Int J Clin Exp Med 2024;17(4):58-66
www.ijcem.com /ISSN:1940-5901/IJCEM0154273

Original Article

Protective effect of glycyrrhizin on articular cartilage in rats with post-traumatic osteoarthritis and its related mechanism

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Received November 6, 2023; Accepted February 10, 2024; Epub April 15, 2024; Published April 30, 2024

Abstract: Objective: To investigate the protective effect of glycyrrhizin on articular cartilage in post-traumatic osteoarthritis (PTOA) and the related mechanism thereof. Methods: Twenty-four Sprague Dawley rats were randomly divided into sham, PTOA, 20 mg·kg^{-1}, and 50 mg·kg^{-1} glycyrrhizin groups, with six rats in each group. The PTOA rat model was established by anterior cruciate ligament transection, and the rats were intragastrically administered with 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin. The pain indexes, pathology, inflammation, apoptosis, extracellular matrix, and toll-like receptor 4/Nuclear factor kappa B (TLR4/NF-κB) signaling pathway proteins were detected after treatment. Results: Compared with the sham group, the pain indexes, proteoglycan content, and the expression of Bcl-2, collagen II, and aggrecan significantly decreased, whereas the Mankin's and Osteoarthritis Research Society International (OARSI) scores, levels of inflammatory factors, and expression of Bax, Caspase-3, MMP-3, MMP-13, ADAMTS5, TLR4, Myd88, and p-p65 were significantly increased in the PTOA group ($P<0.05$). After 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin treatment, the proteoglycan content and expression of Bcl-2, collagen II, and aggrecan were significantly increased, whereas the Mankin's and OARSI scores, levels of inflammatory factors, and expression of Bax, Caspase-3, MMP-3, MMP-13, ADAMTS5, TLR4, Myd88, and p-p65 were significantly decreased ($P<0.05$). The improvement was more significant in the 50 mg·kg^{-1} glycyrrhizin group ($P<0.05$). Conclusion: Glycyrrhizin could inhibit inflammation and articular cartilage degeneration by inhibiting the activation of the TLR4/NF-κB signaling pathway, thereby improving the function of the knee joint in PTOA modeled rats.

Keywords: Glycyrrhizin, post-traumatic osteoarthritis, TLR4/NF-κB pathway, degeneration of cartilage, inflammation

Introduction

Post-traumatic osteoarthritis (PTOA) is caused by trauma with degeneration of the articular cartilage and secondary ossification of cartilage as the main pathological features, and joint pain and dysfunction as the main clinical manifestations [1]. After trauma, cartilage swelling and obvious loss of proteoglycan appear in the joint, followed by gradual chondrocyte necrosis, cartilage thinning, and subchondral bone thickening, initiating the pathological process of osteoarthritis (OA). Early conservative treatment or joint replacement may relieve pain and improve function. However, there is still no ideal treatment for PTOA at present. Various proteolytic enzymes, such as matrix metalloproteinase (MMP), tumor necrosis factor (TNF), interleukin (IL), and polyproteoglycan enzyme (ADAMTSs), play important roles in the early progression of trauma [2]. The toll-like receptor 4/Nuclear factor kappa B (TLR4/NF-κB) signaling pathway regulates the transcription of downstream oxidative stress, cell apoptosis, and inflammatory response-related genes [3, 4]. Furthermore, the TLR4/NF-κB signaling pathway is closely related to the occurrence and development of OA [5, 6]. Therefore, inhibiting the activation of the TLR4/NF-κB signaling pathway is expected to be the primary method to slow the progression of OA.

Glycyrrhizin (also called glycyrrhizic or glycyrrhizinic acid) is a natural triterpenoid saponin derived from the root of licorice, with various anti-inflammatory, antioxidant, cell apoptosis

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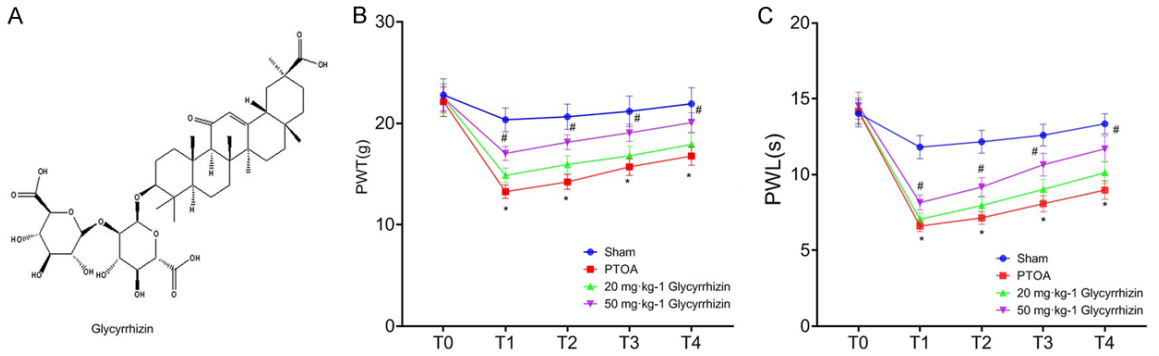

