ORIGINAL RESEARCH

Salidroside Alleviates Cartilage Degeneration Through NF-κB Pathway in Osteoarthritis Rats

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Introduction: Osteoarthritis (OA) is the most common disease, which seriously affects the daily life of the elderly. Currently, no traditional or drug therapy has been shown to explicitly block the progression of OA. Salidroside (Sal) is a bioactive component of *Rhodiola rosea*, which has many beneficial effects on human health. However, the role and mechanism of Sal in OA have not been reported.

Methods: We established an anterior cruciate ligament transection (ACLT)-induced OA Rat model. The rats were divided into five groups (n = 10): Control group; ACLT group; ACLT + Sal (12.5 mg/kg) group; ACLT + Sal (25 mg/kg) group; ACLT + Sal (50 mg/kg) group.

Results: The study showed that Sal could significantly promote the proliferation of chondrocytes in OA rats induced by ACLT and restore the histological alteration of cartilage. Besides, Sal upregulated the levels of Collagen II and Aggrecan, and downregulated the level of MMP-13. Furthermore, Sal could reduce the number of CD4+IL-17$^+$ cells and decrease the levels of IL-17, IKBα and p65, while elevating the number of CD4+IL-10$^+$ cells and the level of IL-10. The decrease of IL-17 further inhibited the dissociation of IKBα to p65, thus reducing the release of TNF-α and VCAM-1. Taken together, Sal alleviates cartilage degeneration through promoting chondrocytes proliferation, inhibiting collagen fibrosis, and regulating inflammation and immune responses via NF-κB pathway in ACLT-induced OA Rats.

Discussion: Collectively, our study investigates the role and mechanism of Sal in OA, which lays a foundation for the application of Sal in OA.

Keywords: osteoarthritis, salidroside, cartilage degeneration, collagen fibrosis, inflammation, immune responses, NF-κB

Introduction

Osteoarthritis (OA) is a chronic arthritis that often occurs in the hip, knee and elbow joints. According to the World Health Organization (WHO), the prevalence of OA is 9.6% in men over 60 years old and 18% in women.[1] OA is characterized by degenerative changes of articular cartilage and secondary osteoporosis.[2,3] The main manifestations of articular cartilage injury caused by OA are loss of cartilage matrix, decrease of chondrocyte, degeneration and exfoliation of cartilage.[4] OA is also caused by the complex interaction of many factors such as inflammation.[5] Currently, no traditional or drug therapy has been shown to explicitly block the progression of OA.[6–8] Surgical intervention may be beneficial when mechanical malformations are present. However, the benefits are limited to advanced OA, and are not considered a long-term solution.[9] Therefore, it is very important to explore new therapeutic methods for the treatment and prevention of OA.

Salidroside (Sal) is a bioactive component derived from *Rhodiola rosea*. Studies have suggested that Sal has various pharmacological effects, including anticancer,[10]

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anti–inflammation,[11] anti-fatigue[12] and anti-apoptosis[13,14] and enhanced immune response.[15] Extensive evidences indicated that Sal ameliorated various diseases, such as diabetic kidney disease,[13] asthma[16] and Parkinson's disease.[17] For example, Kallscheuer et al found that Sal had a positive therapeutic effect on Huntington's disease.[18] Qin et al declared that Sal was a platinum sensitizer to improve the efficacy of chemotherapy for liver cancer.[19] In OA, Liu et al found that Sal alleviated the inflammatory injury induced by LPS through regulating miR-145.[20] Similarly, Zhang and Zhao et al reported that Sal inhibited OA inflammation induced by IL-1β through inhibiting the activation of NF-κB pathway in vitro.[21] However, the role and possible molecular mechanism of Sal in OA have not been reported in animal models.

Inflammatory cytokines are associated with the pathogenesis of OA.[22] Helper T cells (CD4+T cells) and Treg cells participate in the immune balance of the body.[23–25] Th17 (CD4$^+$IL-17$^+$) is a kind of CD4$^+$T cells secreting IL-17 is an important pro-inflammatory cytokine, which can stimulate the production of various inflammatory cytokines, such as IL-6, IL-8 and VCAM-1. CD4$^+$IL-10$^+$ is a regulator T cell that can secrete anti–inflammatory cytokine IL-10. Nuclear factor-kappa B (NF-κB), a regulatory protein, mediates the production of various inflammatory factors. NF-κB plays a key role in the pathogenesis of OA.[26] Zeng et al found that the low-expression of FOXM1 alleviated IL-1β-induced inflammation by inactivating the NF-κB pathway in OA.[26] Besides, Choi et al believed that the NF-κB pathway may be involved in Arg-II-induced OA cartilage injury by up-regulating the levels of MMP3 and MMP13.[27]

In this study, we elucidated the deeper molecular mechanism of Sal in OA, which laid the foundation for Sal as a clinical drug of OA.

