Original Paper

Osthole Inhibits Proliferation and Induces Catabolism in Rat Chondrocytes and Cartilage Tissue

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Key words

Osthole • Chondrocytes • Proliferation • Catabolism • Cartilage tissue

Abstract

Background/Aims: Cartilage destruction is thought to be the major mediator of osteoarthritis. Recent studies suggest that inhibition of subchondral bone loss by anti-osteoporosis (OP) drug can protect cartilage erosion. Osthole, as a promising agent for treating osteoporosis, may show potential in treating osteoarthritis. The purpose of this study was to investigate whether Osthole affects the proliferation and catabolism of rat chondrocytes, and the degeneration of cartilage explants. **Methods:** Rat chondrocytes were treated with Osthole (0 μ M, 6.25 μ M, 12.5 μ M, and 25 μ M) with or without IL-1 β (10ng/ml) for 24 hours. The expression levels of type II collagen and MMP13 were detected by western Blot. Marker genes for chondrocytes (A-can and Sox9), matrix metalloproteinases (MMPs), aggrecanases (ADAMTS5) and genes implicated in extracellular matrix catabolism were evaluated by qPCR. Cell proliferation was assessed by measuring proliferating cell nuclear antigen (PCNA) expression and fluorescence activated cell sorter. Wnt7b/ β -catenin signaling was also investigated. Cartilage explants from two-week old SD rats were cultured with IL-1 β , Osthole and Osthole plus IL-1 β for four days and glycosaminoglycan (GAG) synthesis was assessed with toluidine blue staining and Safranin O/Fast Green FCF staining, collagen type II expression was detected by immunofluorescence. **Results:** Osthole reduced expression of chondrocyte markers and increased expression of MMP13, ADAMTS5 and MMP9 in a dose-dependent manner. Catabolic gene expression levels were further improved by Osthole plus IL-1 β . Osthole inhibited chondrocyte proliferation. GAG synthesis and type II collagen were decreased in both the IL-1 β groups and the Osthole groups, and significantly reduced by Osthole plus IL-1 β . **Conclusions:** Our data suggested that Osthole increases the catabolism of rat chondrocytes and cartilage explants, this effect might be mediated through inhibiting Wnt7b/ β -catenin pathway.

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Introduction

Osteoarthritis (OA) is a degenerative disease primarily characterized by the progressive destruction of the articular cartilage, subchondral bone thickening, osteophyte formation and inflammation of the synovium. Although all of the tissue in the joint is affected, cartilage destruction is deemed to be the major mediator of OA pathogenesis [1]. Articular cartilage is mainly composed of abundant extracellular matrix (ECM) including type II collagen and proteoglycans, and chondrocytes are the primary cell type [2]. Chondrocytes are responsible for the synthesis and catabolism of the ECM [3]. When ECM catabolism and anabolism becomes imbalanced this can cause degenerative diseases such as OA [4].

Cartilage matrix degradation is mediated by matrix metalloproteinases (MMPs) and aggrecanases in response to excess production of pro-inflammatory cytokines. In particular, the production of collagenases such as MMP13 and aggrecanase-2 (ADAMTS5) by potent pro-inflammatory cytokines such as interleukin (IL)-1 β is a major mediator of cartilage destruction [5-7]. OA is usually accompanied by increased expression of collagen type X, Runx2, vascular endothelial growth factor (VEGF) and increased activity of alkaline phosphatase, with decreased expression of collagen type II and aggrecan, and other early chondrocyte marker genes such as Sox9 [8-12].

Many signaling pathways are involved in regulating the normal function of cartilage [13-15], and recently it has been suggested that abnormal Wnt/ β -catenin signaling may contribute to OA pathogenesis [5]. β -catenin apparently plays dual roles in regulating cartilage development and function. Conditional activation of β -catenin in chondrocytes in a transgenic OA mouse model results in articular cartilage degeneration [16], but inhibition of β -catenin in articular chondrocytes also causes OA-like cartilage degradation in a Col2a1-ICAT transgenic mouse model [17]. It seems that optimal expression of the Wnt/ β -catenin signaling pathway is important for balancing cartilage function, which has potential as a therapeutic target for cartilage degradation.

