Curculigoside inhibits osteoarthritis *via* the regulation of NLRP3 pathway

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ABSTRACT

Osteoarthritis (OA) is characterized by degenerative articular cartilage. Nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) plays an important role in inflammation. This study aims to investigate whether protective effects of curculigoside on OA are medicated by the regulation of NLRP3 pathway. Destabilization of the medial meniscus (DMM) was performed to build an OA mouse model. After surgery, OA mice were treated with curculigoside. Immunohistochemistry was conducted to evaluate OA cartilage. In addition, human chondrocytes were isolated and treated with curculigoside. The mRNA and protein expression of iNOS, MMP-9, NLRP3 was detected by PCR and Western blot analysis. Curculigoside inhibited mRNA and protein levels of iNOS and MMP-9 induced by DMM surgery in a dose-dependent manner. Furthermore, the expression of NLRP3, NF-ϰB and PKR was downregulated after curculigoside administration. Moreover, curculigoside reversed the effects of IL-1β on MMP-9, iNOS and type II collagen expression at mRNA and protein levels in human chondrocytes in a dose-dependent manner. In conclusion, curculigoside exhibits beneficial effect on cartilage *via* the inhibition of NLRP3 pathway.

Key words: curculigoside; nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3; osteoarthritis; inflammation.

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Ethical approval: this study was approved by the Ethics Committee of Shanxi Bethune Hospital (protocol no. 200215) and followed the Declaration of Helsinki. All animal study protocols were approved by the Animal Care and Use Committee of Shanxi Bethune Hospital (protocol no. 201410).

Availability of data and materials: the data used to support the findings of this study are available from the corresponding author upon request

Introduction

Osteoarthritis (OA) is a joint disease featuring degenerative articular cartilage and joint inflammation and is one of the main reasons for disability.[1,2] The accompanying pain and potential risks of physiological disability exert a great threat to public health worldwide.[3] Generally, the prevailing strategies for OA focus on pain management and symptom control. However, perplexing factors complicate therapy for OA. Increasing evidence has demonstrated that the degradation of chondrocytes plays a crucial role in the pathogenesis of OA, which induces the anabolic-catabolic imbalance and the degeneration of articular cartilage.[4] Interestingly, the degradation of chondrocytes in OA is associated with inflammasomes, among which nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) is postulated as a key factor in regulating the progression of OA.[5]

NLRP3 inflammasome is an intracellular multi-protein complex that promotes the inflammatory response.[6] NLRP3 is activated by the binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs) or various stimuli, such as apoptosis-associated speck-like protein, ATP, and double-stranded RNA-dependent protein kinase (PKR).[7] NLRP3 renders as a catabolic regulator *via* regulating the expression of catabolic factors, such as matrix metalloproteinases (MMP-1, MMP-3, and MMP-9), inducible nitric oxide synthase (iNOS) and NOS2.[8,9] Therefore, repressing the catabolic metabolism in chondrocytes *via* downregulating the expression of NLRP3 may be a promising strategy for OA.

Curculigoside is the major ingredient of *Curculiginis* and exerts antioxidant, anti-osteoporotic, and anti-inflammation roles as well as boosting the immune system and upregulating the expression of estrogen.[10] It is proved that curculigoside plays an anti-arthritic role *via* blocking NF-κB/NLRP3 signaling pathway in adjuvant arthritis (AA) rats, and downregulating inflammation-related genes such as nuclear factor kappa B (NF-κB) and interleukin 1β (IL-1β).[11] However, the potential mechanisms of curculigoside in regulating NLRP3 in OA are still unknown.

This study aimed to investigate the role of curculigoside in cartilage protection and explore whether curculigoside could inhibit the expression of NLRP3 and catabolism-related genes.

Materials and Methods

Animals

All the animal study protocols were approved by the Animal Care and Use Committee of Shanxi Bethune Hospital (Approval No. 201410). Male C57BL/6 mice (18-22 g) were purchased from Experimental Animal Center of Shanxi Academy of Medical Sciences. Curculigoside was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) as 100 mg/mL stock solution. At 8 weeks, mice were divided into four groups: sham group (no surgery, n=10), OA group (OA models with vehicle surgery, n=10), OA models treated with 10 μg of curculigoside (10 μg group), and OA models treated with 20 μg of curculigoside (20 μg group).

