

# Modified Chuanhu anti-gout mixture, a traditional Chinese medicine, protects against potassium oxonate-induced hyperuricemia and renal dysfunction in mice

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## Abstract

**Objective:** Acute gout is a painful, inflammatory arthritis that features a rapidly escalating inflammatory response resulting from the formation of monosodium urate crystals in the affected joint space. Previously, we found that Chuanhu anti-gout mixture (CAGM) had similar effects as colchicine against gout in the clinic. Subsequently, to improve its effectiveness and efficacy, we modified the original formulation of CAGM. The current study evaluated the effectiveness of the modified formulation in mice.

**Methods:** Potassium oxonate (PO) was used to establish a mouse model of hyperuricemia. Plasma levels of uric acid and creatine were determined using the respective test kits. Hepatic xanthine oxidase (XOD) expression was examined by enzyme-linked immunosorbent assay. To explore the underlying mechanism, renal urate transporter 1 (URAT1) mRNA levels were evaluated by quantitative real-time PCR. Allopurinol and benzbromarone were used as reference drugs.

**Results:** The original CAGM and its modified high-dose formulation significantly reduced serum uric acid and creatine levels in hyperuricemic mice. In addition, the CAGM-treated groups displayed lower mRNA levels of hepatic XOD and renal URAT1.

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**Conclusions:** CAGM and its modified formulation significantly ameliorated PO-induced hyperuricemia in mice, which might be partially attributable to reductions of hepatic XOD and renal URAT1 levels.

### Keywords

Chuanhu anti-gout mixture, hyperuricemia, renal dysfunction, traditional Chinese medicine, xanthine oxidase, urate transporter 1

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### Introduction

Hyperuricemia is a condition of abnormally high levels of plasma uric acid, which exists largely as urate, the ion form, at the pH conditions of body fluids.<sup>1</sup> A balance exists between the amount of purine absorbed from food or synthesized within the body (e.g., through cell turnover) and the amount of urate excreted in urine or through the gastrointestinal tract. In humans, the upper end of the normal range is 360  $\mu\text{mol/L}$  (6 mg/dL) for women and 400  $\mu\text{mol/L}$  (6.8 mg/dL) for men.<sup>2</sup> Persistent hyperuricemia can cause monosodium urate monohydrate crystal deposition in the extracellular fluid of the joints and other sites and induce urate deposition diseases.<sup>3,4</sup> Thus, the plasma uric acid level is strongly associated with the risk of gout development, particularly in men with severe hyperuricemia.<sup>5,6</sup> Moreover, hyperuricemia is a widely accepted risk factor for insulin resistance, prehypertension, diabetes, and cardiovascular disease.<sup>7-9</sup>

Pharmacologically, urate levels are typically lowered by inhibition of urate synthesis or treatment with uricosuric agents. Downstream of the purine catabolic pathway in humans and other uricotelic species, xanthine oxidase (XOD) plays a key role in uric acid biosynthesis by converting hypoxanthine to xanthine and further

converting xanthine to uric acid.<sup>10,11</sup> Allopurinol, which was approved for medical use in the United States in 1966, is an XOD inhibitor used to reduce urate formation under conditions in which urate deposition has already occurred or is predictable. Benzbromarone, a uricosuric agent, inhibits urate transporter 1 (URAT1), enhancing the excretion of uric acid.<sup>12,13</sup>

Chuanhu anti-gout mixture (CAGM) is a traditional Chinese medicine (TCM) developed by our group based on the TCM theory. Since 1999, this TCM has been used in more than 1000 cases of gout, and it exhibits high safety and effectiveness. Our previous randomized, double-blind, double-dummy, non-inferiority trial<sup>14</sup> demonstrated that CAGM was non-inferior to colchicine for treating acute gouty arthritis. In 2000, the therapy was approved for an Invention Patent of China (CN1857317A).<sup>14</sup> To promote patient compliance, a more convenient granule formulation was developed and applied. To enhance its effectiveness and efficacy, the formulation was modified slightly.

The current study was conducted to evaluate the effectiveness of modified CAGM in mice and preliminarily explore the mechanism of its effects on XOD and URAT1.

