



Garcinol Suppresses IL-1 β -Induced Chondrocyte Inflammation and Osteoarthritis *via* Inhibition of the NF- κ B Signaling Pathway

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Abstract— Osteoarthritis (OA), which is characterized as a common degenerative joint disease, is presently the most prevalent chronic degenerative joint disease. Accumulating evidence has shown a biological function for *Garcinol* in a variety of diseases; however, whether it could be used to treat OA remains unclear. In this study, we explored the protective effects of garcinol on the progression of OA and explored the underlying mechanism. *In vitro*, garcinol reduced the expression of pro-inflammatory cytokines, such as IL-6 and tumor necrosis factor alpha (TNF- α). It also decreased the expression of inducible nitric oxide synthase (iNOS), as well as cyclooxygenase-2 (COX-2). Furthermore, garcinol inhibited the expression of thrombospondin motifs 5 (ADAMTS5) and metalloproteinase (MMPs), both of which regulate extracellular matrix degradation. These changes could be attributed to garcinol-related suppression of the IL-1 β -induced NF- κ B signaling pathway. Moreover, we investigated the protective effects of garcinol on the surgical destabilization of the medial meniscus (DMM) of the mouse, an *in vivo* model of OA. Taken together, our data suggest garcinol as a potential future agent for the treatment of OA.

KEY WORDS: garcinol; IL-1 β ; NF- κ B; DMM.

INTRODUCTION

Osteoarthritis (OA) is now the most prevalent chronic degenerative joint disease, affecting tens of millions of

people [7]. Articular cartilage erosion, synovitis and articular hypertrophy, and subchondral bone remodeling are regarded as the main characteristics of OA development [16], resulting in severe joint pain and dysfunction. Currently, pharmaceutical agents such as non-steroidal anti-inflammatory drugs (NSAIDs) and bisphosphonates only alleviate clinical symptoms, and carry their own severe side effects [10]. Therefore, safe and effective drugs that delay the progression of OA represent a critical unmet need.

Although the underlying reason for the initiation of OA is multi-factorial, cartilage destruction seems to be the major cause, and this destruction is a consequence of uncontrolled proteolytic extracellular matrix destruction. Chondrocytes, the sole cell type in

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articular cartilage, maintain a delicate extracellular matrix (ECM) balance between synthesis and degradation, and play an important role in the OA disease processes. Inflammation is reported to trigger the catabolic abilities of chondrocytes, subsequently leading to the degradation of extracellular matrix (ECM). Interleukin (IL)-1 β , a pro-inflammatory cytokine, is reported to be produced and secreted by activated chondrocyte metabolism during the process of OA, and contributing to the development of OA [1, 9]. Studies confirmed that IL-1 β could induce chondrocytes to release multiple proteolytic enzymes, including matrix metalloproteinases (MMPs) [3, 5], which can upregulate anabolism in chondrocytes *via* promoting ECM degradation. In addition, aggrecan, one of the major ECM components of cartilage, could increase the ability of cartilage to resist compressive forces. Accumulating evidence demonstrates the role of IL-1 β in the degradation of aggrecan [22], and aggrecan is cleaved at specific “aggrecanase” sites during the process of OA [35]. This cleavage can be attributed to several members of the ADAMTS family of metalloproteinases, such as ADAMTS4 and ADAMTS5 [23, 30, 32, 38]. Stimulation by IL-1 β highly facilitates the expression of ADAMTS, thereby increasing the degradation of aggrecan [34, 39, 43].

Collagen II has been revealed to be the main component of ECM, and is responsible for supporting cartilaginous structures. Treatment with IL-1 β contributes to the degradation of collagen II, resulting in the degeneration of articular cartilage. On the contrary, collagen X was considered a standard marker of hypertrophic cartilage, and high expression of collagen X can result in ossification [41, 47]. Stimulation with IL-1 β promotes the expression of collagen X, and may thus accelerate the ossification of cartilage, resulting in dysfunction.

In addition, chondrocytes obtained from patients suffering from OA were potent producers of nitric oxide (NO), IL-1 β , TNF- α , IL-6, and IL-8, and elevated levels of tumor necrosis factor- α (TNF- α) and IL-1 β have been found in the synovial fluid and cartilage tissue of OA patients [4, 13]. Therefore, drugs targeting IL-1 β -induced inflammation are considered an effective method for treating OA.

Studies have demonstrated that garcinol (a polyisoprenylated benzophenone extracted from the fruit of *Garcinia indica*) exhibits a wide range of biological activities including antioxidizing, anti-inflammatory, neuroprotective, antimicrobial, and anti-neoplastic effects [2, 6, 14, 19, 20, 24, 36, 44].

Garcinol has also been explored for having a protective effect in various inflammatory situations induced by IL-1 β [18, 42]. We thus hypothesized that it could inhibit IL-1 β -induced inflammatory reactions.

In this study, we demonstrated the inhibitory effects of garcinol on IL-1 β -induced inflammation *via* the NF- κ B signal pathway, and its protective effects on the destabilized medial meniscus (DMM) of mice in an *in vivo* model for OA. Our study demonstrated that garcinol has potential as a novel drug for the treatment of OA.

