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Research Article
Anti-Inflammatory and Analgesic Effects of Chinese Medicine TongFengKang in the Treatment of Gouty Arthritis

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Abstract

Background and Objective: TongFengKang (TFK) is a traditional Chinese medicine prescription for the clinical treatment of acute gouty arthritis. However, the biochemical mechanism of TFK in treating acute gouty arthritis is still unknown. In this study, the anti-inflammatory and analgesic effects of TFK on acute gouty arthritis model rats was investigated and explored its underlying mechanism. **Materials and Methods:** The rat model of acute gouty arthritis was established by injecting MSU into the ankle joint, followed by observing the anti-inflammatory and analgesic effects of TFK and studying its mechanism. The anti-inflammatory activity was evaluated by ankle swelling measurement, NLRP3 inflammatory body and inflammatory cytokine expression. The analgesic effect was evaluated by observing pain-like behavior, establishing writhing, hot plate and formaldehyde pain models and detecting the expression level of PGE2. Western blot was used to examine the protein expression of NLRP3. The levels of PGE2 in serum were measured by Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** Compared with the model group, TFK can significantly inhibit ankle swelling, improve painful gait and relieve the symptoms of acute gout attack. Meanwhile, TFK significantly reduced the protein levels of NLRP3 in rat ankle joints and the production of PGE2 in serum, respectively. In addition, in the writhing, hot plate and formaldehyde pain model rats, the peripheral analgesic effect of TFK is equivalent to that of a non-steroidal anti-inflammatory drug aspirin and the central analgesic effect of TFK is significantly stronger than that of aspirin, confirming that TFK has satisfactory analgesic effects. **Conclusion:** The TFK has reliable anti-inflammatory and analgesic effects on gouty arthritis, probably through inhibiting the NLRP3 inflammatory body and downstream proinflammatory cytokines, which suggests that TFK is a promising herbal formula for the clinical treatment of gouty arthritis.

Key words: Traditional Chinese medicine compound, gouty arthritis, hyperuricemia, immunoinflammatory factor, analgesic effect

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gout is a common metabolic rheumatism. It is a group of clinical syndromes with long-term purine metabolic disorders or decreased uric acid excretion and tissue damage caused by increased levels of blood uric acid. Hyperuricemia is the most important biochemical cause[1]. The clinical manifestations of gout include recurrent inflammatory arthritis, metabolic hyperuricemia and peripheral urate crystal deposition. In the process of disease development, joint stiffness, deformity or function damage may occur in the affected joint, accompanied with renal function damage. Severe hyperuricemia may be accompanied by hyperlipidemia, hypertension and diabetes, leading to coronary atherosclerosis, thus inducing cardiovascular and cerebrovascular diseases. The incidence and severity of gout ranged from 1-4 to 0.1-0.3%, respectively. Gout is more common in men than women, with a ratio of 10.1:3.1[2]. In recent decades, the incidence rate and prevalence of gout in population over 80 years old increased markedly, reaching 11-13 and 0.4%, respectively[3].

The NLRP3 inflammatory body is a kind of polycytoplasmic protein complex, belonging to the Nucleotide-binding Oligomeric Domain-like Receptor (NLRS) family[4]. It is composed of NLRP3 scaffold, apoptosis-related mottled protein containing CARD (ASC) and effector Protein Cysteine Aspartate Specific Proteinase-1 (Caspase-1). It has been documented that NLRP3 inflammatory body is the key link in the reaction process of gouty arthritis[5]. Within the NLRP3 domain, PYD recruited ASC and the precursor caspase-1 to assemble the NLRP3 inflammatory body and induce caspase-1 to autolysis and mature. Activated caspase-1 promotes pro-IL-1β cracking to IL-1β, which would be secreted outside cells to achieve various immune responses[6]. The IL-1β can activate the release of other proinflammatory cytokines and pain mediators, such as TNF-α, PGE2, etc.[7,8]. The above process is crucial to the occurrence and development of gouty arthritis inflammation. At present, colchicine, febuxostat, benzbromarone, non-steroidal anti-inflammatory drugs and allopurinol are commonly used for the routine treatment of gouty arthritis in clinics[9]. However, increasing clinical observations have revealed that these drugs process certain toxic and side effects, such as abnormal stimulating effects on the digestive tract, hematopoietic system and nervous system, on the other hand, their relieving effects on gout pains are short and their long-term effects are quite poor[10-12]. In particular, colchicine has a narrow therapeutic index and no clear distinction between non-toxic dose, toxic dose and lethal dose, which has caused serious confusion in clinical practice[13].

Traditional Chinese medicine (TCM) has been widely used to treat various pathological conditions and complications of gouty arthritis. In recent years, both domestic and international experimental research and clinical application showed that TCM has advantages over Western medicine in the prevention and treatment of gouts, such as definite curative effect, fewer side effects, easy acceptance and ideal overall curative effect[14,15]. TongFengKang (TFK) is a Chinese decoction based on the long-term clinical experience in the treatment of gout and the simplification and development of TCM. The TFK has been authorized by the People's Republic of China for an invention patent (patent number: 201610960459.1).

This study investigated the anti-inflammatory and analgesic effects of TFK through the use of a rat acute gouty arthritis model and several rat pain models. The effects of TFK on the NLRP3 inflammatory body were also explored in the present study, which is helpful in clarifying its pharmacological mechanism.

Study area: The study was carried out in the Key Laboratory of Syndrome Differentiation and Treatment of Gastric Cancer of the State Administration of Traditional Chinese Medicine, in Medical College of Yangzhou University since February, 2023 to August, 2023.

MATERIALS AND METHODS

Laboratory animals: A total number of 50 Wistar rats (200±20 g b.wt.) were purchased from Animal Center of Comparative Medicine, Yangzhou University. All rats were adapted to the dark cycle for 12 hrs in the environment of 22±2 and 55±10% relative humidity for a week and freely supplied with sufficient food and water. These animals were treated and nursed in accordance with the guidelines for the management of experimental animals issued by the National Science and Technology Commission of the People's Republic of China. All experimental procedures were approved by the Ethics Committee of Medical College, Yangzhou University.

Experimental Chinese medicine and reagents

Experimental medical herbs: All materials have been fully validated using *Smilax glabra* Roxb (specification: 1 kg, manufacturer: Anhui Huilong Chinese Medicine Decoction Pipe Co. Ltd., Production Batch No.: 21021, origin: Guangxi), *Euonymus alatus* (Thunb.) Siebold (Specifications: 1 kg, manufacturer: Anhui Huilong Traditional Chinese Medicine Decoction Pieces Co. Ltd., Batch No: 201101, origin: Hubei), *Coix lacryma-jobi var. ma-yuen* (Rom.Caill.) Stapf

(Specifications: 1 kg, manufacturer: Qiyitang Pharmaceutical (Tianjin) Co. Ltd., Batch No. 596210701, origin: Guizhou), *Cynanchum paniculatum* (Bge.) Kitag (Specifications: 1 kg, Manufacturer: Anhui Huilong Traditional Chinese Medicine Decoction Pipe Co. Ltd., Batch No. 210501, Origin: Shandong).

