Exploring the Holistic Effects of Two Chinese Patent Medicines on the Organism Through Urinary Proteomics

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Abstract

[Objective] To investigate the effects of traditional Chinese medicine on the urine proteome of healthy rats.

[Methods] This study established rat gavage models for two traditional Chinese medicines (Compound Danshen Dripping Pills and Huoxiang Zhengqi Oral Liquid), collected urine before and after gavage, and analyzed the extracted urinary proteins using liquid chromatography-tandem mass spectrometry (LC-MS/MS), with subsequent biological pathway analysis of differential proteins.

[Results] The urine proteome could reflect changes before and after 14 days of Compound Danshen Dripping Pills administration, and the biological pathways enriched by differential proteins were associated with its mechanism of action in treating cardiovascular diseases, such as glycolysis and lipid metabolism.

[Conclusion] Urine proteomics can directly and systematically reflect the holistic effects of traditional Chinese medicine on the organism, providing a novel approach for studying the efficacy of traditional Chinese medicine.

Full Text

Exploring the Overall Effects of Two Traditional Chinese Medicines on the Body Through Urinary Proteome

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[Objective] To investigate the effects of traditional Chinese medicine on the urinary proteome of healthy rats.

[**Methods**] This study established intragastric administration rat models using two traditional Chinese medicines (Compound Danshen Dropping Pills and Huoxiangzhengqi Oral Liquid). Urine samples were collected before and after administration, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze extracted urinary proteins and perform biological pathway analysis of differential proteins.
[**Results**] The urinary proteome could reflect changes before and after 14 days of Compound Danshen Dropping Pill administration, and the biological pathways enriched by differential proteins were related to the mechanism of action in treating cardiovascular diseases, such as glycolysis and lipid metabolism.
[**Conclusions**] Urinary proteomics can directly and systematically reflect the overall impact of traditional Chinese medicine on the body, providing a novel method for studying the efficacy of traditional Chinese medicine.

Keywords: proteomics; urine; traditional Chinese medicine

Traditional Chinese medicine (TCM) represents a distinctive feature of Chinese medical science and constitutes an important component of Chinese cultural heritage [1]. TCM is increasingly recognized in current research for its characteristics of minimal side effects, multi-drug combinations, and multi-target effects [2]. Compound Danshen Dropping Pills (CDDP), composed of Danshen (Salvia miltiorrhiza), Sanqi (Panax notoginseng), and Bingpian (borneol) [3], were included in the Chinese Pharmacopoeia in 1990 and are currently widely used in clinical prevention and treatment of angina pectoris in the context of myocardial ischemia and other cardiovascular diseases [4]. Previous studies have demonstrated that the pharmacological mechanisms of CDDP involve antioxidant and anti-inflammatory effects, protection of vascular endothelial function, inhibition of platelet adhesion and aggregation, amelioration of myocardial fibrosis, prevention of microcirculation disorders, and alleviation of myocardial injury [2,5,6]. Huoxiangzhengqi Oral Liquid (HXZQ-OL) is a prepared Chinese medicine derived from the Huoxiangzhengqi formula, which originated from the "Taiping Huimin Heji Jufang" in China' s Song Dynasty. It is commonly used to treat diseases related to dampness syndrome in TCM, such as gastrointestinal disorders like acute gastroenteritis [7].

As an important means and carrier of TCM disease treatment, the multi-component, multi-target, and multi-level characteristics of Chinese medicines also present difficulties for mechanistic research. The lack of holistic systematic evaluation and referenceable standards hinders the modernization and internationalization of TCM. In recent years, the development of multi-omics has provided a scientific and effective approach for exploring the therapeutic efficacy and mechanisms of TCM. Omics research investigates the entire organism, reflecting the overall state of the human body under multi-factor interactions, which aligns with the holistic view and syndrome differentiation and treatment principles of TCM theory. This approach is gradually becoming a novel strategy to effectively drive the modernization of TCM, with proteomics

