The effect of uricase on monosodium urate (MSU) crystallization, IL-1β and TNFα in monosodium urate-induced arthritis

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Abstract
Background: Although the symptoms of gouty arthritis could be controlled by clinical methods, it is often impossible to remove tophi from joints that cause acute gouty arthritis.

Methods: In study, we injected a suspension of MSU into the knee joint of chicken, and then injected uricase solution into the joint after 4, 8 and 12 hours. Since the beginning of the experiment, we monitored the circumference of the diseased joint and local temperature change. Computer tomography (CT) examination was performed to examine the MSU in the knee joints and then measured the levels of TNFα and IL-1β in synovial fluid. Chicken knee joint fluid was collected under polarized light microscope to observe the condition of the crystals and measured the weight of MSU residued in synovial fluid. Finally, the chicken knee joint was dissected and the joint synovium was sliced.

Results: The results showed that injection of MSU suspension into chicken knee joint, significantly increased IL-1β, TNFα levels (p<0.01), circumference of the joint (p<0.05) and the local skin temperature (p<0.05). Moreover, analysis of the joint fluid and synovial slice under the polarized light microscope revealed that crystals significantly reduced. Compared with group II (control group), the residual weight of MSU in the joint fluid of group III and group IV is very small (p<0.05), but there is no significant difference between group III and group IV (p>0.05).

Conclusions: The comprehensive analysis shows that the reaction process between uricase and MSU is similar to that uric acid. Therefore, it provides a new way for clinical treatment of acute gouty arthritis with fewer side effects and significant reduction in the frequency of attacks.

1. Background
Uric acid is the end product of nitrogen metabolism in birds, primates and humans [1], and hyperuricemia is a potential risk of gouty arthritis. Gouty arthritis, especially the joints of the extremities, is caused by poor uric acid excretion in the body [2–4]. Gouty arthritis affects many aspects of human body, such as severe joint pain, impaired movement and mental dysfunction. At the onset of gouty arthritis, the patient experiences severe reactions characterized by severe joint pain, joint swelling and high local skin temperature [5]. In gouty arthritis, the synovial membrane is highly
infiltrated by polarized macrophages, which produce tumor necrosis factor-alpha (TNFα), interleukin-1β (IL-1β) and other pro-inflammatory cytokines\[6, 7\].

In the clinical treatment of hyperuricemia, the xanthine oxidase inhibitors are used to inhibit the production of uric acid such as Allopurinol and Febuxostat. To promote renal uric acid excretion, the most commonly used drugs are Benzobromarone, and probenecid. Puricase and Rasburicase are able to promote uric acid decomposition. Colchicine and non-steroidal anti-inflammatory drugs (NSAIDs) are used in the acute onset of gouty arthritis. Surgical treatment is even needed when severe damage results from joints caused by monosodium urate deposition [9-12]. However, these methods drugs have serious side effects, as they impair endocrine function, decrease liver function, cause gastrointestinal dysfunction and allergic reactions. At the same time, if the patient has liver and kidney dysfunction, most of the above treatments cannot be used. Therefore, limiting the choice of clinical medication also increases the pain of the patient. Moreover, these drugs do not address the causes of gouty arthritis, MSU. Prolonged accumulation of MSU in the joints not only cause recurrent arthritis, but also damages the physiological structure of the joints, causing irreversible damage [11]. According to reports, tophi are formed by the deposition of MSU. Monosodium urate is formed by replacing H + with Na + in the molecular structure, and this molecule is less soluble and thus more likely to form precipitates and deposits in human body [12, 13]. There is no significant difference in molecular structure between the two compounds and the core and functional groups of the two compounds are similar. Therefore, uricase can theoretically react with monosodium urate to form products such as allantoin [13, 14].

2. Methods:
2.1. Experimental procedure
The purpose of this study was to evaluate the effect of uricase on the deposition of monosodium urate (MSU) in the joints. Briefly, the monosodium urate suspension was injected into the joints of chicken, and then continued to inject after 2 and 4 hours. Following this procedure, the circumference of the joint and the local skin temperature were also changed. The uricase solution was continuously injected at 4, 8 and 12 hours after injection of MSU suspension. After 7 days, the intra-articular
condition was examined under CT, and a smear of the joint fluid was examined to assess the status of MSU under a polarized light microscope and detected the levels of TNFα and IL-1β in synovial fluid and measured the weight of MSU resided in synovial fluid. Joint synovial membrane was harvested and sectioned into Hematoxylin-eosin(HE) slices for examination. We anticipated that if the chicken model simulates the human disease state, we expect that the monosodium urate (MSU) in the joints and synovial membranes of the chicken joints will be significantly reduced after the injection of uricase.

