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Activation of Nrf2/HO-1 signal with Myricetin for attenuating ECM degradation in human chondrocytes and ameliorating the murine osteoarthritis

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ABSTRACT

Background: Osteoarthritis (OA), one of the prevailing joint degenerative disorders, contributes to the disability around the world. However, no effective therapeutic was introduced currently. Myricetin was reported to possess the function of anti-inflammatory, anti-diabetic and anti-cancer. Thus, we investigate the protection role of myricetin in OA progression and the potential molecular mechanism in present study.

Methods: Quantitative realtime PCR and western blotting were performed to evaluate the expression of MMP-13, Aggrecan, iNOS, and COX-2 at both gene and protein levels. An enzyme-linked immunosorbent assay was used to evaluate the levels of inflammatory factors (PGE2, TNF-α, and IL-6). The PI3K/AKT, Nrf2/HO-1 and nuclear factor kappa B (NF-κB) signaling pathways were analyzed by western blotting, and immunofluorescence was used to assess the expression of Nrf2, Collagen II and MMP13. The in vitro effect of myricetin was evaluated by intragastric administration into a mouse osteoarthritis model induced by destabilization of the medial meniscus.

Results: Myricetin not only inhibited the generation of inflammatory mediators and cytokines such as nitric oxide (NO), prostaglandin E2 (PGE2), TNF-α and IL-6, but also suppressed the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in human chondrocytes under IL-1β stimulation. Moreover, Metalloproteinase 13 (MMP13) and thrombospondin motifs 5 (ADAMTS5), which resulted in the degradation of cartilage, were also suppressed in chondrocytes with the treatment of myricetin. To explore the potential mechanism, we found out that myricetin suppressed NF-κB signaling pathway through Nrf2/HO-1 axis in human chondrocytes. Besides, myricetin regulated the Nrf2 signaling pathway through PI3K/Akt pathway. In addition, in vivo study demonstrated that myricetin could ameliorated the progression of OA in mice DMM model through PI3K/Akt mediated Nrf2 signaling pathway.

Conclusion: Taken together, our data first demonstrated that myricetin possesses the therapeutic potential on OA through PI3K/Akt mediated Nrf2/HO-1 signaling pathway.

1. Introduction

Articular cartilage is an important component of the musculoskeletal system, which is believed to absorb compression and shear forces during joint movement [1]. Osteoarthritis (OA), the multiple factor disease, is one of the prevailing joint degenerative disorders that contribute to the disability among individuals around the world especially in elderly people [2]. However, there are currently few effective treatments available for OA [3]. This highlights the need for developing new therapeutic concepts and approaches for ameliorating OA progression.

It is widely acknowledged that OA is characterized by cartilage

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degradation, synovial inflammation and subchondral bone remodeling but structural damaged is still the primary hallmark of OA [4]. Chondrocytes, the only cells in articular cartilage, was generally recognized to generate the extracellular matrix including Collagen II and proteoglycans which play a significant role in maintain the function of joint [5]. With the development of OA, inflammatory cytokine and metalloprotease which were released by chondrocytes resulted in ECM degradation and contribute to the process of OA [6]. Interleukin 1β (IL-1β), the major elevated inflammatory cytokines in cartilage tissue of patients, contributes to the catabolic processes in OA [7]. IL-1β promotes the generation of inflammatory mediators and catabolic factors such as cyclooxygenase-2 (COX-2), Prostaglandin E2 (PGE2), nitric oxide (NO), NO synthase (NOS), ADAMTS5, and matrix metalloproteinases (MMPs) which impairs the extracellular matrix (ECM), subsequently contributing to OA progression [8].

Due to previous study, it is clearly that NF-κB exert significant role in IL-1β mediated inflammation in chondrocytes [9]. Chen et, al reported that Baicalin suppresses IL-1β-induced expression of inflammatory cytokines via blocking NF-κB in human osteoarthritis chondrocytes [10]. As we all known, the nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates expression of antioxidant and detoxifying genes including heme oxygenase-1 (HO-1) [11]. Recent evidence demonstrated that Nrf2/HO-1 pathway is involving in inflammation disease. Moreover, NF-κB signaling pathway could be inhibited by Nrf2 and subsequently decreased the generation of inflammatory mediators such as PEG2, MMPs [12–14] [15]. Besides, cartilage in Nrf2-KO mice show severe damage suggesting that Nrf2 may considered as a new potential therapeutic target for OA [16]. Nrf2/HO-1 axis may become a potential therapeutic molecular target for OA.

Myricetin, a naturally occurring flavanol, is commonly consumed in our diet such as vegetables, tea, berries and red wine [17] [18]. And myricetin have been proved safety as dietary supplements according to the study of toxicokinetic of the myricetin and its metabolite in rats [19] Moreover, previous studies demonstrated that myricetin exert various functions such as anti-inflammation [20], anti-cancer [21] and anti-diabetes [22]. Myricetin could decreased the generation of inflammatory enzymes and cytokines lipopolysaccharide-stimulated RAW264.7 macrophages [23].Furthermore, myricetin could inhibit IκBα/NF-κB pathway through activation of Nrf2/HO-1 pathway, resulting in protection function of various disease such as Diabetic Cardiomyopathy [24], brain injury and neurological deficits [18], Multiple sclerosis [25] and Hepatic Steatosis [26]. In addition, previous study shown that Akt regulated the Nrf2/HO-1 signaling pathway in epithelial cells [27]. And recent reports suggest that the anti-cancer effects of myricetin was associated with PI3K/Akt pathway [28,29]. However, the protection effect of myricetin in OA progression is still unknown.

The current study was designed to investigate whether myricetin could protect chondrocytes against IL-1β-induced inflammation response and ECM degradation. In addition, the potential therapeutic effects of myricetin DMM mouse model was also investigated in in vivo experiments.

2. Material and method

2.1. Ethics statement

All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the Animal Care and Use Committee of Wenzhou Medical University.

2.2. Reagents and antibodies

Myricetin (C15H20O8) (purity > 98%) was purchased from MCE China (China). Dimethylsulfoxide (DMSO) and Collagenase II were purchased from Sigma-Aldrich (St Louis, MO, USA). The primary antibody of β-actin (ab8226), Aggrecan (ab3778), MMP13 (ab39012), Collagen II (ab34712), Nrf2 (ab137550), HO-1 (ab13243), ADAMTS5(ab41037), iNOS (ab3523) and Lamin B (ab151735) were purchased from Abcam (Cambridge, UK). The primary antibody of COX-2 (12,282 T), IκBα (4814T) and p65 (8242T) antibodies were obtained from CST (MA, USA). Second antibody, such as Alexa Fluor®488 labeled and Alexa Fluor®594 labeled Goat Anti-Rabbit IgG (H + L) were obtained from Abcam (Cambridge, UK). The 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China). NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit was obtained from Thermo Scientific (Shanghai, China).

2.3. Isolation and culture primary chondrocytes

The study protocol was reviewed and approved by the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical University. All the methods in this study were carried out in accordance with the approved protocol and guidelines. Discarded and de-identified knee cartilage samples were from donors who underwent total knee arthroplasty due to OA in Second Affiliated Hospital, Wenzhou Medical University.

The regions of the cartilage samples with Mankin score [30] of 0–1, which were considered unaffected, were digested by 2 mg/ml 0.1% collagenase II for 4 h at 37 °C. Next, we performed the monolayer culture of primary chondrocytes with DMEM/F12 (Gibco, Invitrogen, Grand Island, NY) with 15% fetal bovine serum (FBS; Gibco, Invitrogen, Grand Island, NY) and antibiotics (1% penicillin/streptomycin) in the incubator at 5% CO2 at 37 °C. And the medium was firstly changed after incubated after 24 h. Harvesting the Chondrocytes by 0.25% Trypsin-EDTA (Gibco, Invitrogen) when confluent. Next, Chondrocytes were passage into 10-cm culture plates at the appropriate density. The complete medium was changed every other day and Chondrocytes no later than first passage were used for follow-up experiments.

2.4. Cell viability analysis

We evaluated the cytotoxicity of myricetin on human chondrocytes by cell counting kit-8 (CCK- 8; Dojindo Co, Kumamoto, Japan) referring to the protocol. As described in the previous study [31], Chondrocytes were treated with myricetin in a dose dependent manner for 24 h or 48 h, and then 100 μl of DMEM/F12 containing 10 μl of CCK-8 solution was added to each well of the plate for 2 h at 37 °C. Micro-plate reader was used to measure the absorbance of wells at 450.

