Original Research Article

Anti-inflammatory effect of clearing-away-heat-promoting-diuresis and Wen-yang herbs on inflammatory signaling pathways in acute gout

Jiyong Huang1, Yan Zhang2*, Kai Chen1, Xiaopeng Li1, Jinghan Liu3
1Department of Immunology and Rheumatology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310005, China, 2Intensive Care Union, The Third Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310005, China, 3The Second Clinical Medical College of Zhejiang Chinese Medical University, Hangzhou 310005, China

*For correspondence: Email: yanzhang73@yandex.ru

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Abstract

Purpose: To investigate the regulatory effect of clearing away, heat-promoting diuresis and Wen yang herbs (CHPW) on acute gout IL-1β inflammatory signaling pathways in rats and cell lines.

Methods: Thirty Sprague-Dawley (SD) rats were randomly divided into five groups: saline (SA group), SA-sodium urate (SA-SU) group, Colchicine-SU (Col-SU) group, CPHW-SU group, and clearing away heat-promoting diuresis-SU (CHP-SU) group. The cell line RAW264.7 groups were RAW264.7-A, RAW264.7-B, RAW264.7-C and RAW264.7-D. For the above cell groups, enzyme-linked immunosorbent assay (ELISA) was used to measure the effects of drugs on macrophage proliferation, and levels of IL-1β and other inflammatory factors, while quantitative real-time polymerase chain reaction (qRT-PCR) was used to assay NALP3 mRNA expression. The protein expressions of p-IRAK4, IRAK4, p-P65, P65, NALP3, and caspase-1 were measured using Western blot assay.

Results: Compared with SA-SU group, CHPW reduced gout symptoms in the joint, and reduced levels of myeloperoxidase (MPO) and IL-1β (p < 0.05). Compared with the SA group, the relative expression of NALP3 increased significantly in the SA-SU group (p < 0.05). The relative expression of NALP3 in CPHW and CHP groups decreased significantly, relative to the SA-SU group (p < 0.05). However, the expressions of p-IRAK4, IRAK 4 and p65 was basically unchanged (p > 0.05). Protein expression levels of NALP3 and caspase-1 were higher in SA-SU group, but lower in CHPW and CHP groups (p < 0.05).

Conclusion: CHPW exerts a protective effect against acute gout by downregulating the IL-1β inflammatory signaling pathway and decreasing the expression levels of NALP3 and p-P65. Thus, CHPW has a potential for use as a new therapeutic agent for gout.

Keywords: Acute gout, Corrigent Wen yang herbs, IL-1β signal pathway, RAW264.7 Monocyte-macrophages

INTRODUCTION

Gout is the most common inflammatory arthritis, and it is characterized by painful and disabling acute attacks [1]. Acute gout is associated with acute gouty arthritis and varying degrees of bone damage. The pathogenesis of acute gout is related to gender, age, region and race,
hyperuricemia, medication, body weight, diet and alcohol, genetics, metabolic syndrome, and mental factors. The main clinical manifestation of gout is acute inflammation of multiple joints, but it is often overlooked because the symptoms disappear after acute exacerbations. However, improper handling of functional activity disorders can affect quality of life. Gout in Chinese medicine belongs to arthralgia, gout, li-jie and other categories. Chinese medicine believes that the main factor involved in acute gout is damp heat. Although CHPW produces a therapeutic effect on acute gout, its mechanism of action is still unclear. Modern medical research has found that the IL-1β inflammatory signaling pathway is pivotal to acute gout. In general, this disease is poorly controlled using immunosuppressive therapies. The suppression of IL-1, particularly IL-1β, is now the standard therapy for a class of inflammatory syndromes termed “autoinflammatory” diseases [2,3]. This study was carried out to investigate the effect of CHPW on acute gout, and its effect on the IL-1β inflammatory signaling pathway.

EXPERIMENTAL

Animals and cell lines

Male SD rats (Sue2013-0003, n = 30) with body weight range of 200 - 250 g were used in the studies. The animals were obtained from Zhayan New Drug Research Center Co.Ltd (Suzhou, China). The experiment was started one week after the rats were fed in our department. The RAW264.7 macrophages were obtained from Chinese Academy of Sciences.

Drugs and reagents

The CHPW was composed from honeysuckle stem (60 g), rhizoma smilacis glabrae (60 g), radix aconiti lateralis preparata (30 g), and Polygonum cuspidatum (30 g). It was provided by the Chinese Medicine and Beverage Factory of Zhejiang University of Traditional Chinese Medicine, Chian). Test kits for uric acid, PBS, myeloperoxidase (MPO), and IL-1β were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); RPMI1640 medium, fetal bovine serum and 0.25 % of pancreatic enzyme were got from GIBCO; Cck-8 kit was obtained from TransGen Biotech (Beijing, China); RIPA buffer, BSA, PVDF membrane and PMSF membrane were products of Sigma. BCA kit was purchased from Beyotime Biotechnology, while qRT-PCR kit was obtained from Tiangen Biotech. Horseradish peroxidase (HRP)-conjugated secondary antibody and GAPDH mouse monoclonal antibody were purchased from MultiSciences Lianke Biotech Co. Ltd.

