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Anethole ameliorates inflammation induced by monosodium urate in an acute gouty arthritis model via inhibiting TLRs/MyD88 pathway

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Abstract

Objective: To assess the effects of anethole on monosodium urate (MSU)-induced inflammatory response, investigate its role in acute gouty arthritis (AGA), and verify its molecular mechanism. **Methods:** Hematoxylin and eosin staining assay and time-dependent detection of degree of ankle swelling were performed to assess the effects of anethole on joint injury in MSU-induced AGA mice. Enzyme-linked-immunosorbent serologic assay was performed to demonstrate the production levels of inflammatory factors (interleukin 1 β [IL-1 β], interleukin 6 [IL-6], interleukin 8 [IL-8], tumor necrosis factor α [TNF- α], and monocyte chemo-attractant protein-1 [MCP-1]) in MSU-induced AGA mice. Western blot assays were used to confirm the effects of anethole on oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activity and the activation of toll-like receptors (TLRs)-myeloid differentiation factor 88 (MyD88) pathway in MSU-induced AGA mice.

Results: We observed that a significant joint injury occurred in MSU-induced AGA mice. Anethole could alleviate the pathological injury of the synovium in MSU-induced AGA mice and suppressed ankle swelling. In addition, we observed that anethole could inhibit MSU-induced inflammatory response and inflammasome activation in MSU-induced AGA mice. Moreover, we discovered that anethole enabled to inhibit the activation of TLRs/MyD88 pathway in MSU-induced AGA mice. Our findings further confirmed that anethole contributed to the inhibitory effects on progression in MSU-induced AGA mice.

Conclusion: It confirmed that anethole ameliorated the MSU-induced inflammatory response in AGA mice *in vivo* via inhibiting TLRs-MyD88 pathway.

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Introduction

Gout, a chronic recurrent inflammatory disease accompanied by disordered purine metabolism and/or decreased uric acid excretion, is characterized by continuous increase of blood uric acid and deposition of monosodium urate (MSU) crystals.¹ Even worse is the accumulation of MSU crystals in the joints or surrounding tissues that induces robust inflammatory responses with drastic pain.² Acute gouty arthritis (AGA) is the main clinical manifestation of gout. In recent years, prevalence of AGA has increased.^{3,4} A study conducted by Gonzalez has reported that MSU crystal deposition in the synovium or joints could increase the expression of inflammatory cytokines, including interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF- α), eventually resulting in the occurrence and development of AGA.⁵ Therefore, inhibiting of MSU crystal deposition-induced inflammatory response is one potential therapeutic method for the treatment of AGA.⁶

Tongfeng prescription, with eupatorium as one of the main ingredients, has been used in the treatment of gout, achieving good results.⁷ Traditional Chinese medicine systems pharmacology database and analysis platform has demonstrated that anethole is the major bioactive component of eupatorium.⁸ Anethole is an aromatic component with many pharmacological properties, including anti-inflammatory, antioxidant, antidiabetic, cardio-protective, gastric-protective and liver-protective attributes.⁹⁻¹¹ A previous study has demonstrated that anethole inhibited the proliferation of prostate cancer cells and induced cell cycle arrest and apoptosis.¹² Meanwhile, studies have also concluded that anethole alleviates lipopolysaccharide (LPS)-induced lung injury by regulating the balance between T helper 17 (Th17) and regulatory T (Treg) cells.¹³ Interestingly, anethole can even improve learning ability and memory impairment in outlier mice, and has antidepressant and anxiety effects. Thus, it is considered as a suitable candidate for the treatment of stress and diseases of the nervous system.¹⁴

Both toll-like receptors (TLRs) and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome are pattern recognition receptors,¹⁵ which play pivotal roles in the initiation and progression of gout.¹⁶ It has been proved that knock out of toll-like receptor 2 (TLR2) and TLR4 could decrease the expression of MSU crystal-induced inflammatory cytokines such as IL-1 β and TNF- α . In addition, another study reported that MSU crystals induced the activation of NLRP3 inflammasome, and the activation and secretion of IL-1 β and IL-18 during the development of gout. It also reported that anethole attenuated the enterotoxin *Escherichia coli*-induced intestinal barrier disruption and intestinal inflammation by inhibiting the activation of TLRs-myeloid differentiation factor 88 (MyD88) signaling pathway.¹⁷ Additionally, anethole also exerted alleviating effects on many inflammatory diseases by blocking TLR4-nuclear factor kappa-B (NF- κ B), TNF- α , mitogen-activated protein kinase (MAPK), and NLRP3 pathways.¹⁸ Recently, anethole has been demonstrated to alleviate adjuvant-induced inflammation and joint injury; hence, it is an effective intervention for the treatment of rheumatoid arthritis. However, the effect of anethole on

gouty arthritis induced by abnormal activation of inflammation in MSU-induced AGA is unclear.

