

Spinosin ameliorates osteoarthritis through enhancing the Nrf2/HO-1 signaling pathway

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ABSTRACT

Osteoarthritis (OA) is a common degenerative joint disease in the elderly, while oxidative stress-induced chondrocyte degeneration plays a key role in the pathologic progression of OA. One possible reason is that the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), which acts as the intracellular defense factor against oxidative stress, is significantly inhibited in chondrocytes. Spinosin (SPI) is a potent Nrf2 agonist, but its effect on OA is still unknown. In this study, we found that SPI can alleviate *tert*-Butyl hydroperoxide (TBHP)-induced extracellular matrix degradation of chondrocytes. Additionally, SPI can effectively activate Nrf2, heme oxygenase-1 (HO-1), and NADPH quinone oxidoreductase 1 (NQO1) in chondrocytes under the TBHP environment. When Nrf2 was silenced by siRNA, the cartilage protective effect of SPI was also weakened. Finally, SPI showed good alleviative effects on OA in mice. Thus, SPI can ameliorate oxidative stress-induced chondrocyte dysfunction and exhibit a chondroprotective effect through activating the Nrf2/HO-1 pathway, which may provide a novel and promising option for the treatment of OA.

Key words: osteoarthritis; Nrf2; cartilage protection; pain relief.

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Introduction

Osteoarthritis (OA) is a chronic, painful inflammation resulting from a combination of mechanical, genetic, metabolic, and inflammatory pathways, characterized by degeneration and destruction of articular cartilage and bone proliferation.¹ With the aging population and the increasing prevalence of obesity in recent years, the incidence of OA is on the rise. Although the stepwise treatment approach for arthritis is widely accepted, patients in advanced stages still require joint replacement surgery.² There is a lack of specific drugs for OA; commonly used clinical treatments such as non-steroidal anti-inflammatory drugs, glucosamine, and chondroitin sulfate provide anti-inflammatory and analgesic effects, but do not reverse the degenerative changes in articular cartilage.³

Prolonged mechanical stress, recurrent inflammation, and metabolic dysregulation contribute to accelerated production of reactive oxygen species (ROS) within chondrocytes. The overproduction of ROS, in turn, induces chondrocyte apoptosis through the initiation of oxidative stress.^{4,5} In addition, several pro-inflammatory cytokines, such as tumor necrosis factor, interleukin-1 β , and interleukin-6, accelerate the degradation of chondrocyte extracellular matrix and upregulate the expression of type II collagen (Col II), which further promotes chondrocyte apoptosis.⁶ Increasing evidence suggests that there is a significant oxidative stress phenomenon within the chondrocytes of OA patients that inhibits the synthesis of collagen and proteoglycans in the cartilage matrix, thereby accelerating the progression of inflammation.^{7,8}

Numerous cellular signaling pathways have been documented in this context. The nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) axis is recognized as one of the established intracellular defense mechanisms against oxidative stress.⁹ Nrf2, a cytoprotective antioxidant nuclear transcription factor, normally binds to Keap1 in a quiescent state and maintains low transcriptional activity.¹⁰ However, when exposed to oxidative stress, activated Nrf2 dissociates and binds to antioxidant response elements, thereby regulating the downstream expression of HO-1. HO-1 attenuates inflammation mediated by pro-inflammatory cytokines by catalyzing heme degradation, ultimately attenuating cytotoxicity induced by oxidative stress and inflammatory responses.¹¹⁻¹⁴

Spinosin (SPI), a flavonoid compound and one of the major active constituents isolated from the seeds of *Zizyphus jujuba* var. *Spinosa*, has been suggested by previous research to mitigate oxidative stress damage in cells by activating the Nrf2/HO-1 pathway and thereby inhibiting the activity of related proteins.^{15,16} However, it remains unclear whether SPI can prevent chondrocyte apoptosis by blocking oxidative stress. Therefore, this study aims to explore the effects of SPI on oxidative stress-induced chondrocyte apoptosis and to elucidate the molecular mechanisms regulated by the Nrf2/HO-1 pathway.

Materials and Methods

Cell culture and treatment

Human normal chondrocytes C28/I2 cell line (HTX2308, Otwo Biotech, Shenzhen, China) were cultured in DMEM containing 10% FBS in a 37°C cell culture incubator with 5% CO₂. When the confluence reached 80%, C28/I2 cells were treated with SPI (HY-N0651, Med Chem Express, USA) for 2 h before tert-Butyl hydroperoxide (TBHP) (100 μ M) (458139; Sigma-Aldrich, Darmstadt, Germany) administration, which is more stable than

H₂O₂ as ROS for *in vitro* studies of OA,¹⁷ for 48 h.

Cell viability assay

After 3 days of cultivation, the medium in each well was aspirated and washed 3 times with PBS. Subsequently, CCK8 solution (HY-K0301; MedchemExpress, Monmouth Junction, NJ, USA) was used to incubate cells for 2 h in a cell incubator. Then, 100 μ L of medium from each well was aspirated into a 96-well plate, followed by testing absorbance value at 450 nm by a microplate reader.

