Original Article

Tissue metabolic changes in rats after administration of Aidi injection by GC-MS

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Abstract: Aidi injection has various pharmacological effects, including tumor angiogenesis inhibition, induction of apoptosis in tumor cells, enhancement of immunity, and relief of chemotherapy-related side effects. In this study, we developed a tissue (liver, kidney and heart) metabolomics method using gas chromatography-mass spectrometry (GC-MS) to evaluate the effect of Aidi injection on rats. The Sprague-Dawley rats were divided into three groups, namely the control (8 mL/kg/day normal saline) group, Low-dose (5 mL/kg/day) Aidi injection-treated group and High-dose (10 mL/kg/day) Aidi injection-treated group, 7 rats per group. Rats were given intraperitoneal injection for 7 days. Tissue (liver, kidney, heart) samples were collected from rats of the 3 groups after 7 day. The tissue samples were protein precipitated with acetonitrile and derivatized for GC-MS analysis. Partial least squares-discriminant analysis (PLS-DA) revealed that Aidi injection induced metabolic perturbations. Compared to the control group, the metabolites (urea, glycerol, hexadecanoic acid, [9, 12] octadecadienoic acid, arachidonic acid, d-ribose, cholesterol, galactose, glucitol, 9h-purine, threitol, tetradecanoic acid) were changed in the Aidi group. The changes of metabolites indicated that Aidi injection-treated rats exhibited induced fatty acid metabolism, energy metabolism, amino acid metabolism, nucleotide metabolism, and urea cycle perturbations. This is the first report to characterize the tissue metabolic changes in rats treated with Aidi injection.

Keywords: Metabolomics, liver, kidney, heart, PLS-DA, Aidi

Introduction

Chinese herbal medicine is widely used to treat cancer, and it has become one of the main ways for cancer comprehensive treatment program. Strengthening the body resistance to eliminate pathogenic factors is a basic principle of Chinese herbal medicine in the treatment of cancer [1]. Aidi injection, a traditional Chinese medicine, has various pharmacological effects, including tumor angiogenesis inhibition, induction of apoptosis in tumor cells, enhancement of immunity and relief of chemotherapy-related side effects [2, 3]. Aidi injection is made from an extraction of Renshen (Radix Ginseng, Araliaceae), Huangqi (Astragalus membranaceus (Fisch.) Bunge, Papilionaceae), Ciwujia (Radix et Caulis Acanthopanacis Senticosoi, Araliaceae) and Banmao (Lytta Vesicatoria, Meloidae) [4, 5]. Its main components include cantharidin, ginsenoside, astrogaloside and acahptopanax senticosus polysaccharide [6, 7].

To more comprehensively investigate the effect of Aidi injection on metabolic pathways, we examined the metabolic profiles of rats that received Aidi injections. To the best of our knowledge, this study is the first to report the tissue metabolic changes in rats after Aidi injections.

Material and methods

Chemicals

Aidi injection (10 mL/ampoule) was purchased from Guizhou Yibai Pharmaceutical Co., Ltd (Guizhou, China), Trimethylchlorosilane (TMCS,
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purity ≥ 99.0%) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, purity ≥ 98.5%) were purchased from Sigma-Aldrich Corp. (Shanghai, China). HPLC-grade acetonitrile and n-hexane were purchased from Tedia Reagent Company (Shanghai, China). Methylhydroxylamine hydrochloride and pyridine were purchased from Aladdin Industrial, Inc. (Shanghai, China).

Instrumentation and conditions

The Agilent 6890N-5975B GC-MS and HP-5MS (0.25 mm × 30 m × 0.25 μm) were obtained from Agilent Technologies Inc. (Santa Clara, CA, USA). Mass detection was conducted first in EI mode with electron energy of 70 eV, then in full-scan mode with m/z 50-550, and finally, by the splitless mode injection [8, 9]. The GC oven was initially set at 80°C and was kept at this temperature for 5 min. The temperature was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 min.