Mice were anesthetized and intraperitoneally injected with pentobarbiturate (0.5 mg/10 g body weight). OA models were constructed by surgical destabilization of the medial meniscus (DMM) in the right knee of the mice. The surgical approach for DMM was a 3 mm longitudinal incision over the distal patella to the proximal tibial plateau. The joint capsule immediately medial to the patellar tendon was incised and opened with microsurgery scissors. Blunt dissection of the fat pad over the intercondylar area was used to expose the intercondylar region. The medial meniscus was surgically removed. The surgical incisions were closed in two layers using absorbable sutures. In sham group and vehicle group, the ligament was visualized but not transected.

Human articular chondrocytes

Normal articular cartilage was collected from enrolled volunteers during knee joint replacement surgery. This study was approved by the Ethics Committee of Shanxi Bethune Hospital (Approval No. 200215). All participants had signed the informed consent. After the surgery, the articular cartilage was treated with 0.25% trypsin and 0.2% collagenase. The released chondrocytes were cultured in DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). The chondrocytes were treated with 10 ng/mL IL-1β (R&D Systems, Minneapolis, MN, USA) for 12 h, or pretreated with 10 μg or 20 μg curculigoside, or 10 ng/mL NLRP3 inhibitor MCC950 (Sigma) and then co-treated with 10 ng/mL IL-1β for 12 h.

Immunohistochemistry

Tissues collected were fixed, dehydrated and embedded with paraffin. The embedded samples were sliced into 5 μm sections and deparaffinizated and then rehydrated. Antigen retrieval was performed by the incubation of the sections in 10 mM citrate buffer (pH 6.0), and the sections were kept at 100°C for 1 h to block endogenous peroxidase. Next, the sections were blocked with 10% bovine serum albumin (BSA). Then the slice was incubated with primary antibodies for NLRP3, PKR, NF-κB, MMP-9 and iNOS (1:100 dilution, all from Abcam, Cambridge, MA, USA) at 4°C overnight. The next day, the slice was washed with phosphate-buffered saline (PBS) and incubated with peroxidase-conjugated secondary antibodies (1:1000 dilution, Abcam) at 37°C for 1 h. For negative control, the slice was incubated with PBS instead of the primary antibody. Finally, the sections were treated with diaminobenzidine (DAB). For each sample, three sections were selected and 5 fields of view per section were evaluated blindly. Immunohistochemical staining density was determined using ImageJ software (ImageJ, Bethesda, MD, USA).

qRT-PCR

Total RNA was collected from human chondrocytes, and reversely transcribed into cDNA using One Step PrimeScript® miRNA cDNA Synthesis kit (Takara Bio, Shiga, Japan). PCR was conducted on ABI PRISM 7500 system with SYBR Green PCR Master Mix (Takara Bio). The primers were purchased from OriGene (Rockville, MD, USA) with the following sequences: NLRP3, GGACTGAAGCACCTGTTGTGCA and TCCTGAGTCTCCCAAGGCATTC; PKR, ACCGTCAGACTCTGGAGGCTTT and GGCTAGATGGTCTGTCGCCAAA; NF-κB GCAGCACTACTTCTTGACCACC and TCTGCTCCTGAGCATTGACGTC; MMP-9, GCCACTACTGTGCCTTTGAGTC and CCCTCAGAGAATCGCCAGTACT; iNOS, GCTCTACACCTCCAATGTGACC and CTGCCGAGATTTGAGCCTCATG; type II collagen (COL2), CCTGGCAAAGATGGTGAGACAG and CCTGGTTTTCCACCTTCACCTG; and GAPDH, GTCTCCTCTGACTTCAACAGCG and ACCACCCTGTTGCTGTAGCCAA. GAPDH served as internal control. Relative mRNA expression was calculated with $2^{-\Delta\Delta CT}$ method.[12] Each experiment was performed in triplicate.