Cell transfection

Transfection of small interfering RNA (siRNA) was performed to knockdown the Nrf2 in C28/I2 cells. Three siRNAs purchased from Sangon Biotechnology (Shanghai, China) were primarily tested to screen out the most efficient one: siRNA1, 5'-UGACA-GAAGUUGACAAUUATT-3'/5'-UAAUUGUCAACUUCUGU-CATT-3'; siRNA2, 5'-GCAGCAAACAAGAGAUGGCAATT-3'/5'-UUGCCAUCUCUUGUUUGCUGCTT-3'; siRNA3, 5'-GAGAAAGAAUUGCCUGUAATT-3'/5'-UUACAGGCAAUUCUUUCUUCTT-3'; negative control (NC), 5'-UUCUCC-GAACGUGUCACGUTT-3'/5'-ACGUGACACGUUCGGA-GAATT-3'. The lipofectamine 2000 reagent kit (11668500; ThermoFisher Scientific, Waltham, MA, USA) was applied in accordance with the manufacturer's instructions to transfect cells with siRNA^{Nrf2} or negative control siRNA (si^{NC}). Western blotting was then performed to detect the knockdown efficiency.

Western blot assay

The whole Cell Lysis Assay Kit (KGB5303; Keygen Biotech, Nanjing, China) was applied to isolate total proteins following the manufacturer's instructions. Protein concentration was then quantified by the BCA protein assay kit (P0012S; Beyotime Biotech, Haimen, China). After electrophoresis on SDS-polyacrylamide gel, proteins were transferred to a PVDF membrane, and then incubated with the primary antibodies including anti-type II collagen (1:1000, A00517-1; Boster Bio, Wuhan, China), anti-MMP1 (1:1000, 10371-2-AP; Proteintech, Rosemont, IL, USA), anti-MMP3 (1:1000, 17873-1-AP; Proteintech), anti-MMP13 (1:1000, 18165-1-AP; Proteintech), anti-Nrf2 (1:1000, 16396-1-AP; Proteintech), anti-HO-1 (1:1000, 10701-1-AP; Proteintech), and anti-NQO1 (1:1000, 11451-1-AP; Proteintech). The anti- β -actin (1:1000, AC026; ABclonal, Wuhan, China) was used as the internal control. The membrane was further incubated with an HRP-conjugated secondary antibody (1:10000, RGAR001; Proteintech) and then visualized with extremely sensitive ECL chemiluminescence kit (P10060; NewCell & Molecular Biotech, China). Finally, the ImageJ software was used to analyze the band density.

Cytoskeleton/nuclear staining

C28/I2 cells were fixed with 4% paraformaldehyde (PFA) for 20 min. After 3 times of washing with PBS, the cells were incubated with Alexa Fluor™ 488 phalloidin (A12379; Thermo Fisher Scientific) at room temperature and sheltered from light for 30 min. After discarding the dye solution, the sample was washed three times with PBS. Subsequently, it was treated with ready-to-use DAPI (KGA1808; Keygen Biotech) solution for 5 min, and then photographed using a fluorescence microscope.

Immunofluorescence assay

C28/I2 cells were fixed with 4% PFA for 20 min, and then permeabilized with Triton X-100 (0.2%) for 20 min. After cleaning, cells were blocked by goat serum (10%) (AR0009; Boster Bio) for 2 h. Then anti-Nrf2 primary antibody (1:100, 16396-1-AP; Proteintech) was used to incubate cells overnight at 4°C. Then the cells were treated with a FITC-conjugated secondary antibody

(1:200, SA00003-2; Proteintech, Wuhan, China) for 1 h, and the nuclei were stained with ready-to-use DAPI (KGA1808; Keygen Biotech) solution for 5 min. Images were acquired using a fluorescence microscope (Zeiss Axiovert 200, objective: 40x, light source: mercury lamp; Carl Zeiss Inc., Hebron, OH, USA). Fluorescence intensity and nuclear translocation were observed.

ROS Assay

Cells were collected and adjusted to a concentration of 1×10^6 /mL. The 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, KGA7308-100; Keygen Biotech) was diluted with serum-free culture medium to a final concentration of 10 μ M. Then the DCFH-DA was used to incubate cells in a 37°C cell culture incubator for 20 min. After that, the cells were washed three times with serum-free cell culture medium to completely remove any DCFH-DA that had not entered the cells. The intracellular ROS was detected by the flow cytometry (Ex = 488 nm, Em = 525 nm, CytoFLEX; Beckman Coulter, Brea, CA, USA).

Animal model

The 12-week-old wild-type C57BL/6 male mice were acquired from the Model Animal Research Center of Nanjing University (Nanjing, China). Firstly, the mice were housed in an SPF grade animal room and kept at a constant temperature, humidity, and light for one week to adapt to the environment. Then, they were randomly divided into sham group, destabilization of the medial meniscus (DMM) group, SPI (20 mg/kg, *per os*, once daily for 8 weeks) group, and SPI treated DMM group.¹⁸ The DMM surgical process is as described in previous studies.¹⁹ In brief, a microsurgical knife was used to cut the medial meniscus ligament which helps the medial meniscus attach to the tibial plateau, while identifying and protecting the lateral meniscus ligament. In the sham group, the medial meniscus ligament was exposed without being cut. The mice were completely free to move after undergoing DMM surgery. The use and experimental protocol of experimental animals have been approved by the Ethics Committee and Institutional Animal Care and Use Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School (approval protocol number: 2020AE0113).

