

## Original Research Article

# Icariin induces autophagy and apoptosis of chondrocytes by inhibiting NF- $\kappa$ B signaling pathway

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### Abstract

**Purpose:** To investigate the effect of icariin on autophagy and apoptosis of chondrocytes, and the associated mechanisms.

**Methods:** The chondrocytes were randomly divided into control (PBS intervention), TNF- $\alpha$  intervention, icariin +TNF- $\alpha$ , and NF- $\kappa$ B inhibition +TNF- $\alpha$ , with 8 strains in each group. The levels of IL-1, IL-6 and IL-12 were assayed by ELISA. The mRNA and protein expressions of ATG5, ATG7, Bax and Bcl-2 cells were determined by polymerase chain reaction (PCR) and Western blotting, while protein expressions of p-p65 and I $\kappa$ B $\alpha$  were assayed using Western blotting.

**Results:** In the cartilage tissue of rats in the icariin +TNF- $\alpha$  group and NF- $\kappa$ B inhibition +TNF- $\alpha$  group, IL-1, IL-6 and IL-12 levels were significantly lower than those in TNF- $\alpha$  treatment group ( $p < 0.05$ ). The AATG5 mRNA and protein in cartilage tissues of rats in icariin +TNF- $\alpha$  and NF- $\kappa$ B inhibition +TNF- $\alpha$  groups were significantly higher than those in TNF- $\alpha$  group. Bax mRNA and protein in cartilage tissues of icariin +TNF- $\alpha$  and NF- $\kappa$ B inhibition +TNF- $\alpha$  groups were downregulated, relative to TNF- $\alpha$  group; on the other hand, Bcl-2 mRNA and protein were significantly higher than those of TNF- $\alpha$  group ( $p < 0.05$ ). In the cartilage tissues of Icarin +TNF- $\alpha$ , NF- $\kappa$ B inhibition +TNF- $\alpha$  groups, P-p65 protein was significantly lower than that of TNF- $\alpha$  ( $p < 0.05$ ).

**Conclusion:** TNF- $\alpha$  enhances the production of a large number of inflammatory factors by cartilage cells, inhibits autophagy of cartilage cells, and promotes cell apoptosis through regulation of NF- $\kappa$ B signaling pathway.

**Keywords:** Icariin, NF- $\kappa$ B signaling pathway, TNF- $\alpha$ , Inflammatory response, Chondrocytes, Autophagy, Apoptosis

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## INTRODUCTION

Osteoarthritis is a chronic degenerative disease which involves the cartilage, subchondral bone and synovium. The main clinical manifestations of osteoarthritis are joint swelling and pain, as well as joint deformity, stiffness and dysfunction.

The disease is more common in the elderly, with higher incidence and higher disability in women than in men, and it seriously affects the physical and mental health of patients [1].

Currently, clinical treatment of osteoarthritis focuses on relieving pain, delaying progression

of the disease, restoring joint function through reconstruction, and improving patients' quality of life. Previous studies have suggested that the pathogenesis of osteoarthritis is associated with joint destruction caused by simple cartilage degeneration. However, in-depth studies have revealed that inflammatory response plays a very important role in the pathogenesis of osteoarthritis [2]. In particular, TNF- $\alpha$  is one of the important inflammatory factors that cause cartilage destruction in osteoarthritis. Studies have shown that TNF- $\alpha$  is distributed in different levels in articular cartilage, subchondral bone and synovium. It promotes the release of fibroblast adhesion molecules and binds to activated vascular endothelial cell adhesion molecules, so that white blood cells in the blood are concentrated in the articular cavity [3]. Chondrocytes are one of the important sources of inflammatory reactions, and they are involved in the synthesis and transformation of extracellular matrix during autophagy and apoptosis [4].

*Herba epimedii* is a traditional Chinese medicinal plant from which is extracted herba epimedin, a bioactive compound with a hormone-like effect which enhances the proliferation and osteogenic differentiation of bone marrow stem cells [5]. The purpose of this study was to determine the effect of icariin on TNF- $\alpha$ -mediated inflammatory response, as well as its effect on autophagy and apoptosis of chondrocytes in a rat model of osteoarthritis.

