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Curcumin attenuates collagen-induced rat arthritis via anti-inflammatory and apoptotic effects

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ABSTRACT

Curcumin is a natural herbal product that has been popularly used to treat autoimmune diseases in China; however, its effects on rheumatoid arthritis and its mechanism are not clear. The main purposes of this study are to explore the therapeutic effects of curcumin on collagen-induced arthritis (CIA) rats and the pharmacological mechanism. In the present study, CIA rats were established by injecting bovine type II collagen. Curcumin and methotrexate were then orally administered daily, and the swelling degree of the hind limb joints was scored every two days. Histopathological changes were observed by hematoxylin-eosin staining. The levels of cytokines (TNF-α, IL-1β, IL-17 and TGF-β) were detected by radioimmunoassay, while the expression of IκBα and COX-2 was detected by Western blot. In addition, cell viability was detected by CCK-8 assay, and the effect of curcumin on macrophage apoptosis was detected by flow cytometry and TUNEL assay. The results indicated that in vivo curcumin attenuated the degree of joint swelling of rats and the further development of joint histopathology. Moreover, it downregulated the levels of cytokines. In vitro curcumin inhibited the degradation of IκBα and reduced the production of COX-2 in LPS-induced inflammatory RAW264.7 cells. Importantly, curcumin significantly induced macrophage apoptosis. In conclusion, in this study, we have demonstrated that curcumin exerts therapeutic effects on arthritis in CIA rats and has a strong pharmacological activity on reducing the inflammatory response in macrophages. Its mechanism may be related to the inhibition of the NF-κB signaling pathway and the promotion of macrophage apoptosis.

1. Introduction

Rheumatoid arthritis (RA), which is characterized by synovial inflammation, joint swelling deformities and potentially premature death, is one of the most common chronic inflammatory systematic autoimmunity diseases [1]. Patients with RA always suffer from joint pain, swelling and potentially lifelong disability, which significantly affect the motor function of the human body and result in a substantial financial burden [2]. Currently, clinical treatment medicines can effectively improve the symptoms of patients. However, these drugs are accompanied by serious adverse reactions, such as gastrointestinal reactions [3]. Therefore, we urgently need new drugs that have fewer side effects and better efficacy. Recently, traditional Chinese herbal medicine has performed well in this regard.

Curcumin, a type of phenolic compound, is a natural herbal medicine that is mainly extracted from turmeric rhizome. Traditionally, it was confirmed that curcumin had various pharmacological activities, such as anti-inflammation, anti-cancer, anti-oxidation, anti-angiogenic, and anti-radiation [4–6]. Its molecular mechanisms are not only related to the inhibition of cell proliferation and metastasis but are also involved in the downregulation of the levels of multifarious factors, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and protease [7–9].

Proinflammatory cytokines, such as TNF-α, IL-1β, IL-17 and TGF-β, related to the pathophysiology of RA, are mainly secreted by activated macrophages, which characteristically exist in the joint tissues of RA patients [10–12]. The excessive elevation of TNF-α will enhance the inflammatory response, activate fibroblasts, and lead to synovial hyperplasia and damage the articular cartilage [13]. IL-1β can induce fibroblast proliferation, which is closely related to the damage of the

Abbreviations: MTX, methotrexate; CIA, collagen-induced arthritis; CII, bovine type II collagen; IFA, incomplete Freund's adjuvant; CUR, curcumin

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joint synovium and enhances the expression of cyclooxygenase-2 (COX-2) in fibroblasts [14]. Both TNF-α and IL-1β promote the activation of genes associated with inflammation, such as NF-κB [14]. Recently, studies have indicated that IL-17 may be a determinant of the pathogenesis of RA and associated with the severity of RA. Moreover, high levels of TGF-β can enhance the bone resorption of osteoclasts and mediate synovial cell proliferation and endothelial angiogenesis [15]. Eventually, these proinflammatory cytokines affect and promote each other to form a strong inflammatory cascade, inducing synovial hyperplasia and enhancing histopathological changes in RA [16]. Moreover, some types of macrophages can activate into osteoclasts and are widely found in the synovium and cartilage of joints, which can cause pathological absorption and destruction of bone and cartilage [17]. Interestingly, the number of macrophages can be regulated by apoptosis. However, TNF-α and IL-1 cause a suppressive rate of apoptosis, which maintains inflammatory macrophage survival [16,18]. Thus, the promotion of macrophage apoptosis in RA joints is a potential new strategy for treating RA.

Recently, clinical studies have indicated that curcumin can ameliorate the symptoms of arthritis patients, reducing joint pain and restoring part of the function [5]. Curcumin may be considered a safe and effective clinical drug in the future due to the advantages of less side effects of the active ingredients of natural plants. However, current studies on the anti-arthritic mechanism of curcumin are not complete. In this study, we used the CIA rat model to explore the therapeutic effect of curcumin in vivo and in vitro. In addition, we evaluated the effect of promoting cell apoptosis with curcumin treatment on LPS-induced RAW264.7 cells.

2. Materials and methods

2.1. Reagents

Curcumin (98% purity) and lipopolysaccharide (99% purity) were purchased from Sigma-Aldrich (#78246, St. Louis, MO, USA) and Solarbio Science & Technology (#L8880, Beijing, China), respectively. Incomplete Freund's Adjuvant (IFA) and bovine type II collagen were obtained from Chondrex (Redmond, WA, USA). The rabbit anti-mouse immunoglobulin G primary antibodies against β-actin (#4967) and IκBα (#4812) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The secondary antibodies (goat anti-rabbit immunoglobulin G horseradish peroxidase) (#ZB-2301) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). The fluorescence secondary antibody for TRITC AffiniPure Goat Anti-Rabbit IgG was purchased from EarthOx (San Francisco, USA). The DAPI Staining Kit was obtained from BOSTER (Wuhan, China). Methotrexate was purchased from Shanghai Xinyi Pharmaceutical Co., Ltd. (Shanghai, China). RPMI Medium1640 (100 U/mL Penicillin and 100 μg/mL streptomycin) was supplied by Beijing Solarbio (Beijing, China). Fetal bovine serum (FBS) was purchased from Beijing Transgen (Beijing, China).

2.2. Animals

Sterile male SD rats (weight between 200 g and 250 g) were obtained from Nanchang University Animal Center, and the entire experiment was performed in a sterile environment with controlled conditions. All agreements were reviewed and ratified by the Nanchang University's Institutional Animal Experiment Ethics Committee.