Histological analysis

At 8 weeks after surgery, the samples of knee joints from mice were fixed with 4% PFA and decalcified with EDTA (10% v/v) for 2 months. Then the samples were embedded in paraffin. The joints were cut into 5 μ m sections and stained with Safranin O/Fast green (BL1105A, Biosharp, Heifei, China). Briefly, after being stained with Weigert staining solution for 4 min, the sections were differentiated twice with an acidic ethanol differentiation solution for 15 s. Subsequently, the sections were immersed in Fast green solution for 3 min, followed by immersion in Safranin O solution for 5 min. Samples were washed with an acetic acid solution for 2 min, and then dehydrated with 95% ethanol and anhydrous ethanol, respectively. The Osteoarthritis Research Society International (OARSI) scoring system was applied to evaluate the degeneration of cartilage.

Immunohistochemistry

Primarily, a citrate buffer (10 mM, pH 6.0) was used for antigen retrieval, followed by endogenous peroxidase blocking by incubating the sections at 100°C for 1 h. Subsequently, the sections were blocked with bovine serum albumin (BSA, 10%) and then incubated with a primary antibody against Col II (1:100, BA0533; Boster Bio) overnight at 4°C. In the negative control group, PBS was used instead of the primary antibody to incubate the sections. The next day, after washing away the primary antibody, a second-

ary antibody conjugated with peroxidase (1:1000, ab6112; Abcam, Cambridge, UK) was applied to incubate samples at 37°C for 1 h. Finally, the sections were treated with diaminobenzidine (DAB) horseradish peroxidase coloration reagent kit (P0203; Beyotime Biotech). The ImageJ software (version 1.8, National Institutes of Health, Bethesda, USA) was used to assess the optical density of Col II in cartilage regions.^{20,21}

Statistical analysis

All experiments are repeated at least three times. Data are exhibited as mean \pm SD. Differences between groups were tested by one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and $p < 0.05$ was identified as statistically significant.

Results

SPI attenuates the ROS and damage in C28/I2 cells induced by TBHP

Primarily, we tested the cell compatibility of SPI with various concentrations. After 3 days of culturing C28/I2 cells, cytoskeletal and nuclear staining revealed that the cell count was highest at a SPI concentration of 25 μ M. Conversely, in the 50 μ M SPI group, the number of C28/I2 cells decreased significantly (Figure 1A). The results of CCK8 test further verified this conclusion (Figure 1B). As the concentration of SPI increased from 0 μ M to 25 μ M, the number of C28/I2 cells in each group appeared to gradually increase without statistical difference. When the concentration of SPI further reached 50 μ M, the number of C28/I2 cells decreased significantly with a statistically significant difference. Therefore, SPI with a concentration of 25 μ M was chosen for the follow-up experiments. Then, we investigated the impact of SPI on ROS in C28/I2 cells using flow cytometry (Figure 2 C,D). The results showed that the ROS expression of C28/I2 cells was significantly increased under TBHP environment, while SPI intervention could significantly reduce ROS levels, indicating that SPI effectively alleviated the oxidative stress state of C28/I2 cells. Subsequently, the influence of TBHP and SPI on the extracellular matrix of C28/I2 cells was examined through Western blot (WB). As shown in Figure 1 E,F, after 2 days of TBHP administration, the expression level of Col II in C28/I2 cells decreased significantly. When further treated with SPI, the C28/I2 cells exhibited approximately twice the expression level of Col II compared to the TBHP group. Subsequently, we also examined the effects of TBHP and SPI on MMPs in chondrocytes. We found that TBHP treatment significantly increased the expression of MMP1 (Figure 1 E,G), MMP3 (Figure 1 E,H), and MMP13 (Figure 1 E,I), and that these levels were normalized by further treatment with SPI. These results suggest that SPI can alleviate TBHP-induced chondrocyte injury.

SPI activates the Nrf2 signaling pathway in C28/I2 cells under oxidative stress

Since SPI is an Nrf2 activator, we investigated SPI-mediated Nrf2 regulation. Under normal circumstances, Nrf2 expression was low in the nucleus of C28/I2 cells, while after SPI treatment, Nrf2 expression became concentrated in the nucleus, and fluorescence intensity significantly increased. We first demonstrated that SPI significantly increased the abundance of Nrf2 protein in C28/I2 cells as its concentrations gradually increased from 0 μ M to 25 μ M (Figure 2 A,B), along with the levels of its downstream targets HO-1 (Figure 2 A,C) and NQO1 (Figure 2 A,D) increasing in a concentration-dependent manner. Additionally, immunofluores-

