Abstract. Osteoarthritis (OA) is a common degenerative joint disease in older adults. A number of previous studies have demonstrated that natural flavonoids can serve as promising therapeutic drugs for OA. Kaempferol, a phytochemical ingredient mainly present in various fruits, has exhibited its prominent anti-inflammatory and antioxidant effects in numerous diseases. However, whether Kaempferol ameliorates the deterioration of arthritis remains to be elucidated. The aim of the present study was to investigate the therapeutic role of Kaempferol on OA in rat chondrocytes. The results revealed that Kaempferol significantly inhibited the interleukin (IL)-1β-induced protein expression of inflammatory mediators such as inducible nitric oxide synthase and cyclo-oxygenase-2. In addition, the common matrix degrading enzymes [matrix metalloproteinase (MMP)-1, MMP-3, MMP-13 and a disintegrin and metalloproteinase with thrombospondin motif-5] induced by IL-1β were also suppressed by Kaempferol, and consequently abolished the degradation of collagen II. Furthermore, the anti-inflammatory effect of Kaempferol was mediated by the inhibition of the mitogen activated protein kinase-associated extracellular signal-regulated kinase and P38 signaling pathways. These results collectively indicated that Kaempferol can potentially prevent OA development and serve as a novel pharmacological target in the treatment of OA.

Introduction

Osteoarthritis (OA) is a common degenerative joint disease in older adults (1). Joint pain, stiffness, motion limitations are the most common symptoms of OA and it is one of the leading causes of disability worldwide (2,3). Recently one comparative analysis confirms that the disease has doubled in prevalence since the mid-20th century (4). To date, however, there are no modifying agents to halt or reverse the progression of OA. The outcomes of most patients with advanced stage of OA are joint replacement. The surgery, however, costs much and causes great social and economic problems (5).

Disintegration of collagen II in cartilage matrix is regarded as the central pathology of OA and it is commonly accepted that inflammation plays a crucial role in the development of OA (6,7). Overproduction of pro-inflammatory cytokines like Interleukin (IL)-1β can lead to increased secretion of catabolic enzymes such as matrix metalloproteinases (MMPs) and disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5) in OA cartilage, which represent the major proteolytic enzymes in the degradation of cartilage (8,9). In addition, IL-1β can also increase the expression of other catabolic factors, including Inducible NOS (iNOS) and Cyclo-oxygenase-2 (COX-2), in chondrocytes (10,11). To this end, we primarily utilize rat chondrocytes treated with IL-1β as our fundamental model of OA.

Numerous studies have confirmed the anti-inflammatory effects of different flavonoids one the treat of OA in vitro or in vivo, indicating that flavonoids can represent good therapeutic method for OA (12). Kaempferol (3, 4', 5, 7-tetrahydroxyflavone) is a natural flavonoid commonly found in a variety of foods such as tea, apples, strawberries, beans, and citrus fruits (13). It has been widely concerned for its antitumor, antioxidative, antibacterial and anti-inflammatory properties (14). One clinical study recently found that Elaeagnus Angustifolia (EA) extract containing 0.21% (w/w) Kaempferol could significantly reduce the symptoms of OA, but its internal anti-inflammatory effect on chondrocytes remains to be fully elucidated (15). The object of this study is to explore its anti-inflammatory effects on IL-1β-induced rat chondrocytes and the internal mechanism.
Materials and methods

Chemicals and reagents. Kaempferol, collagenase, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). Fetal bovine serum (FBS) was obtained from Glico-BRL (Gaithersburg, MD, USA). Recombinant rat IL-1β was obtained from R&D systems (Minneapolis, MN, USA). Primary antibodies specific for MMP-3, COX-2, phosphorylated extracellular signal-regulated kinase (p-ERK), ERK, e-Jun N-terminal kinase (JNK), p-JNK, p38, p-p38 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies specific for iNOS, MMP13, Collagen II was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-MMP1 was obtained from ProteinTech Group (Wuhan, China). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ADAMTS-5, Secondary antibodies and the cell counting kit-8 (CCK-8) were provided from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Rat chondrocyte culture. Animal experiment procedures were approved by the Animal Care and Use Committee of Tongji Medical College (Wuhan, China). 5-day-old Sprague-Dawley rats were used as previously described (16). Briefly, rats were sacrificed by cervical dislocation and articular cartilage was isolated from the knee joints of rats and then cut into pieces. Cartilage pieces were digested in 0.25% trypsin for 30 min and then incubated with 0.25% collagenase II for 8 h at 37°C. Chondrocytes were collected and then cultured in complete culture medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂. Two to three passages were used for our experiment.

