Melatonin Alleviates Acute Gouty Inflammation *In Vivo* and *In Vitro**

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Summary: The aim of this study was to identify the effects of melatonin on acute gouty inflammation and to investigate the underlying mechanisms. We found significantly lower serum melatonin levels in gout patients in the acute phase than in those in the remission phase or in normal individuals. The mRNA expression of melatonin receptor 2 (MT2) was also lower in gout patients than in normal individuals. To verify the *in-vivo* role of melatonin, a gouty arthritis model was established by intraarticular injection of monosodium urate (MSU, 1 mg) crystals into the paws of C57BL/6 mice. Joint inflammation in the mouse model was evaluated by measuring the thickness of the right paw/left paw, and the inflammation index was determined by examining infiltrating neutrophils with haematoxylin and eosin (H&E) staining. Melatonin was found to reduce both paw thickness and the inflammation index in the mouse model, and melatonin also reduced the mRNA levels of interleukin-1 beta (IL-1β), IL-6 and NLR family pyrin domain containing 3 (NLRP3) inflammasome. To mimic gouty inflammation *in vitro*, mouse peritoneal macrophages were stimulated with lipopolysaccharides (LPS) plus MSU. Melatonin was revealed to reduce IL-1β secretion by stimulated macrophages. The mRNA expression levels of IL-1β and IL-6 were also inhibited by melatonin. Western blot analysis showed that the expression of NLRP3, caspase-1 and pro-IL-1β was also inhibited by melatonin. In conclusion, our study demonstrated that melatonin alleviated gouty inflammation *in vivo* and *in vitro*, and the underlying mechanism may involve inhibiting the assembly of the NLRP3 inflammasome.
Key words: melatonin; gout; NLRP3 inflammasome; monosodium urate

Gout is the most common form of non-infectious inflammatory arthritis, with a prevalence of less than 1.14% in the eastern coastal areas of China[1]. It is closely associated with metabolic diseases, cardiovascular disorders, and kidney malfunction. Prevention of gout has great socioeconomic importance[2]. Currently, drugs that are commonly used for the treatment of gout are nonsteroidal anti-inflammatory drugs, glucocorticoids and colchicine. All of these drugs have significant liver and kidney toxicities and can damage glucose tolerance and the digestive tract. Therefore, it is necessary to find safe and effective drugs for the treatment of gouty inflammation. Typical acute gouty inflammation is self-limited inflammation caused by monosodium urate (MSU) crystals deposited in the joints[3]. MSU crystals are recognized by Toll-like receptors (TLR2/4) and CD14, which are anchored on the surface of monocytes/macrophages, providing extracellular and intracellular stimuli and initiating the phagocytosis of MSU crystals by monocytes and macrophages[4]. Joint resident macrophages are stimulated by the phagocytosis of MSU crystals and activate the NLRP3 inflammasome through dual-signal activation. Caspase-1 cleaves pro-IL-1β into mature IL-1β, which attracts neutrophils to the joint, triggering clinical acute inflammatory reactions, such as redness, swelling, heat and pain. Therefore, inhibiting the activation of NLR family pyrin domain containing 3 (NLRP3) in inflammatory cells can alleviate acute gouty inflammation[5].

Melatonin is a natural hormone that is mainly secreted by the pineal gland[6]. Melatonin primarily regulates circadian cycles and improves sleep quality[7]. Recently, melatonin has been confirmed to have immunomodulatory effects and plays an anti-inflammatory role in various models of acute inflammatory diseases, such as acute pancreatitis and sepsis[8]. However, the specific mechanism of the anti-inflammatory effect of melatonin remains unclear. Recent studies have shown that melatonin can inhibit the expression of NLRP3 in a variety of

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cells, such as alveolar macrophages, adipocyte-related macrophages, and intestinal epithelial cells, to exert an anti-inflammatory effect against lipid-associated inflammation and radiation-associated intestinal diseases[9].

However, whether melatonin can be used in the treatment of acute gouty inflammation has not been reported. Thus, we hypothesized that melatonin inhibits acute gouty inflammation by inhibiting NLRP3 inflammasome. In addition, a mouse model and a primary abdominal macrophage model were established to verify this hypothesis *in vivo* and *in vitro*.

