Ergosterol limits osteoarthritis development and progression through activation of Nrf2 signaling

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Abstract. Osteoarthritis (OA) is a common joint disorder characterized by progressive articular cartilage degeneration and destruction and results in gradual disability among middle-aged and elderly patients. Our previous study demonstrated that depletion of nuclear factor erythroid 2-related factor 2 (Nrf2) exacerbated cartilage erosion in an OA model and that activation of the Nrf2 pathway could counter this process. As a downstream target of Nrf2, heme oxygenase (HO) degrades heme to free iron, biliverdin and carbon monoxide (CO), which protects against oxidative stress. Ergosterol (ER), which is extracted from fungi, is a newly discovered Nrf2 activator and displayed efficacy against myocardial injury. The present study aimed to investigate the potential protective effects of ER against cartilage damage during OA. Primary mouse chondrocytes were treated with ER for *in vitro* assays. Furthermore, mice that underwent destabilization of the medial meniscus surgery were orally administered with ER. Western blotting suggested that ER increased protein expression of Nrf2 and HO-1 in primary chondrocytes and articular cartilage from knee joints. Cartilage damage in knee joints was significantly reduced by ER treatment. Western blotting and PCR analysis confirmed that ER could also suppress the expression of MMP-9 and MMP-13 *in vivo* and *in vitro*. The present findings suggested that ER effectively alleviated cartilage degradation and that activation of the Nrf2-heme oxygenase 1 pathway may play a role in ER-mediated cartilage protection against OA.

Introduction

Osteoarthritis (OA) is an aging-associated progressive joint disease characterized by cartilage loss and damage (1,2). However, to date, there is no effective and safe medication available against OA. Most patients with advanced OA require total joint replacement (3). Recently, it was hypothesized that oxidative stress is closely associated with the progression of cartilage degeneration during OA, and promotion of endogenous antioxidant activity was demonstrated to confer protection against OA (4-8). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, which binds to anti-oxidant response elements (AREs) to regulate numerous phase II antioxidant enzymes, including heme oxygenase 1 (HO-1) (9). Our previous study showed that Nrf2 deficiency aggravated the damage of cartilage in an inflammatory model and a post-traumatic model of OA (10). Therefore, the present study aimed to investigate potential drugs targeting Nrf2 activation. Ergosterol (ER), which is extracted from the fungus *Agaricus campestris*, exhibits a wide range of pharmacological properties, including anti-inflammatory and anti-oxidative effects (11,12). Xu *et al* (13) reported that ER increased the expression of Nrf2 and HO-1 in rat hearts and exerted a cardioprotective effect in a lipopolysaccharide-induced sepsis model. However, to the best of our knowledge, the role of ER in OA remains unclear. The aim of the present study was to investigate the chondroprotective effects of ER in a destabilization of the medial meniscus (DMM) surgery-induced OA model and elucidate the underlying mechanisms.

Materials and methods

Chemicals and reagents. ER (cat. no. 45480; purity, 95%) was supplied by Sigma-Aldrich; Merck KGaA. Anti-Nrf2 (cat. no. 12721; 1:1,000), anti-lamin B (cat. no. 13435; 1:1,000) and anti-GAPDH (cat. no. 5174; 1:5,000) antibodies were purchased from Cell Signaling Technology, Inc.. Anti-HO-1 (cat. no. BS6626; 1:1,000), anti-MMP1 (cat. no. BS62563; 1:1,000), anti-MMP3 (cat. no. BS90872; 1:1,000), anti-MMP9

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(cat. no. BS6893; 1:1,000), anti-MMP13 (cat. no. BS6668; 1:1,000), anti-ADAMTS5 (cat. no. BS74041; 1:2,000) and secondary HRP-conjugated goat anti-rabbit (cat. no. BS13278; 1:5,000) antibodies were purchased from Bioworld Technology, Inc.. A nuclear and cytoplasmic protein extraction kit (cat. no. P0028) was obtained from Beyotime Institute of Biotechnology.

Cell culture. Primary mouse chondrocytes were collected from the costal cartilage of 14 male neonatal C57BL/6 mice (age, 6 days; weight, 3.08±0.85 g) obtained from the Comparative Medical Center of Yangzhou University (Yangzhou, China) and housed in specific-pathogen-free conditions under a 12 h light-dark cycle at 25±2°C with 45-50% humidity with food and water available *ad libitum*. Murine cartilage was collected and collagen II immunofluorescence staining was used for identification of chondrocytes, as previously described (14). Following digestion with collagenase D overnight at 37°C, harvested chondrocytes were seeded on a 10-cm dish and incubated in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin with 5% CO_2 at 37°C. To avoid loss of the chondrocyte phenotype with successive passages, cells at 80% confluence were detached and plated in six-well plates at a density of $3x10^5$ cells/well (2.4 cm in diameter) for further assays. Primary mouse chondrocytes pre-treated with IL-1β (10 ng/ml; as previously described) (15,16) for 12 h were cultured in various concentrations (0, 5, 10 or 20 μM) of ER for 24 h at 37°C followed by protein or RNA extraction.

