

7,8-Dihydroxyflavone activates Nrf2/HO-1 signaling pathways and protects against osteoarthritis

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Abstract. The aim of the present study was to investigate the effect of 7,8-dihydroxyflavone (7,8-DHF) against osteoarthritis (OA) and examine its regulatory role in the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway in chondrocytes. Primary mouse chondrocytes were treated with 7,8-DHF to examine the expression of Nrf2 and downstream heme oxygenase 1 (HO-1). The surgical destabilization of the medial meniscus model was used to assess the effectiveness of 7,8-DHF in protecting the cartilage from damage, with knee cartilage harvested from mice for histological analysis. The results revealed that 7,8-DHF activated the Nrf2 signaling pathway in primary chondrocytes. Cartilage degradation in the 7,8-DHF-treated group was reduced significantly compared with that in the vehicle-treated group, according to histological evaluation. The gene expression of matrix metalloproteinase (MMP)1, MMP3, MMP13, interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α were reduced in the cartilage of OA mice following 7,8-DHF treatment. Genetic and protein analyses indicated that the expression levels of HO-1 were upregulated in the cartilage of the knee with OA, and 7,8-DHF treatment further promoted the induction of HO-1. These results suggest that 7,8-DHF may serve as a potential therapeutic agent in OA.

Introduction

Osteoarthritis (OA) is one of the most common types of arthritis, affecting 15% of the population worldwide, which

leads to a reduced quality of life and poses a substantial health threat (1-3). To the best of our knowledge, effective early treatment of OA remains a challenge. Despite many etiological factors having been shown to be involved in the progression of OA, the underlying mechanism remains to be fully elucidated. Oxidative stress is known to serve a critical role in the progression of OA. Our previous studies confirmed that a lack of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a key transcription factor that regulates the antioxidant defense system, can result in cartilage degradation (4). Therefore, the aim of the present study was to establish novel effective strategies by targeting oxidative stress. 7,8-Dihydroxyflavone (7,8-DHF) is a natural flavone, which is widely distributed in plants. 7,8-DHF was first reported to have a therapeutic effect on various central nervous system diseases, including the tropomyosin receptor kinase B (TrkB) agonist (5). Previous studies have reported that 7,8-DHF can independently confer neuroprotection through its antioxidant activity (5-7). Further studies have reported that 7,8-DHF protected C2C12 myoblasts and hamster lung fibroblasts from oxidative stress via ERK/Nrf2/heme oxygenase-1 signaling pathway activation (8,9). The aim of the present study was to examine the regulatory role of 7,8-DHF on the Nrf2 signaling pathway in primary articular chondrocytes, and investigate whether 7,8-DHF can delay the progression of articular cartilage destruction in an animal model of OA.

Materials and methods

Chemicals and reagents. 7,8-DHF (purity >99%) was procured from Sigma-Aldrich; Merck KGaA. Anti-Nrf2 (cat. no. BS1258) and anti-HO-1 (cat. no. BS6626) antibodies were supplied by Bioworld Technology at a dilution of 1:1,000. Anti-actin (cat. no. 4970) and anti-lamin B (cat. no. 13435) antibodies were obtained from Cell Signaling Technology, Inc. at a dilution of 1:5,000. Secondary HRP-conjugated goat anti-rabbit (cat. no. BS13278) antibody was purchased from Bioworld Technology, Inc. and used at a dilution of 1:5,000. The Cell Counting Kit-8 (CCK-8) and nuclear protein extraction kit were purchased from Beyotime Institute of Biotechnology. The superoxidase dismutase (SOD) and malondialdehyde (MDA) assay kits were supplied by Nanjing Jiancheng Bioengineering

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Institute. The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was obtained from Molecular Probes; Thermo Fisher Scientific, Inc.

Cell viability assay. Cell viability was evaluated using a CCK-8 assay. Briefly, chondrocytes were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured with various concentrations of 7,8-DHF (0, 1, 2, 4, 6, 8 and 10 μ M) for 8 h at 37°C. Following incubation, 10 μ l of CCK-8 solution was added to each well and the cells were further incubated for 1 h at 37°C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Analysis of antioxidant enzyme activities. The primary mouse chondrocytes pre-treated with various doses of 7,8-DHF (0, 1, 3 and 9 μ M) for 8 h were cultured in 40 ng/ml H_2O_2 for 24 h at 37°C. The cells were washed in ice-cold phosphate buffer and total proteins were extracted with 100 μ l RIPA for 15 min. The lysates were homogenized using an ultrasonic homogenizer and centrifuged at 13,000 \times g for 5 min at 4°C. The MDA contents in the supernatant were measured by monitoring thiobarbituric acid reacting substances. Using a spectrophotometer, the activities of SOD were measured at a wavelength of 532 nm using the assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Measurement of intracellular reactive oxygen species (ROS). The generation of ROS was detected using the fluorescent probe, H2DCFDA. Following incubation with various concentrations of 7,8-DHF for 8 h, the primary mouse chondrocytes were treated with/without H_2O_2 for 24 h. The cells were incubated with H2DCFDA (10 μ M) for 30 min at room temperature in the dark. The production of ROS in the cells was monitored using a flow cytometer (BD Biosciences) with CellQuest Pro software, version 5.0 (BD Biosciences).

