

Amentoflavone maintaining extracellular matrix homeostasis and inhibiting subchondral bone loss in Osteoarthritis by Inhibiting ERK, JNK and NF- κ B Signaling Pathways

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Abstract

Amentoflavone (AF), a plant biflavone isolated from *Selaginella sinensis* ethanol extract, is characterized by anti-inflammatory and anti-oxidant properties. According to previous studies, inflammation and oxidative stress are closely related to the pathophysiology of osteoarthritis (OA). However, the effects and mechanisms of AF on OA have not been elucidated. To investigate the inhibitory effects and its molecular mechanism of AF on extracellular matrix (ECM) degradation stimulated by IL-1 β as well as subchondral bone loss induced by RANKL in mice chondrocytes. Quantitative PCR was used to detect the mRNA expression of genes related to inflammation, ECM, and osteoclast differentiation. Protein expression level of iNOS, COX-2, MMP13, ADAMTS5, COL2A1, SOX9, NFATc1, c-fos, JNK, ERK, P65, I κ B α was measured by western blotting. The levels of TNF- α and IL-6 in the supernatants were measured by ELISA. The amount of ECM in chondrocytes was measured using toluidine blue staining. The levels of Aggrecan and Col2a1 in chondrocytes were measured using immunofluorescence. Tartrate-resistant acid phosphatase (TRAP) staining, F-actin staining and immunofluorescence were used to detect the effect of AF on osteoclast differentiation and bone resorption. The effect of AF on destabilization of the medial meniscus (DMM)-induced OA mice can be detected in hematoxylin-eosin (H&E) staining, Safranin O green staining and immunohistochemistry. AF might drastically attenuated IL-1 β -stimulated inflammation and reduction of ECM formation by blocking ERK and NF- κ B signaling pathways in chondrocytes. Meanwhile, AF suppressed the formation of osteoclasts and the resorption of bone function induced by RANKL. In vivo, AF played a protective role by stabilizing cartilage ECM and inhibiting subchondral bone loss in destabilization of the medial meniscus (DMM)-induced OA mice, further proving its protective effect in the development of OA. Our study show that AF alleviated OA by suppressing ERK, JNK and NF- κ B signaling pathways in OA models in vitro and DMM-induced OA mice, suggesting that AF might be a potential therapeutic agent in the treatment of OA.

1. Introduction

Osteoarthritis (OA), a chronic bone disease leading to disability and increased economic burden in the elderly [1], is characterized by chondrocyte inflammation and articular cartilage deterioration throughout the joint, both of which are important factors in the disease progression [2]. Despite the unclear pathophysiology, the essential roles of inflammation and inflammatory factors have been confirmed in the progression of OA. Inflammatory factors can increase the generation of inflammatory mediators and accelerate the articular cartilage destruction [3]. To our great knowledge, IL-1 β figures prominently in cartilage ECM degradation because it can stimulate the expression of matrix metalloproteinases (MMPs), as well as disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [4-5]. Moreover, IL-1 β directly promotes the formation of prostaglandin E2 (PGE2) and nitric oxide (NO) by releasing cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), thereby playing an inflammatory role [6]. The major goal of OA treatment is to relieve joint pain, reduce stiffness, maintain cartilage function, and improve the quality of life [7-8]. However, in terms of OA treatment, due to the limited effective approaches, there exists an urgent requirement for safe and effective medication.

To date, an increasing number of studies show that subchondral bone remodeling is crucial to the pathogenesis of OA [9-10]. The primary function of subchondral bone, which includes subchondral trabecular bone and subchondral cortical plate, is to support cartilage mechanically and nutritionally. Subchondral bone has considerable compositional and structural organization changes with OA progresses, which have a negative impact on the biomechanical environment of the overlaying cartilage [11-12]. Osteoclast, as a unique bone resorptive cell, is one of the key cells involved in subchondral bone remodeling [13]. Therefore, reducing subchondral bone remodeling by inhibiting osteoclast differentiation and activation is expected to become a new target for the treatment of OA.

Amentoflavone, isolated from the ethanol extract of *Selaginella sinensis*, is a bi-flavonoid molecule with anti-viral and anti-inflammatory properties. For instance, amentoflavone was discovered to restrict the formation of nitric oxide and suppress the expression of iNOS in macrophages induced by lipopolysaccharide (LPS) [14]. In addition, amentoflavone was capable of preventing acetic acid-induced ulcerative colitis by maintaining the oxidation/antioxidant balance [15]. In glioblastoma, amentoflavone could inhibit tumor growth by modulating the ERK/NF- κ B signaling pathway [16]. Furthermore, amentoflavone reduced epileptogenesis and displayed neuroprotective benefits by suppressing the NLRP3 inflammasome in PTZ-induced kindling mice [17]. In view of the vague effects of AF for OA, we investigate the effects and subtle mechanisms of AF on IL-1 β -induced mouse chondrocytes and RANKL-induced osteoclasts.

2. Materials and methods

2.1 Chemicals and reagents

Amentoflavone (AF) (purity 98.08%), was purchased from MedChemExpress (Shanghai, China) (Cat. No. HY-N0662, CAS No. : 1617-53-4), dissolved in Dimethyl sulfoxide to yield a stock solution (10 mM), and stored at -80°C . Dulbecco's modified Eagle's medium (DMEM)-high glucose and fetal bovine serum (FBS) were provided by Gibco (New York, US). Cell Counting Kit-8 (CCK-8) was acquired from Servicebio (Wuhan, China). Toluidine blue solution was from Solarbio (Beijing, China). Primary antibodies against MMP13, ADAMTS-5, COL2A1, iNOS, COX-2, NFATc1, c-fos and GAPDH were obtained from Abcam (Shanghai, China). Primary antibodies against P65, phospho-P65 (p-P65), ERK, p-ERK, JNK, p-JNK, I- κ B α and phospho-I- κ B (p-I- κ B α) were obtained from Cell Signaling Technology (Danvers, United States). The 4',6-diamidino-2-phenylindole (DAPI) was provided by Beyotime (Shanghai, China). The staining kit for tartrate-resistant acid phosphatase (TRAP) was from Sigma-Aldrich (St. Louis, MO, United States), recombinant mouse RANKL and Recombinant human macrophage colony-stimulating factor (M-CSF) were from BestBio (Shanghai, China).

2.2 Cell isolation and culture

Primary chondrocytes were harvested from the both knee articular surface of C57BL/6 mice. Briefly, bilateral knee joints of mice were dissected into $1 \times 1 \times 1 \text{ mm}^3$ slices and rinsed three times in PBS.

Subsequently, slices of articular cartilage were first treated with 0.25% trypsin–EDTA solution and digested at 37 °C for 1h. After treatment of 0.25% trypsin–EDTA, digestion was performed with 0.2% collagenase type II at 37°C for 4 h. After centrifugation, the supernatant was discarded to obtain the intracellular aggregates and suspended in DMEM-high glucose with 10% FBS and 1% penicillin/streptomycin. Finally, chondrocytes were inoculated at a density of 1×10^5 cells/ml in T-75 cell culture flasks and cultured at 37°C in a humidified 5%CO₂ environment. From the bone marrow of C57BL/6 mice (4–6 weeks old), primary bone marrow macrophage cells (BMMs) were isolated (4–6 weeks old). In a nutshell, cells were extracted from the femur bone marrow and cultivated for 24 hours in a T75 flask using DMEM with M-CSF (30 ng/ml), FBS (10%), and penicillin/streptomycin (1%). Following the removal of non-adherent cells, adherent cells were continued to be cultivated for an additional 3–4 days until they reached complete confluence. The first-passage chondrocytes were utilized in this study. In this experiment, cells were treated with IL-1 β (10 ng/ml) 48 h.

