Original Article

The inhibiting effect of glucosamine sulfate combined with loxoprofen sodium on chondrocyte apoptosis in rats with knee osteoarthritis

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Abstract

Objectives: To explore the efficacy of glucosamine sulfate (GS) combined with loxoprofen sodium (LS) in rats with knee osteoarthritis (KOA) and its effect on chondrocytes. **Methods**: We randomly assigned 40 SPF SD rats to normal group (NG), control group (CG), treatment group (TG), and model group (MG). CG and TG were processed with continuous irrigation of LS and GS. NG and MG were given normal saline. We collected 3 mL of venous blood from the rat's lower limb for the detection of serum IL-1β, IL-6, IL-8, and TNF-α by ELISA. Four weeks after irrigation, 5 rats in each group were randomly selected for anesthesia. The water content was detected, and the chondrocytes were collected. MTT assay was used to detect apoptosis, and Western blot (WB) to measure concentrations of Bax, Bcl-2, Caspase3, Caspase9, TLR4, and NF-kB. **Results**: The levels of IL-1 β, IL-6, IL-8, and TNF-α decreased in CG and TG, but increased in MG (P<0.05). After treatment, the expression of inflammatory factors was highest in MG (P<0.05). **Conclusions**: GS combined with LS showed good efficacy in rats with knee osteoarthritis, which may be achieved by inhibiting the expression of inflammatory factors and knee chondrocyte apoptosis via the TLR4-NF-kB pathway.

Keywords: Chondrocytes, Glucosamine Sulfate, Inflammatory Factors, Loxoprofen Sodium

Introduction

Osteoarthritis (OA) is the most common form of arthritis and the knee is the most commonly affected joint. Knee osteoarthritis (KOA) is a progressive and chronic inflammatory joint disease that causes pain, disability and functional limitations, and mainly affects the middle-aged and older adults[1,2]. KOA worldwide incidence is estimated at 3.1-6.6% and is increasing year by year[3,4]. KOA may cause severe joint pain, joint stiffness, joint function loss, and even paralysis in severe cases[5]. Patients with early KOA may show a good response to conservative treatment, but patients with advanced KOA require artificial joint replacement[6]. The goal of conservative treatment is to prevent further wear and tear of the cartilage. Glucosamine sulfate (GS) is the most common cartilage protector, which can facilitate cartilage synthesis, inhibits articular cartilage degeneration, and inflammation[7]. GS is a normal component of glycosaminoglycans in the cartilage matrix and synovial fluid. This salt may have a role in slowing cartilage breakdown. GS efficacy has been clinically established[8]. However, with the wide application of GS, we found that GS has quite a long response time[9]. A study pointed out that GS may aggravate the patient's condition and seriously compromise the treatment effect[10].

Non-steroidal anti-inflammatory drugs (NSAIDs) are effective in relieving the symptoms of KOA[11]. Loxoprofen sodium (LS) is a propionic acid derivative non-steroidal anti-inflammatory drug that exerts good analgesic and anti-inflammatory effects safely, with only a few side effects[12]. LS is not only effective in the treatment of ankylosing spondylitis[13] but also has a marked analgesic effect in KOA rats[14]. GS combined with NSAIDs in the treatment of hand arthritis can

remarkably enhance the efficacy and shorten the response time[15]. Therefore, we hypothesize that GS combined with LS improves the treatment of KOA. Thus, we established rat models of KOA to explore the effects of GS combined with LS on the inflammatory factors in cartilages, cartilage water content, and cellular changes in rats, aiming to introduce a new *approach* for the *management* of KOA.

Experimental preparation

General data of animals

We bought 40 SPF SD rats from Youcare Pharmaceutical Group Co., Ltd. (Beijing, China, animal license number: SYXK (Beijing) 2018-0027). Rats were in a 1:1 sex ratio, aged 8 weeks, weighing 180±20 g. Rats were regularly raised at 29±2°C (5 rats per cage), with normal access to food and light exposure. In line with the regulations on animal protection and use, this experiment has been approved by Animal Ethics Committee of Ningbo Hangzhou Bay Hospital (WYLS2021-1).

