

Sinenetin ameliorates osteoarthritis progression by modulating the NF- κ B/NLRP3 signaling pathway and suppressing chondrocyte pyroptosis

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ABSTRACT

Sinenetin (SIN) is a natural flavonoid with potential anti-inflammatory effects. This study investigated whether SIN ameliorates osteoarthritis (OA) and explored its underlying mechanisms. *In vitro*, SIN (0.05–1 μ g/mL) protected human OA chondrocytes from IL-1 β -induced viability loss. Transcriptome sequencing and molecular docking identified SERPINA3 as a candidate target of SIN. SIN reduced the expression of NLRP3, GSDMD, and ASC, inhibited pyroptotic morphology, decreased TUNEL-positive cells and LDH release, and lowered IL-1 β and IL-18 secretion in IL-1 β -treated chondrocytes. Knockdown of SERPINA3 reversed these effects, suggesting that SIN acts through SERPINA3 to inhibit the NLRP3 inflammasome. In a rat DMM-induced OA model, SIN (20 mg/kg/day for 6 weeks) alleviated pain, reduced Osteoarthritis Research Society International (OARSI) scores, improved cartilage matrix staining (Safranin O, Masson's trichrome), attenuated synovial hyperplasia and posterior capsule adhesions, and decreased plasma IL-1 β and IL-18 levels. Western blot of rat cartilage confirmed that SIN suppressed GSDMD and NLRP3 expression. These results indicate that SIN ameliorates OA progression by inhibiting SERPINA3-mediated NF- κ B/NLRP3-driven chondrocyte pyroptosis. SIN may be a potential therapeutic candidate for OA.

Key words: sinenetin; osteoarthritis; pyroptosis; inflammation; SERPINA3.

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Ethics approval and consent to participate: all animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The collection and use of human articular cartilage specimens (discarded tissue from total knee arthroplasty) and all animal procedures were approved by the Animal Ethics Committee of Qilu Hospital of Shandong University Dezhou Hospital (Approval No.: 2025095). This study involving human cartilage specimens was conducted in accordance with the ethical principles of the Declaration of Helsinki. Because the human cartilage specimens were anonymized surgical discards and did not involve any identifiable patient information, the requirement for written informed consent was waived by the Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Data availability statement: the data that support the findings of this study are available from the corresponding author upon reasonable request. The datasets generated and/or analyzed during the current study are available in the ICPSR repository (NAHDAP–223101).

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Introduction

Osteoarthritis (OA) is a progressive degenerative condition marked by hyaline cartilage erosion, subchondral bone thickening, synovial lining proliferation, and osteophyte formation. Its substantial prevalence and associated disability make it a leading musculoskeletal disorder in aging populations.¹⁻³

Recent views indicate inflammatory processes contribute significantly to OA pathogenesis. While not being inflammation is not the primary cause of articular cartilage degeneration, the damage caused by its cascading amplification mechanisms accelerates cartilage degeneration, impairs chondrocyte function, and leads to changes in chondrocyte phenotype and degradation of the extracellular matrix (ECM).^{4,5} The breakdown of the ECM precipitates hyaline cartilage erosion and subchondral trabecular compromise, as well as alterations in their corresponding biological properties.⁶

Currently, the treatment for OA remains limited to symptomatic relief, primarily involving administering nonsteroidal anti-inflammatory drugs (NSAIDs) along with chondroprotective agents to alleviate symptoms.⁷ However, long-term use of these medications can lead to severe side effects.⁸ Given the prolonged course of OA, long-term treatment may be required to slow its progression. Thus, a pressing clinical requirement exists to establish a safe, effective approach for long-term OA management. Traditional Chinese Medicine (TCM) serves as a rich heritage of ancient Chinese medical practices with substantial untapped potential.⁹ Studies have shown that TCM, as well as dietary plants, fruits, or their derived products used in certain dietary therapies, play an active role in alleviating arthritis.¹⁰ These natural products are diverse, and flavonoids are among the most extensively studied. They are widely found in fruits, vegetables, nuts, and grains.

In recent years, with the advancement of bioinformatics and the innovation of gene sequencing technologies, there has been deeper research and expansion in understanding the molecular mechanisms underlying articular cartilage metabolism within big data frameworks. In the present work, we initially screened databases and identified the natural small molecule compound sinensetin (SIN), which has anti-inflammatory properties, through the analysis of gene-drug interactions. Previous research found that SIN lowers the release of inflammatory cytokines as well as matrix metalloproteinases in OA chondrocytes, inhibit ECM degradation, and exert a protective effect on chondrocytes.¹¹ Transcriptomic and functional analyses further suggested that SERPINA3 may act as an important mediator of the protective effects of SIN and that SIN-associated upregulation of SERPINA3 inhibits activation of the NF- κ B signaling pathway.¹¹ However, the downstream mechanisms through which the SERPINA3/NF- κ B axis contributes to the chondroprotective effects of SIN remain incompletely understood.

The NF- κ B signaling pathway, a major regulator in inflammation and stress responses, acts as a pivotal signal for NLRP3 inflammasome activation. Priming of the inflammasome can trigger pyroptosis, a form of pro-inflammatory programmed cell death.^{12,13} Pyroptosis can cause cell lysis and lead to the massive release of cellular contents and pro-inflammatory factors, which contribute to the onset and progression of various autoimmune and inflammatory diseases.¹⁴ Multiple studies have confirmed that targeting the NF- κ B/NLRP3 pathway can inhibit ECM degradation and pyroptosis in OA chondrocytes, thereby slowing OA symptom progression.^{15,16}

Drawing on the information above, we hypothesize that SIN may exert its therapeutic effect on OA by inhibiting pyroptosis in OA chondrocytes through the regulation of SERPINA3 expression, thereby further modulating the NF- κ B/NLRP3 signaling

pathway. In the present study, we combined transcriptomic reanalysis, molecular docking, clinical cartilage validation, SERPINA3 knockdown, pyroptosis-related molecular and morphological assessments, and an experimental rat model of OA to investigate this hypothesis. This study therefore extends our previous observations by elucidating a potential downstream mechanism linking SIN-mediated SERPINA3 regulation to NLRP3 inflammasome-associated chondrocyte pyroptosis.