2.3. Cell viability assay

Chondrocyte viability was detected by Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. Briefly, we inoculated the chondrocytes in 96-well plates (70,00 cells/cm²) for 24h. Afterwards, cells were incubated with various concentrations of AF (0, 1, 2, 5, 10, 20 and 40 μ M) for 24 h, 48 h and 72h, respectively. 10 μ l of CCK-8 was added to each well of the plates and incubated at 37°C in a humidified 5%CO₂ environment. After 2 h, the optimal density (OD) value was read at 450nm with microplate reader (Bio-Tek Instruments, USA). Similarly, CCK-8 assay was used to test the cytotoxic effect of AF on BMMs. All experiments were carried out thrice.

2.4. Extraction of RNA and quantitative RT-PCR analysis

Chondrocytes (2×10^5 cells/well) were seed in 6-well plates and treated with IL-1 β for 48 h, with or without AF (1,5,10 μ M). BMMs (1×10^5 cells/ well) were seeded in 6-well plates and stimulated with RANKL (50 ng/ml) and M-CSF (30 ng/ ml) in the presence or absence of AF (5 μ M) for 5–7 days. Total RNA of chondrocytes was extracted with TRIzol (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The A260/A280 was measured to detect purity and quality. Transcribed complementary DNA was reversed from total RNA (1000ng) using reverse transcriptase (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. LightCycler®480 (Roche, Germany) with FastStart Universal SYBR Green Master Mix (Roche, Germany) was used to perform real-time PCR under the following conditions: denaturation at 94 °C for 5 s, then extension at 60 °C for 30s, lasting for 40 cycles. Target mRNA levels were normalized to GAPDH levels and compared with the control group. The specific primers used were as follows: TNF- α forward 5'-GTCAGATCATCTTCTCGAACC-3' and reverse 5'-CAGATAGATGGGC TCATACC-3', IL-6 forward 5'-GACAGCCACTCACCTCTTCA-3' and reverse 5'-TTCACCAGGCAAGTCTCCTC-3'. iNOS forward 5'-CTC TTC GAC GAC CCA GAA AAC-3' and reverse 5'-CAA GGC CAT GAA GTG AGG CTT- 3', COX-2 forward 5'-CAC CCT GAC ATA GAC AGT GAAAG-3' and reverse 5'-CTG GGT CAC GTT GGA TGA GG-3', MMP13 forward 5'-TGTTTGACAGAGCACTACTTGAA-3'and reverse 5'-CAGTCACCTCTAAGCCAAAGAAA-3', ADAMTS-5 forward 5'-GCAGAACATCGACCAACTCTACTC-3'and

reverse 5'-CCAGCAATGCCCACCGAAC-3', COL2A1 forward 5'-CTCAAGTCGCTGAACAACCA-3' and reverse 5'-GTCTCCGCTCTTCCACTCTG-3', SOX9 forward 5'-GCAGGCGGAGGCAGAGGAG-3' and reverse 5'-GGAGGAGGAGTGTGGCGAGTC-3', c-fos Forward 5'-CCAGTCAAGAGCATCAGCAA-3' and Reverse 5'-AAGTAGTGCAGCCCGGAGTA-3', NFATc1 Forward 5'-GAGTACACCTTCCAGCACCTT-3' and Reverse 5'-TATGATGTCGGGAAAGAGA-3', TRAP Forward 5'-TCATGGGTGGTGCTGCT-3' and Reverse 5'-GCCACAGCCACAAATCT-3', DC-STAMP Forward 5'-AAAACCCTTGGGCTGTTCTT-3' and Reverse 5'-AATCATGGACGACTCCTTGG-3', V-ATPase d2 Forward 5'-AAGCCTTTGTTTGACGCTGT-3' and Reverse 5'-TTCGATGCCTCTGTGAGATG-3', Calcitonin receptor Forward 5'-TGCAGACAACTCTTGTTGG-3' and Reverse 5'-TCGGTTTCTTCTCCTCTGGA-3',

GAPDH forward 5'-TCTCCTCTGACTTCAACAGCGAC-3' and reverse 5'-CCCTGTTGCTGTAGCCAAATTC-3'.

Each gene was analyzed in triplicate.

2.5. Western blot analysis

Total proteins were extracted using RIPA lysis buffer. After centrifugation, the protein concentration was measured using the BCA protein detection kit in accordance with the manufacturer's instructions. The same amounts of protein (40 ng) were isolated by 10% SDS-PAGE and transferred onto PVDF membrane (0.45 µm, Millipore, Bedford, MA, United States). After being sealed with 5% defatted milk for 1 h at room temperature, the membrane was incubated with the corresponding primary antibodies at 4°C overnight. The membrane was then cleaned three times with TBST and incubated with secondary antibodies (1:5000) for 2 h at room temperature. Eventually, the bands on the membranes were detected using Enhanced Chemiluminescence (ECL) kit and ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used to quantify.

2.6. High-Density Culture and Toluidine blue staining

To determine the level of chondrocyte ECM in mice, approximately 12×10^6 primary chondrocytes were digested with trypsin and suspended. 10 µl cell suspension was inoculated into each well. Chondrocytes were adhered to the bottom for approximately 1 h at 37°C. 500 µl of DMEM medium with 10% FBS was then added into each well for 24 h. IL-1β and different concentrations of AF (0, 1, 5, and 10 µM) were added, respectively. After 7–9 days, the cells were fixed in 4% paraformaldehyde about 30 min and stained with toluidine blue. The staining intensity was determined using ImageJ software.

2.7. Immunofluorescence staining

Chondrocytes at a density of 3×10^4 cells were inoculated on 24-well plates and incubated for 48 h. Chondrocytes were then fixed at room temperature for 30 min and treated with 0.1 % Triton X-100 (Sigma Aldrich, Germany) for 10 min. After 30 minutes of blockage with 1 % BSA (Sigma Aldrich, Germany), the primary antibody against Aggrecan (1:500), primary antibody against Col2a1 (1:500) and primary antibody against P65 (1:400) were incubated at 4 °C overnight. Afterward, chondrocytes were incubated

with goat anti-rabbit IgG antibody (1:500) at room temperature for 1 h. Finally, chondrocytes were observed and photographed with Leica fluorescence microscope.

2.8. Osteoclast Differentiation Assay

BMMs were seeded in a 96-well plate at a density of 8×10^3 cells/well in DMEM with 30 ng/mL M-CSF, 50 ng/mL RANKL and different concentrations of AF (0, 1, 5, and 10 μ M) for 5 days. Every two days, a new culture media was replaced. When a significant number of mature osteoclasts emerged in the control well, the osteoclasts were selectively stained using the TRAP kit. We counted TRAP-positive cells (≥ 3 nuclei) and measured their area.

2.9. F-actin ring formation evaluation

BMMs were cultured on glass coverslips and treated with RANKL (50 ng/mL) and 0, 1, 5, 10 μ M AF. Following 5-7 days of cell culture, we fixed the mature osteoclasts in 4% paraformaldehyde for 20 minutes, permeabilized them with 0.1% (v/v) Triton X100 (Sigma-Aldrich) for 5 minutes, and then washed them three times with phosphate-buffered saline (PBS). We used 4', 6-diamidino-2-phenylindole (DAPI) to stain the nuclei and phalloidin tetramethylrhodamine isothiocyanate to stain the F-actin. The distribution of F-actin rings was observed using an LSM5 confocal microscope (Carl Zeiss, Oberkochen, Germany), and they were examined using Zeiss ZEN software.

2.10. DMM-Induced OA mice model

All animal experiment procedures were conducted in accordance with the regulations of Animal Ethics Committee of Nanchang University. C57BL/6 mice (10 weeks old, $n = 18$) were anesthetized by intraperitoneal injection of pentobarbital sodium. The attachment between medial meniscus and tibial plateau of the right knee (medial meniscus ligament) was transected with micro scalpel. All animals were randomly divided into three groups: non-DMM group, DMM group, DMM with 5 mg/kg AF group. In the non-DMM group, only the right knee joint was incised without removing the tibial ligament of the medial meniscus. Mice in the AF group were intraperitoneally injected with 5 mg/kg AF every 2 days for 8 weeks. In the non-DMM group and DMM group, mice were given the same dose of PBS. All mice were euthanized, and their knee tissue samples were collected at 8 weeks postoperatively for further analysis.