Methods

Methods of modeling

The modeling in this study was carried out with reference to Fan et al.[16] study. After adaptive feeding for 1 week, we randomly and equally assigned 40 SPF SD rats to the normal group (NG), the control group (CG), the treatment group (TG), and the model group (MG). Rats from NG and CG were not modeled, while rats from TG and MG were selected for KOA modeling. We anesthetized rats through intraperitoneal injection of 10% chloral hydrate (3 mg/kg). After the anesthesia took effect, the rats were fixed on the operating table. All operations were performed in a sterile environment. Following routine disinfection, we shaved the right knee joint of the rat and carefully cut the skin along the medial tibia with the tibial plateau of the knee as the center without causing injury on the articular cartilage. Then we cut off the medial collateral ligament, exposed the knee joint cavity, detached the medial meniscus, and cut it off from the middle position. Finally, we cut off the anterior cruciate ligament in the joint cavity depth, closed the articular cavity, and sutured the incision. After the operation, we gave rats an appropriate amount of penicillin for 3 days to prevent infection.

Drug administration

Five days after the successful modeling, we gave rats in CG and TG an LS (0.3 mg/kg) irrigation mixed with 5 mL normal saline for 7 days. One hour after each LS irrigation, we gave rats 2 mL of normal saline containing GS (100 mg/kg), which lasted for 28 days. Rats in NG and MG were given normal saline at the same dosage for irrigation.

Detection of inflammatory factors

Before the treatment (after successful modeling but before the irrigation) and after the treatment (28 days after the irrigation), we collected 3 mL of venous blood from the lower limb of the rat and placed the sample at room temperature for 30 minutes before centrifugation at 400 × g for 10 minutes to obtain the serum. The enzyme-linked immunosorbent assay (ELISA) was performed to detect serum interleukin-1 β (IL-1 β, Shanghai Lengton Bioscience Co., Ltd., item number: BP-E30419), interleukin-6 (IL-6, Shanghai Kemin Biotechnology Co., Ltd., item number: DXT-EKO412), interleukin-8 (IL-8, Shanghai Hengfei Biotechnology Co., Ltd., item number: E-EL-RO560km-1), and tumor necrosis factor-α (TNF- α, Shanghai Hengfei Biotechnology Co., Ltd., item number: 438204-1). All operations were carried out in strict accordance with the kit instructions.
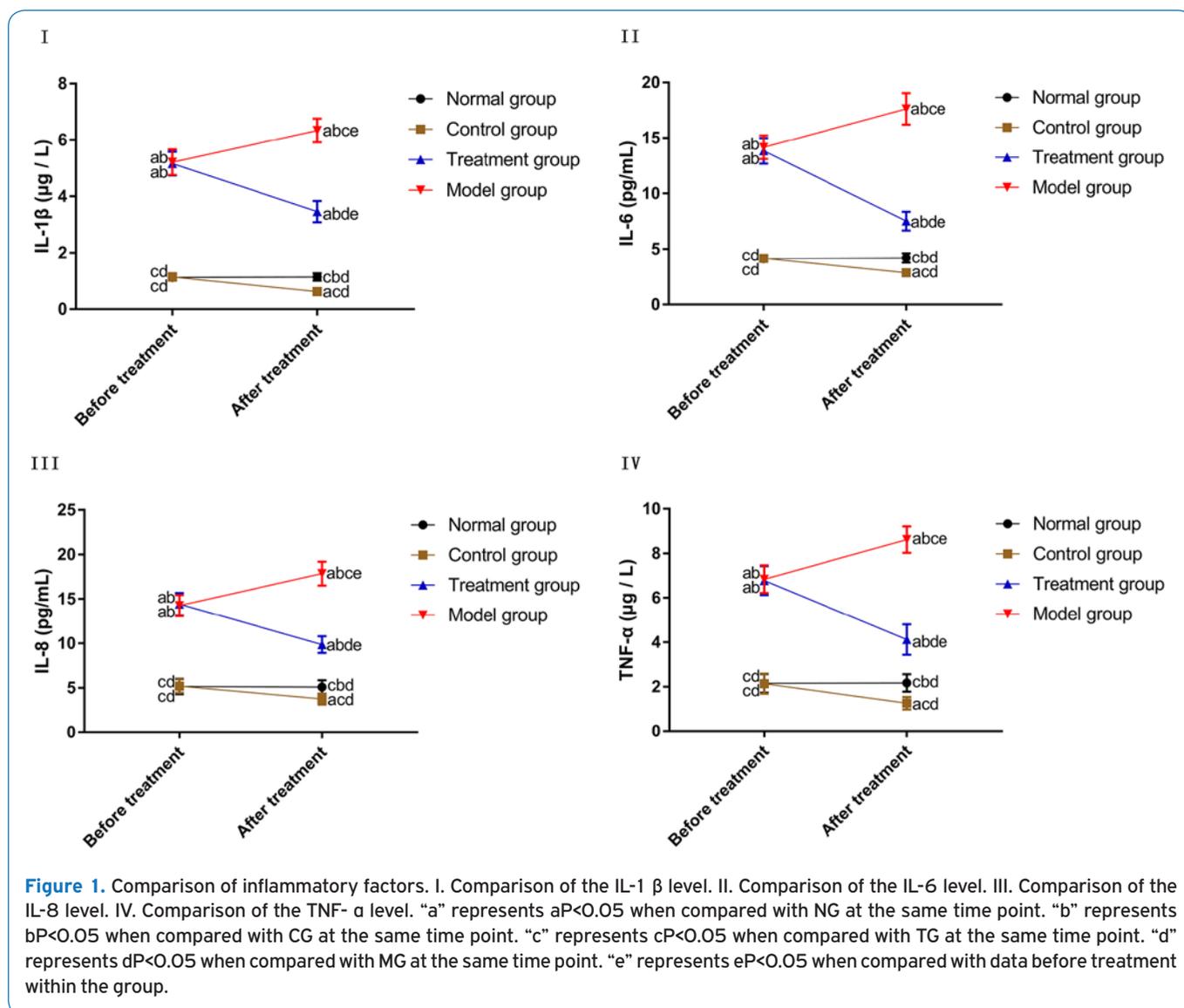
Evaluation of walking gait

