Original Article

Tendon-regulating and bone-setting manipulation promotes the recovery of synovial inflammation in rabbits with knee osteoarthritis via the TLR4–MyD88–NF-κB signaling pathway

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Background: The aim of this study was to clarify the mechanism of tendon-regulating and bone-setting manipulation in the treatment of knee osteoarthritis (KOA).

Methods: A total of 30 adult healthy specific pathogen-free (SPF) New Zealand white rabbits (male; weight 2.0–2.5 kg) were selected and divided into a normal control (NC) group, KOA group, and KOA + manual treatment (MT) group. Each group comprised 10 rabbits. A KOA model was established using the modified Hulth method in the KOA and KOA + MT groups. The 3 groups were fed under the same conditions for 8 weeks. The Lequesne index for KOA was used to evaluate the behavioral status of the model rabbits; hematoxylin and eosin (HE) staining was employed to observe the pathological morphology of the tibial plateau and medial femoral condyle cartilage; the Mankin scoring scale was used to evaluate the cartilage morphology of the model rabbits; Western blot was used to detect the expression levels of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor κB (NF-κB) proteins in the synovial tissue of the model rabbits; the contents of interleukin (IL)-1β, IL-6, and, tumor necrosis alpha (TNF-α) in the synovial fluid of the model rabbits were determined using the enzyme-linked immunosorbent assay (ELISA) method.

Results: Compared with those in the NC group, Lequesne index score, Mankin score of cartilage tissue, protein expression, and content of inflammatory factors were significantly increased in the KOA and KOA + MT groups ($P<0.05$), and these values were significantly higher in the KOA group. Microscopy showed that the cartilage tissue of the experimental rabbits in the KOA and KOA + MT groups was significantly degenerated. Compared with those in the KOA group, the Lequesne index score, Mankin score, protein expression, and inflammatory factor content of the model rabbits in the KOA + MT group were significantly reduced ($P<0.05$), and microscopy showed that cartilage tissue degeneration of the experimental rabbits in the KOA + MT group was significantly improved.

Conclusions: Tendon-regulating and bone-setting manipulation can significantly improve the activity state and motor function of KOA model rabbits and significantly inhibit the expression of the TLR4–MyD88-NF-κB signaling pathway in synovial tissue, thereby reducing knee joint synovial inflammation and delaying the occurrence and development of KOA.

Keywords: Tendon-regulating and bone-setting manipulation; knee osteoarthritis (KOA); synovial inflammation; TLR4–MyD88–NF-κB signaling pathway

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Introduction

Knee osteoarthritis (KOA) is osteoarthritis that occurs in the knee joint. It is a degenerative bone and joint disease common in middle-aged and older adults (1) and has a high disability rate (2). A recent survey showed that among 65-year-old adults, the incidence rate is about 13% and 24% for men and women, respectively (3). In addition, the morbidity of Chinese individuals is significantly higher than that of White individuals (4). Its high incidence represents a heavy burden on patients and their families (5). The cardinal symptoms of KOA are knee joint pain, swelling, impaired mobility, and deformity. Its pathological manifestations are synovial inflammation and articular cartilage degenerative changes (6).

Synovial inflammation, as a typical pathological manifestation in the progression of KOA disease, has become of increasing interest to the academic community in recent years (7). Synovial inflammation was previously considered to be a late pathological manifestation of KOA, but in recent years, with comprehensive and in-depth research, it has been proven to be one of the pathological features of the early and mid-term development of KOA and joint structural damage (8), with the latter being considered the main cause of knee joint pain (9). The main pathological process relates to the immune response theory of synovial tissue, and this is widely acknowledged by the majority of relevant research (10,11). Therefore, aim of this study was to assess the innate immune response pathway of synovial tissue at the molecular biology level to explore new ideas for the treatment of patients with KOA.

In the clinical treatment of KOA, manual treatment has gradually become one of the complementary and alternative treatments to drug therapy. It is a noninvasive biomechanical treatment method (12). In this approach, the articular cartilage cells of the knee joint are stimulated from various angles, which can significantly enhance its anti-inflammatory effect, block the release of interleukin (IL)-1β and other inflammatory factors, and reduce the degeneration and damage to articular cartilage (13,14). Preliminary basic research has found that manual treatment, as a noninvasive biomechanical treatment method, can significantly improve the clinical symptoms of patients with musculoskeletal joint diseases such as KOA, cervical spondylosis, and lumbar disc herniation (6,14,15). Therefore, this study developed a novel means to exploring the mechanism of muscle-strengthening and bone-setting manipulation in the treatment of KOA based on the signaling pathway composed of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor κB (NF-κB). We further used a rabbit model of KOA to explore synovial inflammation and new treatment methods for KOA. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3039/rc).

