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Mechanism Study on the Effect of Dihuang Gutongkang Capsules Improving Knee Osteoarthritis in Rats by Regulating the MYD88/NF - κ B/NLRP3 Signaling Pathway and Reducing Osteoclast Differentiation

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Abstract: ***Objective:*** ***This study aimed to investigate the effects of Dihuang Gutong Kang capsule on osteoclast differentiation in knee osteoarthritis (KOA) rats and its mechanism via the MYD88/NF-κB/NLRP3 signaling pathway.*** ***Methods:*** ***A KOA rat model was established by sodium monoiodoacetate (MIA) induction. Successfully modeled rats were randomized into the model group, low-dose Dihuang Gutong Kang capsule group (16.2 g/kg/d), high-dose Dihuang Gutong Kang capsule group (32.4 g/kg/d), celecoxib group (0.52 g/kg/d), and a blank control group. Cartilage pathology was evaluated by H&E staining; mRNA levels of inflammatory cytokines (IL-6, TNF-α) were measured via qPCR; osteoclast activity was analyzed by TRAP staining; and co-expression of TRAP with MYD88, NLRP3, and p-p65 NF-κB was observed using immunofluorescence co-staining.*** ***Results:*** ***Compared with the blank group, the model group exhibited severe cartilage damage (P<0.001), upregulated IL-6 and TNF-α expression (P<0.001), increased TRAP-positive osteoclasts (P<0.001), and enhanced co-expression of TRAP with MYD88/NLRP3/p-p65 NF-κB (P<0.001). In contrast, high-dose DIHUANG GUTONG KANG CAPSULE significantly alleviated cartilage defects and inflammatory infiltration (P<0.01), reduced IL-6 and TNF-α levels (P<0.001), suppressed TRAP-positive osteoclast numbers (P<0.001), and decreased TRAP+MYD88/NLRP3/p-p65 co-expression (P<0.001) compared to the model group.*** ***Conclusion:*** ***Dihuang Gutong Kang capsule attenuates osteoclast activation and inflammatory responses by inhibiting the MYD88/NF-κB/NLRP3 pathway, thereby ameliorating KOA progression.***

Keywords: Dihuang Gutong Kang capsule, Knee osteoarthritis, Osteoclast, MYD88/NF-κB/NLRP3 signaling pathway.

1. Introduction

Osteoarthritis (OA) is a common chronic degenerative joint disease characterized primarily by degenerative changes in articular cartilage and secondary osteophyte formation [1]. Knee osteoarthritis (KOA), in particular, has a high incidence among the elderly, severely affecting patients' quality of life [2-4]. The pathological process of KOA involves multiple aspects, including inflammatory responses, cartilage damage, and osteoclast activation [5-7]. The NF-κB signaling pathway plays a key role in the inflammatory response [8, 9], while the activation of the NLRP3 inflammasome further exacerbates inflammation and cartilage damage [10]. Excessive activation of osteoclasts leads to the destruction of cartilage and bone tissue, further aggravating KOA.

Rehmannia Gutongkang Capsule is a hospital-prepared formulation of Xi'an Hospital of Traditional Chinese Medicine. It has the effects of nourishing the liver and kidneys, promoting blood circulation to relieve pain, and strengthening tendons and bones. Clinical studies have shown that it has significant therapeutic effects on KOA patients [11-13]. However, its mechanism of action has not been fully elucidated. This study established a KOA rat model to investigate the effect of Rehmannia Gutongkang Capsule on osteoclast differentiation and its mechanism of action via the MYD88/NF-κB/NLRP3 signaling pathway, providing a theoretical basis for its clinical application.

2. Materials and Methods

2.1 Materials

Rehmannia Gutongkang Capsule (Preparation Room, Xi'an Hospital of Traditional Chinese Medicine); Celecoxib Capsules (Sichuan Guowei Pharmaceutical Co., Ltd.); Sodium Iodoacetate (Macklin, Cat. No. I811707-5g); Molpure® Cell/Tissue Total RNA Kit (YEASEN, Cat. No. 19221ES50); PrimeScript RT reagent Kit (Takara Bio, Cat. No. RR047A); TB Green™ Premix Ex Taq™II (Tli RNaseH Plus) (Takara Bio, Cat. No. RR820A); Primary antibodies included TRAP antibody (Huaan Biological, Cat. No. HA500105, 1:100 dilution), NLRP3 antibody (Thermo Fisher, Cat. No. PA5-79740, 1:100 dilution), phospho-NF-κB p65 antibody (Bioswamp, Cat. No. Bs-0982r, 1:100 dilution), and MYD88 antibody (Servicebio, Cat. No. GB111554, 1:100 dilution); Secondary antibodies included FITC-conjugated Goat Anti-Rabbit IgG (Servicebio, Cat. No. GB22303, 1:100 dilution) and HRP-conjugated Goat Anti-Rabbit IgG (Servicebio, Cat. No. GB23303, 1:100 dilution); TSA fluorescence signal amplification used CY3-Tyramide (Servicebio, Cat. No. G1223, 1:500 dilution).

