

## Original Research Article

# Zhihuang Tongfeng decoction ameliorates gouty arthritis via inhibition of NLRP3 inflammasome in rats

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### Abstract

**Purpose:** To investigate the effect and mechanism of action of traditional Chinese medicine, Zhihuang Tongfeng decoction (ZTD), on gouty arthritis in rats.

**Methods:** Monosodium urate (MSU) crystal was injected into the ankle joint of rats to establish an animal model of gouty arthritis. ZTD (4.8, 9.6 and 19.2 g/kg) was administered to the rats. The walking behavior of the rats was observed daily and the gait score computed. Histopathologic severity was scored using three parameters, viz, synovial inflammation, synovial hyperplasia and cartilage surface erosion. The levels of IL-1 $\beta$  and TNF- $\alpha$  in lavage fluid of articular cavities were measured by ELISA. The synovial tissues of the joint of all the rats were obtained and NLRP3 inflammasome analysed by Western blot.

**Results:** The results revealed that compared with control rats, 19.2 g/kg dose of ZTD lowered the owestry disability index of the rats significantly from  $2.3 \pm 0.4$  to  $0.9 \pm 0.3$  ( $p < 0.05$ ). Pathomorphology analysis showed that ZTD attenuated the swelling of toes and the infiltration of inflammatory cells in synovium significantly ( $p < 0.05$ ). Further, ZTD decreased the IL-1 $\beta$  ( $p < 0.05$ ) and TNF- $\alpha$  levels ( $p < 0.05$ ) at all doses, and inhibited NLRP3 ( $p < 0.01$ ), caspase-1 ( $p < 0.01$ ), ASC ( $p < 0.01$ ), IL-1 $\beta$  ( $p < 0.01$ ) and IL-18 ( $p < 0.01$ ) protein expressions in the lavage fluid of articular cavities in MSU crystal-treated rats.

**Conclusion:** The results indicate that ZTD ameliorates gouty arthritis in rats by inhibiting NLRP3 inflammasome, and thus can potentially be developed into a new drug for the treatment of gouty arthritis.

**Keywords:** Zhihuang Tongfeng decoction, Gouty arthritis, NLRP3 inflammasome

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## INTRODUCTION

As one of the most common forms of inflammatory arthritis, gouty arthritis is caused by monosodium urate (MSU) crystal deposition in and around joints [1]. Acute symptoms of gout

patients include redness, swelling, heat and pain [2]. This process is driven by neutrophil influx into the joint and then leads to the attack of acute inflammatory arthritis with severe pain in the affected tissue. Studies have shown that NOD-like receptors containing a PYD3 (NLRP3)

inflammasomes play a critical role in MSU-induced IL-1 $\beta$  secretion in macrophages [3]. There are four kinds of inflammasomes, namely NLRP1, NLRP3, NLRC4, and AIM2. The NLRP3 inflammasomes play an important role in macrophages by cleaving pro-IL-1 $\beta$  into mature IL-1 $\beta$  [4,5]. Monosodium urate can activate the NLRP3 inflammasome and synergistically interact with apoptosis-associated speck-like protein (ASC) to drive caspase-1 release, subsequently leading to maturation and secretion of pro-inflammatory cytokines IL-1 $\beta$  and activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). This process is involved in inflammation response in the gouty arthritis [6]. It has been found that MSU-induced inflammation and pain responses are significantly reduced in NLRP3-deficient mice [7]. However, current gout pain management is far from satisfactory [8]. Hence, safer and more potent drugs are urgently needed for the treatment of gouty arthritis pain.

Nowadays, a number of herbal drugs and their active ingredients have increased interest in the protection against gouty arthritis [9-12]. Traditional Chinese medicine *Zhihuang Tongfeng* Decoction was composed of *Phellodendron chinense* Schneid., *Anemarrhena asphodeloides* Bunge, *Plantago asiatica* L., *Salvia miltiorrhiza* Bge., *Corydalis turtschaninovii* Bess., *Lithospermum erythrorhizon* Sieb. et Zucc., *Asarum sieboldii* Miq., *Arisaema erubescens*(Wall.) Schott., *Kaempferia rotunda* L., *Stephania tetrandra* S.Moore, *Paeonia lactiflora* Pall. and *Dioscorea septemloba* Thunbt. It has been used for the treatment of gouty arthritis for many years, and has achieved obvious curative effect in China. Here, this study aims to investigate the effect of ZTD on gouty arthritis in rats treated with MSU crystal.

