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The protective effect of sophocarpine in osteoarthritis: An *in vitro* and *in vivo* study

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ABSTRACT

Background: Osteoarthritis (OA) is a type of degenerative joint disease affecting millions of individuals worldwide. However, there are currently no great inflammatory treatments available for it. Sophocarpine (SPC), one of the key bioactive compounds derived from *Sophora flavescens*, has shown remarkable anti-inflammatory effects.

Methods: In this study, we evaluated the effect of SPC on preventing the progression of OA and investigated its molecular target involved. In brief, rat chondrocytes were pretreated with SPC and subsequently stimulated with IL-1β. We found that SPC reduced the production of pro-inflammatory cytokines, such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). SPC also inhibited the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) at both the gene and protein level. Moreover, SPC promoted the expression of anabolic factors Sox-9 and aggrecan, while inhibiting the expression of catabolic factors, such as matrix metalloproteinases 13 (MMP-13) and thrombospondin motifs 5 (ADAMTS-5) in rat chondrocytes. Mechanistically, we found that SPC inhibited nuclear factor kappa B (NF-κB) *via* the phosphatidylinositol 3 kinase (PI3K)/AKT pathway. The beneficial effects of SPC were also observed *in vivo* using a rat OA model.

Conclusions: Our findings indicate that SPC may be a potential novel therapeutic in the treatment of OA.

1. Introduction

Osteoarthritis (OA) is a common degenerative joint disease affecting millions of individuals globally [1]. Aging, obesity, abnormal estrogen levels, an abnormal morphology, metabolic diseases are all related to the development of OA [2]. Articular cartilage, subchondral bone, ligaments, capsule and synovium are all believed to play a role in the pathogenesis of OA [3]. Articular cartilage consists of chondrocytes and an extracellular matrix (ECM) [4]. Chondrocytes play an important role in articular cartilage and are responsible for the maintenance of normal synthesis and renewal of the ECM [5]. Type II collagen as well as other collagen types make up the main structure of cartilage [6] and proteoglycans, including aggrecan are embedded in this framework [7]. Pro-inflammatory cytokines, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), can direct chondrocytes to secrete matrix-degrading enzymes including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) that reduce the expression of a key protein, SOX-9, which can regulate ECM synthesis [8]. Upon stimulation of inflammatory cytokines, large quantities of nitric oxide (NO) are produced by inducible nitric oxide synthases (iNOS). This causes upregulation of cyclooxygenase-2 (COX-2) in OA, resulting in an increase in prostaglandin E2 (PGE2) production [9].

Hyaluronic acid, glucosamine, and chondroitin can aid in relieving OA symptoms [10]. Non-steroidal anti-inflammatory drugs (NSAIDs) and joint replacement are still considered the primary treatment options for osteoarthritis. However, this does not protect the cartilage from damage [11]. Thus, it remains a challenge to identify efficient, safe, and inexpensive therapeutics that have a beneficial effect in patients with OA.

Sophocarpine (SPC) is one of the major bioactive compounds derived from the natural plant *Sophora flavescens*, which are widely

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distributed across Asia. Previous studies have demonstrated that SPC exerted beneficial effects in various diseases. It has been shown to have anti-cachectic effects [12], alleviated hepatocyte steatosis [13], and attenuated fibrosis in liver and the heart [14]. Moreover, SPC suppressed pro-inflammatory cytokine release and possessed analgesic properties [15]. In addition, it has been documented that SPC ameliorated dextran sulfate sodium-induced colitis in mice, inhibits non-alcoholic steatohepatitis in rats, and attenuated the formation of osteoclasts [16]. PI3K/AKT/NF-κB signaling pathway is prominent in the regulation and progression of OA as its over-activation has been reported to lead to symptoms of inflammation of chondrocytes [25]. In this study, the efficacy of SPC in the treatment OA, especially on preventing chondrocytes from inducing inflammation *via* PI3K/AKT/NF-κB signaling was investigated.

2. Materials and methods

2.1. Reagents

SPC (purity > 98%), was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamo, Japan). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). ELISA kits for rat PGE2, IL-6, and TNF-α were purchased from R&D systems (Minneapolis, MN, USA). Primary antibodies directed against GAPDH, Type II Collagen, COX-2, iNOS, MMP-3, MMP-13, ADAMTS-5, aggrecan, SOX9, p-AKT, PI3K(110), PI3K(p85), p-P65, P65, p-IκBα, p-IKKα/β were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were from Bioworld (OH, USA). Alexa Fluor®488-labeled and Alexa Fluor®594-labeled goat anti-rabbit IgG (H + L) secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). The nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Healthcare Life Sciences (Hyclone, Logan, UT, USA). TRIzol regent was purchased from Invitrogen (Carlsbad, CA, USA). QuantiTect Reverse Transcription kit was purchased from Qiagen (Valencia, CA, USA), and SYBR Green Master Mix was purchased from Bio-Rad Laboratories (CA, USA).