Sample preparation

A volume of 250 µL of acetonitrile was added to 100 mg of tissue (liver, kidney, heart), the suspension was kept in an ice-bath for 15 min and then centrifuged at 13000 g for 10 min at 4°C. A volume of 150 µL of the supernatant was transferred to a GC vial and evaporated to dryness under a stream of nitrogen gas. Methyl oximation was carried out at 70°C for 24 h after 50 µL of methylhydroxylamine hydrochloride (15 mg/mL in pyridine) was added. Then, a 50 µL volume of MSTFA (with 1% TMCS as the catalyst) was added and kept at 70°C for another hour, and then vortexed after adding 150 µL n-hexane [9].

Metabolomics study

Sprague-Dawley rats (male, 220 ± 20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Rats were housed under natural light-dark cycle conditions with controlled temperature (22°C). All rats were housed at the Laboratory Animal Research Center of Wenzhou Medical University. All experimental protocols were ethically approved by the Administration Committee of experimental animals of Wenzhou Medical University.

Twenty-one Sprague-Dawley rats were divided into three groups, namely the control group, Low-dose Aidi injection-treated group, High-dose Aidi injection-treated group, 7 rats per group. In the Low-dose Aidi injection-treated group, 5 mL/kg/day of Aidi was given to rats by intraperitoneal injection for 7 days. In the High-dose Aidi injection-treated group, 10 mL/kg/day of Aidi was given to rats by intraperitoneal injection for 7 days. In the control group, 8 mL/kg/day of normal saline was given to rats by intraperitoneal injection for 7 days.

Tissue (liver, kidney, heart) samples were separately collected from the 3 groups of rats at 10:00 am on the eighth day. The tissue samples were stored at -80°C until measurement.

Main ingredient in Aidi injection

A volume of 100 µL Aidi injection was added in the eppendorf tube, vortexed after adding methanol 900 µL, and then centrifuged at 13000 g for 10 min. The supernatant was used for UPLC-MS/MS analysis.

UPLC-MS/MS with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) interface and BEH C18 column (2.1 mm × 50 mm, 1.7 μm) were used in our work. Methanol and 0.1% formic acid were used as mobile phases; the flow rate was 0.4 mL/min. A gradient elution program was conducted for chromatographic separation with mobile phase A (0.1% formic acid), and mobile phase B (methanol) as follows: 0-0.2 min (10-10% B), 0.2-1.5 min (10-80% B), 1.5-3.0 min (80-80% B), 3.0-3.5 min (80-10% B), 3.5-5.0 min (10-10% B). Mass spectrometry parameter multiple reaction monitoring (MRM) settings was according to the literature (Liu et al., 2016). The main chemical compositions of Aidi injection acquired through UPLC-MS/MS technique are presented in Figure 1 and Table 1.

Data analysis

The GC-MS data was exported into Microsoft Excel, and the peaks were normalized to the total sum of spectrum prior to multivariate analyses. The resulting data were processed through principal component analysis (PCA) and PLS-DA using the SIMCA-P 11.5 software (Umetrics, Umea, Sweden) [10].
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Figure 1. Representative UPLC-MS/MS chromatograms of Aidi injection.
Tissue metabolic changes after Aidi injection

Statistical analysis

Statistical analysis was conducted using the SPSS software (Version 18.0, SPSS, IBM Corp., Armonk, NY, USA). Independent samples t-test was applied in order to detect significant differences in all metabolites between two groups. P < 0.05 was considered statistically significant.

Results

Metabolomics study

The typical metabolic profiles of tissue (liver, kidney, heart) samples acquired through GC-MS technique are presented in Figure 2. A total of 50 endogenous metabolites were identified in the tissue samples using the NIST 2005 mass spectrometry database.