The walking gait of rats in each group was scored before treatment (TO), 2 weeks after irrigation (T1), and 4 weeks after irrigation (T2). In this study, 0 point represents normal gait; 1 point represents normal gait, with a certain degree of bending of lower limbs; 2 points represents evident abnormalities of lower limbs, with contacts to the ground; 3 points represents limping, with lower limbs not touching the ground. A lower score indicates better gait performance.

Detection of water content of articular cartilage

Four weeks after irrigation, we randomly chose 5 rats in each group for anesthesia and sacrificed them by cervical dislocation. Then we stripped the tibial plateau cartilage of the right knee and weighed the wet weight on the analytical balance. We placed the cartilage in a blast heating machine at 60°C for 12 hours and then measured the dry weight. The water content of cartilage = the difference between the dry weight and the wet weight of cartilage/wet weight of cartilage.

Extraction of chondrocytes

The remaining rats in each group were also anesthetized and sacrificed by cervical dislocation. Then we collected the cartilage tissue of the right knee joint, washed it repeatedly in PBS (Gibco, Carlsbad, CA, the USA), and cut it into pieces (1 mm³). Tissue pieces were first digested by trypsin (0.25%) (Amersco, Framingham, MA, the USA) at 37°C for 30 minutes and then digested with type II collagenase (0.1%) (Sigma, St. Louis, MO, the USA) for 60 minutes, proceeding with centrifugation at 200 × g for 10 minutes to collect chondrocytes. Chondrocytes were cultured in a DMEM medium supplemented by 10% fetal bovine serum (FBS, HyClone, Logan, UT, the USA). Then we inoculated cell suspension (1×10⁶ cells/mL) in a petri dish and placed it in an incubator at 37°C, with 5% CO_2. After 48 hours of incubation, we examined the adherence of cells. The culture medium was changed every 2-3 days. When chondrocytes' adherent growth reached 80%, cells were mixed with trypsin (0.25%) for passage.

