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Original Article

Xanthohumol suppresses ECM degradation in osteoarthritis through the Nrf2/PERK/ATF4/C/EBPβ signaling pathway

Tiansheng Zheng[1,2], Shuo Niu[2], Qingluo Zhou[2], Jishang Huang[2], Jun Yi[2], Runhong Mei[1,3], Xuefeng Yu[1*]

[1] Department of Orthopaedics, the Fourth Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang 330000, Jiangxi, China
[2] Department of Orthopaedics, the First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, Ganzhou, China
[3] Department of Oral and Maxillofacial Surgery, the First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, 330000, Jiangxi, China

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Abstract

In this study, the therapeutic efficacy of Xanthohumol (XH) was evaluated as a preventive agent for extracellular matrix (ECM) degradation in osteoarthritis, using the in vivo monosodium iodoacetate-induced arthritis model in rats, along with the in vitro model of interleukin (IL)-1β-stimulated C28/I2 chondrocytes. With established concentrations of XH, the extent to which this compound may modulate cartilaginous architecture, enzymatic activity, or ECM synthesis was determined. The results clearly show that, in comparison with controls, this drug significantly reduced the catabolism of ECM, exerting a concentration-dependent effect that reduced the production of MMP13 (by 46% in vitro) with enhanced transcriptional production of collagen II (by 38% in vitro) versus controls, as well as exhibiting a cartilage degradation reduction of 31% compared with controls. The results, based on downstream messenger studies, show that this drug reduced transcriptional activation of ER-stress-driven catabolism associated with the PERK/ATF4/C/CEBPβ pathway, with a subsequent, noteworthy increase (by 52%) in the transcriptional activity of Nrf2. Over-expression or activation studies reduced the chondroprotective effects, entirely eliminating the silencing studies of Nrf2. These results clearly indicate that this drug, with its chondroprotective effects, suppresses catabolism, stimulates cartilaginous reinstatement, and reversibly decreases OARSI scores in treated animals. This study indicates that this drug may offer a promising therapeutic modality in modulating ER-stress-driven catabolysis as its pathomechanistic principle in targeted use for osteoarthritis prevention.

Keywords: Musculoskeletal, Natural compounds, Molecular mechanism, ER stress, Oxidative stress , Xanthohumol

1. Introduction

Osteoarthritis (OA) is a progressive or chronic medical condition that involves the progressive loss of cartilage, synovial inflammation, and subchondral bone remodeling. It mainly affects older people [1]. Chondrocytes, key to maintaining the extracellular matrix, play a central role in the development of OA because they produce the main components of the extracellular matrix (ECM), such as type II collagen and proteoglycans. Abnormalities of chondrocytes lead to reduced secretion of ECM and enhanced expression of enzymes responsible for its decomposition, essentially exaggerating inflammatory events, triggering chondrocyte apoptosis, or programmed cell death [2]. OA development involves the decomposition of the ECM through a group of enzymes called matrix metalloproteinases, especially MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13. In addition, inflammatory mediators, for example, TNF-alpha and IL-1 beta, play a key role in triggering rheumatological diseases such as OA. In fact, since OA is a chronic medical condition, to date, there is neither a drug that can halt nor a medicine that can provide a cure for this medical condition. Prevention of inflammatory events is, therefore, a potent therapy [3].

C/EBPβ is a BZIP class transcription factor acting as an important regulator of proliferation, differentiation, and apoptosis, and is known for its overexpression in chondrocytes, causing the development and progression of osteoarthritis [4]. In OA, the process of inflammation and endoplasmic reticulum (ER) stress leads to dysfunctional endoplasmic reticulum, further progressing towards the aggregation of misfolded proteins and the activation of the unfolded protein response (UPR). UPR is known to be a stress-protected mechanism that maintains endoplasmic reticulum homeostasis. It reduces protein synthesis and increases the expression of molecular chaperones. Simultaneously, mitochondrial unfolded protein response (UPRmt) regulates mitochondrial proteostasis and protects cells and organisms against stress. UPRmt is known to be activated in chondrocytes and articular cartilage of OA in mice and human models. Pharmacologically enhan-

* Corresponding author.
E-mail address: yuxuefeng_1958@163.com (X. Yu).
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cing nicotinamide riboside activates the process of UPRmt and improves mitochondrial function and reduces the progression of OA. In addition, higher expression of UPRmt is known to be correlated with lower severity of symptoms in patients suffering from OA, thus making it an important drug target for the treatment of OA [5].

Sensors of endoplasmic reticulum stress, which include protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6), are vital transmembrane receptors that mediate and regulate the unfolded protein response [6]. Stimulation of the PERK/eIF2α/ATF4/CHOP signaling cascade supports the induction of pro-apoptotic pathways in cells that undergo ER stress [7]. C/EBPβ is known to be the downstream mediator of the PERK/eIF2α/ATF4 signaling cascade; downregulation of C/EBPβ expression via knockdown experiments reduces PCD and ER stress in cells treated with oxidized low-density lipoprotein (ox-LDL), especially in RAW264.7 cells [8]. However, recent findings propose that the steady and progressive build-up of ER stress sensor proteins such as PERK, IRE1α, and ATF6, and pro-apoptotic factors such as CHOP, exists in osteoarthritis cartilage. It suggests that the induction of ER stress-mediated UPR signaling is closely implicated in chondrocyte apoptosis and ECM disturbances in OA [9]. Nonetheless, the specific role of ER stress-related responses in the induction of ECM damage in OA remains obscure.

Osteoarthritis can be adequately controlled in the presence of first-line treatments that include nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, central analgesics, and chondroitin. Nonetheless, such treatments are associated with considerable side effects, including those in the gastrointestinal and cardiovascular systems. By contrast, natural products (NPs) are advantageous given their lower rates of side effects, lower likelihood of drug tolerance and dependence, and potential use as alternatives for central analgesics. In addition, natural products are superior to glucosamine chondroitin in offering symptomatic relief for osteoarthritis affecting the knee joint and other areas. However, the use of natural products in osteoarthritis treatment is accompanied by several drawbacks that include complicated chemistry, poor bioavailability, and unclear modes of action. Thus, further investigations are required for understanding the mechanism of action of natural products in the treatment of osteoarthritis [10].

