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Comparison of the different monosodium urate crystals in the preparation process and pro-inflammation

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Abstract

Objectives The deposition of monosodium urate (MSU) crystals within synovial joints and tissues is the initiating factor for gout arthritis. Thus, MSU crystals are a vital tool for studying gout's molecular mechanism in animal and cellular models. This study mainly compared the excellence and worseness of MSU crystals prepared by different processes and the degree of inflammation induced by MSU crystals.

Methods MSU crystals were prepared using neutralization, alkali titration, and acid titration methods. The crystals' shape, length, quality, and uniformity were observed by polarized light microscopy and calculated by the software Image J. The foot pad and air pouch models were used to assess the different degrees of inflammation induced by the MSU crystals prepared by the three different methods at different time points. Paw swelling was evaluated by caliper. In air pouch lavage fluid, inflammatory cell recruitment was measured by hemocytometer, and the level of IL-1β, TNF-α, and IL-18 by ELISA. Inflammatory cell infiltration was assayed by immunohistochemistry of air pouch synovial slices.

Results For the preparation of MSU crystals with the same uric acid, the quantity acquired by the alkalization method was highest, followed by neutralization, with the acid titration method being the lowest. The crystals prepared by neutralization were the longest. The swelling index of the foot pad induced by MSU crystals prepared by acid titration was significantly lower than that of the other methods at 24 h. The inflammatory cell recruitment and level of IL-1β, TNF-α, and IL-18 in air pouch lavage fluid were lowest in animals with crystals prepared by acid titration. IL-1β secretion induced by MSU crystals prepared by acid titration was significantly lower than that of the other two groups, but there was no significant difference in IL-18 secretion between the three groups in THP-1 macrophages and BMDMs.

Conclusions All three methods can successfully prepare MSU crystals, but the levels of inflammation induced by the crystals prepared by the three methods were not identical. The degree of inflammation induced by MSU crystals prepared by neutralization and alkalization is greater than by acid titration, but the quantity of MSU crystals obtained

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by the alkalization method is higher and less time-consuming. Apparently, the window of inflammation triggered by acid titration preparation is shorter compared to other forms of crystal preparation. Overall, MSU crystals prepared by the alkaline method should be recommended for studying the molecular mechanisms of gout in animal and cellular models.

Keywords Gout, MSU crystals, Prepared method, Comparison, Inflammation

Introduction

Gout is the most common inflammatory arthritis in men and impairs the quality of life. Elevation of serum uric acid exceeding the local solubility limits leads to MSU crystal deposition in the articular joints and periarticular tissues, causing clinical manifestations, including severe pain, erythema, and swelling. Hyperuricemia is the vital biological basis of a gout attack, and MSU crystals are the key to acute gout arthritis [1–3]. A rise in the prevalence of gout was observed worldwide, ranging from 1 to 4% and incidence ranging from 0.1 to 0.3%. Han Chinese and some ethnic groups in Asia have a higher prevalence of gout [4–6]. Deposition of MSU crystals in joints and other tissues could lead to chronic joint damage, kidney stones, and renal insufficiency. In addition, gout is an independent risk factor for diabetes, hypertension, and cardiovascular diseases [1, 7].

MSU crystals are the key to gout flare. For in vivo and in vitro studies of urate crystal-induced inflammation, preparation of urate crystals is required. However, various preparation methods are reported in the literature, and their advantages and disadvantages are unknown. The differences in MSU preparation by different methods, such as time requirements, achieved MSU length or yield, and the degree of the inflammatory response, have not been compared. MSU crystals induce classic animal models of gouty arthritis to study the mechanism of gout attacks, drug effects, and their mechanism of action. According to different adjustments of pH value, the preparation of MSU crystals can be a neutralization, acid titration, or alkali titration method [8–10].

The common mouse models of gout include the air pouch model, peritonitis model, a foot gout model, and a gout arthritis model in mice [11–14]. These gout models have different characteristics because of the different sites of inflammation, but they all show the basic characteristics of gout since MSU is their common trigger. In the air pouch model, synovial-like membranes are formed on the back of mice, and MSU crystals are injected into the pouch, giving rise to the pathological characteristics of acute gouty synovial, which is often used to observe the migration and infiltration of leukocytes in inflammatory areas, especially neutrophils [13]. A model where MSU crystals induced paw edema is often used to evaluate the severity of inflammation in research related to drugs and genes [15, 16].

This study compares the three MSU crystal preparation methods and induces inflammation. The three methods can successfully prepare MSU crystals that induce inflammation. However, MSU crystals prepared by the neutralization method have the best mixing ability, and the crystal prepared is fine and does not easily block the needle.

Materials and methods

Reagents

Uric acid, NaOH, NaCl, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA), Monosodium Urate Crystals (Merck,USA), Endotoxin Test Limulus Kit (Rapid Gel Method) (BIOENDO, China).