Reagents: Colchicine (positive control drug) (specifications: 0.5 mg, manufacturer: Xishuangbanna Pharmaceutical Co. Ltd., Batch No. 210825), normal saline (NS) (specifications: 250 mL, manufacturer: Sichuan Kelun Pharmaceutical Co. Ltd.), sodium urate crystal (MSU) (specifications: 15 mg, manufacturer: Sigma, Cat#: U2875), formaldehyde solution (specifications: 500 mL, manufacturer: Sinopharm Chemical Reagent Co. Ltd., Cat#: 10010018), acetic acid (specifications: 500 mL, Manufacturer: Sinopharm Chemical Reagent Co. Ltd., Cat#: 10000218), anti-NLRP3 antibody (specifications: 50 µL, manufacturer: Proteintech, Cat#: 27458-1-AP), anti-caspase-1 antibody (specifications: 50 µL, manufacturer: Boster Biological Technology, Cat#: BM4291), anti-ASC/TMS1 antibody (specifications: 50 µL, manufacturer: ABclonal Technology, Cat#: A22046), anti-β-actin antibody, HRP labeled sheep anti-rabbit second antibody (manufacturer: Thermo Fisher Scientific, USA, Cat#: PA146296, A18865), PGE2 ELISA Kit (specifications: 96T, manufacturer: Beijing Solarbio Science and Technology Co. Ltd. Cat#: SEKR-0053).

Preparations of TFK: The TFK was composed of *Smilax glabra* Roxb (Tu Fu Ling, 30 g), *Euonymus alatus* (Thunb.) Siebold (Gui Jian Yu, 30 g), *Coix lacryma-jobi var. ma-yuen* (Rom. Caill.) *Stapf* (Yi Yi Ren, 30 g), *Cynanchum paniculatum* (Bge.) *Kitag* (Xu Chang Qing, 30 g). Aqueous extract of TFK was prepared in the preparation room of Yangzhou Hospital of Traditional Chinese Medicine. The liquid was collected and stored at 4°C for future use. The filtered TFK decoction was concentrated with a rotary evaporator (RE-3000B, Shanghai Yarong, China) and the final concentration of the medicine was 1 g mL^{-1}.

Induction of gouty arthritis with MSU crystals and administration

MSU crystal preparation: The MSU crystals were prepared by dissolving sodium urate (800 mg) in boiling ultra-pure water (245 mL) containing NaOH (5 mL). After adjusting the pH value to 7.2, the solution was stirred and gradually cool to room temperature and then centrifuged (3000 rpm, 10 min, 4°C). After evaporation and crystallization, the crystals were collected, followed by being heated at 180°C for 2 hrs for disinfection. Needle-like MSU crystals were stored in sterile microtubules and suspended in sterile saline to prepare sodium urate solution (5 mg mL^{-1}) when use.

Animal grouping and drug administration: After 7 day acclimatization, all rats were randomly divided into the following six groups (n = 6 per group): (1) Control group, administration with the same volume of ultra-pure water, (2) model group, administration with the same volume of ultra-pure water, (3) Low-dose TFK group, oral gavage with 5.0 g kg^{-1} TFK per day, (4) Middle-dose TFK group, oral gavage with 10.0 g kg^{-1} TFK per day, (5) High-dose TFK group, oral gavage with 20.0 g kg^{-1} TFK per day and (6) Positive control group, oral gavage with 0.3 mg kg^{-1} colchicine per day. All rats were administrated at the same time for 7 consecutive days.

MSU crystal-induced gouty arthritis in animals: After the administration on day 5, all rats were anesthetized by isoflurane inhalation and then fixed in supine position. After 3-5 min of anesthesia, a needle was inserted into the medial side of the achilles tendon at the direction of 30-45° behind the right ankle joint of each rat. After reaching the ankle joint cavity, 0.1 mL sodium urate solution (5 mg mL^{-1}) was injected into the cavity (except the control group), taking the contralateral eminence of joint capsule as the injection standard. In the control group, 0.1 mL sterile saline was injected instead. Model evaluation was carried out 1 and 2 hrs after injection. When the joints showed obvious swelling, skin redness, pain and other inflammatory reactions, the model was successfully established.

Measurement of ankle swelling: At 2, 6, 12, 24 and 48 hrs after injection of MSU crystals, the degree of inflammatory reaction was quantified by measuring the ankle swelling of each rat using a digital caliper (BM770150, Morcato, Germany). The results were expressed as the measured value of the maximum diameter and circumference of affected joint.

Behavioral assessment of pain response: The joint dysfunction index and pain response behavior were evaluated by the gait score. Gait of the right leg were observed at 6, 12, 24 and 48 hrs after the establishment of the model. The indicators are as follows: Level 0: Normal without claudication, Level 1: Slight claudication, slight bending of the tested joint, Level 2: The tested joint is obviously bent, can only touch the ground and obviously limps and Level 3: The tested joint is completely off the ground, with severe claudication and three-legged gait. The above behavioral assessment was based on Coderre classification[16].

Expression of NLRP3 inflammasome in the ankle joints: The rats in each group were anesthetized and dissected on the seventh day. The ankle joints were collected and ground into powders using liquid nitrogen. The ankle joints samples were mixed with 10 equivalent volumes of RIPA lysis buffer added with 1 mm PMSF (a protease inhibitor). After homogenized in an ice bath for 60 min, the mixture was centrifuged (13,000 rpm, 4°C, 15 min) to extract total proteins. BCA protein assay kit (Beyotime Biotechnology, Shanghai, China) was used to determine the concentration of extracted proteins. Mixed the tissue proteins with a 5× loading buffer and denature it in a metal bath at 100°C for 5 min. Tissue protein (40 μg) was separated by 10/12% SDA-PAGE gel electrophoresis and then were transferred to PVDF membranes (Merck, Germany, 0.45 μm, 26.5 cm×3.75 m). The membranes were blocked with 5% skimmed milk dissolved in PBST (0.05% Tween 20) at room temperature for 1 hr and then incubated with a primary antibody overnight at 4°C. On the second day, after washing with TBST, the membranes were incubated with the corresponding secondary antibody (1:10000) at room temperature for 2 hrs. Protein bands were detected by Universal Hood III gel imaging analyzer (Bio Rad, USA, v4.1). The results of Western blot were expressed by performing and analyzing gel quantification with ImageJ software (v2.3.0/1.53 t). The primary antibodies used in this study included NLRP3, Caspase-1 and ASC with a dilution ratio of 1:1000.

Measurement of inflammatory cytokines: Prostaglandin E2 is arachidonic acid, a lipid mediator produced by enzyme metabolism and a common inflammatory factor in the body. It plays an important role in inflammatory pain by acting on EP receptors[17].