In order to evaluate the metabolic profile changes in rats caused by different dosages (5 and 10 mL/kg/day, for the Low and High groups, respectively) of Aidi, we compared the PCA of the Aidi injection groups (Low and High) with the spectrum from rats in the control group, as illustrated for different tissues in the following figures: Figure 3A (liver), Figure 3C (kidney), Figure 3E (heart); the corresponding load diagram is shown in Figure 3B (liver), Figure 3D (kidney), Figure 3F (heart). We compared the GC-MS spectra from the PLS-DA of the rats in the Aidi injection groups (Low and High) with those of rats in the control group (Figure 4A, 4D, 4G); the corresponding load diagram is shown in Figure 4B, 4E, 4H. Additionally, the PLS-3D results are shown in Figure 4C, 4F, 4I. The PLS-3D score charts in Figure 4 showed that the first principal components of the rats in the Aidi injection groups (Low and High) were distinguished from the rats in the control group. Actually, the results of PLS-3D were better than those of the PCA.

Changes in metabolite

The identification of endogenous compounds that can be used as metabolic biomarkers of Aidi injection poisoning would represent an alternative approach of significant importance to detect hidden effects. In this study, the changes of metabolites between the Aidi injec-
Figure 3. PCA score results of the rat liver, kidney and heart samples (A, C, E) after Aidi injection (0.5, 1.0 g/kg, Low, High), Low (Class 1), High (Class 2), Control (Class 3); the corresponding load diagram (B, D, F).

Discussion

Metabolomics is a newly emerging omics approach to the investigation of metabolic phenotype changes induced by environmental or endogenous factors [11-16]. According to the tissue metabolomics results, Aidi injection-treated rats could be distinguished from rats of the control group, and the High-dose Aidi injection-treated group could be separated from the Low-dose Aidi injection-treated rats.
Figure 4. PLS-DA score results of the rat liver, kidney and heart samples (A, D, G) after Aidi injection (0.5, 1.0 g/kg, Low, High), low (Class 1), high (Class 2), control (Class 3); the corresponding load diagram (B, E, H); PLS-3D score results (C, F, I).
Tissue metabolic changes after Aidi injection

**Table 2.** Relative levels of metabolites in rat liver after Aidi injection

<table>
<thead>
<tr>
<th>NO.</th>
<th>Retention time/min</th>
<th>VIP</th>
<th>Metabolite</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
<th>( P_a )</th>
<th>( P_b )</th>
<th>( P_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.94</td>
<td>1.215</td>
<td>Urea</td>
<td>0.712</td>
<td>1.061*</td>
<td>0.011</td>
<td>0.969*</td>
<td>0.025</td>
<td>0.216</td>
</tr>
<tr>
<td>2</td>
<td>12.89</td>
<td>1.113</td>
<td>Glucose</td>
<td>0.736</td>
<td>0.427**</td>
<td>0.007</td>
<td>0.395**</td>
<td>0.001</td>
<td>0.517</td>
</tr>
<tr>
<td>3</td>
<td>15.19</td>
<td>4.614</td>
<td>D-Glucose</td>
<td>3.772</td>
<td>6.074</td>
<td>0.093</td>
<td>12.339***</td>
<td>0.005</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>15.37</td>
<td>2.803</td>
<td>Galactose</td>
<td>13.464</td>
<td>15.272*</td>
<td>0.048</td>
<td>16.391***</td>
<td>0.006</td>
<td>0.038</td>
</tr>
<tr>
<td>5</td>
<td>17.51</td>
<td>2.176</td>
<td>Hexadecanoic acid</td>
<td>4.023</td>
<td>2.895**</td>
<td>0.005</td>
<td>2.553***</td>
<td>0.002</td>
<td>0.024</td>
</tr>
<tr>
<td>6</td>
<td>19.41</td>
<td>2.580</td>
<td>9,12-Octadecadienoic acid</td>
<td>4.128</td>
<td>2.386**</td>
<td>0.007</td>
<td>2.052***</td>
<td>0.002</td>
<td>0.048</td>
</tr>
<tr>
<td>7</td>
<td>20.97</td>
<td>1.454</td>
<td>Arachidonic acid</td>
<td>2.271</td>
<td>1.575*</td>
<td>0.027</td>
<td>1.382*</td>
<td>0.019</td>
<td>0.332</td>
</tr>
<tr>
<td>8</td>
<td>21.83</td>
<td>1.635</td>
<td>Tetradecanoic acid</td>
<td>6.540</td>
<td>7.637</td>
<td>0.172</td>
<td>8.024*</td>
<td>0.037</td>
<td>0.657</td>
</tr>
<tr>
<td>9</td>
<td>22.72</td>
<td>1.666</td>
<td>D-Ribose</td>
<td>1.661</td>
<td>1.140**</td>
<td>0.009</td>
<td>0.764***</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>10</td>
<td>26.59</td>
<td>1.322</td>
<td>Cholesterol</td>
<td>1.807</td>
<td>1.334**</td>
<td>0.032</td>
<td>1.145**</td>
<td>0.008</td>
<td>0.084</td>
</tr>
</tbody>
</table>

