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Diosmetin ameliorates osteoarthritic inflammation *in vivo* and ECM macromolecules degradation in interleukin-1β-stimulated murine chondrocytes through the Nrf2/NF-κB pathway

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Abstract

Introduction. Osteoarthritis (OA) is a prevailing degenerative disease in elderly population and can lead to severe joint dysfunction. Studies have revealed various pharmacological activities of diosmetin, including the anti-OA efficacy. The present study further investigated its effect on interleukin (IL)-1β-induced OA in chondrocytes.

Material and methods. Primary chondrocytes were isolated from young mice, stimulated with IL-1β (10 ng/mL), and pretreated with diosmetin (10 and 20 μM) to conduct the *in vitro* assays. CCK-8 assay assessed the cytotoxicity of diosmetin whereas the levels of

inflammatory factors (PGE2, nitrite, TNF-α, and IL-6) in homogenized cells were evaluated by ELISA. The levels of inflammatory cytokines, content of extracellular matrix (ECM), and signaling-related proteins (Nrf2, HO-1, and NF-κB p65) were assessed by western blotting. Expression of collagen II, p65, and Nrf2 in the chondrocytes was confirmed by immunofluorescence staining. The chondrocytes treated with IL-1β and diosmetin were transfected with Nrf2 knockdown plasmid (si-Nrf2) to investigate the role of Nrf2. *In vivo* OA mouse model was induced by surgically destabilizing the medial meniscus (DMM). Safranin O staining was conducted to assess the OA severity in the knee-joint tissue.

Results. Diosmetin suppressed the expression of iNOS, COX-2, PGE2, nitrite, TNF-α, IL-6, MMP-13, and ADAMTS-5 induced by IL-1β in chondrocytes. The expression of p-p65, p-IκBα, and nuclear p65 was decreased whereas that of Nrf2 and HO-1 increased by diosmetin treatment in IL-1β-treated chondrocytes. Nrf2 knockdown by siRNA reversed the inhibitory effect of diosmetin on IL-1β-induced degradation of ECM proteins and inflammatory factors in cultured chondrocytes. In the DMM-induced model of OA, diosmetin alleviated cartilage degeneration and decreased the Osteoarthritis Research Society International score.

Conclusions. Diosmetin ameliorates expression of inflammation biomarkers and ECM macromolecules degradation in cultured murine chondrocytes *via* inactivation of NF-κB signaling by activating Nrf2/HO-1 signaling pathway. *(Folia Histochem Cytobiol 2024; 62(2): xx–xx)*

Keywords: mouse; osteoarthritis; chondrocytes; diosmetin; ECM proteins; Nrf2/HO-1; NF-κB

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disorder that leads to unbearable pain, seriously affecting the life quality of more than 250 million people worldwide [1]. Degeneration and inflammation of articular cartilage are the two main features of it [2]. Current treatments for OA mainly focus on the pain management and symptom alleviation [3]. However, those pain-

relief drugs, such as corticosteroids, opioids, and nonsteroidal anti-inflammatory drugs (NSAIDs) have certain side effects, and surgical treatment also carries significant risks, which remain an obstacle to preventing OA [4]. Thus, novel therapeutic targets with fewer side effects are needed.

Increasing studies have revealed inflammatory mechanisms and cartilage extracellular matrix (ECM) damage as the crucial risk factors for the OA progression [5]. It is well known that the key pro-inflammatory cytokine interleukin-1β (IL-1β) is highly expressed in the cartilage tissue of OA patients [6]. It can trigger the secretion of other inflammatory mediators including inducible nitric oxide synthase 2 (iNOS) and cyclooxygenase-2 (COX-2) and proteolytic enzymes including matrix metalloproteinases (MMPs) in OA chondrocytes, resulting in the disorders of the homeostasis between ECM synthesis and degradation [7]. Therefore, inhibition of IL-1β-induced inflammation and ECM degradation has become a potential approach to alleviate the progression of OA.

Diosmetin (3′ ,5,7-trihydroxy-4′-methoxy flavone) is a type of flavonoid, which is abundant in lemon peel and citrus fruits [8]. Hesperidin, an another flavanone derived from *Citrus aurantium L.*, has been reported to repress the OA development [9]. Multiple pharmacological activities of diosmetin have been found, such as anti-cancer [10], anti-oxidant [11], anti-microbial [12], anti-apoptotic [13], anti-estrogenic [14], anti-hyperglycemic [15], and anti-diabetic [16]. Particularly, it has also been reported to be effective in inhibiting inflammatory reaction in various diseases, including myocardial ischemia-reperfusion injury [17], colitis [18], and rheumatoid arthritis [19]. In recent years, the beneficial effects of diosmetin on bone-related diseases have been revealed. For instance, diosmetin regulates bone formation and has an anti-osteoporosis function [20, 21]. Shao *et al*. revealed its inhibitory effect on osteolysis induced by lipopolysaccharide [22]. It can also protect against osteopenia induced by chronic kidney disease [23]. In a very recent study, diosmetin has been shown to prevent the loss of subchondral bone in OA mouse model [24]. However, its specific role in IL-1β-treated chondrocytes has not been detected yet.

In this study, we investigated the effect of diosmetin on IL-1β-induced degradation of ECM proteins and inflammatory response in cultured murine chondrocytes. We also revealed the

possible signaling pathways that participate in the regulatory role of diosmetin in OA. The results of this study may provide novel research option for improving OA treatments.