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin, is a coumarin derivative that is an important component of medicinal plants and herbs [18] that are ingested in Traditional Chinese Medicine (TCM). It has been shown that Osthole has some important therapeutic functions and has a good safety profile compared with other natural products, which makes it a very promising compound for drug discovery. Recently, it had been widely reported that Osthole may play pivotal roles in osteoporosis (OP) by promoting osteoblast differentiation and inhibiting bone resorption [19-23]. Damage to subchondral bone increases the severity of cartilage degeneration when early OA and OP coexist [24]. In an experimental model of osteoporotic OA in rats, cartilage damage was aggravated by OP [25]. It was reported that the anti-OP drug Alendronate had protective effects on cartilage degeneration by suppressing the expression of MMP-13 [26, 27]. Therefore, Osthole is a promising drug for anti-OP therapy in a clinical setting.

Currently, little is known about the way Osthole affects chondrocyte function and its underlying mechanism. In the present study, we investigated the effects of different concentrations of Osthole on catabolism of ECM by chondrocytes and joint degeneration using isolated rat femoral heads. Osthole induced catabolism by increasing the expression levels of MMPs and ADAMTS5 which were responsible for the degradation of ECM including collagen type II and proteoglycans, and further aggravated catabolism by chondrocytes in the presence of IL-1 β in a dose-dependent manner. The expression of collagen type II and proteoglycans from the rat femoral head explants that were treated by IL-1 β , Osthole, and IL-1 β plus Osthole respectively showed that Osthole aggravated the degeneration of cartilage in the presence of IL-1 β . The activity of Wnt7b/ β -catenin pathway was inhibited by Osthole or Osthole plus IL-1 β . All these results suggested that Osthole promoted the catabolism of ECM and inhibited the activity of Wnt7b/ β -catenin pathway.

Materials and Methods

Cell isolation and cell culture

Chondrocytes were isolated from articular cartilage of 24-hour-old SD rats and dispersed in 0.1% collagenase type II (C6885, Sigma-Aldrich) for 3 hours. Chondrocytes were collected and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biowest, France) supplemented with 10% fetal bovine serum (FBS, Biowest, France) and 1% penicillin-streptomycin. Chondrocytes from passage 1 to passage 3 were used in all experiments [28].

Chondrocytes were starved with 1% FBS in DMEM for 24 hours before the interventions with both Osthole and IL-1 β . Chondrocytes were treated with different concentrations of Osthole (0, 6.25 μ M, 12.5 μ M and 25 μ M), 10ng/ml IL-1 β (peprotech, #200-01B), 10ng/ml IL-1 β and 6.25 μ M Osthole (abbreviated IO6.25), 10ng/ml IL-1 β and 12.5 μ M Osthole (IO12.5), 10ng/ml IL-1 β and 25 μ M Osthole (IO25) for 24 hours, cells were harvested for detecting the expression of protein and mRNA.

Cartilage explants culture

To explore the role of Osthole in the catabolism of cartilage over a longer time period cartilage explants from the femoral head isolated from a two-week old SD rat were cultured with IL-1 β (50ng/ml), Osthole (25 μ M) (Nanjing Tcm Institute of Materia Medica, China), and Osthole (25 μ M) plus IL-1 β (50ng/ml), respectively for four days.

Glycosaminoglycan (GAG) synthesis analysis by staining

The explants were fixed in 4% Para formaldehyde, decalcified, dehydrated, embedded in paraffin and cut into slices. The specimens were then dewaxed and hydrated, and then stained with Toluidine blue and Safranin O-Fast Green FCF.

Quantitative analysis of GAG content

Glycosaminoglycan (GAGs) were determined by DMB (dimethylmethylene blue) method using Blyscan sulfated Glycosaminoglycan assay kit (Biocolor Ltd, UK) according to the manufacturer's protocols. Femoral heads isolated from a two-week old SD rat with different treatments(IL-1 β , Osthole, IL-1 β plus Osthole) were lysis in papain extraction reagent at 65°C for 3 hours, samples were sonicated and centrifuged at 10,000g for 10 minutes. About 100ul test samples were mixed with 1ml Blyscan dye reagent, and then place the tubes which contain samples in a shaker for 30 minutes. Samples were centrifuged at 12000g for 10 minutes, the supernatant were drained carefully, and 0.5ml dissociation reagent was added to each tube. Spectrophotometer absorbance measurements were performed at 656 nm for GAGs assays. The supernatant from cartilage explants was collected at 48h and 96h respectively, and analyzed according to the protocols.

