Madecassic Acid Ameliorates the Progression of Osteoarthritis: An in vitro and in vivo Study

Xuejie Fu¹ *, Shuangjian He² *, Liang Wang², Yangyang Xue², Shigang Qiao¹, Jianzhong An¹, Tingting Xia³

¹Institute of Clinical Medicine Research, Suzhou Science & Technology Town Hospital, Suzhou, Jiangsu, People’s Republic of China; ²Department of Orthopedics, Suzhou Science & Technology Town Hospital, Suzhou, Jiangsu, People’s Republic of China

*These authors contributed equally to this work

Correspondence: Tingting Xia, Institute of Clinical Medicine Research, Suzhou Science & Technology Town Hospital, Suzhou, 215153, Jiangsu, People’s Republic of China; Tel +86 18523986726, Email xtt_92@126.com

**Purpose:** Osteoarthritis (OA) places a significant burden on society and finance, and there is presently no effective treatment besides late replacement surgery and symptomatic relief. The therapy of OA requires additional research. Madecassic acid (MA) is the first native triterpenoid compound extracted from Centella asiatica, which has a variety of anti-inflammatory effects. However, the role of MA in OA therapy has not been reported. This study aimed to explore whether MA could suppress the inflammatory response, preserve and restore chondrocyte functions, and ameliorate the progression of OA in vitro and in vivo.

**Methods:** Rat primary chondrocytes were treated with IL-1β to simulate inflammatory environmental conditions and OA in vitro. We examined the effects of MA at concentrations ranging from 0 to 200 µM on the viability of rat chondrocytes and selected 10 µM for further study. Using qRT-PCR, immunofluorescent, immunocytochemistry, and Western blotting techniques, we identified the potential molecular mechanisms and signaling pathways that are responsible for these effects. We established an OA rat model by anterior cruciate ligament transection (ACLT). The animals were then periodically injected with MA into the knee articular cavity.

**Results:** We found that MA could down-regulate the IL-1β-induced up-regulation of COX-2, iNOS and IL-6 and restore the cytoskeletal integrity of chondrocytes treated with IL-1β. Moreover, MA protects chondrocytes from IL-1β-induced ECM degradation by upregulating ECM synthesis related protein expression, including collagen-II and ACAN, and further down-regulating ECM catabolic related protein expression, including MMP-3 and MMP-13. Furthermore, we found that NF-κB/IκBα and PI3K/AKT signaling pathways were involved in the regulatory effects of MA on the inflammation inhibition and promotion of ECM anabolism on IL-1β-induced chondrocytes.

**Conclusion:** These findings suggest that MA appears to be a potentially small molecular drug for rat OA.

**Keywords:** osteoarthritis, madecassic acid, inflammatory responses, cartilage degradation

Introduction

Osteoarthritis (OA) is among the diseases with the most rapid increase in prevalence, which places a significant burden on society and finance, and there is presently no effective treatment besides late replacement surgery and symptomatic relief.¹,² Today, OA can also be defined as a pathology of the “ostechondral unit”, which combined of articular cartilage, calcified cartilage, subchondral cortical and trabecular bone. This unit functions primarily by transferring load-bearing weight over the joint to allow for normal joint articulation and movement.³,⁴ OA is characterized by joint pain and restriction of movement, as well as the presence of effusion and varying degrees of local inflammation.⁵,⁶ Although aging and mechanical instability have been considered the main causes of OA, current research provides a new view of cartilage degeneration caused by inflammatory responses.⁷ In impaired cartilage and synovium, proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are generated.⁸ Several other cytokines including IL-6, IL-15, IL-17, IL-18 and IL-21 have also been shown to be implicated in OA. Moreover, the TGF-β1
signaling pathway has proven to be one of the key factors in cartilage and bone formation. The articular cartilage consists of more than 70% water and organic extracellular matrix (ECM) components, mostly type II collagen and aggrecan. These catabolic cytokines contribute to the degradation of the ECM by encouraging the overproduction of matrix metalloproteinases (MMPs), aggrecanases and chondrocyte apoptosis, which eventually results in cartilage degradation. Due to a lack of chondrocytes and vascular supply, cartilage has limited ability to resist enzymatic damage and initiate an autonomic functional repair process. Consequently, it is crucial to investigate the development of efficient medications that can maintain and repair chondrocyte functions while suppressing the inflammatory response.

Madecassic acid (MA), a triterpenoid found in centella asiatica plants, has a variety of pharmacological properties, such as anti-inflammatory, anti-diabetic, anti-oxidant, neuroprotective, and anticancer effects. According to research, MA can reduce the activation of gammadeltaT17 cells via the Akt/GSK3beta/NFAT pathways, which helps to treat colitis. In addition, MA may offer promise as a solid and hematological tumor anticancer treatment lead. Additionally, MA was regarded as an anti-diabetic drug since studies indicated that it may reduce lipid accumulation, attenuate oxidative and inflammatory stress, improve hemostatic imbalance and glycemic control in diabetic mice. Although MA has been studied extensively in various fields, the study of MA on OA chondrocyte protection and anti-inflammatory effect are poorly studied.