## EXPERIMENTAL

### Reagents and drugs

*Zhihuang Tongfeng* Decoction was prepared according to the standard preparation method of traditional Chinese medicine decoction. Enzyme-linked immunosorbent assay (ELISA) kits of IL-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were obtained from Shenzhen Xin Bo Sheng Biotechnology Co. Ltd (Shenzhen, China). The antibodies of NLRP3 (commodity No. bs-6655R), caspase-1 P20 (commodity No. bs-10442R), ASC (commodity No. bs-6741R), IL-1 $\beta$  (commodity No. bs-0812R) and IL-18 (commodity No. bs-0529R) for rat were purchased from Beijing Bioss Biotechnology Co., Ltd. (China). The antibodies of  $\beta$ -actin were from Wuhan Servicebio Co., Ltd. All other reagents used were standard laboratory

reagents of analytical grade and were purchased locally. Monosodium urate crystals were prepared by crystallization from a supersaturated solution of uric acid (Aldrich Chemical Company, Inc.) under mildly basic conditions according to the method in the previous study [3]. The concentration of MSU crystals suspension was 20 mg/mL.

### Animals

All 60 male rats (Sprague-Dawley, 200 - 220 g) were purchased from Hubei Experimental Animal Center (Wuhan, China) and were housed for one week to adapt to the environment before being used for experiments. All the animals were maintained on standard laboratory conditions of temperature 23  $\pm$  2  $^{\circ}$ C and a 12-h light/12-h dark cycle with free access to commercial feed and pure water for the duration of the study. The rat experiment was approved by Animal Care and Use Committee of Tongren Hospital of Wuhan University (approval ref no. 201405832) and was carried out in compliance with Directive 2010/63/EU on the handling of animals used for scientific purposes [13].

### MSU-induced gouty arthritis model and drug administration

Homogenous suspensions of celecoxib were prepared with distilled water. All rats were randomly divided into six groups of 10 rats each. Group I injected with PBS (control group). Group II injected with 100  $\mu$ L MSU crystals suspension (model group). Group III comprised of MSU crystals-treated rats were administered with celecoxib (0.019 g/kg body weight). Group IV, Group V and Group VI comprised of MSU crystals-treated rats were administered with ZTD (19.2, 9.6 and 4.8 g/kg body weight, respectively). All the administration of rats lasted for 9 days and once daily. At the 7th day of drug administration, rats were anesthetized with 20 % urethane solution, and then MSU crystals suspension (100  $\mu$ L) or PBS (100  $\mu$ L) was injected into the tibiotarsal joint (ankle) of rats.

### Assessment of walking pattern and gait score

The walking behavior of rats was observed every day. According to the Coderre's Method [14], the gait score was calculated to evaluate the Owestry disability index of rats at 2, 6, 12, 24 and 48 h respectively.

### Histological examination

For histological analyses, three ankles of rats were isolated and then embedded in paraffin and

stained with haematoxylin and eosin. Histopathologic severity was scored using three different parameters as previously described [15]: synovial inflammation, synovial hyperplasia and cartilage surface erosion. Histopathological examination was also used to determine the number of infiltrating cells.

### Determination of cytokine levels

After 24 h of the last drug administration, the lavage fluids (including PBS and synovial fluid) were collected. Each lavage fluid of articular cavities was diluted with PBS to a constant volume 1 ml. After that, lavage fluids were centrifuged at 500 g for 10 min and supernatants were stored at -80 °C. Levels of IL-1 $\beta$  and TNF- $\alpha$  in lavage fluid of articular cavities were measured by ELISA kits (according to manufacturer's instructions).

### Western blot analysis

Three synovial of injected ankles after irrigation with PBS were isolated. About 50 mg of frozen rat articular synovium homogenized in 1 mL RIPA buffer, and then centrifuged at 10,000 g for 20 min. Protein concentration of the supernatant were measured by the Bradford method. The volumes equivalent to 30 mg of proteins were analyzed by 10 % SDS-PAGE under non-reducing conditions. After electrophoresis, polyacrylamide gels were blotted onto polyvinylidene fluoride membrane. Membranes were washed in Tris-HCl-buffered saline (TBS, 50 mM, pH 7.5) and incubated in 5% dried skim milk for 2 h and washed twice with tans-buffer (without methanol) and incubated with a primary antibodies, including anti-NLRP3 (1 : 2000), anti-caspase-1 (1 : 2000), anti-ASC (1 : 2000), anti-IL-1 $\beta$  (1 : 2000), anti-IL-18 (1 : 2000) and anti- $\beta$ -actin antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Wuhan Servicebio Co., Ltd.). The immune complexes were detected using chemiluminescence (ECL) system.

