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Urinary metabolomics study on the anti-inflammation effects of flavonoids obtained from Glycyrrhiza

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic disease with pain, swelling, and limitation in the motion and function of multiple joints thus leading to high disability. Previous studies have shown that flavonoids and saponins are the most abundant and active constituents in Glycyrrhiza, which possess a wide range of pharmacological effects such as anti-inflammatory, antioxidant and anti-bacteria. But the mechanisms of those actions are not entirely clear. In order to clarify the mechanisms of those actions, the pharmacodynamical assessments of extraction of water-soluble components and flavonoids and saponins obtained from Glycyrrhiza were investigated. Combining the pharmacodynamical researches, we found that flavonoids obtained from Glycyrrhiza had more significant therapeutic effects on acute inflammation, chronic inflammation and inflammatory pain than that of extraction of water-soluble components and saponins obtained from Glycyrrhiza. The results indicated that flavonoids are the main medicinal ingredients in Glycyrrhiza. In order to further investigate the mechanism of the action of flavonoids in Glycyrrhiza on treating RA, a urine metabolomics method based on ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) was established to observe the metabolic variations in adjuvant-induced arthritis (AIA) rats and investigate the therapeutic effect of flavonoids in Glycyrrhiza on RA. As a result, twenty potential biomarkers were found by comparison with the model group (MG) and flavonoid treated group (FG). We associated these compounds with related metabolic pathways, the results showed that these biomarkers were mainly associated with purine metabolism, taurine and hypotaurine metabolism, tryptophan metabolism, phenylalanine metabolism, tricarboxylic acid cycle (TCA cycle), pantothenate and coenzyme A (CoA) biosynthesis. The results about the pharmacodynamics and metabolomics provided a theoretical basis for clarifying the mechanism of flavonoids in Glycyrrhiza in the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disease that causes pain, stiffness, swelling, and limitation in the motion and function of multiple joints [1]. However, its lesions are not confined to the joints, but also often accompany other organs outside the joint lesions, and RA even can cause the main organ vasculitis and life-threatening [2]. As yet, the etiology of rheumatoid arthritis is not clear which may be related to autoimmune, infection factors, and genetic factors. In recent years, mainly non-steroidal anti-inflammatory drugs are applied for clinical. However, due to the side effects like liver and gastrointestinal disorders [3,4], the clinical uses of these drugs are limited, thus it is necessary to find effect drugs possessing lower side effect.

The Latin name of Glycyrrhiza is *Radix Glycyrrhizae* which mainly contains three types of compounds, flavonoids, saponins and coumarin. Previous studies have shown that flavonoids and saponins are the most abundant and active constituents in Glycyrrhiza [5], which have a wide range of pharmacological effects such as anti-inflammatory, antioxidant and anti-bacteria [6,7]. But the mechanisms of these actions are not entirely clear.

Metabonomics was defined in 1999 by Nicholson, which was mainly used in drug research and development, disease diagnosis, nutrition and environmental science as well as other areas [8,9]. Recently, metabonomics have been utilized to uncover the pharmacological mechanism

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of traditional Chinese medicine. And many analytical techniques such as nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC-MS) have been applied to metabolomics research. In this study, firstly, we compared the therapeutic effects of extraction of water-soluble components from Glycyrrhiza and flavonoids and saponins obtained from Glycyrrhiza on acute inflammation, chronic inflammation and inflammatory pain to find the effective fraction. Then an ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) method was established to gain the global profiling of the endogenous metabolites in the urine of adjuvant-induced arthritis (AIA) rats. The principal component analysis (PCA), as a pattern recognition method, was applied to observe the endogenous metabolites changes after drug treatment. Through analyzing the changes of metabolic pathways, the mechanism of Glycyrrhiza treating on RA will be discussed.

2. Materials and methods

2.1. Materials

Glycyrrhiza was purchased from Changchun Tongrentang Chinese Medicine-Since. Carrageenin was obtained from Aladdin Chemistry Co. Ltd. (No.1126650). Acetic acid was obtained from Beijing Chemical Works (Beijing, China).Complete Freund's adjuvant (CFA) was obtained from Chondrex, Inc. (Redmond, WA, USA). The assay kits for Interleukin-1β(IL-1β), tumor necrosis factor-α(TNF-α), prostaglandin E2(PGE2) and malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Citric acid, kynurenic acid, xanthurenic acid, hippuric acid, uric acid, suberic acid, sebacic acid, homocysteine, taurine, and creatinine were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Leucine enkephalin and sodium formate were obtained from Waters (Milford, USA). Acetonitrile and formic acid, HPLC-grade, were from Tedia Company, INC. (Ohio, USA). Ultrapure water was obtained by using Milli-Q water purification system (Milford, MA, USA).