In order to evaluate the pain inhibition effect of TFK on the gouty arthritis model rats, the levels of inflammatory cytokines was measured. Blood was taken from the abdominal aorta before the rats were sacrificed and the peripheral blood sample of each rat was then centrifuged at 3000 rpm for 15 min at 4°C. The upper serum was transferred to a 1.5 mL micro-centrifuge tube and then stored for use at -20°C. According to the manufacturer's protocol, the levels of PGE2 in the serum was measured using the Enzyme Linked Immunosorbent Assay (ELISA) kit (Beijing Solarbio Science and Technology Co. Ltd.). The absorbance reading is 450 using a microplate reader.

Establishment of pain model in rats and pharmacological intervention

Pain model caused by hot-plate methods: After 7 day acclimatization, 50 female rats were selected for administration. The rats were placed on a hot plate at 55±0.2 and then the time from placing on the hot plate to the first licking of each rat's feet was recorded as the normal pain threshold. The rats with the pain threshold <5 or >30 sec and jumping were discarded. The pain threshold of qualified rats was measured once more and the average value of the 2 times was taken as the pain threshold of each rat.

Due to the instability of animal pain behavior, we increased the sample size to reduce errors, increasing from 6 per group to 10 per group. The eligible rats were randomly divided into the following groups (n = 10 per group): (1) Control group, oral gavage with equivalent amount of ultra-pure water, (2) Low-dose of TFK group, oral gavage with 5.0 g kg^{-1} TFK per day, (3) Middle-dose of TFK group, oral gavage with 10.0 g kg^{-1} TFK per day, (4) High-dose of TFK group, oral gavage with 20.0 g kg^{-1} TFK per day and (5) Positive control group, oral gavage with 30.0 mg kg^{-1} aspirin per day. Each group rats were continuously administered for 5 days. The pain threshold of rats was measured again at the 30, 60 and 90 min after the last administration and the changes of the pain threshold of the rats in each group were compared before and after the administration.

Pain model caused by acetic acid twisting methods: Rats were grouped as mentioned above in 2.9.1. and each group were continuously administered for 5 days according to the dosage. After 30 min of the last administration, 0.9% acetic acid (2 mL/200 g) was injected into the abdominal cavity. The number of times of body twisting reactions (abdominal contraction into "S" shape, body twisting, hind limb extension and peristalsis) were recorded within 20 min[18]. The analgesic effect of the drug was evaluated by calculating the inhibition rate of the drug on the writhing reaction according to the following formula:

$$\frac{\text{Average times of twisting in control group} - \text{Average times of twisting in administration group}}{\text{Average times of twisting in administration group}} \times 100\%$$

Adjuvant arthritis model caused by formaldehyde: Formaldehyde-induced hind paw licking was performed according to the method described by Hunskar and Hole[19]. Rats were grouped as mentioned above in 2.9.1. and each group were continuously administered for 5 days according to the dosage. After 90 min of the last administration, 1.0% formaldehyde 30 μL was injected subcutaneously into the right hind foot of each rat to build an adjuvant arthritis model. After the model was successfully constructed, the rat was immediately placed in a transparent container and the licking and biting time of the feet in the phase I reaction (0-5 min)

and phase II reaction (15-30 min) were monitored and recorded. The cumulative time of each phase was used as an indicator of the intensity of the animal's pain response.

Statistical analysis: Each experiment was repeated at least three times. The data were analyzed by one-way analysis of variance using SPSS 16.0 (SPSS Inc., Chicago, Illinois). The data were shown as the Means±Standard deviations. The $p<0.05$ indicates statistically significant differences.

RESULTS

Ingredients in TFK were analyzed by LC-HRMS: The components in TFK were analyzed and identified using liquid chromatography-high-resolution mass spectrometry (LC-HRMS) and the attribution of each component in TFK was preliminarily determined. A total of 18 compounds were identified, including (-)-Epicatechin-3-(3',4'"-O-dimethyl) gallate, 4'-Hydroxyacetophenone, 4-Methoxybenzoyl acetic acid, Acetovanillone, Astilbin, Neoisoastilbin, Isoastilbin, Engeletin, Neoisoengeletin, Afzelin, Isoengeletin, Quercetin, Demethylwedelolactone, Luteolin, trans-4-Hydroxy-2-nonenoic acid, Naringenin, Kaempferol and Paeonol as shown in Fig. 1.

Effect of TFK on MSU crystal-induced ankle swelling: As is shown in Fig. 2a, compared with the model group, the degree of joint redness and swelling of rats after the TFK treatment is significantly decreased. Within 2 hrs after the establishment of the model, the ankle joints of the experimental rats injected with MSU were significantly swollen compared with these of the control group. There was no significant difference between the MSU-induced groups. The maximum value was reached at about 6 hrs, but gradually decreased from 12 to 48 hrs. Compared with the model group, different doses of TFK can reduce ankle swelling to varying degrees Fig. 2(b-c), especially the middle and high-dose TFK significantly reduced the degree of ankle swelling, the swelling degree of ankle joint is equivalent to that of the colchicine (positive control) group. These data indicated that TFK is able to effectively reduce the ankle swelling in rats caused by the injection of MSU crystals and the therapeutic effect of TFK is similar to that of colchicine, which confirms its clinical use.

TFK relieved pain gait in rats with gouty arthritis: The MSU-induced arthritis rat model was replicated to observe the effects of Res on the gait score to assess the behavioral impact of joint pain. At 12, 24 and 48 hrs after the MSU induction in rats, both TFK and colchicine improved the gait scores, indicating that they inhibited pain and improved joint function to a certain extent (Fig. 3). These results showed that the gait scores of the middle-dose TFK and colchicine groups decreased faster than other groups ($p<0.001$), additionally, no significant difference was detected between the middle-dose TFK and colchicine groups ($p>0.05$). Furthermore, after 48 hrs middle-dose TFK and colchicine treatment, only slight