In this study, we investigated the effects of anethole on AGA. It clearly revealed that anethole restrained inflammatory response in MSU-induced AGA mice by regulating TLRs-MyD88 and NLRP3 pathways. We, therefore, anticipated that anethole could serve as a promising drug for treating gouty arthritis.

Materials and Methods

Animals

Six-week-old female Sprague-Dawley (SD) mice purchased from Shanghai Experimental Animal Center (Shanghai, China) were used in this study. All of them were raised under a 25°C temperature room with a 12-h light-dark cycle with easy accessibility to food and water. All procedures and protocols were approved by the Ethics Committee of the Guizhou University of Traditional Chinese Medicine.

Drugs and antibodies

Anethole (HY-B0900), MSU (HY-B2130A), and colchicine (HY-16569) were purchased from MedChem Express (Shanghai, China). Following antibodies were used in the procedures: NLRP3 (1:1000 dilution, ab263899; Abcam, Cambridge, MA, USA), IL-1 β (1:500 dilution, ab9722; Abcam), caspase-1 (1:1000 dilution, ab286125; Abcam), Pro-caspase-1 (1:1000 dilution, ab179515; Abcam), TLR2 (1:1000 dilution, ab213676; Abcam), TLR4 (1:1000 dilution, 66350-1-Ig; Proteintech Group, Hubei, China), MyD88 (1:1000 dilution, ab219413; Abcam), phospho-NF- κ B (p-NF- κ B) antibody (1:1000 dilution, 30335; CST, MA, USA), NF- κ B (1:1000 dilution, ab239882; Abcam), and β -actin antibody (1:2000 dilution, ab6276; Abcam).

Enzyme-linked-immunosorbent serologic assay (ELISA) kits

ELISA kits were used for the following inflammatory factors: IL-1 β (ab255730; Abcam), IL-6 (ab234570; Abcam), Interleukin 8 (IL-8) (SEKR-0071; Solarbio, Beijing, China), TNF- α (SEKR-0009; Solarbio), monocyte chemo-attractant protein-1 (MCP-1) (SEKR-0024, Solarbio).

AGA rat model and drug treatment

MSU solution, 10 mg (0.4 mL) and 0.1-mL (1 U/mL) polymyxin B were injected into the knee joint cavity of each SD rat to construct an AGA model. The animals were randomly divided into the following five groups: (1) control group (Sham group), (2) untreated MSU group (MSU group), (3) MSU group treated with low dosage of anethole (MSU + anethole 62.5 mg/kg group), (4) MSU group treated with high dosage of anethole (MSU + anethole 125 mg/kg group), and (5) MSU group treated with 0.12-mg/kg colchicine acting as positive control (MSU + colchicine group). The

procedure was performed by intra-articular injection of anethole or colchicine on day 0 and then once daily for 3 weeks. The Sham and untreated MSU groups were treated with saline injection for 3 weeks.

Histologic evaluation

The synovial tissues from anesthetized mice were collected and cut into different pieces after fixing in 4% paraformaldehyde. The cut tissues were washed in water for 2 h and dehydrated in 50%, 70%, 80%, 90%, and anhydrous ethanol. Subsequently, the tissues were separately dehydrated by xylene and paraffin, followed by embedding in paraffin and cut into 4- μ m slices. Next, the slices were dewaxed and stained with hematoxylin and eosin (H&E). After staining, collected sections were sealed in neutral balsam and observed under an optical microscope. The score of degree of joint injury was evaluated by H&E staining in mice from different groups, and the score range was from Sham group (score = 0) to AGA model group (score = 5). The measurement was repeated for three times and the average value was taken for further analysis.

Measurement of degree of ankle swelling

Briefly, the degree of ankle swelling was observed and evaluated at MSU crystals-induced position at 4, 6, 12, 24, and 48 h in successfully obtained animal models. The degree of ankle swelling was evaluated according to the following formula:

$$\text{Girth of ankle joint after MSU crystal injection} - \text{girth of ankle joint in control group} \div \text{girth of ankle joint in control group} \times 100\%$$

The measurement was repeated for 3 times and the average value was taken for further analysis.