2.2. Isolation and culture of chondrocytes

Ten immature SD rats (5 males and 5 females, 10 days) were euthanized with an overdose of sodium pentobarbital, and cartilage was removed from the knee and hip joints. Next, cartilage was minced and washed with phosphate-buffered saline (PBS), containing penicillin-streptomycin solution, then centrifuged at 1000 RPM for 3 min. A total of 6–10 ml of 0.2% type II collagenase was added and digestion was performed for 4–6 h in the incubator maintained at 5% CO_2 at 37 °C. Detached cells were collected and centrifuged at 1000 RPM for 3 min and transferred to a culture flask and incubated (37 °C, 5% CO_2) for 24 h. When up to 80% to 90% confluency, the cells were harvested by using 0.25% Trypsin-EDTA (Gibco, Invitrogen). Then, cells were replanted into 10 cm culture plates at the appropriate density and cell morphology and adherence were evaluated.

2.3. Cell viability assay

The cytotoxicity of SPC to rat chondrocytes was determined by the CCK-8, according to the manufacturer's guidelines. In brief, P3 chondrocytes were seeded in 96-well plates (50,000 cell/cm^2). When cells reached a confluency of 90%–95%, culture media were replaced for medium containing 0, 50, 100, 200, 400, 600, or 800 μM SPC, and cells were cultured for 24 h or 48 h. Then, 10 μl of CCK-8 solution was added to each well and after 2 h, the absorbance was measured at a wavelength of 450 nm. Experiments were performed five times.

2.4. ELISA assay

ELISA kits (R&D Systems, Minneapolis, MN USA) were used, according to the manufacturer's instruction. All assays were performed five times.

2.5. Griess reaction

The production of nitric oxide in the culture medium was detected indirectly by the Griess reaction as previously described [17]. Briefly, Griess reagent was prepared, added to the culture medium, and incubated for 10 min. The absorbance was determined at 543 nm.

2.6. Western blot analysis

Chondrocytes were collected and total proteins were extracted from cells. The protein was isolated using RIPA lysis buffer with 1 mM PMSF (Phenylmethanesulfonyl fluoride) and on the ice for 10 min followed by 15 min centrifugation at 12,000 RPM and 4 °C, and then concentration was measured using the BCA protein assay kit (Beyotime). 40 ng of proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and separated by electrophoresis. Then, proteins were transferred to PVDF membranes (Bio-Rad, USA). Membranes were blocked with 5% nonfat milk for 2 h and incubated with primary antibodies against aggrecan (1:1000), ADAMTS-5 (1:1000), AKT (1:1000), COX-2 (1:1000), GADPH (1:5000), p-IκBα (1:1000), IκBα (1:1000), iNOS (1:1000), MMP-3 (1:1000), MMP13 (1:1000), p65 (1:1000), p-p65 (1:1000), and, PI3K(p110) (1:1000), PI3K(p85) (1:1000), p-AKT (1:1000), p-IKKα/β (1:500) and Sox9 (1:250) overnight, 4 °C. Next, membranes were incubated with appropriate enzyme-linked secondary antibodies for 2 h at room temperature. To visualize immunoblots, enhanced chemiluminescence (ECL) solution was used according to the manufacturer's instructions.

2.7. Immunofluorescence analysis

For assessing ECM synthesis by chondrocytes, type II collagen levels were measured in the cytoplasm. MMP-13 levels were determined in the cytoplasm for assessment of ECM breakdown. Chondrocytes were fixed with 4% paraformaldehyde, then with 0.5%–2% Triton X-100, blocked with 10% Lowlenthal serum, and transferred to a wet box. Chondrocytes were incubated overnight with primary antibodies: collagen II (1:200), MMP-13 (1:200), p65 (1:200). On the next day, the cell washed with PBS, and incubated with Alexa Fluor®488 labeled or Alexa Fluor®594 conjugated second antibodies (1:400) in the dark for 1 h at room temperature. Finally, nuclei were stained with DAPI, anti-fluorescence-quenching agent was used to mount the cover slip. Nail enamel was applied to seal the slides, which were preserved at 4 °C.

2.8. RNA isolation and real-time polymerase chain reaction

Chondrocytes were seeded in DMEM/F-12 in 6-well plates at a density of 3×10^5 cells/ml and incubated for 24 h. After stimulation with IL-1β and SPC at various concentrations (0, 25, 50, and 100 μM), total RNA was isolated from the monolayer of cultured chondrocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 2000). The quality and purity of RNA was determined by A260/A280 ratio. First-strand cDNA was synthesized using 1000 ng of total RNA and a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Real-time PCR (RT-PCR) was performed using the CFX96Real-TimePCR System (Bio-Rad Laboratories, CA, USA), using the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The reaction was

Table 1
Primer sequences used in real-time PCR.