Western blot analysis

Total protein was extracted from human chondrocytes using RIPA buffer (Sangon Biotech Co., Ltd., Shanghai, China). Then

the concentration of the total protein was quantified with BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amount of protein (30 μg) was separated with 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies for NLRP3, PKR, NF-κB, MMP-9, iNOS, COL2 and GAPDH (1:500 dilution, all from Abcam) at 4°C overnight. The membranes were washed and incubated with secondary antibodies (1:2000 dilution, Abcam) at 37°C for 1 h. The bands were pictured with enhanced chemiluminescence kit (ECL, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Quantification of relative protein levels was performed using ImageJ software.

Statistical analysis

All data were analyzed with SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). The difference between two groups was evaluated with Student's *t*-test, while the difference among multiple groups was analyzed with ANOVA. A p-value <0.05 was considered statistically significant.

Results

Curculigoside inhibited the upregulation of MMP-9 and iNOS induced by DMM surgery

The expression of MMP-9 in the articular cartilage of the mice at 8-week treatment with DMM significantly increased compared with the sham group. After exposure to curculigoside for 8 weeks, the expression levels of MMP-9 significantly decreased in a dose-dependent manner (Figure 1 A,B). Meanwhile, significant increase of iNOS was observed in DMM-treated mice, which was alleviated by curculigoside in a dose-dependent manner (Figure 1 C,D).

Curculigoside inhibited the expression of NLRP3, PKR, and NF-κB in OA cartilage

NLRP3 upregulates the expression of iNOS and MMPs *via* binding to PKR and interacting with NF-κB.[7-9] Therefore, we detected the expression of PKR and NF-κB in OA cartilage by

