Caffeic acid phenethyl ester attenuates osteoarthritis progression by activating NRF2/HO-1 and inhibiting the NF-κB signaling pathway

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Abstract. Osteoarthritis (OA) is the most common degenerative disease affecting the joints, and inflammation appears to play a critical role in the initiation and progression of OA. Caffeic acid phenethyl ester (CAPE), a natural flavonoid compound, has anti-inflammatory and antioxidant functions. However, its anti-inflammatory effects on OA and the underlying mechanisms of action of CAPE in the treatment of OA remain elusive. Therefore, the present study investigated the anti-inflammatory effects of CAPE on IL-1β-stimulated chondrocytes *in vitro* and surgically induced rat models of OA *in vivo*. *In vitro*, CAPE reduced the expression of inducible nitric oxide synthase and cyclooxygenase-2 in IL-1β-stimulated chondrocytes, as well as the extracellular secretion of nitric oxide and prostaglandin E2 in the cell culture supernatants. In addition, CAPE attenuated the degradation of extracellular matrix by increasing the expression of aggrecan and collagen II, and decreasing the expression of MMP3, MMP13 and a disintegrin and metalloproteinase with thrombospondin motif-5. Furthermore, CAPE attenuated NF-κB signaling and activated the nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathway in IL-1β-stimulated chondrocytes. *In vivo*, CAPE protected cartilage from destruction and delayed the progression of OA in rats. Taken together, the findings of the present study indicated that CAPE may be a potential therapeutic agent for the prevention or treatment of OA.

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Introduction

Osteoarthritis (OA) is the most common degenerative disease affecting the joints worldwide, and frequently affects the elderly population. The pathological features of OA include the erosion of articular cartilage, subchondral bone sclerosis and synovitis (1,2). Although various studies have reported that sex, age and obesity are the main risks factors for OA, the pathophysiology of OA has not been fully elucidated to date (3,4). It has been demonstrated that inflammatory cytokines play a critical role in the initiation and progression of OA, including interleukin (IL)-1β, tumor necrosis factor (TNF), IL-6, etc. (5,6). Among these, IL-1β is considered the most crucial one (7). The expression level of IL-1β has been found to be increased in the synovial fluid and cartilage tissue of patients with OA (8). IL-1β affects the metabolic process of chondrocytes by increasing the expression of MMPs, which contributes to cartilage degradation (9). IL-1β also induces the production of the inflammatory mediators, nitric oxide (NO) and prostaglandin E2 (PGE2), which leads to bone resorption and extracellular matrix (ECM) degradation (10). Therefore, novel effective agents aiming to suppress IL-1β expression, thus inhibiting IL-1β-induced MMPs and inflammatory mediators, may attenuate the progression of OA (11-14). For example, wogonin, a plant-derived small molecule, has been shown to play a potent anti-inflammatory and cartilage protective role by activating the reactive oxygen species (ROS)/ERK/nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway in chondrocytes of patients with OA (11). Stachydrine, a bioactive alkaloid, has been found to attenuate IL-1β-induced inflammation through the NF-κB signaling pathway in chondrocytes of patients with OA (14). The NF-κB pathway is known to be

involved in IL-1β-induced inflammation. Following stimulation with IL-1β, p65 is translocated from the cytoplasm to the nucleus, where it stimulates the expression of multiple inflammatory or catabolic genes, including inducible NO synthase (iNOS), TNF-α and MMPs (15). NF-κB signaling plays a critical role in the pathogenesis of OA (164). It has been reported that the activation of the NRF2/heme oxygenase-1 (HO-1) signaling inhibits the IL-1β-induced activation of NF-κB in chondrocytes (17). NRF2 is a transcription factor that regulates the cellular response to oxidative stress and affects the expression of superoxide dismutase and HO-1 (18). Previous studies have demonstrated that NRF2 plays a pivotal role in cartilage integrity through anti-oxidative and anti-apoptotic functions (19,20). In addition, an *in vivo* study demonstrated that the deletion of NRF2 led to more severe damage in cartilage (21). Therefore, the activation of the NRF2 signaling pathway and the inhibition of the NF-κB signaling pathway play major chondroprotective roles in alleviating the symptoms of OA.

Caffeic acid phenethyl ester (CAPE), a natural flavonoid compound, is one of the major bioactive ingredients of propolis (22). It is also widely found in fruits, vegetables and grains, and has been shown to exhibit multiple health benefits (23). Previous studies have indicated that CAPE has antioxidant, antitumor, anti-apoptotic, anti-inflammatory and neuroprotective functions (24-27). CAPE has been reported to be a potent inhibitor of NF-κB (28). Liang *et al* (29) reported that CAPE suppressed the proliferation and metastasis of nasopharyngeal carcinoma cells by inhibiting the NF-κB signaling pathway. Lim *et al* (30) reported that CAPE suppressed skin inflammation by inhibiting NF-κB activation. Furthermore, another study demonstrated that CAPE decreased ROS levels and exerted a neuroprotective effect in a mouse model of Alzheimer's disease via the activation of the NRF2/HO-1 signaling pathway (31). In addition, as previously demonstrated, CAPE suppressed the inflammation-triggered myeloperoxidase activity and production of pro-inflammatory cytokines in colitis (32). These results confirm the effectiveness and safety of CAPE as an anti-inflammatory agent. However, the role of CAPE in other age-related and inflammation-related diseases, such as OA, remains unclear. Elmali *et al* (33) reported that CAPE could alleviate unilateral anterior cruciate ligament transection-induced cartilage destruction. However, the chondroprotective effects of CAPE and its underlying mechanisms remain elusive. Therefore, the present study investigated the anti-inflammatory effects and the underlying mechanisms of action of CAPE in the treatment of OA.

Materials and methods

Reagents. CAPE was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. IL-1β was purchased from ProteinTech Group, Inc. The Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology. Griess Reagent Nitrite Measurement kit (cat. no. 13547) was purchased from Cell Signaling Technology, Inc. The ELISA kits for human PGE2 (cat. no. SEKH-0414), Toluidine Blue solution and bovine serum albumin (BSA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Penicillin/streptomycin, fetal bovine serum (FBS) and DMEM were purchased from Gibco (Thermo Fisher Scientific, Inc.).

Primary chondrocyte isolation and culture. Human chondrocytes were isolated from human articular cartilage tissues that were procured from patients with femoral neck fracture. The collection of cartilage tissues from patients was approved by the Ethics Committee of Shenzhen Second People's Hospital (Shenzhen, China). Patients with an average age of 64.8 years (aged 60-68 years, two males and three females) who were subjected to total knee replacement surgery at the Shenzhen Second People's Hospital from January to March, 2021 participated in the study. All patients provided written informed consent. The articular cartilage tissues were washed with PBS and cut into 1 mm^3 pieces. The sections were then cultured with collagenase II (2 g/l) for 4 h at 37˚C (31). Upon centrifugation at 108 x g for 5 min at 24˚C, the cells were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) antibiotics (penicillin/streptomycin) in an atmosphere containing 5% CO_2 at 37˚C. The culture medium was changed every other day. Only cells from the first three passages were used in the present study. Chondrocyte morphology was examined using toluidine blue staining. In brief, the culture medium was aspirated and washed twice with PBS. Toluidine blue solution for 5 min at room temperature, and the same amount of distilled water was added and mixed evenly. Images were acquired using an Olympus microscope (Olympus Corporation) after standing for 15 min at room temperature.

Animal models. Sprague-Dawley male wild-type rats (n=12; 8 weeks old; weighing 300-350 g) were acquired from Cloud-Clone Corp. The rats were maintained in a clean environment at 27˚C under a 12-h light/dark cycle with 50% humidity, and had free access to adequate food and water. The animal protocols and the experimental procedures were in agreement with the Animal Care and Use Committee of Southwest Medical University (approval no. 20211124-043). The model of OA was established by the destabilization of the medial meniscus (DMM) through surgical treatment as previously described (32). Briefly, the rats were anesthetized with 2% (w/v) pentobarbital (40 mg/kg) via intraperitoneal injection. The capsule of the right knee joint was opened though the medial patellar tendon and the medial meniscus tibial ligament was cut using microsurgical scissors. The medial meniscus tibial ligament was not cut in the sham-operated control group. In the present study, the rats were randomly divided into three groups as follows: The sham-operated control group (sham), the OA group (DMM) and the CAPE-treated OA group (DMM + CAPE). Rats in the CAPE treatment group were administered 10 mg/kg/2 days CAPE, for 8 weeks. At the end of the experiment, all animals were sacrificed by cervical dislocation under anesthesia through an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and respiratory arrest was used to confirm animal death. During the course of the experiment, any rats that were unable to eat or drink, had difficulty breathing and had lost 20% of their body weight before the experiment, were regarded as having reached the humane endpoint and were thus immediately euthanized. None of the rats reached the aforementioned end points in this experiment and thus none were euthanized before the end of the experiment.