Gene	Forward primer	Reverse primer
COX-2	5′-GAGAGATGTATCCTCCCACAGTCA-3′	5′-GACCAGGCACCAGACCAAAG-3′
iNOS	5′-CCTTACGAGGCGAAGAAGGACAG-3′	5′-CCTTACGAGGCGAAGAAGGACAG-3′
IL-6	5′-GACAGCCACTCACCTCTTCA-3′	5′-TTCACCAGGCAAGTCTCCTC-3′
TNF-α	5′-GTCAGATCATCTTCTCGA ACC-3′	5′-CAGATAGATGGGCTCATACC-3′

performed in a total volume of 10 μl (4.5 μl diluted cDNA, 0.25 μl forward primer, 0.25 μl reverse primer, and 5 μl SYBR Green Master Mix). Target mRNA levels were normalized to the level of GAPDH which was used as a control. Data were analyzed using the $2^{-\triangle\triangle CT}$ method [18]. Experiments were performed in triplicate and primer sequences are listed in Table 1.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP kit (Thermo Scientific), according to manufacturer protocol. Chondrocytes were exposed to IL-1β for 2 h, alone or in pre-treated with SPC (200 μM), and then samples were subjected to ChIP assay. For immunoprecipitation, primary antibody p65 and normal rabbit IgG were used to incubate with the protein-DNA complex overnight at 4 °C, and then A/G agarose was used to precipitate the fragments. The purified DNA from ChIP samples and input DNA was subjected to real-time PCR analysis for DNA quantification. The specific MMP-13 promoter primers were as follows: forward primer 5′-ACAGTTCAGGCTCAACCTGCTG-3′; reverse primer 5′-GCCC TATCCCTTGATGCCATT-3′.

2.10. Animal experiments

All animal experiments were performed as per the guidelines of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and were approved by the Animal Care and Use Committee of Wenzhou Medical University (Wenzhou, China). In this study, 6-week-old male Sprague-Dawley rats (200 ± 20 g) were used. Animals were acclimatized to the laboratory environment for 1 week prior to start of the experiments. Rats were randomly divided into three groups (n = 15 rats per group), including a sham-operated group (Sham), an OA group (destabilization of the medial meniscus (DMM)), and a SPC-treated OA group (DMM + SPC). In rats in the DMM group and DMM + SPC group, DMM was induced as previously reported [19]. After induction of DMM, rats in the DMM + SPC group received SPC treatment immediately, which was dissolved in 0.5% carboxymethylcellulose sodium, and was given by intragastric administration at a dose of 10 mg/kg/d for 8 consecutive weeks until sacrificed. Meanwhile, rats in the DMM group were given 0.5% carboxymethylcellulose sodium without SPC in a similar fashion.

2.11. Histological analysis

Slides of each joint were stained with safranin O-fast green (S-O). The cellularity and morphology of the cartilage was examined by a separate group of experienced histology researchers who were blind to the grouping using a light microscope and evaluated using the Osteoarthritis Research Society International (OARSI) scoring system for the medial femoral condyle and medial tibial plateau. The destruction of articular cartilage was graded using the Osteoarthritis Research Society International (OARSI) scoring system for medial femoral condyle and medical tibial plateau. OARSI scoring system for each quadrant (including four quadrants of the joint: medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), lateral tibial plateau (LTP)). Then we used a summed OARSI score (0 − 10) from MFC and MTP to evaluate the degree of articular cartilage. Then, we used a summed osteophytes score (0–4) in the tibia to evaluate the osteophytes formation. The details of this scoring system are showed below [20].

2.12. Experimental design

For the *in vitro* study, chondrocytes were treated with 10 ng/ml IL-1β, alone or in pre-treated with SPC at different concentration (50, 100 and 200 μM). A control group was left untreated except for medium change. Cells were harvested after 24 h of incubation. For assessing ECM synthesis by chondrocytes, type II collagen levels were measured in the cytoplasm. MMP-13 levels were determined in the cytoplasm for assessment of ECM breakdown. For p65 staining, the duration of IL-1β treatment was down to 2 h.

For *in vivo* assessment, rats underwent surgical destabilization of the medial meniscus (DMM) as described above. After DMM, SPC administration groups (10 mg/kg/day; received SPC dissolved in carboxymethylcellulose (CMC) by intragastric administration once a day for eight consecutive weeks). Meanwhile, rats in sham groups and DMM group were administered an equivalent volume of CMC. All animals were sacrificed after eight weeks post-surgery, and cartilage tissue sample were collected for histological analysis.