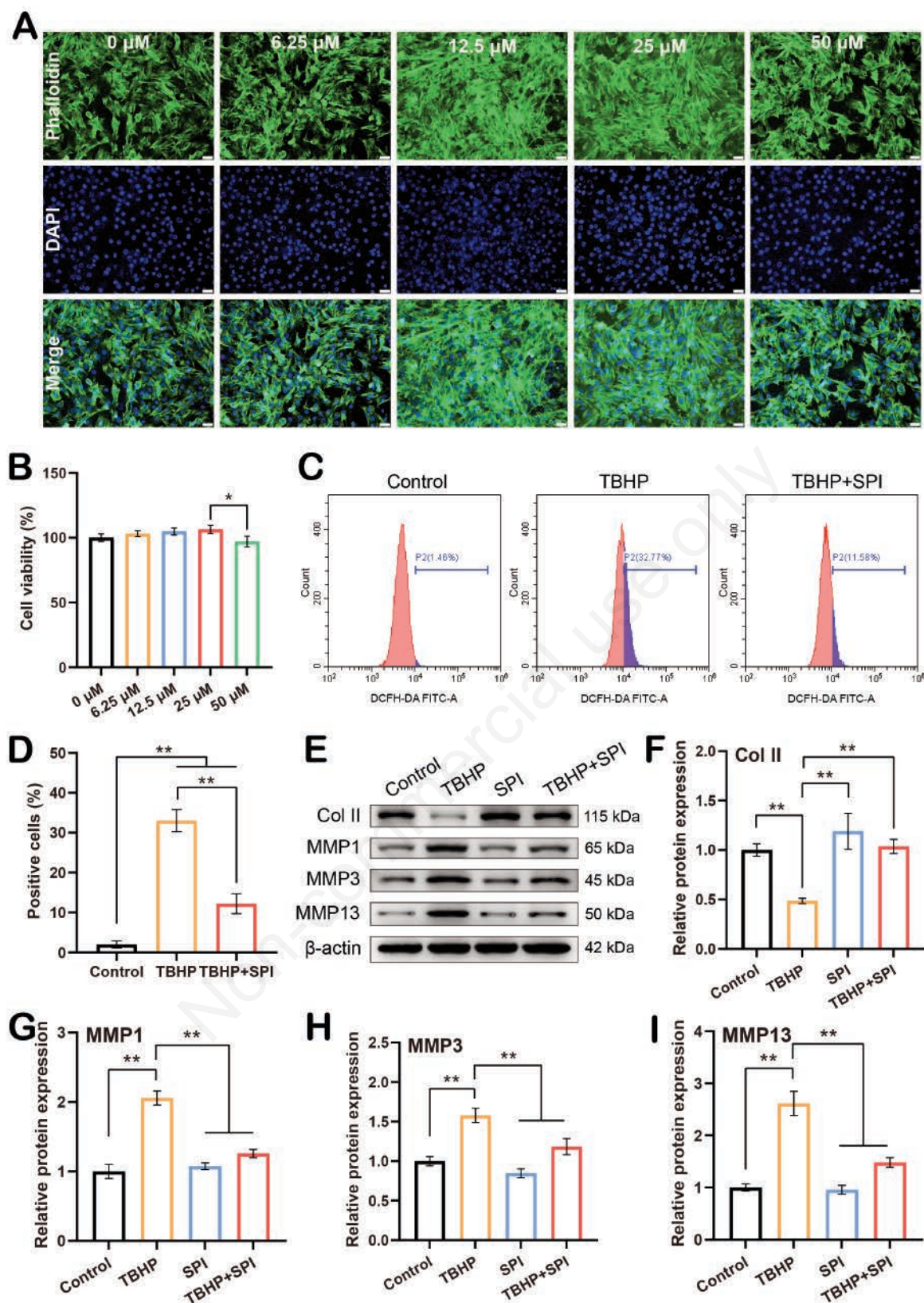


Figure 1. SPI can attenuate the ROS and damage of C28/I2 cells induced by TBHP. **A**) Representative images of C28/I2 cells stained with phalloidin/DAPI after treatment with various doses of SPI for 24 h; scale bars: 200 μm . **B**) Cell viability assessed using CCK8 assay after 24 h of SPI administration (n=3). **C**) Percentage of DCFH-DA-positive C28/I2 cells detected by flow cytometry. **D**) Quantitative analysis of the flow cytometry. **E**) Western blot of Col II, MMP1, MMP3, MMP13, and β -actin protein levels in C28/I2 cells with different interventions. **F-I**) Quantification analyses of Col II (F), MMP1 (G), MMP3 (H), and MMP13 (I) expression levels in C28/I2 cells with different interventions (n=3). * $p < 0.05$, ** $p < 0.01$.

cence staining confirmed that the activation of SPI on Nrf2 in C28/I2 cells was mediated by nuclear translocation (Figure 2E). Subsequently, we further tested whether SPI could reverse the changes in the Nrf2-related signaling pathway induced by TBHP. Results of WB (Figure 2 F-I) showed that after TBHP administration, the levels of Nrf2 in C28/I2 cells decreased significantly, and the expression of proteins such as HO-1 and NQO1 also decreased concurrently. However, after adding SPI the downregulation trend of these proteins was effectively relieved, indicating that SPI can reverse the inhibitory effect of TBHP on Nrf2.

SPI attenuates the damage of C28/I2 cells under oxidative stress through the Nrf2 signaling pathway

To determine if the beneficial regulation of SPI on chondrocytes is attributed to its activation of Nrf2, we investigated the impact of Nrf2 silencing on SPI mediated protein expression regu-

lation. Primarily, we treated cells with various siRNAs targeting Nrf2. The siRNA with the most effective interference (siRNA^{Nrf2-2}), as determined by WB (Fig. 3A and B), was chosen for further experiments. Through further study, we found that siRNA significantly reduced the Nrf2 level (Figure 3 C,G) in C28/I2 cells and partially blocked the cartilage protective effect of SPI on Col II (Figure 3 C,D), MMP1 (Figure 3 C,E), and MMP13 (Figure 3 C,F) expression.