Cell viability assays. A Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability following ER treatment, as detailed in our previous study (14). Briefly, primary chondrocytes were seeded in a 96-well plate at a density of $5x10^3$ cells/well. Following incubation with different concentrations (0, 2, 5, 10, 20 or 40 μM) of ER at 37°C for 24 h, cells were incubated with CCK-8 solution (Bioworld Technology, Inc; 10 μl/well) for 1 h in the dark at room temperature. The absorbance of each well was subsequently measured using a microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 450 nm according to the manufacturer's protocols.

Protein expression analysis. Male mice were kept at 24°C with a 12-h light/dark cycle and food and water access *ad libitum*. Mice were monitored for health and weight every 2 or 3 days. In total 68 knees from 34 mice were used for protein expression analysis. OA was induced by sectioning of the medial meniscotibial ligament, also known DMM surgery. The pre-surgery grouping was separate from the surgery grouping. For pre-surgery, two groups of mice (n=8 per group) that had undergone no sham or DMM surgery were administered saline (vehicle) or ER (25 mg/kg/day) dissolved in saline (0.1 ml/10 g) every day by oral gavage for 2 weeks, and 16 knee joints were harvested for WB analysis to confirm whether ER could increase expression of Nrf2/HO-1 in cartilage. For surgery groups, two groups of mice (n=9 per group) that had undergone DMM surgery on the right knee and sham surgery on the left knee were administered the aforementioned doses of vehicle or ER for 2 weeks. At the eighth week after

surgery, atotal of 36 knees were harvested and formed the four groups (sham surgery + vehicle, sham surgery + ER, DMM surgery + vehicle and DMM surgery + ER). The knees were prepared as described previously (17). Briefly, cartilage tissue obtained from knee joints was harvested using a scalpel blade with a surgical microscope and stored in liquid nitrogen. To obtain a suitable amount of protein, sample pooling was performed. The cartilage collected from each individual knee (including a femur and tibia) was treated as one compartment. Each experimental unit of mouse samples was pooled from three compartments from different mice. When pooling was performed, the experimental unit was regarded as one sample. For animal tissues, the cartilage tissue was extracted in PBS containing 1% Triton X-100, 0.1% SDS, 20 nM sodium orthovanadate, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 5 mM ethylenediaminetetraacetic acid. The homogenates were centrifuged at 12,000 x g for 30 min at 4°C and the protein in the supernatant was used for further study. When analyzing fractionated protein from cultured cells the cytoplasmic and nuclear fractions were separated using the aforementioned nuclear and cytoplasmic protein extraction kit according to the manufacturer's instruction. Briefly, the cultured chondrocytes were washed twice with pre-cooled PBS and harvested by centrifugation at 1,000 x g for 5 min at 4°C, and the cell pellet was lysed in a cytoplasmic extraction reagent. After incubation for 30 min on ice, the homogenate was centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was removed. The nuclear extraction reagent was subsequently added to the precipitate. After incubation for 30 min on ice, nuclear protein fraction was harvested in the supernatant after centrifugation at 10,000 x g for 10 min at 4°C. In a separate total protein extraction, cultured chondrocytes were lysed with pre-cooled RIPA lysis buffer (Sigma-Aldrich; Merck KGaA cat. no. R0278) containing protease and phosphatase inhibitors. The protein concentration of the lysates was measured with a bicinchoninic acid protein quantitation kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (5 μg of protein per lane for tissues and 10 μg of protein per lane for cells) were loaded on SDS-PAGE (10% gel) and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h and then incubated with the aforementioned primary antibodies for 18 h at 4°C. Following three washes and probing with the aforementioned secondary HRP-conjugated goat anti-rabbit antibodies for 2 h at 4°C, membranes were visualized using an Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc. cat. no. 32209) and quantified using ImageJ software (version 1.51; National Institutes of Health).

Gene transcript analysis. In total 24 knees were harvested from 12 mice for gene transcript analysis. Pooling was performed to obtain a suitable amount of cartilage and each experimental unit was a pool of two compartments. The cartilage collected from each individual knee (including a femur and tibia) was treated as one compartment. Total RNA from cartilage in knee joints of mice was isolated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First strand cDNA was synthesized from total RNA using the PrimeScript RT Reagent kit (Promega Corporation) according

Table I. Gene-specific primer sequences used for the quantitative PCR.

