Article

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Salidroside Protects Chondrocytes against IL-1β-Induced Injury and Alleviates Osteoarthritis Progression by Activating the Nrf2 Pathway

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Background: Osteoarthritis (OA) is a common disease that causes pain to many older adults. Because the pathogenesis is not fully elucidated, effective drug therapies are currently lacking. This study aimed to determine how salidroside (Sal)-mediated reduction of osteoarthritis development in mice worked and to identify the underlying mechanism.

Methods: Using *in vitro* experiments, ATDC5 cells were treated with various concentrations of Sal and interleukin (IL)-1β for 24 hours to mimic OA. An enzyme-linked immunosorbent assay (ELISA) was conducted to detect the production of pro-inflammatory cytokines and reactive oxygen species (ROS). Western blotting was performed to observe the nuclear factor-kappa B (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways. In *in vivo* experiments, pathological examination was used to assess the effects of Sal on alleviating OA progression in mice. Nrf2 signaling and its downstream proteins were further tested by immunofluorescence analysis.

Results: The results showed that both pro-inflammatory cytokines and ROS were significantly reduced following Sal treatment in a concentration-dependent manner. Western blotting revealed that Sal could inhibit the expression of the NF-κB/hypoxia-inducible factor-2α pathway and activate the Nrf2/heme oxygenase-1 pathway. *In vivo* experiments showed that the cartilage surface in the saline-treated group eroded to a greater extent than the Sal-treated groups ($p < 0.001$). Immunohistochemistry analysis revealed that matrix metallopeptidase (MMP) 9, MMP13, and a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) decreased expression level. In contrast, collagen-II and aggrecan increased in the Sal-treated groups compared to the saline-treated group.

Conclusions: Our findings indicate that Sal can alleviate OA progression by promoting anti-oxidant expression and inhibiting degradation enzyme expression. These findings suggest that Sal inhibits the NF-κB pathway and its downstream targets through up-regulating the Nrf2 pathway.

Keywords: salidroside; IL-1β-induced injury; osteoarthritis; Nrf2 pathway

Introduction

Osteoarthritis (OA) is a prevalent disease that negatively impacts the elderly population, with 10% of males and 18% of women older than 60 experiencing symptoms [1]. Such a high incidence rate of OA consumes substantial family and social resources [2]. Due to insufficient elucidation of OA pathogenesis, there is still a lack of drugs that can effectively treat this condition [3]. Current drug treatments for OA primarily consist of cyclooxygenase-2 (COX-2) inhibitors, nonsteroidal anti-inflammatory medications, and steroid hormones. However, these drugs only temporarily relieve the symptoms and cannot effectively slow down cartilage degeneration in OA [4]. Moreover, many patients with OA must eventually undergo artificial joint replacement surgery at the late stage. Thus, it is crucial to discern a disease-modifying drug for OA with fewer side effects.

Recently, studies have demonstrated a critical role for oxidative stress in OA pathogenesis [5,6]. Reactive oxygen species (ROS) can directly accelerate chondrocyte apoptosis, inhibit the synthesis of cartilage matrix, and promote interleukin (IL)-1β-mediated cartilage degradation via activating the nuclear factor-kappa B (NF-κB) pathway [7,8]. Therefore, reducing the level of oxidative stress may be a potential therapeutic avenue to inhibit OA cartilage degeneration.

Studies revealed that nuclear factor erythroid 2-related factor 2 (Nrf2) can down-regulate the NF-κB pathway and reduce the generation of pro-inflammatory cytokines [e.g., prostaglandin E-2 (PEG2) and tumor necrosis factor-α (TNF-α)] [9,10]. Several research studies have revealed that up-regulation of Nrf2 signaling provides chondrocyte-protective effects and alleviates OA progression [11–13]. Thus, Nrf2 may be a possible molecular target for the treatment of OA.

Salidroside (Sal), a compound abstracted from *Rhodiola rosea*, has long been discovered to have effects of resisting anoxia [14], anti-apoptosis [15], anti-fatigue [16], anti-tumor [17], protecting the cardiovascular system, and postponing aging [18,19]. Some studies have also confirmed that Sal had an effect in scavenging ROS and anti-inflammatory effects [20–22]. In recent years, the impact of Sal in protecting articular cartilage has been researched at greater depth, and encouraging results have been obtained. An *in vitro* study revealed that Sal alleviated OA inflammation response initiated by IL-1β from down-regulating the NF-κB signal pathway [23]. In addition, Sa *et al*. [24] found that Sal effectively relieves the acute symptoms and signs of OA in a rat model, confirming that Sal has anti-inflammatory and antioxidant effects. Gao *et al*. [25] demonstrated that Sal can stimulate chondrocytes proliferation and hinder OA rats' chondrocytes apoptosis by inhibiting the NF-κB pathway. Moreover, Zhu *et al*. [26] found that Sal can attenuate oxidative stress-induced injury for human umbilical vein endothelial cells by promoting the Nrf2/heme oxygenase-1 (HO-1) pathway. Nevertheless, the effects of Sal on chondrocytes and the underlying mechanism have not been fully clarified. Therefore, this study sought to explore the protective effect of Sal on ATDC5 cells treated with IL-1β, as well as articular cartilage in OA mice, and elucidate the potential mechanism involving the Nrf2 pathway.

Materials and Methods

Chemicals, Reagents, and Antibodies

Sal was produced by Shanghai Aladdin Biotechnology Co., Ltd. (G1817039, Shanghai, China). The following antibodies were purchased from Proteintech Group Inc. (Wuhan, China): Nrf2 (10003757), heme oxygenase-1 (HO-1) (00063937), β-actin (10025459), RelA (p65) (10021394), p-p65 (39692-34509001), inhibitor of nuclear factor-kappa B alpha (IκBα) (0095768) and p-IκBα (BA01162748), matrix metallopeptidase (MMP) 9 (00103171), MMP13 (00098899), superoxide dismutase (SOD) 1 (00099772), thioredoxin-dependent peroxide reductase 3 (PRDX3) (00059927), hypoxia-inducible factor-2α (HIF-2α) (BA01279129), collagen-II (Col II) (00097821), aggrecan (ACAN) (00101410), a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) (AI2111247), horseradish peroxidase (HRP)-labeled sheep anti-mouse/rabbit secondary antibodies (20000757/20000675). Enzyme-linked immunosorbent assay (ELISA) kits were provided by Shanghai Huding Biotechnology Co., Ltd. (Shanghai, China): IL-6 (202106), glutathione (GSH) (202105), TNF-α (202106), ROS (202103), SOD (202104), prostaglandin E-2 (PGE2) (202104), and malondialdehyde (MDA) (202105), IL-1β (202106). The nitric oxide (NO) content detection kit was bought from Solarbio Science & Technology Co., Ltd. (20210513, Beijing, China).

