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An integrative investigation of the therapeutic mechanism of *Ainsliaea fragrans* Champ. in cervicitis using liquid chromatography tandem mass spectrometry based on a rat plasma metabolomics strategy

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ABSTRACT

Cervicitis is an extremely common gynecological disease and can be induced by diverse factors such as Neisseria gonorrhoeae, Chlamydia trachomatis, and Mycoplasma genitalium infections. Long-term unhealed cervicitis may lead to a series of diseases including endometritis, salpingitis, pelvic inflammatory disease, and chorioamnionitis. However, the pathogenesis of cervicitis remains unknown. *Ainsliaea fragrans* Champ. (AFC) has been widely used in clinical treatment of cervicitis. In the present study, we performed an integrative investigation involving histopathology analysis and non-target plasma metabolomics analysis in a cervicitis rat model induced by phenol mucilage, using ultra-performance liquid chromatography coupled with a tandem quadrupole time-of-flight mass spectrometry approach. Based on the integrative investigation, marked metabolomic differences were identified between the cervicitis and control groups using multivariate analysis. As a result, 32 potential biomarkers were identified in the response to cervicitis, and were involved in arachidonic acid metabolism, linoleic acid metabolism, primary bile acid biosynthesis, taurine and hypotaurine metabolism, pantothenate and CoA biosynthesis, and glycerophospholipid metabolism. After treatment, a total of 27 potential biomarkers exhibited altered levels in the AFC group compared to the model group, and 12 metabolites including 1-stearoylglycerophosphoinositol, bolasterone, lysoPC(16:0), lysoPC(20:4), lysoPC(P-16:0), lysoPC(P-18:0), lysoPC(P-18:1), stearoylcarnitine, taurine, lysoPC(17:0), 20-hydroxyeicosatetraenoic acid, and 1-arachidonoylglycerophosphoinositol returned to their normal levels. This study suggested that the therapeutic mechanism of AFC is related to those altered endogenous metabolites.

1. Introduction

Cervicitis is a commonly found disease in adult women and can seriously affect their health. Cervicitis is usually induced by diverse factors such as Neisseria gonorrhoeae, Chlamydia trachomatis [1,2], and Mycoplasma genitalium [3–6] infections. Long-term unhealed cervicitis may lead to various of diseases including endometritis, salpingitis, pelvic inflammatory disease, and chorioamnionitis [7]. Furthermore, it can increase human immunodeficiency virus (HIV) acquisition and transmission [8]. Recent studies have indicated that cervicitis may also be involved in the progression of cervical cancer [9,10].

Ainsliaea fragrans Champ. (AFC), a traditional Chinese herb, has been widely used in clinical practice. Currently, AFC is applied as a very important ingredient in preparations of the 'Xingxiang Tuerfeng tablet' and 'compound Xingxiang Tuerfeng granule', which are common over-the-counter (OTC) medicines [11]. The entire plant has been used for treating gynecological inflammation, such as cervicitis and endometritis, with evident clinical effects [12]. At present, Ouyang, et al. have demonstrated that chlorogenic acids are major bioactive constituents of AFC [13]. Su D, et al. have provided the pharmacokinetic profiles and the tissue regional distribution of mono-caffeoylquinic acids (MCQAs), di-caffeoylquinic acids (DCQAs), and caffeic acid [14]. Chen X, et al. have validated

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that AFC was effective for the treatment of gynecological diseases [15]. However, there is little evidence on how AFC works in clinical treatment, and its underlying therapeutic mechanism remains unknown. Our current study aims to address this question, in an effort to improve clinical treatment of the disease.

In this study, we used metabolomics which can be a powerful tool in establishing a comprehensive metabolic profile of endogenous low-molecular-weight metabolites in a biological organism [16,17]. Metabolomics, has been utilized as an effective and precise tool in disease diagnosis, toxicity evaluation, and biomarker discovery [18]. Nowadays, advanced instrumental platforms, such as liquid chromatography tandem mass spectrometry (LC–MS), nuclear magnetic resonance spectroscopy (NMR), and gas chromatography-mass spectrometry (GC–MS), are frequently utilized to perform the entire metabolic profile. Meanwhile, ultra-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS) has been shown to provide a rapid, accurate, and high throughput analysis for metabolomics [19]. Here, a plasma metabolomics study based on UPLC-QTOF-MS was performed to reveal the mechanism of AFC in the treatment of rat cervicitis for the very first time.

2. Experimental

2.1. Reagents and materials

Deionized water was purchased from Watsons (Hong Kong, China). Methanol and acetonitrile (analytical gradient grade) for liquid chromatography were obtained from Merck (Darmstadt, Germany). HPLC grade formic acid was purchased from Tianjin Kermel Chemical Reagent Company (Tianjin, China). The reference standard of 2-Chloro-L-phenylalanine was used as an internal standard (IS), and was provided by Shanghai Macklin Biochemical Company (Shanghai, China). Its purity was greater than 98%. Phenol was obtained from Sinopharm Chemical Reagent Co., Ltd. Hematoxylin staining and eosin staining solution was purchased from Boster Biological Technology Co., Ltd. The standards for mass calibration (APCI Positive Calibration Solution, APCI Negative Calibration Solution) were purchased from AB Sciex Pte. Ltd.