ELISA

ELISA was performed to evaluate the secretion levels of inflammatory factors (IL-1 β , IL-6, IL-8, TNF- α , and MCP-1) in collected serum samples from MSU-induced AGA mice. According to the manufacturer's protocol, the diluted primary antibody was diluted with carbonate-coated buffer (pH 9.6) until the protein content reached 10 μ g/mL. Diluted antibody, 100 μ L, was added to 96-well plates (Corning® 9018) and incubated at 4°C overnight. On next day, the plates were washed for three times with >250- μ L/well phosphate-buffered saline (PBS) washing buffer (pH 7.4). Subsequently, 100 μ L of diluted rat serum samples (1:5 ratio) were added to the wells. After 2-h incubation, the plates were washed for three times with >250- μ L 0.5% Tween-20 in PBS, and horseradish peroxidase (HRP)-conjugated antibody (Abcam) was added and incubated at 37°C for 1 h. After addition of 100- μ L 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Innoagents, Hangzhou, China), the incubation was continued at 37°C for 30 min. On completion of the reaction, optical density (OD) was measured at 450 nm by a microreader.

Western blot analysis

Radioimmunoprecipitation assay (RIPA) buffer (9800; Cell Signaling, Danvers, MA) was used to lyse and extract total proteins from collected tissues. Protein was quantified by bicinchoninic acid (BCA) method; 20- μ g protein was added to each well and electrophoresed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, samples were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% milk for 2 h at 37°C. Next, incubation of PVDF membranes with anti-NLRP3, anti-IL-1 β , anti-caspase-1, anti-pro-caspase-1, anti-TLR2, anti-TLR4, anti-MyD88, anti-p-NF- κ B, anti-NF- κ B, and anti- β -actin antibodies (Abcam) was completed overnight at 4°C. Then PVDF membranes were washed for at least three to five times and co-incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL) detection reagents, purchased from Amersham Pharmacia Biotech (Tokyo, Japan), were used to detect visualized immunocomplexes. The Image-Pro software was used in this assay to calculate the intensity of each blot.

Statistical analysis

All of the experiments performed in this project were repeated at least for three times, and the data were evaluated using the average value. Analyzed by one-way ANOVA followed by Tukey's test, the data were presented as mean \pm standard deviation (SD). The GraphPad Prism 8.0 software was used for data analysis. $P < 0.05$ was taken as statistically significant.

Results

Effect of anethole on joint injury in MSU-induced AGA mice

In order to reveal the protective effects of anethole on joint injury in AGA, MSU was used to establish the AGA rat model. H&E staining (Figure 1A) and measuring the degree of ankle swelling (Figure 1B) were used to evaluate the effects of anethole on the pathological injury of joint in MSU-induced AGA mice. According to H&E staining results, we discovered that MSU successfully induced the occurrence of AGA in mice (injury score = 5), and 0.12 mg/kg of colchicine treatment (injury score = 2) effectively repaired MSU-induced AGA joint injury. Moreover, we observed that 62.5 mg/kg (injury score = 3) or 125 mg/kg (injury score = 2) of anethole treatment significantly inhibited the synovial injury in MSU-induced AGA in a concentration-dependent manner (Figure 2A). In addition, the degree of ankle swelling indicated that both anethole (62.5 mg/kg or 125 mg/kg) and colchicine (0.12 mg/kg) could significantly inhibit ankle swelling in MSU-induced AGA mice (Figure 2B). The alleviating effects of anethole treatment were displayed in both concentration and time-dependent manners ($P < 0.01$). Therefore, we expected that anethole attenuated MSU-induced AGA.