Figure 1. Comparison of inflammatory factors. I. Comparison of the IL-1 β level. II. Comparison of the IL-6 level. III. Comparison of the IL-8 level. IV. Comparison of the TNF- α level. "a" represents aP<0.05 when compared with NG at the same time point. "b" represents bP<0.05 when compared with CG at the same time point. "c" represents cP<0.05 when compared with TG at the same time point. "d" represents dP<0.05 when compared with MG at the same time point. "e" represents eP<0.05 when compared with data before treatment within the group.

Detection of cell viability

We plated cell suspension (5×10⁴ cells/mL) in 96-well plates and cultured for 24 hours, 48 hours, 72 hours, and 96 hours. After each culture, we introduced 20 μL of the MTT solution (5 mg/mL) (Gibco, Carlsbad, CA, the USA) to each well and incubated the plate for 4 hours. Then we discarded the culture medium, added 200 μL DMSO to each well, and tested each well's absorbance at 570 nm by a microplate reader (Tecan, Switzerland). The cell growth curve was drawn.

Detection of apoptosis

Cell suspension (1×10⁶ cells/mL) was inoculated in a petri dish and pre-cultured in an incubator for 24 hours. We digested cells with trypsin and washed them with PBS 3 times. The cell content was adjusted to 1×10⁶ cells/mL. According to the instructions of the apoptosis kit (Dojindo, Kumamoto, Japan), we mixed cells with Annexin V-FITC (5 μL) and PI (5

μL) and incubated them in a dark place for 15 minutes. Then we measured the apoptosis by the flow cytometry (FCM, BD, Franklin Lakes, NJ, the USA) at 4°C.

Western Blot (WB) detection

Chondrocytes were lysed with lysate (Beyotime Biotechnology, Shanghai, China) to extract the total protein. All proteins were quantified by the BCA kit (Beyotime Biotechnology, Shanghai, China) and adjusted to the same content. Proteins were separated by electrophoresis using 10% SDS-PAGE gel (Beyotime Biotechnology, Shanghai, China). Then proteins were transferred to the PVDF membrane by the semi-dry method and sealed in 5% skimmed milk powder at room temperature for 1 hour. Next, proteins were cultured with Bax, Caspase3, Caspase9, TLR4, NF-kB, p-NF-kB, and β-actin primary antibodies (Abcam, Cambridge, UK) overnight at 4°C. The PVDF membrane was washed
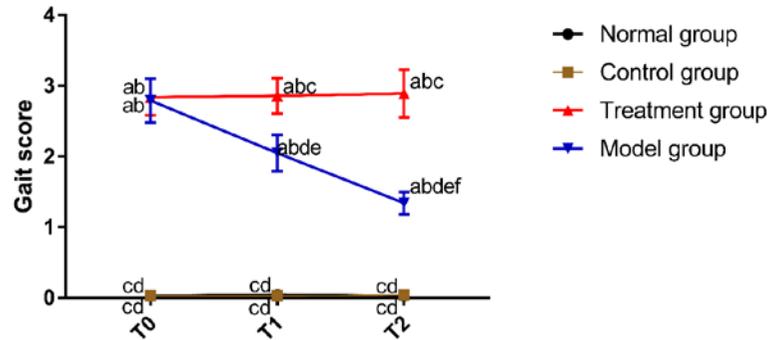
Figure 2. Comparison of the gait score. "a" represents aP<0.05 when compared with NG at the same time point. "b" represents bP<0.05 when compared with CG at the same time point. "c" represents cP<0.05 when compared with TG at the same time point. "d" represents dP<0.05 when compared with MG at the same time point. "e" represents eP<0.05 when compared with T0 within the group. "f" represents fP<0.05 when compared with T1 within the group.
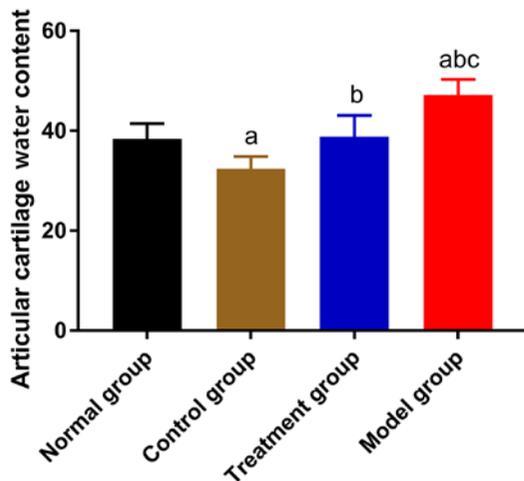