Materials and Methods
Animal Model
Animal experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals, and were approved by The Affiliated Hospital of North Sichuan Medical College (SYXK(川)2018–075). A total of 50 female Sprague-Dawley rats (12-week-old, 250-300 g) were purchased from the Animal Center of the Affiliated Hospital of North Sichuan Medical College. The rats are placed in an environment with a constant temperature of 25 ± 3°C and a humidity of 60%, and free access to food and water in a 12 h light/dark cycle. Bilateral knee anterior

cruciate ligament transection (ACLT) was carried out as previously.[28] Briefly, before ACLT, rats were anesthetized with pentobarbital sodium [0.1mL/100g intraperitoneally (Ip), 40 mg/kg]. After sufficient disinfection, the medial skin of the patellar ligament was incised and the joint capsule was opened to dislocate the patellar bone. Flexing the knee joint exposed the anterior cruciate ligament and cut the anterior cruciate ligament. The Lachman test was used to confirm ACLT.[29] After rinsing with sterile saline, the wound was closed layer by layer and disinfected, and penicillin was injected intramuscularly to prevent infection. After successful modeling, the rats were divided into five groups (n = 10): Control group (Healthy rats without any treatment); ACLT group (Rats were modeled as described above), ACLT model; ACLT + Sal (12.5 mg/Kg) group, OA rats were given 12.5 mg/kg Sal; ACLT + Sal (25 mg/kg), OA rats were given 25 mg/kg of Sal; ACLT + Sal (50 mg/kg), OA rats were given 50 mg/kg of Sal.[30] After 6 weeks, the rats were anesthetized with pentobarbital sodium (0.1 mL/100 g IP, 40 mg/kg) and sacrificed. The synovium was immediately taken out and embedded in paraffin for subsequent experiments.

Histological Analysis
Synovial tissue sections were dewaxed with 4% xylene and dehydrated with gradient alcohol.[31] For H&E staining, tissues were stained with H&E staining kit (toluidine blue, Keygen Biotech. Nanjing, China). For Masson, tissues were stained with Masson trichrome staining kit (toluidine blue, Keygen Biotech. Nanjing, China). For Safranin O staining, tissues were stained with Safranin O solution (ScyTek, Logan, UT, USA). All staining was performed according to the manufacturer's instruction, and the morphological changes were observed with an optical microscope (BX51; Olympus Corp., Tokyo, Japan).

Western Blotting
Proteins isolated from synovial tissues were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore, MA, USA) and sealed with 5% skim milk. Then, the membranes were reacted with primary antibodies overnight at 4°C. The primary antibodies were as follows: PCNA (#2586, 1:1000, Cell Signaling Technology, USA), IKBα (#4812, 1:1000, CST, USA), P65 (#8242, 1:1000, CST, USA), TNF-α (#11948, 1:1000, CST, USA), VCAM-1 (#39036, 1:1000, CST, USA), caspase-3 (#9665, 1:1000, CST, USA), caspase-9 (#9508, 1:1000, CST, USA), Lamin A (#86846, 1:1000, CST, USA), Ki-67 (sc-23900, 1:1000 Santa Cruz

Biotechnology, USA), Collagen II (sc-47764, 1:1000, SCB, USA), Aggrecan (sc-166951, 1:1000, SCB, USA), MMP-13 (sc-515284, 1:1000, SCB, USA). After washing with PBS, the membranes were incubated with anti-rabbit IgG (#7074, 1:2000, CST, USA) for 1 h and visualized with ECL kit (Perkin Elmer Cetus, Foster City, CA, USA).

RT-qPCR

The total RNA isolated from synovial tissues were reverse-transcribed into cDNA by the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher). Then, RT-qPCR was performed with BeyoFast™ SYBR Green qPCR Mix (D7260, Beyotime, Shanghai, China) by using the Bio-Rad CFX96 PCR System (Bio-Rad, CA, USA). Primers were as follows: β-actin,5′-TCACCGAGCGCGGCT-3′ (sense) and 5′-TAAT GTCACGCACGATTTCCC-3′ (antisense); Collagen, 5′-TG ACGCTAAGCTCGACGCG-3′ (sense) and 5′-CCATGCA ATCGCCATTAGC-3′ (antisense); Aggrecan, 5′-ACGTGGC ACGTTGCAATGCT-3′ (sense) and 5′-TACGTACGTGGC ATACGC-3′ (antisense); MMP-13, 5′-TGCATGCCACTGC ACGTACG-3′ (sense) and 5′-GACTACTTGCAACGTACG C-3′ (antisense). β-actin was employed as an internal reference. The fold change is evaluated by the equation $2^{-\Delta\Delta Ct}$.

Enzyme-Linked Immuno Sorbent Assays (ELISA)

The serum was extracted and stored at –80°C for analysis. Inflammatory cytokine (interleukin-17, IL-17) and immunomodulatory factor (interleukin-10, IL-10) were detected by ELISA kits. (Bio-Swamp, Shanghai, China). The OD value was examined at 450 nm with a microplate reader (BioTek Epoch, Winooski, VT, USA).

Flow Cytometry

Single-cell isolated from synovial tissues were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 10% FBS (Whittaker, Walkersville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). After induction with 50 ng/mL PMA (Sigma, St. Louis, MO, USA), 1 μg/mL ionomycin (Sigma, St. Louis, MO, USA) and brefeldin A (BD Bioscience) for 5 h, the cells were incubated with Anti-CD4-PC7, fixed with CytoFix/Cyto Perm buffer (BD Bioscience) and stained with Anti–IL-17-PE or Anti–IL-10-PE (BD Bioscience), respectively. The number of $CD4^+IL\text{-}17^+$ and $CD4^+IL\text{-}10^+$ cells were examined by FACS Canto (BD Biosciences).

