

## Effects of Blood-letting Therapy Combined with Zhuang Medicine Thread Moxibustion on the TLRs/MyD88 Signaling Pathway in Rats with Acute Gouty Arthritis (Postprint)

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### Abstract

**Background** With alterations in environmental and dietary structures, acute gouty arthritis (AGA) has become a common clinical condition that frequently recurs, posing a threat to patient health. In clinical practice, blood-letting therapy combined with Zhuang medicine thread moxibustion demonstrates definite therapeutic efficacy for AGA, but its mechanism of action remains incompletely understood.

**Objective** To investigate the mechanism of blood-letting therapy combined with Zhuang medicine thread moxibustion in the treatment of acute gouty arthritis based on the TLRs/MyD88 signaling pathway.

**Methods** Sixty SD rats were randomly divided into 6 groups: blank group, model group, blood-letting group, medicine thread group, colchicine group, and blood-letting combined with medicine thread group, with 10 rats in each group. With the exception of the blank group, the remaining groups were injected with sodium urate suspension into the right ankle joint cavity to establish the AGA model. At 24 h after modeling, the colchicine group was treated with colchicine suspension by gavage, the blood-letting therapy group was treated with pricking and bloodletting at Ashi points, the medicine thread group was treated with Zhuang medicine thread moxibustion around the lesion, and the blood-letting combined with medicine thread group was first treated with pricking and blood-letting at Ashi points followed by surrounding moxibustion. The swelling degree of the right ankle joint was observed at 6 h, 12 h, 24 h, and 72 h after modeling; pathological changes in the synovial tissue of the right ankle joint were observed by HE staining; ELISA was used to detect the levels of interleukin-10 (IL-10), interleukin-8 (IL-8), and cyclooxygenase-2 (COX-2) in rat serum; Western blot

was used to detect the protein expression of MyD88 and IKK- $\beta$  in the synovial tissue of the right ankle joint.

**Results** Following intervention, all experimental groups demonstrated reduced ankle joint swelling compared with the model group ( $P < 0.05$ ). Compared with the model group, serum levels of IL-8 and COX-2 decreased while IL-10 level increased in the blood-letting group, medicine thread group, colchicine group, and blood-letting combined with medicine thread group ( $P < 0.05$ ), with comparable efficacy observed between the blood-letting combined with medicine thread group and the colchicine group. Additionally, the protein expression levels of MyD88 and IKK- $\beta$  were significantly decreased in all treatment groups compared with the model group ( $P < 0.01$ ), with no significant difference between the blood-letting combined with medicine thread group and the colchicine group.

**Conclusion** Blood-letting therapy combined with Zhuang medicine thread moxibustion can ameliorate AGA arthritic symptoms through regulation of the TLRs/MyD88 signaling pathway, suggesting that blood-letting therapy combined with Zhuang medicine thread moxibustion may be a potential alternative therapy for AGA.

## Full Text

### Effect of Blood-Pricking Therapy Combined with Zhuang-Medicine Thread Moxibustion on the TLRs/MyD88 Signaling Pathway in Rats with Acute Gouty Arthritis

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## Abstract

**Background:** With changes in environmental and dietary structures, acute gouty arthritis (AGA) has become a common clinical disease. AGA is prone to recurrent attacks that harm patient health. In clinical practice, blood-pricking therapy combined with Zhuang-medicine thread moxibustion demonstrates definite therapeutic efficacy for AGA, though its mechanism of action remains unclear.

**Objective:** To investigate the therapeutic mechanism of blood-pricking therapy combined with Zhuang-medicine thread moxibustion for AGA based on the TLRs/MyD88 signaling pathway.

**Methods:** Sixty SD rats were randomly divided into six groups (n=10 each): blank, model, blood-pricking, Zhuang-medicine thread moxibustion, colchicine, and combined therapy groups. Except for the blank group, all groups received intra-articular injection of monosodium urate suspension into the right ankle joint to establish the AGA model. At 24 hours post-modeling, the colchicine group received colchicine suspension via gavage, the blood-pricking group underwent Ashi point pricking and bloodletting, the Zhuang-medicine thread moxibustion group received encircling moxibustion at the lesion site, and the combined therapy group received both blood-pricking followed by encircling moxibustion. Right ankle swelling was observed at 6h, 12h, 24h, and 72h post-modeling. Synovial tissue pathology was examined via HE staining. Serum IL-10, IL-8, and COX-2 levels were measured by ELISA. MyD88 and IKK- $\beta$  protein expression in synovial tissue was detected by Western blot.