2.13. Statistical analysis

Experiments were at least performed five times. Data are presented as the mean ± S.D. Statistical analyses were performed using SPSS statistical software version 16.0. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey's test for comparison between groups. Non-parametric data (like OARSI score) were analyzed by the Kruskal–Wallis H test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of sophocarpine on chondrocyte viability

The chemical structure of SPC is shown in Fig. 1A. The cytotoxicity of SPC on chondrocytes was tested by CCK-8 assay. No significant reduction in cell viability was observed after treatment with different concentrations of SPC (0, 50, 100, 200 μM) for different time periods (24 h or 48 h) (Fig. 1B). Therefore, we selected SPC doses of 50, 100 and 200 μM as treating concentrations in consequent experiments.

3.2. Sophocarpine inhibits the production of inflammatory mediators in IL-1β-stimulated chondrocytes

The effect of SPC on IL-1β-induced (10 ng/ml) inflammation in chondrocytes was evaluated. Both gene and protein levels of COX-2 and iNOS were reduced by SPC treatment in a dose-dependent manner (Fig. 2A, D). As shown in Fig. 2B, SPC concentrations of 100 and 200 μM significantly inhibited COX-2 and iNOS protein levels compared to IL-1β-treated chondrocytes ($P < 0.01$). In addition, PGE2 and NO were significantly reduced ($P < 0.01$) when compared to the IL-1β cells treated with 100 and 200 μM SPC (Fig. 2C). Moreover, IL-6 and TNF-α, which were upregulated by IL-1β treatment ($P < 0.01$), were also inhibited by SPC in a dose-dependent manner (Fig. 2C). Moreover,

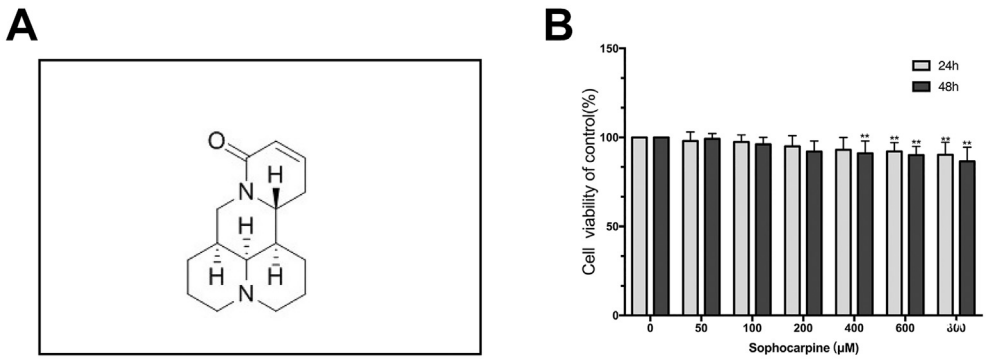

Fig. 1. The effect of sophocarpine on chondrocyte viability. (A). Chemical structure of sophocarpine. (B). The cytotoxic effect of sophocarpine on chondrocytes was determined at various concentrations for 24 and 48 h using a CCK8 assay. Data are presented are the means ± S.D. of five independent experiments. **P < 0.01 *vs.* control group, n = 5.

at the concentration of 50 μM, SPC inhibited the mRNA expressions of IL-6 and TNF-α but have no inhibitory effect on protein expressions. At concentrations of 100 and 200 μM, SPC significantly inhibited TNF-α and IL-6 generation at both the protein and mRNA level. (Fig. 2D) Together, these findings suggested that SPC inhibited the production of these inflammatory cytokines at both the gene and protein level in a dose-dependent manner.

3.3. Sophocarpine protects chondrocytes from IL-1β-induced ECM degradation

Stimulation of IL-1β (10 ng/ml) resulted in reduced levels of aggrecan and SOX-9, whereas ADAMTS-5, MMP-3, and MMP-13 were increased at the protein level (Fig. 3A). However, SPC pretreatment reversed these data to a certain extent. Levels of SOX-9 and aggrecan were increased, whereas ADAMTS-5, MMP-3, and MMP-13 levels were reduced in a dose-dependent manner when compared to the IL-1β-treated group (Fig. 3B). Furthermore, immunofluorescence analysis showed that, in contrast to the IL-1β group, treatment with SPC inhibited IL-1β-stimulated MMP-13 generation and collagen II degradation (Fig. 3C–F). Thus, these data consistently showed that treatment with SPC protected chondrocytes from IL-1β-induced ECM degradation.

3.4. Sophocarpine inhibits NF-κB activation in IL-1β-stimulated chondrocytes

We next explored the mechanism involved in SPC protection of chondrocytes. Our findings indicated that protein levels of p-IκBα and p-P65 were down-regulated by SPC in contrast to IL-1β-treated (10 ng/ml) cells (Fig. 4A, C). Moreover, we investigated the translocation of P65 from the cytoplasm to nucleus and found that treatment with SPC inhibited the translocation of P65 to the nucleus (Fig. 4B). ChIP assay was conducted in chondrocytes treated with IL-1β for 2 h. The results showed that p65 could directly bind to the MMP-13 promoter after IL-1b treatment, and SPC can decrease this phenomenon. (Fig. 4D) Therefore, we concluded that SPC treatment reduced IL-1β-induced over-activation of NF-κB signaling in chondrocytes.

