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Urinary metabonomic study of the surface layer of *Poria cocos* as an effective treatment for chronic renal injury in rats

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ABSTRACT

Ethnopharmacological relevance: *Poria cocos* Wolf (Polyporaceae) is a well-known medicinal fungus. The epidermis of the sclerotia ("Fu-Ling-Pi" in Chinese) is used as a diuretic and traditionally used for promoting urination and reduce edema.
Aim of the study: Traditional Chinese medicines (TCM) treat many diseases through multi-components, multi-ways and multi-targets. However, the molecular mechanisms of TCM are not yet well understood. In the present work, ultra performance liquid chromatography-based metabonomics analysis was applied to investigate the urinary metabolite profiling of the renoprotective effect of FLP on adenine-induced chronic kidney disease (CKD) rat model and involved possible mechanism.
Material and methods: A metabonomic approach based on ultra performance liquid chromatography coupled with quadrupole time-of-flight high-sensitivity mass spectrometry and a novel mass spectrometry$^{Elevated\ Energy}$ data collection technique was developed. The resulting dataset was analyzed by principal component analysis and partial least squares discriminant analysis. The identification of all potential biomarkers was performed using reference standard by comparing their mass spectra, MS^E fragments information, isotopic pattern and MassLynx i-FIT algorithm.
Results: By partial least squares-discriminate analysis, 15 biomarkers in rat urine were identified and 11 of them were related to the pathway of adenine metabolism and amino acid metabolism. Among these biomarkers, eight biomarkers like adenine, L-acetylcarnitine, 8-hydroxyadenine, hypoxanthine, creatine, methionine, phytosphingosine and phenylalanine were reversed to the control level in FLP-treated group and six biomarkers like 2,8-dihydroxyadenine, indole-3-carboxylic acid, 3-methyldioxyindole, ethyl-N_2-acetyl-L-argininate, 3-*O*-methyldopa and xanthurenic acid were reversed to high control group by FLP, which indicates that the urinary metabolic pattern significantly changed after FLP treatment.
Conclusions: Our study indicates that FLP treatment can ameliorate CKD by intervening in some dominating metabolic pathways, such as adenine metabolism and amino acid metabolism. The metabonomic results not only supplied a systematic view of the development and progression of CKD and mechanism studies of FLP but also provided the theoretical basis for the prevention or treatment of CKD.

1. Introduction

Metabonomics is defined as the quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to patho-physiological stimuli or genetic modifications (Nicholson et al., 1999). Metabonomics is based on the determination of global metabolite profiles in biological fluids and tissues with subsequent data analysis via a range of multi-variate statistical approaches (Nicholson et al., 2002). As a powerful analytical platform, the application of metabonomics has dramatically

Abbreviations: FLP, Fu-Ling-Pi; CKD, chronic kidney disease; TCM, traditional Chinese medicine; MS^E, mass spectrometry$^{Elevated\ Energy}$; CRF, chronic renal failure; UPLC Q-TOF/HSMS, ultra performance liquid chromatography/quadrupole time-of-flight high-sensitivity mass spectrometry; PCA, principal component analysis; PLS-DA, partial least squares-discriminate analysis; BPI, base peak intensity.

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increased in the fields of disease diagnosis (Kaori et al., 2010; Li et al., 2010), safety and toxicity evaluation (Griffin, 2004; Chen et al., 2006), therapy and biomarker discovery (Zhao et al., 2012b, 2012f) and plant genotype discrimination (Taylor et al., 2002). Metabolic responses of living systems lead to alterations of low molecular mass metabolites, thus changing the metabolic profile, such as the presence or concentrations of metabolites.

With the development of "omics" sciences, metabonomics, as a potential translational tool, provides a promising opportunity for herb medicine study. Various studies demonstrate that traditional Chinese medicine (TCM) is playing an important role in the treatment of many complex diseases and are being accepted by more and more people (Zhang et al., 2010; Wang et al., 2011). The constitutions of TCM are highly complex, mostly unknown and varying as the practices of planting, harvesting, storage and manufacturing change. TCM's multi-components can hit multiple targets (Anonymous, 2003). Metabolomics method meets the requirements for the evaluation of multi-component TCM *in vivo* by simultaneous analysis of hundreds and thousands of variables, and establish connection between TCM's multi-components and its multi-component therapeutics (molecular pharmacology) (Wang et al., 2005). The method and designation of metabonomics is well coincident with the integrity and systemic feature of TCM. The systemic thinking and strategy of the metabonomics and its aim at grasping integral function have provided unprecedented enlightenment for the modern TCM research.