2.11. Histological assessment

Knee joints in each group were fixed with 4% paraformaldehyde for 24 h. After fixation, the knee joints were decalcified with 10% EDTA for 4 weeks and embedded in paraffin. Paraffin blocks containing knee joints from mice were cut to a thickness of 5 μ m in varied planes. The sections were then subjected to hematoxylin-eosin (H&E) staining, Safranin O-Fast Green staining and immunohistochemical staining. Articular cartilage injury was assessed by the Osteoarthritis Research Society International (OARSI) scoring system, ranging from 0 (normal) to 6 ($>80\%$ loss of cartilage). Xylene was used for dewaxing and dehydrated with gradient ethanol. After extracting the antigen in the sodium citrate buffer, the slices were incubated with anti-MMP13, anti-Col2a1 antibody (BOSTER; 1:200) at 4 $^{\circ}$ C overnight.

After rinsing with PBS trice (5 min each time), the tissues were incubated with secondary antibody (1:200, Solarbio, China) for 30 min at 37 °C, followed by 3 trice PBS washes. Subsequently, 3, 3'-diaminobenzidine tetrahydrochloride (DAB) kit (ZSGB-BIO, China) was then added and stained for 5-7 min to observe the antibody-antigen complex. Results were observed under microscope (Nikon, Tokyo, Japan).

2.12. Statistical analysis

Data were expressed as mean $\pm$ standard deviation. All experiments were performed at least thrice. All analyses were performed using Student t-test with GraphPad Prism 6.02 software. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of AF on chondrocytes and BMMs viability in mice

The chemical structure of AF was shown in **Fig 1A**. CCK-8 assay was used to detect the cytotoxicity of AF on chondrocytes, and the results showed that AF concentrations below 10 μ M did not affect cell viability within 24 h, 48h and 72h (**Fig 1B**), respectively. In addition, the effects of AF on precursor of osteoclasts (BMMs) was shown in **Fig 1C**. Therefore, AF (1, 5, 10 μ M) is suitable for all subsequent studies.

3.2. AF suppressed the degradation of extracellular matrix in IL1- β -induced chondrocytes in mice

To confirm the effect of AF on ECM degradation in IL-1 β -induced mouse chondrocytes, immunofluorescence and toluidine blue staining were exerted to assess the ability of chondrocytes to secrete ECM. As shown in **Fig2A-C**, Immunofluorescence results indicated that the levels of Aggrecan and Col2a1 in the IL-1 β -stimulated group was lower than that in the control group. However, AF significantly rescued IL-1 β -stimulated Aggrecan and Col2a1 degradation. Toluidine blue staining results proved that the ability of ECM secretion in chondrocytes was significantly decreased after IL-1 β treatment, but promoted by AF in a dose-dependent manner (**Fig2D-E**). These results indicated the obviously protective effect of AF on ECM degradation in IL1- β -induced chondrocytes in mice.

3.3. AF inhibits osteoclastogenesis induced by RANKL and formation of F-actin rings

To measure the effect of AF on RANKL-induced osteoclastogenesis, BMMs were stimulated with M-CSF and RANKL in the presence of various concentrations of AF (0, 1, 5, and 10 μ M). Interestingly, the mature osteoclast formation was significantly and concentration-dependently inhibited in the AF-treated BMMs (**Fig.3A-C**). Additionally, the formation of F-actin rings is essential for osteoclast function and reflects bone resorptive function of osteoclast. Therefore, we started by looking at how AF affected the formation of F-actin rings in RANKL-induced osteoclasts derived from BMMs. In cells treated with various concentrations of AF, F-actin ring formation was suppressed in a concentration-dependent manner (**Fig3D-E**).

3.4. AF regulates ECM-related gene expression and Osteoclast-related Gene Expressions

We assessed the effect of AF on iNOS, COX-2, MMP13 and COL2A1 mRNA expression as well as COL2A1, MMP13 and ADAMTS5 protein expression in IL-1 β -induced mice chondrocytes. The quantitative PCR results indicated that compared with control group, IL-1 β showed a significant upregulation of MMP13, COX-2 and iNOS mRNA expressions and downregulation of COL2A1 mRNA expressions. Whereas, AF inhibited the IL-1 β -induced mRNA expression of MMP13, COX-2 and iNOS and upregulated COL2A1 mRNA expressions (**Fig.4A**). the result of western blot was basically consistent with the quantitative PCR results (**Fig.4C-D**). Furthermore, we investigated how AF affected the expression of genes involved in osteoclast differentiation, such as NFATc1, c-fos, TRAP, DC-STAMP, calcitonin receptor, and V-ATPase d2. The quantitative PCR results revealed that osteoclast-specific gene expressions were elevated by M-CSF and RANKL treatment. However, AF diminished the expression of these genes (**Fig.4B**). the result of western blot was basically consistent with the quantitative PCR results (**Fig.4E-F**).

3.5. AF inhibited the activation of JNK/ERK and NF- κ B signaling pathways in IL1- β -induced mice chondrocytes

On the basis of the crucial roles of JNK, ERK and NF- κ B signaling pathways in cartilage degeneration, Western Blot was used to investigate whether these signaling pathways were involved in inflammation response. As expected, Western Blot revealed that IL-1 β treatment dramatically increased the phosphorylation expression of P65 and I κ B α , and down-regulated the expression of I κ B α (an inhibitor of NF- κ B), suggesting activated NF- κ B signaling pathway in chondrocytes. In contrast, AF strongly diminished the expression of p-P65 and p-I κ B α and enhanced the expression of I κ B α in a dose-dependent manner in IL1- β -induced chondrocytes in mice (**Figure 5A-B**). In addition, JNK and ERK phosphorylation in mice chondrocytes could be greatly promoted by IL-1 β stimulation, and suppressed by AF treatment at various concentrations (**Figure 5C-D**). Taken together, these results indicated that AF attenuated inflammation response in IL-1 β -induced chondrocytes by inhibiting NF- κ B JNK and ERK signaling pathways. Similarly, p65 immunofluorescence staining result revealed that AF exposure reduced the nuclear translocation of p65 in mice chondrocytes (**Figure 5E**).

3.6. AF blocked JNK/ERK and NF- κ B signaling pathways in BMMs

Given the significance of NF- κ B, JNK and ERK pathways in osteoclastogenesis, Western Blot and immunofluorescence staining were used to investigate whether AF inhibited osteoclast differentiation through these signaling pathways. Compared with the control group, the protein level of I κ B α , an inhibitor of NF- κ B, was significantly increased in the AF group, indicating that AF inhibited the NF- κ B pathway. We further investigated how AF affected the activation of ERK and JNK in BMMs because these signaling pathways are equally crucial for osteoclast differentiation. When compared to control group, phosphorylated ERK and JNK were both greatly diminished after AF treatment (**Figure 6A-B**). Meanwhile, immunofluorescence staining result indicated that AF greatly inhibited the nuclear translocation of p65

in BMMs (**Figure 6C**). Collectively, these data suggest that AF blocks osteoclastogenesis via NF- κ B, JNK and ERK signaling pathways.

3.7. AF ameliorated OA progression in DMM-Induced OA mice model

DMM-induced OA mice model was established to investigate the impact of AF on OA *in vivo*. Knee joint samples were subjected to hematoxylin-eosin (H&E) staining, Safranin O-Fast Green staining and immunohistochemical staining. H&E staining and Safranin O-Fast Green staining revealed more proteoglycan loss and cartilage erosion in the DMM group than those in the sham group. Curiously, AF significantly inhibited proteoglycan loss and cartilage destruction compared with the DMM group (**Figure 7A-B**). Consistent with the staining results, the OARSI score of the DMM group was higher than that of the sham group, and that of the AF treated group was lower than that of the DMM group (**Figure 7E**). Immunohistochemical staining was performed to examine the effects of AF on ECM during OA, and AF was found to inhibit the degradation of MMP13 and Col2a1 (**Fig.7C-D**). The percentags of MMP13 and Col2a1-positive cells in each section were quantified by Image J(**Fig.7F-G**), suggesting that AF can ameliorate the development of OA in DMM-induced OA mice model.