Immunofluorescence

Specimens embedded in paraffin were dewaxed and hydrated. After being incubated with 0.1% trypsin for 30 min at RT. The specimens were then washed in PBS three times, permeabilized with 0.1% Triton X-100 in PBS for 30 min at RT, washed in PBS three times, incubated in PBST with 1% BSA for 60 min to block unspecific binding of the antibodies, incubated with primary antibody Col2a1 (SC-52658) in 1% BSA for 60 min at RT, washed with PBS three times, and incubated with secondary antibody in 1% BSA for 30 min at RT. Nuclei were stained with DAPI (Cat. No A1001, Applichem, Germany) at a concentration of 10 ng/ml. The pictures were taken under an OLYMPUS IX71 inverted microscope.

Western blotting

Cells were lysed with lysis buffer (Beyotime, P0013B) containing phenylmethylsulfonyl fluoride (PMSF). Lysates was centrifuged at 12,000 g at 4°C for 10 min and protein concentrations were determined using a BCA Protein Assay Kit (Cat. No 23227, Pierce, USA). Proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane using a standard protocol. The membrane was incubated with primary antibodies and probed with the respective secondary antibodies. Enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA) was used for protein visualization. The following antibodies were used: Col2a1 (SC-52658), β -catenin

(#9582S, CST), TCF4 (#2569, CST), proliferating cell nuclear antigen (PCNA) (SC-25280), MMP13 (SC-30073), MMP9 (#3852S, CST) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118, CST). The gray values of the resulting protein bands were determined by Image J software to allow an estimate of protein concentration.

Analysis of cell cycle distribution

The effect of Osthole on cell cycle distribution was determined by flow cytometric analysis of the DNA content of the nuclei of cells following staining with propidium iodide (P4170, Sigma). Rat chondrocytes were seeded in a 10 cm dish, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of Osthole or dimethyl sulfoxide (DMSO, control), and cells were incubated for 24 h at 37°C. Cells were harvested and washed with 1×PBS three times, and then fixed by 70% ethanol overnight at 4°C. The cells were then treated with 100 μ g/ml RNaseA and 100 μ g/ml propidium iodide for 30 min at 37°C. Stained cells were transferred to FACS tubes and detected using a flow cytometry (Beckman Coulter).

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the cultured cells with Trizol reagent (Cat. No 15596-026, Invitrogen). The first strand cDNA was synthesized with RT Reagent Kit (takara code DRR037A). qPCR was performed with SYBR® Premix Ex Taq™ (Cat.# RR420R). Delta-delta Ct method was used to analyze the result. Primers' sequences are listed in Table 1. All the experiments were independently repeated three times.

The data are expressed as mean $\pm$ SD. Statistical correlation of data was checked for significance by One-Way ANOVA and Post Hoc Turkey HSD for multiple comparisons. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Table 1. Primer sequences for Real-time quantitative PCR. MMP9= matrix metalloproteinase 9; Acan= aggrecan; ALP= Alkaline phosphatase; Col10a1= Type x collagen; VEGF= vascular endothelial growth factor; RUNX2= runt-related transcription factor 2; Sox9= SRY (sex determining region Y)-box 9; OCN= Osteocalcin; ADAMTS5= A disintegrin and metalloproteinase with thrombospondin motifs 5, IL-1 β = interleukin 1 beta