Cell viability assay. Cell viability was estimated by the CCK-8 assay. Briefly, rat chondrocytes were seeded in a 96-well plate (2x10³/well). After 24 h of cell adherence, chondrocytes were treated with different concentrations of Kaempferol (0, 5, 10 and 20 µM) for 24 h. Each well was incubated with 100 µl culture medium containing 10 µl CCK-8 solution for 1 h at 37°C with 5% CO₂, and then the absorbance was detected at 450 nm using a micro plate reader (Bio-Rad, Richmond, CA, USA).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. Following the respective treatments, total RNA in each group was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. An NanoDrop spectrometer (Thermo Fisher Scientific, Inc.) was used here to detect the concentration and purity of the total RNA. RNA samples were then reverse-transcribed to cDNA via RT-PCR using a ReverTra Ace qPCR RT kit (Toyobo Life Science, Osaka, Japan), as per manufacturer’s instructions. The expression of mRNA was determined quantitatively using SYBR-Green Realtime PCR Master Mix (Toyobo Life Science) with the following thermocycling conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The GAPDH gene was used as an internal control. Each set of samples included a template-free control and data were analyzed using the 2^-ΔΔCq method (17). Primers used in this study were as follows: GAPDH forward, 5'-CTCATGACC ACAGTCCATGC and reverse, 5'-TTCAAGCTCTGGGATG ACCTT-3'; and MMP13 forward, 5'-AAGATGGGA GTGCC TGATG-3' and reverse, 5'-CCAGTGTAAGGTAGATGGGA AC-3'.

Western blotting analysis. Chondrocytes were co treated with Kaempferol (0, 5, 10 and 20 µM) and IL-1β (10 ng/ml) for 24 h, then cells were lysed with cold RIPA Lysis Buffer supplemented with 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Wuhan Boster Biological Technology, Ltd.). BCA protein assay kit (Wuhan Boster Biological Technology, Ltd.) was used here to detect protein concentration in the lysates. Equal amounts of protein (25 µg) were fractionated on 10% SDS-PAGE gels and transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). Membranes were firstly hatched in blocking buffer containing 5% bovine serum albumin powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h, and then incubated overnight with primary antibodies at 4°C. The primary antibodies are as follows: MMP1 (cat. no. 10371-2-AP; 1:1,500 dilution), MMP-3 (cat. no. 14351; 1:1,000 dilution), MMP13 (cat. no. sc-30073; 1:500 dilution), iNOS (cat. no. sc-7271; 1:500 dilution), COX-2 (cat. no. 12882; 1:1,000 dilution), p-ERK (cat. no. 4370; 1:1,000 dilution), ERK (cat. no. 4695; 1:1,000 dilution), JNK (cat. no. 9258; 1:1,000 dilution), p-JNK (cat. no. 9255; 1:1,000 dilution), p38 (cat. no. 8690; 1:1,000 dilution), p-p38 (cat. no. 4511; 1:1,000 dilution), Collagen II (cat. no. sc-28887; 1:500 dilution), ADAMTS-5 (cat. no. BA3020; 1:500 dilution), GAPDH (cat. no. BM3876; 1:500 dilution). After wash three times with TBST for 15 min, blots were then incubated with HRP-conjugated secondary antibodies (goat Anti-Rabbit IgG, BA1054; 1:5,000 dilution; and goat Anti-Mouse IgG, BA1050, 1:5,000 dilution) for 1 h at room temperature. The immunoreactive bands were visualized using an ECL System (Wuhan Boster Biological Technology, Ltd.). GAPDH was used as an internal control. The western blots were repeated three times and representative bands were presented.

Immunofluorescence staining. Chondrocytes were firstly treated with 20 µM Kaempferol in the presence of 10 ng/ml IL-1β for 24 h. Then, cells were fixed with 4% paraformaldehyde for 15 min. After fully washed with phosphate buffered solution (PBS), the fixed cells were blocked in PBS containing 5% FBS and 0.3% Triton X-100 for 1 h and then incubated overnight at 4°C with anti-collagen II antibody (cat. no. sc-28887; 1:300 dilution; Santa Cruz Biotechnology Inc.). Finally, the fixed cells were washed and incubated with CY3-goat anti-rabbit IgG (cat. no. BA1032; 1:100 dilution; Wuhan Boster Biological Technology, Ltd.) and observed under a standard fluorescence microscope.