Statistical Analysis

SPSS 21.0 (Chicago, Illinois, USA) was used for statistical analysis (n=5). The measured data are expressed as mean ± standard deviation (x ± s), and the data consistent with the normal distribution were analyzed by t-test. Multiple groups of data were analyzed by one-way ANOVA. The count data are expressed as a percentage or ratio and verified by chi-square test. $P < 0.05$ was considered to be statistically significant.

Results

Sal Improved Cartilage Injury by Promoting Chondrocytes Proliferation in OA Rats

To investigate the role of Sal in cartilage injury, H&E staining and Western blotting were performed. As shown in Figure 1A, the normal chondrocytes were arranged neatly, with clear tidal lines and complete matrix in the control group. However, in the ACLT-induced OA group, chondrocytes were severely damaged and exfoliated. Sal treatment (12.5, 25 and 50 mg/kg) dose-dependently ameliorated cartilage injury (Figure 1A). Besides, Western blotting showed that chondrocytes proliferation was obviously inhibited in the ACLT-induced OA group. Interestingly, Sal (12.5, 25 and 50 mg/kg) treatment dose-dependently promoted the proliferation of chondrocytes compared to the ACLT-induced OA group (Figure 1B). In addition, ACLT also induced chondrocytes death in ACLT-induced OA group, and Sal treatment (12.5, 25 and 50 mg/kg) suppressed chondrocytes death in a dose-dependent manner (Figure 1C). Thus, we declared that Sal improved cartilage injury by promoting ACLT-induced chondrocytes proliferation in OA rats.

Sal Alleviated Cartilage Injury by Inhibiting the Proliferation of Collagen Fibers in OA Rats

Safranin O staining and Masson staining were used to further confirm the healing effect of Sal on cartilage alteration. As shown in Figure 2A, Safranin O staining showed that the cartilage area of the control group was red and the bone area was blue. However, in OA group, the cartilage tissues were severely damaged and the red area almost disappeared. Intriguingly, Sal treatment (12.5, 25 and 50 mg/kg) dose-dependently ameliorated cartilage injury in cartilage tissue (Figure 2A). Besides, Masson staining showed that the collagen fibers were blue, while muscle fibers were red in the control group. However, in OA group,
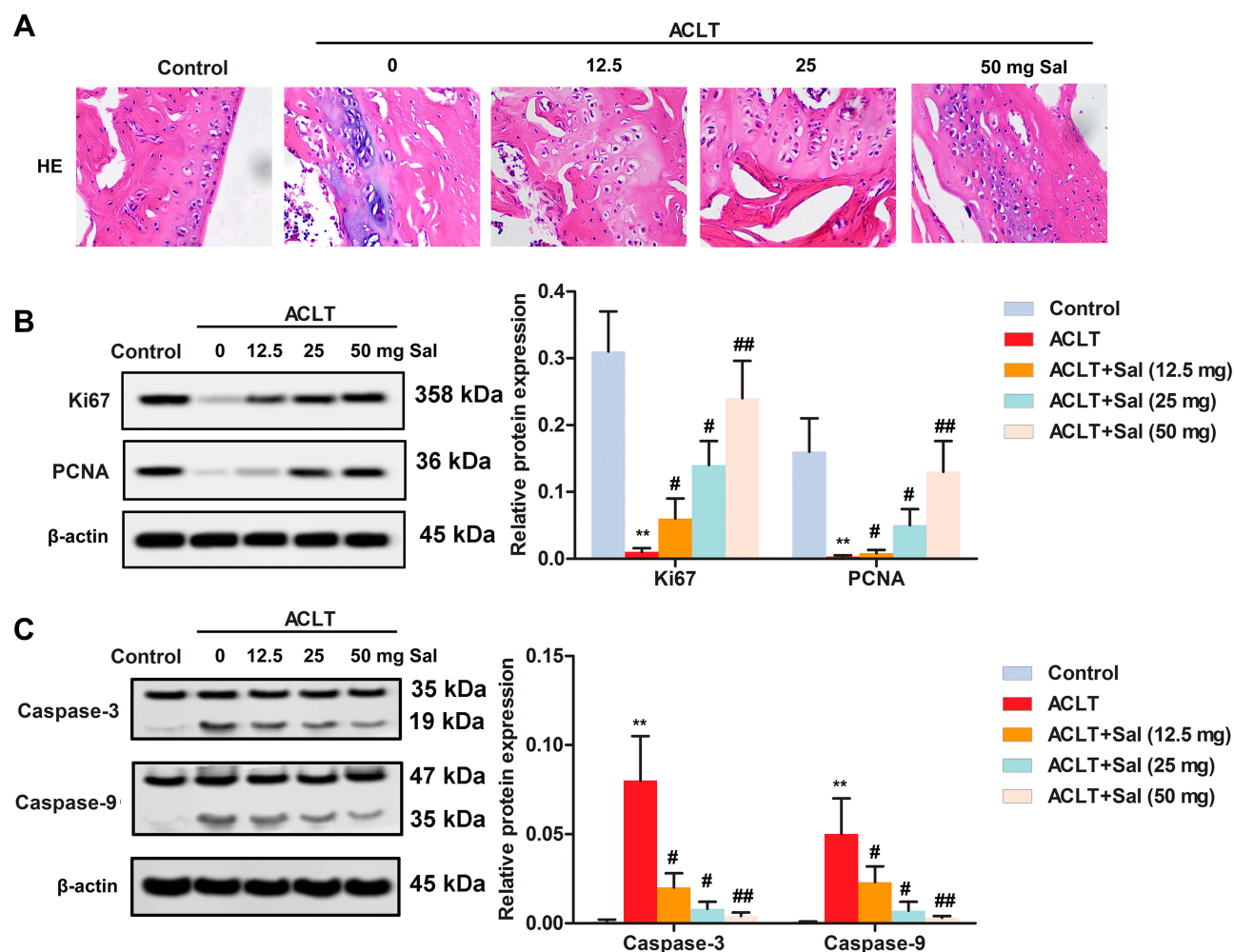
Figure 1 Sal improved cartilage injury by promoting chondrocytes proliferation in OA rats. Rats were divided into five groups (n=10): Control group (Healthy rats were given saline treatment); ACLT, ACLT model; ACLT + Sal (12.5 mg/kg), OA rats were treated with 12.5 mg/kg Sal; ACLT + Sal (25 mg/kg), OA rats were treated with 25 mg/kg Sal; ACLT + Sal (50 mg/kg), OA rats were treated with 50 mg/kg Sal. (**A**) Cartilage injury was analyzed by H&E staining. (Magnification × 400). (**B**) Levels of proliferation marker proteins (Ki67 and PCNA) were measured by Western blotting. (**C**) Levels of apoptosis marker proteins (Caspase-3 and Caspase-9) were measured by Western blotting. β-actin was employed as an internal reference. (**P < 0.01 vs control group, #P < 0.05, ##P < 0.01 vs ACLT group).