2.2. Drug and instrument
All chemicals were of analytical purity. IL-1β and TNFα kit, uric acid, uricase, and other reagents were purchased from Sigma. CT equipment purchased from Toshiba Corporation of Japan, model number is PICKER PQS 16. Polarized light microscope purchased from Olympus, model number is CX41.

2.3. Synthesis of monosodium urate crystals
4 g of uric acid was dissolved in NaOH (9 ml / 0.5 N) in 800 ml of H₂O, adjusted to pH 8.9 and then and heated at 60℃. The, solution was left overnight at 4℃, after which it was washed, sterilized, and needle crystals were recovered and suspended in in sterile saline (40 mg/ml) [4].

2.4. Animals
The white leghorns, which were purchased from Guangdong Xinxin Dahua Agricultural Egg Co., Ltd., weighing about 200 ± 20 g, were adapted to the laboratory environment for 7 days in darkness for one week. They were allowed free access to standard feed and clean drinking water. Animals are euthanized by intravenous injection of Pelltobarbitalum Natricum(200 mg / kg I.p).

2.5. The experiments on uricase in vitro
Divided into 1–9 groups, one group contains 3 test tubes, they have the same settings, including conditions, liquid volume, etc. Groups 1–3 are blank control groups, and groups 4–9 are experimental groups. The 4–6 groups and the 7–9 groups are both experimental groups, but the dosage of urase is different.
1–3 groups plus 1 ml MSU suspension + 1 ml saline water, 4–6 groups plus 1 ml MSU suspension + 1 ml (5 mg/ml) uricase solution, 7–9 group plus 1 ml MSU suspension + 1 ml (10 mg/ml) uricase solution. The samples were stored at 40℃, after which the liquid was removed after 3 days, filtered,
dried, weighed and counted.

2.6. Experimental grouping and experimental animal preparation
Chicken were divided into four groups of six animals. The first group (Group I) was the blank group and intra-articular injection of 1 ml saline. The second group (Group II) was control group. Intraarticular injection of monosodium urate (MSU) crystal suspension caused inflammation. The third and fourth groups (Group III and Group IV) were treated with uricase (5 mg / 10 mg, neglecting body weight) for chickens with inflammation induced by monosodium urate crystals. Uricase was suspended in a solution of pH 8.8 and injected into the joints 4, 8, and 12 hours after the last urate injection; CT irradiation at the joint was performed as described by Lisa, et al. [17], and MSU was observed under a polarized light microscope [18]. The synovial membrane was harvested to prepare slips for examination [19].

2.7. Statistical analysis
Results are expressed as mean ± S.D. and statistical analysis was performed using ANOVA to determine significant differences between groups, followed by Student’s Newman–Keul’s test. \( p < 0.05 \) implied significant differences. The statistical analyses were performed using GraphPad Prism 5.0 software.

3. Results
3.1. In vitro experimental results
It was found after the NO. 1–3 group test tubes were placed for 3 days, a precipitate was formed. After shaking the test tube, the liquid became turbid. The suspension of the NO. 4–9 groups became clear, and a small amount of tiny white floc was present, and the turbulent test tube liquid was not turbid. Table 1 shows the oxidation of MSU by uricase (5 mg/ml) and uricase (10 mg/ml). Analysis of the residue after the reaction, revealed that there was a significant difference between the control group and the 4–9 group, \( p < 0.05 \), so the results were significant. In contrast, there was no difference between 4–6 groups and 7–9 groups, \( p > 0.05 \), so the results were significant. These results indicate that uricase can significantly oxidize monosodium urate into soluble substances. However, the doses of uricase 5 mg and 10 mg did not significantly differ in the oxidation of MSU for a sufficient period of time. (Table 1)

3.2. Several parameters were altered after injection of uricase.
3.2.1. Under CT examination, all the chicken joints were invisible (including control group and experimental group) (Fig. 1)

3.2.2. (1). (a) shows that Group II had crystals deposition; (b) shows that has no visible crystal deposition could be seen after injection of uricase in Group III and Group IV, indicating that intra-articular injection of uricase did not increase crystal deposition. So Thus, uricase can oxidize monosodium urate in the body, But there is no significant differences between group III and IV. (2) Residual weight of MSU in synovial fluid. (Fig. 2)