Preparation of drugs

CHPW preparation: 60 g of honey suckle stem, 60 g of rhizoma smilacis glabrae and 30 g of radix aconiti lateralis preparata were added to an appropriate amount of water, and heated for 1 h. Then, 30 g of Polygonum cuspidatum was added to the mixture, followed by boiling for another 30 min. Thereafter, the final volume of the supernatant was 100 mL. The preparation of clearing away heat-promoting diuresis (CHP) followed the same procedure used for CHPW, but there was no radix aconiti lateralis preparata. The concentration of colchicine was 0.015 mg/mL. The water extracts of CHPW and CHP were each vacuum-dried and dissolved in PBS to yield a concentration of 36 mg/mL. The sodium uric acid (SU) concentration was 500 mu/mL.

Animal grouping and treatments

Thirty SD rats were randomly divided into 5 groups (six rats in each group): SA group treated with normal saline only; SA-SU group, i.e., saline administration, followed by SU (SA-SU group); Col-SU group i.e., colchicine administration followed by SU; CHPW-SU group, i.e., CHPW administration followed by SU, and CHP-SU group, i.e., CHP administration followed by SU. The SA, Col, CHPW or CHP were administered for 2 days at a dose of 1 mL/100 g body weight, twice a day. On the third day, SU was injected into the stomach before administration of the respective drug in the stomach, and 0.2 mL of SU solution was injected into the right sacral cavity of the rat. After 72 h of SU injection, the animals were sacrificed, and the right ankle (0.5 cm proximal and distal) was excised for paraffin sectioning. The animal studies received ethical approval from the institutional animal ethics committee and followed international guidelines for the conduct of animal studies. The RAW264.7 cells were incubated in RPMI1640 medium with 10 % FBS. Four (4) groups of RAW264.7 mononuclear cell lines (5 x 10^5 cells/mL) were treated as follows: RAW264.7-A without any treatment; RAW264.7-B treated with SU for 24 h; RAW264.7-C: treated with CHPW for 1 h, followed by treatment with SU for 24 h; and RAW264.7-D treated with CHP for 1 h, and then with SU for 24 h.

Determination of the effect of CHPW on rat model of acute gout

To assess joint swelling index, the joint swelling was continuously examined 3 days before saline
administration, or at 10, 24, and 72 h after infection with SU. The circumference of the point 0.5 mm below the right posterior ankle joint in each group was measured. Joint swelling index (S) was calculated as in Eq 1.

\[ S = \frac{(J_t - J_i)}{J_i} \]  

where Jt and Ji are joint circumference at a given timepoint and initial joint circumference, respectively. Ji was determined as the mean of the circumference of the joint during three days of saline administration and before saline administration.

The inflammation index was measured before injection of saline or SU, and at 10, 24 and 72 h after the injection of SU. The dysfunction index was measured before injection of saline or sodium urate, and at 10, 24 and 72 h after the injection of SU. The serum MPO and IL-1β contents were measured according to instructions (Nanjing Jiancheng Bioengineering Institute, China).

**Assessment of the effect of CHPW on the secretion and proliferation of inflammatory factors**

The proliferation of RAW264.7 cells was measured using the CCK-8 assay. In this assay, 100 µL RAW264.7 cells (5 × 10^5 cells/mL) were grown in 96-well plates for 72 h, and treated with CHPW/CHP extract over a range of concentrations [(A, 1/2A, 1/4A and 1/8A (A = 1.44 mg/mL)]. After 3 h, cell growth was measured using CCK-8 assay (Dojindo, Kumamoto, Japan) according to the kit instruction. For each concentration of CHPW/CHP, mean values of the absorbance from eight wells were calculated.

**RT-PCR**

Total RNA extraction was carried out using TRIzol reagent according to the manufacturer’s instructions. The qRT-PCR amplification was performed for 180 s at 95 °C, followed by 40 cycles at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 3 min. The target mRNA levels were calculated based on the CT method, and normalized to β-actin. All reactions were run in triplicate. The primer sequences for each gene were as follows:

- mNALP3 forward: AAACCCACCATGTGCAAGGA,
- mNALP3 reverse: TCCCATAAGTGCCTCGTCAAA;
- β-actin forward: ATGTGGATCAGCAAGCAGGA,
- β-actin reverse: AAGGGTGTAACGCCAGCTCA.