MATERIALS AND METHODS

Animal model

C57BL/6 mice (male; 5–8 weeks old) were provided by the Animal Center of Chinese Academy of Sciences (Shanghai, China) and randomly divided into three groups (n = 12 in each group): Sham; DMM; and DMM + diosmetin. All the mice were anesthetized using 1% sodium pentobarbital (60 mg/kg). To establish an OA model, the right knee joint capsule of the mouse was exposed, and then medial meniscotibial ligaments was sectioned to induce the destabilization of the medial meniscus (DMM) as described by Glasson *et al.* [25]. Mice in the Sham group received arthrotomy without transecting the meniscotibial ligament. Finally, the right knee joint capsule and skin were sutured using surgical absorbable suture. Two weeks after the DMM surgery, diosmetin (10 mg/kg; purity: 99.80%; 520-34-3; MedChemExpress, China) dissolved in sterile saline was subcutaneously injected into the mice in the DMM + diosmetin group every other day for 7 consecutive days. The treatment dose was used according to the previous study [22]. Meanwhile, the mice in the Sham and DMM groups only received l ml of sterile 0.9% NaCl. At the end of the drug treatment, all the mice were euthanized using an overdose injection of pentobarbital sodium (100 mg/kg), and the knee joints of all mice were resected for the subsequent experiments. All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethical Review System for Laboratory Animal Welfare of the Wuhan Myhalic Biotechnology Co., Ltd (approval number: HLK-20207112; approval date: 2023.7.26).

Safranin O staining

The collected knee joints were fixed in paraformaldehyde (4%; Sigma-Aldrich, MO, USA) for 24 h and then decalcified in EDTA (10%; Sigma-Aldrich). Followed by paraffin embedding, the specimens were sectioned into slices (5 μm-thick) and stained with safranin

O/fast green (Solarbio, Beijing, China) [26]. Then, the Osteoarthritis Research Society International (OARSI) scoring system was used to grade the severity of OA (between 0 and 15) in each group according to Glasson *et al*. recommendations [27].

Cell culture and treatment

Primary chondrocytes were isolated from C57BL/6 mice (2 weeks old; Animal Center of Chinese Academy of Sciences). Briefly, the articular cartilage was separated from the knee joint of the mice after euthanasia (cervical dislocation under inhalation anesthesia) and cut into 1–3 mm pieces, which were then treated with trypsin (0.25%; Gibco, CA, USA) for 10 min and collagenase type II (0.25%; Gibco) for 1.5 h at 37°C and centrifuged for 5 min at 1000 *g* to obtain the cell pellet. Then, the collected chondrocytes were cultured in Dulbecco's modified Eagle's medium (Gibco) containing penicillin/streptomycin (1%; Gibco) and fetal bovine serum (10%; Gibco) at 37°C. Cells were passaged when the confluency reached 80–90%, and the second-generation chondrocytes were selected. Chondrocytes were stimulated with IL-1β (10 ng/mL; PeproTech, Drive Cranbury, NJ, USA) for 24 h and then the cells were treated with diosmetin (10 and 20 μM) for 48 h. In rescue assay, cells were transfected with si-Nrf2 (sc-37030; Santa Cruz, USA) and its control using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA). Transfection efficiency was verified using western blotting analysis.

CCK-8 assay

Cell counting kit-8 (Beyotime, Haimen, Jiangsu**,** China) was used to assess the cytotoxicity of diosmetin to mouse chondrocytes. Chondrocytes were seeded on 96-well plates and treated with diosmetin (5, 10, 20, or 30 μM) for 24 h and 48 h. Then, the CCK-8 solution (10 μL) was added. After 4 h of incubation, the absorbance at 450 nm was determined using a microplate reader (Thermo Fisher).

Western blotting

Total protein extraction from the treated mouse chondrocytes was conducted using RIPA lysis buffer (Beyotime), and the BCA protein assay kit (Beyotime) was used to evaluate the protein concentration. Proteins (50 μg) were separated by SDS-PAGE (10% gels) and electro-

transferred onto PVDF membrane (Millipore, USA), which was subsequently blocked with 5% skim milk. Primary antibodies against iNOS (ab178945; 1:1000; Abcam, UK), COX-2 (ab179800; 1:1000), collagen II (ab34712; 1:1000), aggrecan (FNab00213; 1:500; FineTest, China), MMP-13 (ab39012; 1:3000), ADAMTS-5 (ab41037; 1:250), p-p65 (ab76302; 1:1000), p65 (ab32536; 1:1000), p-IκBα (ab133462; 1:10000), IκBα (ab32518; 1:1000), Nrf2 (abs130481; 1:500; Absin Bioscience, China), HO-1 (ab52947; 1:2000), GAPDH (ab181602; 1:10000), and lamin B (ab151735; 1:2000) were incubated at 4°C with the membranes overnight. Then, goat anti-rabbit secondary antibody (ab205718; 1:2000) was added. Finally, electrochemiluminescence plus reagent (Invitrogen, China) was prepared for visualizing the protein bands, and the intensity of protein bands was quantified with Image Lab 3.0 software (Bio-Rad, USA).

ELISA

Mouse chondrocytes were seeded on 6-well plates and treated with IL-1β (10 ng/mL) for 24 h and diosmetin (10 and 20 μM) for 48 h. Then the levels of PGE2, Nitrite, TNF-α, and IL-6 in the cell culture supernatants were measured by ELISA kits (Invitrogen) according to the manufacturer's protocol.

Immunofluorescence

The chondrocytes were fixed with 4% paraformaldehyde for 10 min and permeabilized with Triton X-100 (0.1%; Sigma-Aldrich) for 15 min. After rinsing with phosphate buffer saline, cells were incubated with primary antibodies against Collagen II (ab34712), p65 (ab32536), and Nrf2 (abs130481; 1:100; Absin Bioscience) at 4°C overnight. Then, the secondary antibodies were added (ab150077; 1:200) for 1 h at room temperature. Then, the nuclei of cells were stained with 4',6-diamidino-2'-phenylindole (Sigma-Aldrich) for 30 min. At the end, an Olympus IX73 fluorescence microscope (Olympus, Tokyo, Japan) was used for analyzing the results.

Statistical analysis

SPSS18.0 software (IBM, USA) was used to conduct the statistical analysis, and the data are

presented as mean ± SD. Significant differences ($P < .05$) were analyzed using Student's *t*-test or one-way analysis of variance followed by the Tukey *post hoc* tests. Nonparametric data (OARSI scores) were evaluated using the Kruskal-Wallis H test. All the experiments were repeated at least three times.