Gene name	GeneBank accession no.	mRNA sequences(5'-3')
Actin-F	NM_031144.3	CCCGCGAGTACAACCTTCT
Actin-R		CGTCATCCATGGCGAACT
Acan-F	NM_022190.1	GCAGGGATAACGGACTGAAG
Acan-R		GAGTAAAGTGGTCATAGTTCAGCTTG
Sox9-F	XM_003750950.2	ATCTTCAAGGCGCTGCAA
Sox9-R		CGGTGGACCCCTGAGATTG
Col10a1-F	XM_001053056.3	CCCTATTGGACCACCAGGTA
Col10a1-R		ACTGCTTGGCTGGGAGGT
ADAMTS5-F	NM_198761.1	AGCCATCCTGTTTACCAGAG
ADAMTS5-R		CATTCCCAGGTTGTACAT
ALP-F	NM_013059.1	GCACAACATCAAGGACATCG
ALP-R		TCAGTTCTGTCTTGGGGTACAT
VEGFa-F	NM_031836.3	GCAATGATGAAGCCCTGGAG
VEGFa-R		CGCTCCAGGATTTAAACCGG
OCN-F	M25490.1	CCCAATTGTGACGAGCTAGC
OCN-R		CTGTGCCGTCCATACTTTTCG
MMP9-F	NM_031055.1	CCTCTGCATGAAGACGACATAA
MMP9-R		GGTCAGGTTTAGAGCCACGA
Wnt7b-F	NM_001009695.1	AATGAGGCAGGCAGAAAGG
Wnt7b-R		CCGACACTCCGTGACACTTA
Runx2-F	NM_001278483.1	AAGGAGCACAAACATGGCTG
Runx2-R		TCTTAGGGTCTCGGAGGGAA
IL-1 β -F	NM_031512.2	ATCTCACAGCAGCATCTCGA
IL-1 β -R		TAGCAGGTCGTCATCATCCC

Results

Effects of Osthole on the expression of chondrocyte marker genes

ECM molecules such as aggrecan and type II collagen in cartilage tissue cooperatively maintain the function and integrity of cartilage. The synthesis of aggrecan and type II collagen is regulated by MMP13 and ADAMTS5 respectively [29]. Sox9 is a master transcription factor in chondrocytes which regulates the formation of cartilage ECM.

To assess the changes in ECM content secreted collagen type II was evaluated by western blot, and the expression levels of aggrecan and Sox9 were detected by quantitative PCR. The results indicated that the expression levels of these genes exhibited a tendency to decrease slightly after stimulation with Osthole for 24hrs (Fig. 1A, 1B, 1C, left). In the presence of IL-1 β , Osthole facilitated the down-regulation of type II collagen, aggrecan and Sox9 (Fig. 1A, 1B, 1C, right).

The expression of MMPs and ADMATS5 in rat chondrocytes treated by Osthole, IL-1 β and Osthole plus IL-1 β

The effect of Osthole on chondrocytes was further verified by checking the expression of MMPs. The matrix degradation enzymes such as MMP13 are usually induced under inflammatory conditions [30, 31]. Osthole significantly stimulated the expression of MMP13 in rat chondrocytes in a dose-dependent manner (Fig. 2A, left). Similar effects were also

Fig. 1. The effect of Osthole on the degeneration of rat chondrocytes. Chondrocytes were treated with increasing concentrations of Osthole (0, 6.25 μ M, 12.5 μ M and 25 μ M) or different concentrations of Osthole (0, 6.25 μ M, 12.5 μ M and 25 μ M) plus IL-1 β (10ng/ml), 10ng/ml IL-1 β and 6.25 μ M Osthole (I06.25), 10ng/ml IL-1 β and 12.5 μ M Osthole (I012.5), 10ng/ml IL-1 β and 25 μ M Osthole (I025) for 24 hrs. (A) The protein expression levels of type II collagen were analyzed by western Blot. (B-C) mRNA levels of both Sox9 and aggrecan were detected by qPCR. Data was expressed as mean $\pm$ SD from three separate experiments. *p < 0.05 versus control. The Y axis indicates the values normalized to the control value.

Fig. 2. Rat primary chondrocytes were cultured with different concentrations of Osthole or Osthole plus IL-1 β for 24hrs. (A) The protein expression levels of MMP13 were analyzed by western Blot. (B) The protein expression levels of MMP9 were analyzed by western Blot. (C) mRNA levels of ADAMTS5 were analyzed by qPCR. Data is expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control. The Y axis indicates the levels normalized to the control value.

seen on the expression levels of ADAMTS5 and MMP9 after being incubated with Osthole for 24hrs (Fig. 2B, 2C, left). Furthermore, the expression levels of these genes in the Osthole plus IL-1 β groups were elevated with respect to those in the Osthole-treated groups (Fig. 2A, 2B, 2C, right).