2.3. Establishment of collagen-induced rat arthritis model

According to our previous study [19], the CIA model was established via the following steps: Bovine type II collagen (CII) was emulsified with an equal volume of IFA. The CII-containing emulsion (0.2 mL) was then intradermally injected into the tail root of the rats to induce inflammation (primary immunization). On day 7 after the primary immunization, the same volume of this emulsion was intradermally injected into the left hind limb of the rats to enhance the primary immunization (second immunization). The animals without the signs of swelling joint were removed. With the exception of the normal control group (Sham), the rats of the collagen-induced arthritis model were randomly divided into 4 groups (n = 8 per group): the collagen-induced arthritis model control group (CIA control), the methotrexate group (MTX) and two curcumin groups (CUR 200 mg/kg and 100 mg/kg).

2.4. Treatment and evaluation of collagen-induced arthritis

Two CUR groups were orally administered curcumin in normal saline solution at 200 mg/kg and 100 mg/kg (body weight, the same below), respectively; the MTX group was orally administered 0.3 mg/kg methotrexate solution; the Sham and CIA control groups were only orally administered the same volume of normal saline. All CUR group rats were treated with curcumin every day after arthritis episodes (the day after the secondary immunization), once per day until the end of the experiment (day 18 of the primary immunization). The swelling degree of each rat's right hind limb ankle joint was scored and recorded every 2 days until the end of the experiment.

2.5. Histopathology and assessment of joints

The histopathological analysis of the joints, evaluation methods and standards of the joint health status for this experiment were based on our previous study [19]. The hind limb joint swelling degree was scored as follows: 0, no swelling and redness; 1, mild swelling and redness; 2, moderate swelling and redness and accompanied by minor changes in joint activity; 3, severe swelling and redness, accompanied by joint stiffness and activity was significantly limited.

2.6. Radioimmunoassay

Five milliliters of each blood sample was taken from all subjects to detect the levels of cytokines in the serum. After the blood was agglutinated, all samples were centrifuged at 1024*g* for 15 min, and the serum was subsequently collected and stored at −80 °C. The rat joint synovium was removed from each group and minced mechanically in sterile phosphate buffered saline (PBS). The synovium supernatant was collected via centrifugation at 1024*g* for 15 min. The protein content of the synovium homogenate and serum supernatant was assayed using the BCA Protein Assay Kit (Beijing Solarbio, Beijing, China). The levels of TNF-α, IL-17, IL-1β and TGF-β in the synovium homogenate and the levels of TNF-α and IL-17 in the serum supernatant were detected via a radioimmunoassay kit (Beijing SINO-UK Institute of Biological Technology, Beijing, China).

2.7. Cell culture and cell viability assay

The mouse macrophage-like RAW264.7 cells were acquired from the Autoimmune Disease Research Center of Nanchang University and cultured in RPMI Medium 1640 (100 U/mL Penicillin and 100 μg/mL streptomycin) that contained 10% FBS in a humidified incubator with 5% CO_2 at 37 °C. Moreover, the cytotoxicity of curcumin on RAW264.7 cells was detected via the Cell-Counting Kit-8 (CCK-8, TransGen Biotech, Beijing, China) assay. In brief, the cells (2×10^4 cells/well) were seeded into 96-well plates with RPMI Medium 1640 that contained 2% FBS overnight at 37 °C and incubated with curcumin of different concentrations (experimental group) for 12 h (n = 6). The curcumin was diluted with dimethyl sulfoxide (DMSO) and mixed in medium with different concentrations (0.1, 1, 10, or 100 μmol/L). The cells without curcumin served as the Sham group. Ten microliters of CCK-8 reagents was added to each well and incubated in an incubator

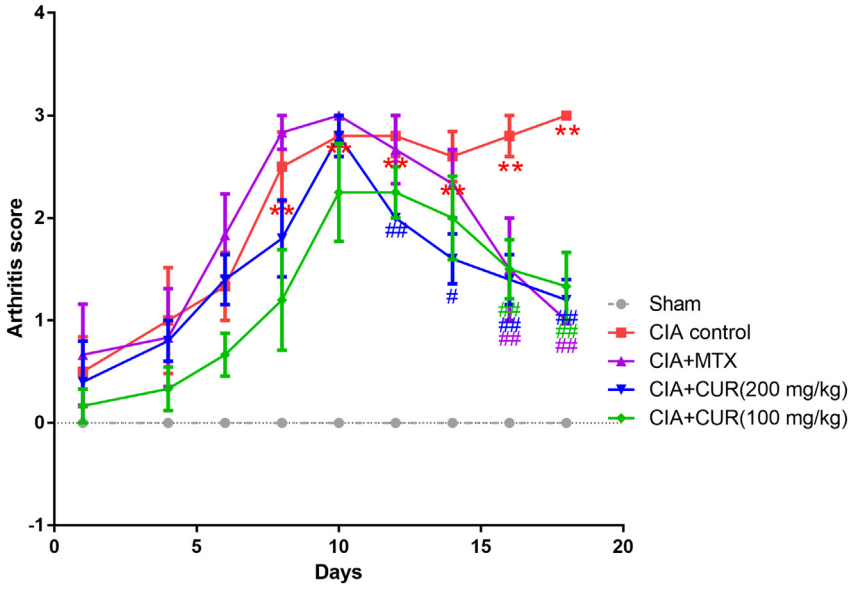

Fig. 1. Effect of curcumin on severity of arthritis in collagen-induced arthritis (CIA) rats. The rats were orally administered curcumin from days 7 to 18 after model establishment. The arthritis score of the right hind limb ankle joint in different groups was scored every 2 days from day 2 of the beginning of the experiment. Sham group, normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; CIA + MTX, methotrexate-treated rats (0.3 mg/kg) + CIA; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. This experiment was performed three times (n = 8). $^{**}p < 0.01$, compared with the Sham group; $^{\#}p < 0.05$, compared with the CIA control group; $^{\#\#}p < 0.01$, compared with the CIA control group.

with 5% CO_2 at 37 °C for 2 h. Finally, the absorbances of all wells were detected at 450 nm using a microplate reader (HBS-1096A, Detie, Nanjing, China). The cell viability was counted using the following formula: cell viability = absorbance of experimental group/absorbance of sham group × 100%. All experiments were conducted three times, and six wells with the same concentration were analyzed in each group.