Osteoarthritis has been described as an inflammatory, traumatic, and degenerative joint disease for which, as of yet, there is no cure. There is an imbalance between anabolic and catabolic activities in cartilage tissue due to chronic inflammation and oxidative stress, which is recognized as the central mediator of OA [11]. Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and nitric oxide, generated due to oxidative stress, are known to be cytotoxic and lead to damage of macromolecules like DNA, proteins, and lipids, and thereby catalyze cartilage breakdown [12]. In addition, these species regulate the signaling pathways of cells and control cartilage breakdown and chondrocyte apoptosis. Transcription factor NRF2 is very important in intracellular redox balance and regulates the induction of antioxidant proteins such as Heme Oxygenase-1 in cells. Activation of NRF2 and AREs or antioxidant response genes exists as a promising target for the treatment of OA mediated due to chronic insults to joint tissues. Natural products, especially flavonoids, can activate NRF2 and act as novel potential drug treatments for OS and OA due to scavenging of ROS and halting in progression of OA. Some reports have evidenced that natural products can work as protective agents for OA. Bergamottin, for example, present in grapefruit and pomelo, can halt OA progression mediated due to modulation of Sirtuin 1/NF-κB signaling [13]. In addition, it has been evidenced that Cucurbitasin B, derived from Cucurbitaceae, can halt the progression of OA mediated by to prevention of assembly of the inflammasome. In addition, it has been reported that Tanshinone IIA, derived from Salvia miltiorrhiza, can halt the progression of OA mediated by to prevention of angiogenesis of subchondral bone [14].

Xanthohumol (XH) (Fig. 1) is known as an endogenously existing prenylated flavonoid derived from beer and hops, and has been demonstrated to be a strong potentiator of the estrogenic action of flavonoids [15]. Estrogen deficiency has been implicated in the onset of osteoarthritis (OA) [16]. Menopausal hormone therapy in the treatment of OA can be accompanied by dangerous side effects [17]. Phytoestrogens such as XH have been shown to provide protective action in the prevention of OA development [18]. In earlier work, it has been reported that XH reduces inflammation, inhibits the expression of MMP-13, and prevents ECM degradation in OA chondrocytes (CTs) [19]. Nonetheless, the specific mechanism of action of XH in inhibiting ECM breakdown in OA untreated cells remains unclear. There is current evidence that C/EBPβ stimulates the action of active MMP-13 in OA CTs [20], [21], and that XH can act as an Nrf2 activator [22].

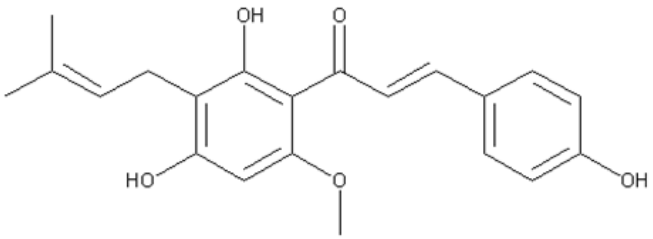

Fig. 1. Chemical structure of XH.

1.1. Literature Review

In relation to this area of interest, review articles focusing on other potential therapeutic targets for the treatment of OA include that of Sheng et al. [23], which discussed the protective role of NRF2 in OA, particularly in relation to its role in regulating antioxidant enzyme expression and in inhibiting the NF-κB signaling pathway. Another review discussed on this topic is that of Hu and Ecker [24], which looked further into the role of MMP-13 in OA and its very important role in cartilage breakdown mediated by the cleavage of type II collagen. In this review, the authors explained that the role of MMP-13 is regulated at the molecular and epigenetic levels and that it remains a promising target for therapy because, at present, no specific treatment has been developed for the repair of cartilage in OA patients. Extending this analysis to the role of MMP-13, Alwan and Ghal [25] analyzed the level of MMP-1, MMP-9, and MMP-13 in 69 RA patients and reported that the level of these enzymes increased significantly compa-

red to controls. In this analysis, it has been noted that sex hormones play no role in the regulation of the expression of MMPs in RA patients, and this makes it evident that the role of MMP-13 is independent of sex parameters.

In the medical field, Wang et al. [26] studied the mechanism of C/EBPβ in the breakdown of cartilage in osteoarthritis and its effect on the expression of aggrecanase ADAMTS5, and how 5,7,3',4'-tetramethoxyflavone (TMF) protects against the breakdown of extracellular matrices (ECM) by activating Sirt1/FOXO3a signaling pathways that regulate C/EBPβ. They concluded that TMF protects and induces the expression of aggrecan and reduces the expression of ADAMTS5 in chondrocytes of OA, making it very useful in protecting against the breakdown of the ECM in OA. Likewise, in OA, Zhang et al. [27] investigated the role of Xanthohumol in the regulation of inflammation and ECM breakdown and reported that XH inhibits the expression of inflammatory factors like TNF-α and IL-6 and reduces the expression of catabolic enzymes like MMP-3 and MMP-13. In addition, the authors reported that HO-1 signaling and C/EBPβ nuclear translocation were increased in the presence of XH, and HO-1 knockdown abolished the protective action of XH. Significant findings indicate that HO-1 signaling and the C/EBPβ nuclear translocation pathway play important roles in OA progression.

Further expanding the potential of XH and in relation to the therapy of OA, Chen et al [28] investigated the effects of XN on this condition, showing that it inhibits important markers of inflammation like iNOS, TNF-α, and COX-2. In addition, it was seen that it increases the expression of type II collagen and aggrecan and reduces the expression of MMP-13. Further, it has been reported that it triggers the Nrf2 signaling pathway and inhibits the NF-κB signaling pathway in chondrocytes. Based on these observations, it can be further suggested that, like XH, it may be another promising candidate for halting the progression of OA. In this regard, Bendele et al. [29] described another candidate known as AQU-019, an allosteric inhibitor of MMP-13, which was engineered for increased potency, plasma stability, and oral bioavailability as a putative disease-modifying drug for the treatment of osteoarthritis. Based on the results obtained in this study and others discussed above, the chondroprotection afforded by AQU-019 in the MIA rat model of OA suggests that this approach may be useful in slowing the progression of this condition, in that current treatments are only focused on alleviating symptoms and do not target the progression of the disease. All of this supports the hypothesis that inhibiting MMP-13 may be an approach worth exploring in the therapy of OA.

1.2. Research Gaps and Novelties

There are several gaps in the current understanding of xanthohumol (XH), which is still a potential treatment for osteoarthritis (OA), that need further investigation. Firstly, despite the protective properties of XH, the exact molecular mechanism underlying ECM inhibition in this case is still unclear. In addition, the role of some signaling pathways, such as PERK/ATF4/C/CEBPβ and Nrf2, in regulating ECM in OA still needs further investigation. Secondly, despite potential properties of natural products, including XH, their poor availability and unclear molecular mechanism of action are some factors that impede their further use in the treatment of OA. In addition, further investigation is required for a better understanding of the interaction between such natural products and OA-related pathways. Long-term in vivo observations are also required for such potential treatments as xanthohumol. In vitro studies are very informative in this case, whereas large-scale in vivo and human observations are required for confirmation of the potential use of XH for treatment of OA in the future.

