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Metabonomic study of Wu-tou decoction in adjuvant-induced arthritis rat using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

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ABSTRACT

A urinary metabonomics method based on the ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) had been established to investigate the holistic efficacy of Wu-tou decoction (WTD), a traditional Chinese medicine (TCM) formula used to treat rheumatoid arthritis (RA), in adjuvant-induced arthritis (AIA) rat model. Multivariate statistical approaches, such as principal component analysis (PCA) and orthogonal projection to latent structures squares-discriminant analysis (OPLS-DA) were used to distinguish healthy control group, AIA model group and WTD treated group and find potential biomarkers. There was a clear separation among the three groups in PCA model. Sixteen potential biomarkers had been identified using OPLS-DA, and 11 of them was considered to be in response to therapeutic effects of WTD involved in tryptophan metabolism, phenylalanine metabolism, tricarboxylic acid (TCA) cycle, bile acid biosynthesis, steroid hormone biosynthesis and valine metabolism. In this study, WTD also showed good anti-inflammatory and antioxidant activities *in vivo*, and it could suppress histopathological changes of AIA rats. There might be a correlation between these results and the regulation of the disturbed metabolites in urine. This study demonstrates that metabonomics is a powerful methodology to gain insight in the mechanism of TCM formula in therapy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of synovial joints. Pain and bone erosion are its predominant problem and central feature. It is also an autoimmune disease, and characteristic production rheumatoid factor and anti-citrullinated peptide antibody are used to diagnose the disease [1]. The histopathology of RA showed pronounced angiogenesis, cellular hyperplasia, an influx of inflammatory leucocytes and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines [2]. RA is also associated with an increased cardiovascular CV burden [3]. Oxidative stress has been proposed to correlate with the degree of inflammation in patients with RA and contribute to the joint tissue damage [4]. Although RA has been studied for many years, the pathogenesis is still unclear and no drug could cure RA completely. Non-steroidal anti-inflammatory drugs, glucocorticoids and immunosuppressants are commonly used as therapeutic approach. However, various side effects, like gastrointestinal and liver disorders, happened when using these drugs. Because of these side effects, safe and effective new drugs for RA are required. Herb medicines, which have been used for thousands of years in many countries, provide an immense drug library for searching new drug leads, especially traditional Chinese medicine (TCM) which is regarded naturally and harmlessly and accepted gradually in the world in recent years [5,6].

Formulas have been used for centuries to enhance therapeutic efficacy and reduce adverse effects in TCM. Wu-tou decoction (WTD), a TCM formula designed by Chinese medicine sage Zhang Zhongjing, is composed of Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata, and it was applied to treat RA, rheumatic arthritis and pain of joints for more than a thousand years. Previous research in our laboratory showed that WTD contained numerous alkaloids, triterpene saponins and some monoterpene glycosides, flavones and flavones glycosides [7]. In recent years, researchers found

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that WTD could decrease the abdomen capillary permeability and reduce dimethylbenzene-induced ear edema and acetic acid-induced writhing numbers in mice [8]. It could also down-regulate the level of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in serum, decrease the blood viscosity and erythrocyte aggregation index, reduce the peripheral blood $CD4^+$ percentage and increase the peripheral blood $CD8^+$ percentage in adjuvant-induced arthritis (AIA) rat model [9–11].

Unlike western medicine, TCM is a "system-to-system" treatment mode, and it focuses on both the pathological and physiological changes [12]. Metabonomics was defined by Nicholson as "quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" in 1999 [13]. The systemic feature of metabonomics is consistent with the holistic characteristics of TCM. In recent years, metabonomics was used to evaluate therapeutic effects, toxicity and side effects of many Chinese herbal medicines and TCM prescriptions [14–17]. Nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and other hyphenated technologies have been applied for metabolomics analysis [18–20]. Compared with NMR, MS is generally more sensitive, and it can reduce mass spectral complexity coupled with separation techniques, such as UPLC. Recently, the application of high resolution MS in metabonomics is wide, since this technique could acquire a more specific and accurate mass in metabolite identification. Thus, in this study, an UPLC-Q-TOF-MS method was established to gain the global profiling of the endogenous metabolites in urine of AIA rats. The metabolite pathway changes after the treatment with WTD were investigated using pattern recognition approaches, and further understanding of how WTD could modify the symptom RA may be obtained.