2.8. Western blot

The joints' synovium was lysed in a RIPA lysate buffer (Appliedgen Technologies Inc., Beijing, PR China) and centrifuged at 1024*g* for 15 min. RAW264.7 cells were seeded into a 6-well culture plate (1×10^6 cells/well) in RPMI-1640 medium that contained 1% FBS overnight. After incubation with curcumin (10. 0, 1.0 or 0.1 μmol/L) for 30 min, the cells were stimulated by LPS (10 μg/mL) for 12 h, and the protein was ultimately extracted from the RAW264.7 cells. The synovium of the joints and RAW264.7 cell proteins were separated by 12% polypropylene gel electrophoresis and transferred to a nitrocellulose acetate membrane. After blocking, the membrane was incubated with the corresponding primary antibody overnight at 4 °C and then incubated with the secondary antibody (horseradish peroxidase conjugated) at room temperature for 1 h. Finally, the immunoreactive protein was detected by the chemiluminescence assay (Amersham Imager 600, GE, USA) using the ECL Advance Western blot kit (TransGen Biotech, Beijing, China).

2.9. Immunofluorescence staining

Immunofluorescence staining was improved from the previous study [20]. RAW264.7 cells (2×10^4 cells/well) were seeded on sterile glass coverslips in 6-well plates and cultivated with RPMI Medium 1640 (100 U/mL Penicillin and 100 μg/mL streptomycin) that contained 2% FBS overnight in 37 °C. The cells were incubated with 10 μmol/L curcumin for 30 min, followed by stimulation with LPS (10 μg/mL) for 12 h. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde for 20 min. The fixed cells were penetrated with 0.1% Triton X-100 for 20 min and then incubated with anti-IκBα primary antibody in a wet box overnight at 4 °C. After washing, the cells were incubated for 1 h in a dark condition with FITC secondary antibody. Finally, the cells were incubated with DAPI and anti-fading buffer. Images of the protein fluorescence signal were obtained under fluorescence microscopy (Olympus TH4-200, Tokyo, Japan).

2.10. Detection of apoptosis by flow cytometry

Apoptosis was detected by the Annexin V-FITC/PI Apoptosis Detection Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. RAW264.7 cells (1.0×10^6 cells/well) were cultured in 24-well plates with RPMI Medium 1640 that contained 2% FBS overnight at 37 °C and incubated with 10 μmol/L curcumin for 24 h. After centrifugation at 1280*g* for 10 min, the cells were resuspended in precooled 100 μL of Annexin V binding buffer; 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were then added separately into the suspension for 15 min at room temperature in the dark. Finally, 400 μL of Annexin V binding buffer was added into the suspension and analyzed on a flow cytometer (FACSJazz, BD, USA).

2.11. TUNEL assay for the synovial macrophage apoptosis of rat joints

Apoptotic macrophages of rat ankle joints were labeled in situ using the TdT-mediated dUTP nick end labeling (TUNEL) Apoptosis Assay Kit (Living Biotechnologies Co., Ltd., Beijing, China). The ankle joints of the five groups (n = 6) were fixed in 10% neutral buffered formalin, and all slides were decalcified and dehydrated, embedded in paraffin and cut into a thickness of 5 μm.

After deparaffinization, the joint tissue sections were incubated in proteinase K (1 mg/mL) for 30 min at 37 °C and then blocked with 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase. Fifty microliters of the terminal deoxynucleotidyl transferase (TdT) reaction solution was added to each sample to label the end of the tissue fragmented DNA and incubated at 37 °C for 60 min in the dark. The tissue was then incubated with the streptavidin linked to horseradish peroxidase for 30 min under the same conditions. In the presence of diaminobenzidine (DAB), apoptotic cells are stained dark brown at room temperature for 2 min. All samples were then counterstained with hematoxylin stain (Boster Biological Technology Co. Ltd., Wuhan, China). The samples were then dehydrated in ethanol and xylene and sealed.

The percentage of apoptotic cells was determined using light microscopy. The brown nuclei represent apoptotic cells, and the blue nuclei indicate viable cells. Six areas of interest were selected from each sample and analyzed. The number of apoptotic cells and viable cells in each region of interest was counted three times and then averaged. The percentage of positive-macrophage cells was calculated using the following formula: Percentage of positive-macrophage cells = (number of apoptotic cells / total number of cells) × 100% (total number of