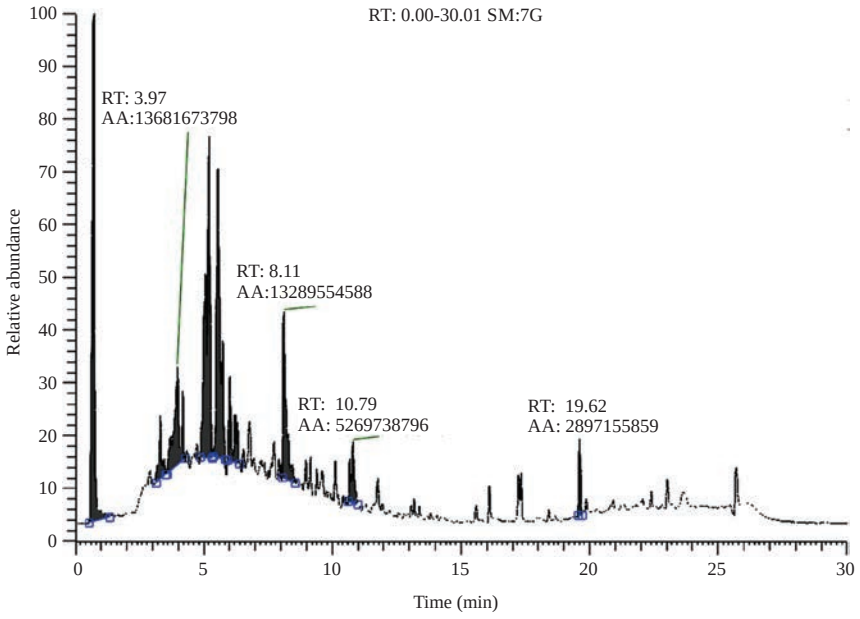

Fig. 1: Total ion chromatograms (TIC) of TFK in positive mode

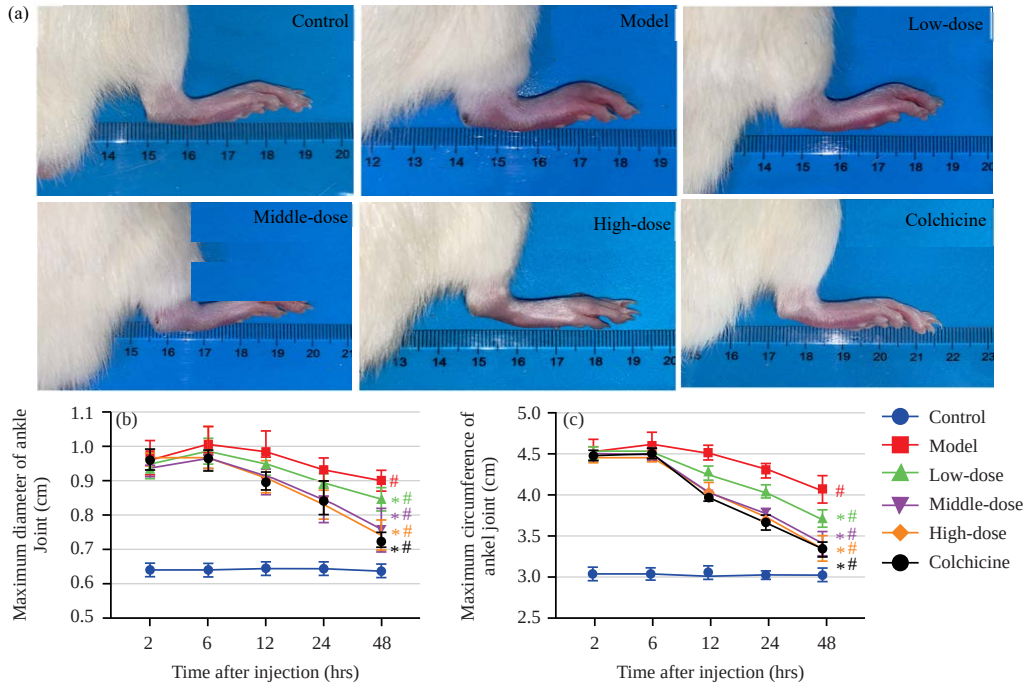

Fig. 2: Effect of TFK on MSU (a) Photographs of the ankles of the rats after the TFK administration, (b) Maximum diameter of ankle joint after modeling and (c) Maximum circumference of ankle joint after modeling

*p<0.05, compared to model group, #p<0.05, compared to control group (n = 6 per group), one-way analysis of variance is used and Dunnett's Test is used for pairwise comparison

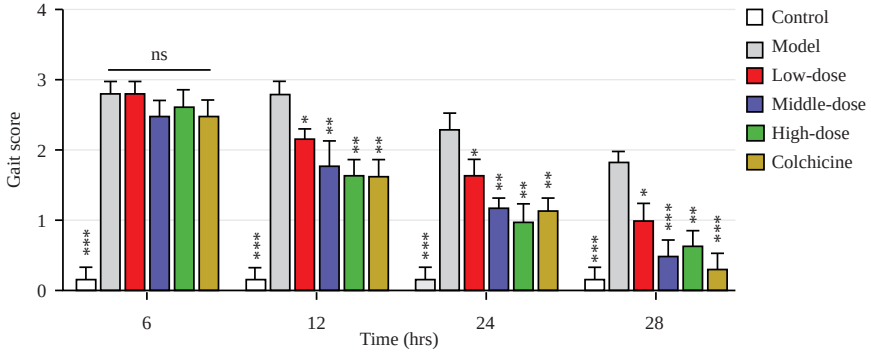

Fig. 3: Gait scores of the rats with acute gouty arthritis at 6, 12, 24 and 48 hrs after modeling

*p<0.05, **p<0.01 and ***p<0.001, compared to model group (n = 6 per group) and One-way analysis of variance is used and Dunnett's Test is used for pairwise comparison

claudication and joint dysfunction were observed, which indicated a better curative effect Fig. 4.

TFK increased rat latency time to respond to heat-induced pain: Hot plate test is a common method for screening central analgesics. The first licking of the paw was recorded as the withdrawal latency. The increase of the time interval of withdrawal latency indicates the analgesic activity. As 20 to further study the effect of TFK on central analgesia, we used the reaction time of licking feet on the hot plate 55±0.2 as a reference for the pain threshold of the rats to thermal stimulation pain. After 30, 60 and 90 min of the administration, compared with the control group, different doses of TFK significantly increased the pain threshold. The pain threshold reached the maximum level 60 min after the administration. Especially in the middle-dose TFK group, the percentage increase of the pain threshold was higher than that of the classic non-carrier anti-inflammatory drug aspirin, indicating that TFK had a significant central analgesic effect.

Analgesic effect of TFK on acetic acid writhing in rats: In order to confirm the analgesic mode of TFK, we further use an

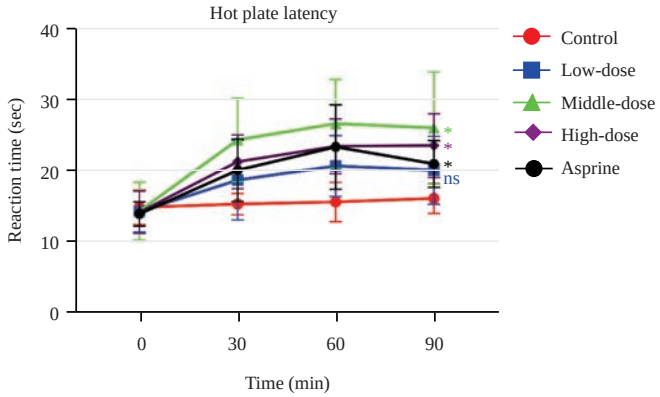

Fig. 4: Pain reaction time of licking feet caused by thermal stimulation on the hot plate after 30, 60 and 90 min of the TFK administration

*p<0.05, compared to control group (n = 10 per group), one-way analysis of variance is used and Dunnett's test is used for pairwise comparison