Figure 3. Comparison of the water content of articular cartilage. "a" represents aP<0.05 when compared with NG. "b" represents bP<0.05 when compared with CG. "c" represents cP<0.05 when compared with TG.

by TBST and incubated with goat anti-rabbit IgG second antibody for 1 hour. Finally, we added ECL reagent (Beyotime Biotechnology, Shanghai, China) for color development. The bands were scanned, and the WB images were collected. The relative expression of the protein = the gray value of the target protein/the internal reference's gray value (β-actin).

Statistical analysis

Statistical analysis was performed with SPSS22.0. All tests were performed three times to determine the average value, which was represented by the `c±s. The comparison of measurement data between multiple groups was analyzed

by the one-way analysis of variance (ANOVA); the pairwise comparison of measurement data between any two groups was analyzed by the LSD test; the comparison of measurement data between multiple time points was analyzed by the repeated measures ANOVA and bonferroni post-test. P<0.05 represents a statistically significant difference.

Results

Comparison of inflammatory factors

Before treatment, CG and NG were not different in the levels of IL-1 β, IL-6, IL-8, and TNF-α; MG and TG were not different in the levels of such inflammatory factors (P>0.05). The levels of such inflammatory factors were higher in MG and TG than in CG and NG before treatment (P<0.05). After treatment, the levels of inflammatory factors were not changed in NG (P>0.05). The levels of inflammatory factors in CG and TG were lower after treatment than those before treatment, while the levels of those factors in MG were higher after treatment than those before treatment (P<0.05). After treatment, the expression of inflammatory factors was the highest in MG, followed by TG, NG, and CG (P<0.05) (Figure 1).

Comparison of the gait score

In NG, CG, and MG, there were no marked differences in gait scores between T0, T1, and T2 (P>0.05). In TG, the gait score was the highest at T0, decreased at T1, and the lowest at T2 (P<0.05). At T0, NG and CG were not different in the gait score, and MG and TG were also not different in the gait score (P>0.05). The gait score was lower in NG and CG than in MG and TG at T0 (P<0.05). At T1 and T2, there was no difference in the gait score between CG and NG (P>0.05). The gait score was lower in CG and NG than in MG and TG T1 and T2, with a lower gait score in MG than in TG (P<0.05) (Figure 2).
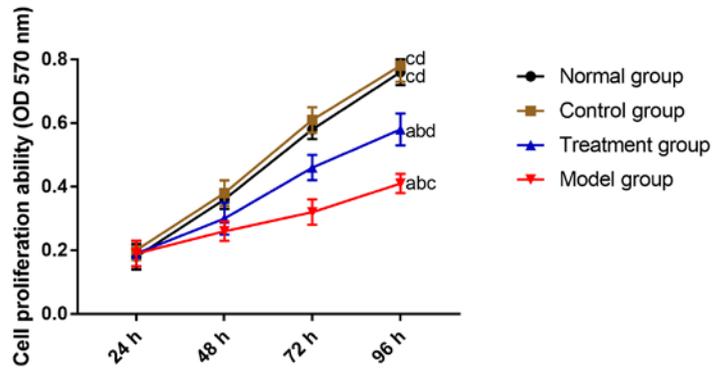
Figure 4. Comparison of the chondrocyte activity. "a" represents aP<0.05 when compared with NG. "b" represents bP<0.05 when compared with CG. "c" represents cP<0.05 when compared with TG. "d" represents dP<0.05 when compared with MG.
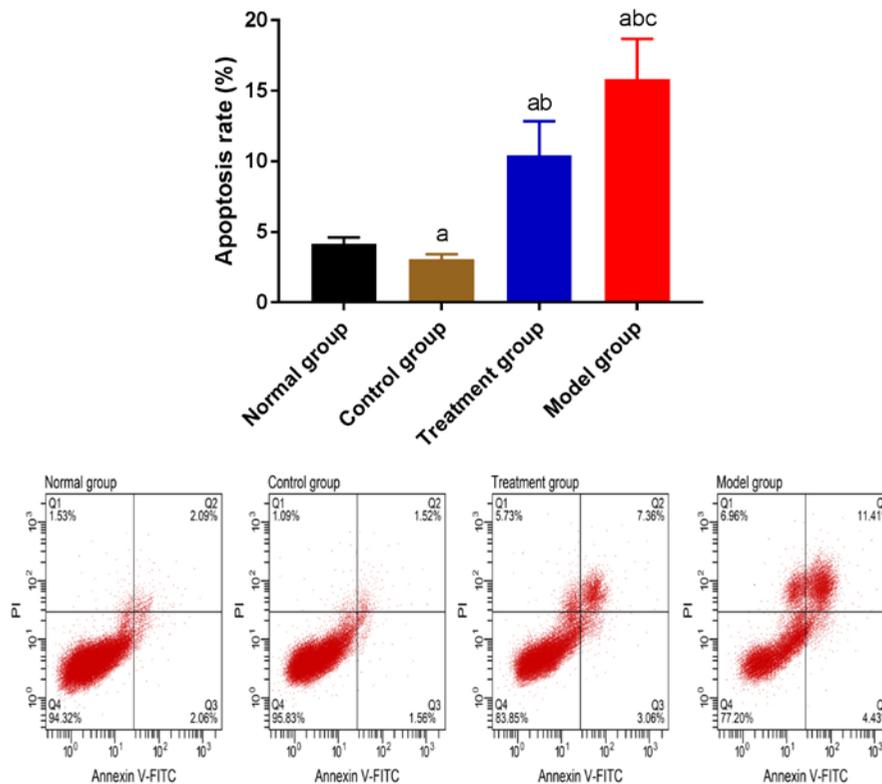

