Untargeted serum metabolomics reveals Fu-Zhu-Jiang-Tang tablet and its optimal combination improve an impaired glucose and lipid metabolism in type II diabetic rats

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A B S T R A C T

Fu-Zhu-Jiang-Tang tablet, a six-herb preparation, was proved to show beneficial effects on type II diabetes patients in clinical. This study aims to optimize the component proportion of the six-herb preparation and explore the serum metabolic signatures of type II diabetes rats after treatment with Fu-Zhu-Jiang-Tang tablet and its optimal combination. The component proportion of the preparation was optimized using uniform experimental design and machine learning techniques. Untargeted GC–MS metabolomic experiments were carried out with serum samples from model group and treatment groups. Data were normalized, multivariate and univariate statistical analysis performed and metabolites of interest putatively identified. 23 metabolites were significantly changed by Fu-Zhu-Jiang-Tang tablet treatment and the majority of these were decreased, including various carbohydrates (glucose, mannose, fructose, allose and gluconic acid), unsaturated fatty acids (palmitic acid, 9-octadecenoic acid, oleic acid, arachidonic acid), alanine, valine, propanoic acid, 3-hydroxybutyrate, along with pyrimidine and cholesterol. Increased concentrations of oxalic acid, leucine, glycine, serine, threonine, proline, lysine and citrate were observed. In the optimal combination-fed group, 21 metabolites were significantly affected and strikingly, the magnitudes of changes here were generally much greater than that of Fu-Zhu-Jiang-Tang tablet treated rats. 18 metabolites affected in both groups included various carbohydrates (mannose, glucose, allose, fructose and gluconic acid), unsaturated fatty acids (palmitic acid, 9-octadecenoic acid, oleic acid and arachidonic acid), short-chain fatty acids (oxalic acid, 3-hydroxybutyrate), and amino acids (alanine, valine, leucine, glycine, proline and lysine), as well as pyrimidine. Metabolites exclusively affected in optimal combination treated rat included succinic acid, cysteine and phenylalanine, whilst four metabolites (propanoic acid, citrate, serine and threonine) were only altered in Fu-Zhu-Jiang-Tang tablet treated rat. Our investigation demonstrated Fu-Zhu-Jiang-Tang tablet and its optimal combination treatments were able to ameliorate impaired glucose and lipid metabolism, down-regulate the high level of glucose to a lower level and reverse abnormal levels of metabolites in serum of type II diabetes rats. However, the optimal combination treatment was able to maximize the magnitude of changes in some metabolites. These findings may be helpful in clarifying the anti-diabetic mechanism of FZJT tablet and its optimal combination.

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1. Introduction

Type II diabetes is a long term metabolic disorder that is characterized by high blood sugar, insulin resistance, and relative lack of insulin [1]. Since 1960, rates of type II diabetes have increased markedly in parallel with obesity. As of 2013, there were approximately 368 million people diagnosed with the disease compared to around 30 million in 1985 [2]. Type II diabetes is associated with a ten-year-shorter life expectancy. Current treatments for type II diabetes can improve glycaemia and some may even delay the onset of diabetes. However, several antidiabetic drugs are associated with adverse effects. For example, treatment with metformin led to gastrointestinal symptoms, whereas treatment with sulphonylureas or insulin resulted in increase in body weight [3]. Therefore, searching into traditional medicine in order to re-evaluate old remedies

to find new natural entities to be used as anti-diabetic products cannot be ignored.

Fu-Zhu-Jiang-Tang tablet, which was proved to show beneficial effects on patients with type II diabetes in clinical, was a six-herb preparation, including *Pueraia lobata* (PL), *Morus alba* (MA), *Panax notoginseng* (PN), *Astragalus membranaceus* (AM), *Momordica charantia* (MC) and *Lycium chinense* (CL). Until now, a number of articles have been published on the use of the six individual herbs for treating diabetes [4-7]. Our previous study characterized and determined 39 major constituents of Fu-Zhu-Jiang-Tang tablet using UHPLC-Q-TOF/MS and HPLC-UV [8]. However, the mechanism underlying the anti-diabetic effect of the six-herb preparation—FZJT tablets remains elusive.

Metabolomics is defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [9]. As a versatile tool, metabolic techniques have already been widely applied to detect chemical and environmental toxicology [10], diagnosis of carcinogenesis [11] and metabolic syndromes such as type II diabetes [12,13]. As a systemic approach, metabolomic adopts a ‘top-down’ strategy to reflect the function of organisms from terminal symptoms of metabolic network and understand metabolic changes of a complete system caused by interventions in holistic context. This research strategy is well coincident with the integrity and systemic feature of traditional Chinese medicine (TCM). Currently, it has been increasingly employed for assessing therapeutic effects and toxic effects of many herbal TCM and TCM prescriptions [14]. Although extensive reports have advocated the use of nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass spectrometry (LC–MS) for metabolomic study, gas chromatography coupled with mass spectrometry (GC–MS) is another commonly used technique for metabolomic analysis [15]. NMR technology tends to have low sensitivity and can detect only highly abundant analytes. The LC–MS method has inferior response and limitation of metabolite identification in detecting some small molecule compounds, such as fatty acids, amino acids and carbohydrates. In contrast, GC–MS can perform simultaneous analysis on numerous biochemicals including amino acids, organic acids, carbohydrates and fatty acids. Meanwhile, the availability of many structure databases can be the advantage for GC–MS based metabolomic study.

In this study, untargeted GC–MS based metabolomic approach was established to metabolic profiling of the serum signatures of Fu-Zhu-Jiang-Tang tablets and its optimal combination treated type II diabetes rats. Schematic diagram of the GC–MS based untargeted metabolomic approach was shown in Fig. 1. Biochemical measurement and pancreas histopathology study were carried out to ensure the success of the type II diabetes model. The GC–MS data were normalized, multivariate and univariate statistical analysis performed and metabolites of interest putatively identified. The metabolic pathways of Fu-Zhu-Jiang-Tang tablets and its optimal combination treated type II diabetes rats were elucidated based on data deconvolution and pattern recognition.