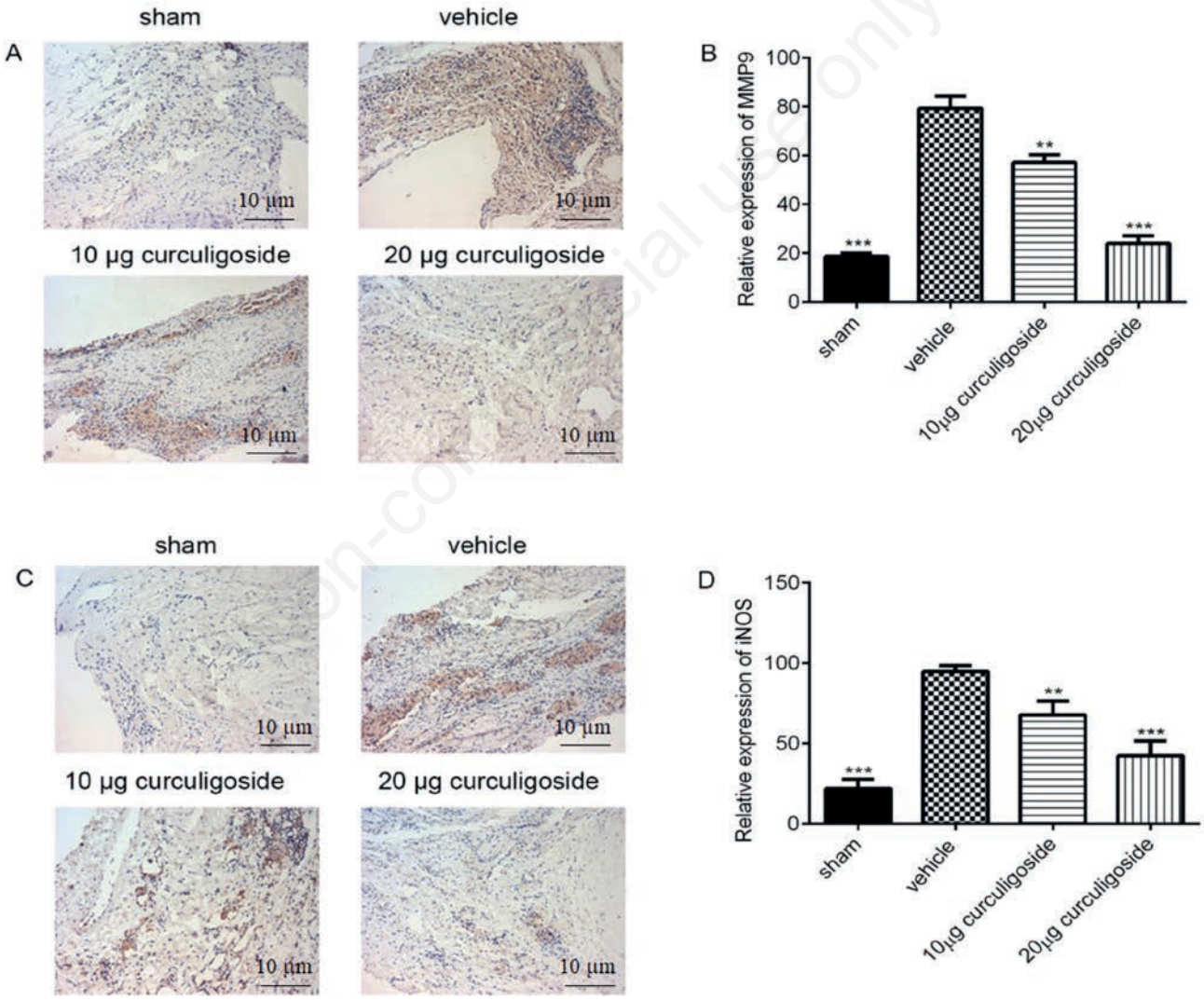

Figure 1. Curculigoside mediated the downregulation of MMP-9 and iNOS induced by destabilization of the medial meniscus (DMM). **A**) At 8-week DMM surgery, the staining density of MMP-9 significantly increased while, after exposure to curculigoside, it significantly decreased in the mice. **B**) Quantitative analysis of the expression of MMP-9. **C**) The staining intensity of iNOS significantly increased in mice with DMM surgery, but decreased after the treatment of curculigoside. **D**) Quantitative analysis of the expression of iNOS. **p<0.01; ***p<0.001 compared to the DMM+vehicle group (n=3).

immunohistochemistry. The expression of NLRP3, PKR, and NF-κB in mice subjected to DMM significantly increased compared with the sham group, which was reversed after being treated with 10 and 20 μg curculigoside. The expression levels of NLRP3, PKR, and NF-κB in curculigoside group were similar to those in the sham group (Figure 2A-F).

Curculigoside inhibited the upregulation of NLRP3 induced by IL-1β

To investigate the potential roles of curculigoside in protecting cartilage, human chondrocytes were treated with IL-1β. qRT-PCR and Western blot were performed to determine the expression of NLRP3, PKR, and NF-κB in human chondrocytes exposed to 10 μg