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
iNOS	GACGAGACGGATAGGCAGAG'	CACATGCAAGGAAGGGAACT
COX-2	GAGAGATGTATCCTCCCACAGTCA	GACCAGGCACCAGACCAAAG
MMP3	TGCGTGGCAGTTTGCTCAGCC	GAATGTGAGTGGAGTCACCTC
MMP13	GGCTCCGAGAAATGCAGTCTTTCTT	ATCAAATGGGTAGAAG TCGCCATGC
Adamts5	GGTCAAGGTCCCATGTGCAAC	GAATGCGGCCATCTTGTCATC
Aggrecan	AGGATGGCTTCCACCAGTGT	GGCATAAAAGACCTCACCCTCC
Collagen II	CTGTCCTTCGGTGTCAGGG	CGGCTTCCACACATCCTTAT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

iNOS, inducible nitric oxide synthase; *COX-2*, cyclooxygenase-2; *Adamts5*, a disintegrin and metalloproteinase with thrombospondin motif-5.

Cell transfection with NRF2 small-interfering RNA (siRNA). Negative control (NC) siRNA and NRF2 siRNA were purchased from Shanghai GenePharma Co., Ltd. Chondrocytes were plated and cultured in six-well plates for 24 h, and then transfected with 80 nM NC and NRF2 siRNA for 48 h using Lipofectamine® 2000 siRNA transfection reagent (Thermo Fisher Scientific, Inc.). The sequences of the NRF2 and NC siRNAs were as follows: 1#NRF2 siRNA: sense, 5'-GGGAGG AGCUAUUAUCCAUTT-3' and antisense, 5'-AUGGAUAAU AGCUCCUCCCTT-3'; and 2#NRF2 siRNA sense, 5'-GCC CAUUGAUGUUUCUGAUTT-3' and antisense, 5'-AUCAGA AACAUCAAUGGGCTT-3'; and 3#NRF2 siRNA sense, 5'-GCCUGUAAGUCCUGGUCAUTT-3' and antisense, 5'-AUGACCAGGACUUACAGGCTT-3'; and NC siRNA sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; NC FAM-siRNA sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

CCK-8 assay. The cytotoxicity of CAPE on human chondrocytes was evaluated using CCK-8 assay. In brief, chondrocytes were plated into 96-well plates in serum-free medium for 24 h, and then treated with various concentrations of CAPE (0, 1, 3, 5, 10, 20 and 40 μM) for 24 h. The chondrocytes were then washed with PBS, and 100 μl DMEM containing 10 μl CCK-8 solution was added to each well, followed by incubation at 37˚C for 3 h. The optical density value of the wells was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Griess reaction and ELISA. Chondrocytes were plated and cultured in 6-well plates, and pre-treated with CAPE (0, 10 or 20 μM). After 24 h, IL-1β (10 ng/ml) was added, followed by additional 24 h of incubation at 37˚C. The content of NO and PGE2 in each well was detected using the Griess reaction and ELISA kits, respectively, according to the manufacturer's protocols.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the chondrocytes using TRIzol® reagent (Takara Bio, Inc.), and 1 μg total RNA was used to synthesize cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Inc.), and the conditions were as follow: 15 min at 37˚C, followed by 5 sec at 85˚C. The total volume of q-PCR was 10 μl, including 5 μl 2X SYBR Master Mix (Beyotime Institute of Biotechnology), 0.25 μl each primer and 4.5 μl diluted cDNA. qPCR was conducted in a CFX96Real-Time PCR System, and the thermocycling conditions were as follows: 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. GAPDH was used as the internal control for normalization. The relative mRNA of the target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (36). The primer sequences of the target genes are presented in Table I.

Western blot analysis. The cells were lysed by radio immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Inc.) containing 1 mM PMSF to extract total protein. The protein concentration was detected with a BCA protein assay kit (Beyotime Institute of Biotechnology). Subsequently, 40 μg denatured protein solution were separated by 12% SDS-PAGE (Wuhan Servicebio Technology Co., Ltd.) and transferred to a PVDF membrane (Merck KGaA), which was incubated in methanol for 1 min at room temperature. The methanol was then removed and the membrane was equilibrated in transfer buffer until ready to use. Upon transfer at 100 V for 1 h at 4˚C, the membrane was blocked with 5% skimmed milk for 1 h at room temperature and then incubated with primary antibodies against GADPH (1:1,000; cat. no. AP0066; Bioworld Technology, Inc.), MMP3 (1:5,000; cat. no. 66338-1-Ig; ProteinTech Group, Inc.), MMP13 (1:1,000; cat. no. 18165-1-AP; ProteinTech Group, Inc.), a disintegrin and metalloproteinase with thrombospondin motif-5 (Adamts5; 1:500; cat. no. A2836; ABclonal Biotech Co., Ltd.), iNOS (1:500; cat. no. 18985-1-AP; ProteinTech Group, Inc.), cyclooxygenase-2 (COX-2; 1:500; cat. no. 12375-1-AP; ProteinTech Group, Inc.), lamin B1 (1:1,000; cat. no. AF1408; Beyotime Institute of Biotechnology), NRF2 (1:2,000; cat. No. 16396-1-AP; ProteinTech Group, Inc.), HO-1 (1:1,000; cat. no. 10701-1-AP; ProteinTech Group, Inc.), p65 (1:1,000; cat. no. 8242; Cell Signaling Technology Inc.), phosphorylated (p)-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology Inc.), IκBα (1:1,000, cat. no. 4814; Cell Signaling Technology Inc.), p-IκBα (1:1,000, cat. no. 2859; Cell Signaling Technology Inc.) and collagen II (1:500; cat. no. PA5-99159, Thermo Fisher Scientific, Inc.) overnight at 4˚C. Subsequently, the membranes

were incubated with a HRP-conjugated goat anti-rabbit (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology Inc.) or goat anti-mouse IgG (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology Inc.) secondary antibody for 2 h at room temperature. Finally, an ECL reagent (cat. no. 34580; ThermoFisher Scientific, Inc.) was used to detect the blots. The ECL signal was captured using Odyssey FC Imager (Gene Company, Ltd.), and GAPDH and Lamin B1 were used as loading controls for normalization. Quantitative analysis was performed using ImageJ 1.53e software (National Institutes of Health).

Immunofluorescence. Chondrocytes were seeded in a glass plate, cultured for 12 h, pre-treated with or without CAPE (20 μM) for 24 h, and then co-incubated with or without IL-1β (10 ng/ml) for 24 h. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. The cells were then treated with 0.1% TritonX-100 for 5 min and blocked with 5% BSA for 1 h at room temperature. The cells were then incubated with primary antibodies against collagen II (1:100; cat. no. PA5-99159, Thermo Fisher Scientific, Inc.), MMP3 (1:100; cat. no. 66338-1-Ig; ProteinTech Group, Inc.), p65 (1:400; cat. no. 8242; Cell Signaling Technology Inc.) and NRF2 (1:500; cat. no. 16396-1-AP; ProteinTech Group, Inc.) overnight at 4˚C. The following day, the cells were incubated with Alexa Fluor® 647-conjugated goat anti-rabbit IgG (1:500; cat. no. A0468; Beyotime Institute of Biotechnology) and Alexa Fluor® 647-conjugated goat anti-mouse IgG secondary antibodies (1:500; cat. no. A0473; Beyotime Institute of Biotechnology) for 1 h at room temperature in the dark and exposed to DAPI (Beyotime Institute of Biotechnology) for 5 min at room temperature. Finally, the cells were observed under a confocal microscope (ZEISS GmbH) and analyzed using ImageJ 1.53e software.

Molecular docking of CAPE with the Kelch-like ECH-associated protein 1 (Keap1)-NRF2 complex. The structure data file (SDF) of CAPE (Pubchem ID: 5281787) was downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and converted to PDB format on OpenBabel 3.1.1. The structure of Keap1-NRF2 complex was downloaded from the RCSB Protein Data Bank (https://www.rcsb.org/). AutoDockTools1.5.7 software was used to modify the receptor protein, such as water removal and hydrogenation, and also to modify ligand small molecule, such as hydrogenation. Next, the molecular docking analysis of receptor protein and ligand small molecule was performed using AutoDockTools. Eventually, ligand binding flexibility with the binding pocket residues were drawn using PYMOL 2.5.2.