RESULTS

Diosmetin inhibits IL-1β-induced markers of inflammation in chondrocytes

To confirm the optimal concentrations of diosmetin, CCK-8 assay was conducted to evaluate the cell viability of chondrocytes under the treatment with diosmetin (5, 10, 20, and 30 μM). Chondrocytes viability had no significant alteration when the cells were treated with 5, 10, and 20 μM of diosmetin for 24 and 48 h; however, 30 μM of diosmetin slightly reduced the viability of cells after 48 h (Fig. 1A). Therefore, diosmetin at 10 μM and 20 μM concentrations was used for the subsequent experiments. After the stimulation of chondrocytes by IL-1β, the protein levels of enzymes producing inflammatory mediators (iNOS and COX-2) were increased in the chondrocytes. Subsequent treatment of cells with diosmetin remarkably reduced the protein levels of iNOS and COX-2 (Fig. 1B, C).

To further check the effect of diosmetin on inflammatory biomarkers induced by IL-1β in chondrocytes, ELISA was performed to further assess the changes in PGE2, nitrite, TNF-α, and IL-6 levels in the supernatants. Consistently, the treatment with diosmetin suppressed the release of PGE2, Nitrite, TNF-α, and IL-6, indicating its anti-inflammatory role in the *in vitro* OA model (Fig. 1D–G).

Diosmetin suppresses IL-1β-induced ECM molecules' degradation in chondrocytes

In this part of our study, we further explored the effect of diosmetin on ECM molecules degradation. We found (Fig. 2) that the levels of collagen II and aggrecan proteins were decreased while the levels of MMP-13 and ADAMTS-5 proteins were increased in the chondrocytes stimulated by IL-1β, suggesting severe degradation of ECM molecular components in chondrocytes. By contrast, diosmetin promoted collagen II and aggrecan expression and restrained MMP-13 and ADAMTS-5 expression in IL-1β-treated chondrocytes

(Fig. 2A–C). Immunofluorescence also showed decreased collagen II expression after IL-1β stimulation, while collagen II expression was restored in the cells incubated with IL-1β and diosmetin (Fig. 2D), indicating that diosmetin had inhibitory effect on ECM molecules' degradation in IL-1β-treated chondrocytes.

Diosmetin inactivates NF-κB signaling pathway in IL-1β-treated chondrocytes

To investigate the potential mechanisms of diosmetin action, expression of signaling-related proteins was evaluated in cultured chondrocytes. Relative to the control group, IL-1β upregulated p-p65 and p-IκBα expression and downregulated IκBα expression, and diosmetin reversed these alterations (Fig. 3A, B). Additionally, the protein level of nuclear p65 in the chondrocytes was increased by IL-1β and was reduced by diosmetin (Fig. 3C, D). Consistent with that, immunofluorescence showed that diosmetin decreased the fluorescence of nuclear p65 induced by IL-1β in the chondrocytes (Fig. 3E), suggesting that diosmetin inhibited NF-κB signaling by blocking NF-κB p65 nuclear translocation.

Diosmetin activates Nrf2/HO-1 signaling pathway in IL-1β-treated chondrocytes

Western blotting revealed that the levels of nuclear Nrf2 and HO-1 proteins were not affected by IL-1β in chondrocytes. However, diosmetin treatment markedly enhanced the protein levels of nuclear Nrf2 and HO-1 in IL-1β-treated chondrocytes (Fig. 4A, B). Consistently, enhanced fluorescence intensity of nuclear Nrf2 was observed in the IL-1β + diosmetin group relative to the IL-1β group (Fig. 4C), suggesting that diosmetin activated Nrf2/HO-1 pathway in IL-1β-treated chondrocytes.

Diosmetin inhibits IL-1β-induced changes in chondrocytes *via* activation of Nrf2/HO-1 signaling pathway

In this part of our study, chondrocytes were treated with IL-1β (10 ng/mL) and diosmetin (20 μM) and then transfected with Nrf2 knockdown plasmid (si-Nrf2) or its control (si-NC) to investigate how Nrf2/HO-1 signaling possibly influences the diosmetin action in OA. Transfection efficiency was then confirmed by western blotting, which showed that the protein levels of nuclear Nrf2 and HO-1 were markedly reduced in the si-Nrf2-treated

chondrocytes si-Nrf2 compared with the cells transfected with control plasmid (Fig. 5A, B). Interestingly, the downregulated protein levels of nuclear p65 and p-p65 induced by diosmetin were restored by Nrf2 knockdown (Fig. 5C, D). In addition, the elevated protein levels of inflammatory markers (iNOS and COX-2) and ECM degradation markers (MMP-13 and ADAMTS-5) induced by IL-1β were decreased by diosmetin, whereas Nrf2 knockdown counteracted these diosmetin effects (Fig. 5E, F). Moreover, the effect of diosmetin on the levels of inflammatory biomarkers PGE2, nitrite, TNF-α, and IL-6 in the supernatants of IL-1β-treated cells was also abolished by Nrf2 knockdown (Fig. 5G–J). These results suggested that diosmetin may inhibit NF-κB signaling-mediated inflammatory response by activating Nrf2.

Diosmetin improves osteoarthritis progression in destabilized medial meniscus (DMM) mouse model

The OA mouse model was established by the DMM surgery to investigate the role of diosmetin in protecting articular cartilage. According to the results of Safranin O staining, the mice in the DMM group presented early OA-like manifestations, including erosion of the cartilage and massive loss of proteoglycan staining with higher OARSI score compared to the control (Fig. 6). In contrast, diosmetin treatment significantly slowed the OA progression in mouse model, which was evidenced by less cartilage erosion, smoother cartilage surface, and more proteoglycan staining than the DMM group. In addition, the increase of OARSI score in the DMM group was suppressed by diosmetin as well, indicating its protective effect against pathological osteoarthritic changes (Fig. 6A, B).

