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Metabolomics analysis of Danggui Sini decoction on treatment of collagen-induced arthritis in rats

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent joint inflammation leading to bone and cartilage damage and even disability. However, the pathogenesis of RA is multi-factorial and to a large degree, remains unknown. Danggui Sini decoction (DSD), a traditional Chinese medicine (TCM) formula, has been widely used as a remedy for rheumatoid arthritis (RA) in recent years. In our study, ^{1}H-nuclear magnetic resonance (^{1}H NMR) based metabolomics analysis of 7 potential biomarkers, including taurine (**1**), urea (**2**), betaine (**3**), pyruvate (**4**), hippurate (**5**), succinate (**6**) and acetone (**7**) was performed to investigate the progression of RA and assess the efficacy of DSD in collagen-induced arthritis (CIA) rats. According to pathway analysis using identified metabolites and correlation construction, taurine and hypotaurine metabolism, gut microbiota metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, the citrate cycle (TCA cycle) and lipid metabolism were recognized as being the most influenced metabolic pathways associated with RA. As a result, deviations of metabolites 1, 3, 4, 5, 6 and 7 in CIA rats were improved by DSD, which suggested that DSD mediated the abnormal metabolic pathways synergistically. In summary, the efficacy and its underlying therapeutic mechanisms of DSD on RA were systematically investigated and expect to provide a new insight in relevant studies of other TCM formulas.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by persistent synovitis, systemic inflammation, renal injury and progressive damage to articular cartilage and the underlying bone [1,2]. The effects of RA often mainly manifest as swelling and pain in the small movable joints of the hands and feet and if not treated, they will be deformed [3]. Currently, despite many drugs such as glucocorticoids, tumor necrosis factor-alpha (TNF-α) inhibitors and disease-modifying antirheumatic drugs (DMARDs) have been introduced to treat the RA, their therapeutic effects are still unsatisfactory, and they carry serious side effects such as Cushing's syndrome, diabetes, hepatotoxicity and even malignant tumors [4,5]. Therefore, alternative therapies for RA are needed.

Alternative therapies using Traditional Chinese medicine (TCM) suggest new approaches to the management of RA. Since the pathogenesis of RA is a multistage process, it could be problematic by using a single agent. Therefore, the synergistic interactions among the active ingredients present in TCM may help to protect against RA. Danggui Sini decoction (DSD) is a classic TCM formula consisting of seven herbs: *Angelica sinensis*, *Cinnamomi cassia*, *Paeonia lactiiflora*, *Tetrapanax papyriferus*, *Asarum heterotropoides*, *Glycyrrhiza uralensis* and *Ziziphus jujuba* Mill. DSD traditionally used in China to treat the arthritis, diabetic peripheral neuropathy and coronary atherosclerotic heart disease [6]. Recently, pharmacological studies have shown that DSD has anti-inflammatory and anti-oxidant activities and has been widely used clinically to treat the RA [7]. However, the underlying mechanisms of DSD treating RA haves not been elucidated. It is universally acknowledged that the holistic therapeutic effects of herbal formulae based on multiple components acting on multiple targets inevitably make understanding their mechanisms of action challenging [8]. To provide the deeper insight into an explanation of the

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therapeutic effects of DSD on RA, applicable strategies for complex systems are in great demand.

Metabolomics is an integral technology for understanding the function of biological systems based on the global metabolite profiles of pathological stimuli or drug treatments in biological samples such as urine, plasma or tissue in living systems. This technology provides diagnostic information and presents mechanistic insights into the biochemical effects of toxins and drugs, as well as providing information on the variations in metabolic networks that characterize pathological states in both animals and humans [9]. Depending on a series analysis of a variety of different samples and the combination of multivariate statistical methods, metabolomics can be used to characterize drug efficacy in organisms [10]. Various analytical techniques, such as gas chromatography mass spectrometry (GC–MS), liquid chromatography mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) have been applied in the study of metabolomics [11]. Compared with LC–MS and GC–MS methods, ^{1}H NMR-based metabolomics is a more powerful technique to reveal the overall metabolic profile of bio-fluids or tissue extracts, as it offers vital information on the structure of metabolites in a non-invasive and non-destructive way [12,13]. Previous studies have indicated that, ^{1}H NMR analysis provided an effective approach for evaluating the therapeutic and toxic effects of TCM formula [14–16].

The most widely used model for RA is the collagen-induced arthritis (CIA) in rats, since they share both immunological and pathological features with RA [17]. In this study, urine metabolomics based on ^{1}H NMR was applied to investigate the metabolic profiles and potential biomarkers in the CIA rats after DSD treatment. And that the most relevant pathways related to RA and potential therapeutic targets were further revealed by pathway analysis and correlation network construction. To our knowledge, it's the first time to investigate the therapeutic mechanism of DSD on RA using the metabolomics approach and expect to provide a new insight in relevant studies of other TCM formulas.

