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Metabolomics coupled with system pharmacology reveal the protective effect of total flavonoids of Astragali Radix against adriamycin-induced rat nephropathy model

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ABSTRACT

Astragali Radix (AR) has been used in the traditional Chinese medicine (TCM) in the treatment of various renal diseases for many years. In this study, a NMR based metabolomic approach coupled with biochemical assay and histopathological inspection had been employed to study the protective effect of total flavonoids (TFA) in AR against adriamycin-induced nephropathy using rats model. Multivariate analysis revealed that 11 of perturbed metabolites could be reversed by TFA, and the MetaboAnalyst analysis revealed that the anti-nephrotic syndrome effect of TFA was probably related with regulation of alanine, aspartate and glutamate metabolism, citrate cycle, pyruvate metabolism, cysteine and methionine metabolism and glyoxylate and dicarboxylate metabolism. The regulatory effects on the gene expression (ACE, nephrin, podocin) suggested that the anti-nephrotic syndrome effect of TFA was also related with the protection of renal filtration function and regulation of blood pressure. The system pharmacology analysis revealed 43 potential targets for TFA, and suggested that the protective effect of TFA on the nephrotic syndrome was probably related with the regulation of immune and renin-angiotensin system. These metabolic changes and the associated pathways, as well as the compound-target-disease network provide insights into the mechanisms of TFA for the treatment of nephrotic syndrome, and further studies are needed to validate the bioactive compounds responsible for the anti-nephrotic syndrome effect of TFA.

1. Introduction

Nephrotic Syndrome (NS) is a chronic metabolic disorder influenced by interactions between environmental and genetic factors [1]. Nephrotic Syndrome shows symptoms of proteinuria, hypoalbuminemia and hyperlipidemia. Chronic degenerative diseases such as hypertension, hyperlipidemia, diabetes mellitus and atherosclerosis promote renal function decline [2]. The pathogenesis of NS is very complex and not entirely clear, which is involving the circulatory and metabolic systems. Previous studies suggested that renal inflammation, apoptosis, oxidative stress and podocyte injury are associated with the development and progression of NS pathological features [3–5]. Among the anti-nephrotic syndrome medications available, the immunosuppression and glucocorticoid drugs are the preferred therapeutic for mild to moderate NS. However, immunosuppression therapy can induce many serious side effects, such as fungal infection, diverse organ failure (cardiac, renal, and ear) and a fast relapse following any stoppage in therapy [6]. The glucocorticoids can induce hypertension and diabetes mellitus, which limited the use of glucocorticoids in NS therapy [7]. In addition, immunosuppression and glucocorticoid drugs were not universally effective in all patients, although they are the recommended medication for NS in clinical practice [8,9].

Traditional Chinese medicine (TCM) showed unique therapeutic effect in the treatment of various diseases, as it had the advantages of little side effect, low cost and low recurring rate [10]. Various plant-derived natural extracts, such as *Bupleurum chinense* DC. [11], *Astractylodes macrocephala* Koidz. [12], *Codonopsis pilosula* (Franch.) Nannf. [13], have been evaluated for potential application in the treatment of NS. Astragali Radix (AR), it is the dried root of *Astragalus membranaceus* (Fisch.) Bge. or *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao., which is widely used to treat weakness, wounds, anemia, fever, multiple allergies, chronic fatigue, loss of appetite, various renal diseases [14]. In addition, the extracts of AR also showed other bioactivities, such as anti-

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inflammatory, anti-cancer, diuretic action, and adjusting immune function of organism [15]. Previous studies [16] showed that the AR extracts exhibited anti-nephrotic syndrome effect in some aspects: increasing kidney blood perfusion and glomerular filtration rate, reducing podocytes injury and urinary protein, delaying glomerular sclerosis. As the total flavonoids in AR (TFA) showed bioactivities of antioxidant, anti-apoptosis and strengthen the immune system [17], we speculated that the TFA may be effective for NS.

The present study aimed to evaluate the protective role of TFA using adriamycin-induced rat nephropathy model, as well as its underlying mechanism based on a new strategy, which integrating untargeted metabolomics and system pharmacology. To our knowledge, this is the first study that deeply assessed the metabolic regulation of TFA against NS.

2. Materials and methods

2.1. Reagents and plant materials

Sodium-3-(trimethysilyl) propionate-2,2,3,3-d4 (TSP) was purchased from Cambridge Isotope Laboratories Inc (Andover, MA, USA). D_2O and CD_3OD were purchased from Norell (Landisville, PA, USA). Adriamycin hydrochloride for injection (lot 1412E1, 10 mg per bottle) was provided by Shenzhen Main Luck Pharmaceutials Inc. (Shenzhen, China). Astragali Radix was purchased from Shanxi Datong Materials Market (Hunyuan, China) on September 2016, and identified as the roots of *A. membranaceus* var. *mongholicus* by Professor Xuemei Qin. The reagents for qPCR were purchased in Sangon Biotech Co., Ltd. (Shanghai, China). All others chemicals were of analytical grade.