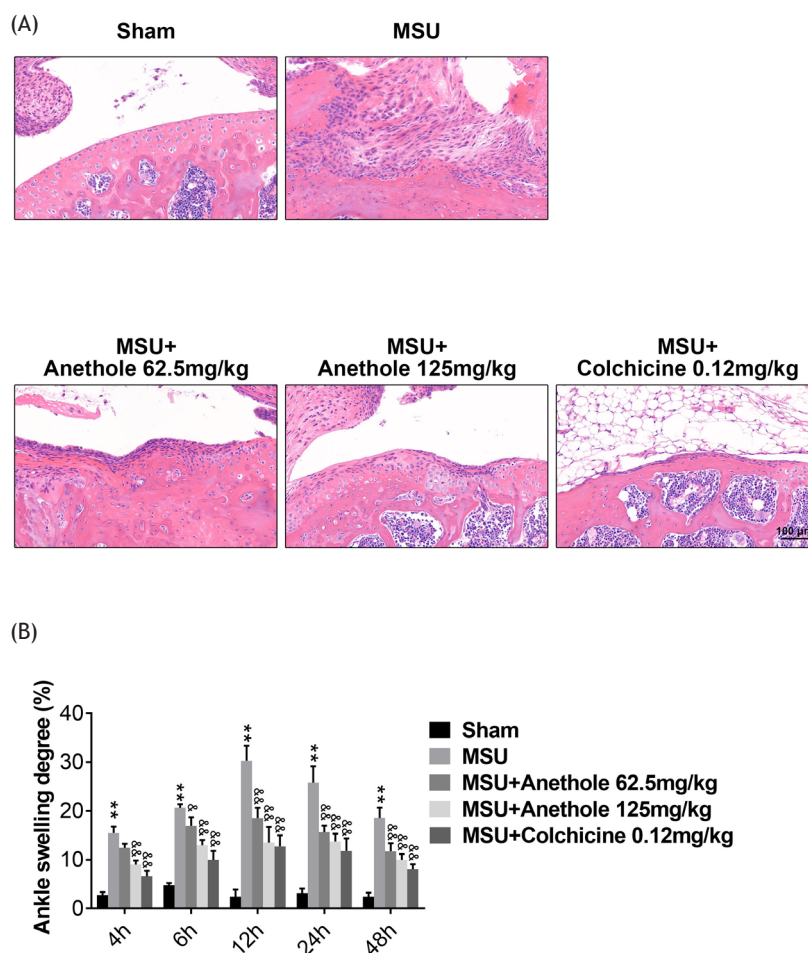


Figure 1 Anethole alleviated joint injury in MSU-induced AGA mice. (A) H&E staining of the joint in MSU-induced AGA mice. (B) Concentration and time-dependent detection of ankle swelling degree in MSU-induced AGA mice. Results were presented as mean \pm SD. MSU vs. Sham group, ** $P < 0.01$. MSU vs. MSU + anethole (62.5 mg/kg and 125 mg/kg) or MSU + colchicine group, $^{##}P < 0.01$.

Anethole suppressed inflammation in MSU-induced AGA

We focused on the effects of anethole on the levels of inflammatory factors in MSU-induced AGA. It was considered that the production and secretion of inflammatory factors was an important indicator for the initiation and progression of AGA. We, therefore, discovered the expression levels of IL-1 β , IL-6, IL-8, TNF- α , and MCP-1 in MSU-induced AGA upon anethole treatment. We observed through immunoblot assays that the protein levels of IL-1 β , IL-6, IL-8, TNF- α , and MCP-1 were up-regulated after induction of MSU whereas anethole or colchicine treatment significantly decreased the protein expression levels of IL-1 β , IL-6, IL-8, TNF- α , and MCP-1. In addition, compared with the high-dosage (125 mg/kg) treatment group, the low dosage (62.5 mg/kg) of anethole treatment would not significantly affect the production of IL-1 β and IL-8. Therefore, the results revealed that anethole could inhibit inflammatory response in MSU-induced AGA in a concentration-dependent manner.

Anethole inhibited the activation of NLRP3 inflammasome in MSU-induced AGA mice

The activation of NLRP3 inflammasome is known to participate in the initiation and development of inflammation, and we therefore investigated whether anethole suppressed joint injury in MSU-induced AGA. By immunoblot assay, we determined the expression levels of NLRP3 inflammasome-associated markers, including NLRP3 and its downstream proteins IL-1 β , caspase-1, and pro-caspase-1. We discovered that induction of MSU effectively increased pro-caspase-1-caspase-1 ratio as well as expression levels of both NLRP3 and IL-1 β ($P < 0.01$). In addition, anethole treatment (62.5 mg/kg or 125 mg/kg) and colchicine treatment (0.12 mg/kg) significantly decreased the pro-caspase-1-caspase-1 ratio as well as the expression levels of NLRP3 and IL-1 β ($P < 0.01$; Figure 3). Therefore, we believed that anethole restrained the activation of NLRP3 inflammasome in MSU-induced AGA.