**2. Materials and methods**

2.1. Chemical and reagents

N-methyl-N-({trimethylsilyl})-trifluoracetamide(MSTFA) with 1% trimethyl- chlorosilane (TMCS), methoxylamine hydrochloride and pyridine were purchased from Shanghai Macklin Biochemical Co. L-proline, L-valine, L-serine, D-fructose, D-mannose, L-alanine, L-cysteine, L-leucine, L-lysine, L-threonine, oxalic acid and palmitic acid were purchased from Shanghai Ryon Biological Technology Co. Ribitol, glycine, cholesterol, citrate, and succinic acid were obtained from Sinopharm Chemical Reagent Co. n-Heptane was obtained from Aladdin industrial Co. Distilled water was produced by a Milli-Q Reagent Water System. Streptozotocin was purchased from Sigma Co.

The leaf of Moraceae *Morus alba* L. was collected in Jiaying City, Zhejiang Province (lot: 140801). The root of Leguminosae *Pueraia montana var. lobata* (Willd.) Sanjappa & Pradeep was collected in Taizhou City, Zhejiang Province (lot: 140701). The root and rhizome of Araliaceae *Panax notoginseng* (Burk.) F.H.Chen. were collected in Wenshan City, Yunnan Province (lot: 140801). The root of Leguminosae *Astragalus membranaceus* (Fisch.) Bunge. was collected in Wumeng City, Neimenggu Province (lot: 140301). The root bark of Solanaceae *Lycium chinense* Mill. was collected in Zhongning City, Ningxia Province (lot: 140801). The fruit of Cucurbitaceae *Momordica charantia* L. was collected in Guilin City, Guangxi Province (lot: 140702). All of these plants were authorized by an expert in the field. Voucher specimens have been deposited in the herbarium of Jiangsu Key Laboratory of Chinese Medicine Processing, Nanjing University of Chinese Medicine. The extraction procedures for *Morus Alba*, *Corixa chinensis* and *Astragalus membranaceus* were as follows: first, 1 kg of crude drug was extracted with water (×8) for twice to yield a crude extract. Second, the crude extract was concentrated and spray-dried to give the final product. For preparation of *Pueraia montana var. lobata* and *Panax notoginseng* extracts, 1 kg of crude drug was extracted with 60% ethanol-water (×8) for twice. After that, the extracts were combined together, concentrated and spray-dried to yield the final product. Apart from the extraction solvent (40% ethanol-water six times instead), the remaining procedures for preparation of *Momordica charantia* extract were the same as that of other extracts. Fu-Zhu-Jiang-Tang total fraction (lot: 150202) was donated by Research Center of By-Health Co. The proportion of *Momordica charantia* (MC), *Pueraia montana var. lobata* (PL), *Morus Alba* (MA), *Corixa chinensis* (CL), *Astragalus membranaceus* (AM) and *Panax notoginseng* (PN) in Fu-Zhu-Jiang-Tang total fraction was about 3:3:1:1:1:1. In this study, FZJT total fraction without pharmacological excipients instead of the tablets was employed for the animal experiment.

2.2. Animal handling

The ethical use of animals was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine, China. Male Sprague-Dawley rats (n=84), weighing 180±20 g, were obtained from Slaccas Experiment Animal Company (Shanghai, China), and maintained at a constant temperature (25±1 °C) on a 12 h light/12 h dark cycle with feeds and water were provided ad libitum. All the rats were fed with common pellet diets for 2 weeks after arrival, and after fasted for 3−4 h, blood samples were obtained from tail veins just prior to glucose administration (0) and 0.5, 1 and 2 h after glucose loading (2.5 g/kg b.w.), and values were estimated using the one-touch glucometer. Then the rats were randomly divided into fourteen groups: (1)Normal group: normal SD rats (n=6), (2)Model group: high-fat diet combined with STZ-induced diabetic rats (n=6), (3)FZJT tablets treated group: high-fat diet combined with STZ-induced diabetic rats treated with FZJT total fraction (540 mg/kg b.w., n=6), (4)Ten different combinations-fed group: high-fat diet combined with STZ-induced diabetic rats treated with ten different combinations of the six components of FZJT tablets (see Table 1), (5)Optimal combination-fed group: high-fat diet combined with STZ-induced diabetic rats treated with optimal combinations of six components (540 mg/kg b.w., n=6). This group was performed after other groups were accomplished. High-fat diets were consisted of normal pellet diet (52.6%), lard (10%), sucrose (15%), egg yolk powder (15%), casein (5%), choles.
terol (1.2%), sodium cholate (0.2%), calcium bicarbonate (0.6%) and calcium carbonate (0.4%).

All the rats had free access to food and water. After 4 weeks of high fat diets, the rats were subjected to a 12-h fast. Type II diabetes was induced by intraperitoneal injection of freshly prepared STZ solution dissolved in citrate buffer (pH 4.5) at the dose of 40 mg/kg b.w., and the rats continued to be fed with high-fat diets for one week. Then the blood glucose levels were determined, the rats were considered to be type II diabetic when fasting blood glucose levels exceeded 16.7 mmol/L. After that, type II diabetic rats were treated

Table 1
The significantly changed endogenous metabolites in serum by one-way ANOVA.