2. Materials and methods

2.1. Materials and chemicals

The assay kits for bovine type II collagen (CII) and incomplete Freund's adjuvant (IFA) were purchased from Sigma-Aldrich (St. Louis, USA). ELISA kits of rat TNF-α and 4% paraformaldehyde were obtained from Boster Biological Technology (Wuhan, China). Dexamethasone acetate tablets were purchased from Tongji Pharmacy Co., Ltd. (Nanning, China). Ferulic acid, cinnamic acid, glycyrrhizic acid, liquiritin and isoliquiritin were purchased from Chengdu Must Biotechnology Co., Ltd (Purity > 98% (HPLC), Chengdu, China). D_2O and Sodium-3-(trimethylsilyl) propionate-2, 2, 3, 3-d4 (TSP) were purchased from Qingdao Tenglong Bio-technology Company (Qingdao, China). Formic acid (HPLC grade) was obtained from Tedia (Fairfield, OH, USA). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). EDTA was bought from Solarbio Co.,Ltd (Purity > 99.5%, Beijing, China). Purified water was produced using the Milli-Q ultra-pure water system (Millipore, Billerica, MA, USA).

2.2. Preparation of DSD samples

The herbs of DSD were purchased from Nanning Shengyuantang Chinese Herbal Medicine Co., Ltd. (Nanning, China) and authenticated by Associate Professor Changming Mo of Guangxi Botanical Garden of Medicinal Plants. The herbs were first cleaned, dried and then cut into small pieces. The 100 g sample was extracted twice with distilled water: the former used 600 ml at 100 °C for 2 h, and the latter used 400 ml at 100 °C for 1.5 h. After filtration, the filtrate was evaporated in a vacuum to obtain a 1.0 g/ml stock solution, which was subsequently stored at −20 °C. The chemical profile was characterized by UPLC-QTOF–MS; the corresponding analytical methods and results were demonstrated in supplementary material-(1).

2.3. Rats and treatments

Twenty-four female Sprague-Dawley rats (180–220 g) were purchased from the Experimental Animal Center of Guangxi Medical University (Guangxi, China; Approval No.: GXMU2010032418). The rats were housed in plastic cages in a controlled room with 55 ± 15% humidity, a temperature of 22 ± 3 °C, and a daily light cycle of 06:00–18:00. This research was conducted according to protocols approved by the Institutional Ethics Committee of Guangxi Medical University.

The rats were divided equally and randomly into four groups: the normal group (NG), model group (MG), dexamethasone group (DG) and DSD treated group (TG). After 1 week for acclimation, except the normal group, the rats were injected intradermally with 400 μl bovine type II collagen (CII) emulsified in an equal volume of incomplete Freund's adjuvant (IFA) at the base of the tail (day 0). 7 days after the primary immunization, rats were boosted intradermally with 100 μl CII in IFA for the second time. The progression of CIA was evaluated by measuring thickness of the right hind joint, body weight every seven days with a vernier calliper (GB/T21389-2008, Guilin) and an electronic balance (JSC-1000, Zhejiang). To assess the effect of DSD on the established CIA model rats, all drugs were administered to them once daily via gavage from day 20 to day 40 of the experiment. Rats in the normal and model groups received distilled water (1 ml/100 g, i.g.). The dose of DSD and dexamethasone we selected was 16.2 g/kg and 0.07 mg/kg in order to equivalent to the actual clinic dosage based on the conversion coefficient 0.018 of the body surface area conversion factor between rats (200 g) and human (70 kg). All rats were executed in 24 h after the last drug administration. Urine was collected for 24 h in metabolism cages at ambient temperature and 10 μl (10 mg/mL) NaN_3 was added for antisepsis before collection. Plasma samples were isolated by centrifugation for 10 min at 3500 rpm at 4 °C (Fichal centrifuge TDL-5A, Shanghai, China). The fresh urine and serum samples were stored at −80 °C until further analysis.

2.4. Biochemical assay and histological study

Levels of TNF-α in the serum were measured using commercially available ELISA kits according to the manufacturers' instructions. Joints were fixed with 4% paraformaldehyde for 24 h and decalcified with 10% EDTA for 30 days. After that, the joints were incised longitudinally, embedded in paraffin, stained with HE, and examined histopathological changes under a microscope (OLYMPUS microscope CX31, Olympus Microsystems Corp., Japan). Images were obtained at an original magnification of 40× (Olympus DP12 Microsystems Digital Imaging, Olympus, Japan).

2.5. Urine sample preparation and ^{1}H NMR assay

The urine was centrifuged at 13,000 rpm at 4 °C for 10 min. A total of 400 (μL) of the supernatant was placed in 5-mm NMR tubes and mixed with 50 μl D_2O (containing 2.0 mg/mL TSP). The prepared samples were kept at 4 °C until NMR analysis. NMR spectral measurements were acquired on a 600 MHz NMR spectrometer (Varian INOVA, Varian, Inc., USA) operating at a 600.14-MHz ^{1}H frequency and a temperature of 303 K using a Carr-Purcell-Merboom-Gill (CPMG) pulse sequence. For each sample, the presaturation pulse for water peak suppression and the 16 FIDs were collected into 16k data points using a spectral width of 9615.1 Hz, an acquisition time of 1.7039 s, a relaxation delay of 10.0000 s, and a total T2 echo delay time of 25 ms. ^{1}H–^{1}H correlation spectroscopy (COSY), a two-dimensional (2D) NMR method, was used to confirm the assignments.