Highlight box

Key findings

- Tendon-regulating and bone-setting manipulation can inhibit the TLR4–MyD88–NF-κB pathway in synovial tissue, thereby reducing the content of IL-1β, IL-6, and TNF-α in synovial fluid, reducing knee joint synovial inflammation, and delaying the occurrence and development of KOA.

What is known and what is new?

- What is known from clinical experience is that tendon-regulating and bone-setting manipulation can reduce synovial inflammation and delay the development of KOA.
- What's new is that the tendon-regulating and bone-setting manipulation can inhibit the TLR4–MyD88–NF-κB pathway in synovial tissue, thereby reducing the content of IL-1β, IL-6, and TNF-α in synovial fluid, reducing knee joint synovial inflammation, and delaying the occurrence and development of KOA.

What is the implication, and what should change now?

- This means that we have a new research direction on the mechanism of tendon-regulating and bone-setting manipulation of knee osteoarthritis and synovial inflammation, and we should now follow this finding with further research.

Methods

Experimental materials and reagents

Instruments and reagents

The following instruments and reagents were used: an automatic ice maker (Changshu Xueke Electric Co., Ltd., Jiangsu, China), an electrophoresis instrument (Shanghai Tianneng Technology Co., Ltd., Taiyuan, China), an electrophoresis tank (Shanghai Tianneng Technology Co,

Ltd.), a membrane transfer instrument (Shanghai Tianneng Technology Co., Ltd.), a horizontal shaker (Haimen Qilin Bell Instrument Manufacturing Co., Ltd., Jiangsu, China), a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA, USA), a centrifuge 5415 (Eppendorf, Hamburg, Germany), an MV-100 Vortex Mixer (Wuhan Saiweier Biological Technology Co., Ltd., Wuhan, China), a DHP-9012 electric heating thermostat (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China), β-Actin (Zs-BIO, Evanston, IL, USA), goat anti-mouse immunoglobin G (IgG; Zs-BIO), TLR4 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), MyD88 antibody (Santa Cruz Biotechnology), NF-κB p65 antibody (Santa Cruz Biotechnology), radioimmunoprecipitation assay (RIPA) Lysis Solution (BioSharp, Tallinn, Estonia), phenylmethylsulfonyl fluoride (PMSF; BioSharp), sodium dodecyl-sulfate (SDS; Solarbio, Beijing, China), glycine (Solarbio), polyacrylamide gel electrophoresis (PAGE) gel accelerator (Solarbio), Tris (Solarbio), Tween-20 (Solarbio), polyvinylidene fluoride (PVDF) membrane (MilliporeSigma, Burlington, MA, USA), APS (BBI Life Sciences Advanced Technologies, St. Petersburg, FL, USA), acrylamide (BBI Life Sciences Advanced Technologies, St. Petersburg, FL, USA), bis-acrylamide (Solarbio), methanol (Shanghai Su Yi, Shanghai, China), phosphate-buffered saline (PBS) buffer powder (Zs-BIO), prestained protein marker (Thermo Fisher Scientific), Western primary antibody secondary antibody removal solution (Beyotime, Jiangsu, China), enhanced chemiluminescence (ECL) ultra-sensitive luminescence kit (Thermo Fisher Scientific), a rabbit tumor necrosis factor alpha (TNF-α) Quantitative Detection Kit (enzyme-linked immunosorbent assay, ELISA; Quanzhou Ruixin Biotechnology Co. Ltd., Quanzhou, China), a Rabbit Interleukin 6 (IL-6) Quantitative Detection Kit (ELISA; Quanzhou Ruixin Biotechnology Co., Ltd.), and a Rabbit Interleukin 1β (IL-1β) Quantitative Detection Kit (ELISA; Quanzhou Ruixin Biotechnology Co., Ltd.).