Histopathological analysis. The knee joint tissue was fixed with 4% paraformaldehyde for 24 h at 4˚C and then decalcified with 10% EDTA solution for 2 weeks. The tissue was then dehydrated, cleared, embedded in paraffin blocks and sliced to obtain frontal serial sections at a thickness of 5 μm. The slides were then stained with Safranin O/Fast Green (S/O) (cat. no. G1053; Beijing Solarbio Science & Technology Co., Ltd.). In brief, the slides were strained with Fast Green for 2 min, washed with water, and soaked in 1% hydrochloric acid and alcohol for 10 sec at room temperature. Then strained with Safranin O for 5 sec, rapid dehydrated in absolute ethanol for 5 sec, 2 sec and 10 sec, and sealed with neutral resin (Sinopharm Group Chemical Reagent Co., Ltd.) at room temperature. The extent of cartilage degeneration was assessed using a Nikon E100 microscope (Nikon Corporation) and evaluated using the Osteoarthritis Research Society International (OARSI) scoring system as described previously (37).

Immunohistochemical assay. Immunohistochemical assay was performed as previously described (38). Briefly, the slides were deparaffinized and rehydrated. For antigen repair, the slides were incubated with 0.4% pepsin (Sangon Biotech Co., Ltd.) in 5 mM HCl at 37˚C for 20 min, and then blocked with 5% BSA for 30 min. The slides were then incubated with primary antibodies against collagen II (1:50; cat. no. PA5-99159, Thermo Fisher Scientific, Inc.), MMP3 (1:500; cat. no. 66338-1-Ig; ProteinTech Group, Inc.), MMP13 (1:500; cat. no. 18165-1-AP; ProteinTech Group, Inc.) and NRF2 (1:500; cat. no. 16396-1-AP; ProteinTech Group, Inc.) overnight at 4˚C. Finally, the slides were incubated with HRP-conjugated goat anti-rabbit (1:50; cat. no. A0208; Beyotime Institute of Biotechnology, Inc.) or goat anti-mouse IgG (1:50; cat. no. A0216; Beyotime Institute of Biotechnology, Inc.) secondary antibody for 50 min at room temperature, and images were captured using an Olympus BX51 microscope (Olympus Corporation).

Statistical analysis. Data are expressed as the mean ± SD. All experiments were repeated at least three times. Statistical analyses were performed using GraphPad Prism version 8.0 software (GraphPad Software, Inc.). Comparisons between two groups were performed using an unpaired two-tailed Student's t-test. Comparisons among multiple groups were done using one-way ANOVA test followed by a post-hoc Test (Bonferroni). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of CAPE on human chondrocytes. The molecular chemical structure of CAPE is illustrated in Fig. 1A, and the morphology of the human chondrocytes are illustrated in Fig. 1B and C, respectively. To measure the cytotoxic effect of CAPE on human chondrocytes, the chondrocytes were treated with various concentrations of CAPE (0, 1, 3, 5, 10, 20 and 40 μmol/l) for 24 h. CAPE cytotoxicity was detected using CCK-8 assay. The results revealed that there was no obvious cytotoxic effect of CAPE on human chondrocytes when the cells were incubated with 1, 3, 5, 10 or 20 μM CAPE (Fig. 1D). However, the viability of the chondrocytes was significantly inhibited following treatment with 40 μM CAPE (Fig. 1D). Therefore, CAPE was used at 10 and 20 μM in the subsequent experiments.

CAPE blocks the levels of IL-1β-induced inflammatory mediators in human chondrocytes. To examine the effects of CAPE on the IL-1β-induced inflammation of chondrocytes, the expression and production levels of COX-2, iNOS, NO and PGE2 were measured using RT-qPCR, western blot analysis and ELISA. The results revealed that CAPE at the

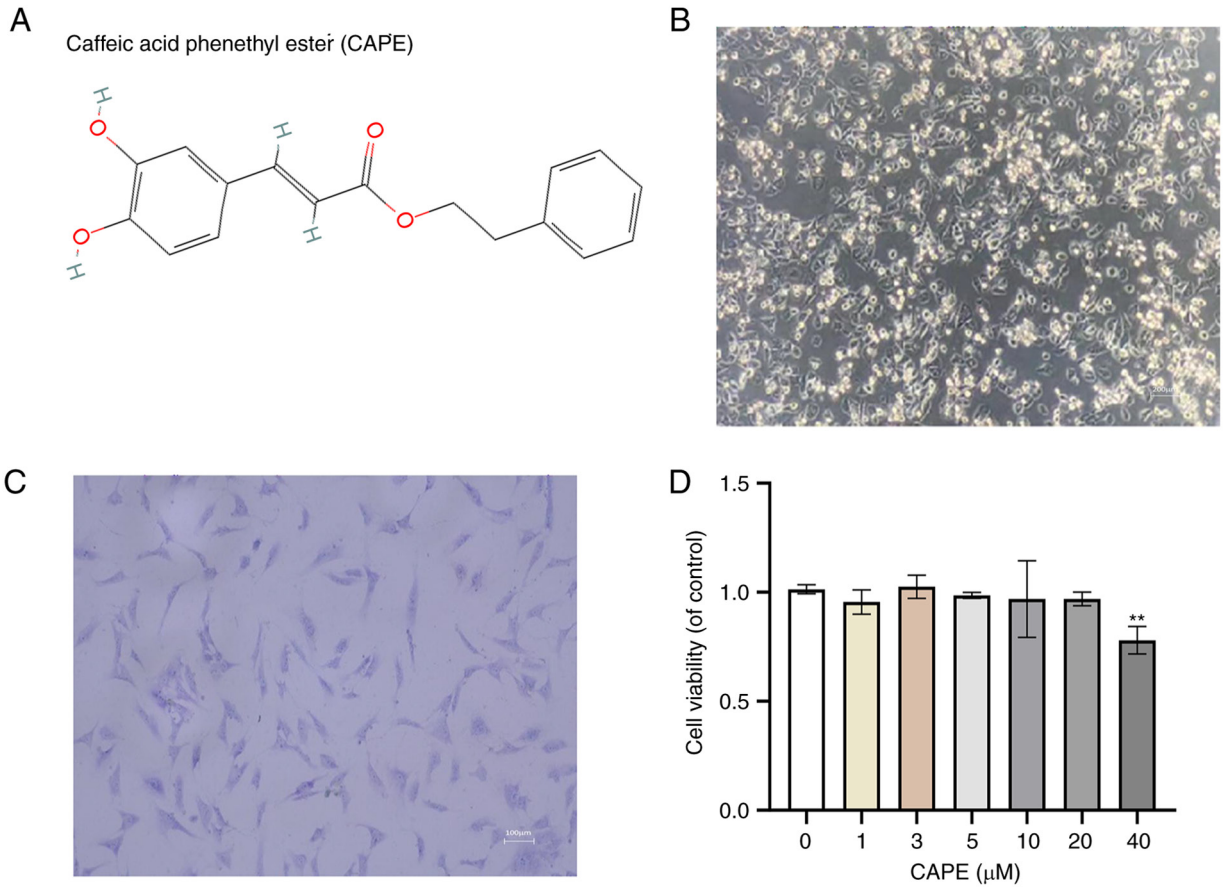

Figure 1. Cytotoxic effects of CAPE on human chondrocytes. (A) Chemical structure of CAPE. (B) Cell morphology of human primary chondrocytes under light microscopy. Scale bar, 200 μm. (C) Toluidine blue staining in human primary chondrocytes. Scale bar, 100 μm. (D) The cytotoxic effects of CAPE on human chondrocytes were determined using Cell Counting Kit-8 assay. **P<0.01, vs. control. CAPE, caffeic acid phenethyl ester.

concentrations of 10 and 20 μM inhibited the expression of COX-2 and iNOS stimulated by IL-1β at the protein and mRNA level (Fig. 2A-C). In addition, the results of Griess reaction and ELISA revealed that CAPE at concentrations of 10 and 20 μM suppressed the production of NO and PGE2 stimulated by IL-1β (Fig. 2D and E). Therefore, these results indicated that CAPE inhibited the expression and production of IL-1β-induced inflammatory mediators in human chondrocytes, particularly at the concentrations of 10 and 20 μM.

CAPE attenuates the IL-1β-induced degradation of the ECM in human chondrocytes. To determine the effects of CAPE on IL-1β-induced ECM synthesis and degradation in chondrocytes, the effects of CAPE on the expression of collagen II, aggrecan, MMP3, MMP13 and Adamts5 were examined using RT-qPCR, western blot analysis and immunofluorescence. The results revealed that the mRNA expression levels of collagen II and aggrecan were increased by CAPE at 10 and 20 μM under IL-1β stimulation, while the mRNA expression levels of MMP3, MMP13 and Adamts5 were inhibited (Fig. 3A). In addition, the protein level of collagen II was increased by CAPE at 10 and 20 μM following IL-1β stimulation, while the protein levels of MMP3, MMP13 and Adamts5 were inhibited (Fig. 3B). The immunofluorescence staining of collagen II and MMP3 revealed that CAPE activated collagen II expression and inhibited MMP3 expression following IL-1β stimulation (Fig. 3C and D). Therefore, these data indicated that CAPE attenuated the IL-1β-induced ECM degradation in human chondrocytes by preventing the degradation of collagen II and the production of ECM degradative enzymes.

