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Original Research Article

Effect of *Hedyotis diffusa* Willd extract on gouty arthritis in rats

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Abstract

Purpose: *To investigate the effect of Hedyotis diffusa Willd extract (HDWE) on gouty arthritis in rats.*
Method: *Monosodium urate (MSU) crystal was injected into the ankle joint of rats to establish a rat model of gouty arthritis. HDWE (4.8, 9.6 and 19.2 g/kg) was administered to the rats treated with MSU crystals. The walking behavior of the rats was observed daily, and the gait score was calculated to evaluate the Oswestry disability index of rats. Levels of IL-1β and TNF-α in lavage fluid of articular cavities were measured using enzyme linked immunosorbent assay (ELISA) kits. The synovial tissues of joint of control, model and 19.2 g/kg HDWE group rats were obtained and NLRP3 inflammasome was analysed by Western blot.*
Results: *The results showed that HDWE ameliorated the symptoms of gouty arthritis and gait score in rats significantly ($p < 0.05$). Further pharmacological experiments showed that all doses of HDWE decreased the levels of inflammatory cytokines IL-1β and TNF-α ($p < 0.05$), and inhibited NLRP3, caspase-1, ASC, IL-1β and IL-18 protein expressions of the lavage fluid of articular cavities in MSU crystal-treated rats ($p < 0.01$).*
Conclusion: *The results indicate that HDWE exhibits a significant effect in ameliorating gouty arthritis via inhibition NLRP3 inflammasome, and thus is a potential new drug choice for the treatment of gouty arthritis.*

Keywords: *Hedyotis diffusa, Caspase, Gouty arthritis, Inflammatory cytokines, NLRP3 inflammasome*

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INTRODUCTION

As an inflammatory arthritis, gouty arthritis is caused by monosodium urate (MSU) crystal deposition in and around the joints [1]. Acute symptoms of gouty arthritis include redness, swelling, heat, pain, and even joint functional loss [2]. This process is driven by neutrophil influx into the joint and leads to the attack of acute inflammatory arthritis with severe pain in the affected tissue. Studies have shown that NOD-like receptors containing a PYD3 (NLRP3) inflammasomes play a critical role in MSU-induced IL-1β secretion in macrophages [3]. There are four kinds of inflammasomes, namely NLRP1, NLRP3, NLRC4, and AIM2. The NLRP3

inflammasomes play an important role in macrophages by cleaving pro-IL-1β into mature IL-1β [4,5]. MSU activates the NLRP3 inflammasome and synergistically interact with apoptosis-associated speck-like protein (ASC) to drive caspase-1 release, subsequently leading to maturation and secretion of pro-inflammatory cytokines IL-1β and activation of nuclear factor-κB (NF-κB). This process is involved in inflammatory response in the gouty arthritis [6]. It has been found that MSU-induced inflammation and pain responses are significantly reduced in NLRP3-deficient mice [7]. Regretfully, current gout pain management is far from being satisfactory [8]. Hence, safer and more potent drugs are needed for the treatment of gouty arthritis pain.

Nowadays, a number of herbal drugs and their active ingredients attract increased interest in the protection against gouty arthritis [9-12]. *Hedyotis diffusa Willd* has been used for the treatment of gouty arthritis for many years, and has achieved obvious curative effect in China. This study employed MSU crystals-treated rats to evaluate the effect of HDWE on gouty arthritis in rats.

EXPERIMENTAL

Reagents and drugs

Hedyotis diffusa Willd extract (HDWE) was prepared by our lab according to the standard preparation method of traditional Chinese medicine decoction. Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and tumor necrosis factor-alpha (TNF-α) were obtained from Shenzhen Xin Bo Sheng Biotechnology Co. Ltd. (Shenzhen, China). The antibodies of NLRP3 (No. bs-6655R), caspase-1 P20 (No. bs-10442R), ASC (No. bs-6741R), IL-1β (No. bs-0812R) and IL-18 (No. bs-0529R) for rat were purchased from Beijing Bioss Biotechnology Co., Ltd (China). The antibodies of β-actin were from Wuhan Servicebio Co., Ltd. All other reagents used were standard laboratory reagents of analytical grade and were purchased locally. Monosodium urate (MSU) crystal were prepared by crystallization of a supersaturated solution of uric acid (Aldrich Chemical Company, Inc.) under mildly basic conditions according to the method used in a previous study [3]. The concentration of MSU crystals suspension was 20 mg/mL.