1.MATERIAL AND METHOD

1.1 Patients and Specimen Collection

Twenty-four acute gout patients and 29 gout patients in remission who met the American Rheumatism Society (ACR) diagnostic criteria of gout in 2015 were enrolled in this study between April 2019 to July 2019. Additionally, 9 normal individuals were recruited as controls. The patients were all male, aged 22 to 62. Moreover, there were no age differences among the 3 groups. All participating patients gave their written informed consent. This study was approved by the Ethics Committee of Shanghai Huashan Hospital. Venous blood samples were collected from patients in the outpatient department, and sera were isolated after the samples were centrifuged at room temperature at 1500 r/min for 20 min. Additionally, peripheral blood mononuclear cells (PBMCs) were isolated using lymphoprep density gradient centrifugation (Accurate Chemical, USA).

1.2 Reagents and Antibodies

Melatonin (purity>98%) was acquired from Selleck (USA). MSU was obtained from Sigma Company (USA). Alpha-modified minimal essential medium (α-MEM), fetal bovine serum (FBS), RPMI 1640 and penicillin-streptomycin were obtained from Thermo Fisher Scientific (Australia). The haematoxylin and eosin (H&E) staining kit was obtained from Beyotime Biology Co. (China). Primary antibodies against NLRP3, P-65, caspase-1 and pro-IL-1β were purchased from Cell Signaling Technologies (USA).

1.3 MSU Crystal Preparation

MSU crystals were produced by a previously described method[10]. Uric acid (800 mg) was first dissolved in 155 mL of boiling Milli-Q water and 5 mL of sodium hydroxide, and the solution was then adjusted to pH 7.2 and cooled gradually at room temperature. After centrifugation (3000 g, 2 min, 4°C), part of the water was vaporized and crystallized for 2 h at 180°C to sterilize the sample. Finally, the crystals were collected and stored under sterile conditions.

1.4 ELISA Measurement of Serum Melatonin

ELISA was conducted according to the manufacturer's instructions (Abcam, UK). Briefly, samples were bound to monoclonal antibodies. Biotinylated mouse anti-melatonin antibodies were added to form immune complexes and attach to the plate. Horseradish peroxidase-labelled streptomycin was added to bind to biotin. Then, the enzyme substrate o-phenylenediamine (OPD) was added for visualization. After the addition of sulfuric acid, the reaction was terminated. The absorbance (*A*) value was measured at 492 nm, and since the melatonin concentration was positively correlated with the *A* value, the melatonin concentration in the sample could be determined using the standard curve.

1.5 Animal Grouping and Establishment of the Mouse Model of Gout

Male C57B/6L mice aged 6–8 weeks (20–25 g, Vitonlihua, China) were housed in a standard transparent plastic cage (5–6/cage) and allowed free access to food and drink. All tests were conducted in a temperature control room between 9 a.m. and 5 p.m. Animal care and handling procedures were approved by the Ethics Committee of Shanghai Huashan Hospital. First, the left and right paw thickness of each mouse was measured by a Vernier calliper and recorded as baseline data. To investigate the role of melatonin in MSU-induced acute gouty inflammation, the mice were randomly separated into three subgroups. In the blank group, the mice were injected with 50 μL of normal saline only in the left paw. In the model control group, the mice were injected with 50 μL of normal saline in the left paw and 1 mg of MSU crystals in 50 μL of normal saline in the right paw. In the model plus melatonin group, 50 μL of normal saline was injected into the left paw, and 1 mg of MSU and melatonin (15 mg/Kg) were injected into the right paw. Peak inflammation in the joint usually occurred 8 h after injection of the MSU suspension. Mice were sacrificed at the time of peak inflammation, and the right paw tissue was fixed with 4% paraformaldehyde. After decalcification, pathological H&E staining was performed. The inflammation index of the mouse paw was defined as the thickness of the right paw/left paw as measured by a Vernier calliper.