The main instruments used in this study were: tabletop centrifugal machine (2-6E, Sigma, St. Louis, MO, USA), low-temperature centrifuge (MIKRO200R, Hettich zentrifugen, Erfurt, Germany), microplate reader (Epoch, BioTek, Winooski, VT, USA), cell incubator (371, Thermo Scientific, Waltham, MA, USA), vertical electrophoresis apparatus (165-8001, Bio-rad, Hercules, CA, USA), gel imager (Chemilmager5500, Alpha Innotech, San Diego, CA, USA), protein transfer tank (170-3940, Bio-rad, Hercules, CA, USA), optical microscope (BX61, Olympus, Tokyo, Japan), invert microscope (CKX41, Olympus, Tokyo, Japan), fluorescence microscope (BX51, Olympus, Tokyo, Japan), vertical flow ultra clean workbench (AVC-3D1, Esco, Singapore).

Chondrocyte Culture and Differentiation

ATDC5 cells (14632, SUNNCELL, Wuhan, China) were used in the present experiments. The cells had been detected with no mycoplasma infection before use. Dulbecco's modified Eagle's medium (DMEM)/F-12 (8123355, Gibco, Carlsbad, CA, USA) mixed with 5% fetal bovine serum (FBS) (UP202207, Upsilon, Boulder, CO, USA,), and 1% bovine serum albumin (BSA) (EZ4567D106, Biofroxx, Einhausen, Germany) was used to maintain the ATDC5 cells in monolayer culture with 6000 cells/cm^2 in 6-well plates. The differentiation medium was comprised of DMEM/F-12 (1:1) with GlutaMAX I (35050061, Gibco, Carlsbad, CA, USA), including 5% FBS, 1% antibiotics, sodium pyruvate, 1% selenium, and insulin transferrin. After incubated for 6 days to grow to confluency, the culture medium was added with β-glycerophosphate (10 mM) (1300032301, Sigma-Aldrich, St. Louis, MO, USA) and L-ascorbate-2-phosphate (50 µg/mL) (2400282301, Sigma-Aldrich, St. Louis, MO, USA). The differentiation time of the ATDC5 cells was 2 weeks, and the replacement time of the culture medium was 2 days.

Cells Viability Assay

The ATDC5 cells were seeded in a 96-well plate containing 1×10^4 cells per well and incubated with Sal solution at concentrations of 0, 20, 40, 80, and 160 µM for 24, 48, and 72 hours. At each time point, 10 µL of cell counting kit-8 (CCK-8) reagent (BCCB5327, Sigma-Aldrich, St. Louis, MO, USA) mixed with 100 µL of DMEM/F12 was put into each well and incubated with 5% CO_2 for 3 hours at 37 °C. The absorbance of all the wells was detected using a microplate photometer (3131, Thermo Fisher, Waltham, MA, USA) at 450 nm of optical density (OD).

Western Blotting

Sal was pretreated in ATDC5 cells at doses of 0, 20, 40, and 60 µM for 4 hours, and then the cells were stimulated with IL-1 (10 ng/mL) for 24 hours to mimic OA on a culture dish. The entire protein from the chondrocytes

was extracted by radioimmunoprecipitation assay (RIPA) buffer (96854, CWBIO, Beijing, China) and phenylmethylsulfonyl fluoride (PMSF) (22061243, Biosharp, Beijing, China) and quantified by BCA protein assay kit (40537, CWBIO, Beijing, China). The proteins of cell lysates separated with SDS-PAGE loading buffer (20344, CWBIO, Beijing, China) were transferred to a polyvinylidene fluoride (PVDF) membrane (0000198133, Millipore, Dublin, Ireland) and blocked in a 5% non-fat milk. After incubating the membranes with primary antibodies (1:1000 dilution) against Nrf2, HO-1, β-actin, p-p65, COX-2, p65, IκB, and p-IκB for an overnight period at 4 °C, the membranes were then incubated with an HRP-conjugated secondary antibody for an additional hour at room temperature. Image J software (version 1.51J8, NIH, Bethesda, MD, USA) was applied to quantify the relative intensities of the blots.

Nrf2-Small Interfering RNA (Nrf2-siRNA) Transfection

The specific *Nrf2-siRNA* (2328448) and negative control *siRNA* (220642) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected by Lipo3000 Transfection Reagent (239038, Invitrogen, San Diego, CA, USA) according to the manufacturer's instruction. The nucleotide sequences of *Nrf2-siRNA* and negative control *siRNA* were 5′-UCAAUGUGUGCAUACCG-3′ and 5′-AAGCUGUCCCUGUUGGG-3′, respectively.

ELISA and Measurement of NO

ATDC5 cells were cultured with Sal at concentrations of 0, 20, 40, and 60 µM for 4 hours, then treated with IL-1β (10 ng/mL) for 24 hours to mimic OA *in vitro*. Supernatants were collected to detect the value of IL-6, GSH, TNF-α, ROS, SOD, PGE2, and MDA using an ELISA kit. The OD value was analyzed at 450 nm through a microplate reader (Epoch, BioTek, Winooski, VT, USA). The NO content in supernatants was measured using a NO detection kit based on the Griess reaction [27].

OA Model

The experimental protocols were approved by the Hengyang Central Hospital Medical Ethics Committee (2021HS016). Twenty-four C57BL/6 male mice weighing 24 +/– 3 g were purchased from the Guangdong Laboratory Animal Centre of Southern Medical University in China (Guangzhou, China) when they were 12 weeks old. Mice were adaptively fed for one week. Destabilization of the medial meniscus (DMM) was carried out as previously reported to produce an OA model [28]. 1% pentobarbital sodium (40 mg/kg, Ayrton Saunders, Cheshire, UK) was used to anesthetize the mice. The right knee joint was exposed by incising the skin along the medial patellar ligament, followed by opening the joint capsule and exposure of the medial tibial plateau, then the medial meniscotibial ligament and medial meniscus were transected. Mice were randomly divided into 3 groups after DMM surgery: DMM only (n = 6), DMM + Sal 20 (n = 6), and DMM + Sal 40 (n = 6). Sham surgery involved skin incision and exposure of the meniscus without medial meniscotibial ligament transection in 6 mice.

Animal Treatment and Specimen Preparation

Following DMM surgery, the DMM + Sal 20 group and DMM + Sal 40 group received Sal treatment (20 and 40 mg/kg, respectively) via intragastric administration once every 2 days for 8 weeks. The sham and DMM groups received only physiological saline (10 mL/kg). At 10 weeks post-operative, the mice were euthanized by CO_2 asphyxia. The knee joints of mice were dissected, fixed in 4% paraformaldehyde (20210423, Solarbio, Beijing, China) for 24 hours, and decalcified with 14% ethylenediaminetetraacetic acid (EDTA) (EZ5679D161, Biofroxx, Einhausen, Germany) for 5 weeks at room temperature. Next, the specimens were longitudinally embedded in paraffin, and 2 µm serial sections were cut from the medial compartment in the sagittal plane.

