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Metabolomic strategy to study therapeutic and synergistic effects of tanshinone IIA, salvianolic acid B and ginsenoside Rb1 in myocardial ischemia rats

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ABSTRACT

Aim of the study: Tanshinone IIA (T), salvianolic acid B (S) and ginsenoside Rb1 (G) are the three major active ingredients of Compound Danshen Formula (CDF) for its protective effects on myocardial ischemia (MI). In this study, we aimed to investigate therapeutic and synergistic effects of TSG (combination of T, S and G) on MI rats with metabolomic strategy.
Materials and methods: MI model were induced in Sprague-Dawley rats by left anterior descending coronary artery ligation. MI rats were respectively administrated T, S, G, TSG and CDF. Plasma was analyzed by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Partial least squares discriminate analysis (PLS-DA) models were built to evaluate the therapeutic and synergistic effects of TSG at whole level. 22 MI biomarkers in rat plasma were also investigated to explain that.
Results: TSG brings nearly equal therapeutic effects on MI as CDF and it plays more stable regulated action on those 22 identified metabolites than single compound.
Conclusions: Overall, there were few methods for the study of synergistic effects of Chinese medicine. Our results suggested that metabolomics offers a new idea for Chinese medicine research.

1. Introduction

Chinese Herbal Formula (CHF) is defined as a mixture of two or more herbs after prescriptive processing for special disease and syndrome prescriptions. In clinical practice, CHF was increasingly used to treat chronic disease for the roles of multi-component and multi-target. However, the two properties of CHF are regarded as weakness as well as strength, in that being that mechanisms of action of CHF remain unclear for so many herbs in a formula.

Compound Danshen Formula (CDF) is a classical formula to improve coronary and cerebral circulation for at least 30 years in China (Wei et al., 2007). The formula mainly derived from the herbs of *Radix Salviae Miltiorrhizae* (Danshen in Chinese), *Radix Notoginseng* (Sanqi) and *Borneolum Syntheticum* (Bingpian). There were three preparations based on the formula in the 2010s edition of Chinese pharmacopoeia (Editorial Committee of Pharmacopoeia of Ministry of Health P.R. China, 2010), known as Compound Danshen Tablet (CDT), Compound Danshen Dripping Pill (CDDP) and Compound Danshen Granule (CDG). Although CDDP passed Investigational New Drug (IND) exam by Food and Drug Administration (FDA) in United States in 1993, application of CDF was still limited in the United States and many European countries because of complexity of components.

Presently, more than 100 compounds have been isolated and identified in Danshen and Sanqi, but only a small portion of compounds were reported to be responsible for their biological effects (Lv et al., 2010a; Li et al., 2005; Ye et al., 2003; Yu et al., 2007). It means that most of components of CDF are non active ingredients, maybe, they are not necessary in the formula. Thus, we hope to build a simplified formula with active ingredients of CDF to improve acceptance of western people. After searching the articles (Fu et al., 2007; He et al., 2008a,b; Wang et al., 2008; Xu et al., 2009; Yang et al., 2008), we found that tanshinone IIA (T), salvianolic acid B (S) and ginsenoside Rb1 (G) were the major active ingredients of CDF for cardioprotection, moreover, their mechanisms of action have been elucidated clearly: T protects cardiac myocytes via inhibiting oxidative stress-induced apoptosis, promoting angiogenesis and up-regulating (vascular endothelial growth factor) VEGF expression due to the enhancement of hypoxia-inducible factor-1alpha mRNA expression; S exerts beneficial cardioprotective effects by augmenting VEGF expression, promoting angiogenesis, recovering the normal expressions of sarco/endoplasmic reticulum ATPase 2a and phospholamban, and inhibiting the activation of platelet during myocardial ischemia and reperfusion; G takes protective effects

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on myocardial ischemia and reperfusion injury by mediating the activation of the PI3K pathway and phosphorylation of Akt.

The concept of metabolomics, which is first mentioned by Nicholson et al. (1999), is "the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications". It is a new tool to pinpoint changed metabolites in the body in response to a disease process or drug therapy (Constantinou et al., 2007; Julian, 2004; Nebert and Vesell, 2006; Yang et al., 2004). In our previous metabolomic study on intervention effects of CDF on myocardial ischemia (MI) model (Lv et al., 2010b), 22 biomarkers of MI were identified. Meanwhile, it was proved that CDF showed a well reverse effect on the MI model. In the present study, metabolomic strategy was selected to evaluate therapeutic and synergistic effects of the new formula TSG (combination of T, S and G) on MI.