<table>
<thead>
<tr>
<th>No.</th>
<th>t_{25}(min)</th>
<th>Endogenous metabolites</th>
<th>Normal group</th>
<th>Model group</th>
<th>FZJT tablets treated group</th>
<th>Optimal combination-fedgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.445</td>
<td>Propanoic acid</td>
<td>4.255±0.279</td>
<td>5.130±0.822</td>
<td>3.832±0.698</td>
<td>5.619±0.681</td>
</tr>
<tr>
<td>2</td>
<td>8.383</td>
<td>Alanine</td>
<td>0.163±0.039</td>
<td>0.294±0.114</td>
<td>0.173±0.048</td>
<td>0.148±0.044</td>
</tr>
<tr>
<td>3</td>
<td>9.36</td>
<td>Oxalic acid</td>
<td>0.999±0.115</td>
<td>1.214±0.192</td>
<td>1.601±0.397</td>
<td>1.928±0.370</td>
</tr>
<tr>
<td>4</td>
<td>9.942</td>
<td>3-Hydroxybutyrate</td>
<td>0.662±0.118</td>
<td>1.005±0.359</td>
<td>0.312±0.050</td>
<td>0.352±0.053</td>
</tr>
<tr>
<td>5</td>
<td>11.789</td>
<td>Valine</td>
<td>0.550±0.158</td>
<td>0.772±0.182</td>
<td>0.484±0.117</td>
<td>0.569±0.075</td>
</tr>
<tr>
<td>6</td>
<td>12.587</td>
<td>Pyrimidine</td>
<td>0.323±0.070</td>
<td>0.323±0.047</td>
<td>0.274±0.015</td>
<td>0.260±0.033</td>
</tr>
<tr>
<td>7</td>
<td>13.224</td>
<td>Urea</td>
<td>3.480±0.297</td>
<td>3.063±0.101</td>
<td>3.198±0.169</td>
<td>3.118±0.661</td>
</tr>
<tr>
<td>8</td>
<td>14.609</td>
<td>Leucine</td>
<td>0.807±0.089</td>
<td>0.668±0.107</td>
<td>0.765±0.108</td>
<td>0.974±0.129</td>
</tr>
<tr>
<td>9</td>
<td>15.316</td>
<td>Glycine</td>
<td>0.370±0.051</td>
<td>0.312±0.036</td>
<td>0.439±0.047</td>
<td>0.401±0.146</td>
</tr>
<tr>
<td>10</td>
<td>15.544</td>
<td>Succinic acid</td>
<td>0.032±0.015</td>
<td>0.026±0.004</td>
<td>0.032±0.005</td>
<td>0.053±0.005</td>
</tr>
<tr>
<td>11</td>
<td>16.845</td>
<td>Serine</td>
<td>0.212±0.084</td>
<td>0.241±0.036</td>
<td>0.159±0.085</td>
<td>0.259±0.098</td>
</tr>
<tr>
<td>12</td>
<td>17.395</td>
<td>Threonine</td>
<td>0.206±0.064</td>
<td>0.250±0.022</td>
<td>0.200±0.012</td>
<td>0.223±0.015</td>
</tr>
<tr>
<td>13</td>
<td>18.815</td>
<td>β-aminoisobutyric acid</td>
<td>0.017±0.005</td>
<td>0.027±0.082</td>
<td>0.009±0.008</td>
<td>0.043±0.007</td>
</tr>
<tr>
<td>14</td>
<td>19.507</td>
<td>Proline</td>
<td>0.543±0.041</td>
<td>0.444±0.056</td>
<td>0.551±0.041</td>
<td>0.551±0.100</td>
</tr>
<tr>
<td>15</td>
<td>20.030</td>
<td>Cysteine</td>
<td>0.089±0.019</td>
<td>0.089±0.018</td>
<td>0.093±0.011</td>
<td>0.118±0.033</td>
</tr>
<tr>
<td>16</td>
<td>20.943</td>
<td>Phenylalanine</td>
<td>0.111±0.0018</td>
<td>0.102±0.014</td>
<td>0.112±0.013</td>
<td>0.144±0.028</td>
</tr>
<tr>
<td>17</td>
<td>21.822</td>
<td>Fructose</td>
<td>0.142±0.020</td>
<td>0.155±0.035</td>
<td>0.127±0.014</td>
<td>0.112±0.016</td>
</tr>
<tr>
<td>18</td>
<td>23.369</td>
<td>Citrate</td>
<td>0.073±0.052</td>
<td>0.080±0.029</td>
<td>0.110±0.052</td>
<td>0.068±0.037</td>
</tr>
<tr>
<td>19</td>
<td>23.678</td>
<td>Lysine</td>
<td>0.225±0.037</td>
<td>0.152±0.026</td>
<td>0.212±0.029</td>
<td>0.249±0.059</td>
</tr>
<tr>
<td>20</td>
<td>24.147</td>
<td>Mannose</td>
<td>0.436±0.139</td>
<td>3.052±1.432</td>
<td>0.560±0.390</td>
<td>0.475±0.160</td>
</tr>
<tr>
<td>21</td>
<td>24.279</td>
<td>d-Allose</td>
<td>27.683±3.553</td>
<td>89.781±7.83</td>
<td>38.431±3.764</td>
<td>37.684±8.709</td>
</tr>
<tr>
<td>22</td>
<td>24.45</td>
<td>Glucose</td>
<td>5.557±0.404</td>
<td>19.999±3.57</td>
<td>7.459±0.799</td>
<td>7.460±1.792</td>
</tr>
<tr>
<td>23</td>
<td>25.05</td>
<td>Gluconic acid</td>
<td>0.424±0.204</td>
<td>4.642±2.069</td>
<td>0.633±0.607</td>
<td>0.389±0.200</td>
</tr>
<tr>
<td>24</td>
<td>25.375</td>
<td>Palmitic acid</td>
<td>0.603±0.034</td>
<td>0.972±0.241</td>
<td>0.532±0.062</td>
<td>0.543±0.038</td>
</tr>
<tr>
<td>25</td>
<td>26.937</td>
<td>0-Octadecenoic acid</td>
<td>0.631±0.132</td>
<td>0.907±0.245</td>
<td>0.496±0.064</td>
<td>0.404±0.077</td>
</tr>
<tr>
<td>26</td>
<td>27.158</td>
<td>Oleic acid</td>
<td>0.576±0.176</td>
<td>0.622±0.154</td>
<td>0.428±0.020</td>
<td>0.522±0.223</td>
</tr>
<tr>
<td>27</td>
<td>28.247</td>
<td>Arachidonic acid</td>
<td>0.147±0.061</td>
<td>0.148±0.055</td>
<td>0.108±0.016</td>
<td>0.094±0.007</td>
</tr>
<tr>
<td>28</td>
<td>33.081</td>
<td>Cholesterol</td>
<td>0.543±0.112</td>
<td>0.907±0.334</td>
<td>0.643±0.055</td>
<td>0.483±0.202</td>
</tr>
</tbody>
</table>

* P<0.05 vs. normal group.
* P<0.05 vs. model group.
* P<0.05 vs. FZJT tablets treated group.

for eight weeks as described above. In this period, the rats continued to be fed with high-fat diets.