In the current investigation, rat primary chondrocytes were treated with IL-1β to simulate inflammatory environmental conditions and in vitro OA. In addition, it was investigated whether MA therapy might reduce OA in vivo and in vitro. After identifying the cytotoxicity of MA on chondrocytes, its effect on inflammatory cytokine expression, ECM synthesis, and catabolism-related protein expression was examined in IL-1β-treated rat primary chondrocytes. To investigate the therapeutic properties of MA on OA in vivo. We established an OA rat model induced by anterior cruciate ligament transection (ACLT). The animals were then periodically injected with MA into the knee articular cavity. Additionally, simultaneous verification of the potential molecular mechanisms was simply performed.

Materials and Methods

Chemicals and Reagents
MA was obtained from Selleck Chemicals (Houston, TX, USA). Recombinant IL-1β was obtained from Peprotech (Cranbury, NJ, USA). Primary antibodies against collagen-II and MMP-13 were purchased from Abcam (Cambridge, MA, USA). MMP-3 was obtained from Absin Bioscience (Shanghai, China). ACAN was obtained from Bioss Inc (Beijing, China). IκBα and phospho-IκBα, NF-κB and phospho-NF-κB, and Akt and phospho-Akt, PI3K and phospho-PI3K were obtained from Cell Signalling Technology Co. (Boston, MA, USA). GAPDH was purchased from Proteintech (Chicago, IL, USA). Second antibodies of anti-mouse/rabbit HRP-linked IgG were purchased from CST Co. Gibco provided cell culture reagents (Grand Island, NY, USA).

Primary Chondrocytes Isolation
Rat chondrocytes were collected from male Sprague-Dawley (SD) rats, as previously published. Collagenase (2 mg/mL) was used to break down a knee cartilage block into little pieces. For around 3 hours, the digested material was incubated at 37°C. A cell strainer with a pore size of 70 μm was used to filter the cell suspensions. The cells were cultured in DMEM/F12 medium supplied with 10% fetal bovine serum (FBS). In the following research, chondrocytes from the third culture passage were used.

Cell Viability
Experimental cells were cultured in 96-well plates at a density of 4 × 10³ cells per well. After 12 hours, a serum-free medium was employed to starve cells for 12 hours. The cells were subsequently treated for 48 hours with varying doses of MA (0, 5, 10, 20, 50, 100, 200 μM) with 2% FBS of DMEM/F12 medium. After that, CCK-8 solution was added with serum-free medium for 1 hour incubation. Then, the absorbance was measured at 450 nm using a microplate read (Spectramax 190, Molecular Devices, USA).
Animals and Experimental Groups
We conduct animal feeding and related animal experiments in Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences. All animal experiments met the requirements of the Guide for the Animal Care and Use Committee of Suzhou Institute of Biomedical Engineering and Technology Chinese Academy of Sciences (Approval file ID 2022-B22). ACLT was done on the right knee joint on male Sprague-Dawley rats (200 ± 20g) to lead OA. Eight weeks after ACLT, rats were randomly categorized into three groups of five each: the sham group, the OA group, and the OA+ MA group. Rat knee joints were given intra-articular injections of 1 mg/kg of MA every day for one week, then once every three days for another three weeks. Four weeks after the injection, the rats were sacrificed and the right knee joints were taken off and put in a solution of 4% paraformaldehyde. Hematoxylin-eosin and safranin O-fast green stains were used on these joints.

Real-Time PCR
Using an RNA-Quick Purification Kit, according to the manufacturer’s guidelines, total RNA was extracted (Yishan, China). Using a PrimeScript chemical kit with gDNA Eraser, total RNA was reverse-transcribed into cDNA. The cDNA products were then amplified with SYBR green PCR Premix and specific primers. The CFX96 Real-Time PCR Detection System was used to conduct real-time RT-PCR experiments. The $2^{-\Delta\Delta C_t}$ method was used to determine the target gene’s expression level in relation to the housekeeping gene GAPDH. Supplementary Table 1 displays the primer sequences which listed as Supplementary Material.

Western Blotting Assay
The chondrocytes were treated with a lysis buffer containing PMSF after 48 hours of varied treatments. SDS-PAGE was used to separate the proteins, which were subsequently transferred to polyvinylidene fluoride membranes. Primary antibodies were then incubated. Primary antibodies and their dilution ratio were showed as following. Collagen-II (ab188570, 1:1000), MMP-13 (ab39012, 1:3000), MMP-3 (abs146188, 1:1000), ACAN (bs-1223R, 1:1000), IκBα (CST#4814, 1:1000), p-IκBα (CST#2859, 1:1000), NF-κB (CST#8242, 1:1000), p-NF-κB (CST#3033,1:1000), AKT (CY5561, 1:1000), p-AKT (CY6569, 1:1000), PI3K (CST#4249, 1:1000), p-PI3K (CST#4228, 1:1000) and GAPDH (10494-1-AP, 1:2000). The membranes were cleaned three times with TBST and then incubated with corresponding secondary antibody (1:1000). Primary antibodies and secondary antibodies were incubated and then used to detect the target proteins. To ensure repeatability, all Western blots were conducted at least three times under identical experimental circumstances. Densitometry values for the bands for each protein of interest were determined to calculate fold changes representing protein expression levels under each treatment condition. In each experiment, values were standardized to the loading control.