2.5. Limulus Amebocyte Lysate test (LAL test)

We dilute the Standard Endotoxin(10EU/ml)to 1EU/ml Standard Endotoxin, then we mixed a limulus amebocyte lysate (LAL) (0.5EU) with 0.1 ml dissolved water and 0.1 ml Standard Endotoxin as a positive control 1, and mixed a LAL (0.5EU) with 0.1 ml dissolved water 、 0.1 ml Standard Endotoxin and 0.1 ml sample for test as a positive control 2. Next, we dissolved one LAL (0.5EU) in 0.2 ml dissolved water as negative control. And we respectively dissolved one LAL (0.5EU) in 0.1 ml sample for test and 0.1 ml dissolved water to test endotoxin in drug. Finally, we put the above test tube in a 37 °C water bath, and inversion tube to observe the reaction after 60 min. (+):The gel in the test tube does not deform and cannot fall off the tube wall; (—):The gel in the test tube cannot be kept intact and will fall off the tube wall; (±) One positive , one negative in two tests.

2.6. Immunofluorescence

As described in the previous study [31], The cells in each well were washed by PBS for three-times and then were fixed by 4% paraformaldehyde. 0.5% Triton for 5 min was applied in this study to permeate chondrocytes. Before incubated with primary antibodies: Nrf2(1:200), Collagen II (1:200), MMP-13 (1:200) and P65, in a humid

chamber overnight at 4 °C, chondrocytes were incubated by 10% bovine serum albumin for 1 h at 37 °C. Then, chondrocytes were incubated with Alexa Fluor®488/594 labeled conjugated second antibodies (1:400) for 1 h at 37 °C and nuclear of Chondrocytes were labeled with DAPI for 5 min. Finally, each sample was observed using fluorescence microscope (Olympus Inc., Tokyo, Japan), and the fluorescence intensity was quantified by Image J software 2.1 (Bethesda, MDUSA).

2.7. Western blot assay

We use RIPA lysis buffer containing with1 mM PMSF (Phenylmethanesulfonylfluoride) to extract the total protein in chondrocytes and measured the concentration of extracted protein by using the BCA protein assay kit (Beyotime) according to protocol. The protein was prepared for western blots according to the previous study [32] [33]. In addition , the nuclear protein was extracted by the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit , which is a reagent-based protocol that enables the stepwise lysis of cells, separation of the cytoplasm from the intact nuclei and then extraction of nuclear proteins away from genomic DNA and mRNA. The primary antibody: Nrf2(1:500), HO-1(1:5000), β-actin (1:1000), collagen II (1:1000), Aggrecan (1:1000), MMP13 (1:1000), iNOS (1:1000), COX-2 (1:1000), p65 (1:1000) and IκBα (1:1000) were used for this study.

2.8. Real-time PCR

After treatment, total RNA was extracted from Chondrocytes using the TRIzol method and quantified as describes [34]. Total RNA was Transcribed into cDNA by PrimeScript-RT reagent kit, then the cDNA was amplificated by the PrimeScript-RT reagent kit and SYBR Premix Ex Taq (Sangon). The expression of target genes in different groups were measured using the △△Ct method. The primers of COX-2 (F) 5′-GAGAGATGTATCCTCCCACAGTCA-3′ (R) 5′-GACCAGGCACCAGACCAAAG-3′; iNOS (F) 5′-CCTTACGAGGCGAAGAAGGACAG-3′, (R) 5′-CAGTTTGAGAGAGGAGGCTCCG-3′; IL-6 (F) 5′-GACAGCCACTCACCTCTTCA-3′, (R) 5′-TTCACCAGGCAAGTCTCCTC-3′; TNF-α (F) 5′-GTCAGATCATCTTCTCGA ACC-3′, (R) 5′-CAGATAGATGGGCTCATACC-3′ β-actin (F) 5′-TGCCACTCAGAAGACTGTGG-3′, β-actin (R) 5′-TTCAGCTCTGGGATGACCTT-3′ were refer to previous study [15].

2.9. The measurement of NO, PGE2, TNF-α, IL-6

After treatment, the NO level in cell culture supernatants was measured by Griess reagent as described [35]. Elisa kits (R&D Systems, Minneapolis, MN) was used to determine the level of PGE2, NO, TNF-α, IL-6 in cell culture supernatants. All experiments were performed for five times.

2.10. Surgical induction of OA by destabilization of the medial meniscus

Thirty-six C57BL/6 male wild-type (WT) mice (8 weeks age) were random classified into three groups, including sham group (n = 12), DMM group (saline intragastric administration after surgery, n = 12) and myricetin group (myricetin intragastric administration after surgery, n = 12). OA mice was induced by DMM of the right knee as described in the previous study [36]. Briefly, mice were anesthetized by 2% (w/v) pentobarbital (40 mg/kg), and the anterior attachment of the medial meniscus to the tibial plateau was transected to induce the instability of the right knee joint. Left knee joints were left intact in this study. In addition, sham operation was performed just by incision of the cutaneous and muscular of knee in present study. After surgery, the 20 mg/kg myricetin in 0.5% carboxymethylcellulose (CMC) was immediately given by intragastric administration in myricetin group and 0.5% CMC given by intragastric administration alone in sham group and DMM group every two days for 8 weeks until the mice were sacrificed.

2.11. Safranin o-fast green staining

The mice were sacrificed by over dosage injection of 10% pentobarbital and knee joints were harvested from 8 weeks post-surgery. The specimens were fixed in 4% paraformaldehyde > 48 h and decalcified

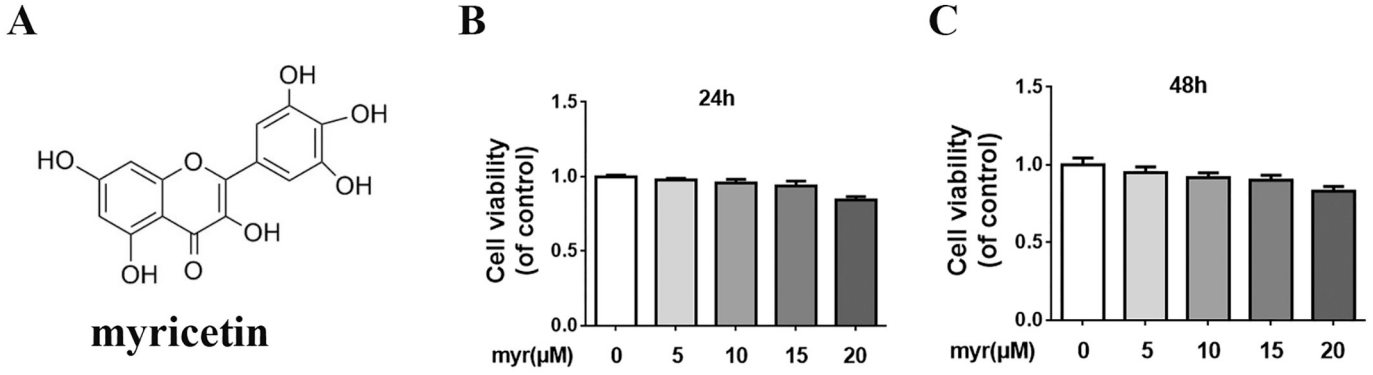

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Table1 LAL test

	Standard Endotoxin	Standard Endotoxin+Myr(10μM)	Standard Endotoxin+Myr(20μM)	Dissolved Water	Myr (10μM)	Myr (20μM)
LAL (0.5EU)	+	+	+	-	-	-

Fig. 1. Potential cytotoxicity of myricetin in human chondrocytes.
Human chondrocytes were treated with myricetin in a dose dependent manner for 24 h or 48 h. (A) The chemical structure of myricetin. (B, C) Cell viability of chondrocytes were determined by Cell Counting Kit-8 (CCK8). (D) Limulus Amebocyte Lysate Test(LAL test)of Myricetin. The experiment was repeated three times, with a representative example shown; Significant differences between groups are indicated as *** P < 0.001, **P < 0.01, *P < 0.05, ns p > 0.05.