Figure 5. Comparison of the chondrocyte apoptosis. "a" represents aP<0.05 when compared with NG. "b" represents bP<0.05 when compared with CG. "c" represents cP<0.05 when compared with TG.

Comparison of the water content of articular cartilage

Four weeks after irrigation, the water contents of knee cartilages in NG and TG were not different (P>0.05), and both were higher than that in CG and lower than that in MG (P<0.05) (Figure 3).

Comparison of the chondrocyte viability

According to the MTT assay results, there was no difference in chondrocyte proliferation between CG and NG (P>0.05). Chondrocyte proliferation was stronger in CG and NG than in MG and TG (P<0.05), with a higher proliferation rate in TG than in MG (P<0.05) (Figure 4).
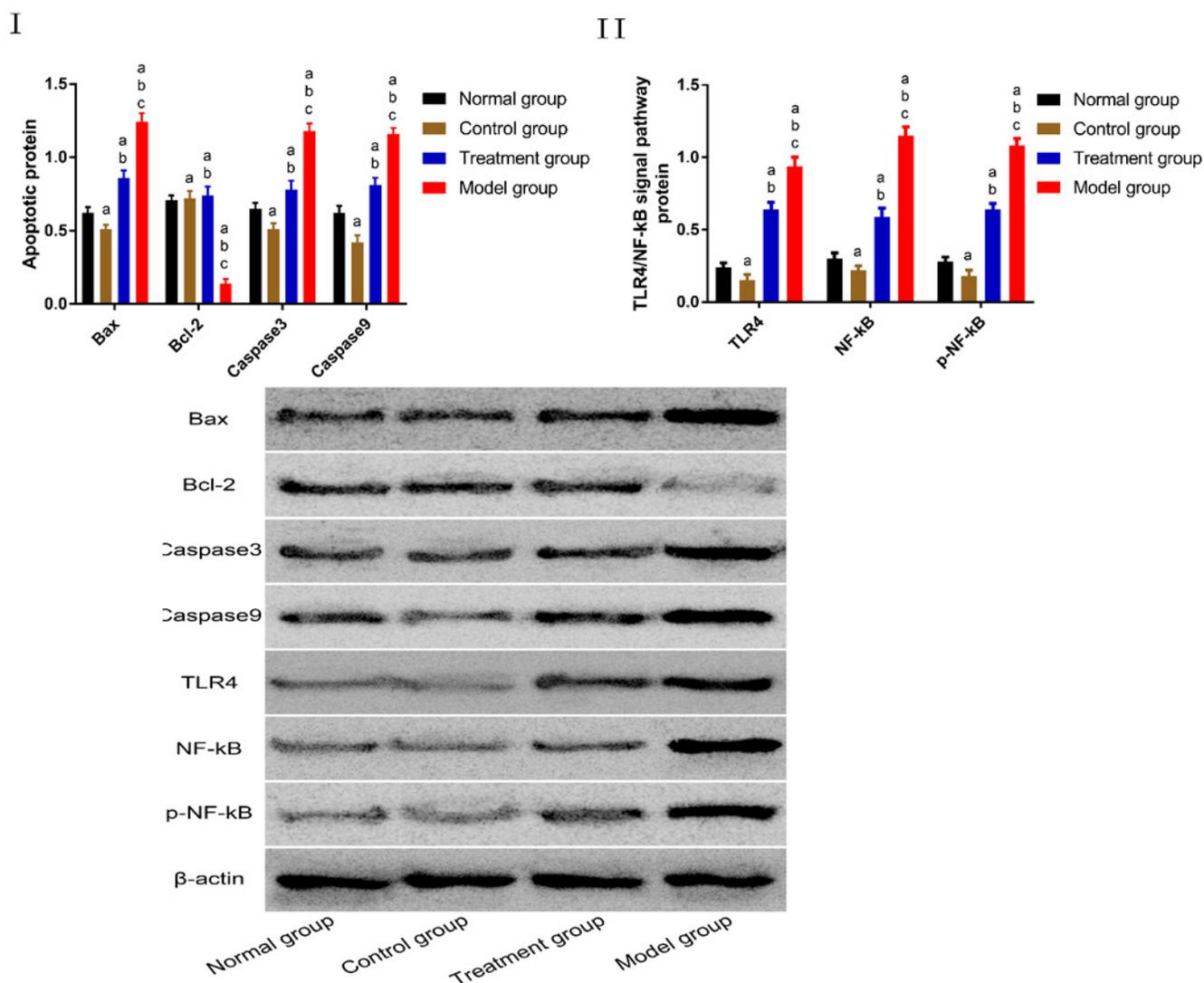
Figure 6. Comparison of the proteins expression in chondrocytes. I. Comparison of apoptosis-related proteins. II. Comparison of TLR4/NF-kB signal pathway-related proteins "a" represents aP<0.05 when compared with NG. "b" represents bP<0.05 when compared with CG. "c" represents cP<0.05 when compared with TG.

Comparison of the chondrocyte apoptosis

The results of the FCM revealed that the apoptosis rate of chondrocytes was the highest in MG, followed by TG, NG, and CG (P<0.05) (Figure 5).

Comparison of the expression of proteins in chondrocytes