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 1.0. Compared with control group, \( P_a \) (Low-Control), \( P_b \) (High-Control), \( P_c \) < 0.05 and \( P < 0.01; \) Compared with Low-dose Aidi injection treated group, \( P_a \) (High-Low), \( P < 0.05 \) and \( **P < 0.01 \).

**Table 3.** Relative levels of metabolites in rat kidney after Aidi injection

<table>
<thead>
<tr>
<th>NO.</th>
<th>Retention time/min</th>
<th>VIP</th>
<th>Metabolite</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
<th>( P_a )</th>
<th>( P_b )</th>
<th>( P_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.89</td>
<td>1.276</td>
<td>Glycerol</td>
<td>1.439</td>
<td>1.166*</td>
<td>0.048</td>
<td>1.179*</td>
<td>0.031</td>
<td>0.715</td>
</tr>
<tr>
<td>2</td>
<td>13.05</td>
<td>1.047</td>
<td>2-aminoheptanoic acid</td>
<td>1.083</td>
<td>1.118</td>
<td>0.534</td>
<td>0.823*</td>
<td>0.026</td>
<td>0.351</td>
</tr>
<tr>
<td>3</td>
<td>13.22</td>
<td>1.887</td>
<td>L-Proline</td>
<td>1.778</td>
<td>1.744</td>
<td>0.112</td>
<td>1.294***</td>
<td>0.007</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>14.82</td>
<td>1.013</td>
<td>Glucitol</td>
<td>0.433</td>
<td>0.370*</td>
<td>0.043</td>
<td>0.300***</td>
<td>0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>17.04</td>
<td>2.336</td>
<td>9-Purine</td>
<td>1.870</td>
<td>1.499**</td>
<td>0.007</td>
<td>1.161***</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td>17.45</td>
<td>1.544</td>
<td>Hexadecanoic acid</td>
<td>2.877</td>
<td>2.937</td>
<td>0.079</td>
<td>3.368*</td>
<td>0.032</td>
<td>0.275</td>
</tr>
</tbody>
</table>

Note: VIP was acquired from the PLS-DA model with a threshold of 1.0. Compared with control group, \( P_a \) (Low-Control), \( P_b \) (High-Control), \( P < 0.05 \) and \( **P < 0.01; \) Compared with Low-dose Aidi injection treated group, \( P_a \) (High-Low), \( *P < 0.05 \) and \( **P < 0.01 \).

Compared to the control group, urea, glycerol, hexadecanoic acid, 9,12-octadecadienoic acid, arachidonic acid, d-ribose and cholesterol were decreased in the Low and High Aidi injection groups, while galactose was increased (Table 2). In addition, compared to the control group, glycerol, glucitol, 9-Purine were decreased in the Low and High Aidi injection groups (Table 3). Also, compared to the control group, threitol, hexadecanoic acid, 9,12-octadecadienoic acid, tetradecanoic acid, d-ribose, cholesterol were increased in the Low and High Aidi injection groups (Table 4).