Immunofluorescences and Immunocytochemistry
Chondrocytes were cultured under various conditions for 48 hours before being rinsed in ice-cold PBS. After that, the cells were immersed in paraformaldehyde for 20 minutes. Then, samples were permeabilized for 15 minutes and blocked with bovine serum albumin, and primary antibodies were then incubated overnight at 4°C. The samples were then exposed to fluorescent second antibody, followed by a 5 minute incubation with DAPI for nuclear staining. The DAB kit was used to color the immunocyte stain. After washing with PBS, images were captured at room temperature using a Zeiss confocal microscope or an Olympus inverted fluorescent microscope (CKX53).

Histological Analysis
Knee samples were put in 4% paraformaldehyde for 48 hours at 4 °C and then put in decalcifying solution for 4 weeks to remove the calcium. Embedded samples were sliced into 10 μm slices and stained with H&E and Safranin-O/green. The guidelines of the Osteoarthritis Research Society International (OARSI) were used to score microscopic histology. The quantification of safranin-O was measured by Image J software.
Statistical Analysis
At least three trials have produced comparable outcomes. All data are displayed as mean ± SD. One-way ANOVA was used in the statistical analysis using GraphPad Prism 8 to decide whether there were any differences between the groups. *p*-values <0.05 were considered statistically significant.

Results
Cytotoxicity of MA on IL-1β-Induced Chondrocytes

Figure 1A illustrates the chemical structure of MA. Chondrocytes were treated with MA at various dosages (0, 5, 10, 20, 50, 100, 200 μM) for 48 hours to detect the cytotoxic effect of the compound, and a CCK-8 assay was carried out to determine cell viability. As shown in Figure 1B, after 48 h of treatment, MA dramatically suppressed the viability of chondrocytes at 100 and 200 μM when compared to the DMSO (0.1%) group. The 10 μM solution significantly increased chondrocytes’ viability. There was no significant difference in the viability at the concentrations of 5, 20 and 50 μM. Based on these results, the best dose of MA for future studies is 10 μM.

MA Suppress the IL-1β Induced Inflammatory Reaction and Cytoskeleton Destruction

Inflammatory factors emerged as the primary contributors to cartilage injury as OA progressed. We analyzed the effect of MA on IL-1β-activated inflammatory activity in rat chondrocytes. As demonstrated in Figure 2A, the IL-6, iNOS and Cox2 mRNA expression were significantly upregulated in the IL-1β-treated group compared with the DMSO group. The addition of MA reduced the expression of these inflammatory genes substantially. Further, we analyzed the effect of MA on IL-1β-induced cytoskeleton structure change in chondrocytes. Figure 2B shows that following IL-1β treatment, the cytoskeletal structure of chondrocytes was damaged, microfilaments and pseudopodia were diminished, and the skeleton’s conformation was reversed when compared to the control group. The cytoskeleton was partly rebuilt after MA therapy. Microfilaments and pseudopods are in good condition. These results indicated that MA effectively alleviated the inflammatory response and restored the cytoskeletal integrity of IL-1β-induced chondrocytes.

MA Protects Chondrocytes Against ECM Degradation Triggered by IL-1β

To determine the role of MA in IL-1β-induced ECM degradation of chondrocytes. Chondrocytes were treated with IL-1β in the presence and absence of MA. IL-1β treatment resulted in abnormal expression of ECM synthesis and degradation-related proteins. As demonstrated in Figure 3A, when compared with the control group, IL-1β significantly up-regulated ECM degradation-related genes MMP-3 and MMP-13 expression and down-regulated ECM synthesis-related genes collagen-II and ACAN expression levels. However, MA could restore those gene expressions. As demonstrated in Figure 3B and C, immunofluorescence and immunocytochemistry assay of Collagen-II, ACAN and MMP-13 showed
that MA inhibited the expression of MMP-13 and enhanced Collagen-II and ACAN expression. As additional confirmation of the results, Western blots (Figure 3D and E) demonstrated that MA enhanced the collagen-II and ACAN protein expression, while the protein expression of MMP-3 and MMP-13 was drastically decreased in the MA-treated group when compared to the IL-1β-treated group in rat chondrocytes. These results imply that MA may successfully protect chondrocytes from ECM degradation caused by IL-1β.