and metabolomics being the most widely applied. Metabolomics analyzes metabolites in biological fluids, cells, and tissues to detect subtle changes in biological pathways, thereby exploring potential disease pathogenesis and drug targets and further elucidating drug mechanisms of action [8]. However, the intrinsic connection between chemical components of TCM and metabolite changes remains unclear, making it difficult to exclude the influence of drug metabolism on end products. In contrast, proteomics reveals fundamental laws of organismal activity from the perspective of "holistic" protein activities at different levels—whether from an entire organism, tissue, or cell. Utilizing proteomics technologies for high-throughput, multi-target screening of TCM formulas can associate the multi-component, multi-target, and multi-pathway characteristics of TCM formulas with protein expression. Changes in protein expression and content at the protein level can reveal alterations in the overall functional state of the organism. Compared with metabolomics, proteomics primarily reflects the organism's overall response to drugs, reducing interference from the drug itself and drug metabolism. Academician Zhu Chen' s team applied proteomics to study the multi-component, multi-target synergistic mechanism of Realgar-Indigo Naturalis formula in treating acute promyelocytic leukemia, and used modern medical methods to elucidate the TCM classic "Jun-Chen-Zuo-Shi" (sovereign-minister-assistant-courier) compatibility principle [9]. This research provides an exemplary model for studying TCM formula mechanisms.

Urine, as a non-invasive and easily obtainable biological fluid, is becoming an ideal sample for proteomics research. Urine is not regulated by homeostatic mechanisms and can accommodate and accumulate more changes within the body [10]. Additionally, the relatively low complexity of the urinary proteome facilitates detection of low-abundance protein changes [11], demonstrating potential for sensitively reflecting organismal changes. Therefore, urinary proteomics can more sensitively reflect the impact of drugs on the entire organism. This study utilized animal models to investigate the effects of TCM on healthy rats, avoiding confounding factors such as individual differences in age, sex, diet, and living environment [12]. Urine samples collected before and after intragastric administration were analyzed using label-free quantitative proteomics to explore proteomic changes after administration of the two medicines, providing a novel method for studying TCM mechanisms of action.

1.1 Experimental Animals and Model Establishment

Nine SPF-grade male Sprague-Dawley rats (170–190 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed under standard conditions (room temperature 22$±$1 °C, humidity 65–70%). Animal experiments were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University (Approval No.: CLS-EAW-2020-034).

Model establishment method: The nine rats were randomly divided into two

groups, Group A (n=5) and Group B (n=4). Group A received CDDP dissolved in normal saline via intragastric administration. The effective daily dose for adults weighing approximately 60 kg is 81 mg. Based on the equivalent dose conversion ratio between humans and rats according to body surface area (rat dose is approximately 6.25 times the human dose in mg/kg), the effective dose for rats was calculated to be approximately 8.44 mg/kg—equivalent to 1.69 mg/day. Intragastric administration ensured each rat received the same dose daily for 2 weeks. This experiment used self-control, with urine samples collected before administration serving as the control group (designated D0) and samples collected after 14 days of administration serving as the experimental group (designated D14). Group B received HXZQ-OL via intragastric administration. The effective daily dose for adults weighing approximately 60 kg is 20 ml. Using the same conversion method, the effective dose for rats was calculated to be approximately 2.08 ml/kg—equivalent to 0.42 ml/day. Intragastric administration ensured each rat received the same dose daily for 3 days. Similarly using self-control, urine samples collected before administration served as the control group (designated D0) and samples collected after 3 days of administration served as the experimental group (designated D3).

1.2 Urine Collection

Two sampling time points were set before and after drug administration to collect urine samples. On the day before collection, rats were individually placed in metabolic cages overnight for 12-hour urine collection. During collection, water was provided but food was withheld to avoid urine contamination. A total of 18 samples were collected. Urine was centrifuged at 3000×g for 30 minutes and stored at -80 °C.

1.3 Urinary Protein Extraction and Proteolysis

Frozen urine samples (4 ml) were thawed and centrifuged at 12,000×g for 30 minutes at 4 °C to remove cell debris. The supernatant was precipitated with 3 volumes of ethanol overnight, then centrifuged at 12,000×g for 30 minutes. Protein pellets were resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, and 50 mmol/L Tris). Protein concentration was measured using the Bradford method. Urinary protein digestion was performed using the Filter-Aided Sample Preparation (FASP) method [13]. Urinary proteins were loaded onto 10 kDa ultrafiltration tubes (PALL), washed twice with UA (8 mol/L urea), 0.1 mol/L Tris-HCl (pH 8.5), and 25 mmol/L NH_4HCO_3 solution. Proteins were denatured with 20 mmol/L dithiothreitol (DTT, Sigma) at 37 °C for 1 hour, then alkylated with 50 mmol/L iodoacetamide (IAA, Sigma) in the dark for 30 minutes. After two washes with UA and NH_4HCO_3 solution, trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 ratio and incubated overnight at 37 °C. After overnight incubation, the digested filtrate was collected by centrifugation as the peptide mixture. Peptides were desalted using HLB columns (Waters, Milford, MA), vacuum-dried, and stored

at -80 °C.