Animals

Sixty male rats (Sprague-Dawley, 200 – 220 g) were purchased from Hubei Experimental Animal Center (Wuhan, China) and were housed for one week to adapt to the environment before being used for experiments. All the animals were maintained on standard laboratory conditions of temperature 23 ± 2 ℃ and a 12-h light/12-h dark cycle with free access to commercial food and pure water for the duration of the study. The rat experiment was approved by the Animal Care and Use Committee of Wuhan No. 1 Hospital (approval ref no. 20190936), and was carried out in compliance with Directive 2010/63/EU on the handling of animals used for scientific purposes [13]. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals.

Establishment of MSU-induced gouty arthritis model and drug administration

Homogenous suspensions of celecoxib was made with distilled water. Fresh solution was prepared before each experiment. All the rats were randomly divided into six groups of 10 rats each. Group I was injected with PBS and served as a normal (control) group. Group II injected with 100 μL MSU crystal suspension served as a model group. Group III comprised of MSU crystals-treated rats were administered with celecoxib (0.019 g/kg body weight). Group IV, Group V and Group VI comprised of MSU crystals-treated rats were administered HDWE (19.2, 9.6 and 4.8 g/kg body weight, respectively). All drug administrations of different group rats lasted for 9 days once daily. At the 7th day of drug administration, rats were anesthetized with 20% urethane solution, and then MSU crystals suspension (100 μL) or PBS (100 μL) was injected into the tibiotarsal joint (ankle) of rats.

Assessment of walking behavior and gait score

The walking behavior of rats was observed every day. According to the Coderre's Method [14], the gait score was calculated to evaluate the Oswestry disability index of rats at 2, 6, 12, 24 and 48 h.

Determination of cytokine levels in the joint of MSU crystals-treated rats

After the last drug administration, the lavage fluids (including PBS and synovial fluid) were collected. Each lavage fluid of articular cavities was diluted with PBS to the volume of 1 mL. The lavage fluids were centrifuged at 500 g for 10 min and supernatants were stored at -80 ℃ before biochemical determinations. Levels of IL-1β and TNF-α in lavage fluid of articular cavities were measured with ELISA kits (according to manufacturer's instructions).

Western blotting analysis

Three synoviums of injected ankles after being irrigated with PBS were isolated. About 50 mg of frozen rat articular synovium were homogenized in 1 ml RIPA buffer, and then centrifuged at 10,000 g for 20 min. Protein concentrations of the supernatants were measured with the Bradford method. The volumes equivalent to 30 mg of proteins were analyzed by 10 % SDS-PAGE under non reducing conditions. After electrophoresis, polyacrylamide gels were blotted onto polyvinylidene fluoride membrane. Membranes were washed in Tris-HCl-buffered saline (TBS, 50 mM, pH 7.5) and incubated in 5% dried skim milk for 2 h and washed twice with tans-buffer (without methanol) and incubated with a primary antibodies, including anti-NLRP3 (1:2000), anti-caspase-1 (1:2000), anti-ASC (1:2000), anti-IL-1β (1:2000), anti-IL-18 (1:2000) and anti-β-actin antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Wuhan Servicebio Co., Ltd.). The immune complexes were detected using chemiluminescence (ECL) system.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using Student's t-test for two groups or a one-way ANOVA for three or more multiple groups (GraphPad Software San Diego, CA, USA). For all results, $p < 0.05$ was considered statistically significant.

