Tropical Journal of Pharmaceutical Research April 2021; 20 (4): 741-748
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i4.12

Original Research Article

Zhitong Jiangu decoction mitigates osteoarthritis in rabbits via regulation of NF-κB signaling pathway

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Sent for review: 26 November 2020 *Revised accepted: 20 March 2021*

Abstract

Purpose: *To investigate the effect of Zhitong Jiangu decoction (ZJD) on osteoarthritis rabbits, and the mechanism of action involved.*
Methods: *Chondrocytes were obtained from the knees of osteoarthritic rabbits. These chondrocytes were randomly assigned to 7 groups: sham, 5, 10 and 20 % normal serum groups; 5 % ZJD, 10 % ZJD and 20 % ZJD groups. The gross and histopathological features of the rabbit cartilage were examined by microscopy. Each group was treated with a different concentration of rabbit normal serum or rabbit drug-containing serum. The protective effect of different concentrations of ZJD on the cells were determined. Cell proliferation and concentrations of IL-1 and MMP-3 were determined using cell counting kit (CCK) 8 and enzyme-linked immunosorbent assay (ELISA), respectively. The mRNA and protein expressions of inhibitor of nuclear factor-κB kinase α (IKK-α) and nuclear factor kappa B p65 (NF- κB p65) were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunoblotting, as appropriate.*
Results: *Gross and histopathological examinations of rabbit cartilage showed that osteoarthritis was successfully established in rabbit knee joint. Cell proliferation significantly and time- and concentration-dependently increased in drug-containing serum groups, relative to sham and normal serum-containing groups. However, expressions of NF-κB p65, IL-1, MMP-3 and IKK-α were markedly and time- and concentration-dependently reduced in drug-containing serum groups, relative to sham and normal serum-containing groups ($p < 0.05$).*
Conclusion: *These results indicate that ZJD mitigates osteoarthritis in rabbits via regulation of NF-κB signaling pathway. Thus, it can potentially be developed for the management of osteoarthritis.*

Keywords: *Chondrocytes, MMP-3, NF- κB, Osteoarthritis, Zhitong Jiangu decoction*

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INTRODUCTION

Osteoarthritis, a chronic degenerative osteoarthrosis, is the main cause of disability in the elderly. It mostly affects the knee. Joint damage, inflammation, age, obesity, joint mechanics and heredity are risk factors for osteoarthritis [1]. The disease is characterized by degradation of articular cartilage, synovial inflammation, callus formation, as well as

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changes in articulating surfaces of bone and subchondral bone [2]. Therefore, a major strategy for the treatment of osteoarthritis is to stop or delay the degeneration of articular cartilage.

Zhitong Jiangu decoction (*ZJD*), a preparation used in Traditional Chinese Medicine (TCM), has been reported to be effective against osteoarthritis [3,4]. It inhibited synovial hyperplasia in a rabbit model of osteoarthritis [5]. *Zhitong Jiangu* decoction (*ZJD*) has been reported to increase the thickness of non-calcified cartilage, slow cartilage degeneration and improve joint function [5,6]. Moreover, it has been speculated that the preparation confers protection on chondrocytes via inhibition of inflammation and mRNA expressions of IKK-α, NF-κB, MMP-1 and tumor necrosis factor α (TNF-α) [7-9]. At present, the precise molecular mechanism underlying the effect of *ZJD* remains largely unknown. The present research was aimed at studying the influence of *ZJD* on osteoarthritic rabbits, and the mechanism involved.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was product of Gibco. Diaminobenzidine (DAB) and two-step kit were purchased from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd. Cell counting kit 8 (CCK8), Tris buffer and ELISA kits were bought from Sigma-Aldrich Ltd. Safranin O staining kit was obtained from Wellbio. TRIzol was product of Invitrogen. UltraSYBR mixture was bought from Beijing Kangwei Century Biotechnology Co. Ltd. White rabbit IKK-α and NF-κB-p65 mRNA primers were products of Shanghai Shenggong Bioengineering Technology Service Co. Ltd. Horse-radish peroxidase (HRP) goat anti-mouse IgG and HRP goat anti-rabbit IgG were purchased from Proteintech, while β-actin was bought from PeproTech. Microplate reader was product of Bio-Tek.