**Results:** Compared with the model group, all treatment groups showed reduced ankle swelling ( $P<0.05$ ). Serum IL-8 and COX-2 levels decreased while IL-10 increased in the blood-pricking, Zhuang-medicine thread moxibustion, colchicine, and combined therapy groups ( $P<0.05$ ), with comparable efficacy between the combined therapy and colchicine groups. MyD88 and IKK- $\beta$  protein expression levels were significantly lower in all treatment groups compared with the model group ( $P<0.01$ ), with no significant difference between the combined therapy and colchicine groups.

**Conclusions:** Blood-pricking therapy combined with Zhuang-medicine thread moxibustion can improve AGA inflammatory symptoms by regulating the TLRs/MyD88 signaling pathway. This combination therapy may represent a potential alternative treatment for AGA.

**Keywords:** acute gouty arthritis; blood-pricking therapy; Zhuang-medicine thread moxibustion; TLRs/MyD88 pathway

## Introduction

Gouty arthritis (GA) is a metabolic disease caused by deposition of monosodium urate monohydrate (MSU) crystals in joint cavities [1]. Acute episodes manifest as red, swollen joints with elevated local skin temperature and severe pain, characterized by sudden onset, frequent recurrence, and poor prognosis. Approximately 1.1% of China's population suffers from gouty arthritis, with incidence rates rising annually and a trend toward younger onset. Research indicates that increasing GA incidence correlates with higher rates of comorbidities including obesity, hypertension, hyperlipidemia, diabetes, and kidney disease [2], severely impacting patient health, quality of life, and economic burden [4,5].

Traditional Chinese Medicine (TCM) attributes acute gouty arthritis attacks to the intermingling of toxic heat and phlegm-dampness in joints, representing an excess heat pattern. Blood-pricking therapy, a TCM external treatment, involves rapid needling at lesion sites to release a certain amount of blood, thereby clearing heat and activating blood circulation. Zhuang-medicine thread moxibustion is an ethnic medical therapy characteristic of the Lingnan region, utilizing medicated threads to apply moxibustion at relevant acupoints. Through the warmth and medicinal effects stimulating the points, this therapy unblocks pathways, restores the synchronization of the three qi, draws out heat, disperses stasis, and reduces inflammation and pain. Modern research suggests that both Zhuang-medicine thread moxibustion and blood-pricking therapy may exert anti-inflammatory effects by upregulating local anti-inflammatory factors, reducing inflammatory cell infiltration, improving local tissue necrosis in GA, and promoting recovery of damaged joint function. Both therapies are simple to perform, demonstrate definite efficacy, produce minimal adverse reactions, and impose lighter economic burdens, making them particularly suitable for widespread implementation in primary hospitals.

## Materials and Methods

### Experimental Animals

Sixty SPF-grade male SD rats weighing  $200 \pm 20$ g were purchased from Hunan Slack Experimental Animal Company (SCXK(Xiang)2019-0004). The study was approved by the Guangxi University of Traditional Chinese Medicine (DW20210426-055). Animals were housed under controlled conditions: temperature  $22 \pm 1^\circ\text{C}$ , humidity  $50 \pm 10\%$ , 12h:12h light-dark cycle, with quiet environment maintenance.

### Reagents and Materials

Colchicine (Hefei Bomei Biotechnology, Model QC8624-100mg); Monosodium urate (Sigma-Aldrich, Model U2875-5g); Hematoxylin-eosin staining kit (Wuhan Baiqiandu Biotechnology); ELISA kits for COX-2, IL-8, and IL-10 (Biowamp); BCA protein quantification kit (BIOSHARP).

## Experimental Groups

After 7 days of adaptive feeding, 60 rats were randomly divided into six groups (n=10 each): blank, model, blood-pricking, Zhuang-medicine thread moxibustion, colchicine, and combined therapy groups. All rats were housed individually in the SPF animal facility with free access to food and water, with regular cleaning and disinfection.

## Model Establishment

Rats were anesthetized with isoflurane via a small animal anesthesia machine. Using a 1ml syringe, 0.2ml of monosodium urate suspension was injected into the right ankle joint cavity at a 30-40° angle from the posterior aspect. Successful injection was confirmed by a distinct loss-of-resistance sensation and bulging of the contralateral joint capsule. The blank group received 0.2ml normal saline injection into the right ankle joint cavity.