4. Discussion

OA, a chronic joint disease, is characterized by degeneration of articular cartilage, joint dysfunction and pain [18]. Persistent inflammatory response features in OA progression, which can be hastened by various inflammatory factors[19]. OA has been identified as one of the leading causes of impairment in the elderly. Currently, medications figure prominently in OA treatment. Despite the successfully reduced short-term pain by several oral drugs, the major side effects still remain, such as digestive system disorders, kidney damage, cardiovascular disease, etc [20-21]. Consequently, it is of urgent requirement for novel drugs to prevent or relieve the pain and symptoms of OA, as well as side effects during the treatment[22]. Recent studies have found the essential role of plant-derived chemicals in the treatment of inflammation-related disorders. As an illustration, amentoflavone, has exhibited its anti-apoptotic, antioxidative, and anti-inflammatory properties in various diseases. Pharmacological effects of AF on numerous diseases have been reported in vivo and in vitro experimental models. Amentoflavone protects rats from ulcerative colitis by altering cytokine profiles and NF- κ B signaling pathway [15]. The anti-apoptotic, anti-inflammatory, and antioxidatant properties of amentoflavone can protect hippocampus neurons and prevent seizures in epileptic mice[23]. In addition, amentoflavone can also slow down tumor development in hepatocellular carcinoma by blocking ERK signaling pathway [24] and enhance apoptosis in breast cancer cells by inhibiting fatty acid production [25]. However, the pharmacological effects of AF on OA remain unclear. In our study, we investigated the effects of amentoflavone on IL-1 β -induced chondrocyte inflammation and RANKL-induced osteoclastogenesis, we found that amentoflavone suppressed IL-1 β -induced inflammatory responses and RANKL-induced osteoclastogenesis in mice by targeting the NF- κ B, JNK and ERK signaling pathways. The potential protective mechanism is illustrated in **Figure 8**. In addition, in vivo studies have confirmed

that AF slow down the OA progression in DMM mice models. As a result, AF has potential application value in OA treatment.

Chondrocytes maintain a balance between synthesis and degradation of extracellular matrix (ECM) in normal joints [26]. ECM is composed of type II collagen and aggrecan, which is responsible for cartilage structure [27]. Previous research has shown that blocking the production of inflammatory mediators NO and PGE2 could contribute to delaying the onset of OA [28]. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) can inhibit type II collagen and proteoglycan synthesis while activating MMPs, which are considered to be important contributors to OA [29-30]. MMPs are a group of proteolytic enzymes working in tissue remodeling [31]. Among the MMPs, MMP13 has been implicated in the progression of OA since it effectively and irreversibly tears down type II collagen, the major structure of ECM [32]. In addition, IL-1 β also promotes the degradation of aggregated proteoglycans by regulating the expression of ADAMTS [33]. In the process of OA, IL-1 β -stimulated inflammatory responses is involved in several signaling pathways, such as PI3K/AKT/mTOR pathway [34], ROS/MAPK/Nrf2 Signaling Pathway [35], Wnt/ β -catenin signaling pathway [36] and JAK2/STAT3 signaling pathway [37]. These signaling pathways have potential mechanism of action during OA in previous studies.

There is substantial evidence that subchondral bone remodeling and articular cartilage are both involved in the development of OA [38, 2]. Osteoclasts, as unique bone resorptive cells, are the main contributors to subchondral bone remodeling [39]. As a result, focusing only on articular cartilage may not be enough to prevent the progression of OA. suppressing osteoclast differentiation and activation has emerged as a novel therapeutic strategy for the treatment of OA [40]. In this study, when RANKL binds to its receptor RANK during osteoclast differentiation, NF- κ B, ERK and ERK signaling can be triggered, which causes the activation and nuclear translocation of osteoclast transcription factors. However, AF significantly inhibit the activation of NF- κ B, ERK and ERK signaling pathways, as well as nuclear translocation of P65.

Furthermore, NF- κ B, JNK and ERK signaling pathways have been extensively studied in IL-1 β -stimulated inflammatory responses [41-43]. Based on the protective effects of AF on inflammatory responses, we aimed to investigate NF- κ B, JNK and ERK signaling pathways, which are known to play a vital role in the regulation of inflammatory response [44-46]. Inhibition of NF- κ B activation has been shown to have a therapeutic impact on OA in previous investigations [47]. NF- κ B inhibitor (I κ Bs) commonly binds to NF-B, which ordinarily exists in cytoplasm. I κ B protein is phosphorylated and destroyed in response to IL-1 β activation, and p65 is translocated from the cytoplasm to nucleus to regulate inflammatory mediators [48-50]. In this study, AF was found to suppress the activation of the NF- κ B, JNK and ERK signaling pathways in mouse chondrocytes by drastically reducing p65, JNK and ERK phosphorylation. However, whether other pathways are implicated in AF in the evolution of OA remains to be elucidated.

The mice DMM model was established to explore the chondroprotective impact of AF on OA. AF treatment effectively retard the progression of OA. Immunohistochemistry revealed a considerable increase in aggrecan levels and a decrease in MMP13 levels, indicating the alterations in the ECM of

cartilage. Ex-vivo models should be established in the future, so as to preferably understand the underlying mechanism of AF in OA. Moreover, we should also attempt to clarify whether AF impacts the progression of OA through other signaling pathways.

Abbreviations

OA, osteoarthritis; AF, Amentoflavone; ECM, extracellular matrix; IL-1 β , interleukin-1 beta; RANKL, receptor activator of nuclear factor- κ B ligand; qPCR, quantitative real-time polymerase chain reaction; COL2a1, type II collagen; SOX9, sex-determining region Y box 9; ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs 5; TNF- α , tumor necrosis factor- α ; MMPs, matrix metalloproteinases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; TRAP, Tartrate-resistant acid phosphatase; PGE2, prostaglandin E2; NO, nitric oxide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M-CSF, macrophage colonystimulating factor; BMMs, bone marrow macrophage cells; DMEM, dulbecco's modified Eagle's medium; PBS, Phosphate Buffer Saline, FBS, fetal bovine serum; PVDF, polyvinylidene fluoride; CCK-8, cell counting kit-8; EDTA, ethylenediaminetetraacetic acid; H&E, hematoxylin and eosin; DMM, destabilized medial meniscus; OARSI, Osteoarthritis Research Society International.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Institutional Review Board of the First Affiliated Hospital of Nanchang University, and written informed consent was obtained from all study participants.

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Authors contributions

JL and JH designed the study. XH, MZ and GX carried out experiments. LH, JY and SL contributed to analysing the data. JH, XH and KQ collected patient specimens and related information. JL wrote the manuscript. SW and SF supervised the overall project. SW and SF revised the paper. All authors reviewed the results and approved the final version of the manuscript.

Data Availability

Data on the findings of this study are available from the corresponding author upon request.

Conflict of Interest

The authors declared no conflict of interest.