Immunohistochemistry (IHC) and Immunofluorescence Analysis

The sagittal slices were blocked with 1% sheep serum for 30 min at 37 °C and then treated with 3% hydrogen peroxide for 10 minutes. The sections were treated with primary antibodies for 1 hour at 37 °C, then with secondary antibodies that had been HRP-labeled for 30 minutes at 37 °C. For immunofluorescence, the sections were incubated with primary antibodies and followed by Alexa 488 dye-labeled secondary antibodies (110802, Earthox, San Diego, CA, USA). A fluorescence microscope (BX51, Olympus, Tokyo, Japan) was used to acquire the images, and Image J software (version 1.51J8, NIH, Bethesda, MD, USA) was used to detect the positive cells.

Histological Analysis

Slides for each joint were stained with hematoxylin and eosin (HE) (20210905, Solarbio, Beijing, China) and toluidine blue (TB) (21210822, Solarbio, Beijing, China) to assess the cartilage and subchondral bone morphology. Two blinded observers evaluated slides using an optical microscope (BX61, Olympus, Tokyo, Japan). The Osteoarthritis Research Society International (OARSI) scoring system was used to grade the pathological changes of the cartilage [29].

Image Drawing

The software Adobe Illustrator CC (AI) (version 2015.0.0, Adobe Systems Incorporated, San Jose, CA, USA) was used to draw a schematic figure to illustrate the molecular signaling pathways of Sal-mediated alleviation of osteoarthritis progression.

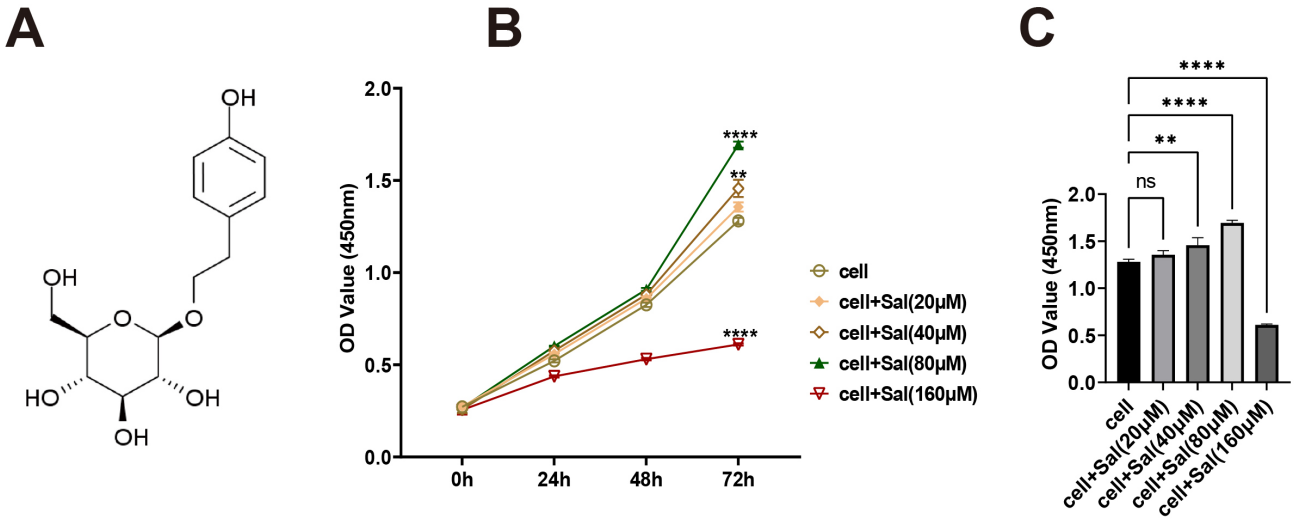

Fig. 1. Effect of salidroside (Sal) on chondrocyte survival *in vitro*. (A) The chemical structure of Sal. (B) The cytotoxic effect of Sal on chondrocytes was determined at different concentrations for 0, 24, 48, and 72 hours using a cell counting kit-8 (CCK-8) assay. (C) The viability of cultured chondrocytes was compared after 72 hours of treatment. The results were presented as the mean ± standard error (SE) (n = 3). ns, no significance; $^{**}p < 0.01$; $^{****}p < 0.0001$.

Statistical Analysis

The data were presented as the mean ± standard error (SE) and statistically analyzed using GraphPad Prism software (version 9.0, GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was used to detect the differences between all groups, and Dunnett's multiple comparisons test was applied to compare the control and treatment groups. Significant differences between the compared groups were established for p values less than 0.05.

Results

Effects of Sal on Chondrocyte Viability

Fig. 1A depicts the molecular formula for Sal's structural makeup. To observe whether Sal has an adverse impact on cell viability, a CCK-8 assay was conducted on ATDC5 cells with treatment of Sal solution at increasing concentrations (0, 20, 40, 80, and 160 μM) for 24, 48, and 72 hours. The results showed that the viability of ATDC5 cells was unaffected by Sal in the concentration range between 0–80 μM after 72 hours of treatment, but significantly affected under concentration of 160 μM (Fig. 1B,C). This suggested no toxicity was observed towards mouse chondrocytes in response to Sal treatment at concentrations ≤80 μM. Because previous study showed that Sal had good pharmacological effects at concentrations 12.5, 25, and 50 μM [23], Sal concentrations of 20, 40, and 60 μM were used in our subsequent experiments.

Sal Attenuates IL-1β-Mediated Activation of Pro-Inflammatory Cytokine Expressions in Chondrocytes by Suppressing the NF-κB Pathway

In our experiments, we assessed the effect of Sal on IL-1β-mediated inflammatory activity in ATDC5 cells. The concentration of IL-6, PGE2, TNF-α, and NO gradually decreased as the Sal concentration increased (Fig. 2A–D). The western blotting revealed that the value of COX-2 protein expression decreased significantly in the Sal treatment groups (Fig. 2E,F). These results demonstrate that Sal could reduce pro-inflammatory cytokines in chondrocytes mediated with IL-1β. To reveal the molecular mechanism of Sal in reducing the inflammatory response, the western blotting was completed to evaluate the variation of the NF-κB pathway in ATDC5 cells. Fig. 2G–K showed that the value of IκBα, p-IκBα, p65, and p-p65 increased remarkably upon IL-1β activation, whereas they were significantly inhibited following Sal treatment. HIF-2α, an inducer of catabolic factors in chondrocytes, was also found to be down-regulated following treatment with Sal (Fig. 2L).