MATERIALS AND METHODS

Reagents

Garcinol (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) for storage at a concentration of 50 mM, then further diluted in cell culture medium until DMSO comprised <0.1% of the total volume. Recombinant human IL-1 β (Peprotech) was dissolved in water. Dulbecco's Modified Eagle Medium (DMEM/F12), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Rockville, MD, USA). Type II collagenase was purchased from Sigma-Aldrich (St. Louis, MO, USA). 0.25% trypsin-EDTA was obtained from Gibco (Rockville, MD, USA). Safranin-O and Fast Green were purchased from Solarbio (Beijing, China). MTT kit and BCA were bought from the Beyotime Institute of Biotechnology (Shanghai, China).

Primary Mice Chondrocytes Culture

Chondrocytes were isolated from the knee joints and costal cartilage of C57BL/6 mice (2-day old). The costal cartilage and articular cartilage were extracted carefully under aseptic conditions and treated with 0.25% type II collagenase for 6 h at 37 °C, with 5% CO₂. Later, the digested cartilage tissues were washed twice by PBS, and then seeded into tissue culture flasks for culture in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in an incubator maintained at 37 °C with 5% CO₂. The medium was changed after 24 h of incubation. When chondrocytes in culture demonstrated 70–90% confluency, we harvested the cells by using 0.25% trypsin-EDTA (Gibco, Invitrogen).

Cytotoxicity Assays

Mouse chondrocytes were seeded in 96-well plates at a density of 8000 cells/well. After culturing for 24 h, the medium was changed with fresh culture medium containing various concentrations of garcinol (0, 2, 4, 8, 16, 32, and 64 μM) according to the time until cell adherence. Each garcinol concentration was repeated in 6 wells. At each experimental end point, 10 μl MTT solution was added to each well, and chondrocytes were further incubated for 4 h at 37 °C with 5% CO₂. Later, the medium was changed to 200 μl DMSO, allowed to dissolve for 10 min, and then measured using the Enzyme standard instrument at OD 570 nm (Thermo Scientific, Multiskan GO, Waltham, MA, USA).

Quantitative Real-time PCR

Total RNA was extracted from the chondrocytes in 6-cm culture plates using TRIzol reagent. Chondrocytes at a density of 2×10^5 cells/well were seeded onto a six-well plate and treated with IL-1 β , with or without various concentrations of garcinol, and an untreated group was used as a control. At the end of the study, total RNA was extracted from each well using RNAiso Plus (Takara Bio, Otsu, Japan). First-strand cDNA was synthesized using PrimerScript RT cDNA Synthesis kit (Takara Bio) and 1 μg of extracted RNA template. qPCR was then carried out using the SYBR Green-based Real-time PCR Master Mix (Takara Bio) with the following cycling parameters: 42 cycles of 94 °C for 20 s; 60 °C for 20 s; and 72 °C for 30 s. The results were normalized to β -actin, an internal housekeeping gene. The specific primers (based on the mouse sequences) used are shown in Table 1.

Western Blot Analysis

Cellular proteins were treated with the indicated conditions in an incubator maintained at 37 °C with 5% CO₂. At the end of the experiment, cells were washed by PBS twice, and later collected by cell lysis buffer. After exposure to cell lysis buffer for 30 min on ice, the samples were then centrifuged at 15000g for 10 min at 4 °C. The protein-containing supernatant was collected, and we measured protein concentration using a bicinchoninic acid (BCA) protein assay kit. After normalizing the protein concentration, the sample was mixed with sodium dodecyl sulfate-sampling buffer, followed by boiling at 95 °C for 5 min. To continue, protein (40 μg) was loaded onto 10–12% SDS-PAGE gels and subsequently transferred to a 0.22- μm PVDF membrane. The PVDF membranes were later incubated with blocking buffer for 1 h. The blocked membranes were then incubated with primary antibodies against collagen X (1:1000), collagen II (1:1000), INOS (1:1000), COX-2 (1:1000), Aggrecan (1:1000), ADAMT5 (1:1000), MMP-13 (1:1000), MMP-3 (1:1000), p65 (1:1000), P-p65 (1:1000), I κ B α (1:1000), and actin (1:1000) overnight at 4 °C, then incubated with the secondary antibody for 1 h. Finally, the bands were detected *via* ECL Western Blotting Substrate and visualized by the Bio-Rad ChemiDoc system.

Immunofluorescence Microscopy

Chondrocytes (5×10^5 cells/mL) were seeded on a 6-well plate with glass coverslips in advance and incubated for 24 h. At the end of the experiment, the glass coverslips were washed three times in PBS. Later, chondrocytes were fixed with 4% paraformaldehyde for 30 min and permeabilized by treatment with Triton X-100 for 10 min at room temperature. Following

Table 1. Sequences of Primers Used in Real-time Polymerase Chain Reaction (Real-time PCR)

| Gene | Forward primer | Reverse primer |
|---------------------------------|---------------------------------|----------------------------------|
| <i>TNF-α</i> | 5'-CAGGCGGTGCCTATGTCTC-3' | 5'-CGATCACCCCGAAGTTCAGTAG-3' |
| <i>IL-6</i> | 5'-TAGTCCTTCTACCCCAATTTC-3' | 5'-TTGGTCCTTAGCCACTCCTTC-3' |
| <i>INOS</i> | 5'-GTTCTCAGCCCAACAATACAAGA-3' | 5'-GTGGACGGGTCGATGTAC-3' |
| <i>MMP3</i> | 5'-ACATGGAGACTTTGTCCCTTTG-3' | 5'-TTGGCTGAGTGGTAGAGTCCC-3' |
| <i>MMP9</i> | 5'-GCAGAGGCATACTTGTACCG-3' | 5'-TGATGTTATGATGGTCCCCTTG-3' |
| <i>MMP13</i> | 5'-TGTTTGACAGCACTACTTGAA-3' | 5'-CAGTCACCTCTAAGCCAAAGAAA-3' |
| <i>β-actin</i> | 5'-AGC CAT GTA CGTAGC CAT CC-3' | 5'-CTC TCA GCA GTG GTG GTG AA-3' |

of variance (ANOVA) followed by a Tukey's *post hoc* test was used to assess statistical differences between different experimental groups. *P* values < 0.05 were considered significant.