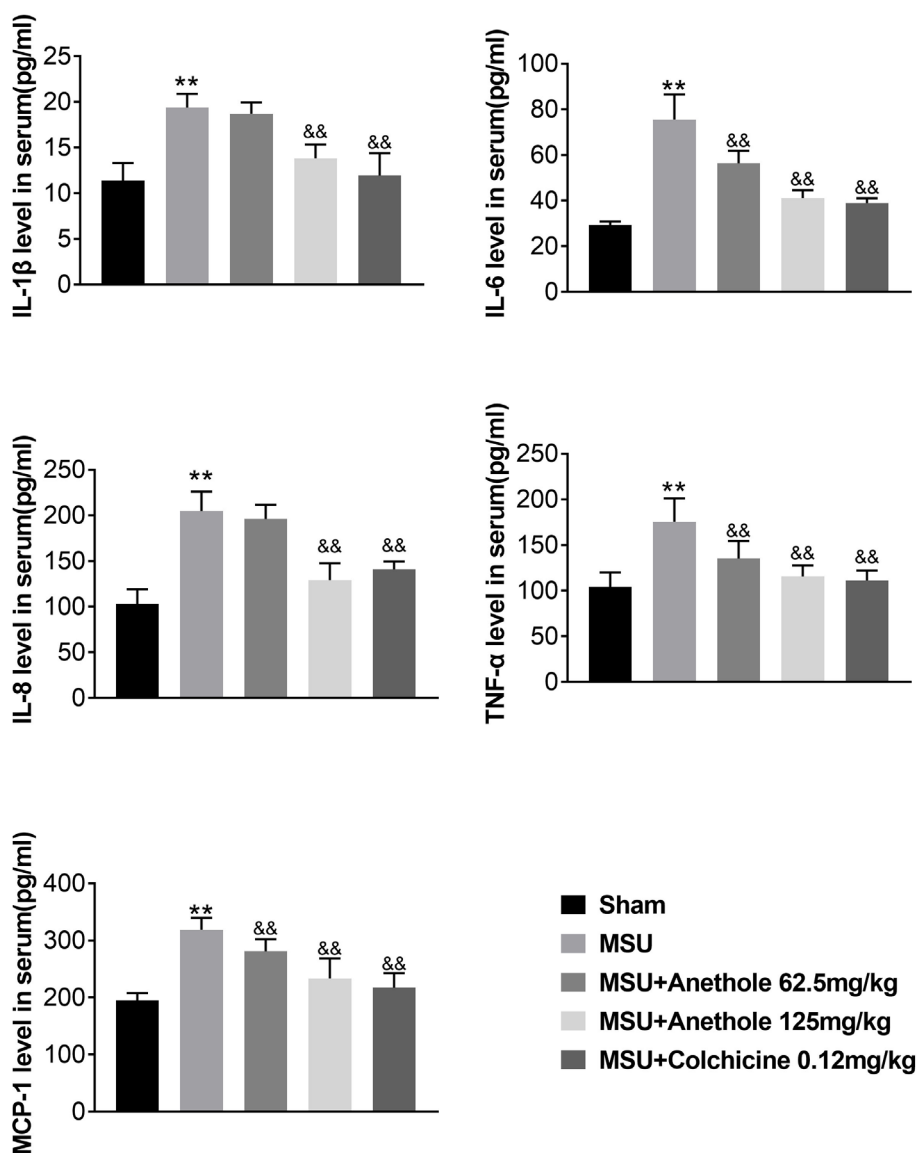


Figure 2 Anethole inhibited MSU-induced inflammatory responses in MSU-induced AGA mice. ELISA analyzed the effects of anethole on the secretion of inflammatory factors (IL-1 β , IL-6, IL-8, TNF- α , and MCP-1) in MSU-induced AGA mice. Results were presented as mean \pm SD. MSU vs. Sham group, ** $P < 0.01$. MSU vs. MSU + anethole (62.5 mg/kg and 125 mg/kg) or MSU + colchicine group, $^{\&\&}P < 0.01$.

Anethole blocked the activation of TLRs/MyD88/NF- κ B pathways in MSU-induced AGA mice

Abnormal activation of TLRs/MyD88/NF- κ B pathways was involved in the initiation and progression of AGA. Herein, we tried to evaluate the potential effects of anethole on TLRs/MyD88/NF- κ B pathways in MSU-induced AGA. Through immunoblot assays, we found that MSU could effectively up-regulate p-NF- κ B-NF- κ B ratio as well as expression levels of TLR2, TLR4, and MyD88 ($P < 0.01$) whereas anethole treatment (62.5 mg/kg or 125 mg/kg) and colchicine treatment (0.12 mg/kg) significantly decreased the p-NF- κ B-NF- κ B ratio as well as the expression levels of TLR2, TLR4, and MyD88 ($P < 0.01$; [Figure 4](#)).