Glycerol is generally obtained from plant and animal sources where it occurs as triglycerides. Triglycerides are esters of glycerol with long-chain carboxylic acids. Hexadecanoic acid is the first fatty acid produced during fatty acid synthesis and is the precursor to longer fatty acids. Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of membranes of the cells of the body, and is abundant in the brain, muscles, and liver. Cholesterol, given that it composes about 30% of all animal cell membranes, is required to build and maintain cell membranes and modulates membrane fluidity over the range of physiological temperatures.

The changes of metabolites (urea, glycerol, hexadecanoic acid, 9,12-octadecadienoic acid, arachidonic acid, D-ribose, cholesterol, galactose, glucitol, 9-Purine, threitol, tetradecanoic acid), increased or decreased, indicate that Aidi injection-treated rats had induced fatty acid metabolism, energy metabolism, amino acid metabolism, nucleotide metabolism, urea cycle perturbations. These findings may be useful for evaluating new evidence in Aidi injection studies.

**Conclusion**

In this study, we developed a tissue metabolomic approach by GC-MS to evaluate the effect of Aidi injection in rats. PLS-DA revealed that Aidi injection induced metabolic perturbations. We demonstrated that metabolomic methods based on GC-MS could provide a useful tool for
Table 4. Relative levels of metabolites in rat heart after Aidi injection

<table>
<thead>
<tr>
<th>NO.</th>
<th>Retention time/min</th>
<th>VIP</th>
<th>Metabolite</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
<th>( P_{\alpha} )</th>
<th>( P_{\beta} )</th>
<th>( P_{\gamma} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.02</td>
<td>1.233</td>
<td>Urea</td>
<td>2.350</td>
<td>2.765</td>
<td>0.291</td>
<td>3.868**</td>
<td>0.040</td>
<td>0.445</td>
</tr>
<tr>
<td>2</td>
<td>12.82</td>
<td>1.066</td>
<td>Threitol</td>
<td>0.028</td>
<td>1.112&quot;</td>
<td>0.003</td>
<td>1.048&quot;</td>
<td>0.001</td>
<td>0.176</td>
</tr>
<tr>
<td>3</td>
<td>17.48</td>
<td>1.712</td>
<td>Hexadecanoic acid</td>
<td>7.134</td>
<td>8.224&quot;</td>
<td>0.023</td>
<td>8.859&quot;</td>
<td>0.007</td>
<td>0.209</td>
</tr>
<tr>
<td>4</td>
<td>19.38</td>
<td>2.977</td>
<td>9,12-Octadecadienoic acid</td>
<td>7.729</td>
<td>9.995&quot;</td>
<td>0.036</td>
<td>7.903*</td>
<td>0.102</td>
<td>0.047</td>
</tr>
<tr>
<td>5</td>
<td>21.87</td>
<td>1.227</td>
<td>Tetradecanoic acid</td>
<td>1.594</td>
<td>1.885&quot;</td>
<td>0.019</td>
<td>2.261&quot;&quot;</td>
<td>0.008</td>
<td>0.035</td>
</tr>
<tr>
<td>6</td>
<td>22.77</td>
<td>3.190</td>
<td>D-Ribose</td>
<td>15.312</td>
<td>20.574&quot;</td>
<td>0.045</td>
<td>21.053&quot;</td>
<td>0.034</td>
<td>0.185</td>
</tr>
<tr>
<td>7</td>
<td>26.58</td>
<td>2.113</td>
<td>Cholesterol</td>
<td>6.806</td>
<td>8.754&quot;</td>
<td>0.028</td>
<td>9.572&quot;*</td>
<td>0.006</td>
<td>0.142</td>
</tr>
</tbody>
</table>

Note: VIP was acquired from the PLS-DA model with a threshold of 1.0. Compared with control group, \( P_{\alpha} \) (Low-Control), \( P_{\beta} \) (High-Control), \( P_{\gamma} \) (Low-Control), \( P_{\gamma} \) (High-Control), \( * \) \( P < 0.05 \) and ** \( P < 0.01 \); Compared with Low-dose Aidi injection treated group, \( P_{\alpha} \) (High-Low), \( P_{\beta} \) (Low-Control), ** \( P < 0.05 \).

**References**


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