Western blot analysis. The proteins lysed from the primary mouse chondrocytes and the cartilage tissue extracted from the knee joints were measured and normalized for western blot analysis, as previously described (10). Each experimental unit for the mice consisted of a pool of two cartilage compartments. Nuclear extraction was performed according to the manufacturer's protocols of the nuclear protein extraction kit (Beyotime Institute of Biotechnology). Briefly, normalized volumes of samples (20 μ g protein) were separated by 10% SDS-PAGE at 100 V for 100 min in Tris-glycine-SDS running buffer (Thermo Fisher Scientific, Inc.). The gel was transferred for 90 min at 300 mA to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Membranes were then incubated for 2 h with blocking buffer (5% skimmed milk in TBST) at room temperature, and incubated overnight with the anti-Nrf2 (1:1,000; cat. no. BS1258; Bioworld Technology, Inc.), anti-HO-1 (1:1,000; cat. no. BS6626; Bioworld Technology, Inc.) antibodies and anti-actin (1:5,000; cat. no. 4970; Cell Signaling Technology, Inc.) antibodies at 4°C. Subsequently, the membranes were washed three times in TBST, then incubated with secondary HRP-conjugated goat anti-rabbit (1:5,000 cat. no. BS13278; Bioworld Technology, Inc.) at room temperature for 1 h and finally washed an additional three times in TTBS. The membranes were visualized using the enhanced

chemiluminescence assay (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Densitometry of protein bands was performed for western blot analysis. Bands were semi-quantified by reverse image scanning densitometry with Photoshop (version 6.0; Adobe Systems, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was extracted from the knee cartilage of mice using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following homogenization. The cartilage, which was snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction, was ground with a pestle and liquid nitrogen-chilled mortar prior to TRIzol extraction. Each experimental unit consisted of a pool of 1-2 cartilage compartments and, when pooling was performed, the experimental unit was regarded as a single unit. The purity and yield of the RNA was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies; Thermo Fisher Scientific, Inc.). cDNA was synthesized from the RNA with PrimeScript RT Master mix (Takara Bio, Inc.). Gene expression was measured using a 7500 real-time PCR system in the presence of SYBR Premix Ex Taq reagents, according to the manufacturer's protocol. The thermocycling conditions were as follows: Denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 5 sec, followed by an annealing/extension step at 60°C for 30 sec. The primer sequences are presented in Table I. Relative quantification was defined as $2^{-\Delta\Delta C_q}$ (11). qPCR was performed in triplicate for each sample.

Experimental OA and 7,8-DHF treatment. A total of 92 wild-type B6 mice (8-10 weeks old) were used in the experiments and were housed in standard mouse cages (10 animals per cage) in specific-pathogen-free conditions under a light-dark cycle of 12:12 h at 25 \pm 2°C with standard mouse food (RM3; Special Dietary Systems) and water *ad libitum*. Animal health and behavior were monitored every 12 h. When pain/distress were observed, the animals were treated with Buprenex (Reckitt & Colman Pharmaceuticals, Inc.; 0.1-2.0 mg/kg), in addition to crushed or wet food. If pain or distress continued, the mouse was sacrificed regardless of the scheduled endpoints. The criteria that determined discomfort/distress/pain were any three of the following signs: Abnormal posture, a slow, careful or abnormal (waddling) gait, low activity levels, slow eating, cowering or vocalizing on handling, a change in eye or coat appearance, and weight loss. Sacrifice was confirmed by one of the following criteria: Cervical dislocation following no response to tail or toe pinch, no respiration or heartbeat following continuous monitoring for 30 sec, or rigor mortis. All procedures were performed in accordance with the Nanjing Medical University Institutional Animal Care and Use Committee (Nanjing, China) guidelines. The mice were anesthetized by inhalation of ether during OA surgery. OA was induced following sectioning of the medial meniscotibial ligament, also known as the coronary ligament, which connects the medial meniscus to the periphery of the tibial plateau, and mice undergoing sham surgery were used as a control (12,13). 7,8-DHF (Sigma-Aldrich; Merck KGaA) was subsequently dissolved in ethanol and phosphate-buffered saline (PBS). 7,8-DHF was administered by intraperitoneal injections (5 mg/kg) once a week postoperatively. The knee

Table I. Gene-specific primer sequences for qPCR.

Gene	qPCR primer (5'-3')
IL-1 β	Forward: ATGGCAGAAGTACCTAAGCTCGC
IL-1 β	Reverse: ACACAAATTGCATGGTGAAGTCA GTT
IL-6	Forward: ACACACTGGTTCTGAGGGAC
IL-6	Reverse: TACCACAAGGTTGGCAGGTG
TNF- α	Forward: ATGAGCACAGAAAGCATGATCC GC
TNF- α	Reverse: CCAAAGTAGACCTGCCCGGACTC
MMP-1	Forward: GCCACAAAGTTGATGCAGTT
MMP-1	Reverse: GCAGTTGAACCAGCTATTAG
MMP-3	Forward: ATGAAAATGAAGGGTCTTCCGG
MMP-3	Forward: GCAGAAGCTCCATACCAGCA
MMP-13	Forward: ATGCATTCAGCTATCCTGGCCA
MMP-13	Reverse: AAGATTGCATTTCTCGGAGCCTG
HO-1	Forward: ACATCGACAGCCCCACCAAGTT CAA
HO-1	Reverse: CTGACGAAGTGACGCCATCTGT GAG
Nrf2	Forward: TCTCCTCGCTGGAAAAGAA
Nrf2	Reverse: AATGTGCTGGCTGTGCTTTA
β -actin	Forward: TGACGGGGTCACCCACACTGTGC CCATCTA
β -actin	Reverse: CTAGAAGCATTTGCGGTGGACGA TGGAGGG

IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; HO-1, heme oxygenase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; qPCR, quantitative real-time polymerase chain reaction.

joints of the mice were harvested 4 and 8 weeks postoperatively, and were stained with Safranin O/Fast Green for assessment using the OARSI scoring system for histologic alterations (14). The mounted slides were placed in 70% ethyl alcohol for 15 min and then stained with 0.04% safranin O/sodium acetate buffer (pH 4.0), for 10 min at 22°C.

Cell culture. Murine knee articular cartilage was obtained from 5-day-old B6 mice and digested with collagenase D, as previously described (15). The chondrocytes isolated from articular cartilage of one litter were seeded onto a 10-cm dish at a density of 5×10^5 cells/per dish. On reaching 80% confluency, the cells were detached and randomly plated in six-well plates (10^5 /well), and cultured in Ham's F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) containing 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA), 100 IU/ml penicillin (Sigma-Aldrich; Merck KGaA), 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine at 37°C. Only the first passage cells were used for 7,8-DHF treatment, as indicated below.

Luciferase assays. 293 cells (Shanghai Institutes for Biological Sciences, China) were maintained in Dulbecco's modified

Eagle's medium (DMEM; Hyclone; GE Healthcare, Chicago, IL, USA) + 200 μ M geneticin (G-418; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) at 37°C with 5% CO₂. The HO-1 promoter was amplified by PCR from RAW cell genome DNA using the following primer sequences: HO-1, forward 5'-GGAAGATCTCTGCAGAGCCCCACTGGAG-3' and reverse 5'-CCCAAGCTTGGAACAGCAACGCTGT-3'. The PCR product was subsequently inserted into the pGL3 vector at the *Hind*III and *Bgl*III sites. A non-specific oligo was used to construct a control plasmid. The 293 cells (3×10^5 cells/well) seeded in 24-well plates were transfected with the HO-1-ARE-promoter-driven luciferase plasmid (Beyotime Institute of Biotechnology). Transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h of transfection, the cells were treated with 1, 3 and 9 μ M of 7,8-DHF for 8 h at 37°C. The relative luciferase activity was measured by normalizing HO-1-ARE-promoter-driven firefly luciferase activity to Renilla luciferase activity with a luciferase assay system (Promega Corporation).

Statistical analysis. All data are expressed as the mean \pm standard deviation. Data following Gaussian distribution were further analyzed using Student's t-test and data without Gaussian distribution was analyzed via the Mann-Whitney U test. Differences between groups were analyzed using one-way analysis of variance and Duncan's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All data were analyzed using SPSS 16.0 (SPSS, Inc.) software.

Results

7,8-DHF activates the Nrf2 signaling pathway in primary mouse chondrocytes. Primary mouse chondrocytes were treated with various doses of 7,8-DHF for 8 h (9). The cytotoxicity of 7,8-DHF was analyzed in primary mouse chondrocytes and non-toxic concentrations were used in the subsequent experiments (Fig. 1A). To examine whether 7,8-DHF can activate Nrf2 signaling in chondrocytes, the effect of 7,8-DHF on the expression of the Nrf2 downstream protein in primary mouse chondrocytes was examined. The results indicated that the expression of HO-1 was upregulated by 7,8-DHF in a dose- and time-dependent manner (Fig. 1E-J). Furthermore, 7,8-DHF treatment caused the nuclear translocation of Nrf2 and activated HO-1 promoter transactivation activity (Fig. 1B-D). Therefore, these findings suggested that 7,8-DHF effectively activated Nrf2 signaling in primary chondrocytes.

7,8-DHF regulates the activities of antioxidant markers. To investigate whether 7,8-DHF affects oxidative stress damage, the level of intracellular ROS, the content of MDA and the activity of SOD were measured. The H₂O₂-induced ROS fluorescence intensity was markedly weakened in the chondrocytes pretreated with 7,8-DHF (Fig. 2A). 7,8-DHF treatment suppressed the expression level of MDA (Fig. 2B) and increasing the activity of SOD (Fig. 2C). The results suggested that 7,8-DHF attenuated H₂O₂-induced oxidative stress in primary chondrocytes.