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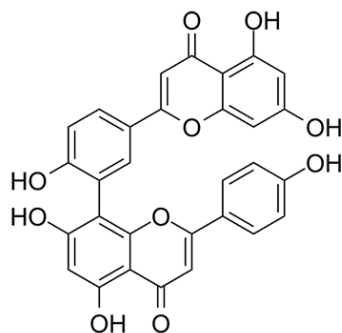
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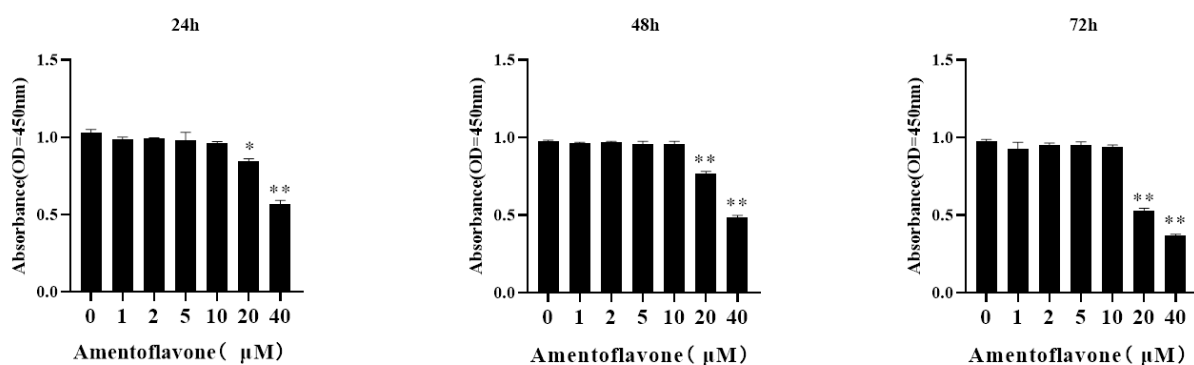
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Figures

A



B



C

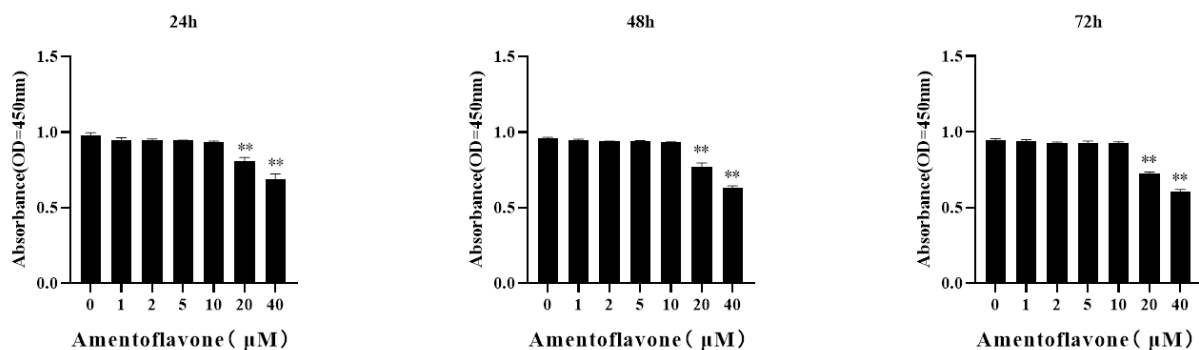


Figure 1

The chemical structure of amentoflavone and the cytotoxicity assay of amentoflavone on mice chondrocytes and BMMs. (A) The chemical structure of AF. (B-C) The cytotoxic effects of AF on chondrocytes and bone marrow macrophages (BMMs) were determined at increasing concentrations for 24, 48 and 72h. The values presented are the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control group.

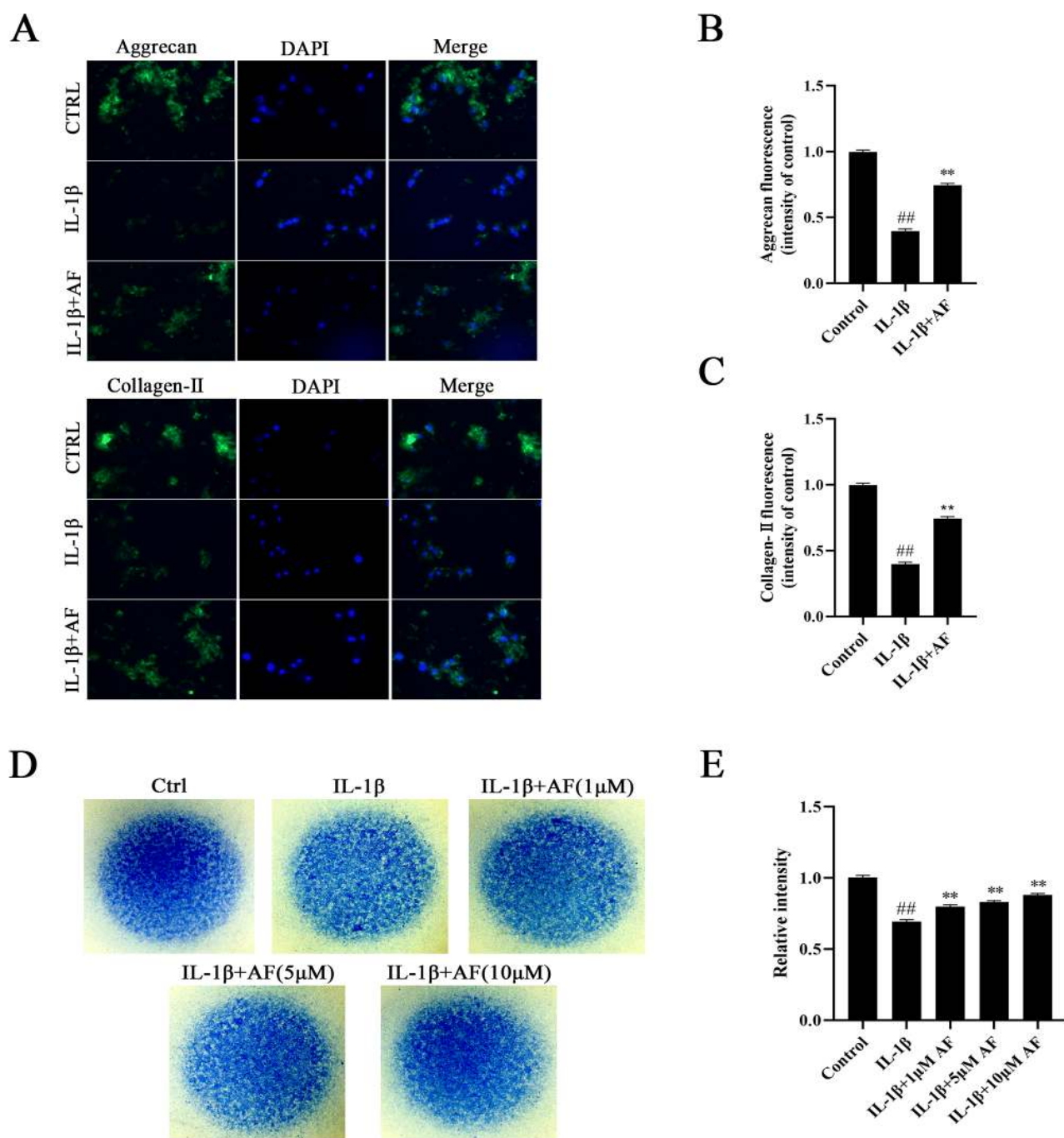


Figure 2

AF suppress the degradation of extracellular matrix in IL1- β -induced mice chondrocytes. (A) Aggrecan and Collagen-II in chondrocytes were determined using immunofluorescence. (B-C) The fluorescence intensities of Aggrecan and Collagen-II were determined using ImageJ software. (D) Toluidine blue staining results for chondrocytes cultured with IL-1 β (10 ng/mL) and various concentrations of AF (0, 1, 5,

and 10μM) for 7–9 days by high density culture. (E)The relative intensity of blue staining. ##p < 0.01 versus the control group and *p <0.05, **p <0.01 versus the IL-1β treatment group.