The expression of catabolic genes in chondrocytes were increased by Osthole and further enhanced in the presence of IL-1 β

The expression levels of degenerative genes such as collagen type X (ColX), VEGF and alkaline phosphatase in chondrocytes were up-regulated after the chondrocytes were induced with the proinflammatory factor IL-1 β . The mRNA levels of these genes were increased in Osthole-treated groups compared to control group (Fig. 3A), the changes in the expression levels of these genes were increased further after rat chondrocytes were treated with Osthole plus IL-1 β (Fig. 3B).

Cell proliferation was inhibited in chondrocytes treated by Osthole

It is well known that Osthole can induce G2/M phase arrest in several tumor cells [32-34]. The effect of Osthole on the cell cycle in chondrocytes is still unclear. To address this

Fig. 3. The mRNA levels of ALP, OCN, RUNX2, ColX and VEGF in rat primary chondrocytes were evaluated by quantitative PCR after chondrocytes were incubated with Osthole or Osthole plus IL-1 β for 24hrs. (A) The mRNA levels of ALP, OCN, RUNX2, ColX and VEGF in chondrocytes were analyzed after being treated with Osthole. (B) The mRNA levels of these genes were assessed by qPCR after the chondrocytes were cultured with Osthole plus IL-1 β . Data was expressed as mean $\pm$ SD from three separate experiments. *p < 0.05 versus control. The Y axis indicates the values normalized to those of the control value.

question, cell cycle profiles were analyzed by PI staining and flow cytometry. As shown in Fig. 4, treatment with different concentrations of Osthole resulted in the accumulation of cells at G0/G1 phase and a corresponding decrease in those in the S phase. This anti-proliferative role was further exhibited by the down-regulated PCNA protein levels in cells treated with Osthole compared to the control (Fig. 4A). Both the increasing percentage of cells in S phase and high expression level of PCNA that were stimulated by IL-1 β were reversed in the presence of Osthole, and the inhibition of proliferation was in a concentration dependent manner (Fig. 4B).

The activity of Wnt7b/ β -catenin pathway were inhibited by Osthole

In the present experiment, western blots showed that expression levels of components of the Wnt/ β -catenin pathway including β -catenin and TCF4 were obviously inhibited in the Osthole treated groups. The expression levels of both β -catenin and TCF4 were slightly increased after stimulation by IL-1 β alone compared to the control group. The activity of this pathway in the presence of IL-1 β was reversed by different concentrations of Osthole (Fig. 5A). The proportion of β -catenin/GAPDH and TCF4/GAPDH were measured and compared. Osthole decreased the expression levels of β -catenin and TCF4 consistently, further results showed that IL-1 β stimulated the activity of Wnt/ β -catenin signaling by elevating the

Fig. 4. The effect of Osthole on the proliferation of rat chondrocytes was evaluated by FACS and PCNA expression after treatment with Osthole or Osthole plus IL-1 β for 24hrs. (A) The expression of PCNA was investigated in combined group. Data was expressed as mean $\pm$ SD from three separate experiments, and (B) Chondrocyte cell cycle analysis. * $p < 0.05$ versus control.

Fig. 5. The influence of Osthole on the Wnt7b/ β -catenin pathway. (A) After being treated with different concentrations of Osthole or Osthole plus IL-1 β for 24hrs, both the expression of β -catenin and TCF4 were repressed. (B) Quantification of β -catenin and TCF4 protein were normalized to GAPDH using Image J software. Data is expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control. (C) mRNA levels of Wnt7b and IL-1 β in chondrocytes treated with Osthole or Osthole plus IL-1 β . The Y axis indicates the value normalized to the control value.

expression levels of β -catenin and TCF4, and the expression levels in the combined group (treated by Osthole and IL1 β) were lower than that in the control group (Fig. 5B). We