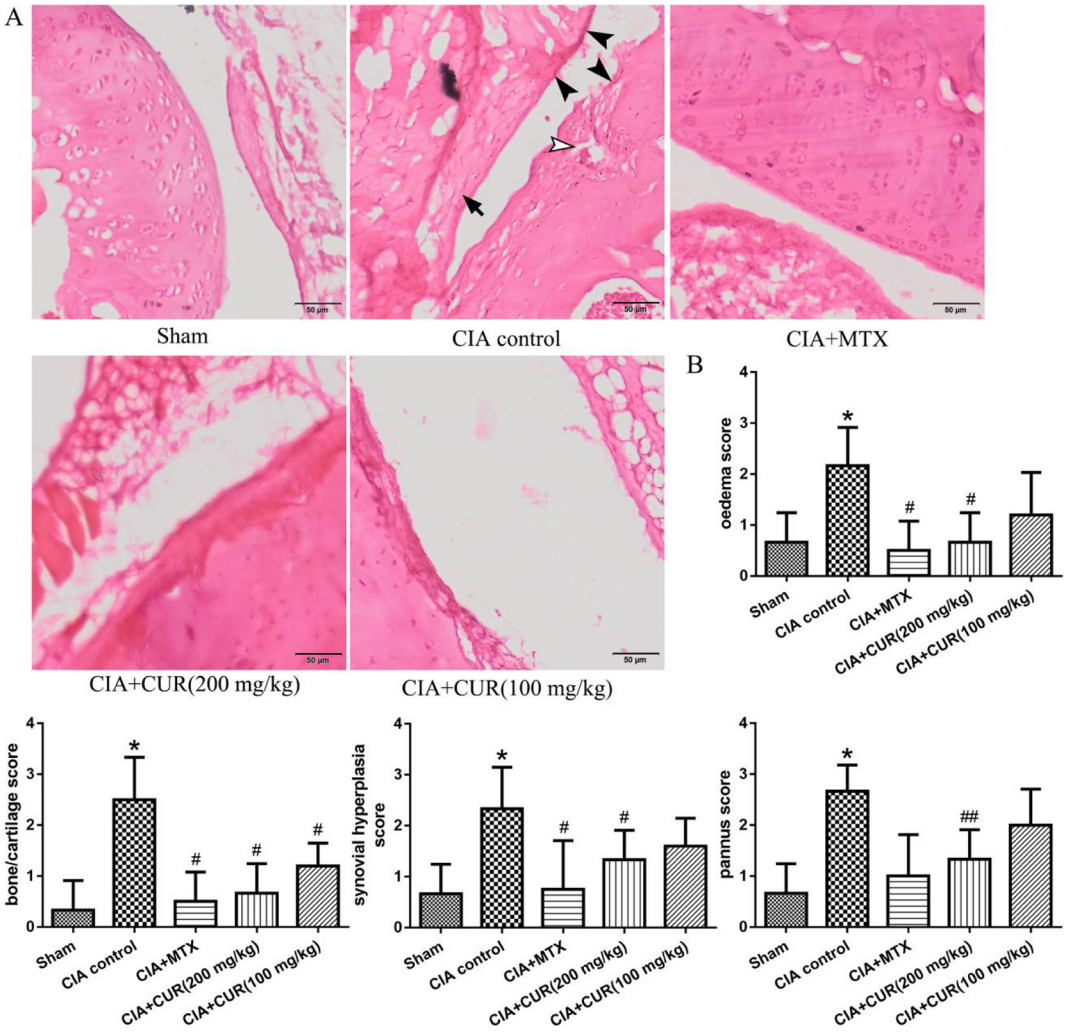

Fig. 2. (A) Curcumin alleviates the histopathologic changes of rheumatoid arthritis in CIA rats. Typical photomicrographs of H&E staining (magnification, 200×) indicated the synovial tissue changes in tibia-talus joints of rats. (B) Histopathological scores were obtained from H&E staining of rat tibia-talus joint sections in each group for all four parameters, including edema, bone/cartilage destruction (open arrowheads), synovial hyperplasia (solid arrows), and pannus formation (solid arrowheads). Sham group, normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; CIA + MTX, methotrexate-treated rats (0.3 mg/kg) + CIA; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. This experiment was performed three times (n = 6). $^{*}p < 0.05$, compared with the Sham group; $^{\#}p < 0.05$, compared with the CIA control group; $^{\#\#}p < 0.01$, compared with the CIA control group.

cells = number of apoptotic cells + number of viable cells).

2.12. Statistical analysis

All experiments were performed three times. All data were analyzed by SPSS 17.0 software and expressed as the means ± SEM. Except for the results of the flow cytometry using two-sample t-test, the comparison of the results among each independent group was performed via One-way analysis of variance with Tukey's post hoc test. A p-value < 0.05 was considered to indicate a significant difference.

3. Results

3.1. Effect of curcumin on severity of arthritis in CIA rats

The severity of arthritis in CIA rats can be determined from the arthritis score, which was evaluated from day 1 to day 18 (Fig. 1). In the CIA control group, the arthritis score increased and plateaued between day 8 and day 14. The treatment of CIA rats with curcumin (200 and 100 mg/kg) or MTX decreased the severity of arthritis after day 10.

3.2. Curcumin alleviates joint histopathological changes in CIA rats

The rat tibia-talus joints stained with H&E-staining showed that the CIA control rats had significant histopathological changes of arthritis, including edema, bone/cartilage destruction, synovial hyperplasia, and pannus formation. However, treatment with curcumin or MTX almost completely prevented the joint histopathological changes in the CIA rats (Fig. 2).

3.3. Effects of curcumin on TNF-α, IL-17, IL-1β and TGF-β in the synovium of the joints in CIA rats

Compared with the Sham group, the levels of TNF-α, IL-17, IL-1β and TGF-β in the CIA rat synovium were increased. However, the treatment of CIA rats with 200 mg/kg curcumin or MTX significantly inhibited the level of these inflammatory cytokines in the synovium of the joints ($p < 0.05$) (Fig. 3).

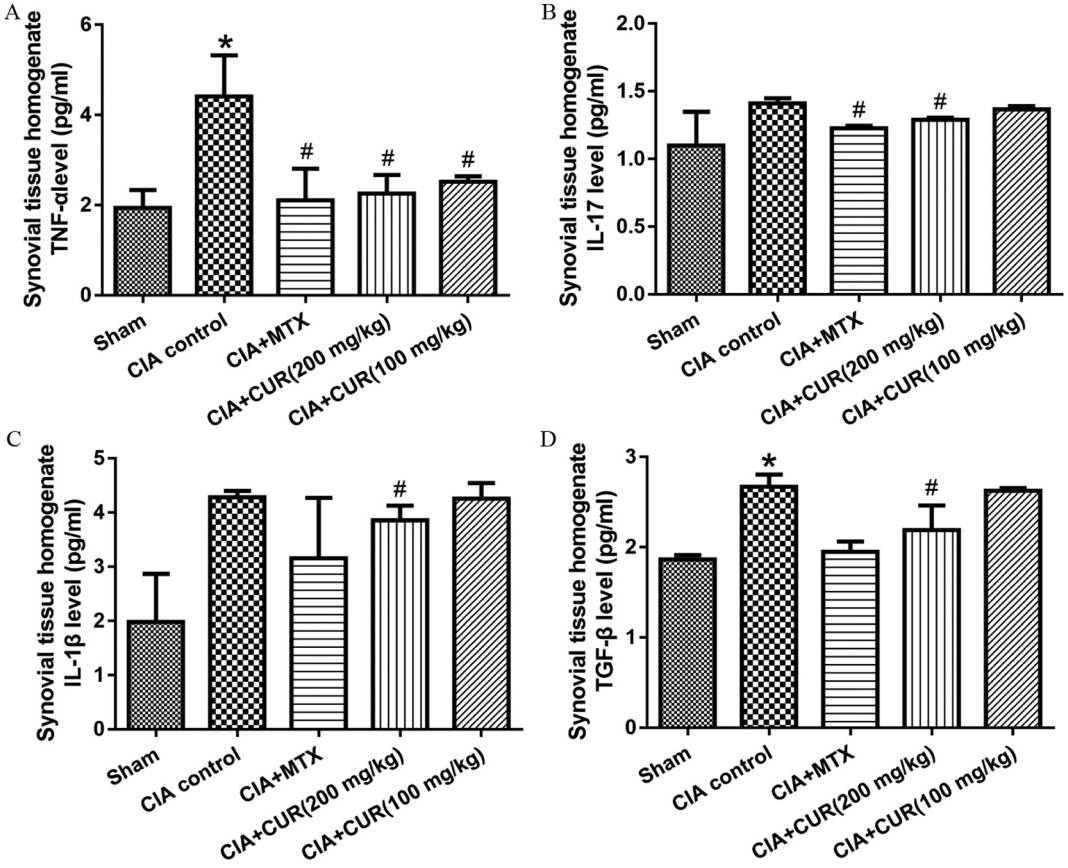

Fig. 3. Effects of curcumin on the levels of TNF-α, IL-17, IL-1β and TGF-β in the synovium of the joints in CIA rats. The levels of these cytokines in synovium homogenate were detected by radioimmunoassay. (A) TNF-α level, (B) IL-17 level, (C) IL-1β level, and (D) TGF-β level in synovial tissue homogenate. Sham group: normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; CIA + MTX, methotrexate-treated rats (0.3 mg/kg) + CIA; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. This experiment was performed three times (n = 6). $^{*}p < 0.05$, compared with the Sham group; $^{\#}p < 0.05$, compared with the CIA control group.