Statistical analysis. All experiments were performed three times using independent samples. Data were presented as the mean ± standard deviation. Data were analyzed using GraphPad Prism version 6.00 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons of multiple groups were performed using one- and two-way analysis of variance.
analysis with a post hoc Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*Effects of Kaempferol and IL-1β on chondrocytes viability and MMP13 expression.* The potential toxicity of Kaempferol on chondrocytes was evaluated by CCK-8 assay. As shown in Fig. 1A, there were no significant difference between the viability of cells in the control and that in the 5-20 μM of Kaempferol treatment. To select the appropriate concentration of IL-1β used in vitro model of OA, chondrocytes were treated with various concentrations of IL-1β (2.5-40 ng/ml) for 24 h. The protein level and mRNA expression of MMP13 were used as an indicator here. As shown in Fig. 1B, the expression level of MMP13 peaks at the 10 ng/ml of IL-1β. These results indicated that 10 ng/ml was the appropriate concentration of IL-1β for further experiments.
Effects of Kaempferol on IL-1β-induced COX2 and iNOS expression. Pro-inflammatory mediators such as COX2 and iNOS play a key role in the pathogenesis of OA. So we investigated the effects of Kaempferol on IL-1β-induced COX2 and iNOS expression. The expression of COX2 and iNOS were measured by western blot. As shown in Fig. 2, chondrocytes stimulated with IL-1β (10 ng/ml) showed enhanced production of COX2 and iNOS. However, chondrocytes treated with Kaempferol can significantly inhibit the IL-1β-induced expression of COX2 and iNOS.

Effects of Kaempferol on IL-1β-induced MMPs, ADAMTS5 expression. It is widely known that MMPs and ADAMTS5 are the major catabolic enzymes of the cartilage matrix. Therefore, we investigated the effects of Kaempferol on the protein production of MMP-1, MMP-3, MMP-13, and ADAMTS5. As shown in Fig. 3, IL-1β significantly increased the expression of MMPs and the degradation of collagen II. However, Kaempferol (5, 10 and 20 µM) dose-dependently decreased the protein expression levels of MMP1, MMP3, MMP13 and ADAMTS5 induced by IL-1β.

Effects of Kaempferol on IL-1β-induced collagen II degradation. Collagen II is one of the major components of cartilage matrix. So we explored the effects of Kaempferol on IL-1β-induced collagen II degradation. The expression of collagen II was measured by western blot and Immunohistochemistry. As shown in Fig. 4, IL-1β obviously increased the degradation of collagen II and Kaempferol (5, 10 and 20 µM) could suppress this process in protein levels. The fluorescent results also contributed to the same outcome. Compared to the control group, the positive expression areas...
of collagen II were significantly lower in the IL-1β group. Compared to the IL-1β group, there were significantly increased levels of collagen II in chondrocytes treated with 20 µM Kaempferol.

Effects of Kaempferol on mitogen activated protein kinase (MAPK) signaling pathway. MAPK pathways participate in the process of cartilage degradation. To identify the signaling pathway involved in the anti-inflammatory effects of Kaempferol, chondrocytes were co treated with Kaempferol (0, 5, 10 and 20 µM) and IL-1β (10 ng/ml) for 30 min. As shown in Fig. 5, IL-1β upregulated the protein level of P-P38, P-ERK, P-JNK, while Kaempferol could downregulated the activation of P38 and ERK. It is interesting that we did not observe significant change of JNK pathway.

Discussion

OA is conventionally defined as a typical non-inflammatory joint disease, but nowadays it is increasingly accepted that it is an inflammatory disease (18,19). Mounting evidence has demonstrated that inflammation contributes to the symptoms and the progression of OA (20-22). Pro-inflammatory cytokines, such as IL-1β, is known to be capable of eliciting inflammatory responses. Various studies have confirmed that IL-1β can block the synthesis of the key structural proteins in chondrocytes such as type-II collagen and aggrecan (23). Besides IL-1β also increases the expression of MMPs and aggrecanases, which are a class of proteinases involved in the cartilage degradation (24). Furthermore, IL-1β can also increase the expression of iNOS and COX-2 in chondrocytes, which inhibit cartilage matrix synthesis and promote its degradation (25). Thus, IL-1β is selected here to develop a cellular OA model and treatment that can reverse IL-1β induced inflammation responses may provide new venues for the therapy for OA. In this study, we certified that Kaempferol inhibited IL-1β induced iNOS and COX-2 production and the degeneration of collagen II. Meanwhile, Kaempferol could inhibit IL-1β-induced MMP-1, MMP-3, MMP-13 and ADAMTS5 expression in rat chondrocytes in vitro. These
findings collectively demonstrate the anti-inflammatory effects of Kaempferol against OA. However, in this study we failed to elucidate its protective effects in OA in animal experiments. Further research is required to clarify its mechanism in vivo.