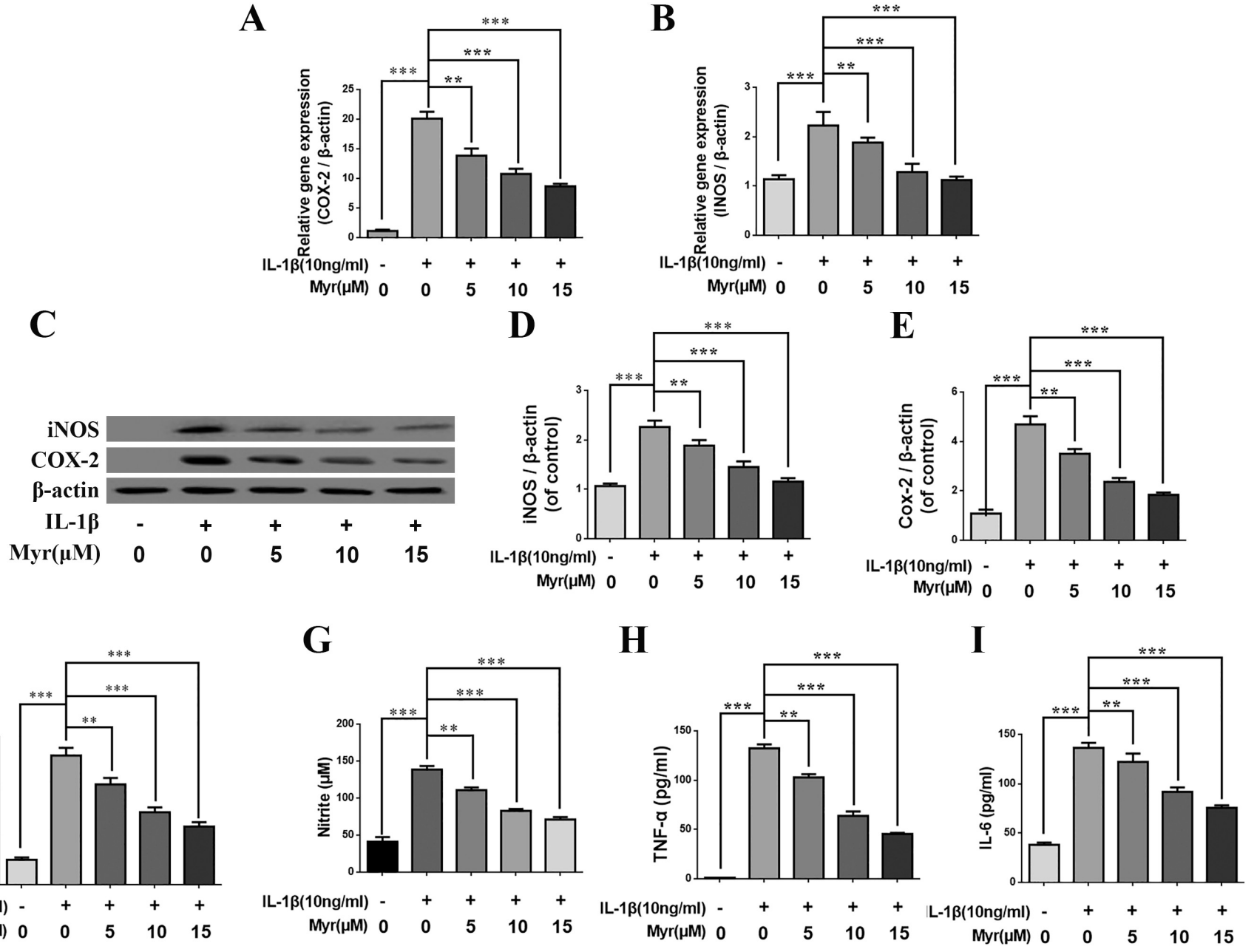

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Fig. 2. Myricetin suppressed the generation of inflammatory mediators in IL-1β induced human chondrocytes.
Human chondrocytes were treated with myricetin in a dose dependent for 24 h and with or without the stimulation of IL-1β. (A, B) The mRNA expression of iNOS and COX-2 in chondrocytes were evaluated by Real time PCR. (C) The protein expression of iNOS and COX-2 in chondrocytes were determined by western blots. (D, E) Quantitative western blot analysis of iNOS and COX-2 in chondrocytes. (F—I) Effect of myricetin on IL-1β-induced PGE2, Nitrite, TNF-α and IL-6 generation in chondrocytes. The experiment was repeated three times, with a representative example shown. The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

for > 30 days, then dehydrated and embedded in paraffin. The sample were cut into sections (5 μm thick). Safranin o-fast green staining assess the OA development. The construction of knee joint and the Chondrocytes' morphology and cellularity were observed by another three two-blinded experienced histology researchers using a microscope (Leica) and the Osteoarthritis Research Society International (OARSI) scoring system for medial femoral condyle and medial tibial plateau was evaluated [37].

2.12. Statistical analysis

All experiments were performed at least three times. The results were expressed as mean ± S.D. Statistical analyses were performed using SPSS statistical software program 20.0. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test or *t*-tests (and nonparametric tests) for comparison between control and treatment groups. Nonparametric data (Pfirrmann grading) were analyzed by the Kruskal–Wallis H test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Myricetin potential cytotoxicity for chondrocytes

To investigate the role of myricetin in human chondrocytes, firstly, we access the cytotoxic of myricetin in human chondrocytes in a dose dependent manner for 24 h or 48 h using cck8 assay kit. As shown in Fig. 1B and C, myricetin exerted no significant cytotoxic effect on human chondrocytes in a dose dependent manner. Besides, LAL test showed that myricetin can directly applied to cultured cells (Fig. 1D).

3.2. Myricetin regulate the generation of inflammatory mediators and cytokines in Chondrocytes with IL-1β stimulated

Inflammatory mediators contribute to the ECM degradation in cartilage, thus, we investigate the effect of myricetin on regulation of inflammatory mediators. As shown in Fig. 2, mRNA and protein expression of iNOS and COX-2, which were recognized as indicators of degree of inflammation, were remarkedly increased in chondrocytes under IL-1β stimulation, whereas the increments were suppressed with the myricetin in a dose dependent manner (Fig. 2A-E). In addition, generation of endogenous NO and PGE2 was increased with IL-1β stimulation. And myricetin remarkedly decreased the production of NO and PGE2 in a dose-dependent manner (Fig. 2F, G). As we all known, with the development of OA, various inflammation cytokines were increased in cartilage and synovium tissue. We also observed that tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) production was significantly increased when chondrocytes were stimulated with IL-1β. Meanwhile, myricetin suppressed the secretion of TNF-α and IL-6 in chondrocytes in a dose dependent manner by enzyme-linked immunosorbent assay (ELISA) (Fig. 2H, I). In a conclusion, these data suggest that myricetin inhibit the generation of inflammatory mediators and cytokines in a dose-dependent manner.

3.3. Myricetin protect chondrocytes against extracellular matrix degradation induced by IL-1β

Disruption of the balance between synthesis and degradation of extracellular matrix (ECM) contribute to the progression of OA. Thus, in present study, we explore the role of myricetin in protecting chondrocytes against ECM degradation. As shown in Fig. 3A, western blot results showed that IL-1β remarkedly inhibited the synthesis of aggrecan, which consist of the extracellular matrix of chondrocytes but increased the generation of MMP13, one of the MMPs family which blamed for the degradation of ECM (Fig. 3A-C) ($p < 0.01$). After treatment with myricetin, IL-1β induced alterations of extracellular matrix in chondrocytes was inhibited by myricetin. To further confirm the protection effects of myricetin, the mRNA expression of related genes was measured. The results showed that myricetin promoted the expression of aggrecan and Collagen II but inhibited the expression of ADAMTS5 and MMP13 in IL-1β stimulated chondrocytes. In addition, double immunofluorescence of Collagen II (green) and MMP 3 (red) shown showed that intensity of Collagen II was decreased and fluorescence intensity of MMP13 (MMPs family) was increased in chondrocytes with IL-1β treatment, whereas, this trend described above was partially inhibited by myricetin (Fig. 3H-J) ($p < 0.01$). Taken together, these dates suggest that myricetin could protect chondrocytes against extracellular matrix degradation induced by IL-1β.

3.4. Myricetin inhibited IL-1β induced NF-κB activation in human chondrocytes

Several studied have showed that inflammation-related changes were involving in the progression of OA and NF-κB was confirmed involving in the OA development [38] [39]. Therefore, we measured the expression of Inflammation related proteins, such as IκBα, and NF-κB (p65) in chondrocytes under the IL-1β treatment by western blot. IκBα was an upstream target of NF-κB, its degradation contributes to the activation of the NF-κB pathway. As shown in Fig. 4, western blot results shown that IL-1β stimulation could obviously promote degradation of IκBα in the cytoplasm and upregulated the expression of p65 in the nuclear of Chondrocytes (Fig. 4A). Then, with the treatment of myricetin, the degradation of IκBα was inhibited and the expression of p65 in nuclear of Chondrocytes was also decreased. This data suggests that myricetin inhibit IL-1β induced activation of NF-κB in human chondrocytes. Moreover, in this study, we also observed the translocation of p65 during IL-1β induced NF-κB activation in chondrocytes according to the immunofluorescence staining of p65. As shown in Fig. 4D, the fluorescence intensity of p65 in nuclear of chondrocytes increased significantly comparing to the control group, this phenomenon suggests that NF-κB signaling pathway was activated in IL-1β stimulated chondrocytes. However, myricetin could notably inhibit the trend of nuclear translocation in chondrocytes as shown above in Fig. 4D. NF-κB nuclear translocation triggers release of inflammatory mediators. Thus, myricetin could inhibit the generation of inflammatory mediators and cytokines by suppressing the NF-κB activation in chondrocytes.

3.5. Myricetin activated the Nrf2/HO-1 signaling pathway in IL-1β stimulated human chondrocytes

In this study, as shown in Fig. 5A, western blot results shown that myricetin increased the expression of Nrf2 in nuclear and increased HO-1 expression in cytoplasm in a dose dependent manner in IL-1β stimulated human chondrocytes. Besides, there was no significant alteration of Nrf2 and HO-1 could be observed in chondrocytes under pathological condition(Fig. 6A, B)($p > 0.05$). Translocation of Nrf2 from cytoplasm to nuclear indicate the activation of Nrf2. In addition,

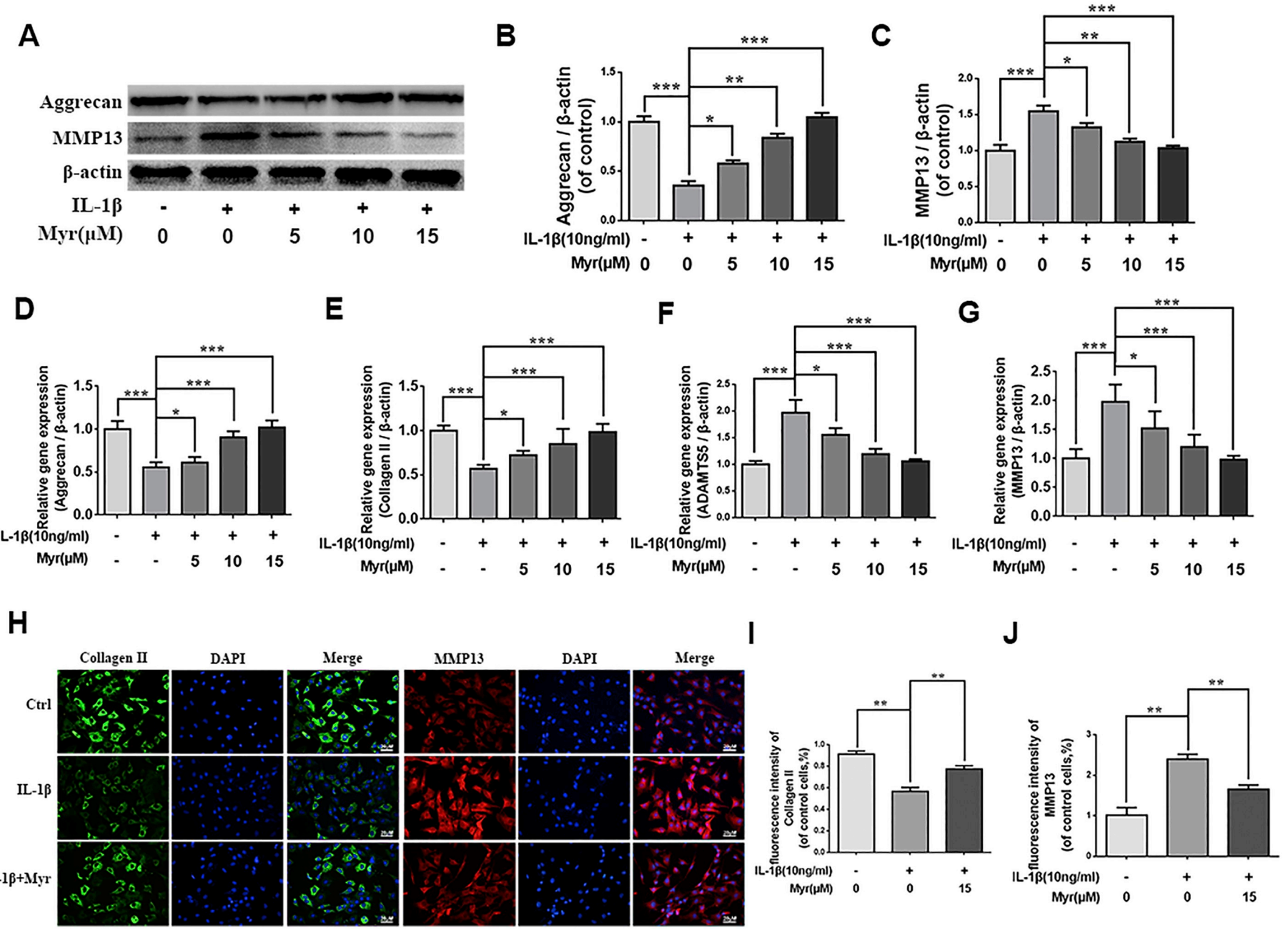

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Fig. 3. The effect of Myricetin on synthesis and degradation of the ECM in IL-1β induced human chondrocytes.
Human chondrocytes were treated with myricetin in a dose dependent for 24 h and with or without the stimulation of IL-1β. (A) The protein expression of Aggrecan and MMP13 in chondrocytes were determined by western blots. (B, C) Quantitative western blot analysis of Aggrecan and MMP13 in chondrocytes. (D-G) The mRNA expression of Aggrecan, Collagen II, ADAMTS5 and MMP13 in chondrocytes were evaluated by Real time PCR. (H) Immunofluorescence of Collagen II and MMP 13 in chondrocytes were evaluated (original magnification × 200, scale bar: 50 μm). (I, J) Quantitative of the fluorescence intensity of Collagen II and MMP13 by image J. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

we use immunofluorescence staining of Nrf2 to text the translocation of Nrf2 in chondrocytes after myricetin treatment. The results showed that myricetin promote Nrf2 translocate into nuclear, which is consistent with the western blot results (Fig. 5D). Previous studies reported that myricetin could activate the Nrf2/HO-1 in myocardial cell and neurocyte except chondrocytes. In a conclusion, myricetin could activate the Nrf2/HO-1 signaling pathway in chondrocytes.

3.6. Myricetin inhibit the inflammation response and ECM degradation though Nrf2/HO-1 signaling pathway in IL-1β stimulated human chondrocytes

As shown in Fig. 6A, western blots results showed that Nrf2-siRNA could suppressed the upregulated expression of Nrf2 in nuclear of chondrocytes and the expression of HO-1 in cytoplasm of chondrocytes with the treatment of myricetin. Moreover, nuclear accumulation of p65 was subsequently increased in nuclear of myricetin treated chondrocytes with Nrf2-siRNA pre-treated. This data suggested that the activation of Nrf2 by myricetin could be inhibited by Nrf2-siRNA. What' more, with the inhibition of Nrf2 in chondrocytes, western blot and real time PCR results showed that although the IL-1β stimulated chondrocytes were treated with the myricetin, the mRNA and protein expression of aggrecan and collagen II was obviously inhibited and the expression of MMP13 and ADAMTS5 was remarkedly increased with the treatment of Nrf2-siRNA. Besides, comparing to myricetin group, not only the generation of inflammation mediators such as PEG2 and Nitrites were notably upregulated in IL-1β induced chondrocytes, but also the production of TNF-α and IL-6 were increased. Therefore, Nrf2/HO-1 pathway involving in the protection effects of myricetin against inflammation response and ECM degradation.