2. Experiment

2.1. Materials and animals

HPLC grade acetonitrile was purchased from JT Baker (NJ, USA). Spectroscopic grade formic acid and leucine enkephalin were purchased from Sigma/Aldrich (MO, USA). Distilled water was purified "in-house" using a Milli-Q20 system Millipore (MA, USA). Compound Danshen Formula was purchased from Leiyunshang Pharmaceutical Co., Ltd. (Shanghai, China). Tanshinone IIA, salvianolic acid B and ginsenoside Rb1 were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Fifty male Sprague–Dawley rats (200 ± 15 g) were purchased from the Slac Laboratory Animal Co., Ltd. (Shanghai, China). The animals were housed in stainless steel metabolic cages with free access to food and tap water under standard conditions of humidity ($50 \pm 10\%$), temperature ($25 \pm 2\,^{\circ}C$) and light (12 h light/12 h dark cycle). All animals were handled with humane care throughout the experiment.

2.2. MI model and drug administration

MI model was induced by left anterior descending coronary artery ligation (Ytrehus, 2006). In order to ascertain that the MI model is successful, electrocardiograms (ECG) were recorded by MPA 2000 bio-signal analysis system (Alcott Biotech Co. Ltd., Shanghai, China) for at least 5 min until they were well balanced. Meanwhile, serum concentrations of lactate dehydrogenase (LDH) and creatine kinases (CK) were measured by UV-1100 ultraviolet spectrophotometer (Beijing Rayleigh Analytical Instrument Corporation, Beijing, China). 7 rats died during the 24 h postoperative period because of acute pumps failure or lethal arrhythmias. 43 rats survived, including 37 MI rats and 6 sham rats (without ligation). 30 of 37 MI rats were randomly treated with five medicines ($n = 6$), which were CDF (300 mg/kg/d), T (10 mg/kg/d), S (20 mg/kg/d), G (10 mg/kg/d), and TSG (5 mg T + 10 mg S + 5 mg G per kg every day). Treated rats were consecutively oral administrated for 7 d; sham ($n = 6$) and MI ($n = 7$) rats were received 0.2 mL saline each time. Rats were fasted overnight before administrations with free access to water. During the administrated period, one rat in MI group died.

All 42 survived rats were put to death after blood was collected from ophthalmic venous plexus on the 9th day. The experiment was carried out in accordance with guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Shanghai, China. The study protocol was approved by the Animal Care and Use Committee of Second Military Medical University.

2.3. Sample preparation

1 mL blood of each rat was respectively added into 2.5 mL heparin-coated tubes and then centrifuged at $2789.1 \times g$ for 10 min at 4 °C. 100 μL of the supernatant was added to 300 μL acetonitrile and then the mixture was shaken vigorously for 30 s. After centrifugation at $9562.5 \times g$ for 10 min at 4 °C, the supernatant was stored at −80 °C until analysis.

2.4. UPLC–Q-TOF-MS conditions

Metabolomics analysis was performed on an ACQUITY^{TM} UPLC system coupled to a Micromass Q-Tof Micro^{TM} (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization source. A 2.1 mm i.d. × 100 mm ACQUITY^{TM} 1.7 μm column (Waters, Milford, MA, USA) was used. The column was maintained at 45 °C. The mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. Injection volume was 5 μL. The gradient duration program was: 0–1.5 min, 5% B; 1.5–9 min, 5–100% B. The flow rate was 0.4 mL/min. The parameters of mass detection were set as followed: desolvation gas, 400 L/h; cone gas, 20 L/h; desolvation temperature, 250 °C; source temperature, 100 °C; capillary voltage, 3000 V; cone voltage, 30 V; collision energy, 5 eV. Leucine enkephalin was used as the lock mass (m/z 556.2771 in the positive mode, and 554.2615 in the negative mode). The mass range was 50–1000 m/z.

2.5. Data processing

The LC–MS data were exported by Micromass MarkerLynx^{TM} applications manager version 4.1 software (Waters Corporation, Milford, MA, USA). Before multivariate analysis, the data of each sample was normalized to total area to correct for the MS response shift from the first injection to the last injection due to the long duration, overnight or longer, of an LC–MS analysis in metabolomic studies. After this operation, the sum of the ion peak area within each sample was set to 10,000. Then, partial least squares discriminant analysis (PLS-DA) was performed by the SIMCA-P 11 version (Umetrics AB, Umeå, Sweden). The significance was expressed by using one-way analyses of variance (ANOVAs) of the SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA), followed by Duncan post hoc tests. P values less than 0.05 were considered significant.

3. Results

3.1. Electrocardiograms and serum enzymes

The ECG alterations, especially the degrees of T wave and ST-segment alterations, are generally considered as a main index to evaluate animals' MI. In this study, we found that T waves of ECG in MI model rats were inversed and ST-segments were depressed after MI induced (Fig. 1). Furthermore, serum enzyme activities have also been cited as important parameters in the assessment of MI (Zheng et al., 2004). As shown in Table 1, compared with sham rats, the concentrations of LDH and CK in MI model rats were significantly increased. All together, the ECG and enzymatic results presented here demonstrated that both myocardial tissue and cell in model rats have damaged.

3.2. Biomarkers of MI

In our previous metabolomic study (Lv et al., 2010b), a total of 160 out of 2700 ions (sum of ions in both modes) were different between sham and MI groups. Among the perturbed ions, 39 were predicted by searching Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome