NMR-based Metabonomic Studies on Intervention Effects of Zuojin Pill on Stomach Heat Syndrome in Rats

Meng-Juan GONG\textsuperscript{a}, Shu-Mei WANG\textsuperscript{b}, Sheng-Wang LIANG\textsuperscript{c}, Zhong-Jie ZOU\textsuperscript{d,*}

\textsuperscript{1}School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou 510006, China
\textsuperscript{2}Key Laboratory of Digital Quality Evaluation of Chinese Materia Medica of State Administration of TCM, Guangdong Pharmaceutical University, Guangzhou 510006, China
\textsuperscript{3}Engineering & Technology Research Center for Chinese Materia Medica Quality of the Universities of Guangdong Province, Guangdong Pharmaceutical University, Guangzhou 510006, China
\textsuperscript{a}gongmengjuan@139.com, \textsuperscript{b}2395903468@qq.com, \textsuperscript{c}swliang371@163.com, \textsuperscript{d}zouzhongjie@139.com

*Corresponding author

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Abstract. Zuojin Pill (ZJP), composed of Rhizoma Coptidis and Fructus Euodiae, is commonly used for the treatment of stomach heat syndrome (SH) in traditional Chinese medicine, but the underlying molecular mechanisms remain unclear. The present study was performed to investigate the protective effect and the potential mechanisms of ZJP at metabolic level in a rat model of SH. Proton nuclear magnetic resonance (\textsuperscript{1}H NMR) based metabonomic approach was developed to profile SH-related metabolic perturbations in rat urine and feces. Distinct clustering and a clear separation of the model group from control and ZJP groups was observed. Furthermore, seven and five potential biomarkers associated with SH in rat urine and feces, respectively, which were mainly involved in energy metabolism and gut microbiota metabolism, were identified. ZJP could reverse the pathological process of SH through regulating the perturbed metabolic pathways. These findings offered new insights into in-depth understanding of the pathogenesis of SH and laid scientific foundation for the traditional use of ZJP in treating SH-related gastrointestinal diseases.

Introduction

In traditional Chinese medicine (TCM), stomach heat syndrome (SH), characterized by scorching pain in epigastric region, acid regurgitation, preference for cold drinks, reddish tongue with yellow fur, slippery and rapid pulse, halitosis, etc [1], is usually seen in acute and chronic gastritis, digestive ulcer, stomach cancer, and so forth [2]. Up to now, the molecular pathophysiological processes that underlie this TCM syndrome remain largely elusive.

Zuojin Pill (ZJP), consisting of two Chinese medicines: Rhizoma Coptidis and Fructus Euodiae, is originally recorded in \textit{Danxi Xinfa} and has been effectively used in the treatment of SH for centuries [3]. Previous studies have reported that ZJP could significantly reduce the
SH-induced gastric lesions in rats via modulation of inflammatory cytokines [3]. Further studies are needed to fully elucidate the efficacy of ZJP in treating SH.

Using a holistic approach, metabonomics has been exploited for the study and characterization of TCM syndromes in several experimental models [4]. In our previous study, seventeen potential biomarkers were identified in serum of rats with SH and ZJP could partially restore the homeostasis of the disturbed metabolic pathways [2]. These results inspired us to identify new biomarkers in other biological samples such as urine and feces.

In the current investigation, the SH-related metabolic perturbations in rat urine and feces were explored using NMR analysis and multivariate statistical analysis. Furthermore, the restorative effect of ZJP and its possible mechanisms of action were systematically evaluated based on changes in potential biomarkers.

Materials and Methods

Chemicals and Reagents

Rhizoma Coptidis, Fructus Euodiae and Fructus Capsici were purchased from Tongrentang Group Co., Ltd. (Beijing, China) and authenticated by Professor Bin Han (School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, China). Other materials, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the Decoction of ZJP and the Suspension of Fructus Capsici

The decoction of ZJP (0.14 g/mL, expressed as the weight of raw materials) and the suspension of Fructus Capsici (80 mg/mL) were prepared according to our published method [2].