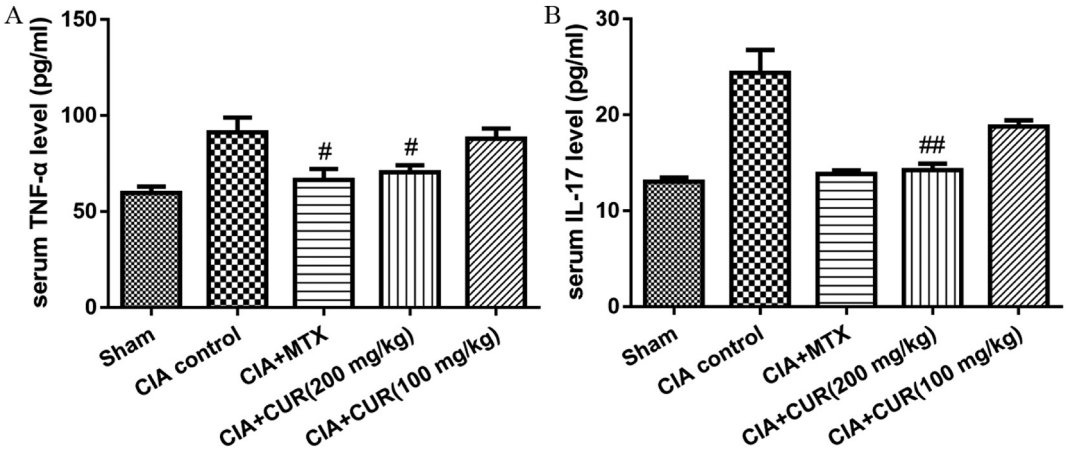

Fig. 4. Effects of curcumin on serum levels of TNF-α and IL-17 in CIA rats. The levels of these cytokines in serum supernatant were detected by radioimmunoassay. (A) TNF-α level in serum, and (B) IL-17 level in serum. Sham group, normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; CIA + MTX, methotrexate-treated rats (0.3 mg/kg) + CIA; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. This experiment was performed three times (n = 6). $^{*}p < 0.05$, compared with the Sham group; $^{\#}p < 0.05$, compared with the CIA control group; $^{\#\#}p < 0.01$, compared with the CIA control group.

3.4. Effects of curcumin on serum levels of TNF-α and IL-17 in CIA rats

Compared with the Sham group, the levels of TNF-α and IL-17 in the serum of the CIA control group were increased. Nevertheless, the levels of TNF-α and IL-17 in the serum of the rats treated with 200 mg/kg curcumin or MTX were obviously reduced compared to the CIA control group ($p < 0.05$) (Fig. 4).

3.5. Effect of curcumin on cell viability in RAW264.7 cells

The cytotoxicity of curcumin in RAW 264.7 cells was first detected using the CCK-8 assay. The cell viability was not impacted under the

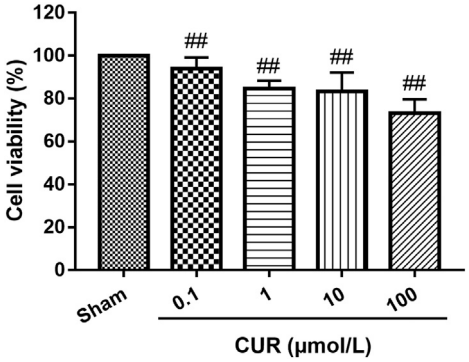

Fig. 5. The cytotoxicity of curcumin on RAW264.7 cells was detected by Cell-Counting Kit-8 assay. Curcumin was diluted with DMSO and mixed in medium with different concentrations (2, 10, 50, or 100 μmol/L) to treat cells. The cells without curcumin treatment served as the Sham group, which was set to 100% as the control group. This experiment was performed three times (n = 6). Sham, negative control treated without curcumin; CUR (0.1, 1, 10, 100 μmol/L), the cells treated with curcumin at different concentrations. $^{\#\#}p < 0.01$, compared with the Sham group.

concentration of 10 μmol/L treatment with curcumin (Fig. 5). However, curcumin had an increasing cytotoxicity with the increase in the curcumin concentration. Therefore, in our subsequent in vitro experiments, curcumin was used in RAW 264.7 cells at a concentration of 0 to 10 μmol/L.

3.6. Curcumin inhibits the degradation IκBα and the expression of COX-2 in the synovium of the joints in CIA rats and LPS-induced RAW264.7 cells

IκBα is an important mediator for the expression of various inducible inflammatory genes and negatively regulates the transcriptional activity of NF-κB. Immunofluorescence staining and Western blot analysis were employed to detect the level of IκBα. The results indicated that the degradation of IκBα in LPS-induced RAW264.7 macrophages and the synovium of the joints in CIA rats was significantly increased compared to the Sham group. Nevertheless, the pretreatment with curcumin could substantially inhibit the degradation of IκBα in LPS-induced RAW264.7 macrophages (Fig. 6A, D) and the synovium of the joints in the CIA rats (Fig. 7A).

COX-2 is a key enzyme that promotes inflammation in rheumatoid arthritis, and the expression of COX-2 is regulated by the NF-κB activity. For this reason, a further study was conducted to investigate whether curcumin can inhibit the expression of COX-2 via NF-κB. The results