SPI ameliorates DMM-induced OA in mice

In order to further explore the therapeutic effect of SPI *in vivo*, we used a DMM model to induce OA. Results of safranin O/fast green staining revealed that DMM led to substantial cartilage erosion and loss, resulting in an increase in the OARSI score. However, SPI treatment inhibited cartilage erosion and significantly reduced the OARSI score (Figure 4 A,B). Immunohistochemical staining of joint sections showed a decrease in Col II in the carti-

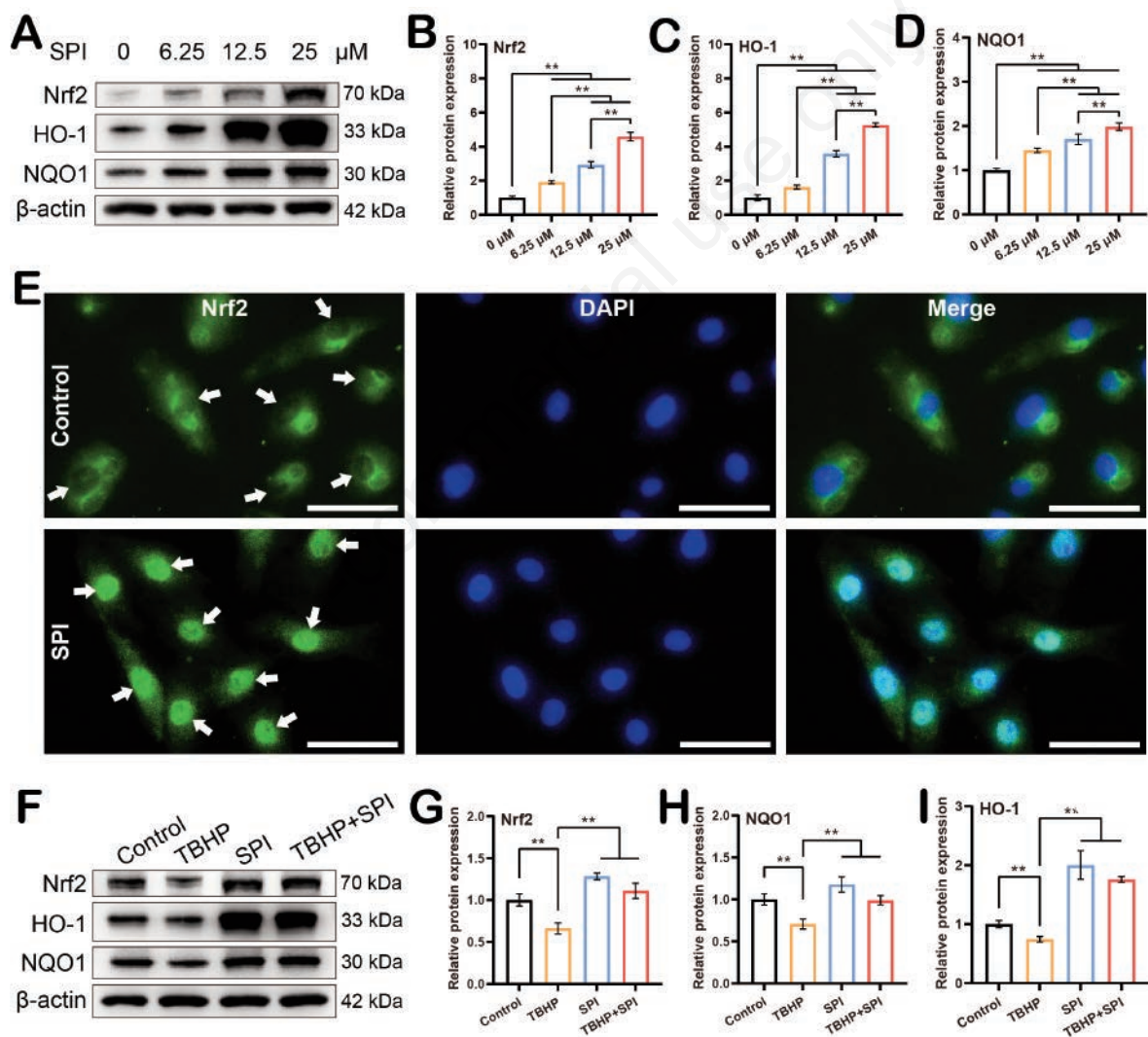


Figure 2. SPI can activate the Nrf2 signaling pathway in C28/I2 cells under TBHP environment. **A)** Western blot of Nrf2, HO-1, NQO1, and β -actin protein levels in C28/I2 cells under various interventions. **B-D)** Quantification analyses of Nrf2 (**B**), HO-1 (**C**), and NQO1 (**D**) expression levels in C28/I2 cells with different interventions (n=3). **E)** Representative images of Nrf2 immunofluorescence staining in C28/I2 cells with different interventions (the white arrow points to the cell nucleus); scale bars: 50 μ m. **F)** Western blot of Nrf2, HO-1, NQO1, and β -actin protein levels in C28/I2 cells with different interventions. **G-I)** Quantification analyses of Nrf2 (**G**), HO-1 (**H**), and NQO1 (**I**) expression levels in C28/I2 cells with different interventions (n=3). ***p*<0.01.

lage of DMM mice, which was alleviated by SPI treatment (Figure 4 C,D). These data indicate that SPI inhibits DMM induced cartilage erosion *in vivo*.