### Statistical analysis

Data are expressed as mean  $\pm$  standard error of measurement (SEM). The statistical analysis was performed using Student's t-test for two groups or a one-way ANOVA for three or more multiple groups (GraphPad Software San Diego, CA, USA).  $P < 0.05$  was considered statistically significant.

## RESULTS

### ZTD improved the walking behavior and gait score in MSU crystal-treated rats

After the injection of MSU crystals into the ankle joint, joint swelling appeared immediately. Compared with control group ( $0.0 \pm 0.1$ ), the oswestry disability index of model group rats increased significantly at hour 2, 6, 12, 24 and 48 h (all  $p < 0.01$ , respectively). Celecoxib and ZTD (19.2 g/kg) both ameliorated the oswestry disability index and walking behavior significantly respectively ( $p < 0.01$ , Table 1).

### Foot appearance and histological assessment of synovial tissue of joint

As shown in Figure 1, injection of MSU crystal suspension into the ankle joint obviously increased the swelling of toes and infiltration of inflammatory cells. Treatments with ZTD and celebrex significantly attenuated the swelling of toes and the infiltration of inflammatory cells in synovium.

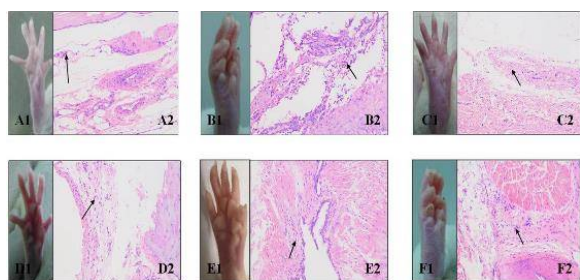
### ZTD attenuated the levels of pro-inflammatory cytokines in MSU crystal-treated rats

To identify the alterations of pro-inflammatory cytokines after ankle injection of MSU crystals suspension, the levels of pro-inflammatory cytokines in the lavage fluid of articular cavities were collected at 24 h later of the last drug administration.

**Table 1:** Effect of *Zhihuang Tongfeng* decoction on gait score in gouty arthritis rats

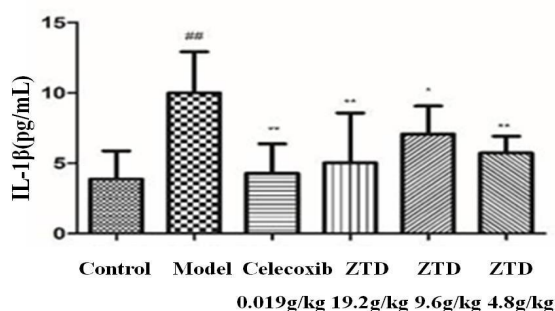
Group	Dosage (g/kg)	Owestry disability index				
		2h	6h	12h	24h	48h
Control	-	0.0 $\pm$ 0.1	0.0 $\pm$ 0.1	0.0 $\pm$ 0.1	0.0 $\pm$ 0.1	0.0 $\pm$ 0.1
Model	-	2.9 $\pm$ 0.3 <sup>##</sup>	2.7 $\pm$ 0.7 <sup>##</sup>	2.5 $\pm$ 0.5 <sup>##</sup>	2.4 $\pm$ 0.5 <sup>##</sup>	2.3 $\pm$ 0.4 <sup>##</sup>
Celecoxib	0.019	2.4 $\pm$ 0.6 <sup>~</sup>	1.8 $\pm$ 0.4 <sup>~</sup>	1.4 $\pm$ 0.4 <sup>~</sup>	1.2 $\pm$ 0.3 <sup>~</sup>	1.0 $\pm$ 0.2 <sup>~</sup>
ZTD-H	19.200	2.5 $\pm$ 0.6 <sup>~</sup>	2.0 $\pm$ 0.5 <sup>~</sup>	1.7 $\pm$ 0.4 <sup>~</sup>	1.1 $\pm$ 0.3 <sup>~</sup>	0.9 $\pm$ 0.3 <sup>~</sup>
ZTD-M	9.600	2.6 $\pm$ 0.7 <sup>~</sup>	2.3 $\pm$ 0.5 <sup>~</sup>	2.0 $\pm$ 0.5 <sup>~</sup>	1.9 $\pm$ 0.4 <sup>~</sup>	1.6 $\pm$ 0.4 <sup>~</sup>
ZTD-L	4.800	2.8 $\pm$ 0.4 <sup>~</sup>	2.6 $\pm$ 0.6 <sup>~</sup>	2.2 $\pm$ 0.6 <sup>~</sup>	2.0 $\pm$ 0.5 <sup>~</sup>	1.9 $\pm$ 0.4 <sup>~</sup>