2.3. Optimization the proportion of the six components

Uniform design (UD) method makes experiment points uniformly scattered in the range of experiment parameters for getting more information by less experiment. Similar to orthogonal experimental design (OD), UD defines experiment points by some specially designed UD tables, the details of which can refer to ref. [16]. But compare to OD, UD just takes uniform into consideration, so its points have better representative. UD has widely application scopes in traditional experimental design and compound design. In this study, the uniform experiment design was adopted to optimize the proportion of six components derived from FJZT tablets. As shown in Table S1, ten combinations (U10*) of the six components were designed for the animal experiment.

Machine learning techniques were employed in modeling composition–activity relationship, including multiple linear regressions (MLR) and radical function artificial neural network (RBFANN). Multiple linear regression (MLR) attempts to model the relationship between two or more explanatory variables and a response variable by fitting a linear equation to observed data [17]. Every value of the independent variable x is associated with a value of the dependent variable y. Radial basis function network is an artificial neural network that uses radial basis functions as activation functions. The output of the network is a linear combination of radial basis functions of the inputs and neuron parameters. Radial basis function networks have many uses, including function approximation, time series prediction, classification, and system control [17]. Two algorithms were implemented by in-house program in MATLAB 7.1 (Mathwork Inc., Natick, MA, USA).

2.4. Sample collection and preparation

After treatment with FJZT tablets or ten different combinations of the six components for 8 weeks, the rats were subjected to a 12-h fast. Upon anesthetizing using pentobarbital, abdominal aorta blood samples were collected from the rats. The blood samples were divided into two aliquots. Serum was obtained from one aliquot by centrifugation at 3000 rpm for 15 min after the blood samples clotted, then analyzed using a Hitachi 7100 automated biochemical analyzer (Hitachi Co., Japan) to test for HDL, LDL, TG, TC, HbA1c and insulin. The other blood samples were collected using 5 mL anticoagulant tubes. Tubes were vortexed to mix the anticoagulant with the blood, then centrifuged at 3000 rpm for 15 min. The obtained serum was transferred into Eppendorf tubes and stored at −80 °C for metabolic analysis.

The pretreatment procedure for serum samples were as follows: 100 μL aliquots of serum was spiked with the internal standard (5 μL of 1 mg mL−1 of ribitol), followed by adding precipitation solvents (200 μL) into the tube. After vigorous shaking for 1 min and incubation on ice for 10 min, the mixture was centrifuged at 4500g for 15 min to precipitate the protein. 200 μL aliquots of supernatant was transferred into a GC vial and evaporated to dryness by using nitrogen blowing device at room temperature. The derivatization was carried out using methoxylamine hydroxide (75 μL, 15 mg mL−1) at 70 °C for 1 h, followed by MSTFA (75 μL) with 1% TMCS as catalyst at room temperature for 1 h 150 μL aliquots of n-Heptane was added to dilute the solution after the derivatization and the supernatant was sent for GC–MS analysis.

2.5. Oral glucose tolerance

The rats in all groups were fasted for 12 h and then administered glucose (2.5 g/kg b.w.) orally. Blood samples were obtained from tail veins just prior to glucose administration and 0.5, 1, 2 h after glucose loading, and values were estimated using the one-touch glucometer. The rats were considered to be type II diabetic when fasting blood glucose levels exceeded 16.7 mmol/L.

2.6. Serum biochemistry

Serum biochemical analyses were performed on an automatic biochemical analyzer including total cholesterol (TC), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) and triglyceride (TG), HbA1c and insulin levels. One-way ANOVA was conducted to compare the clinical biochemical data from different groups.

2.7. Pancreas histopathology

The typical pancreas tissues in the normal group, model group, FJZT tablets treated group and optimal combination-fed group were fixed in 10% buffered formalin and then embedded in paraffin wax. Sections with the thickness of 5 μm were obtained with a microscope and then stained with the routine hematoxylin and eosin (H&E) method for assessments. The pictures were taken with 200-fold magnification.

2.8. GC–MS analysis and method validation

The system consisted of an Agilent 5975C Series mass spectrometer coupled to an Agilent 7890A gas chromatography (Agilent Technologies, Atlanta, GA, USA) with an autosampler. A 2.0 μL of sample solution was injected with splitless mode to HP-5MS column (30 m × 0.25 mm) with helium as the carrier gas at a flow of 0.8 mL min−1. The injector temperature was set at 270 °C. The temperature of the ion source was adjusted to 230 °C and that of quadrupole was set at 150 °C. Electron ionization mass spectra were recorded at 70 eV, 2 scan s−1. Full-scan mass spectra were acquired from m/z 60 to 600 with a scan rate of five times per second. The initial temperature of column was kept at 70 °C for 5 min. Then the temperature was ramped at 10 °C min−1 to 100 °C where it was held for 5 min, and then to 250 °C at a rate of 10 °C min−1. Subsequently, the temperature was held at 250 °C for 10 min.

To evaluate linearity of presented method, sixteen stock solutions of standard compounds (~1.000 g L−1) were prepared and then diluted to relative concentrations of 0.500, 0.250, 0.125, 0.0625, and 0.03125 g L−1. Pretreatment and derivatization were performed according to sample preparation section. After GC–MS analysis, the intensities of endogenous metabolites were normalized by internal standard. The normalized intensities were used to calculate linear correlation coefficients. The lower limit of quantification (LLOQ) was determined based on at least 10 times of signal-to-noise ratio.

For intra-day precision, the relative standard deviation (R.S.D.) of six independently processed replicates was calculated. All the samples were treated as described above. The repeatability of the proposed method was determined by injection of six parallel QC sample solutions. For the inter-day assay precision and accuracy, six replicates of QC samples were analyzed daily for 3 days. The repeatability of the proposed method was determined by injection of six parallel sample solutions. The freeze-thaw stability of samples was evaluated by comparing the stored samples with freshly prepared samples. Briefly, the serum samples were frozen in refrigerator at −80 °C and then thawed after 6 h. Blank serum samples spiked with known amounts of the standard compounds at low, medium and high levels were prepared and analyzed. The average recoveries of the method were estimated by the formula: Recovery.
(%) = (amount found – original amount)/amount spiked × 100%, and R.S.D. (%) = (S.D./mean) × 100%.

