GC/MS-based metabolomics strategy to analyze the effect of exercise intervention in diabetic rats

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Abstract
Metabolomics was used to explore the effect of exercise intervention on type 2 diabetes. The rat model of type 2 diabetes was induced by an injection of streptozocin (30 mg/kg), after fed with 8-week high-fat diet. The rats were divided into three groups: the control group, the diabetic model group (DM) and the diabetes + exercise group (DME). After exercise for 10 weeks, blood samples were collected to test biomedical indexes, and 24-h urine samples were collected for the metabolomics experiment. In the DME group, fasting blood glucose (FBG), both total cholesterol (TC) and total plasma triglycerides (TG), were decreased significantly, compared with those in the DM group. Based on gas chromatography-mass spectrometry (GC/MS), a urinary metabolomics method was used to study the mechanism of exercise intervention on diabetes mellitus. Based on the principal component analysis (PCA), it was found that the DM group and control group were separated into two different clusters. The DME group was located between the DM group and the control group, closer to the control group. Twelve significantly changed metabolites of diabetes mellitus were detected and identified, including glycolate, 4-methyl phenol, benzoic acid, 1H-indole, arabinitol, threitol, ribonic acid, malic acid, 2,3-dihydroxy-butanolic, aminomalonic acid, L-ascorbic acid and 3-hydroxy hexanedioic acid. After exercise, seven metabolites were significantly changed, compared with the control group, the relative contents of benzoic acid, aminomalonic acid, tetrabutyl alcohol and ribonucleic acid in the diabetic exercise group decreased significantly. The relative contents of 2,3-dihydroxybutyric acid, L-ascorbic acid and 3-hydroxy adipic acid increased significantly. L-ascorbic acid and aminomalonic acid which related with the oxidative stress were significantly regulated to normal. The results showed that exercise could display anti-hyperglycemic and anti-hyperlipidemic effects. The exercise had antioxidation function in preventing the occurrence of complications with diabetes mellitus to some extent. The work illustrates that the metabolomics method is a useful tool to study the mechanism of exercise treatment.

Introduction
The prevalence of type 2 diabetes (T2D) continues to increase. Within the next 20 years, the number of people affected by this disease is expected to reach almost 600 million worldwide (1). T2D is accompanied by a host of risk factors including dyslipidemia, hypertension and cardiovascular disease (2), thus putting a severe burden on our global health care systems.

Exercise plays a crucial role in the prevention and treatment of several chronic diseases, including glucose intolerance states, type 2 diabetes (3, 4) and diseases of
the cardiovascular system (5, 6). Moreover, it has been demonstrated that exercise improves the quality of life in the general population (7). Nevertheless, T2D patients are encouraged to exercise regularly as a means of improving social integration and cardiovascular health (8). Thus, a better understanding of the effects of exercise on the metabolic response in T2D patients will allow clinicians to prescribe exercise to their patients with greater clarity.

Urine is mainly composed of small hydrophilic molecules such as sugar, organic acids, amino acids, soluble lipids, organic amines and inorganic salts small enough to successfully pass through the human reticular endothelial filtration system. On the other hand, the non-invasive collection of urine is conducive to many metabolomic applications. Metabolomics enables the systematic assessment of the abundant changes of low-molecular-weight compounds present in biological samples, using high-throughput sample analysis techniques and computer-assisted multivariate pattern recognition techniques (9). Metabolomics is enriching our current understanding of both the physiologic and physiopathologic processes underlying diabetes mellitus (10, 11, 12). Moreover, recent metabolomic-based studies have described the first metabolic signatures of exercise in human plasma (13, 14, 15). For example, Lewis et al. (13) described the metabolic changes in tricarboxylic acid cycle, fatty acid oxidation and lypolisis in the plasma of healthy subjects exposed to different intensities and durations of exercise.

The aim of this study is to analyze the metabolic changes induced by 10 weeks of aerobic exercise performed by T2D rats. A comprehensive GC-MS untargeted metabolomics approach was applied to urine samples taken from all rats.

Materials and methods

Animals and induction of diabetes

Induction of diabetes

Thirty male Sprague-Dawley rats, weighing 200-220g were bred in the Center of Experimental Animal, Xuzhou Medical University, where an SPF level laboratory (24±1°C; humidity of 45% to 55%, 12:12 dark/light cycle) was founded, as authorized by the Jiangsu Province Government. Animals were allowed food and water ad libitum before and during the experiment.