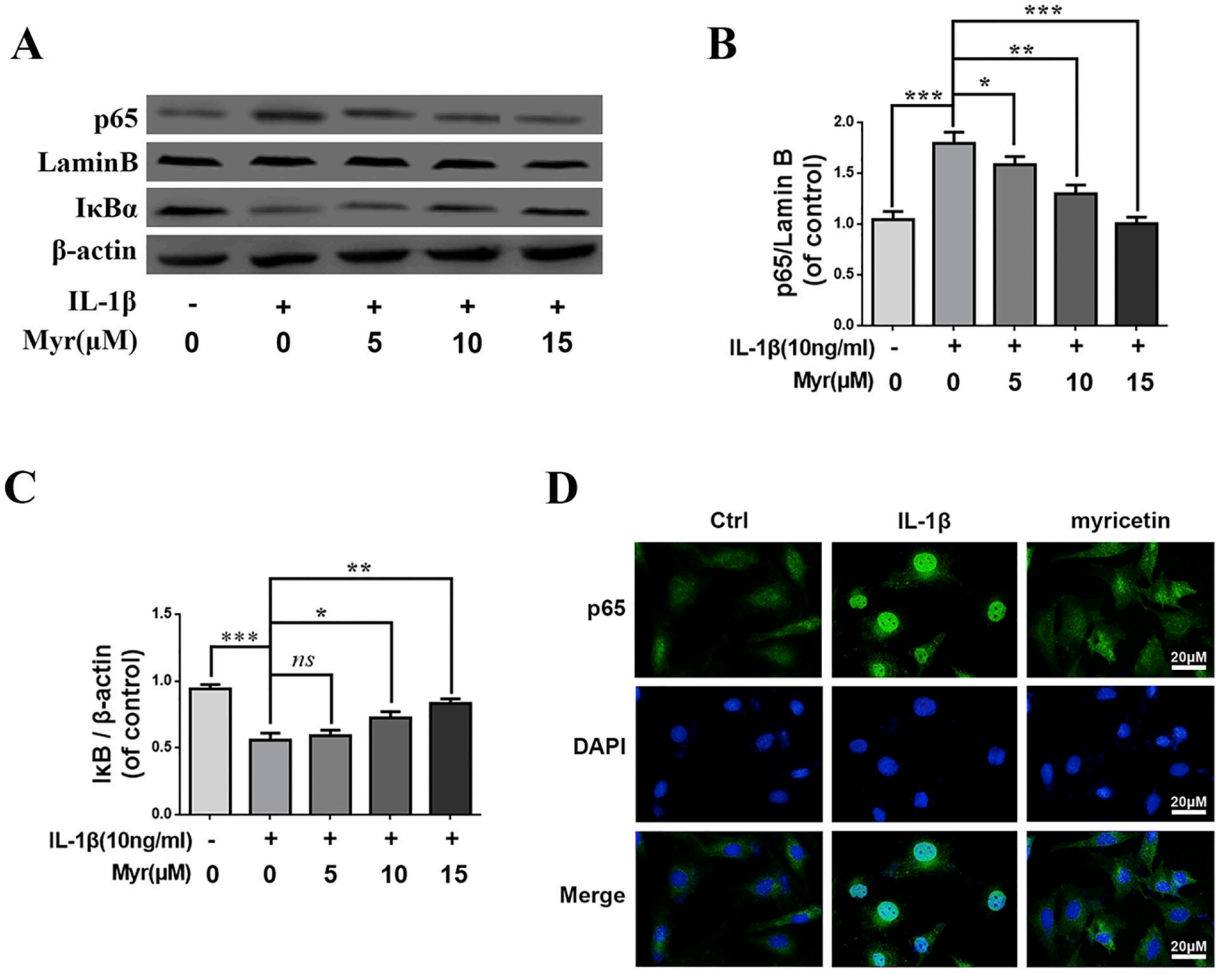

Fig. 4. Myricetin inhibits the activation of NF-κB signaling pathway in IL-1β induced in human chondrocytes.
Human chondrocytes were treated with myricetin in a dose dependent for 24 h and with or without the stimulation of IL-1β. (A) The protein expression of p65 in nuclear of chondrocytes and the expression of IκBα in cytoplasm of chondrocytes were determined by western blots. (B, C) Quantitative western blot analysis of p65 and IκBα in chondrocytes. (D) Immunofluorescence of p65 were measured to detect the translocation of p65 in chondrocytes. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

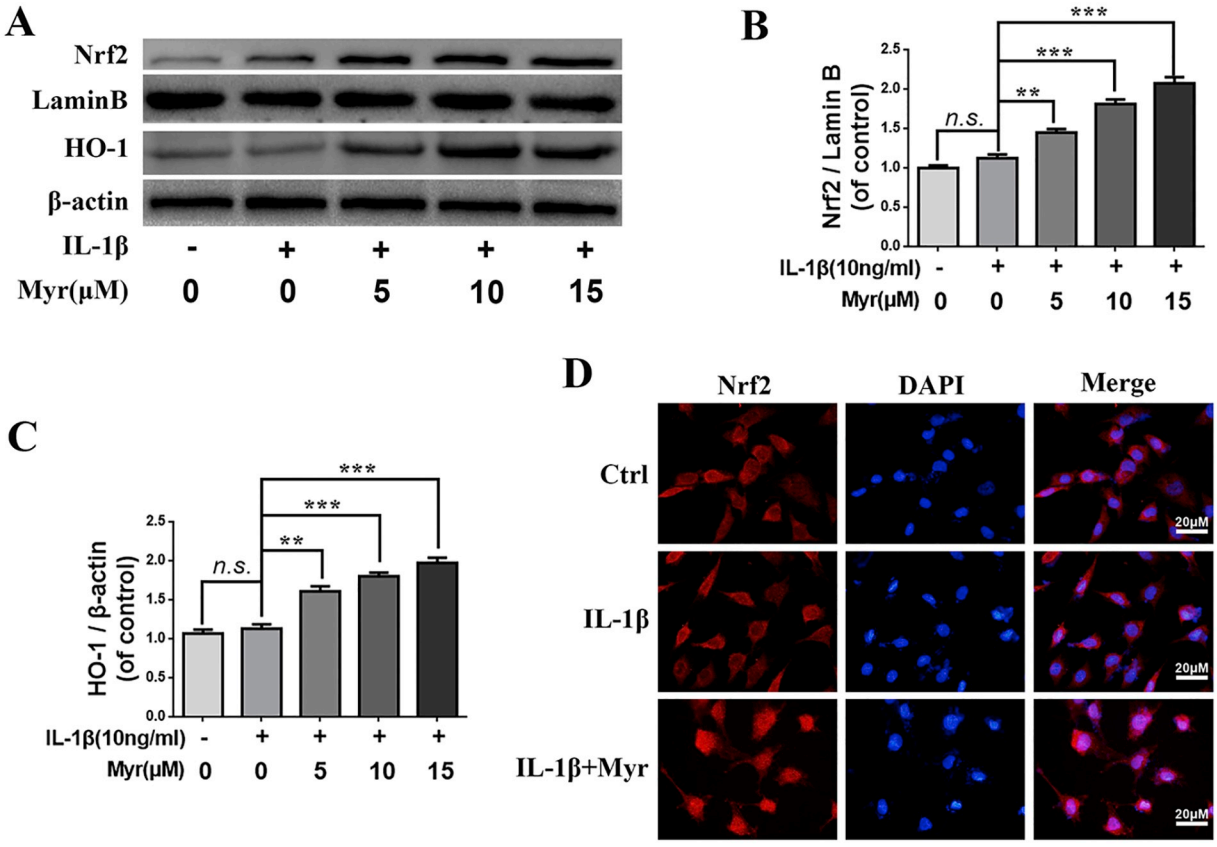

Fig. 5. Myricetin activates the Nrf2/HO-1 signaling pathway in IL-1β induced in human chondrocytes.
Human chondrocytes were treated with myricetin in a dose dependent for 24 h and with or without the stimulation of IL-1β. (A) The protein expression of Nrf2 in nuclear of chondrocytes and the expression of HO-1in cytoplasm of chondrocytes were determined by western blots. (B, C) Quantitative western blot analysis of Nrf2 and HO-1 in chondrocytes. (D) Immunofluorescence of Nrf2 were measured to detect the translocation of Nrf2 in chondrocytes. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