RESULTS

HDWE improved the walking behavior and gait score in MSU crystals-treated rats

After the injection of MSU crystals into the ankle joint, joint swelling was observed immediately. Compared with control group (0 ± 0.1), the Oswestry disability index of model group rats increased significantly at hour 2 (2.9 ± 0.3), hour 6 (2.7 ± 0.7), hour 12 (2.5 ± 0.5), hour 24 (2.4 ± 0.5) and hour 48 (2.3 ± 0.4) (all $p < 0.01$,). Celecoxib and HDWE (19.2 g/kg) ameliorated the Oswestry disability index and walking behavior significantly at different time points respectively ($p < 0.01$) (Table 1).

HDWE attenuated the levels of pro-inflammatory cytokines in MSU crystals-treated rats

To identify the alterations of pro-inflammatory cytokines after ankle injection of MSU crystals suspension, we determined the levels of pro-inflammatory cytokines in the lavage fluid of articular cavities collected at 24 h later of the last drug administration. Compared with control group, IL-1β and TNF-α levels of the lavage fluid of articular cavities in MSU crystals-treated rats were increased significantly ($P < 0.01$). Celecoxib and HDWE (19.2 g/kg) both ameliorated them significantly ($P < 0.01$).

HDWE suppressed caspase-1 activation and IL-1β and IL-18 secretion

In order to understand the effect of HDWE on joint, the effect of HDWE on the NLRP3 inflammasome protein expression was investigated.

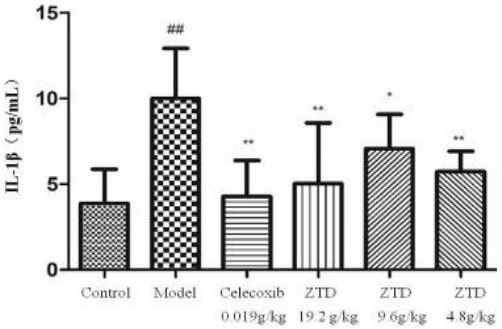

Figure 1: Effect of HDWE on IL-1β level in the lavage fluid of articular cavities in MSU crystals-treated rats

Table 1: Effect of HDWE on gait score in gouty arthritis rats

Group	Dose (g/kg)	Oswestry disability index				
		2h	6h	12h	24h	48h
Control	-	0.0±0.1	0.0±0.1	0.0±0.1	0.0±0.1	0.0±0.1
Model	-	2.9±0.3$^{\#\#}$	2.7±0.7$^{\#\#}$	2.5±0.5$^{\#\#}$	2.4±0.5$^{\#\#}$	2.3±0.4$^{\#\#}$
Celecoxib	0.019	2.4±0.6*	1.8±0.4**	1.4±0.4**	1.2±0.3**	1.0±0.2**
HDWE-H	19.200	2.5±0.6*	2.0±0.5**	1.7±0.4**	1.1±0.3**	0.9±0.3**
HDWE-M	9.600	2.6±0.7	2.3±0.5*	2.0±0.5*	1.9±0.4*	1.6±0.4**
HDWE-L	4.800	2.8±0.4	2.6±0.6	2.2±0.6	2.0±0.5*	1.9±0.4*

Compared with control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with model group, $^{*}p < 0.05$, $^{**}p < 0.01$. HDWE-H: high dose of HDWE, HDWE-M: middle dose of HDWE, HDWE-L: low dose of HDWE

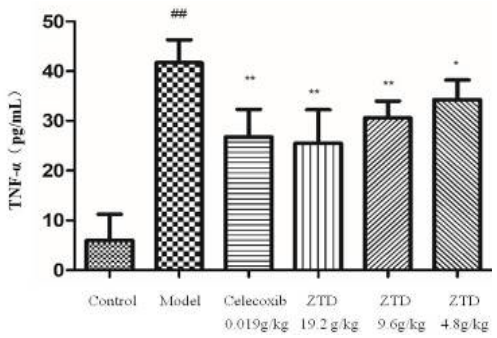

Figure 2: Effect of HDWE on TNF-α level in the lavage fluid of articular cavities in MSU crystals-treated rats