CAPE prevents IL-1β-induced NF-κB activation in human chondrocytes. To explore the anti-inflammatory mechanisms of CAPE in IL-1β-stimulated chondrocytes, the expression of p65, p-p65, IκBα and p-IκBα was detected using western blot analysis and immunofluorescence. The results revealed that the phosphorylation of p65 was significantly increased by IL-1β stimulation, while it was significantly inhibited by pre-treatment with CAPE (Fig. 4A and B). Additionally, pre-treatment with CAPE inhibited the protein level of IkBα and the phosphorylation level of IkBα in IL-1β-stimulated chondrocytes (Fig. 4A and B). The immunofluorescence staining of p65 revealed that pre-treatment with CAPE decreased the expression of p65 in the nucleus of IL-1β-stimulated chondrocytes (Fig. 4C). Therefore, these data suggested that CAPE prevented the IL-1β-induced NF-κB activation by inhibiting the translocation of p65 and the degradation of IκBα in human chondrocytes.

CAPE upregulates the NRF2/HO-1 signaling pathway in IL-1β-stimulated human chondrocytes. To investigate the role

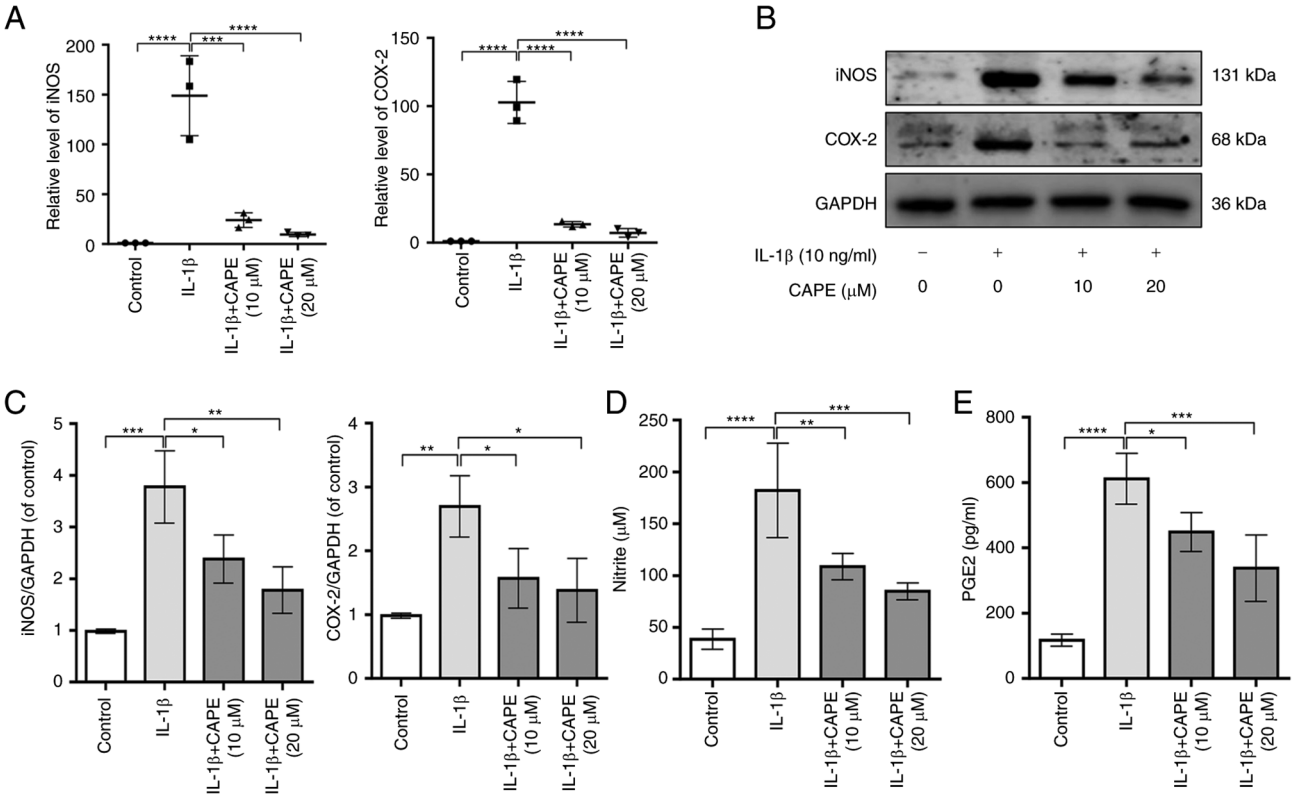

Figure 2. CAPE blocks the levels of IL-1β-induced inflammatory mediators in human chondrocytes. (A) The mRNA expression of COX-2 and iNOS was detected using reverse transcription-quantitative PCR. The protein expression of COX2 and iNOS was (B) detected using western blotting and (C) quantified. (D) The production of nitric oxide was detected via the Griess reaction. (E) The production of prostaglandin E2 was detected using ELISA. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CAPE, caffeic acid phenethyl ester; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.

of the NRF2/HO-1 signaling pathway in the anti-inflammatory effects of CAPE, the expression of NRF2 and HO-1 in IL-1β-stimulated human chondrocytes was detected using western blot analysis and immunofluorescence. The results revealed that CAPE increased the expression of NRF2 in a concentration-dependent manner (Fig. S1). Additionally, the results revealed that the protein levels of NRF2 and HO-1 were increased by 10 and 20 μM CAPE under IL-1β stimulation (Fig. 5A and B). The immunofluorescence staining of NRF2 revealed that pre-treatment with CAPE increased the expression of NRF2 in the nuclei of IL-1β-stimulated chondrocytes (Fig. 5C). Therefore, these data suggested that CAPE exerted anti-inflammatory effects in IL-1β-stimulated chondrocytes through the activation of the NRF2/HO-1signaling pathway.

To further confirm that the anti-inflammatory effects of CAPE are mediated via the NRF2/HO-1 signaling pathway in IL-1β-stimulated chondrocytes, NRF2 siRNA was transfected into chondrocytes to silence the expression of NRF2 (Fig. S2). The results revealed that the silencing of NRF2 inhibited the protein levels of NRF2 and HO-1 which had been increased by pre-treatment with CAPE in the IL-1β-stimulated chondrocytes, which suggested that the CAPE-induced activation of the NRF2/HO-1 signaling activation was inhibited by the silencing of NRF2 (Fig. 6A and B). The silencing of NRF2 increased the phosphorylation levels of p65 in the nuclei of IL-1β-stimulated chondrocytes which had been decreased by pre-treatment with CAPE; this suggested that the CAPE-mediated suppression of NF-κB signaling was abolished by the silencing of NRF2 (Fig. 6A and B). Furthermore, downregulation of NRF2 could inhibit the protein levels of iNOS and COX-2 by pre-treatment with CAPE in IL-1β-induced chondrocytes, which suggested that the CAPE-mediated suppression of inflammation was abolished by downregulation of NRF2 (Fig 6A and B). In addition, the silencing of NRF2 in IL-1β-stimulated chondrocytes increased the protein levels of MMP3, MMP13 and Adamts5, whereas it decreased the protein levels of collagen II upon pre-treatment with CAPE (Fig. 6C and D). Therefore, these data indicated that CAPE regulated NF-κB signaling and the inflammatory response though the NRF2/HO-1 signaling pathway in IL-1β-stimulated chondrocytes.

Molecular docking between CAPE and the Keap1-NRF2 complex. In order to evaluate whether there is any affinity between CAPE and Keap1-NRF2 complex, computational molecular docking analysis was performed. The structures of CAPE and the Keap1-NRF2 complex (PDB ID: 2FLU) were obtained and analyzed (Fig. 7A and B). It was found that CAPE interacted with and docked at the Keap1-NRF2 complex binding site. High-affinity (-7.39 kcal/mol) hydrogen binding events were observed between the residues of Arg415, Val465 and Val512 in CAPE and Keap1-NRF2 complex (Fig. 7C). Therefore, these results indicated that CAPE probably inhibited the development of OA by interacting with the Keap1-NRF2 complex, inhibiting ubiquitination and promoting nuclear translocation of NRF2.