Animal experiments

Thirty 6-month-old adult healthy specific pathogen-free (SPF) male New Zealand white rabbits were purchased from the Animal Breeding Center of Anhui Medical University, and each weighed between 2.0 and 2.5 kg. Random numbers were generated using the standard=RAND() function in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) to divide 30 New Zealand white rabbits into the normal control (NC), KOA, and KOA + manual treatment (MT) groups. There were 10 rabbits in each group. This animal experiment was carried out at Anhui University of Traditional Chinese Medicine (Meishan Road Campus).

Empirical method

Animal model establishment

A protocol was prepared before the study without registration. Experiments were performed under a project license (No. AHUCM-rabbits-2019010) granted by the Medical Ethics Committee of Anhui University of Traditional Chinese Medicine and conducted in compliance with the National Institutes of Health Guidelines for the care and use of animals. During the experiment, the room temperature was constant, and a relative humidity of 45% to 60% was maintained. The animals were kept in single cages, fed with standard feed, and given water freely. The modified Hulth method was used to establish the KOA rabbit model. During the experiment, due to individual differences in animals, postoperative infection, and other reasons, 1 rabbit each died in the KOA + MT and the KOA groups, while the other experimental animals survived.

Modeling was started after 1 week of suitable feeding, and New Zealand rabbits requiring surgery were fasted for 12 h before modeling. During the operation, 3% pentobarbital sodium solution was used for ear vein anesthesia, and the dosage was 3.0 mL/kg. The skin of the animal was then prepared using a special operating table and sterile area. The inner side of the rabbit's right knee joint was sterilized and a longitudinal 2-cm surgical incision was made. After that, the skin and subcutaneous tissue were separated layer by layer, and finally the joint capsule of the knee joint was incised. The joint cavity was opened, the medial collateral ligament was disconnected, and the patella was pushed outward to dislocate the knee joint. The anterior cruciate ligament was severed, and the medial meniscus was removed completely. After the lateral separation and confirmation of drawer test positivity, bleeding in the joint cavity was stopped, and it was then washed with saline and sutured layer by layer. For 3 consecutive days after the operation, 200,000 U/d of penicillin was injected intramuscularly into the rabbits to prevent infection.

Intervention method

The rabbits in the KOA + MT group were fixed in position, and when their lower limbs were relaxed, they were treated with tendon-regulating and bone-setting manipulation. The interventions were performed by the same researcher. The order of treatments was randomly assigned to avoid

Video 1 The treatment process of model rabbits in the KOA + MT group. KOA + MT, knee osteoarthritis + manual treatment.

human factors interfering with experimental results. First, the muscles of the rabbits' right lower limb were used for tendon manipulation through pinching and twisting from top to bottom for 5 min. Second, both sides of the knee joint were kneaded around the patella 50 times. The technique applied was as continuous, uniform, light, and gentle as possible. Finally, the bone setting method was used; that is, the inner and outer sides of the knee joint were pressed with the thumbs of both hands to bend and extend the knee joint 10 times. This was done once every other day, 20 min each time, and for 20 consecutive treatments (see *Video 1*).

After treatment, Lequesne index of severity for osteoarthritis (16) was used to measure and evaluate the pain, gait, range of motion, and swelling. Of the affected knee joint of each group of experimental animals before and after treatment. The curative effect of the treatment of KOA with the manipulation of muscles and bones was evaluated at the molecular biology level.

Material extraction and preservation method

After the animal was anesthetized, 1 mL of normal saline was drawn with a 1 mL syringe and injected into the knee joint cavity, the knee joint was repeatedly flexed and extended at least 10 times, and the same amount of joint fluid was drawn as that of the normal saline solution. The extracted liquid was allowed to stand before it was centrifuged, and the supernatant was removed and transferred to the refrigerator at −20 ℃ and frozen for ELISA testing. The knee joint was taken as the center of the surgical area for removal of the synovial tissue of the knee joint. After separation, it was quickly put into a sterile Eppendorf (EP) tube and placed in liquid nitrogen for quick freezing. Once these steps were completed, the tubes were moved to a −80 ℃ refrigerator and frozen for Western blot detection. Finally, the New Zealand rabbits of each group were euthanized using air embolization while being under anesthesia. A 3% pentobarbital sodium solution (3.0 mL/kg) was administered via the rabbits' ears for venous anesthesia, and the edge of ears of the experimental rabbits were used to insert the air needle for sampling (the experimental rabbits were killed while unconscious). The femurs and tibias were cut off 1 cm above and below the knee joint of the right hind limb. After being washed with normal saline, the rabbits were fixed in 4% paraformaldehyde for 24 to 48 h for hematoxylin and eosin (HE) staining and sectioning and observed under a light microscope.