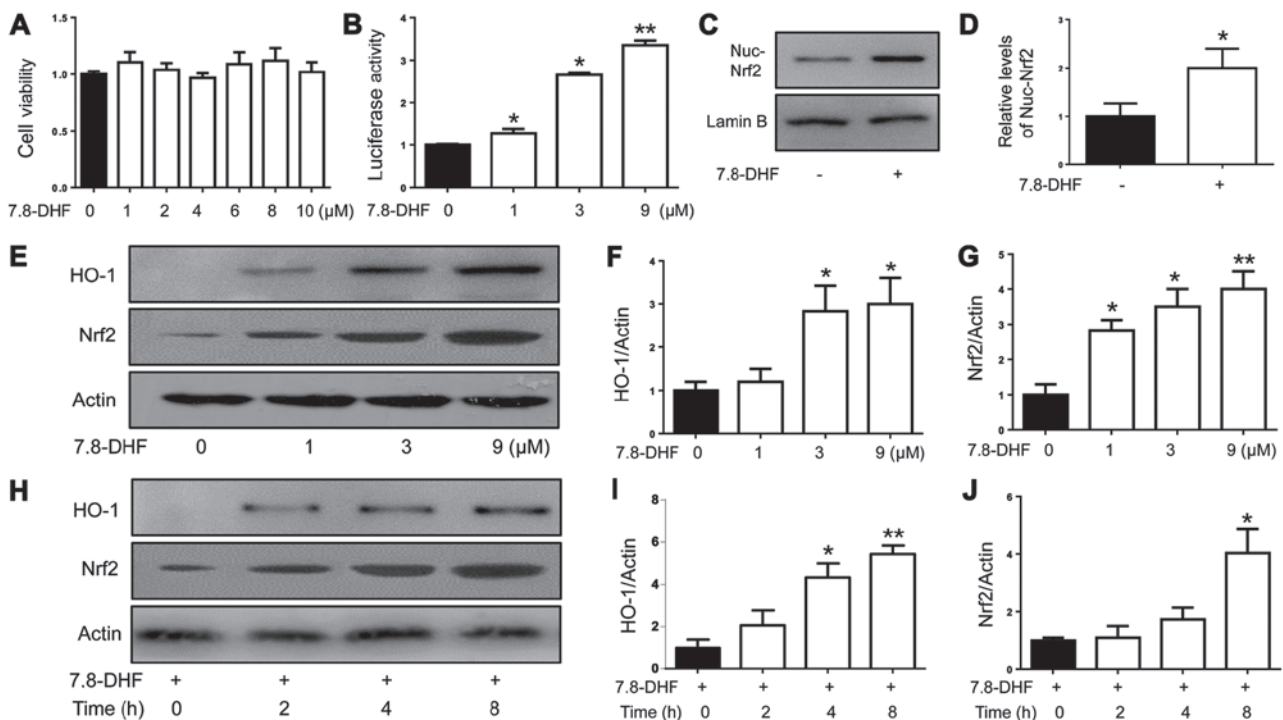


Figure 1. Induction of the expression of Nrf2 and HO-1 with 7,8-DHF treatment in primary mouse chondrocytes. (A) Cell viability assay. Primary mouse chondrocytes were treated with various doses of 7,8-DHF for 8 h. A Cell Counting Kit-8 assay was used to measure the viable cells in triplicate. (B) 293 cells were transiently transfected with HO-1 promoter reporter plasmid and a *Renilla* luciferase plasmid for 24 h. The cells were treated with various doses of 7,8-DHF for 8 h. The luciferase activity was measured and normalized to *Renilla* luciferase activity. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated control. (C) Primary mouse chondrocytes were treated with 7,8-DHF (9 μM) for 8 h. The cell nuclear fractions were isolated to be assayed for Nrf2, with lamin B serving as a control. (D) Quantitative analysis of nuclear expression levels of Nrf2. * $P < 0.05$ compared with the untreated controls. (E) Primary mouse chondrocytes were treated with various doses of 7,8-DHF for 8 h. Quantitative analysis of the expression levels of (F) HO-1 and (G) Nrf2. * $P < 0.05$ and ** $P < 0.01$ compared with untreated control. (H) Primary mouse chondrocytes were treated with 7,8-DHF (9 μM) at different time points and HO-1 and Nrf2 were assayed by western blot analysis. Quantitative analysis of the expression levels of (I) HO-1 and (J) Nrf2. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated controls. 7,8-DHF, 7,8-dihydroxyflavone; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1.

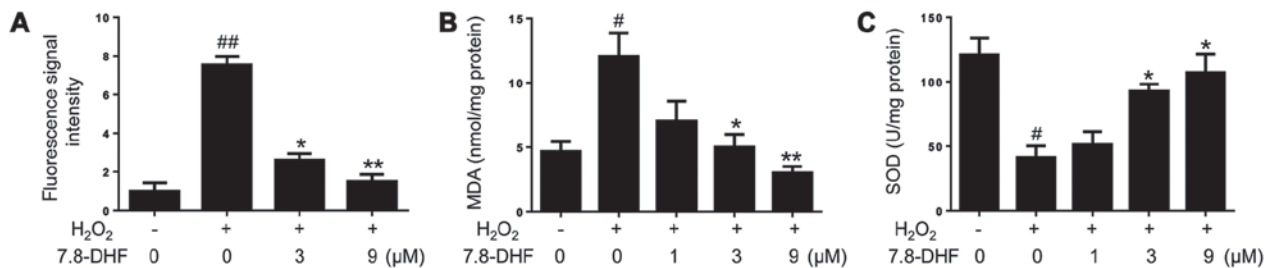


Figure 2. 7,8-DHF ameliorates oxidative stress in primary mouse-treated chondrocytes. Chondrocytes were pretreated with different concentrations of 7,8-DHF for 8 h and incubated with 40 ng/ml H₂O₂ for 24 h. (A) Effect of 7,8-DHF treatment on the level of intracellular ROS. (B) Effects of 7,8-DHF on MDA content. (C) Effects of 7,8-DHF on SOD activities. # $P < 0.05$ and ## $P < 0.01$ compared with the untreated control. * $P < 0.05$, ** $P < 0.01$ compared with H₂O₂-treated group. SOD, superoxidase dismutase; MDA, malondialdehyde; 7,8-DHF, 7,8-dihydroxyflavone; ROS, reactive oxygen species.