2.2 Model Establishment and Grouping

Thirty-six rats were provided by Shaanxi Lilihongrui Biotechnology Co., Ltd., production license number: SCXK

(Shaan) 2023-002. After one week of adaptive feeding, 30 rats were randomly selected to establish the KOA model. The KOA model was induced by intra-articular injection of sodium iodoacetate solution (40 mg/mL, 50 μL). Two weeks after modeling, three rats were randomly selected to confirm successful model establishment (if significant swelling and deformity of the left knee joint, loss of smoothness in the articular surfaces of the distal femur and tibial plateau, significant synovial thickening, cartilage edema or cartilage defects, and even exposure of subchondral bone or joint deformation were observed, it was considered successful modeling). The successfully modeled rats were randomly divided into a model group, a low-dose Rehmannia Gutongkang Capsule group (16.2 g/kg/d), a high-dose Rehmannia Gutongkang Capsule group (32.4 g/kg/d), and a celecoxib group (0.52 g/kg/d), with six rats in each group. The blank control group consisted of non-modeled rats raised normally.

2.3 H&E Staining

Mouse knee joint tissues were washed with normal saline, fixed in 4% paraformaldehyde for 30–50 minutes, and paraffin sections were prepared. Tissue sections were dried in a 45°C incubator and routinely dewaxed. After soaking in distilled water for 5 minutes, sections were stained with hematoxylin for 5 minutes, rinsed with tap water for 3 seconds, differentiated with 1% hydrochloric acid ethanol for 3 seconds, stained with 5% eosin for 3 minutes, rinsed with tap water, and air-dried. Tissue sections were observed under an optical microscope.

2.4 qPCR

Primers for IL-6 and TNF-α were designed using Primer 5.0 and synthesized by Shanghai Biotechnology Co., Ltd. The reaction was performed in a 20 μL system containing 10 μL of TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus), 0.8 μL each of forward and reverse primers (10 μM), 2.0 μL of template, and 6.4 μL of distilled water. The reaction program was as follows: pre-denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Primer sequences are shown in Table 1.

Table 1: Primer Sequences

Primer Name	Forward	Reverse
β-actin	gggaaatcgtgcgtgacatt	gcggcagtggccatctc
IL-6	cttccagccagttgccttcttg	tggtctgttgtgggtggtatcc
TNF-α	gcccagaccctcacactcag	ccgcttggtggtttgctacg

2.5 TRAP Staining

After dewaxing and hydration in graded series, paraffin sections underwent microwave-mediated heat-induced antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific binding sites were blocked with bovine serum albumin (BSA). Rabbit-derived TRAP primary antibody (1:100 dilution) was added and incubated overnight at 4°C, followed by HRP-conjugated goat anti-rabbit secondary antibody (1:100) incubated at 37°C for 30 minutes. Color development was performed using the DAB chromogenic system, and nuclei were stained with hematoxylin. After dehydration through graded alcohols, sections were mounted with neutral resin. Positive signals (brown-yellow) were localized in the cytoplasm. Images were captured under a microscopic imaging system (400×), and the percentage of positive area was quantified using Halo image analysis software.

2.6 Immunofluorescence

The immunofluorescence staining procedure for paraffin sections was as follows: sections were deparaffinized and hydrated; slides were placed in antigen retrieval solution and subjected to microwave-assisted antigen retrieval for 20 minutes, cooled, and rinsed three times with PBS; sections were placed in 3% hydrogen peroxide and incubated at room temperature in the dark for 25 minutes, rinsed three times with PBS, then BSA blocking solution was applied and incubated at room temperature for 30 minutes; diluted primary antibody working solution was added and incubated overnight at 4°C, followed by three PBS washes; secondary antibody was applied and incubated at 37°C for 30 minutes, followed by three PBS washes; DAPI counterstain was added and incubated at room temperature for 10 minutes; slides were washed three times with PBS, 5 minutes each. Sections were mounted with anti-fade mounting medium and observed under a fluorescence microscope.