CAPE attenuates OA progression in a rat model of DMM. To examine the effects of CAPE on OA progression *in vivo*, a

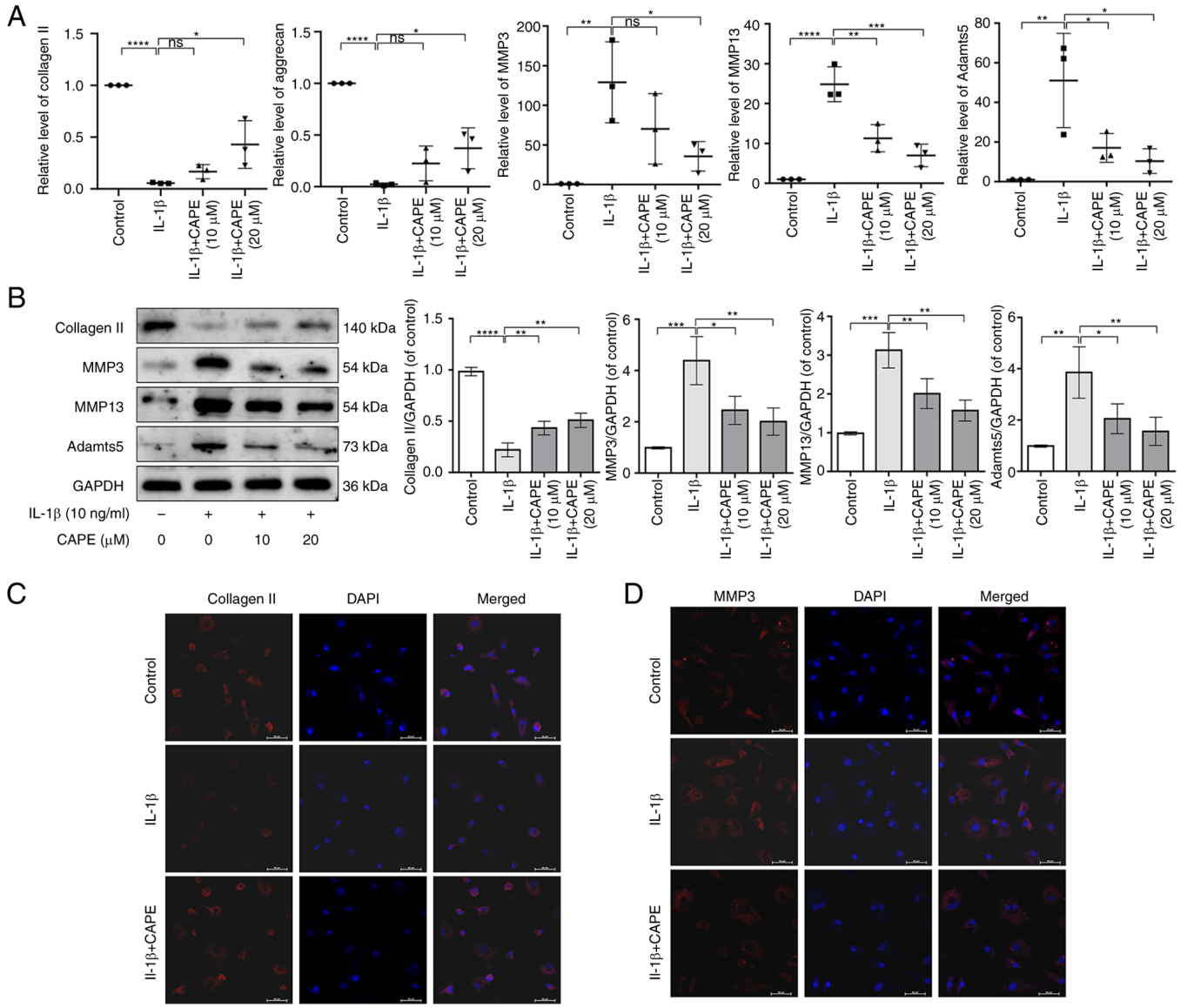

Figure 3. CAPE attenuates the IL-1β-induced extracellular matrix degradation in human chondrocytes. (A) The mRNA expression of collagen II, aggrecan, MMP3, MMP13 and Adamts5 was detected using reverse transcription-quantitative PCR. (B) The protein expression of collagen II, MMP3, MMP13 and Adamts5 was detected using western blot analysis and quantified. (C) Collagen II and (D) MMP3 expression was detected by immunofluorescence. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CAPE, caffeic acid phenethyl ester; Adamts5, a disintegrin and metalloproteinase with thrombospondin motif-5.

model of OA was established using rats, followed by an intraperitoneal injection of 10 mg/kg CAPE. S/O staining revealed that the articular cartilage exhibited a normal, red-dyed area and a smooth surface in the sham control group, whereas the thickness of the articular cartilage was significantly reduced in the DMM group (Fig. 8A). Compared with that in the DMM group, the red-dyed area was thicker and the surface of articular cartilage was smoother in the CAPE-treated group (Fig. 8A), which suggested that CAPE played a role in attenuating the degradation of cartilage matrix. The immunohistochemistry of MMP3, MMP13, collagen II and NRF2 was also performed in the OA model. In the DMM group, the expression of MMP3 and MMP13 was markedly increased compared with that in the sham group, while the DMM + CAPE group exhibited a decreased expression of MMP3 and MMP13 (Fig. 8B). In the DMM group, the expression of collagen II was markedly decreased compared with that in the sham group, while the DMM + CAPE group exhibited a high expression (Fig. 8B). In the DMM group, the expression of NRF2 was not markedly different from that in the sham group, while the DMM + CAPE group showed increased expression in the cell nuclei (Fig. 8B).

Discussion

OA is a common degenerative disease affecting the joints; however, there are currently no effective therapeutic drugs for the clinical treatment of OA (39). Currently, OA is considered to be a chronic and low-grade inflammatory disease. A better understanding of the inflammatory pathophysiology of OA may lead to the identification of novel potential therapeutic drugs (40). Natural products have already exhibited potential for use in the treatment of OA by inhibiting inflammatory processes (41). CAPE, a natural polyphenolic product, is

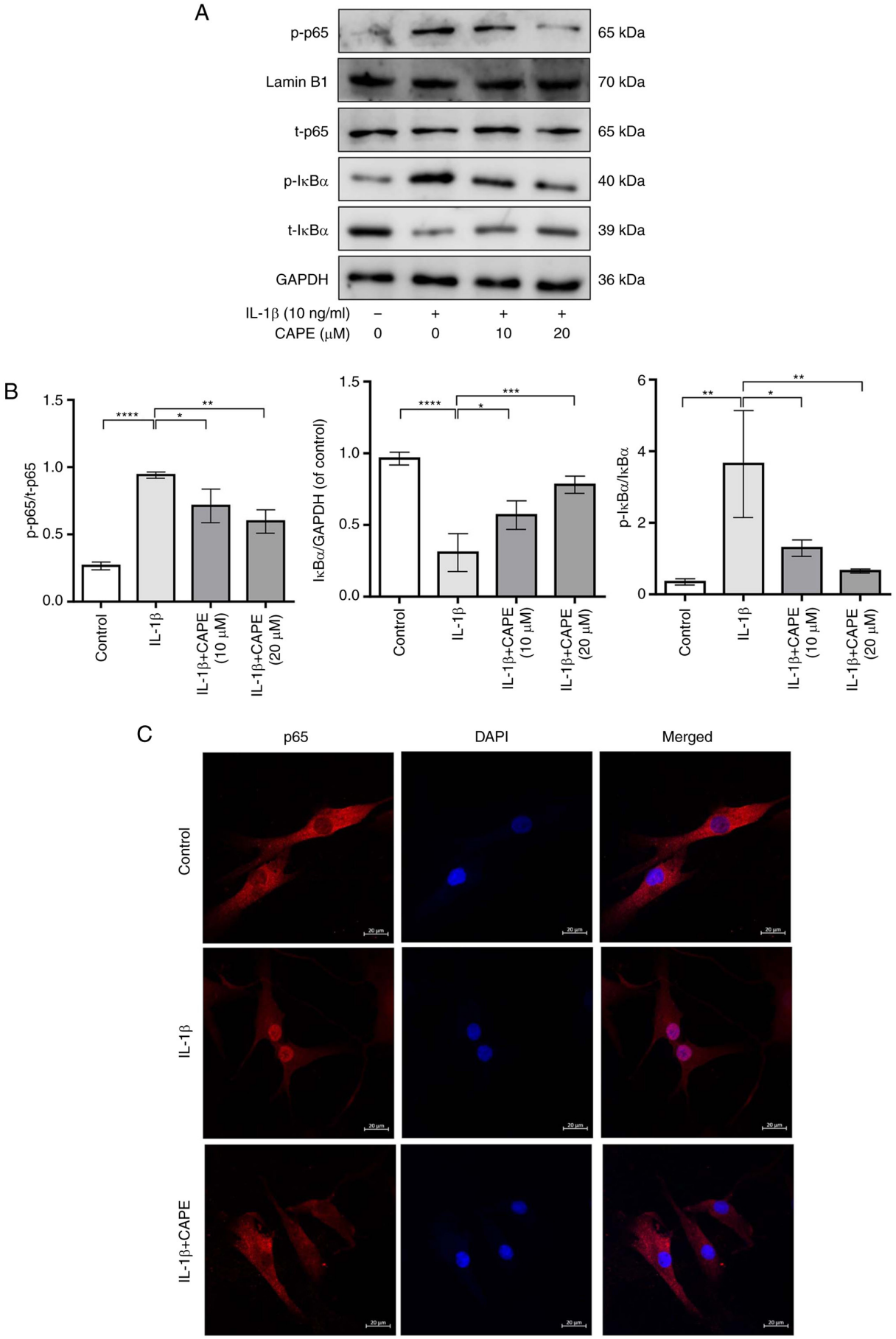

Figure 4. CAPE prevents IL-1β-induced NF-κB activation in human chondrocytes. The protein expression of p65, p-p65, IκBα and p-IκBα was (A) detected using western blot analysis and (B) quantified. (C) The nuclear translocation of p65 was observed using immunofluorescence. Scale bar, 20 μm. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CAPE, caffeic acid phenethyl ester; p, phosphorylated.