Despite the above shortcomings, the current study brings in some novel features that can contribute to advancement in this area. The role of XH in relieving endoplasmic reticulum stress and activating Nrf2, the ultimate oxidative stress protective mechanism, in combination, is definitely novel for use in the treatment of OA. The approach of addressing both oxidative stress and endoplasmic stress together, which are core-driving factors in OA, indicates that this novel compound may be very effective in this area. Another novel approach of this compound is its potential in inhibiting the C/EBPβ-induced ECM damage mechanism, which has never been thoroughly discussed in any of the past literature. Another novel aspect of this compound is that it can act as a disease-modifying OA drug (DMOAD). In this case, this compound protects against ECM damage in both in vitro and in vivo models, and this can be another advancement in the use of natural products for OA treatment.

1.3. Paper Organization

This work is divided into three main sections: Materials and Methods, Results, and Discussion, and concludes with Remarks. Materials and Methods describe the experimental designs used in the study, such as animal models and cell cultures, and discuss the use of experimental biochemistry analysis of experimental results in order to allow for the verification of the results and form the basis for drawing conclusions later. Results explain the role of XH in degrading the extracellular matrix and present for the first time the efficiency of in vivo and in vitro modulation of the main signaling pathways like PERK/ATF4/C/EBPβ and Nrf2, in different cells and conditions. Conclusion discusses the experimental results together with the current values in the literature concerning the role of XH in the potential alleviation of both endoplasmic reticulum stress and oxidative stress, and discusses further directions in experimental and scientific efforts.

2. Material and Methods

2.1. General information

This project was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Gannan Medical University (Approval ID: 211506) and carried out in compliance with the Declaration of Helsinki as well as other international principles of laboratory animal care, such as European Economic Community regulation (EEC Directive 86/609/EEC [30]). Male Sprague-Dawley rats, aged 6–8 weeks, were provided by the Laboratory Animal Center of Gannan Medical University, and weighed approximately $220 \pm 20g$, and were used as models. Animals were kept in a temperature-regulated environment $(20 \pm 1°C)$ with controlled humidity $(50\% \pm 5\%)$ in addition to light cycle regulation (12-hour day/night). Free access was permitted for water as well as chow. Cells were cultured under standard cell culture practices. Xanthohumol (XH) was acquired with

high purity (≥98% tested with high-performance liquid chromatography (HPLC) from Solarbio Company with catalog # SX8070 (Beijing, China). However, its concentration was dissolved in both sodium carboxymethylcellulose (SCMC) $(0.5\% v/v)$ as well as DMSO, depending on the experiment.

2.2. Generation of Monosodium Iodoacetate (MIA)-induced rat OA models

Rats were randomly divided into four groups (n = 10 per group): negative control (NC) group, monosodium iodoacetate (MIA)-induced osteoarthritis (OA) group, OA with Xanthohumol (XH) (50 mg/kg), and OA with Xanthohumol (100 mg/kg). Deeply anesthetic conditions were achieved through intraperitoneal injection of 3% (w/v) pentobarbital (45 mg/kg). After that, the skin around the right knee joint was shaved and disinfected with 75% ethanol. Further injection of MIA (2 mg in 10 µL sterile water) was performed in the OA group, whereas the negative control group received its vehicle (10 µL sterile water). Finally, xanthohumol was orally administered once daily (50 mg/kg or 100 mg/kg) by gavage for a total of 40 days, as previously reported [31]. During this stage, none of the animals were terminated. After completion of the experiment, all animals were terminated through spinal dislocation under deep pentobarbital (3% w/v, 45 mg/kg) anesthesia. Finally, the skin area surrounding the right knee joint was once again prepared, after which the joint cartilage (JC) was collected.

2.3. Histological and Immunohistochemical (IHC) evaluation

Cartilage tissue (CT) decalcification was carried out using a 10% ethylenediaminetetraacetic acid (EDTA) solution for one month. After decalcification, the tissue was processed through a series of graded ethanol solutions (70%, 80%, 90%, and 100%) followed by xylene treatment to remove the ethanol. The specimens were then embedded in paraffin wax, and 4 µm-thick sections were cut and mounted on pre-coated polylysine slides. The sections were stained using hematoxylin and eosin (HE) and Safranin O-Fast Green (OFG). Medial joints were scored in a blinded manner using the OA Research Society International (OARSI) grading system, with scores ranging from 0 to 6 (n = 3) [32], [33].

For immunohistochemistry (IHC), the prepared sections were incubated with primary antibodies targeting key proteins: anti-MMP-13 (1:100, cat. no. AF5355, Affinity Biosciences, Cincinnati, OH, USA), anti-collagen II (1:100, cat. no. AF0135, Affinity Biosciences), anti-C/EBPβ (1:100, cat. no. AF6202, Affinity Biosciences), anti-p-PERK (1:100, cat. no. DF7576, Affinity Biosciences), and anti-Nrf2 (1:100, cat. no. AF0639, Affinity Biosciences). Immunoreactive protein bands were visualized using a horseradish peroxidase (HRP) detection system (1:500, cat. no. A0208, Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.4. Cell culture

Human normal cartilage tissue (CT) cell line, C28/I2, was purchased from Procell (Wuhan, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Catalog #12491015, Gibco, Waltham, MA, USA) with addition of 10% fetal bovine serum (FBS) (Catalog #A3161001C, Gibco), as well as addition of 100U/mL penicillin, streptomycin (100U/mL), with standard conditions of temperature (37°C) and atmospheric tension of CO2 (5%). CT cells were treated with human recombinant interleukin-1β (IL-1β) with a final concentration of 10 ng/mL (Catalog #IL038, Sigma, St. Louis, MO, USA). ER stress was generated with tunicamycin (TM) with a final concentration of 1 µM (Catalog #5.04570, Sigma) along with a 24-hour culture temperature of 37°C

2.5. Flow cytometric detection of apoptosis

An Annexin V apoptosis assay (cat. no. C1065L, Beyotime) was used for evaluating the rate of apoptosis in cartilage tissue (CT). An explanation of this protocol involves the use of IL-1β-preleased C28/I2 cells that were cultured with Xanthohumol (Xh) concentrations of 5µM and 20µM for a period of 24 hours [28]. After that, the wells were washed with phosphate-buffered saline (PBS) three times, then re-suspended in Annexin-V binding solution, and further stained with Annexin-V-FITC, as well as propidium iodide (PI) for a further 15 minutes in the dark. Analysis of cell death was performed via flow cytometry with a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA.