2.9. Multivariate analysis

First, the raw data were acquired and aligned using the Agilent ChemStation software package based on the m/z value and the retention time. All of the peak areas in each sample were normalized to the peak area of internal standard ribitol. The data matrix from GC–MS was imported into the SIMCA-P program (version 11.5, Umetrics) for multivariate analysis. Principal component analysis (PCA) was used to obtain an overview of variations among the different groups. Two strategies were employed to define the potential differential metabolites for distinguishing the FZJT tablets treated group and normal group, including multivariate and univariate methods. In the univariate method, the relative amounts of metabolites in the different groups were compared by one-way ANOVA, and those with a P < 0.05 were chosen. For the multivariate mode, a PLS-DA S-plot was used to find the ions in the GC–MS data with a variable importance in the projection (VIP) of more than 0.75. The parameters of the models, such as the R²X, R²Y, and Q²Y were analyzed to ensure the quality of the multivariate models and to avoid the risk of over-fitting. Identification of the interested peaks was performed by searching in NIST v1.0.0.12 mass spectra library and comparing with the peaks of standards.

The Heml 1.0 software packages were used to make a heat map to represent the relative amount of differential metabolites, and to analyze their hierarchical cluster. A Spearman correlation analysis was performed to disclose the associations between differential metabolites. The correlation network was constructed using the Cytoscape 3.3.0 software package. Highly correlated metabolites ([correlation coefficient] > 0.5) are connected with a line.

3. Results

3.1. Optimization the proportion of six components

The uniform experiment design was adopted to optimize the proportion of the six components in FZJT tablets. Ten herbal combinations were designed and selected for the animal experiment. As shown in Fig. S1A, after treatment with the ten combinations, the glucose tolerance of the type II diabetes rats were alleviated. To model the composition-activity relationship, the combination of the six components can be represented by a vector \([X_1, X_2, X_3, X_4, X_5, X_6]\) and the glucose concentration at 0.5 h after glucose load was set as \(Y\). Therefore, the relationship between biological activity and chemical composition of the herbal combinations can be represented by a mathematic function: \(Y = f(X)\). Based on the experimental data set, multiple linear regression algorithms were employed to establish a linear model below:

\[
Y = 19.43 + 0.39X_1 - 0.47X_2 - 0.20X_3 - 0.52X_4 - 0.26X_5
\]

In the upper function, \(Y\) is glucose concentration at 0.5 h after glucose load. \(X_1, X_2, X_3, X_4\) and \(X_5\) represent the proportion of components MC, PL, LC, PN and MA in the combination, respectively. The predictive result of MLR model v.s. actual result is illustrated in Fig. S2A. As shown in Table S2, the mean error of prediction (MEP, \(-24.52\%\)) and relative standard error of prediction (RSEP, \(-6.45\%\)) of this linear model are relatively high, which indicated that linear model may not properly map the sophisticated relationship between the ten combinations and their bioactivity. Subsequently, one non-linear machine learning technique (i.e. radial basis artificial neural network) was employed to establish the model. In RBFANN implementation, radius (spread) value will greatly affect the accuracy of model. According to computational results of cross-validation, optimal radius value for the RBFANN was 0.55. The predictive result of RBFANN v.s. actual value is illustrated in Fig. S2B. As shown in Table S2, RBFANN model have smaller MEP (~10.7%) and RSEP, which was near 1.3%. This result reveals that the non-linear approach is more suitable to develop the model. Furthermore, RBFANN model was applied to search for the optimal proportions of the six components. The optimal proportion for MC, PL, LC, PN, MA and AM in the preparation were calculated as 1.443: 1.986: 0.186: 2.671: 1.414: 2.300. At this proportion, the optimal glucose concentration at 0.5 h was calculated as 7.71, which were in consistent with the actual value 7.45.

3.2. Biochemical measurements and tissue histopathology

As shown in Fig. S3, fasting blood glucose of diabetic rats was significantly higher than that of normal rats. After the oral dose of glucose, the blood glucose levels in type II diabetic rats remained higher even after 2 h. FZJT tablets and its optimal combinations treatment resulted in significantly decrease in serum glucose level. The hypoglycemic activity of the optimal combination demonstrated almost an equal to that of the FZJT tablets in type II diabetic rats.

In the case of diabetes, the levels of TC, TG, HbA1c, insulin and LDL in the type II diabetic rats were significantly increased, whereas the levels of HDL were significantly decreased, when compared to the normal rats (see Fig. S4). The insulin level of model group was elevated due to the insulin resistance compensation. High-density lipoprotein was responsible for removing fat molecules from cells which want to export fat molecules. These fats carry include cholesterol, phospholipids and triglycerides. Decreasing concentrations of HDL particles are strongly associated with increasing accumulation of fat molecules within the walls of arteries [18]. The blood TC, TG, LDL insulin and HbA1c levels were significantly reduced (p < 0.05) in rats after FZJT tablets and its optimal combinations treatment, while HDL levels were elevated, when compared to the model group (see Fig. S4). In addition, HDL level of the optimal combination-fed group was significantly increased, when compared to the FZJT tablets treated group. The results demonstrated that the optimal combination-fed group had almost an equal to that of the FZJT tablets treated group in reducing the blood lipids level.

Histology of pancreas in experimental rats was assessed after eight weeks of treatment. Normal islets with normal cellular characteristics were observed for the normal group (see Fig. 2A). Fig. 2B displayed degenerative and necrotic changes with shrunk islets of Langerhans in the diabetic group. The black arrows in Fig. 2B indicated severe inflammatory infiltration. Pancreatic tissue of FZJT tablets treated diabetic rat presented the restoration of the normal cellular population and size of islets (see Fig. 2C) and mild degrees of inflammatory infiltration. As shown in Fig. 2D, tissues from the optimal combination-fed group illustrate shrunk islets of Langerhans and mild degrees of inflammatory infiltration.