RESULTS

Effects of Garcinol on Chondrocyte Viability

The chemical structure of garcinol is shown in Fig. 1 a, and representative images of the cell morphology of chondrocytes are displayed in Fig. 1 b. In order to estimate the cytotoxicity of garcinol, chondrocytes were treated with or without garcinol at different concentrations (0, 0.5, 1, 2, 4, 8, 16, and 32 μ M) for 24 h and 48 h, and the cellular viability was ultimately observed by MTT assay. Treatment with garcinol at the indicated concentrations, ranging from 0 to 8 μ M, at 24 h and 48 h showed no significant variation in cell viability (Fig. 1c). Therefore, we chose to use garcinol (2, 4, or 8 μ M) in all subsequent experiments.

Protective Effect of Garcinol on IL-1 β -Induced Inflammation in Mouse Chondrocytes

In order to investigate the protective effect of garcinol in chondrocytes under inflammatory condition induced by IL-1 β , mouse chondrocytes were treated with IL-1 β at various concentrations of garcinol for 24 h, then RT-PCR and western blot analyses were performed to explore the expression levels of RNA and protein. The results demonstrated that IL-1 β up-regulated the expression of inflammation-related marker genes: TNF- α , IL-6, and INOS, while treatment with garcinol restored them to baseline (Fig. 2a). In addition, at the protein level, garcinol decreased IL-1 β -induced INOS and COX-2 in a dose-dependent manner (Fig. 2b).

Garcinol Alleviated IL-1 β -Induced ECM Degradation in Mouse Chondrocytes

To evaluate the function of garcinol on IL-1 β -induced degradation of the ECM, we investigated the effect of garcinol on ECM components in chondrocytes

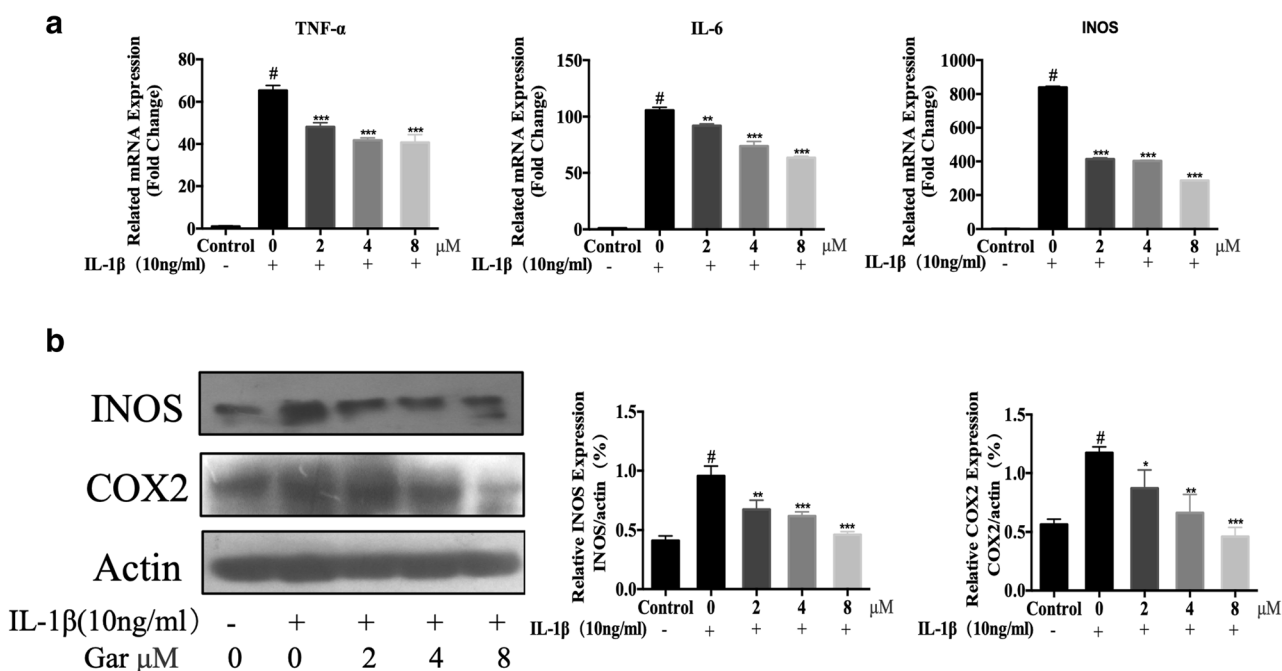


Fig. 2. Protective effects of garcinol on IL-1 β -induced inflammation in mouse chondrocytes. **a** Garcinol dose-dependently suppressed IL-1 β -induced inflammation-related marker genes. The expression of TNF- α , IL-6, and INOS in chondrocytes was assessed by real-time PCR. The expression of target genes was normalized to β -actin, and then expressed as fold change relative to control group. **b** Garcinol dose-dependently suppressed IL-1 β -induced expression of INOS and COX-2. Total cellular extracts from chondrocytes treated with or without IL-1 β in media containing garcinol for 24 h were subject to western blot analyses using specific antibodies against INOS and COX-2. β -actin served as an internal loading control. Data are presented as mean \pm SD. Significant differences among groups are indicated as [#]*P* < 0.01 vs. control group; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. IL-1 β alone treatment group, *n* = 3.