Mice

Male C57BL/6 mice were housed in temperature-controlled rooms and received water and food ad libitum. All mice used for the experiments were aged eight weeks. All experimental procedures were approved by the Affiliated Hospital of Qingdao University (AHQU-MAL20190908).

Preparation and identification of MSU crystals

Neutralization MSU crystals were prepared by the neutralization method as described previously [9]: 0.25 g of uric acid was added to 45 mL of deionized water, then 300 µL of 5 M NaOH was added, stirred, and heated at 250 ℃ until the uric acid was completely dissolved. After adding 1 mL of 5 M NaCl, the solution was allowed to stand at room temperature for seven days. The crystals were washed three times with absolute ethanol and dried naturally. Then, they were sterilized by heating at 180 ℃ for 2 h before each experiment.

Alkali titration MSU crystals were prepared by alkali titration as described previously [17]. Uric acid (4 g) was dissolved in 800 mL of sterile demineralized water at 60 ℃. The pH was adjusted to 8.9 with 0.5 M NaOH, and the initial temperature was maintained until complete dissolution. The solution obtained was kept in a refrigerator (4 ℃) for 24 h, when the white precipitate was decanted, and subjected to drying at 100 ℃ for 6 h. After drying, the crystals were sterilized at 180 ℃ for 2 h.

Acid titration MSU crystals were prepared by acid titration as described previously [13]. Briefly, 1.0 g of uric acid

was dissolved in 200 mL of boiling distilled water containing 6.0 mL of 1 M NaOH. After adjusting the pH of the solution to 7.2 with HCl, crystals that formed were sterilized by heating at 180 °C for 2 h. The solution was gradually cooled by stirring at room temperature and stored overnight at 4℃. After drying, the crystals were sterilized at 180℃ for 2 h.

MSU crystals' morphological and birefringence properties were assessed by standard light microscopy and polarized light microscopy (Nikon, ECLIPSE LV100POL, Japan). The length of crystals (under four fields of view) was imaged by an upright fluorescence microscope (ZEISS, Imager.M2, Germany).

Mouse air pouch model
Eight-week-old wild-type mice were used for in vivo experiments. Air pouches were created by two dorsal subcutaneous injections of 5 mL and 3 mL of sterile air (days 0 and 3) under isoflurane anesthesia. On day 7, PBS or MSU (3 mg/mL, diluted in 1mL of PBS) were injected directly into the air pouch. At different time points, the air pouch exudate cells were harvested by lavage with 3 mL of PBS containing 25 units/mL heparin and 10% fetal bovine serum (FBS). The cells were retrieved from the lavage fluid, and lavage fluid was retained for assay.

Acute gout foot model
MSU crystals (20 mg/mL, 20 μL) were injected subcutaneously into the right hind paws of mice, and the left paws were injected with PBS as control. Paw swelling was evaluated at different time points (4, 8, 12, 24, 48 and 72 h). Paw swelling index was defined as the diameter difference between the right and left paws.

Table 1 MSU crystal preparation procedures

Method		Neutralization	Alkali titration	Acid titration
Reagent	Uric acid(g)	0.25	4	1
	NaOH(M)	5	0.5	1
	Volume(mL)	0.3	Adjust PH to 8.9	6
	HCL	-	-	Adjust PH to 7.2
	ddH$_2$0(mL)	45	800	194
Condition	PH	7.2	8.9	7.2
	Temperature	250℃	60℃	180℃
	Stored	26℃ for 7 days	4℃ for 24 h	4℃ for 24 h
Endotoxin removal		180℃ for 2 h	180℃ for 2 h	180℃ for 2 h
Endotoxin content		<0.25EU/mL	<0.25EU/mL	<0.25EU/mL
Time(day)		8	2	2

MSU, monosodium urate. PH, Pondus Hydrogenii. ddH$_2$0, double distilled water. HCL, hydrogen chloride. NaOH, sodium hydroxide

Bone marrow-derived Macrophages cells (BMDMs) isolation and culture
Bone Marrow-derived Macrophages Cells (BMDMs) were isolation fromC57BL/6 mouse. Bone marrow cells were recovered from tibia and femoral bones of mice and plated at the density of 2×10^6 cells in 6-well plates. Cells are cultured in 2 mL PRMI 1640 media with10% FBS and 200 μg/ml GM-CSF. Every two days, the media renewed after cells were washed 3 times with PBS until complete differentiation of BMDMs. BMDMs were primed 3 h with ultrapure lipopolysaccharide (LPS; 20 ng/mL), then stimulated 8 h with or without MSU (200 μg/mL). After the MSU stimulation, supernatants were collected for cytokine quantification. THP-1 (human monocytic leukemia) line was purchased from Procell, Wuhan. THP-1 cells were incubated with 50 ng/mL of PMA for 24 h and then stimulated with or without MSU (200 μg/mL) for 8 h. Cell supernatant was collected and IL-1β and IL-18 concentration was detected by Enzyme-linked immunosorbent assays (ELSIA).