the blue areas of collagen fibers are markedly enlarged. Similarly, Sal treatment (12.5, 25 and 50 mg/kg) dose-dependently reduced the accumulation of collagen fibers and inhibited fibrosis in cartilage tissue (Figure 2B). The results of Safranin O staining were consistent with H&E staining. Therefore, this study demonstrated that Sal alleviated cartilage injury by inhibiting the proliferation of collagen fibers in ACLT-induced OA rats.

Sal Alleviated Cartilage Injury by Regulating the Levels of Extracellular Matrix Proteins (Collagen II, Aggrecan, MMP-13) in OA Rats

RT-qPCR showed that Collagen II and Aggrecan were decreased and MMP-13 was increased in OA group. However, different doses of Sal (12.5, 25 and 50 mg/kg)

remarkedly increased the levels of Collagen II and Aggrecan, while decreasing MMP-13 level (Figure 3A). Consistent with these results, Western blotting showed that Sal dose-dependently increased the levels of Collagen II and Aggrecan, while decreasing the level of MMP-13 (Figure 3B). Taken together, these results demonstrated that Sal alleviated cartilage injury by regulating the levels of extracellular matrix proteins (Collagen II, Aggrecan, MMP-13) in ACLT-induced OA rats.

Sal Ameliorated Cartilage Injury by Regulating Inflammation and Immune Responses in OA Rats

ELISA assay showed that the level of IL-17 in peripheral blood was increased, while the level of IL-10 was decreased
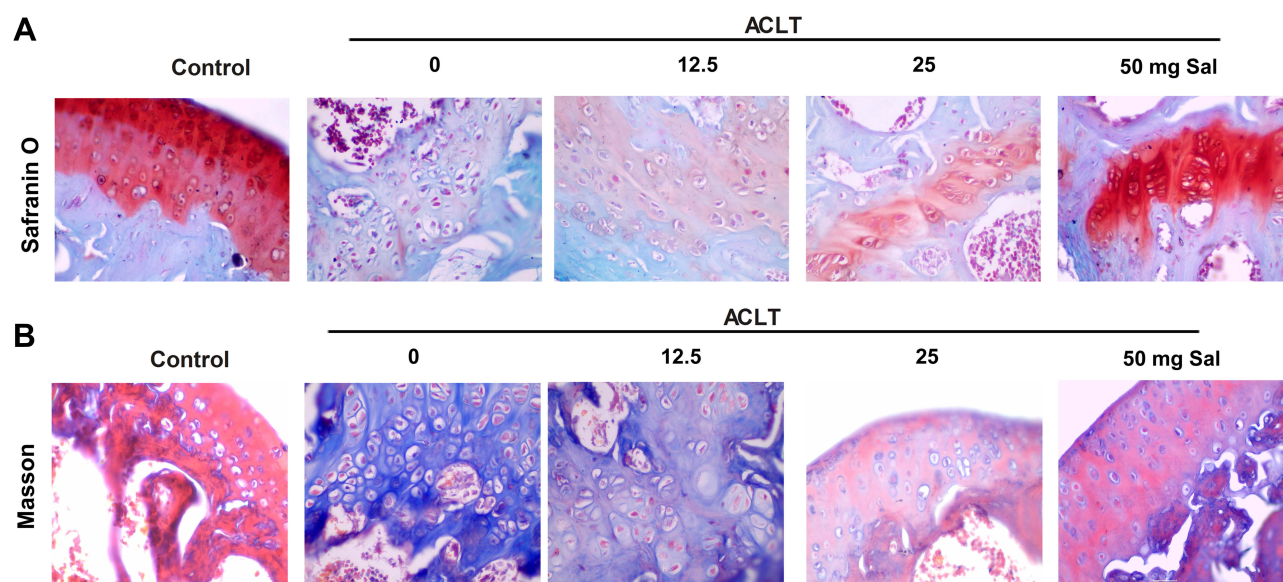
A