1.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Peptides were resuspended in 0.1% formic acid, and concentration was determined using the BCA assay kit. Peptide concentration was diluted to 0.5 g/ L. A pooled peptide sample was prepared by taking 9 L from each sample and separated using the high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific) according to the manufacturer' s instructions. Ten fractions were collected by centrifugation, vacuum-dried, and resuspended in 0.1% formic acid. iRT (Biognosis) was added at a 10:1 sample-to-iRT volume ratio. For each sample (individual experimental samples and ten fractions), 1 g was analyzed using the EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA). Parameters were set as follows: elution time 90 minutes with a gradient of mobile phase A (0.1% formic acid) and mobile phase B (80% acetonitrile). Eluted peptides were detected using the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA). Data-dependent acquisition (DIA) mass spectrometry data were collected for all samples, with each sample analyzed in duplicate.

1.5 Data Analysis

The ten fractions obtained from reversed-phase chromatography were analyzed in DDA mode. DDA results were imported into Proteome Discoverer software (version 2.1) for database searching. The PD search results were used to establish the DIA acquisition method, with window width and quantity calculated based on m/z distribution density. Individual peptide samples were analyzed in DIA mode. Spectronaut X software was used to process and analyze mass spectrometry data. DIA raw files from each sample were imported for database searching. High-confidence protein criteria were peptide q-value < 0.01, with protein quantification based on the peak area of all fragment ions from secondary peptides.

1.6 Statistical Analysis

Each sample was analyzed in two technical replicates, and the resulting data were used for statistical analysis. Urinary proteins identified before and after intragastric administration were compared for differential protein screening. Screening criteria for differential proteins were as follows: fold change ≥ 1.5 or ≤ 0.67, and two-tailed paired t-test P-value < 0.05.

1.7 Random Grouping Analysis

Samples from the CDDP group before (n=5) and after (n=5) administration were randomly divided into two groups. Among all possible random combinations, the average number of differential proteins was calculated under the same screening conditions. The same procedure was applied to the HXZQ-OL group.

1.8 Bioinformatics Analysis

Unsupervised hierarchical clustering analysis (HCA) was performed using the Wukong platform (https://www.omicsolution.org/wkomic/main/) [14]. Functional enrichment analysis of identified differential proteins was conducted using DAVID 6.8 (https://david.ncifcrf.gov/) across three aspects: biological process, cellular localization, and molecular function [15]. The functions of differential proteins were searched in reported studies based on the public database (https://pubmed.ncbi.nlm.nih.gov).

2.1 Urinary Proteome Changes

After intragastric administration, LC-MS/MS analysis was performed on 18 urinary protein samples (before and after administration) collected from both groups of rats. A total of 611 proteins were identified ($ $2 unique peptides, protein-level FDR $< 1\%$). Unsupervised hierarchical clustering analysis clearly distinguished samples before and after 14 days of CDDP administration but could not significantly separate samples before and after 3 days of HXZQ-OL administration. [Figure 1: see original paper] shows the detailed unsupervised clustering results.

Comparing post-administration urine with pre-administration urine in both groups, differential proteins were screened using the criteria: fold change FC ≥ 1.5 or ≤ 0.67, two-tailed paired t-test $P < 0.05$. The results showed that 84 differential proteins were identified after CDDP administration, and 64 differential proteins were identified after HXZQ-OL administration, compared to pre-administration. Detailed information on differential proteins is listed in (CDDP group) and (HXZQ-OL group).

2.2 Results of Random Grouping of Urine Samples

Given that the number of proteomic features identified in samples exceeded the number of samples, differences between groups might occur randomly. A random grouping statistical analysis strategy was developed to confirm whether these differential proteins were caused by disease. Pre-administration (n=5) and post-administration (n=5) samples from the CDDP group were randomly divided into two groups. Among all random combination types, the average number of differential proteins calculated across all random iterations was 20 (see Supplementary Table 1). These results indicate that randomly generated differential proteins numbered 20, giving the screening credibility of 76%. This suggests that under the current drug dosage, the urinary proteome did not show particularly significant changes, possibly reflecting the mild nature of TCM effects. The same procedure was applied to pre- and post-administration urine samples from the HXZQ-OL group, yielding an average of 18 differential proteins from random combinations (see Supplementary Table 2).