## Treatment Protocol

Interventions were administered at 24h, 48h, and 72h post-modeling. Body weight was measured before each treatment, with gavage volume standardized at 1ml per 100g body weight. The colchicine group received 5% colchicine suspension via gavage. The blood-pricking group underwent Ashi point needling at the most swollen area of the right ankle using a #6 injection needle, with approximately 0.1ml blood released per rat. The Zhuang-medicine thread moxibustion group received plum-blossom encircling moxibustion around the most swollen area of the right ankle, with six moxibustion points evenly distributed around the central swelling. The combined therapy group received both blood-pricking followed by Zhuang-medicine thread encircling moxibustion as described above. All groups except the colchicine group received pure water gavage.

## Sample Collection

After treatment completion, rats were anesthetized with 10% chloral hydrate via intraperitoneal injection. The abdominal cavity was opened to expose the abdominal aorta for blood collection. Serum was separated by centrifugation, aliquoted into EP tubes, and stored at -80°C for ELISA detection. Rats were then euthanized, and the right ankle joint cavity was fully opened to identify the synovial tissue (yellowish, translucent, smooth, and resilient tissue beneath articular cartilage). Synovial tissue from seven randomly selected rats per group was preserved in cryovials in liquid nitrogen for Western blotting. The remaining three rats' right ankle joints were placed in centrifuge tubes with 4% paraformaldehyde for pathological examination.

## Outcome Measures

**Ankle Swelling Assessment** Right ankle swelling was measured at 6h, 12h, 24h, 48h, and 72h post-modeling. Before each intervention, the transverse diameter of the right ankle's medial and lateral malleoli was measured using vernier calipers, with three consecutive measurements taken per rat and averaged.

**Histopathological Examination** Synovial tissue was fixed in 4% paraformaldehyde, dehydrated through graded ethanol series, embedded in paraffin, and sectioned at 4 $\mu$ m thickness. Sections were stained according to the HE staining kit protocol, mounted with neutral resin, and examined under microscopy for pathological changes.

**Western Blot Analysis** Synovial tissue was lysed to extract total protein. Protein concentration was determined using the BCA kit. Samples were loaded, electrophoresed, and transferred to membranes, which were blocked with 5% skim milk for 1 hour. Membranes were incubated overnight at 4°C with primary antibodies against MyD88, IKK- $\beta$ , and GAPDH, washed five times with TBS-T buffer, then incubated with corresponding secondary antibodies at 37°C for 1 hour. After washing, protein bands were visualized using ECL and analyzed.

**Statistical Analysis** SPSS 21.0 was used for statistical analysis. Measurement data are expressed as mean $\pm$ standard deviation. Inter-group comparisons were performed using LSD test.  $P < 0.05$  was considered statistically significant.

## Results

### General Condition of Rats

Blank group rats exhibited normal feeding, good mental status, naturally glossy fur, free movement, and quick responses. Model group rats showed poor mental status, reduced food intake, rough and dull fur, elevated local skin temperature with redness and swelling at the right ankle, and significantly decreased activity. All treatment groups showed marked improvement compared with the model group. Colchicine group rats developed diarrhea, while rats in the blood-pricking, Zhuang-medicine thread moxibustion, and combined therapy groups had normal stool.

### Ankle Swelling Degree

The right ankle circumference remained unchanged in the blank group across all time points. In remaining groups, ankle swelling peaked at 12h post-modeling then gradually decreased. Compared with the model group, ankle swelling was significantly reduced in the blood-pricking, Zhuang-medicine thread moxibustion, colchicine, and combined therapy groups at 48h and 72h ( $P < 0.05$ ). At 72h post-modeling, no significant difference existed between the combined therapy and blank groups ( $P > 0.05$ ). Detailed data are presented in .

## Synovial Histopathology

Blank group rats showed intact synovial tissue and cellular structure with orderly collagen fiber arrangement and no inflammatory cells. Model group rats exhibited severe synovial damage with extensive necrosis and detachment, accompanied by massive inflammatory cell infiltration. All treatment groups showed varying degrees of improvement in inflammatory cell infiltration. Representative images are shown in [Figure 1: see original paper].

## Serum Inflammatory Cytokine Levels

Compared with the blank group, the model group showed significantly elevated IL-8 and COX-2 levels with decreased IL-10 ( $P < 0.01$ ). Compared with the model group, the colchicine and combined therapy groups demonstrated significantly reduced IL-8 and COX-2 with increased IL-10 ( $P < 0.01$ ), while the blood-pricking and Zhuang-medicine thread moxibustion groups showed similar but less pronounced changes ( $P < 0.05$ ). Significant differences existed between the blood-pricking/Zhuang-medicine groups and the colchicine group ( $P < 0.01$ ), but not between the combined therapy and colchicine groups ( $P > 0.05$ ). Detailed results are presented in .