According to the western blot results, the Bcl-2 concentration was higher in TG, NG, and CG than in MG (P<0.05), and TG, NG, and CG were not different in the Bcl-2 concentration. The concentrations of pro-apoptotic proteins (Bax, Caspase3, and Caspase9) and TLR4/NF-kB signal pathway-related proteins (TLR4, NF-kB, and pNF-kB) were the lowest in CG, followed by NG, TG, and MG. More details are shown in Figure 6.

Discussion

The pathological features of KOA include deformation and destruction of articular cartilage, the formation of osteophytes at the edge of the joint, synovial hyperplasia, and so on[17]. During the pathogenesis of KOA, the biosynthesis and decomposition of proteoglycan in the articular cartilage matrix were impaired, and inflammatory factors were massively activated and released, causing a series of injuries to the surrounding tissues[18]. Therefore, improving the structure of knee cartilage is one of the keys to the treatment of KOA. GS is a frequently studied structure-modifying drug that has a recognized therapeutic effect on KOA[19]. LS, as a NSAID, is also widely used for anti-inflammation and analgesia in patients with rheumatic arthritis, lumbago,

and omarthritis[20]. This study aims to explore the effects of GS combined with LS on the chondrocytes of KOA rats, broadening the understanding of the clinical therapeutic value of GS combined with LS.