## EXPERIMENTAL

### Reagents and equipment

Icariin and chloral hydrate were purchased from Sigma. Trypsin and PBD buffer were bought from Hyclone Company, USA. Fetal bovine serum was product of Gibco, USA, while PVDF membrane was obtained from MILLIPORE Inc., USA. Trizol was purchased from Invitrogen, USA. Assay kits for IL-1, IL-6 and IL-12 ELISA were purchased from Wuhan Bainlai Biotechnology Co. Ltd. Rabbit anti-rat primary antibodies for ATG5, ATG7, Bax, Bcl-2, p-p65 and I $\kappa$ B $\alpha$  were purchased from Santa Cruz.

This research was approved by the Animal Ethical Committee of Wuhan Central Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430014, PR China, according to Principles of Laboratory Animal Care, (approval number was 20198631) [6].

Super clean workbench was bought from Suzhou Jingtai Co. Ltd, while CO<sub>2</sub> thermostatic incubator was purchased from Shanghai lishen Scientific Instrument Co. Ltd. Centrifuge was product of Hunan Xiangyi Laboratory Instrument Co. Ltd. Inverted microscope was purchased from Olympus in Japan. Swim meter was bought from Bio-Rad Company, US. Automatic Biochemical analyzer was purchased from Shanghai Technology Co. Ltd. Flow cytometer was product of Beckman. Fluorescence quantitative PCR instrument was purchased from Real-Time System.

### Animals and culture of chondrocytes

Twenty-five healthy SPF rats weighing 180 – 220 g, were obtained from the Animal Experiment Center of Tongji Hospital of Huazhong University of Science and Technology. Under chloral hydrate anesthesia, cartilage tissues were taken from the knee joints of rats, and digested with 0.25 % trypsin. Following centrifugation, the chondrocytes were placed in DMEM/F12 medium containing 10 % fetal bovine serum, and inoculated in a culture bottle at a concentration of  $1 \times 10^6$ /mL, followed by culturing in an incubator at 37 °C and 5 % CO<sub>2</sub>. Chondrocytes at logarithmic growth were divided into control group (PBS intervention), TNF- $\alpha$  intervention group, icariin +TNF- $\alpha$  group, and NF- $\kappa$ B inhibition +TNF- $\alpha$  group, with 8 strains in each group.

### Various assay methods used

Enzyme linked immunosorbent assay (ELISA) was used to determine the levels of IL-1, IL-6 and IL-12 in rat cartilage cells. The expression of ATG5 and ATG7 mRNA in rat cartilage tissue was determined using PCR. Total RNA of rat cartilage tissue in each group was extracted with Trizol method. Then, 2  $\mu$ L RNA solution was subjected to 1 % agarose gel electrophoresis in order to determine the purity and integrity of the RNA. Subsequently, reverse transcription was carried out to convert each RNA to cDNA.

Reverse transcription and PCR was used at 42 °C for 50 min, and heated at 95 °C for 5 min in order to inactivate the reverse transcriptase. After the reaction, the cDNA products were kept in the refrigerator at -20 °C prior to use. The Ct value of a target gene was obtained, and the relative quantitative analysis of the gene data was conducted using the  $2^{-\Delta\Delta Ct}$  formula, with GAPDH as the internal reference gene.

The protein expressions of ATG5 and ATG7 in rat cartilage were assayed with Western blotting. Chondrocytes at logarithmic growth in each

group were collected, and total protein was extracted using protein quantification kit. Equal amounts of protein were separated on SDS-polyacrylamide gel electrophoresis, and then transferred to PVDF membrane. The membrane was blocked by incubation with 5 % non-fat milk at room temperature for 2 h, followed by incubation of the membrane with primary antibodies for ATG5 (1: 5000 dilution) and ATG7 (1: 10000 dilution) overnight in a refrigerator at 4 °C. Then, the PVDF membrane bound to primary antibodies was washed with TTBS, and incubated with horse radish peroxidase-conjugated secondary antibody at room temperature for 1 - 2 h. The bands were analyzed using enhanced chemiluminescence.

The mRNA expressions of Bax and Bcl-2 in rat cartilage were determined using PCR, while the protein expressions of Bax, Bcl-2, p-p65, IκBα were determined with Western blotting, using the same procedure described above.