B

Figure 2 Sal alleviated cartilage injury by inhibiting the proliferation of collagen fibers in OA rats. Rats were divided into five groups (n=10): Control group (Healthy rats were given saline treatment); ACLT, ACLT model; ACLT + Sal (12.5 mg/kg), OA rats were treated with 12.5 mg/kg Sal; ACLT + Sal (25 mg/kg), OA rats were treated with 25 mg/kg Sal; ACLT + Sal (50 mg/kg), OA rats were treated with 50 mg/kg Sal. (**A**) Cartilage injury was analyzed by Safranin O staining. (**B**) Cartilage injury was analyzed by Masson staining. (magnification × 400).

compared to the control group (Figure 4D). Interestingly, treatment with different doses of Sal (12.5, 25 and 50 mg/kg), the level of IL-17 was dose-dependently decreased, while the level of IL-10 was increased. Besides, flow cytometry analysis further confirmed that ACLT increased the number of $CD4^+IL-17^+$ cells (Th17), and decreased the number of $CD4^+IL-10^+$ cells. Sal treatment (12.5, 25 and 50 mg/kg) reduced the number of $CD4^+IL-17^+$ cells and increased the number of $CD4^+IL-10^+$ cells to varying degrees (Figure 4A–C). Totally, these results indicated Sal ameliorated cartilage injury by regulating inflammation and immune responses in OA rats.

Sal Ameliorated Cartilage Injury by the Regulation of NF-κB Pathway in OA Rats

To clarify the potential molecular mechanism of Sal in cartilage repair, Western blotting was performed. As shown in Figure 5A–C, ACLT induction increased the level of IKBα compared with the control group. Increased IKBα then dissociated to NF-κB p65, and further promoted the release of TNF-α and VCAM-1. However, Sal treatment (12.5, 25 and 50 mg/kg) inhibited the up-regulation of IKBα induced by ACLT, and then decreased the level of NF-κB p65, TNF-α and VCAM-1. Collectively, this study demonstrated that Sal ameliorated cartilage injury by the regulation of NF-κB pathway in OA rats.

Discussion

As the most common arthropathy, OA seriously affects the daily life of the elderly.[32] Articular cartilage degeneration is the leading cause of OA. The main drugs for the treatment of OA are acetaminophen, NSAIDs, celecoxib and VA692.[33–35] Recently, studies on the treatment of OA have focused on Chinese herbal extracts. Evidence from Wang et al revealed that Agkistrodon, an ethanol extract of *Agkistrodon acutus*, improved chondrogenic injury of OA rats by enhancing cell survival, restraining cell apoptosis and abnormal expression of collagen II and MMP13.[36] As reported by Zeng et al, curcumin, a component extracted from *Curcuma longa*, alleviated inflammation of OA by down-regulating the level of MMP3, inhibiting cell proliferation and inducing cell apoptosis.[37] At this point, we elucidated the role of Sal in OA in vivo. The results demonstrated Sal has a significant therapeutic effect on OA rats. Our results were consistent with previous research in vitro.[20,21]