2.6. Lentivirus (LV) infection

The plasmid of LV-C/EBPβ and LV-short hairpin RNA (shRNA)-Nrf2 was designed, tracked, and validated by OBiO Corporation (Shanghai, China). The target sequences of three designed shRNAs for the C/EBPβ gene were: 1# ACAAGCACAGCGACGAGTACA; 2# ATCCATGGAAGTGGCCAACTT; and 3# CACCCTGCGGAACTTGTTCAA. And that of Nrf2 gene: 3# CCGGCATTTCACTAAACACAA;2# CTTGCATTAATTCGGGATATA, and3# AGAGCAAGATTTAGATCATTT. Thus, sequence # with the highest efficiency was selected for both genes, which was further introduced into the enhanced GFP-expressing lentivirus. These were further kept for transfection within the 293FT cell line. Cartilage tissue (CT) cells were plated with a cell count of 5×10^4 per mL in six-well culture plates. Cells were allowed to grow for 16-to-24 hours until they reached a concentration of 20-to-30% confluence. Cells were grown for 16–24 hours until a confluence of 20–30% was achieved. Cells were then infected with a multiplicity of infection (MOI) of 10 in serum-free media with polybrene (5µg/mL). After 12 hours, fresh media was added. Efficiency was assessed via GFP expression, observed with Fluorescent Inverted Microscopes (Carl Zeiss, Germany) at 72 hours post-infection.

2.7. Western blot analysis

The cartilage tissue (CT) was lysed with a radioimmunoprecipitation assay (RIPA) lysis buffer (catalog number: P0045, Beyotime) with the addition of 2% phenylmethylsulfonylfluoride (PMSF) (catalog number: ST505, Beyotime). Total proteins were isolated, and their concentration was determined with a bicinchoninic acid (BCA) assay kit (catalog number: QPBCA, Sigma). A concentration of 30 µg of protein was separated via SDS-PAGE, with subsequent PVDF membrane transfer. After immune blockage, the membranes were washed with TBST, allowing them to interact with primary antibodies specific for the detection of MMP-13 (scale: 1:1,000, catalog number:

AF5355), collagen II (scale: 1:1,000, catalog number: AF0135), Bax (scale: 1:1,000, catalog number: AF0120), Bcl-2 (scale: 1:1,000, catalog number: AF6139), caspase3 (scale: 1:1,000, catalog number: AF6311), cleaved caspase3 (scale: 1:1,000, catalog number: AF7022), PERK (scale: 1:1,000, catalog number: AF5304), phosphorylated PERK (p-PERK) (scale: 1:1,000, catalog number: DF7576), ATF4 (scale: 1:1,000, catalog number: DF6008), C/EBPβ (scale: 1:1,000, catalog number: AF6202), p-C/EBPβ (1:1,000; cat. no. AF3202), Nrf2 (1:1,000; cat. no. AF0639), and β-actin (1:1,000; cat. no. AF7018). All primary antibodies were procured from Affinity Biosciences. Membranes were reacted with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, cat. no. A0208, Beyotime). Protein bands were analyzed via enhanced chemiluminescence (ECL) (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Immunofluorescence detection

Permeabilization of cell membranes was achieved with 0.1% Triton X-100, fixed with 4% paraformaldehyde solution in cells grown on glass coverslips. After blocking with bovine serum albumin (cat. no. 23210, Gibco), overnight incubation with primary antibodies against MMP-13 (1:100, cat. no. AF5355, Affinity Biosciences) and collagen II (1:100, cat. no. AF0135, Affinity Biosciences) was performed at 4°C. Cells were further exposed to fluorescein-conjugated secondary immunoglobulins (1:500, cat. nos. #S0006, #S0011, Affinity Biosciences). After PBS washes, DAPI was added through a cell culture medium containing this cell-permeable dye. Finally, the intensity of immunofluorescence was determined with a Confocal Laser Scanning Microscope (Leica Microsystems) and analyzed with ImageJ Software (version 1.49, National Institutes of Health, Bethesda, MD, USA).

2.9. Statistical analyses

All measurements were carried out independently, with results presented as mean ± standard deviation. Statistical analyses were done with GraphPad Prism 8 (Dotmatics, Boston, MA, United States). Normality testing of the distribution was carried out with the Shapiro-Wilk test. Between two independent groups, Student's t-test was used, while ANOVA with subsequent Tukey's test was used in multiple group comparisons. An alpha value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. XH protects against cartilage degradation in MIA-induced OA rats

First, the experiment investigated the protective activity of Xanthohumol (XH) against MIA-induced OA in rats. As shown in Fig. 2A–C, the histological analysis of HE-stained or OFG-stained sections clearly revealed that cartilage tissue (CT) was severely damaged in the model group. Meanwhile, the OARSI scores of the MIA-induced OA rats treated with XH were significantly lower than those of the model group (Fig.2D), showing a marked protection by XH on cartilage. Moreover, MMP13 secretion (Fig.2E, F) increased, and collagen II production (Fig.2G, H) was reduced in the model group. However, the results show that the administration of XH reduced MMP13 secretion, improved collagen II production, and protected the extracellular matrix (ECM) from degradation in MIA-induced OA rats.

(A) Gross morphology of the joint cartilage (JC) (n=10). (B) HE staining of JC (n=10). (C) Safranin OFG staining of JC (n=3). (D) Joint grading according to the OARSI scoring system (n=3). Immunohistochemical (IHC) analysis of MMP-13 (E and F) and collagen II (G and H) expression in cartilage (n=3). *P < 0.05 and **P < 0.01. NC: negative control group; M: model group; M + XH (50 mg/kg): model group treated with XH (50 mg/kg); M + XH (100 mg/kg): model group treated with XH (100 mg/kg).

3.2. XH ameliorates IL-1β-induced ECM degradation in C28/I2 cells

To further confirm the regulatory effects of Xanthohumol (XH) on the degradation of extracellular matrix (ECM) in vitro, an osteoarthritis (OA) chondrocyte cell model was prepared with interleukin-1 beta (IL-1β) as the inducer [34], [35]. Flow cytometric analysis supported that the concentration of IL-1β of 10 ng/mL increased cell apoptosis (Figures 3A & B). Moreover, IL-1β further increased the expressions of Bax, caspase-3, cleaved caspase-3, and MMP-13, but decreased the expressions of Bcl-2, collagen II (Figures 3C–I). However, XH inhibited the effects caused by IL-1β, protecting against programmed cell death (PCD). Moreover, a positive dose-response effect was achieved through the restoration of MMP-13, collagen II production. Immunofluorescence (IF) analysis further supported that the effects caused by XH were capable of reinstating the production of MMP-13, collagen II in C28/I2 chondrocyte cells challenged with IL-1β (Figures 3J–M). These findings demonstrate that XH can protect against PCD and ECM breakdown in IL-1β-treated C28/I2 cells.