Figure 1. Effects of glycyrrhizin on PWT and PWL of rats in each group. A: Chemical structure of glycyrrhizin. B, C: Mechanical pain experiments analyzing PWL and PWT. PWT: paw withdrawal threshold, PWL: paw withdrawal latency. Data are presented as mean ± SD (n=6) *vs.* Sham group, $^{*}P<0.05$; *vs.* PTOA group, $^{\#}P<0.05$ (ANOVA combined with Tukey's test).

inhibition, and immunomodulation properties [7, 8]. Glycyrrhizin also has a protective effect on OA [9]. Meanwhile, glycyrrhizin inhibits the TLR4/NF-κB signaling pathway [10, 11]. We, therefore, aimed to intragastrically administer glycyrrhizin to PTOA modeled rats to investigate the protective effect thereof and to analyze whether the mechanism is related to the TLR4/NF-κB signaling pathway.

Materials and methods

Animals and materials

Twenty-four healthy male Sprague Dawley rats (weight, 200-220 g) were purchased from Shanghai Slack Experimental Animal Co., Ltd. (animal certificate No. SCXK20070005). Glycyrrhizin was purchased from the China National Institute for Food and Drug Control, Beijing (Batch No. 160904, purity 99.9%). The chemical structural formula of glycyrrhizin is shown in **Figure 1A**. The experimental protocols were approved by the appropriate institutional review committee (Ethical approval No. 20220913) and met the guidelines of the responsible governmental agency.

Grouping and intervention

The rats were randomly divided into sham, PTOA, and 20 mg/kg and 50 mg/kg glycyrrhizin groups, with six rats in each respective group. The PTOA model was established by the anterior cruciate ligament transection (ACLT) method [12]. Rats were anesthetized with 1% pentobarbital sodium, and the knee joint of the right lower limb was exposed. The lateral parapatellar skin was cut longitudinally with eye scissors, approximately 1 cm from the proximal end of the talus patella of the right knee joint, and the patella was turned outward to fix. The cruciate ligament was exposed and cut to avoid loss of articular facet cartilage. The knee joint was straightened, the patella reset, and the wound closed layer by layer.

A drawer test was performed, with a positive result indicating success of the operation. On the second day after the successful surgery, glycyrrhizin (20 mg/kg or 50 mg/kg) was intragastrically administrated to the PTOA modeled rats, once a day for 4 weeks [13]. The PTOA and sham groups were intragastrically administrated equal volumes of normal saline once a day for 4 weeks. In the sham group, the joint cavity was incised without damaging the ligament.

Pain behavior test

The paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) of rats were measured by the von Frey fiber method and a heat sting instrument 1 day before operation (T0) and 1, 2, 3, and 4 weeks after the operation (T1-T4). Each rat was tested 3 times, on average, at 5-min intervals.

Histopathological analysis

The rats were sacrificed after the last pain behavior measurement. After dissecting the knee joint, a part of the joint tissue was fixed in 4% paraformaldehyde solution, decalcified and sliced. The rest of the cartilage tissue of the knee joint was carefully scraped with a blade,

and then placed in a cryogenic tube and stored in liquid nitrogen. The joint tissue was embedded with paraffin and sliced into 5-μm-thick slices. Hematoxylin and eosin (H&E) staining (Beijing Tiangen Biotechnology Co., Ltd.) was utilized to observe the pathological change and safranine O-fast green staining (US Sigma) was used to detect the proteoglycan changes in the cartilage matrix after dewaxing the sections. The histological score was determined according to modified Mankin's [14] and Osteoarthritis Research Society International (OARSI) scores [15].

Enzyme linked immunosorbent assay (ELISA)

The cartilage tissues were ground and washed with buffer solution. Protease inhibitor was added and centrifuged (1,2000×g for 5 min) to obtain the supernatant. The levels of inflammatory factor TNF-α, IL-1β, IL-6, and NO in rat cartilage tissue were determined using an ELISA kit (Beijing Solebo Technology Co., Ltd.).