7,8-DHF alleviates OA-associated cartilage damage. The vehicle-treated OA mice exhibited a loss of knee joint cartilage with exposed subchondral bone, whereas the 7,8-DHF-treated group exhibited a greater extent of cartilage damage alleviation (Fig. 3A). The 7,8-DHF-treated group exhibited a lower staining score of 1.70 ± 1.10 for the tibia ($P < 0.05$) 4 weeks following surgery (Fig. 3B). In the 7,8-DHF-treatment group, the stained sections of the joints harvested at 8 weeks had lower scores of 2.20 ± 0.93 for the femur and 2.45 ± 1.40 for the tibia, indicating that 7,8-DHF significantly protected the cartilage from damage in the femoral condyle and medial tibial plateau (Fig. 4A and B).

Therefore, 7,8-DHF had a protective effect on the cartilage of the OA mouse model.

7,8-DHF attenuates OA-associated mRNA expression. The cartilage of the knee joints was used to examine changes in the OA-associated mRNA expression levels via RT-qPCR analysis. Consistent with the histopathological results, the cartilage of the OA group exhibited significantly higher mRNA expression levels of TNF- α , IL-1 β , IL-6, MMP-1, MMP-3 and MMP-13 compared with that in the sham group, and the upregulated gene expression levels were suppressed by 7,8-DHF (Fig. 5A-F).

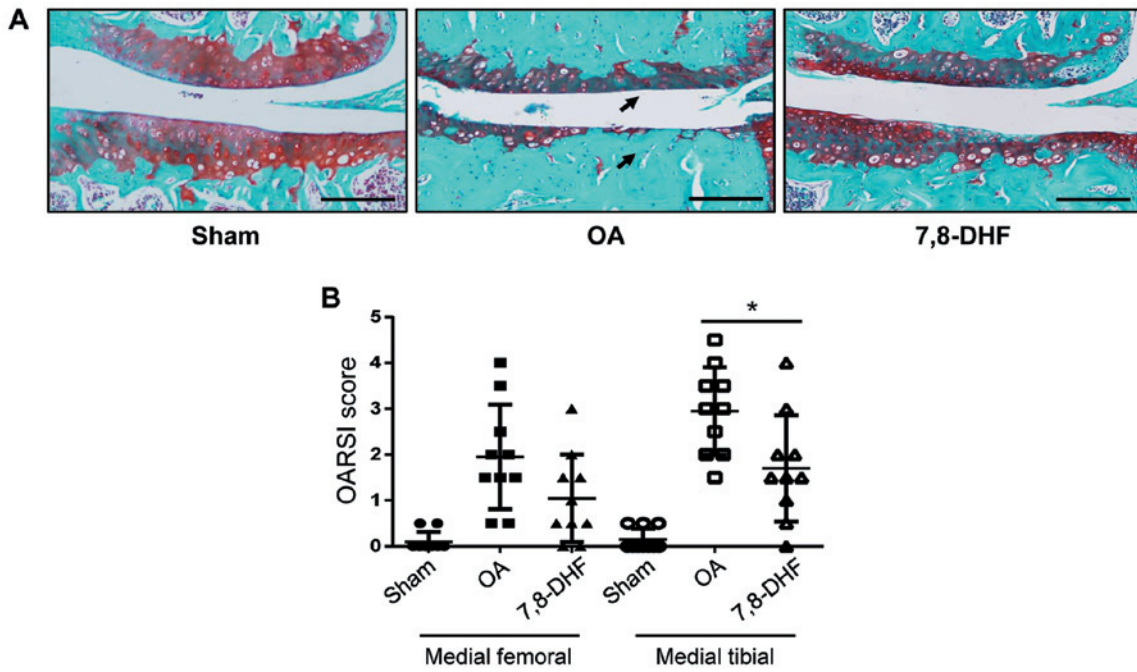


Figure 3. Histological features and scoring for structural damage in mouse knee articular cartilage 4 weeks following DMM model establishment. OA was induced in B6 mice following surgical DMM, and 7,8-DHF or vehicle was subsequently administered by intraperitoneal injection. (A) Representative frontal sections (6- μ m) were stained with Safranin O/Fast Green (n=10 mice per group). Images were captured at x200 magnification. Arrows indicate the damage-affected cartilage areas. Scale bars=100 μ m. (B) Histological scoring of OA. *P<0.05 compared with the OA group. 7,8-DHF, 7,8-dihydroxyflavone; OA, osteoarthritis; DMM, destabilization of the medial meniscus.