Materials and Methods

Reagents and Materials

Sinensetin (purity $\geq 99.67\%$) was procured from MedChemExpress (Monmouth Junction, NJ, USA). Recombinant human IL-1 β (catalog no. 200-1B) was obtained from PeproTech (Monmouth Junction, NJ, USA), and the Cell Counting Kit-8 (CCK-8) was sourced from Dojindo Laboratories (Kumamoto, Japan). Human IL-1 β and IL-18 ELISA kits were purchased from Absin (Shanghai, China), whereas TUNEL staining, LDH release, and cell cycle analysis kits were supplied by Beyotime Biotechnology (Shanghai, China). Primary antibodies against ASC, NLRP3, GSDMD, SERPINA3, GAPDH, and β -actin were acquired from Proteintech (Wuhan, China), and secondary antibodies conjugated with Alexa Fluor 488 or 594 were obtained from Beyotime.

Chondrocyte extraction, culture, and identification

Human articular cartilage specimens were collected as discarded tissue from patients undergoing total knee replacement arthroplasty. The samples were minced into approximately 1 mm³ pieces and subjected to enzymatic dissociation using 0.1% type II collagenase over an 8-h period under 37°C conditions within culture dishes. Following digestion, the resulting cell suspension was filtered, and the filtrate was centrifuged to pellet the cells. The pelleted cells were then resuspended in DMEM supplemented with 10% fetal bovine serum. Upon reaching $\sim 90\%$ confluence, the cells were passaged. For all experiments, chondrocytes at the third passage and in the logarithmic growth phase were utilized. After cultures had stabilized, cellular identity was confirmed via immunofluorescence staining for type II collagen combined with 1% toluidine blue staining.

Cell viability assay

CCK-8 assays were employed to evaluate SIN-mediated influence on chondrocyte viability. Briefly, 100 μ L of cell suspension was dispensed per well into 96-well plates. Following a 24-h incubation period, varying concentrations of SIN were introduced into designated wells, and cells were cultured for another 24 h. Post-intervention, supernatants were discarded, and each well received 90 μ L of fresh culture medium along with 10 μ L of CCK-8 reagent. Plates were subsequently incubated at 37°C for 2 h. Absorbance at 450 nm was then recorded using a microplate reader. Initial screening allowed calculation of viability percentages across experimental conditions. To mimic an OA-like environment, chondrocytes were exposed to IL-1 β , after which graded doses of SIN were applied. Viability changes were measured using identical procedures to determine the optimal therapeutic concentration window.

Target screening and validation

For transcriptomic analysis, RNA-sequencing data from IL-1 β -stimulated chondrocytes treated with or without SIN were obtained from our previously generated dataset (GSE201564).¹¹ RNA sequencing was performed by LC Bio Technology Co., Ltd. (Hangzhou, China) on the Illumina NovaSeq 6000 platform using paired-end 150-bp (PE150) sequencing. The significant difference between samples was analyzed using R software (version 4.2.1). Genes with FC >2 or FC <0.5 ($|\log_2\text{FC}| \geq 1$) and $p < 0.05$ were defined as DEGs, and genes with an expression abundance >10 were further considered for candidate-target screening. Molecular docking was subsequently performed to evaluate the potential binding affinity between SIN and SERPINA3. Public genomic datasets (GEO) were then interrogated to identify OA-related expression patterns, and trends for SERPINA3 were examined across clinical samples for validation. In parallel, cartilage specimens from healthy donors and OA patients were harvested and subjected to immunoblotting to compare SERPINA3 protein levels between the two populations.

Transmission electron microscopy (TEM) scanning

Chondrocytes exhibiting robust growth were chosen for experimentation. Following SIN treatment, cells were harvested without prior washing by immediately adding electron microscopy fixative. The suspension was centrifuged at 1000 rpm for 5 min, after which the supernatant was discarded and replaced with fresh fixative. Specimens were then allowed to fix for 2 h at ambient temperature. Finally, samples were transferred to an electron microscopy facility, where they underwent embedding, ultrathin sectioning, mounting onto copper grids, and TEM imaging.

LDH release detection in cell supernatant

Chondrocyte suspensions were prepared and plated in 96-well culture plates. Once cellular adherence was established, the original culture medium was exchanged for serum-free medium across all experimental groups. Upon completion of the treatment period, supernatants were harvested, aliquoted into microcentrifuge tubes, and centrifuged at 400 g for 5 min. Following centrifugation, 120 μL of each supernatant was transferred to fresh 96-well plates. Subsequently, 60 μL of LDH detection reagent was introduced per well, followed by a 30-min incubation. Absorbance at 490 nm was then recorded, and the percentage of LDH release was determined.

Establishment and grouping of the OA model

All animal work complied with the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed under controlled environmental conditions: ambient temperature maintained at 23 $\pm$ 2°C, relative humidity at 45–55%, and a 12-h light/12-h dark photoperiod. Standard rodent chow and water were provided *ad libitum*. Male Wistar rats (body weight ~200 g) received anesthesia, after which knee joint instability was induced *via* destabilization of the medial meniscus (DMM) procedure. Sham-operated animals underwent skin incision and muscle dissection only, without meniscus destabilization. A total of 18 male Wistar rats were included. The sample size was predetermined in the original animal experiment. Six rats per group were selected based on the group sizes commonly used in comparable DMM-induced OA studies and the expected variability of the histological Osteoarthritis Research Society International (OARSI) scores, while also minimizing animal use in accordance with the 3Rs principle. Following acclimatization, the rats were allocated in a 1:1:1 ratio to three experimental groups: a

sham-operated control, an OA group receiving DMM plus saline vehicle, and a SIN-treated group receiving DMM plus SIN (20 mg/kg/day *via* oral gavage for 6 weeks). The allocation sequence was generated by an investigator who was not involved in the surgical procedures, drug administration, or outcome assessment. During OARSI scoring, the investigators were blinded to the group allocation to minimize assessment bias. The present study used the same previously reported animal cohort and archived joint tissues; therefore, no additional animals were enrolled specifically for the current mechanistic analyses.¹¹ In the present study, we re-analyzed the behavioral and histological data from this cohort and used the archived joint tissues to perform additional mechanistic experiments focusing on NF- κB /NLRP3-mediated pyroptosis.