2.2. Preparation of drugs

The counter-current extraction was performed by using weighted Glycyrrhiza with distilled water at a sample-to-solvent ratio 1:10 (w/v) for 1 h. Afterward, the residue was counter-currently extracted twice with the same multiple of water for 40 min. The filtrates were immersed with 70% alcohol overnight. Finally the filtrates were combined, concentrated and lyophilized to obtain extract powder of water-soluble components from Glycyrrhiza. The flavonoids of Glycyrrhiza were purified thought the combination of DM130 macroporous resin and polyamide resin [10]. And the saponins of Glycyrrhiza were purified thought D101 macroporous resin [11].

2.3. Experimental design

2.3.1. Acetic acid-induced abdominal writhes

According to the dose of each drug in Section 2.2, the mice were previously treated with different extractions for 8 days. After the last administration for 30 min, the mice were injected intraperitoneally with 0.6% acetic acid (0.1 mL/10 g body weight) [12]. The number of writhing in mice within 20 min after injection was recorded.

2.3.2. Carrageenin-induced inflammation mice model

According to the dose of each drug in Section 2.2, the mice were previously treated with different extractions for 7 days. Briefly, 30 min after the last intragastric administration, the initial volume of left hind paw (V_0) was measured. Then, the mice were injected with 1% aqueous solution of carrageenin (0.1 mL) by microinjectors [13]. The volume of left hind paw (V) in each group was measured at 30, 60, 90, 120, 180 and 240 min after injection. The inhibitory rates were calculated as follows:

1. The paw swelling (PS, %) = $(V_t - V_0)/V_0 \times 100\%$, where, V_0 = the average initial volume before injecting the aqueous solution of carrageenin per group; V_t = the average volume of joint swelling at different time per group.
2. Inhibitory rates (%) = $(PS_{0\text{-}MG} - PS_{0-t})/PS_{0\text{-}MG} \times 100\%$, where, PS_{0-MG} = the average paw swelling of MG; PS_{0-t} = the average paw swelling at different time per treated group.

All the rats were sacrificed. And their whole blood was collected and centrifuged at 3000 rpm/min for 10 min at 4 °C to get the serum sample. The levels of PGE2 and MDA were measured in the serum of carrageenin-induced inflammation mice.

2.3.3. Adjuvant-induced arthritis

The model group and the treated groups were injected 0.1 mL CFA (10 mg/mL) in the left hind toe of the rats. Healthy control group was injected with 0.1 mL saline in the same site. Two weeks after the immunization, all the animals were administered intragastrically as indicated in Section 2.2 for three weeks. Body weights before modeling and after modeling at 35 days were detected. Then all the rats were sacrificed. And their whole blood was collected and centrifuged at 3000 rpm/min for 10 min at 4 °C to get the serum sample. The levels of TNF-α and IL-1β were measured in the serum of adjuvant arthritis rats. At the same time, the right hind joints were fixed in formalin solution, decalcified with nitric acid for 2 weeks, then paraffin embedding was carried out according to the standard method. Joint sections were cut, dewaxed, dehydrated and stained with haematoxylin and eosin (H&E) for general evaluation. Arthroscopic sections were scored from inflammatory infiltration, synovial proliferation, cartilage erosion, and bone destruction. Above each assignment was rated by 0–3 level evaluations: 0 point as asymptomatic, 1 point as mild symptoms, 2 points as moderate symptoms, 3 points as severe symptoms, and the total score of 12 points as previously described [14,15].

2.4. Animals and treatments

The Kunming mice (weights 20 ± 2 g) and Sprague Dawley (SD) rats (weights 200 ± 20 g) were provided by Experimental Animal Center of Jilin University (China). Animals were housed in a barrier system with standard food and tap water in an air-conditioned room under light-dark cycle of 12 h per day at 22–24 °C and 50–60% relatively humidity. All the animals were acclimated for 7 days before the experimentation. Then, all the animals were randomly divided into 5 groups respectively containing 10 rates and respectively named normal control group (NG), model group (MG), Glycyrrhiza treated group (RG), flavonoid treated group (FG) and saponin treated group (SG). The mice in the Glycyrrhiza treated group were administered intragastrically with extraction of water-soluble components from Glycyrrhiza at a dose of 1.1 g crude drugs per kilogram per day (equal to10 mL/kg/day); flavonoid treated group and saponin treated group were respectively administered intragastrically with the purifications from Glycyrrhiza at a dose of 1.1 g crude drugs per kilogram per day (equal to 10 mL/kg/day). The rats in the Glycyrrhiza treated group were administered intragastrically with the extractions of Glycyrrhiza at a dose of 1.56 g crude drugs per kilogram per day (equal to10 mL/kg/day); flavonoid treated group and saponin treated group were respectively administered intragastrically with the purifications from Glycyrrhiza at a dose of 1.56 g crude drugs per kilogram per day (equal to10 mL/kg/day). The animals in the healthy control and model groups were administered with the same amount of distilled water.