3.5. Sophocarpine regulates the PI3K/AKT signaling pathway in IL-1β-stimulated chondrocytes

We next evaluated whether PI3K/AKT are upstream signaling molecules that are influenced by SPC. Our data showed that PI3K (P110), PI3K (P85), p-AKT, and p-IKKα/β were consistently down-regulated by SPC treatment in a dose-dependent manner when compared to IL-1β-treated (10 ng/ml) cells (Fig. 5). Thus, it can be deduced that SPC exerted inhibition of IL-1β-induced activation of the PI3K/AKT/NF-κB signaling pathway.

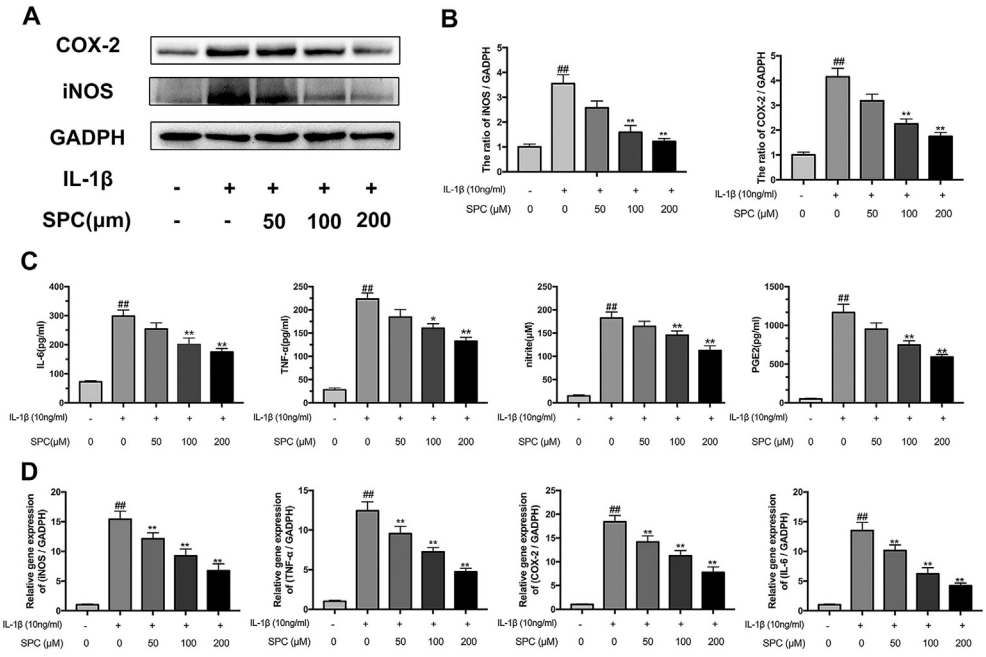

Fig. 2. Effects of Sophocarpine on IL-1β-induced inflammatory mediators. Protein levels of COX-2 and iNOS were determined by Western blot analysis (A–B). Inflammatory mediators in response to IL-1β stimulation, including IL-6, TNF-α and PGE2 were determined by ELISA assay, whereas NO levels were determined by the Griess reaction (C). Real-time PCR analysis of COX-2, iNOS, TNF-α, and IL-6 (D). Data are presented as the mean ± S.D. of five independent experiments. ##P < 0.01 compared to the control group. *P < 0.05, **P < 0.01 compared to the IL-1β-treated group.