Enzyme-linked immunosorbent assays (ELSIA)
According to the manufacturer's instructions, the level of IL-1β, IL-18 and TNF-α measured by ELISA kits (Elabscience, China).

Statistical analysis
Prism 8.1.1 (GraphPad Software) and SPSS 24.0 were used for statistical analysis. The data were expressed as mean±SEM. Differences between experimental groups were tested using the unpaired t-test. $p < 0.05$ was considered statistically significant.

Results
The comparisons of three MSU crystals-preparation procedure
The comparisons among the three MSU crystal-preparation procedures are revealed in Table 1. Uric acid and NaOH were all used by the three methods, but extremely corrosive HCl was only used by the acid method. When the uric acid was completely dissolved, it was allowed to stand and dry for different times. The neutralization procedure was relatively complicated and time-consuming and required the crystals to stand at room temperature for seven days, washed three times with absolute ethanol, and then dried naturally. The endotoxin in MSU crystals was removed by dry heat methods. MSU crystals were placed at 180℃ for 2 h and then to detect endotoxin content by Rapid gel method and endotoxin content was less than 0.25 EU/mL.

The identification of MSU crystals
MSU crystals prepared by three different methods were all blue-yellow birefringent needle-like crystals by

standard light and polarized light microscope, are similar to those of clinical patients and commercial MSU crystals (Fig. 1A). The quantity and length of MSU crystals were calculated. The results showed that the length of MSU crystals prepared by the acid titration was 126.83 ± 6.31 μm, which was longer than that of alkali titration (94.83 ± 6.62 μm, $p < 0.05$) and neutralization (87.83 ± 6.97 μm, $p < 0.05$, Fig. 1B). Also, the length uniformity of MSU crystals prepared by acid titration is significantly lower than the other two methods (Fig. 1C). The quantity of MSU crystals prepared by the alkali titration was 0.69 ± 0.05 g/1 g uric acid, which was higher than that of neutralization (0.46 ± 0.04 g/1 g uric acid, $p < 0.05$) and the acid titration (0.51 ± 0.03 g/1 g uric acid, $p < 0.05$, Fig. 1D).

The comparison of MSU-induced inflammation in the paw

To compare the difference in the gout inflammatory response induced by MSU crystals prepared by these three methods, foot pad models of MSU crystal-induced inflammation were assessed at different time points. After injection of the MSU crystals prepared by three methods, the swelling index of the foot pad increased at 4 h and peaked at 24 h, and was still high at 72 h after injection of MSU crystals, although it had returned to a similar level to that observed at 72 h (Fig. 2A-B). Compared with acid titration, the swelling index of the foot pad induced by MSU crystals prepared by neutralization and alkali titration was significantly increased at 24 h ($p < 0.05$, Fig. 2A-B). At 24 h, while there was a trend between the acid titration group and the neutralization group ($p = 0.095$) when the heat pain index was measured by a heat pain meter at 52 ℃, there was no statistical difference between three groups (Fig. 2C).