Target gene	Primer sequence (5'-3')
MMP-9	Forward: TGGCTTTTGTGACAGGCACTTC
	Reverse: CGGTGGTGTTCTCCAATGTAAGAG
MMP-13	Forward: ATGCATTCAGCTATCCTGGCCA
	Reverse: AAGATTGCATTTCTCGGAGCCTG
ACTB	Forward: TGACGGGGTCACCCACACTGTGCCCATCTA
	Reverse: CTAGAAGCATTTGCGGTGGACGATGGAGGG

to the manufacturer's protocols. mRNA expression of MMP-9 and MMP-13 was measured on a 7500 Real-Rime PCR system with SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.). ACTB was used as a reference gene (18). Gene-specific primer sequences used in the present study are listed in Table I. The expression levels of genes were calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Luciferase assays. The HO-1 promoter was amplified by PCR from RAW cell genome DNA and the product was then inserted into a pGL3 vector (Promega Corporation) at the *Hin*dIII and *Bgl*II sites (20). DNA from RAW cells was extracted using the TIANamp Genomic DNA Kit (cat. no. DP304; Tiangen Biotech Co., Ltd.) and amplified with AmpliTaq Gold™ DNA Polymerase (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The thermocycling conditions were as follows: 2 min at 94˚C; 30 cycles of 30 sec at 94˚C, 30 sec at 56˚C and 30 sec at 72˚C, and a final 5 min at 72˚C. All the constructs were subjected to 4% agarose gel electrophoresis in an ABI Prism 377 DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). A nonspecific oligonucleotide was used to construct a control plasmid. The primer sequences for HO-1 were as follows: Forward, 5'-GGAAGA TCTCTGCAGAGCCCCACTGGA G-3' and reverse, 5'-CCC AAGCTTGGAACAGCAACGCTGT-3'. All the constructs were confirmed by sequencing. 293 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were transfected with the HO-1-ARE-promoter-driven luciferase plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following transfection for 24 h, the cells ($5x10^3$ cells/well in a 96 well plate) were treated with different concentrations of ER (0, 10 or 20 μM) for 24 h at 37˚C. The Dual-luciferase reporter assay system (Promega Corporation) was used to measure luciferase activity by normalizing firefly luciferase activity to *Renilla* luciferase activity.

OA model and histological analysis. A total of 86 C57BL/6 male mice (age, 10 weeks; weight, 24.78±4.05 g) purchased from the Comparative Medical Center of Yangzhou University (Yangzhou, China) were kept at 24˚C in standard mouse cages (5 animals per cage) with a 12-h light/dark cycle and food and water access *ad libitum*. Mice were monitored for health and weight every 2 or 3 days. When pain or distress were observed, the animals were treated with buprenex (0.1-2.0 mg/kg; Reckitt & Colman Pharmaceuticals, Inc.), which was added to crushed or wet food. If pain or distress continued, the mice were sacrificed regardless of the scheduled endpoints. The criteria that determined discomfort/distress/pain were any three of the following signs: Abnormal posture, slow, careful or abnormal (waddling) gait, low activity levels, slow eating, cowering or vocalizing on handling, change in eye or coat appearance and weight loss. Animal death following sacrifice was confirmed by one of the following criteria: No response to tail or toe pinch, no respiration or heartbeat following continuous monitoring for 30 sec or rigor mortis. Mice were euthanized using 100% CO_2 anesthesia using an air displacement rate of 20% of the chamber volume/min. Experiment duration was 8 weeks. The animal research was performed in accordance with Nanjing Medical University Institutional Animal Care and Use Committee guidelines (approval no. IACUC 1903044). The mice were anesthetized with intraperitoneal ketamine hydrochloride (120 mg/kg) and xylazine hydrochloride (5 mg/kg). OA was induced by sectioning of the medial meniscotibial ligament, also known as the coronary ligament, which anchors the medial meniscus (MM) to the tibial plateau (21). Forty mice were divided into four groups with 10 mice per group (sham surgery + vehicle, sham surgery + ER, DMM surgery + vehicle and DMM surgery + ER). The sham surgery (ligament was exposed but not transected) was performed on the left knee of the same mice that underwent DMM on the right knee. Mice were administered with saline as vehicle or ER (25 mg/kg/day) dissolved in saline by oral gavage for 2 weeks immediately after surgery. Mice were sacrificed at 8 weeks post-DMM surgery. Knee joints were dissected free of skin or excess muscle and fixed with 10% buffered formalin for 24 h at 22˚C. Obtained sections (5 μm) 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. Sectioned murine joint tissues were observed under an Olympus BX51 light microscope and photographed by a computer-operated Olympus DP72 digital camera (Olympus Corporation). Sections of knee joints (10 slides per joint) were evaluated by an assessor experienced in this technique and blinded to the origin of the sample using the Osteoarthritis Research Society International scoring system (0-6 subjective scoring system) where the higher the score, the more severe the joint degeneration (22).