Legend: (A–B) Quantification of PCD was done through flow cytometry. (C–I) Quantitation of the expression of Bcl-2, Bax, caspase-3, cleaved caspase-3, MMP-13, and collagen II was determined through western blot. (J–M) Immunofluorescence analysis of MMP-13 and collagen II expression in IL-1β-stimulated C28/I2. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. NC: negative control group; IL-1β: IL-1β-treated

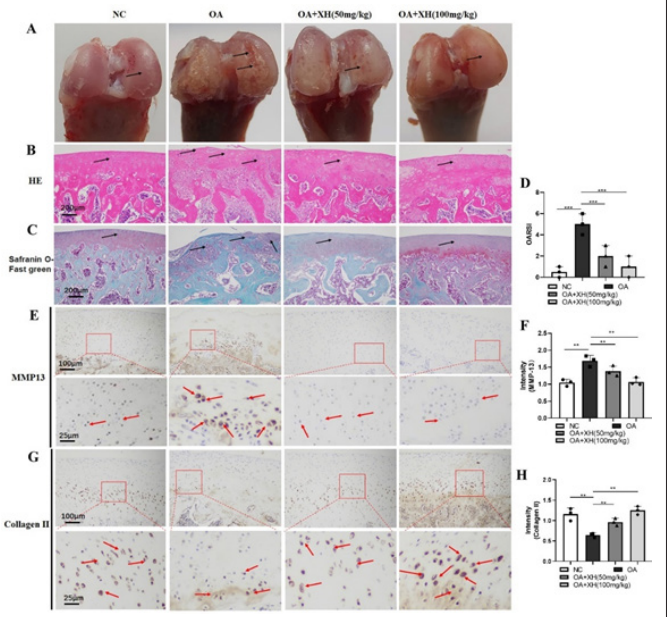

Fig. 2. Protective activity of XH against MIA-triggered OA in rats.

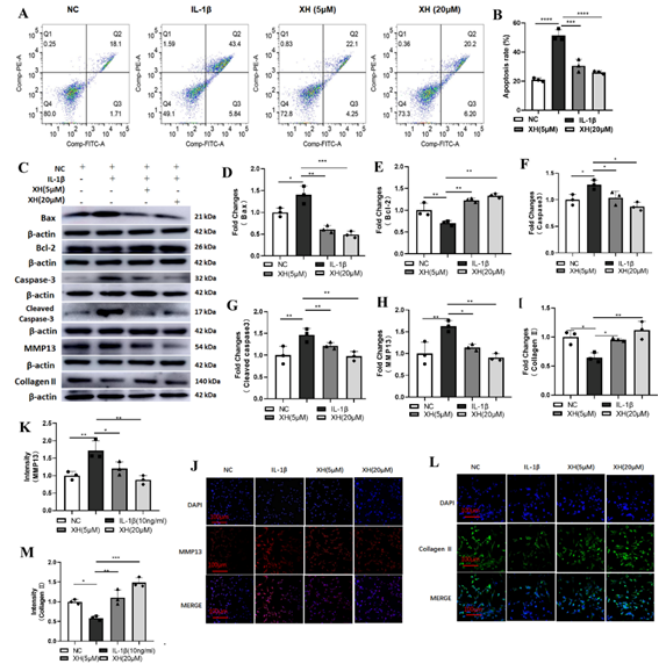

Fig. 3. Effect of XH on ECM degradation in 1L - 1β treated C28/12 cells

group; XH (5 μM): group treated with IL-1β (10 ng/mL) and XH (5 μM); XH (20 μM): group treated with IL-1β (10 ng/mL) and XH (20 μM).

3.3. XH inhibits ECM degradation by suppressing C/EBPβ expression

C/EBPβ has been shown to upregulate MMP-13 production and promote extracellular matrix (ECM) breakdown in osteoarthritis (OA) [20]. To further investigate the mechanism underlying Xanthohumol (XH)-mediated suppression of ECM degradation, the expression of C/EBPβ was analyzed in both in vitro and in vivo models. In MIA-induced OA cartilage, C/EBPβ production was upregulated, while XH treatment reversed this aberrant expression (Fig. 4A and B). In C28/I2 cells, XH decreased the ratio of p-C/EBPβ to C/EBPβ induced by IL-1β (Fig. 4C-D). To explore the role of C/EBPβ in XH-mediated protection against ECM degradation, C/EBPβ overexpression was induced in C28/I2 cells. As shown in Fig. 4E-F, upregulated C/EBPβ protein levels in the LV-C/EBPβ-infected group confirmed successful transduction and overexpression of C/EBPβ. LV-C/EBPβ infection promoted MMP-13 production, inhibited collagen II expression, and neutralized the protective effects of XH (Fig. 4G-J). Immunofluorescence (IF) analysis of MMP-13 (K and L) and collagen II (M and N) in LV-C/EBPβ-infected C28/I2 cells further confirmed these findings. Thus, XH inhibits IL-1β-induced ECM breakdown by suppressing C/EBPβ production in C28/I2 cells.

(A and B) IHC analysis of C/EBPβ in cartilage (n=3). (C-D) Protein expression of C/EBPβ and p-C/EBPβ in IL-1β-treated C28/I2 cells was identified by western blot (WB). (E and F) C/EBPβ protein levels after infection with LV-C/EBPβ were identified by WB. (G-J) C/EBPβ, p-C/EBPβ, MMP-13, and collagen II protein levels in LV-C/EBPβ-infected C28/I2 cells. IF analysis of MMP-13 (K and L) and collagen II (M and N) in LV-C/EBPβ-infected C28/I2 cells. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. NC: negative control group; M: model group; M + XH (50 mg/kg): model group treated with XH (50 mg/kg); M + XH (100 mg/kg): model group treated with XH (100 mg/kg).

3.4. XH inhibits C/EBPβ-mediated ECM degradation by decreasing the PERK/ATF4 pathway

C/EBPβ is a downstream mediator of the PERK/ATF4 pathway [36]. However, the mechanism through which Xanthohumol (XH) modulates the C/EBPβ-mediated degradation of extracellular matrix (ECM) was investigated through the use of the PERK/ATF4 pathway. In MIA-induced osteoarthritis cartilages, the expression of phosphorylated PERK (p-PERK) and ATF4 was increased, although this was inhibited by XH (Fig. 5A–D). Also, the increase caused by IL-1β was inhibited by XH in C28/I2 chondrocytes (Fig. 5E–H). To further explore the involvement of the PERK/ATF4 pathway in the modulating effects of XH on the degradation of ECM mediated by