Compared with control group, <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ ; compared with model group, <sup>~</sup> $p < 0.05$ , <sup>~</sup> $p < 0.01$ ; high dose of ZTD-H, ZTD-M: middle dose of ZTD-H, ZTD-L: low dose of ZTD-H

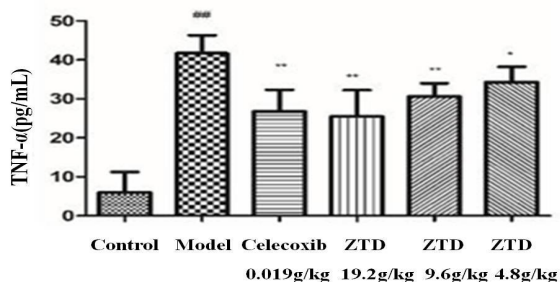


**Figure 1:** Inflammatory responses were attenuated by Zhihuang Tongfeng Decoction (ZTD) and Celebrex in the MSU crystals-treated rats. A1 and A2: PBS-treated rats; B1 and B2: MSU crystals-treated rats; C1 and C2: Celebrex-treated rats; D1 and D2: 19.2 g/kg ZTD-treated rats; E1 and E2: 9.6 g/kg ZTD-treated rats; F1 and F2: 4.8 g/kg ZTD-treated rats. N = 6; Arrows hinting to inflammatory cells; original magnification 200x. Scale bars: 100 μm.

Compared with control group, IL-1β and TNF-α levels of the lavage fluid of articular cavities in MSU crystals-treated rats increased significantly ( $P < 0.01$ ). Celecoxib and ZTD (19.2 g/kg dose) both ameliorated IL-1β and TNF-α levels significantly ( $p < 0.01$ , Figure 2 and Figure 3).



**Figure 2:** Effect of ZTF on IL-1β level of the lavage fluid of articular cavities in MSU crystal-treated rats.

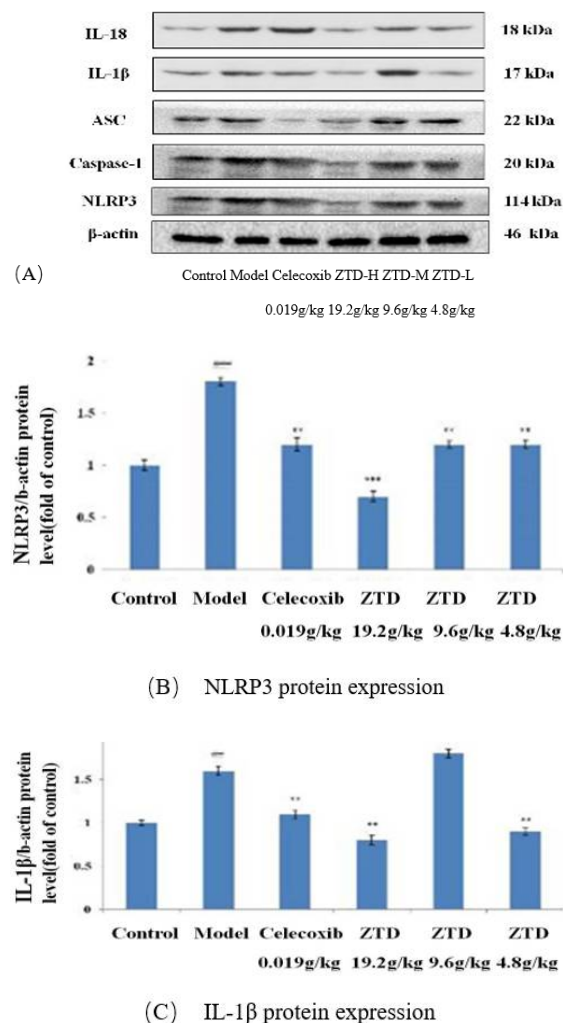


**Figure 3:** The effect of ZTF on TNF-α level of the lavage fluid of articular cavities in MSU crystal-treated rats

**ZTD suppressed caspase-1 activation and IL-1β and IL-18 secretion**

In order to understand the effect of ZTD on joint, the effect of ZTD on the NLRP3 inflammasome protein expressions was investigated. Compared