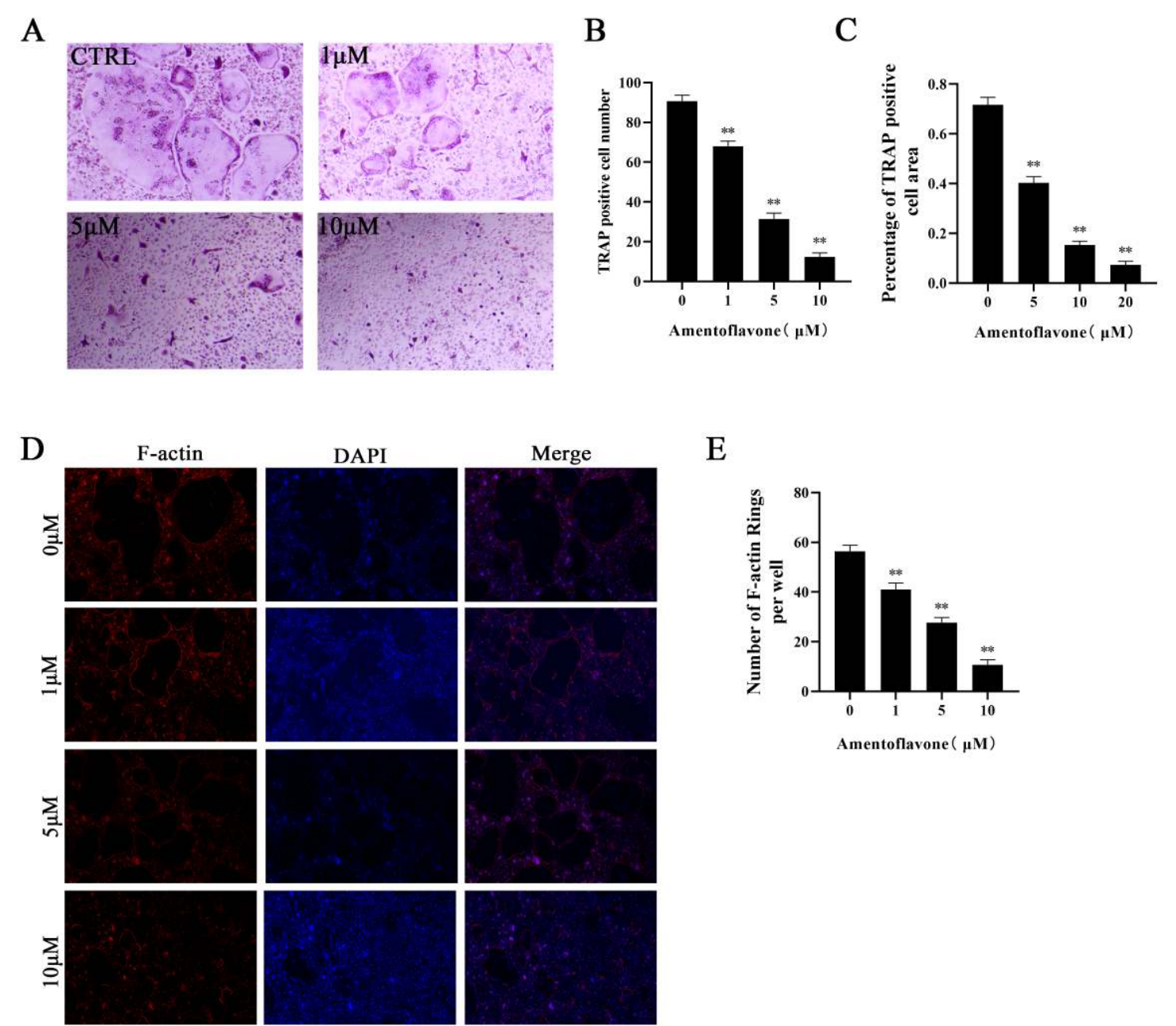


Figure 3

AF inhibits osteoclastogenesis induced by RANKL and formation of F-actin rings. (A) BMMs were cultured with various concentrations AF (0, 1, 5 and 10 μM) and RANKL for 5-7 days, stained by Tartrate-resistant acid phosphatase (TRAP). (B-C) TRAP-positive multinuclear cells numbers and area. (D) Confocal microscopy results of F-actin rings. (E) Quantification of F-actin rings. The values presented are the mean±SD of three independent experiments. *p <0.05, **p <0.01 versus the control group.

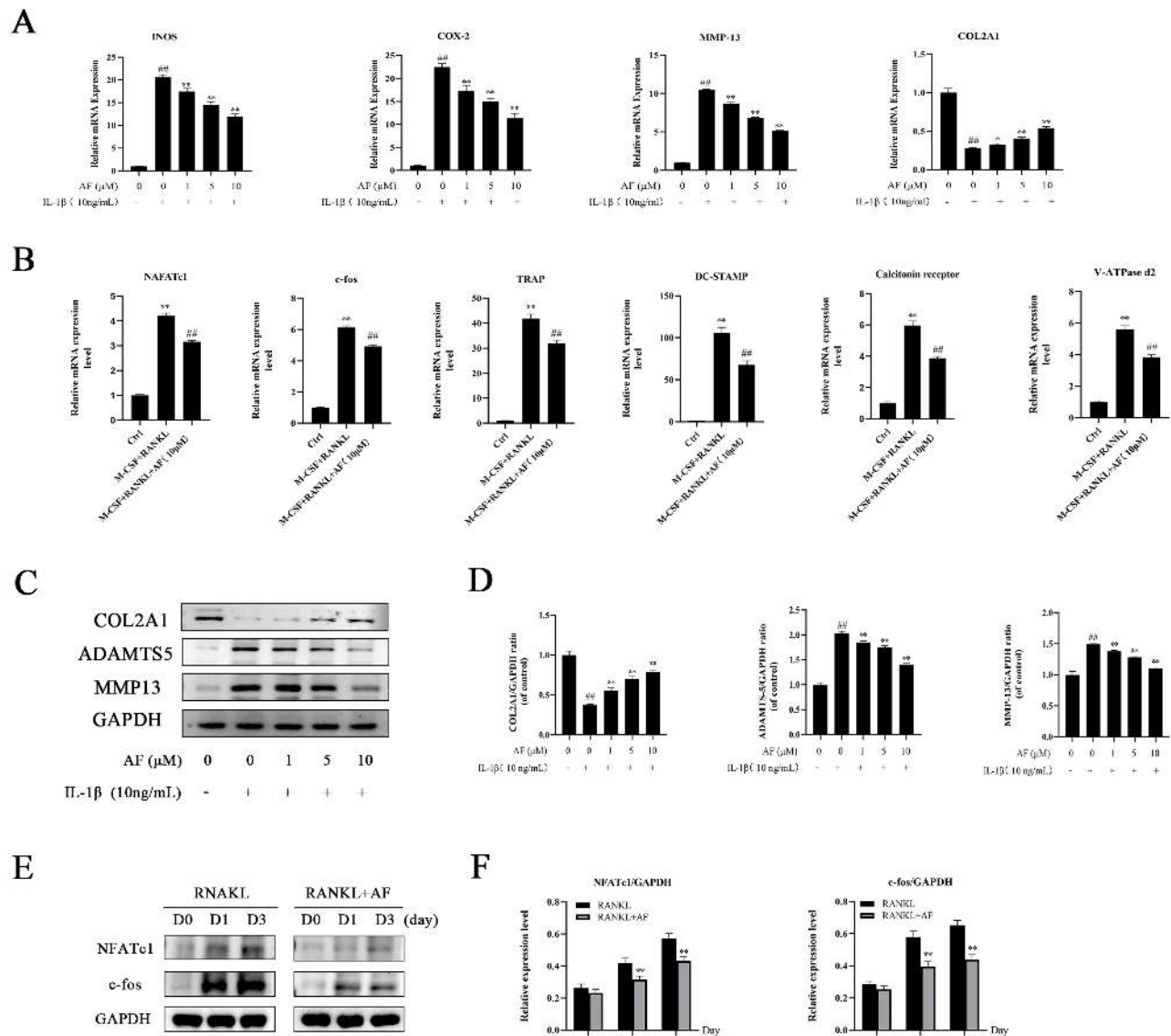


Figure 4

AF regulates ECM-related gene expression and Osteoclast-related Gene Expressions. (A) The mRNA expression of iNOS, COX-2, MMP13 and COL2A1 were analyzed using quantitative PCR. (B) The osteoclast-related gene expressions (c-fos, NFATc1, TRAP, DCSTAMP, calcitonin receptor, and V-ATPase d2) (C) Mice chondrocytes were pretreated with or without AF for 2 h followed by 0 or 10 ng/ml IL-1β for 48 h. (D) Quantification analysis of western blotting. (E) BMMs were pretreated with or without AF for 2 h followed by 0 or 50 ng/ml RANKL for 48 h. (F) Quantification analysis of western blotting. ##p < 0.01 versus the control group and *p < 0.05, **p < 0.01 versus the IL-1β treatment group.