Salidroside is the bioactive component of *Rhodiola rosea*, which has various beneficial effects on human health. Numerous studies have mentioned that Sal was involved in the treatment of many diseases, such as non-alcoholic steatohepatitis[38] and Alzheimer's disease.[39] During OA, chondrocytes undergo massive apoptosis. Previous studies have shown that Sal promoted chondrocyte proliferation, inhibited IL-1β-induced apoptosis, and removed reactive oxygen species (ROS) and nitric oxide (NO) from
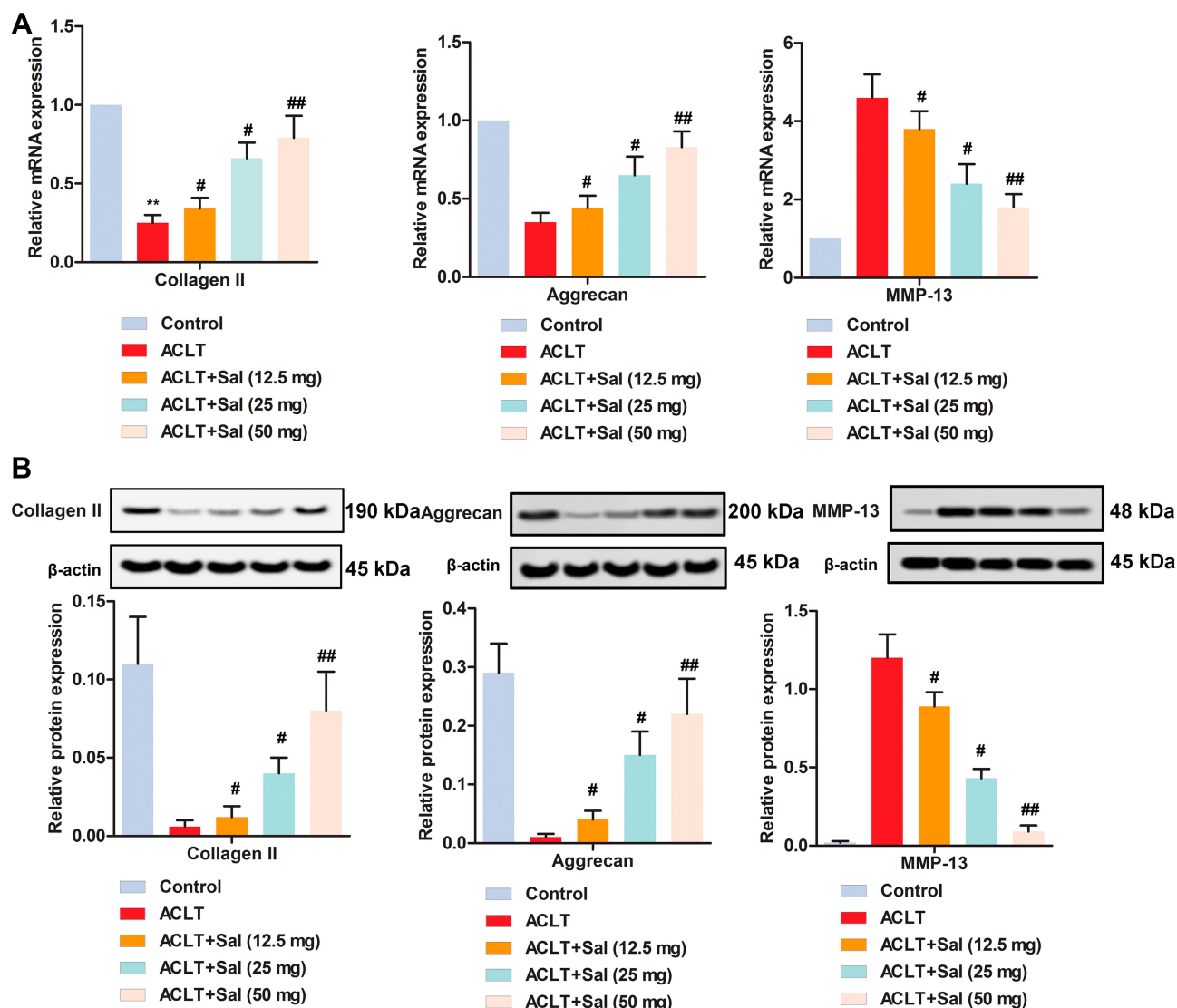
Figure 3 Sal alleviated cartilage injury by regulating the levels of extracellular matrix proteins (Collagen II, Aggrecan, MMP-13) in OA rats. Rats were divided into five groups (n=10): Control group (Healthy rats were given saline treatment); ACLT, ACLT model; ACLT + Sal (12.5 mg/kg), OA rats were treated with 12.5 mg/kg Sal; ACLT + Sal (25 mg/kg), OA rats were treated with 25 mg/kg Sal; ACLT + Sal (50 mg/kg), OA rats were treated with 50 mg/kg Sal. (**A**) Relative mRNA expression levels of Collagen II, Aggrecan and MMP-13 were measured by RT-qPCR. (**B**) Relative protein levels of Collagen II, Aggrecan and MMP-13 were measured by Western blotting. β-actin was employed as an internal reference. (**P < 0.01 vs control group, #P < 0.05, ##P < 0.01 vs ACLT group).

chondrocytes.[40] Similarly, this study found that Sal promoted the proliferation of chondrocytes in OA rats and inhibited the expression of apoptotic proteins (cleaved caspase 3 and cleaved caspase 9). The main factors for the progression of OA are chronic inflammation and progressive structural changes in joint tissues. Among them, inflammatory cytokines and proteases are the two main factors that change the microenvironment of the cell by regulating the composition of the extracellular matrix of cartilage, and further interfere with cell function. It is worth noting that Sal inhibited the production of matrix metalloproteinases induced by IL-1β in human OA chondrocytes, and had a protective effect on LPS-induced