3.2.3. (c) shows crystal deposition on the synovial membrane of Group II; (d) shows that there is no crystal deposition in Group III and Group IV, indicating that it shows that both concentrations of uricase have an effect on the oxidation of MSU in the joint. But there is no significant differences between group III and IV. (Fig. 3)

3.3. Changes in local temperature in the joints
The peak temperature was recorded at 8 hours. Under the action of uricase, compared with Control group (Group II), the temperature of Group III and Group IV changes significantly, and the temperature of group IV was slightly lower than that of group III. However, the difference was not significant, indicating that the injection of uricase into the joint can remove monosodium urate and also relieve local fever at the joint but doubling the concentration of uricase is slightly decreased the local temperature the cooling effect is only slightly improved. And there was no significant change in the temperature of group I (blank group). (Fig. 4)

3.4. Changes in the circumference of the joint.
It can observed found monosodium urate injection into the joint caused inflammation, which in turn induced swelling of the joint. Compared with the control group, the joint circumference of group II and III slightly increased, and there was no significant difference between group II and group III, so P > 0.05, indicating that uricase has a sustained release effect on joint swelling caused by monosodium urate-induced arthritis. The effect of high concentration of uricase on the slow release of joint swelling compared with low concentration of uricase was not obvious. And there was no significant change in the circumference of the joint of group I (blank group). (Fig. 5)

3.5. Changes in TNFα and IL-1β levels.
Compared with the control group, the increase of TNFα and IL-1β levels were significantly higher in group II and group III was significant, P < 0.05, and the results were significant. This indicating indicates that joint injection of uricase into joints can effectively inhibit the increase expression of TNFα and IL-1β. While in contrast, there was not big difference in TNFα and IL-1β expression in group II and group III, TNFα and IL-1β don’t increased obviously, so P > 0.05. It shows that compared with the control group, although uricase can inhibit the increase of TNFα and IL-1β, there is no significant difference between 5 mg / ml and 10 mg / ml of uricase. And there was no significant change in TNFα and IL-1β levels of group I (blank group) (Fig. 6)

4. Discussion:
Urate is a key factor inducing acute gouty arthritis, and it is also a major factor inducing recurrent gout and joint deformities [2, 3]. It plays a key role in joint swelling, temperature rise, and joint limitation of gouty arthritis. Previous researchers have isolated urate from the joints of the limbs of patients with hyperuricemia [18], and it affects the development of gouty arthritis. With the exception of humans, primates, and birds, almost all animals synthesize and secrete uric acid oxidase which function is to decompose uric acid into water-soluble products such as allantoin [1]. Currently, artificially recombinant uricase is used in clinical trials, such as rasburicase and pegloticase. However, there are many restrictions on the use of uricase, such as large side effects, and uricase injected into the blood can induce an immune response. In addition, as the number of uses increases, the effect of the enzyme will become lower and eventually useless [26, 27].

To this end, Specific pathogen Free (SPF) chickens were used to make animal models to induce acute attacks of gouty arthritis, which could simplify the acute onset of gouty arthritis. Some reports have documented events that urate crystals were added to phagocytes in vitro, that is, the crystals were rapidly absorbed by leukocytes, followed by degranulation, phagosome membrane lysis, and cell rupture with release of the crystals. It is assumed that a hydrogen donor site on the crystal is attached to an acceptor site on the outer surface of the cell membrane lipid bilayer. The interaction between the crystal and the cell membrane can also alter cell metabolism and cause the secretion of inflammatory mediators [23]. Because only MSU is used to induce arthritis [2, 4], this model can
simulate symptoms such as joint swelling, local joint temperature rise, and joint limitation in acute episodes of gouty arthritis, and can be viewed as a simple arthritis. Both IL-1β and IL-1beta are the important indicators [20, 22]. TNFα is the earliest and most important inflammatory medium produced by activated macrophages. It can activate neutrophils and lymphocytes, increase the permeability of vascular endothelial cells, regulate the metabolic activity of other tissues and promote the synthesis and release of other cytokines [24, 25]. Interleukin-1β (IL-1β) is a pivotal proinflammatory cytokine that plays important roles in regulating immune responses and in inducing a series of inflammatory reactions in response to infection.