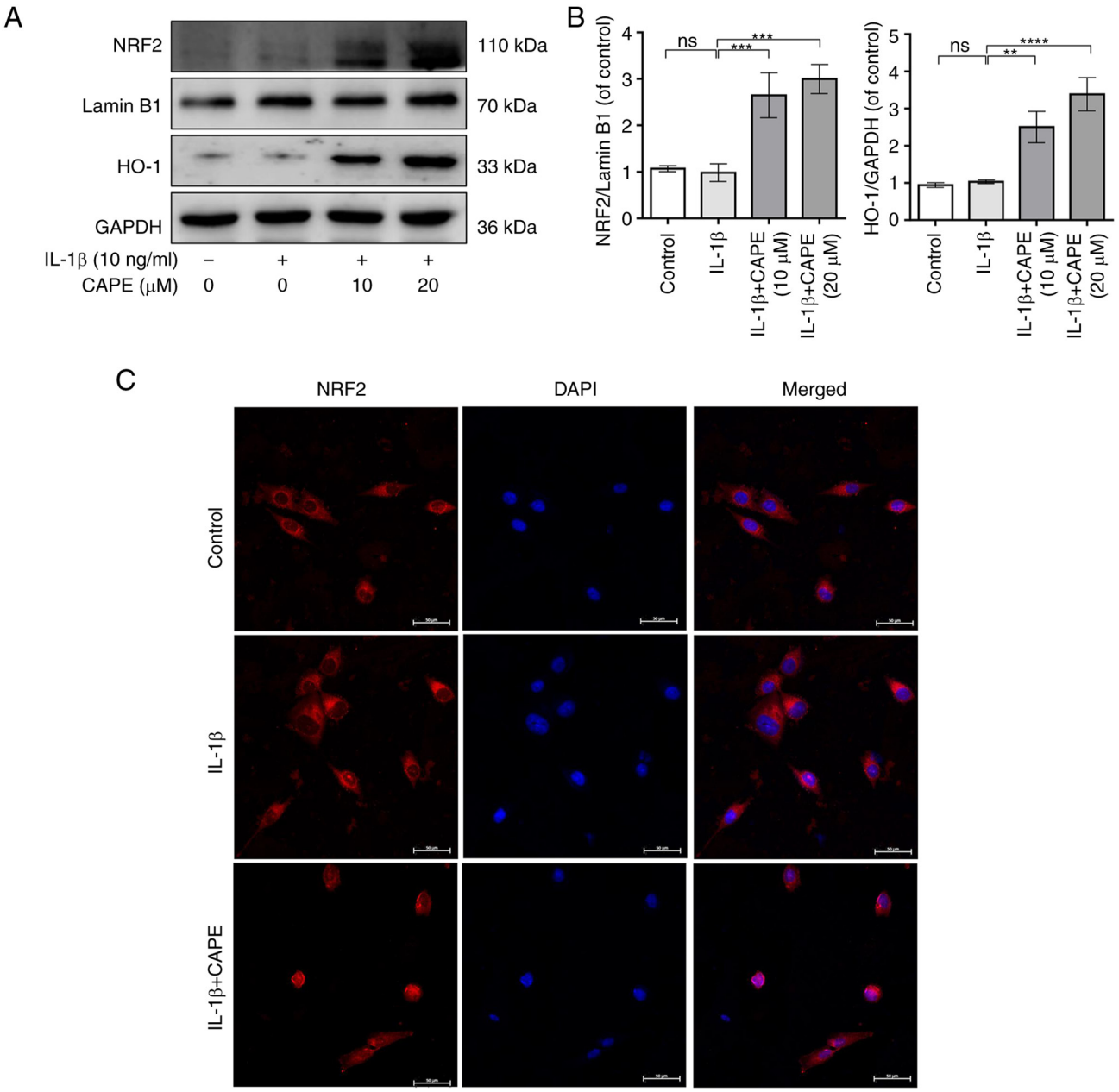

Figure 5. CAPE upregulates the NRF2/HO-1 signaling pathway in IL-1β-induced human chondrocytes. The protein expression of NRF2 and HO-1 was (A) detected using western blot analysis and (B) quantified. (C) The nuclear translocation of NRF2 was observed using immunofluorescence. Scale bar, 50 μm. **P<0.01, ***P<0.001 and ****P<0.0001. CAPE, caffeic acid phenethyl ester; NRF2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1.

extracted from the bark of conifer trees and propolis (22). It has been reported that CAPE has antioxidant, antimicrobial, anti-inflammatory, anti-cancer and immunomodulatory functions (42-44). Numerous studies have recommended the use of CAPE for the treatment of heart, kidney, liver and neurological diseases, as well as for diabetes and cancer (42-44). A previous study demonstrated that intra-articular injections of CAPE significantly decreased cartilage destruction *in vivo*, which indicated that CAPE was potentially useful as a therapeutic drug (33). However, the mechanisms through which how CAPE protects cartilage and attenuates OA are currently unknown. To the best of our knowledge, the present study identified for the first time, the effects of CAPE on IL-1β-induced human chondrocytes *in vitro* and explored the potential molecular mechanisms involved in the prevention of OA.

Chondrocytes are the only cell type in cartilage. Multiple pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6 can influence the functions of chondrocytes and may thus be implicated in the pathogenesis process of OA (45). It is well-accepted that IL-1β can be used to mimic OA by inducing an inflammatory response in chondrocytes (7-9). In the present study, IL-1β was used to induce OA in chondrocytes, and it was found that IL-1β significantly increased the inflammatory responses of chondrocytes.

CAPE has been reported to possess an anti-inflammatory function (44). CAPE has been found to attenuate amyloid-β oligomer-induced neurodegeneration, neuroinflammation and memory impairment in mice by inhibiting oxidative stress through the NRF2/HO-1 signaling pathway (31). A previous study also demonstrated that CAPE exerted protective effects against