Experimental rabbits

Healthy New Zealand white male rabbits (n = 15) weighing 1.8 - 2.2 kg (mean weight = 2.0 ± 0.2 kg) were obtained from the Animal Laboratory of Hunan Academy of TCM. The rabbits were housed in metal cages under standard conditions and allowed *ad libitum* access to feed and water. They were exposed to 12-h light/12-h dark cycle, and maintained at an average temperature of 22 ± 1 ℃ and 40 – 50 % humidity. The rabbits were acclimatized to the laboratory conditions for 10 days prior to commencement of study. Study permission was received from the Animal Ethical Committee of The Affiliated Hospital of Hunan Academy of TCM, Changsha, China (approval no. 2018-0035). The study procedures were implemented in adherence to international guidelines [10].

Experimental design

Ten rabbits were numbered according to weight and randomly divided into two groups: ZJD-containing serum group (n=5) and blank control group (n =5). Based on the formula of equivalent dose conversion for different animals [11], the dose of ZJD for the rabbits was calculated as 4.48 g/kg body weight. The rabbits in the serum group were daily given intragastric administration of twice the equivalent dose, i.e., 8.96 g/kg administered as 10 mL/kg/day (the concentration of raw medicine containing ZJD was 0.896 g/mL). The blank control group was intragastrically administered an equivalent dose of normal saline (10 ml/kg/day), once a day for 7 days. One hour after intragastric administration on the 8th day, 10 % chloral hydrate was used for intraperitoneal anesthesia at a dose of 3.5 ml/kg. Sera were derived from abdominal aorta blood containing the drug ZJD, and from normal rabbits (normal sera). These were inactivated in a constant temperature water bath at 56℃ for 30 min, and kept refrigerated at -20℃ for later use. The serum samples were filtered and sterilized when needed.

Establishment of rabbit model of osteoarthritis

The remaining five rabbits were used to make rabbit model of osteoarthritis by injection of 4 % papain into the knee joint, once every 3 days, in a total of 3 injections [12].

Gross examination of rabbit knee joint

Two weeks after establishment of rabbit model of osteoarthritis, the rabbits were euthanized via induction of air embolism. The knee joint of each rabbit was excised and the cartilage of femoral condyle and tibial plateau were macroscopically examined.

Histopathological examination

Histological examination of distal femoral cartilage was performed using H & E staining, and safranin O cartilage staining. Histopathological changes were assessed based on the degree of inflammatory cell infiltration, damage, underlying bone destruction and

articular cartilage damage. The extent of cartilage damage was also determined using Mankin score [13].

Isolation and culture of chondrocytes

Chondrocytes were isolated via enzymatic digestion of bone tissue. The cartilage of each rabbit was excised under sterile conditions, cut into bits and crushed to a volume < 0.3 mm³. Then, 2 mL of 0.25 % trypsin was added for digestion, and the mixture was shaken vigorously on a shaking incubator at 37 ℃ for 20 min. Then, 2 mL of 0.02 % type Ⅱ collagenase was added to the digest in Ham's F-12 medium and maintained for 24 h at 37 °C in an atmosphere of 5 % CO_2 and 95 % oxygen. The cell suspension was sieved using a cell strainer to get rid of debris. The filtrate was subjected to centrifugation at 15,000 rpm for 7 min and the sediment was rinsed in DMEM. The cells were inoculated in a culture flask at a density of 4 × 10^4 cells/mL and maintained in DMEM containing 10 % FBS and 1 % streptomycin/penicillin at 37 ℃ in an incubator with 5 % CO_2 and 95 % oxygen. The culture medium was changed every 2 to 3 days, and cell growth was observed under a microscope every 24 h. Knee articular chondrocytes were identified with collagen immunofluorescence staining [14,15]. The third-generation chondrocytes were employed in this research.