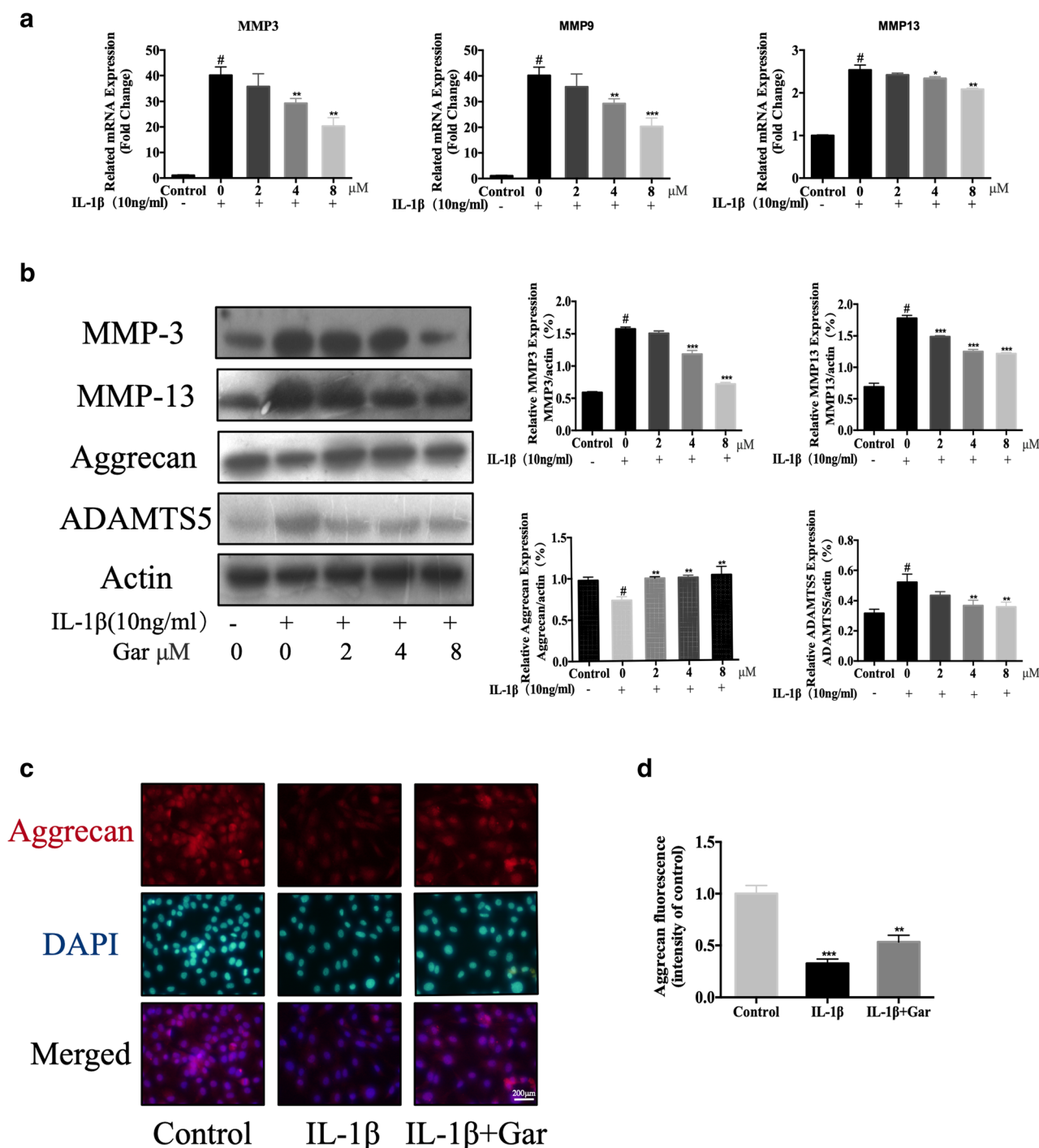


Fig. 3. Garcinol alleviated IL-1 β -induced ECM degradation in mouse chondrocytes. **a, b** IL-1 β increased the expression of MMP3, MMP9, and MMP13, while treatment with garcinol attenuated the expression at both the gene and protein levels. Expression of target genes was normalized to β -actin, and then expressed as fold change relative to controls group. **b** Treatment with IL-1 β degraded aggrecan and increased the expression of ADAMTS-5, while treatment with garcinol reversed this effect. **c** Representative aggrecan was detected by immunofluorescent and DAPI staining. **d** The fluorescence intensity of aggrecan was analyzed by image J. Data are presented as mean \pm SD. Significant differences among groups are indicated as $^{\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. IL-1 β alone treatment group, $n = 3$.

by using qPCR and western blot analysis. As the figure shows, IL-1 β increased the gene expression of MMP3, MMP9, and MMP13, while garcinol prevented this expression in a dose-dependent manner (Fig. 3a, b). We also demonstrated that stimulation with IL-1 β (10 ng/mL) led to the acceleration of ADAMTS-5 (Fig. 3b), while garcinol inhibited this expression. As ADAMTS-5 has been demonstrated to act in cleaving aggrecan, we next investigated the effect of garcinol on IL-1 β -induced degradation of aggrecan, and the data revealed a protect role for garcinol (Fig. 3b). Additionally, the results of our immunofluorescence staining for aggrecan (Fig. 3c, d) confirmed the protective effect of garcinol on IL-1 β -induced ECM degradation.