2.3.1 Functional Analysis of Differential Proteins in the CDDP Group

Functional enrichment analysis of differential proteins identified in the CDDP group was performed using the DAVID database (https://david.ncifcrf.gov/) across three aspects: biological process, cellular component, and molecular function (Figure 2 [Figure 2: see original paper]). The results showed that these differential proteins were primarily involved in biological processes such as proteolysis, positive regulation of peptidyl-tyrosine phosphorylation, positive regulation of nitric oxide biosynthesis, glycosaminoglycan metabolism, response to ethanol, and ganglioside catabolism. In terms of cellular component, these differential proteins were mostly derived from extracellular and plasma membrane regions. In molecular function, these differential proteins predominantly exhibited endopeptidase activity, β-N-acetylhexosaminidase activity, and collagen binding. To identify the main metabolic pathways involved by differential proteins, KEGG pathway enrichment analysis was performed. The results showed that eight metabolic pathways were significantly enriched, including glycosphingolipid biosynthesis, glycosaminoglycan degradation, atherosclerosis, and glycolysis.

2.3.2 Functional Analysis of Differential Proteins in the HXZQ-OL Group

Similarly, functional analysis revealed (Figure 3 [Figure 3: see original paper]) that differential proteins identified in the HXZQ-OL group were primarily involved in biological processes such as ossification, negative regulation of megakaryocyte differentiation, nucleosome assembly, cellular redox homeostasis, acute-phase response, negative regulation of endopeptidase activity, and cellular detoxification. In cellular component, these differential proteins were still derived from extracellular regions and extracellular matrix. In molecular function, differential proteins mainly exhibited calcium ion binding, protein heterodimerization activity, thioredoxin peroxidase activity, and complement binding. KEGG pathway enrichment analysis showed that four metabolic pathways were significantly enriched, including glycolysis and response to alcohol.

This study established rat intragastric administration models using two common traditional Chinese medicines. Through label-free LC-MS/MS identification and analysis of urine collected before and after administration, we investigated the effects of TCM on rat urinary proteins. Statistical analysis results showed that 84 differential proteins were identified in the CDDP group and 64 differential proteins in the HXZQ-OL group when comparing post-administration urine with pre-administration urine. Unsupervised clustering results demonstrated clear differences between samples before and after 14 days of CDDP administration. However, samples before and after 3 days of HXZQ-OL administration could not be significantly distinguished, which we consider may be due to limitations in administration duration or protein identification numbers resulting in no significant differences between pre- and post-administration samples in the HXZQ-OL group. This suggests that to further observe the effects of TCM on

the body, administration duration should be extended or drug dosage increased. We also consider that the minimal side effects of TCM may be due to its very subtle influence on the body, thereby achieving fine-tuning therapeutic effects. Furthermore, previous studies evaluating CDDP therapeutic effects found no significant differences in heart tissue morphology and blood biochemical indicators in healthy rats after 28 consecutive days of intragastric administration compared to pre-administration [2]. Our study found that urinary proteomics could significantly distinguish samples before and after 14 days of CDDP administration, demonstrating that urinary proteomics can reveal differences earlier than tissue morphological changes, reflecting the sensitivity of urine.

Further literature review of the biological function analysis results for the CDDP group differential proteins revealed that some biological processes and metabolic pathways are related to the mechanism of CDDP in treating myocardial ischemia. When coronary blood flow is insufficient, myocardial ischemia leads to metabolic disorders and reduced energy supply to myocardial cells and the organism; therefore, metabolic regulation is considered an effective therapeutic approach for ischemic heart disease [16]. Studies using plasma metabolomics have found that CDDP can alleviate metabolic disorders induced by acute myocardial ischemia, including glycolysis and lipid metabolism [17]. Additionally, other studies have shown that CDDP has significant vasodilatory effects that can relieve angina pectoris and regulate atherosclerosis, with mechanisms related to mediating platelet activation, cGMP-PKG signaling pathways, and vascular smooth muscle contraction [18]. Consequently, CDDP has been widely used in China as a supplement to nitroglycerin [19] to improve symptoms of coronary atherosclerotic heart disease and angina pectoris.

This study demonstrates that urinary proteomics can comprehensively and systematically reflect the overall effects of traditional Chinese medicine on the body. This suggests that urinary proteomics has potential for application in pharmacological research of TCM and provides an innovative method for studying TCM efficacy.

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Note: Figure translations are in progress. See original paper for figures.