Statistical analysis. All data are presented as the mean ± SEM. All assays were repeated at least three times independently. Statistical analysis was performed using Mann-Whitney
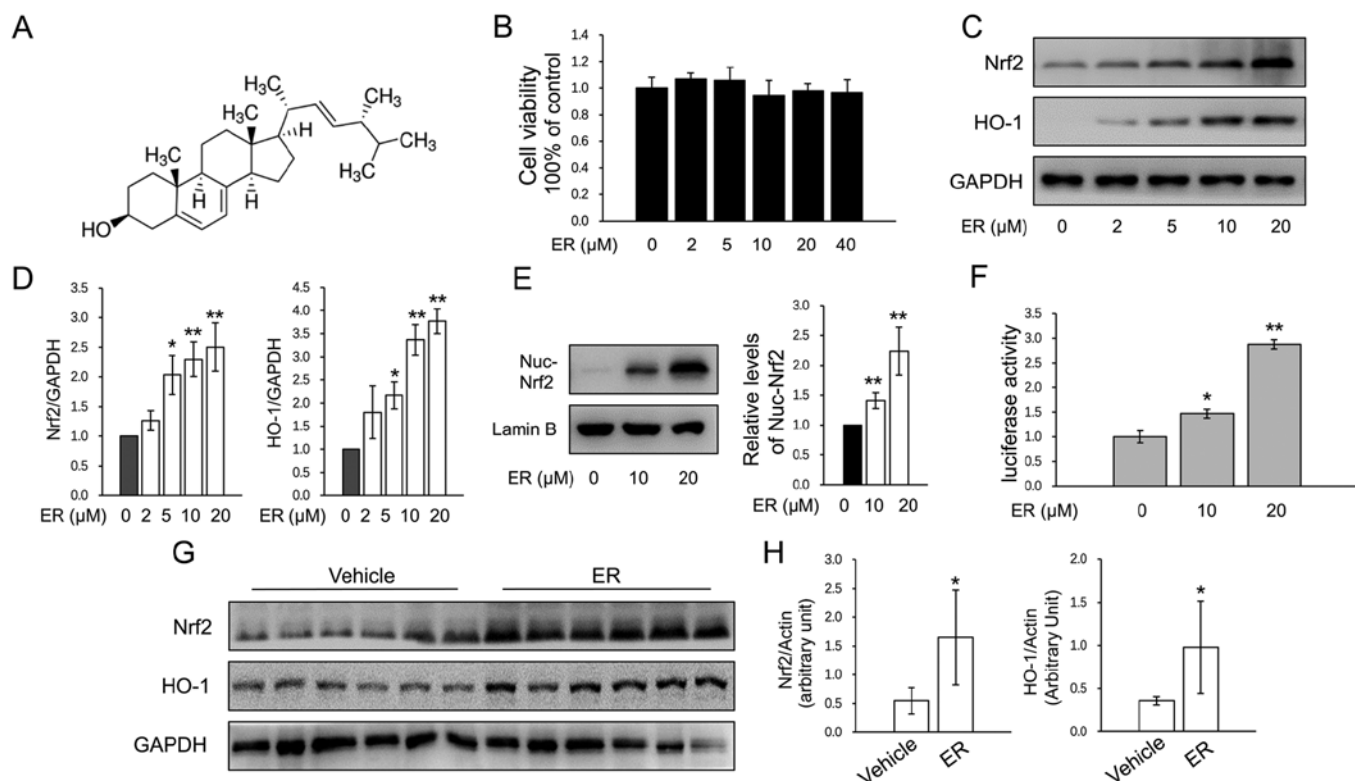
Figure 1. ER activates the Nrf2 signaling pathway *in vitro* and *in vivo*. (A) Structure of ER. (B) Cell viability assay. (C) Nrf2 and HO-1 expression levels were assessed using western blot analysis. (D) Quantitative analysis of Nrf2 and HO-1 protein levels. *$P<0.05$ and **$P<0.01$ vs. untreated control. (E) Cell nuclear fractions were isolated from primary murine chondrocytes for western blot analysis. **$P<0.01$ vs. untreated control. (F) Luciferase assay using 293 cells transfected with a Renilla luciferase plasmid and an HO-1 promoter reporter plasmid, followed by treatment with increasing doses of ER for 24 h. *$P<0.05$ and *$P<0.01$ vs. plasmid control. (G) Western blot analysis of Nrf2 and HO-1 expression from the cartilage of knee joints from vehicle- and ER-treated groups. (H) Quantitative analysis of Nrf2 and HO-1 protein levels. **$P<0.05$ vs. vehicle-treated group. ER, ergosterol; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; vehicle, saline.