Garcinol Protected against IL-1 β -Induced Hypertrophic Conversion

As for articular cartilage, collagen II was revealed to be the main component of ECM, responsible for supporting cartilaginous structure and tensile strength, while collagen X is a marker of hypertrophic cartilage, and high expression of collagen X can result in ossification [41, 47]. We thus explored the effect of garcinol on collagen II and X. Our results showed that IL-1 β led to the upregulation of collagen X (Fig. 4a), the primary maker of hypertrophic conversion; however, treatment with garcinol inhibited this acceleration. Conversely, IL-1 β induced the degradation of collagen II while garcinol reversed this effect (Fig. 4a, c).

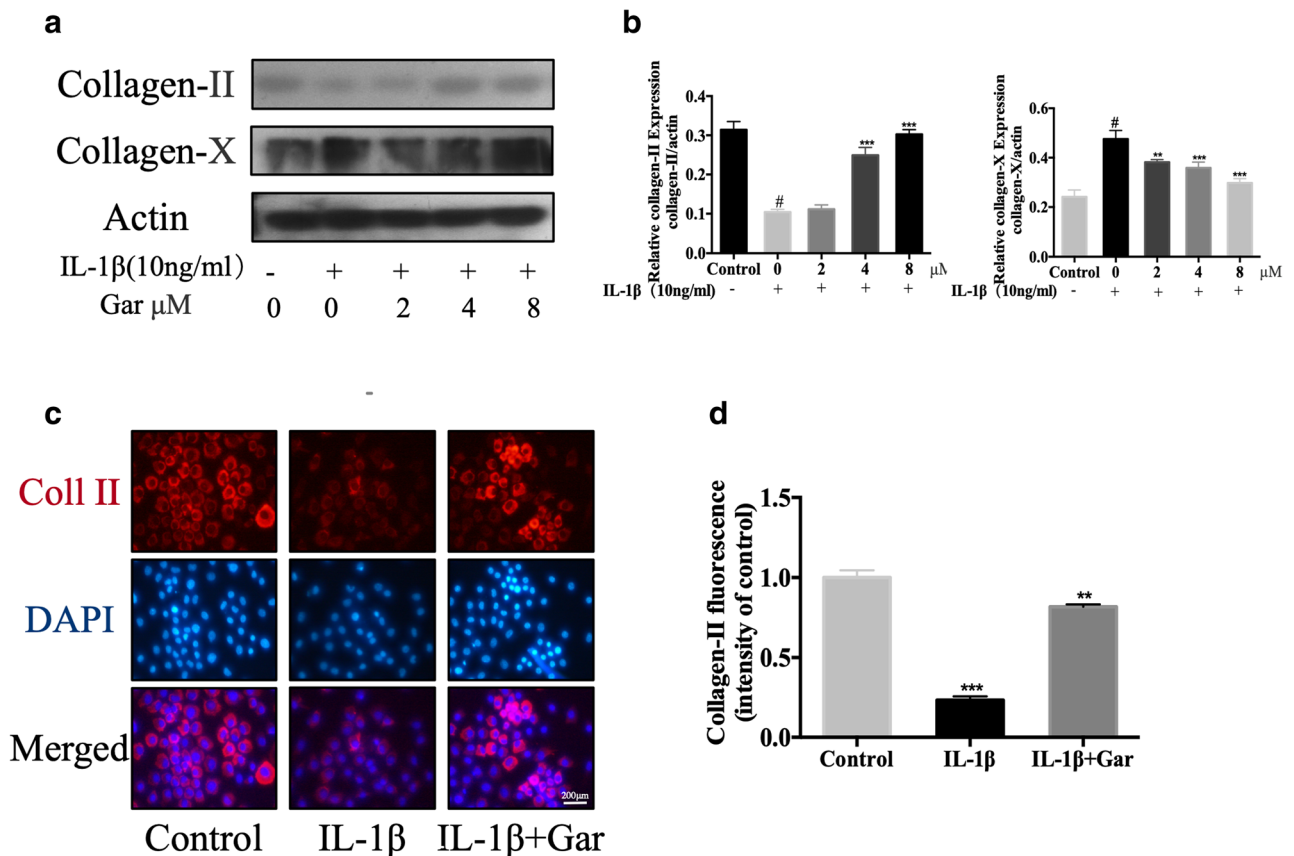


Fig. 4. Garcinol protected against IL-1 β -induced hypertrophic conversion. **a** IL-1 β -induced collagen X was decreased in a dose-dependent manner, with an opposite effect on collagen II as shown by western blot. **b** The expression of collagen X and collagen II relative to actin were determined by ImageJ. **c** Representative collagen II was explored by immunofluorescent and DAPI staining. **d** The fluorescence intensity of collagen II was analyzed using image J. Data are presented as mean \pm SD. Significant differences among groups are indicated as [#] $P < 0.01$ vs. control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. IL-1 β alone treatment group, $n = 3$.