### Statistical analysis

Measurement data are expressed as mean ± standard deviation (SD). Statistical analysis was done with SPSS20.0 software package. Independent sample *t*-test was used to compare means between two groups, while analysis of variance (ANOVA) was used for multiple group comparisons. Values of  $p < 0.05$  were regarded as indicative of statistically significant differences.

## RESULTS

### Inflammatory factors in cartilage tissue of rats

The levels of IL-1, IL-6 and IL-12 in the cartilage tissue of rats in the TNF- $\alpha$  treatment group were significantly higher than those in the control group ( $p < 0.05$ ), while IL-1, IL-6 and IL-12 levels in cartilage tissue of rats in the icariin +TNF- $\alpha$  group and NF- $\kappa$ B inhibition +TNF- $\alpha$  group were significantly lower than those in the TNF- $\alpha$  treatment group ( $p < 0.05$ ). These results are shown in Table 1.

### Effect of icariin on TNF- $\alpha$ -mediated chondrocyte autophagy

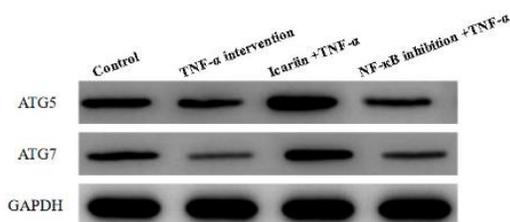
The mRNA and protein expressions of ATG5 and ATG7 in the cartilage tissue of rats in the TNF- $\alpha$  intervention group were significantly lower than the corresponding levels in the control group ( $p < 0.05$ ), but they were significantly higher in the cartilage tissue of rats in the icariin +TNF- $\alpha$  group and NF- $\kappa$ B inhibition +TNF- $\alpha$  group than in

the TNF- $\alpha$  group ( $p < 0.05$ ). These results are shown in Figure 1 and Table 2.

**Table 1:** Levels of inflammatory factors in cartilage tissue of rats in each group (mean ± SD, n = 8)

Group	IL-1 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)
Control	145.26 ± 10.33	65.24 ± 5.33	50.10 ± 5.98
TNF- $\alpha$	482.16 ± 17.65*	210.74 ± 15.20*	154.26 ± 12.36*
I + T	253.44 ± 12.67#	126.02 ± 10.78#	103.68 ± 10.20#
NF + T	302.16 ± 13.58#	148.56 ± 11.22#	120.74 ± 11.55#

\* $P < 0.05$ , compared with the control group; # $p < 0.05$ , compared with TNF- $\alpha$  group; I + T, Icarin +TNF- $\alpha$ ; NF + T, NF- $\kappa$ B inhibition +TNF- $\alpha$



**Figure 1:** Effect of icariin on protein expressions of ATG5 and ATG7 in TNF- $\alpha$ -treated chondrocytes

**Table 2:** Effect of icariin on protein expressions of ATG5 and ATG7 in TNF- $\alpha$ -treated chondrocytes (mean ± SD, n = 8)

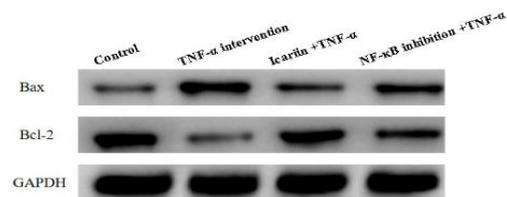
Group	ATG5		ATG7	
	mRNA	Protein	mRNA	Protein
Control	1.25 ± 0.13	0.42 ± 0.03	1.05 ± 0.06	0.32 ± 0.05
TNF- $\alpha$	0.67 ± 0.08*	0.30 ± 0.04*	0.85 ± 0.07*	0.20 ± 0.04*
I + T	5.72 ± 1.78#	0.68 ± 0.08#	3.88 ± 1.06#	0.58 ± 0.06#
NF + T	3.16 ± 1.02#	0.40 ± 0.06#	2.08 ± 0.89#	0.38 ± 0.07#

\* $P < 0.05$ , compared with the control group; # $p < 0.05$ , compared with TNF- $\alpha$  group. I + T, Icarin +TNF- $\alpha$ ; NF + T, NF- $\kappa$ B inhibition +TNF- $\alpha$

### Effect of icariin on TNF- $\alpha$ -mediated chondrocyte apoptosis

As shown in Figure 2 and Table 3, the mRNA and protein expressions of Bax in the cartilage tissues of rats in the TNF- $\alpha$  intervention group were significantly higher than those in the control group, but the mRNA and protein expressions of Bcl-2 were significantly lower than those in the control group ( $p < 0.05$ ). In contrast, the mRNA and protein expressions of Bax in the cartilage tissues of the icariin +TNF- $\alpha$  group and NF- $\kappa$ B

inhibition +TNF- $\alpha$  group were significantly lower than those in the TNF- $\alpha$  intervention group, but the mRNA and protein expressions of Bcl-2 were markedly higher than those in the TNF- $\alpha$  intervention group ( $p < 0.05$ ).



