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# Serum metabolomics study of Traditional Chinese medicine formula intervention to polycystic ovary syndrome

Caixia Lu[a,1], Xinjie Zhao[b,*,1], Yan Li[a], Yanjie Li[b], Chengkun Yuan[a], Fang Xu[a], Xiaoyu Meng[a], Lihui Hou[a,**], Guowang Xu[b]

[a] Department Gynaecological and Obstetrical of First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin 150040, China
[b] Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a most common, heterogeneous, complex endocrinopathy disease. Traditional Chinese medicine (TCM) has been used in the treatment of PCOS for many years. However, the mechanism underlying TCM remains obscure and challenging. In this study, 30 PCOS subjects were separated into normoinsulinemic group (NI = 13) and hyperinsulinemic group (HI = 17), and treated for three menstrual cycles with TCM Formula, Bushen Huatan Formula (BHF). A metabolomics approach based on ultra-high-performance liquid chromatography (UPLC) coupled with linear ion trap Orbi-trap mass spectrometer (LTQ Orbi-trap MS) is used to investigate serum metabolic changes of TCM intervention to PCOS. After BHF intervention for three menstrual cycles, the serum levels of glycerophosphorylethanolamine (GPEA), creatine, creatinine decreased in both NI and HI groups. Furthermore, in NI group, the main manifestation was the changes of phospholipid metabolism. While in HI group, lysine, phenol sulfate, phe–phe etc. decreased, and ornithine, proline, betaine, acetylcholine etc. increased. Combined with clinical biochemical data, BHF was proved effective to PCOS by reducing the inflammatory reaction and oxidative stress. This study also illustrates that the LC–MS based metabolomic approach is a helpful tool to evaluate curative effect and to understand the mechanisms of TCM.



## 1. Introduction

Polycystic ovary syndrome (PCOS) is a most common, heterogeneous, complex endocrinopathy disease, which affect 5–10% of reproductive age females [1]. As an intractable disorder, the mechanism of PCOS is complicated. Insulin resistance and hyperinsulinemia play a critical role in pathogenesis of PCOS. Approximately 50–70% of women with PCOS have insulin resistance, independently of obese [2]. Insulin resistance is also linked to a higher risk of cardiovascular disease and diabetes mellitus in PCOS patients [3]. It is necessary for PCOS patient to handle reproductive dysfunction and insulin resistance. TCM has been used in the treatment of PCOS for many years, and lots of studies have been published to indicate the effectiveness to PCOS, and improved reproductive and endocrine metabolic disorders [4]. However, the mechanism underlying Chinese medicine remains obscure.

Metabolomics, as a member of omics family, unbiasedly and integratedly, quantify and identify small metabolite molecules in a biological system [5]. Owing to the dynamic cornerstone, metabolomics provides novel insights on what has happened in organism [6]. Metabolomics approaches have been used to investigate pathogenesis and to find potential diagnostic markers of PCOS [7,8]. In our previous research, we found the increase in serum levels of unsaturated free fatty acids, sulfated steroids, glycated amino acid and the decreased serum levels of lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs) in PCOS subjects [8]. Metabolomics approaches were also used to investigate composition and curative mechanism of Chinese medicine [9], although the metabolomics study of the TCM treatment on PCOS is still absent.

Bushen Huatan Formula (BHF), a TCM formula, consists of Astragali Radix, Poriacocos Wolf, Atractylodeslancea, Radix Salvia miltiorrhiza, Rhizoma Coptidis and Herba Epimedium. BHF was reported to ameliorate insulin resistance, and also to improve the

* Corresponding author. Fax: +86 411 84379559.
** Corresponding author. Fax: +86 451 82119168.
*E-mail addresses:* xj_zhao1@126.com (X. Zhao), houlihui2007@sina.com (L. Hou).
[1] These two authors contributed equally to this work.

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function of ovarian ovulation in PCOS patients [10]. In this study, after three menstrual cycles of treatment with BHF the serum metabolic changes of PCOS patients were investigated by using metabolomics approach based on UPLC coupled with LTQ Orbi-trap MS.

## 2. Methods

### 2.1. Patients

The study was conformed to the Helsinki Declaration on human experimentation approved by the Ethics Committee of Heilongjiang University of Chinese Medicine (HZYLL2013KT00201). All patients were signed informed consent. The PCOS patients were recruited from June, 2011 to February, 2014 in clinic of Heilongjiang University of Chinese Medicine. All subjects had age between 18 and 35 year, 2 years of menstrual history and BMI $\geq$23 kg/m$^2$, and all subjects were diagnosed according to modified Rotterdam criteria [11]: (i) oligomenorrhea or amenorrhea; (ii) ovarian presence of $\geq$12 antral follicles ($\leq$9 mm) and/or the volume >10 mL on transvaginal scanning; and (iii) and/or clinical/biochemical hyperandrogenism. Exclusion criteria are as follows: (i) subjects known to accept any intervention affecting reproductive or metabolism function during three months, namely oral contraceptive drugs, antiandrogens, GnRH agonists and antagonist gonadotropins, anti-obesity medications, insulin-sensitizing agents and Chinese herbal medicines; (ii) subjects with other endocrine dysfunctions including Cushing's Syndrome, 21-hydroxylase deficiency, hyperprolactinemia, thyroid disorders and diabetes; and (iii) subjects with severe absence of heart, liver or kidney dysfunction and mental illness. A total 30 patients were included in the study. And the subjects were separated into 2 groups, normoinsulinemic group (NI = 13) and hyperinsulinemic group (HI = 17) according to the fasting and 2-h insulin levels after the load of 75 g glucose [12].