Garcinol Inhibited IL-1 β -Induced Activation of the NF- κ B Pathway in Mouse Chondrocytes

The NF- κ B signaling pathway is reported to be a vital signaling pathway in the development of OA, due to its ability to elicit matrix degradation and anti-inflammation, and inhibit ECM synthesis [31]. To investigate the deep molecular mechanisms of garcinol in IL-1 β -induced inflammation, we further explored its role on the NF- κ B signaling pathway. As Fig. 5 a shows, phosphorylation of p65 was markedly upregulated in the IL-1 β group compared with the control group. However, garcinol decreased this acceleration in a dose-dependent manner. Moreover,

IL-1 β significantly stimulated I κ B α degradation, while garcinol attenuated this degradation. Confocal microscopy also indicated that IL-1 β induced the translocation of p65 from the cytoplasm to the nucleus, while treatment with garcinol suppressed translocation (Fig. 5c).

Garcinol Ameliorated Cartilage Degeneration of OA in Mice Models

Combined with the *in vitro* data, we also established a DMM-induced OA model in mice to investigate whether garcinol has preventive effects against osteoarthritis *in vivo*. The treatment group received an

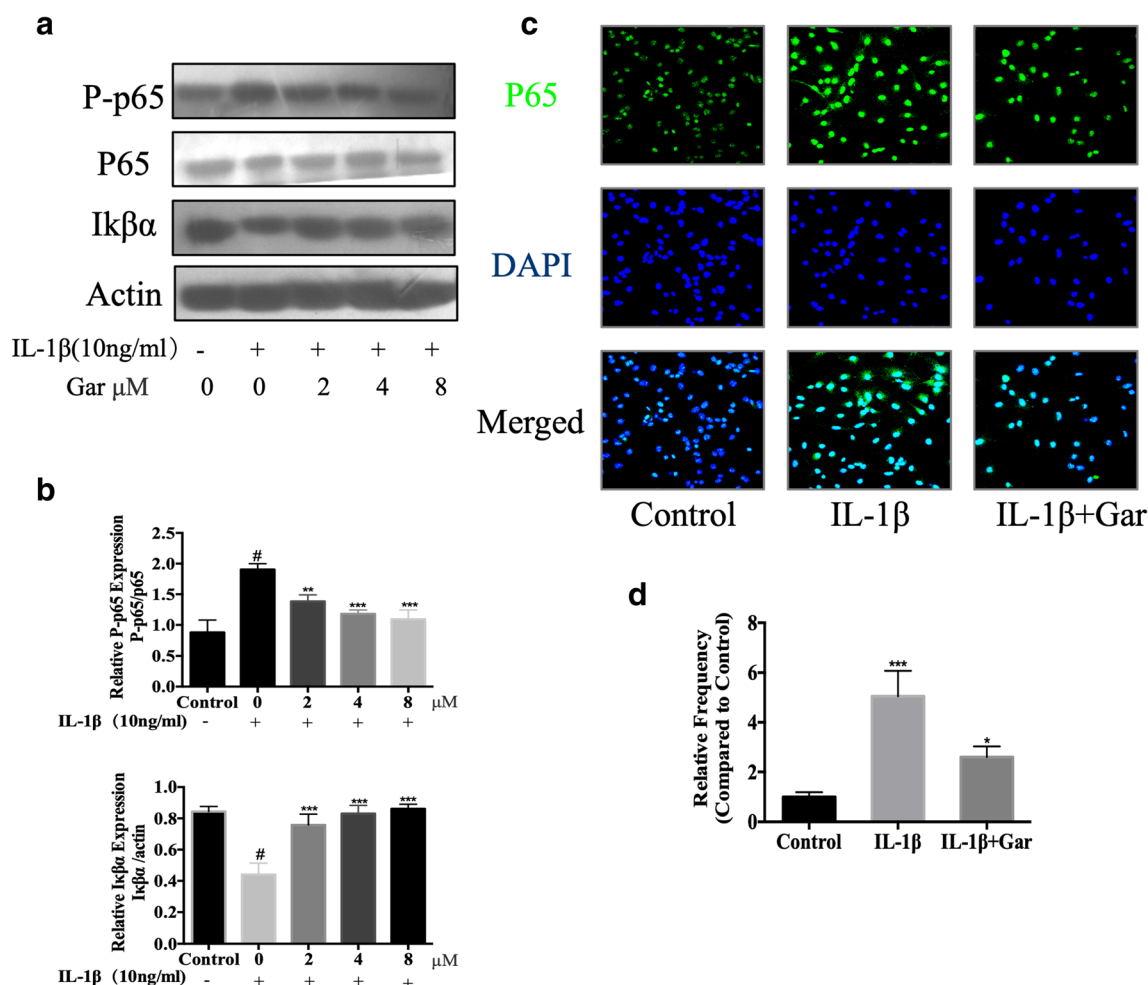


Fig. 5. Garcinol inhibited IL-1 β -induced activation of the NF- κ B signaling pathway in mouse chondrocytes. **a** IL-1 β promoted I κ B α degradation and p65 phosphorylation, while garcinol alleviated both in a dose-dependent manner. **b** The expression of P-p65 relative to total p65 and I κ B α relative to actin were determined by ImageJ. **c** IL-1 β promoted p65 nuclear translocation and localization, while garcinol reversed this effect. Representative immunofluorescence images of p65 localization (green) from chondrocytes. Nuclei were counterstained with DAPI (blue). **d** The fluorescence intensity of p65 was analyzed using image J. Data are presented as mean \pm SD. Significant differences among groups are indicated as # P < 0.01 vs. control group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. IL-1 β alone treatment group, n = 3.

intraperitoneal injection of garcinol (10 mg/kg), while the sham and DMM groups received 5% DMSO *via* intraperitoneal injection every other day in the 6 weeks following surgery. As shown in Fig. 6 a, the cartilage surface was smooth and stained red by Safranin-O staining in the sham control group, while the OA group showed severe cartilage erosion and cartilage destruction. However, the treatment with garcinol rescued this destruction. The OARSI score was higher in the DMM groups, while the garcinol treatment group received

lower OARSI scores (Fig. 6b). To verify whether the protective effect of garcinol *in vivo* is related to collagen II degradation, we performed immunofluorescent staining for collagen II. Our results showed that the fluorescence intensity of collagen II was significantly decreased in the DMM group as compared to sham; however, the treatment with garcinol reversed the degradation (Fig. 7a). In conclusion, our data suggest that garcinol had the ability to attenuate the progression of OA in mice DMM models.