2.7 Data Analysis

IBM® SPSS 25.0 statistical software was used for data analysis. Data are expressed as mean ± standard deviation (mean ± SD). Intergroup comparisons were performed using one-way ANOVA, with $P < 0.05$ considered statistically significant.

3. Results

3.1 Effect of Rehmannia Gutongkang Capsule on Pathological Cartilage Damage in KOA Rats

As shown in Figure 1, compared with the blank control group, the model group exhibited extensive cartilage surface defects, chondrocyte degeneration and necrosis, trabecular bone necrosis, increased osteoclasts, inflammatory cell infiltration, fibrous tissue proliferation, neovascularization, hemorrhage, and pseudocyst formation. Compared with the model group, the low-dose Rehmannia Gutongkang Capsule group showed a certain degree of alleviation in pathological changes, while the high-dose Rehmannia Gutongkang Capsule group and the celecoxib group exhibited significant alleviation of pathological alterations. The results indicate:

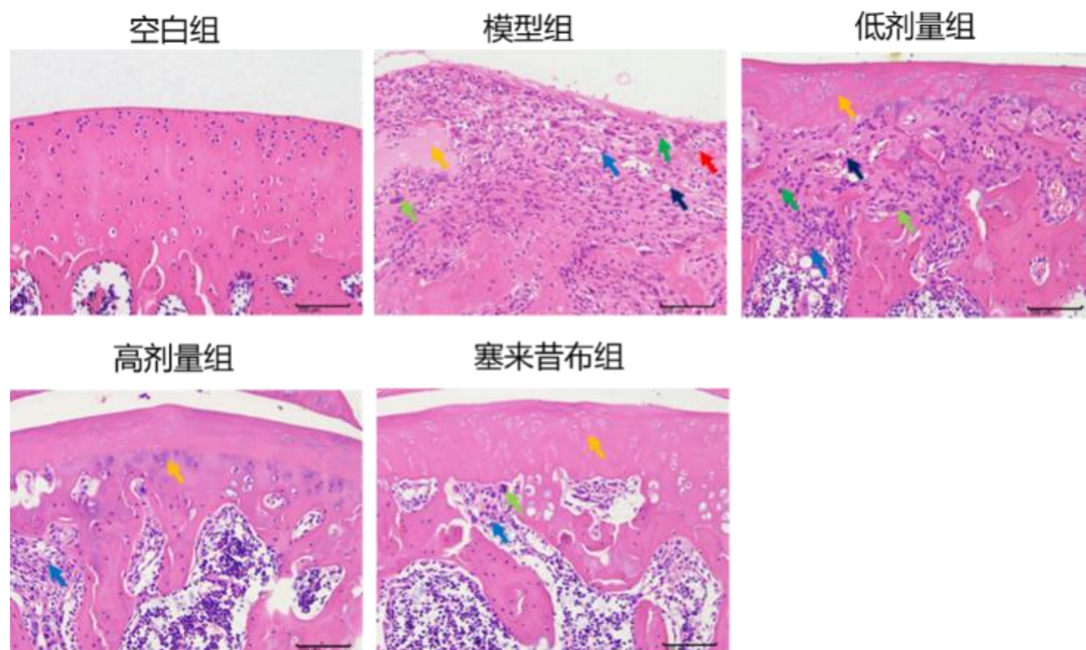


Figure 1: Effect of Rehmannia Gutongkang Capsule on Pathological Cartilage Damage in KOA Rats (×200)

Note: Necrotic chondrocytes (↑) proliferating osteoclasts (↑) macrophages (↑) fibroblasts (↑) neovascularization (↑) hemorrhage (↑) (×200)

3.2 Effect of Rehmannia Gutongkang Capsule on Inflammatory Responses in the Knee Joints of KOA Rats

As shown in Figure 2, compared with the blank control group, the mRNA expressions of IL-6 and TNF-α in the knee cartilage tissues of rats in the model group were significantly increased ($P<0.001$). Compared with the model group, both the high-dose Rehmannia Gutongkang Capsule group and the celecoxib group significantly reduced the expression of these two inflammatory factors ($P<0.01$), whereas the low-dose group showed no significant effect. The results indicate:

