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Chaihu-Shu-Gan-San regulates phospholipids and bile acid metabolism against hepatic injury induced by chronic unpredictable stress in rat

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ABSTRACT

Chaihu-Shu-Gan-San (CSGS) is a famous classic traditional Chinese medicines (TCM) formula for treatment of liver stagnancy recorded in a famous book of traditional Chinese medicine, Jing Yue Quan Shu published in 1624. It has been extensively accepted as an antidepressant in China and its mechanism of action is still not clear. Previously we have found that hepatic injury happens in chronic unpredicted mild stress (CUMS). Thus, the protection of CSGS against hepatic injury induced by CUMS treatment was explored by metabonomics study and gene expression of the rat liver tissue. The results indicated that CSGS improved 8 of the 18 perturbed potential biomarkers in liver tissues of rats treated with CUMS, and involved in regulating phospholipids and bile acid metabolism against hepatic injury induced by CUMS in rat. The expressions of two apoptosis associated genes (*Bcl-2* and *Bax*) and four genes (*Pnpla6*, *Pla2g15*, *Baat* and *Gad1*) related to the perturbed metabolic pathways were further investigated by quantitative real-time polymerase chain reaction (qRT-PCR). Both metabonomics and studies of genetic influences on metabolites demonstrated that CSGS inhibited hepatocyte apoptosis, and regulated phospholipids and bile acid metabolism against hepatic injury induced by CUMS in rat. Exploring the protection of CSGS against hepatic injury related to depression further clarify the relationship between CUMS-induced depression and hepatic injury, and also provide a novel insight to understand the underlying antidepressive mechanism of CSGS.

1. Introduction

The practice of traditional Chinese medicine (TCM) is mainly guided by the cumulative empirical experience of its practitioners. TCM is widely used for the treatment of various diseases in China and many other Asian countries [1]. Many clinical therapies are often ineffective and its side-effect. TCM is regarded as a complementary or alternative therapy for different diseases especially in chronic diseases. Chaihu-Shu-Gan-San (CSGS) is a famous classic TCM formula for treatment of liver stagnancy recorded in ancient Jing Yue Quan Shu published in 1624 (Ming Dynasty of China) and has been extensively accepted as an antidepressant in China [2]. CSGS contains seven Chinese herbs with the proportions of 4:4:3:3:3:3:1 by weight in sequence, including the roots of *Bupleurum chinense* DC., the pericarps of *Citrus reticulata* Blanco., the roots of *Paeonia lactiflora* Pall., the rhizoma of *Cyperus rotundus* L., the fruits of *Citrus aurantium* L., the rhizoma of *Ligusticum chuanxiong* Hort., and the roots and rhizoma of *Glycyrrhiza uralensis* Fisch. The antidepressant effect of CSGS has been evaluated using urinary, serum and hippocampus metabonomics strategies on a rat model of chronic unpredicted mild stress (CUMS) [3,4], which not only confirm the antidepressant-like effect of CSGS on the CUMS-induced depression rats, including the improvements of CSGS on the spontaneous activities, sucrose preference, and the contents of serotonin and other neurotransmitters of CUMS-induced depression rats, but also elucidate its synergistic action through the mediation of multiple metabolic pathways. However, understanding its active constituents and mechanism of action against depression is still challenging. Recently, we have found that chronic unpredicted stimulation has led to hepatic injury with disturbance in metabolite profiling and gene expression in rat liver tissues of CUMS induced depression [5]. The findings just coincides the concept of TCM theory, which believes bad motion can harm liver function [6]. In TCM practice, relieving liver stagnancy is considered as the effective therapy for depression [7] and the representative formulas include CSGS, Xiao-Yao-San and YueJu Pill. Therefore, exploring the protection of CSGS against hepatic injury related depression would further reveal the relationship between depression and liver, and also provide a novel insight to understand the underlying mechanism of antidepressants.

Metabonomics is one of the methods of systems biology, which focus on the comprehensive metabolic profile of endogenous low-

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molecular-weight metabolites in a biological organism [8,9]. The method has been successfully applied in the field of disease biomarker discovery, disease diagnosis, drug bioactivity and toxicity evaluation and disease pathogenesis [10–13]. Therefore, metabonomics provides an opportunity to enable us for better understanding the metabolic pathways, which can clarify the mechanism of TCM [14]. Mass spectrometry (MS) and magnetic resonance spectroscopy (^{1}H NMR) are two mainly analytical techniques applied to metabolomic studies [15,16]. In the MS-based metabolomic technique, ultra-high performance liquid chromatography-quadrupole time-of-flight high-definition mass spectrometry (UPLC-QTOF/HDMS) is suitable for the analysis of untargeted and targeted metabolic profiles owing to its high reproducibility of retention time compared with traditional MS techniques [17,18]. MS^E technique was first applied to metabolite identification in 2005 [19], in which two scanning functions are simultaneously used for collection data. MS^E with two scanning functions can simultaneous obtained full-scan accurate precursor ion, mass fragment and neutral loss information [20]. In agreement with the holistic opinion of TCM, UPLC-QTOF/HDMS-based on metabolomic technique has demonstrated to possess enormous potential in therapeutic effects and toxicity evaluation of TCM such as geniposide [21], Chuanwu [22], and so on. Here a liver-targeted metabonomics using reverse phase and hydrophilic interaction chromatography coupled with MS^E was applied to investigate the protection effect of CSGS against hepatic injury of rat with CUMS treatment. Meanwhile, the gene expressions related to hepatocyte apoptosis and the perturbed metabolic pathways were investigated by quantitative real-time polymerase chain reaction (qRT-PCR). To the best of our knowledge, the mechanism of antidepressant from the protection of CSGS against hepatic injury related depression is reported for the first time.