U test or one-way ANOVA followed by Tukey's test using GraphPad Prism software (version 6.01; GraphPad Software, Inc.). Datasets containing a mixture of paired and unpaired samples were analyzed using mixed ANOVA followed by Bonferroni correction using SPSS 22.0 (SPSS, IBM Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

ER activates the Nrf2 pathway in chondrocytes and cartilage. The chemical structure of ER is presented in Fig. 1A. The cytotoxicity of ER was measured by CCK-8 assay, and concentrations <40 μM were used in primary murine chondrocytes (Fig. 1B). ER significantly increased the protein expression of Nrf2 and HO-1 in chondrocytes in a dose-dependent manner (Fig. 1C and D). Nuclear protein was extracted for assays and the results showed that ER upregulated nuclear Nrf2 expression (Fig. 1E). ER treatment caused a significant increase in luciferase activity (Fig. 1F), which indicated that ER activated the HO-1 promoter transactivation activity. To investigate the effects of ER on Nrf2 and HO-1 expression in the cartilage of knee joints, mice that had undergone no treatment were administered ER (25 mg/kg/day) for 2 weeks and cartilage samples were harvested for western blot analysis. The results revealed that the expression levels of both of Nrf2 and HO-1 were significantly higher in the cartilage of the ER-treated

group compared with the expression levels in the saline-treated group (Fig. 1G and H).

ER inhibits the expression of MMPs in chondrocytes. MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) play a role in cartilage destruction during OA. Both MMP-9 and MMP-13 are important proteolytic enzymes in ECM breakdown, and abnormal up-regulation of these enzymes can induce excess catabolism in cartilage, gradually leading to cartilage breaking down (23,24). To investigate the potential therapeutic effects of ER on OA, the protein expression of matrix-degrading enzymes were further examined. As predicted, IL-1β increased the protein expression levels of MMP-1, MMP-3, MMP-9, MMP-13 and ADAMTS-5 in the cells (Fig. 2). Although IL-1β induced expression of MMP-1 and MMP-3 were not regulated by ER (Fig. 2A-C), significant inhibition of matrix-degrading enzymes MMP-9 and MMP-13 was observed at the protein level (Fig. 2D and E). ADAMTS-5 expression also did not appear to be regulated by ER (Fig. 2F). Considering their roles in the cartilage degradation network (25,26), it was hypothesized that ER may suppress MMP-9 and MMP-13 expression in chondrocytes and reduce cartilage breakdown.