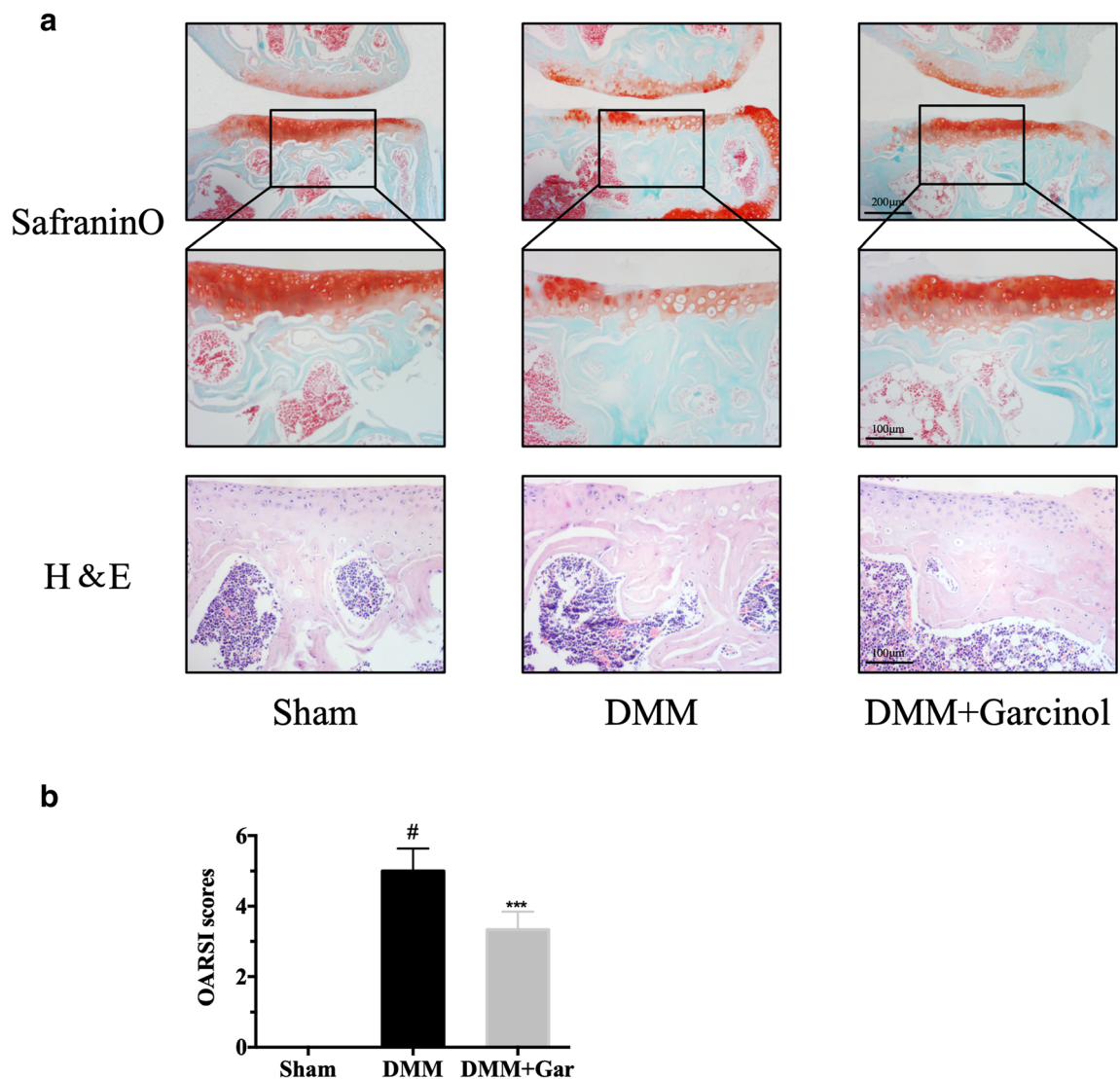


Fig. 6. Garcinol alleviates cartilage degeneration in the mouse DMM model. **a** Safranin-O staining of the cartilage and synovitis from different experimental groups. **b** Representative images of H&E. **c** Diagrams show the cartilage OARSI scores. Data are presented as mean ± SD. Significant differences among groups are indicated as [#] $P < 0.01$ vs. control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. IL-1 β alone treatment group, $n = 3$.