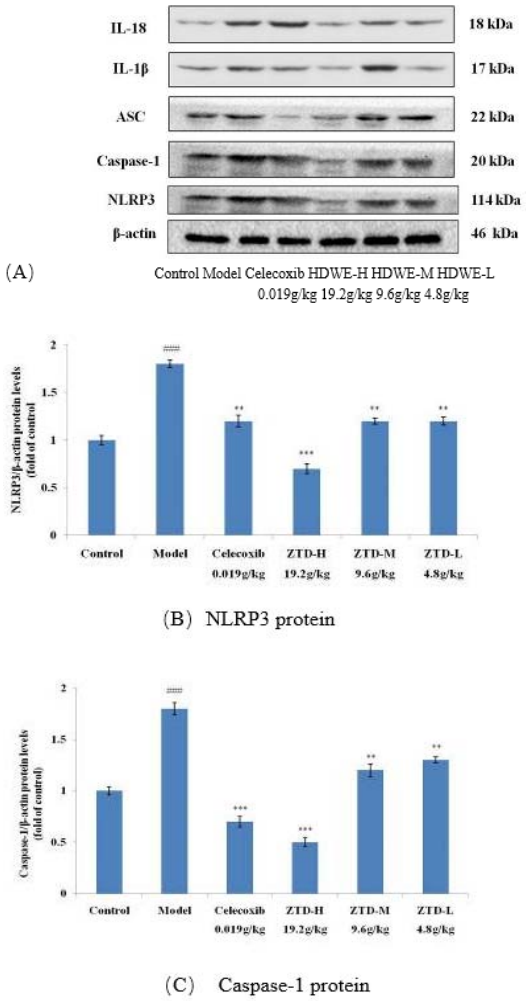

Figure 3: HDWE (4.8, 9.6 and 19.2 g/kg) and celecoxib decreased the expressions of inflammation-associated proteins in the synovium of MSU crystals-treated rats. A - C: Protein expressions of control, NLRP3 and caspase-1 P20, were determined by western blotting and the bar chart indicated the relative protein. expression. $^{###}P < 0.001$ vs. control rats; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. MSU crystals-treated rats

Compared to the control group, the protein expressions of NLRP3, caspase-1, ASC, IL-1β and IL-18 proteins were all up-regulated in the MSU-treated group ($p < 0.01$), suggesting that the inflammatory status might be involved in the articulation dysfunction. As shown in Figure 3 and Figure 4, the results showed that HDWE (4.8, 9.6 and 19.2 g/kg) and celebrex decreased NLRP3, caspase-1, ASC, IL-1β and IL-18 protein expression in rats respectively (all $p < 0.01$).