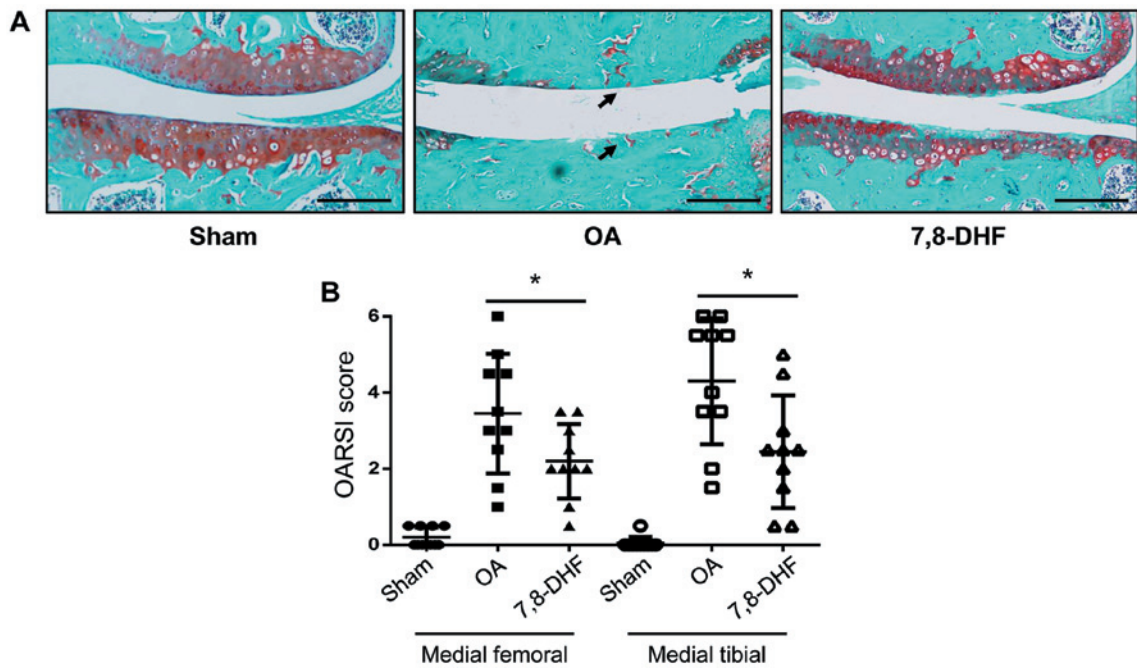


Figure 4. Histological features and scoring for structural damage in mouse knee articular cartilage at 8 weeks following destabilization of the DMM model. OA was induced in C57BL/6J mice following surgical DMM, 7,8-DHF or vehicle was administered by intraperitoneal injection. (A) Representative frontal sections (6- μ m) were stained with Safranin O/Fast Green (n=10 mice per group). Images were captured at x200 magnification. Arrows indicate the damage-affected cartilage areas. Scale bars=100 μ m. (B) Histologic scoring of OA. *P<0.05 compared with the OA group. 7,8-DHF, 7,8-dihydroxyflavone; OA, osteoarthritis; DMM, destabilization of the medial meniscus.

7,8-DHF increases the expression of HO-1 and Nrf2 in vivo. The expression of HO-1 and Nrf2 in the knee cartilage obtained from mice was further examined, in order to investigate whether Nrf2-HO-1 was substantially upregulated in

the 7,8-DHF-treatment group. The results of the present study confirmed that the protein and gene expression levels of HO-1 and Nrf2 increased following 7,8-DHF treatment (Fig. 6A-E). This was consistent with the data *in vitro*, indicating that