Cytokine quantification by ELISA

Plasma concentrations of IL-1 β and IL-18 were quantified by sandwich ELISA assay. Blood was drawn from each rat and promptly spun at 1500 g for 15 min. The resulting pale yellow, transparent supernatant was extracted. Each sample from the different groups was subjected to dilution in strict accordance with the manufacturer's stipulated protocols. The concentrations IL-1 β and IL-18 concentrations were calculated using a standard curve generated from the provided standard samples.

Western blot

RIPA lysis buffer was employed to isolate total protein from chondrocytes and knee cartilage, with lysate concentrations measured via BCA methodology. Equal amounts of each lysate were resolved on 8–12% SDS-polyacrylamide gels, then electrotransferred onto PVDF membranes under chilled conditions. Nonspecific binding was blocked by incubating membranes in 5% non-fat dry milk for 2 h at ambient temperature. Blots were then probed overnight at 4°C with primary antibodies targeting the proteins of interest. Following three TBST rinses, secondary antibody incubation proceeded for 2 h at room temperature. Immunoreactive signals were visualized using enhanced chemiluminescence (ECL) substrate on a gel documentation system. Band intensities were normalized to GAPDH as the loading control.

Histological analysis

Following the 6-week intervention period, all animals were humanely sacrificed using a lethal dose of anesthetic, after which knee joints were harvested. Harvested tissues underwent fixation in 4% paraformaldehyde (48 h) followed by decalcification in 10% EDTA (30 days). Upon adequate tissue softening, samples were paraffin-embedded and cut into 5- μm -thick serial sections. Hematoxylin and eosin (H&E) staining, Safranin O-Fast Green staining, and Masson's trichrome staining were performed to evaluate the cartilage and joint soft tissue.

TUNEL staining of rat knee joint sections

After deparaffinization and hydration of the tissue sections, proteinase K solution (without DNase) was applied for tissue repair. The TUNEL detection solution was following the manufacturer's protocol and placed onto tissue sections. Subsequent incubation was performed at 37°C under dark conditions for 60 min. After incubation, nuclei were counterstained with DAPI for 5 min. Sections were then visualized and imaged using a fluorescence microscope.

Statistical analysis

Statistical evaluation was performed with SPSS version 20.0. Quantitative values are presented as means $\pm$ SD. Intergroup differ-

ences were assessed *via* one-way ANOVA, followed by Tukey's *post-hoc* test for pairwise comparisons. A threshold of $p < 0.05$ was adopted to denote statistical significance.

Results

SIN protects chondrocyte viability and targets SERPINA3

We first examined the effect of SIN on human OA chondrocyte viability using CCK-8 assays. SIN at concentrations up to 1 $\mu\text{g/mL}$ did not reduce viability, whereas concentrations $\geq 5 \mu\text{g/mL}$ significantly decreased viability (Figure 1A). IL-1 β treatment alone markedly lowered cell viability; however, SIN at 0.05–1 $\mu\text{g/mL}$ reversed this effect, indicating a protective role (Figure 1A). Transcriptome sequencing of SIN-treated chondrocytes identified 1,355 differentially expressed genes (639 upregulated, 716 downregulated; Figure 1B). Using $p < 0.05$, gene expression abundance > 10 , and a fold change (FC) ≥ 2 or ≤ 0.5 (*i.e.*,

$|\log_2 \text{FC}| \geq 1$) as the screening criteria, seven significantly differentially expressed genes were identified. Among them, BASP1, SERPINA3, and TMEM230 were the three most markedly upregulated candidate genes. Among the three most upregulated candidates (BASP1, SERPINA3, TMEM230), molecular docking revealed the strongest binding affinity between SIN and SERPINA3 (-6.6 kcal/mol ; Figure 1C). To further validate SERPINA3 expression in OA, we analyzed GEO datasets (GSE55235 and GSE1919) and found that SERPINA3 expression was lower in OA patients than in normal controls (Figure 1D). Western blot analysis of clinical cartilage specimens confirmed that SERPINA3 protein levels were reduced in OA cartilage compared with normal controls (Figure 1E). Together, these results suggest that SERPINA3 is a candidate target of SIN in OA chondrocytes.

SIN inhibits chondrocyte pyroptosis via the NLRP3 inflammasome

We next examined whether SIN affects pyroptosis in IL-1 β -induced chondrocytes. Western blot analysis showed that SIN treatment reduced the levels of NLRP3, GSDMD, and ASC.