One week after feeding adaptation, the rats were randomly divided into control group and DM and were given standard diet and high-fat diet for 8 weeks, respectively. The rats were fasted for 12 h and subjected to a single intraperitoneal injection of 30 mg/kg STZ, which was freshly dissolved in 100 mmol/L sodium citrate buffer (pH 4.5). Normal rats received sodium citrate buffer only. On the third day of STZ administration, the FBG value was determined by a reagent kit. Rats with FBG levels higher than 13.9 mmol/L were considered to be diabetic. All animal procedures were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Chaohu University (Anhui China).

Treadmill exercise protocol

Before beginning the formal 10-week exercise protocol, animals were familiarized to treadmill running (5–20 min/day) for five consecutive days. After this period of habituation, the exercised animals performed 5 days of consecutive treadmill exercise (60 min/day) with 22 m/min speeds (16). At the beginning of 60 min exercise, to warm up the rats, treadmill speed had been set at 5 m/min and progressively increased to 22 m/min. At the final of 60 min exercise, the speed progressively decreased to 5 m/min to cool down. Mild electrical shock was used the negligible amount to motivate animals to run. Control animals did not carry out treadmill exercise but were placed on a nonmoving treadmill for 60 min/day for 5 days a week. Exercised animals were studied 24 h after their last exercise session.

Sample collection

After 10 weeks of exercise, samples were collected from the rats in three groups. Rats were placed in metabolic cages to collect urine samples, and approximately 4 mL of urine per rat was obtained. The collected urine samples were stored in a refrigerator at −80°C. Twelve hours after urine sample collection, after ether anesthesia, the blood was collected from orbital into the heparin sodium tube and centrifuged, plasma was used for the detection of TC, total triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL), by blood lipid test kit (Nanjing Jiachen Technology Co., Ltd. China), fasting serum insulin (FINS) was determined by ELISA, at the same time, the tail blood was taken to measure FBG by JPS-S Yicheng blood glucose meter and then 2 g/kg glucose solution intragastric administration to make oral glucose tolerance test. The blood glucose values (BG) were measured at 30, 60 and 120 min after exercise.
Table 1  After 10 weeks, the levels of fasting blood glucose (FBG), FINS, HOMA-IR and body weight in different groups (mean ± s.d.).

<table>
<thead>
<tr>
<th>Group</th>
<th>FBG (mmol/L) Before</th>
<th>FBG (mmol/L) After</th>
<th>Body weight (g)</th>
<th>FINS (ng/mL)</th>
<th>HOMA-IR (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.83 ± 0.56</td>
<td>5.91 ± 1.73</td>
<td>477.63 ± 29.94</td>
<td>1.20 ± 0.17</td>
<td>0.315 ± 0.013</td>
</tr>
<tr>
<td>Diabetic model group</td>
<td>13.45 ± 2.12a</td>
<td>15.86 ± 5.78a</td>
<td>452.76 ± 18.13a</td>
<td>1.54 ± 0.09a</td>
<td>1.085 ± 0.023a</td>
</tr>
<tr>
<td>Diabetic exercise group</td>
<td>13.87 ± 2.05ab</td>
<td>6.46 ± 1.91abc</td>
<td>449.71 ± 32.14ab</td>
<td>1.29 ± 0.20abc</td>
<td>0.370 ± 0.017abc</td>
</tr>
</tbody>
</table>

*P < 0.05 vs control; *P < 0.05 vs model; *P < 0.05, before exercise vs after exercise.
FINS, fasting insulin; HOMA-IR, homeostasis model assessment.
Metabolomics and exercise

Diabetes and exercise

Fig. 1. Oral glucose tolerance test (OGTT) curves of rats in different groups.

- Control
- Model
- Exercise

Fig. 2. Changes of blood lipid in diabetic rats after exercise.

Table 2. Levels of biochemical indexes in different groups (mean ± s.d.).

