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Metabolomics research on the hepatoprotective effect of *Angelica sinensis* polysaccharides through gas chromatography–mass spectrometry

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ABSTRACT

Angelica sinensis polysaccharides (ASP) have an established hepatoprotective effect, but the mechanism for this effect remains unclear. A novel approach using biochemical parameters coupled with metabolomics based on gas chromatography–mass spectrometry (GC–MS) and chemometrics was established in this study to explain the hepatoprotective effect mechanism of ASP. The superoxide dismutase activity, malonaldehyde content, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase in plasma were measured. Pathological changes in the liver were observed. Plasma and liver homogenate obtained from mice were analyzed using GC–MS. Distinct changes in metabolite patterns in the plasma and liver homogenate after being induced by carbon tetrachloride and drug intervention were observed using principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA). Potential biomarkers were found using PLS-DA and *T*-test. The results of the pathological changes observed in the liver, the biochemical parameters in plasma, and the metabolomics of the plasma and liver homogenate all showed that liver injury was successfully reproduced, ASP exhibited hepatoprotective effect, and the medium dose of ASP exhibited the best. Nine endogenous metabolites in the liver homogenate and ten endogenous metabolites in the plasma were all considered as potential biomarkers. They were considered to be in response to hepatoprotective effects of ASP involved in the amino acids metabolism, energy metabolism, and lipids metabolism. Therefore metabolomics is a valuable tool in measuring the efficacy and mechanisms of action of traditional Chinese medicines.

1. Introduction

Angelica sinensis (AS) is the root of *A. sinensis* (Oliv.) Diels, one of the most widely used materials in traditional Chinese medicine (TCM) [1]. It is mainly distributed in Gansu province in China [2]. Pharmacological tests have revealed that AS can be used to treat irregular menstruation [3], amenorrhea, dysmenorrhea, anemia, gastrointestinal disease, cardiovascular disease, chronic bronchitis, asthma, rheumatism, hypertension, and other diseases in females [4]. Over 70 kinds of compounds have been identified from AS, including polysaccharides, essential oils, organic acids, and ester [5,6]. Among these compounds, *A. sinensis* polysaccharides (ASP) are an important group of pharmacologically active substances against liver injury [7]. Superoxide dismutase (SOD) and malonaldehyde (MDA) were the two important biochemical parameters to evaluate the hepatoprotective effect indirectly [8]. In the CCl_4-induced liver damage model, when the reactive oxygen species (ROS) in liver cells significantly increased, the SOD would be consumed excessively, and MDA accumulation would be in large quantities. Treated by ASP [9], SOD and MDA would be restored to normal. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GT)

Abbreviations: AS, *Angelica sinensis*; GC–MS, gas chromatography–mass spectrometry; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; ASP, *Angelica sinensis* polysaccharides; LASPG, low dose group of ASP; MASPG, middle dose group of ASP; HASPG, high dose group of ASP; CCl_4, carbon tetrachloride; HE, hematoxylin and eosin; MDA, malonaldehyde; SOD, superoxide dismutase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase; MSTFA, N-methy-N-(trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; VIP, variable importance in the projection; VLDL, very low density lipoproteins; NMR, nuclear magnetic resonance; LC–MS, liquid chromatography–mass spectrometry; CSV, comma-separated value; TCM, traditional Chinese medicines.

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in the plasma were the three important biochemical parameters to evaluate the hepatoprotective effect directly [10]. In the CCl_4-induced liver damage model, membrane disintegration of hepatocytes with subsequent release of ALT, AST, and γ-GT marker enzymes of hepatotoxicity, centrilobular necrosis and steatosis are some of the consequences of CCl_4-induced lipid peroxidation [11]. Treated by *Lycium barbarum* polysaccharides [12], ALT, AST, and γ-GT would be also restored to normal. So the above 5 biochemical parameters in plasma have been used to evaluate the hepatoprotective effect of polysaccharides. However, the exact hepatoprotective effect mechanism of ASP remains unknown.

Metabolomics is a new discipline in systems biology. According to the "holism" philosophy, metabolomics techniques can provide important information for TCM research [13,14]. Multifarious metabolic characteristics of normal, pathological, or drug-treated subjects can be revealed by metabolomics, and this method has been used to explore the therapeutic effect mechanism of TCMs [15,16].

Metabolomics have been studied through numerous methods. Compared with NMR and LC–MS-based metabolomics methods, GC–MS-based metabolomics [17] has numerous advantages, such as the availability of many structure databases, higher sensitivity, better ability of material separation, and easier identification of metabolites.