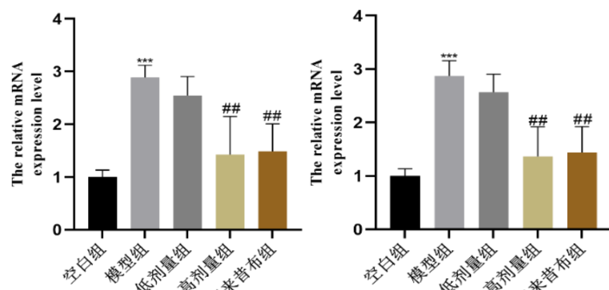


Figure 2: Effect of Rehmannia Gutongkang Capsule on IL-6 and TNF-α in the Knee Joints of KOA Rats

Note: A: ; B: . Compared with the blank control group, P<0.05, P<0.01, P<0.001; compared with the model group, #P<0.05, ##P<0.01, ###P<0.001.

3.3 Effect of Rehmannia Gutongkang Capsule on Osteoclast Differentiation in the Knee Joints of KOA Rats

As shown in Figure 3, compared with the blank control group, the expression of TRAP-positive cells in the knee bone tissue of rats in the model group was increased, with extremely significant statistical significance ($P<0.001$). Compared with the model group, the high-dose Rehmannia Gutongkang Capsule group and the celecoxib group both showed reduced TRAP-positive expression in knee bone tissue, with extremely significant statistical significance ($P<0.001$). The low-dose Rehmannia Gutongkang Capsule group also showed reduced TRAP-positive expression, with statistical significance ($P<0.05$). The results indicate:

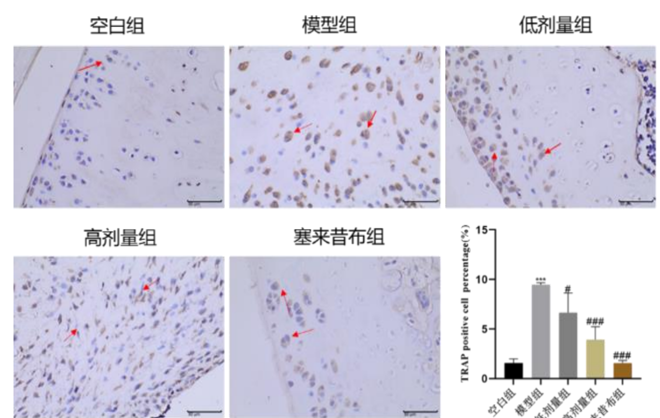


Figure 3: Effect of Rehmannia Gutongkang Capsule on Osteoclast Differentiation in the Knee Joints of KOA Rats

Note: Compared with the blank control group, P<0.05, P<0.01, P<0.001; compared with the model group, #P<0.05, ##P<0.01, ###P<0.001.

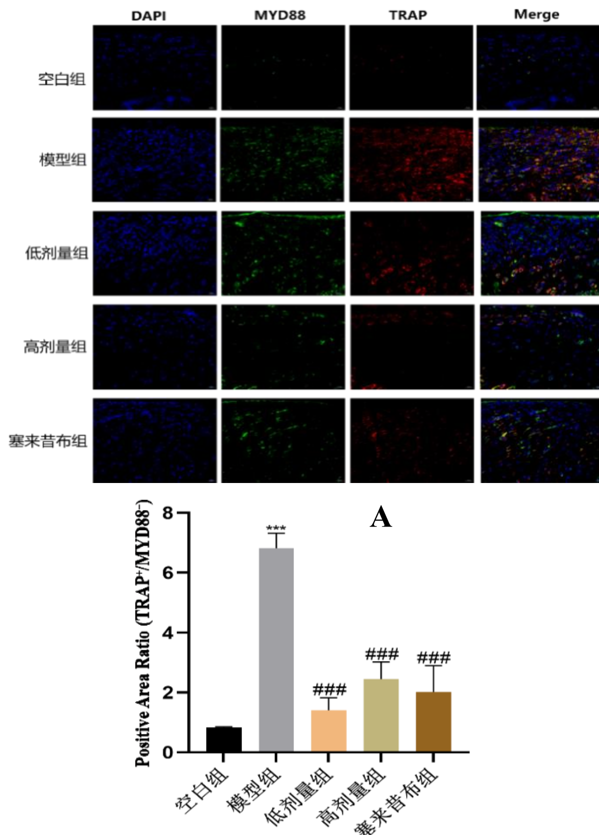
3.4 Effect of Rehmannia Gutongkang Capsule on the MYD88/NF-κB/NLRP3 Signaling Pathway in Osteoclasts in the Knee Joints of KOA Rats