The comparison of MSU-induced inflammation in the air pouch

A mouse air pouch model was also used to assess different degrees of inflammation induced by MSU crystals prepared by the three methods and clinical MSU
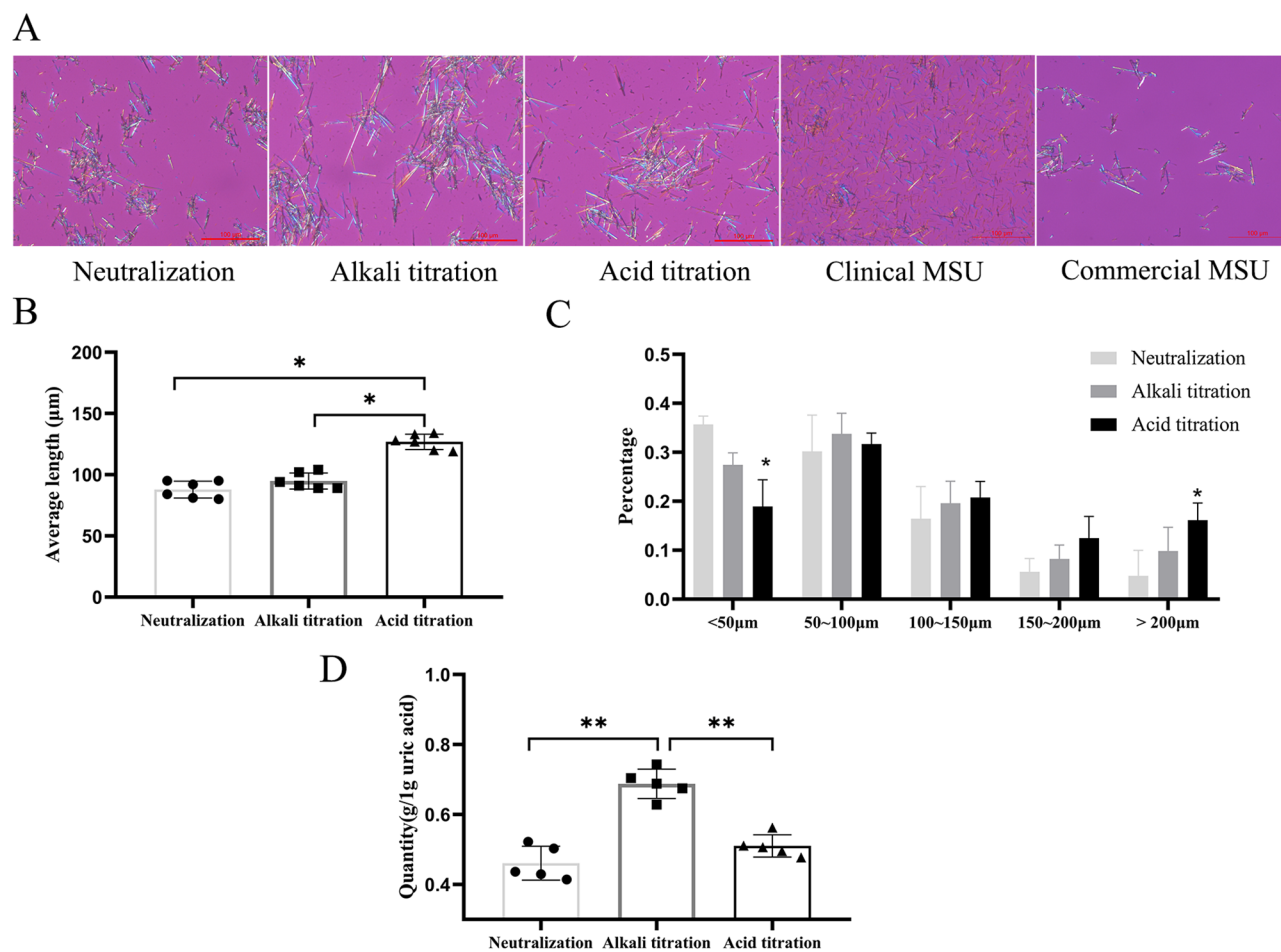

Fig. 1 MSU crystal identification results. **A**, Polarized light microscope observation of the morphology of MSU crystals (×200). **B**, The average of lengths of MSU crystals (n = 4). **C**, The ratio of different lengths of MSU crystals (n = 3). **D**, The quantity of MSU crystals (n = 5). *p < 0.05,**p < 0.01. MSU, monosodium urate
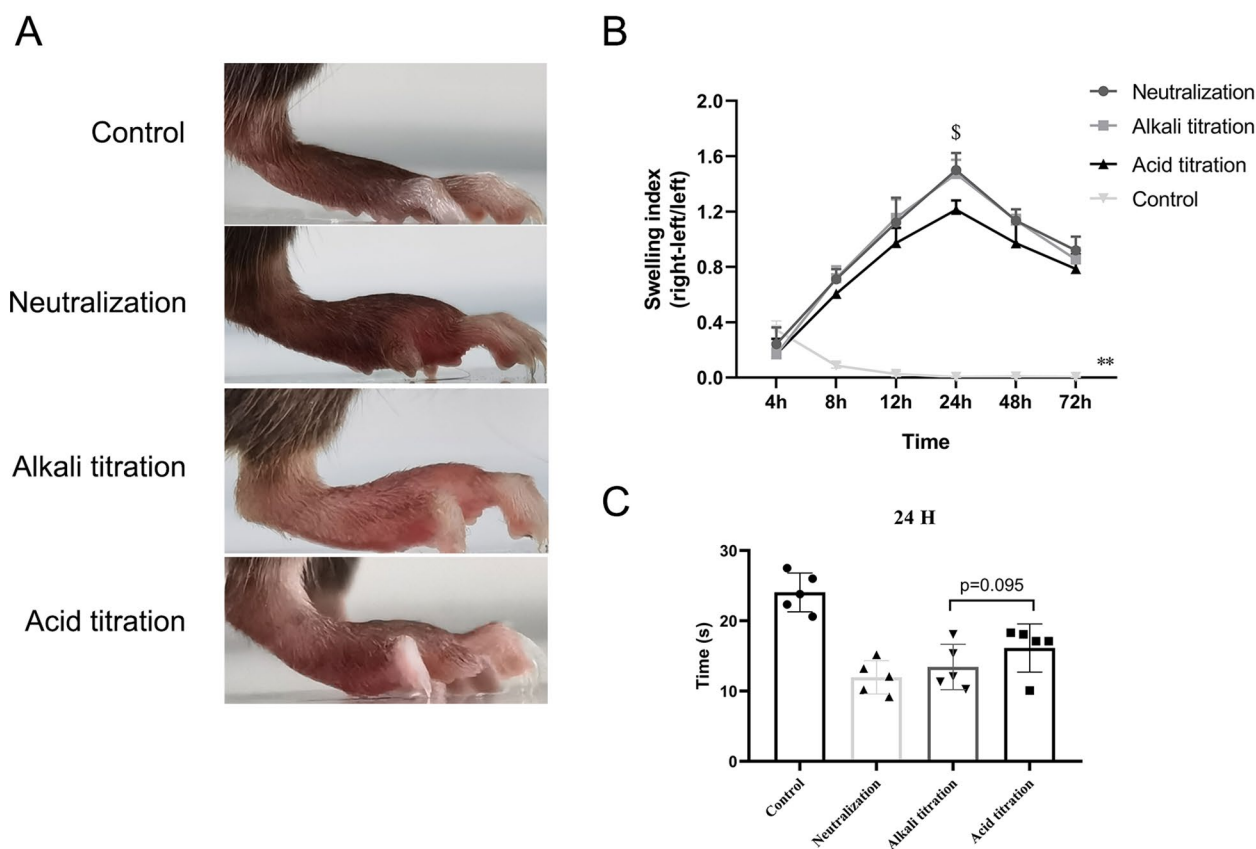
Fig. 2 The comparison of MSU-induced inflammation in the paw. MSU crystals suspension (20 mg/mL, 20 μL) was injected into the right footpad of mice and PBS as control into the left footpad of mice. An electronic caliper was used to test foot thickness. Paw swelling index was defined as the diameter difference between the right and left paws. **A-B**, Time course of swelling index (right-left) (n=4–6).**C**, *p＜0.05,**p＜0.01. MSU, monosodium urate