Cell grouping

When the chondrocytes attained 80 % confluency, they were randomly assigned to 7 groups: sham, 5, 10 and 20 % normal serum groups; 5 % *ZJD*, 10 % *ZJD* and 20 % *ZJD* groups. Cells in sham group were treated with 0.5 % volume fraction of FBS, while those in 3 normal serum groups were treated with 5, 10 or 20 % volume fractions of normal rabbit serum. Moreover, 5, 10 or 20 % volume fraction of serum containing *ZJD* was added to the 3 *ZJD* groups, as appropriate.

Determination of proliferative potential

This was done using CCK-8 kit. The cells were inoculated in 96-well plates (2 × 10^4/mL), and maintained at 37 °C for 24 h in a medium of 5 % CO_2 and 95 % oxygen, followed by addition of CCK-8. The mixture was cultured for 4 h, after which OD was read at 450 nm. The assay was done in triplicate at 24 h intervals.

Determination of levels of IL-1 and MMP-3 in cell suspension

The cells were rinsed in PBS and lysed with ice-cold RIPA buffer containing protease blocker. The resultant lysate was spun at 15, 000 rpm for 15 min at 4 °C, and the supernatant was subjected to assay of IL-1 and MMP-3 using ELISA.

Quantitative RT-PCR

Total RNA extraction from cells of each group was done with TRIzol RNA extraction reagent. The RNA was reverse-transcribed to cDNA, followed by qRT-PCR. The relative mRNA expression levels of IKK-α and NF-κB p65 were calculated using $2^{-\Delta\Delta Ct}$ method, with GAPDH as internal standard. Table 1 shows the nucleotide sequences of primers employed.

Determination of protein expression levels

Protein expressions of IKK-α and NF-κB p65 in chondrocytes were measured using Western blotting.

Statistical analysis

Results are presented as mean ± SD. Groups were compared using Tukey, Welch and Games-Howell tests, where applicable. Statistical analysis was performed with SPSS version 24. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

Table 1: Sequences of primers used for PCR

Gene	Primer	Sequence	Length
GAPDH	Sense	5′- TGGAATCCACTGGCGTCTTCAC -3′	168bp
	Anti-sense	5′- AGGATGCGTTGCTGACAATCTTGA -3′	
IKKα	Sense	5′- ACAAAGAGCAGCAATGTTAAGCC -3′	149bp
	Anti-sense	5′- ACAAAGAGCAGCAATGTTAAGCC -3′	
NFKB-p65	Sense	5′- ATGCCAATGCCCTCTTCGACT -3′	164bp
	Anti-sense	5′- CGTGACTTCCAGCAGATCCCT -3′	

RESULTS

Results of gross examination of rabbit knee joint

The articular cartilage surface of normal rabbit appeared light pink. Cartilage defect, joint effusion and swelling of synovial membrane were absent. However, the cartilage surface of osteoarthritic rabbits was white, with articular cavity effusion and synovial swelling. The rough surface was characterized by reduced brightness and visible marginal cartilage defect (Figure1).

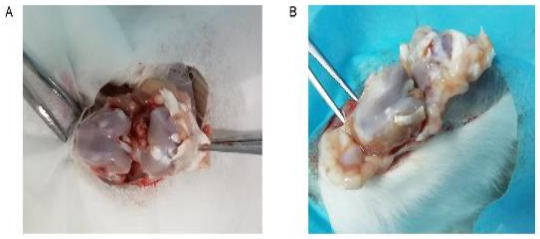

Figure 1: Gross morphology of rabbit knee joint. **(A):** Normal knee joint; and **(B):** knee joint of osteoarthritis rabbit

Histopathological features of cartilage of rabbit

Hematoxylin and eosin (H & E) staining of rabbit distal femoral cartilage revealed diffused increase in the number of chondrocytes as well as uneven distribution of chondrocytes in each layer (Figure 2 A and B). Similarly, Safranin O cartilage staining revealed a slight decrease in staining of the matrix and irregular cracks on the joint surface (Figure 2C and D). The modified Mankin's score was 3.