DISCUSSION

In recent years, increasing plant-based natural agents have caught much attention of researchers and have been confirmed as potential drugs to treat OA, such as Astilbin extracted from Dimorphandra Mollis [28], murine OA model), β-HIVS derived from Lithospermum erythrorhizon [29], murine OA model), and Xanthohumol isolated from hops [30], rat OA model). Diosmetin from *Citrus aurantium L.* has been shown to protect cartilage in a

surgically-induced OA mouse model [24]. We for the first time confirmed the anti-OA effect of diosmetin occurs *via* Nrf2/NF-κB signaling pathways. Inflammation has been proved to be a critical factor in the pathogenesis of OA [28]. IL-1β is often used as an inducer of OA in articular chondrocytes, and it was shown to stimulate the secretion of inflammatory cytokines IL-6 and TNF-α, leading to an inflammatory response [29]. Diosmetin is recognized as an anti-inflammatory agent in various diseases. For example, in atopic dermatitis model, it has been reported to inhibit the production of nitric oxide (NO) and reduce the expression of iNOS [30]. Yu *et al.* also demonstrated its inhibitory effect on iNOS expression in cerulein-induced acute pancreatitis [31]. Similarly, we found that the levels of iNOS and COX-2 proteins and their products were elevated in mouse chondrocytes under the stimulation by IL-1β, and their levels were significantly reduced by diosmetin treatment, suggesting the anti-inflammatory action of diosmetin in the IL-1β-induced OA *in vitro* model. It has been reported that PGE2, nitrite, TNF-α, and IL-6 are upregulated in OA animal models [32, 33]. PGE2, nitrite, TNF-α, and IL-6 are regarded as important catabolic factors that can trigger the degradation of ECM macromolecules in chondrocytes [7, 34]. In our study, diosmetin remarkably reduced levels of those catabolic factors in IL-1β-treated chondrocytes, suggesting that during OA *in vivo* diosmetin may suppress the ECM degradation and protect chondrocytes and articular cartilage against inflammation-induced dysfunction.

Many studies revealed that impaired ECM metabolism also contributes to OA progression [35]. Under IL-1β stimulation in chondrocytes, key ECM degradative enzymes, including MMPs and aggrecanases, are upregulated, and collagen II and aggrecan are downregulated, which contribute to ECM molecules' degradation [29]. Consistently, here, IL-1β-induced murine chondrocytes showed decreased expression of collagen II and aggrecan and increased levels of MMP-13 and ADAMTS-5, while diosmetin reversed the changes, suggesting its suppressive effect on IL-1β-induced ECM degradation.

As an upstream regulatory factor of inflammation-related factors (iNOS, IL-1β, and TNF-α and other) and cartilage-degrading enzymes (ADAMTs and MMPs), NF-κB signaling has been widely reported to participate in inflammatory processes and degradation of ECM in articular cartilage and chondrocytes [36, 37]. After stimulation by IL-1β, the IκB subunit can

be activated, resulting in the transport of NF-κB p65 to the nucleus [38, 39]. Diosmetin has been previously reported to exert anti-inflammatory effect by decreasing the p-JNK and p-NF-κB protein expression in the aorta of hypertensive rats [40]. Chen *et al.* reported that diosmetin inhibited inflammatory response in synoviocyte-like MH7A cells by inhibiting Akt and NF-κB pathways, suppressing features of rheumatoid arthritis *in vitro* model *[19*]. Here, we found that diosmetin reversed the upregulated p-p65, p-IκBα, nuclear p65 expression and downregulated IκBα expression induced by IL-1β in chondrocytes, indicating that probably *via* inactivation of NF-κB signaling diosmetin inhibits markers of inflammation and ECM degradation in IL-1β-exposed chondrocytes.

Nrf2 is demonstrated to be responsible for the inactivation of NF-κB pathway [41], and emerging evidence has implied the key role of Nrf2 pathway in alleviating IL-1β-induced ECM molecule's degradation in chondrocytes *via* the inhibition of NF-κB pathway [42, 43]. Consistent with the previous study showing the effect of diosmetin on activation of Nrf2 in thoratic aorta of hypertensive rats [40], also in our in vitro OA model, diosmetin upregulated the expression of Nrf2 and HO-1 protein in IL-1β-treated chondrocytes, which confirmed the beneficial involvement of Nrf2/HO-1 pathway. Furthermore, we were the first to show that Nrf2 knockdown in IL-1β-treated chondrocytes significantly abolished the inhibitory effect of diosmetin on NF-κB signaling activation, inflammation, and ECM molecule's degradation, indicating that diosmetin mitigates OA development *via* the inactivation of NF-κB signaling by promoting Nrf2/HO-1 pathway.

To verify the anti-inflammatory effect of diosmetin elucidated in the *in vitro* OA model, we conducted a histological analysis of the articular cartilage of mice subjected to the classical DMM OA model. The protective effect of diosmetin against IL-1β-induced inflammatory in the *in vitro* model of OA has been confirmed in the *in vivo* model. Diosmetin dampened pathological osteoarthritic changes including cartilage erosion and massive loss of proteoglycans. In addition, the reduced OARSI score was shown in diosmetin-treated OA mice, which confirmed the therapeutic effect of diosmetin in murine OA model.

In conclusion, the current study revealed that diosmetin effectively alleviated OA progression under *in vivo* and *in vitro* conditions acting *via* the inactivation of NF-κB signaling pathway

by activating Nrf2/HO-1 signaling (Fig. 7). The results of our study proved that diosmetin could be considered as a potential drug for OA treatment.

Article information and declarations

Data availability statement

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

Ethics statement

All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethical Review System for Laboratory Animal Welfare of the Wuhan Myhalic Biotechnology Co., Ltd (approval number: HLK-20207112; approval date: 2023.7.26).