**Figure 2:** Effect of icariin on TNF- $\alpha$ -mediated changes in chondrocyte Bax and Bcl-2 proteins

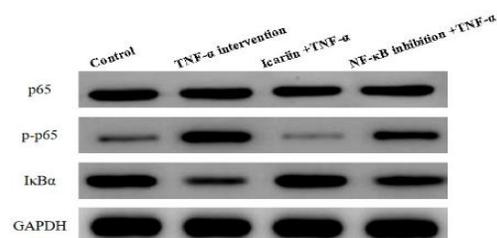
**Table 3:** Effect of icariin on Bax and Bcl-2 proteins in TNF- $\alpha$ -treated chondrocytes (mean  $\pm$  SD, n = 8)

Group	Bax		Bcl-2	
	mRNA	Protein	mRNA	Protein
Control	1.00 $\pm$ 0.01	0.39 $\pm$ 0.02	1.00 $\pm$ 0.01	0.60 $\pm$ 0.09
TNF- $\alpha$	3.89 $\pm$ 1.02*	0.71 $\pm$ 0.06*	0.20 $\pm$ 0.03*	0.32 $\pm$ 0.05*
I + T	1.45 $\pm$ 0.08#	0.45 $\pm$ 0.05#	0.71 $\pm$ 0.08#	0.50 $\pm$ 0.07#
NF + T	2.66 $\pm$ 1.03#	0.50 $\pm$ 0.07#	0.48 $\pm$ 0.07#	0.47 $\pm$ 0.06#

\* $p < 0.05$ , compared with the control group; # $p < 0.05$ , compared with TNF- $\alpha$  group. I + T, Icarin + TNF- $\alpha$ ; NF + T, NF- $\kappa$ B inhibition + TNF- $\alpha$

### Effect of icariin on NF- $\kappa$ B signaling pathway

The p-p65 protein level in the cartilage tissue of rats in the TNF- $\alpha$  intervention group was significantly higher than that in the control group, and the expression of NF- $\kappa$ B was significantly lower than that in the control group ( $P < 0.05$ ). However, the p-p65 protein expressions levels in the cartilage tissue of the icarin + TNF- $\alpha$  group and NF- $\kappa$ B inhibition + TNF- $\alpha$  group were significantly lower than those in the TNF- $\alpha$  intervention group, and I $\kappa$ B $\alpha$  was significantly lower than that in the TNF- $\alpha$  intervention group ( $p < 0.05$ ). These results are shown in Figure 3 and Table 4.



**Figure 3:** Effect of icariin on NF- $\kappa$ B signaling-related proteins

**Table 4:** Effect of icariin on NF- $\kappa$ B signaling-related proteins (mean  $\pm$  SD, n = 8)

Group	p65/p-p65	I $\kappa$ B $\alpha$
Control	0.39 $\pm$ 0.05	0.68 $\pm$ 0.10
TNF- $\alpha$	0.82 $\pm$ 0.15*	0.37 $\pm$ 0.08*
Icarin + TNF- $\alpha$	0.42 $\pm$ 0.09#	0.70 $\pm$ 0.12#
NF- $\kappa$ B inhibition + TNF- $\alpha$	0.56 $\pm$ 0.08#	0.57 $\pm$ 0.07#

\* $P < 0.05$ , compared with the control group; # $p < 0.05$ , compared with TNF- $\alpha$  group

## DISCUSSION

It is believed in Western medicine that osteoarthritis is a chronic inflammatory disease involving multiple mechanisms associated with cytokines and inflammatory mediators, leading to imbalance between normal degradation and synthesis of chondrocytes, subchondral bone and extracellular matrix [7]. In traditional Chinese medicine, osteoarthritis belongs to the category of "bone numbness and knee pain", and its pathogenesis is due to the *response of the positive and deficient constitution to cold and dampness, phlegm and blood stasis*. The symptoms of osteoarthritis are joint pain accompanied by the pathological characteristics of *qi stagnation and blood stasis, deficiency of liver and kidney, and blocking of dampness and heat, resulting in incompatibility of qi and blood, and obstruction of meridians and collaterals* [8].