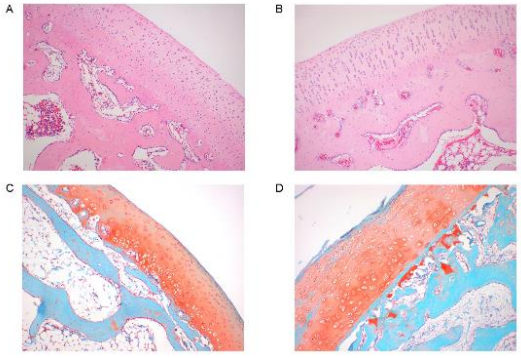

Figure 2: Histopathological features of cartilage of rabbits. **(A):** H & E staining of cartilage of normal rabbit; **(B):** H & E staining of cartilage of osteoarthritic rabbit; **(C):** Safranin O staining of cartilage of normal rabbit; and **(D):** Safranin O staining of cartilage of osteoarthritic rabbit

Morphological characteristics of isolated chondrocytes

Chondrocytes isolated from cartilage of osteoarthritis rabbit adhered gradually to the wall of the culture flask within 24 h of separation. Most of the primary chondrocytes remained spherically suspended in the culture medium with strong refractive properties (Figure 3 A). After 72 h of culture, primary chondrocytes were completely adherent to the wall, with most of their nuclei appearing round or oval. The cells were polygonal, triangular, or short spindle-shaped. There were protrusions between cells, and colony formation units were observed (Figure 3 B and C). After 9 days, monolayer cells were formed, which spread to cover the bottom of the culture flask. The nuclei were larger, and the cytoplasm was uniform and closely arranged. The entire monolayer cells formed a "paving stone"-like structure (Figure 3 D). After the first passage, the adherent time of chondrocytes was significantly reduced, but cell proliferation was markedly enhanced. Most of the cells were triangular and spindle shaped, with only a few appearing polygonal (Figure 3 E). The observed morphological changes were characteristic of chondrocytes.

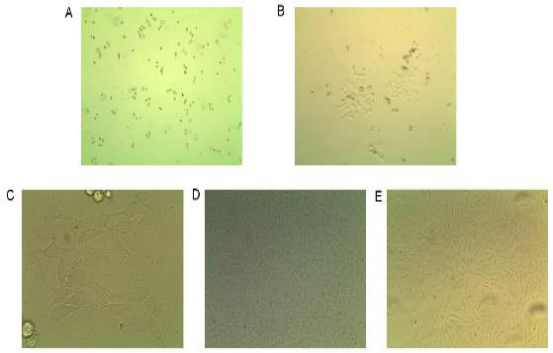

Figure 3: Morphological characteristics of isolated chondrocytes. **(A):** Appearance of primary cells after 24 h (× 100); **(B & C):** Appearance of primary cells after 72 h (b: × 100; c: × 400); **(D):** "Paving stone"-like structure of isolated chondrocytes (× 100); and **(E):** First generation of chondrocytes (× 100)

Type II collagen immunocytochemical staining

As shown in Figure 4 A and B, the nuclei of isolated chondrocytes appeared brown or brownish-yellow. The results of type II collagen immunofluorescence staining showed that type II collagen was mainly distributed in the cytoplasm and membrane of isolated chondrocytes (Figure 4 C – E).

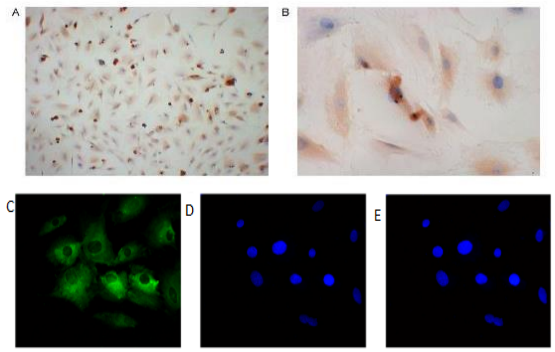

Figure 4: Immunohistochemical features of chondrocytes. **(A and B):** Type II collagen (a: ×100; b: ×400); **(C):** Type II collagen positive signal pattern; **(D):** staining of cell nucleus; and **(E)**: Type II collagen immunofluorescence staining (×400)

Proliferation of chondrocytes

The proliferation of chondrocytes was significantly lower in sham group than in normal serum-containing groups (p < 0.05). Cell proliferation was significantly and time- and concentration-dependently increased in drug-containing serum groups, relative to sham and normal serum containing groups (p < *0.05;* Figure 6 and Figure 7).