Western blot

A protein extraction kit (Beijing Solebo Technology Co., Ltd.) was used to extract the cartilage tissue protein in strict accordance with kit instructions. Protein concentration was determined by the bicinchoninic acid method in strict accordance with the kit instructions, and 40 μg protein samples were taken for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and blocked in dissolved skim milk at room temperature for 2 h. Bax, Caspase-3, Bcl-2, MMP3, MMP13, ADAMTS5, Collagen II, Aggrecan, TLR4, human myeloid differentiation factor 88 (MyD88), p-p65, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal (1:1,000, British Abcam) primary antibodies were incubated overnight at 4°C. The secondary antibody, horseradish peroxidase-labeled IgG (1:2,000), was incubated for 1 h at room temperature. A chemiluminescence kit was used for detection in the chemiluminescence system, and the results were analyzed by Image-Pro software. The ratio of gray value of the protein to the internal reference GAPDH was used as the relative expression level of the protein.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software. The Shapiro-Wilk test was performed to determine the normal distribution of the data. All data were expressed as mean ± standard deviation (SD). Repeated measurements and one-way analysis of variance followed by Tukey's test were used in comparison with datasets obeying normal distribution. Otherwise, a nonparametric Mann-Whitney U test was performed. $P<0.05$ was considered statistically significant.

Results

Effect of glycyrrhizin on pain behavior

All rats completed modeling and completed the experiment. There was no statistically significant difference in PWT and PWL in each group before the operation ($P>0.05$). The PWT and PWL in the PTOA group were lower than those in the sham group at T1-T4 ($P<0.05$). The PWT and PWL in the 50 mg·kg^{-1} glycyrrhizin group were higher than those in the PTOA group ($P<0.05$, **Figure 1B**, **1C**).

Effect of glycyrrhizin on pathological damage of PTOA rat cartilage

H&E and safranine O-fast green staining (**Figure 2A**) showed that the articular structure was normal, the chondrocytes had normal arrangement, and uniform chromatin distribution was present in the sham group. Conversely, the cartilage layer was thin, the structure was not clear, the cell arrangement was disordered, and the chondrocytes and the staining intensity were reduced in the PTOA group. The cartilage destruction and osteophyte formation were reduced after treatment with glycyrrhizin. In addition, the Mankin's and OARSI scores in the PTOA group were significantly higher than those in the sham group, whereas the percentage of proteoglycan was significantly lower than that in the sham group ($P<0.05$). The Mankin's and OARSI scores in the 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin groups were significantly lower than those in the PTOA group, whereas the percentage of proteoglycan was significantly higher than that in the PTOA group ($P<0.05$). The improvement of Mankin's and OARSI scores in the 50 mg·kg^{-1} glycyrrhizin group was more significant, compared with the 20 mg·kg^{-1} glycyrrhizin group ($P<0.05$, **Figure 2B-D**).

Effect of glycyrrhizin on the inflammation of PTOA cartilage tissue

Inflammation is the most important pathological reaction in PTOA, and IL-1β, IL-6, TNF-α, and