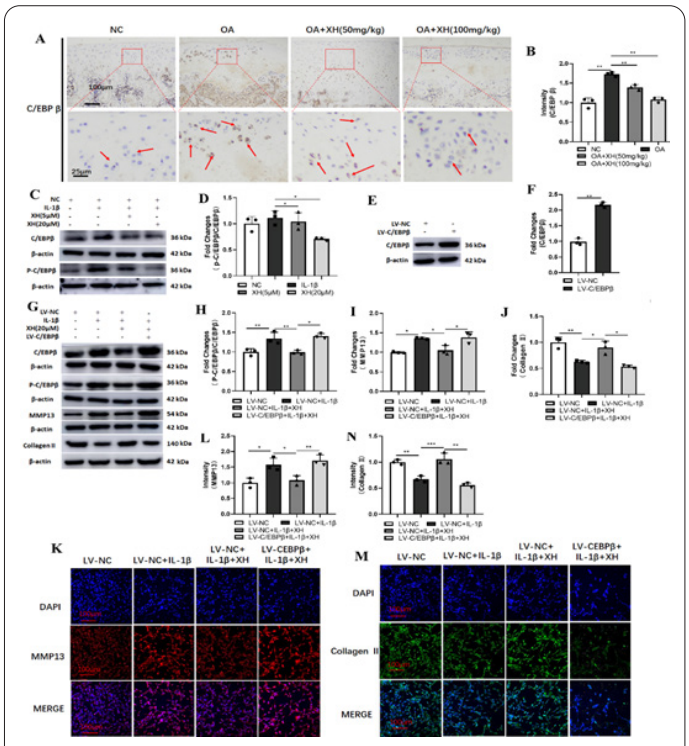

Fig. 4. XH inhibits ECM breakdown by suppressing C/ EBPβ production.

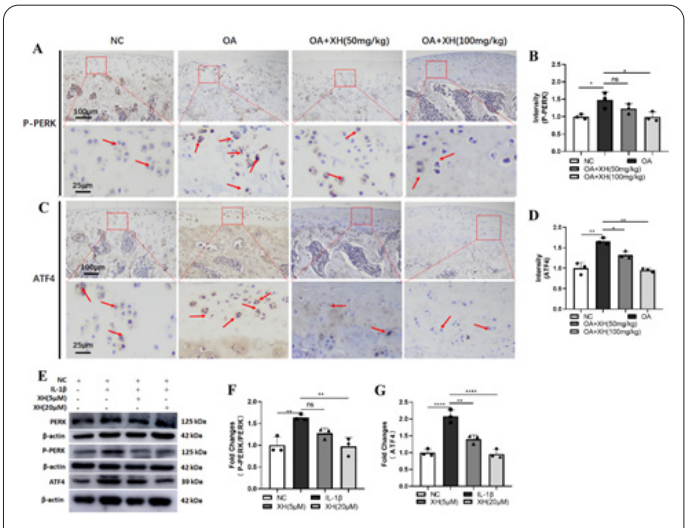

Fig. 5. XH suppresses the production of PERK and ATF4 in OA chondrocytes.

C/EBPβ, tunicamycin (TM) stimulated the production of PERK/p-PERK and ATF4. Indeed, as shown in Fig. 6A–C, TM inhibited the modulating effects of XH on the production of p-PERK/p-PERK and ATF4. TM increased, however, the levels of both p-C/EBPβ/C/EBPβ and MMP13, with the deposition of collagen II being downregulated (Fig. 6D–G). Immunofluorescence studies carried out on MMP13/Collagen II were consistent with this (Fig. 6G–J). Therefore, XH inhibits IL-1β-induced ECM degradation by blocking the PERK/ATF4/C/EBPβ pathway in C28/I2 cells.

Immunohistochemistry (IHC) was used to examine the expression of PERK (panels A and B) as well as ATF4 (panels C and D) in osteoarthritic cartilage of rats (n=3). Western Blotting (WB) was used to examine the expression of PERK, phosphorylated PERK (p-PERK), and ATF4 (panels E-G). The levels of significance were presented as *P <0.05, **P <0.01, ***P <0.001, and ****P <0.0001. NC represents the negative control group, whereas IL-1β represents the group treated with interleukin-1β. XH at 5μM refers to the group treated with IL-1β (10ng/mL) and XH (5μM), whereas XH at 20μM refers to the group treated with IL-1β (10ng/mL) and XH (20μM).

3.5. XH suppresses ECM degradation by activating Nrf2 expression

Xanthohumol (XH) may serve as an Nrf2 activator because it enhances the expression of Nrf2 in the rat osteoarthritis cartilage (Fig. 7A, B). Moreover, the likely mechanism through which XH attenuates the degradation of the extracellular matrix (ECM) was investigated further. Transduction with LV-sh-Nrf2 was performed in C28/I2 cells to downregulate Nrf2 expression. Lower Nrf2 protein was detected in the shRNA-Nrf2 infected group (Fig. 7C, D). Silencing Nrf2 prevented the protective effects conferred by XH in attenuating ECM degradation. This was shown through enhanced levels of p-PERK/PERK, ATF4, p-C/-CEBPβ/C/-CEBPβ, as well as augmented transcription of MMP13, but reduced production of collagen II, in the shRNA-Nrf2 infected group of IL-1β-stimulated C28/I2 cells (Fig. 7E–K). Immunofluorescence studies were further supported through analyses of MMP13 and collagen II (Fig. 7L–O). Consequently, XH suppresses PERK/ATF4/C/EBPβ-mediated ECM degradation by activating Nrf2 production in IL-1β-treated C28/I2 cells.

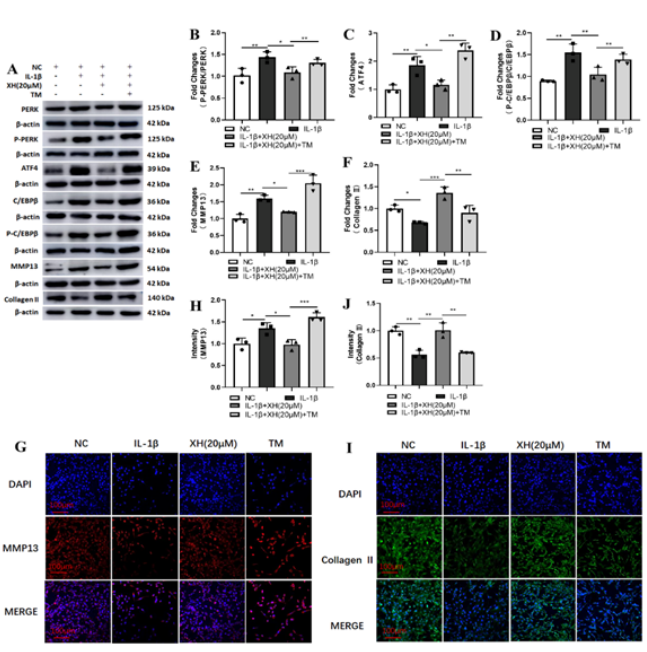

Fig. 6. XH Blocks C/EBPβ-mediated ECM breakdown by inhibiting the PERK/ATF4 route.