## MyD88/TLRs Signaling Pathway Protein Expression

Compared with the blank group, MyD88 and IKK- $\beta$  protein expression was significantly elevated in the model group ( $P < 0.01$ ). All treatment groups showed significantly reduced MyD88 and IKK- $\beta$  expression compared with the model group ( $P < 0.01$ ). Representative Western blot results are shown in [Figure 2: see original paper].

## Discussion

The development of acute gouty arthritis results from multiple factors including diet, genetics, and environment [9]. In TCM, AGA belongs to the categories of “bi syndrome” and “white tiger joint wind.” The concept of “gout” was first proposed by Zhu Danxi in *Gezhi Yulun · Tongfeng Lun*, which states: “Gout mostly occurs because blood receives heat...cold and coolness externally strike, hot blood encounters cold, sweat and turbidity coagulate, thus causing pain.” The pathogenesis primarily involves congenital deficiency or external pathogenic invasion, with evil qi stagnating in joints, or internal injury from diet, excessive consumption of rich fatty foods generating heat and phlegm over time, and the intermingling of stasis, toxin, and phlegm-turbidity obstructing joints. Western medicine considers MSU crystal deposition the key factor in AGA pathogenesis. Macrophages recognize and phagocytose MSU, activating inflammatory signal transduction pathways and releasing inflammatory factors that produce intense inflammatory reactions, clinically manifesting as localized redness, swelling, elevated skin temperature, and severe pain.

This study employed the classic Coderre method to establish the AGA rat model by injecting MSU into the right ankle joint. The right ankle manifestations closely matched clinical AGA symptoms, and combined with synovial histopathological changes showing varying degrees of tissue damage, necrosis, and inflammatory cell infiltration, confirmed successful model establishment. Model group rats developed progressive right ankle swelling that peaked at 12h post-modeling. After treatment, the colchicine, blood-pricking, Zhuang-medicine thread moxibustion, and combined therapy groups all alleviated joint swelling to varying degrees. At 72h post-modeling, no significant difference existed between the combined therapy and blank groups ( $P>0.05$ ), indicating the combined therapy was most effective in improving ankle swelling. Colchicine group rats developed diarrhea during treatment, likely representing gastrointestinal side effects, while rats in other treatment groups maintained normal stool, suggesting blood-pricking therapy and Zhuang-medicine thread moxibustion effectively improve AGA symptoms with fewer adverse reactions.

The *Suwen·Tiaojing Lun* records: “When blood is excessive, drain the abundant meridian and release the blood,” while *Lingshu·Xiaozhen Jie* states: “When full, drain it; when the qi opening is exuberant, it should be drained. When stale, remove it; this means removing blood vessels.” These classical texts indicate that blood-pricking therapy treats excess patterns of blood stasis, heat-toxin, damp-heat, and qi stagnation by clearing heat, activating blood, regulating qi-blood, and generating new tissue after removing stasis. In the Lingnan region, the unique geographical environment and climate produce diseases with damp-heat-stasis-toxin characteristics. Zhuang medicine believes “cold hands, hot back, swelling in plum,” referring to swelling-type diseases that can be treated with plum-blossom encircling moxibustion centered on the most swollen area to soften hardness, disperse nodules, activate blood, reduce swelling, detoxify, and reduce inflammation. From the perspective of integrated TCM and ethnic medicine, blood-pricking therapy combined with Zhuang-medicine thread moxibustion is applicable for AGA treatment.

During acute gouty arthritis attacks, MSU crystal phagocytosis induces cell membrane dissolution, lysosome and inflammatory mediator release, while chemokines including MCP-1, CXCL8, and IL-8 recruit neutrophils to inflammatory tissues, causing persistent inflammatory reactions [11]. In the immune system, cytokines are crucial messenger and effector molecules participating in immune responses during inflammation and infection. TLRs are involved in innate immunity; upon activation, they promote massive release of inflammatory factors such as IL-1 $\beta$  and IL-8, generating inflammatory cascades in AGA. Therefore, investigating whether blood-pricking therapy and Zhuang-medicine thread moxibustion exert beneficial effects on AGA-related inflammatory factors and reduce inflammatory responses can determine the therapeutic value of these treatments.