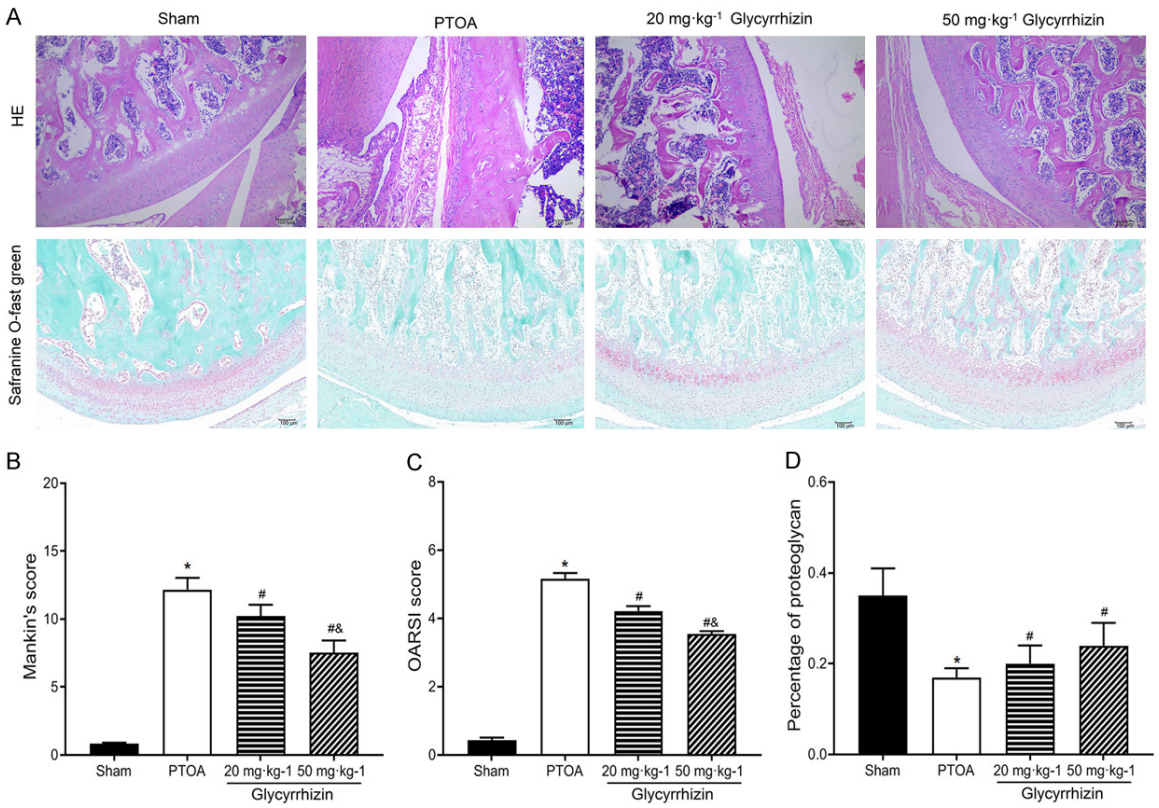

Figure 2. Effects of glycyrrhizin on pathological damage of PTOA rat cartilage. A: H&E and safranine O-fast green staining. B: Comparison of Mankin's score of cartilage tissue in each group. C: Comparison of OARSI score of cartilage tissue in each group. D: Comparison of the percentage of proteoglycan in cartilage tissues of each group; *vs.* Sham group, $^{*}P<0.05$; *vs.* PTOA group, $^{\#}P<0.05$. Data are presented as mean ± SD (n=6); *vs.* 20 mg·kg^{-1} glycyrrhizin group, $^{\&}P<0.05$ (ANOVA combined with Tukey's test).

NO are closely related to the occurrence and development of PTOA [16]. To evaluate the effect of glycyrrhizin on PTOA cartilage inflammation, the levels of inflammatory cytokines, IL-1β, IL-6, TNF-α, and NO, were detected. The results showed that, compared with the sham group, the levels of IL-1β, IL-6, TNF-α, and NO were increased in the PTOA group ($P<0.05$). Furthermore, compared with that in the PTOA group, the levels of IL-1β, IL-6, TNF-α, and NO were significantly lower in the 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin groups ($P<0.05$). The indexes in the 50 mg·kg^{-1} glycyrrhizin group were lower than those in the 20 mg·kg^{-1} glycyrrhizin group ($P<0.05$, **Figure 3**).

Effect of glycyrrhizin on the apoptosis of PTOA in rats

In the PTOA process, chondrocyte necrosis and apoptosis occur secondary to inflammation. The apoptosis of chondrocytes represents the degree of tissue damage to a certain extent [17]. Therefore, the expression of Bax, Bcl-2, and Caspase-3, markers of cell apoptosis and necrosis, were detected. Our results showed that, compared with the sham group, the expression of Bax and Caspase-3 were significantly increased, whereas Bcl-2 was significantly decreased in the PTOA group ($P<0.05$). Compared with the PTOA group, the expressions of Bax and Caspase-3 were significantly decreased, whereas Bcl-2 was significantly increased in the 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin groups ($P<0.05$); changes were more obvious in the 50 mg·kg^{-1} glycyrrhizin group ($P<0.05$, **Figure 4A**).

Effects of glycyrrhizin on extracellular matrix (ECM) degradation

ECM degradation is another typical pathological reaction of PTOA. ECM degradation and collagen loss gradually occur with the aggravation of inflammatory reactions in the process of PTOA [18]. Therefore, the expression of MMP-3,