Author contributions

Liang Qian was the main designer of this study. Liang Qian, Chuang Li, Hong Liu, Hui Zhou and Tao Tan collected and analyzed the data. Liang Qian, Chuang Li, Hong Liu, Hui Zhou and Tao Tan drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declared no competing interests in this study.

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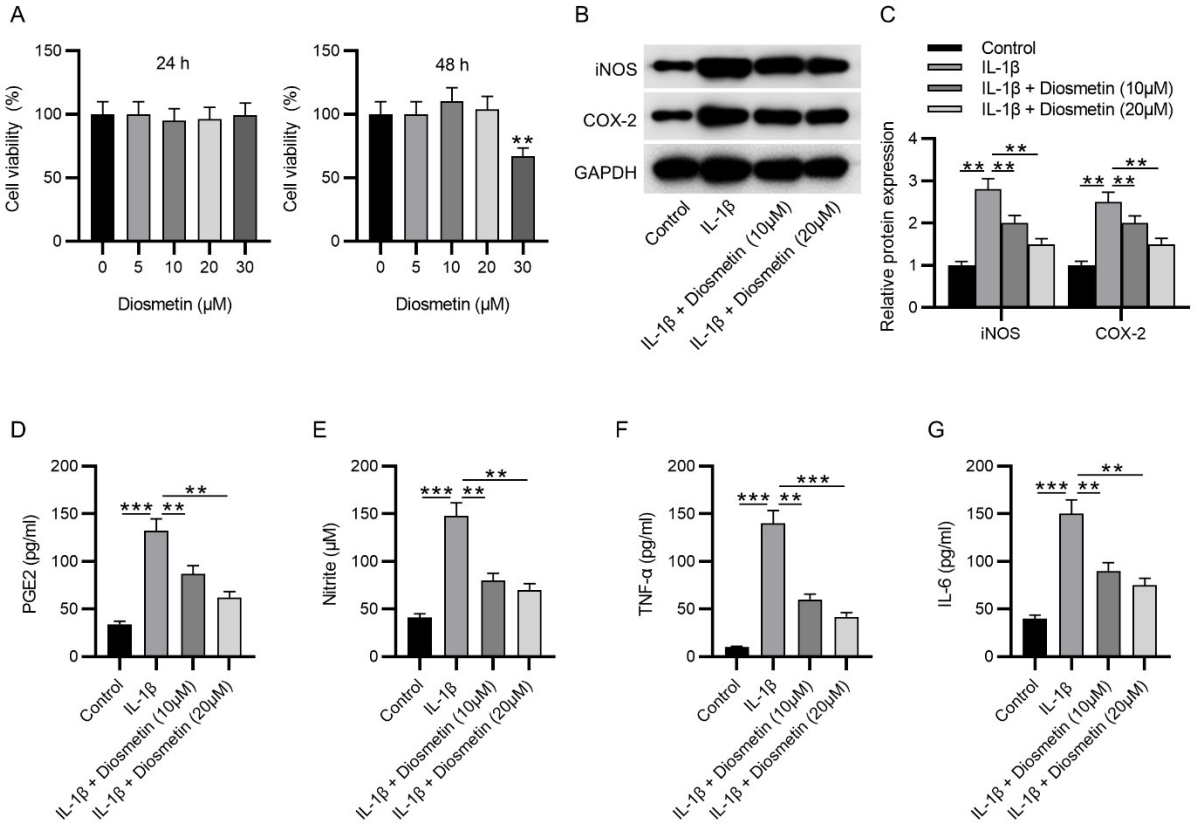

Figure 1. Diosmetin inhibits IL-1β-induced inflammation in cultured murine chondrocytes. **A.** CCK-8 assessed the viability of chondrocytes under the treatment of diosmetin (5, 10, 20, and 30 μM). **B, C.** Western blotting of iNOS and COX-2 expression in chondrocytes treated with IL-1β and diosmetin. **D, G.** The levels of inflammatory markers in the supernatants of chondrocytes treated with IL-1β and diosmetin were measured by ELISA as described in Methods. $^{**}P < 0.01$, $^{***}P < 0.001$.

