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Original article

Quercetin-3-O-β-D-glucuronide attenuates osteoarthritis by inhibiting cartilage extracellular matrix degradation and inflammation

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ABSTRACT

Objective: Osteoarthritis (OA) is a chronic degenerative joint disease characterized by cartilage damage. In order to find a safer and more effective drug to treat OA, we investigated the role of quercetin-3-O-β-D-glucuronide (Q3GA) in OA.

Methods: We used qRT-PCR and western blots to detect the effects of Q3GA on extracellular matrix (ECM) and inflammation related genes and proteins in interleukin-1β (IL-1β) induced chondrocytes. We determined the effect of Q3GA on the NF-κB pathway using western blots and immunofluorescence. Moreover, the effect of Q3GA on the Nrf2 pathway was evaluated through molecular docking, western blots, and immunofluorescence experiments and further validated by transfection with Nrf2 siRNA. Subsequently, we established a rat model of OA and injected Q3GA into the joint cavity for treatment. After 5 weeks of Q3GA administration, samples were obtained for micro-computed tomography scanning and histopathological staining to determine the effects of Q3GA on OA rats.

Results: We found that Q3GA reduced the degradation of ECM and the expression of inflammatory related proteins and genes in primary chondrocytes of rats induced by IL-1β, as well as the expression of nitric oxide (NO) and reactive oxygen species (ROS). It inhibited the activation of the NF-κB pathway by increasing the expression of Nrf2 in the nucleus. In addition, Q3GA inhibited cartilage degradation in OA rats and promoted cartilage repair.

Conclusion: Q3GA attenuates OA by inhibiting ECM degradation and inflammation via the Nrf2/NF-κB axis.

The translational potential of this article: The results of our study demonstrate the promising potential of Q3GA as a candidate drug for the treatment of OA and reveal its key mechanisms.

Abbreviations: OA, osteoarthritis; Q3GA, quercetin-3-O-β-D-glucuronide; IL-1β, interleukin-1β; ECM, extracellular matrix; NO, nitric oxide; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; Nrf2, nuclear factor erythroid 2-related factor 2; Col2a1, collagen II; ACAN, aggrecan; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinase; DMEM, Dulbecco's modification of Eagle's medium Dulbecco; FBS, fetal bovine serum; qRT-PCR, quantitative real-time polymerase chain reaction; 3D, three-dimensional; Keap1, Kelch-like ECH associated protein 1; MNX, medial meniscal transection; PEG, polyethylene glycol; micro-CT, micro-computed tomography; BV/TV, bone volume/total tissue volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; S&F, Safranin O-fast green; H&E, hematoxylin-eosin; OARSI, Osteoarthritis Research Society International; COX2, Cyclooxygenase-2; iNOS, inducible nitric oxide synthase; HO-1, heme oxygenase-1.

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1. Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease that is becoming increasingly common in the aging population. OA has causing substantial medical and healthcare costs as well as economic burden owing to unemployment and early retirement, seriously affecting people's lives [1,2]. Many risk factors trigger OA, which is now considered a disease of the whole joint. The manifestations of joint structural damage include cartilage loss, osteophyte formation, subchondral bone changes, meniscus changes, inflammation, and pain [3].

The study of the articular cartilage is an important aspect of OA. Articular cartilage is composed of unique chondrocytes and the extracellular matrix (ECM), which mainly comprises collagen II (Col2a1), aggrecan (ACAN), and other proteoglycans. Collagen networks have tensile strength, whereas charged proteoglycans have compression elasticity and hydrophilic glycosaminoglycan side chains [4,5]. In the early stages of OA, the ECM of cartilages is destroyed, thereby exposing chondrocytes. Subsequently, proteoglycan depletion and erosion of the collagen network reflect irreversible disease progression [6]. Together with the induction of multiple aggrecanases families, including a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMPs) families, which cleave the aggrecan core protein [7].

The pathogenesis of OA is multifactorial and complex, involving various cytokines and signaling pathways [2]. Inflammation promotes the development of OA and plays an important role in its pathogenesis. An increase in inflammatory cytokines promotes cartilage degradation, and damaged joints further trigger inflammation [8]. Changes in reactive oxygen species (ROS) levels are also related to the progression of OA. The excessive production of ROS in OA exacerbates the progression of OA [9].

Medication remains the main treatment method for OA, and clinical drugs include non-steroidal anti-inflammatory drugs, analgesics, glucocorticoids, and hyaluronic acid [10]. These drugs have side effects on gastrointestinal and cardiovascular diseases and cannot effectively alleviate cartilage degradation [11]. Although the progression of OA is slow, it may ultimately lead to severe pain and disability, necessitating joint replacement surgery [12]. Drugs capable of reducing cartilage degradation and restoring cartilage construction are lacking; therefore, the development of safer and more effective drugs for the treatment of OA is pertinent.

Quercetin-3-O-β-D-glucuronide (Q3GA) is one of the main metabolites in the circulatory system after human consumption of quercetin-rich foods, and exists in many Chinese herbal medicines [13]. However, compared to the long history of application and extensive research on quercetin, research on Q3GA remains unexplored. Studies have shown that Q3GA exhibits antioxidant, anti-arteriosclerotic, and antiviral activities in both *in vivo* and *in vitro* models [14]. Q3GA enhances the anti-inflammatory properties of M2a macrophages [15], inhibits aging in primary human cells [16], and inhibits collagenase production [17]. Nevertheless, it is still unclear whether Q3GA can treat OA and its mechanism of action. Therefore, this study aimed to evaluate the role of Q3GA in OA progression through a series of *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Reagents

Q3GA (molecular formula: $C_{21}H_{18}O_{13}$, MW: 478.36, CAS No. 22688-79-5) was obtained from Chengdu Alfa Biotechnology Co. Ltd. (Sichuan, China; ABL1913). Recombinant rat IL-1β (501-RL) and recombinant human IL-1β (201-RL) were purchased from R&D Systems (Minneapolis, USA). SW1353 cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12; 11320033), Dulbecco's modified Eagle's medium (DMEM; C11995500BT) and fetal bovine serum (FBS; 10099141) were purchased from Gibco (NY, USA). Primary antibodies against ACAN (ab36861) and ADAMTS5 (ab41037) were purchased from Abcam (Cambridge, UK); iNOS (18985-1-AP), COX2 (12375-1-AP), p65 (66535-1-Ig), Nrf2 (16396-1-AP), HO-1 (10701-1-AP), and LaminB1 (12987-1-AP) from Proteintech (Wuhan, China); p-p65 (WL02169) from Wanleibio (Shenyang, China); Col2a1(AF0135) and MMP13 (AF5355), MMP3 (AF0217), IκBα (AF5002) and GAPDH (AF7021) from Affinity (Melbourne, USA). The secondary antibodies goat anti-rabbit IgG (10004301) and goat anti-mouse IgG (10004302) were purchased from MyBioScience (Nanjing, China).

2.2. Cultivation of primary chondrocytes and SW1353 cell line and cell viability assay

Chondrocytes were isolated from Sprague–Dawley rats aged 5–7 days, as previously described [18,19]. The articular cartilage was carefully removed and cut, digested with 0.25% trypsin for 30 min, and then digested with 0.2% collagenase II (Yeasen, Shanghai, China; 40508ES60) at 37 °C for 12 h. The primary chondrocytes were cultured in DMEM containing 10% FBS. Only chondrocytes from passages 1 or 2 were used in the present study to avoid phenotypic loss. The human chondrosarcoma cell line SW1353 has similar phenotypes to chondrocytes and is often used in OA research [20]. SW1353 cells are cultured in DMEM/F-12 medium containing 1% streptomycin, 1% penicillin, and 10% FBS in 5% CO_2 at 37 °C.

The MTT assay was used to determine the effect of Q3GA on the viability of chondrocytes. Chondrocytes and SW1353 cells were cultured overnight in 96-well plates (5000 cells/well) and treated with different concentrations of Q3GA (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μM) for 24 or 48 h. Thereafter, MTT was added to the 96-well plates. After 4 h, the medium was discarded, and DMSO (150 μl) was added to each of the wells. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Shanghai, China).