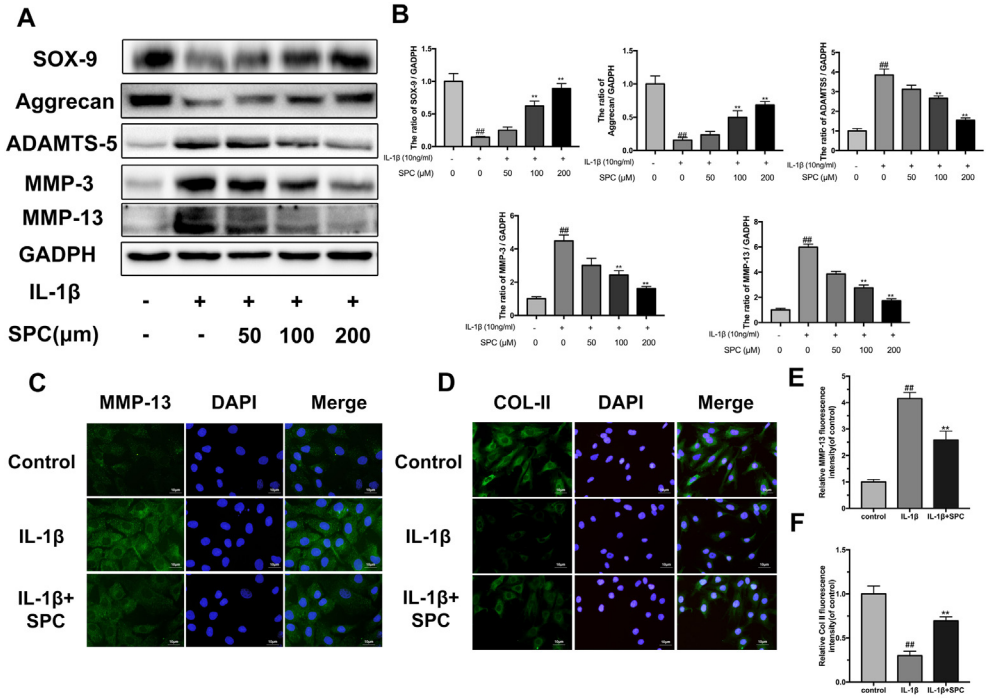

Fig. 3. Effects of sophocarpine on IL-1β-induced extracellular matrix degradation. Western blot analysis and quantification analysis showing protein levels of SOX-9, aggrecan, ADAMTS-5, MMP-3, MMP-13, and GAPDH after different treatments. (A–B). MMP-13 and collagen II were evaluated by immunofluorescence analysis (C–F), combined with DAPI staining to identify nuclei (original magnification ×400, scale bar: 10 μm). (E–F) Data are presented as the mean ± S.D. of five independent experiments. $^{\#\#}P < 0.01$ compared to the control group. *P < 0.05, **P < 0.01 compared to the IL-1β-treated group.

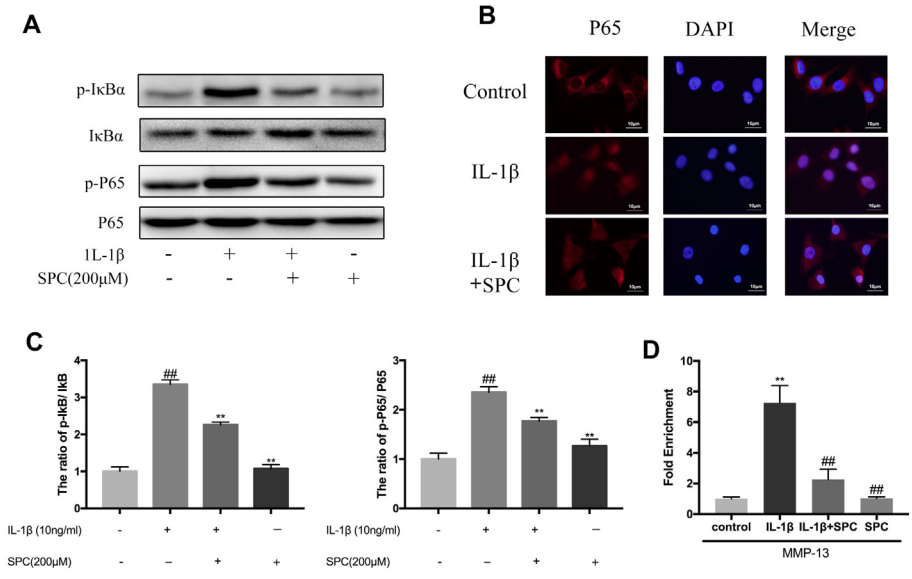

Fig. 4. Effects of sophocarpine on IL-1β-induced activation of the NF-κB pathway in chondrocytes. Western blot analysis (A) and quantification analysis (C) showing the protein level of p-IκBα, p-P65, IκBα, and P65 after different treatments. (B). P65 translocation was determined by immunofluorescence analysis, combined with DAPI staining to identify nuclei (original magnification ×400, scale bar: 10 μm). (D). CHIP samples were performed real-time PCR and analyzed with a fold enrichment method. Data are presented as the mean ± S.D. of five independent experiments. $^{\#\#}P < 0.01$ compared to the control group. *P < 0.05, **P < 0.01 compared to the IL-1β group.