MA Protects Chondrocyte via Inhibiting NF-κB/IκBα and Regulating PI3K/AKT Signaling Pathways

Previous studies have reported that NF-κB contributes to IL-1β-provoked cell damage. In our study, we used Western blotting assay to evaluate the influence of MA on the IL-1β-induced chondrocytes. As demonstrated in Figure 4A and B,
Figure 3 Effects of MA on the ECM degradation of chondrocytes. (A) The mRNA expression of Collagen-II, ACAN, MMP-3 and MMP-13. (B) Immunofluorescence image of Collagen II, bar = 50 μm. (C) Immunocytochemistry image of ACAN and immunofluorescence image of MMP-13, bar = 100 μm. (D) The protein expression of collagen-II, ACAN, MMP-3 and MMP-13 in chondrocytes. Expressions have been normalized to GAPDH. (E) Relative intensity of protein expression. The data are presented as mean ± SD. Significant differences are represented as *p < 0.05, **p < 0.01 compared with the DMSO group, #p < 0.05, ##p < 0.01 compared with IL-1β group, n = 3.
compared to the DMSO group, p/t-IκBα expression was significantly increased in the IL-1β treated group, while MA treatment inhibited the phosphorylation of IκBα. The down-stream target of IκBα is NF-κB. We further analyzed the phosphorylation level of NF-κB (p65). In comparison to the DMSO group, IL-1β induction dramatically increased the phosphorylation level of p65, while MA could restore the p/t-p65 value. PI3K/AKT is an upstream pathway of NF-κB. As demonstrated in Figure 4C and D, IL-1β stimulation inhibits the phosphorylation levels of PI3K and AKT, while MA treatment restores the p/t-PI3K and p/t-Akt. These findings reveal that NF-κB/IκBα activation was suppressed and PI3K/AKT was activated by MA following IL-1β conditioning, and further implying that MA may inhibit inflammation and promote ECM synthesis by inhibiting the phosphorylation of NF-κB and up-regulating the phosphorylation of PI3K/AKT.

MA Attenuated Cartilage Degradation

We used an OA model with ACLT to test MA's anti-arthritic efficacy in vivo. Eight weeks after ACLT, rats were randomly categorized into three groups of five each: the sham-operated group, the OA group, and the OA+ MA group. The HE and Safranin-O/green stains in Figure 5A showed a regular morphology and structure of articular cartilage in the sham-operated group, while the OA group had a disordered articular structure and thinner cartilage layers, after the introduction of MA, the structure and thickness of the articular cartilage were more complete and thicker after MA therapy. As shown in Figure 5B, quantification of safranin-O revealed a higher cartilage expression in the MA therapeutic group than in the OA group. Meanwhile, after MA therapy, the OARSI score was lower than in the OA group (Figure 5C). These findings revealed that MA inhibited cartilage matrix degradation and delayed cartilage surface degeneration, implying that MA may be useful in slowing the progression of OA.
Discussion

OA is the pathology of the osteochondral unit, which places a significant burden on society and finance. Age, joint damage and obesity are the principal predisposing risk factors for OA. The increased expression of inflammatory mediators changes the cartilage homeostasis by favoring the catabolic activity of chondrocytes, resulting in disruption, loss of the cartilage matrix and impairing the repair ability. Current therapeutic options, such as physical therapy and pain relieving medicines, just alleviate symptoms and frequently have undesirable side effects. As a result, an effective treatment is urgently needed to prevent the progression of OA. MA has been shown to have anti-inflammatory properties. Studies found that MA inhibited LPS-induced inflammation in macrophages. MA also been reported to have anti-colitis effect by suppressing inflammatory signaling pathway. However, its ability to protect OA chondrocytes has yet to be investigated. As a result, we investigated the anti-inflammatory and protective effects of MA on rat OA chondrocytes. Results suggested that MA suppresses IL-1β-induced inflammation and chondrocyte ECM degradation, reducing cartilage degeneration in rat OA.

Now, it is becoming increasingly obvious that chronic inflammation plays key roles in OA. A lot of animal studies supported that IL-1β and TNF control the degeneration of articular cartilage matrix, and several other cytokines including IL-6, IL-15, IL-17, IL-18 and IL-21 have also been shown to be implicated in OA. Specifically, proinflammatory cytokines such as IL-6, TNF-α, iNOS and COX2 contribute to the progression of OA by promoting the synthesis of MMPs, which in turn accelerates matrix degradation. IL-6 has been found to be upregulated in the synovial fluid and serum of OA patients. In addition, when coupled with IL-1, IL-6 increases the expression of MMP-1 and MMP-13 while decreasing the expression of collagen-II. Another effective treatment for OA is COX-2 inhibition. iNOS in chondrocytes synthesizes nitric oxide, which is a major inflammatory mediator in OA. Consistent with those studies mentioned above, our current investigation found that MA reduced the production of IL-6, iNOS and COX-2 in IL-1β-induced chondrocytes. The cytoskeleton structure can reflect the overall state of the cells. Cytoskeleton staining results further confirmed that MA can restore the chondrocytes’ cytoskeletal structure damage caused by IL-1β. Our study showed that MA can protect the cytoskeleton structure of chondrocytes and has an anti-inflammatory activity on chondrocytes stimulated by IL-1β.
The imbalance of synthesis and degradation of extracellular matrix is a direct manifestation of cartilage degradation. The inflammatory mediators increase the synthesis of MMPs. Among which, stromelysin MMP-3 and collagenases MMP-13 play crucial roles in OA. MMP-3 is largely produced by synoviocytes, whereas MMP-13 is primarily produced by chondrocytes that live in cartilage. MMP-13 destroys both collagen and aggrecan, hence performing a dual role in matrix breakdown. Moreover, MMP-3 is well-known activator of other MMPs, including MMP-13. The in vitro study showed that IL-1β significantly induced higher expression of MMP-3 and MMP-13. The main components of the cartilage ECM are collagen-II and ACAN, and its reconstruction has been proven to be a potential therapy in OA progression. Our study results also proved that MA enhanced the collagen-II and ACAN expression, which was inhibited by IL-1β in chondrocytes. These results were consistent with recent works and further proved the importance of ECM reconstruction in OA. However, the underlying mechanism of how MA exhibited the anti-inflammation and anti-ECM degradation remains unclear.