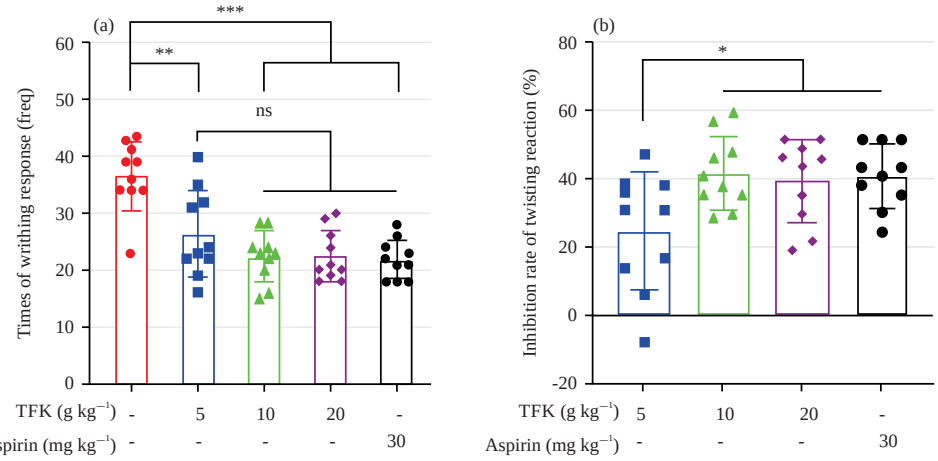

Fig. 5(a-b): (a) Total number of acetic acid writhing reaction in each group, (b) Inhibition rate of acetic acid writhing reaction in administration groups

*p<0.05, **p<0.01 and ***p< 0.001 (n = 10 per group), one-way analysis of variance is used and Dunnett's test is used for pairwise comparison

acetic acid writhing method to examine whether TFK also has an effect on peripheral analgesia. Inflammatory pain caused by 0.9% acetic acid injection leads to writhing reaction in rats, which is a classic peripheral pain model. As is shown in Fig. 5, TFK significantly prolonged the latency of body twisting in rats and significantly reduced the number of body twisting, compared to the control group (Fig. 5a, **p<0.01 and ***p<0.001). In terms of the twisting reaction inhibition rate, the middle- and high-dose TFK administration exerted slightly better effects than that of the low-dose TFK administration, which is equivalent to that of aspirin (Fig. 5b and *p<0.05).

Analgesic effect of TFK on adjuvant arthritis induced by formalin: To further verify the anti-inflammatory and analgesic effects of TFK, established a model of adjuvant arthritis pain induced by formaldehyde was established. The results in Fig. 6(a-b) showed that different doses of TFK could reduce the phase I and phase II pain response in rats and the analgesic effects of the middle- and high-dose TFK groups were particularly significantly better than that of aspirin. Therefore, it is suggested that the analgesic mechanism of TFK is related to both peripheral and central analgesia, which is consistent with the results of the above two pain models.

TFK inhibited the expression of NLRP3 inflammasome in ankle joint: The NLRP3 Inflammasome has been proposed as an important target of gouty arthritis. In this study, Western blot experiments were conducted on three target proteins including NLRP3, Caspase1 and ASC, to confirm whether TFK regulates the activation level of NLRP3 inflammasomes (Fig. 7a).

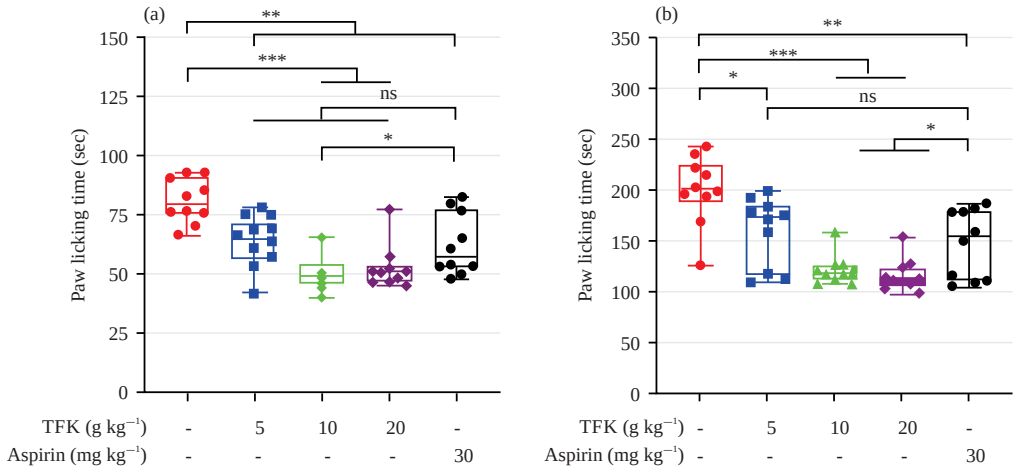

Fig. 6(a-b): Licking reaction time caused by formaldehyde, (a) Phase I reaction time (0-5 min) of acute inflammatory pain and (b) Phase II reaction time (15-30 min) of acute inflammatory pain

*p<0.05, **p<0.01 and ***p<0.001 (n = 10 per group), one-way analysis of variance is used and Dunnett's Tests is used for pairwise comparison