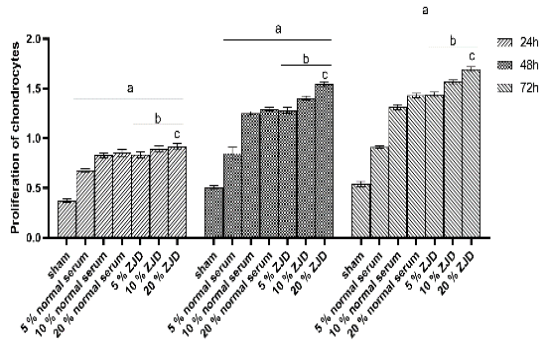

Figure 6: Proliferation of chondrocytes at 24, 48 and 72 h of culture. [a]P < 0.05, vs sham group; [b]p < 0.05, vs normal serum-containing groups; [c]p < 0.05, compared with 5 and 10 % *ZJD* groups

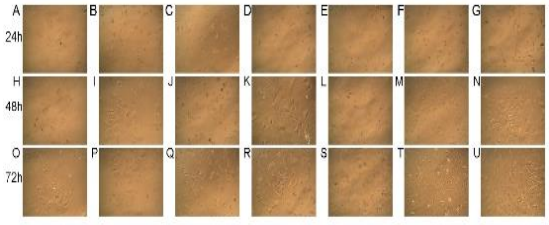

Figure 7: Microscopic observation of chondrocytes in each group after drug intervention (×100). **A, H and O:** Sham group; **B, I and P:** 5 % normal serum group; **C, J and Q:** 10 % normal serum group; **D, K and R:** 20 % normal serum group; **E, L and S:** 5 % *ZJD* drug serum group; **F, M and T:** 10 % *ZJD* drug serum group; **G, N and U:** 20 % *ZJD* drug serum group

Levels of expression of IL-1 and MMP-3

As presented in Figure 8 and Figure 9, the levels of expression of IL-1 and MMP-3 were markedly higher in sham group than in normal serum-containing groups (p < 0.05). Interleukin 1 (IL-1) and MMP-3 expression levels were significantly and time- and concentration-dependently reduced in drug-containing serum groups, relative to sham and normal serum-containing groups (p < 0.05).

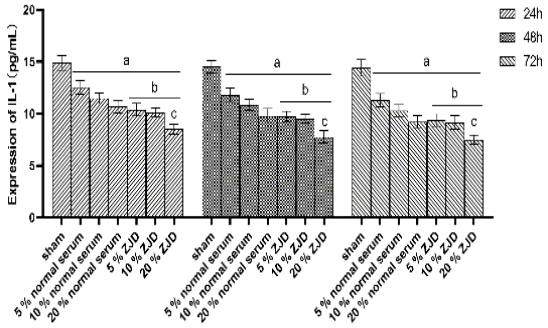

Figure 8: Levels of expression of IL-1 at 24, 48 and 72 h of culture. [a]P < 0.05, vs sham group; [b]p < 0.05, vs normal serum-containing groups; [c]p < 0.05, compared with 5 and 10 % *ZJD* groups