crystals or commercial MSU crystals. Compared to control groups, the total number of cells and the levels of IL-1β, TNF-α, IL-18 in air pouch lavage fluid were all markedly increased at 4, 8, and 12 h after MSU crystal injection (Fig. 3). At 4 h after MSU injection, the total number of cells in the air pouch lavage fluid was all increased and maintained at a high level at 12 h (Fig. 3A-B). However, the total number of cells induced by MSU crystals prepared by acid titration was significantly lower than by alkali titration or neutralization (p<0.05), also lower than clinical and commercial MSU crystals (p<0.05) at 8 h (Fig. 3B). After MSU crystal stimulation, the secretion of IL-1β in the air pouch lavage fluid increased significantly, peaked at 8 h, and decreased at 12 h in each group and the level of IL-1β in the acid titration group was significantly lower than the other groups at 8 and 12 h (Fig. 3**CD**). The changes in the concentration levels of IL-18 and TNF-α were largely consistent with those of IL-1β (Fig. 3E-H), but the level of IL-18 in the acid titration group was significantly lower than the other two groups only at 12 h and the level of IL-18 in the commercial MSU group was significantly higher than

that in the prepared MSU groups (Fig. 3F). TNF-α level in the acid titration group was significantly lower than the other two the prepared MSU groups and commercial MSU groups only at 4 h (Fig. 3H), Furthermore, the levels of TNF-α were significantly higher in the commercial MSU group than in the other groups at 8 h (Fig. 3H).