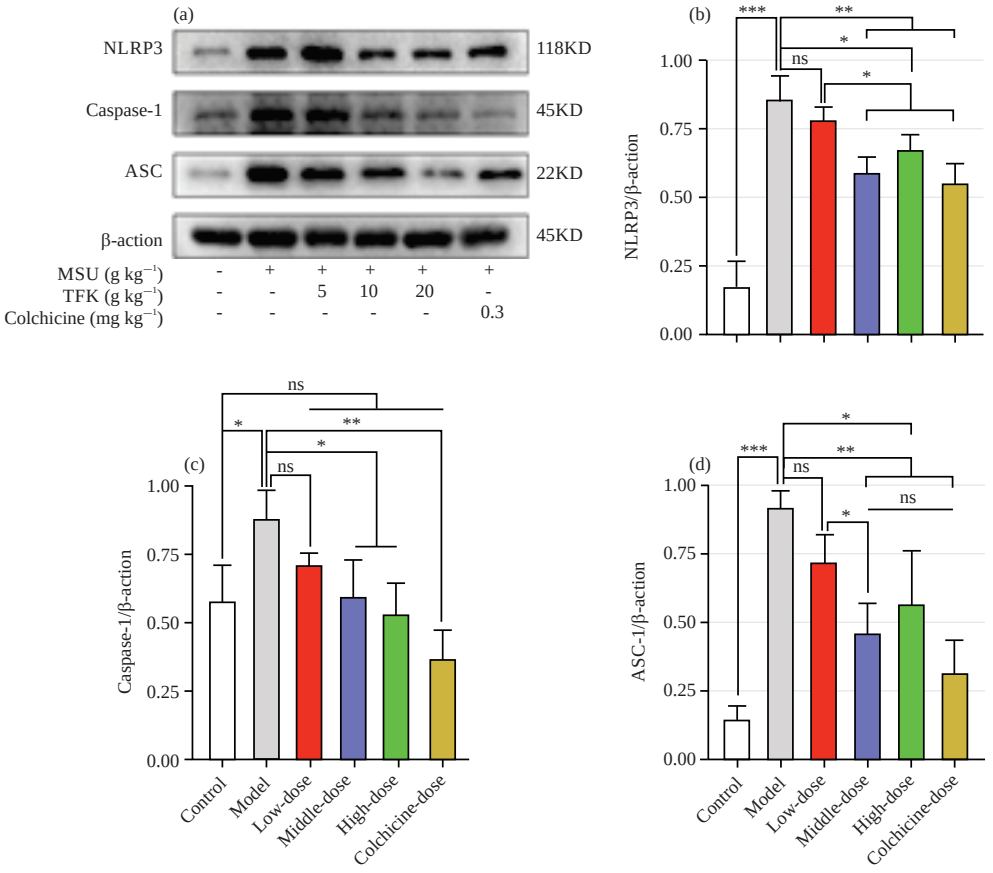

Fig. 7(a-d): Western blot detection of TFK inhibiting the expression of NLRP3 inflammasome in ankle joint, (a) Expression of NLRP3, Caspase-1 and ASC in the ankle joints of the MSU crystal-induced rats were determined by Western blot and (b-d) Protein expressions were normalized to β-actin

*p<0.05, **p< 0.01 and ***p<0.001 (n = 6 per group), one-way analysis of variance is used and Dunnett's Tests is used for pairwise comparison

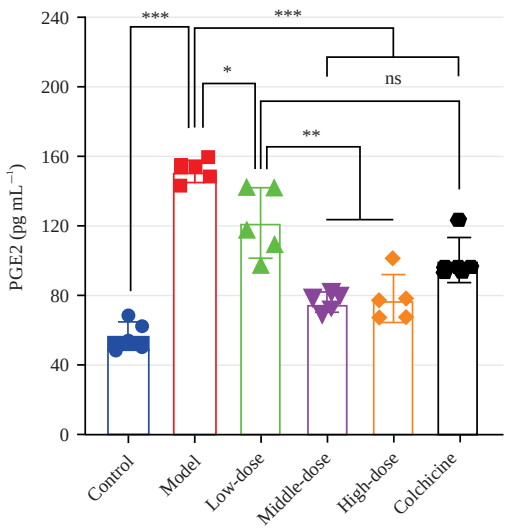

Fig. 8: Serum PGE2 levels of each MSU crystal-induced rat group after the TFK administration

*p<0.05, **p< 0.01 and ***p<0.001 (n = 6 per group), one-way analysis of variance is used and Dunnett's Test is used for pairwise comparison and Colchicine: Positive control

The expression of NLRP3, Caspase-1 and ASC in the ankle joints were significantly increased in the experimental model of gouty arthritis compared with those of the control group, which indicates that NLRP3 inflammasome was activated in the rat model of gout arthritis. Furthermore, compared with the model group, the low-dose TFK did not significantly reduce the levels of the three cytokines (p>0.05). The middle-dose TFK, high-dose TFK and positive control groups showed obvious inhibition of NLRP3, Caspase-1 and ASC, no significant difference was detected among them (*p<0.05 and **p< 0.01). This shows that the middle and high dose of TFK can inhibit the activation of NLRP3 inflammasome in the ankle joint Fig. 7(b-d). It has been revealed that the potential anti-inflammatory mechanism of TFK may be through inhibiting the assembly and activation of NLRP3 inflammasomes, affecting the maturation and activation level of Caspase-1, thereby regulating downstream inflammatory factors.

Effects of TFK on prostaglandin E2 production: Prostaglandin E2 (PGE2) is the main inflammatory mediator of rheumatoid arthritis and osteoarthritis, which is closely related to inflammatory pain. As shown in Fig. 8, elevated serum levels of PGE2 (p<0.001) was found in all MSU crystal-induced rats. Compared to the model group, the TFK treatment led to a decrease in serum PGE2 levels (p<0.01). The reduction of the PGE2 levels in the low-dose group was similar to that of the colchicine group, without statistical difference between the two groups. However, the middle- and high-dose TFK administration is superior to the low-dose TFK and colchicine administration in this respect. The decrease in the serum PGE2 levels indicated that TFK had a significant analgesic effect on gouty arthritis and the middle- and high-dose TFK is superior to colchicine.

DISCUSSION

The present study further studied the anti-inflammatory and analgesic effects of TFK and explored its potential mechanism. In this study, the effects of TFK on an MSU-induced gouty arthritis rat model were first tested. It was found that TFK can effectively inhibit the degree of swelling of affected joints and inhibit local inflammatory reaction, indicating that TFK has satisfactory anti-inflammatory and detumescence effects. Next, through observing the pain behavioral characteristics of rats with gouty arthritis, it was found that TFK can significantly reduce their pain gait and improve pain-like behavior, suggesting that TFK has a good analgesic effect. In order to further understand the analgesic effect of TFK and explore its potential action mechanism, we established three pain models and used chemical stimulation, thermal stimulation and inflammatory stimulation to reveal the inhibitory effect of TFK on different types of pain stimuli through observing the corresponding pain responses of rats. The experimental results showed that TFK significantly inhibited different types of pain, displaying a satisfactory analgesic effect and its action mechanism might be related to peripheral and central analgesia. Lastly, the NLRP3 inflammatory body in the ankle joints of the rats with gouty arthritis was examined. It was found that TFK could effectively inhibit its expression and significantly reduce the levels of the downstream inflammatory factors, which further confirmed the anti-inflammatory and immune effects of TFK. Taken together with the effective control of TFK on clinical symptoms of gouty arthritis in this study, TFK is believed to have great potential for the treatment of gouty arthritis in the future.

The TCM decoction TFK investigated in this study is based on the long-term clinical treatment of gouty arthritis. *Smilax glabra* Roxb (Tu Fu Ling) is a kind of plant rhizome with the functions of relieving pain, reducing uric acid, clearing damp and promoting diuresis. *Euonymus alatus* (Thunb.) Siebold (Gui Jian Yu) plays a role in detoxifying, detumescence, promoting blood circulation and removing

stasis. *Coix lacryma-jobi var. ma-yuen* (Rom. Caill.) Stapf (Yi Yi Ren) has the effect of reducing swelling, clearing damp and removing arthralgia. *Cynanchum paniculatum* (Bge.) Kitag (Xu Chang Qing) possesses the functions of detoxification, wind-dispelling and dehumidification and anti-inflammatory analgesic efficacy. Modern pharmacological studies showed that the extract of *Cynanchum paniculatum* leaves exhibits highly effective anti-hyperuricemia and anti-inflammatory effects and its anti-gout arthritis mechanism is closely related to the regulation of NALP3 inflammatory body[21]. Neoastilbin, a flavonoid extracted from the rhizome of *Smilax glabra*, can inhibit the NF-κB and NLRP3 inflammasome-mediated pathways, which play an important role in the treatment of gouty arthritis[22]. Coixol, a plant polyphenol extracted from coix seed, was found to be able to reduce the expression of IL-1β, IL-6, IL-18, TNF- α, NO, iNOS and COX-2 in macrophage and this anti-inflammatory mechanism is related to the ability of activating NLRP3 inflammatory body[23]. These studies together have proved the therapeutic effects of TFK on gouty arthritis by inhibiting NLRP3 inflammatory body from the pharmacological aspect.