Animal Handling and Sample Collection

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Guangdong Pharmaceutical University.

Male Sprague-Dawley (S.D.) rats (weighing 180±20 g) were purchased from the Experimental Animal Center of Sun Yat-Sen University (Guangdong, China). Rats were housed in an environmentally controlled condition (22±2°C, relative humidity of 50±10%) with a 12-h light/dark cycle and allowed free access to food and water. After one week of acclimation, the animals were transferred to individual metabolic cages and randomly divided into three groups with 6 rats per group. The control group was given distilled water orally at about 2 mL twice daily (at 9:00 am and 15:00 pm) for consecutive 6 days and once on day 7. The SH model group received the suspension of Fructus Capsici by oral gavage at the dose of 10 mL/kg body weight twice daily for consecutive 6 days followed by oral administration of absolute ethanol (1 mL) once on day 7 after rats were fasted and allowed free access to water for 12 h [5]. The ZJP pretreatment group received the suspension of Fructus Capsici and absolute ethanol in the same manner as SH model group and was treated with the decoction of ZJP (1.4 g/kg body weight) by gastric instillation once a day from day 4 to day 7 (1 h prior to the administration of suspension of Fructus Capsici and absolute ethanol). The dose level in this study was chosen based on our previous experiment [2]. 24-h urinary samples and fecal
samples acquired between 09:00 and 11:00 were stored at -80°C until further analysis. After all rats were euthanized, the stomachs were removed rapidly, opened along the greater curvature and rinsed with cold saline to remove the gastric contents and blood clots. The flattened stomach samples were photographed and the ulcer area (mm²) was measured using ImageJ software (National Institutes of Health, USA). Thereafter, each stomach was immersed in 10% formalin solution for histological evaluation (H&E).

**Urinary and Fecal Sample Preparation for Metabonomic Analysis**

For urine samples, 200 μl of buffer solution (0.2 M Na₂HPO₄ and 0.2 M NaH₂PO₄, pH7.4) was mixed with 400 μl urine to minimize variations in the pH of the urine samples. The samples were allowed to stand for 20 min prior to centrifugation at 4000 rpm for 10 min at 4°C to remove any precipitates. Aliquots of the supernatant (500 μl) from each sample were transferred into 5 mm NMR tubes followed by adding 50 μl of D₂O containing 0.05% (w/v) of sodium 3-trimethylsilyl [2,2,3,3-d₄] propionate (TSP-d₄). The TSP was used as a chemical shift reference (δ0) and the D₂O acted as a field frequency lock for the spectrometer. Fecal water was extracted according to published methods with minor modifications [6]. Briefly, 100 mg of feces was homogenized in 800 μl of buffer solution (0.2 M Na₂HPO₄ and 0.2 M NaH₂PO₄, pH7.4) and 200 μl of D₂O containing 0.05% (w/v) of TSP-d₄. The homogenates were sonicated at ambient temperature (298 K) for 30 min to destroy bacterial cells and then centrifuged at 12000 rpm for 10 min at 4°C. Aliquots of 550 μL were then pipetted into 5 mm NMR tubes for subsequent analysis.

**1H NMR Spectroscopic Analysis and Metabolite Identification**

All 1H NMR spectra were randomly measured at 298 K on a Bruker AVANCE III 500 MHz spectrometer (BrukerBiospin, Rheinstetten, Germany) operating at 500.13 MHz 1H frequency. 1H NMR spectra of urine and feces samples were acquired using a standard 1D nuclear Overhauser enhancement spectroscopy (NOESY)-presaturation pulse sequence. A relaxation delay of 3 s, 2 s and an acquisition time of 3.28 s, 1.64s were used for urine and feces samples, respectively. For each sample, 128 free induction decays (FIDs) were collected into 64 k data points over a spectral width of 10,000 Hz.

To assign the metabolite resonances, we matched the acquired data (i.e., chemical shifts, coupling constant and multiplicity) to reference spectra recorded in the Human Metabolome Database, which contained thousands of NMR spectra collected on purified reference metabolites and previous reports [7, 8].