HE staining was used to observe pathological changes of cartilage tissue

After the general observation of the KOA group, the medial femoral condyle of the right knee joint of the rabbit was cut, the soft tissue and blood around the cartilage were removed, and the cartilage tissue was rinsed. Care was taken to not damage the cartilage surface during this process. After fixation in 4% paraformaldehyde for 24 to 48 h, decalcification, and dehydration, transparency, paraffin embedding, and HE staining were performed after tissue sectioning. After mounting, the tissue morphology was observed under a microscope and photographed for recording.

Mankin score for the evaluation of cartilage morphology

We examined the histological morphology and structure of each group of knee joints after HE staining under a microscope and used the modified Mankin articular cartilage pathology scoring standard (17) to evaluate the cartilage tissue structure, staining, tide line, and cell number; with the cumulative score corresponding to the degree of cartilage degeneration. The maximum score is 14 points, and the higher the score, the more serious the degeneration of articular cartilage. The specific classification criteria are the following: normal cartilage = 0–1 point, early osteoarthritis = 2–6 points, midterm osteoarthritis = 7–10 points, and advanced osteoarthritis = 11–14 points.

Western blot detection method was used to detect the protein expression of TLR4, MyD88, and NF-κB in the synovial tissues of rabbit knee joints in each group

In this study, the tissue was cut, and RIPA cell lysate was

Table 1 Lequesne index score of the 3 groups of model rabbits ($\bar{x}$±s)

Group	N	Lequesne index score (points)
NC group	10	0.00±0.00
KOA group	9	6.38±0.63*
KOA + MT group	9	3.17±0.51*▲

*, compared with the normal control group, P<0.05; ▲, compared with the KOA group, P<0.05. NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment.

added for lysis and centrifuged to collect total tissue protein. Then, SDS-PAGE was proportionally added to a 5× protein loading buffer and heated in a boiling water bath for 10 min to fully denature the protein. After electrophoresis, the membrane was transferred to the prepared PVDF membrane, and then the PVDF membrane was put into a Western blocking solution, slowly shaken on a shaker, and blocked at room temperature for 2 h. The PVDF membrane was diluted with primary antibody diluent and incubated at 4 ℃ slowly overnight. The secondary antibody was then labeled with horseradish peroxidase (HRP), diluted with the diluent, and incubated at room temperature for 2 h. After the 2 incubations, the PVDF membrane was washed PBS with Tween20 for 10 min, 3 times each time. The PVDF membrane protein was placed face up in the central position of the exposure plate of the automatic exposure instrument, and the mixed ECL solution was added to achieve a full reaction. Finally, relevant software was used to analyze the results.

ELISA method was used to assess the contents of IL-1β, IL-6, and TNF-α in the synovial fluid of rabbit knee joints in each group

The relevant solutions were prepared as required, returned to room temperature, placed into a standard well with the well set at 0, placed into a blank well after the sample was emptied, and then added to a standard well; following this, 50 μL of sample dilution with different concentrations was added to the standard well set at 0, the blank well was emptied, and 50 μL of joint fluid was added to the sample well. HRP-labeled detection antibody (100 μL) was added to the remaining wells except for the blank wells. After being covered, these mixed liquids were incubated in a 37 ℃ water bath or incubator for 60 min and were washed, allowed to stand, and patted dry, and this was repeated five times. Substrates A and B were mixed 1:1, 100 μL of mixed solution was added to each well, and wells were incubated in a 37 ℃ water bath or incubator for 15 min. Stop solution (50 μL) was added to all wells, and the absorbance value [optical density (OD) value] of each well was read on a microplate reader. Finally, the standard was taken as concentration as the ordinate (6 standard wells, plus 1 zero value hole, 7 concentration points in total), and the corresponding absorbance value (OD value) was taken as the abscissa. Using computer software and 4-parameter logistic curve fitting (4-pl), we created a standard curve equation, which was used to calculate the concentration value of the sample from its absorbance value (OD value). The concentration of the original sample was multiplied by the dilution factor to obtain the final concentration of the diluted sample.