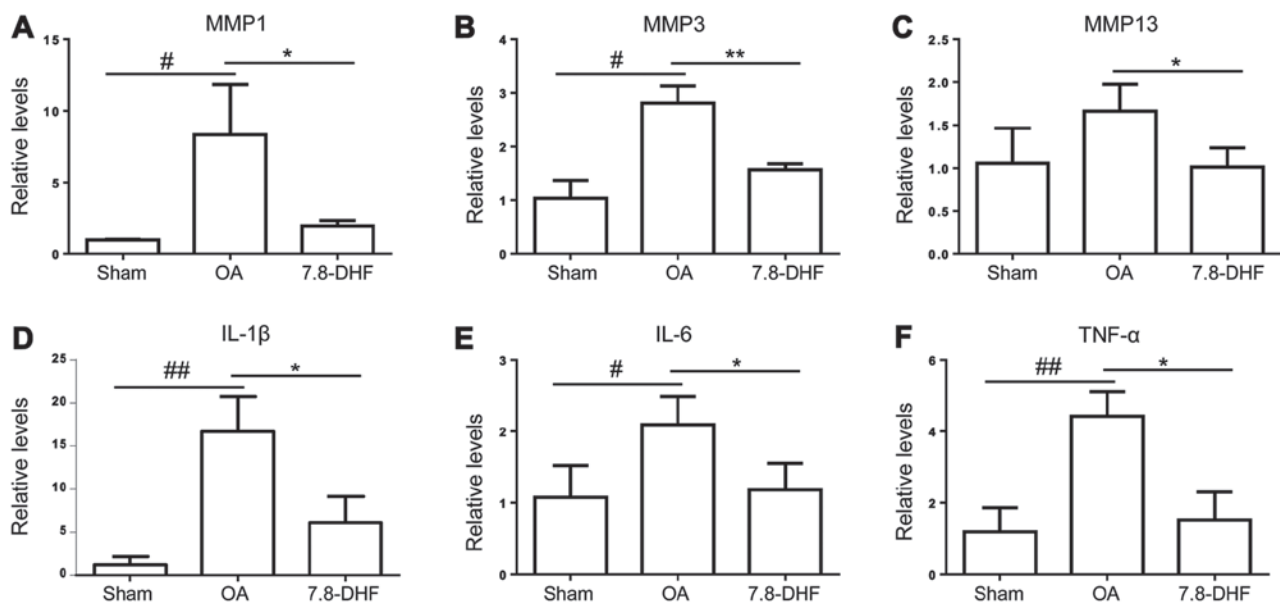


Figure 5. Effects of 7,8-DHF on OA-associated mRNA expression. Relative gene expression levels of (A) MMP-1, (B) MMP-3, (C) MMP-13, (D) IL-1 β , (E) IL-6 and (F) TNF- α in the knee joint cartilage of mice. # P <0.05 and ## P <0.01 compared with the sham group. * P <0.05 and ** P <0.01 compared with the OA group. 7,8-DHF, 7,8-dihydroxyflavone; OA, osteoarthritis; MMP, matrix metalloproteinases; IL, interleukin; TNF- α , tumor necrosis factor α .

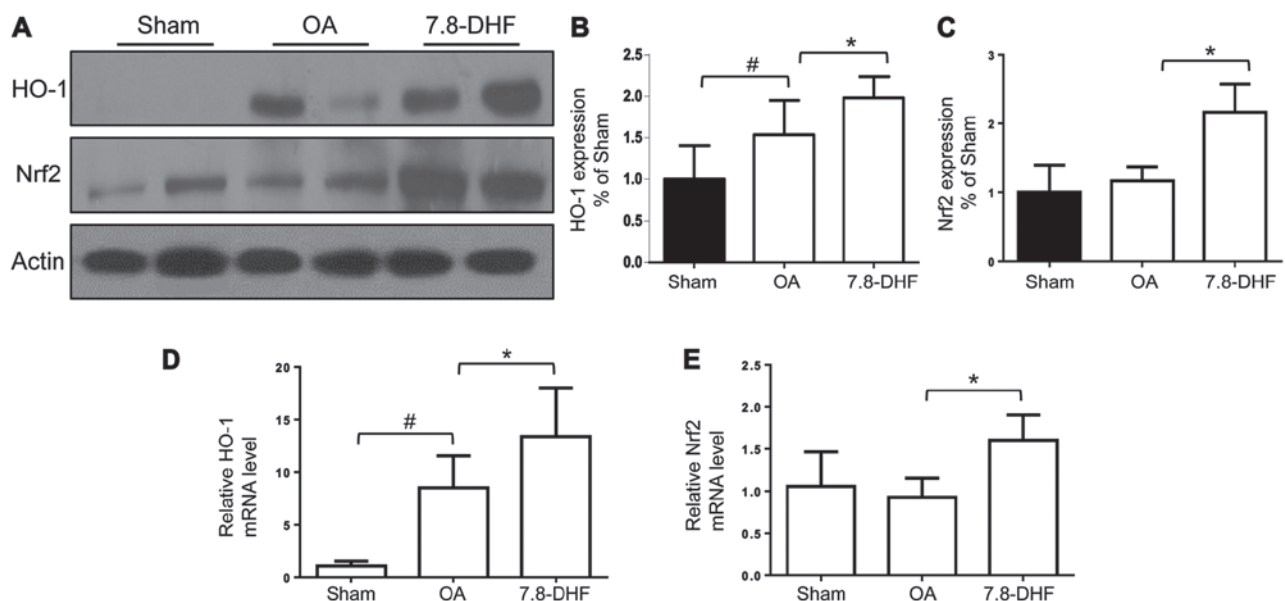


Figure 6. Effects of 7,8-DHF on the expression of Nrf2/HO-1. (A) Representative western blot analysis and the expression ratio of (B) HO-1 and (C) Nrf2 to Actin ($n=6$; * P <0.05). Gene expression levels of (D) HO-1 and (E) Nrf2 in cartilage from mice knee joints of the sham, OA and 7,8-DHF-treated groups. # P <0.05 compared with the sham group. * P <0.05 compared with the OA group. 7,8-DHF, 7,8-dihydroxyflavone; Nrf2, nuclear factor-(erythroid-derived 2)-like 2; HO-1, heme oxygenase-1.

7,8-DHF may serve a protective role in cartilage erosion in OA mice via Nrf2 signaling pathway activation.