2.2. Extract preparation

Astragali Radix (2.5 kg) which was smashed and soaked in distilled water at room temperature, was extracted by refluxing (1:10, w/v) first for 2 h and then (1:8, w/v) 2 h. The filters were combined and concentrated in a rotary evaporator. The concentrated solution was further separated using AB-8 macroporous resin, which was eluted sequentially with distilled water, 10% ethanol, 35% ethanol. The 35% ethanol eluate was concentrated and portioned three times with ethyl acetate to afford the TFA. Then the TFA extract was stored at −20 °C before use.

2.3. NMR and LC–MS analysis of TFA

For NMR analysis, about 5 mg of TFA was dissolved in CD_3OD. After centrifuging for 15 min at 13000 rpm, the supernatant (600 mL) was transferred into a 5 mm NMR tube for NMR analysis. The NMR spectral was measured using zgpr sequence at 298 K on Bruker Avance 600-NMR spectrometer (600.13 MHz proton frequency, Bruker, Germany) equipped with a Bruker 5 mm double resonance BBI probe.

TFA was analyzed on the UHPLC-Q-Exactive-Orbittrap-HRMS equipment (Thermo Fisher, USA). A sample containing 5 g/mL of TFA in methanol was passed through a 0.22 mm pore size syringe filter, and an aliquot of 2 μL was injected into the column. The chromatographic separation was performed on the Thermo Fisher U 3000 system equipped with an ACQUITY UPLC® $HSST_3$ (2.1 × 100 mm, 1.8 um, Waters USA). The elution was performed using acetonitrile (solvent A) and water (solvent B) at a flow rate of 0.3 mL/min, and the gradient was optimized as follows: 0–3 min, 80% B; 3–6 min, 80%–73% B; 6–10 min, 73% B; 10–12 min, 73%–57% B; 12–16 min, 57%–40% B, 16–20 min, 40%–0% B, and the re-equilibration time of gradient elution was 5 min. The Z-spray ionization source was maintained at 300 °C with spray voltages of 3.5 kV in the positive ionization mode. Additional operating parameters were: capillary temperature 320 °C, lens voltage 55 kPa, mass resolution 70000, and mass scan range 150–1500 m/z in the full-scan mode.

2.4. Animal experiments

Forty male Sprague-Dawley (SD) rats (200 ± 20 g, animal licence No. SCXK 2016-0014) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. Rats were housed in isolation and ventilation cages (IVC) in a climate-controlled room with 12 h of light-dark illumination cycle at 23 ± 1.5 °C and 45 ± 15% humidity.

After acclimatization for 7 days, 24 h of urine were collected and recorded the volume. The urine protein content was determined by BCA (bicinhoninine acid) method.

According to the content of 24 h urine protein, unqualified (Upro >80 and Upro <60) rats were excluded. Then, the qualified rats were randomly divided into three groups (n = 10), including the Control group, Model group and TFA group. Model group and TFA group were intravenous injected with adriamycin (4 mg/kg, 2 mL/kg) at the first day, while the Control group were injected with the same volume of normal saline. A week later, Model group and TFA group were treated with adriamycin (1 mg/kg) again, and the Control group were treated with normal saline. From the 14 day, the TFA group received intraperitoneal injection (3 mL/kg) of TFA (1.1 mg/kg), while the Control group and Model group received same volume of water. All the rats were allowed free access to the feed and water in the process of experiment, and the entire experiment was lasted for 49 days.

On the day 50, all the rats were anesthetized with 10% urethane, and the blood samples were collected from femoral artery and collected into 10 mL EP tubes. The blood samples were allowed to stand at room temperature to coagulate, then the samples were isolated by centrifugation for 15 min at 3500 rpm at 4 °C. The heart, liver, spleen, lung, left kidney and thymus were removed and put into the cryopreservation tube. All of the samples were stored at −80 °C refrigerator for further analysis. The right kidneys were soaked in 10% neutral formalin for histopathological examination.

2.5. Histopathological study and biochemical assay

The formalin-fixed left kidney tissues (>48 h soaked) were dehydrated and immersed in paraffin, and cut into sections of 3–5 μm. These sections were stained with hematoxylin and eosin (H&E) and histopathologically evaluated under the microscopy. Renal tubular injury (RTI) [18] was assessed and given a score defined as tubular injury score from 0 to 4 (0 = normal, 1 = sight damage, 2 = moderate damage, 3 = severe damage, 4 = most severe damage).

The Biochemical parameters, which included serum albumin (ALB), serum urea nitrogen (BUN), creatinine (CREA), total cholesterol (TCHO), total triglycerides (TG), total protein (TP) were determined on Automatic Analyzer (PRIME60i, Therom Fisher, USA). A value of $p < 0.05$ using t-test was considered to be significant.

2.6. qRT-PCR

Total RNA was extracted from the kidney tissue by Trizol method. Firstly, the concentration of RNA was determined (at 150–500 mg/L), the cDNA was amplified by 1st-Strand cDNA synthesis method according to the instructions. Then the Quantitative Real-time PCR (qRT-PCR) amplification reaction was carried out according to the instructions. The reaction system (25 μL) was composed of 2 μL cDNA sample, 12.5 μL of SYBR Premix Ex Taq (2×), 0.5 μL of PCR Forward Primer (10 μM), 0.5 μL of PCR Reverse Primer