Statistical analysis

The sample size of each experimental group was determined based on previous research and sample size calculations. SPSS 23.0 (IBM, Corp., Armonk, NY, USA) was used for statistical analyses. Continuous numerical variables are described as mean ± standard deviation ($\bar{x} \pm s$). Data that met independence, normal distribution, and homogeneity of variance conditions were compared using 1-way analysis of variance, and data that did not meet the above 3 conditions were compared using the nonparametric rank-sum test. A P value <0.05 indicated a statistically significant difference (the results of statistical analysis of all experimental data are based on 95% confidence interval analysis).

Results

Behavior observation

After the treatment, the following results were recorded for each group of rabbits.

In the NC group, the rabbits' knee joints had a normal appearance, there was no obvious swelling or deformation, the muscles around the knee joint were soft, the rabbits' activities were normal, and they could jump freely.

In the KOA group, the rabbit knee joints were hyperplastic and swollen, quadriceps and medial femoris muscles were increased in muscle tension, indurations and cords were palpable around the knee joint, the affected limb was limp and unable to bear weight, and the center of gravity shifted to the healthy side during activities.

In the KOA + MT group, after manual treatment, the appearance of the rabbits' knee joints was improved, muscle

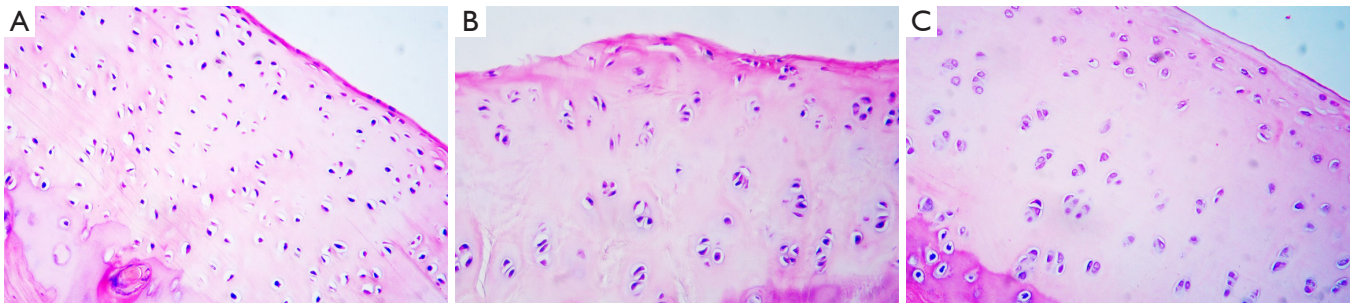

Figure 1 HE-stained sections of cartilage tissue. (A) HE staining of cartilage tissue in the NC group (×400); (B) HE staining of cartilage tissue in the KOA group (×400); (C) HE staining of cartilage tissue in the KOA + MT group (×400). HE, hematoxylin and eosin; NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment.

Table 2 Mankin score of the 3 groups of model rabbits ($\bar{x}$±s)

Group	N	Mankin score (points)
NC group	10	0.53±0.29
KOA group	9	7.76±1.48*
KOA + MT group	9	3.09±1.17*▲

*, compared with the normal control group, P<0.05; ▲, compared with the model group, P<0.05. NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment.

tension around the knee joint was reduced, induration and cord disappeared or decreased, the affected limb was appropriately weight-bearing, and the performance of activity was improved.

The Lequesne index of severity for osteoarthritis was used to score the general state of the experimental rabbits in each group. Based on the statistical analysis (*Table 1*), it was found that the difference in the Lequesne scores between the 3 groups was statistically significant (P<0.05). Compared with those of the NC group, the scores of the KOA group and the treatment group were significantly higher (P<0.05), and compared with those in the KOA group, the scores of the treatment group were significantly lower (P<0.05). These results indicate that the overall morphology of the rabbits in the KOA group showed the most obvious changes. After manual treatment, the overall morphology of the rabbits in the treatment group was significantly improved.

Histological analysis

HE staining of the cartilage tissue

The following was observed by microscopy using HE staining.

In the NC group, the cartilage tissue surface was smooth; the chondrocytes and matrix were arranged evenly, neatly and tightly; the number and morphology of chondrocytes were normal; the stained tissues were distinct; and the surface moisture lines were complete (*Figure 1A*).