3.6. Sophocarpine alleviated the development of osteoarthritis in a rat destabilization of the medial meniscus model

An OA model in rats was established to evaluate the effect of SPC *in vivo*. The DMM model was established and SPC treatment was given for 8 consecutive weeks. As shown in Fig. 6A, OA was evaluated by safranin O-fast green to evaluate the protecting effects of SPC on articular cartilage. OARSI scores and osteophytes scores showed a significant difference after SPC treatment in DMM model rats when compared to OA group (Fig. 6B–C). The cartilage surface was smooth in the sham control group. The OA group showed cartilage erosion when compared to the sham control group (Fig. 6A). However, in the DMM + SPC group, the cartilage surface appeared smoother than that of the DMM group. In addition, the degeneration of cartilage was also observed in the DMM + SPC group, hypocellularity and some proteoglycan loss were observed compared to the sham group. However, compared with the DMM group, the articular surface remains basically smooth, which can prove SPC can slow down the degeneration of the articular surface. Consistent with the histological results, the OARSI scores and osteophytes scores of rats in the DMM group (Fig. 6B–C) were markedly higher compared to those of the sham control group ($P < 0.01$). In contrast, rats in the SPC group had lower scores compared to that of rats in the OA group ($P < 0.01$). In conclusion, SPC treatment ameliorated OA *in vivo*.

4. Discussion

OA has been shown to be an inflammatory arthropathy and more than just a simple wear-and-tear disease. NSAIDs have been largely used for relieving clinical symptoms, which inevitably resulted in several side effects [11]. Thus, identifying novel, effective and safer drugs for the treatment of OA is of utmost importance. SPC, however, has

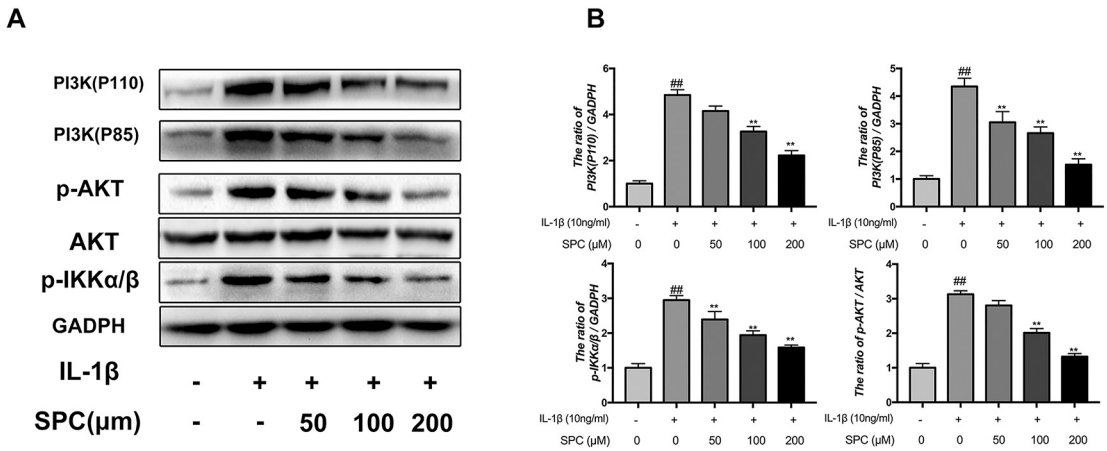

Fig. 5. Sophocarpine reduces IL-1β-induced phosphorylation of IKKα/β by inhibiting PI3K/AKT signaling. Western blot analysis (A) and quantification analysis (B) showing the protein level of P110, P85, p-AKT, AKT, p-IKKα/β, and GAPDH after different treatments. Data are presented are the mean ± S.D. of three independent experiments. $^{\#\#}P < 0.01$ compared to the control group. *P < 0.05, **P < 0.01 compared to IL-1β group.

demonstrated anti-inflammatory effects in several fields [12]. In this study, we evaluated the effects of SPC in down-regulating inflammatory mediators *in vitro* and in inhibition of OA progression *in vivo*. SPC administration had low cytotoxic effects on chondrocytes, however, no significant side effects have been observed in a rat model at the dosages evaluated in this study.

Inflammation takes great part in the progression of OA [21]. The synovium and chondrocytes are likely to release inflammatory mediators, such as IL-1β, which may initiate inflammatory responses [22]. In this study, IL-1β (10 ng/ml) was used for the induction of inflammation in chondrocytes. SPC treatment not only effectively down-regulated the inflammatory mediators in chondrocytes, including TNF-α, IL-6, PGE2 and NO, but also inhibited the degeneration of aggrecanase and collagenase. These findings suggested that anti-inflammatory activity may