Abundant evidence suggests that inflammation-related pathways PI3K/AKT and NF-κB have been linked to the pathophysiology of OA. To corroborate the therapeutic efficacy of MA, the PI3K/AKT and NF-κB signaling pathway in IL-1β-stimulated chondrocytes were analyzed by Western blotting. Compared with the IL-1β group, MA effectively suppresses the phosphorylation level of IκBα and NF-κB. NF-κB is a downstream target of IκBα, and the IκBα phosphorylation enhances NF-κB pathway activity. Furthermore, recent research has shown that reducing the degradation of IκBα can prevent the activation of NF-κB. Accumulating data supports that PI3K/Akt plays a key role in OA progression. PI3K molecules are classified into three types (I, II, III). The most studied of these is PI3K class I, which contributes to a variety of bioactivities. In PI3K signaling, Akt is a critical messenger. PI3K/Akt is involved in a variety of biological activities, including cell cycle, cell survival, inflammation, metabolism, and apoptosis. According to earlier studies, human OA cartilage tissue has significantly lower levels of the PI3K/AKT signaling pathway than healthy cartilage. The activation of Akt phosphorylation can enhance collagen-II production. PI3K/AKT not only participates in ECM anabolism but also plays a crucial role in ECM catabolism. Another study found that activating PI3K can reduce MMP-13 expression. However, other investigations have shown that specific PI3K and AKT inhibitors can drastically lower MMP expression. These findings highlight the fact that the PI3K/AKT pathway functions as a double-edged sword in ECM metabolism. Our study found that under the IL-1β-stimulation conditions, the phosphorylation level of PI3K and AKT was significantly decreased, while MA effectively up-regulated the phosphorylation level of PI3K and Akt. NF-κB is one of the downstream of PI3K/AKT. Our study implied that MA could inhibit inflammation and promote ECM synthesis via inhibiting the phosphorylation of NF-κB and up-regulating the phosphorylation of PI3K/AKT.

Thus, as illustrated in Figure 6, MA could inhibit the activation of NF-κB signaling pathways which induced by IL-1β. Moreover, MA could promote the PI3K/AKT pathway which inhibited by IL-1β, the PI3K/AKT further promotes the phosphorylation of NF-κB, which targeting in the overexpression of inflammatory factors and inhibition of ECM anabolism. Both of our in vitro and in vivo studies demonstrated that MA could inhibit inflammation and promote ECM synthesis. We hypothesize that MA's inhibition effect on NF-κB pathway was stronger than the promotion effect on PI3K/AKT and finally resulting in the inflammation inhibition and ECM synthesis promotion. Thus, further research was needed to determine the function of PI3K/AKT in cartilage homeostasis under the treatment of MA. Furthermore, our in vivo animal experiments also confirmed the protective effect of MA against OA, where it inhibited the degradation of articular cartilage and decreased its erosion. Our study results indicated that MA has protective effect on rat articular cartilage. Subchondral cortical is an important component of the osteochondral unit, and the role of MA in subchondral bone is also worth further investigation.

There are several drawbacks in this study. Firstly, our study was based on rat chondrocytes and the OA model, rather than human chondrocytes and the OA model as the source of human chondrocytes is limited. Secondly, further research is required to determine the effect of MA in regulating the PI3K/AKT in OA. Therefore, there is room for improvement in future studies.

Conclusion
Our study shows that MA attenuates IL-1β-activated rat chondrocytes inflammation and ECM degradation by inhibiting NF-κB/IκBα and promoting PI3K/AKT signaling pathways. In vitro and in vivo results support MA as a potential therapeutic agent for rat OA.
Figure 6 Schematic illustration of the potential protective mechanism of MA in OA. On the one hand, MA inhibited the activation of NF-κB signaling pathways which induced by IL-1β. (labeled as “a” in red font). On the other hand, MA could promote the PI3K/AKT pathway which inhibited by IL-1β. (labeled as “b” in blue font). MA’s inhibition effect on NF-κB pathway was stronger than the promotion effect on PI3K/AKT, and finally resulting in the inflammation inhibition and ECM anabolism in IL-1β-induced chondrocyte.

Abbreviations
OA, osteoarthritis; MA, Madecassic acid; ACLT, anterior cruciate ligament transection; ACAN, aggrecan; MMPs, matrix metalloproteinases; ECM, extracellular matrix; TNF-α, tumor necrosis factor-α; IL, interleukin; FBS, fetal bovine serum; OARSI, Osteoarthritis Research Society International; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.