No peritonitis was reported in rats during the modeling. We processed rat models, and normal rats with GS combined with LS and then detected the levels of inflammatory factors in the blood. Before treatment, TG and MG were not different in the inflammatory factors, and CG and NG were not different in the inflammatory factors. Still, the expression levels of inflammatory factors were higher in TG and MG than in CG and NG. These results suggest that the increased expression of inflammatory factors is a major cause of knee joints' inflammatory injury during the pathogenesis of KOA. After treatment, the expression levels of inflammatory factors were decreased in both TG and CG groups, indicating that the joint therapy can inhibit the release of inflammatory factors, which was mainly achieved by LS. A previous study suggested that LS can inhibit cyclooxygenase, reduce interleukin and prostaglandin synthesis, and thus exert an anti-inflammatory effect[21]. The results of this study also support this view. Here we noted a gradual decrease in the gait score in TG, suggesting that rats in TG were gradually recovering from KOA. This may be a reasonable consequence of GS. As a necessary component for the synthesis of proteoglycans in articular cartilage matrix, GS can produce polymer structural glycoproteins to inhibit the production of superoxide free radicals and reduce the oxidative stress damage of cells, and hence cure osteoarthritis[22]. In our study, the MG group, showed an increase in the expression of inflammatory factors which was accompanied by an increase in the gait score, suggesting that with the progression of KOA, the damage to the knee joints caused by inflammatory factors gets more severe. Articular cartilage is a kind of non-vascular tissue, which gets its nutritional support mainly from the exchange between chondrocytes and the surrounding nutrient-rich synovial fluid[23]. We detected the highest water content of the knee cartilage in MG, suggesting that synovium secreted a large amount of synovial fluid during the occurrence of osteoarthritis, which leads to decreased osmotic pressure in the joint cavity. Proteoglycans in the cartilage matrix, with negative electricity, have a strong water-absorbing capacity and hence increase the water content. Such findings are consistent with the results of a previous study[24] that explored the water content of knee cartilage. In our study, the water content of knee cartilage was lower in TG than in MG and lower in CG than in NG, suggesting that GS combined with LS reduced the secretion of substances such as joint effusion to secure a stable environment of the knee cartilage, which facilitates the recovery from the disease. All the above results indicate that GS combined with LS is effective and safe and may protect the knee joints of healthy people. Nevertheless, further studies are required to confirm this finding.

We also performed the MTT, FCM, and WB assays to explore the effects of GS combined with LS on knee cartilage. In this study, TG had a higher chondrocyte proliferation rate and a lower apoptosis rate than MG,

and CG had a lower chondrocyte apoptosis rate than NG. Thus, we hypothesize that GS combined with LS may also affect the progression of KOA by inhibiting the apoptosis of chondrocytes. Chondrocytes can synthesize and secrete matrix and fiber. A previous study revealed that a large number of chondrocytes would be subjected to apoptosis in the process of KOA[25]. Inhibited apoptosis of chondrocytes can strengthen the knee cartilage's structural integrity and minimize the injuries caused by pathological changes of KOA. Ding et al.[26] demonstrated that the TLR4-NF-kB signaling pathway plays a critical part in osteoarthritis. We also detected the protein expression of TLR4 and NF-kB in chondrocytes in the 4 groups. The concentrations of TLR4 and NF-kB were lower in TG than in MG and lower in CG than in NG. Therefore, we hypothesize that GS's inhibition of chondrocyte apoptosis combined with LS may be achieved through the TLR4-NF-kB pathway.

In summary, GS combined with LS has good efficacy for KOA rats, which may be achieved by inhibiting the expression of inflammatory factors and knee chondrocyte apoptosis via the TLR4-NF-kB pathway. Given the differences between the human body structure and the rat body structure, the effects of GS combined with LS on human knee chondrocytes need to be investigated by human experiments. In addition, whether GS combined with LS inhibits the expression of inflammatory indicators and the apoptosis of knee chondrocytes through the TLR4-NF-kB pathway needs to be verified by more experiments. Here we did not detect markers for oxidative stress reaction such as superoxide dismutase and malondialdehyde in rats, so the effects of GS combined with LS on the oxidative stress response of KOA are not clear.

Authors' contributions

ML and WL conceived and designed the study, and drafted the manuscript. ML, FX and WL collected, analyzed and interpreted the experimental data. WL revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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