ATDC5 cell damage.[20,21] According to reports, Sal had an inhibitory effect on IL-1β-induced OA inflammation by inhibiting the activation of the NF-κB pathway in vitro.[21] Similarly, this study found that Sal reduced the number of CD4 $^+$ IL-10 $^+$ cells in OA rats and increased the number of CD4 $^+$ IL-10 $^+$ cells, thereby regulating the inflammation and immune response in OA rats. The fibrosis of articular cartilage, which is dominated by type III collagen deposition, can affect the local cellular metabolism and biomechanical characteristics of joints during OA. Based on this, the present study found that Sal inhibited collagen fibrosis and reduced cartilage degeneration in OA rats by NF-κB pathway.
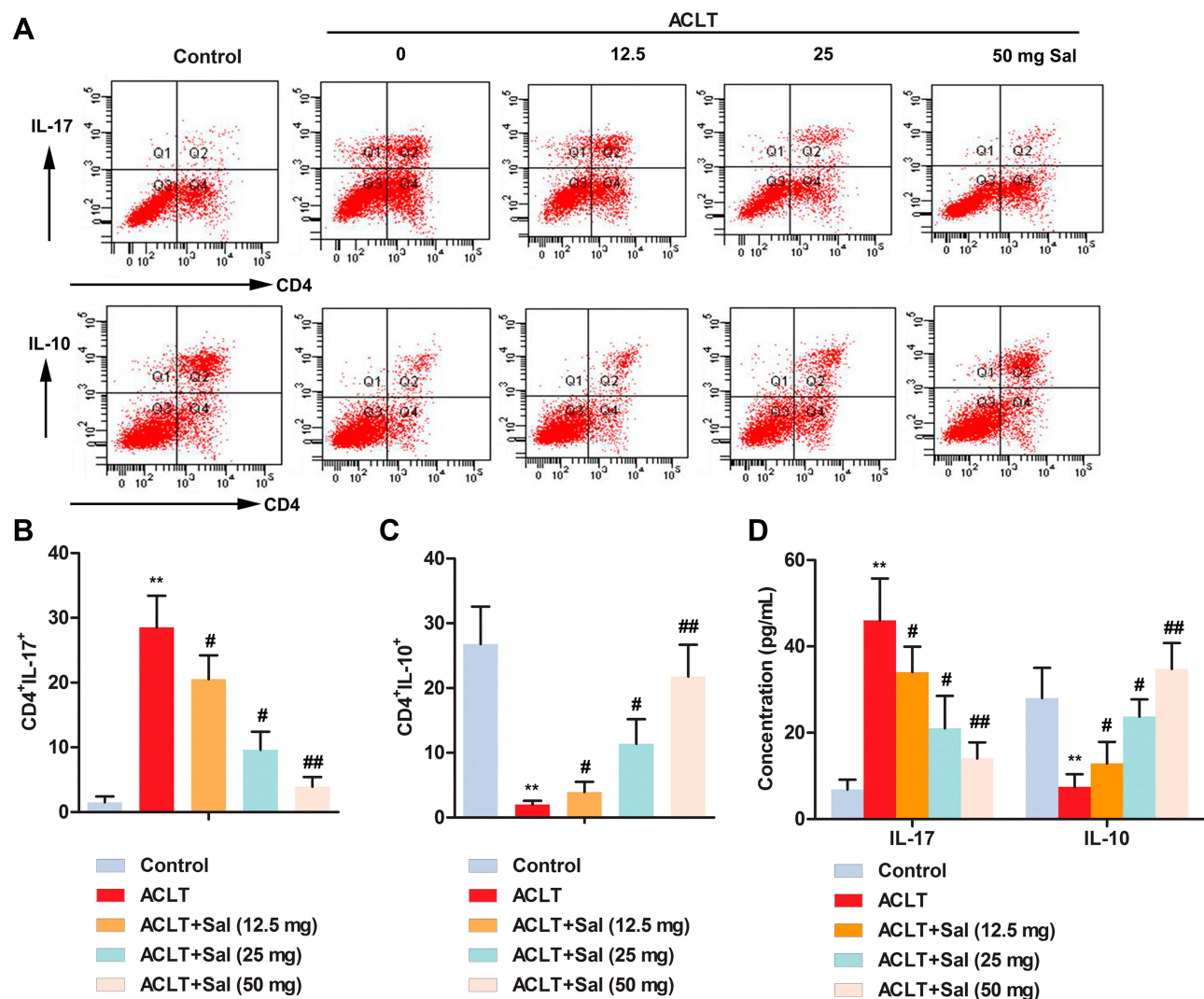
Figure 4 Sal ameliorated cartilage injury by regulating inflammation and immune responses in OA rats. Rats were divided into five groups (n=10): Control group (Healthy rats were given saline treatment); ACLT, ACLT model; ACLT + Sal (12.5 mg/kg), OA rats were treated with 12.5 mg/kg Sal; ACLT + Sal (25 mg/kg), OA rats were treated with 25 mg/Kg Sal; ACLT + Sal (50 mg/kg), OA rats were treated with 50 mg/kg Sal. (**A–C**) The number of CD4$^+$IL-17$^+$ and CD4$^+$IL-10$^+$ cells was examined by flow cytometry. (**D**) Levels of IL-17 and IL-10 were measured by ELISA. (**P < 0.01 vs control group, #P < 0.05, ##P < 0.01 vs ACLT group).

NF-κB is an important inflammatory response regulator.[41] Abnormal activation of NF-κB pathway is implicated in the degeneration of OA chondrocytes.[42] NF-κB mediates the release of various inflammatory factors, such as IL-6, IL-1β, IFN-γ, TNF-α and MMPs. As reported by Wang et al, Umbelliferone (Umb) mitigated LPS-induced acute lung injury (ALI) by attenuating inflammatory cell infiltration and production of inflammatory cytokines, and enhancing antioxidant activity via inactivating TLR4/MyD88/NF-κB pathway.[43] Besides, Hu et al declared that follistatin-like protein 1 elevated the levels of MMP-13, IL-1β, TNF-α and IL-6 by activating the NF-κB pathway in chondrocyte.[44] We found that Sal treatment markedly decreased the levels of MMP-13 and TNF-α. MMP-13 was an important regulator that