NLRP3 inflammatory body is a cytoplasmic multi-protein complex. Overactivation of NLRP3 inflammatory body will lead to the development of inflammatory diseases and cancer[24]. In innate immunity, NLRP3 induces inflammation and cell death against both pathogens (PAMP) and endogenous activator (DAMP). The activation of NLRP3 inflammatory bodies is mediated by two key steps: Initialization (signal 1) and activation assembly (signal 2). Initialization of NLRP3 inflammatory body by activating the NF-κB pathway can up-regulates the proteins related to inflammatory corpuscles (including inflammatory corpuscle sensor protein, IL-1β and IL-18). The activation assembly process triggers the aggregation of inflammatory body sensing protein and inflammatory body junction protein, thereafter recruiting Caspase-1. Activated Caspase-1 promotes IL-1β and IL-18 hydrolyze and mature, facilitating the cutting of Gasdermin D (GSDMD). The cut GSDMD then forms a hole on the cell membrane, causing proinflammatory cell death, namely cell charring[25]. The IL-1β is considered as a typical multifunctional cytokine, acting alone or in combination with other cytokines to affect almost all types of cells, which is essential for cell defense and tissue repair in almost all tissues and is also associated with pain, inflammation and autoimmunity. As the downstream of the NLRP3 signal pathway, IL-1β activates COX-2, facilitates the arachidonic acid metabolism pathway and promotes PGE2 synthesis[26], causing local redness, swelling, heat and pain of joints and eventually leading to the occurrence and development of gouty arthritis. The results of this study showed that the therapeutic doses of TFK can effectively inhibit the synthesis of NLRP3 inflammatory body-related protein and reduce the levels of the downstream inflammatory factor IL-1β. Furthermore, the changed expression of PGE2 induced by TFK indicates that TFK has significant inhibitory effects on both upstream and downstream of this signal pathway. In addition, in previous research, it was also found that TFK could significantly reduce the levels of IFN- γ, IL-1, IL-2, IL-4, IL-13, TNF-α and MCP-1 in the blood of the model rats, which together indicates that besides inhibiting the NLRP3 signal pathway, TFK also has other potential anti-gout mechanisms with multi-targeted effects.

It is also worth noting that TFK seems to have relieving effects on different types of pain. Various physical and chemical properties of the pain stimulation were used in this study, including chemical stimulation (the body twisting method), thermal stimulation (the hot plate method) and inflammatory pain of adjuvant arthritis. Acetic acid writhing test is a classic method to study peripheral analgesics. Acetic acid-induced writhing reaction is a sensitive procedure for establishing a peripheral analgesic model. This reaction was shown to be associated with local peritoneal receptors[27]. Since this method is mainly used to evaluate the peripheral analgesic, the experimental results can preliminarily conclude that the therapeutic doses of TFK has obvious peripheral analgesic effects and these analgesic effects are equivalent to that of aspirin. The PGE2 is an important inflammatory and analgesic mediator produced by the enzymatic metabolism of arachidonic acid. In addition, COX is the key enzyme in the synthesis of prostaglandins. The IL-1β can induce the production of COX-2, which is therefore closely related to the regulation of peripheral inflammatory pain. In this study, TFK can significantly reduce the levels of PGE2 in serum, which is consistent with the results of the acetic acid writhing test and adjuvant inflammatory pain II reaction, indicating that TFK has a strong peripheral analgesic effect on gouty arthritis. Hence, it can be speculated that the mechanism of the peripheral analgesic effect of TKF is related to the inhibition of the NLRP3 signal pathway and the reduction of PGE2 serum levels. The hot plate test, first described in 1944, can be used to determine heat thresholds in rats[28]. The hot plate test and other tests of applying thermal stimulation to the hind paw are considered to integrate the supraspinal pathway. Because rats with spinal cord transection will not retract their hind legs

in the hot plate test, the nature of thermal stimulation pain caused by the hot plate test is considered as central pain[29]. In hot plate test, the therapeutic doses of TFK showed satisfactory central analgesic effects in the rats, which was significantly superior to aspirin that is primarily effective in peripheral analgesia. In combination with the phase I reaction results of adjuvant arthritis pain, our data showed that TFK possesses certain central analgesic effects which however are not as strong as its peripheral analgesic effects. The mechanism of the central analgesic effects of TFK is speculated that TFK may be able to activate opioid receptors, simulating endogenous opioid peptides to block the pain transmission pathway[30]. This study aims to investigate the anti-inflammatory and analgesic effects of TFK in gouty arthritis and its potential mechanisms. The TFK may be a promising clinical treatment of gouty arthritis and provide an experimental basis for future anti-gout drug development and targeted therapy. It is of great significance for the prevention and treatment of gout, hyperuricemia and its complications. Its limitation is that the study of TFK's anti-inflammatory mechanism only focuses on the NLRP3 inflammasome and its downstream signaling molecules. Other potential anti-inflammatory pathways have not yet been explored. At the same time, only a preliminary exploration of TFK's central and peripheral analgesic effects has been conducted. However, there is no in-depth study on which factors and signaling pathways are affected to play a central and peripheral analgesic role. The exploration of TFK's anti-inflammatory and analgesic mechanisms is still at a relatively superficial level and needs to be further explored.

CONCLUSION

The purpose of this study was to investigate the anti-inflammatory and analgesic effects of TFK on gouty arthritis. TFK was found to reduce the swelling and pain of rats caused by MSU crystals, significantly inhibit the immune inflammatory reaction caused by gout and synergistically decrease the pain reaction from both central and peripheral aspects, thus improving the clinical symptoms of gout. The potential anti-gouty arthritis effects of TFK may be attributed to the inhibition of NLRP3 inflammatory body and the down-regulation of downstream proinflammatory cytokines. The present study suggested that TFK may be a promising therapeutic formula for the prevention and treatment of gouty arthritis in clinical settings and lays an experimental foundation for the early search and development of anti-gout drugs with improved therapeutic effects.

SIGNIFICANCE STATEMENT

The TFK is a traditional Chinese medicine formula for the clinical treatment of acute gouty arthritis. This study aims to investigate the therapeutic effect of TFK on acute gouty arthritis model rats and explore its potential anti-inflammatory and analgesic mechanisms. The results indicate that TFK has reliable anti-inflammatory and analgesic effects on gouty arthritis and its potential mechanism of action is by inhibiting NLRP3 inflammasomes and downstream inflammatory factors. At the same time, its analgesic effect is combined by central and peripheral effects, indicating that TFK may be a promising clinical treatment of gouty arthritis and provide an experimental basis for future anti-gout drug development and targeted therapy. Further research is still needed on relevant potential anti-inflammatory and analgesic mechanisms.

