As a main contributor in oxidative stress, reactive oxygen species (ROS) are a double-edged sword, functioning as a component of cell signaling at low doses but as a cytotoxic agent at high doses [17]. Excessive ROS, which were produced by oxidative stress, damage DNA, and ROS scavengers are capable of suppressing apoptosis resulting from a variety of causes [14, 33]. Oxidative stress has been reported to play an important role in the pathology of OA. In abnormal conditions, Danshen prevents oxidative stress [27, 36]. In our study, the antioxidant system, which was impaired in OA, was rescued by Danshen. This result was consistent with a previous report [3]. Through regulation of the antioxidant system, Danshen also has a protective effect against ischemic-reperfusion injury [7].

Chondrocyte apoptosis has been identified as an important event in the growth of articular cartilage [19] and a crucial contributor to OA [18, 23]. Hence, we explored whether Danshen impacted chondrocyte apoptosis. In our study, Danshen was found to inhibit SNP-induced chondrocyte apoptosis *in vitro*, and it rescued apoptosis-related proteins impacted by SNP. Our previous study also showed that Danshen inhibited *in vivo* chondrocyte apoptosis [28]. Thus, the inhibition of chondrocyte apoptosis may contribute to the protective effects of Danshen on OA. Recently, salvianolic acid B, a hydrophilic component of Danshen, has also been reported to promote cell growth and attenuate the dedifferentiation status of articular chondrocytes [31].

The mechanism of the OA-ameliorating effect of Danshen was further investigated in our study. Our data showed that the JAK2/STAT3 and AKT pathways were activated by Danshen, and treatment with corresponding inhibitors treatment abrogated the apoptosis-inhibition effect of Danshen. This information reveals that the JAK2/STAT3 and AKT pathways are implicated in the OA-ameliorating effect of Danshen. However, in the report of Zhang *et al.* [34], AG490 could decrease ACLT-induced apoptosis. The actual reason for the differences between the present study and this previous report needs further exploration. The JAK2/STAT3 and AKT signaling pathways are not the only signaling pathways by which Danshen ameliorates OA. Our previous study

shows that the NF- κ B pathway is also involved in the protective effects of Danshen against OA [28]. Other pathways, such as PTEN, AMPK, and ERK, are also downstream signaling pathways of Danshen [12, 22, 32] and may be also implicated in the OA-ameliorating effect of Danshen. However, further evidence is needed. Through the JAK2/STAT3 and AKT signaling pathways, Danshen may also exert protective effects against ischemic-reperfusion injury, fibrosis, and cancer [7, 9, 15, 21, 30].

In conclusion, Danshen causes chondrocytes to be distributed homogeneously and ameliorates cartilage injuries *in vivo*. Moreover, Danshen exerts notable effects against oxidative stress and apoptosis induced by SNP *in vitro*. Further study revealed that the JAK2/STAT3 and AKT signaling pathways are involved in the OA-ameliorating effect of Danshen. Taken together, Danshen ameliorates osteoarthritis cartilage injury by activating the JAK2/STAT3 and AKT pathways. The present study proposes a novel therapeutic role of Danshen in the management of osteoarthritis.

Acknowledgments

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