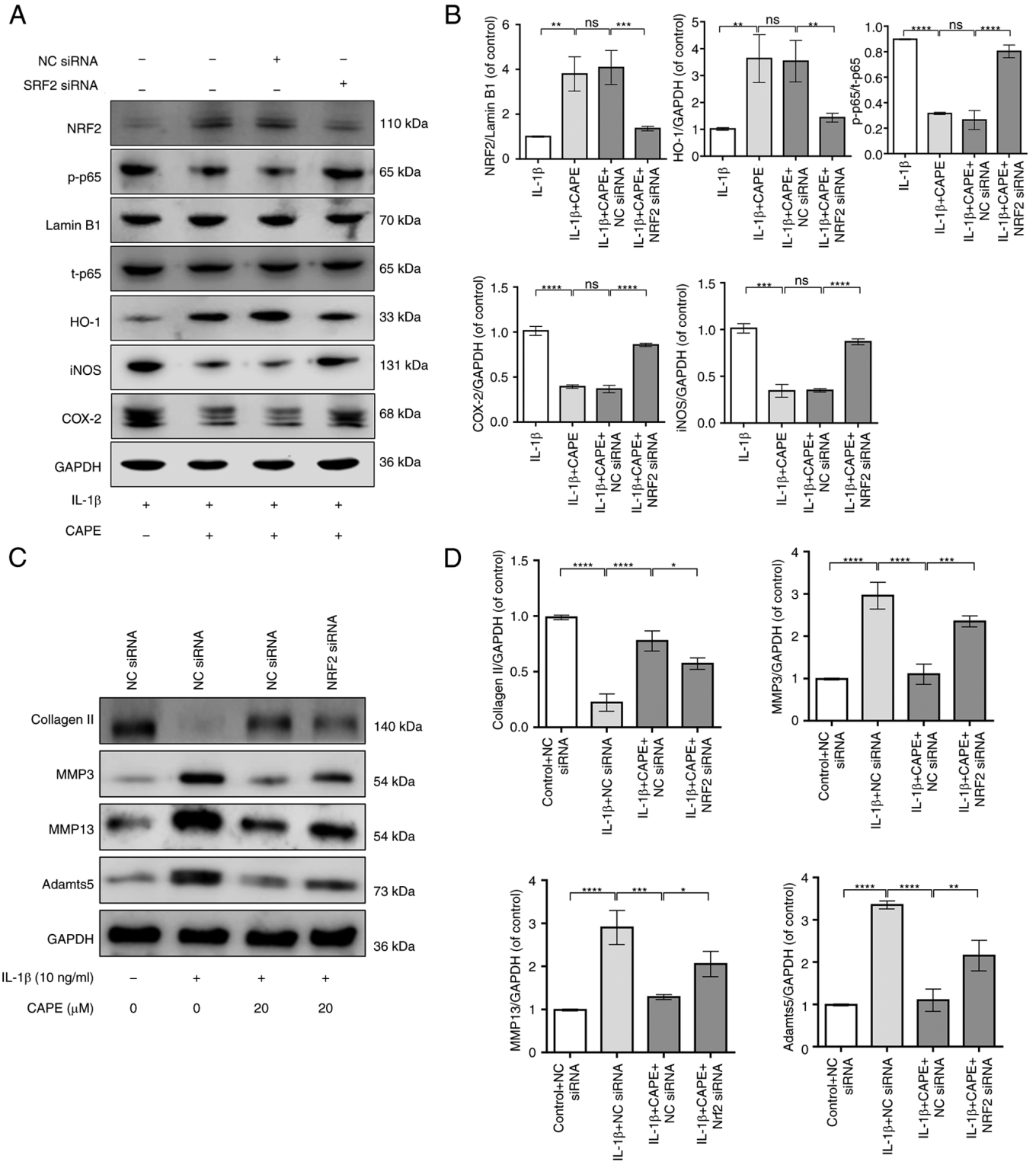

Figure 6. Downregulation of NRF2 inhibits the anti-inflammatory effects of CAPE on human chondrocytes. The protein expression of NRF2, HO-1, p-p65, iNOS and COX-2 was (A) detected using western blot analysis and (B) quantified. (C) The protein expression of collagen II, MMP3, MMP13 and Adamts5 was (C) detected using western blotting and (D) quantified. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CAPE, caffeic acid phenethyl ester; NRF2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2.

Helicobacter pylori-induced gastritis in Mongolian gerbils, which was attributed to its ability to suppress inflammatory mediators, including TNF-α, IL-2, IL-6, IL-8 and iNOS through the inhibition of NF-κB activation (46). In addition, CAPE has been shown to prevent colitis-associated cancer by post-transcriptionally inhibiting the NOD-, LRR- and pyrin domain-containing protein 3 inflammasome (47). The present study found that CAPE inhibited the levels of IL-1β-induced inflammatory mediators, including NO, PGE2, iNOS and COX-2 in human chondrocytes. Therefore, the results of the present study suggested that CAPE exerted its anti-inflammatory effects by suppressing the levels of inflammatory mediators in IL-1β-stimulated chondrocytes.

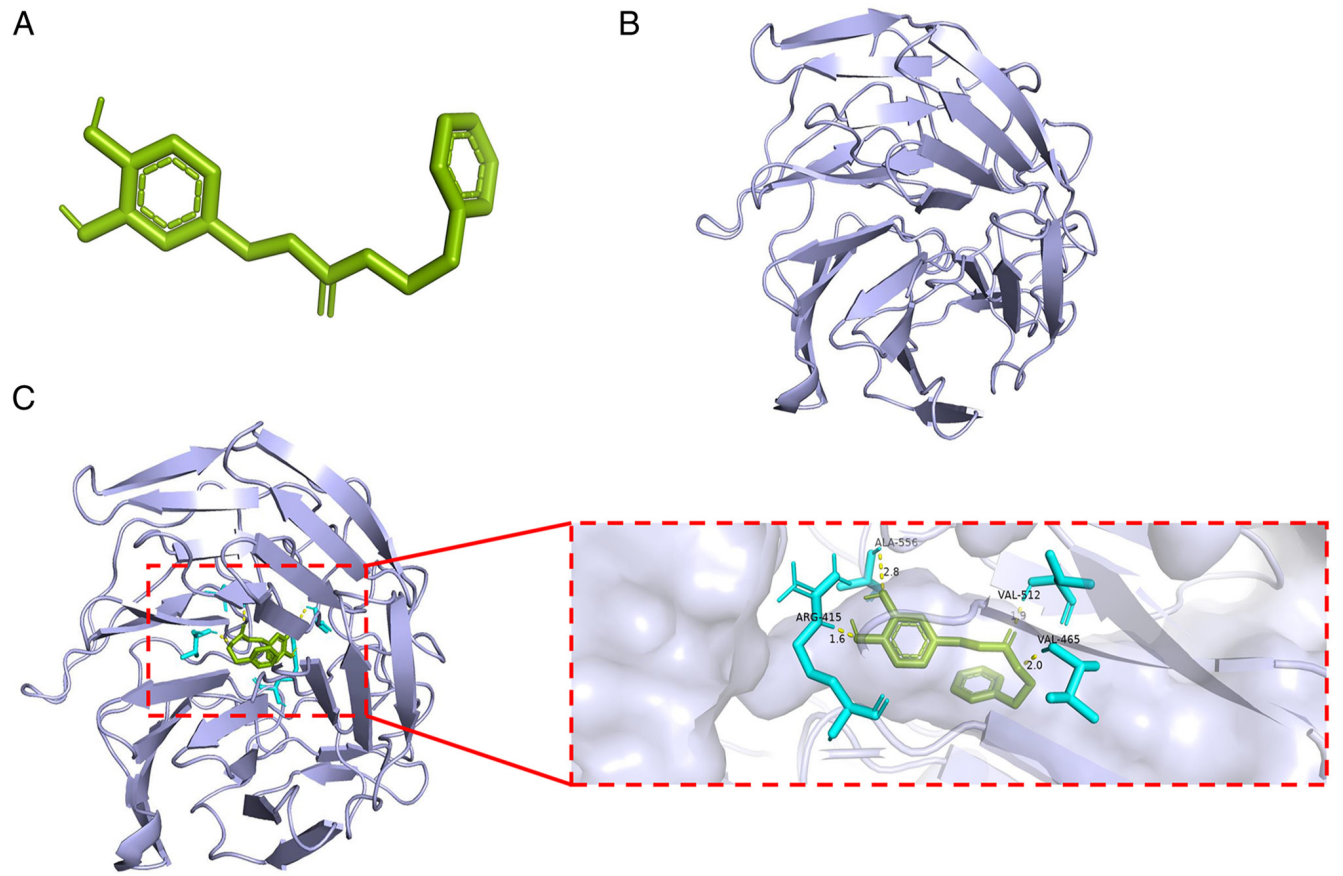

Figure 7. Caffeic acid phenethyl ester interacts with Keap1-NRF2 complex in a docking study. (A) Modular structure of CAPE. (B) The ribbon modular structure of Keap1-NRF2 complex. (C) The high interaction energy (-7.39 kcal/mol) interaction between CAPE and Keap1-NRF2 complex. NRF2, nuclear factor erythroid 2-related factor 2.