The comparison of MSU-induced inflammation in macrophages
Then we compared the inflammation effects of the three methods prepared MSU crystals using the bone marrow-derived macrophages (BMDMs) and THP-1 macrophages. IL-1β secretion in the acid titration group was significantly lower than the other two group in BMDMs (Fig. 4A). In THP-1 macrophages, the secretion of IL-1β in the acid titration group was significantly lower than the alkali titration group (Fig. 4C). However, there was no significant difference in the level of IL-18 between the groups (Fig. 4B, D).
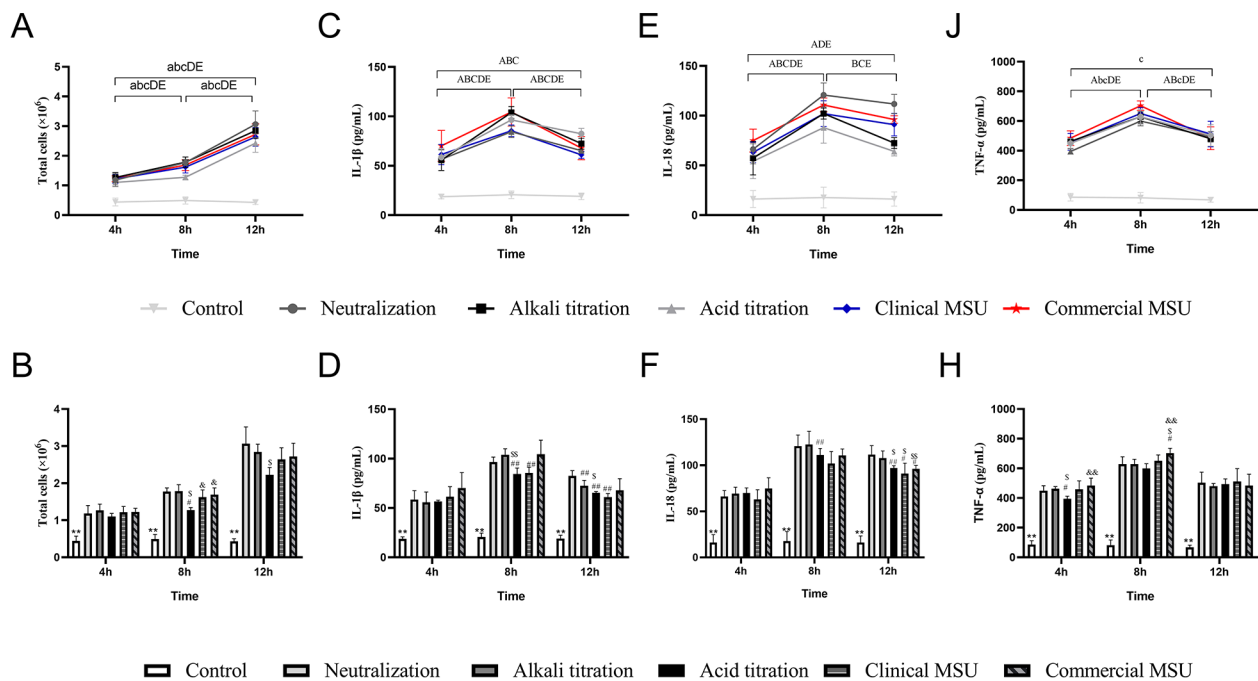
Fig. 3 The degree of inflammation in the prepared MSU -induced air pouch model. 3 mg/1mL MSU suspension was injected into the air pouch, and the lavage fluid harvested at 4 h, 8 h, 12 h (n = 4–6 for each group). **A-B**, Total cell number was counted by a hematocytometer. **C-H**, Levels of IL-1β, IL-18 and TNF-α measured by enzyme-linked immunosorbent assays (ELSIA) in air pouch lavage fluid at 4,8, and 12 h. Unpaired t-test was used for each group. IL, interleukin. TNF, tumor necrosis factor. MSU, monosodium urate. [a] $p < 0.05$, [A] $p < 0.01$ for different time points of the Neutralization group; [b] $p < 0.05$, [B] $p < 0.01$ for different time points of the Alkali titration group; [c] $p < 0.05$, [C] $p < 0.01$ for different time points of the Acid titration group; [d] $p < 0.05$, [D] $p < 0.01$ for different time points of the Clinical MSU group; [e] $p < 0.05$, [E] $p < 0.01$ for different time points of the Commercial MSU group; *$p < 0.05$, **$p < 0.01$ for control group compared to other groups; [#]$p < 0.05$, [##]$p < 0.01$ for the Neutralization group compared to other groups; [$]$p < 0.05$, [$$]$p < 0.01$ for the Alkali titration group compared to other groups; [&]$p < 0.05$, [&&]$p < 0.01$ for the Acid titration group compared to other groups

Discussion

Our study revealed that three methods could successfully prepare monosodium urate (MSU) crystals, but ease of preparation varies, the degree of inflammation caused by them is different. The prepared MSU crystals were basically consistent with clinical MSU crystals or commercial MSU crystals in terms of shape and degree of inflammation, except at individual time points. The degree of inflammation induced by MSU crystals prepared by neutralization and alkalization methods is greater than by the acid titration method, but the quantity of MSU crystals obtained by the alkaline method was higher and less time-consuming. Overall, MSU crystals prepared by the alkaline method should be recommended to study the molecular mechanism of gout in animal and cellular models.

Monosodium urate (MSU) monohydrate ($NaC_5H_3N_4O_3 \cdot H_2O$), in which a urate molecule binds to sodium and a water molecule, is one of the most common forms of crystalline urate, the main sediment in gouty arthritis [18]. MSU crystals viewed by microscope were needle-shaped, with a triclinic structure containing three unequal axes, none of which are perpendicular to the others [19]. The general physicochemical process of MSU crystallization formation is similar to that of other crystal formation processes, and the formation conditions depend on the urate concentration and other related factors, such as the solubility limit, approximately 6.8 mg/dL (405 μmol/L) under normal physiological conditions [20, 21]. Beyond this concentration, supersaturation causes the formation of MSU crystals to begin, with further changes in urate solubility or other excitation events and further expansion of crystallization occurring, depending on local conditions. Multiple factors have been found to influence the formation of MSU crystals, including temperature, sodium and cation concentrations, pH, mechanical stress, cartilage composition, uric acid-binding antibodies, and cartilage and synovial fluid components [21–23]. When the conditions affecting MSU solubility change (e.g., increased concentration and/or decreased temperature), MSU molecules accumulate in solution [24]. Given the importance of MSU crystals in studying the pathogenesis of gout diseases, the preparation method of urate crystals is very important.