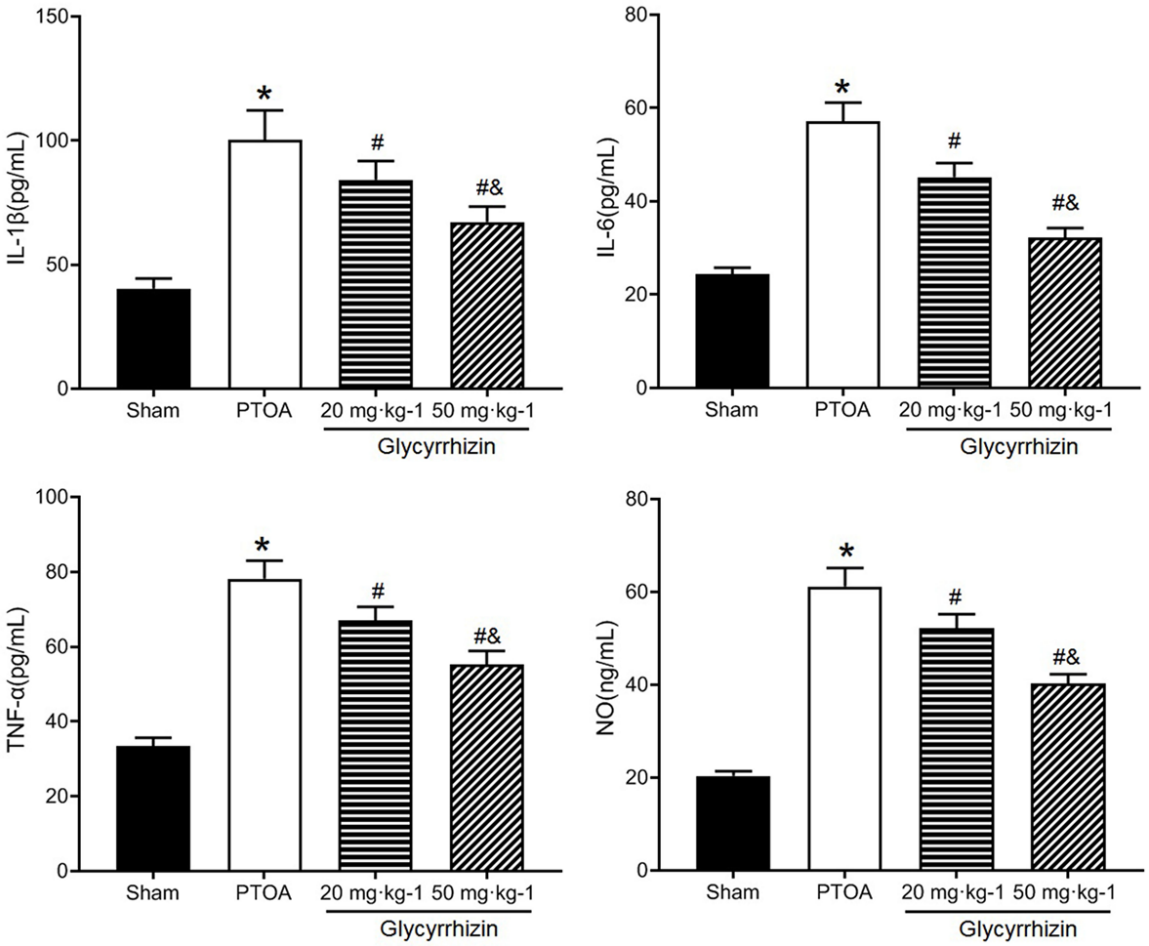

Figure 3. Effect of glycyrrhizin on inflammatory factors in PTOA rat cartilage. Data are presented as mean ± SD (n=6) *vs.* Sham group, $^{*}P<0.05$; *vs.* PTOA group, $^{\#}P<0.05$; *vs.* 20 mg·kg^{-1} glycyrrhizin group, $^{\&}P<0.05$ (ANOVA combined with Tukey's test).

MMP-13, ADAMTS5, collagen II, and aggrecan were detected. Compared with the sham group, the expression of MMP-3, MMP-13, and ADAMTS5 were significantly increased, whereas collagen II and aggrecan were significantly decreased in the PTOA group ($P<0.05$). Compared with the PTOA group, the expression of MMP-3, MMP-13, and ADAMTS5 were significantly decreased, whereas collagen II and aggrecan were significantly increased in the 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin groups ($P<0.05$). Changes in the 50 mg·kg^{-1} glycyrrhizin group were more obvious than those in the 20 mg·kg^{-1} group ($P<0.05$, **Figure 4B**).