In the KOA group, the cartilage surface was severely damaged; the surface was thinned, defective, uneven, and cracked; the stained tissue structure lacked organization; the chondrocytes were arranged disorderly; the number of chondrocytes was reduced; shape changes were present; and the tide line was interrupted or had disappeared (*Figure 1B*).

In the KOA + MT group, the stained cartilage tissue structure seen under the microscope was basically intact, the cartilage surface was thinned but basically smooth, the number of cartilage cells was slightly reduced, the arrangement was slightly disordered, a small number of cartilage cells had congregated, and the tide line was basically intact (*Figure 1C*).

Mankin score of cartilage tissue

There was a statistically significant difference in the Mankin score between the 3 groups (P<0.05). Compared with those in the NC group, the scores of the KOA and KOA + MT groups were significantly higher (P<0.05), and compared with those in the KOA group, the scores of the KOA + MT group were significantly lower (P<0.05). This indicated that the articular cartilage tissue damage of the model rabbits in the KOA group was the most severe. After manual treatment, the degree of cartilage tissue damage in the treatment group improved significantly (*Table 2*).

Western blot analysis of protein

The protein expression results of TLR4, MyD88, and NF-κB detected in the 3 groups are shown in *Figures 2,3*.

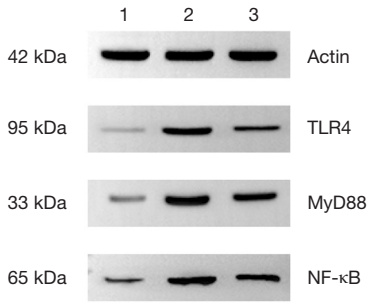

Figure 2 Three histone expression profiles of TLR4, MyD88, and NF-κB in synovial tissue. 1= NC group; 2= KOA group; 3= KOA + MT group. NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor κB.

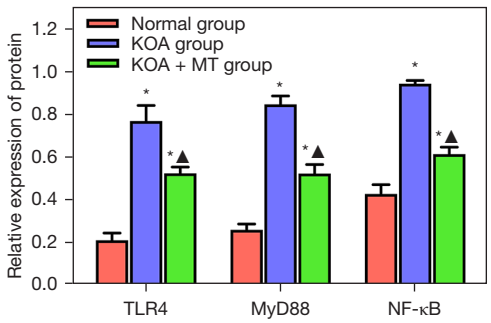

Figure 3 Relative expression of TLR4, MyD88, and NF-κB in synovial tissue. *, compared with the normal control group, P<0.05; ▲, compared with the KOA group, P<0.05. NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor κB.

Compared with the NC group, the KOA and KOA + MT groups had significantly higher protein expression (P<0.05). Compared with the KOA group, KOA + MT group had significantly reduced expression of the 3 proteins (P<0.05). These results showed that the protein expressions of TLR4, MyD88, and NF-κB in rabbit knee synovial tissue were significantly increased after modeling. After manual treatment, the protein expression of TLR4, MyD88, and NF-κB in the rabbit synovial tissue of the KOA + MT group was significantly inhibited.

ELISA analysis of inflammatory factor

The concentrations of IL-1β, IL-6, and TNF-α in the articular fluid detected using ELISA are shown in *Figure 4*. The levels of IL-1β, IL-6, and TNF-α in the articular fluid in the KOA and KOA + MT groups were significantly higher than those in the NC group (P<0.05), and the levels of IL-1β, IL-6, and TNF-α in the KOA + MT group were significantly lower than those in the KOA group (P<0.05). This indicated that the contents of IL-1β, IL-6, and TNF-α in the rabbit knee fluid increased significantly after modeling, and the release of inflammatory cytokines IL-1β, IL-6, and TNF-α in the synovial tissues of rabbits in the KOA + MT group decreased significantly after manual treatment.

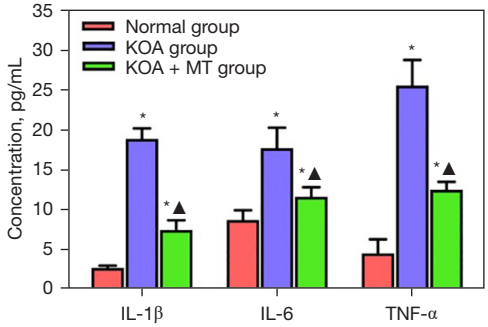

Figure 4 Concentrations of 3 inflammatory cytokines IL-1β, IL-6, and TNF-α in synovial fluid. *, compared with the normal control group, P<0.05; ▲, compared with the KOA group, P<0.05. NC, normal control; KOA, knee osteoarthritis; KOA + MT, knee osteoarthritis + manual treatment; IL, interleukin; TNF- α, tumor necrosis factor alpha.