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REFERENCES

1. Tausche, A.K. and M. Aringer, 2016. GichtL: Gouty arthriti. Z. Rheumatol., 75: 885-898.
2. Chopra, A., 2015. Disease burden of rheumatic diseases in India: COPCORD perspective. Indian J. Rheumatol., 10: 70-77.
3. Singh, J.A. and A. Gaffo, 2020. Gout epidemiology and comorbidities. Semin. Arthritis Rheumatism, 50: S11-S16.
4. Shao, B.Z., Z.Q. Xu, B.Z. Han, D.F. Su and C. Liu, 2015. NLRP3 inflammasome and its inhibitors: A review. Front. Pharmacol., Vol. 6. 10.3389/fphar.2015.00262.
5. Cabău, G., T.O. Cri an, V. Klück, R.A. Popp and L.A.B. Joosten, 2020. Urate induced immune programming: Consequences for gouty arthritis and hyperuricemia. Immunol. Rev., 294: 92-105.
6. Biasizzo, M. and N. Kopitar-Jerala, 2020. Interplay between NLRP3 inflammasome and autophagy. Front. Immunol., Vol. 11. 10.3389/fimmu.2020.591803.
7. Choi, H.M., D.H. Oh, J.S. Bang, H.I Yang, M.C. Yoo and K.S. Kim, 2010. Differential effect of IL-1β and TNF-α on the production of IL-6, IL-8 and PGE_2 in fibroblast-like synoviocytes and THP-1 macrophages. Rheumatol. Int., 30: 1025-1033.

8. Park, J., H.M. Choi, H.I. Yang, M.C. Yoo and K.S. Kim, 2012. Increased expression of IL-1 receptors in response to IL-1β may produce more IL-6, IL-8, VEGF, and PGE_2 in senescent synovial cells induced *in vitro* than in presenescent cells. Rheumatol. Int., 32: 2005-2010.
9. Robinson, P.C. and S. Horsburgh, 2014. Gout: Joints and beyond, epidemiology, clinical features, treatment and co-morbidities. Maturitas, 78: 245-251.
10. Raskin, J.B., 1999. Gastrointestinal effects of nonsteroidal anti-inflammatory therapy. Am. J. Med., 106: 3S-12S.
11. Cozza, J., T.V.C. Do, S. Ganti and J. Depa, 2021. The ugly side of colchicine. J. Invest. Med. High Impact Case Rep., Vol. 9. 10.1177/23247096211029744.
12. White, W.B., K.G. Saag, M.A. Becker, J.S. Borer and P.B. Gorelick *et al*., 2018. Cardiovascular safety of febuxostat or allopurinol in patients with gout. New Engl. J. Med., 378: 1200-1210.
13. Finkelstein, Y., S.E. Aks, J.R. Hutson, D.N. Juurlink and P. Nguyen *et al*., 2010. Colchicine poisoning: The dark side of an ancient drug. Clin. Toxicol., 48: 407-414.
14. Pu, M.J., C.J. Yao, L.M. Liu, L.J. Ren, Y.l. Li and Y. Xie, 2021. Traditional Chinese medicine for gouty arthritis: A protocol for meta-analysis. Medicine, Vol. 100. 10.1097/MD.0000000000023699.
15. Liang, H., P. Deng, Y.F. Ma, Y. Wu and Z.H. Ma *et al*., 2021. Advances in experimental and clinical research of the gouty arthritis treatment with traditional Chinese medicine. Evidence-Based Complementary Altern. Med., Vol. 2021. 10.1155/2021/8698232.
16. Coderre, T.J. and P.D. Wall, 1987. Ankle joint urate arthritis (AJUA) in rats: An alternative animal model of arthritis to that produced by Freund's adjuvant. Pain, 28: 379-393.
17. Kawahara, K., H. Hohjoh, T. Inazumi, S. Tsuchiya and Y. Sugimoto, 2015. Prostaglandin E_2-induced inflammation: Relevance of prostaglandin E receptors. Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids, 1851: 414-421.
18. Vogel, H.G., 2007. Analgesic, Anti-inflammatory, and Anti-pyretic Activity. In: Drug Discovery and Evaluation: Pharmacological Assays, Vogel, H.G. (Ed.), Springer, Berlin, Heidelberg, ISBN: 978-3-540-71420-0, pp: 983-1116.
19. Hunskaar, S. and K. Hole, 1987. The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. Pain, 30: 103-114.
20. Chogtu, B., K.L. Bairy, S.M. Satyam, R. Pirasanthan and S. Gupta, 2013. Analgesic modulation of tramadol, amitriptyline and gabapentin in male and female Wistar rats. Res. J. Pharm. Biol. Chem. Sci., 4: 70-78.
21. Rahmi, E.P., E. Kumolosasi, J. Jalil, F. Buang and J.A. Jamal, 2021. Extracts of *Andrographis paniculata* (Burm.f.) nees leaves exert anti-gout effects by lowering uric acid levels and reducing monosodium urate crystal-induced inflammation. Front. Pharmacol., Vol. 12. 10.3389/fphar.2021.787125.
22. Xu, W., F. Li, X. Zhang, C. Wu, Y. Wang, Y. Yao and D. Xia, 2022. The protective effects of neoastilbin on monosodium urate stimulated THP-1-derived macrophages and gouty arthritis in mice through NF-κB and NLRP3 inflammasome pathways. Molecules, Vol. 27. 10.3390/molecules27113477.
23. Hu, Y., Q. Zhou, T. Liu and Z. Liu, 2020. Coixol suppresses NF-κB, MAPK pathways and NLRP3 inflammasome activation in lipopolysaccharide-induced RAW 264.7 cells. Molecules, Vol. 25. 10.3390/molecules25040894.
24. Wang, L. and A.V. Hauenstein, 2020. The NLRP3 inflammasome: Mechanism of action, role in disease and therapies. Mol. Aspects Med., Vol. 76. 10.1016/j.mam.2020.100889.
25. Paik, S., J.K. Kim, P. Silwal, C. Sasakawa and E.K. Jo, 2021. An update on the regulatory mechanisms of NLRP3 inflammasome activation. Cell. Mol. Immunol., 18: 1141-1160.
26. Migliorini, P., P. Italiani, F. Pratesi, I. Puxeddu and D. Boraschi, 2020. The IL-1 family cytokines and receptors in autoimmune diseases. Autoimmunity Rev., Vol. 19. 10.1016/j.autrev.2020.102617.
27. Jaiswal, S.R. and S.D. Sontakke, 2012. Experimental evaluation of analgesic and anti-inflammatory activity of simvastatin and atorvastatin. Indian J. Pharmacol., 44: 475-479.
28. Woolfe, G. and A.D. MacDonald, 1944. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). J. Pharmacol. Exp. Ther., 80: 300-307.
29. Giglio, C.A., H.L.A. Defino, C.A. da-Silva, A.S. de-Souza and E.A. Del Bel, 2006. Behavioral and physiological methods for early quantitative assessment of spinal cord injury and prognosis in rats. Braz. J. Med. Biol. Res., 39: 1613-1623.
30. Al-Hasani, R. and M.R. Bruchas, 2011. Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology, 115: 1363-1381.