Sal Attenuates IL-1β-Activated Oxidative Stress in Chondrocytes by Up-Regulating the Nrf2/HO-1 Pathway

Elevated ROS levels can cause damage to cellular molecules, and the antioxidant enzymes increase to counteract this damage. Thus, we evaluated the ROS and antioxidant capacity level of a cultured chondrocyte cell line in response to Sal treatment. The production of ROS and MDA vastly increased, followed by IL-1β activation; however, the treatment of Sal decreased the production of ROS and MDA in the cultured chondrocytes (Fig. 3A,B). Further experiments showed that antioxidants, such as GSH

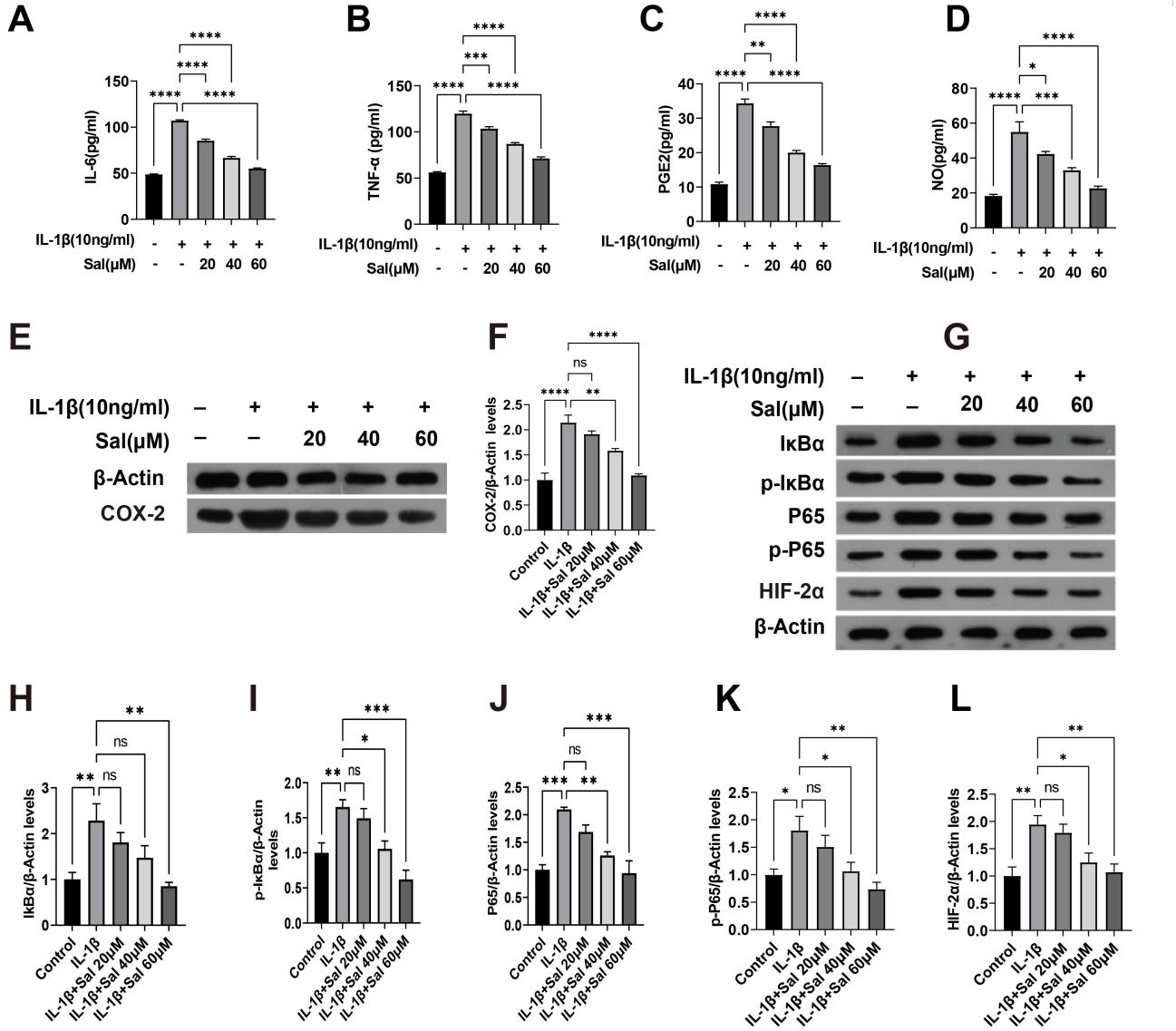

Fig. 2. Sal attenuates interleukin (IL)-1β-mediated activation of pro-inflammatory cytokine expression in chondrocytes by suppressing the nuclear factor-kappa B (NF-κB) pathway. ADTC5 chondrocytes (1×10^5 cells/well) were pretreated with various concentrations of Sal (0, 20, 40, and 60 μM) for 4 hours, followed by an incubation with IL-1β (10 ng/mL) for 24 hours. (A–C) The concentration of IL-6, tumor necrosis factor-α (TNF-α), and prostaglandin E-2 (PGE2) were evaluated by an enzyme-linked immunosorbent assay (ELISA). (D) The level of nitric oxide (NO) was determined by the Griess reaction. (E,F) The cyclooxygenase-2 (COX-2) protein expression level was quantified by western blotting. (G) Western blotting was used to evaluate the expression of inhibitor of nuclear factor-kappa B alpha (IκBα), p-IκBα, p65, p-p65, and hypoxia-inducible factor-2α (HIF-2α) in cultured chondrocytes treated with IL-1β and various concentrations of Sal. (H–L) The level of IκBα, p-IκBα, p65, p-p65, and HIF-2α protein expression was quantitatively analyzed using Image J software (version 1.51J8, NIH, Bethesda, MD, USA). The results were presented as the mean ± SE (n = 3); ns, no significance; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

and SOD in chondrocytes, increased with the administration of Sal, which showed that Sal could maintain the redox homeostasis (Fig. 3C,D).