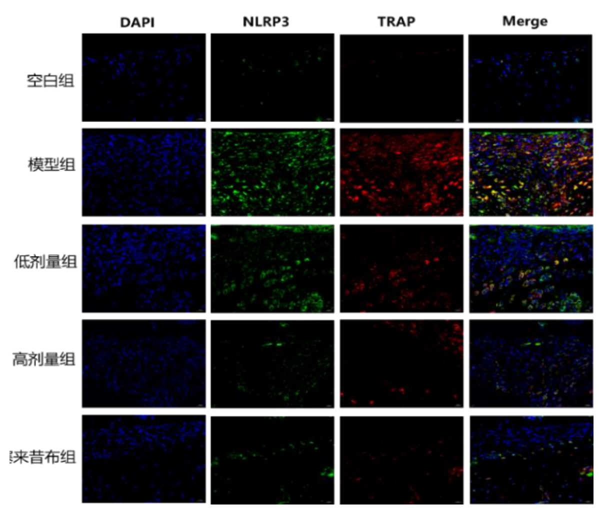
As shown in Figure 4A, compared with the blank control group, the co-expression of TRAP+MYD88 in the knee joint tissues of rats in the model group was increased, with extremely significant statistical significance ($P<0.001$). Compared with the model group, the celecoxib group, the low-dose Rehmannia Gutongkang Capsule group, and the high-dose Rehmannia Gutongkang Capsule group all showed reduced co-expression of TRAP+MYD88, with extremely significant statistical significance ($P<0.001$).

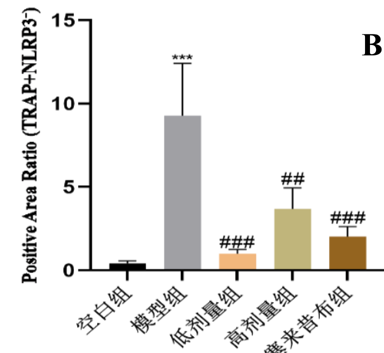
As shown in Figure 4B, compared with the blank control group, the co-expression of TRAP+NLRP3 in the knee joint tissues of rats in the model group was increased, with extremely significant statistical significance ($P<0.001$). Compared with the model group, the celecoxib group and the

high-dose Rehmannia Gutongkang Capsule group showed reduced co-expression of TRAP+NLRP3, with extremely significant statistical significance (P<0.001); the low-dose Rehmannia Gutongkang Capsule group also showed reduced co-expression, with significant statistical significance (P<0.01).

As shown in Figure 4C, compared with the blank control group, the co-expression of TRAP+p-p65 in the knee joint tissues of rats in the model group was increased, with statistical significance (P<0.05). Compared with the model group, the celecoxib group and the high-dose Rehmannia Gutongkang Capsule group showed reduced co-expression of TRAP+p-p65, with statistical significance (P<0.05); the low-dose Rehmannia Gutongkang Capsule group showed no significant change in TRAP+p-p65 co-expression, with no statistical significance (P>0.05). The results indicate:







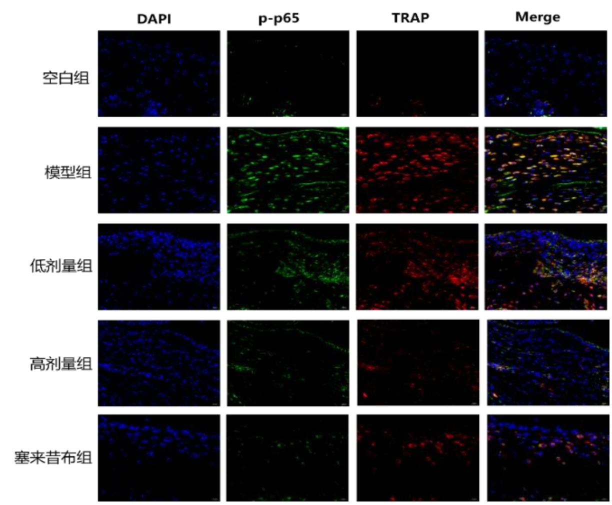


Figure 4: Immunofluorescence detection (×40)

Note: Compared with the blank control group, *P<0.05, P<0.01,*P<0.001; compared with the model group, #P<0.05, ##P<0.01, ###P<0.001.