Discussion

The utilization of pharmacological interventions to prevent oxidative stress-induced chondrocyte damage represents a promising and valuable therapeutic approach to alleviate and reverse cartilage damage in OA. Among the numerous pathways associated with oxidative stress, the Nrf2-related pathway has recently garnered significant attention.¹⁵ In our study, we verified that Nrf2 expression is suppressed during the advancement of OA, resulting in the accumulation of MMPs and ultimately compromising chondrocyte function. As a natural flavonoid, SPI has low cytotoxicity and was recently discovered to effectively activate Nrf2.

Therefore, our research investigated the feasibility and therapeutic efficacy of treating OA with SPI. Our results indicate that 25 μ M SPI inhibited TBHP-induced overexpression of MMP1, MMP3, and MMP13 in C28/I2 cells, and increased the expression of Col II. Further investigation revealed that SPI activated the nuclear translocation of Nrf2 in C28/I2 cells under TBHP induction, thereby regulating MMPs and Col II through Nrf2. *In vivo* studies demonstrated that SPI enhanced the gait of DMM mice and elevated the levels of joint cartilage Col II and proteoglycans, indicating a partial reversal of cartilage damage in OA mice.

Under normal conditions, chondrocytes secrete proteoglycans that are degraded by MMPs to maintain essential cellular turnover and balance. In addition to inducing apoptosis by modulating various pathways such as Nrf2, NF- κ B, and MAPK, previous studies have confirmed that accumulated reactive ROS can damage the extracellular matrix in two ways: firstly, oxidative stress can increase the expression of catabolic markers like MMP3, MMP13, and ADAMTS4, hastening degradation; secondly, it can decrease