We have investigated the expression of IL-1β, TNFα and the symptoms of acute arthritis induced by monosodium urate, and have investigated the relation between joint circumference, joint temperature, synovial fluid TNFα and IL-1β and arthritis symptoms, before both before and after onset of clinical symptoms of arthritis. With the development of arthritis, the local temperature of the joints gets higher and higher, and the circumference turns longer and longer, and at the same time, the level of TNFα and IL-1β increase, reaching the highest level after 8 hours approximately. We found that the degree of TNFα and IL-1β produced in the joint depends on the severity of the disease, and the joint circumference becomes longer and the temperature rises with the increase of TNFα and IL-1β, so the level of TNFα and IL-1β is closely related to inflammation. Therefore, in the use of MSU-induced arthritis, the production of detectable T TNFα and IL-1β is closely related to the onset of clinical symptoms. Although TNFα is not a diagnostic and therapeutic indicator of gout, it is a crucial indicator that cannot be ignored in human arthritis [22].

Studies have shown that the use of recombinant uricase can decompose uric acid in serum, reduce the level of serum uric acid to reduce the probability of gouty arthritis [26, 27]. The occurrence and development of gouty arthritis probably result from the excessively high level of serum uric acid which becomes monosodium urate after encountering monosodium ion. Monosodium urate is hardly dissolved in the blood and will gradually deposit on joints and other parts [2–4]. If it deposits on joints, arthritis can be induced. Intravenous injection of uricase is an important way to reduce uric acid levels in current clinical trials, but there are many limitations. From the analysis of the molecular
structure of monosodium urate and uric acid, the core genes and functional bonds of the two have not changed, which provides a theoretical basis for the reaction of uricase and monosodium urate.

The principle of the reaction between uric acid and monosodium urate is shown in Fig. 7, represented by yellow and green arrows. There is a dynamic balance between monosodium urate and synovial fluid, and the separated monosodium urate ion immediately produces a chemical reaction when it encounters uricase [13]. Although monosodium urate is difficult to dissolve, it still has dissolution equilibrium with the aqueous solution. For example, monosodium urate is broken down into monosodium urate anion and monosodium positive ion (Monosodium urate ⇌ Monosodium urate ion + Na+). After encountering uricase, under the catalysis of uricase, monosodium urate ion reacts with oxygen to form 5-hydroxyisourate (C5H4N4O4) (Monosodium urate ion + H2O + O2 → 5-hydroxyisourate + H2O). The resulting 5-hydroxyisourate is automatically decomposed into allantoin (C4H8N4O4) and carbon dioxide (5-hydroxyisourate + H2O → Allantoin + CO2) due to unstable existence. Allantoin is easily soluble in water and excreted through the urinary system. If uricase is injected into the joint, many adverse reactions are reduced at the same time, and the effect may not decrease with the increase of the number of times. Because uricase enters the bloodstream and spreads throughout the body with blood circulation, it is easier to trigger the immune system. To this end, we injected uricase into the joints that induced inflammation with MSU, and observed the correlation between its efficacy and the symptoms of experimental animals and the level of IL-1β and TNFα. It is also encouraging that with the continuous injection of uricase into the joints, the symptoms of the experimental animals improved significantly, including reduced joint swelling, decreased temperature and decreased TNFα and IL-1β level. This treatment effect is similar to the treatment of arthritis by nonsteroidal anti-inflammatory drugs (NSAIDS)[29], so uricase can inhibit or alleviate urate-induced inflammation.

However, in the experiment, according to the observed experimental phenomena and analysis of the experimental data, it was found that the therapeutic effect of 10 mg uricase was not significantly different from that of 5 mg uricase (p > 0.05), as shown in Fig. 4,5,6. The reason for this phenomenon
may be the "saturation" of the activity of uricase to break down monosodium urate or the inhibitory effect of degradation products on uricase. But unfortunately this is only our inference, and no research has proved this conjecture.

In this study, chicken knee joints were observed under CT and no significant results were obtained in all groups. As reported by Lisa et al. [17], CT can be used to diagnose gouty arthritis. Although 50 mg / ml monosodium urate can be observed on a polarized light microscope as shown in Fig. 8, the concentration of monosodium urate suspension injected into the joints of chicken was 50 mg / ml, which was not much different from the concentration of joint fluid. At the same time, the injected monosodium urate suspension was greatly diluted with synovial fluid, leading to its density was more similar to that of synovial fluid, which could not be distinguished under CT. A report by Lisa et al. [17] suggests that the test object is a patient who has been exchanging gouty joint fluid for some time, and thus the density and size of monosodium urate deposition have reached a level different from that of joint fluid, so CT detection can be used. Furthermore, according to Alexis et al. [28], CT or ultrasonography is not sensitive to the detection of patients with early gout.