2.3. Total RNA extraction and qRT–PCR

Chondrocytes were cultured overnight in a 6-well plate. The cells were pretreated with Q3GA for 2 h and then stimulated with IL-1β (10 ng/ml), followed by incubation for 48 h. Total RNA was extracted from the chondrocytes using Trizol reagent (Vazyme, Nanjing, China; R401), and the concentration was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, USA). According to the manufacturer's instructions, RNA (1 μg) was reverse-transcribed into cDNA using reverse transcriptase, followed by qPCR. Relative mRNA expression was calculated according to the $2^{-\triangle\triangle Ct}$ method with *Gapdh* for normalization. The forward and reverse primer sequences used in this study are listed in Supplementary Material Table S1.

2.4. Western blots

Chondrocytes and SW1353 cells were pretreated with Q3GA for 2 h, followed by stimulation with IL-1β and incubation for 48 h. Proteins from the NF-κB pathway were extracted 30 min after IL-1β stimulation. Total intracellular proteins were extracted using the RIPA lysis buffer (Beyotime, Shanghai, China; P0013B), and nuclear and cytoplasmic proteins were extracted using a kit (Beyotime, Shanghai, China; P0027). The proteins were then separated using SDS-PAGE and transferred onto PVDF membranes. The PVDF membranes were sealed with 5% skim milk and incubated overnight with primary antibodies at 4 °C. The protein bands were incubated with secondary antibodies at room temperature for 1 h, and placed on an imaging system (Bio-Rad Laboratories, Hercules, CA) for imaging. Image Lab software was used for the quantitative analysis.

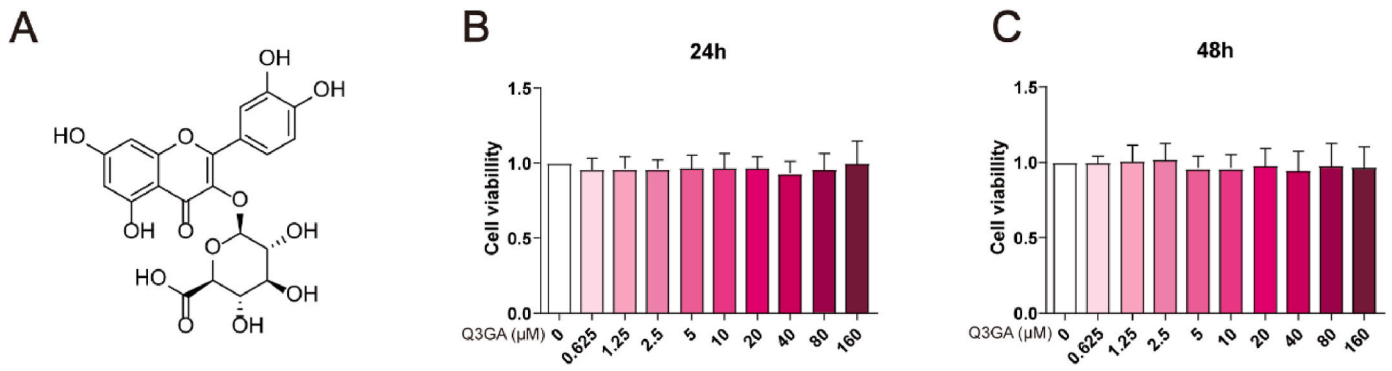


Figure 1. Effects of quercetin-3-O-β-D-glucuronide (Q3GA) on the viability of chondrocytes. (A) Chemical structure of Q3GA. (B, C) MTT kit was used to determine the cytotoxic effects of different concentrations of Q3GA (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 μM) on chondrocytes for 24 and 48 h, n = 5.

2.5. Measurement of nitric oxide content in the culture supernatant

The nitric oxide (NO) levels in the culture supernatant were detected using the Griess assay (Beyotime, Shanghai, China; S0021S), following the manufacturer's protocol. The chondrocytes were cultured overnight in 96-well plates (3×10^4/well). After the cells were treated with Q3GA and IL-1β, 50 μl of the cell supernatant was transferred into a new 96-well plate, followed by addition of 50 μl of Griess Reagent I and 50 μl of Griess Reagent II. Absorbance was measured at 540 nm using a microplate reader.

2.6. Detection of intracellular ROS

Intracellular ROS levels were detected using an ROS assay kit (Beyotime, Shanghai, China; S0033S). Chondrocytes were cultured overnight in 96-well plates (5000 cells/well) and treated with different concentrations of Q3GA and IL-1β. Thereafter, the DCFH-DA probes were added and incubated for 30 min. The ImageXpress® Micro system (Molecular Devices, Shanghai, China) was used for observation, and quantitative analysis was performed using the Image J software.

2.7. Immunofluorescence

After treatment with Q3GA and IL-1β, the chondrocytes were fixed with 4% paraformaldehyde for 30 min, penetrated with 0.5% Triton X-100 for 20 min, and sealed with 5% BSA for 1 h. Thereafter, the cells were incubated with primary antibodies against p65 (1:200) and Nrf2 (1:200) overnight at 4 °C. Alexa Fluor® 488 AffiniPure goat anti-mouse IgG (1:200; Yeasen, Shanghai, China; 33206ES60) and goat anti-rabbit IgG (1:200; Yeasen; Shanghai, China; 33106ES60) were used as secondary antibodies. Nuclei were stained with DAPI (1:1000; Beyotime, Shanghai, China; C1006) for 10 min. The ImageXpress® Micro system was used to capture fluorescence images.

2.8. Molecular docking

The three-dimensional (3D) structure of Q3GA was searched using the PubChem database and created using the ChemOffice software. The Keap1-Nrf2 Complex (PDB ID:4XMB), JNK (PDB ID: 3V6R), PI3K (PDB ID:3LJ3), TLR4 (PDB ID:2Z62), and SIRT3 (PDB ID:4O8Z) were downloaded from the RCSB protein database and imported into the PyMOL software to remove water molecules and original ligands. The Q3GA and protein molecules were then imported into the AutoDock Vina software for processing. Using the original ligand of the protein molecule as the center, the corresponding position and size of the docking box were set, and the protein molecules were set as the receptor and Q3GA as the ligand for molecular docking. Finally, the molecular docking results were evaluated and imported into the PyMOL software for visualization.

2.9. siRNA transfection

The control and Nrf2 siRNAs were prepared by RiboBio (Guangzhou, China). The control and Nrf2 siRNAs were transfected into chondrocytes using the Lipofectamine™ 3000 transfection reagent (Invitrogen, USA; L3000008) according to the manufacturer's instructions. After transfection, the chondrocytes were induced with IL-1β (10 ng/ml) and Q3GA (5 μM) for 48 h for subsequent experiments.

2.10. Animal experiments

All animal experimental protocols were approved by the Animal Ethics Committee of the China Pharmaceutical University. Male Sprague–Dawley rats (200 ± 10 g) were obtained from Shanghai Sippe-Bk Lab Animal Co. Ltd. After one week of adaptive feeding with free access to food and water, 30 rats were randomly classified into five groups: control, OA, OA + Q3GA (0.25, 0.5, and 1 mg/kg). Q3GA was dissolved in 40% polyethylene glycol 400 (PEG400). The rat model of OA was established using medial meniscal transection (MNX), as previously described [21,22]. Briefly, a longitudinal medial parapatellar incision was made at the knee joint of the right leg of each rat. Subsequently, the medial meniscus was removed after cutting off its anterior ligament. Finally, the articular capsule and the skin were sutured. The control group underwent the same incision without ligament transection or meniscectomy. After establishment of OA, each rat was administered penicillin for three consecutive days. One week after surgery, each rat in the OA + Q3GA group received an intra-articular injection of 60 μl Q3GA twice a week for 5 consecutive weeks. The control and OA groups were injected with 40% PEG400 without Q3GA. Six weeks after surgery, all the rats were euthanized, and the right knee joint as well as the adipose tissue were removed and fixed in 4% paraformaldehyde for subsequent experiments.

2.11. Evaluation using micro-computed tomography scanning

Specimens fixed in paraformaldehyde were subjected to micro-computed tomography (micro-CT) scanning using Scanco viva CT 80 (Scanco Medical AG, Switzerland) as described previously [23], with the following parameters: resolution, 21 μm; voltage, 70 kV; electric current, 114 μA. After the scanning was completed, the femur disappeared, and the tibia appeared 200 layers above and below the boundary points. We set the 50 layers from the disappearance of the medial epiphyseal line to the distal end of the femur as the region of interest, performed 3D image reconstruction, and analyzed the degree of cartilage damage. Bone volume/total tissue volume (BV/TV), trabecular number (Tb.N), and separation (Tb.Sp) were evaluated.