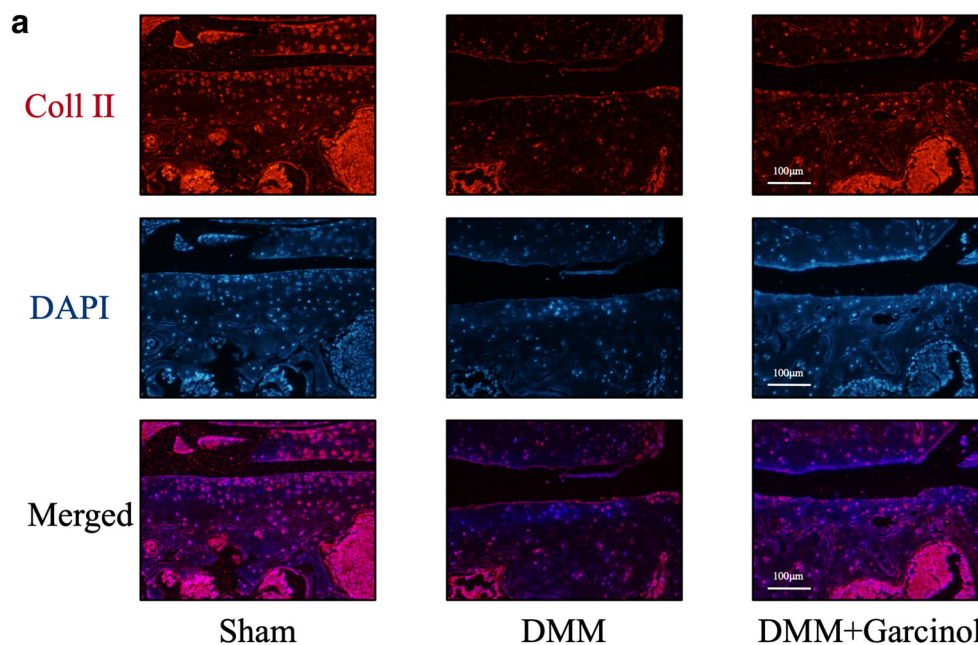


Fig. 7. Garcinol alleviates OA progression in the mouse DMM model. **a** Immunofluorescent staining of collagen II expression in the cartilage samples.

DISCUSSION

Osteoarthritis (OA) is a chronic degenerative disease with features including joint dysfunction, cartilage degradation, and pain [26, 29], and is now highly prevalent among older individuals. Accumulating evidence indicates the important role of inflammation in the initiation and development of OA [28]. However, existing drugs for OA, such as NSAIDs, do not effectively ameliorate symptoms while displaying several side effects [25, 45]. Therefore, development of a safe agent which can inhibit cartilage degradation in OA is urgent. Recently, studies have demonstrated that plant-derived compounds are potent in the treatment of inflammation-related diseases. For instance, garcinol, the main active component derived from *Garcinia indica* (*G. indica*), has displayed antioxidant and anti-inflammatory effects in various diseases [8, 27]. In this study, we explored the effects of garcinol on inflammation which was induced by IL-1 β . Our data showed that garcinol dramatically inhibited IL-1 β -induced inflammation in chondrocytes. The primary mechanism contributing to the garcinol suppression of IL-1 β -induced events was NF- κ B activation in chondrocytes.

The extracellular matrix (ECM), which consists of type II collagen and aggrecan, primarily maintains the structure of cartilage [11]. Uncontrolled proteolytic extracellular matrix destruction results in cartilage destruction,

regarded as the material cause of OA. In addition, as the only cell type in cartilage, chondrocytes maintains the balance of ECM between synthesis and degradation in normal joints. However, inflammatory factors such as IL-1 β break this homeostasis. Inflammatory reactions lead to the upregulation of matrix metalloproteinases (MMPs), such as MMP3 and MMP13, resulting in the degradation of ECM. In addition, IL-1 β also degrades aggrecan, the major ECM component of cartilage, and this degradation contributes to the acceleration of ADAMTS. Our study demonstrated that garcinol promoted the synthesis of the main components of ECM, which were decreased by IL-1 β . Furthermore, treatment with garcinol also reduced factors which could promote the catabolism of ECM, such as MMPs and ADAMTS-5.

Collagen II is the main component of the ECM and supports cartilaginous structure, while collagen X is considered as a standard marker of hypertrophic cartilage and high expression of collagen X can result in ossification [41, 47]. Our data demonstrated that garcinol ameliorated IL-1 β -induced degradation of collagen II and decreased the expression of collagen X, which was induced by IL-1 β , recovering inflammation-induced joint dysfunction.

The NF- κ B signaling pathway is reported to be a vital signaling pathway in the process of OA due to its ability to elicit matrix degradation, contribute to inflammation, and inhibit ECM synthesis [31]. NF- κ B is normally located in

the cytoplasm and is bound to I κ B α , but stimulation with IL-1 β frees NF- κ B and promotes its translocation from the cytoplasm to the nucleus, subsequently facilitating the expression of inflammatory genes such as MMPs, iNOS, COX2, and IL-6 [31]. Our data revealed that garcinol inhibited p65 phosphorylation and nuclear translocation, demonstrating a suppressive role of garcinol on NF- κ B in chondrocytes.

The surgical destabilization of the medial meniscus (DMM) was confirmed as an instability model to investigate OA [15]. Mice developed calcification of the cartilage surface, osteophyte formation, and cartilage erosion after receiving the surgical DMM. However, our data showed that treatment with garcinol recovered the destruction induced by DMM.

In summary, our study demonstrated that garcinol inhibited chondrocyte inflammation *in vitro* and relieved DMM-induced cartilage degeneration *in vivo*. The primary mechanism contributing to the garcinol suppression of IL-1 β -induced events was NF- κ B activation in chondrocytes. These findings demonstrate that garcinol has potential as a novel drug for the treatment of OA.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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