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Urinary metabonomics elucidate the therapeutic mechanism of *Orthosiphon stamineus* in mouse crystal-induced kidney injury

Songyan Gao [a,1], Wei Chen [b,1], Zhongjiang Peng [b], Na Li [a], Li Su [a], Diya Lv [a], Ling Li [a], Qishan Lin [c], Xin Dong [a,*], Zhiyong Guo [b,**], Ziyang Lou [a,***]

[a] School of Pharmacy, Second Military Medical University, Shanghai 200433, China
[b] Changhai Hospital, Second Military Medical University, Shanghai 200433, China
[c] Proteomics/Mass Spec Facility, Center for Functional Genomics, University at Albany, Rensselaer, NY 12144, USA

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ABSTRACT

Ethnopharmacological relevance: *Orthosiphon stamineus* (OS), a traditional Chinese herb, is often used for promoting urination and treating nephrolithiasis.
Aim of the study: Urolithiasis is a major worldwide public health burden due to its high incidence of recurrence and damage to renal function. However, the etiology for urolithiasis is not well understood. Metabonomics, the systematic study of small molecule metabolites present in biological samples, has become a valid and powerful tool for understanding disease phenotypes. In this study, a urinary metabolic profiling analysis was performed in a mouse model of renal calcium oxalate crystal deposition to identify potential biomarkers for crystal-induced renal damage and the anti-crystal mechanism of OS.
Materials and methods: Thirty six mice were randomly divided into six groups including Saline, Crystal, Cystone and OS at dosages of 0.5 g/kg, 1 g/kg, and 2 g/kg. A metabonomics approach using ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF/MS) was developed to perform the urinary metabolic profiling analysis. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were utilized to identify differences between the metabolic profiles of mice in the saline control group and crystal group.
Results: Using partial least squares-discriminant analysis, 30 metabolites were identified as potential biomarkers of crystal-induced renal damage. Most of them were primarily involved in amino acid metabolism, taurine and hypotaurine metabolism, purine metabolism, and the citrate cycle (TCA). After the treatment with OS, the levels of 20 biomarkers had returned to the levels of the control samples.
Conclusions: Our results suggest that OS has a protective effect for mice with crystal-induced kidney injury via the regulation of multiple metabolic pathways primarily involving amino acid, energy and choline metabolism.

1. Introduction

Renal stone disease is one kind of disease characterized by intermittence nephric colic and hematuria. Due to its high incidence of recurrence and harm towards renal function, kidney stones seriously jeopardize overall public health for society and quality of life for affected individuals. The prevalence of urinary stone disease ranges from 1% to 20% (average 10%) worldwide due to various factors, such as geography, climate, race, metabolic dysfunction and diet (Amato et al., 2004; Tiselius, 2003). Several epidemiologic studies have found that the formation of kidney stones increases the risk of chronic kidney disease (CKD) (Rule et al., 2009, 2011), and is associated with cardiovascular diseases, such as myocardial infarction and angina pectoris (Ando et al., 2013; Domingos and Serra, 2011; Rule et al., 2010). The development of

Abbreviations: OS, *Orthosiphon stamineus*; UHPLC-Q-TOF/MS, ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; TCA, Tricarboxylic acid cycle; CKD, chronic kidney disease; RSD, relative standard deviation; i.p., intraperitoneally; i.g., intragastrically; Scr, serum creatinine; BUN, blood urea nitrogen; QC, quality control; ESI, electrospray ionization; ANOVA, analysis of variance; TIC, total ion chromatogram; VIP, variable importance value; EIC, extracted ion chromatogram; AAA, aromatic amino acids; ROS, reactive oxygen species

* Corresponding author.
** Corresponding author.
*** Corresponding author. Tel./fax: +86 21 81871335.
E-mail addresses: dongxinsmmu@126.com (X. Dong), drguozhiyong@163.com (Z. Guo), ziyanglou@163.com (Z. Lou).
[1] These authors contributed equally to this work.

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a urinary stone is a multistep process involving crystal nucleation, growth, formation and aggregation. However, the mechanism underlying it is not well understood.

Orthosiphon stamineus (Labiatae) has many synonyms, such as, *Clerodendranthus spicatus*, *Orthosiphon spicatus*, and *Orthosiphon grandiflorum*, of which the main active ingredients include flavonoids, terpenes and phenolic acids (Ameer et al., 2012). Previous studies have demonstrated OS could alleviate renal calcium oxalate deposition and urinary calcium excretion in an oxalate-stone-forming rat model (Akanae et al., 2010; Zhong et al., 2012); however, no studies have been performed systematically to evaluate the anti-urolithiasis effect and mechanism of OS.

Metabonomics, a new emerging approach of systems biology, focuses on the investigation of all endogenous metabolic responses in complex living systems, and has been used for the research of natural and alternative medicine. In the present study, we employed a metabonomics-based approach to explore urinary metabolomic changes in a murine model of crystal induced kidney injury and evaluated the protective effect of OS.

2. Materials and methods

2.1. Chemicals and reagents

Chromatographic grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). Ultrapure water was prepared using a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA). The following compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA): kynurenic acid, L-lysine, L-phenylalanine, L-tyrosine, L-tryptophan, taurine, and citrate. Glyoxylic acid was obtained from TCI (Tokyo, Japan). All other chemicals were of analytical grade. The Cystone (Himalaya®, India) powders were dissolved in saline to obtain a concentration of 72 mg/ml.

