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Metabonomic study on the anti-osteoporosis effect of *Rhizoma Drynariae* and its action mechanism using ultra-performance liquid chromatography–tandem mass spectrometry

Xinyu Liu, Shangshang Zhang, Xiumei Lu, Shuning Zheng, Famei Li, Zhili Xiong*

School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China

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ABSTRACT

Ethnopharmacological relevance: Rhizoma Drynariae (RD) is an effectively traditional Chinese medicine which is usually used in treating osteoporosis, bone fracture, streptomycin ototoxicity and hyperlipemia. Up to now, studies on pharmacological mechanism of RD mostly focus on cell and gene level, little is known about its metabonomics study. The aim of this study is to establish the rats plasma metabonomic profiles of control, model and treatment group, then to investigate the anti-osteoporosis effect of RD and its action mechanism.

Method: A total of 21 Wistar rats was divided into three groups: control group, model group and treatment group. The model and treatment rats were injected prednisolone for 12 weeks, at the same time the treatment rats were orally administered RD extract at a therapeutic dose (10 g/kg, expressed as the weight of raw material) once daily throughout the experimental period, control group and model group were orally gavaged approximately volume normal saline solution. After 12 weeks, all plasma samples of three groups were collected and their metabolic profiling changes were analyzed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). The resulting dataset was analyzed by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The identification of all potential biomarkers was performed using reference standard by comparing their mass spectra, MS/MS fragmentation and retention time. Furthermore, clinical biochemistry and biomechanics study were also carried out to ensure the success of the osteoporosis model and to investigate the anti-osteoporosis effect of RD.

Results: Obvious separation trend between control and model group was found in PCA score plot, the anti-osteoporosis effect of RD can be indicated in PLS-DA score plot among these three groups. Six potential metabolite biomarkers, Lysophosphatidylcholines (C16:0 LPC, C18:0 LPC, C18:1 LPC and C18:2 LPC), tryptophane and phenylalanine, which were proved to be related with osteoporosis, were identified in the rats plasma. Compared with control group, level of all biomarkers increased significantly in model group, while that was much closer to normal in treatment group.

Conclusion: The anti-osteoporosis effect of RD has been reliably confirmed by the metabonomics method. The osteoporosis might be prevented by RD via intervening antioxidant–oxidation balance, tryptophane metabolism and phenylalanine metabolism in vivo in rats.

1. Introduction

Osteoporosis is a metabolic bone disease characterized by low bone mass, impaired micro-architecture and susceptibility to fracture. One reason for osteoporosis is long-term glucocorticoid therapy, and glucocorticoids are considered to have the most detrimental effect on bone tissue metabolism. It is a classical method to establish the osteoporosis model via injecting a high dose of glucocorticoid to rats.

Rhizoma Drynariae (RD), the dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. (Gu-Sui-Bu in Chinese), has been widely used as a kidney-tonifying and anti-osteoporosis herb for the treatment of

Abbreviations: ALP, alkaline phosphatase; BGP, bone-γ-carboxyglutamic acid protein; BMD, bone mineral density; BPI, base peak intensity; ESI, electrospray ionization source; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; RD, *Rhizoma Drynariae*; RE, relative error; ROS, reactive oxygen species; RSD, relative standard deviations; TCM, traditional Chinese medicine; TIC, total ion current.

* Corresponding author at: School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China. Tel.: +86 24 23986290; fax: +86 24 23986289.
E-mail address: bearry200@126.com (Z. Xiong).

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nephrasthenia syndrome (Du and Meng, 2004), osteoporosis (Ma et al., 1992; Deng et al., 2007; Zhang et al., 2008) and bone fracture (Wong et al., 2007) for thousands of years in China. Furthermore, previous studies have shown that it also has anti-inflammatory (Anuja et al., 2010), prevention for ototoxicity of aminoglycoside antibiotics treatment (Huang et al., 2009), antiatherosclerosis (Chen and Guan, 2006) effects. Until now, studies on the pharmacological mechanism of RD mostly focus on cell and gene level (Jeong et al., 2004; Zhang et al., 2009). However, little is known about its metabonomics study.

Metabonomics is a quantitative approach to study "the dynamic metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999), which based on the analysis of entire pattern of low molecular weight compounds rather than focusing on individual metabolite. This research strategy is well coincident with the integrity and systemic feature of TCM. Recently, it has been increasingly used as a versatile tool for assessing therapeutic effects and toxic effects of many herbal TCM and TCM prescriptions (Gu et al., 2010; Ma et al., 2010; Sun et al., 2010; Lu et al., 2011). A number of analytical tools have been currently employed including ^{1}H NMR spectroscopy (Zhao et al., 2010), LC/MS (Lai et al., 2009), CE/MS (Balderas et al., 2010) and GC/MS (Xu et al., 2010). UPLC/MS technology, which leads to better chromatographic peak resolution, considerable shorter analysis time and higher sensitivity compared with LC/MS, has been considered to have a bright future in the research of metabonomics (Bruce et al., 2008; Gika et al., 2008; Chen et al., 2009).