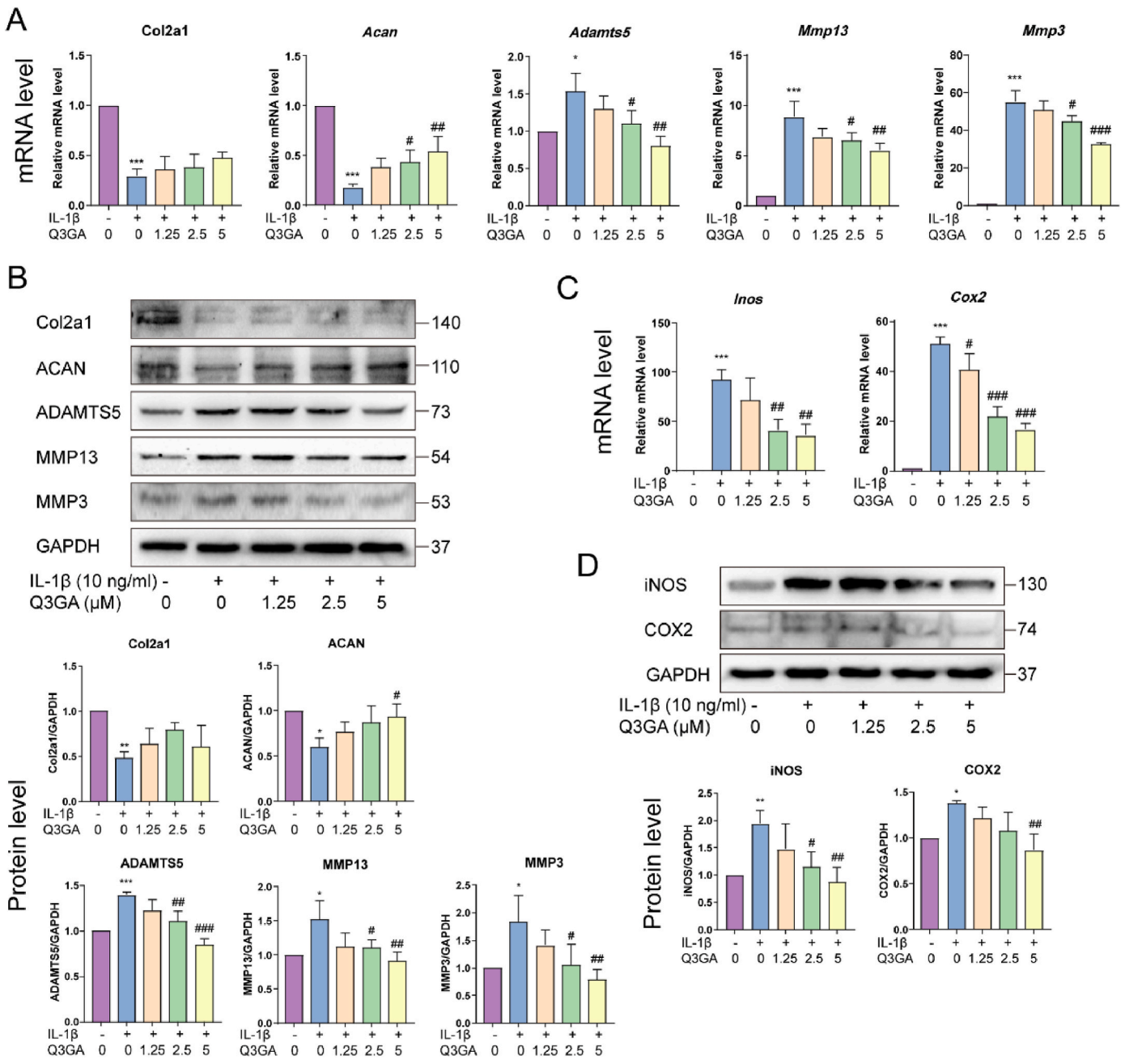


Figure 2. Q3GA on the expression of genes and proteins in the extracellular matrix (ECM) and inflammation in chondrocytes. (A) QRT–PCR was used to detect the mRNA expression of Col2a1, Acan, Adamts5, Mmp13, and Mmp3, n = 3. (B) Western blots were used to detect the protein expression of Col2a1, ACAN, ADAMTS5, MMP13, and MMP3, n = 3. (C) QRT–PCR was used to detect the mRNA expression of Inos and Cox2, n = 3. (D) Western blots were used to detect the protein expression of iNOS and COX2, n = 3. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001, compared with the IL-1β induced group.

2.12. Histopathology and immunohistochemical analysis

Specimens were decalcified with 10% EDTA and embedded in paraffin. Sagittal plane sections of the specimens were cut to the medial compartment of the knee joint at a thickness of 5 μm and stained with Safranin O-fast green (S&F), hematoxylin & eosin (H&E), and Masson. The specimens were evaluated based on the guidelines of the Osteoarthritis Research Society International (OARSI) [24]. Immunohistochemical staining was performed using antibodies against ACAN, ADAMTS5, MMP13, and Nrf2 (1:100). Finally, the images were scanned and analyzed using a scanner, and the number of positively stained cells in each sample was measured.

2.13. Statistical analysis

The quantitative data of all experiments are reported in the form of mean ± standard deviation. Data analysis was performed using GraphPad Prism 8.0.2. One-way analysis of variance (ANOVA) and the Tukey's range test were used to analyze the significant differences between the groups, and $P < 0.05$ was taken to indicate statistical significance.