Discussion

As a common clinical manifestation of gout, AGA is a type of inflammatory arthritis and is characterized by redness, tenderness, and joint swelling.¹⁹ To some degree, AGA is considered as a metabolic disease triggered by disordered metabolism of uric acid.²⁰ Briefly, uric acid crystallizes in the form of MSU crystals and precipitates in joints, tendons, and the surrounding tissues. This leads to the activation of innate immune responses, which finally aggravate the progress of AGA.²¹ Epidemiological studies have proved that inflammation caused by deposition of MSU crystals in joints has become one of the main causes of AGA.²² At present, anti-inflammatory drugs, colchicine, adrenocortical

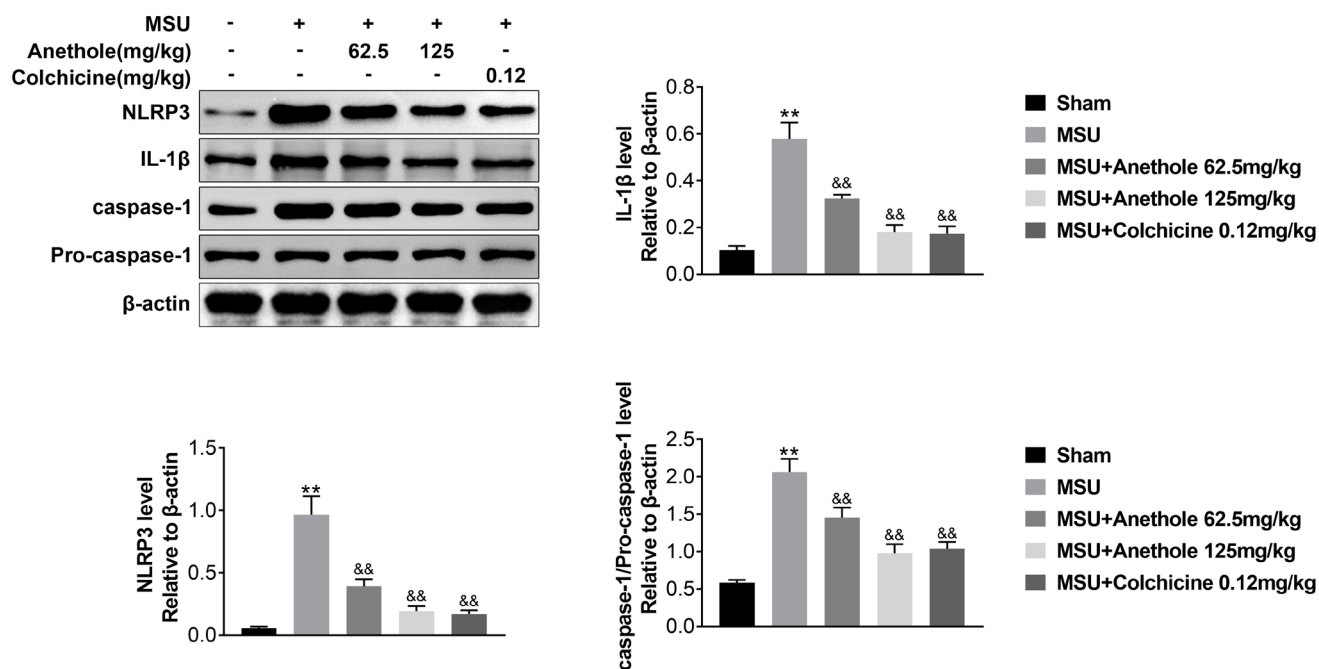


Figure 3 Anethole inhibited the activation of NLRP3 inflammasome in MSU-induced AGA mice. Western blot analysis demonstrated the effects of anethole on the expression levels of NLRP3, IL-1 β , caspase-1, and pro-caspase-1 in MSU-induced AGA mice. All experiments were repeated for three times. Data were presented as mean \pm SD. MSU vs. Sham group, ** $P < 0.01$. MSU vs. MSU + anethole (62.5 mg/kg and 125 mg/kg) or MSU + colchicine group, $^{##}P < 0.01$.

hormones, and other biological agents are the main medicines used to treat AGA. However, the long-term use of these drugs leads to serious adverse reactions and drug resistance as well as lack of safety and complications in patients.²³ Growing evidences have revealed that traditional Chinese medicines are potential medications having low toxicity and could be promising agents in the treatment of AGA.² In this study, we found a useful agent anethole, which had the potential to inhibit inflammation, and demonstrated that it exerted anti-inflammatory effects in MSU crystals-induced AGA.