Studies show that for endotoxin-induced fever in rabbits, blood-pricking combined with cupping therapy not only inhibits GM-CSF, IL-1 $\beta$ , TNF- $\alpha$ , and

IL-6 levels but also provides superior anti-inflammatory and fever-reducing effects compared with cupping alone. Shen et al. demonstrated that Chinese medicine combined with blood-pricking therapy effectively reduced pro-inflammatory factors TNF- $\alpha$ , IL-8, and IL-1 $\beta$  levels in gouty arthritis patients, facilitated uric acid excretion, and improved clinical symptoms. In research on Zhuang-medicine thread moxibustion combined with acupuncture for knee osteoarthritis [14], IL-1 $\beta$  and IL-18 levels significantly decreased, joint inflammation improved, and articular cartilage repair function recovered. In this study, IL-10, a typical anti-inflammatory cytokine, inhibits pro-inflammatory signals and immune responses, reduces inflammation, and improves clinical symptoms [14]. IL-8, a chemokine, participates throughout the inflammatory response process. COX-2 is an inducible enzyme regulating cell growth, migration, proliferation, autophagy, apoptosis, and immune responses, promoting prostaglandin synthesis and showing high expression during tissue damage and inflammatory reactions, thereby inducing significant pain [18].

ELISA results showed elevated IL-8 and COX-2 levels with decreased IL-10 in model group rats, indicating excessive inflammatory reactions in the right ankle joint of AGA rats. After intervention, AGA rats showed reduced IL-8 and COX-2 levels with increased IL-10, suggesting blood-pricking therapy and Zhuang-medicine thread moxibustion inhibit AGA inflammatory responses. No significant difference existed between the colchicine and combined therapy groups ( $P > 0.05$ ), indicating comparable efficacy, with the combined therapy showing superior results compared with either therapy alone. This study confirms that blood-pricking therapy combined with Zhuang-medicine thread moxibustion effectively inhibits pro-inflammatory factor secretion and promotes anti-inflammatory factor release in AGA rats, providing reliable evidence for this treatment approach.

Toll-like receptors (TLRs) are a well-characterized pattern recognition receptor family. Except for TLR3, all TLRs utilize the myeloid differentiation primary response 88 (MyD88)-dependent signaling pathway [19]. TLRs are type I transmembrane proteins, and MyD88 is a crucial signal transduction protein and key adaptor molecule mediating TLR innate immune response pathways. It is essential for the inflammatory mediator NF- $\kappa$ B in the classical activation pathway, while I $\kappa$ B kinase  $\beta$  (IKK- $\beta$ ), the main catalytic subunit of the IKK complex, is required for NF- $\kappa$ B activation, thereby inducing secretion of many pro-inflammatory factors and producing effects important for other immune responses [20,21]. The TLR/MyD88 signal transduction pathway is one of the most studied pathways in gouty arthritis pathogenesis. During acute gouty arthritis attacks, activated Toll-like receptors trigger innate immune responses and initiate downstream signaling events through MyD88-dependent pathways, regulating expression of various inflammatory genes. Signals are transmitted through the TIR domain of MyD88, activating transcription factors and promoting release of multiple inflammatory factors including IL-8, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , generating inflammatory reactions [22]. Research indicates that gout attacks are associated with MSU activation of type I transmembrane protein

receptors and their downstream signaling pathways, and that isovitexin can reduce joint swelling indices, decrease inflammation, and improve AGA symptoms [23]. Thus, the TLRs/MyD88 signaling pathway represents a therapeutic target for improving AGA conditions and reducing inflammation.

This study demonstrates that blood-pricking therapy combined with Zhuang-medicine thread moxibustion significantly inhibits MyD88 and IKK- $\beta$  protein expression in AGA rat synovium. Combined with the observed regulation of inflammatory cytokine levels, this treatment enhances inhibition of activated inflammatory factors and promotes anti-inflammatory factor secretion, indicating that blood-pricking therapy combined with Zhuang-medicine thread moxibustion can inhibit the TLRs/MyD88 signaling pathway, regulate inflammatory factors, and exert therapeutic effects on AGA.

In summary, this study shows that blood-pricking therapy combined with Zhuang-medicine thread moxibustion better reduces joint swelling and alleviates inflammatory reactions through downregulating MyD88 and IKK- $\beta$  expression. Multi-target antagonism of the TLRs/MyD88 inflammatory signaling pathway may represent one anti-inflammatory mechanism for treating AGA. Traditional Chinese medicine and ethnic medicine constitute our unique treasure. We hope this study provides new insights and theoretical foundations for applying blood-pricking therapy combined with Zhuang-medicine thread moxibustion for acute gouty arthritis in clinical practice, promoting further development of TCM and ethnic medicine.

## Author Contributions

Danmeng Zhu: Conceptualization, manuscript writing, and revision. Yuying Huang, Tong'an Luo, and Changyuan He: Data collation and analysis. Rilan Chen: Quality control, review, and overall supervision.

## Conflict of Interest

The authors declare no conflict of interest.

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