Evidence indicates that Nrf2/HO-1 signaling has an important protective effect on chondrocytes from oxidative stress-induced injury. Previous studies found that Sal can attenuate oxidative stress-induced injury for human umbilical vein endothelial cells and nerve cells by promoting the Nrf2/HO-1 pathway [26,30]; however, it is unclear how Sal regulates the Nrf2/HO-1 pathway in chondrocytes. Therefore, western blotting was performed to evaluate the Nrf2/HO-1 pathway to reveal the antioxidative mechanism of Sal on chondrocytes. The results indicated that Sal significantly promotes Nrf2 and HO-1 protein expression in chondrocytes (Fig. 3E–G). Under IL-1β-activated oxidative stress conditions, the expression of Nrf2 and HO-1 in-

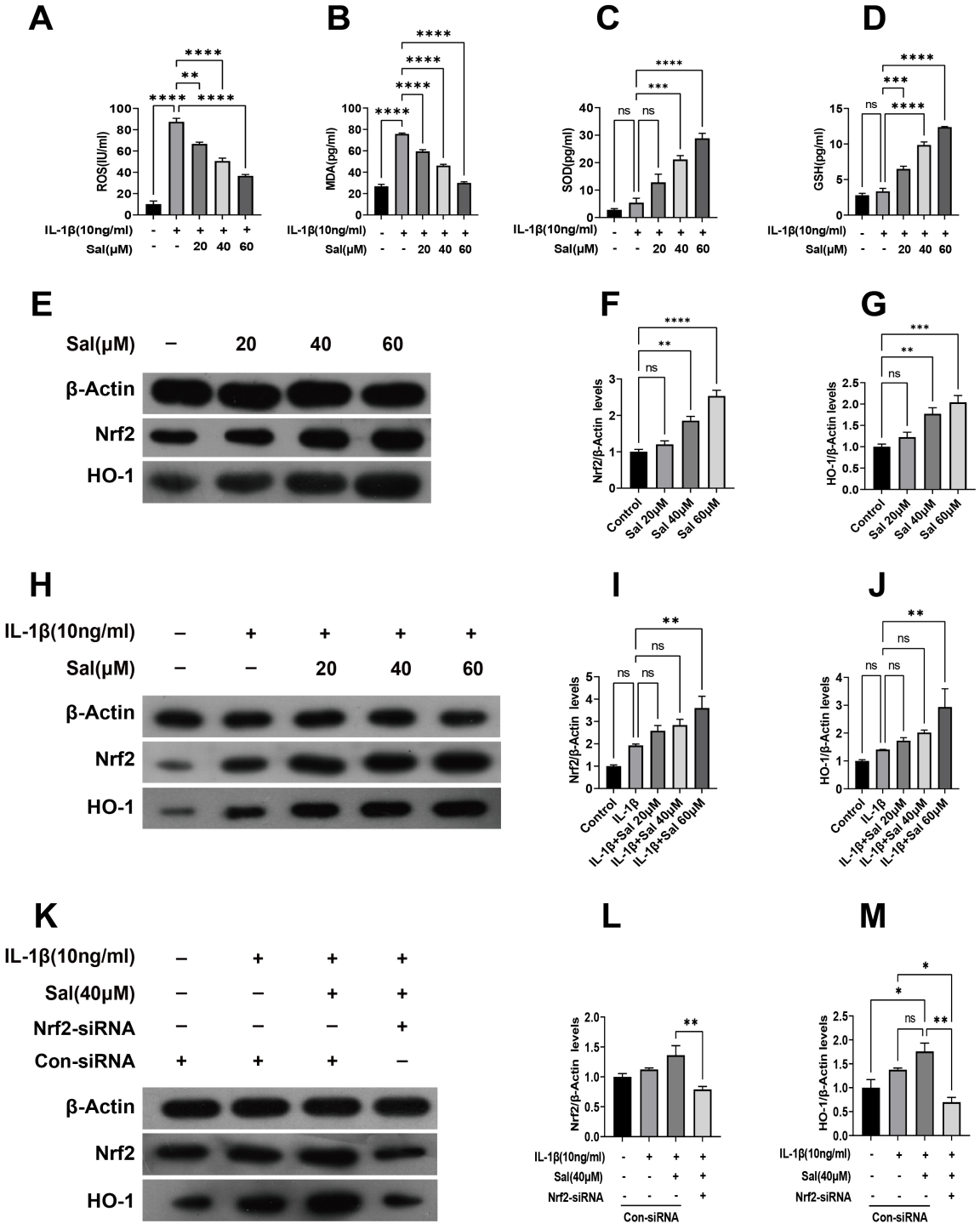

Fig. 3. Sal attenuates IL-1β-activated oxidative stress in chondrocytes by up-regulating the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway. ADTC5 chondrocytes (1×10^5 cells/well) were pretreated with various concentrations of Sal (0, 20, 40, and 60 μM) for 4 hours and then incubated with IL-1β (10 ng/mL) for 24 hours. (A,B) The level of reactive oxygen species (ROS) and malondialdehyde (MDA) was largely increased following IL-1β treatment. In contrast, adding Sal decreased the ROS and MDA levels in cultured chondrocytes. (C,D) The expression of antioxidant enzymes, including glutathione (GSH) and superoxide dismutase (SOD) in chondrocytes, were substantially increased with the administration of Sal in a concentration-dependent manner. (E–G) The western blotting results indicated that Sal significantly up-regulated the protein expression of Nrf2 and HO-1. (H–J) The expression of Nrf2 and HO-1 was highly promoted in Sal-treated chondrocytes compared to IL-1β-treated chondrocytes. (K–M) The expression of HO-1 was significantly reduced after cells transfected with Nrf2-small interfering RNA (*Nrf2-siRNA*). The results were presented as the mean ± SE (n = 3); ns, no significance; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