1.6 Culture of Peritoneal Macrophages Isolated from the Mouse Abdominal Cavity

Resident mouse peritoneal macrophages were collected by lavage with RPMI 1640. Each mouse was first intraperitoneally injected with 2 mL of starch broth (100 mL of 6% starch broth mixed with 1.0 g of peptone, 0.5 g of sodium chloride, and 0.3 g of beef extract) 2–4 days before the procedure. The mice were then sacrificed and soaked in 75% alcohol for 1–2 min. After disinfection, 5 mL of RPMI 1640 supplemented with penicillin-streptomycin was injected into the abdominal cavity to collect peritoneal macrophages. After centrifugation at 1000 r/min for 5 min, the cells

were washed once with RPMI 1640 and centrifuged twice. RPMI 1640 containing 10% FBS was used to resuspend the cells for further analysis.

1.7 Western Blotting

The cells were lysed in freshly prepared radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. After protein levels were quantified by the BCA method, equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was then blocked at room temperature with 5% bovine serum albumin (BSA) for 1 h and incubated overnight with diluted anti-NLRP3, anti-IL-1β and anti-caspase-1 primary antibodies at 4°C (Abcam, UK). After being washed three times with Tris-buffered saline containing Tween-20 (TBST), the membrane was then incubated with IgG monoclonal antibodies (Abcam, UK) diluted in 5% skimmed milk at room temperature for 1 h. The film was incubated with ECL matrix solution for 1 min, and signal development was analysed using INTAS Science Imaging (Germany).

1.8 Real-time Quantitative PCR (RT-qPCR)

A TRIzol-based one-step method was used to extract total RNA from macrophages, which was quantified using a spectrophotometer (Omega Plate Reader, BMG Labtech GmbH, Germany) according to the manufacturer's protocol (Invitrogen, USA). The extracted RNA was then reverse-transcribed into complementary DNA (cDNA) using a real-time polymerase chain reaction (RT-PCR) kit (Fermentas, Germany). Primers and PCR conditions were optimized with increasing cDNA to analyse the PCR products obtained at the logarithmic stage. Relative mRNA expression was calculated with the $2^{-\Delta\Delta CT}$ method. The primers used are listed below: mouse IL-1β 5′-TAC ATC AGC ACC TCA CAA GC-3′ and 5′-AGA AAC AGT CCA GCC CAT ACT-3′; mouse IL-6 5′-ACC AAG ACC ATC CAA TTC ATC-3′ and 5′-CTG ACC ACA GTG AGG AAT GTC-3′; mouse NLRP3 5′-GAC TGC GAG AGA TTC TAC AGC-3′ and 5′-CCT CCT CTT CCA GCA AAT AGT- 3′; mouse β-actin 5′-CGA TGA CAT CCG TAA AGA CC-3′ and 5′-AAC AGT CCG CCT AGA AC-3′; human MT1 5′-CCT GGT CAT CCT GTC GGT GTAT-3′ and 5′-CAG ATG TAG CAG TAG CGG TTGAT-3′; human MT2 5′-TGT TCT TGG TGA GTC TGG CATTG-3′ and 5′-GCA GAT GTA GCA GTA GCG GT-3′; human β-actin 5′-CTC CAT CCT GGC CTC GCT GT-3′ and 5′-GCT GTC ACC TTC ACC GTT CC-3′.

1.9 Statistical Analysis for Inter-Group Comparisons

The SPSS 19.0 software package was used for statistical analysis, and all data were expressed as the mean±standard error of the mean (SEM) or standard deviation (SD). A unpaired, 2-tailed Student's *t* test was used for comparisons between two groups. One-way ANOVA was used for comparisons between multiple groups, and the LSD-T test was used for pairwise comparisons within groups. $P<0.05$ was regarded as statistically significant. Figures were prepared using GraphPad Prism version 5.0.

2 RESULTS

2.1 Plasma Melatonin Expression in Gout Patients

Peripheral blood was collected from gout patients at 9–11 o'clock and analysed by ELISA. The level of plasma melatonin was significantly decreased in gout patients compared with normal individuals (acute gout: 8.012±2.65 pg/mL; remission: 9.601±1.06 pg/mL; normal: 10.66±1.25 pg/mL; fig. 1A). Additionally, we also isolated PBMCs from patients to analyse the expression of melatonin receptors (MT1 & MT2). Similar to melatonin, MT2 was significantly downregulated in gout patients compared with normal individuals (figs. 1B, 1C). These results suggest that melatonin is involved in systemic acute inflammation associated with clinical gout.