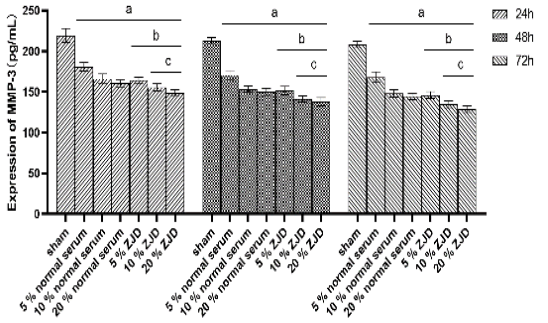

Figure 9: Expression of MMP-3 at 24, 48 and 72 h culture. [a]P < 0.05, vs sham group; [b]p < 0.05, vs normal serum-containing groups; [c]p < 0.05, compared with 5 and 10 % *ZJD* groups

mRNA and protein levels of NF-κB p65 and IKK-α

Markedly higher mRNA and protein expressions of NF-κB p65 and IKK-α were seen in sham group than in normal serum-containing groups (p < 0.05). In drug-containing serum groups, the mRNA and protein concentrations of these factors were significantly and time- and

concentration-dependently downregulated, relative to sham and normal serum-containing groups ($p < 0.05$; Figure 10 and Figure 11).

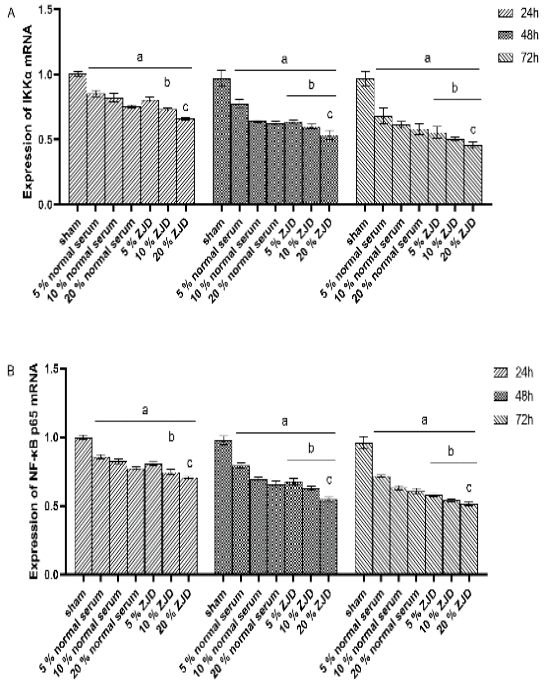

Figure 10: mRNA levels of NF-κB p65 and IKK-α. mRNA expressions of IKK-α (A) and NF-κB p65 (B). [a]$P < 0.05$, compared with sham group; [b]$p < 0.05$, vs normal serum-containing groups; [c]$p < 0.05$, compared with 5 and 10 % ZJD groups

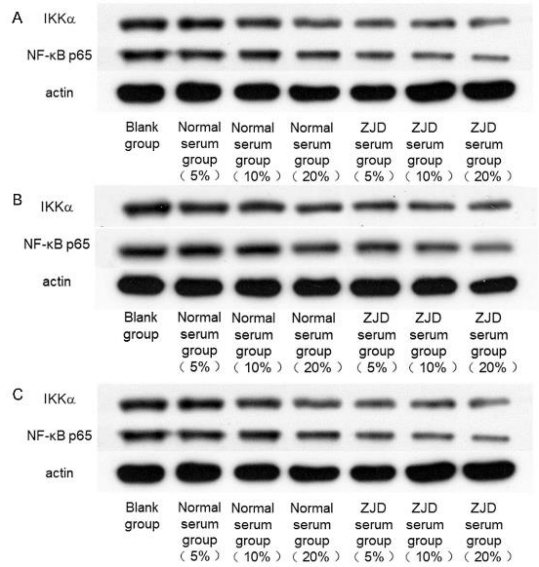

Figure 11: Effect of treatments on apoptosis. NF-κB p65 and IKK-α protein levels after 24 h (A); after 48 h (B), and after 72 h of culture (C)