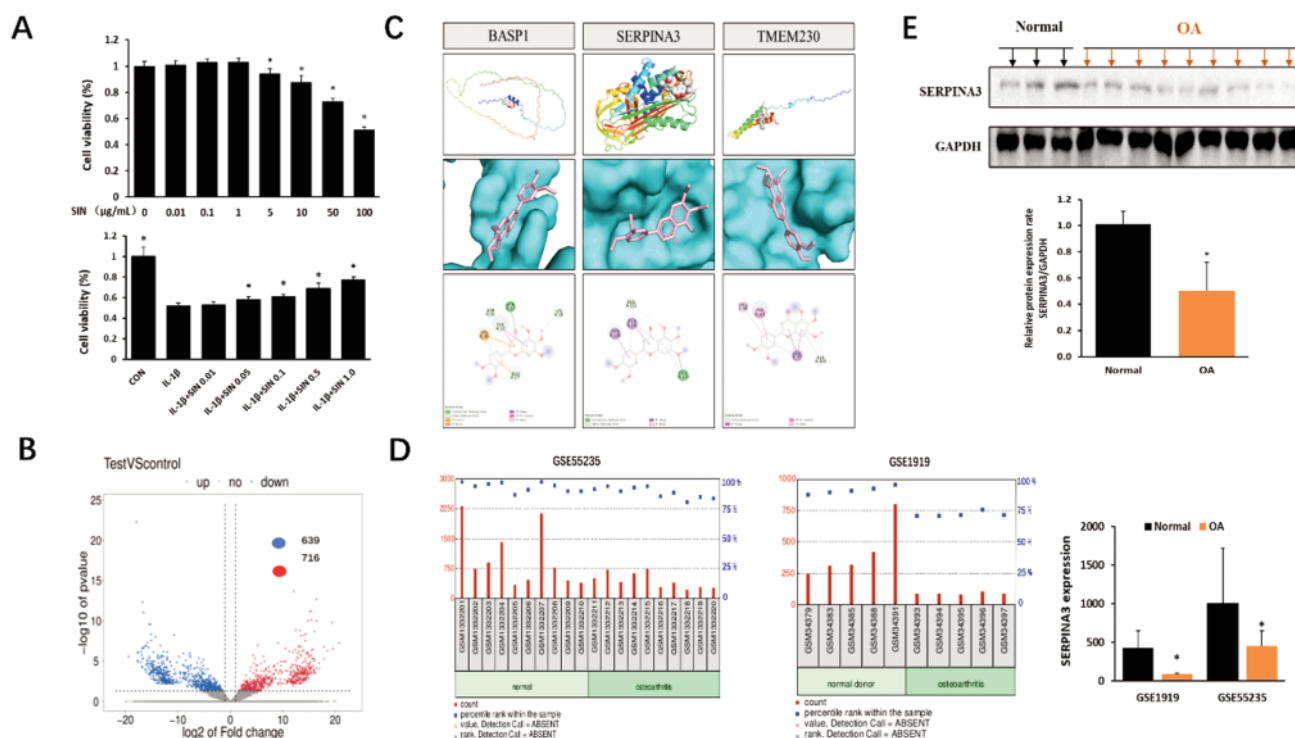


Figure 1. Effect of SIN on chondrocyte viability and target screening and validation of SIN. **A**) Chondrocyte viability was assessed using the CCK-8 assay following treatment with different concentrations of SIN, either alone or in combination with IL-1 β . **B**) Volcano plot showing the differentially expressed genes identified by transcriptomic sequencing in SIN-treated chondrocytes, including 639 upregulated genes and 716 downregulated genes. **C**) Molecular docking analysis of SIN with the three candidate proteins BASP1, SERPINA3, and TMEM230. **D**) SERPINA3 expression in normal and OA samples from the GSE55235 and GSE1919 datasets. **E**) Western blot analysis and quantitative assessment of SERPINA3 protein expression in clinical knee cartilage samples from normal controls and patients with OA. Quantitative data are presented as the mean $\pm$ SD from at least three independent biological replicates. Statistical comparisons among multiple groups in panel A were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Comparisons between two groups in panels D and E were performed using an unpaired two-tailed Student's *t*-test; $*p < 0.05$ was considered statistically significant.

Knockdown of SERPINA3 by lentivirus increased these proteins, and subsequent SIN treatment reversed the increase (Figure 2A). Transmission electron microscopy revealed that IL-1 β -treated chondrocytes exhibited typical pyroptotic morphology: cell swelling, numerous vacuoles, and membrane protrusions. SIN treatment reduced these changes, and the cells appeared relatively intact (Figure 2B). TUNEL staining showed few positive cells in normal chondrocytes, whereas IL-1 β induced abundant TUNEL-positive cells with nuclear condensation. SIN treatment significantly reduced the number of TUNEL-positive cells (Figure 2C). LDH release, a marker of membrane rupture, was increased by IL-1 β , and SIN treatment reduced LDH release in a dose-dependent manner (Figure 2D). Together, these data suggest that SIN inhibits IL-1 β -induced chondrocyte pyroptosis through SERPINA3-dependent modulation of the NLRP3 inflammasome.

SIN ameliorates pain and cartilage degeneration in OA rats

We then evaluated the *in vivo* effects of SIN using a rat DMM-induced OA model. In the hot plate test, SIN treatment

prolonged the latency of heat-stimulated response compared with the OA model group, indicating pain relief (Figure 3A). Gross observation of knee joints showed smooth, intact cartilage in the sham group. OA model joints presented disorganized internal structures, rough cartilage surfaces, and osteophyte formation (black arrows). SIN-treated joints showed improved cartilage condition with only mild overgrowth (Figure 3B). OARS1 scoring confirmed that SIN significantly reduced the OA score compared with the OA model group (Figure 3B). Safranin O-Fast Green staining revealed a well-defined cartilage-subchondral bone boundary and abundant matrix in the sham group. OA specimens exhibited severe surface attrition and irregularity, while SIN-treated specimens showed improved cartilage thickness and smoothness (Figure 3C). Masson's trichrome staining demonstrated abundant collagen (blue) in sham cartilage. OA cartilage showed marked collagen loss and disorganized soft tissues with increased fibrosis. SIN treatment increased collagen staining and improved tissue organization (Figure 3D). HE staining of the posterior joint capsule showed clear structures and no adhesions in the sham group. OA specimens displayed disorganized soft tissues, loss of joint capsule recesses, reduced joint space, and synovial