Discussion

Synovial inflammation, as an aseptic inflammation in the pathogenesis of KOA, has been given increasing attention in relation to the pathological manifestations and clinical treatment of KOA (18). In many knee arthroscopy and imaging examinations, it has been found that as the degree of synovial inflammation increases, the severity of clinical symptoms and pathological manifestations of KOA patients also increases (19). One study has shown (20) that the occurrence of KOA is related to a variety of inflammatory mediators and signal pathways. Inhibiting the secretion of inflammatory mediators and the expression of inflammatory signaling pathways can significantly improve the clinical symptoms of KOA and delay the progression of KOA. Therefore, finding an effective method to treat KOA from the perspective of inhibiting the secretion of inflammatory mediators and the expression of inflammatory signal

pathways is important.

There are many mediators and cell signal transduction pathways involved in synovial inflammation. TLRs are very important recognition receptor proteins in the innate immune system and one of the important molecules in the basic research of inflammation and immunity (6). When the synovium of the knee joint is stimulated to cause inflammation, the innate immune system can be activated in 2 ways: pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP). Both PAMP and DAMP can be recognized by TLRs, and at the same time activate downstream NF-κB and other inflammatory signaling pathways, thereby activating macrophages to inhibit inflammation and promote tissue repair physiological functions (21,22). A previous study found that TLRs are expressed very slightly in normal synovial tissues and articular cartilage but are highly expressed in synovial tissues of patients with OA (11). Among them, the expression of TLR4 is the most obvious (11), and TLR4 expression can be increased by NF-κB signal transduction, which is activated by MyD88 (6,11). TLR signal transduction pathways are mainly divided into 2 types: MyD88–dependent and MyD88–independent pathway. TLR4 is the only receptor that can activate the above 2 pathways at the same time (23). MyD88 is the first adaptor protein found to be compatible with the TLR signal transduction pathway. Its role is mainly to transmit upstream information, and it is a key protein in inducing diseases (6). Previous studies (24,25) demonstrated that MyD88 is composed of an N-terminal death domain and a C-terminal kinase domain. In the MyD88-dependent pathway, the kinase structure at the C-terminus recognizes the kinase structure that binds to TLR4, and the N-terminus binds to TNF receptor-associated factor 6 (TRAF6). TRAF6 is composed of the helical coil structure of the TRF-N domain and the highly conformed TRF-C domain. The ubiquitin ligase in the TRF-N domain synthesizes the polyubiquitin chain linked to lysine 63 (K63), which is ubiquitinated TRAF6. The ubiquitin ligase forms a complex with UBCI3 and UEV1A. Specifically, TAK1X is activated, and TAK1 activates downstream inhibitory and mitogen-activated protein kinase pathways. Inhibitor of kappa B kinase α (IKK), inhibitor of kappa B kinase β (IKKβ), and inhibitor of kappa B kinase Y (IKKY) to form a complex that phosphorylates the inhibitor of kappa B (IKB) protein, thereby degrading the IKB protein, causing the release of NF-κB and rapid nuclear translocation. Activated NF-κB rapidly transmits signals to the nucleus, promoting the transcription of genes including *IL-1*β, *IL-6*, and *TNF*-α, thereby further activating nuclear transcription factors and increasing the secretion and release of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. Il-1β, IL-6, TNF-α, and other inflammatory cytokines also activate NF-κB-induced kinase and mitogen-activated protein kinase kinase 1 (MEKK1), which activates phosphorylated IKK, further degrades IKB protein, activates NF-κB, and enhances signaling (26). In summary, TLR4 activates the dependent MyD88 pathway to combine MyD88 with TRAF6 to activate NF-κB, thereby activating the signal transcription expression in the next-level inflammatory cells, activating the inflammatory transcription program, and, thus, promoting the production of IL-1β, IL-6, and TNF-α. Inflammatory factors are released in large quantities, causing the synovial tissue to produce a cascade of inflammation, and finally triggering KOA. Therefore, we chose to observe the protein expression of the TLR4–MyD88–NF-B signaling pathway in the synovial tissue of the KOA model rabbits and then to assess the changes of inflammation and the degree of cartilage degeneration in the synovium of the KOA model rabbits.