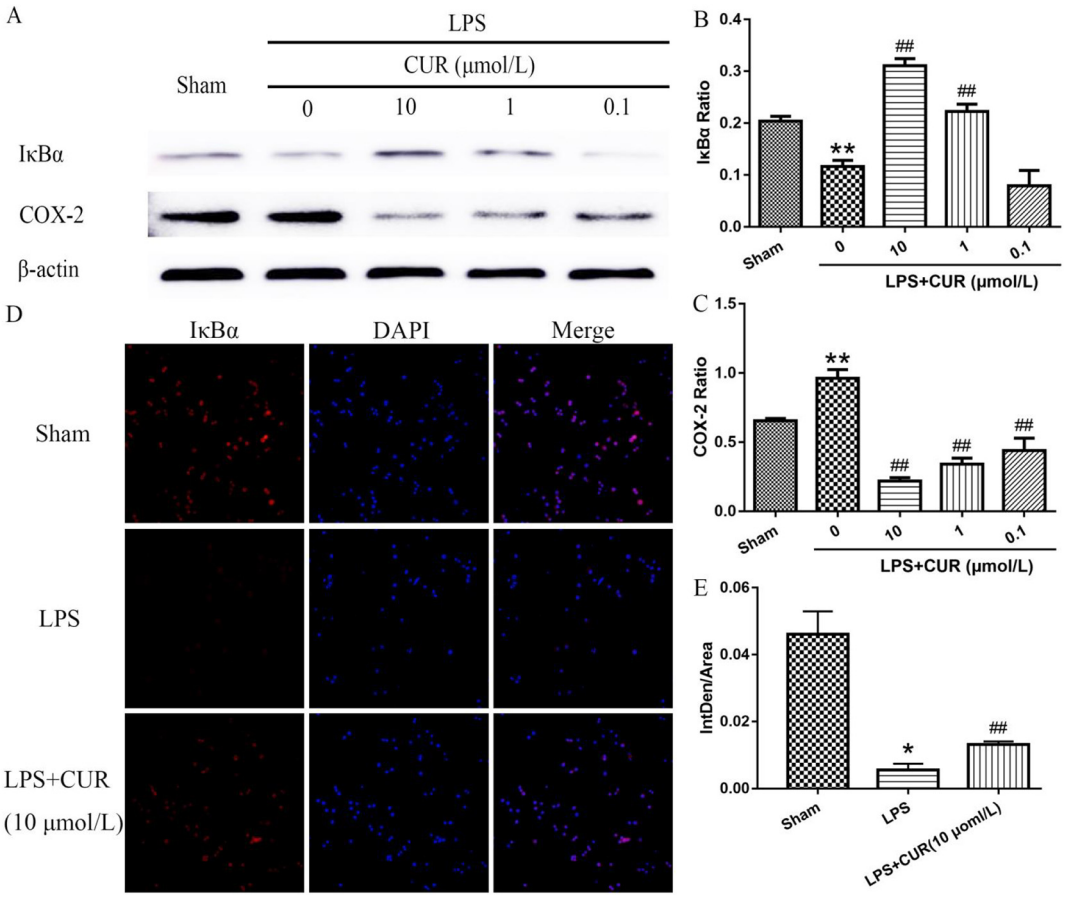

Fig. 6. Curcumin can inhibit the degradation IκBα and the expression of COX-2 in LPS-induced RAW264.7 cells. RAW264.7 cells (1×10^6 cells/well) were stimulated with LPS (10 μg/mL) for 12 h with or without pretreatment with curcumin for 30 min (IκBα) or 12 h (COX-2). (A) The extracts of the cells were measured by Western blot. Sham group, cells treated with DMSO; LPS + CUR(0 μmol/L), cells stimulated with LPS without pre-treatment with curcumin; LPS + CUR(10 μmol/L), cells stimulated with LPS with pre-treatment with curcumin(10 μmol/L); LPS + CUR(1 μmol/L), cells stimulated with LPS with pre-treatment with curcumin(1 μmol/L); LPS + CUR(0.1 μmol/L), cells stimulated with LPS with pre-treatment with curcumin(0.1 μmol/L); (B and C) The relative contents of the IκBα and COX-2 proteins were quantified through IMAGEJ. (D) RAW264.7 cells were incubated with 10 μmol/L curcumin for 30 min, followed by stimulation with LPS (10 μg/mL) for 12 h. The level of IκBα was detected by immunofluorescence staining. Sham group, negative control treated with DMSO; LPS, positive control stimulated with LPS only; LPS + CUR, experimental group treated with curcumin (10 μmol/L) and LPS. *Red*, IκBα; *Blue*, DAPI. (E) Immunofluorescence staining results were semi-quantitatively determined using IMAGEJ. $^{*}p < 0.05$, compared with the Sham group; $^{**}p < 0.01$, compared with the CIA control group; $^{\#\#}p < 0.01$, compared with the CIA control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

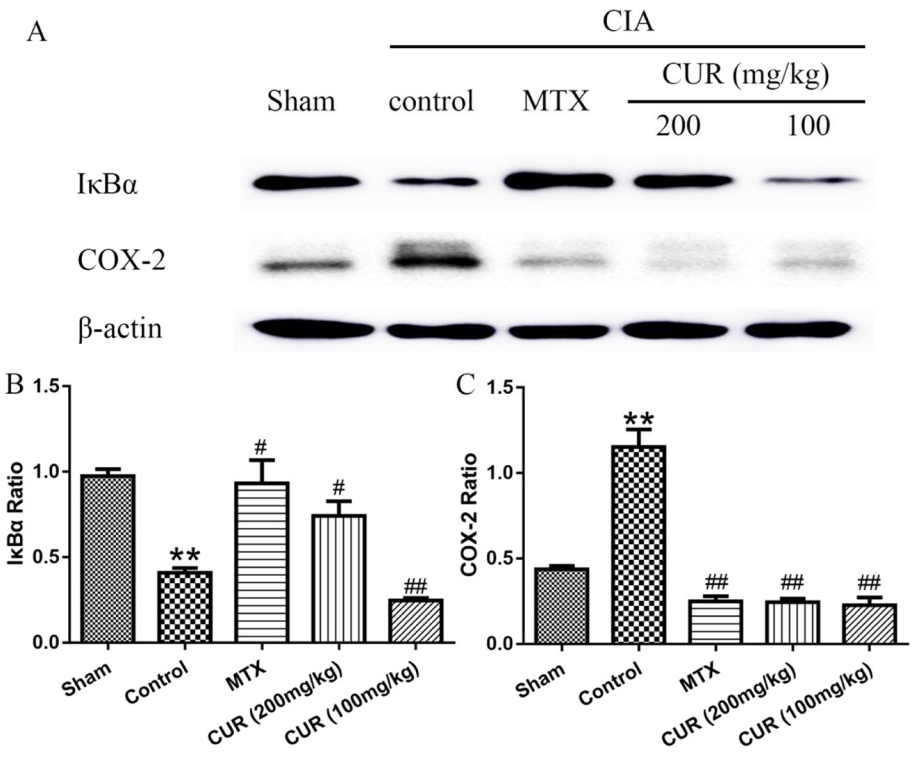

Fig. 7. Curcumin can inhibit the degradation IκBα and the expression of COX-2 in the synovium of the joints in CIA rats. The synovium of the joint was removed and lysed in a RIPA lysate buffer. (A) The supernatants of all synovium were measured by Western blot. (B and C) The relative contents of the IκBα and COX-2 proteins were quantified through IMAGEJ. Sham group, normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; MTX group, methotrexate-treated rats (0.3 mg/kg) + CIA; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. Every experiment was performed three times (n = 6). $^{**}p < 0.01$, compared with the Sham group; $^{\#}p < 0.05$, compared with the CIA control group; $^{\#\#}p < 0.01$, compared with the CIA control group.