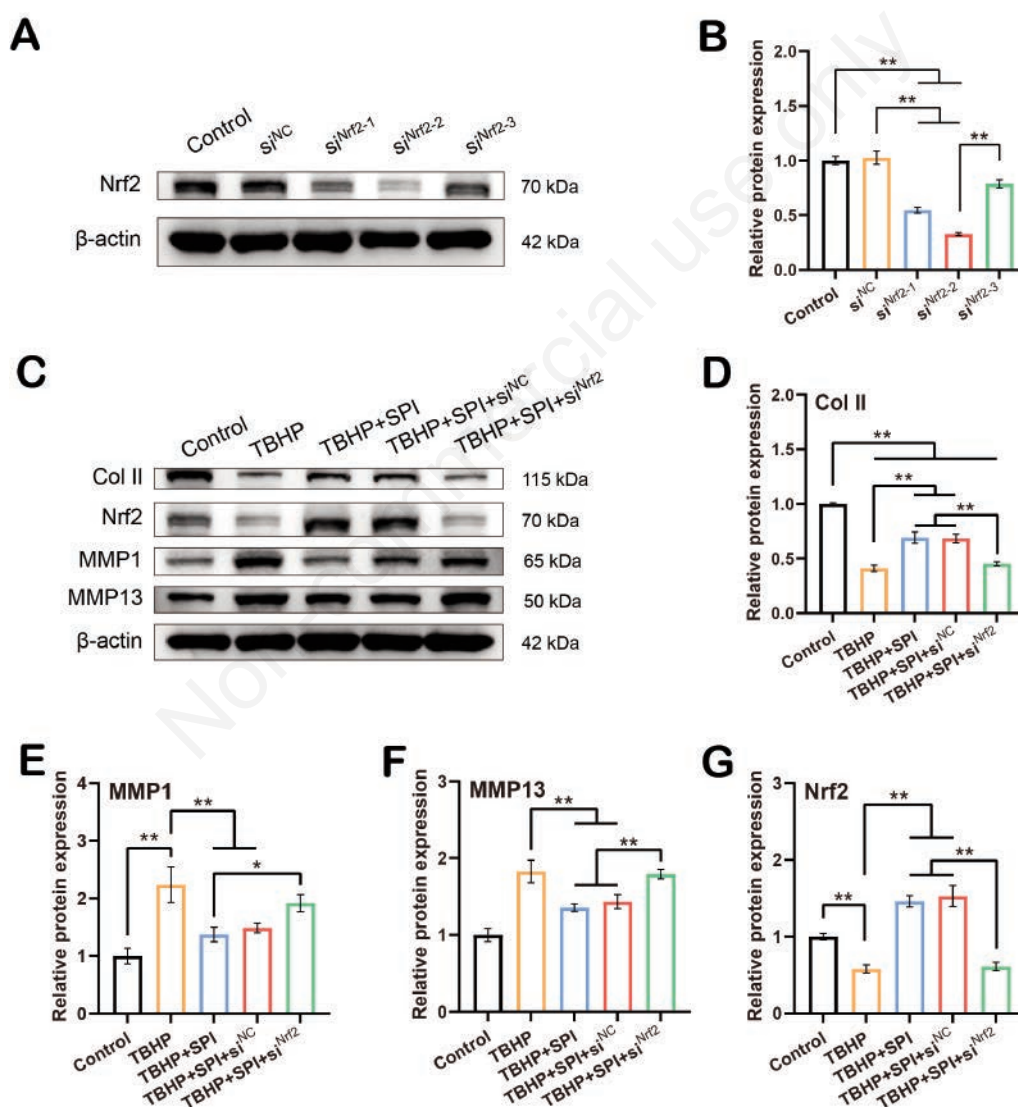


Figure 3. SPI attenuates the damage of C28/I2 cells induced by TBHP through the Nrf2 signaling pathway. **A)** Western blot of Nrf2 and β -actin protein levels in C28/I2 cells with different interventions. **B)** Quantification analysis of Nrf2 expression levels in C28/I2 cells with different interventions (n=3). **C)** Western blot of Col II, Nrf2, MMP1, MMP13, and β -actin protein levels in C28/I2 cells with different interventions. **D-G)** Quantification analyses of Col II (**D**), Nrf2 (**E**), MMP1 (**F**), and MMP13 (**G**) expression levels in C28/I2 cells with different interventions (n=3). * $p < 0.05$, ** $p < 0.01$.

the expression of anabolic markers such as Col II and proteoglycans, further accelerating cartilage degeneration.²²⁻²⁴ Additionally, apart from directly contributing to cartilage damage, oxidative stress may also trigger inflammation in OA. Oxidative stress generated in chondrocytes by lipopolysaccharide stimulation can lead to the activation of the NLRP3 inflammasome, resulting in the release of pro-inflammatory cytokines such as tumor necrosis factor- α , interleukin-1 β , and interleukin-6. These cytokines can stimulate chondrocytes to secrete fibrinolysins, directly or indirectly inducing an inflammatory response.^{25,26} Therefore, effective strategies for the treatment of OA should focus on preventing and mitigating the effects of oxidative stress on chondrocytes and the extracellular matrix.

In normal chondrocytes, Nrf2 maintains a low level of transcription. However, during early stages of oxidative stress, it binds to antioxidant response elements and activates the Nrf2/HO-1 signaling pathway to regulate the activity of downstream antioxidant enzymes, thereby exerting an antioxidant effect. Numerous studies have shown that trichostatin A can target the Nrf2/HO-1 pathway to inhibit the expression of various catabolic markers such as MMP3, MMP13, and ADAMTS4 induced by pro-inflammatory cytokines.^{27,28} Therefore, this pathway holds potential value in elucidating the pathogenesis of OA and developing targeted therapeutics for clinical management. SPI, a flavonoid compound and one of the main active ingredients in *Ziziphi Spinosae* Semen, has been shown to alleviate inflammation and reduce apoptosis by ameliorating oxidative stress and inhibiting COX-2 protein overexpression, thereby ameliorating symptoms of Alzheimer's disease.

However, there are few reports on the role of SPI in OA.^{29,30} Our investigation into the therapeutic utility of SPI against OA elucidates the pivotal role of oxidative stress in the pathogenesis of OA and highlights the therapeutic potential of modulating the Nrf2 pathway for disease management. The increased generation of ROS within chondrocytes, a defining feature of OA, triggers a series of cellular processes culminating in apoptosis and extracellular matrix degradation. The Nrf2/HO-1 axis, which is essential for cellular defense against oxidative stress, serves as a critical regulator in mitigating chondrocyte apoptosis and matrix degradation. Our findings indicate that SPI activates this protective pathway, thereby alleviating ROS-mediated cellular damage. This is in line with the increasing body of literature that supports antioxidant modalities in the treatment of OA. The ability of SPI to counteract TBHP-induced chondrocyte dysfunction through modulation of the Nrf2 pathway provides robust evidence for its therapeutic potential and is consistent with the emerging paradigm shift towards harnessing endogenous antioxidant mechanisms in OA therapy. Crucially, the delineated dose-dependent response of chondrocytes to SPI, with optimal therapeutic efficacy achieved at a concentration of 25 μ M, underscores the need for precise dosing in prospective therapeutic regimens. The dose-response relationship not only influences the therapeutic approach but also sheds light on the complex interplay between pharmacological interactions and cellular mechanistic responses. The demonstrated *in vivo* efficacy of SPI, as evidenced by improved cartilage integrity and reduced severity of OA in destabilized medial meniscus mouse models, further supports its potential as an innovative treatment