2.2. Preparation and quality control of OS extract

OS was purchased from Anguo (Hebei, China) and authenticated by Prof. Lian-na Sun (School of Pharmacy, Second Military Medical University, Shanghai, China). A voucher specimen was deposited in the Chinese *Material Medica* specimen room of the Pharmacy School, Second Military Medical University. The whole dried OS plant was ground to powder by a disintegrator. Next, the powder (200 g) was extracted with 1000 ml of 75% ethanol (v/v) for 2 h by an ultrasonic method at room temperature. The extraction solution was filtrated and concentrated using a rotary evaporator at 60 °C until dry. The yield of ethanol extract was 9.6%, as calculated by final weight. The residue was dissolved in saline to obtain the concentration of 50 mg/ml for the following experiments.

To test the reproducibility of OS extraction, six OS metabolites, including isosinensetin, eupatorin, salvigenin, caffeic acid, rosmarinic acid and ursolic acid, were measured from the final products by UHPLC-Q-TOF mass spectrometry. The relative standard derivation (RSD) values of the peak areas of each component were less than 5% in six sets of experiments, indicating that the OS extraction is reproducible. Detailed methodology is listed in Supplemental materials section.

2.3. Animal experiment and sample collection

All animal studies were performed in accordance with the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Ethical Committee for the Experimental Use of Animals at Second Military Medical University (Shanghai, China). Thirty six wild-type male C57B/L6 mice at seven to eight weeks old were purchased from the Shanghai SLAC laboratory Animal Co., Ltd. After conditional housing for one week, these mice were randomly divided into Saline, Crystal, Cystone and three doses of OS groups with 6 mice in each group. To establish the crystal renal injury model, all mice excluding the saline control group were i.p. injected with glyoxylate at a dosage of 100 mg/kg once daily for six days. Four hours after each glyoxylate injection, the mice in the OS groups (total 18 mice and 6 mice for each dosage) were i.g. administrated with OS extract at a dosage of 0.5 g/kg,1 g/kg, and 2 g/kg. The mice in the Cystone group were i.g. administrated with Cystone at a dosage of 1.2 g/kg, which served as a positive reference control (Patel et al., 2012), while mice from the Saline and Crystal groups were given saline.

After the last gavages, all mice were placed into metabolic cages for 24 h to collect urine samples. At the end of the study, blood samples were collected by retro-orbital puncture and kidneys were harvested after in situ cardio-perfusion and fixed in formalin for further analysis. After clotting at 4 °C for 2 h, the venous blood was centrifuged at 4000 rpm for 5 min; the sera were harvested for biochemical analysis. All urine and sera samples were immediately stored at −80 °C prior to analysis.

2.4. Histological and biochemistry analysis

The kidneys were fixed in 10% buffered formalin and paraffin-embedded. Sections 3 μm thick were used to conduct von Kossa staining according to the instructions of the commercial kit (Jiemei Gene, Shanghai, China). Five random light microscope images of von Kossa staining were collected. Thirty views were gathered from each group for semi-quantitative examination by image analysis software ipp6.0 (Media Cybernetics, Washington, USA).

The levels of calcium, phosphate and creatinine in urine, the levels of serum creatinine (Scr), and blood urea nitrogen (BUN) were measured by a BC-2800Vet animal auto biochemistry analyzer (Shihai, Guangdong, China).

2.5. Sample preparation

Prior to analysis, a 100 μl aliquot of urine sample was thawed at 4 °C followed by the addition of 300 μl of acetonitrile to precipitate the proteins. The resulting solution mixture was spun at 13,000 rpm for 15 min at 4 °C. The clear supernatant (150 μl) was transferred to a sampling vial for UHPLC–MS analysis. A QC sample was prepared by pooling aliquots from all urine samples collected in the course of the study.

2.6. UHPLC-Q-TOF/MS profiling analysis

UHPLC-Q-TOF/MS analysis was performed on an Agilent 1290 Infinity LC system equipped with an Agilent 6530 Accurate Mass Quadrupole Time-of-Flight mass spectrometer (Agilent, USA). Chromatographic separations were performed at 40 °C on an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters, Milford, MA). The mobile phase consisted of 0.1% formic acid (A) and ACN modified with 0.1% formic acid (B). The optimized UPLC elution conditions were 0–2 min, 5% B; 2–10 min, 5–15% B; 10–14 min, 15–30% B; 14–17 min, 30–95% B; 17–19 min, 95% B, and the posttime was 6 min for equilibrating the system. The flow rate was set to 0.4 ml/min and the injection volume was 2 μl. The autosampler was maintained at 4 °C.

An electrospray ionization source (ESI) was operated in both positive and negative modes. The optimized conditions were as follows: capillary voltage, 4 kV in positive mode and 3.5 kV in negative mode; drying gas flow, 11 l/min; gas temperature, 350 °C; nebulizer pressure, 45 psig; fragment voltage, 120 V; skimmer voltage, 60 V. The mass spectrum was collected in profile mode

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