maintained the metabolic balance of chondrocytes. TNF-α induced the expression of ADAMTS-4 and ADAMTS-5, and degraded collagen and proteoglycan. Besides, we found that Sal treatment promoted the production of collagen II and aggrecan, which contributed to reduce the damage to cartilage structure and prevent the destruction of the cartilage matrix. Furthermore, our results showed that Sal reduced the number of CD4$^+$IL-17$^+$ cells, elevated the number of CD4$^+$IL-10$^+$ cells and suppressed the levels of IKBα and p65. These results demonstrated that Sal reduced IL-17 level and increased IL-10 level. The decrease of IL-17 further inhibited the dissociation of IKBα, and consequently reduced the production of TNF-α and VCAM-1. Taken together, Sal alleviated cartilage degeneration by promoting chondrocytes proliferation, inhibiting
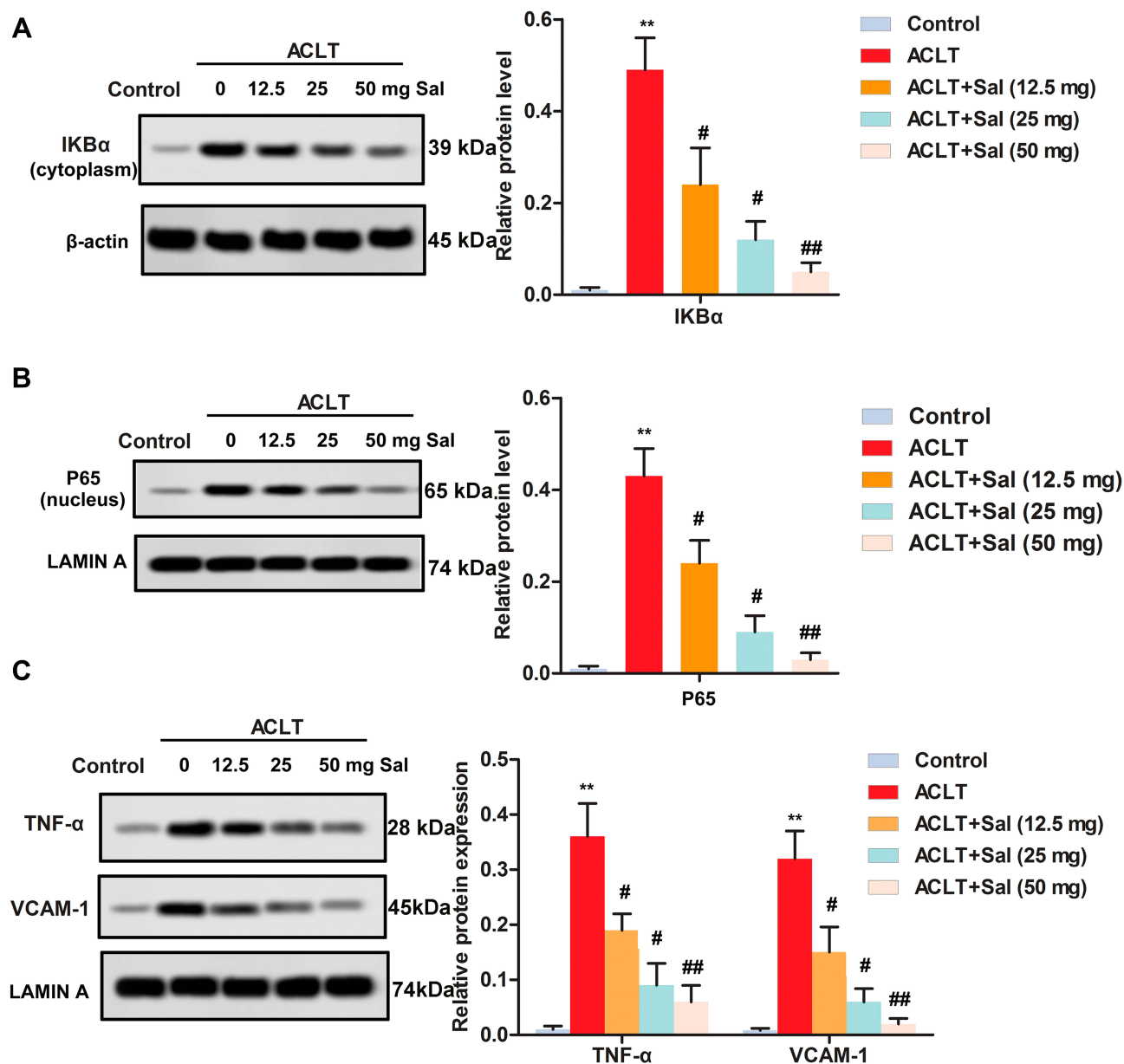
Figure 5 Sal ameliorated cartilage injury by the regulation of NF-κB pathway in OA rats. Rats were divided into five groups (n=10): Control group (Healthy rats were given saline treatment); ACLT, ACLT model; ACLT + Sal (12.5 mg/kg), OA rats were treated with 12.5 mg/Kg Sal; ACLT + Sal (25 mg/Kg), OA rats were treated with 25 mg/kg Sal; ACLT + Sal (50 mg/kg), OA rats were treated with 50 mg/Kg Sal. (**A–C**) Levels of IKBα, p65, TNF-α and VCAM-1 were measured by Western blotting. (**A**) IKBα level. (**B**) p65 level. (**C**) Levels of TNF-α and VCAM-1. β-actin and Lamin A were employed as an internal reference, respectively. (**P < 0.01 vs control group, #P < 0.05, ##P < 0.01 vs ACLT group).

collagen fibrosis, and regulating inflammation and immune responses via NF-κB pathway in OA rats.

Conclusion

In conclusion, our results revealed the role and mechanism of Sal in OA in vivo. In the current study, we proved that Sal (12.5, 25 and 50 mg/kg) significantly promoted chondrocyte proliferation and inhibited chondrocyte apoptosis. Besides, Sal alleviated collagen

fibrosis and modulated ACLT-induced inflammation and immune response in OA rats through the NF-κB pathway. Collectively, our study investigates the role and mechanism of Sal in OA, which lays a foundation for Sal as a clinical drug of OA.

Disclosure

Hui Gao and Lu Peng are co-first authors. The authors report no conflicts of interest in this work.

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