2.2 Melatonin Reduced the Inflammation Index in the Paws of Mice

To explore the role of melatonin in gout treatment, a mouse model of acute gout was established by MSU injection into the paw. The mice were sacrificed 8

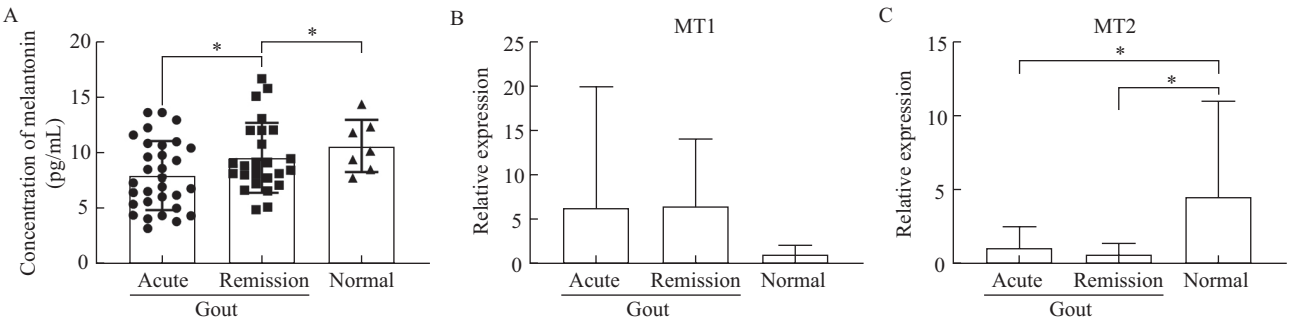

Fig. 1 Comparison of serum melatonin levels among different groups of individuals

A: Significantly lower serum melatonin was measured in patients in the acute phase (n=24) and remission phase (n=29) than in normal individuals (n=9). B, C: the mRNA expression of melatonin receptors (MT1 and MT2) in PBMCs of gout patients and normal individuals. ($^*P<0.05$)

h after MSU administration, and the thickness of the right paw in each group was determined. The thickness of the mouse paw in the model control group was significantly upregulated compared with that in the blank group (P<0.01), indicating the successful establishment of acute inflammation in the mouse paw. Interestingly, melatonin treatment plus MSU stimulation significantly reduced paw thickness in model mice (P<0.01), which indicated that melatonin could inhibit MSU-induced acute paw inflammation in mice (fig. 2A). Then, we investigated neutrophil infiltration in synovial tissues by H&E staining. Similar to the effect on paw thickness, melatonin treatment significantly reduced the infiltration of inflammatory cells. Overall, melatonin effectively inhibited the occurrence of acute inflammation (fig. 2B).

2.3 Melatonin Inhibited the mRNA Expression of IL-1β, IL6 and NLRP3

To determine the the mechanism by which melatonin regulates gout, a real-time PCR analysis was performed on the paw samples. The mRNA expression of IL-1β, NLRP3 and IL-6 in the model plus melatonin group (paw samples) was significantly downregulated compared with that in the model plus control group (fig. 3).

2.4 Melatonin Inhibited the Expression of IL-1β and IL-6 in Peritoneal Macrophages

Based on the findings of the real-time PCR, we further focused on NLRP3 inflammasomes. Mouse peritoneal macrophages were isolated, cultured, and divided into two groups: the DMSO solvent control group and the melatonin intervention group. Both