2. Materials and methods

2.1. Materials

Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata were purchased from Beijing Huamiao Chinese medicine Engineering Development Center (Beijing, China). Leucine enkephalin was obtained from Waters (Milford, USA). Acetonitrile and formic acid (Fisher Scientific, Loughborough, UK) were HPLC-grade. Ultrapure water was produced by a Milli-Q plus (Milford, MA, USA) water purification system. Valine, phenylalanine, kynurenic acid, 2-methylhippuric acid, citric acid and cholic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Complete Freund's adjuvant (CFA) was purchased from Chondrex, Inc (Redmond, WA, USA). Rat IL-1β and TNF-α ELLSA kits were obtained from Dakewe Biotech Co., LTD (Beijing, China). The kits for superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. WTD extraction

Powder of Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata in the proportions of 2:3:3:3:3 with a total weight of 700 g was immersed in water 10 times of their total weight for 1 h, and then heated to refluxing for 1.5 h. Eight times of water was added for another 1.5 h refluxing after filtering. The filtered extraction solutions were combined and concentrated using a rotary evaporator at 60 °C and then lyophilized.

2.3. AIA model construction and treatment

Twenty-four male Wistar rats, weighing 205–240 g, obtained from Experimental Animal Center of Jilin University (China) were randomly divided into 3 groups, namely healthy control group (HG), AIA model group (AG) and WTD treated group (WG). Standard laboratory chow was available ad libitum through the whole experiment. Prior to initiation of experimentation, all the rats were acclimated for 7 days, and then the rats in AG and WG were injected 0.1 mL CFA containing 10 mg mL^{-1} dead *Mycobacterium tuberculosis* bacteria in the left hind footpad and the rats in HG were injected 0.1 mL saline. Fourteen days after the immunization, the rats in WG were administered with WTD extraction intragastrically at a dose of 9.8 g crud drug per kilogram per day (equal to 10 mL/kg/day) for 21 days. The rats in other groups were given water intragastrically as controls. After 3 weeks administration, all the rats were sacrificed, blood was collected for the following analysis and the right hind joints were excised for histologic examination.

2.4. Serum biochemical parameters

The whole blood of rats was centrifuged at 3000 × *g* for 10 min at 4 °C to get the serum sample. The serum samples were stored at −80 °C. Prior to analysis, the serum samples were thawed at 4 °C. ELISA kits were applied to evaluate TNF-α and IL-1β levels in rat serum. SOD and GSH-Px activities and MDA level were measured by using commercially available kits.

2.5. Histologic examination

Joint tissues were fixed in 10% neutral buffered formalin, and then decalcified for two weeks. Joints were processed for paraffin embedding as per the standard protocol. Joints sections were subsequently cut, deparaffinized, dehydrated, and stained with haematoxylin and eosin (H&E) for general evaluation. Inflammatory infiltration, synovial proliferation, cartilage erosion, and bone destruction graded from 0 to 3 were evaluated as previously described [21,22].

2.6. Urine sample preparation

Samples of 24-h urine were collected on the 21st day after administration and stored at −80 °C. Prior to UPLC-MS analysis, urine samples were thawed at room temperature. After centrifuging at 10,000 rmp for 10 min, the supernatant was diluted at a ratio of 1:9 with water, and then filtered through a 0.22 μm filter membrane.

2.7. UPLC-MS conditions

Metabonomics analysis was performed using a Waters Acquity UPLC system coupled with a Q-TOF SYNAPT G2 High Definition Mass Spectrometer (HDMS) (Waters, UK). Separation was performed on a Waters ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 mm × 50 mm) kept at 40 °C and at a flow rate of 0.5 mL min^{-1}. 0.1% aqueous formic acid (v/v) (A) and acetonitrile (B) were used as mobile phase. The gradient elution of B was performed as follows: 5–20%B at 0–3 min, 20–40%B at 3–4 min, 40–100%B at 4–6 min, 100%B at 6–7 min, 100–5%B at 7–7.1 min and then kept at 5%B for 4 min. The sample inject volume was 5 μL. During the whole analysis, all the samples were maintained at 4 °C. The ESI source in both positive and negative ion mode was used in MS analysis. The source temperature was 120 °C, and desolvation gas temperature was 350 °C. Nitrogen was used as cone and desolvation gas. The flow rates of cone and desolvation gas were set at 50 L h^{-1} and 700 L h^{-1}, respectively. Capillary, cone and extraction cone voltages were set