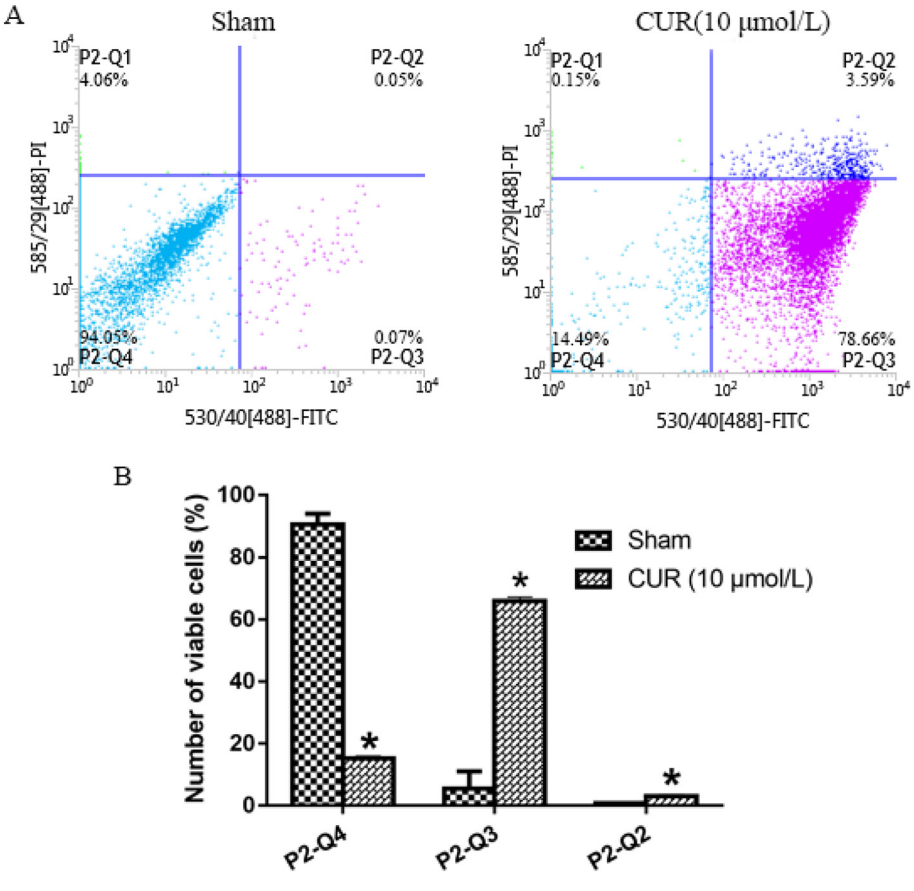

Fig. 8. The apoptosis of RAW264.7 cells was promoted by curcumin. (A) RAW264.7 cells (1.0×10^6 cells/well) were cultured in 24-well plates with DMSO in the presence or absence of curcumin (10 μmol/L) for 24 h and stained with Annexin V-FITC/PI. Apoptosis was analyzed using flow cytometry. The proportion of the cell number (%) is demonstrated in each quadrant. (B) The number of viable cells (%) was standardized against the Sham. Sham group, treated with DMSO only; CUR (10 μmol/L), treated with curcumin (10 μmol/L); P2-Q1, necrotic cells; P2-Q2, late apoptotic cells; P2-Q3, early apoptotic cells; P2-Q4, viable cells. This experiment was performed three times (n = 5). $^{*}p < 0.05$, compared with the Sham group.

showed that the expression of COX-2 was increased in the LPS-induced RAW264.7 cells and the synovium of the joints in the CIA rats compared to the Sham group. However, the pretreatment with curcumin could greatly reduce the expression of COX-2 in LPS-induced RAW264.7 macrophages (Fig. 6A) and the synovium of the joints in the CIA rats (Fig. 7A).

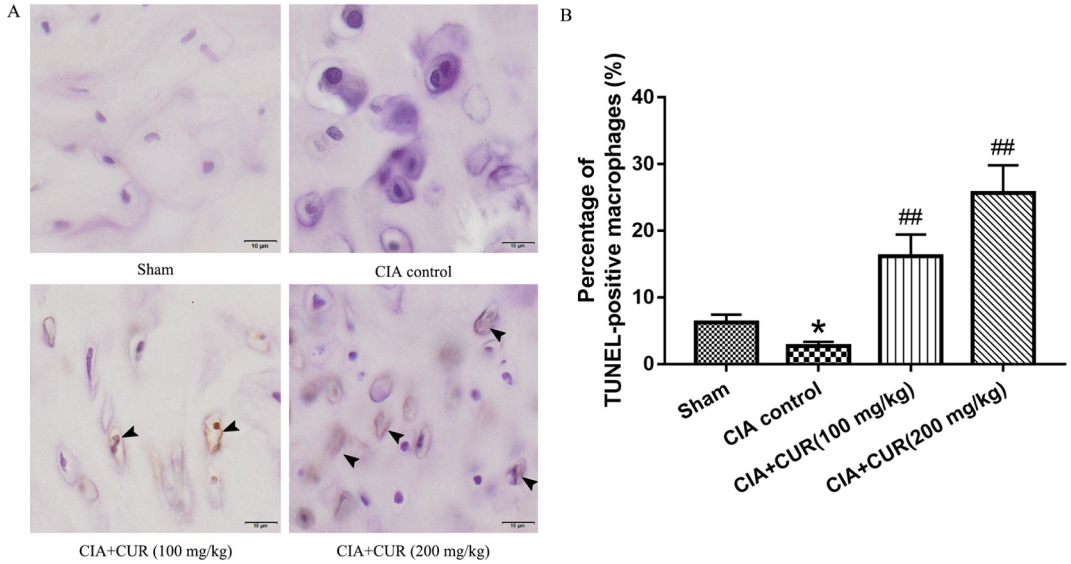

Fig. 9. Apoptotic macrophages of CIA rat ankle joints were labeled in situ using TUNEL staining. The paraffin sections of the ankle joints of each group of rats were dewaxed, stained with TUNEL reagent and then counterstained with hematoxylin. Apoptotic synovial macrophages appear dark brown (indicated by black arrow head), whereas normal cells appear blue (magnification, 1000×). (A) Sham group, normal rats with saline treatment; CIA control group, collagen-induced arthritis rat models; CUR + CIA (200 mg/kg), rats with curcumin treatment (200 mg/kg) + CIA; CUR + CIA (100 mg/kg), rats with curcumin treatment (100 mg/kg) + CIA. (B) The histogram shows the ratio of TUNEL positive/negative macrophages in each group. The experiment was performed three times, and six fields of view were selected for analysis in each group. $^{**}p < 0.01$, compared with the Sham group; $^{\#\#}p < 0.01$, compared with the CIA control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. The effect of curcumin on the apoptosis of RAW264.7 cells

The enhanced apoptosis of macrophages in the joint is therapeutically beneficial. To investigate the therapeutic effect and the mechanism of curcumin on joint inflammation in rheumatoid arthritis, annexin V–FITC and propidium iodide (PI) double staining was used to assess the effect of curcumin on RAW264.7 macrophage apoptosis. Compared with the Sham group (early apoptotic cells: 0.05%; late apoptotic cells: 0.07%) the percentages of early apoptotic cells (Annexin V-FITC positive/PI negative P2-Q2: 3.59%) and late apoptotic cells (Annexin V-FITC positive/PI negative P2-Q3: 78.66%) treated with curcumin were substantially increased ($p < 0.05$) (Fig. 8).