Ethics Statement
We conduct animal feeding and related animal experiments in Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences. All animal experiments met the requirements of the Guide for the Animal Care and Use Committee of Suzhou Institute of Biomedical Engineering and Technology Chinese Academy of Sciences (Approval file ID 2022-B22).

Acknowledgments
This work was supported by the National Natural Science Foundation of China (11902059); Chongqing Postdoctoral Science Foundation Project (cstc2019jcyj-bshX0054); Gusu Health Talents Program of Suzhou Municipal Health Commission (GSWS2021070); Key clinical diseases project of Suzhou Municipal Health Commission (LCZX202135). Talent introduction project of Suzhou Science and Technology Town Hospital (yjxm2020004).
Disclosure
The authors report no conflicts of interest in this work.

References
1. Wolf J, Franca EB, Assuncão AA. The burden of low back pain, rheumatoid arthritis, osteoarthritis, and gout and their respective attributable risk factors in Brazil: results of the GBD 2017 study. *Rev Soc Bras Med Trop.* 2020;53(suppl 1):e0285. doi:10.1590/0037-8682-0285-2021
2. Mahmoudian A, Lohmander LS, Mobasher A, et al. Early-stage symptomatic osteoarthritis of the knee - time for action. *Nat Rev Rheumatol.* 2021;17(10):621–632. doi:10.1038/s41584-021-00673-4
3. Molfetta L, Casabella A, Rosini S, et al. Role of the Osteochondral Unit in the Pathogenesis of Osteoarthritis: focus on the Potential Use of Clodronate. *Curr Rheumatol Rev.* 2022;18(1):2–11. doi:10.2174/157399711766621106094117
4. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nat Rev Rheumatol.* 2016;12(11):632–644. doi:10.1038/nrrheum.2016.148
5. Krishnan Y, Grodzinsky AJ. Cartilage diseases. *Matrix Biol.* 2018;71-72:51–69. doi:10.1016/j.matbio.2018.05.005
6. Palazzo C, Nguyen C, Lefevre-Colau -M-M, et al. Risk factors and burden of osteoarthritis. *Ann Phys Rehabil Med.* 2016;59(3):134–138. doi:10.1016/j.rehab.2016.01.006
7. Goldring MB, Otero M. Inflammation in osteoarthritis. *Curr Opin Rheumatol.* 2011;23(5):471–478. doi:10.1097/BOR.0b013e328349e2b1
8. Berenbaum F, Walker C. Osteoarthritis and inflammation: a serious disease with overlapping phenotypic patterns. *Postgrad Med.* 2020;132(4):377–384. doi:10.1080/00325481.2020.1730669
9. He J, Cao W, Azem I, et al. Epigenetics of osteoarthritis: histones and TGF-beta1. *Clin Chim Acta.* 2020;510:593–598. doi:10.1016/j.cca.2020.08.011
10. Rezus E, Burlati A, Cardonea A, et al. From Pathogenesis to Therapy in Knee Osteoarthritis: bench-to-Bedside. *Int J Mol Sci.* 2021;22(5):2697. doi:10.3390/ijms22052697
11. Heinegard D, Saxne T. The role of the cartilage matrix in osteoarthritis. *Nat Rev Rheumatol.* 2011;7(1):50–56. doi:10.1038/nrrheum.2010.198
12. Whittaker JL, Truong LK, Dhiman K, et al. Osteoarthritis year in review 2020: rehabilitation and outcomes. *Osteoarthritis Cartilage.* 2021;29(2):190–207. doi:10.1016/j.joca.2020.10.005
13. Thielen N, Neeffes M, Wiegertjes G, et al. Osteoarthritis-Related Inflammation Blocks TGF-beta’s Protective Effect on Chondrocyte Hypertrophy via (de)Phosphorylation of the SMAD3/2 Linker Region. *Int J Mol Sci.* 2021;22(15):8124. doi:10.3390/ijms22158124
14. Barison MJ, Nogoceke R, Josino R, et al. Functionalized Hydrogels for Cartilage Repair: the Value of Secretome-Instructive Signaling. *Int J Mol Sci.* 2022;23(11):6010. doi:10.3390/ijms23116010
15. Li MH, Xiao R, Li JB, et al. Regenerative approaches for cartilage repair in the treatment of osteoarthritis. *Osteoarthritis Cartilage.* 2017;25(10):1577–1587. doi:10.1016/j.joca.2017.07.004