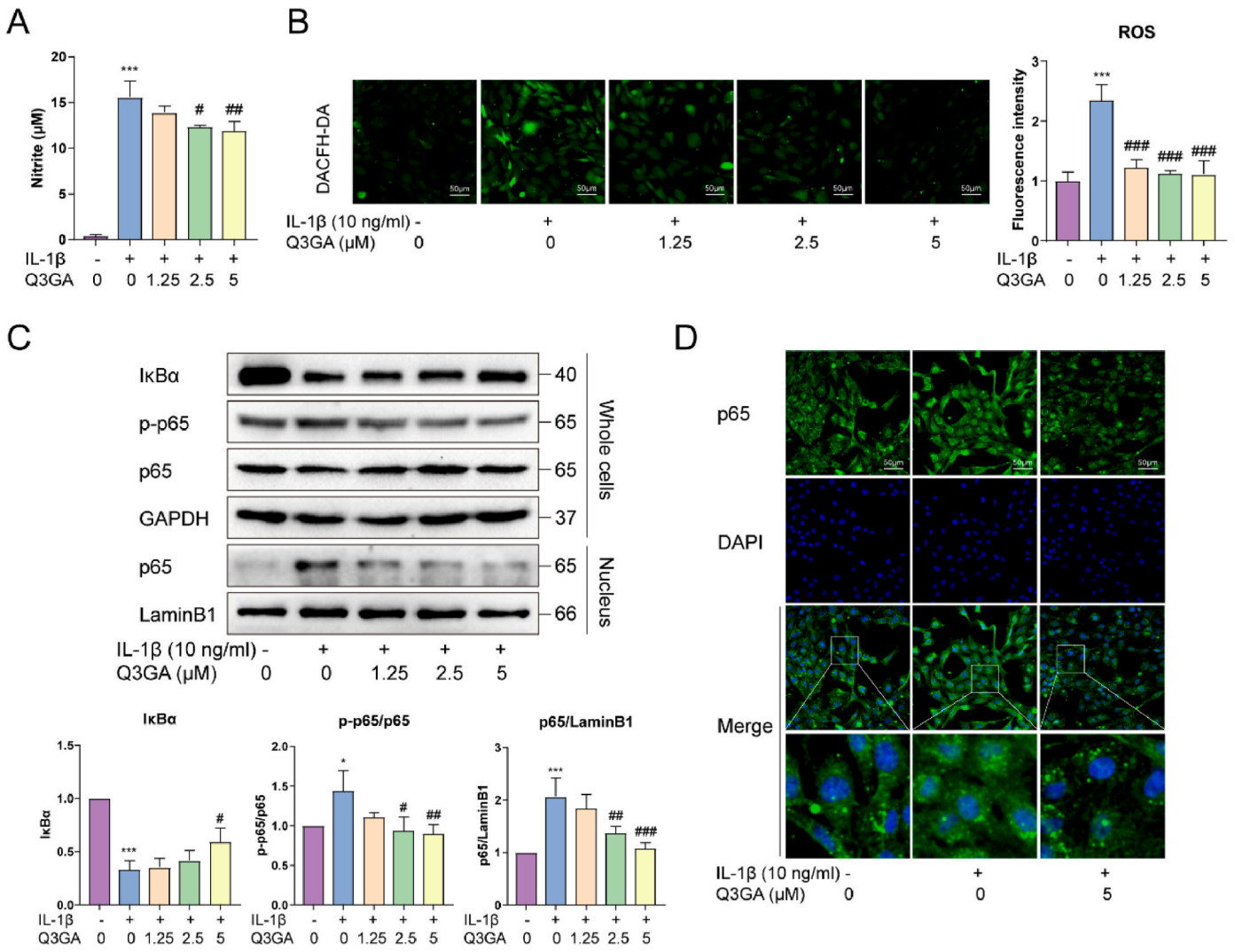


Figure 3. The effect of Q3GA on the NF-κB pathway in IL-1β-induced chondrocytes. (A) The level of NO in the supernatant of chondrocytes, n = 3. (B) The level of ROS in chondrocytes (scale bar: 50 μm). (C) The protein expression levels of IκBα, p-p65, and p65 in whole cells, as well as the expression level of p65 in the nucleus, n = 3. (D) The nuclear translocation of p65 was detected using immunofluorescence staining (scale bar: 50 μm). *$P < 0.05$, and ***$P < 0.001$, compared with the control group; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$, compared with the IL-1β induced group.

3. Results

3.1. The effect of Q3GA on the viability of chondrocytes

To evaluate the cytotoxicity of Q3GA (Fig. 1A), chondrocytes were treated with different concentrations of Q3GA for 24 and 48 h (Fig. 1B and C). We found that Q3GA had no effect on chondrocytes at concentrations less than or equal to 160 μM for 24 or 48 h. We also found that Q3GA had no effect on SW1353 cells at concentrations less than or equal to 80 μM for 48 h (Supplementary Material Fig. S1A).

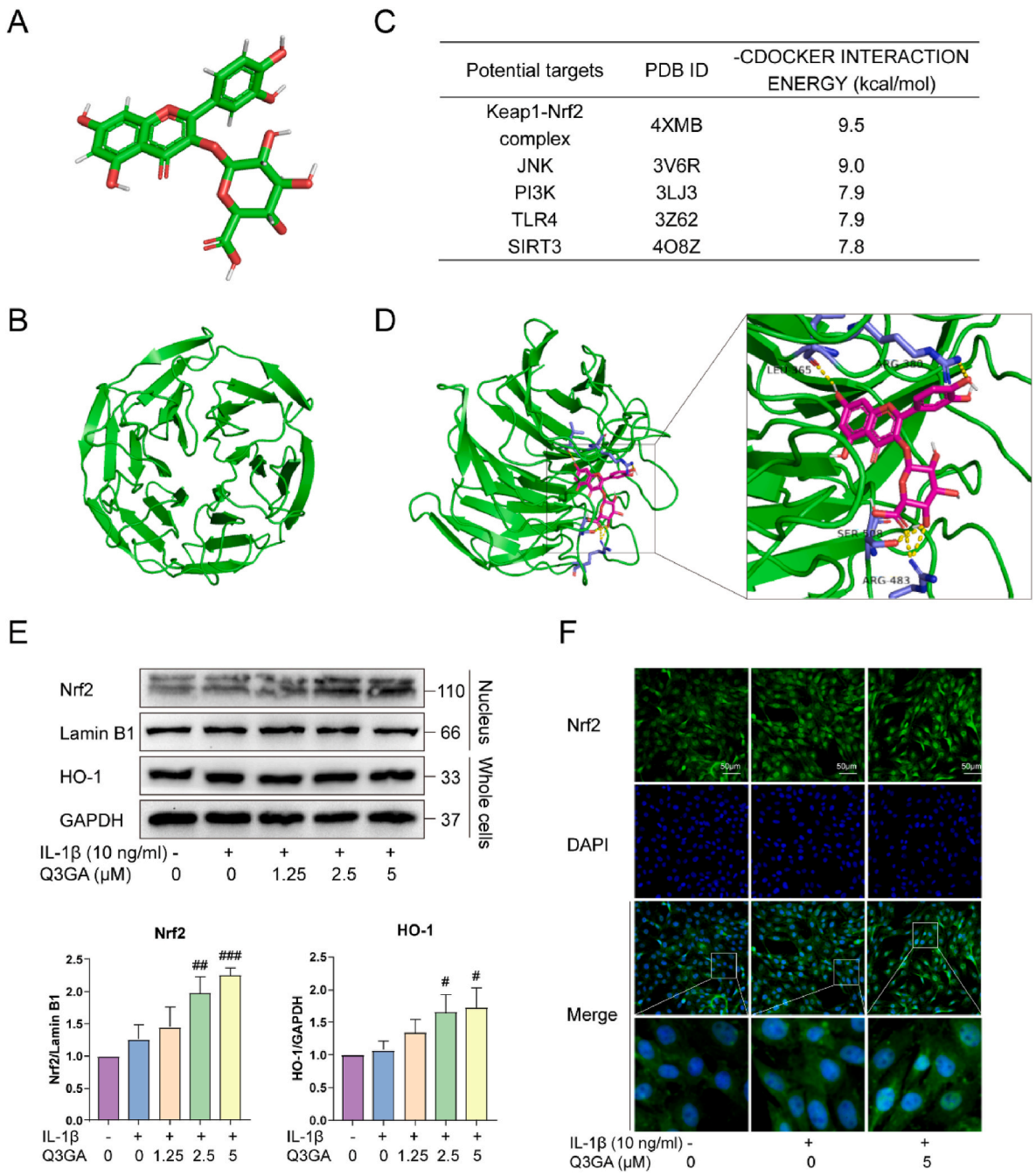
3.2. Q3GA reverses ECM degradation in IL-1β-induced chondrocytes

To investigate the effect of Q3GA on the OA-related phenotype of chondrocytes, we detected the gene and protein expression of an ECM-related anabolic metabolic factor (Col2a1 and ACAN) and catabolic metabolic factors (ADAMTS5, MMP13, and MMP3). The results showed that compared with chondrocytes treated only with IL-1β, those treated with Q3GA exhibited no significant increased expression of Col2a1, but increased expression of ACAN and decreased expression of ADAMTS5, MMP13, and MMP3 (Fig. 2A and B). Q3GA increased the expression of ACAN and decreased the expression of MMP3 in SW1353 cells, consistent with primary chondrocytes (Supplementary Material Figs. S2A and B). These results indicate that Q3GA could inhibit ECM degradation in chondrocytes.

3.3. Q3GA inhibits inflammation in IL-1β-induced chondrocytes

We detected the gene and protein expression of the inflammation-related factors iNOS and COX2. Chondrocytes induce an inflammatory response under IL-1β stimulation with increased expression of iNOS and COX2. However, Q3GA inhibits the expression of iNOS and COX2, indicating the potential of Q3GA to inhibit IL-1β-induced inflammatory responses in chondrocytes (Fig. 2C and D).

NO and ROS are important regulatory factors of OA [25]. NO was detected using the Griess method to detect nitrite content. Q3GA inhibited the enhanced production of NO in chondrocytes (Fig. 3A). Furthermore, the fluorescent probe DCFH-DA was used to detect ROS production in chondrocytes, which was also inhibited by Q3GA (Fig. 3B). Collectively, these results indicate that Q3GA affects the expression of other factors by inhibiting the production of NO and ROS in chondrocytes.



Potential targets	PDB ID	-CDOCKER INTERACTION ENERGY (kcal/mol)
Keap1-Nrf2 complex	4XMB	9.5
JNK	3V6R	9.0
PI3K	3LJ3	7.9
TLR4	3Z62	7.9
SIRT3	4O8Z	7.8

Figure 4. The effect of Q3GA on the Nrf2 pathway in IL-1β-induced chondrocytes. (A) The 3D structure of Q3GA. (B) The 3D structure of the Nrf2 protein. (C) Molecular docking results of Q3GA with potential candidate proteins. (D) A combined 3D structure of Q3GA and Nrf2. (E) Western blots revealed the protein expression of Nrf2 in the nucleus and HO-1 in whole cells, n = 3. (F) Immunofluorescence assay was used to detect the expression of Nrf2 (scale bar: 50 μm). $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$, compared with the IL-1β-induced group.