3.7. Myricetin regulated the Nrf2/HO-1 signaling in chondrocytes via PI3K/Akt pathway

In order to investigate the potential relationship between myricetin and Nrf2 in human chondrocytes, and previous study showed that PI3K/Akt was involving in the regulation of Nrf2/HO-1 signaling pathway, we hypothesis that myricetin may exert protective role in chondrocytes through PI3K/Akt mediated Nrf2/HO-1signaling pathway. As shown in Fig. 7A, the results indicate that p-Akt/Akt was markedly increased in human chondrocytes with the treatment of myricetin. Then we use Ly294002, the PI3K/Akt inhibitor, to explore the potential mechanism of myricetin. When treated with Ly294002, the increased expression of p-Akt/Akt was revered, and the increment of HO-1 by myricetin was inhibited (Fig. 7C). Besides, as shown in Fig. 7F, the expression of Nrf2 was downregulated and P65 was increased in nuclear of human chondrocytes. These results suggest that inhibition of PI3K/Akt signaling abolished the activation of Nrf2/HO-1 pathway and the suppression of NF-κB by myricetin.

3.8. Myricetin ameliorates the progression of OA in mice DMM model through PI3K/Akt mediated Nrf2/HO-1 signaling pathway

In order to test the protection of myricetin in OA development in vivo, we established a mice DMM model to simulate the OA progression in vivo. After surgery, the 20 mg/kg myricetin in 0.5% carboxymethylcellulose (CMC) was immediately given by intragastric administration in myricetin group and 0.5% CMC given by intragastric administration alone in sham group and DMM group every two days for 8 weeks until the mice were sacrificed. Besides, SO staining was widely used for proteoglycans and glycosaminoglycans staining, which are the main components of ECM in cartilage. The SO staining results showed that ECM of cartilage was significantly decreased in DMM group than sham group, whereas the ECM of cartilage in myricetin group were better preserved (Fig. 8A). Destruction of structure of cartilage contribute to the progression of OA. We detected that articular surface in sham group was smoother than that in DMM group. However, articular surface in myricetin group was better preserved than DMM group (Fig. 8A). Osteoarthritis Research Society International (OARSI) scores were also determined in this study. Consistent with the results of SO staining, the OARSI scores of the DMM group were markedly higher than those of the sham control group (Fig. 8B) ($P < 0.01$). In contrast, the myricetin group exerts lower OARSI scores than the DMM group (Fig. 8B) ($P < 0.01$).

In order to investigate whether PI3K/Akt mediated Nrf2 is involving in the protection effect of myricetin in OA development, we measured the translocation of Nrf2 in cartilage cells in vivo by immunofluorescence staining and p-Akt expression by immunochemical staining. As shown in Fig. 8C, Nrf2 was almost localized in cytoplasm of cartilage cells in sham group and DMM group (Fig. 8C, D) ($P > 0.05$), whereas Nrf2 translocated to the nuclear of numerous cartilage cells in myricetin group (Fig. 8C, D) ($P < 0.01$). In addition, the expression of p-Akt was higher in myricetin group than DMM group.

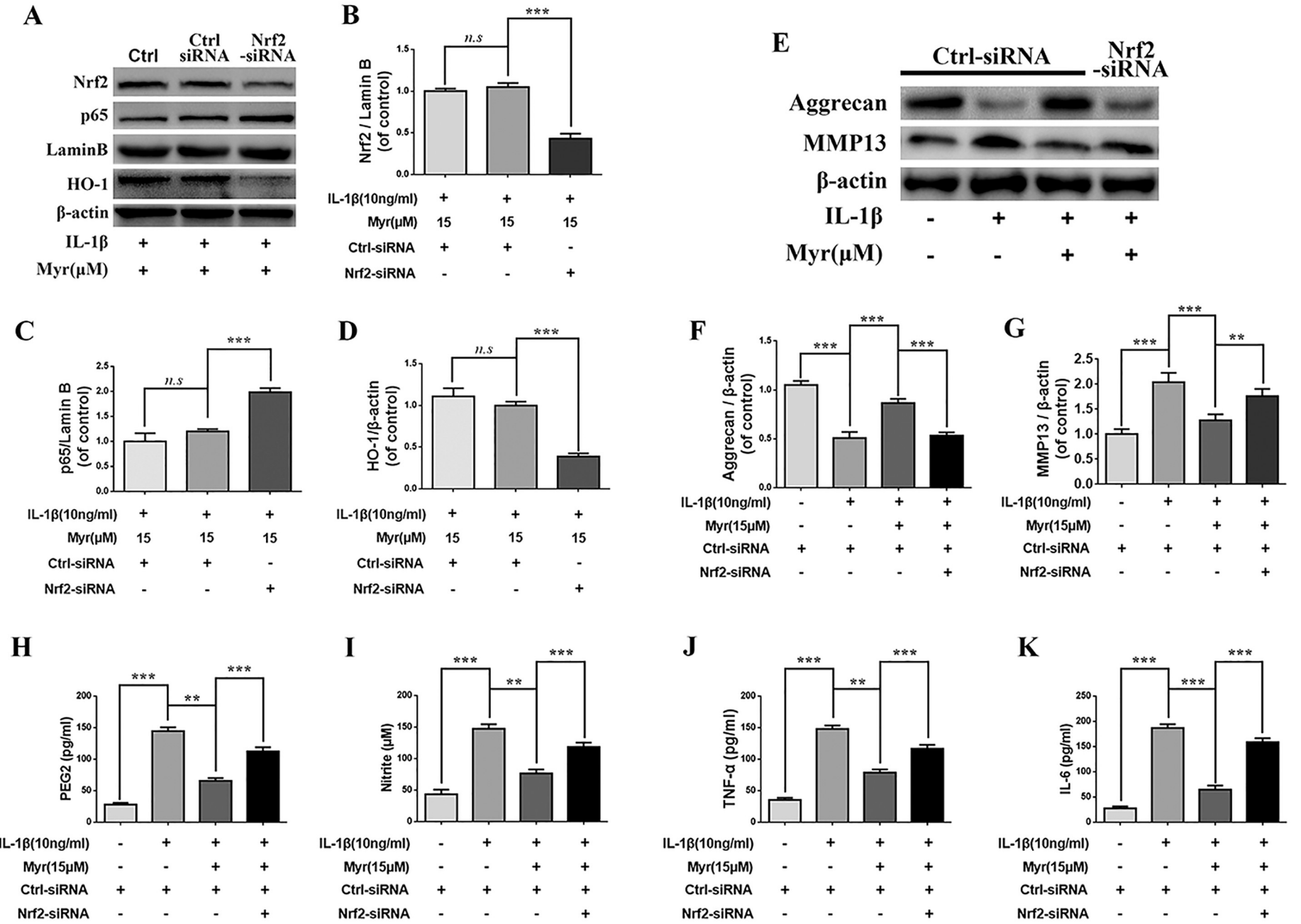

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Fig. 6. Myricetin inhibit the activation of NF-κB via activation of Nrf2/HO-1 signaling pathway in IL-1β stimulated human chondrocytes
Human chondrocytes were untreated (DMEM 10% FBS) or treated with IL-1β (12*h*), with IL-1β (12 h) plus myricetin (24 h), or with IL-1β (12 h) plus myricetin (24 h) combined with Nrf2-siRNA(24 h). (A) The expression of Nrf2 and p65 in nuclear of chondrocytes and HO-1 in cytoplasm of chondrocytes were evaluated by western blot. (B-D) Quantitative western blot analysis of Nrf2, p65 and HO-1 in chondrocytes. (E) The expression of Aggrecan and MMP1 in chondrocytes were evaluated by western blot. (F, G) Quantitative western blot analysis of Aggrecan and MMP13 in chondrocytes. (H−K) The generation of PGE2, Nitrite, TNF-α and IL-6 generation in chondrocytes were evaluated by Elisa assay. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

In this study, we focused on the therapeutic effects of myricetin on osteoarthritis, and evaluate the effects of myricetin on chondrocytes with IL-1β stimulation, but the effects of myricetin on chondrocytes in physiological condition are unrevealed. Therefore, more studies were planned to explain the question that whether myricetin influence ECM homeostasis, e.g. ECM synthesis and degradation, and reduce the inflammation in physiological condition (without IL-1β stimulation). The results showed that myricetin can promote the expression of p-Akt and activate the Nrf-2/HO-1 signaling pathway, and has no effect on the release of inflammation related cytokines and ECM homeostasis in physiological condition (Supplementary Fig. 1). It suggests that myricetin can activate Nrf2 pathway and inhibit NF-κB pathway, but have no difference on the inflammation and ECM homeostasis of chondrocytes in a non-pathological state.