In the present work, metabonomics study based on UPLC–MS/MS was applied to investigate the plasma metabolite profiling of osteoporosis rats induced by prednisolone, the anti-osteoporosis effect of RD and its action mechanism for the first time. Potential biomarkers related with osteoporosis were identified, and their metabolic pathways were also discussed. Furthermore, clinical biochemistry and biomechanics study were also carried out to ensure the success of the osteoporosis model and to investigate the anti-osteoporosis effect of RD. Therefore, metabonomics, as a new "omics" science, could be a promising scientific platform for therapeutic evaluation and action mechanism study of TCM.

2. Experimental

2.1. Materials and reagents

RD (collected in Yunnan, China) was purchased from Weikang Drug Store (Shenyang, China), and authenticated as the dried rhizome of *Drynaria fortunei* (Kunze) J.Sm. by Professor Jincai Lu of Traditional Chinese Medicine College, Shenyang Pharmaceutical University. Prednisolone crude drug (purity, 98.81%) was purchased from Wuhan Shunda Fine Chemical Co., Ltd. (Wuhan, China).

Acetonitrile of HPLC grade was purchased from Tedia (Fairfield, OH, USA). Formic acid of HPLC grade was supplied by Dikma Crop. (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use. The reference standards of phenylalanine, tryptophane and lysophosphatidylcholines (C16:0 LPC, C18:0 LPC, C18:1 LPC and C18:2 LPC) were supplied by Sigma Corporation (St. Louis, MO, USA).

The Kits used in the experiment were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Preparation of ethanol extract of Rhizoma Drynariae

RD, the content of naringin (pharmacological active component, 0.74%) was determined referring to the authentic method (Pharmacopeia of the People's Republic of China, 2010).

Powdered RD (100 g) was extracted with 75% ethanol (1200 mL) under thermal reflux for 1.5 h and then filtered. The extraction was repeated three times. The extracted solution was combined and ethanol was removed under reduced pressure. The residue was dissolved in 0.5% sodium carboxylmethyl cellulose to give an extract with a concentration of 1.5 g/mL (expressed as the weight of raw materials).

2.3. Animals and treatment

Animal experiment was carried out in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University (Shenyang, China) and the animal study was approved by the Animal Ethics Committee of the Institution. A total of 21 Wistar rats (200–250 g, animal licence No. SCXK-(military) 2007-004) was obtained from Experimental Animal Center of Shenyang Pharmaceutical University (China). All rats were kept under standard animal conditions with regulated temperature (17–25 °C), humidity (45–60%) and 12 h/12 h light/dark cycle. Free access to food and drinking water was guaranteed throughout the study period. All animals were allowed to acclimatize for 7 days prior to treatment. After that they were separated randomly into three groups (n = 7/group) as follows: model group, prednisolone at the dose of 15 mg/kg was injected intramuscularly to the rats twice every week for 12 weeks; treatment group was injected prednisolone at the same dose as the model group, and they were orally gavaged ethanol extract of RD at a dose of 10 g/kg every day for 12 week continuously; control group and model group were orally gavaged approximately volume normal saline solution during experimental period.

2.4. Collection and preparation of plasma sample

Blood was collected from the suborbital vein at 24th h after oral gavaged of RD extract at 12th week. Blood samples were collected into heparinized tubes and immediately centrifuged at 13,000 rpm for 10 min. The plasma was transferred into clean tube and stored at −80 °C until analysis.

Each 200 μL aliquot of plasma sample was mixed with 400 μL of acetonitrile and vortexed to precipitate the proteins. After centrifugation at 13,000 rpm for 10 min, the supernatant was transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dried residue was then reconstituted in 100 μL of acetonitrile–water (10:90, v/v). After vortexed for 30 s, the content was transferred to 2 mL glass vials and an aliquot of 5 μL was injected for UPLC–MS/MS analysis.

2.5. Clinical biochemistry and biomechanics study

The content of alkaline phosphatase (ALP) in plasma was analyzed on an Automatic Clinical Chemistry Analyzer (Hitachi, Japan) and the content of bone-γ-carboxyglutamic acid protein (BGP) was analyzed on an Automatic Chemical Luminescence Immunoassay Analyzer (Siemens, Germany).

After treated for 12 weeks, all rats were executed for bones. The wet bone degreased by chloroform-methanol (2:1, v/v) for 48 h, then stoving for 6 h at 120 °C. The dry bone was ashed in Muffle Furnace for 5 h at 900 °C. The fracture force of rats' dry tibia was tested on Fracture Instrument. Changes on bone mineral density (BMD) at rats' femur and proximal femur were monitored with Dual-energy X-ray Bone Density Screening (NORLAND Corporation, USA).

2.6. Protein oxidation level study

The protein concentration in plasma and the carbonyl content of protein were determined by biuret method and DNPH