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Integrative drug efficacy assessment of Danggui and European Danggui using NMR-based metabolomics

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ABSTRACT

Danggui (DG) is a commonly used herbal drug in traditional Chinese medicine, and usually adulterated with European Danggui (EDG) due to the increasing demand. In present study, global metabolic profiling with NMR coupled with integrative drug efficacy evaluation methods was performed to compare and discover underlying blood-enriching regulation mechanisms of DG and EDG on blood deficiency rats induced by acetyl phenylhydrazine (APH). Totally, the contents of 12 key metabolites in serum and 4 in urine of DG group, 7 in serum and 4 in urine of EDG group were significantly reversed in comparison with model group. DG was more effective than EDG as revealed by the relative distance, efficacy index and similarity analysis. The metabolism pathways analysis showed that the better effect of DG maybe related with the regulatory effect on valine, leucine and isoleucine biosynthesis, synthesis and degradation of ketone bodies, glycine, serine and threonine metabolism, as well as nicotinate and nicotinamide metabolism. The results presented here showed that metabolomic coupled with efficacy index and similarity analysis made it possible to disclose the subtle biological difference between DG and EDG, which highlight the potential of metabolomic approach to quantitatively compare the pharmacological effect of the herbal drugs.

1. Introduction

Radix Angelicae Sinensis, also called Danggui (DG) in China, is archived in the Chinese Pharmacopoeia and recorded in Chinese medical classics in "Shennong's Herba" that DG is regarded as female's ginseng and extensively applied to the treatment of gynecological disorders as well as to nourish and tonify blood [1]. Besides its medical property, DG has also been recommended as a kind of edible plant with its diverse applications in flavoring and cosmetics. According to the Chinese Pharmacopoeia, only the roots of *Angelica sinensis* can be used as DG in TCM [2]. However, due to the increasing demand for DG, adulteration with European Danggui (EDG), which is also cultivated in Gansu Province, was frequently encountered in the market place, as a result of their similar appearances in shape. In our previous investigation, the chemical components of DG and EDG were compared by ^{1}H NMR based fingerprinting approach, and the results showed that there existed big chemical difference between DG and EDG [3]. However, no significant differences on the hemogram indexes existed between the two drug treated groups in the animal blood deficiency model. The reason maybe that the traditional hemogram indexes were not sensitive enough to discriminate their blood enriching effect between DG and EDG, and thus more sensitive methods are needed.

Metabolomics, is an emerging field of biochemical research as well as the comprehensive assessment and simultaneous profiling of endogenous metabolic changes in living systems [4,5]. Metabolomics largely relies on other advanced instrumental analysis, such as gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) spectroscopy. With rapidity, non-selectiveness, reproducibility, and stability and providing detailed structural information of metabolites, including chemical shifts and coupling constants, NMR emerges as an ideal tool to identify endogenous metabolites of drug efficacy, such as Genipin [6], Astragali Radix [7], Gegen Qinlian Decoction [8]. In the present study, a NMR-based metabolomic strategy combined with relative distance, efficacy index and similarity analysis, was further applied to search the subtle biological difference and quantitatively compare the pharmacological effect between DG and EDG.

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2. Materials and methods

2.1. Materials, solvent and chemicals

Crude dried roots of *Levisticum officinale* Koch and *A. sinensis* were bought from Gansu Province, China, and authenticated by Prof. Xue-Mei Qin. All the voucher specimens are deposited in the herbarium of Modern Research Center for Traditional Chinese Medicine of Shanxi University.

D_2O was bought from Norell (Landisville, PA, USA). Sodium 3-trimethylsilyl [2,2, 3, 3-d_4] propionate (TSP) and NaOD was from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). *N*-Acetyl phenylhydrazine (APH) was purchased from Sangon Biotech (Shanghai, China) Co., Ltd.

2.2. Collection of serum and urine

The details of animal experiment is described in our previous paper [3]. At the end of the experiment, femoral artery blood was collected into 1.5 mL heparinized Eppendorf centrifuge tubes. Then, the blood samples were immediately centrifuged at 3000 r/min for 10 min to afford the serum samples, which were separated and stored at −80 °C until analysis. Samples of 12 h urine were collected on day 13 using metabolic cages on ice. All urine samples were immediately centrifuged at 3000 r/min for 10 min, then the supernatants were separated and stored at −80 °C until analysis.

2.3. Sample preparation for ^{1}H NMR analysis

Serum samples were thawed and then prepared as follows: 450 μL serum was mixed with 350 μL D_2O (Deuterium Oxide) and the mixture was centrifuged at 4 °C and 13,000 r/min for 20 min. 600 μL supernatants were transferred into 5 mm NMR tubes for ^{1}H NMR analysis. Urine samples were thawed prior to use. A total of 500 μL of each sample was diluted with 200 μL of phosphate buffer (0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4) containing D_2O for the purpose of field lock and 0.01% sodium 3-trimethylsilyl-(2, 2, 3, 3-d_4)-1-propionate (TSP) as a chemical shift reference. The mixture was centrifuged at 4 °C and 13,000 r/min for 20 min, and an aliquot of 600 μL supernatant was transferred into 5 mm NMR tubes for NMR analysis.