Discussion

Excessive oxidative stress is a common pathological mechanism, which has been demonstrated to be involved in the progression of OA (16-18). Excessive oxidative stress can increase the secretion of inflammatory cytokines and alter the function of multiple signaling pathways in chondrocytes, causing cell dysfunction, extracellular matrix degradation and the programmed cell death

or necrosis of chondrocytes during OA (19-21). Nrf2 is known to regulate antioxidant protein expression, which protects against the oxidative damage induced by inflammation and injury. Usually, Nrf2 resides in the cytoplasm and translocates to the nucleus during conditions of stress. Nrf2 binds to the antioxidant response elements, which are located in the promoter region, and one of the target genes is HO-1, which counteracts the oxidative damage in various tissue injuries (22-24). The expression of HO-1 has been reported to be upregulated in numerous inflammatory diseases, as a physiological response to oxidant damage in the aforementioned conditions. Maicas *et al*

reported that HO-1 was expressed at a high level in mice with K/BxN serum-induced rheumatoid arthritis (RA) (25). Although Kobayashi *et al* demonstrated that the expression of HO-1 was enhanced in the joint tissues of patients with RA compared with that in patients with OA (26), the present study demonstrated that the level of HO-1 was higher in cartilage with OA compared with that in normal mice cartilage, which indicated that activation of the Nrf2/HO-1 signaling pathway may have a cytoprotective effect in the cartilage once OA develops.

MMPs and proinflammatory cytokines are considered to promote cartilage OA injuries (27-29). In accordance with previous studies, the present study indicated that the increased expression of MMP-1, MMP-3 and MMP-13 in the cartilage of mice with OA was suppressed following 7,8-DHF treatment. The findings reported by Choi *et al* (30) indicated that 7,8-DHF inhibited the expression of MMP-1 in Hs68 cells. Therefore, the suppressive effect of 7,8-DHF on MMPs depends on the cell type. Proinflammatory cytokines also serve major roles in the pathogenesis of OA. qPCR analysis indicated that 7,8-DHF decreased the expression of IL-1 β , IL-6 and TNF- α in the arthritic cartilage. This result was consistent with the results of a previous study that the lipopolysaccharide (LPS)-induced production of TNF- α , prostaglandin E2, NO and IL-6 was inhibited by 7,8-DHF in RAW264.7 cells (31). Park *et al* (32), further reported that 7,8-DHF may reduce the generation of pro-inflammatory mediators and cytokines by inhibiting the MAPK and nuclear factor (NF)- κ B signaling pathways in LPS-stimulated BV2 microglial cells.

In addition to the antioxidant benefits, HO-1 serves an important regulatory role in the inflammation and catabolism in chondrocytes. Associated studies have confirmed that HO-1 is involved in the production of MMPs and proinflammatory cytokines. The upregulation of HO-1, which is induced by BTB domain and CNC homology 1 deficiency, decreased the gene expression of MMP-13 and A disintegrin and metalloproteinase with thrombospondin motifs 5 in response to cytokine treatment in primary mouse articular chondrocytes (33). Rousset *et al* also reported that the increased expression of HO-1 caused a significant decrease in the expression of MMP-1 stimulated by IL-1 β in human C-20/A4 chondrocytes (34). As an inducible isoform of HO, HO-1 degrades heme to carbon monoxide, biliverdin and Fe²⁺. Carbon monoxide can suppress the synthesis of inflammatory mediators, including pro-inflammatory cytokines and nitric oxide (35). Previous studies have reported that the overexpression of HO-1 inhibited the generation of IL-6 and activation of the NF- κ B signaling pathway in cultured human tracheal smooth muscle cells (36).

7,8-DHF is an occurring flavone found in natural plants. It has been reported to act as a potent and selective small-molecule agonist of TrkB. The protective effects of 7,8-DHF on cells by scavenging intracellular ROS has been reported in HT-22 hippocampal cells and PC12 pheochromocytoma cells (37,38). 7,8-DHF has also been demonstrated to protect lung fibroblasts against oxidative damage by activating the ERK/Nrf2/HO-1 and PI3K/Akt signaling pathways (9). Our previous study indicated that Nrf2 deficiency caused more severe cartilage degradation in the knee joints of mice with OA, however, the damage was reduced by activating the Nrf2/HO-1 signaling pathway. The results of the present study indicated that 7,8-DHF enhanced the expression and translocation of Nrf2

into the nucleus, leading to an enhanced expression of HO-1 in primary mouse chondrocytes. The expression of HO-1 in the knee cartilage of mice with OA was also upregulated following 7,8-DHF treatment by administering once-a-week intraperitoneal injections postoperatively. These were in line with the findings *in vitro*. These results suggested that 7,8-DHF may protect against oxidative damage in OA cartilage.

OA is an age-associated joint disease and is characterized by the slow progression of cartilage degradation. Therefore, mice were sacrificed at different time points, in order to properly mimic this stepwise development of OA. The histological results obtained 4 and 8 weeks following surgery demonstrated that 7,8-DHF effectively reduced cartilage damage in the cartilage of OA mice. As 7,8-DHF was administered to mice at the same time of the onset of OA in the study, whether 7,8-DHF is protective in pre-arthritic knees remains to be elucidated.

In conclusion, the present study demonstrated for the first time, to the best of our knowledge, the protective effect of 7,8-DHF treatment against cartilage degradation in the development of OA. This effect was associated with activation of the Nrf2-HO-1 signaling pathways. Therefore, these results suggest that 7,8-DHF can be considered as a potential therapeutic option in the treatment of human OA.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DWC, WF and JQ conceived and designed the study. DWC, WF, JL, LHJ, SCC, TBY, CY, HX and DWG performed the experiments. DWC, WF wrote the manuscript. JQ reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All experiments related to the use of animals were approved by the Institutional Animal Care and Use Committee of The Sir Run Run Hospital of Nanjing Medical University (Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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