3.3. Metabolic profiling of serum samples

Total ion chromatograms (TICs) of serum samples from normal group, model group, FZJT tablets treated group and optimal combination-fed group were shown in Fig. 3. For validation of the developed method, 16 metabolites were validated with authentic standards. The peak areas of these standard compounds were used to calculate linear correlation coefficients in concentration interval. As shown in Table S3, the linearity of those compounds was generally high \((r^2 > 0.99)\). Six injections of a sample were carried out to validate the precision of injection. The intra-day precision of the proposed method was less than 10.7%. The inter-day precision was evaluated by analyzing six portions of sample that were prepared twice a day on consecutive three days, and the result was less
than 9.8% (see Table S4). The repeatability of the proposed method was determined by injection of six parallel treated samples, and the R.S.D was less than 9.8%. Meanwhile, the freeze-thaw cycles induced less than 9.1% alteration of the relative intensity of each peak. The developed method had good accuracy with the recoveries ranging from 91.10% and 106.82% (see Table S5). Our results demonstrated that the method was sensitive, precise, and accurate enough for metabolomic analysis.

To analyze the differences in the metabolic profile, GC–MS data from different groups were imported into the SIMCA-P 11.5 software Package. 3D PCA score plots in Fig. S5 revealed the ten combinations-fed groups (green dots) surrounded the normal group (blue dots), while the optimal combination-fed group (gold dots) and the normal group were overlapped, indicating that the optimal combination treatment tended to shift the endogenous metabolites to the normal state. 3D PCA score plots in Fig. S6 demonstrated the optimal combination-fed group (gold dots) and the FZJT tablets treated groups (red dots) were overlapped, which meant the metabolic states of FZJT tablets treated group and the optimal combination-fed group were close to each other.

Subsequently, partial least squares-discrimination analyses (PLS-DAs) were applied to the classification of normal group, model
group, FZJT tablets treated group and optimal combination-fed group. The PLS-DA score plot in Fig. 4 showed the model group (black dots) was clearly separated from the normal group (blue dots). The cumulative R$^2$Y and Q$^2$ of the model were 0.958 and 0.853, while the R$^2$Y-intercepts and Q$^2$-intercepts were 0.457 and $-0.198$, respectively (see Table S6). There were also distinct differences between the FZJT tablets treated group (red dots) and the model group (see Fig. 5A). The cumulative R$^2$Y and Q$^2$ of the model were 0.955 and 0.897, respectively, whereas the R$^2$Y-intercepts and the Q$^2$-intercepts were 0.381 and $-0.215$ (see Table S6), respectively. No overfitting was observed according to the results of 200 times of chance permutation (see Fig. S7A and Fig. S7B). Moreover, the score plot of the PLS-DA model demonstrated a significant difference in the metabolic profiles between the optimal combination-fed group (yellow dots) and the model group (see Fig. 5B). A remarkable difference in the metabolic profile of between optimal combination-fed group (yellow dots) and the FZJT tablets treated group (red dots) was also observed (see Fig. 6). The cumulative R$^2$Y and Q$^2$ of the model were 0.944 and 0.836, while the R$^2$Y-intercepts and the Q$^2$-intercepts were 0.887 and 0.425 (see Table S4), respectively. The permutation curves also showed no overfitting of the model (see Fig. S7C and Fig. S7D).

3.4. Differential metabolites in different groups and their comparisons

Two strategies were employed to define the potential differential metabolites for distinguishing the FZJT tablets treated group and normal group, including multivariate and univariate methods. In the univariate method, the relative amounts of metabolites in the different groups were compared by one-way ANOVA, and those with a P < 0.05 were chosen (see Table 1). For the multivariate mode, a PLS-DA model was used to find the ions in the GC–MS data with a variable importance in the projection (VIP) of more than 0.75 and the results were shown in Table S7. These metabolites were tentatively identified by comparing with the NIST v1.0.0.12 database and standard compounds.

After integrating the data, 19 differential metabolites were observed (P < 0.05, or VIP > 0.75) in model group, as compared with the normal group (see Table S7). 23 metabolites were significantly changed after FZJT tablets treatment and the majority of these were decreased, including various carbohydrates (glucose, mannose, fructose, allose and gluconic acid), unsaturated fatty acids (palmitic acid, 9-octadecenoic acid, oleic acid, arachidonic acid), alanine, valine, propanoic acid, 3-hydroxybutyrate, along with pyrimidine and cholesterol. Increased concentrations of oxalic acid, leucine, glycine, serine, threonine, proline, lysine and citrate were observed. In the optimal combination-fed group, 21 metabolites were sig-

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nificantly affected and strikingly, the magnitudes of changes here were generally much greater than that of Fu-Zhu-Jiang-Tang tablet treated group. 18 metabolites affected in both groups included various carbohydrates (mamnose, glucose, allose, fructose and gluconic acid), unsaturated fatty acids (palmitic acid, 9-octadecenoic acid, oleic acid and arachidonic acid), short-chain fatty acids (oxal acid, 3-hydroxybutyrate), and amino acids (alanine, valine, leucine, glycine, proline and lysine), as well as pyrimidine. Metabolites exclusively affected in optimal combination-fed group included succinic acid, cysteine and phenylalanine, whilst four metabolites (propanoic acid, citrate, serine and threonine) were only altered in FZJT tablets treated group.

To analyze these differential metabolites globally, the Heml 1.0 software packages were used to make a heat map according to a ratio of the content of each metabolite in each group to the highest content of this metabolite in four groups (see Fig. 7). Meanwhile, high correlated metabolites were visualized by Cytoscape 3.3.1 software packages according to their spearman correlation coefficients (see Fig. S8). Highly positive correlated metabolites are connected with a blue line, whereas high negative correlated metabolites are connected with a red dashed line. Overall, treatment with FZJT tablets and its optimal combination resulted in significant changes of endogenous metabolites in type II diabetes rats (Fig. 8).