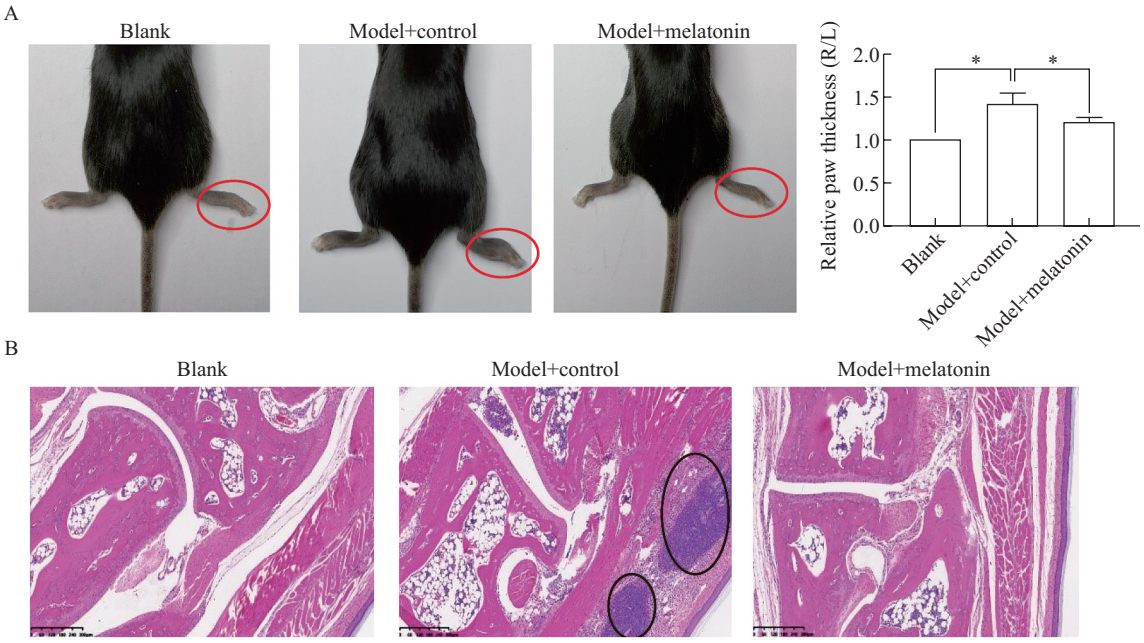

Fig. 2 Melatonin inhibited MSU-induced acute inflammation in the paws of mice.
A: the paw thickness of the right paw (as indicated in the circles) in the model melatonin group and model control group (normalized to the right control paw thickness $^{*}P$<0.05); B: H&E staining showed that inflammatory cells infiltration (as indicated in the circles, ×40) in the model melatonin group was significantly less than that in the model control group.

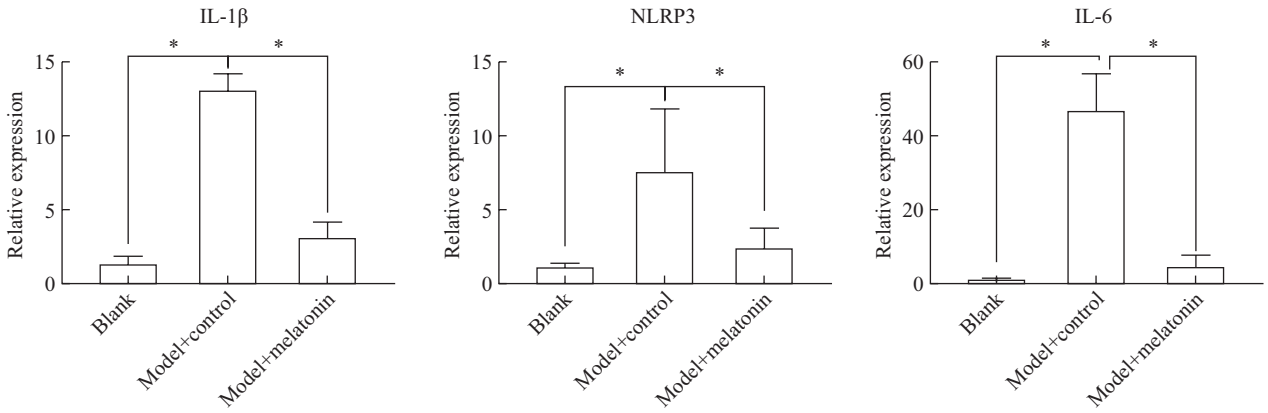

Fig. 3 The mRNA expression of IL-1β, IL-6 and NLRP3 decreased in the model melatonin group.
RNA was extracted from paw samples in each group and the mRNA expression of IL-1β, IL-6 and NLRP3 was compared between groups ($^{*}P$<0.05).