Fig. 6. The influence of Osthole on GAG synthesis and expression of type II collagen. Cartilage explants from rat femoral heads were treated with IL-1 β , Osthole or Osthole plus IL-1 β for four days. (A) The GAG content was determined by Safranin O-Fast Green FCF and Toluidine blue staining (100 $\times$). The region of cartilage degeneration was marked by arrows which was pale in staining. (B) The GAG in the medium and tissue was analyzed by DMB method. Data is expressed as mean $\pm$ SD from three separate experiments. [#]p < 0.05 versus control(48h), ^{*}p < 0.05 versus control(48h). (C) The expression of type II collagen was detected by immunofluorescence (100 $\times$), nuclei was stained with DAPI (100 $\times$).

also found that Wnt7b exhibited the similar expression patterns to that of β -catenin and TCF4, the endogenous expression of IL-1 β was not changed by the treatment of Osthole or Osthole plus IL-1 β , demonstrating that Osthole did not suppress endogenous IL-1 β to inhibit β -catenin and TCF4 and Wnt7b (Fig. 5C).

The components of extracellular matrix was inhibited by Osthole

To explore the role of Osthole in the catabolism of cartilage over a longer time period experiments were performed on cartilage explants over a period of four days. GAG and collagen are the main components of ECM in cartilage. GAG plays an important role in cartilaginous tissues to support and transmit mechanical loads. The synthesis of GAG is usually analyzed by toluidine blue and Safranin O-Fast Green FCF staining, and the GAG content and release were measured by using the DMB method. The expression levels of

type II collagen were assessed by immunofluorescence. We found both the synthesis of GAG content and the expression of type II collagen were decreased in both the IL-1 β groups and the Osthole groups, Osthole further aggravated the degeneration of ECM in the presence of IL-1 β (Fig. 6A, 6B). The change of GAG release was contrary to that of GAG content, which IL-1 β stimulated GAG release into the medium [35].

Discussion

Osthole has shown promise as a treatment for OP and this suggests that it might also be important in OA. However, the effect of Osthole on chondrocytes was unknown. In this study, we investigated whether Osthole also had a role in rat ECM degeneration involving chondrocytes. ECM degeneration is considered to be the result of increased expression of proteolytic enzymes; ADAMTS is believed to be the main enzyme responsible for aggrecan, GAG, and proteoglycan loss [36]. MMP-13 is a collagenase which was responsible for the degradation of type II collagen. The expression level of MMP13 is differentially up-regulated in OA cartilage [37].

From the results presented in our study, the expression levels of MMP13 and ADAMTS5 are significantly elevated after the treatment with different concentrations of Osthole, and such effects were more evident in the presence of IL-1 β . MMP9 is usually activated in osteoarthritic chondrocytes and contributes to cartilage destruction [38, 39]. Its expression was also induced by Osthole in rat chondrocytes, and the expression pattern was the same as MMP13. High expression levels of these proteolytic enzymes associated with Osthole treatment in chondrocytes suggested chondrocyte degeneration.

OA is characterized by the degradation of collagenous and noncollagenous ECM components in articular cartilage. ECM components in chondrocytes represented by type II collagen and aggrecan were analyzed. We found that the changes of type II collagen expression levels were contrary to that of MMP13. Furthermore, Osthole also regulated the mRNA levels of A-can, which was consistent with type II collagen. Sox9 is an essential transcription factor for maintaining cartilage phenotype, which regulates the expression of type II collagen [40]. It seemed that Osthole decreased the expression of Sox9 in a concentration dependent manner, and the reduction of this gene level was more evident after chondrocytes were treated with Osthole plus IL-1 β .

Cartilage degeneration was also accompanied by the altered expression of hypertrophic marker genes including type x collagen, Osteocalcin, Runx2, VEGF and ALP [41]. We analyzed the mRNA levels of these genes. A slight increase in the expression levels of these genes was found in groups treated by different concentrations of Osthole. The expression levels were promoted significantly with Osthole plus IL-1 β .