Inflammatory cytokines are involved in the pathogenesis of cartilage degeneration and osteoarthritis. Cytokines such as IL-1 and TNF- $\alpha$  are important mediators of osteoarthritis. The cytokine TNF- $\alpha$  is secreted by macrophages, fibroblasts and chondrocytes. It increases the release of growth factors in synovial membrane, endothelial cells and fibroblasts, and enhances the formation of osteoarthritis pannus [9,10]. In addition, TNF- $\alpha$  stimulates chondrocytes to secrete fibrin lysosomal activator, and accelerates arthritis injury. It stimulates connective tissue cells and polymorphonuclear cells to produce small inflammatory mediators such as prostaglandins. At the same time, TNF- $\alpha$  upregulates the expressions of IL-1, IL-6 and IL-12, resulting in an inflammatory reaction cascade.

The NF- $\kappa$ B family is involved in a wide range of biological activities including cell differentiation, proliferation, autophagy and apoptosis, and plays an important role in most immune responses and inflammatory responses [11]. Several studies

have now demonstrated the effect of NF- $\kappa$ B on chondrocytes. It is known that NF- $\kappa$ B and MAPK are ERK1/2 involved in the expression of type II collagen, leading to chondrocyte destruction and apoptosis [12,13]. Other scholars have found that TNF- $\alpha$  promotes the transformation of chondrocytes into mast cells by regulating NF- $\kappa$ B signaling pathway, and also damage the structure of articular cartilage [14].

Icariin is a natural substance extracted from *Herba epimedii*. It exerts a variety of pharmacological effects. Increasing attention has been paid to its therapeutic effects and associated mechanisms in the endocrine, nervous and cardiovascular systems. Modern studies of traditional Chinese medicine have found that icariin exerts powerful pharmacological effects which enhance the phagocytosis by macrophages and enhance the body's specific immunity [15-18]. Moreover, the pharmacological effect of icariin on the skeletal system is effective in the treatment of osteoporosis.

Recent studies have reported that icariin enhanced chondrocyte proliferation and chondrogenic differentiation of bone marrow mesenchymal stem cells, and reduced the destruction of articular cartilage [19]. The results of this study showed that the levels of IL-1, IL-6 and IL-12 in the cartilage tissues of rats in the TNF- $\alpha$  intervention group were significantly higher than those in the control group, and that intervention with icariin significantly inhibited the pro-inflammatory response of TNF- $\alpha$  and the overexpression of related factors, suggesting that icariin may inhibit chondromalacia by inhibiting inflammatory response of cells.

Further analysis showed that icariin significantly reduced the TNF- $\alpha$ -induced autophagy inhibition and the apoptosis induced by inflammatory factors. Therefore, it is not unreasonable to suggest that icariin may eliminate TNF- $\alpha$ -induced autophagy inhibition and reduce apoptosis. In order to unravel its mechanism of action, it was demonstrated that p-p65 protein levels in the cartilage tissues of icariin + TNF- $\alpha$  and NF- $\kappa$ B inhibition +TNF- $\alpha$  groups were significantly lower than that in the TNF- $\alpha$  intervention group.

## CONCLUSION

This study has demonstrated that TNF- $\alpha$  promoted the production of a large number of inflammatory factors by chondrocytes through regulation of the NF- $\kappa$ B signaling pathway, inhibition of chondrocyte autophagy and enhancement of apoptosis. However, icariin

inhibited the NF- $\kappa$ B signaling pathway, reduced the formation of TNF- $\alpha$  and inflammatory metabolites, activated autophagy, and inhibited apoptosis, thereby protecting the chondrocytes.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Yi Yu conceived and designed the study, Jun Xiong, Hui Zou, Yi Yu collected and analysed the data, while Jun Xiong wrote the manuscript. Jun Xiong and Hui Zou contributed equally to this work as co-first author.

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