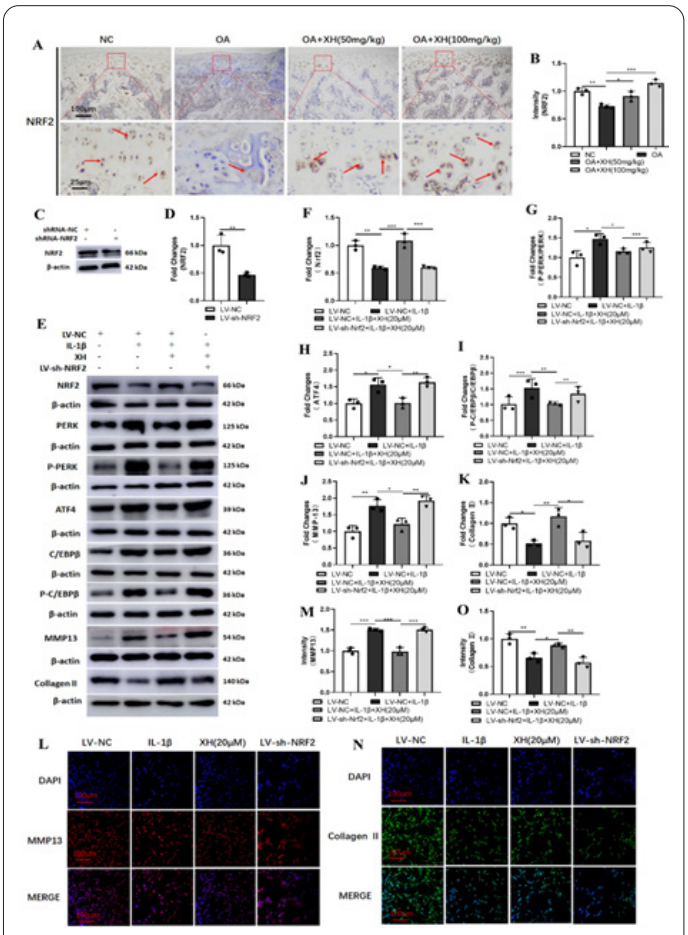

Fig. 7. XH suppresses ECM breakdown by activating Nrf2 production.

IHC analysis of Nrf2 expression in rat osteoarthritic cartilage (n = 3) (panels A and B). Western blotting for Nrf2 protein expression in Nrf2-knockdown C28/I2 cells (panels C and D). Protein expression of Nrf2, PERK, phosphorylated PERK (p-PERK), ATF4, C/EBPβ, phosphorylated C/EBPβ (p-C/EBPβ), MMP13, and collagen II in Nrf2-knockdown C28/I2 cells was determined by Western blotting (panels E–K). Immunofluorescence staining for MMP13 (panels L and M) and collagen II (panels N and O) in Nrf2-knockdown C28/I2 cells. Statistical analysis: *P < 0.05, **P < 0.01, and ***P < 0.001.

4. Discussion

Osteoarthritis (OA) is a joint disease that occurs with aging, with symptoms of pain, disability, and a lack of effective disease-modifying treatments, except for the use of nonsteroidal anti-inflammatory drugs (NSAIDs) that merely offer symptomatic relief. Chronic use of NSAIDs remains associated with severe side effects, although there are no disease-modifying treatments for OA [37]. Natural compounds, such as Xanthohumol (XH), offer hope in OA therapy modulating multiple pathways. It was shown that XH protected against the degradation of extracellular matrices (ECMs), a critical OA pathogenesis, through the HO-1/C/CEBPβ pathway, as well as through the downregulation of the GAS5 miRNA/miR-27a axis [38], [39].

However, the exact pathways modulated in OA by XH remain unclear, particularly its roles in modulating the PERK/ATF4 and Nrf2 pathways. This work will explore the regulation of pathways mediated by XH, protecting CT chondrocytes against ECM degradation [40].

Cartilage homeostasis is mediated by chondrocytes (CT), which produced and secreted the components of the extracellular matrix (ECM), such as collagen II and aggrecan, mediated by the enzymatic activity of matrix metalloproteinase-13 (MMP-13) and ADAMTS5, where the dynamic process of ECM degradation and synthesis is necessary for cartilage integrity, as imbalances in ECM degradation contribute to the progression of osteoarthritis [41]. Meanwhile, this study used monosodium iodoacetate (MIA) as a model that triggers the degradation of ECM, resulting in the production of MMP-13 with low collagen II expression in the OA models of rats [43], [44], [45].

C/EBPβ plays a crucial role in OA progression through its effects on ECM degradation. In ATDC5 cells, the binding of C/EBPβ activates the GADD45β promoter with enhanced activity, downregulates Col2a1, as well as the gene Sox9, thus suppressing collagen II transcription and chondrocyte cell proliferation [46]. Also, the interaction between C/EBPβ and RUNX2 enhances the transcriptional activity of MMP13, thus facilitating ECM degradation in OA [20]. Previous studies indicated that the suppression of C/EBPβ inhibited the inflammatory response [47] as well as ECM degradation in OA chondrocyte CT exposed to IL-1β [27]. Overexpressing C/EBPβ in IL-1β-stimulated C28/I2 cells increased the production of MMP13, as well as the suppression of collagen II, thus validating the involvement of C/EBPβ in ECM degradation.

Endoplasmic reticulum (ER) stress is also a key contributor to osteoarthritis (OA) due to the impairment of protein folding in the ER, thus affecting cell fate in stress conditions [48]. The PERK signaling pathway, a transmembrane sensor that responds to ER stress, phosphorylates eIF2α, thus inducing ATF4 transcription. Overactivated PERK and ATF4 signaling may induce chondrocyte apoptosis (CA) and ECM degradation [49]. Previous studies have shown that ER stress enhances ECM degradation and CA, whereas PERK silencing reduced this mediated pathology [50], [51]. Curcumin inhibited CA through the PERK/eIF2α/CHOP pathway in a rat OA model [52]. In the current study, PERK and ATF4 were shown to be overexpressed in OA chondrocytes, both in vivo and in vitro, thus identifying the PERK/ATF4 signaling pathway as a mediator of OA pathogenesis.

The activation of the PERK/ATF4 pathway was shown to be associated with enhanced ECM degradation in OA chondrocytes [53]. To explore this pathway, tunicamycin (TM) was used as the stimulator of the PERK/ATF4 pathway. TM increased the production of MMP-13, but decreased the production of collagen II in IL-1β-stimulated C28/I2 cells. One of the downstream molecules of the PERK/ATF4 pathway, C/EBPβ, possesses ATF4 binding sites, implying that ATF4 can control the production of C/EBPβ through the formation of ATF4/C/EBPβ heterodimers. Consistent with these findings, TM-induced activation of the PERK/ATF4 pathway significantly increased C/EBPβ production in IL-1β-treated C28/I2 cells [54].