ER alleviates cartilage erosion in experimental OA. To determine the protective effects of ER against OA, a murine model of OA was induced by transecting the medial meniscotibial
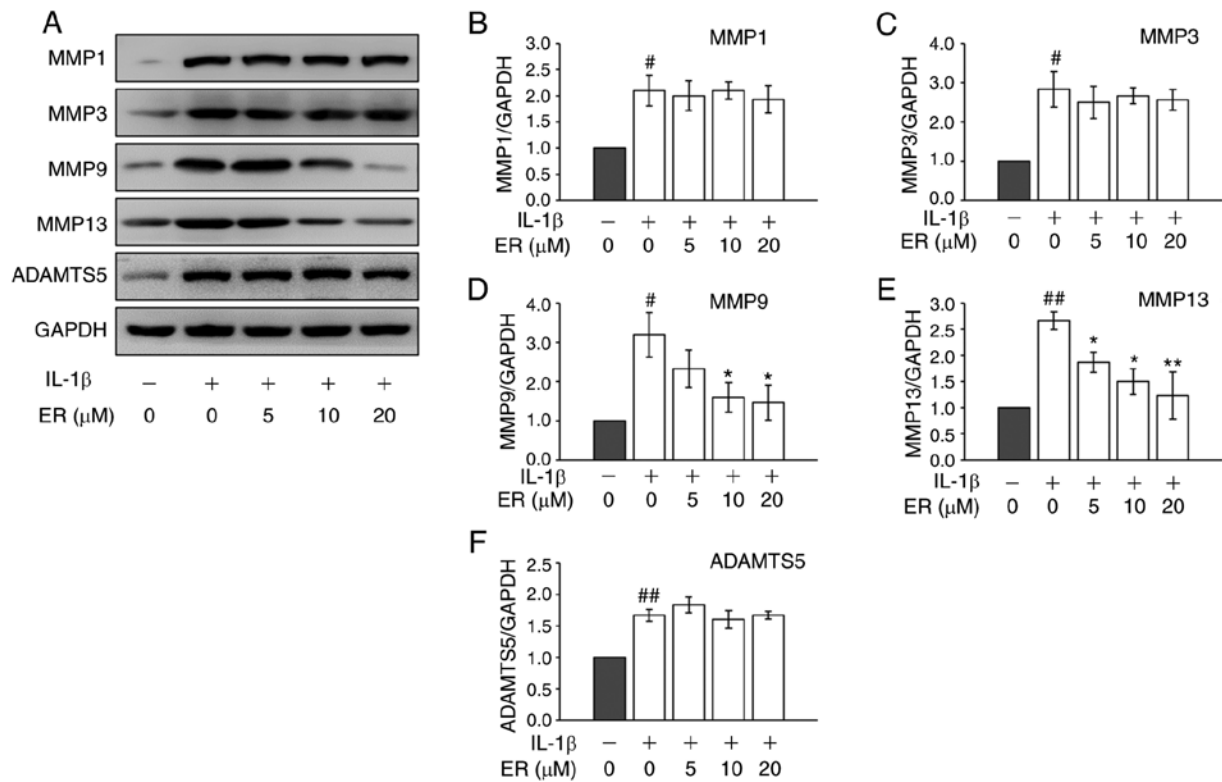
Figure 2. Effects of ER on IL-1β-induced MMPs and ADAMTS-5 expression. (A) Protein expression levels of MMPs and ADAMTSs were determined by western blot analysis. Quantitative analysis of (B) MMP1, (C) MMP3, (D) MMP9, (E) MMP13 and (F) ADAMTS5 levels. Bands are representative of three separate experiments. [#]P<0.05 and [##]P<0.01 vs. untreated control. [*]P<0.05 and [**]P<0.01 vs. IL-1β treatment alone. ER, ergosterol; ADAMTS, and a disintegrin and metalloproteinase with thrombospondin motifs.
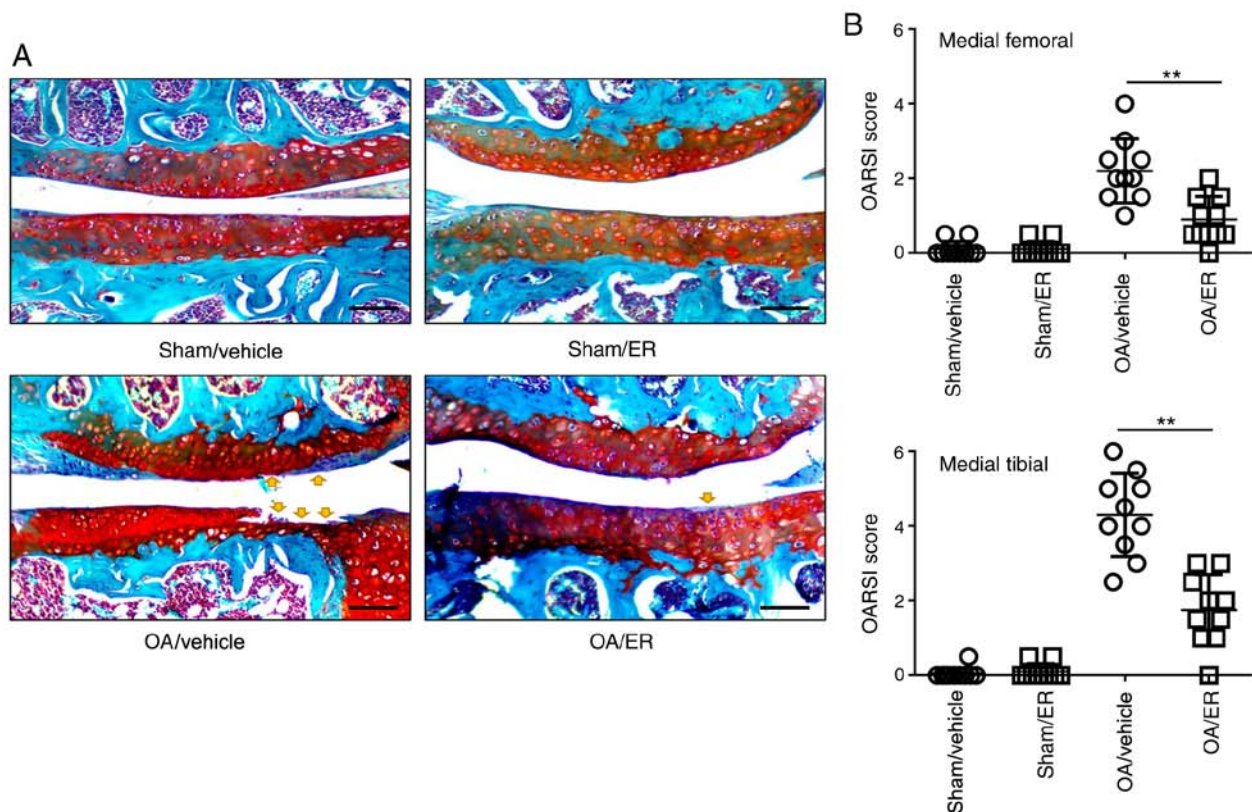