## Material and methods

### Reagents

TRIZOL and potassium oxonate (PO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Allopurinol was purchased from Jiulian Co., Ltd (Hefei, China). Sodium carboxymethylcellulose (CMC) was purchased from Guichi Co., Ltd (Hangzhou, China). Phosphate-buffered saline was purchased from Solarbio Company (Beijing, China). Benzbromarone was purchased from Heumann Pharma GmbH (Nurnberg, Bavaria, Germany). Absolute ethyl alcohol was purchased from the fine chemical plant of the Laiyang Economic and Technological Development Zone (Yantai, Shandong Province, China). Trichloromethane was purchased from Shuangshuang Chemical Plant (Yantai, Shandong Province, China). Isopropanol was purchased from Fuyu Fine Chemical Industry (Tianjin, China). Diethyl pyrocarbonate (DEPC) was purchased from Sangon Biotech (Shanghai, China). A TRUEscript 1st strand cDNA synthesis kit and SYBR Green qPCR mix were purchased from Aidlab Biotechnologies Co., Ltd. (Beijing, China). Creatinine, urea nitrogen, and reagent kits were purchased from Nanjing Jiancheng Biological Company (Nanjing, China).

### Plant material

Unmodified CAGM was supplied by the Affiliated Hospital of Qingdao University. The original CAGM formulation consists of 15 g of *Dioscorea nipponica*, 15 g of *Polygonum cuspidatum*, 30 g of honeysuckle rattan, 15 g of, 15 g of glabrous greenbrier, 15 g of *Radix saposhnikoviae*, 15 g of *Radix clematidis*, 15 g of *Rhizoma chuanxiong*, 15 g of coix seed, 6 g of *Glycyrrhizae radix et rhizoma*, and 2 g of sodium alginate. The modified formulation contains 10 g of

cassia twig, 0.3 g of borneol, 15 g of *D. nipponica*, 15 g of *P. cuspidatum*, 30 g of honeysuckle rattan, 15 g of *Radix cyathulae*, 15 g of glabrous greenbrier, 15 g of *Radix saposhnikoviae*, 15 g of *Radix clematidis*, 15 g of *Rhizoma chuanxiong*, 15 g of coix seed, 6 g of *Glycyrrhizae radix et rhizoma*, and 2 g of sodium alginate. The TCM granules were prepared by Qingdao Huanghai Pharmaceutical (Qingdao, China).

Doses were determined according to the animal dose conversion formula as follows:  $DB = DA \times (KB/KA)$ , where DB is the unknown dose for animal B, DA is the known dose for animal A, and KB and KA are the dose conversion coefficients for animals B and A, respectively. According to some provisions of the State Administration of Traditional Chinese Medicine of the People's Republic of China, the low, medium, and high doses applied to the mice were set as 2.5-, 5-, 10-fold the result calculated using the animal dose conversion formula. As a result, the low, medium, and high doses of modified CAGM were 2.7, 5.4, and 10.75 g/mL, respectively. Only one dose (10.14 g/mL) of the original CAGM was applied.

### Animals

Male Kunming mice ( $25 \pm 2$  g) were purchased from the animal center of Dawu Fucheng (Qingdao, China). The animals were maintained under controlled environmental conditions at a constant temperature ( $23 \pm 1^\circ\text{C}$ ), humidity ( $60 \pm 10\%$ ), and 12-h:12-h light/dark cycle. The mice were acclimatized for 1 week prior to any experimental procedures and given standard rat chow and water *ad libitum*. All procedures pertaining to animal care and treatments strictly adhered to the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Ethical approval for the study was provided by

the Experimental Animal Welfare Ethics Committee of The Affiliated Hospital of Qingdao University.

### **Hyperuricemic mice and drug administration**

The mouse model of hyperuricemia was established via intraperitoneal injections of PO (300 mg/kg), a uricase inhibitor.<sup>15</sup>

Eighty mice were randomly divided into eight groups ( $n=10$ ) as follows: Group Con, normal mice that received oral vehicle (0.8% CMC); Group PO, hyperuricemic mice that received oral vehicle (0.8% CMC); Group ALP, hyperuricemic mice that received allopurinol (65 mg/kg/day); Group BBM, hyperuricemic mice that received benzbromarone (32.5 mg/kg/day); Group o-CAGM, hyperuricemic mice that received original CAGM (202.8 g/kg/day); Group m-CAGM-l, hyperuricemic mice that received low-dose modified CAGM (54 g/kg/day); Group m-CAGM-m; hyperuricemic mice that received modified CAGM at the medium dose (108 g/kg/day); and Group m-CAGM-h, hyperuricemic mice that received modified CAGM at the high dose (215 g/kg/day). All medications were administered twice daily for 7 consecutive days.