**Western blot assay**

Total protein was extracted with RIPA solution as described in the manual kit instructions. A 50-µg protein sample was separated using SDS-PAGE, and electro-transferred onto a PVDF membrane. The membrane was incubated overnight at 4 °C with primary antibodies for P-IRAK4 (1:500), IRAK4 (1:1000), p-P65 (1:500), P65 (1: 500), NALP3 (1:500), Caspase1(1:500) and GAPDH (1 :5000). Then, the membrane was incubated with secondary antibody (1:10000) at 25 °C for 1 h. The protein bands were visualized using enhanced chemiluminescence substrate, and the relative expression of each protein was calculated from optical density values.

**Statistical analysis**

Data were analyzed with SPSS 26.0 software. All data are presented as mean ±SD, and were compared using t-test. Statistical significance was fixed at \( p < 0.05 \).

**RESULTS**

**Effect of CHPW on symptoms of acute gout rats, and the mechanism involved**

As shown in Figure 1, Figure 2, and Figure 3, at 10 h after treatment, joint swelling index was significantly reduced after treatment in Col-SU, CHPW and CHP groups, relative to SA-SU group. These indices of gout were significantly mitigated in the CHPW group, when compared with Col-SU group after 72 h of treatment. Compared with the SA-SU group after 24 h of treatment, the inflammation index of the CHPW group was significantly reduced, and was lower than that of Col-SU rats 72 h after the treatment. With respect to joint dysfunction index, the CHPW group was significantly lower after 24 h of treatment than the SA-SU group (Figure 2 and Figure 3 show data for 4 groups only, since the SA group data was zero).

**Expressions of MPO and IL-1β**

Compared with the SA group, the MPO expression in the Col-SU, CHPW-SU and CHP-SU groups were significantly increased (\( p < 0.05 \); Table 1). Similarly, IL-1β expression in the Col-SU, CHPW-SU and CHP-SU groups were significantly increased, relative to the SA group (\( p < 0.05 \); Table 1).
Figure 1: Effect of the treatments on joint swelling; *p < 0.05, vs. SA group; #p < 0.05, vs. SA-SU group; @p < 0.05 vs. Col-SU group

Figure 2: Levels of inflammatory indices of joints: Comparison of groups at the same time points: *p < 0.05, vs. SA-SU group; #p < 0.05, vs. Col-SU group; @p < 0.05, vs. CHPW-SU group

Figure 3: Dysfunction index of joints in each group: Comparison of groups at the same time; *p < 0.05, vs. SA-SU group

Table 1: Expression levels of MPO and IL-1β

<table>
<thead>
<tr>
<th>Group</th>
<th>MPO</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>46.56±20.35</td>
<td>167.00±67.85</td>
</tr>
<tr>
<td>SA-SU</td>
<td>90.33±33.38*</td>
<td>499.34±60.14*</td>
</tr>
<tr>
<td>Col-SU</td>
<td>52.35±28.61*</td>
<td>263.33±84.31*</td>
</tr>
<tr>
<td>CHPW-SU</td>
<td>67.07±24.99*</td>
<td>315.72±66.18*</td>
</tr>
<tr>
<td>CHP-SU</td>
<td>67.08±25.00*</td>
<td>315.73±66.18*</td>
</tr>
</tbody>
</table>

MPO: myeloperoxidase; *p < 0.05, compared with the SA-SU group

Effect of different drugs on sodium urate-induced proliferation of macrophages

Based on data obtained via CCK 8 kit detection (Figure 4), the proliferation of macrophages decreased significantly when the concentration of CHPW or CHP extract was higher than 1.44 mg/mL. Therefore, the concentration of CHPW or CHP extract used in the subsequent experiments was 1.44 mg/mL.

Figure 4: Growth of cells in each group. No SU; +2 mg/mL SU, 0.288, 0.144, 7.2 and 36 mg of CHPW or CHP extract

Levels of IL-1β

As shown in Figure 5, the levels of IL-1β were lower in all groups treated with 0.72 mg/mL of CHPW or CHP extract. Therefore, a concentration of 0.72 mg/mL was used for subsequent experiments.