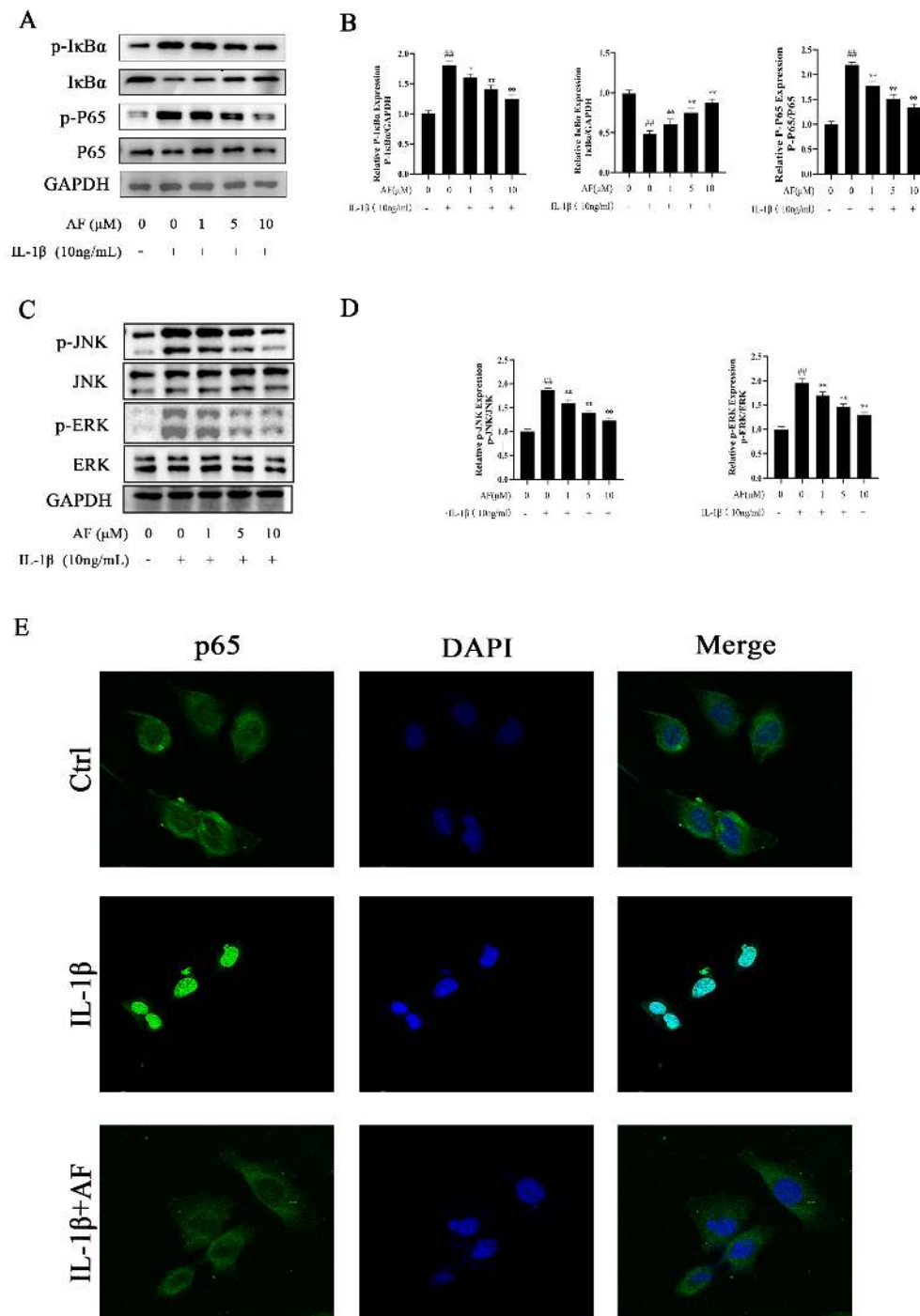


Figure 5

Effect of AF on NF-κB, JNK and ERK signaling pathways in IL-1β-stimulated mice chondrocytes. (A-B) The levels of p-p65, p65 IκBα and p-IκBα were examined by western blot and quantification analysis. (C-D) The levels of JNK, p-JNK, ERK and p-ERK were examined by western blot and quantification analysis. (E) Nuclear translocation of p65 in mice chondrocytes was determined using immunofluorescence. ##p < 0.01 vs. control group; **p < 0.01 vs IL-1β-stimulated group.

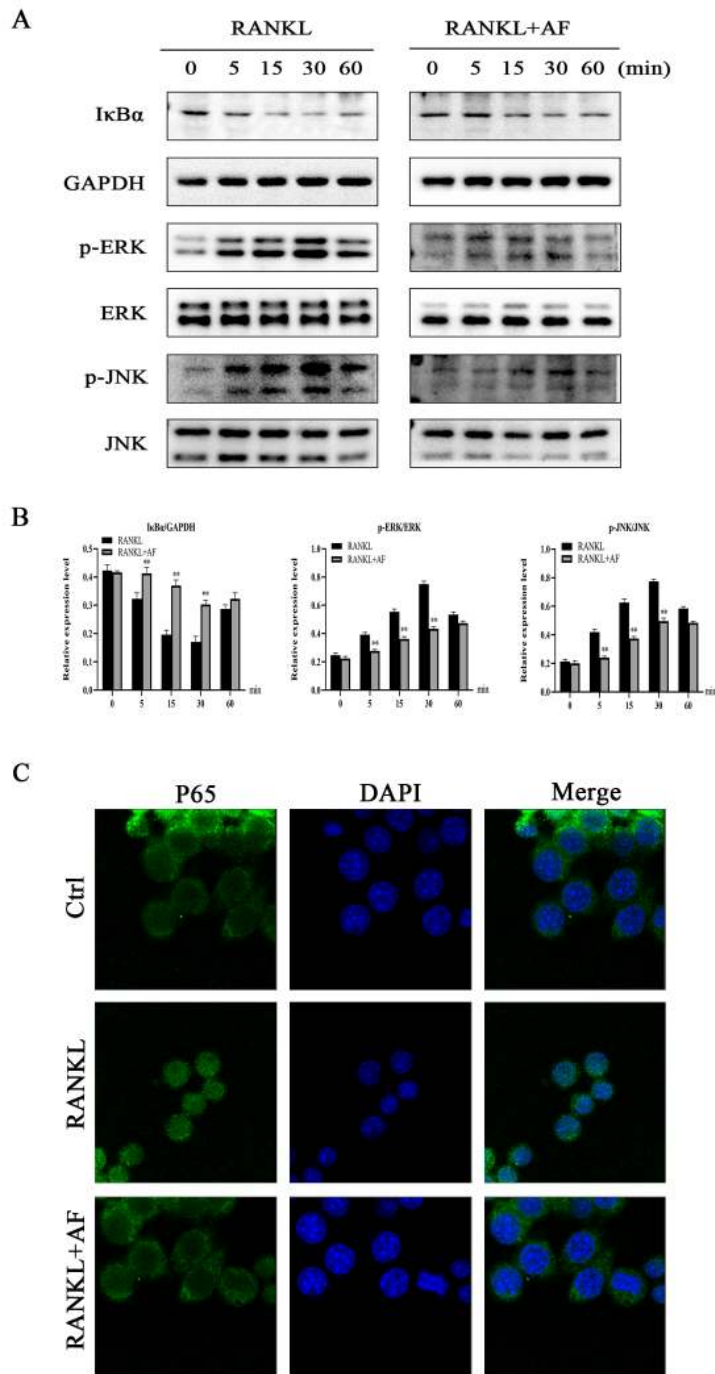


Figure 6

AF blocked JNK/ERK and NF- κ B signaling pathways in BMMs. (A) The levels of I κ B α , JNK, p-JNK, ERK and p-ERK were examined by western blot (B) Quantification analysis of western blotting. (C) Nuclear translocation of p65 in BMMs was determined using immunofluorescence. (* $p < 0.05$; ** $p < 0.01$).