groups were stimulated with 100 ng/mL LPS for 12 h, followed by 200 μg/mL MSU for an additional 6 h. The melatonin intervention group was treated with 100 nmol/L or 200 nmol/L melatonin 1 h before MSU administration (pre-treatment) or were cotreated with MSU for 6 h (co-stimulation). Both pre-treatment and co-stimulation with melatonin significantly repressed the expression of IL-1β and IL-6 in the cell culture medium of peritoneal macrophages (fig. 4A). Similarly, the mRNA expression of IL-1β and IL-6 in the melatonin intervention group was significantly downregulated (fig. 4B).

2.5 Melatonin Inhibited the Protein Expression of NLRP3, Pro-IL-1β and Caspase-1 in Peritoneal Macrophages

Western blot analysis indicated that the expression of NLRP3, pro-IL-1β and caspase-1 was significantly downregulated in the melatonin (100 nmol/L) intervention group compared with the DMSO solvent control group (fig. 5).

3 DISCUSSION

Melatonin is an endogenous indoleamine secreted by the pineal gland[11]. In vertebrates, circulating melatonin is mainly produced by the pineal gland and can also be produced by other organs, such as the gastrointestinal tract, skin and retina[12]. Melatonin is also a multifunctional molecule involved in many physiological processes, including the initiation of sleep, mood regulation, control of sexual behaviour and autonomic nervous system[13–16]. In addition, melatonin has anti-inflammatory and antioxidant functions and can also affect energy metabolism. Melatonin stimulates the release of proinflammatory cytokines and other mediators, but under certain conditions, it can inhibit proinflammatory factors, such as cyclooxygenase 2, NLRP3, and Toll-like receptor 4[17]. Melatonin also