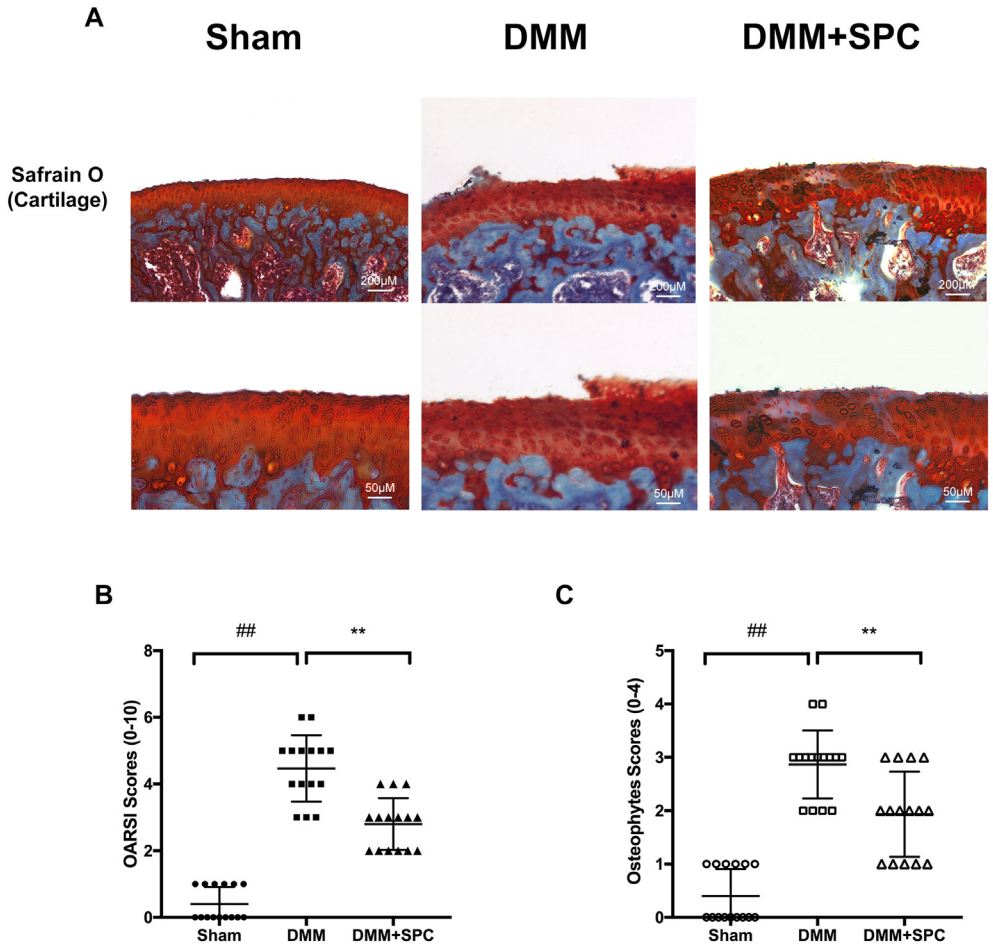

Fig. 6. Effects of sophocarpine on osteoarthritis *in vivo*. Safranin O-fast green (S-O) staining (original magnification ×100 or ×400, scale bar: 200 μm or 50 μm) (A). Osteoarthritis Research Society International (OARSI) scores were evaluated and analyzed (B). Osteophytes scores were evaluated and analyzed (C). $^{\#\#}P < 0.01$ compared to the control group. *P < 0.05, **P < 0.01 compared to the DMM group.

be the main mechanism by which SPC functions in OA.

The NF-κB pathway plays a crucial role in inflammation due to its ability to promote the transcription of inflammatory mediators [23]. To explore whether SPC exerted anti-inflammatory effects *via* the NF-κB pathway, we determined IκB and IKKα/β at protein level as well as the nuclear translocation of P65. The data suggested that NF-κB inactivation was consistent with down-regulation of inflammatory mediators by treatment with SPC, which confirmed the involvement of NF-κB in OA. Furthermore, the PI3K/AKT pathway, an upstream pathway, which can activate NF-κB, is also down-regulated by SPC in IL-1β-induced chondrocytes, indicating that SPC may target a component that is located more upstream. However, additional studies must be performed for unraveling the precise mechanisms of SPC function. Furthermore, several upstream signaling, such as Wnt/β-catenin and Hedgehog pathway, have been reported to have a dramatic impact on the progression of osteoarthritis [26]. But the effect of SPC on this pathway is still unknown, so we wish to research in a future study.

In vivo, SPC was orally gavaged at 10 mg/kg/d. Pharmacokinetic and pharmacodynamic studies of SPC in rats and rabbits have been described in a previous study [24]. In this study, DMM rats were used as an osteoarthritic model. Histological analysis indicated that SPC prevented the joint from degeneration. Characteristics of OA such as cartilage erosion are overtly reversed by SPC treatment. Although no obvious troublesome effects were observed at the treatment dosage tested in our study, whether SPC interferes with the biological functions of other systems remains to be elucidated. In addition, the application of SPC in human OA must be investigated in further study.

In summary, our study has verified the potential of SPC in protecting cartilage from OA inflammatory degeneration both *in vitro* and *in vivo*. Thus, SPC may serve as a novel anti-inflammatory agent for the treatment of OA.

Compliance with ethical standards

The study was in accordance with the Declaration of Helsinki and Tokyo.

Conflict of interest

The authors declare that they have no conflict of interest.

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