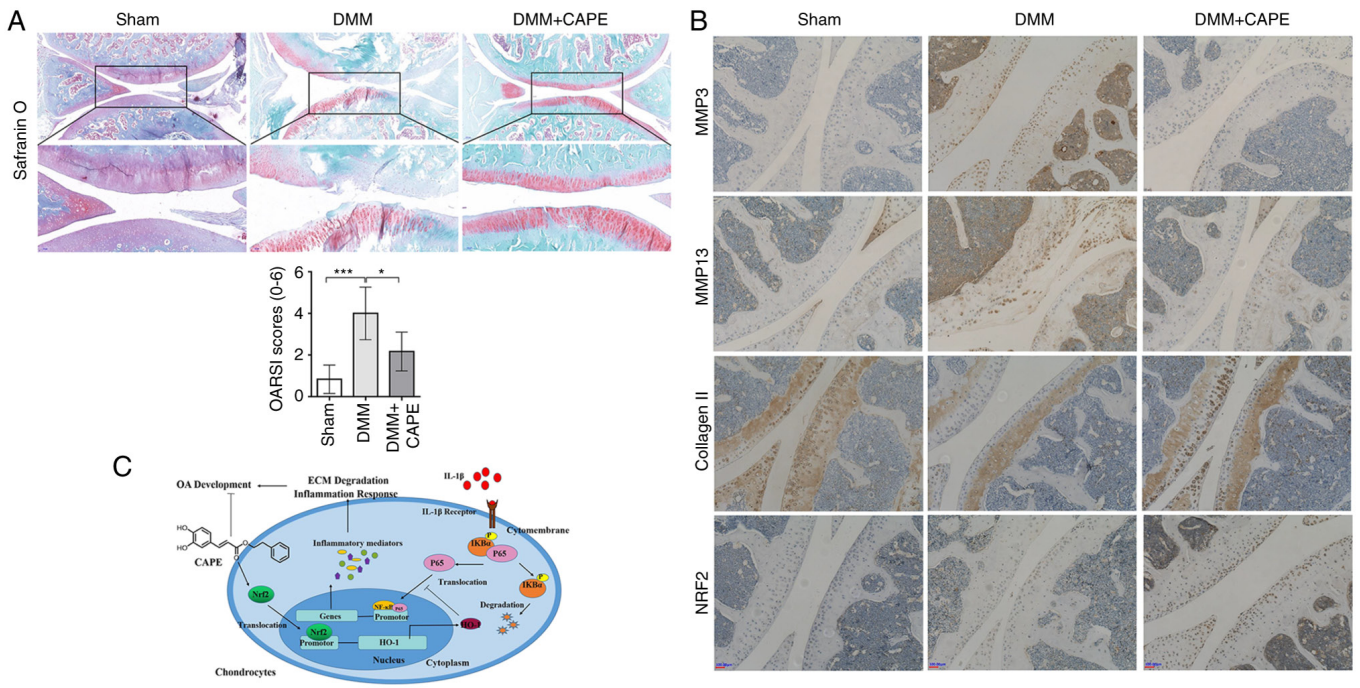

Figure 8. CAPE alleviates OA progression in a DMM rat model. (A) Degeneration of articular cartilage was observed using the Safranin O/Fast Green staining. Upper panel scale bar, 200 μm; lower panel scale bar, 100 μm. The images on the bottom panel are a larger image of the boxed areas in the respective top panels. (B) Immunohistochemistry of MMP3, MMP13, collagen II and NRF2 was employed to assess the effect of CAPE on cartilage in the DMM models. Scale bar, 100 μm. (C) Schematic illustration of the potential protective effects and the underlying mechanism of CAPE in OA development. *P<0.05 and ***P<0.001. CAPE, caffeic acid phenethyl ester; OA, osteoarthritis; DMM, destabilization of the medial meniscus; NRF2, nuclear factor erythroid 2-related factor 2.

The ECM degradation of articular cartilage is one of the main features in the process of OA development (48). During the development of OA, chondrocytes produce and secrete ECM degradative enzymes, including family members of MMPs and Adamts, in response to inflammatory factors, which can promote the degradation of the ECM (49-51), while the generation of collagen II and aggrecan is decreased (51). The present study found that the levels of ECM degradative enzymes, including MMP3, MMP13 and Adamts5, were increased in IL-1β-stimulated chondrocytes, while the level of collagen II was decreased under IL-1β stimulation. Pre-treatment with CAPE inhibited the generation of ECM degradative enzymes and promoted the generation of collagen II in IL-1β-induced chondrocytes, which indicated that CAPE could prevent the ECM degradation of articular cartilage. In addition, in the model of DMM OA, CAPE mitigated the progression of OA, which was consistent with the results of the *in vitro* experiments, suggesting that CAPE may be an effective drug for the treatment of OA.

Previous studies have indicated that the NF-κB transcription factor plays a central role in the pathogenesis of OA (52,53). Notably, NF-κB is activated by pro-inflammatory factors, excessive mechanical stresses associated factors and ECM degradation products during the development of OA. NF-κB then promotes the transcription of catabolic genes, including the MMPs and Adamts families, and triggers the expression of pivotal inflammatory factors of OA, including iNOS, COX-2 and PGE2 (16,54). Therefore, the inhibition of NF-κB provides an effective strategy with which to protect cartilage from damage in OA. The results of the present study demonstrated that pre-treatment with CAPE inhibited the translocation of p65 from the cytoplasm to the nucleus in IL-1β-stimulated chondrocytes, indicating that CAPE exerted an anti-inflammatory effect by inhibiting the activation of the NF-κB signaling pathway to protect cartilage from destruction.

NRF2 is a crucial transcription factor that mediates the response to ROS and inflammation (55). The activation of NRF2 has been reported to repress the activity of NF-κB, leading to a decrease in the production of inflammatory factors (56,57). Increasing evidence has indicated that the NRF2/HO-1 signaling pathway plays a pivotal role in the protection of joint cartilage during OA pathogenesis (58). The activation of the NRF2/HO-1 signaling pathway inhibits the production of MMPs and inflammatory factors to reduce the degradation of ECM in OA chondrocytes (19,59,60). A previous study reported that CAPE exerted its anti-inflammatory effects by inducing the activation of the NRF2/HO-1 signaling pathway; CAPE attenuated 2,4,6-trinitrobenzene sulfonic acid-induced colitis via the activation of the NRF2 signaling pathway in the rat inflamed colon (61). CAPE has also been shown to protect the periodontal status, which is attributed to its ability to suppress inflammation through activation of the NRF2/HO-1 signaling pathway and the inhibition of the NF-κB signaling pathway (62). Additionally, CAPE has been found to prevent liver fibrosis via the upregulation of NRF2 (63). In the present study, CAPE reduced the inflammatory response and attenuated the degradation of ECM via the upregulation of NRF2, the activation of the NRF2/HO-1 signaling pathway and the inhibition of the NF-κB signaling pathway. In the physiological state, NRF2 was ubiquitinated by binding with Keap1 in the cytoplasm. Under stimulation, NRF2 dissociates from Keap1 and enters the nucleus to form a coactivator complex, which can improve the driving function (64). Therefore, in the present study, the interaction between CAPE and Keap1/NRF2 complex was analyzed by computational molecular docking. The results of the present study indicated that CAPE interacting with Keap1 led to the release of NRF2 from the Keap1/NRF2 complex, which is essential for NRF2 activation.

Although the effects of CAPE on OA have been analyzed, there are relatively few studies available on its mechanisms of action. In the study by Elmali *et al* (33), it was reported that CAPE attenuated unilateral anterior cruciate ligament transection-induced cartilage destruction *in vivo*. In the study by Pichler *et al* (65), it was found that CAPE reduced the mRNA levels of IL-1β and MMP-13 under treatment with the galectin mixture, Gal-1/-3/-8. As CAPE has been previously described as an effective inhibitor of the NF-κB-dependent expression of Gal-7 in breast cancer cells, Pichler *et al* (65) hypothesized that the function of CAPE in galectin-induced chondrocytes occurred via the NF-κB signal pathway. However, this was not confirmed experimentally. In the study by Wang *et al* (66), the role of CAPE in the apoptosis of TNF-α induced chondrocytes and in the expression of MMP-2 and MMP-9 *in vitro* was investigated. However, they did not fully elucidate the role and related mechanisms of CAPE in OA. Therefore, these studies only simply described the role of CAPE in OA, but did not discuss the mechanisms. The present study investigated the anti-inflammatory effects of CAPE and the anti-degradation of the ECM in IL-1β-stimulated chondrocytes *in vitro* and *in vivo* for the first time (to the best of our knowledge), and confirmed experimentally that CAPE participated in OA regulation as an NF-κB inhibitor by regulating the NRF2/HO-1 signaling pathway. Therefore, the findings of the present study are in agreement with those from previous studies (33,65,66), and is comprehensive and novel to a certain extent. However, there are several limitations to the present study. Firstly, the binding between CAPE and the Keap1/NRF2 complex needs to be confirmed by co-immunoprecipitation. In addition, in terms of morphology, the present study only observed the morphology of normal chondrocytes and did not use OA chondrocytes as a positive control. Furthermore, the effects of CAPE need to be tested in NRF2-knockout mice. Therefore, further studies are required to confirm and further elaborate on the present findings.

In conclusion, the present study provided novel insight into the potential protective effects of CAPE in OA. CAPE attenuate IL-1β-induced inflammation and ECM degradation through activation of the NRF2/HO-1 signaling pathway and the inhibition of the NF-κB signaling pathway (Fig. 8C). In addition, the intraperitoneal injection of CAPE ameliorated the degradation of cartilage matrix *in vivo*. Therefore, CAPE may have potential for use in the treatment of 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

QY and WeiS conceived and designed the experiments. YC, DH, LJ and QH performed the experiments. WeiS, JY and WX conducted the literature search and the processing of the figures. JY, WX, JX, QY and WeichaoS analyzed the data and wrote the manuscript. QY and WeichaoS confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All individuals provided informed consent for the use of human specimens in clinical experiments. The present study was approved by the Ethics Committees of Shenzhen Second People's Hospital (approval no. ethic NO.:20211215005-FS01). The animal protocols and experimental procedures were in agreement with and were approved by the Animal Care and Use Committee of Southwest Medical University (approval no. 20211124-043).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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