Hyperuricemia (elevated uric acid level) is the largest single risk factor for AGA, and MSU crystals are deposits of uric acid, which induces inflammation and is closely associated with pathological injury in AGA.²⁴ By H&E staining and measuring the degree of ankle swelling, we discovered that MSU crystals significantly induced joint tissue injury in mice. However, anethole displayed alleviating effects on joint injury in MSU crystals-induced mice. Anethole's protective effects appeared in a concentration and time-dependent manner. It was reported that the secretion of pro-inflammatory factors played a significant role in the development of AGA, but anethole possessed multiple pharmacological effects, including anti-inflammatory and anti-oxidative effects.^{9,25} We established through ELISA that anethole had stronger anti-inflammatory effects and could decrease the expression levels of inflammatory cytokines (i.e., IL-1 β , IL-6, IL-8, TNF- α , and MCP-1) caused by the stimulation of MSU crystals. NLRP3 inflammasome had a crucial role in AGA inflammation.²⁶ Studies demonstrated that MSU crystals precipitated in joint tissues and induced the activation of NLRP3 inflammasome, resulting in the secretion of proinflammatory cytokine IL-1 β and release of various cytokines.²⁷ In this

study, the Western blot analysis revealed that anethole could suppress the activity of NLRP3 inflammasome and down-regulate the expression levels of NLRP3, IL-1 β , caspase-1, and pro-caspase-1 in a concentration-dependent manner in MSU-induced AGA mice. However, the precise inhibitory mechanism of anethole needs to be further studied.

Recently, innate immunity has been proved to be directly involved in the development of AGA, and the recognition and phagocytosis of MSU crystals lead to the activation and signal transduction of intracellular TLRs/MyD88/NF- κ B signaling pathways and regulation of inflammatory cytokines, such as IL-6 and IL-1 β .¹⁶ Therefore, suppressing the activation of TLRs/MyD88-mediated inflammatory pathways may contribute to the treatment of AGA. For example, Abdollahi-Roodsaz et al. reported that TLR2 and TLR4 were involved in the pathogenesis of MSU crystals-induced AGA, and down-regulation of TLR2 and TLR4 would suppress inflammation and alleviate joint injury in AGA.²⁸ Wang et al. reported that steroidal saponin, a bioactive compound extracted from the rhizome of *Dioscorea collettii*, could attenuate MSU-induced inflammation by reducing the release of cytokines by blocking the activation of TLR4/NF- κ B pathway.²⁹ In addition, Ouyang et al. demonstrated that active flavonoids from *Lagotis brachystachya* significantly inhibited the progression of MSU crystals-induced AGA by restraining the activation of TLR4/MyD88/NF- κ B pathway and NLRP3 inflammasome activity.³⁰ In this study, we established that anethole could down-regulate the expression levels of TLR2, TLR4, MyD88, p-NF- κ B, and NF- κ B, and displayed an inhibitory effect on the activation of TLRs/MyD88 signaling pathway in MSU-induced AGA mice. The results of this study, which are consistent with the results of previous studies, established that anethole