16. Xu X, Wang Y, Wei Z, et al. Madecassic acid, the contributor to the anti-colitis effect of madecassoside, enhances the shift of Th17 toward Treg cells via the PPARgamma-PTEN/Akt/GSK3beta/NFAT pathway. *Cell Death Dis.* 2019;10(3):e3283. doi:10.1038/s41419-020-02969-x
17. Won JH, Shin J-S, Park H-J, et al. Anti-inflammatory Effects of Madecassic Acid via the Suppression of NF-κB Pathway in LPS-Induced RAW 264.7 Macrophage Cells. *Planta Med.* 2010;76(3):251–257. doi:10.1055/s-0029-1186142
18. Hsu YM, Hung Y-C, Hu L, et al. Anti-Diabetic Effects of Madecassic Acid and Rotundic Acid. *Nutrients.* 2015;7(12):10065–10075. doi:10.3390/nu7125512
19. Arancibia-Radich J, González-Blázquez R, Alcalá M, et al. Beneficial effects of mirttilla extract and madecassic acid on insulin sensitivity and endothelial function in a model of diet-induced obesity. *Sci Rep.* 2019;9(1):599. doi:10.1038/s41598-018-36555-1
20. Tabasserum R, Vaibhav K, Shrivastava P, et al. Centella asiatica attenuates the neurobehavorial, neurochemical and histological changes in transient focal middle cerebral artery occlusion rats. *Neurosci Res.* 2013;73(4):925–933. doi:10.1016/j.neures.2013.07.002
21. Siddiqui S, Khan F, Jamali KS, et al. Madecassic Acid Reduces Fast Transient Potassium Channels and Promotes Neurite Elongation in Hippocampal CA1 Neurons. *CNS Neurol Disord Drug Targets.* 2020;19(1):12–26. doi:10.2174/187152731866619111105508
22. Zhang H, Zhang M, Tao Y, et al. Madecassic Acid inhibits the mouse colon cancer growth by inducing apoptosis and immunomodulation. *J BUON.* 2014;19(2):372–376.
23. Valdeira ASC, Darvishi E, WoldeMICHAEL GM, et al. Madecassic Acid Derivatives as Potential Anticancer Agents: synthesis and Cytotoxic Evaluation. *J Nat Prod.* 2019;82(8):2094–2105. doi:10.1021/acs.jnatprod.8b00864
24. Chen H, Qin J, Shi H, et al. Rhofolin ameliorates osteoarthritis via the Nrf2/NF-kappaB axis: in vitro and in vivo experiments. *Osteoarthritis Cartilage.* 2022;30(5):735–745. doi:10.1016/j.joca.2022.01.009
25. Wang C, Gao Y, Zhang Z, et al. Safflower yellow alleviates osteoarthritis and prevents inflammation by inhibiting PGE2 release and regulating NF-kappaB/SIRT1/AMPK signaling pathways. *Phytomedicine.* 2020;78:153305. doi:10.1016/j.phymed.2020.153305
26. Xia T, Zhao R, Liu W, et al. Effect of substrate stiffness on hepatocyte migration and cellular Young’s modulus. *J Cell Physiol.* 2018;233(9):6966–7006. doi:10.1002/jcp.26491
27. Chow YY, Chin KY. The Role of Inflammation in the Pathogenesis of Osteoarthritis. *Mediators Inflamm.* 2020;2020:8293921. doi:10.1155/2020/8293921
28. van Doorna MCM, Meehoff GA, Vliet Vlieland TPM, et al. A clinical practice guideline for physical therapy in patients with Hip or knee osteoarthritis. *Musculoskeletal Care.* 2020;18(4):575–595. doi:10.1016/j.msc.1492
29. Goh SL, Persson MSM, Stocks J, et al. Relative Efficacy of Different Exercises for Pain, Function, Performance and Quality of Life in Knee and Hip Osteoarthritis: systematic Review and Network Meta-Analysis. *Sports Med.* 2019;49(5):743–767. doi:10.1007/s40279-019-01082-0
30. Katz JN, Arant KR, Loeser RF. Diagnosis and Treatment of Hip and Knee Osteoarthritis: a Review. *JAMA.* 2021;325(6):568–578. doi:10.1001/ jama.2020.22171
31. Yun X, Fang Y, Lv C, et al. Inhibition of the activation of gammadeltaT17 cells through PPARgamma-PTEN/Akt/GSK3beta/NFAT pathway contributes to the anti-colitis effect of madecassic acid. *Cell Death Dis.* 2020;11(9):752. doi:10.1038/s41419-020-02969-x
32. Robinson WH, Lepus CM, Wang Q, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol. 2016;12(10):580–592. doi:10.1038/nrrheum.2016.136