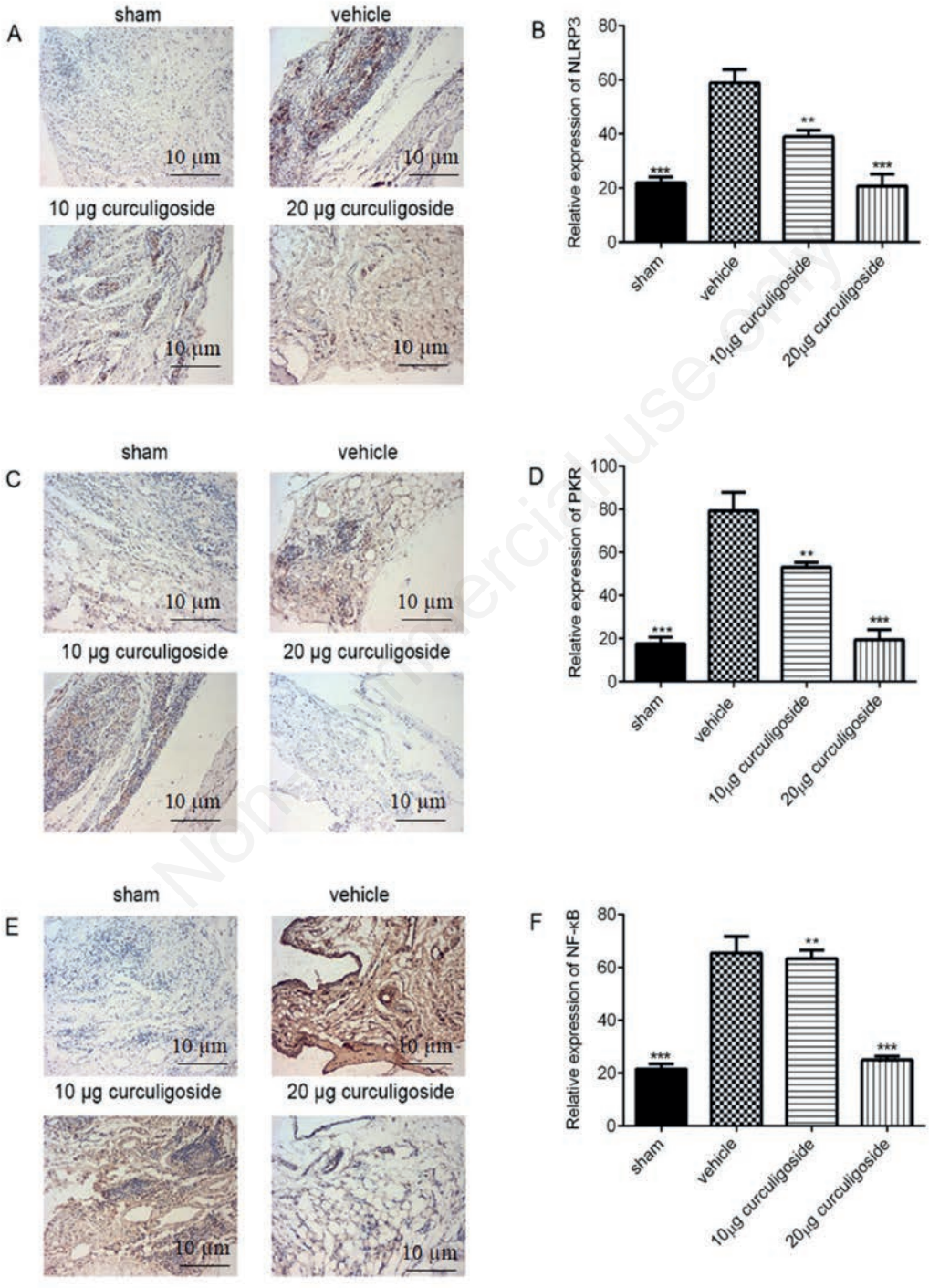

Figure 2. Curculigoside suppressed the expression of NLRP3, PKR and NF-κB in OA cartilage. **A-F**) The expression of NLRP3, PKR, and NF-κB in DMM-treated mice significantly increased compared to the sham group, but decreased after treatment with 10 and 20 μg curculigoside. **p<0.01; ***p<0.001 compared to the DMM+vehicle group (n=3).

or 20 μg curculigoside. IL-1β treatment induced the upregulation of NLRP3, PKR, and NF-κB mRNA expression. The administration of 10 and 20 μg curculigoside significantly downregulated mRNA levels of NLRP3, PKR, and NF-κB in a dose dependent manner (Figure 3A). Meanwhile, protein levels of NLRP3, PKR, and NF-κB in chondrocytes treated with IL-1β decreased by 10 and 20 μg curculigoside in a dose dependent manner (Figure 3 B,C).

NLRP3 may contribute to the regulation of catabolic factors by curculigoside

To investigate potential role of NLRP3 in protective effects of curculigoside on OA, human chondrocytes were treated with 10 ng/mL NLRP3 inhibitor, MCC950. The expression of MMP-9 and iNOS significantly increased while the expression of COL2 significantly decreased after the treatment of IL-1β compared with control group. In human chondrocytes treated with 20 μg curculigoside, the mRNA expression of MMP-9 and iNOS was significantly downregulated while that of COL2 was upregulated compared with IL-1β group, and their levels were similar to those in MCC950 group (Figure 4A). Western blot analysis confirmed the expression changes of these catabolic factors at protein levels by curculigoside and MCC950 (Figure 4 B,C).