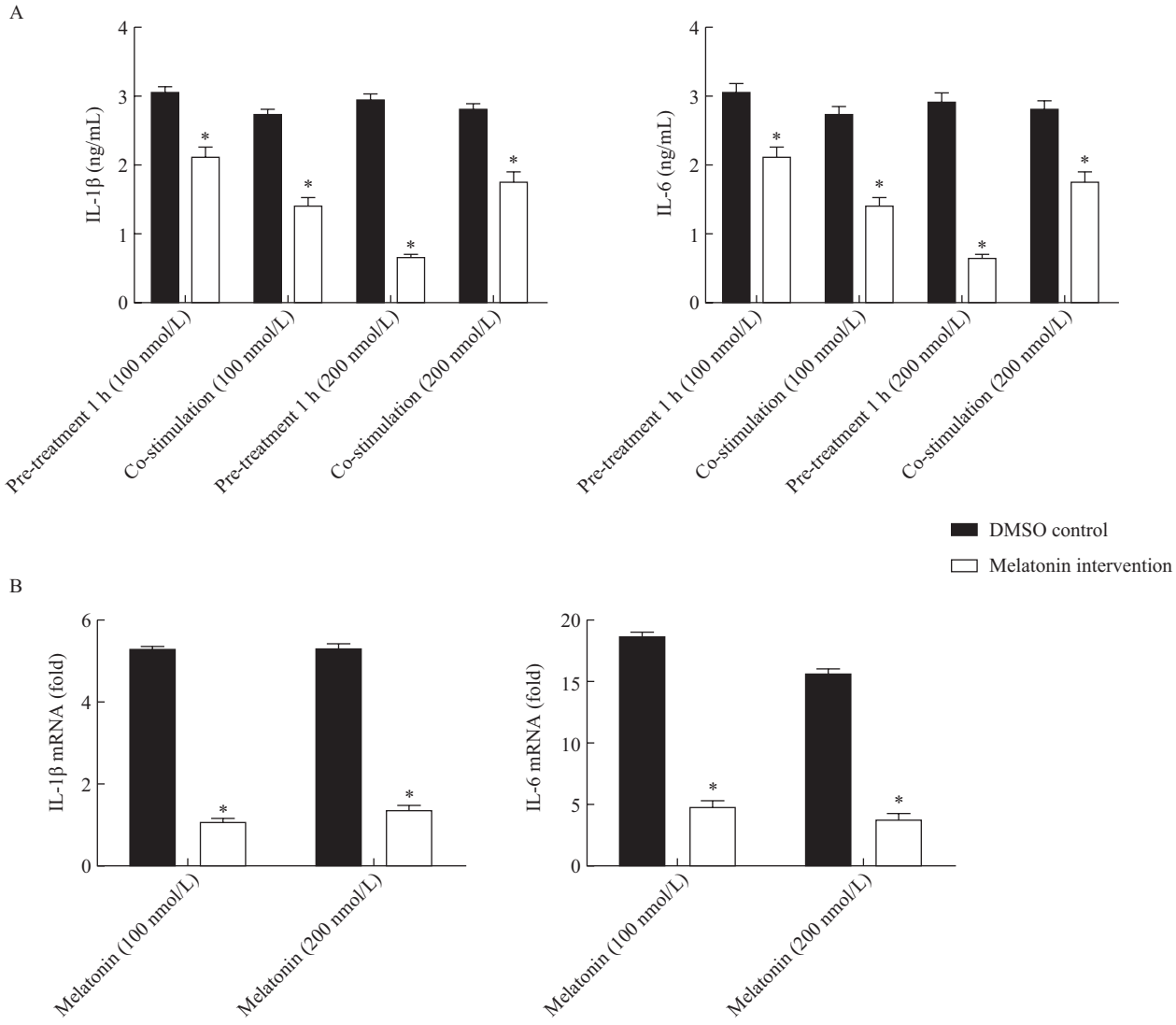

Fig. 4 Melatonin inhibited the mRNA expression of IL-1β and IL-6 in peritoneal macrophages.
Peritoneal macrophages were stimulated with LPS for 12 h, followed by treatment with MSU for an additional 6 h. A: the levels of IL-1β and IL-6 secreted by macrophages in the melatonin intervention group and DMSO control group; B: the mRNA expression of IL-1β and IL-6 in the melatonin intervention group and DMSO control group ($^*P<0.05$)

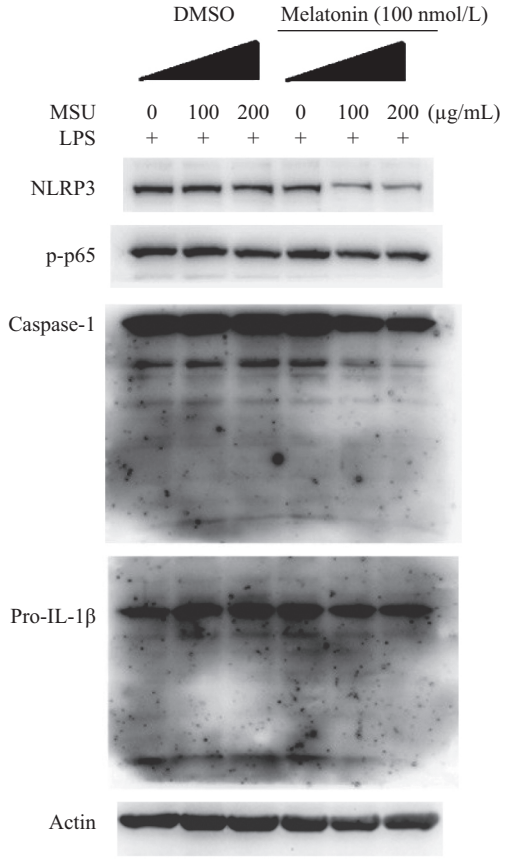

Fig. 5 Melatonin inhibited the protein expression of NLRP3, caspase-1 and pro-IL-1β in peritoneal macrophages.

functions by directly scavenging free radicals. For example, in mastitis, melatonin inhibits LPS-induced inflammation and oxidative stress, thus playing a therapeutic role[18, 19]. In hepatic ischaemia-reperfusion injury, melatonin promotes liver regeneration through monocyte-derived IL-6 and downstream IL-6/gp130-STAT3 signalling[20].