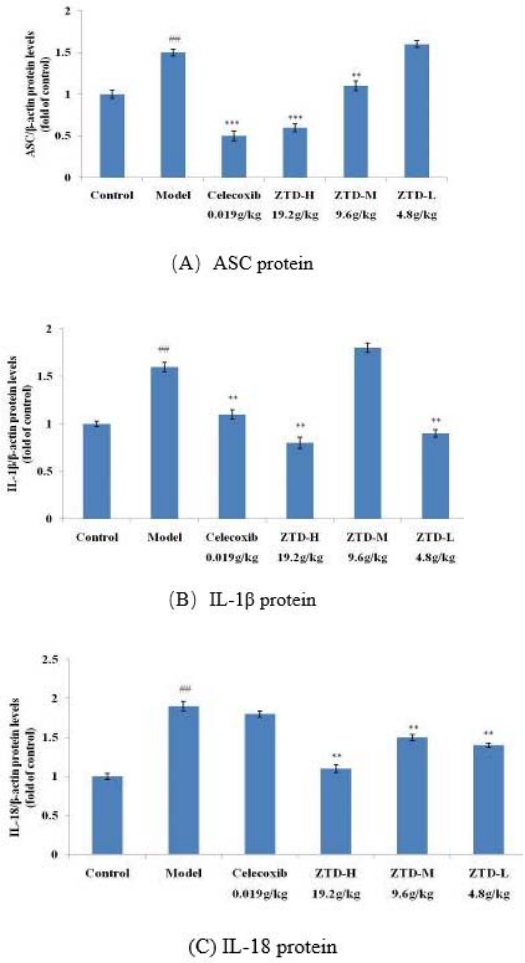

Figure 4: HDWE (4.8, 9.6 and 19.2 g/kg) and celecoxib decreased the expressions of inflammation-associated proteins in the synovium of MSU crystals-treated rats. A - C: Protein expressions of ASC, IL-1β and IL-18, as evaluated by western blotting. $^{###}P < 0.001$ vs. control rats; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. MSU crystals-treated rats

DISCUSSION

Gout is caused mainly by the accumulation of MSU crystals in the joints, and it is characterized by IL-1β-driven acute inflammation which is associated with the infiltration of monocyte-mediated neutrophils in the joints [15]. Evidence shows that crystals from the synovial fluid of

patient with gout are composed of MSU crystals [16]. MSU crystal is widely recognized as a danger signal to promote an acute inflammation in the joint cavity, and MSU crystal is most frequently employed to develop an animal model of gouty arthritis [17,18]. Neutrophil recruitment and activation in both joint fluid and synovial membrane is a hallmark the acute inflammatory response to MSU crystals in acute gouty arthritis [19]. In the present study, MSU crystals-induced joint swelling and thermal hyperalgesia elevating in the present study were obviously observed in rats, suggesting the acute inflammation in the joint cavity. HDWE treatment attenuate the pain threshold value and the joint swelling degree in MSU crystals-treated rats significantly.

The objectives for gout treatment include managing the symptoms of acute attack and preventing further attacks by reducing uric acid levels in the blood. The most commonly used therapies for acute gout in general practice non-steroidal anti-inflammatory drugs (NSAIDs), colchicine, celecoxib and corticosteroids. Although these drugs have certain therapeutic effects, they present serious side effects, such as liver and kidney damage and severe gastrointestinal reactions [20,21]. Moreover, the treatment of gout is often a long-term process. Therefore, it is urgent to search for more safer drugs.

The NLRP3 inflammasome is a cytosolic protein complex composed of NLRP3, ASC, and caspase-1, and it is assembled in response to both microbial infection and endogenous ‘‘danger signal’’ [22]. Activation of NLRP3 inflammasome promotes the maturation and release of several pro-inflammatory cytokines, such as interleukin-1β(IL-1β) and IL-18, so it plays critical roles in the initiation of inflammation and the development of immune responses [23-24]. Among several of the inflammasomes, NLRP3 inflammasome is relatively well studied and its activation is linked to age related metabolic diseases and auto-inflammatory diseases [25-26]. Therefore, much attentions have been given to find active constituents that can act as specific NLRP3 inflammasome inhibitor [27].

In the present study, differential gene signal network diagram of gene chip analysis revealed that caspase-1, IL-1β and IL-18 genes occupied the core position in the signal network, which suggested NLRP3 inflammasome participated in the pathogenetic process of gouty arthritis. Moreover, we found that HDWE inhibited NLRP3 inflammasome activation, and MSU induced IL-1β and IL-18 production and neutrophil infiltration in vivo, suggesting that HDWE ameliorated the gouty arthritis induced by MSU crystals through inhibiting NLRP3 inflammasome.

CONCLUSION

These findings indicate that HDWE inhibited gouty arthritis through reducing swelling and pain and blocking anti-inflammatory pathway, and it provide new drug choice for the treatment of gouty arthritis in future.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. LanFang Wang and Fang OuYang performed the experiment. Yan Ma wrote the manuscript. Rui Sun and ShiWei Tan wrote the proposal and designed the manuscript. Liu Xiao conducted data analysis. QuanWei Yang modified the manuscript. LanFang Wang and Fang OuYang contributed equally to this work, and they are co-first authors. Liu Xiao and QuanWei Yang are co-corresponding authors.

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