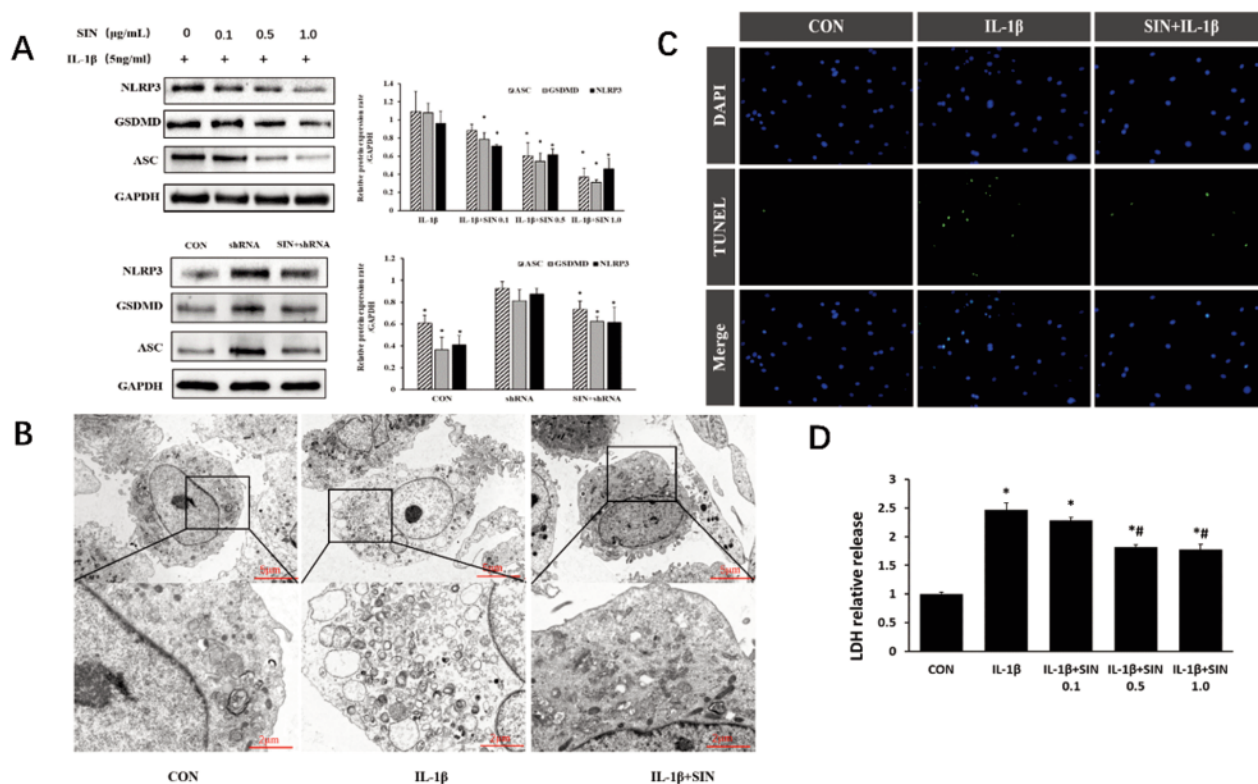


Figure 2. Effect of SIN on chondrocyte pyroptosis. **A)** Western blot analysis and densitometric quantification of NLRP3, GSDMD, and ASC protein expression in IL-1 β -stimulated chondrocytes treated with different concentrations of SIN, and in SERPINA3-knockdown chondrocytes subsequently treated with SIN. **B)** Representative transmission electron microscopy images showing ultrastructural changes in chondrocytes under different treatment conditions. **C)** TUNEL staining and quantitative analysis of chondrocytes. TUNEL-positive cells are shown in green, and nuclei counterstained with DAPI are shown in blue. **D)** LDH release from chondrocytes was measured using an LDH assay kit to evaluate cell membrane damage. All experiments were independently repeated at least three times, and representative images are shown. Statistical comparisons among multiple groups in panels A, C, and D were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test; * p <0.05 was considered statistically significant.

hyperplasia. SIN treatment attenuated these changes, preserving recesses and reducing synovial hyperplasia (Figure 3E). These results indicate that SIN ameliorates joint structural damage and reduces pain in OA rats.

SIN suppresses pyroptosis and inflammatory cytokines in OA rat joints

We further assessed pyroptosis and inflammation in rat knee joints. TUNEL staining of joint sections showed minimal positive signals in the sham group, a marked increase in OA specimens, and a significant reduction in SIN-treated animals (Figure 4A). Thus, SIN preserves chondrocyte chromatin integrity in OA cartilage. Western blot analysis of rat articular cartilage confirmed that GSDMD and NLRP3 levels were elevated in OA specimens, and SIN treatment reduced these levels (Figure 4B). Plasma cytokine measurements by ELISA revealed that OA induction significantly raised IL-1 β and IL-18 levels compared with sham controls, whereas SIN treatment significantly lowered both cytokines (Figure 4C). These data indicate that SIN suppresses pyroptosis-associated systemic inflammation in OA rats.