4. Discussion

This study established a rat model of knee osteoarthritis (KOA) to investigate the effect and mechanism of Rehmannia Gutongkang Capsule on osteoclast differentiation by regulating the MYD88/NF-κB/NLRP3 signaling pathway.

Cartilage damage is a core pathological feature of KOA, and its repair relies on precise regulation of inflammatory signals. In this study, HE staining revealed that Rehmannia Gutongkang Capsule significantly improved pathological cartilage damage in KOA rats, manifested as reduced cartilage defect area and decreased synovial hyperplasia. This finding is consistent with the results of Liu et al. [14], who reported that inhibiting the TLR4/MyD88/NF-κB/NLRP3 pathway suppressed chondrocyte pyroptosis and reactive oxygen species (ROS)-induced macrophage inflammation, thereby delaying OA pathological progression. Additionally, Li et al. [15] demonstrated that inhibiting the P2X7 receptor suppressed chondrocyte pyroptosis and inflammatory

responses via the NF-κB/NLRP3 pathway. These studies provide indirect evidence supporting the cartilage-protective effect of Rehmannia Gutongkang Capsule.

Inflammatory signaling pathways play a central role in the pathological process of KOA. As key mediators, the overexpression of IL-6 and TNF-α can activate downstream signaling pathways (such as JAK/STAT and MAPK), further aggravating cartilage damage and bone resorption. In this study, qPCR detection revealed that Rehmannia Gutongkang Capsule significantly reduced the expression levels of IL-6 and TNF-α in the joints of KOA rats, indicating a significant anti-inflammatory effect. This result is consistent with multiple studies: Shang Lanqing et al. [16] found that catalpol from Rehmannia exerts anti-inflammatory effects by reducing the mRNA and protein levels of inflammatory factors such as IL-1, IL-6, and TNF-α; Man et al. [17] confirmed through machine-learning analysis of data from 600 KOA patients that IL-6 and TNF-α are key indicators of systemic inflammation and are significantly associated with KOA pain and functional impairment.

Osteoclast activation is a key driver of bone resorption and subchondral bone sclerosis (sclerotic osteoarthritis) in KOA. In this study, TRAP staining revealed that Rehmannia Gutongkang Capsule significantly inhibited osteoclast differentiation and reduced the number of osteoclasts in KOA rats. This finding is consistent with the results of Ding et al. [18], who demonstrated that dihydroartemisinin attenuated osteoclast formation and bone resorption by inhibiting the NF-κB, MAPK, and NFATc1 signaling pathways; Guo et al. [19] also reported that metformin inhibited osteoclast - mediated abnormal subchondral bone remodeling via the AMPK/NF-κB/ERK pathway. Rehmannia Gutongkang Capsule likely reduces RANKL-induced NFATc1 activation by inhibiting the MYD88/NF-κB/NLRP3 pathway, thereby blocking osteoclast differentiation.

The MYD88/NF-κB/NLRP3 pathway is a core regulatory network for inflammatory responses and pyroptosis, and its excessive activation is closely associated with cartilage damage, bone resorption, and pain in KOA. In this study, immunofluorescence co-staining revealed that Rehmannia Gutongkang Capsule significantly reduced the expression levels of MYD88, p-p65 NF-κB, and NLRP3, indicating that it inhibits osteoclast activation by regulating this signaling pathway. The study by Chen et al. [20] reported that nicorandil suppresses the TLR4/MyD88/NF-κB/NLRP3 signaling pathway to reduce pyroptosis in rats with myocardial infarction. Research by Li et al. [21] demonstrated that tanshinone IIA attenuates pyroptosis in rats with coronary microembolization by inhibiting the TLR4 / MyD88 / NF-κB / NLRP3 pathway. These studies provide direct evidence for the therapeutic effect of Rehmannia Gutongkang Capsule via the MYD88/NF-κB/NLRP3 pathway.

In summary, Rehmannia Gutongkang Capsule significantly improved joint inflammation and cartilage damage in KOA rats by inhibiting the MYD88/NF-κB/NLRP3 signaling pathway, providing an important experimental basis for its clinical application. Future studies should further explore its specific mechanisms of action and conduct larger-scale clinical trials to verify its long-term efficacy and safety.

Fund Project

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