Effects of glycyrrhizin on the TLR4/NF-κB signaling pathway in PTOA cartilage

The TLR4/NF-κB signaling pathway is a classic inflammatory response pathway and has been proven to play an important role in PTOA [4, 5]. However, glycyrrhizin may inhibit the TLR4/NF-κB signaling pathway in diseases [11]. Therefore, the expressions of TLR4/NF-κB signaling pathway proteins were detected after the action of glycyrrhizin. The results showed that, compared with the sham group, the expression of TLR4, Myd88, and p-p65 in the PTOA group were significantly upregulated ($P<0.05$). Compared with the PTOA group, the expression of TLR4, Myd88, and p-p65 decreased significantly in the 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin groups ($P<0.05$). The expressions of TLR4, Myd88, and p-p65 in the 50 mg·kg^{-1} glycyrrhizin group were obviously lower than those in the 20 mg·kg^{-1} group ($P<0.05$, **Figure 5**).

Discussion

Compared with the sham group, the pain indexes, proteoglycan content, and the expression of

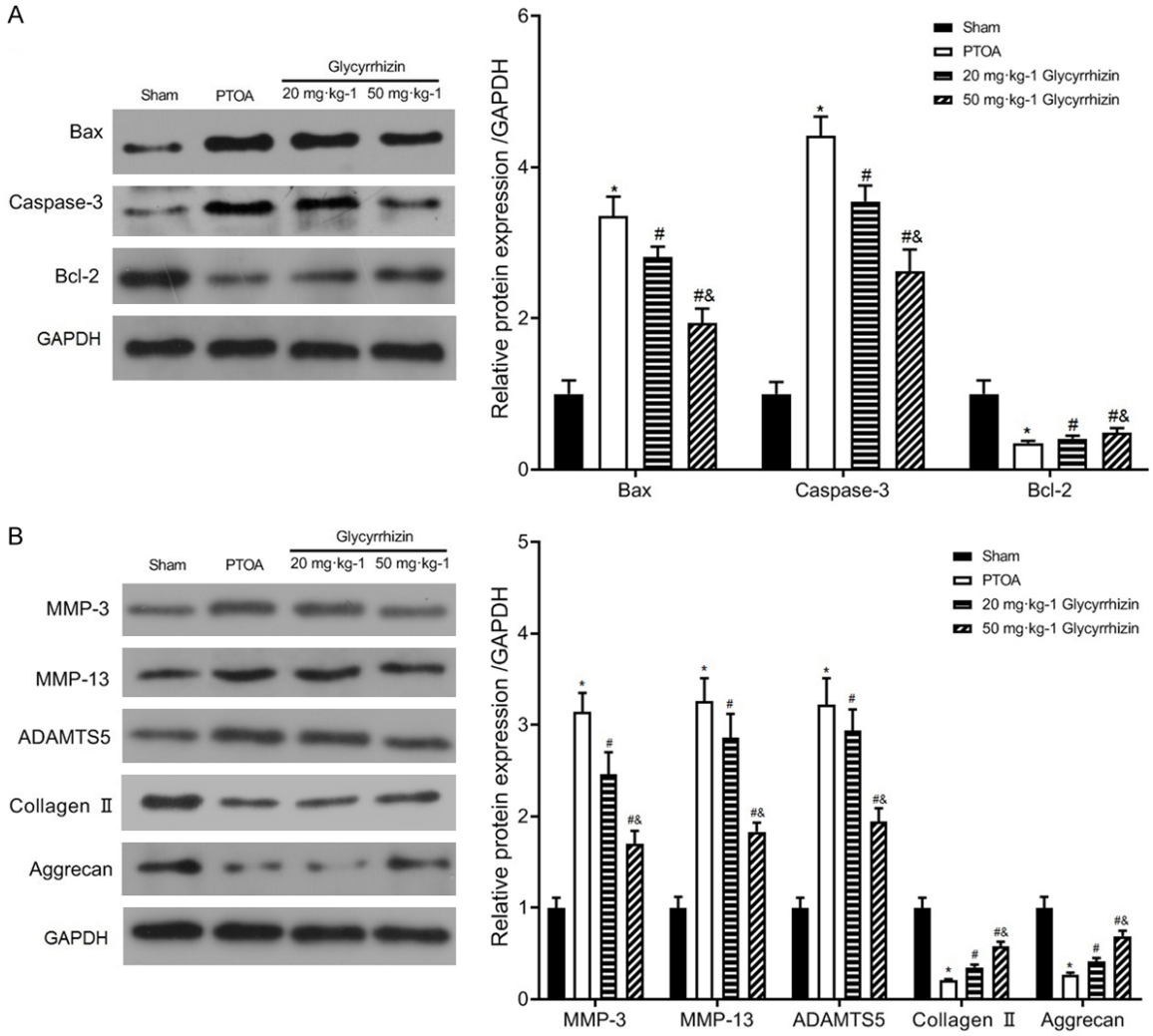

Figure 4. Effects of glycyrrhizin on the apoptosis and ECM of PTOA rat cartilage. A: The expression of Bax, Caspase-3, and Bcl-2 in the cartilage tissues of each group detected by western blot. B: The expression of MMP-3, MMP-13, ADAMTS5, collagen II, and aggrecan in cartilage tissues of each group detected by western blot. Data are presented as mean ± SD (n=6) *vs.* Sham group; $^{*}P<0.05$; *vs.* PTOA group, $^{\#}P<0.05$; *vs.* 20 mg·kg^{-1} glycyrrhizin group, $^{\&}P<0.05$ (ANOVA combined with Tukey's test).