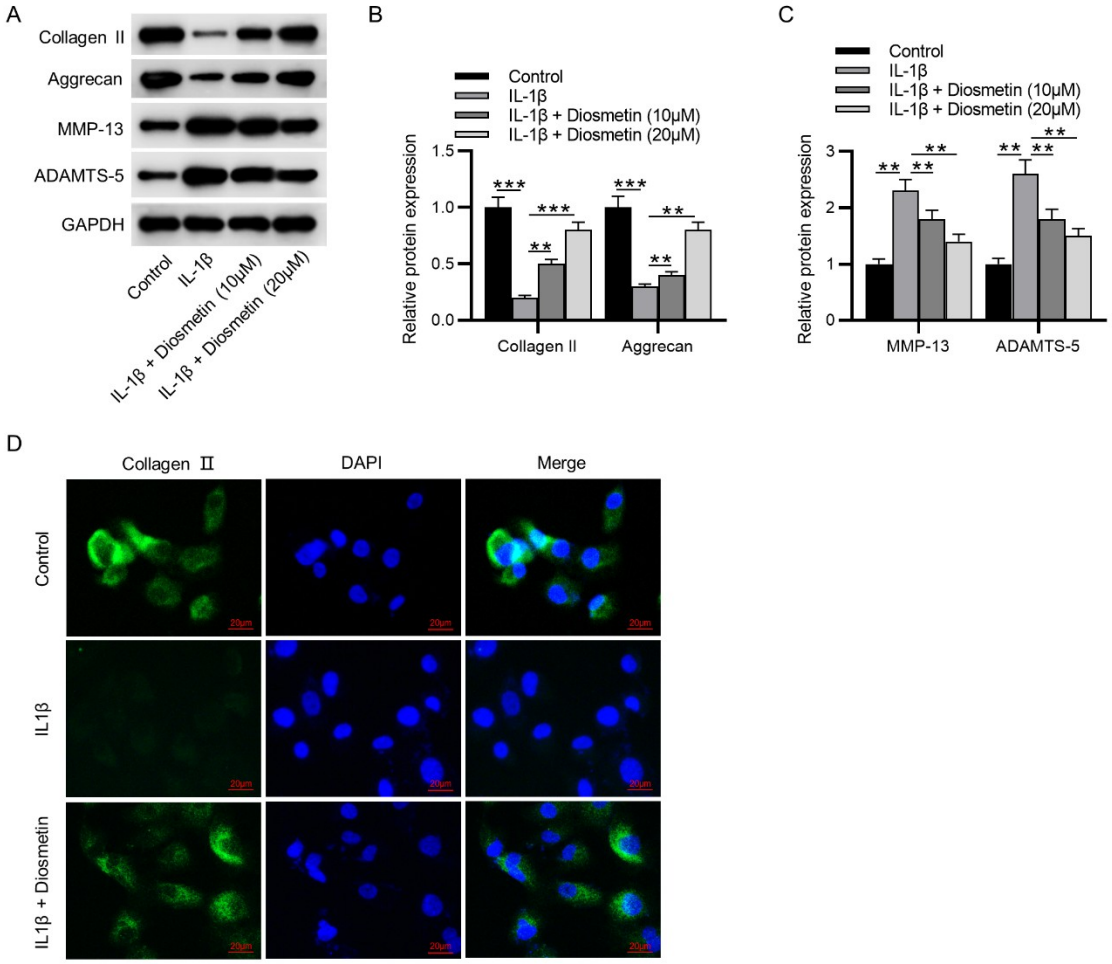

Figure 2. Diosmetin suppresses IL-1β-induced ECM degradation in chondrocytes. **A–C.** Western blotting of ECM-related proteins in chondrocytes treated with IL-1β and diosmetin. **D.** Immunofluorescence of collagen II expression in chondrocytes treated with IL-1β and diosmetin. $^{**}P < 0.01$, $^{***}P < 0.001$.

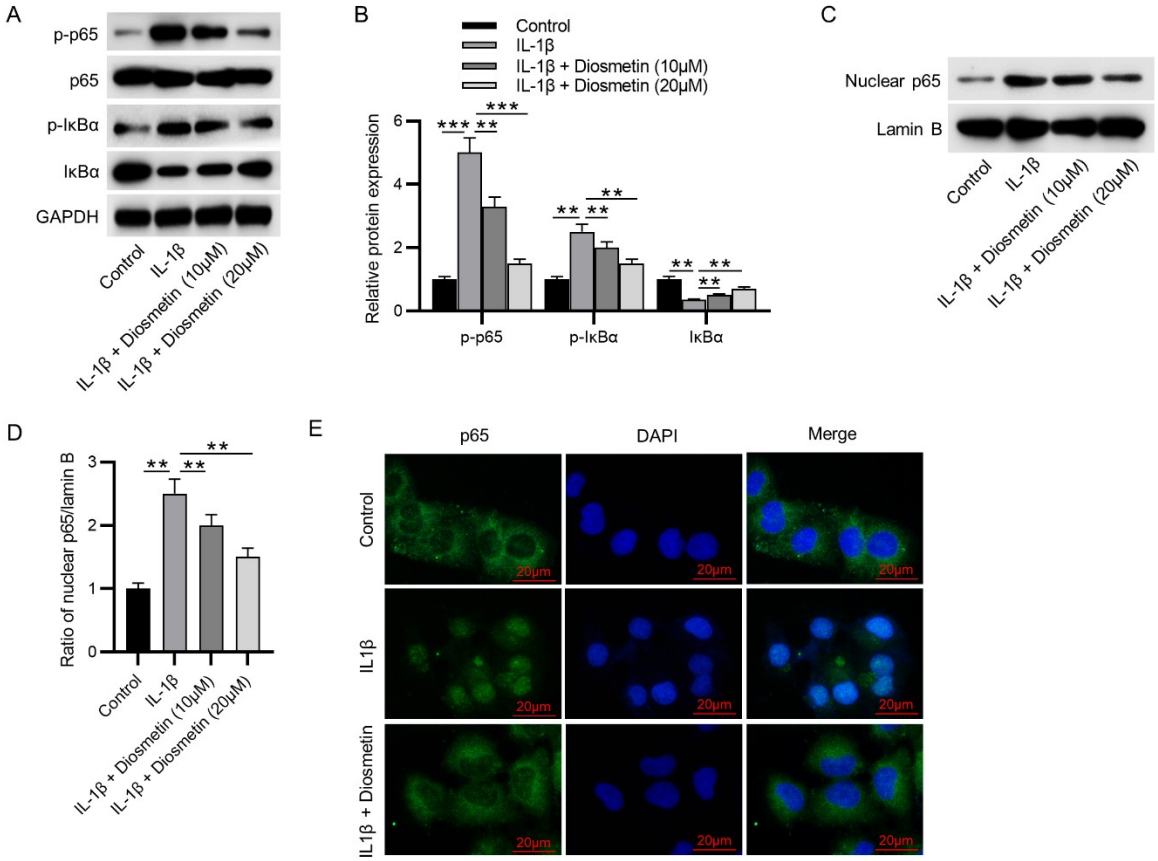

Figure 3. Diosmetin inactivates NF-κB signaling pathway in IL-1β-treated chondrocytes. **A–D.** Western blotting of NF-κB signaling-related proteins in chondrocytes treated with IL-1β and diosmetin. **E.** Immunofluorescence of p65 expression in chondrocytes treated with IL-1β and diosmetin. $^{**}P < 0.01$, $^{***}P < 0.001$.