Recent studies also showed that melatonin could inhibit the expression of NLRP3 in a variety of cells and play an anti-inflammatory role. For example, melatonin inhibits the expression of NLRP3 in adipocytes and inhibits inflammation in adipose tissue[19–21]. In both acute lung injury (ALI) and brain injury, melatonin inhibits the activation of NLRP3 in macrophages in septic mice and prolongs the survival time of the mice[22–23]. However, whether melatonin plays a role in the acute inflammation associated with gout remains unclear. Our study was the first to demonstrate that the low plasma level of melatonin and the down-expression of MT2 in PBMCs were associated with the onset of gout, suggesting that melatonin was involved in this process. Furthermore, we confirmed that melatonin treatment in the joint cavity could alleviate inflammation in the paws of mice. *In vitro*, mouse peritoneal macrophages were isolated and cultured, and melatonin repressed the expression of IL-1β, IL-6, NLRP3, caspase-1 and pro-IL-1β in macrophages, which indicated that melatonin inhibited the formation of NLRP3 inflammasomes.

NLRP3 inflammasomes are intracellular poly-protein complexes that play important roles in inflammatory caspase activation by promoting the secretion of IL-1 and IL-18[24]. To date, five different inflammasomes have been clearly identified. Among them, NLRP3 is the most classic and well-studied inflammasome[25]. NLRP3 is a NOD-like receptor that is composed of three parts (NRR-Nacht-PYD), and it is largely expressed on macrophages, monocytes and neutrophils and acts as a pattern recognition receptor (PRR) to initiate inflammatory pathways[26]. Activation of the NLRP3 inflammasome requires two signals: microbial molecules or endogenous cytokines that upregulate NLRP3 and pro-IL-1 expression through activation of the NF-κB pathway. Thus, activation of NLRP3 inflammasome-associated immune pathways amplifies NF-κB-mediated inflammatory responses. In addition, oligomerization of inflammasomes is affected by adenosine triphosphate (ATP) and activates caspase-1 to enzymatically hydrolyse pro-IL-1β, releasing IL-1β to mediate inflammation and activate the expression of inflammatory factors *in vivo*[27, 28]. Previous studies have confirmed the close association between gout and the NLRP3 pathway. The initiation of gouty inflammation is closely associated with the activation of NLRP3 in macrophages. Studies have shown that inhibiting macrophages with sodium chlorophosphate can significantly reduce acute gouty inflammation in mice, suggesting that macrophages drive the development of inflammation[29]. Macrophages play important roles in the inflammatory response, and the inflammasome pathway in macrophages is an important component of the first line of defence of the innate immune system. During bacterial infection, immune cells such as macrophages are first stimulated by pathogen-associated molecular patterns (PAMPs) to induce the expression of inflammatory cytokines such as IL-6 and TNF-α and simultaneously upregulate NLRP3 and IL-1β. The activation induced by PAMPs is the sensitization signal (also known as the first signal) in macrophages. Then, sensitized macrophages are activated by a second signal secreted by bacteria or host cells, leading to the activation of caspase-1 and IL-1β and inducing the occurrence of inflammatory cell death, which further amplify the inflammatory response[30].

In conclusion, our study explored the mechanism by which melatonin alleviates acute gouty arthritis. We successfully established a mouse model of acute gouty arthritis with MSU administration and demonstrated that melatonin could effectively reduce inflammation in model mice. To further explore the mechanism, we extracted peritoneal macrophages from the mouse

abdominal cavity and treated these cells with or without melatonin. The expression of IL-1β, IL-6, NLRP3, caspase-1, and pro-IL-1β was significantly decreased by melatonin treatment. Therefore, we verified that melatonin could inhibit MSU-induced activation of NLRP3 and the subsequent expression of downstream pathway molecules, including the cytokine IL-1β. Our findings suggest that melatonin may be a potential drug for treating or preventing acute gouty inflammation.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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