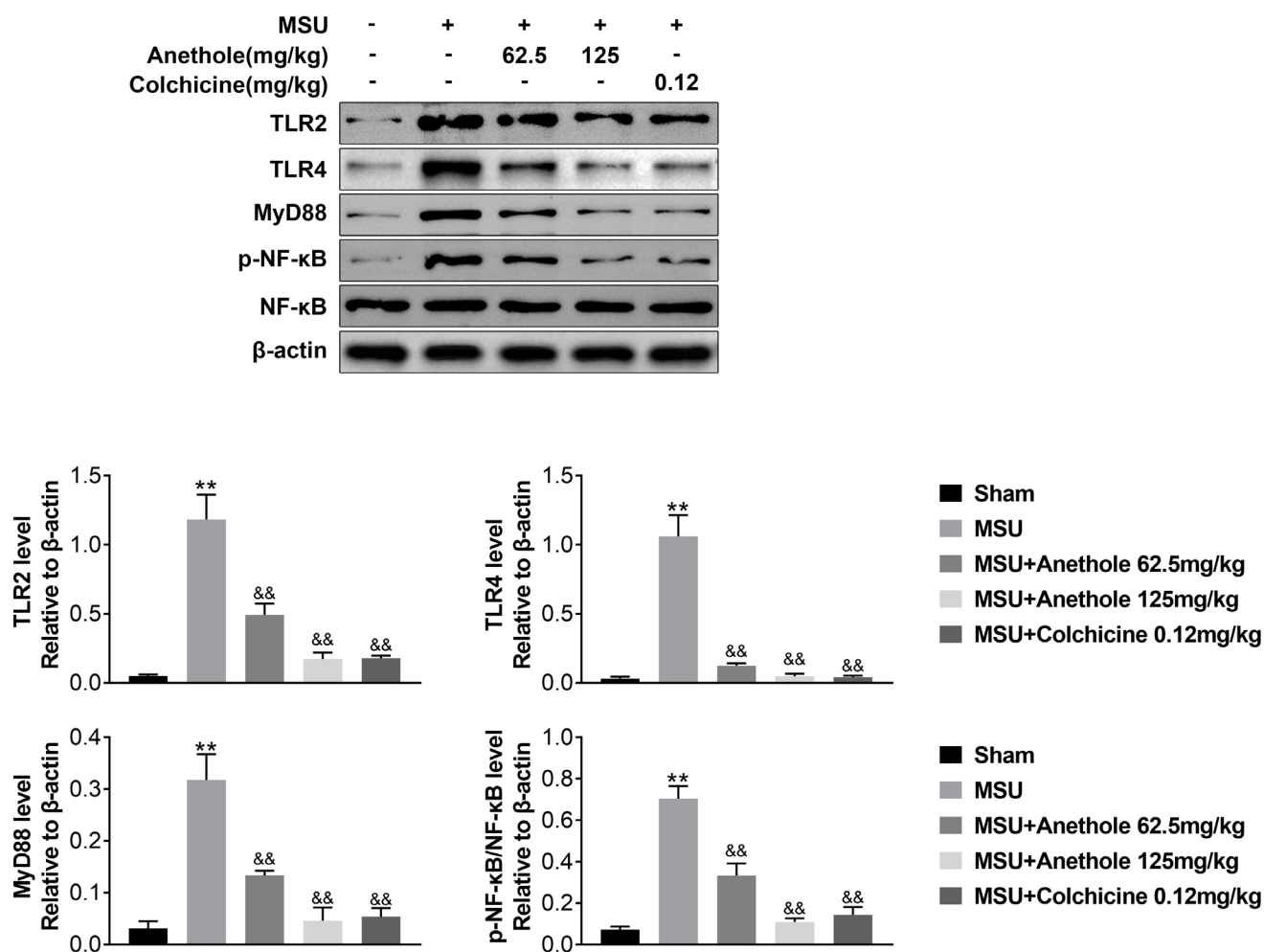


Figure 4 Anethole inhibited the activation of TLRs/MyD88 signaling pathway in MSU-induced AGA mice. Western blot analysis demonstrated the effects of anethole on the expression levels of TLR2, TLR4, MyD88, p-NF-κB, and NF-κB in MSU-induced AGA mice. All experiments were repeated for three times. Data were presented as mean ± SD. MSU vs. Sham group, **P < 0.01. MSU vs. MSU + anethole (62.5 mg/kg and 125 mg/kg) or MSU + colchicine group, ^{##}P < 0.01.

is a promising agent for inhibiting the activation of TLRs/MyD88 pathway and could serve as a therapeutic target for AGA treatment.

Conclusions

In summary, we established an MSU-induced AGA animal model and explored the therapeutic effects of anethole on MSU-induced AGA mice. The *in vivo* study confirmed that anethole alleviated joint injury by decreasing the release of cytokines, such as IL-1β, IL-6, IL-8, TNF-α, and MCP-1, and inhibiting the activation of TLRs/MyD88 and NLRP3 inflammasome pathways in MSU-induced AGA mice. Therefore, we confirmed that anethole could be used as a potential agent for treating AGA.

Funding

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Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Data Availability

The authors declared that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Author Contributions

All authors contributed to the conception and designing of the study. Material preparation and experiments were performed by Yuepeng Cao and Qin Zhong. Data collection and analysis was done by Fang Tang, Xueming Yao, and Xiaodong Zhang. The first draft of the manuscript was prepared by Zhengqi Liu, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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