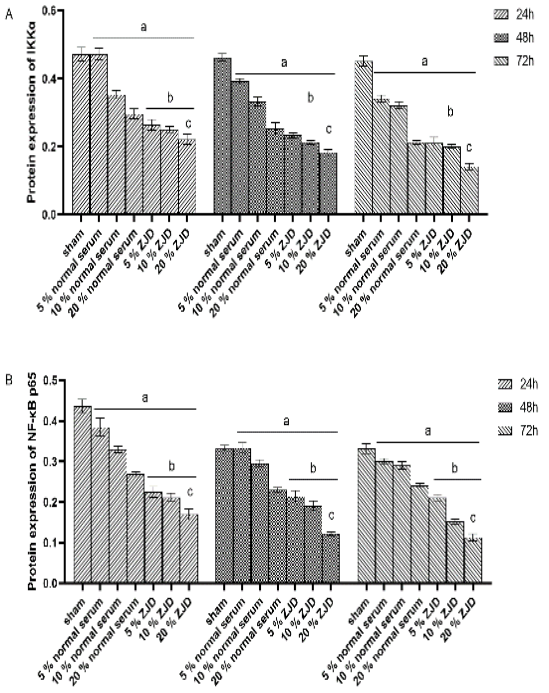

Figure 12: IKK-α and NF-κB p65 protein concentrations at 24, 48 and 72 h of culture. **(A):** IKK-α protein level; and **(B):** protein expression level of NF-κB-p65

DISCUSSION

Osteoarthritis, a degenerative disease of the bones and joints, occurs mainly in the knee joint, hip joint, spine and other parts of the body in middle-aged and the elderly people. It is mainly caused by pain and joint dysfunction. Pain of long duration induces local inflammation of joint soft tissue, thereby causing damage to cartilage [1]. Early osteoarthritis is characterized by painful joints, stiffness and hypertrophy. Several factors may be involved in etiology of knee osteoarthritis. The NF-κB signaling pathway which is implicated in aging and inflammation, is involved in the pathophysiological changes of osteoarthritis [16]. Activated NF-κB causes damage to the extracellular matrix, thereby setting in motion the pathological processes that culminate in osteoarthritis. The NF-κB signaling pathway regulates homeostasis of articular chondrocytes in various ways, and it has been reported to modulate apoptosis of chondrocytes via regulation of cell proliferation and apoptosis-related genes [17]. In a previous study, NF-κB promoted apoptosis of chondrocytes via upregulation of the expressions of MMP-3, IL-1, IL-6 and TNF-α [18]. Moreover, MMP-3 and IL-1 are downstream products of the NF-κB signal pathway, which expressions are important in the

etiology of osteoarthritis. The results of this study suggest that *ZJD* may protect chondrocytes via inhibition of NF-κB expression.

In Traditional Chinese Medicine (TCM), knee osteoarthritis is classified as *bi* syndrome, sinew *bi* and *li jie feng*. *Zhitong Jiangu* decoction (*ZJD*) is used in TCM to treat osteoarthritis [3,4]. It promotes blood circulation, relieves pain, removes invisible phlegm, *dredges meridians*, and strengthens the muscles and bones. The crude drug has been shown to inhibit synovial hyperplasia in rabbit model of knee osteoarthritis. A study reported that it increased the thickness of non-calcified cartilage, slowed cartilage degeneration, and improved joint function [4]. Downregulated expressions of MMP-1 and TNF-α have been demonstrated to inhibit inflammatory reactions in osteoarthritis rabbits [3,5-9].

In this study, after the successful establishment of knee osteoarthritis, different volume fractions of *ZJD*-containing serum were used for treatment. The results suggest that *ZJD* may confer protection on chondrocytes and promote their proliferation.

CONCLUSION

These findings suggest that *ZJD* mitigated osteoarthritis in rabbits via regulation of the NF-κB signaling pathway. The results provide supportive experimental evidence for the use of *ZJD* in the treatment of knee osteoarthritis in TCM.

DECLARATIONS

Acknowledgement

This work was funded by the National Natural Science Foundation of China (no. 81603482) and Hunan Scientific Research Program of TCM (no. 201908).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. Xiaodong Li, Xuyi Tan and Haien Luo conducted the literature search, performed the experiments and drafted the manuscript. Xinping Su and Kejian Zhu participated in the design and conduction of the experiment. Canyu He, Gang Huang and Daowei Zhang performed the statistical analysis and assisted in writing of the manuscript. All authors read and approved the final manuscript.

Data availability statement

The raw data generated in this study are available on request.

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