**Data Analysis**

The obtained spectra of urine and feces samples were processed with a 0.3 Hz line-broadening factor prior to Fourier transformation. All the spectra were manually corrected for phase and baseline distortions using MestReNova software (Mestrelab Research S.L, Santiago de Compostela, Spain). The spectra of urine and feces were referenced to the chemical shift of TSP at δ0. Integration was performed over δ0.5-9.0 region with the bucket width set to 0.01 for all obtained spectra. The regions of δ4.50-5.98 and δ4.70-5.15 were removed from urine and feces spectra, respectively, to eliminate the influence of water. The regions containing ethanol (δ1.16-1.21 and 3.64-3.69) and its metabolite acetate (δ1.91-1.94) were also excluded from all samples. Finally, all remaining regions of the spectra were then normalized to the total integrated area of the spectra to reduce any significant concentration differences.
The resulting dataset was imported into SIMCA-P 12.0 software (Umetrics, Umea, Sweden) for orthogonal partial least-squares discriminant analysis (OPLS-DA).

Results

Protective Effect of ZJP Against SH-induced Gastric Damages

Compared to the control group, rats in the model group experienced severe macroscopic gastric mucosal injuries in the form of elongated bands of hemorrhagic lesions in the glandular portion of the stomach. Attenuated gastric lesions were observed in the ZJP pretreatment group, as evidenced by significant decrease in ulcer area (266.90±56.96 and 37.55±14.71 mm² for the model and ZJP group, respectively). Histopathological examination showed severe disruption to the superficial region of the gastric gland with epithelial cell loss and intense hemorrhage in the model group, while these damages in rats pre-treated with ZJP were rarely found (Fig. 1).

Figure 1. Histopathological evaluation of the SH-induced gastric mucosa damage in rats (H&E staining; magnification ×100). Control (A), model (B) and ZJP pretreatment (C) groups.

Metabolic Variations Associated with SH in Rats

Representative 1H NMR spectra of rat urine and feces were shown in Figs. 2 and 3. OPLS-DA models were constructed with NMR spectral data from rat urine and feces samples. As shown in the OPLS-DA score plots (Fig. 4A and B), good separation between the control and SH model groups was clearly seen, suggesting that urine and feces metabolic profiles of rats with SH were significantly changed compared with those of healthy controls. The model parameters were as follows: R²Y=0.96, Q²=0.84 for urine; R²Y=0.99, Q²=0.81 for feces. In general, excellent models were obtained when values of R²Y and Q² were above 0.8 [9].

According to the VIP values from the OPLS-DA models (VIP≥1) and the P values from univariate statistical analysis (P<0.05), seven and five endogenous metabolites associated with SH were identified as potential biomarkers in rat urine and feces, respectively (Fig. 5).

Figure 2. Typical 1H NMR spectra of rat urine. Control (A), model (B) and ZJP pretreatment (C) groups. Keys: 1, succinate; 2, 2-oxoglutarate; 3, citrate; 4, creatine; 5, creatinine; 6, taurine; 7, uridine.
Figure 3. Typical 1H NMR spectra of rat feces. Control (A), model (B) and ZJP pretreatment (C) groups. Keys: 1, n-butyrate; 2, propionate; 3, threonine/lactate; 4, β-glucose; 5, uracil.

Figure 4. Metabolic variations illustrated by OPLS-DA score plots derived from 1H NMR spectra of rat urine (A, C and E) and feces (B, D and F) samples. ■, control group; ●, SH model group, ▲, ZJP pretreatment group.
Influence of ZJP on Metabolic Pattern of Rats with SH

The OPLS-DA score plots derived from NMR spectra of rat urine and feces samples in the control, model and ZJP pretreatment groups showed a tendency recovering to healthy control group in ZJP pretreatment group as well as an obvious separation between SH model group and ZJP pretreatment group (Fig. 4C-F). Moreover, all potential biomarkers associated with SH in rats were significantly reversed by ZJP except creatinine and uridine in urine and β-glucose in feces (Fig. 5). The above results, which were also in accordance with those in Fig. 1, suggested that there was really no doubt that pretreatment of ZJP in rats with SH induced substantial and characteristic changes in their metabolic profiles.