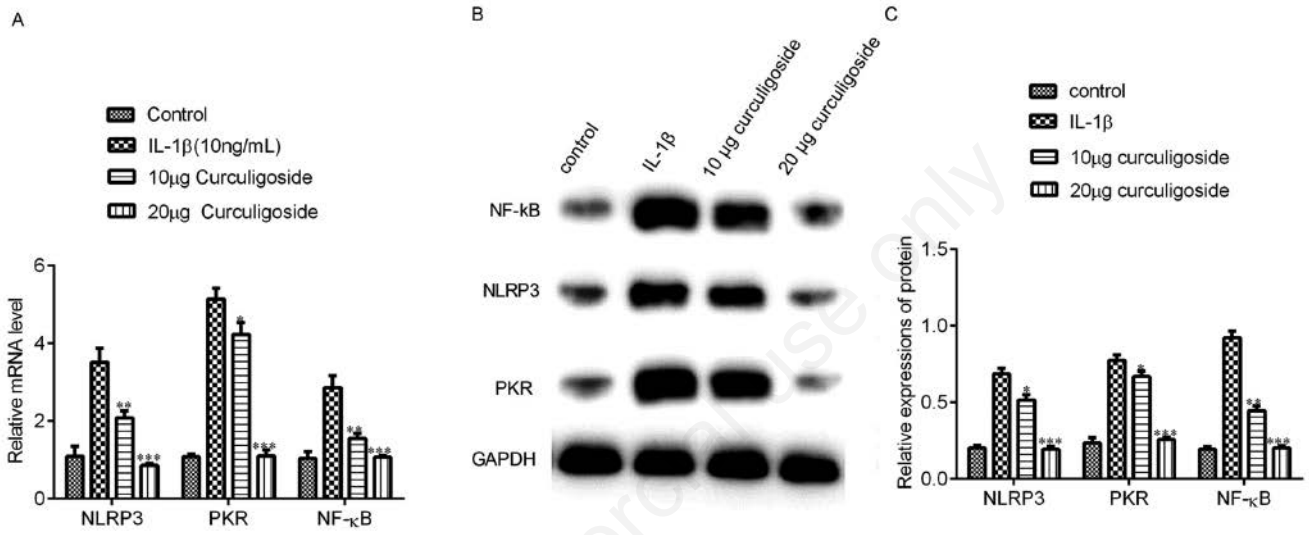

Figure 3. Curculigoside inhibited the upregulation of NLRP3 induced by IL-1β in human chondrocytes. **A**) The mRNA levels of NLRP3, PKR, and NF-κB significantly increased after the treatment with IL-1β compared with control group, but the changes reversed in a dose-dependent manner after curculigoside administration. **B**) Western blot analysis of NLRP3, PKR and NF-κB in the different groups; GAPDH was the loading control. **C**) Densitometric analysis of the protein levels of NLRP3, PKR and NF-κB in the different groups. *p<0.05; **p<0.01; ***p<0.001 compared to IL-1β group (n=3).