2. Experimental

2.1. Reagents and materials

Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, France). Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Formic acid (HPLC grade) was purchased from Tedia (Fairfield, USA). Leucine-enkephalin was purchased from Sigma Aldrich (St. Louis, MO, USA). TRIzol Reagent was purchased from Invitrogen. PrimeScript™ RT and SYBR premix Ex Taq™ II kit were obtained from TaKaRa. The alanine aminotransferase (ALT) assay kits and aspartate aminotransferase (AST) assay kits were made in Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All the herb specimens are kept in the laboratory at institute of Medicinal Plant Development (IMPLAD). The preparation method of CSGS extract is consistent with the previous reports [5].

2.2. Experimental design

Forty male adult wistar rats (200 ± 20 g) were obtained from Institute of Laboratory Animal Science (Beijing, China). Rats were housed in cages for a week to adapt to the environment, including 12 h light – 12 h dark cycles from 6 AM – 6 PM (lamp), relative humidity (10%) and constant temperature control (20 ± 3 °C), free access to commercial diet and water. The Ethics Committee of the Institute of Medicinal Plant Development approved the all experimental procedures. Rats were randomly assigned 4 groups: (1) Control group, (2) CUMS group, (3) CSGS treated group (CUMS + CSGS group), (4) positive group oraled with fluoxetine (CUMS + fluoxetine group). The CSGS dose was 2.5 g/kg which based on the previous studies of tail-suspending and force swimming tests [3]. The oral dose of fluoxetine is 20 mg/ kg. The CUMS procedure included a series of stimuli which described in our previously reports [5]. The process description is as follows [5]: (1) noise stimulus at 110 dB, (2) immobilization for 5 h, (3) food with holding for 48 h, (4) water intake forbidden for 48 h, (5) thermal stimulus in 45 °C water for 5 min, (6) stroboflash. 2 flashs per second for 4 h, (7) swimming in 15 °C water for 5 min, and (8) electric shock to pelma (electric current for 1 mA, 2 s pershock, 2 shocks per minute). These stimulated in unpredictable ways with one or two in each rat every day. Each stimulus for three times at least 28 days.

On the 28th day, sodium pentobarbital was applied to anaesthetize all the rats. Serum samples were collected, and centrifuged at 3000 *rpm* for 15 min at 4 °C. The liver were removed and subpackaged quickly, and frozened in liquid nitrogen immediately. And then, the tissue samples were kept at −80 °C until analysis. The repackaging of tissue samples will be used in the study of metabonomics and qRT-PCR analysis respectively.

2.3. Biochemical indicator detection

Blood biochemical indicator (ALT and AST) were measured by spectrophotometry using ultraviolet spectrophotometry (Mapada, UV-3100, China) according to the operating manual of enzymatic kits.

2.4. Tissue samples extraction

Metabolites in the liver tissue were extracted by tissue homogenate method. The extraction procedure of tissue samples was performed as described in our previous report [5]. The operating procedures were as following: 250 mg liver sample was added 1500 μL chilled methanol-water. Dynamoelectric homogenizer (IKA, Staufen, Germany) was used for homogenizing the tissue samples 30 s, and then the homogenate were centrifuged (13000 *rpm*, 15 min, 4 °C). The upper clear liquor was used as polar solvent extract. The precipitate was deal with 1500 μL chloroform-methanol (4:1, v/v) in the same manner as above. The supernatant fluid after the treatment was used as nonpolar solvent extracts. The above two supernatant samples were dried under N_2, and then were stored at −80 °C.

Before LC–MS analysis, the nonpolar solvent extract samples were dissolved in 500 μL of acetonitrile-water (2:8, v/v). And then, 5 μL was used for UPLC analysis by BEH C18 column. Meanwhile, the polar solvent extracts were dissolved in 500 μL of acetonitrile-water (1:1, v/v). And 5 μL was used for UPLC analysis by HILIC column.

2.5. Metabonomics analysis

2.5.1. Data acquisition

Waters ACQUITY UPLC System equipped with a binary solvent delivery system was applied to chromatographic separation analysis. Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) and BEH HILIC column (100 mm × 2.1 mm, 1.7 μm) were applied to the analysis of nonpolar and polar samples respectively. The conditions for UPLC was optimized, including column temperature (40 °C), flow rate (0.40 mL/min), mobile phase [(A) 0.1% (by volume) formic acid in water and (B) acetonitrile]. The liquid chromatography method was performed as described in the previous report [5]. The gradient program for nonpolar extracts of liver was optimized as follows: 10% B from 0 to 1 min, 50–95% B from 1 to 6 min, 95–98% B from 6 min to 8 min, and 100% B from 8 to 12 min. The gradient program for polar extracts of liver was optimized as follows: 99% B from 0 to 1 min, 99–70% B from 1 to 10 min, and 99% B from 10 to 15 min.

A Q-TOF analyzer in a SYNAPT HDMS system was applied to collect mass spectrometry data, both in positive and negative ion modes. The Mass parameter setting was performed as described in the previous report [5]. The mass spectrometric data were collected in centroid mode from *m/z* 50–1200 with a scan time of 0.3 s and an interscan delay of 0.02 s over a 15 min analysis time. Specific parameter settings were as follows: capillary voltage: 3.0 kV (positive ion mode); 2.5 kV (negative ion mode), cone voltage: 35 V, source temperature: 120 °C, cone gas flow: 50 L/H, desolvation gas temperature: 300 °C, desolvation