Synovial inflammation and KOA treatment are mainly divided into drug therapy, nondrug therapy, and surgical treatment (27). Currently, there is no effective way to completely cure KOA, and knee replacement is the only treatment for patients with advanced KOA, but it is unlikely to become a common treatment because of its characteristics of high harm, high cost, and low safety (28). The drugs used in the clinical treatment of KOA are mainly nonsteroidal anti-inflammatory drugs (NSAIDs), but long-term use of these drugs can lead to liver and kidney damage, gastrointestinal complications, and other side effects (29-31). According to previous studies, biomechanical stimulation can significantly affect the expression of inflammatory mediators and proteases and has a significant anti-inflammatory effect on damaged tissues (32,33). Nogueira *et al.* (34) tested the expression of TLR4 in periodontal ligament (PDL) cells without biomechanical stimulation by adding TLR4 antibody to the cell culture dish which resulted in little change in the expression of TLR4 protein. In subsequent research, the stimulation of biomechanical load reduced the amount of TLR4 protein significantly, and the nuclear translocation of NF-κB was eliminated. This result fully indicates that the expression of TLR4 and NF-κB in damaged tissues will be significantly inhibited if there are external stimulating factors such as biomechanics. Other studies (35,36) have also shown that when cells are stimulated by mechanics and TLR4 ligand, the phosphorylation level of NF-κB

increases significantly. Biomechanical stimulation also has a significant effect on the expression of inflammatory factors. Leong *et al.* (37) found that low-intensity chondrocyte biomechanical stimulation *in vitro* inhibits the inflammation and catabolism induced by IL-1 and TNF-α. Therefore, we chose biomechanical intervention to intervene in the protein expression of the TLR4–MyD88–NF-κB signaling pathway in knee synovial tissue to further observe the changes of synovial inflammation and the degree of cartilage degeneration in KOA model rabbits.

Manual therapy is one of the common nondrug treatments of KOA in clinical practice (29). It is defined as a treatment method that manually adjusts joints and loosens soft tissues to provide comfort and is a noninvasive biomechanical stimulation method (38). Compared with drug therapy and surgery, manual treatment has the advantages of lower cost, higher safety, and easier application (39). Previous studies (40-43) have shown that manual treatment can relieve the pain, stiffness, and functional status of patients with KOA by boosting blood circulation around the joints, improving muscle tension, and increasing joint flexibility, and that it is an effective biomechanical treatment method.

Therefore, in this study, a KOA rabbit model was established using the modified Hulth method, the TLR4–MyD88–NF-κB signaling pathway in the synovial tissue was used as the entry point, and tendon-regulating and bone-setting manipulation was used as a biomechanical treatment method to carry out animal experimental research. After manual therapy, it was found that compared with those in the NC group, the Lequesne index score, Mankin score of cartilage tissue, protein expression, and content of inflammatory factors were significantly increased in the KOA group and the KOA + MT group ($P<0.05$). Compared with those in KOA group, the Lequesne index score, Mankin score, protein expression, and inflammatory factor content of the model rabbits in the KOA + MT group were significantly decreased ($P<0.05$). This result clearly shows that tendon-regulating and bone-setting manipulation can significantly reduce synovial inflammation, slow down the degeneration of articular cartilage, and improve the KOA model rabbits' activity level by regulating the expression of the TLR4–MyD88–NF-κB signaling pathway in synovial tissue.

Conclusions

This study found that tendon-regulating and bone-setting manipulation, as a noninvasive biomechanical intervention method, can significantly improve the activity state and motor function of KOA model rabbits; significantly reduce the protein activities of TLR4, MyD88, and NF-κB; and inhibit the TLR4–MyD88–NF-κB pathway in synovial tissue. This intervention method can affect the expression of the NF-κB signaling pathway, thereby reducing the content of IL-1β, IL-6, and TNF-α in synovial fluid, reducing knee joint synovial inflammation, and delaying the occurrence and development of KOA.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. AHUCM-rabbits-2019010) granted by the Medical Ethics Committee of Anhui University of Traditional Chinese Medicine, in compliance with National Institutes of Health Guidelines for the care and use of animals.

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