Figure 3. ER protects against OA. The right knees of mice underwent destabilization of the medial meniscus surgery and the left knees underwent sham surgery. Subsequently, mice were administered with ER or vehicle for 2 weeks after surgery. n=10/group. (A) Representative sections of knee joints from experimental mice stained with Safranin O/fast green. Yellow arrows indicate destruction-affected cartilage areas. (B) Histological scoring of OA. The scores were for femoral and tibial samples on the y-axes of representative graphs. [**]P<0.01 as indicated. ER, ergosterol; OA, osteoarthritis; vehicle, saline.
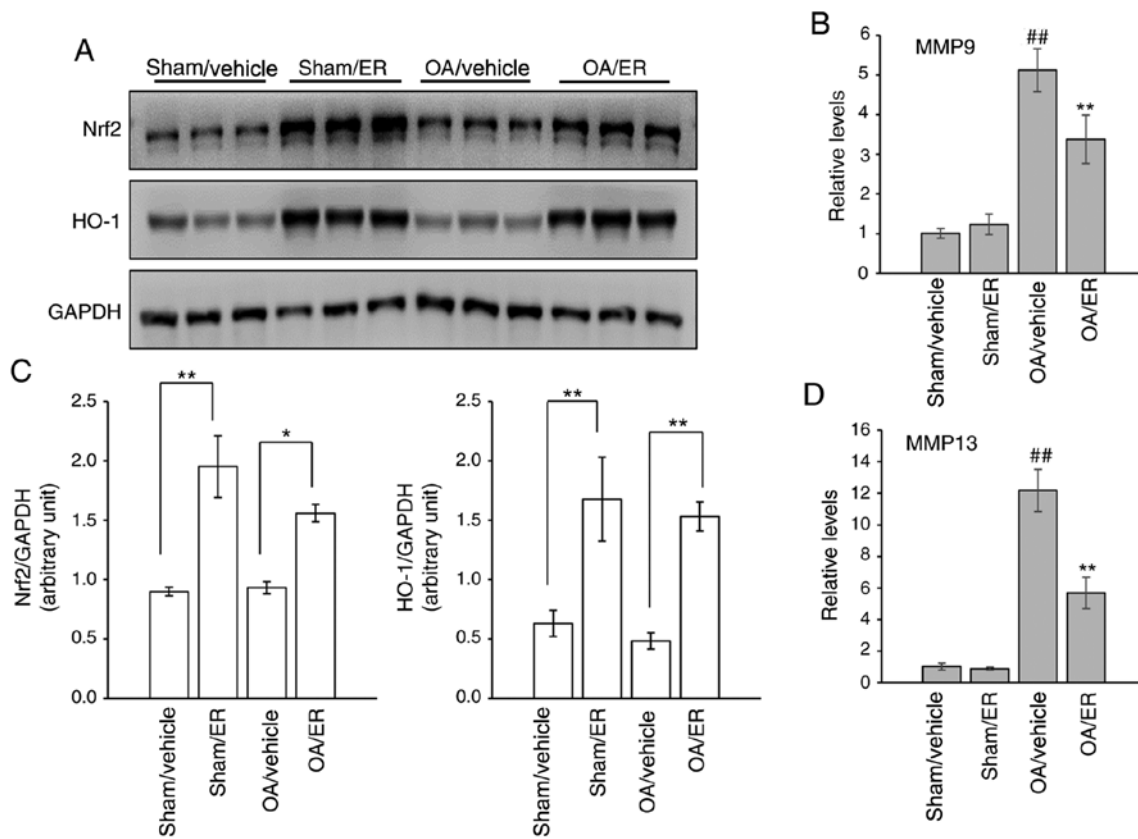
Figure 4. Effects of ER on the protein expression levels of Nrf2, HO-1, and the mRNA expression levels of MMP-9 and MMP-13 in experimental OA. (A) Western blot analysis of Nrf2 and HO-1 expression in the cartilage from murine knee joints. (B) mRNA expression of MMP-9. ##$P<0.01$ vs. the sham/vehicle group. **$P<0.05$ vs. OA/vehicle group. (C) Quantitative analysis of Nrf2 and HO-1 protein levels. *$P<0.05$ and **$P<0.01$ as indicated. (D) mRNA expression of MMP-13. ##$P<0.01$ vs. the sham/vehicle group. **$P<0.01$ vs. OA/vehicle group. ER, ergosterol; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; DMM, destabilization of the medial meniscus; OA, osteoarthritis.

ligament. ER (25 mg/kg/day) or vehicle was administered for 2 weeks post-operation. Mice were sacrificed at 8 weeks post-DMM surgery. The knee joint tissues were collected for Safranin O/Fast Green staining. Histological sections were assessed using OARSI scores in a blinded manner. The data showed that the ER-treated group significantly improved femur and tibia proteoglycan loss or cartilage damage compared with the vehicle-treated group at 8 weeks post-DMM surgery. Knee cartilage harvested from mice administrated with ER or vehicle that underwent sham surgery showed no damage, indicating the ER administration had no effect on an undamaged knee. These results suggested that that oral administration of ER in mice effectively delayed the progression of OA (Fig. 3A and B).