The preparation of MSU crystals is often the first step in studying gout-related mechanisms [25]. In most experiments, a small number of MSU crystals used natural tophi extracted from the human body, and most of them
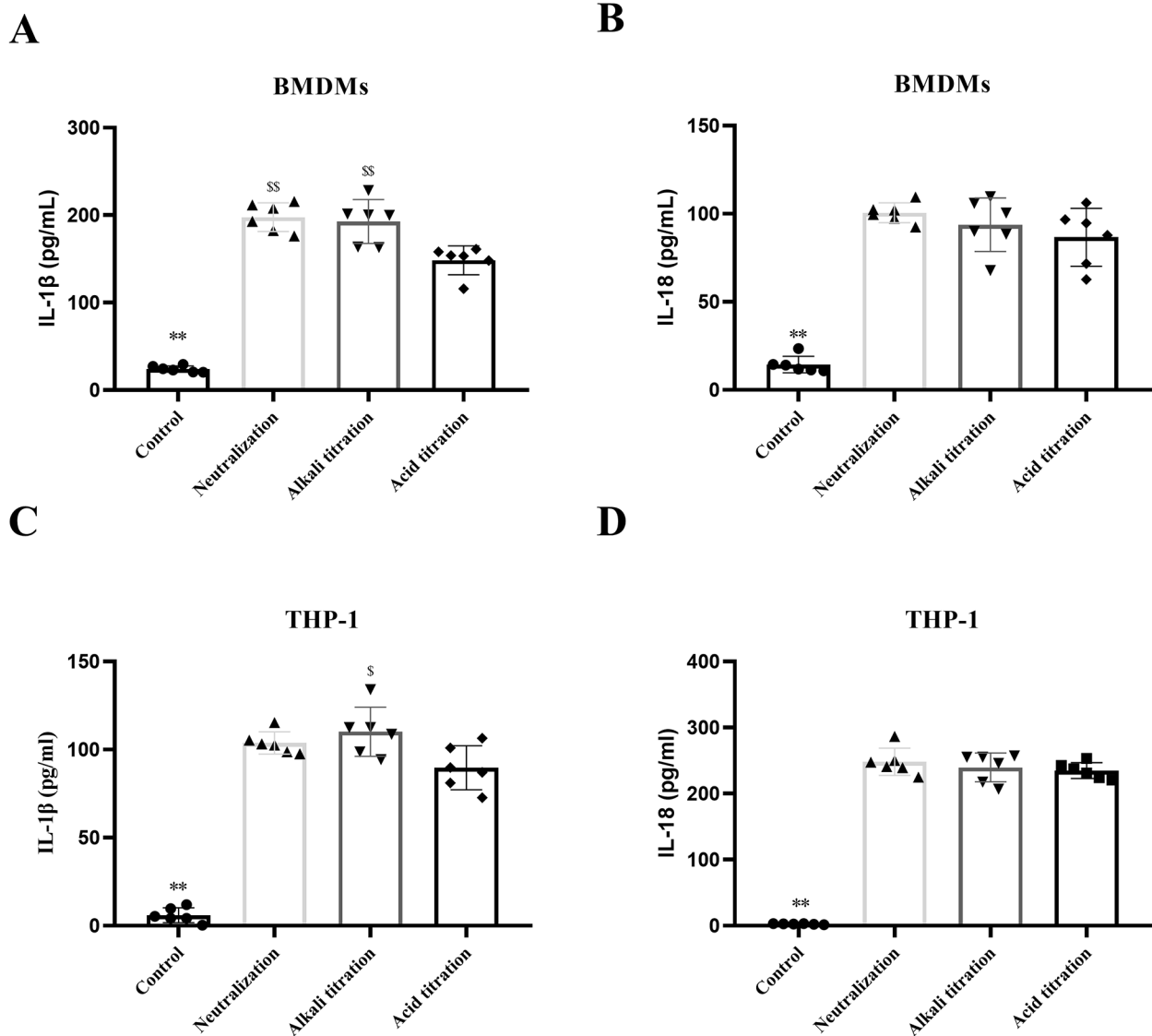
A