Discussion

Inflammation has become a significant risk factor for the increasing prevalence and mortality of age-related diseases, as many age-associated conditions involve inflammatory mechanisms.^{17,18} The global incidence of chronic conditions such as OA continues to increase.¹⁹ While the precise etiology and pathogenesis of OA remain incompletely defined, inflammatory reactions and chondrocyte loss within cartilage tissue are known to serve as key drivers in disease progression.^{16,20,21} A growing body of evidence indicates that chronic low-grade inflammation accelerates OA development and intensifies pain perception.²² The persistent inflammatory milieu within OA joints contributes to peripheral and central sensitization, manifested as mechanical allodynia and hyperalgesia in periarticular structures.²³ Recent investigations support inflammation as a core mediator underlying OA-associated pain.²⁴ In addition, inflammatory mediators can induce the expression of matrix-degrading enzymes in articular cartilage, triggering ECM breakdown and cartilage injury, thereby shifting metabolism toward catabolism.²⁵ When the homeostatic balance between cartilage anabolism and catabolism is disrupted, uncontrolled degradation of ECM and collagen impairs cartilage structure, ultimately leading to the onset and progression of OA.²⁶

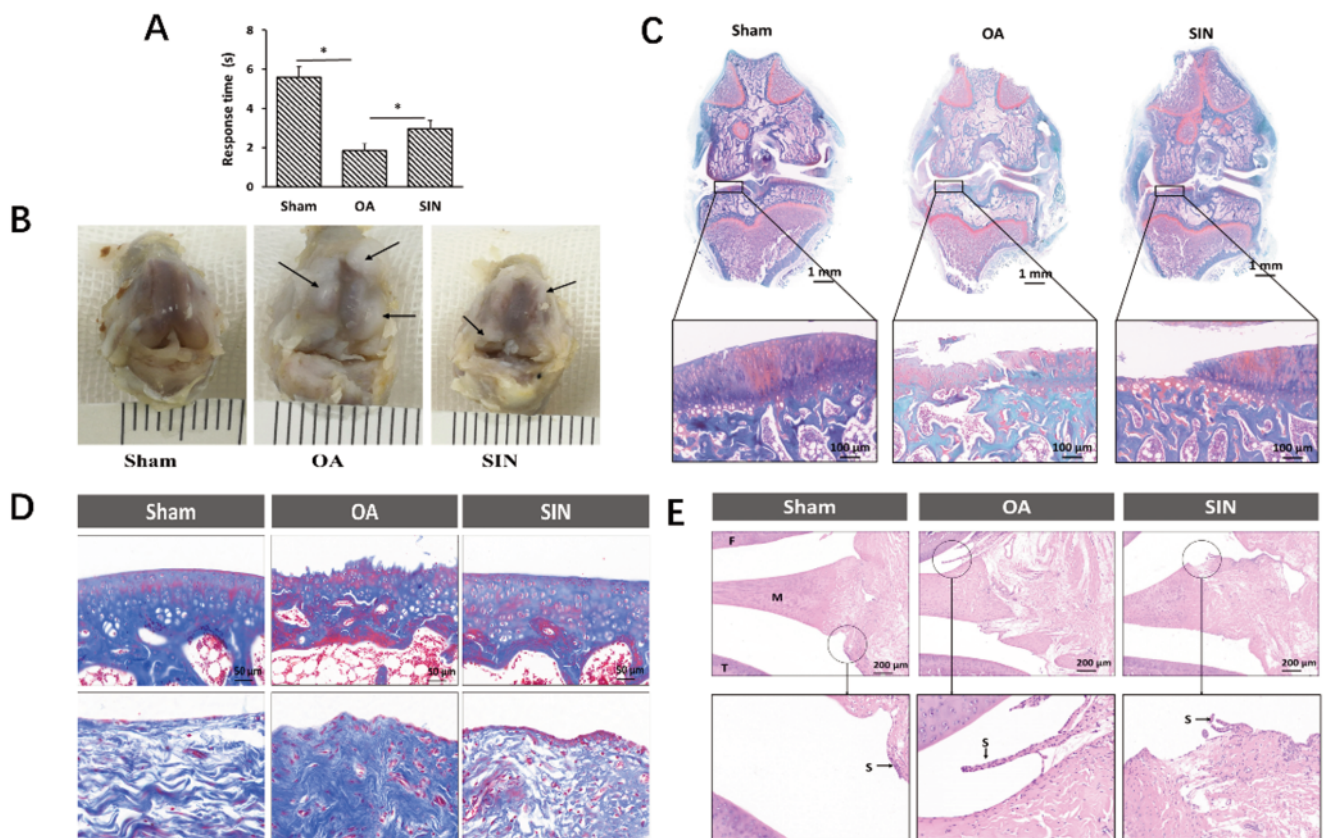


Figure 3. Effect of SIN on the knee joints of OA rats. **A)** Comparison of the response latency in the hot plate test among the sham, OA, and SIN-treated groups. **B)** Representative gross images of rat knee joints and corresponding Osteoarthritis Research Society International (OARSI) scores. Black arrows indicate osteophyte formation. **C)** Representative Safranin O-Fast Green-stained sections of rat knee joints. **D)** Representative Masson's trichrome-stained sections showing the articular cartilage and periarticular soft tissues of rat knee joints. **E)** H&E staining of sagittal sections showing pathological changes in the posterior joint capsule. Each group comprised 6 rats ($n=6$ per group). All the above results are shown as mean $\pm$ SD. F, femur; T, tibia; M, meniscus; S, synovial tissue; $*p<0.05$ was considered statistically significant.

Pyroptosis, initially identified in 2001 and formally named by Cookson and colleagues, represents a pro-inflammatory mode of programmed cell death with essential functions in inflammatory responses and immune regulation.²⁷⁻²⁹ This form of cell death is initiated by inflammasome activation, resulting in cellular lysis and the release of intracellular components in a manner analogous to necrosis, thereby further amplifying local inflammatory reactions.^{30,31}

Following activation, inflammasomes drive the cleavage of caspase-1, which in turn converts the inactive pro-forms of pro-IL-1 β and pro-IL-18 into their biologically mature cytokines. These mature inflammatory mediators are subsequently secreted *via* membrane pores formed during cell lysis.³²⁻³⁴ During pyroptotic cell death, inflammasomes act as critical molecular scaffolds to facilitate the maturation and release of IL-1 β and IL-18.³⁵ As one of the most abundantly secreted inflammatory cytokines, IL-1 β contributes significantly to cartilage degeneration and joint damage in the course of OA progression.³⁶ Likewise, IL-18 can be synthesized by multiple cell types implicated in OA pathogenesis, such as chondrocytes, osteoblasts, synoviocytes, and macrophages.³⁷ Studies have shown increased concentrations of IL-18 in the serum, synovial fluid, and articular cartilage of indi-

viduals with OA, with greater abundance in advanced OA relative to early disease.³⁸ Further research demonstrated that IL-18 content in synovial fluid is tightly linked to the severity of post-traumatic OA, implying a link between its expression and disease progression.³⁹ Similarly to IL-1 β , IL-18 is capable of eliciting intracellular signals through the NF- κ B pathway, thereby promoting the expression of inflammatory cytokines and matrix metalloproteinases. These factors subsequently suppress the biosynthesis of extracellular matrix constituents and exacerbate the degradation of cartilage matrix.⁴⁰ Such events ultimately establish a self-sustaining inflammatory vicious cycle involving multiple pro-inflammatory cytokines and mediators.