ER promotes expression of Nrf2/HO-1 and suppresses gene expression of MMPs in experimental OA. The expression levels of Nrf2 and HO-1 in the cartilage of knee joints were measured by western blot analysis to determine whether activation of the Nrf2 signaling pathway in response to ER was similar to that observed *in vitro*. The results showed that protein expression levels of Nrf2 and HO-1 were increased in the cartilage of mice administrated orally with ER compared with the respective vehicle-treated groups (Fig. 4A and C). The effect of ER on the expression of MMP-9 and MMP-13 in articular cartilage was further assessed. Gene transcript analysis results showed that the mRNA expression levels of

MMP-9 and MMP-13 were increased in the knee cartilage from the OA/vehicle group compared with the sham/vehicle group, and this upregulation was suppressed by ER (Fig. 4B and D), similar to the aforementioned *in vitro* assay results.

Discussion

Excessive oxidative stress is associated with OA and triggers chondrocyte senescence and apoptosis, extracellular matrix (ECM) degradation, dysfunction of the subchondral bone and synovial inflammation during OA (27). The transcription factor Nrf2 regulates the expression of a set of genes that counteract oxidative stress. Under physiological conditions, Nrf2 is tethered in the cytoplasm by its inhibitor, Kelch-ECH associated protein 1 (KEAP1), which controls its proteasomal degradation. During increased oxidative or electrophilic stress, Nrf2 is released from KEAP1 and translocates to the nucleus, binding to AREs located in the promoter regions to activate its target genes, including HO-1, which is an important defense against reactive oxygen species-mediated damage in various tissue injuries (28). A previous study revealed that HO-1 expression markedly decreased in the articular cartilage of wild-type mice with age, and that maintenance of HO-1 expression had the potential to protect against OA development (29). The present study confirmed that ER enhanced the nuclear translocation of Nrf2, promoted HO-1 promoter transactivation and upregulated the expression of genes downstream of Nrf2,

indicating that ER activated the Nrf2 signaling pathway, which contributed to preventing cartilage degeneration.

Common medication for OA aims to relieve patient's joint pain of to improve quality of life (30,31). Trichostatin A, a histone deacetylase inhibitor, was used to activate Nrf2 signaling pathways, subsequently leading to a significantly decreased severity of cartilage damage in DMM surgery-induced OA mice in our previous study (10). While disease-modifying OA drugs (DMOAD) require long-term use, the search for potential drugs targeting Nrf2 activation continues, considering the side effects caused by trichostatin A (32). In the present study, ergosterol significantly activated the Nrf2 pathway in primary chondrocytes. The present study further demonstrated that oral administration of ER in mice significantly increased the expression of Nrf2 and HO-1 in murine knee cartilage, exerting protective effects on the cartilage during OA. Additionally, the oral administration of ER may make it more suitable for clinical use as a potential DMOAD in comparison with injectable therapeutic agents.

When measuring the expression of ECM-degrading proteases, the present study found that ER significantly inhibited the expression levels of MMP9 and MMP13 in chondrocytes and cartilage, which were enhanced during OA and play an important role in articular cartilage damage. Previous studies reported that upregulation of Nrf2 downstream proteins such as HO-1 can reduce the expression of MMPs and inhibit the production of proinflammatory cytokines (29,33-35). Hence, the inhibition of MMP9 and MMP13 may be partially attributed to ER-induced Nrf2 signaling activation.

Several limitations of the present study should be noted. First, the mice used in the experiments were relatively young (~10 weeks old) with immature skeletons. Therefore, the possibility that other late developmental events might affect the effectiveness of ER in the OA model could not be excluded. Second, since ER was only administered at the same time as OA onset in the experiment, it could not be determined whether ER was protective in pre-arthritic knees. A middle-stage OA model should be used in future investigations to fully elucidate the preventive effect of ER. Although to the best of our knowledge there are no direct links between ER and Nrf2 expression in the existing literature, with further research into the physiological effect of ER, more cross-pathways may be found to help understand the potential regulation of Nrf2 protein expression by ER. The present study made an assumption that ER may alter the expression of Nrf2 gene through epigenetics, such as microRNA (miRNA/miR). Previous studies have shown that miR-144, miR-28, miR-93 and other miRNAs can regulate Nrf2 gene expression (36-38), while miR-125a, miR-378 and other miRNAs have been shown to be regulated by ER (39-41). A possibility that ER mediated the expression of Nrf2 through certain miRNA cannot be ruled out in the present study.

In conclusion, the present study found that ER served a regulatory role in anti-oxidative damage and reduction of catabolism in cartilage tissues, suggesting that ER could be considered a promising effective option for the treatment of OA.

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DC, XW and JQ conceived and designed the study. DC, HY, JL, SC and LJ performed the experiments. DC and HY wrote the paper. JQ and XW reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments performed with the use of animals were approved by the Animal Ethical and Welfare Committee of Nanjing Medical University Institutional Animal Care and Use Committee (approval no. IACUC 1903044).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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