B

C

D

Fig. 4 The extent of inflammation in the prepared MSU-induced BMDMs. Primary bone marrow mononuclear cells from C57/ BL6 mice were cultured with GM-CSF for 7 days, stimulated for 3 h with LPS (20 ng/mL) and 8 h with MSU crystals (200 μg/mL), and cell supernatants were collected. THP-1 cells were incubated with 50 ng/mL of PMA for 24 h and then stimulated with or without MSU (200 μg/mL) for 8 h. **A, C**. The levels of IL-1β; **B, D**. The level of IL-18. IL, interleukin. TNF, tumor necrosis factor. MSU, monosodium urate. BMDMs, bone marrow-derived macrophages. ******$p<0.01$ for control group compared to other groups; $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.01$ for the Alkali titration group compared to other groups

were artificially prepared and synthesized. It is necessary to explore an efficient and facile method to prepare MSU crystals. However, there are few previous studies on the elaboration of the characteristics of different MSU crystal preparation methods and comparing their advantages and disadvantages. Our study reported three main urate preparation methods: neutralization, alkaline, and acid methods [13, 17, 26–28]. This study intends to compare these three different preparation methods, from their degree of simplicity, the uniformity of uric acid crystal length, crystal acquisition rate, and the degree of the

induced inflammatory response, to obtain simple, efficient MSU crystals with better inflammatory effect.

We compare the lengths and quantities of MSU crystals prepared from the three MSU crystal preparation methods. The mouse foot pad and air pouch model were used to compare the differences in the inflammatory response induced by crystals from the three different MSU preparation methods in mice. The results showed that Urate crystals were prepared using the same quality of uric acid, with the highest quality obtained by alkaline titration, followed by the acid method, and the lowest by neutralization. The lengths of MSU crystals prepared by

the three preparation methods were classified according to <50 μm, 50~100 μm, 100~150 μm, 150~200 μm and >200 μm, and the proportion of MSU crystals in each length interval in the three preparation methods was counted. The uniformity of urate crystals prepared by acid titration is relatively poor, and the crystals with large lengths account for more, the proportion of the crystals larger than 200 μm and shorter than <50 μm was significantly higher than the remaining two groups. The uniformity of MSU crystals prepared by the alkali titration and neutralization methods was better. And the average length of MSU crystals obtained by acid titration is the longest, and no significant difference was observed in the shape of crystals obtained with the other two methods. In an in vivo study, compared with injecting larger MSU crystals into the knee, injecting smaller MSU crystals into the knee of dogs resulted in a stronger inflammatory response, higher phagocytosis rate, and increased leukocyte infiltration [29]. Ashika Chhana also found that human cartilage homogenate will increase the formation of MSU crystals, promote the formation of smaller crystals, and enhance the inflammatory response [30]. These studies suggest that altering the size of MSU crystals may affect the inflammatory response elicited [21, 30]. In this study, the window of inflammation triggered by acid titration preparation is shorter compared to other forms of crystal preparation. Certain previous studies have shown that the window of inflammation induced by MSU crystals is not exactly the same. TNF-α level in cell culture supernatants reached peak at 15 or 18 h, and MSU crystal-induced IL-1β secretion levels peaked at 6 or 8 or 48 h [16, 31–33] .

There are some limitations to this study. Firstly, the current study weakly investigated the inflammation triggered by MSU crystals, leaving out the inflammasome pathway, the key intracellular signaling activated by these crystals, and later time points of investigation. Secondly, the detection indicators are not comprehensive enough.

In conclusion, by comparing three urate crystal synthesis methods, the alkali titration method has high repeatability, good uniformity, simple production procedures, few steps, is less time-consuming, and the induced inflammation model is stable. The window of inflammation triggered by acid titration preparation is shorter compared to other forms of crystal preparation. But it needs to show the differences among the time points in the groups. It has an important significance for the in vivo studies, especially those testing anti-inflammatory drugs. It will help us to better study the mechanism of gout and formulate treatment strategies in the future. However, the differences in the gouty inflammation induced by MSU prepared by different methods remain poorly studied. Exploring a simple, efficient, time-saving, and highly inflammatory MSU crystal is significant for

animal and cell experiments. At present, we only discuss the above three MSU crystal preparation methods, and we may find better MSU crystal preparation methods in the future.

Conclusion

The three methods can successfully prepare MSU crystals, but the degree of provoked inflammation is different. The degree of inflammation induced by MSU crystals prepared by neutralization and alkalization is greater than by the acid titration, but the quantity of MSU crystals obtained by the alkaline method was higher and less time-consuming. Overall, MSU crystals prepared by the alkaline method should be recommended to study the molecular mechanism of gout in animal and cellular models.

Author contributions

Zhen Liu and Lin Han designed the study, Fei Yan, Maichao Li, Youlin Fan, Xuefeng Wang and Yuwei He performed the experiments, Fei Yan, Hui Zhang, Xuan Yuan and Zhaotong Jia analyzed the data and wrote the paper.

Data Availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Affiliated Hospital of Qingdao University (AHQU-MAL20190908).

Consent for publication

All authors agree to be published in this journal.

Competing interests

The authors declare no competing financial interests.

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