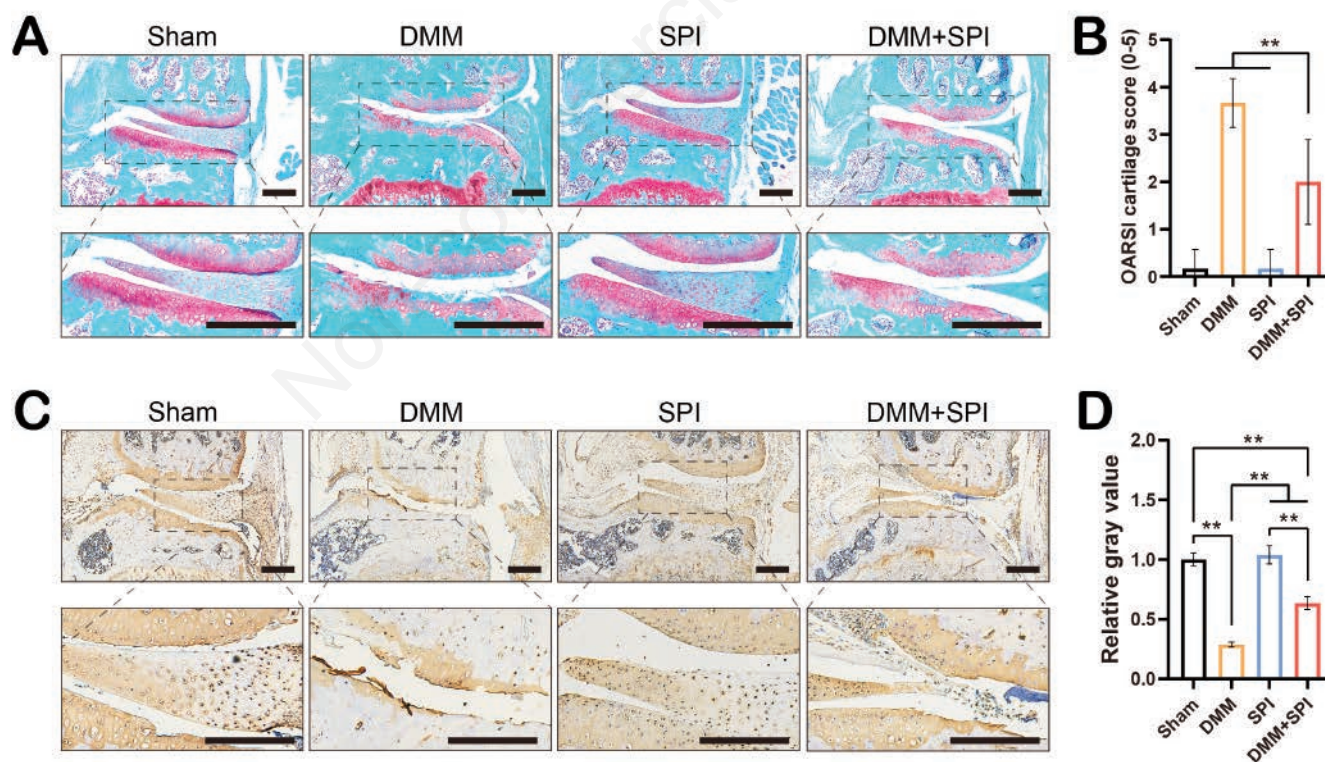


Figure 4. SPI can alleviate DMM-induced OA in mice. **A)** Representative images of Safranin-O/Fast green staining of knee joints in mice with different interventions; scale bars: 200 μ m. **B)** The analysis of OARSI cartilage scoring on the knee joints of mice subjected to different interventions (n=6). **C)** Representative images of Col II Immunohistochemistry staining in mice knee joints with different interventions. **D)** Quantitative analysis of Col II expression levels in mice knee cartilage with different interventions (n=6); scale bars: 200 μ m. ****** p <0.01.

approach for OA. These observations, coupled with the regulated activity of Nrf2 and its downstream mediators, provide a promising avenue for OA therapeutic strategies that go beyond symptomatic relief and target the fundamental oxidative stress mechanisms inherent in OA pathogenesis.

The multifaceted etiology of OA, characterized by a combination of genetic, metabolic, inflammatory, and mechanical factors, necessitates a comprehensive therapeutic strategy.^{31,32} Although SPI has demonstrated potential in modulating a crucial pathway linked to OA, the intricate nature of the disease demands an integrated treatment approach that considers its various aspects. Consequently, future investigations should consider the synergistic application of SPI alongside other therapeutic interventions, potentially including mechanical strategies to reduce joint stress, metabolic modulation, and agents targeting the inflammatory dimensions of OA. The translational viability of these findings will depend on thorough clinical validation. While the demonstrated efficacy of SPI in animal models of OA lays the groundwork for its therapeutic application, the heterogeneity inherent in human OA, shaped by diverse genetic predispositions, comorbid conditions, and environmental influences, poses potential challenges to its straightforward clinical translation. Therefore, carefully designed clinical trials to evaluate the safety, therapeutic efficacy, and optimal dosage of SPI in human OA cohorts are essential.

There are still some limitations in our study. Firstly, this study did not use human chondrocytes for *in vitro* experiments, so there may be differences in drug dosage. Secondly, our research on the treatment mechanism of SPI is relatively superficial. Thirdly, we only explored the therapeutic effects of SPI without combining it with delivery systems or regenerative scaffolds. In the future, we hope to combine the SPI with functional tissue-engineered scaffolds and study their therapeutic effects and exhaustive mechanisms on human chondrocytes.

In conclusion, we demonstrate for the first time that SPI has significant effects on cartilage protection and anti-OA both *in vitro* and *in vivo*. These functions are mainly achieved by activating the Nrf2/HO-1 signaling pathway. Therefore, SPI may provide a novel and effective strategy to treat OA.

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