4. Discussion

4.1. Optimization the proportion of the six components

Fu-Zhu-Jiang-Tang tablet is an effective proved herbal prepa-
ration on treating type II diabetes in clinical. The chemical basis for the six-herb preparation was very clear. Our previous study identified 39 compounds in FZJT tablets using UHPLC-Q-TOF- MS, including 13 flavonoids, 12 fatty acids, 8 triterpenoid saponins, 2 phenols and 4 unknown compounds [8]. Among them, 17 compounds were confirmed by comparing with standards. Moreover, an HPLC-UV method was developed for quantitative determination of ten major compounds in FZJT tablets, including 3′-hydroxypropuerarin, puerarin, 3′-methoxypuerarin, daidzin, rutin, astragalin, daidezin, ginsenoside Rg1, astragaloside IV and 20(S)-ginsenoside Rg3.

The proportion of the six components of FZJT tablets were arranged by clinical experience, which may not be the optimal. Therefore, uniform experimental design is employed to optimize the proportion of the six components. Uniform design is based on uniform distribution in number theory. It makes experiment points uniformly scattered in the range of experiment parameters for getting more information by less experiment [19], which can save cost and enhance efficiency for animal experiment. The glucose concentration at 0.5 h after glucose load was selected as the optimization target, which revealed the glucose tolerance of the rats after treatment.

The data of the uniform design experiment was modeled by both linear and non-linear machine learning techniques. Compared with the linear model using multiple linear regression algorithm, the non-linear model employing radial basis artificial neural network algorithm showed smaller mean error of prediction (MEP) and relative standard error of prediction (RSEP), indicating RBFANN was superior to MLR in modeling the combination-activity relationship. Therefore, RBFANN model was used to search for the optimal combination with minimal value of glucose concentration at 0.5 h. The optimal proportion of MC, PL, LC, PN, MA and AM in the preparation were calculated as 1.443: 1.986: 0.186: 2.671: 1.414: 2.300, which was different from the initial proportion (about 3:3:1:1:1:1) of FZJT tablets. Validation experiment was also carried out to ensure the anti-diabetic effects of the optimal combination. The real glucose concentration at 0.5 h after glucose load was in well agreement with that of the calculated value. Moreover, the levels of other biochemical parameters such as TC, HDL, LDL, TG, HbA1c and insulin in the optimal combination-fed group were comparable with that of the FZJT tablets treated group, indicating FZJT tablets and its optimal combination treatment improve glucose and lipids metabolism of type II diabetic rats in a similar manner.

4.2. Differential metabolites in different groups

Metabolomic approach was employed to monitor the dynamic changes in the endogenous metabolites of serum from different experimental groups. Streptozotocin is a naturally occurring che-
mal that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals, which is a routine agent for preparation of diabetes model[20]. The success of the type II diabetes model was evidenced by the fasting blood glucose levels above 16.7 mmol/L and degenerative and necrotic changes with shrunken islets of Langerhans. Except for several amino acids (e.g. leucine, glycine, proline, phenylalanine and lysine), urea and succinic acid, all other metabolites including carbohydrates (e.g., glucose, fructose, mannose and D-αllose), lipids (e.g., palmitic acid, 9-octadecenoic acid, and cholesterol), amino acids (e.g., alanine, valine, serine, thre-
nine), ketone body (e.g., 3-hydroxybutyrate), short chain fatty acids (e.g., propanoic acid, oxalic acid) were elevated in serum of the model group. These metabolomic alterations were related to suppression of glycolysis and TCA cycle, disruption of lipid metabolism, and promotion of ketogenesis, which were consistent with the previous reports [13].

4.3. Biochemical interpretation

Carbohydrates are the major source of fuel for metabolism, being used as an energy source (glucose being the most important in nature). The elevation of carbohydrates such as glucose, fructose, allose and mannose in model group is associated with the high fat diet and STZ-caused insulin resistance. After treatment with FZJT tablets treated group and its optimal combination, the levels of glucose, fructose, allose and mannose were significantly reduced. However, the serum insulin concentration was down-regulated. This phenomenon can be explained by the enhancement of insulin sensitivity of peripheral tissues. Meanwhile, the compo-
ent of Momordica charantia has been reported to increase insulin secretion of the pancreas, decrease intestinal glucose uptake, and increase uptake and utilization of glucose in peripheral tissues, which may also contribute to the decreased carbohydrate level after FZJT tablets and its optimal combination treatment. Glucose can be oxidized to pyruvate via glycolysis to provide acetyl-CoA for TCA cycle. Oxalic acid is a competitive inhibitor of the lactate dehydro-
genase enzyme (LDH) in the glycolytic pathway, which catalyses the conversion of pyruvate to lactic acid. The levels of oxalic acid were dramatically elevated in FZJT tablets and its optimal combi-
nation treated group, which led to the reduced production of lactic acid. Propanoic acid, being a three-carbon molecule, can be oxys-
dized to yield propionyl-CoA, a precursor for succinyl-CoA, which can be converted to pyruvate and enter into gluconeogenesis. The level of propanoic acid was elevated in model group, which may promote gluconeogenesis.

Fatty acid molecules are broken down in the mitochondria and undergo beta-oxidation to generate acetyl-CoA, which enters the TCA cycle. Cholesterol and unsaturated fatty acids including palmitic acid, 9-octadecenoic acid and oleic acid were significantly elevated in model group. Compared with the normal group, the LDL level was up-regulated, whereas the HDL level was down-regulated in the model group, which were consistent with that in the literature [13]. The dysfunction of insulin inevitably causes sig-

significant dyslipidemia including both lipoproteins and fatty acids [21]. The level of arachidonic acid was unchanged in type II diabetic rats; however, it was significantly down-regulated after FZJT tablets and its optimal combination treatment. Under normal metabolic conditions, arachidonic acid will not cause inflammation unless lipid peroxidation products are mixed in. As a precursor, arachidonic acid is metabolized to both proinflammatory and anti-inflammatory eicosanoids after the inflammatory response [22]. Histology of pancreas revealed that the degree of inflammatory infiltration was alleviated by FZJT tablets and its optimal combination treatment, which was in agreement with the down-regulation of inflammatory precursor arachidonic acid.