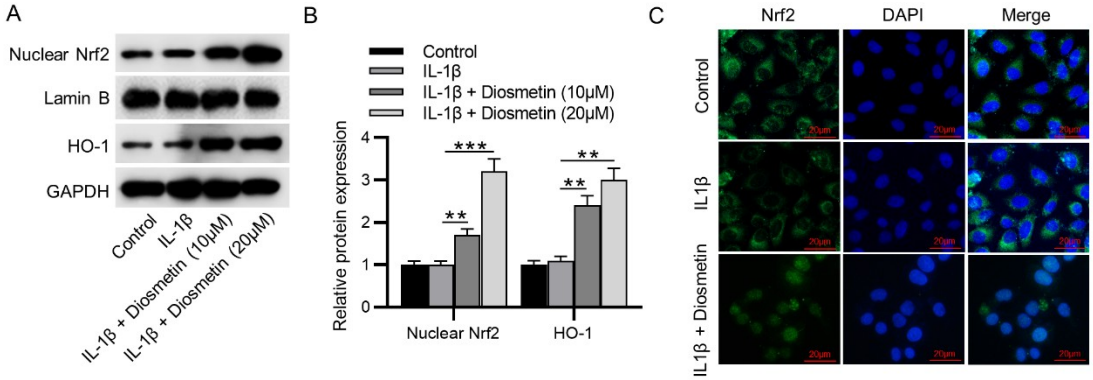

Figure 4. Diosmetin activates Nrf2/HO-1 signaling pathway in IL-1β-treated chondrocytes.

A, B. Western blotting of Nrf2/HO-1 signaling-related proteins in chondrocytes treated with IL-1β and diosmetin. **C.** Immunofluorescence of Nrf2 expression in chondrocytes treated with IL-1β and diosmetin. $^{**}P < 0.01$, $^{***}P < 0.001$.

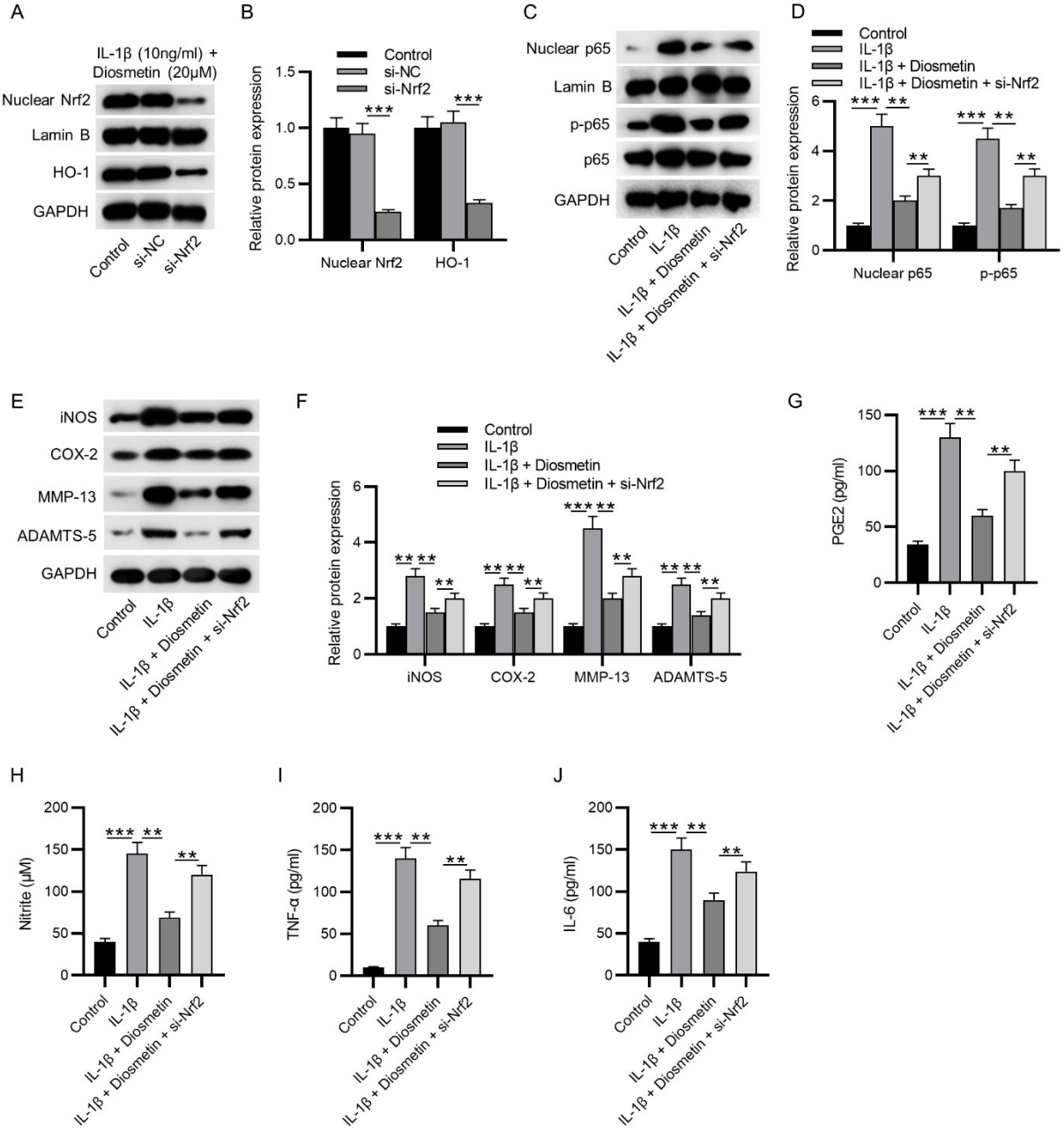

Figure 5. Diosmetin inhibits IL-1β-induced markers of OA in chondrocytes *via* activation of Nrf2/HO-1 signaling pathway. **A, B.** Western blotting of NF-κB and **(C, D)** Nrf2/HO-1

signaling-related proteins, as well as **(E, F)** inflammation and ECM-related proteins. **G–J.** The levels of inflammatory markers in IL-1β-treated chondrocytes pretreated with diosmetin and transfected with siRNA targeting Nrf2 were assessed by ELISA. $^{**}P < 0.01$, $^{***}P < 0.001$.