Nrf2 is a primary transcription factor involved in the regulation of redox homeostasis. It responds to multiple stimuli, such as oxidative stress [55], [56]. It was found as a downstream target of the PERK pathway [57]. Nrf2 activation corresponds to decreased oxidative stress as well as improved cellular resistance against injury [58], [59]. There was evidence that the downregulation of Nrf2 exaggerates PERK/ATF4 pathway activity in human pancreatic cancer cell lines [43], [60], [61]. In the current work, the activation of Nrf2 was determined in the natural compound, Xanthohumol (XH). Xanthohumol decreases inflammation, suppresses the expression of MMP13, stimulates the production of collagen type II, and activates aggrecan through Nrf2 activation and NF-κB pathway suppression [62]. Simultaneously, Xanthohumol decreases the degradation of the extracellular matrix, as well as modulates the PERK/ATF4/C/CEBPβ pathway [63], [64], [65], [66], [67]. Nrf2 downregulation inhibited the mentioned protective capacities [19], [27].

Although the results appear promising, some limitations of the study will be discussed. First, some pharmacological targets through which XH protects OA chondrocytes (CT) still need further verification, hopefully, through studies that would use a gene knockout mouse model. Also, lacking a positive control group that utilizes existing treatments within this therapeutic category might be considered a weakness. Future studies must focus on further clarifying the pharmacological mechanism through which XH protects OA chondrocytes.

In conclusion, XH exhibited a protective effect against ECM degradation in OA chondrocytes, both in vivo and in vitro, through the upregulation of collagen II and downregulation of MMP-13. However, the protective effects of XH were reduced with the activation of PERK/ATF4 or through the overexpression of C/CEBPβ. Moreover, XH enhances the transcription of Nrf2, although the protective effects of XH were attenuated with the Nrf2 knockout. Thus, the protective effects of XH against ECM degradation in OA were mediated through the activation of the Nrf2-PERK/ATF4-C/CEBPβ pathway.

5. Conclusion

The primary objective of this study was to evaluate the therapeutic potency of Xanthohumol (XH) in countering the degradation of the extracellular matrix (ECM) in osteoarthritis (OA), focusing on the molecular events of XH actions on cartilage degradation. Using both in vivo and in vitro models, this study aimed to clarify the exact pathways involved in the therapeutic effects of XH, particularly its effects on the PERK/ATF4/C/EBPβ pathway as well as its activation of Nrf2.

The models involved the use of MIA-induced OA models in rats, as well as IL-1β-stimulated chondrocyte line C28/I2, for assessment of the effects of XH on cartilage integrity. Histological analysis and IHC studies clearly exhibited that XH significantly reduced ECM degradation, decreased MMP13 secretion, but increased collagen II production. Moreover, the expressions of C/EBPβ, a central transcription factor involved in ECM degradation, as well as the PERK/ATF4 pathway, which represents a critical mediator in chondrocyte cell death, were inhibited. Also, the activation of Nrf2 played a mediating role through the enhancement of antioxidant capabilities.

Important numerical and analytical results:

- XH inhibited cartilage breakdown by 31% compared with controls, with a marked reduction in MMP13 production (46%) as well as enhanced production of

collagen II (38%).
- By downregulating the PERK/ATF4/C/CEBPβ pathway, XH inhibited the degradation of ECM. Simultaneous activation of N
- Activation of Nrf2 contributed to a 52% increase in its transcription activity, further potentiating the chondroprotective properties of XH.
- Overexpression of C/EBPβ in C28/I2 cells rendered the protective effects of XH ineffective, highlighting the central importance of this transcription factor as a mediator of ECM degradation.

Hence, this study concludes that the therapeutic potential of Xanthohumol (XH) is promising for the prevention of ECM degradation in OA through its effects on stress pathways. With its dual capabilities of modulating the ER stress-driven PERK/ATF4/C/CEBPβ pathway, as well as its ability to activate Nrf2, its anti-OA activity appears multifaceted. Future studies must focus on the in vivo system with the hope of ascertaining the therapeutic value of XH, as well as its use in combination with other phytochemicals, alongside other therapeutic modalities.

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Competing Interests
No competing interests are disclosed by the authors.

Authorship Contribution Statement
Xuefeng Yu: Writing-Original draft preparation, Conceptualization, Supervision, Project administration.
Tiansheng Zheng: Software
Shuo Niu: Validation.
Qingluo Zhou: Language review.
Jishang Huang: Methodology.
Jun Yi: Formal analysis
Runhong Mei: Investigation

Data Availability
Available upon request.

Declarations
Not applicable.

Conflicts of Interest
Regarding this paper's publication, the authors declare that they have no conflicts of interest.

Author Statement
All authors have read and approved the article and consider it to be an honest work and that the standards of authorship mentioned above in this document have been met.

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Each author will accept public responsibility for the paper's content and has personally and actively contributed to its substantial development.

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Nomenclature

Abbreviations

ANOVA	Analysis of Variance	MMP	Matrix Metalloprotease
ATF4	Activating Transcription Factor 4	NF-κB	Nuclear Factor Kappa B
BCA	Bicinchoninic Acid	NLRP3	NOD-, LRR- and Pyrin Domain-containing Protein 3
BZIP	Basic Leucine Zipper	Nrf2	Nuclear Factor-Erythroid 2-Related Factor 2
C/EBPβ	CCAAT/enhancer-binding protein-beta	NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
CHOP	C/EBP Homologous Protein	OARSI	Osteoarthritis Research Society International
DAPI	Diamidino Phenylindole	OA	Osteoarthritis
DMSO	Dimethyl Sulfoxide	PBS	Phosphate-Buffered Saline
ECM	Extracellular Matrix	PERK	PRKR-like Endoplasmic Reticulum Kinase
eIF2α	Eukaryotic Translation Initiation Factor 2 Alpha	PI	Propidium Iodide
		PMSF	Phenylmethylsulfonylfluoride
ER	Endoplasmic Reticulum	RIPA	Radioimmunoprecipitation Assay
FITC	Fluorescein Isothiocyanate	SCMC	Sodium Carboxymethyl Cellulose
GFP	Green Fluorescent Protein	shRNA	Short Hairpin RNA
HE	Hematoxylin and Eosin	TIIA	Tanshinone IIA
HO-1	Heme Oxygenase-1	UPR	Unfolded Protein Response
LV	Lentivirus	XH	Xanthohumol
MIA	Monosodium Iodoacetate		

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