Make a conclusion, myricetin protect cartilage cells and ameliorate the progression of OA in mice DMM model through PI3K/Akt mediated Nrf2/HO-1 signaling pathway.

4. Discussion

OA is a leading cause blame for chronic disability among elderly population around the world and disruption of chondrocytes homeostasis contribute to the progression of OA. With the remarkable progress of OA therapeutic, a prevailing phenomenon is that Non-steroidal anti-inflammatory drug (NSAID) widely applied for attenuating the symptom induced by OA, whereas no effective medicine introduced for ameliorating OA progression until now. In this study, it was interesting to observed that myricetin, a natural compound extracted from edible berries, exerts therapeutic effects in OA progression through mediating the PI3K/Akt mediated Nrf2/HO-1 signaling pathway. The results of this study provide a potential application of myricetin for OA therapeutic.

This study first demonstrated that myricetin could ameliorated OA via inhibiting inflammation response, suppressing ECM degradation but promoted generation of Collagen II in chondrocytes. Accumulated evidences shown that NF-κB play a significant role in the pathological of OA [40,41]. IL-1β is upregulated in OA joints and has been shown to increase the expression of cartilage matrix degrading proteases and other inflammatory mediators [42]. Consistent with our results, with the treatment of IL-1β, IκB kinase (IKK) is activated and subsequently leads to IκB degradation, which promote the transfer p65 into nuclear of cells [43]. Then, p65 translocation promotes the generation of inflammatory mediators, cytokines and catabolic enzymes. In chondrocytes, NO was degraded by iNOS thereby increased the secretion of MMPs and reducing the synthesis of Collagen II and aggrecan, finally contribute to the degradation of ECM. In addition, COX-2 promoted the production of [44]. Besides, cytokines such as TNF-α, IL-6, increased in cartilage tissues and synovial tissues [45,46], were also significantly upregulated in chondrocytes under IL-1β stimulation (Fig. 2). All these mediators contribute to the progression of OA. In MMPs family, MMP13 was recognized as the most influential role in the degradation of cartilage and evaluated in our study. In present study, myricetin suppressed the generation of inflammatory mediators such as PEG2, COX-2, MMPs as well as secretion of TNF-α and IL-6 in chondrocytes. Thus, our data indicate that regulation of mediators by myricetin was associated with the inhibition of NF-κB signaling pathway in chondrocytes.

In order to investigate the potential mechanism of myricetin in OA development, further experiments were performed. Previous studies have revealed the association between myricetin and Nrf2 [18,23,24], but no light was shed in OA. Intriguingly, with the treatment of Nrf2-siRNA, the effect of myricetin in the inhibition of the inflammatory response was offset. Meanwhile, myricetin induced translocation of p65 was also suppressed by Nrf2-siRNA. These results suggest that myricetin could inhibit IL-1β induced NF-κB activation by activation of Nrf2 in human chondrocytes, and subsequently decreased the generation of matrix degrading proteases and other inflammatory mediators. As far as I known, massive evidences shown that there was a crosstalk between Nrf2 and NF-κB. Due to the recent studies, inflammation in lung was enhanced in Nrf2-KO mouse comparing to normal mouse and Pan er, al reported that absence of Nrf2 may induce severe inflammation through activation of NF-κB and downstream proinflammatory cytokines in astrocytes [47]. Moreover, other studies demonstrate that activation of HO-1 could inhibit NF-κB pathway in endothelial cells [48]. Taken conclusion, these findings indicated that myricetin may protect chondrocytes through Nrf2/HO-1 pathway.

Previous study have reported that myricetin could activate the Nrf2/HO-1 pathway in divers cells such as cardiomyocytes [49], hepatocyte [26] and SHSY5Y cells [50]. However, it is not clear how dose myricetin activate Nrf2 in chondrocytes. PI3K/Akt was involved in the function exhibited by myricetin as reported by recent study [51] suggest that PI3K/Akt may act as the link between the myricetin and Nrf2. Consistent with our results, myricetin may exhibit the function of inhibiting inflammation response and ECM degradation through PI3K/Akt mediated Nrf2 signaling pathway.

DMM mouse model possess the similarity between model in animal and Human OA [52]. Intragastric administration of myricetin (20 mg/kg) every two days can effectively inhibited the degradation of articular cartilage matrix, suppressed the disruption of the joint structure comparing to DMM group.

However, there was some limitation for the application of myricetin in OA progression. Besides, we found out that myricetin at the dose of 20 mg/kg every two days can confer protective effect in mice DMM model, which was a widely acknowledged model for OA in vivo experiments. According to the surface area principle, about 200 mg myricetin should be consumed for a person of 60 kg weight. The content of myricetin in edible berries is between 14 and 142 mg/kg. It seems that it is impossible to achieve this dosage by general diet. However, the dietary supplements containing myricetin is promising for OA protection. In addition, it is true that chondrocytes tend to dedifferentiate into fibroblasts when they are cultured [53]. Nowadays, some methods have been coming up with to deal with this trouble, for example, collagen and alginate hydrogels was efficient to prevent chondrocyte dedifferentiation according to the recent study by Jin [54]. Due to our previous study, we found out that the characterization of chondrocytes within three generations will not change and the expression of chondrogenic genes, including SOX9, type II collagen, and aggrecan was almost remained [34]. In this study, chondrocytes no later than first passage were used for follow-up experiments to prevent this phenomenon.

In a conclusion, our data provides first detail study that myricetin inhibit the inflammation response, ECM degradation in human chondrocytes through PI3K/Akt mediated Nrf2/HO-1/NF-κB axis. Moreover, oral administration of myricetin on mice could ameliorate the