Bcl-2, collagen II, and aggrecan were significantly decreased, whereas the Mankin's and OARSI scores, levels of inflammatory factors, and expression of Bax, Caspase-3, MMP-3, MMP-13 ADAMTS5, TLR4, Myd88, and p-p65 were significantly increased in the PTOA group. After 20 mg·kg^{-1} and 50 mg·kg^{-1} glycyrrhizin treatment, the proteoglycan content and expression of Bcl-2, collagen II, and aggrecan were significantly increased, whereas the Mankin's and OARSI scores, levels of inflammatory factors, and expression of Bax, Caspase-3, MMP-3, MMP-13, ADAMTS5, TLR4, Myd88, and p-p65 were significantly decreased. The improvement was more significant in the 50 mg·kg^{-1} glycyrrhizin group.

When joint trauma occurs, the articular cartilage degenerates, secondary hyperplasia leads to joint dysfunction, and approximately 12% of patients develop secondary OA [19]. PTOA is mainly manifested as knee pain, swelling, movement disorders, and disability, which seriously affect the patient's quality of life and increases the economic burden on the family [20]. Early conservative treatment or joint replacement may relieve pain and improve function. However, there is still no ideal treat-

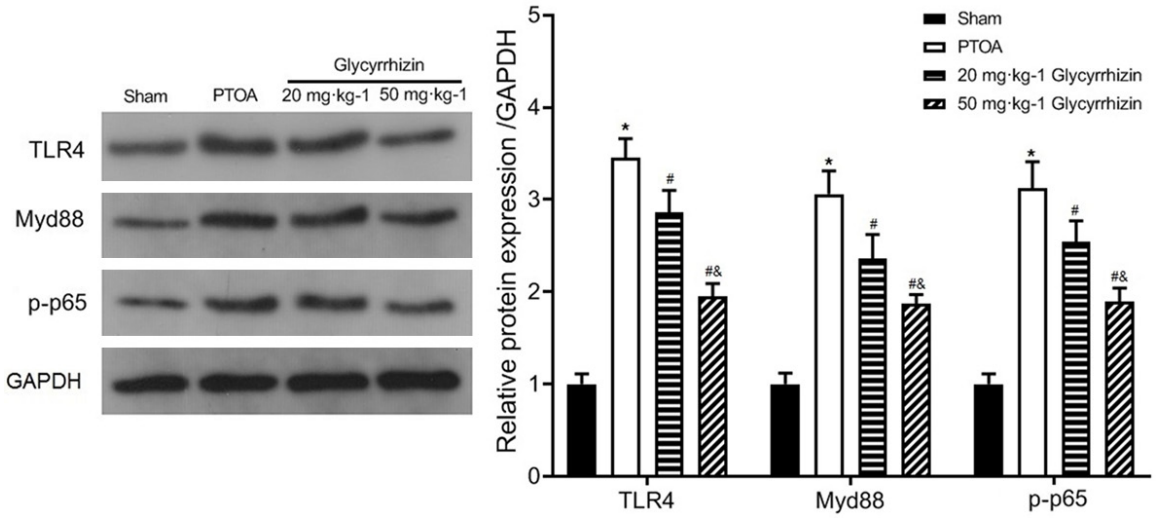

Figure 5. Effect of glycyrrhizin on TLR4/NF-κB signaling pathway in PTOA rat cartilage. Data are presented as mean ± SD (n=6) *vs.* Sham group, $^{*}P<0.05$; *vs.* PTOA group, $^{\#}P<0.05$. *vs.* 20 mg·kg^{-1} glycyrrhizin group, $^{\&}P<0.05$ (ANOVA combined with Tukey's test).

ment for PTOA at present. Therefore, new effective drugs or therapies for the early prevention and treatment of PTOA are urgently required.