33. Zhen G, Guo Q, Li Y, et al. Mechanical stress determines the configuration of TGFbeta activation in articular cartilage. Nat Commun. 2021;12(1):1706. doi:10.1038/s41467-021-21948-0

34. Martel-Pelletier J, Barr AJ, Cicuttini FM, et al. Osteoarthritis. Nat Rev Dis Primers. 2016;2:16072. doi:10.1038/nrdp.2016.72

35. Hou CH, Tang C-H, Chen P-C, et al. Thrombospondin 2 Promotes IL-6 Production in Osteoarthritis Synovial Fibroblasts via the PI3K/AKT/ NF-kappaB Pathway. J Inflamm Res. 2021;14:5955–5967. doi:10.2147/JIR.S314747

36. Kapoor M, Martel-Pelletier J, Lajeunesse D, et al. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol. 2011;7(1):33–42. doi:10.1038/nrrheum.2010.196

37. Wen ZH, Lin Y-Y, Chang Y-C, et al. The COX-2 inhibitor etoricoxib reduces experimental osteoarthritis and nociception in rats: the roles of TGF-beta1 and NGF expressions in chondrocytes. Eur J Pain. 2020;24(1):209–222. doi:10.1002/ejp.1478

38. Ahmad N, Ansari MY, Haqqi TM. Role of iNOS in osteoarthritis: pathological and therapeutic aspects. J Cell Physiol. 2020;235(10):6366–6376. doi:10.1002/jcp.29607

39. Lai WF, Wong WT. Roles of the actin cytoskeleton in aging and age-associated diseases. Ageing Res Rev. 2020;58:101021. doi:10.1016/j.arr.2020.101021

40. Gentili C, Cancetta R. Cartilage and bone extracellular matrix. Curr Pharm Des. 2009;15(12):1334–1348. doi:10.2174/138161209787846739

41. Guilak F, Nims RJ, Dicks A, et al. Osteoarthritis as a disease of the cartilage pericellular matrix. Matrix Biol. 2018;71-72:40–50. doi:10.1016/j.matbio.2018.05.008

42. Malemud CJ. Inhibition of MMPs and ADAM/ADAMTS. Biochem Pharmacol. 2019;165:33–40. doi:10.1016/j.bcp.2019.02.033

43. Mehana EE, Khafaga AF, El-Blehi SS. The role of matrix metalloproteinases in osteoarthritis pathogenesis: an updated review. Life Sci. 2019;234:116786. doi:10.1016/j.lfs.2019.116786

44. Pengas I, Eldridge S, Assiotis A, et al. MMP-3 in the peripheral serum as a biomarker of knee osteoarthritis, 40 years after open total knee meniscectomy. J Exp Orthop. 2018;5(1):21. doi:10.1186/s40634-018-0132-x

45. Knauper V, Will H, López-Otin C, et al. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. J Biol Chem. 1996;271(29):17124–17131. doi:10.1074/jbc.271.29.17124

46. Kapoorn M, Martel-Pelletier J, Lajeunesse D, et al. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol. 2011;7(1):33–42. doi:10.1038/nrrheum.2010.196

47. Han G, Zhang Y, Li H. The Combination Treatment of Curcumin and Probucol Protects Chondrocytes from TNF-alpha Induced Inflammation by Inhibiting the PI3K/AKT Pathway. J Biol Chem. 2018;293(35):17061–17072. doi:10.1074/jbc.M118.006667

48. Qi Y, Cheng X, Gong G, et al. Synergistic neuroprotective effect of schisandrin and nootkatone on regulating inflammation, apoptosis and autophagy via the PI3K/AKT pathway. Food Funct. 2020;11(3):2427–2438. doi:10.1039/C9FO02927C

49. Fisch KM, Gamini R, Alvarez-Garcia O, et al. Identification of transcription factors responsible for dysregulated networks in human osteoarthritis cartilage by global gene expression analysis. Osteoarthritis Cartilage. 2018;26(1):1531–1538. doi:10.1016/j.joca.2018.07.012

50. Greene MA, Loeser RF. Function of the chondrocyte PI-3 kinase-Akt signaling pathway is stimulus dependent. Osteoarthritis Cartilage. 2015;23(6):949–956. doi:10.1016/j.joca.2015.01.014

51. Tewari D, Patni P, Bishayee A, et al. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: a novel therapeutic strategy. Semin Cancer Biol. 2022;80:1–17. doi:10.1016/j.semcancer.2019.12.008

52. Han G, Zhang Y, Li H. The Combination Treatment of Curcumin and Probucol Protects Chondrocytes from TNF-alpha Induced Inflammation by Inhibiting the PI3K/AKT Pathway. J Biol Chem. 2018;293(35):17061–17072. doi:10.1074/jbc.M118.006667

53. Qi Y, Cheng X, Gong G, et al. Synergistic neuroprotective effect of schisandrin and nootkatone on regulating inflammation, apoptosis and autophagy via the PI3K/AKT pathway. Food Funct. 2020;11(3):2427–2438. doi:10.1039/C9FO02927C

54. Fisch KM, Gamini R, Alvarez-Garcia O, et al. Identification of transcription factors responsible for dysregulated networks in human osteoarthritis cartilage by global gene expression analysis. Osteoarthritis Cartilage. 2018;26(1):1531–1538. doi:10.1016/j.joca.2018.07.012

55. Greene MA, Loeser RF. Function of the chondrocyte PI-3 kinase-Akt signaling pathway is stimulus dependent. Osteoarthritis Cartilage. 2015;23(6):949–956. doi:10.1016/j.joca.2015.01.014

56. Han G, Zhang Y, Li H. The Combination Treatment of Curcumin and Probucol Protects Chondrocytes from TNF-alpha Induced Inflammation by Enhancing Autophagy and Reducing Apoptosis via the PI3K-Akt-mTOR Pathway. Oxid Med Cell Longev. 2021;2021:5558066. doi:10.1155/2021/5558066

57. Hui W, Litherland GJ, Elias MS, et al. Leptin produced by joint white adipose tissue induces cartilage degradation via upregulation and activation of matrix metalloproteinases. Ann Rheum Dis. 2012;71(3):455–462. doi:10.1136/annrheumdis-2011-200372