Chronic kidney disease (CKD) is becoming a worldwide public health problem. The number of patients with CKD continues to grow worldwide (Janjua and Mahan, 2011). TCMs are gaining more attention for the treatment of CKD all over the world, due to their specific theory and long historical clinical practice. *Poria cocos* Wolf (Polyporaceae) is a well-known traditional East-Asian medicinal fungus that grows around the roots of pine trees in China, Japan, Korea, and North America (Lee and Jeon, 2003). It has frequently been prescribed as one of the chief ingredients in composite prescriptions in TCM. Nearly 10% of the traditional Chinese medicinal prescriptions or prescriptions admitted to Chinese Pharmacopoeia contain *Poria cocos* (The State Pharmacopoeia Commission of PR China, 2010). It is prepared from the dried sclerotia of *Poria cocos* Wolf as Fuling in China and Hoelen in Japan. The inner parts of the sclerotia of *P. cocos*, called "Fu-Ling" in Chinese, are used to treat chronic gastritis, acute gastroenteric catarrh, gastric atony, edema, nephrosis, dizziness, nausea and emesis (Ríos, 2011).

As reported previously, the chemical constituents of Fu-Ling mainly include triterpenes, polysaccharides and steroids (Sekiya et al., 2003; Chen and Chang, 2004; Wang et al., 2004; Akihisa et al., 2007; Zheng and Yang, 2008a, 2008b; Ríos, 2011). However, the triterpenoid compounds are the main components of the epidermis ("Fu-Ling-Pi" in Chinese) of the sclerotia (Tai et al., 1993, 1995; Yang et al., 2009). The Fu-Ling-Pi (FLP) was described that it promotes urination and leaves out dampness, the problems caused by stagnance of dampness such as edema and urinary dysfunction (The State Pharmacopoeia Commission of PR China, 2010). We have previously reported the ethanol and aqueous extracts of the surface layer of *Poria cocos* possessed a diuretic activity (Zhao et al., 2012d), which is consistent with another previous report (The State Pharmacopoeia Commission of PR China, 2010).

However, the mechanism of FLP treatment on CKD is not yet clear at the biochemistry level. We applied metabonomics analysis combined with biochemical techniques and pathology examinations to explore the renoprotective effects of FLP and its molecular mechanism of action.

Mass spectrometry (MS) and nuclear magnetic resonance spectroscopy are two analytical tools commonly used in metabonomics studies (Boudonck et al, 2009). In the MS-based metabonomics, ultra performance liquid chromatography coupled with quadrupole time-of-flight MS (UPLC Q-TOF/MS) has gained more application due to the high resolution of chromatographic peaks, increased analytic speed and sensitivity for complex mixtures (Jia et al, 2008; Zhao et al., 2012c, 2012e). In our previous study, a metabonomic approach based on UPLC Q-TOF high-sensitivity MS (UPLC Q-TOF/HSMS) was developed to characterize the metabolic profile associated with adenine-induced chronic renal failure (CRF) and demonstrated that the utility of metabolic profiling combined with multivariate analysis was a powerful tool to investigate CRF pathogenesis (Zhao et al., 2012a, 2012c, 2012e, 2013b). In the current study, metabonomics study based on UPLC Q-TOF/HSMS and a novel mass spectrometry$^{\text{Elevated Energy}}$ (MS^E) data collection technique was applied to investigate the urinary metabolite profiling of the renoprotective effect of FLP on adenine-induced CKD rat model and involved possible mechanism. Potential biomarkers related with CKD were identified, and their metabolic pathways were also discussed. This research proved that MS^E can simultaneous acquire precursor ion information and fragment ion data at high and low collision energy in one analytical run, which facilitated the fast structural characterization of metabolites. Metabonomics could be a promising scientific platform for therapeutic evaluation and action mechanism study of TCM.

2. Experimental

2.1. Chemicals and reagents

Adenine (batch No.: A8626, Purity 99.0%) and formic acid solution (ref. BCBB6918, purity 50%) were purchased from Sigma Chemical Co., Ltd (Sigma Corp., St. Louis, MO, USA). Creatinine (batch No.: 100877-200901, Purity 99.8%) was obtained from the National Institutes for Food and Drug Control (Beijing, China). LC-grade methanol and acetonitrile were purchased from the Baker Company (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Ultra high purity water was prepared using a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA). Other chemicals were of analytical grade and their purity was above 99.5%.

2.2. Preparation of ethanol of FLP

FLP was collected from Shaanxi Province in March 2012, and was identified by Prof. Y.Z. Wang (College of Life Sciences, Northwest University, Xi'an, Shaanxi, People's Republic of China). FLP was dried according to the method of Chinese Pharmacopoeia (The State Pharmacopoeia Commission of PR China, 2010). A voucher specimen (120304) was deposited at Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, Northwest University, Xi'an, Shaanxi. FLP was ground to powder (about 20 meshes) by a disintegrator, and the powder (2 kg) was extracted three with 15 L 95% ethanol for 0.5 h by ultrasonic method (Yang et al., 2009). The extracts were combined together and filtrated, and then the filtrate was concentrated in vacuum using a rotary evaporator to give dried powder.

2.3. Animals and sample collection

The study was conducted in accordance with the Regulations of Experimental Animal Administration, which was issued by the State Committee of Science and Technology of the People's Republic of China and ethical approval reference number (No. SYXK 2010-004). All procedures and care of the rats were in accordance with the institutional guidelines for animal use in research. Male Sprague-Dawley rats were obtained from the Central Animal Breeding House of Fourth Military Medical