3.4. Q3GA inhibits the NF-κB pathway in IL-1β-treated chondrocytes

NF-κB is a set of transcription factors that can be activated by different types of pro-inflammatory cytokines and participate in the inflammation, differentiation, proliferation, and survival of mammalian cells [2]. NF-κB also plays an important role in the occurrence and development of OA [9]. Therefore, we investigated whether the NF-κB signaling pathway is involved in the effect of Q3GA on the ECM of chondrocytes. Western blots results showed a decrease in the expression level of IκBα and an increase in the expression level of p-p65/p65 in IL-1β-induced chondrocytes, indicating activation of the NF-κB signaling pathway. Notably, Q3GA treatment significantly increased the expression level of IκBα and decreased the expression level of p-p65/p65. Subsequently, we extracted the p65 nuclear protein from the chondrocytes and found that p65 expression increased in the nuclei of IL-1β-induced chondrocytes, but decreased after Q3GA treatment, indicating that Q3GA inhibits the phosphorylation of p65 as well as its entry into the nucleus (Fig. 3C). Immunofluorescence results showed that in IL-1β-treated chondrocytes, p65 was translocated from the cytoplasm to the nucleus, whereas Q3GA weakened this translocation (Fig. 3D). These results imply that Q3GA inhibits the NF-κB pathway in chondrocytes.

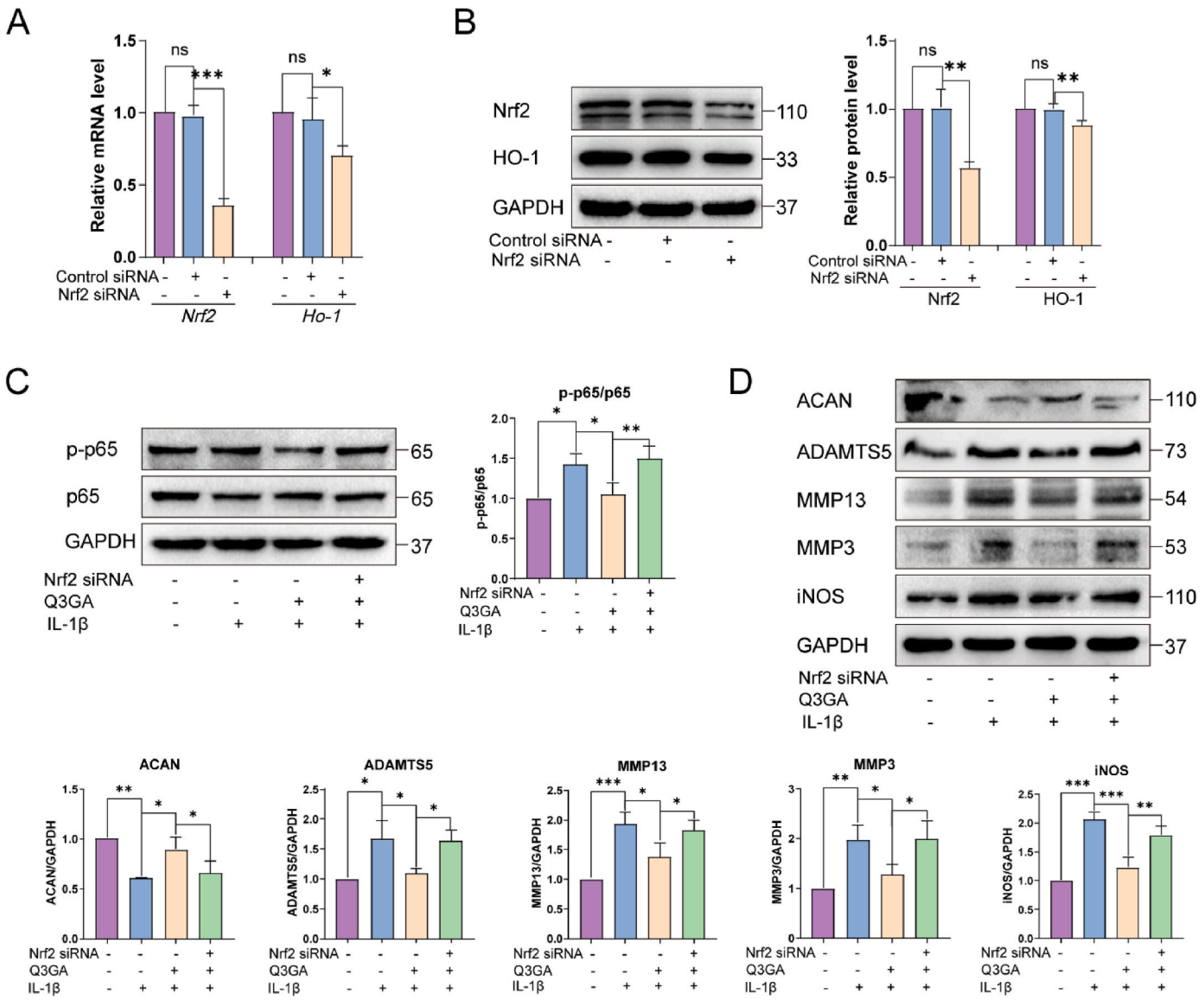


Figure 5. The effect of knockdown of Nrf2 on IL-1β-induced chondrocytes. (A) The mRNA expression of *Nrf2* and *Ho-1* in chondrocytes treated with or without Nrf2 siRNA was detected using qRT–PCR, n = 3. (B) The protein expression of Nrf2 and HO-1 in chondrocytes treated with or without Nrf2 siRNA was detected using western blots, n = 3. (C) The protein expression of p-p65 and p65 in chondrocytes was detected using western blots, n = 3. (D) The protein expression of ACAN, ADAMTS5, MMP13, MMP3, and iNOS was detected using western blots, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.5. Q3GA activates the Nrf2 pathway in IL-1β-treated chondrocytes

We used a molecular docking assay to screen potential targets for the action of Q3GA on chondrocytes. We selected several potential candidate proteins such as the Keap1–Nrf2 complex [26], JNK [27], PI3K [28], TLR4 [29], and SIRT3 [30]. We searched the 3D structure of Q3GA from the PubChem database and downloaded the required protein structure from the RCSB protein database (Fig. 4A and B). The highest -CDOCKER energy score (9.5 kcal/mol) was observed for the binding between Q3GA and the Keap1–Nrf2 complex, indicating the highest affinity between these structures (Fig. 4C and D). Oxidative stress and inflammation can be ameliorated by the activation of Nrf2, the activity of which is negatively regulated by Keap1, which is currently the main target for activating Nrf2 in organisms [31]. After the simulation, Q3GA formed hydrogen bonds with Arg-483, Arg-380, Leu-365, and Ser-508, indicating that Q3GA effectively binds to the Kelch domain of Keap1 and interacts with Nrf2.

Furthermore, we evaluated the expression of the Nrf2 pathway-related proteins. Western blots results showed that Q3GA promotes the expression of Nrf2 in the nucleus and its downstream effector protein, HO-1, in whole cells (Fig. 4E). Immunofluorescence also showed that Q3GA promotes Nrf2 expression in the nucleus (Fig. 4F). These results indicate that Q3GA activates the Nrf2 pathway in chondrocytes.

3.6. Q3GA affects IL-1β-induced chondrocytes by regulating the Nrf2/NF-κB pathway

Although we demonstrated that Q3GA activates the Nrf2 pathway and inhibits the NF-κB pathway, the effect of Q3GA-mediated regulation of the Nrf2/NF-κB pathway on chondrocytes was still unclear.