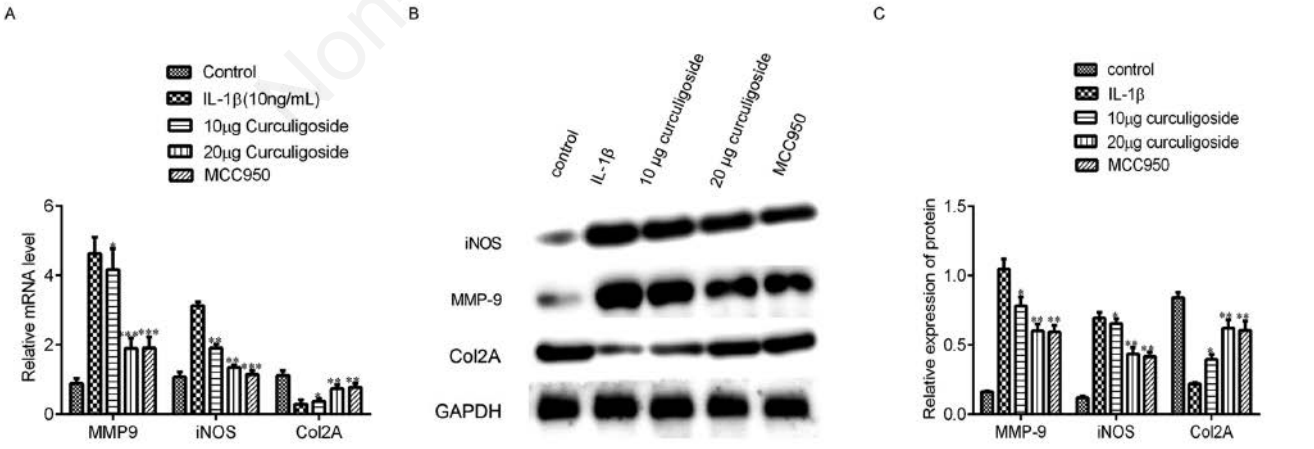

Figure 4. NLRP3 was involved in the regulation of catabolic factors by curculigoside in human chondrocytes. **A**) The mRNA levels of MMP-9, and iNOS significantly increased and the mRNA levels of COL2 decreased in human chondrocytes after treatment with IL-1β compared to the control group, but the changes reversed in a dose-dependent manner after curculigoside administration or treatment with MCC950. **B**) Western blot analysis of MMP-9, iNOS and COL2 in the different groups. GAPDH was loading control. **C**) Densitometry analysis of the protein levels of MMP-9, iNOS and COL2 in the different groups. *p<0.05; **p<0.01; ***p<0.001 compared to IL-1β group (n=3).

Discussion

The main cause of OA is cartilage abnormalities.[13] Previous study reveals that the degradation of extracellular matrix in articular cartilage induces the apoptosis of chondrocytes, which is regulated by ERK/NF-κB pathway.[14] Curculigoside, a key component of curculiginis rhizome, exhibits protective effects on arthritis *via* blocking NF-κB/NLRP3 signaling pathways.[15] NLRP3 inflammasome is reported to participate in the pathogenesis of various arthritics and is a promising biomarker for OA.[16] However, the underlying mechanisms that NLRP3 regulates the progression of OA have not been elucidated.

Curculigoside was found to protect human amniotic fluid-derived stem cells against osteoclastogenesis *via* promoting their osteogenic differentiation, suggesting its beneficial role in the treatment of bone disorders.[17] In the present study, C57BL/6 mice were subjected to DMM to establish an OA model. Based on this model, we explored whether curculigoside could alleviate cartilage lesions induced by DMM.

Cytokines such as IL-1β, TNFα and IL-6 promote inflammation and contribute to a variety of diseases.[18-20] Pro-inflammatory cytokines upregulate the expression of a set of aggrecanases and collagenases *via* activating the NF-κB pathway, which degrades the components of joint tissues.[21] Therefore, targeting pro-inflammatory cytokines-associated pathways may be a promising strategy for OA therapy. A marine bioactive compound LD-1227 mediates the upregulation of TNF-alpha, MMP-13, MMP-1 and Col10A1 and the activation of NF-κB induced by IL-1β, and inhibits cartilage degradation of human chondrocytes.[22] IGF-1 and PDGF-bb inhibit the IL-1β-mediated activation of NF-κB, COX-2, and MMPs, and the apoptosis in chondrocytes. Oleocanthal inhibits the progression of OA *via* the MAPKs/NF-κB/MMP13/ADAMTS-5 pathways.[23] In the present study, curculigoside inhibited the IL-1β-induced inflammatory response and the increase of MMP-9 and iNOS and NF-κB.

PKR plays a dominant role in inflammation, which is initiated by NLRP3. Moreover, NLRP3 interacts with PKR to mediate the expression of IL-1β and IL-8.[24] In present study, curculigoside treatment significantly reduced the expression of NLRP3, NF-κB and PKR. To investigate the potential role of NLRP3 in OA, human chondrocytes was treated with the NLRP3 inhibitor, MCC950. Curculigoside attenuated the increase of iNOS and MMP-9 and the decrease of COL2 induced by IL-1β at both mRNA and protein levels, similar to MCC950. These results suggest that NLRP3 plays an important role in NLRP3/NF-κB axis and curculigoside treatment could block pro-inflammatory cytokine-associated catabolic factors *via* inhibiting NLRP3, thus exhibiting a protective effect on chondrocytes. In conclusion, curculigoside exhibits beneficial effect on cartilage *via* the inhibition of NLRP3 pathway.

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