2.2. Preparation of extract

The raw material of the AFC plant was purchased from herbal medicine market located in Zhangshu city and identified by Vice Director of Pharmacists Bei Wu, Nanchang Institute for Food and Drug Control. AFC plant (400 g) was extracted twice (1 h at a time) with a boiled methanol: water solution (70:30, *v/v*). The decoction was then merged together, filtered, and dried using a water bath at 65 °C to obtain the extract powder. The extract powder was dissolved with 888 mL water to obtain the extract solution which contains crude drug of 0.45 g/mL.

2.3. Animal handling and treatment

Specific pathogen free (SPF) Sprague–Dawley rats (female, 180–220 g) were supplied by Hunan Silaikejingda Laboratory Animal Co., Ltd. (Changsha, PR China). All the rats were housed for 7 days under standard laboratory conditions (22 ± 2 °C, RH 50 ± 20%, and natural light-dark cycle) prior to the experiment. Afterwards, 80 rats were randomly divided into 4 groups (control group, sham group, model group, and AFC group). Before model creation, the AFC group rats were given AFC extract for 3 days by oral administration, and the AFC dose was 4.5 g/kg/d body weight. At the same time, the other groups were given physiological saline by oral administration. The model and AFC group rats were then induced with cervicitis as a result of 0.2 mL of 25% hydroxybenzene mucilage [20,21]. Meanwhile, the sham group underwent similar modeling using physiological saline. The model establishment of the model group rats and AFC group rats was sustained for 5 days. During the process of modeling, the AFC group was given the AFC extract treatment, and the other groups were given physiological saline. Animal welfare and experimental procedures strictly complied with the laws and guidelines, and every effort was made to reduce the pain of the rats. All animal experiments were approved by The Animal Ethics Association of Jiangxi University of Traditional Chinese Medicine.

2.4. Histopathological analysis

At the end of the experiment, cervix tissues were taken from the rats of each group, and fixed in formalin: saline solution (10:90, *v/v*). The fixed tissues were then embedded in paraffin. Sections (thickness, 5 μm) were cut and stained with hematoxylin and eosin (HE). Histopathological analysis of each group was then performed by examination under an Olympus BX43 microscope (Olympus Corporation, Japan).

2.5. Plasma sample collection and preparation

After 24 h of the last AFC administration, eye orbital venous blood was collected from all groups. The plasma samples were obtained by centrifugation (4 °C, 4500 r/min) for 10 min and stored at −80 °C until preparation.

A working internal standard (IS) solution of 2-chloro-L-phenylalanine (5.24 μg/mL) was prepared in methanol. Plasma samples (50 μL) were added into 200 μL of the working IS solution, the mixture was vortexed for 3 min for sedimentation of the protein. Finally, the supernatant was extracted after centrifugation at 15000 rpm, 4 °C for 10 min and stored at −20 °C. A total of 800 μL rat plasma from each group (aliquots of 10 μL plasma from each sample) was added to 3200 μL of the working IS solution to create a quality control (QC) sample for method validation.

2.6. Chromatography and mass spectrometry

Chromatographic separation of plasma samples was performed on a ACQUTITY UPLC™ HSS T3 column (100 mm × 2.1 mm, 1.7 μm; Waters Corp.) using an ACQUITY H-CLASS instrument which was equipped with an automatic degasser, a quaternary pump, and an autosampler. The conditions for UPLC were optimized, including column temperature (30 °C), flow rate (0.35 mL/min), injection volume (5 μL), and mobile phase [(A) 0.1% (by volume) formic acid in water and (B) acetonitrile]. The 25 min binary gradient elution conditions were optimized as follows: 0–3 min, 5–20% B; 3–5 min, 20–40% B; 5–9 min, 40–60% B; 9–16 min, 60–65% B; 16–18 min, 65–80% B; 18–21 min, 80–95% B; 21–23 min, 95–5% B; and 23–25 min, 5% B (the last two minutes were applied to allow for column re-equilibration).

UPLC-QTOF-MS/MS (5600), a hybrid triple time-of-flight mass spectrometer equipped with a Duo Spray™ ion source, was utilized to detect plasma samples in both positive and negative ionization modes with high resolution (AB SCIEX, Foster City, CA, USA). The optimized MS conditions were as follows for the positive mode: ion spray voltage floating, 4500 V; declustering potential (DP),80 V; collision gas (CE), 35 eV, collision energy spread (CES) was (±) 10 eV; turbo spray temperature (TEM),500 °C; nebulizer gas (Gas 1) of 50 psi; heater gas (Gas 2), 50 psi; curtain gas, 25 psi. Nitrogen was used as a nebulizer and auxiliary gas. Optimized conditions in the negative mode were as follows: ion spray voltage, −4500 V; ion source temperature, 500 °C; curtain gas, 25 psi; GS 1, 50 psi; GS 2, 50 psi; and DP, −100 V. In the information dependent acquisition (IDA) experiment, parameters were as follows: CE,–30 eV,