Therefore, we transfected the chondrocytes with Nrf2 siRNA and evaluated its impact. Firstly, we found that transfection with Nrf2 siRNA in qRT–PCR and western blots experiments significantly reduced Nrf2 levels in chondrocytes (Fig. 5A and B). The level of HO-1 had decreased, but may be influenced by the regulation of other proteins. Additionally, in Q3GA-treated chondrocytes, transfection with Nrf2 siRNA resulted in an increase in the ratio of p-p65/p65, indicating that Q3GA may regulate the NF-κB signaling pathway through Nrf2 (Fig. 5C). Furthermore,

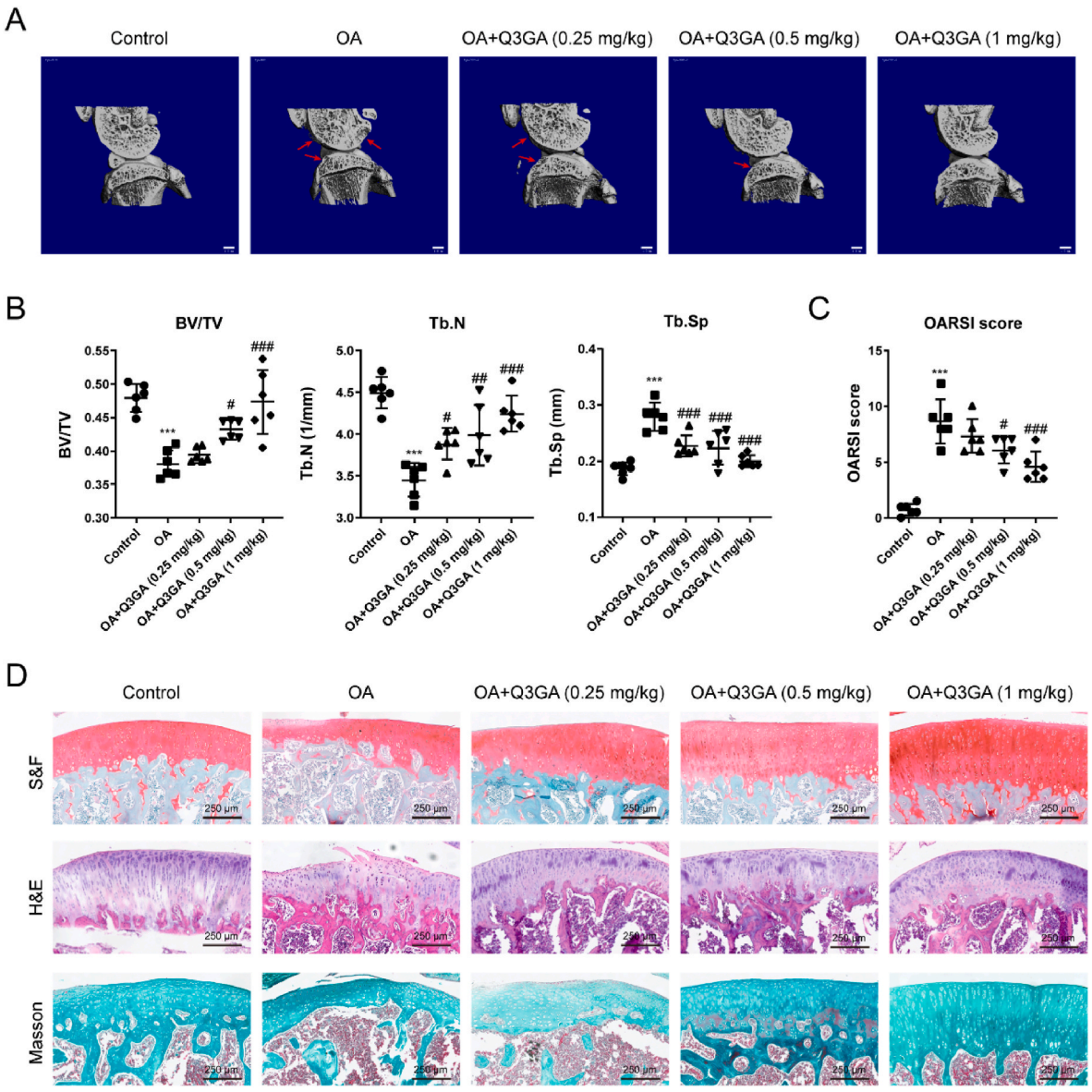


Figure 6. Q3GA ameliorates OA in the MNX rat model. (A) The 3D micro-CT images of the sagittal view of the medial compartment of the knee joint six weeks after surgery, scale = 1 mm. (B) Quantitative analysis of BV/TV, Tb.N, Tb.Sp, n = 6. (C) The OARSI score for assessing the degree of cartilage damage, n = 6. (D) Cartilage staining of S&F, H&E, and Masson (scale bar: 250 μm). ***$P < 0.001$, compared with the control group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, compared with the OA group.

transfection with Nrf2 siRNA significantly increased the expression of ADAMTS5, MMP13, MMP3, and iNOS, while reducing the expression of ACAN in Q3GA-treated chondrocytes (Fig. 5D). Transfection with Nrf2 siRNA promoted ECM degradation and inflammation in IL-1β-induced chondrocytes. Q3GA treatment did not reverse ECM degradation or inflammation, indicating that Q3GA regulates the OA phenotype through the Nrf2/NF-κB signaling pathway.

3.7. Q3GA promotes cartilage repair in OA rats

We investigated the effect of Q3GA on OA *in vivo* using an MNX rat model. One week after surgery, Q3GA (0.25, 0.5, or 1 mg/kg) was injected into the joint cavity twice weekly for 5 weeks. Micro-CT scanning and 3D reconstruction revealed that the cartilage of the femur and tibia in the control group was smooth and undamaged, whereas significant damage and osteophyte formation were observed in the cartilage of the femur and tibia in the OA group. Q3GA significantly reduced ECM degradation and cartilage destruction in OA rats (Fig. 6A and B). Moreover, the OARSI score was higher in the OA group with significant cartilage destruction. Q3GA improved the inhibition of bone resorption, reduced subchondral bone reconstruction, and alleviated bone destruction (Fig. 6C). In addition, S&F, H&E, and Masson staining confirmed that the cartilage surfaces of the femur and tibia in the control group were smooth and flat, with clear boundaries between the cartilage and subchondral bone and intact tidal lines. The rats in the OA group showed cartilage degradation, surface damage, and cracks. Rats in the Q3GA treatment group demonstrated significantly improved cartilage structure compared to those in the OA group, with no significant damage or cracks in the cartilage (Fig. 6D).

Immunohistochemical staining showed that Q3GA increased the expression of ACAN and decreased the expression of ADAMTS5 and MMP13, indicating that Q3GA reduces ECM degradation in the knee joints of OA rats (Fig. 7A). In addition, Q3GA increased the expression of Nrf2 in the cartilage, consistent with the *in vitro* results, indicating that Q3GA alleviates cartilage degradation in OA rats through the Nrf2 pathway (Fig. 7B).