We have shown in this study that uricase can indeed treat MSU-induced inflammation. Uricase breaks down urate, which improves symptoms and suppresses the increase in TNFα and IL-1β. IL-1β is a key factor. Of course, if combined with anti- TNFα, anti- TNFα treatment will be better. Uricase joint injection is a new way to treat arthritis induced by urate deposition. It can bring long-term benefits to patients, if combined with the therapy of lowering serum uric acid.

Conclusion:
New research evidence suggests that uricase can dissolve urate, which is tophi. Therefore, it is very important to understand the reaction process of uricase and urate. At the same time, it is the most important purpose to study the curative effect of uricase. Although the cause of urate accumulation is not very clear, the composition of the final product urate is clear. In this study, we studied the effect of urase on urate, both in vitro and in vivo. Except that the urate in the joint is not visible under CT irradiation, other observations are obvious and meaningful. We conducted qualitative and quantitative studies, in which the urate in the experimental group was significantly dissolved, and the
control group did not significantly decrease. Our observations are at multiple levels. Rough conjectures and studies have been conducted on the reaction process. Molecular level studies have not been performed on the details of the urate accumulation process and uricase dissolution. But this study can provide a new idea for the treatment of recurrent gouty arthritis.

Declarations

**Availability of data and materials**: All the data generated and analyzed in the present study are available from the corresponding author upon reasonable request.

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**Author Contributions**: Data curation, CYM, FX, WR, JDD, HYQ, LCJ and XMY; Formal analysis, CYM, FX, JDD, WR, YQ He, XMY and QG; Methodology, CYM, FX, JDD, WR, LCJ, ZSP, ZSY and QG; Software, XGQ, WR, TH, ML and HQX; Writing – original draft, CYM, FX; Writing – review & editing, CYM, FX, JDD, WR and QG.

**Ethics declarations**

**Ethics approval and consent to participate**: The study was approved by the Animal Ethics Committee of the Animal Center of Guangzhou Medical University (reference number: GY2019-053) and written informed consent was obtained from all study participants.

**Competing interests**: The authors declare that they have no competing interest.

**Consent for publication**: Not applicable

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Table

Table 1

| Parameters | Group 1-3: Control group | Group 4-6: 1ml MSU+1ml uricase (5 mg/ml) | Group 7-9: 1ml MSU+1ml uricase (10 mg/ml) |
|------------|--------------------------|----------------------------------------|----------------------------------------|
| Weight mg  | 39.5±0.2                 | 1.8±0.3 a,*                            | 1.2±0.2 a,*                            |
Comparisons were made with control group

*Statistical significance at: p < 0.05.

Figures

Figure 1

represents all groups, including experimental and control groups that under CT examination

(INCLUDING EXPERIMENTAL AND CONTROL GROUPS), the MSU were invisible. (1)
(1) shows the results of the chicken joint fluid examination, (a.1) and (a.2) shows that Group II had crystals deposition; (b.1) and (b.2) shows that no visible crystal deposition could be seen after injection of uricase in Group III and Group IV. 

(2) Residual weight of MSU in synovial fluid. * Compared with the control group (group II), \( p < 0.05 \).
Figure 3
shows the results of the analysis of the coverslips of dissected synovial membrane the chicken joint; (c) shows crystal deposition on the synovial membrane of Group II; (d) shows that there is no crystal deposition in Group III and Group IV.

Figure 4
The monosodium urate injection into the joint caused joint inflammation thereby increasing the local temperature. Values are expressed as mean ± S.D. (n = 6). Compared with the control group, the * symbol represents statistical significance at: p < 0.05.
Figure 5

Depicts the effect of uricase injection into the joint circumference after uric acid monosodium-induced arthritis. Values are expressed as mean ± S.D. (n = 6). * Compared with the group II, p < 0.05. ** Compared with the group II (p < 0.05) and group III (p > 0.05).
Figure 6

Depicts the effect of intra-articular injection of uricase on elevated the levels of TNFα and IL-1β caused by monosodium urate-induced acute arthritis. Values are expressed as mean ± S.D. (n = 6). * Compared with the group I, p < 0.05. # Compared with the group II (p < 0.05) and group IV (p < 0.05).
Figure 7

Schematic diagram showing the reaction between monosodium urate and uricase. Uricase can react with ions produced by monosodium urate, similar to the reaction of uricase with uric acid. Monosodium urate ⇄ monosodium urate ion + Na⁺ (1) Monosodium urate ion + H₂O + O₂ → 5-hydroxyisourate + H₂O (2) 5-hydroxyisourate + H₂O → Allantoin + CO₂ (3)
Figure 8
Schematic illustration of a 50 mg/ml monosodium urate suspension under a polarized light microscope.