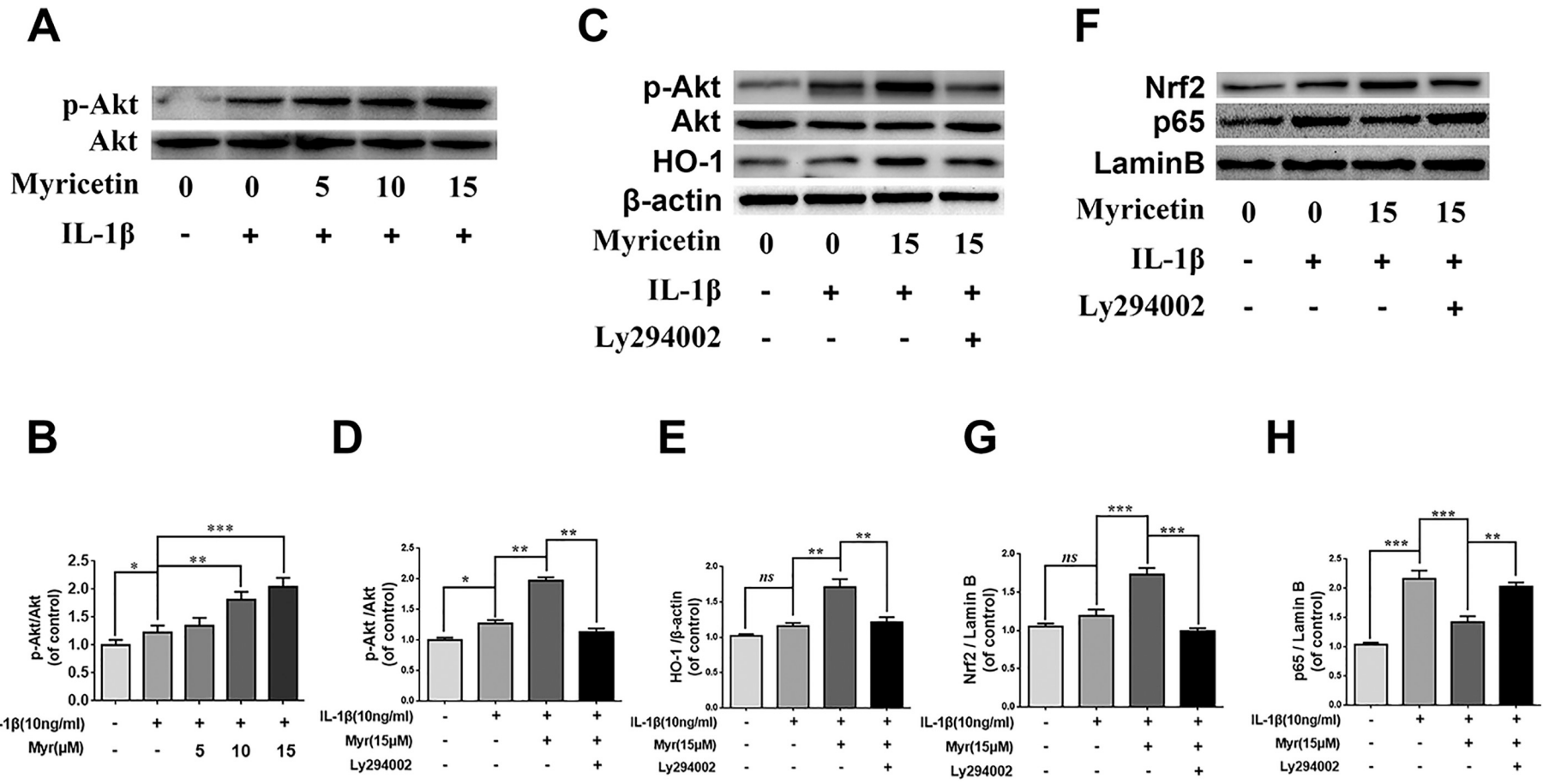

Fig. 7. Myricetin mediated Nrf2/HO-1 signaling pathway through PI3K-Akt pathway.

Human chondrocytes were untreated (DMEM 10% FBS) or treated with IL-1β (12 h), with IL-1β (12 h) plus myricetin (24 h), or with IL-1β (12 h) plus myricetin (24 h) combined with Ly294002 (4 h). (A) The expression of p-Akt and Akt in chondrocytes were evaluated by western blot. (B) Quantitative western blot analysis of p-Akt/Akt in chondrocytes. (C) The expression of Nrf2 and p65 in nuclear of chondrocytes were evaluated by western blot. (D, E) Quantitative western blot analysis of Nrf2 and p65 in nuclear of chondrocytes. (F) The expression of p-Akt, Akt and HO-1 in chondrocytes were evaluated by western blot. (G, H) Quantitative western blot analysis of p-Akt/Akt and HO-1 in chondrocytes. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

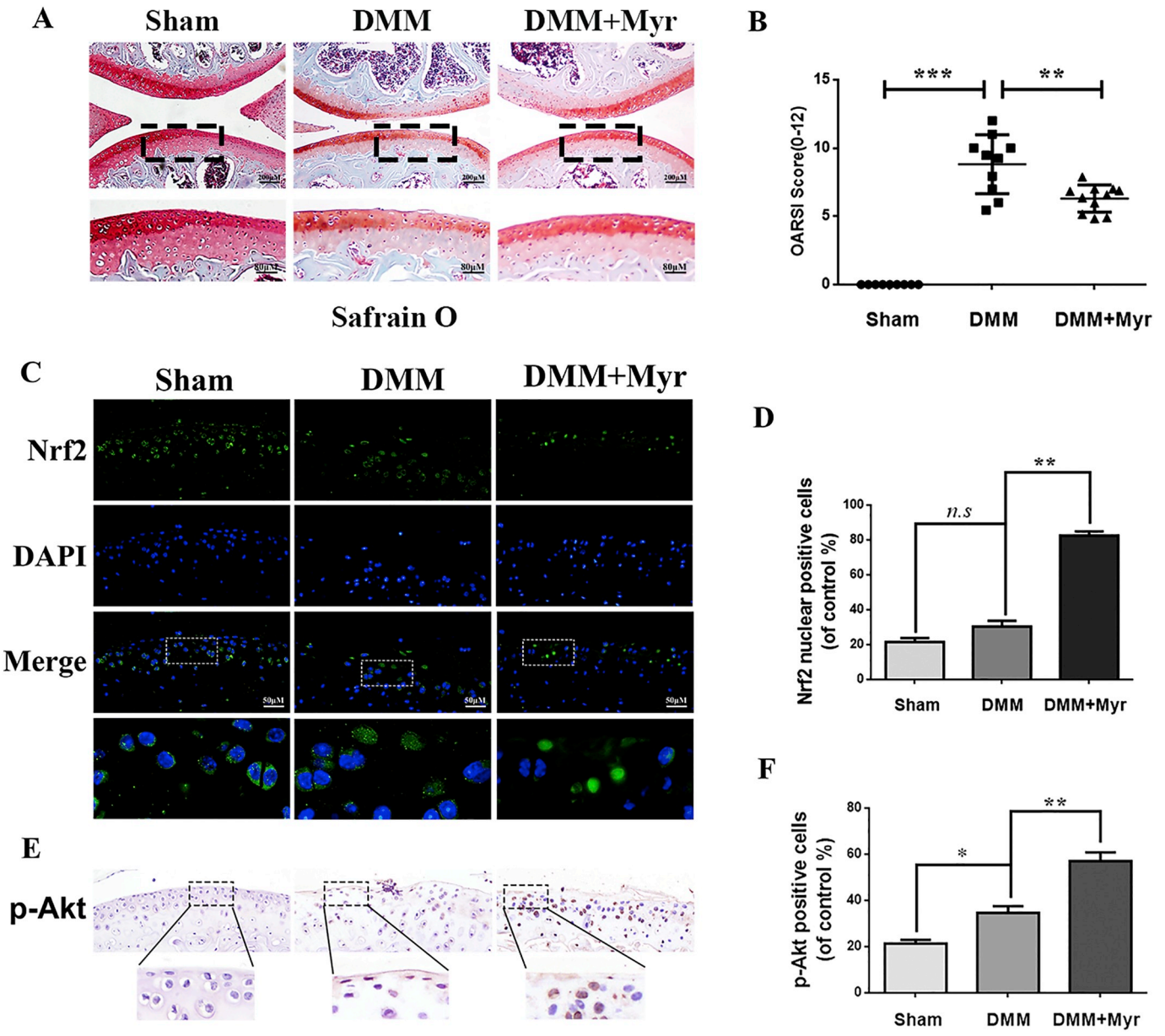

Fig. 8. Myricetin ameliorate the progression of OA in mice DMM model via Nrf2/HO-1 signaling.
The OA in mice was established by destabilization of medial meniscus (DMM) surgery. Briefly, the anterior attachment of the medial meniscus to the tibial plateau was transected to induce the instability of the right knee joint. At 8 weeks, OA development were evaluated by safranin O staining (SO; original magnification 40×, scale bar: 50 μm). (A) Representative S−O staining of cartilage from different experimental groups at 8 weeks post-surgery (bar: 200 μm, 80 μm). (B) OARIS scores of cartilage and the scores of synovitis in three groups. (C) Immunofluorescence staining of Nrf2 expression in the cartilage samples (original magnification × 400, scalebar: 50 μm). (D) The percentage of nuclear Nrf2 positive cells of total chondrocytes each section. (E) Representative p-Akt immunohistochemical staining of cartilage from different experimental groups at 8 weeks post-surgery (bar: 200 μm, 80 μm). The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $p > 0.05$.

progression of OA in mice DMM model. Thus, our study suggests myricetin possesses the therapeutic potential on osteoarthritis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105742.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Author contribution statement

Xiaozhou Ying, Xiangyang Wang and Long Chen contributed to the conception of the study. Xiangxiang Pan, Tingting Chen and Zengjie Zhang contributed significantly to analysis and manuscript preparation; Xiaowei Chen and Chengshu Chen performed the cell experiment, data analyses and wrote the manuscript; Zengjie Zhang and Tingting Chen performed the animal experiment, data analyses and wrote the manuscript.

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