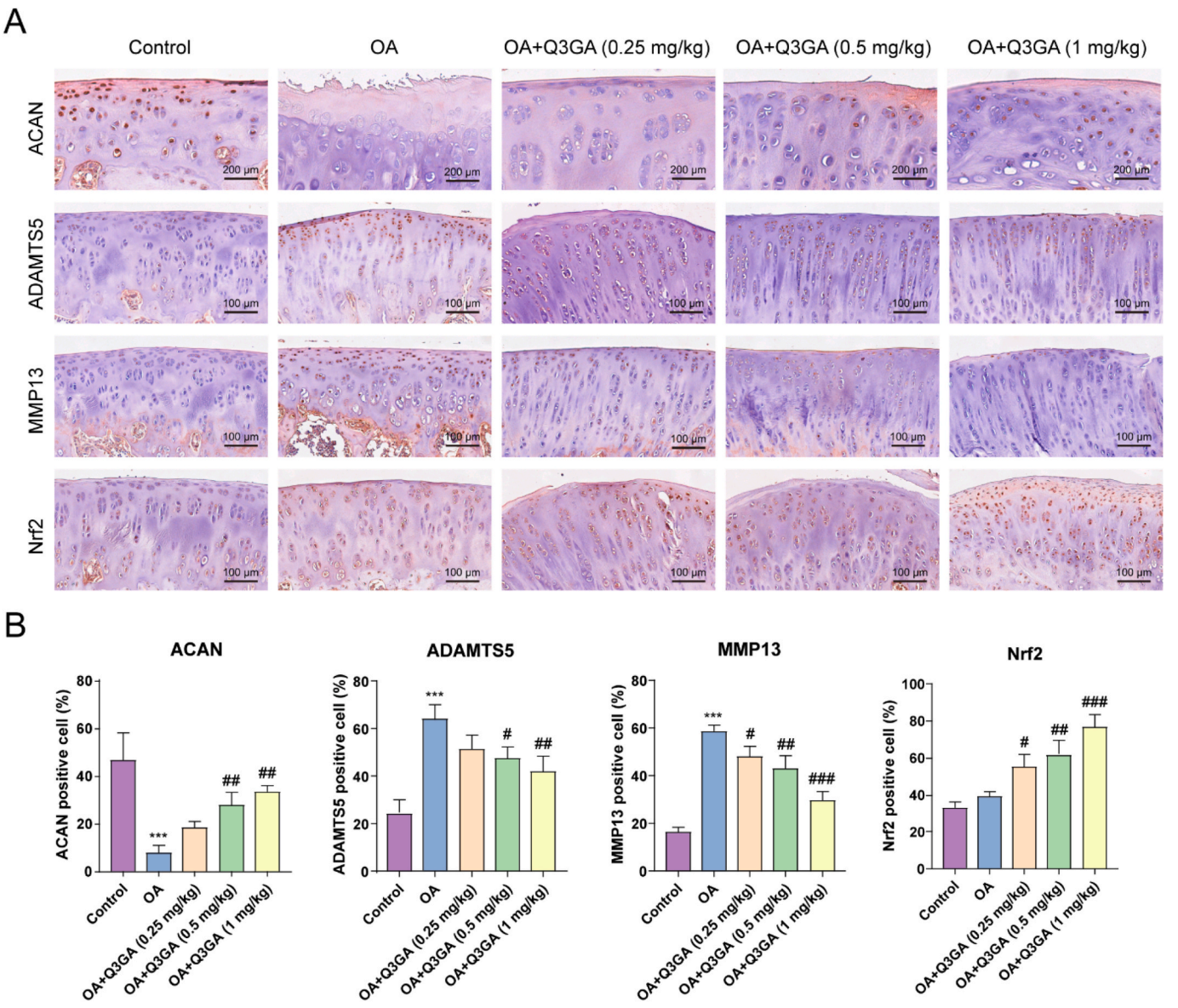


Figure 7. Q3GA inhibits ECM degradation in OA rats. (A) Immunohistochemical staining assay for ACAN, ADAMTS5, MMP13, and Nrf2 in the cartilage. (B) Quantitative analysis of ACAN, ADAMTS5, MMP13, and Nrf2 using immunohistochemistry staining, (scale bar: 100 μm). ***$P < 0.001$, compared with the control group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, compared with the OA group.

4. Discussion

OA is a chronic degenerative joint disease characterized by cartilage loss, osteophyte formation, subchondral bone changes, meniscus changes, inflammation, and pain. Development of OA is associated with an upregulation of the protein-coding genes related to inflammation and catabolic reactions, mainly through signal transduction involving NF-κB, MAPKs, and other inflammation- and stress-induced pathways [2,32]. Many chondrocytes also exhibit increased production of ROS, cytokines, and other pro-inflammatory products [33].

IL-1β is one of the main inducers for the establishment of OA models *in vitro*, and it is expressed in chondrocyte injury and inflammatory synovial cells [25]. IL-1β induces the expression of many pro-inflammatory and injury-mediating biomarkers in OA, such as iNOS and COX. IL-1β is involved in the catabolism of OA tissues mainly by upregulating MMPs, such as MMP3 and MMP13. ROS, hydrogen peroxide, and NO have also been found to be the main regulatory factors for chondrocyte metabolism and other important cellular events in normal and diseased states [34]. ECM has been regarded as an important indicator of OA in many studies [23]. Q3GA can reduce inflammation and inhibit the production of collagenase and has the potential for the treatment of OA [14,17]. We tested the effect of Q3GA on chondrocytes and found that Q3GA alleviates IL-1β-induced inflammation and ECM degradation in chondrocytes. Compared with previous studies on Quercetin in OA, Q3GA has less cytotoxicity and good therapeutic effect, and has obvious therapeutic effect on OA at 5 μM [22]. However, Q3GA did not significantly increase the expression of Col2a1, and the reasons still need further exploration.

The NF-κB pathway is involved in the pathological process of OA [35]. Inhibitors of NF-κB (IκB) proteins prevent NF-κB phosphorylation and activate NF-κB in the cytoplasm. Various inflammatory signals can activate IκB kinases (IKKs) and inhibit their expression by regulating the phosphorylation of IκB. The NF-κB complex is transferred to the nucleus and triggers transcription of the downstream target genes [36]. We found that Q3GA increases IκB expression and inhibits p65 phosphorylation, along with inhibition of the expression of p65 in the nucleus, indicating that Q3GA inhibits the NF-κB pathway in chondrocytes.

Furthermore, we searched for several potential targets of Q3GA that

act on the NF-κB pathway through literature review. Activation of Nrf2 can reduce the activity of NF-κB, leading to a decrease in the production of cytokines, thereby protecting tissues from the harmful effects of inflammation [37]. Nrf2 is a key transcription factor that controls many aspects of cellular oxidative and toxic damage [38]. It is particularly important to regulate the transcription of antioxidant proteins that are responsible for clearing ROS and providing protection against the accumulation of toxic metabolites. The most studied Nrf2 target genes include NAD (P) H dehydrogenase quinone 1 (NQO1), heme oxygenase-1 (HO-1), and ferritin, which maintain a reducing environment within cells [39]. We conducted molecular docking and found that Q3GA binds well with Nrf2–keap1, indicating that Nrf2 is a potential target for Q3GA. Western blots and immunofluorescence confirmed that Q3GA further inhibits ECM degradation and inflammation by activating Nrf2 and inhibiting the NF-κB pathway. Moreover, the inhibitory effect of Q3GA on ECM degradation could be reversed by Nrf2 siRNA. Some studies have shown that Q3GA plays an anti-inflammatory role in human Keratinocyte and Melanoma cells through NF-κB and AP-1 pathways [40], and plays an anti-inflammatory role by inhibiting JNK and ERK signaling pathways in LPS-challenged RAW264.7 cells [41]. This study indicates that Q3GA ameliorates IL-1β-induced degradation and inflammation of chondrocyte ECM through the Nrf2/NF-κB axis. However, Q3GA may have other pathways that act on chondrocytes, and its more direct targets need further exploration.

There are many animal models of OA, and MNX can cause asymmetric weight bearing in the knee joint of rats; make direct contact between the femur and tibia; and cause cartilage wear, inflammation, and pain and is characterized by a good fit with human OA [21,42]. After one week of modeling, the surgical wound healed, and the rats showed early features of OA, with increased cartilage wear and inflammation. After five weeks of administration, the wear in the OA group was more severe, and in severe cases, the rats showed inconvenience in movement. Through micro-CT and histopathological staining, it was found that Q3GA could effectively protect the structure of the joint cartilage in OA rats, and improve cartilage damage and degradation, thereby delaying OA progression. This indicates that Q3GA can be used for the treatment of OA and has great potential for the development of drugs for OA.

There are several limitations to this study. The therapeutic effect of Q3GA on OA in this study is limited to rat models, and further clinical studies on human patients with OA need to be investigated. Are there any other signaling pathways that have a more significant impact besides the Nrf2/NF-κB pathway? The mechanism of action of Q3GA still needs further research. The impact of Q3GA on other phenotypes of OA, such as synovitis, cell apoptosis, and macrophage polarization, deserves further investigation.

5. Conclusion

In this study, we investigated the effect of Q3GA on OA and its underlying mechanisms. We found that Q3GA inhibits ECM degradation and inflammation in chondrocytes stimulated by IL-1β through the Nrf2/NF-κB axis. Q3GA treatment significantly alleviated OA progression in the MNX rat model. This indicates that Q3GA is a promising drug for the treatment of OA.

Credit author statement

H. J. Mao and Y. W. Feng conducted experiments and wrote this article, J. Feng and Y. Yusufu. assisted in the experiment, M. H. Sun and L. Yang provided guidance for the experiment, and Q. Jiang provided supervision and financial support for the experiment.

Declaration of competing interest

All authors disclosed no relevant relationships.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2024.01.007.