PCA and PLS-DA are the indispensable pattern recognition methods in the metabolomics research. PCA is an unsupervised multivariable statistical method. It is firstly carried out to investigate whether two groups can be separated and to find out their metabolic distinction. PLS-DA is a supervised multivariable statistical method. It is used to sharpen an already established (weak) separation between groups of observations plotted in PCA [18,19].

In this work, the hepatoprotective effects of ASP are studied by evaluating superoxide dismutase (SOD) activity, malonaldehyde (MDA) content, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GT) in the plasma and pathological changes. Moreover, a metabolomics strategy based on GC–MS is employed to access the metabolic response of ASP in mice with CCl_4-induced liver injury.

2. Experimental

2.1. Chemicals

A. sinensis was purchased from Minxian County, Gansu Province, China and authenticated by Dr. Yanming Wei (School of Veterinary Medicine, Gansu Agriculture University, Lanzhou, China). Soybean oil, O-methyl hydroxyllmine hydrochloride, N-methyl-N-(trimethylsilyl) trifluoroacetamie (MSTFA), Trimethylchlorosilane (TMCS), and docosane (used as internal standard) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Assay kits for MDA, SOD, ALT, AST and γ-GT were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). All the reagents were analytical grade or chromate-graphic grade.

2.2. Extraction of ASP

Crude polysaccharide was extracted according to the following optimized procedure: 100 g of every AS sample was prepared, and 838 mL water was put in; 229 mL solution was obtained after concentrated in a rotary evaporator under reduced pressure; according to the formula: alcohol density $(\%) = V_{\text{anhydrous alcohol}}/(V_{\text{anhydrous alcohol}} + V_{\text{solution}})$, the alcohol density was 65.80%, and the reflux extraction time was set at 120 min. Then crude polysaccharide was purified according to the following procedure: protein was then removed using papain (mass fraction = 1%) and Sevage reagent [chloroform: n-butanol, 4:1 (v/v)] (volume fraction = 20%), while pigments were removed using hydrogen peroxide with the concentration of 30% (volume fraction = 2.5%). In the end, purified polysaccharide fraction ASP was obtained.

2.3. Animal studies

50 six-week-old male Kunming mice (20.0 ± 2.0 g) (SCXKZ (Gan) 2009-0004) were purchased from Experimental Animal Center of Lanzhou University (China). All animals were kept in a barrier system with controlled conditions of 22 ± 0.5 °C, 50 ± 2.0% RH, and on a 12/12-h light/dark cycle. The mice were also allowed free access to basal pellet diet and tap water.

After one week of feeding, the mice were randomly divided into five groups (10 mice for each group): control group, liver injury group, low dose group of ASP (LASPG), middle dose group of ASP (MASPG), and high dose group of ASP (HASPG). ASP was dissolved in normal saline for use. Mice in the control and liver injury groups were given normal saline; whereas those in the LASPG, MASPG, and HASPG groups were given ASP (60, 120, and 240 mg/kg/day, respectively) [20,21]. Drugs and normal saline were orally administered once daily for three successive days. Starting from the 4th day, liver injury was induced using 0.1% CCl_4 in soybean oil at 20 mL/kg through intraperitoneal injection in mice from the liver injury group, LASPG, MASPG, and HASPG, whereas the mice in the control group was only injected with soybean oil. After liver injury was established, mice in LASPG, MASPG, and HASPG were administered with ASP at the dosage of 60, 120, and 240 mg/kg/day, respectively; normal saline was used for the control and liver injury groups. Each mouse was administered with an orally accurate volume of 1 mL/100 g.

2.4. Sample preparation

All mice were sacrificed after 36 h of being induced with CCl_4. Blood were collected into heparinized tubes, and then centrifuged at 3000 rpm and 4 °C for 10 min. The plasma was collected. One part of the plasma was used to detect SOD activities, MDA content, ALT, AST, and γ-GT according to the instructions; the other part was frozen at −80 °C until GC–MS analysis. One part of the left lobe of the liver tissue was fixed in 10% formalin for observations of pathological changes. The remaining liver tissue was made into liver homogenate with twice the weight of normal saline and was frozen at −80 °C until GC–MS analysis.

Animal welfare and experimental procedures were always performed according to the guide for the Care and Use of Laboratory Animals and were also approved by the Animal Ethics Committee of Gansu Agricultural University.

2.5. Determination of biochemical parameters in plasma

MDA content, SOD activity, ALT, AST, and γ-GT in plasma were measured using commercially available kits.

2.6. Pathological changes

After 72 h of being fixed in 10% phosphate-buffered formalin, the liver blocks were embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E staining).