to the control group, the expressions of NLRP3, caspase-1, ASC, IL-1β and IL-18 proteins were all up-regulated in the MSU-treated group (all  $p < 0.01$ ), suggesting that inflammatory status might be involved in the articulation dysfunction. As shown in Figure 4A - C, the results showed that ZTD (4.8, 9.6 and 19.2 g/kg) and and celebrex decreased NLRP3, caspase-1, ASC, IL-1β and IL-18 protein expressions in rats ( $p < 0.01$ ).



**Figure 4:** Expressions of inflammation-associated proteins in the synovium of MSU crystals-treated rats by Western blot. A - C: The protein expressions of NLRP3 and IL-1β were measured by western blotting and the bar chart indicated the relative protein expression; \*\*\*  $p < 0.001$  vs. control rats;  $p < 0.05$ ,  $** p < 0.01$  and  $** p < 0.001$  vs. model rats

**DISCUSSION**

Gout is caused by the accumulation of MSU crystals in joints, and it is characterized by IL-1β-driven acute inflammation, which is associated with the infiltration of monocyte-mediated neutrophils in the joints [16]. Evidence shows that crystals from the synovial fluid of patient with

gout are composed of MSU crystal. Monosodium urate crystal is widely recognized as a danger signal to promote inflammation in the joint cavity, and MSU crystal is most frequently employed to develop an animal model of gouty arthritis [17]. Neutrophil recruitment and activation in joint fluid and synovial membrane is a hallmark the acute inflammatory response to MSU crystals in acute gouty arthritis [18]. In the present study, MSU crystals-induced joint swelling and thermal hyperalgesia elevating in the present study were observed in rats, suggesting acute inflammation in the joint cavity. And ZTD treatment attenuate the pain threshold value and the joint swelling degree in MSU crystal-treated rats significantly.

The objectives of treating gout include managing the symptoms of acute attacks and preventing further attacks by reducing uric acid levels in the blood. The most commonly used therapies for acute gout in general practice include the use of non-steroidal anti-inflammatory drugs (NSAIDs), colchicine, celecoxib and corticosteroids. Although these drugs have certain therapeutic effects, they present serious side effects, such as liver and kidney damage and severe gastrointestinal reactions [19]. Moreover, the treatment of gout is often a long-term process. Therefore, there is an urgent need to search for safer drugs.

NLRP3 inflammasome is a cytosolic protein complex composed of NLRP3, ASC, and caspase-1, and assembled in response to both microbial infection and endogenous "danger signal". The activation of NLRP3 inflammasome promotes the maturation and release of several proinflammatory cytokine, such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18. Therefore, it plays critical roles in the initiation of inflammation and the development of immune responses [20]. Among the inflammasomes, NLRP3 inflammasome is relatively well studied and its activation is linked to age related metabolic diseases and autoinflammatory diseases [21].

Therefore, much attention has been given to find active constituents that can act as specific NLRP3 inflammasome inhibitor. In this study, ZTD inhibited NLRP3 inflammasome activation, and MSU induced IL-1 $\beta$  and IL-18 production and neutrophil infiltration *in vivo*, suggesting that ZTD ameliorated the gouty arthritis induced by MSU crystals by inhibiting NLRP3 inflammasome.

## CONCLUSION

The findings of this study indicate that the traditional Chinese medicine, ZTD, suppresses

MSU crystal-induced swelling and pain in rats and also exerts anti-inflammatory effect by suppressing NLRP3 inflammasome activation. Therefore, ZTD provide a new potential strategy for the management of gouty arthritis.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this study.

### 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 the authors. Xiong Wang and Li Ma performed the experiments. Jun Wu wrote the manuscript. Jie Ping and Yong-gang Chen wrote the proposal and designed the manuscript. Dan Luo and Yuan Wang conducted data analysis. Jin-hu Wu prepared ZTD. Xiang-you Li modified the manuscript. Xiong Wang and Jun Wu contributed equally to this work and they are co-first authors. Yong-gang Chen, Jin-hu Wu and Xiang-you Li are co-corresponding author.

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