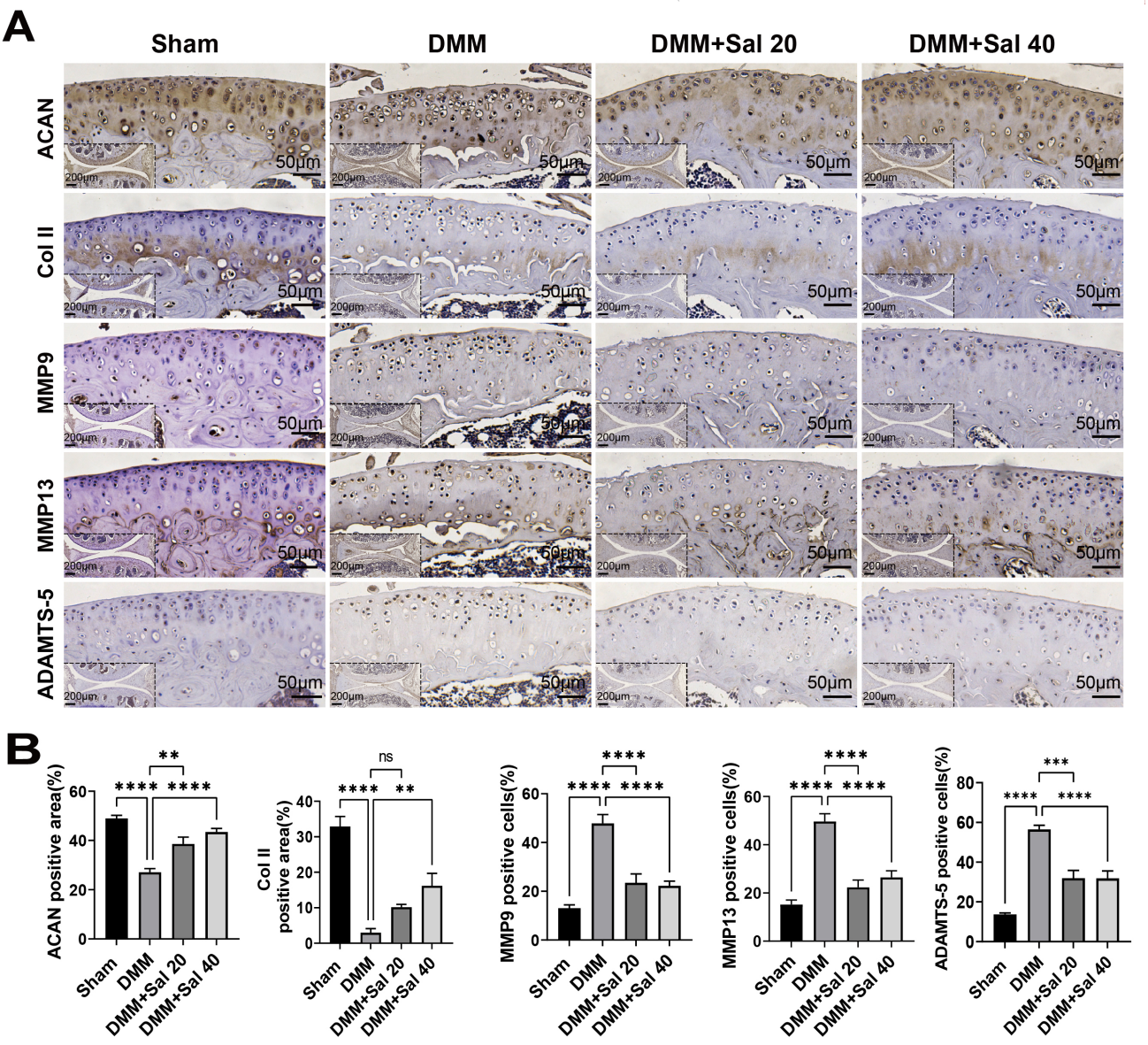

Fig. 4. Sal suppresses extracellular matrix (ECM) degradation in a mouse destabilization of the medial meniscus (DMM) model. (A) Matrix metallopeptidase (MMP) 9, MMP13, a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), collagen-II (Col II), and aggrecan (ACAN) expression in osteoarthritis (OA) mouse cartilage following Sal treatment were visualized by immunohistochemistry (IHC) staining. (B) Quantification of cells positive for MMP9, MMP13, ADAMTS-5, and positive area of Col II and ACAN. The results were presented as the mean ± SE; scale bar: 50 μm; ns, no significance; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

creased with increasing Sal concentration (Fig. 3H–J); however, transfection of *Nrf2-siRNA* resulted in a reversal of the results (Fig. 3K–M). Knockdown of Nrf2 led to the loss of the promotional effect of Sal on HO-1, confirming that Sal has an enhancing effect on the Nrf2/HO-1 pathway.

Sal Suppresses Extracellular Matrix (ECM) Degradation in a Mouse DMM Model

Matrix degradation enzymes (e.g., MMP9, MMP13, and ADAMTS-5) play a critical role in the progression of OA. To explore the performance of Sal in suppressing ECM degradation, immunohistochemistry (IHC) staining was conducted to visualize the expression of MMP9, MMP13, ADAMTS-5, Col II, and ACAN in OA mouse cartilage following Sal treatment. The data revealed that compared with the DMM group, the expressions of these matrix degradation enzymes decreased. In contrast, Col II and ACAN increased in the Sal-administered groups (Fig. 4A). Quantifying positive cells or positive areas of each IHC confirmed the matrix protective effects of Sal (Fig. 4B).

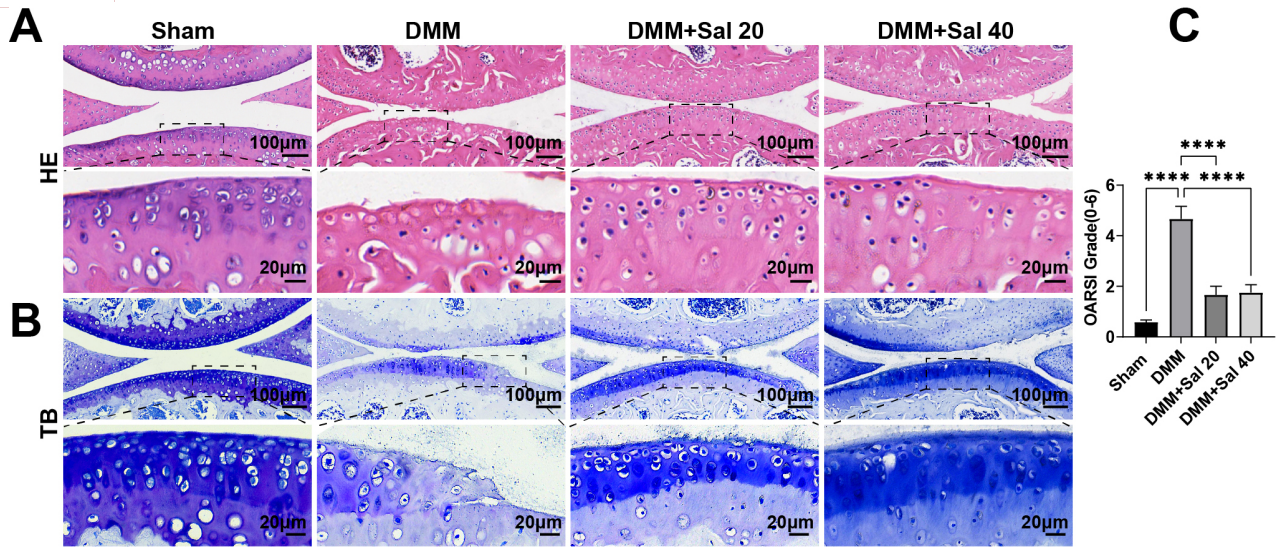

Fig. 5. Sal attenuates osteoarthritis (OA) progression in a mouse DMM model. (A) Hematoxylin and eosin (HE) staining and (B) toluidine blue (TB) staining of the articular cartilage were used to observe the morphological variations among the sham group, the DMM group, and the Sal treatment groups. (C) The Osteoarthritis Research Society International (OARIS) scores system evaluated tibial plateau cartilage. The results were presented as the mean ± SE; scale bar: 100 μm or 20 μm; ****$p < 0.0001$.