References

[1] Martel-Pelletier J, Barr AJ, Cicuttini FM, Conaghan PG, Cooper C, Goldring MB, et al. Osteoarthritis. Nat Rev Dis Prim 2016;386:376–87.
[2] Yao Q, Wu X, Tao C, Gong W, Chen M, Qu M, et al. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. Signal Transduct Targeted Ther 2023;8(1):56.
[3] Martel-Pelletier J, Wildi LM, Pelletier JP. Future therapeutics for osteoarthritis. Bone 2012;51:297–311.
[4] Heinegard D, Saxne T. The role of the cartilage matrix in osteoarthritis. Nat Rev Rheumatol 2011;7(1):50–6.
[5] Houard X, Goldring MB, Berenbaum F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. Curr Rheumatol Rep 2013;15(11):375.
[6] Jakkula E, Melkoniemi M, Kiviranta I, Lohiniva J, Raina SS, Perala M, et al. The role of sequence variations within the genes encoding collagen II, IX and XI in non-syndromic, early-onset osteoarthritis. Osteoarthritis Cartilage 2005;13(6):497–507.
[7] Fosang AJ, Beier F. Emerging Frontiers in cartilage and chondrocyte biology. Best Pract Res Clin Rheumatol 2011;25(6):751–66.
[8] Motta F, Barone E, Sica A, Selmi C. Inflammaging and osteoarthritis. Clin Rev Allergy Immunol 2023;64:222–38.
[9] Lepetsos P, Papavassiliou KA, Papavassiliou AG. Redox and NF-κB signaling in osteoarthritis. Free Radical Biol Med 2019;132:90–100.
[10] Toyoda E, Maehara M, Watanabe M, Sato M. Candidates for intra-articular administration therapeutics and therapies of osteoarthritis. Int J Mol Sci 2021;22(7):3594.
[11] Yao XD, Zhang JM, Jing XZ, Ye YP, Guo JC, Sun K, et al. Fibroblast growth factor 18 exerts anti-osteoarthritic effects through PI3K-AKT signaling and mitochondrial fusion and fission. Pharmacol Res 2019;139:314–24.
[12] Mao L, Wu W, Wang M, Guo J, Li H, Zhang S, et al. Targeted treatment for osteoarthritis: drugs and delivery system. Drug Deliv 2021;28(1):1861–76.
[13] Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MR, Williamson G. Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. Free Radic Res 2001;35(6):941–52.
[14] Li F, Sun XY, Li XW, Yang T, Qi LW. Enrichment and separation of quercetin-3-O-β-D-glucuronide from lotus leaves (*nelumbo nucifera* gaertn.) and evaluation of its anti-inflammatory effect. J Chromatogr, B: Anal Technol Biomed Life Sci 2017;1040:186–91.
[15] Derlindati E, Dall'Asta M, Ardigo D, Brighenti F, Zavaroni I, Crozier A, et al. Quercetin-3-O-glucuronide affects the gene expression profile of M1 and M2a human macrophages exhibiting anti-inflammatory effects. Food Funct 2012;3(11):1144–52.
[16] Yang HH, Hwangbo K, Zheng MS, Cho JH, Son JK, Kim HY, et al. Quercetin-3-O-β-D-glucuronide isolated from *Polygonum aviculare* inhibits cellular senescence in human primary cells. Arch Pharm Res (Seoul) 2014;37(9):1219–33.
[17] Mandrone M, Coqueiro A, Poli F, Antognoni F, Choi YH. Identification of a collagenase-inhibiting flavonoid from *Alchemilla vulgaris* using NMR-based metabolomics. Planta Med 2018;84(12–13):941–6.
[18] Zou ZL, Sun MH, Yin WF, Yang L, Kong LY. Avicularin suppresses cartilage extracellular matrix degradation and inflammation via TRAF6/MAPK activation. Phytomedicine 2021;91:153657.
[19] Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. Nat Protoc 2008;3(8):1253–60.
[20] Lin Z, Miao J, Zhang T, He M, Wang Z, Feng X, et al. JUNB-FBXO21-ERK axis promotes cartilage degeneration in osteoarthritis by inhibiting autophagy. Aging Cell 2021;20(2).
[21] Alves-Simoes M. Rodent models of knee osteoarthritis for pain research. Osteoarthritis Cartilage 2022;30(6):802–14.
[22] Hu Y, Gui ZP, Zhou YN, Xia LG, Lin KL, Xu YJ. Quercetin alleviates rat osteoarthritis by inhibiting inflammation and apoptosis of chondrocytes, modulating synovial macrophages polarization to M2 macrophages. Free Radical Biol Med 2019;145:146–60.
[23] Xie JW, Wang Y, Xiao K, Xu H, Luo ZY, Li L, et al. Alpha defensin-1 attenuates surgically induced osteoarthritis in association with promoting M1 to M2 macrophage polarization. Osteoarthritis Cartilage 2021;29(7):1048–59.
[24] Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage 2010;18:S17–23.
[25] Mallah AH, Amr M, Gozen A, Mendenhall J, Van-Wie BJ, Abu-Lail NI. Interleukin 1β and lipopolysaccharides induction dictate chondrocyte morphological

properties and reduce cellular roughness and adhesion energy comparatively. Biointerphases 2022;17(5):051001.
[26] Shao Z, Wang B, Shi Y, Xie C, Huang C, Chen B, et al. Senolytic agent Quercetin ameliorates intervertebral disc degeneration via the Nrf2/NF-kB axis. Osteoarthritis Cartilage 2021;29(3):413–22.
[27] Ismail HM, Miotla-Zarebska J, Troeberg L, Tang X, Stott B, Yamamoto K, et al. Brief Report: JNK-2 controls aggrecan degradation in murine articular cartilage and the development of experimental osteoarthritis. Arthritis Rheumatol 2016;68(5):1165–71.
[28] Sun K, Luo J, Guo J, Yao X, Jing X, Guo F. The PI3K/AKT/mTOR signaling pathway in osteoarthritis: a narrative review. Osteoarthritis Cartilage 2020;28(4):400–9.
[29] Gomez R, Villalvilla A, Largo R, Gualillo O, Herrero-Beaumont G. TLR4 signalling in osteoarthritis-finding targets for candidate DMOADs. Nat Rev Rheumatol 2015;11(3):159–70.
[30] He Y, Wu Z, Xu L, Xu K, Chen Z, Ran J, et al. The role of SIRT3-mediated mitochondrial homeostasis in osteoarthritis. Cell Mol Life Sci 2020;77(19):3729–43.
[31] Zhang Y, Shi Z, Zhou Y, Xiao Q, Wang H, Peng Y. Emerging substrate proteins of kelch-like ECH associated protein 1 (Keap1) and potential challenges for the development of small-molecule inhibitors of the Keap1-nuclear factor erythroid 2-related factor 2 (Nrf2) protein-protein interaction. J Med Chem 2020;63(15):7986–8002.
[32] Liu-Bryan R, Terkeltaub R. Emerging regulators of the inflammatory process in osteoarthritis. Nat Rev Rheumatol 2015;11(1):35–44.
[33] Loeser RF. Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis Cartilage 2009;17(8):971–9.
[34] Choi MC, Jo J, Park J, Kang HK, Park Y. NF-κB signaling pathways in osteoarthritic cartilage destruction. Cells 2019;8(7):734.
[35] Guo Q, Chen X, Chen J, Zheng G, Xie C, Wu H, et al. STING promotes senescence, apoptosis, and extracellular matrix degradation in osteoarthritis via the NF-κB signaling pathway. Cell Death Dis 2021;12(1):13.
[36] Arra M, Swarnkar G, Alippe Y, Mbalaviele G, Abu-Amer Y. IκB-ζ signaling promotes chondrocyte inflammatory phenotype, senescence, and erosive joint pathology. Bone Res 2022;10(1):12.
[37] Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochem Soc Trans 2015;43(4):621–6.
[38] Kobayashi A, Ohta T, Yamamoto M. Unique function of the Nrf2–Keap1 pathway in the inducible expression of antioxidant and detoxifying enzymes. Methods Enzymol 2004;378:273–86.
[39] Dhakshinamoorthy S, Jaiswal AK. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. Oncogene 2001;20:3906–17.
[40] Ha AT, Rahmawati L, You L, Hossain MA, Kim JH, Cho JY. Anti-Inflammatory, antioxidant, moisturizing, and antimelanogenesis effects of quercetin 3-O-β-D-glucuronide in human keratinocytes and melanoma cells via activation of NF-κB and AP-1 pathways. Int J Mol Sci 2021;23:433.
[41] Park JY, Lim MS, Kim SI, Lee HJ, Kim SS, Kwon YS, et al. Quercetin-3-O-β-D-glucuronide suppresses lipopolysaccharide-induced JNK and ERK phosphorylation in LPS-challenged RAW264.7 cells. Biomol Ther 2016;24(6):610–5.
[42] von Loga IS, El-Turabi A, Jostins L, Miotla-Zarebska J, Mackay-Alderson J, Zeltins A, et al. Active immunisation targeting nerve growth factor attenuates chronic pain behaviour in murine osteoarthritis. Ann Rheum Dis 2019;78(5):672–5.