Glycyrrhizin is a small molecule inhibitor of high mobility group box 1 (HMGB1), which can effectively inhibit inflammation, and has high safety and tolerance [21]. Glycyrrhizin has been clinically used in the treatment of various skin diseases and malignant tumors [7, 8, 20]. In recent years, glycyrrhizin has also been found to have a protective effect on arthritis. Jiang et al. demonstrated that glycyrrhizin remarkably suppresses the IL-1β-induced level of NO, prostaglandin E2, TNF-α, IL-6, and the production of cyclooxygenase-2, inducible nitric oxide synthase, MMP3, MMP13, and ADAMTS5; inverts the degradation of aggrecan and collagen II; and inhibits PI3K/AKT phosphorylation and NF-κB mobilization in human OA chondrocytes [22]. Hu et al. found that glycyrrhizin lowers the levels of inflammatory and catabolic mediators, alleviates cartilage degeneration, and improves TMJOA by regulating the HMGB1-RAGE/TLR4-NF-kB/AKT signaling pathway [13]. However, the effect of glycyrrhizin on PTOA is not clear at present. Our PTOA rat model was constructed by the ACLT method [12] and treated with glycyrrhizin. The results showed that, in the PTOA rat model, the cartilage tissue was severely damaged, inflammatory factors were significantly increased, and the expression of collagen II and aggrecan were significantly decreased; whereas those of MMP-3, MMP-13, ADAMTS5, and apoptosis protein were significantly increased, indicating that the PTOA rat model was successfully constructed. Glycyrrhizin can effectively improve the pain threshold and joint pathological injury, alleviate chondrocyte apoptosis and inflammatory reaction, inhibit abnormal degradation of cartilage ECM, and promote the synthesis of ECM components in the PTOA rat model. Glycyrrhizin, therefore, has a significant therapeutic effect on PTOA, which is consistent with the conclusions of existing studies on the treatment of OA. However, the experimental design of glycyrrhizin concentration and action time are limited, so the optimal intervention concentration and intervention time require further discussion.

TLR4 is a receptor that recognizes pathogen-related molecular patterns and can recognize "endogenous danger signals" caused by injury or stress [23, 24]. TLR4 activates NF-κB through the downstream MyD88-dependent signal transduction pathway, and the phosphorylated NF-κB plays a transcriptional regulatory role in the nucleus, promoting cytokine IL-1β, IL-6, and TNF-α release [25]. IL-1β and TNF-α are considered the most important pathogenic factors of synovial inflammation and cartilage injury [6], so the activation of the TLR4/NF-κB signaling pathway is related to the occurrence and development of OA. We found that the level of inflam-

matory factors and the expression of TLR4, Myd88, and p-p65 in cartilage tissue were increased in the PTOA rat model, suggesting that the TLR4/NF-κB signaling pathway is activated therein, which may be one of the important molecular mechanisms leading to sustained joint inflammation, joint damage, and sensitivity to pain. Additionally, there is evidence that blocking the TLR4/NF-κB signaling pathway can significantly reduce inflammation. Zhang et al. indicated that curcumin could block the TLR4/NF-κB signal pathway and reduce inflammation levels to prevent knee wounds in OA rats [26]. Liu et al. identified and confirmed that the TLR4/MyD88/NF-κB signaling pathway is an essential inflammatory signaling pathway in the Duhuo Jisheng Decoction underlying OA treatment. Glycyrrhizin has been found to inhibit the TLR4/NF-κB signaling pathway [27]. Li et al. found glycyrrhizic acid suppresses the expression of Myd88, HMGB1, TLR4, and NF-κB, as well as reduces serum concentrations of IL-1β, IL-6, and TNF-α in diabetic mice with cerebral I/R injury [28]. However, there is still a lack of clinical and experimental data to support the fact that glycyrrhizin improves PTOA through the TLR4/NF-κB signaling pathway. In this study, glycyrrhizin may have inhibited the release of IL-1β, IL-6, and TNF-α; the expression of TLR4, Myd88, and p-p65; and the activation of the TLR4/NF-κB signaling pathway to improve PTOA progression by inhibiting synovial inflammation and articular cartilage damage. However, whether the protective effect of glycyrrhizin on articular soft bone is through the TLR4/NF-κB signaling pathway or the involvement of other pathways cannot be confirmed at present. Further studies should verify this by blocking the TLR4/NF-κB signaling pathway.

Conclusion

Glycyrrhizin may inhibit inflammation and articular cartilage degeneration by inhibiting the activation of the TLR4/NF-κB signaling pathway, thereby improving the function of the knee joint in PTOA rats. Therefore, glycyrrhizin is expected to be a therapeutic agent for PTOA, but its pathogenesis and clinical application require further investigation.

Disclosure of conflict of interest

None.

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