MAPK, including JNK, p38, and ERK signaling pathways, is the central node of multiple signal transduction pathways. A large number of experiments suggested that it was a key upstream signaling pathways in the regulation of inflammatory mediators production involved in the pathogenesis of OA (26). Activation of the MAPK signaling pathway may lead to the secretion of several matrix-degrading proteinases, including the MMPs and the aggrecanases, leading to articular cartilage breakdown. IL-1β, the major proinflammatory cytokine involved the progression of OA, can easily activated the MAPK pathways (27,28). Thus, in our study, we investigated whether the anti-inflammatory effects of Kaempferol were through the attenuation of MAPK. It was observed that the JNK, p38, ERK signaling pathways were obviously activated upon IL-1β stimulation. However, Kaempferol treatment dose-dependently suppressed the phosphorylation of p38 and ERK activation without markedly affecting JNK activation. Therefore, our results indicated that Kaempferol may inhibit IL-1β-stimulated inflammation responses by suppressing the MAPK related ERK and P38 pathways.

Kaempferol, a natural flavonoid extracted from various fruits, has exerted its anti-inflammatory properties in various diseases. Previous studies have reported that Kaempferol inhibits the expression of iNOS in activated macrophages and decreases LPS-induced COX-2 level in RAW 264.7 cells (29,30). Kaempferol can also inhibit IL-1β-induced the production of MMPs in rheumatoid arthritis synovial fibroblasts (31). Interestingly another in vivo study reported that kaempferol could not inhibit MMP-13 induction in IL-1β treated SW1353 cells at 5–25 µM (32). The SW1353 chondrosarcoma cell line is widely used as a candidate in vitro system for exploring chondrocytes anabolism (33). In contrast, however, we found Kaempferol strikingly inhibited MMP-13 induction in IL-1β treated rat chondrocytes at 10–20 µM as well as other MMPs. The cause of the different results may explain to that SW1353 cells may not be a very good alternative for studying chondrocytes anabolism in vitro (34). It was interesting that a recent study reported that kaempferol (25-100 µM) could downregulate the IL-1β-induced COX2 and iNOS expression via NF-κB pathways in chondrocytes (35). However, in the present study, we observed that kaempferol at 10–20 µM concentrations strikingly inhibited Cox2 and iNOS production as well as other inflammation responses.

The limitation of this study was that we failed to elucidate its protective effects in OA in animal experiments. Further research was required to clarify its anti-inflammation effects in vivo. Besides, in present study our data simply showed the inhibition of Kaempferol on the production of inflammatory mediators was possibly by alleviating of activation of the p38 and ERK signaling pathway, but we failed to conduct more experiments to provide detailed insight about the protective effects of kaempferol in OA. Thus, deeper investigation is necessary to ascertain its intrinsic mechanism.

Collectively, our study provided a new insight into the protective effects of kaempferol in OA. Kaempferol could suppress the IL-1β-induced inflammatory reactions such as iNOS2, COX2, MMPs and ADAMTS5. Moreover, cartilage degeneration could also be reversed by kaempferol. Furthermore, Our results also indicate that the downregulation of MMPs with kaempferol treatment was putatively mediated by the MAPK related ERK pathway.

Figure 5. Effects of kaempferol on the MAPK signaling pathway. Cells were treated with IL-1β (10 ng/ml) in the absence or presence of kaempferol (5, 10 and 20 µM) for 30 min. (A) The protein levels of P38, ERK and JNK were determined by western blot analysis. (B) Relative protein expression was then quantified using Image-J software. The results are representative of three separate experiments. *P<0.05 vs. control group; †P<0.05 vs. IL-1β group. IL, interleukin; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated.
and P38 pathway. These findings indicated that kaempferol may serve as a potent anti-arthritic agent for the treatment of OA.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HY and XH conceived the idea for the study. XH carried out the research. QP, ZM, PW, RZ, XM and JC analyzed and interpreted the data. HY and XH wrote the manuscript.

Ethics approval and consent to participate

Animal experiment procedures were approved by the Animal Care and Use Committee of Tongji Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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