Since pyroptosis contributes to OA progression by degrading structurally stable cartilage ECM, secreting pro-inflammatory cytokines, and accelerating chondrocyte degeneration,⁴¹ blocking the pyroptosis pathway may represent a promising therapeutic approach for improving OA. Accumulating evidence indicates that blockade of NLRP3 inflammasome activation can reduce cartilage degradation, reverse subchondral bone remodeling, and suppress chondrocyte pyroptosis, thereby slowing the progression of OA.⁴²

Our previous study established the anti-inflammatory and chondroprotective effects of SIN in OA. Specifically, SIN reduced

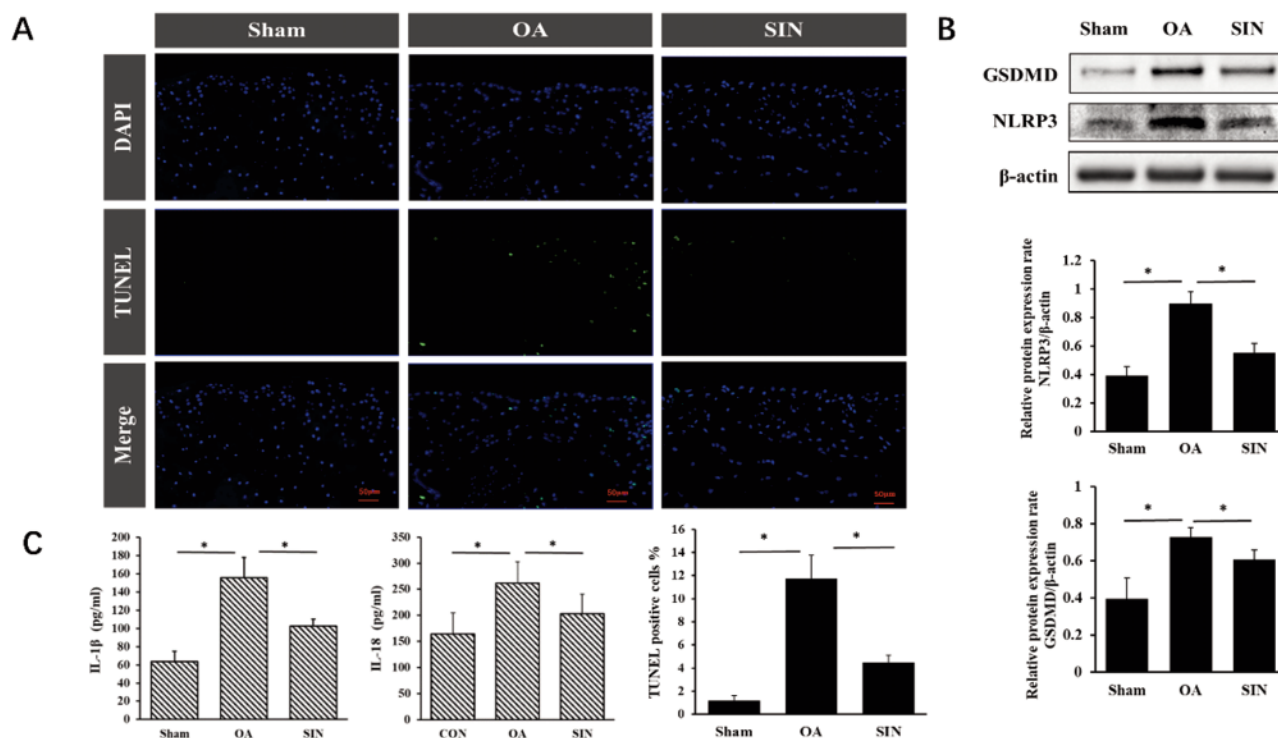


Figure 4. Effect of SIN on pyroptosis and inflammatory cytokines in the knee joints of OA rats. **A)** TUNEL staining of rat knee joint tissue sections: TUNEL-positive cells are shown in green, and nuclei are stained blue with DAPI. **B)** Western blot analysis and densitometric quantification of the pyroptosis-related proteins GSDMD and NLRP3 in rat articular cartilage. **C)** Plasma levels of IL-1 β and IL-18 in the sham, OA, and SIN-treated groups were measured using ELISA. All experiments and quantitative measurements were performed at least three times, and representative images are shown. Quantitative data are presented as the mean $\pm$ SD. Statistical comparisons among the three groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test; * p <0.05 was considered statistically significant.

the expression of inflammatory mediators, including COX-2, iNOS, TNF- α , and IL-6, decreased the levels of the matrix-degrading enzymes MMP-9 and MMP-13, preserved type II collagen, and attenuated NF- κ B activation in a SERPINA3-associated manner. In the DMM-induced rat model, SIN also improved cartilage histopathology and reduced OARSI scores.¹¹ However, the previous study primarily focused on inflammatory mediator production, extracellular matrix catabolism, and general cartilage degeneration, and did not determine whether inflammasome-associated pyroptosis contributed to the protective effects of SIN.