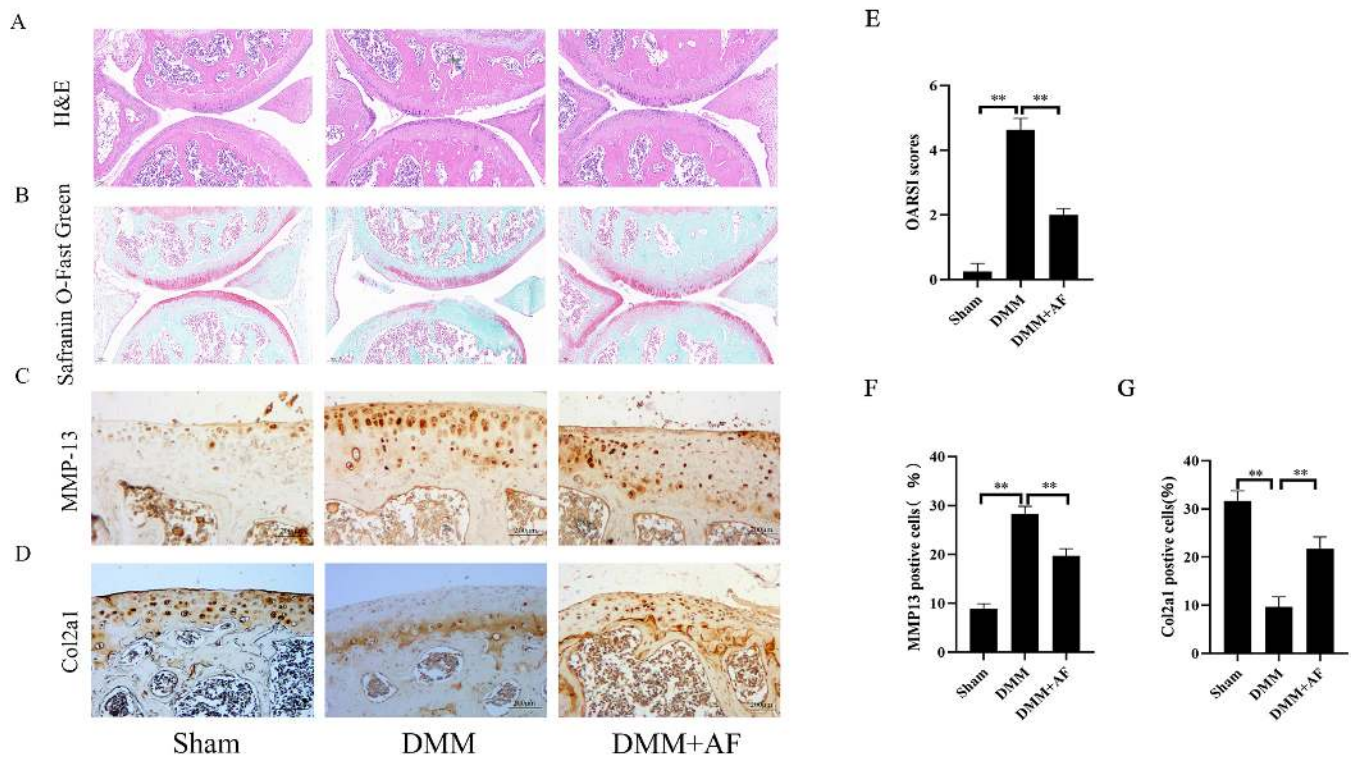


Figure 7

AF ameliorated OA progression in DMM-Induced OA mice model. (A-B) The effect of AF on DMM-stimulated cartilage degeneration was observed by HE Staining and Safranin O-Fast Green staining. (E) Quantitative analysis of OARSI scores. (C-D) Immunohistochemical (MMP13 and Col2a1) staining of mice chondrocytes in articular cartilage and quantitative analysis. (* $p < 0.05$; ** $p < 0.01$).

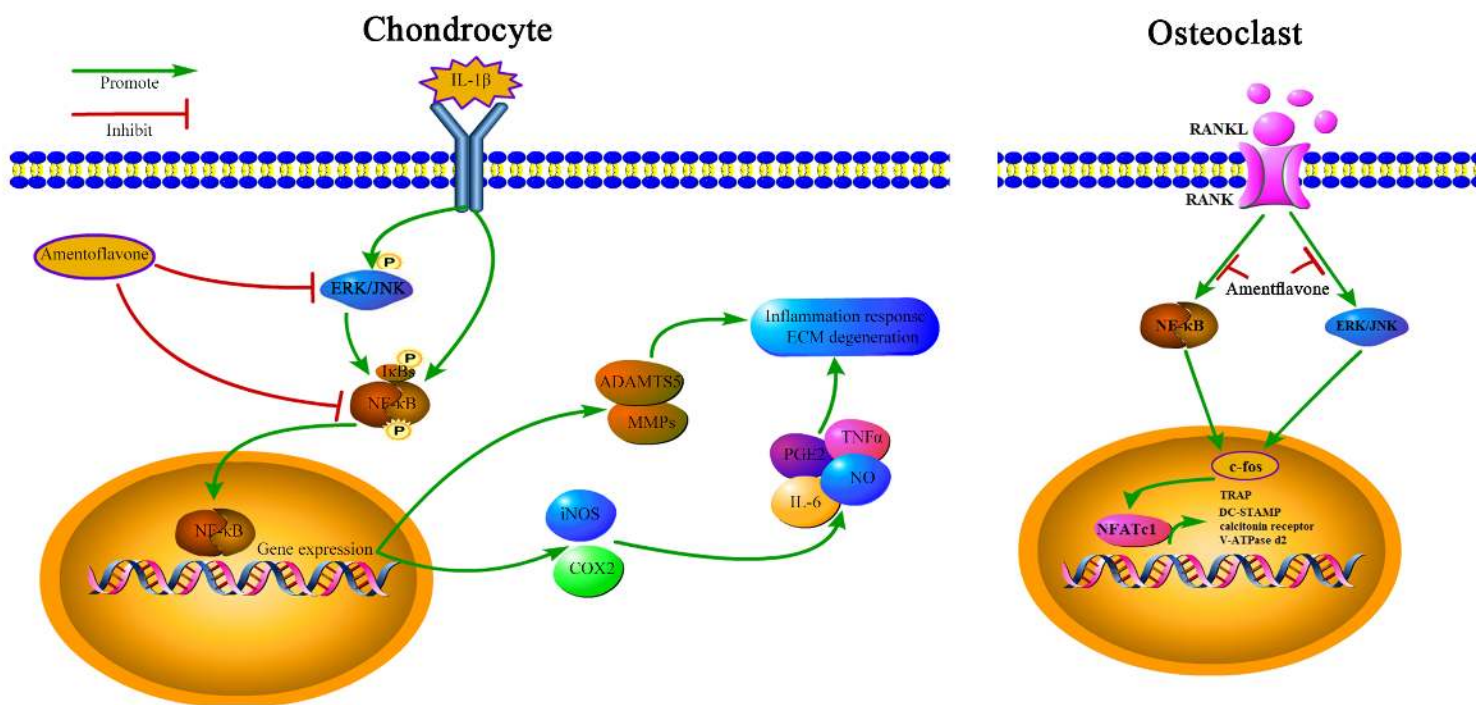


Figure 8

Schematic diagram of the potential protective mechanism and effects of amentoflavone in the development of osteoarthritis.