Sal Attenuates OA Progression in a Mouse DMM Model

To validate whether Sal can attenuate OA progression, we established a DMM model in mice and intragastrically administered Sal for 8 weeks. The results of HE and TB (Fig. 5A,B) staining showed that the cartilage surfaces in the DMM group were severely eroded, with a thinner hyaline cartilage layer, thickening of the calcified cartilage layer, and fewer chondrocytes. However, in the Sal treatment groups, cartilage surface erosion was mild, and both the hyaline cartilage and the calcified cartilage layers were more robust than that of the DMM group. Moreover, compared with the DMM group, the OARSI grade was significantly lower in the Sal treatment groups, indicating that Sal can attenuate OA progression (Fig. 5C).

Sal Attenuates OA Progression via Increasing Antioxidant Expression in vivo

To further confirm whether Sal can promote antioxidant expression *in vivo*, we used immunofluorescence combined with 4′,6-diamidino-2-phenylindole (DAPI) (1155MG010, Biofroxx, Einhausen, Hessen, Germany) staining to observe protein expression of Nrf2, HO-1, PRDX3, and SOD1. As shown in Fig. 6A, Nrf2 expression was up-regulated and transported into the nucleus of DMM mice treated with Sal, compared with sham and DMM mice treated with saline. The antioxidant enzymes (e.g., HO-1, PRDX3, and SOD1) expressed of a notably higher degree in Sal treatment groups than in the DMM group (Fig. 6B). Together, the results of the present study suggest that the effect of Sal promoting Nrf2/HO-1 pathway contribute to suppress OA development in mice.

Discussion

OA results from the destruction of balance between the decomposition and regeneration of chondrocytes, subchondral bone, and extracellular matrix caused by the amalgamated effects of biological and mechanical factors [31]. The pathophysiological factors leading to OA focus on two of the most extensive research pathways: NF-κB and Nrf2. NF-κB signaling is activated in chondrocytes in the processes of aging and inflammation, which promotes the release of inflammation-related factors, including MMPs, inducible nitric oxide synthase (iNOS), IL-1β, and TNF-α, leading to cartilage ECM degradation and OA progression [32]. Nrf2 activity protects cartilage by inhibiting oxidative stress and counteracting NF-κB-driven inflammatory response [10,23–25]. Thus, the up-regulating expression of Nrf2 might represent a promising method to treat OA. The present study revealed that Sal can inhibit IL-1β initiated pro-inflammation in chondrocytes and attenuate OA progression in mice through the Nrf2 pathway.

Several *in vivo* and *in vitro* experiments were conducted to investigate the effect of Sal in inhibiting the progression of OA. The concentration of Sal chosen for the treatment of OA was calculated from previously reported studies [22,25]. We found that Sal had not reduced the cell viability under a concentration of 80 μM. To simulate the effects of OA on chondrocytes *in vitro*, an IL-1β administration model was used on a chondrocyte cell line. To this end, IL-1β mingled with culture medium at a final concentration of 10 ng/mL [23].

Inflammatory cytokines promote the development of OA since local inflammation may induce chondrocyte apoptosis and activate the abnormal expression of

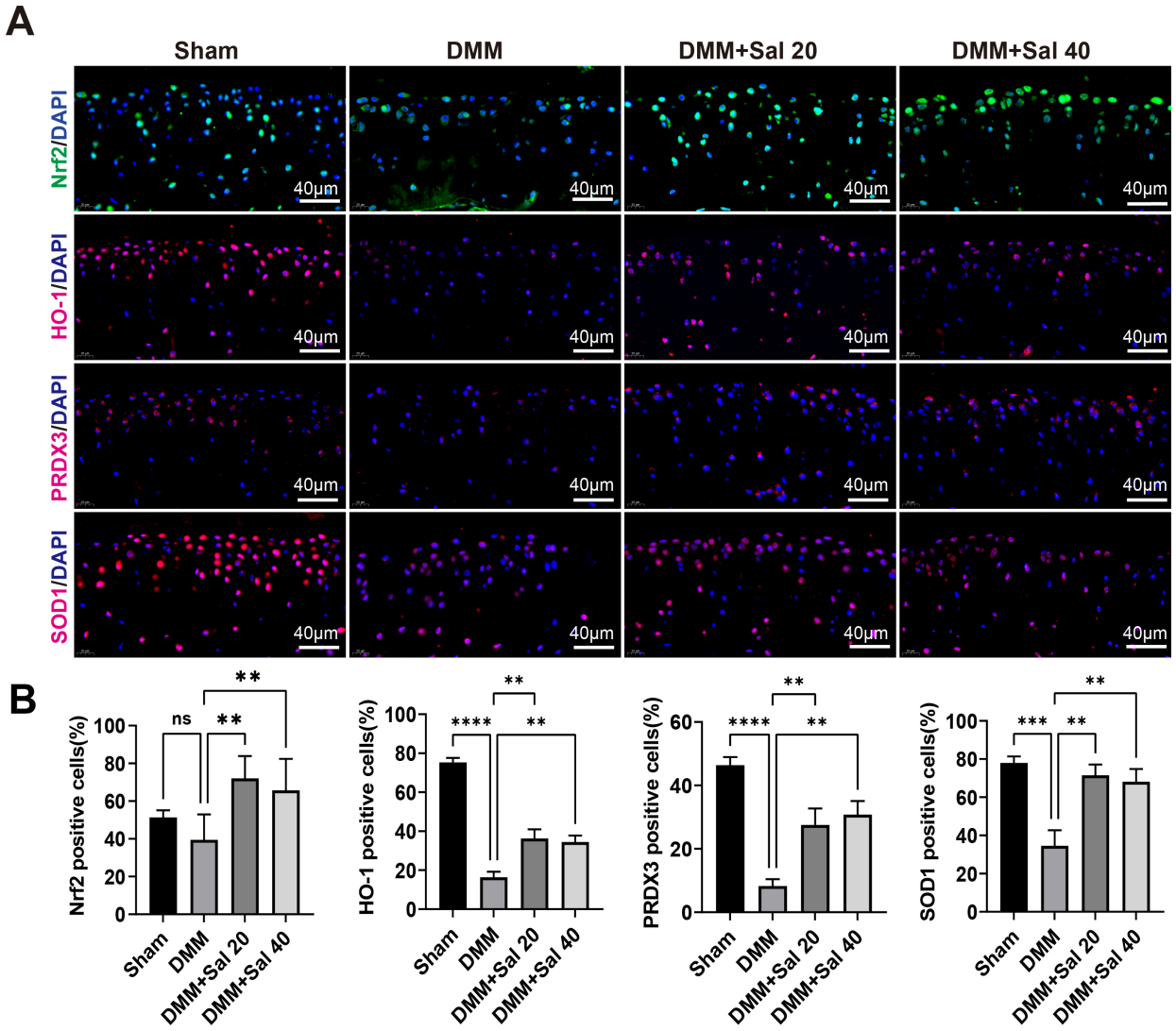

Fig. 6. Sal attenuates OA progression via increasing antioxidant expression *in vivo*. (A) The protein expression of Nrf2, HO-1, thioredoxin-dependent peroxide reductase 3 (PRDX3), and superoxide dismutase (SOD) 1 in the OA model were visualized by an immunofluorescence analysis combined with 4′,6-diamidino-2-phenylindole (DAPI) staining. (B) Quantifying Nrf2, HO-1, PRDX3, and SOD1 positive cells in the cartilage. The results were presented as the mean ± SE; scale bar: 40 μm; ns, no significance; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