Citric acid was an intermediate in the TCA cycle, a central metabolic pathway for production of ATP in animals. The entire set of reactions of the TCA cycle takes place in mitochondria. Citric acid can be transported out of the mitochondria and into the cytoplasm, and then broken down into acetyl-CoA for fatty acid synthesis and into oxaloacetate [23]. No significantly difference was observed for the citric acid levels between the normal and model group. However, the level of citric acid was significantly elevated in the FZJT tablets treated group, indicating FZJT tablets treatment could supply of precursor molecules for TCA cycle. Succinic acid serves as an electron donor to the electron transport chain and was another intermediate in the TCA cycle. Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Compared with the normal group, the level of succinic acid was reduced in the model group, which implied the impaired activity of TCA cycle. However, the level of succinic acid was dramatically up-regulated in the optimal combination-fed group. The elevation of succinic acid led to the activation of TCA cycles and promotion of catabolism.

Ketone bodies including acetoacetate, 3-hydroxybutyrate and acetone are usually overproduced in diabetic rats. The impaired insulin sensitivity, combined with the inappropriately high glucagon concentrations, induce the liver to produce glucose at an inappropriately increased rate, causing acetyl-CoA resulting from the beta-oxidation of fatty acids, to be converted into ketones bodies. The occurrence of high levels of ketone bodies in the blood of uncontrolled type II diabetes is known as ketosis, and in its extreme form in out-of-control type II diabetes, as ketoacidosis [24]. Compared with the normal group, the level of 3-hydroxybutyrate were significantly elevated in the model group. After treatment with FZJT tablets and its optimal combination, a significant reduction in the levels of 3-hydroxybutyrate was observed, which was ascribed to the improved insulin sensitivity and decreased ketogenesis.

Phenylalanine is a direct precursor to fumarate and acetylacetyl-CoA, which can be converted into ketone bodies, for example, 3-hydroxybutyrate. The level of phenylalanine in the optimal combination-fed group was significantly elevated. However, ketogenesis was reduced in the optimal combination-fed group. The accumulation of phenylalanine may provide adequate precursors to fumarate in the TCA cycle.

Gluconeogenesis takes place mainly in the liver. The glucose produced passes into the blood to supply other tissues. Alanine is an important gluconeogenic precursor and plays a key role in glucose-alanine cycle between tissues and liver [25]. The level of alanine was significantly increased in model group, which was suggestive of enhanced activity of gluconeogenesis. The levels of
alanine were restored to the normal level by FZJT tablets and its optimal combination treatment. Glycine, serine and threonine are precursors of glucose, because they can be converted to pyruvate. The three amino acids were high correlated. The levels of the three amino acids were elevated in FZJT tablets and its optimal combination treated group, which may supply of precursors for gluconeogenesis. Valine can be converted to succinyl-CoA. Proline can be converted to \( \alpha \)-ketoglutarate. Both succinyl-CoA and \( \alpha \)-ketoglutarate were citric acid cycle intermediates. In the model group, the level of valine was up-regulated, while the level of proline was down-regulated. After treated with FZJT tablets and its optimal combination, the levels of valine and proline were restored to normal levels. Leucine and lysine, which are degraded entirely or in part to acetoacetyl-CoA and/or acetyl-CoA, can yield ketone bodies in the liver. These are the ketogenic amino acids. Their ability to form ketone bodies is particularly evident in uncontrolled diabetes mellitus, in which the liver produces large amounts of ketone bodies from both fatty acids and the ketogenic amino acids. The levels of leucine and lysine were significantly reduced in the model group, as compared to the normal group, indicating they were consumed to be transformed into ketone bodies. After treated with FZJT tablets and its optimal combination, the levels of leucine and lysine were restored to normal levels, indicating the reduced activity of ketogenesis.

Urea serves an important role in the metabolism of nitrogen-containing compounds. Being practically neutral and highly soluble in water, urea is a safe vehicle for the body to transport and excrete excess nitrogen. The decrease of urea level in serum of model group may be due to the polyuria, which was one of the routine complications of type II diabetes [26]. Although classified as a non-essential amino acid, cysteine may be essential for metabolic disease. Due to the ability of thiol to undergo redox reactions, cysteine has antioxidant properties. Cysteine’s antioxidant properties are typically expressed in the tripeptide glutathione, which occurs in organisms. The level of cysteine was significantly increased in optimal combination-fed group, as compared to the model group, which may protect internal environment from oxidative stress.

Collectively, the FZJT tablets and its optimal combination treatment were able to reverse abnormal levels of metabolites, which were related to carbohydrates and lipid metabolism, TCA cycle and ketogenesis. However, compared with FZJT tablets treatment, the optimal combination treatment was able to up-regulate the levels of succinic acid, propanoic acid, phenylalanine, leucine, and cysteine to a higher level, and down-regulate the level of fructose, arachidonic acid and cholesterol to a lower level. These subtle variations revealed the different regulation mechanism of FZJT tablets and its optimal combination.

5. Conclusions

An untargeted metabolomic approach based on GC–MS and multivariate statistical technique has been successfully applied to investigate the regulation mechanism of FZJT tablets and its optimal combination on type II diabetes rats. The PLS-DA scores plot showed the complete distinction of normal group, model group, FZJT tablets treated group and its optimal combination-fed group. Furthermore, the serum levels of metabolites such as glucose, palmitic acid and so on were brought to the normal level by FZJT tablets and its optimal combination treatment. These results indicated the FZJT tablets and its optimal combination exerted antidiabetic effects through regulation of carbohydrates metabolism, lipid metabolism, TCA cycle and ketogenesis pathway. Meanwhile, the optimal combination treatment was able to maximize the magnitudes of changes in some metabolites. The study demonstrates that the untargeted metabolomics based on GC–MS and multivari-

ate method is a promising tool in elucidation of the regulation mechanism of TCM preparation.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

All procedures performed in studies were in accordance with the ethical standards of Animal Ethics Committee of Nanjing University of Chinese Medicine and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.11.012.

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