Figure 5: Effect of different concentrations of CHPW and CHP on IL-1β content. -: No SU; +: 2 mg/mL SU, 0.18, 0.36, 0.72 and 1.44 mg/mL CHPW or CHP extract

mRNA expression of NALP3 in macrophages

As shown in Figure 6, the relative expressions of NALP3 mRNA in AW264.7-B group was significantly higher than that in the RAW264.7-A group. The NALP3 level was markedly decreased in RAW264.7-C and RAW264.7-D groups, when compared to RAW264.7-B group (p < 0.05).
**Huang et al**

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Figure 6: Relative expressions of NALP3 gene in cells with different treatments

**Expressions of P-IRAK4, IRAK4, p-P65, P65, NALP3 and caspase-1 in macrophages**

As depicted in Figure 7 and Figure 8, the results for TLR2/4-MyD88-NF-κB pathway showed that the expression of the inflammatory signal p-P65 was increased in RAW264.7-B cells, but it was decreased slightly in RAW264.7-C and RAW264.7-D groups of cells. The basal expression levels of p-IRAK4, IRAK4 and P65 did not change significantly. The expression levels of two typical inflammatory proteins NALP3 and cleaved caspase-1 in the NALP3-ASC-caspase-1 pathway, were up-regulated in RAW264.7-B cells, but were decreased in the RAW264.7-C and RAW264.7-D groups.

Figure 7: Relative expressions of representative proteins of the two signal pathways, as assayed with Western blotting

**Figure 8: Quantified expression levels of representative proteins in the two signal pathways**

**DISCUSSION**

Gout is thought to be one of the most painful acute symptoms caused by monosodium urate (MSU) crystal deposits in the joints [4,5]. Sodium urate (SU) crystals cause acute mobilization of inflammatory cytokine IL-1β which is present in neutrophils and monocytes [6]. The clinical manifestations of gout are divided into four stages: asymptomatic hyperuricemia, acute gout arthritis, interphase, and chronic local gout. The acute symptoms of gout are mediated by internal white blood cells and macrophages [7]. Acute gout often recurs. Thereafter, it may cause kidney damage and joint deformity, which seriously affects quality of life of the affected
patients; it is closely related to the occurrence of hyperlipidemia, hypertension and atherosclerosis [8].

Acute gout may also be caused by inflammatory factors. It activates the NARP3 inflammasome, leading to release of IL-1β and other pro-inflammatory factors, causing joint inflammation and joint pain. The active IL-1β is released into the extracellular joint fluid in gout. The IL-1 receptors on endothelial cells and resident macrophages are activated within the joint under the mediation of nuclear factor kappa light chain enhancer of activated B cell signaling pathway, leading to production of proinflammatory cytokines. Therefore, IL-1β plays a key role in acute gout. Many studies have found that urate crystals mediate the main signaling pathways of IL-1β, i.e., Toll-like receptor dependent signaling pathway and NALP 3 inflammatory body-dependent signaling pathway [9-13]. Therefore, the symptoms of acute gout may be alleviated by controlling the IL-1β inflammatory signaling pathway.

At present, acute gout is treated with non-steroidal anti-inflammatory drugs, immunosuppressive agents and glucocorticoids. Colchicine effectively suppresses acute gouty arthritis, but the side effects are also very high. Traditional Chinese Medicine (TCM) (Gezhi Yu Lun. Gout) thought that gout was the heat of one’s own blood, followed by feelings of cold and wet. In Synopsis of the Golden Chamber, gout was defined as "wet turbid" which should be treated with heat and dampness. Previous studies have shown that the use of CHP relieved internal heat or fever. The pain relieved by CHP not only shortened the course of the disease, but also reduced its recurrence [14]. The components of CHP are honeysuckle vine, Smilax glabra and Polygonum cuspidatum. The honeysuckle vine clears heat, detoxifies and dredges the collaterals, while Smilax glabra clears heat and promotes diuresis, thereby relieving joint pain and removing damp evil. The compatibility of the three components accentuates the effect of clearing heat and dampness. Wen yang herb aconite is used to disperse cold and warm meridians to help Yin and yang in balance.

This study showed that CHPW decreased IL-1β levels, reduced the release of macrophage inflammatory factor, improved joint swelling, alleviated joint inflammatory index and reduced joint dysfunction index or alleviated symptoms of gout arthritis.

The expression of NALP3 mRNA protein in RAW264.7-B was significantly increased. This is one of the proteins involved in acute gout. Indeed, NALP3 and p-P65 are the inflammation proteins. Sodium urate crystals caused high expression levels of these proteins, but their expressions were decreased after treatment with CHPW and CHP. Thus, these TCM herbs inhibited the expressions of inflammatory response proteins associated with TLR2/4-MyD88-NF-κB pathway and NALP3-ASC-caspase-1 pathway; regulated IL-1β inflammatory signaling pathway, and controlled IL-1β level, thereby relieving acute gout inflammatory reactions and acute gout. Wen yang herb effectively prevented adverse drug reactions, leading to effective regulation of the IL-1β inflammatory pathway, and suppression of gout.

CONCLUSION

The results obtained in the present study demonstrate that CHPW and CHP regulate IL-1β inflammatory signaling pathway and ameliorates symptoms of acute arthritis. Following treatment with CHPW or CHP, the expression levels of NALP3 and p-P65 proteins are down-regulated. Thus, CHPW and CHP may be new therapeutic approaches for acute gout.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

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