2.4. NMR measurement and data preprocessing

The one and two dimensional NMR spectra of the serum and urine samples were acquired on a Bruker 600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a Bruker 5 mm PA BBO probe, operating at 600.13 MHz ^{1}H frequency and a temperature of 25 °C. Samples were analyzed using Carr–Purcell–Meibom–Gill (CPMG) spin–echo pulse sequence (for serum) to attenuate broad signals from proteins and lipoproteins due to their long transverse relation time, and Nuclear Overhauser Effect Spectroscopy (NOESY, RD-90°-t1-90°-tm-90°-acquire) (for urine) with water suppression. Each ^{1}H NMR spectrum of serum consisted of 64 scans requiring 5 min acquisition time with the following parameters: spectral width = 12019.2 Hz, spectral size = 65536 points, pulse width (PW) = 12.6 μs (90°), and relaxation delay (RD) = 1.0 s. Each ^{1}H NMR spectrum of urine consisted of 64 scans with the following parameters: SW = 12345.7 Hz, SZ = 65536, PW = 14 μs (90°), and RD = 1.0 s. ^{1}H-^{1}H-correlated spectroscopy (COSY) was acquired using the cosygpprqf pulse sequence, which consisted of 25 scans with 1.5 s RD and 6602.1 and 6601.5 Hz spectral widths in both dimensions. The heteronuclear single quantum coherence (HSQC) spectrum was obtained using the hmqcgpqf sequences with 110 scans. All HSQC spectra were obtained with 1.5 s RD using spectral widths of 6602.1 Hz in the ^{1}H dimension and 36219.4 Hz in the ^{13}C dimension.

The 1D and 2D dimensional spectra were processed using MestReNova software (version 8.0.1, Mestrelab Research, Santiago de Compostella, Spain). All the spectra were manually phased and baseline corrected. The spectra of serum, which were referenced internally to the chemical shift of creatine at δ 3.04 ppm, were divided and the signal integral computed in 0.01 ppm intervals across the region δ 0.60–9.00 ppm. The region of δ 4.68–5.20 ppm was removed to eliminate the effects of imperfect water saturation. The spectra of urine were referenced to the chemical shift of TSP at δ 0.00 ppm. The spectra were divided and the signal integral computed in 0.01 ppm intervals across the region δ 0.60–9.60 ppm. The regions of δ 4.60–4.96 ppm and δ 5.52–6.12 ppm were removed to eliminate the influence of water and urea. The resulted data matrix were then normalized to the total sum of the spectra prior to multivariate analysis.

2.5. Data analysis

2.5.1. Multivariate data analysis

Multivariate data analysis was performed with the software package SIMCA-P 13.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) was performed with the mean-centered data to generate an overview for group clustering and to search for possible outliers [9]. Results were visualized in the form of the score plots, where each point represented an individual sample (or its metabonome). Partial least-squares discriminant analysis (PLS-DA), with R2X representing the explained variations and Q2 for the model predictabilities, applies PLS to discriminate between groups of samples that are defined as separate response variables [10]. Orthogonal projection to latent structure with discriminant analysis (OPLS-DA), with the ability to separate predictive from non-predictive (orthogonal) variation, was further conducted with Pareto scaling [11]. Besides, S- and VIP- plots constructed from OPLS-DA were employed to choose potential biomarkers based on their variable importance of projection (VIP > 1) statistics [12]. For quantitative data analysis of changes of endogenous metabolites by the treatments of DG and EDG, relative amounts of metabolites were evaluated based on the integrated regions (buckets) from the least overlapping NMR signals of metabolites. These semi-quantitative data were expressed in the form of mean ± standard error of the mean (SEM) and were also subjected to classical one-way ANOVA analysis using SPSS 16.0 software to investigate the differences between DG and EDG at the same dose. Differences among groups were considered to be statistically significant if $p < 0.05$.

2.5.2. The calculation of relative distance and similarity analysis

The relative distances between other groups and control group from 3 D PLS-DA score plot, used as a quantitative method, were calculated with the average value (*x*-axis, *y*-axis, and *z*-axis) of all samples of the control group as the referenced point [13,14]. To compare the drug efficacy comprehensively, efficacy index was calculated with Eq. (1), where X_i, M_i, C_i represent relative contents evaluated on the basis of the integrated regions (buckets) from the least-overlapping NMR signals of metabolites in drug-treated group, model group and control group, respectively.

$$\mathrm{EI(Efficacy\ Index)} = \sum_{i=1}^{n} \left| \frac{X_i - M_i}{C_i - M_i} \right| \times 100\% \quad (1)$$

Similarity analysis is employed to evaluate drug efficacy using the included angle cosine. Every single spectrum can be viewed as a set of values (taken as X_i or Y_i) of corresponding relative peak areas of key metabolites, which are considered a vector (taken as *X* or