The above results including ECM degradation and the accelerated expression of hypertrophic genes suggested that chondrocyte degeneration was induced by the up-regulated expression of MMPs and ADAMTS5. Meanwhile, we also investigated cartilage explants from rat femoral heads that were cultured with IL-1 β , Osthole or Osthole plus IL-1 β . The GAG content from cartilage tissue and expression of Col2a1 were detected. The effects of Osthole on cartilage explants over the longer time period of four days were consistent with that of rat chondrocytes. The release of GAG into the medium was usually assessed in the pathogenesis of OA. The concentration of GAG in the medium was significantly increased by Osthole plus IL-1 β compared to that of IL-1 β or Osthole, both of which were higher than that of control group. All these findings indicated that Osthole induces cartilage degeneration, especially in the presence of IL-1 β .

Wnt/ β -catenin signaling is essential for the function of chondrocytes, and imbalance of this pathway can lead to OA-like pathology in cartilage [16, 17]. To investigate Wnt/ β -catenin signaling, we examined protein expression levels of pathway components. We found that Osthole down-regulated β -catenin expression in a dose-dependent manner. We also detected the expression of down-stream molecules TCF4, which is deemed as a mediator

that contributes to cartilage degeneration through regulating the activity of the NF- κ B pathway [42]. Protein expression levels of TCF4 were also decreased with Osthole treatment, which indicated that Wnt/ β -catenin signaling was blocked by treatment of chondrocytes with Osthole. To further unravel the correlation between the signaling pathway and chondrocyte degeneration the chondrocytes were stimulated with IL-1 β , which increases MMPs expression by activating Wnt/ β -catenin signaling pathway [43]. The expression levels of β -catenin and TCF4 were higher in IL-1 β -treated groups compared to the control group. The increased expression levels of these two proteins were reversed by the combination of Osthole with IL-1 β . Although IL-1 β is involved in regulating the chondrocyte metabolism, the endogenous expression level of IL-1 β was not altered by treatment with Osthole or Osthole plus IL-1 β .

It was reported that the up-stream molecules Wnt7b play a part in regulating the cartilage degeneration [44]. To determine whether Wnt7b was involved in the regulation of chondrocytes by Osthole, the expression of Wnt7b were examined. The results showed that the expression pattern of Wnt7b was decreased in a manner consistent with that of β -catenin and TCF4. Taken together, these findings indicated that Osthole may induce cartilage degeneration by inhibiting the Wnt7b/ β -catenin pathway. Interestingly, the regulation of this pathway by Osthole or Osthole plus IL-1 β were different from that of the proteolytic enzymes and ECM, the expression levels of which were consistent in the presence of Osthole or Osthole plus IL-1 β . Maybe this is because either increased or decreased activity of Wnt/ β -catenin can lead to chondrocytes differentiation resulting from an imbalance in catabolism and anabolism [16, 17]. In our study, we found the activity of this pathway in chondrocytes treated by IL-1 β with different concentrations of Osthole remained below the normal level. Further research is needed to fully reveal how the effects of Osthole are enhanced by IL-1 β in terms of cartilage degeneration about their different effects on Wnt/ β -catenin signaling.

The number of chondrocytes can be altered by proliferation and apoptosis [45]. There is a very low level of proliferation in osteoarthritic chondrocytes in contrast to normal articular chondrocytes [43]. Proliferation is essential for maintaining the normal function of chondrocytes. Cell proliferation is also associated with degeneration in primary chondrocytes [46, 47]. Wnt/ β -catenin signaling molecules also regulate chondrocytes proliferation [48]. The increased inflammation that occurs in OA also causes chondrocyte apoptosis [49]. We did not investigate apoptosis in this study so we cannot say whether apoptosis was increased or decreased by Osthole treatment, this would be an interesting study for the future. In our experiment, Osthole induced the arrest of cell cycle in the G0/G1 phase. The decreased percentage proportion of G0/G1 phase induced by IL-1 β was further increased by the treatment of IL-1 β plus Osthole. All these findings indicated that Osthole inhibited the proliferation of chondrocytes and the effect was more obvious in an inflammatory condition.

Based on these results presented in our study, it suggests that Osthole induced chondrocyte catabolism and inhibited the activity of Wnt7b/ β -catenin and proliferation of chondrocytes, especially in the presence of IL-1 β .

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Disclosure Statement

The authors declare that they have no competing interests.

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