### **Collection of blood, liver tissue, and kidney tissue**

After sacrificing the animals, whole blood and liver samples were collected 1 h after PO injection. The serum was obtained by incubating the samples at 25°C for approximately 1 h, followed by centrifugation at  $10,000 \times g$  for 5 min. Plasma uric acid and creatinine levels were determined using an automatic biochemical analyzer (Siemens ADVIA 2400, Munich, Germany). Liver and kidney tissues were isolated at 4°C and stored at -80°C until analysis.

### **Determination of XOD levels**

A mouse liver ELISA XOD kit was purchased from NeoBiolab (Shanghai, China). A standard curve was made prior to determining XOD levels in liver samples according to the manufacturer's instructions. Briefly, the standard solution (50  $\mu$ L) was added to a microtiter plate and incubated for 1 h at room temperature. The wells were then emptied and washed 3–5 times with 300–400  $\mu$ L of wash solution per well. Conjugate (100  $\mu$ L per well) was added and mixed, and then the plate was covered and incubated for 1 h at 37°C in a humidified chamber. The wells were washed five times with wash solution. After the final wash, the plate was inverted and blotted dry via tapping on absorbent paper. Substrate A (50  $\mu$ L) was added to each well, followed by the addition of 50  $\mu$ L of substrate B. The plates were covered and incubated for 10–15 min at room temperature. Thereafter, 50  $\mu$ L of stop solution were added to each well and mixed. The optical density (OD) at 450 nm was immediately read, and a standard curve was constructed using graph paper or statistical software, giving the following formula:  $Y = 27.04X - 0.92$ ,  $R^2 = 0.9881$ . The same procedure was used to read the OD of the sample at 450 nm, and the calculated curve equation was used to determine the sample concentration.

### **Reverse transcription-polymerase chain reaction (qRT-PCR) of URAT1**

Each sample (0.05 g) was added to 500  $\mu$ L of cracking liquid according to the manufacturer's instructions to extract total RNA. Total RNA (1  $\mu$ L), diluted with DEPC in water, was analyzed at 260/280 nm absorbance (A) as  $A_{260}/A_{280}$  to determine its purity.

The concentration was calculated according to different samples in advance using 2  $\mu$ g of sample according to the

**Table 1.** Primer sequences for URAT1 and GAPDH.

	primer pairs (5'-3')
URAT1-F	GCTACCAGAATCGGCACGCT
URAT1-R	CACCGGGAAGTCCACAATCC
GAPDH-F	GCCCTCCAAGGAGTAAGAAA
GAPDH-R	GCCCCTCCTGTTATTATGG

Abbreviation: URAT1, urate transporter 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

manufacturer's instructions for reverse transcription synthesis of cDNA.

The primer sequences are shown in Table 1. The reaction system (25  $\mu$ L) was compounded using two double SYBR qPCR mixes (12.5  $\mu$ L), forward primer (0.5  $\mu$ L), reverse primer (0.5  $\mu$ L), DEPC in water (10.5  $\mu$ L), and DNA samples (1  $\mu$ L). For each sample (three complex holes), two-step PCR was conducted with the following parameters: 40 cycles of denaturation for 3 min, extension at 94°C for 10 s, and annealing at 60°C for 35 s; for melting curve analysis, temperatures of 60–95°C were used. This process was repeated three times for each sample.

### Statistical analysis

All data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and expressed as the mean  $\pm$  standard error of the mean. Statistical analysis was performed using one-way analysis of variance with Tukey's test for multiple comparisons. A *P* value of <0.05 was considered statistically significant.