The present study extends these findings by linking the previously identified SERPINA3/NF- κ B pathway to NLRP3 inflammasome-associated chondrocyte pyroptosis. Our experimental results demonstrated that SIN not only inhibits NF- κ B phosphorylation but also suppresses the downstream NLRP3 pathway, effectively alleviating pyroptosis in OA chondrocytes. Following knockdown of the target gene SERPINA3, phosphorylation status of I κ B α and P65 was elevated, nuclear shuttling of P65 was strengthened, and levels of pyroptosis-associated proteins were upregulated. However, SIN treatment led to an upregulation of SERPINA3 expression, and the NF- κ B/NLRP3 inflammatory axis was once again inhibited. These findings strongly suggest that SIN effectively suppresses IL-1 β -induced activation of the NF- κ B/NLRP3 signaling pathway, reducing the release of inflammatory and destructive factors and inhibiting pyroptosis. The animal experiments analyzed in this article were performed using the same cohort of OA rats as in our previous study on SIN.¹¹ In that work, we mainly reported the effects of SIN on pain behavior and overall cartilage degeneration. In the present study, we re-analyzed the previously collected behavioral and histological data and used the archived joint tissues from this cohort to conduct additional molecular, histological and biochemical experiments aimed at elucidating NF- κ B/NLRP3-mediated chondrocyte pyroptosis. No additional animals were used beyond this previously described cohort.

In our *in vitro* analyses performed on rat knee joint samples and human cartilage tissue, SIN displayed a series of protective actions. It notably suppressed the production of inflammatory cytokines, raised type II collagen levels in articular cartilage, and strengthened expression of the target gene SERPINA3. SIN also blocked activation of the NF- κ B/NLRP3 signaling pathway and lowered pyroptosis-associated protein levels. These data collectively support that SIN mitigates OA progression, likely by modulating the NF- κ B/NLRP3 axis to hinder pyroptotic cell death.

Of note, SIN is a polymethoxyflavone predominantly distributed in *Orthosiphon stamineus* (often referred to as Java tea) as well as in citrus peels. This herb grows extensively across Southeast Asia and tropical regions, where its foliage has long been used to prepare traditional herbal teas. In local folk medicine, this plant has been traditionally applied to manage rheumatism, edema, gout, and urinary tract inflammation. Similarly, citrus-related products have a long history of use in TCM for alleviating indigestion, bronchitis, asthma, and various inflammatory disorders. Chenpi (dried citrus peel) is widely consumed as a daily tea; moreover, studies have verified its beneficial effects against OA in TCM prescriptions. The present findings are consistent with an expanding body of evidence showing that natural flavonoids protect articular cartilage by simultaneously suppressing inflammatory signaling and extracellular matrix catabolism. Nobiletin, another citrus-derived polymethoxyflavone, has been reported to inhibit IL-1 β -induced phosphorylation of IKK α / β , I κ B α , and NF- κ B p65, reduce the production of inflammatory and catabolic mediators, restore type II collagen and aggrecan expression, and attenuate cartilage degeneration in a mouse model of OA.⁴³ Similarly, the citrus flavanone naringenin reduced TLR4 and TRAF6 expression and

NF- κ B phosphorylation, thereby alleviating inflammatory mediator production, chondrocyte apoptosis, and matrix degradation in experimental OA.⁴⁴ These observations suggest that suppression of NF- κ B signaling may represent a shared chondroprotective mechanism among citrus-derived flavonoids.

Several natural flavonoids have also been shown to interfere directly or indirectly with inflammasome activation and chondrocyte pyroptosis. Icariin suppressed NLRP3/caspase-1 signaling, reduced IL-1 β and IL-18 release, alleviated chondrocyte pyroptosis, and preserved collagen expression in LPS-stimulated rat chondrocytes and an experimental rat OA model.⁴⁵ Quercetin similarly reduced oxidative stress-dependent NLRP3 activation and chondrocyte pyroptosis, accompanied by decreased apoptosis and extracellular matrix degradation in both *in vitro* and *in vivo* OA models.⁴⁶ In addition, intra-articular administration of baicalein reduced inflammatory mediator production, MMP-13 and ADAMTS-5 expression, type II collagen loss, and NLRP3 inflammasome activation in DMM-induced OA rats.⁴⁷ Interestingly, baicalein did not substantially alter the evaluated oxidative stress markers, suggesting that different flavonoids may converge on NLRP3 through distinct upstream regulatory mechanisms. Beyond articular cartilage, chrysin has been reported to ameliorate synovitis and synovial fibrosis by suppressing PERK/TXNIP/NLRP3 signaling and disrupting the interaction between TXNIP and NLRP3 in osteoarthritic fibroblast-like synoviocytes.⁴⁸ Collectively, these studies indicate that the effects of flavonoids on the NLRP3 inflammasome are not restricted to chondrocytes but may extend to other joint-resident cells involved in the whole-joint pathology of OA. Based on our current results, we suggest that this study may provide a theoretical basis for exploring TCM herbal teas as potential interventions for OA and other chronic musculoskeletal diseases. This study clarifies the potential role and underlying mechanism of SIN in the management of OA. Our data demonstrate that SIN targets the NF- κ B/NLRP3 axis and modulates SERPINA3 expression, thereby effectively suppressing pyroptosis in OA chondrocytes. SIN also mitigates pyroptosis-associated inflammation, blocks extracellular matrix catabolism, and maintains cartilage structural integrity, thus delaying the pathological progression of OA. Its capacity to restrain the release of IL-1 β and IL-18 further highlights its strong anti-inflammatory activity. Collectively, these findings identify SIN, a TCM-derived natural compound, as a promising therapeutic candidate for OA. This work not only deepens mechanistic understanding of SIN's protective effects against OA but also provides a theoretical basis for developing SIN and related compounds as novel, well-tolerated long-term treatments for OA and other chronic inflammatory disorders.

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