3.8. Curcumin enhances the apoptosis of synovial macrophages of rats

To investigate whether curcumin can ameliorate activated macrophage infiltration in CIA rats by causing the apoptosis of macrophages, TUNEL in situ staining was applied to detect the proportion of apoptotic macrophages. As shown in Fig. 9, few apoptotic cells (2.7 ± 0.7%) were able to be detected in the synovium or cartilage of the ankle joint of the CIA rats compared with the Sham group and numerous macrophages were infiltrated. In contrast, more TUNEL-positive macrophages were found in the cartilage edge of the rat joints that were treated with curcumin (16.2 ± 3.2% in 100 mg/kg group and 19.9 ± 4.2% in 200 mg/kg group) compared to the CIA control group ($p < 0.05$).

4. Discussion

Curcumin has been confirmed to have anti-inflammatory and antioxidant activities and is under development as a potential therapeutic drug for autoimmune disorder [4]. Previous studies have demonstrated that curcumin had substantial efficacy in improving the Disease Activity Score (DAS) in RA patients [21]. However, the precise mechanism of action of curcumin has not been fully elucidated. In our present study, it was demonstrated that curcumin exerts therapeutic effects on arthritis in CIA rats and has a strong pharmacological activity on reducing the inflammatory response in macrophages. According to Qiaoding Dai et al. [22], curcumin can improve CIA-induced inflammation and synovial hyperplasia by the mammalian target of rapamycin (mTOR) pathway. However, the mechanism of this study may be related to inhibiting the NF-κB signaling pathway and promoting macrophage apoptosis. This study provides further evidence on the development of natural botanical drugs for the treatment of RA by curcumin.

RA is an autoimmune disease characterized by chronic joint inflammation and destruction of the joints. The joint histopathological changes are linked to inflammatory cell activation, proliferation, proinflammatory cytokine release, continuous inflammatory cell infiltration in the synovium and activated macrophage (osteoclast precursors) erosion in cartilage. The previous in vitro and in vivo studies have confirmed that the NF-κB signaling pathway plays an important role in the pathogenesis of various rheumatic disorders, such as RA [23,24]. Edema, bone/cartilage destruction, synovial hyperplasia, and pannus formation are processes in which the activity of NF-κB is crucial [20,25,26]. IκBα, a nuclear IκB protein, inhibits the release and nuclear translocation of NF-κB and negatively regulates NF-κB-mediated expression of proinflammatory cytokines (e.g., TNF-α, IL-17, IL-1β and TGF-β), protease and inducible enzymes (e.g., COX-2) [27]. Therefore, IκBα may be a potential therapeutic target in RA. In this study, as shown in Figs. 6 and 7, curcumin inhibited the degradation of IκBα in the synovium of CIA rats in vivo and reduced the expression of COX-2. In addition, the treatment of CIA rats with curcumin reduced the severity (arthritis score) of CIA and the histopathological changes (e.g., edema, bone/cartilage destruction, synovial hyperplasia, and pannus formation), as well as inhibited proinflammatory cytokine release (TNF-α, IL-17, IL-1β and TGF-β). These inflammatory cytokines and COX-2 are involved in the pathogenesis of RA by inducing the penetration of immune cells and stimulating the release of many proteases, which are closely related to the pathogenesis of RA [2,28]. Therefore, the inhibition of the NF-κB activity by curcumin treatment can slow the disease course and pathological changes of RA.

LPS-stimulated RAW264.7 cells are classical inflammatory cell models used to explore the intracellular anti-inflammatory mechanisms of curcumin [29]. In this study, LPS, as a stimulus, can cause the

phosphorylation and degradation of IκB proteins and result in the release of NF-κB, which can translocate into the cell-nucleus to regulate the transcription of genes involved in the expression of various pro-inflammatory cytokines and inducible enzymes (e.g., COX-2). As shown in Fig. 6, IκBα in the cytoplasm of the curcumin-treated cells was less degraded compared with the LPS-stimulated RAW264.7 cell group, and the expression of COX-2 was also inhibited, further confirming curcumin reduces intracellular inflammatory activity by inhibiting NF-κB activation. This result is consistent with previous studies explored by other methods [30], but we also innovatively found that curcumin can promote apoptosis in RAW264.7 cells (Fig. 8).

Apoptosis plays a key role in the regulation of the amount of activated macrophages, which can cause synovial hyperplasia, pannus formation, inflammatory cell infiltration and bone/cartilage destruction [16,18,27]. Previous studies indicate that the insufficient apoptosis of inflammatory cells causes the pathogenesis of RA. In the synovial tissue of RA patients, there are abundant macrophages, and insufficient apoptosis causes the increased proliferation of synovial fibroblasts and chronic inflammatory cell infiltration in RA joints [16]. Therefore, targeting macrophage apoptosis may be a new approach for the therapy of RA. In previous work, curcumin has been reported to induce the apoptosis of various cancer cells by suppressing NF-κB activity [31]. However, in our study, curcumin induces macrophage apoptosis in both RAW264.7 cells and the cartilage edge of rats' joints. These findings are obtained at a safe curcumin concentration. The results thus indicate that curcumin ameliorates joint histopathological changes of RA by inducing the apoptosis of macrophages, and the mechanism of curcumin induced apoptosis might involve NF-κB activity.

In conclusion, curcumin exerts therapeutic effects on arthritis in CIA rats by reducing the inflammatory response in CIA rat joints and inducing the apoptosis of macrophages. Thus, the above two beneficial effects of curcumin together improve the symptoms of rheumatoid arthritis in rats. The mechanism of action of curcumin may be related to the inhibition of NF-κB activity. Therefore, this study provides further evidence on the development of curcumin for the treatment of RA.

Conflict of interest

The author declares that there are no conflicts of interest.

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