inflammation-associated genes, such as catabolic genes [33]. An ELISA analysis of chondrocytes cultured under treatment with IL-1β and Sal showed that the expression level of pro-inflammatory cytokine (e.g., PGE2, IL-6, and TNF-α) increased when stimulated with IL-1β. At the same time, it decreased when Sal was administered in a dose-dependent manner. Moreover, COX-2 expression showed a similar pattern. The results of the immunoblotting assay indicated that Sal could suppress IL-1β activation of pro-inflammatory cytokine expression through the down-regulating NF-κB pathway, which was identical to the effects of previous studies [23–25].

Cartilage matrix degradation in the progress of OA involves over-expression of several matrix degradation enzymes, including MMP9, MMP13, and ADAMTS-5. Thus, experiments were performed to observe the expression of these enzymes in cartilage tissue. The results demonstrated that the positive cells of MMP9, MMP13, and ADAMTS-5 increased following DMM surgery, indicating a successful OA model. Moreover, compared with the DMM surgery group, Sal treatment decreased the expressions of these catabolic enzymes and Col II and ACAN degradation. Evidence further demonstrated that HIF-2α could directly induce catabolic enzyme expression in chondrocytes, such as MMPs, ADAMTS-4, and nitric oxide synthase-2 [34]. The western blotting results of present experiments demonstrated that Sal could down-regulate the expression of HIF-2α. Considering this, the current results suggest that Sal can maintain cartilage homeostasis by decreasing the expression of catabolic enzymes via inhibiting HIF-2α.

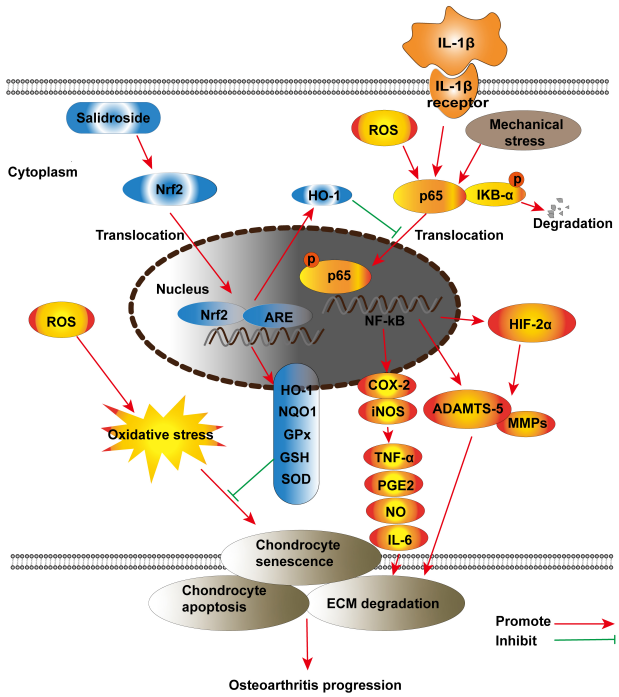

Fig. 7. Schematic representation of Sal-mediated alleviation of osteoarthritis progression. Enhanced NF-κB activity induced by IL-1β, ROS, and mechanical stress results in excess production of pro-inflammatory cytokines [e.g., TNF-α, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2)]. These pro-inflammatory cytokines further promoted the production of catabolic factors, cartilage inflammation, and chondrocyte apoptosis, resulting in articular cartilage erosion [8]. Reduced Nrf2 activity results in impaired antioxidant defense, which is characterized by a decline in HO-1, SOD, glutathione peroxidase (GPx), catalase, glutathione (GSH), and nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1 (NQO1) levels, leading to oxidative stress [10]. Both inflammation and oxidative stress can cumulatively induce structural damage to the joint, leading to OA progression. Enhanced Nrf2 activity can inhibit the NF-κB pathway and subsequently decrease the generation of inflammatory mediators [11]. The schematic representation shows that Sal prevents OA through up-regulating Nrf2 expression, and promoting it transcolate to nucleus for binding antioxidant response element (ARE), thereby inhibiting the NF-κB pathway and enhancing the scavenging capability of ROS in chondrocytes.

The age-related imbalance between ROS production and chondrocytes' antioxidant capacity is important to cartilage decomposition and chondrocyte death [35]. Therefore, we assessed antioxidant expression and capability in IL-1β and Sal-treated cells. The data showed that the level of ROS increased following IL-1β administration, and Sal treatment largely attenuated this expression. Using *in vivo* studies, immunofluorescence was conducted to visualize the expression of antioxidants and their regulators. The expression of antioxidants (e.g., HO-1, SOD1, and PRDX3) was significantly affected by DMM surgery, whereas Sal administration maintained these antioxidants increased expression. Therefore, the results demonstrated that Sal can prevent OA by reestablishing the balance between oxidants and antioxidants.

Nrf2 is a pivotal transcription factor in regulating oxidative stress and maintaining homeostasis of intracellular redox [36–40]. In this study, western blotting experiments showed that Sal can up-regulate the Nrf2/HO-1 pathway in a concentration-dependent manner. In the OA model experiment, Sal promoted the protein expression of Nrf2 and induced Nrf2 nuclear translocation, which further activated the production of Nrf2-regulated antioxidant enzymes. These results demonstrated that Sal can re-establish the oxidant and antioxidant balance in chondrocytes through up-regulating the Nrf2 pathway. Fig. 7 (Ref. [8,10,11]) describes the mechanism of Sal inhibiting cartilage degeneration.

Conclusions

Our findings demonstrate that Sal re-established the balance between ROS and antioxidant production, and inhibited degradation enzyme expression, thereby attenuating OA progression. In addition, Sal suppresses the expression of the NF-κB pathway and its downstream targets through up-regulating the Nrf2 pathway. Our experimental results provide essential evidence supporting Sal as a potential drug candidate for the treatment of OA. Although Sal can effectively attenuate OA progression, further safety assessments and clinical trials are needed to verify these findings before clinical use.

Availability of Data and Materials

The data and materials used to support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

XPL conceived the study, performed statistical analysis and wrote the manuscript. JP and HL performed the experiments. XPL and XCJ performed the pathological analysis and writing-review. All authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The experimental protocols were approved by the Hengyang Central Hospital Medical Ethics Committee (2021HS016).

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Conflict of Interest

The authors declare no conflict of interest.

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