Figure 5. Relative levels of potential biomarkers associated with SH in rat urine (A) and feces (B). *represents P<0.05 compared with the model group.

Discussion

In TCM theory, SH is usually caused by excessive intake of pungent and warm foods, such as Fructus Capsici, which transforms into heat and fire, or by emotional upsets and stagnation of qi which transforms into fire and attacks the stomach [1]. So, heat-related pathogenic factors
such as Fructus Capsici and ethanol could be used to induce various pathological changes in experimental animals that mimicked the state of SH to some extent in patients [5].

Citrate, 2-oxoglutarate and succinate are key intermediate products of tricarboxylic acid (TCA) cycle which involves not only the glucose aerobic oxidation but also the major pathways for fat and amino acid metabolisms. Consistent with our previous study that revealed the slowdown of glycolytic activity in rats with SH [2], the model group showed decreased urinary excretion of citrate, 2-oxoglutarate and succinate in the current investigation, indicating the suppression of TCA cycle activity caused by SH. Creatine plays an essential role as phosphocreatine in regenerating adenosine triphosphate in skeletal muscle to energize muscle contraction, while phosphocreatine undergoes irreversible cyclization and dehydration to form creatinine [10]. Hence, the elevated levels of urinary creatine and creatinine in rats with SH might reflect the synthesis of creatine was accelerated and creatine and phosphocreatine shuttle system provided an alternative energy source.

Taurine is an antioxidant and an organic osmolyte capable of protecting and stabilizing cells [11]. The increase in urinary taurine might be attributed to an intrinsic self-defense against the oxidative damage triggered by SH as mentioned above.

Uridine is the principal circulating pyrimidine nucleoside and serves as a precursor of membrane phospholipids via the Kennedy pathway as a building block of uridine-5'-triphosphate (UTP) [12]. Reduction in urinary level of uridine possibly contributed to the SH-induced gastric mucosal injuries as evidenced by histopathological examinations.

Disturbance in gut microbiota induced by SH has been reported in our previous study by assessing the gut microbial-host co-metabolites in rat serum [2]. As gut microbiota composition modified fecal metabolic profiles [13], a NMR-based metabonomic approach was applied to fecal samples in the present work with the aim of further probing dysbiosis of gut microbiota associated with SH. Short-chain fatty acids (SCFAs), predominantly acetate, propionate and butyrate, are primarily generated from anaerobic fermentation of dietary fibers and/or other resistant carbohydrates by gut microbes including Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria [14]. SCFAs, especially butyrate, are regarded as an important energy source for colonic epithelial cells of the host. SCFAs also have immunomodulatory effects and protect the gut from inflammation [13]. Rats with SH showed depleted levels of β-glucose, butyrate and propionate in feces, manifesting the reduced fermentation of indigestible fibers and changes in the intestinal microbial population. Additionally, the relatively low concentrations of fecal lactate found in the SH model group suggested an imbalance of lactate-producing (e.g., Bifidobacteria) and lactate-utilizing bacteria (e.g., Eubacterium hallii, Anaerostipes caccae) in the gut [15].

With the exception of creatinine and uridine in urine and β-glucose in feces, ZJP effectively attenuated the alterations of all the urinary and fecal potential biomarkers associated with SH in rats. These findings implied that ZJP exerted beneficial effect on SH through partially restoring the balance of the perturbed metabolic pathways, including energy metabolism and gut microbiota metabolism.

In conclusion, these results provided novel insights into in-depth understanding of the specific physiopathologic state of SH and the elucidation of the underlying mechanisms of action also helped put the traditional use of ZJP for SH-related gastrointestinal diseases on a solid scientific footing.
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References