## Results

### *Modified CAGM significantly reduced plasma uric acid levels in hyperuricemic mice*

To evaluate the effectiveness of modified CAGM against hyperuricemia in mice,

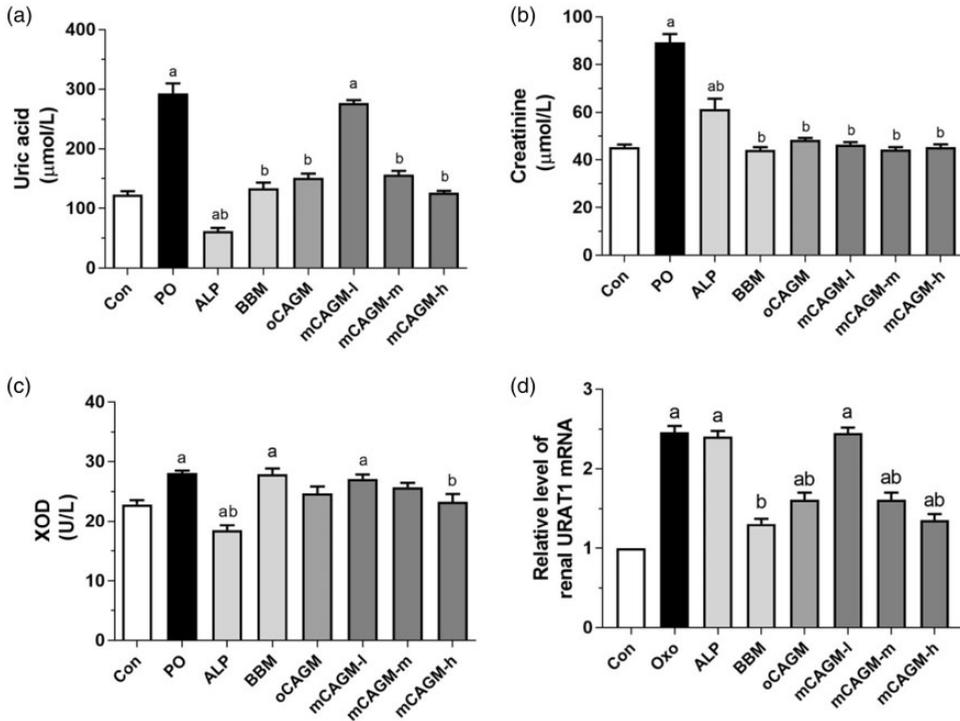
plasma uric acid levels were measured using allopurinol, benzbromarone, and original CAGM as positive controls. The results revealed that PO injection induced a significant increase in uric acid levels in mice ( $P < 0.05$  vs. Group Con; Figure 1a) that was effectively reversed by the administration of allopurinol, benzbromarone, and original CAGM ( $P < 0.05$  vs. Group PO; Figure 1a). Compared with the findings in Group PO, plasma uric acid levels were significantly decreased in hyperuricemic mice treated with medium and high doses of modified CAGM ( $P < 0.05$  vs. Group PO; Figure 1a), whereas the low dose of modified CAGM exerted no significant protective effect ( $P = 0.87$  vs. Group PO; Figure 1a).

### *Modified CAGM significantly reduced serum creatinine levels in hyperuricemic mice*

To determine the effect of modified CAGM on renal function in mice, serum creatinine levels were measured. The results indicated that PO injection significantly augmented serum creatinine levels compared with its levels in Group Con ( $P < 0.05$  vs. Group Con; Figure 1b). All treated groups exhibited lower serum creatinine levels than Group PO ( $P < 0.05$  vs. Group PO; Figure 1b).

### *Modified CAGM significantly inhibited hepatic XOD levels in hyperuricemic mice*

To observe the effect of modified CAGM on the hepatic capacity to produce urate in mice, hepatic XOD levels were detected via enzyme-linked immunosorbent assay. The results revealed that hepatic XOD levels were significantly higher in hyperuricemic and BBM-treated hyperuricemic mice than in Group Con mice ( $P < 0.05$  vs. Group Con; Figure 1c) and significantly



**Figure 1.** Modified Chuanhu anti-gout mixture (CAGM), a traditional Chinese medicine, protects against potassium oxonate-induced hyperuricemia and renal dysfunction in mice. a) Modified CAGM significantly reduced the plasma levels of uric acid in hyperuricemic mice. b) Modified CAGM significantly reduced serum creatinine levels in hyperuricemic mice. c) Modified CAGM significantly reduced hepatic xanthine oxidase (XOD) levels in hyperuricemic mice. d) Modified CAGM significantly reduced renal urate transporter 1 (URAT1) mRNA levels in hyperuricemic mice. Values are presented as mean  $\pm$  standard error of the mean. <sup>a</sup> $P < 0.05$  vs. Group Con; <sup>b</sup> $P < 0.05$  vs. Group PO.

Abbreviations: Con, normal mice that received oral vehicle (0.8% CMC); PO, hyperuricemic mice that received oral vehicle; ALP, hyperuricemic mice that received allopurinol; BBM, hyperuricemic mice that received benzbromarone; o-CAGM, hyperuricemic mice that received original CAGM; m-CAGM-l, hyperuricemic mice that received low-dose modified CAGM; m-CAGM-m, hyperuricemic mice that received medium-dose modified CAGM; and m-CAGM-h, hyperuricemic mice that received high-dose modified CAGM.

lower in ALP-treated hyperuricemic mice ( $P < 0.05$  vs. Group Con; Figure 1c). Moreover, among the CAGM groups, only high-dose modified CAGM significantly reduced hepatic XOD levels in hyperuricemic mice ( $P < 0.05$  vs. Group PO; Figure 1c).

#### *Modified CAGM significantly decreased renal URAT1 mRNA levels in hyperuricemic mice*

To observe the effect of modified CAGM on the renal capacity to excrete urate in mice, the mRNA expression of renal

URAT1 was detected by qRT-PCR. The results illustrated that renal URAT1 mRNA levels were significantly higher in hyperuricemic and ALP-treated hyperuricemic mice than in Group Con mice ( $P < 0.05$  vs. Group Con; Figure 1d). Compared with the findings in Group PO, renal URAT1 mRNA levels were significantly lower in BBM-treated hyperuricemic mice ( $P < 0.05$  vs. Group PO; Figure 1d). Moreover, all CAGM groups excluding the low-dose modified CAGM group displayed significantly reduced renal URAT1 mRNA levels ( $P < 0.05$  vs. Group PO; Figure 1d).

## Discussion

Because of rapid economic growth and improved living standards, the prevalence of gout in China has increased each year. Epidemiological studies indicated that the incidence of gout is 1.14% in the coastal cities of Shandong province in China<sup>16</sup> and 1.4% in Europe.<sup>17</sup> Hyperuricemia and subsequent gout are becoming common diseases that seriously affect health. Currently available treatments for acute gouty arthritis include colchicine, non-steroidal anti-inflammatory drugs, and corticosteroids targeting interleukin-18, the long-term use of which may have side effects.

Chinese herbal compounds with high efficacy and low risks of adverse reactions have attracted increasing attention from researchers worldwide. Our previous clinical trial<sup>14</sup> demonstrated that CAGM is an effective treatment for acute gouty arthritis. To improve patient compliance, a granule formulation was developed. To enhance efficacy, borneol and sodium alginate were added to the formulation. Designed on the basis of the TCM theory, CAGM was demonstrated to conform to modern pharmacology. Chemokines and inflammatory factors such as interleukin (IL)-1 $\alpha$  may be

inhibited by CAGM treatment. Moreover, the current study found that CAGM and its modified formulation inhibited the hepatic production and enhanced the excretion of plasma uric acid by regulating the levels of hepatic XOD and renal URAT1 in hyperuricemic mice.

XOD is a key hepatic enzyme involved in regulating uric acid that plays an important role in the pathogenesis of hyperuricemia and gout. Allopurinol, an XOD inhibitor, reduces uric acid levels, mitigating the symptoms of gout. In this study, XOD levels were significantly reduced by allopurinol treatment in hyperuricemic mice. Similar effects were observed for original CAGM treatment and high-dose modified CAGM treatment, further indicating that the mechanism underlying the urate-lowering effect of CAGM is at least partially related to the regulation of XOD.

URAT1 plays an important role in the excretion of uric acid. It was found that benzbromarone reduces uric acid levels by decreasing URAT1 mRNA levels and enhancing uric acid excretion.<sup>18</sup> URAT1 mRNA levels were significantly lower in the original CAGM and medium- and high-dose modified CAGM groups than in the PO-injury hyperuricemic group. This finding indicates that the mechanism underlying the protective effect of CAGM is at least partially attributed to the inhibition of URAT1.

Notably, a previous study revealed that the addition of sodium alginate to resveratrol, a key effective component of CAGM, significantly enhanced its anti-inflammatory and antioxidant effects.<sup>19</sup> Specifically, the combination of sodium alginate and resveratrol significantly reduced the synovial levels of IL-1 $\beta$ , C-C chemokine receptor type 5 (CCR5), and C-X-C motif chemokine 10 (CXCL10) compared with the effects of colchicine. The combination of

sodium alginate and resveratrol was also superior to resveratrol alone in terms of the serum and synovial levels of IL-1 $\beta$ , CCR5, and CXCL10. Additionally, resveratrol, with or without sodium alginate, clearly reduced NLRP3 expression in synovial tissues.

In conclusion, CAGM and its modified formulation protect against PO-induced hyperuricemia in mice, which may be at least partially attributable to reductions in hepatic XOD and renal URAT1 levels. Importantly, CAGM and its modified formulation did not impair renal function given the absence of increased creatinine levels. Further studies should focus on establishing a standard formulation for CAGM granules and determining the mechanism in more detail.

### Declaration of Conflicting Interest

The authors declare that there is no conflict of interest.

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