All 30 patients were treated with BHF for three menstrual cycles. BHF were produced by Jiang Yin pharmaceutical company (Jiang Yin, China) to confirm the concordance of intervention. Fasting blood samples were collected at day 3 of menstrual period before treatment and the end of treatment. All serum samples were stored at −80 °C until analysis.

### 2.2. Clinical biochemical analysis

Steroid hormones including luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), prolactin (PRL), testosterone (T), sulfated-dehydroepiandrosterone (DHEAS), androstenedione (AND), and sex hormone binding globulin (SHBG) were measured with radio-immunoassay (Abbott Laboratories, North Chicago, IL, USA). Other parameters including fasting plasma glucose (FPG), fasting insulin (FIN), plasma glucose and insulin at 30 min, 60 min, 90 min, 120 min, 180 min during the OGTT, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB) and lipoprotein (Lpa) were assessed with chemiluminescence (Hitachi, Ltd., Tokyo, Japan). Homeostasis model of assessment for insulin resistence index (HOMA-IR) = fasting serum insulin $\times$ fasting serum glucose/22.5. Quantitative insulin sensitivity check index (QUICK) I = 1/(log (fasting serum insulin) + log(fasting serum glucose)).

### 2.3. Metabolomics analysis

After thawed at 4 °C, each 150 μL serum was deproteinized with 4 fold of acetonitrile with 11 internal standards in volume, then the supernatant was divided into two equal aliquots and dried in a vacuum centrifuge. 11 internal standards were choline-d4, carnitine C2: 0-d3, carnitine C10: 0-d3, carnitine C16: 0-d3, cholic acid-d4, chenodeoxycholic acid-d4, LPC 19: 0, phenylalanie-d5, tryptophan-d5, Palmitic acid-d3 and stearic acid-d3 (Sigma–Aldrich, St. Louis, MO, USA).

ACQUITY UPLC system (Waters Corporation Milford, MA, USA) coupled with LTQ Orbi-trap MS (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the metabolic profiling in both ESI positive and ESI negative ion modes. In positive ion mode, the separation of metabolites was conducted on a 2.1 $\times$ 100 mm ACQUITYTM 1.7 μM BEH C8 column, and the mobile phase contained water with 0.1% formic acid (A) and acetonitrile (B). The linear elution gradient program was used as follows: 5% B kept 1.0 min, then linearly increased to 100% B at 24 min, and held for 4 min. In negative ion mode, the metabolite separation was performed on 2.1 $\times$ 100 mm ACQUITY$^{TM}$ 1.8 μm HSS T3 column, and the mobile phase contained 6.5 mM ammonium bicarbonate water solution (C) and 6.5 mM ammonium bicarbonate in 95% methanol and water (D). The linear elution gradient program was 2% D kept 1.0 min, then linearly increased to 100% D at 18 min, and held for 4 min. The flow rate was 350 μL/min and column temperature was 50 °C. The injection volume was 5 μL.

Mass spectrometry detections were set as the following: capillary temperature 325 °C, source voltage 4.5 kV and −4.0 kV for positive ion mode and negative ion mode, respectively. The mass scan range was from 80 to 1000. The resolution of the MS was set to 15,000.

The metabolites were identified by accurate mass, MS/MS ion fragment pattern and retention time of LC, and then were validated by available standard.

### 2.4. Data analysis

Metabolomics data were acquired using the SIEVE 1.2 version workstation (Thermo Fisher Scientific, Waltham, MA, USA). After removing zero value with 80% rule [13], all ions were calibrated with a suitable internal standard. Then the metabolomics data were analyzed with the SIMCA-P 11.0 version; software (Umetrics, Umea, Sweden) for multivariate statistics. Principal component analysis (PCA) and orthogonal signal correction (OSC) partial least-squares-discriminant analysis (PLS-DA) were performed after the Pareto scaling (mean centering and scaled to square root of variance). Univariate analysis of the clinical and metabolomics data was performed with SPSS 17.0 version software (SPSS Inc., Chicago, USA). Paired Wilcoxon Mann–Whitney test was used to detect the differences before and after intervention. Wilcoxon Mann–Whitney test is carried out for the difference between NI and HI group before intervention. The statistical significance is set at $p < 0.05$.

## 3. Results and discussion

### 3.1. Comparison of clinical characteristics

BHF, as an active TCM formula, active ingredients including astragalus polysaccharides, astragaloside IV, berberine, etc. has been demonstrated to be effective in phlegmatic hygrosis (obesity) PCOS patients based on TCM theory, to ameliorate insulin resistance [14], and also to improve the function of ovarian ovulation in PCOS patients [10]. In our study, after BHF intervention for three menstrual cycles, body weight, Body Mass Index (BMI), waist circumference and Hip circumference decreased significantly compared to before intervention. Acanthosisnigrican score, FPG, HOMA-IR, LDL-C, ApoB and Apob/ApoA1 also decreased. The level