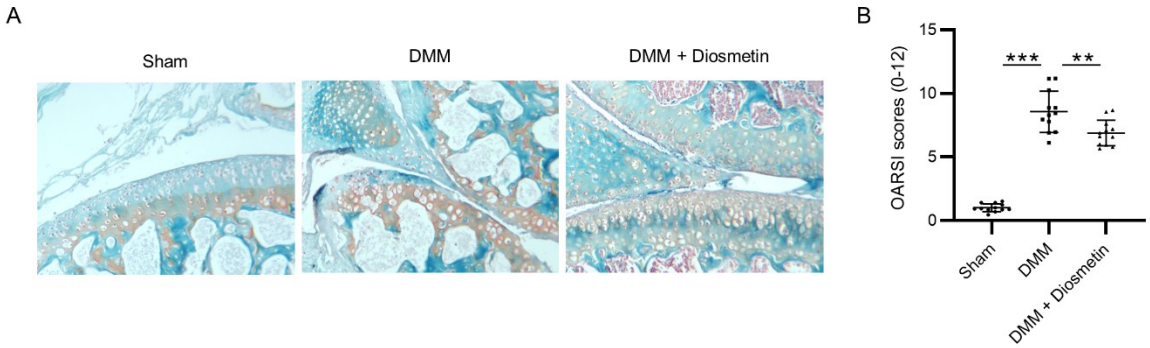

Figure 6. Diosmetin improves osteoarthritis progression in destabilization of the medial meniscus mouse model. **A.** Safranin O staining of the articular cartilage from different experimental groups. **B.** Osteoarthritis Research Society International (OARSI) scores of cartilages from different experimental groups. $^{**}P < 0.01$, $^{***}P < 0.001$.

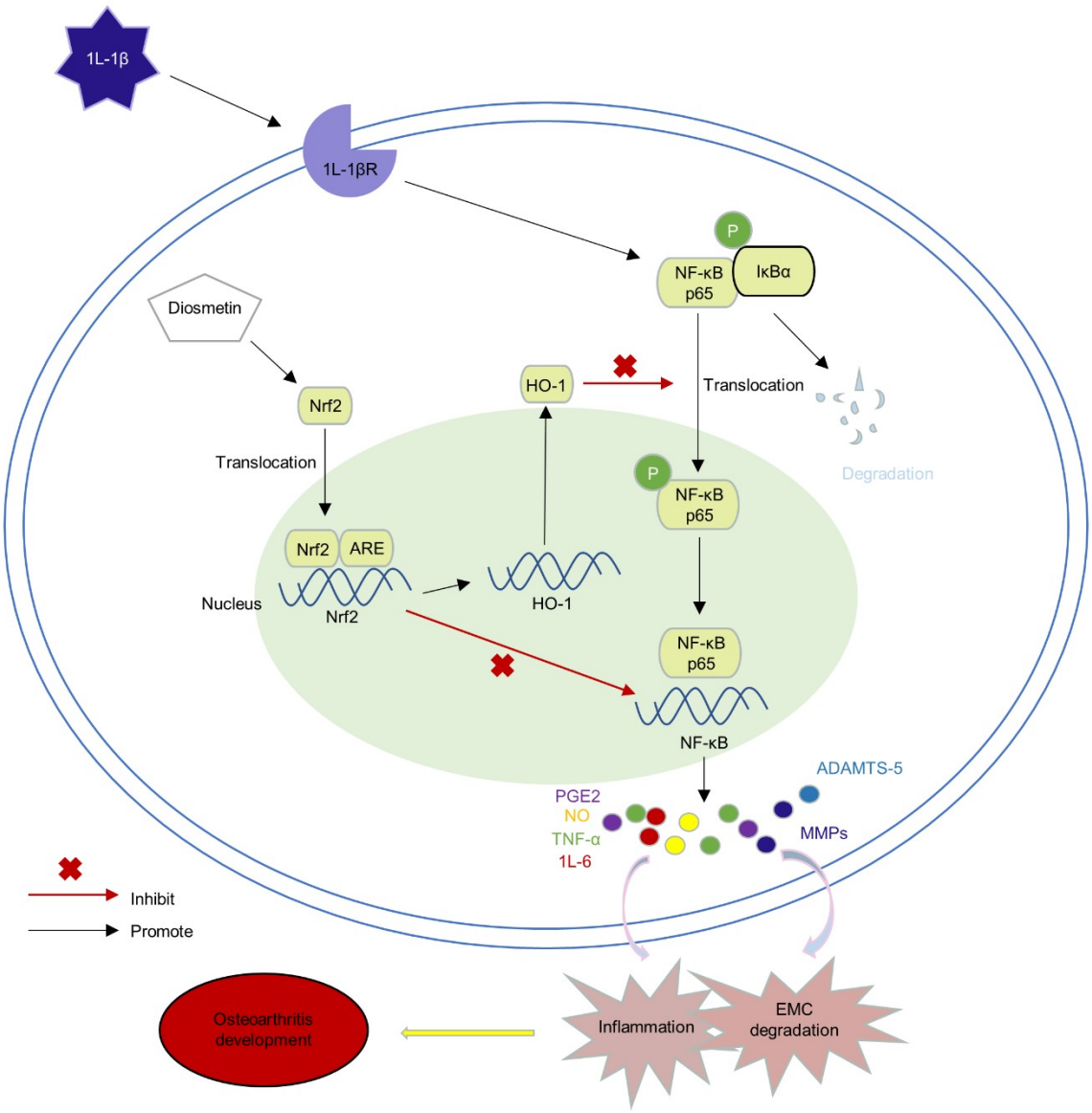

Figure 7. Graphical summary of diosmetin effects on IL-1β-induced murine chondrocytes.