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.5 ± 3.9a</td>
<td>37.9 ± 8.7b</td>
<td>13.5 ± 1.1</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>Diabetic model group</td>
<td>95.3 ± 13.4a</td>
<td>81.3 ± 10.6a</td>
<td>15.8 ± 1.3</td>
<td>25.8 ± 3.2</td>
</tr>
<tr>
<td>Diabetic exercise group</td>
<td>39.4 ± 4.3b,c</td>
<td>47.0 ± 7.2bc</td>
<td>14.1 ± 1.6</td>
<td>23.1 ± 3.0</td>
</tr>
</tbody>
</table>

- P < 0.05 vs control;
- P < 0.05 vs model;
- FBG before exercise vs after exercise.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, total plasma triglyceride.
contents of 2,3-dihydroxybutyric acid, L-ascorbic acid and 3-hydroxy adipic acid increased significantly.

**Discussion**

Type 2 diabetes mellitus (DM) is a chronic metabolic disease. The disorder of glucose metabolism associated with insulin resistance is the main feature, accompanied by disorders of lipid metabolism. In this study, a high-fat diet with small dose of streptozotocin was used to establish the model of diabetes. The results of biochemical indexes showed that the BG and blood lipid of diabetes mellitus group had no significant difference between 30 days after modeling, and maintained hyperglycemia and hyperlipidemia, which indicated that the modeling method was stable and reliable. After exercise, BG and blood lipids were significantly decreased in rats, and the results showed that exercise had the effect of lowering BG and blood lipid.

After analysis of the endogenous substances in the urine of diabetic rats, 12 metabolites were found to be related to diabetes mellitus. The metabolites of 4-methyl phenol, benzoic acid and 1H-indole are related to the metabolic disorder of intestinal microflora. Several studies have shown that diabetes is related to the metabolic disorder of intestinal microflora; 4-methyl phenol and 1H-indole are metabolites produced from proteins in food by intestinal microflora, benzoic acid is the product of Omega-oxidation decomposition of phenyl fats (18, 19). In this study, we found that the relative levels of
4-methylphenol, benzoic acid and 1H-indole in diabetic rats were significantly increased. After exercise, these metabolites showed a significant tendency to adjust to the control group, indicating that exercise may have a regulatory role on intestinal microflora metabolic disorders consistent with other research results (20, 21).

Oxidative stress is the marked imbalance for oxidative agents when compared to antioxidants, meaning there is a significant increase of oxidants, hence the name oxidative stress. In this study, we found that L-ascorbic acid and aminomalonic acid were the metabolites that reflect the oxidative stress status in the diabetic-related metabolites (22), among them aminomalonic acid is a kind of metabolite of glycine oxidation (23, 24). Diabetic patients are often accompanied by oxidative stress, persistent hyperglycemia leads to increased mitochondrial superoxide anion generation in endothelial cells, leaving the body in oxidative stress, excessive oxygen free radical damage and increasing the risk of diabetes mellitus. After exercise, aminomalonic acid and L-ascorbic acid were significantly adjusted to the control group; these data showed that exercise could decrease the blood sugar and regulate the oxidative stress in the body. Oxidative stress can increase the occurrence and development of complications in diabetic patients, the effect of exercise on the metabolism of metabolites associated with oxidative stress in vivo suggests that exercise may, to some extent, prevent the occurrence and development of diabetes complications.

2,3-Dihydroxybutyric acid is one of the metabolites of threoneine (25). Threonine is an essential amino acid for human beings and is a glucogenic amino acid. It is not well clear that the metabolic pathways associated with 2,3-dihydroxybutyrate and diabetes. 3-Hydroxy adipic acid is one of the metabolites of fatty acids, butanetraol, and arabinitol is one of the metabolites of sugar polyols, malic acid is the intermediate product of the three Krebs cycle of sugars, and the mechanisms involved in these metabolites and diabetes need to be further investigated. Based on the GC-MS method, this study investigated the urine metabolomics of exercise therapy for type 2 diabetes mellitus. It provides a further experimental reference for elucidating the mechanism of exercise therapy for diabetes.

**Conclusion**

In this study, we developed a type 2 diabetic rat model using STZ combined with a high-fat diet, and studied the urine metabolites of exercise therapy for type 2 diabetes mellitus by combining the relative indexes and the GC-MS method. The results showed that exercise can reduce blood sugar and blood lipid. Using GC-MS technique to detect endogenous metabolites in rat urine, through PCA, there are significant differences between control group and DM group, found 12 metabolites associated with diabetes. The PCA results showed that the DME group was separated from the DM group. After exercise, seven of the differential metabolites associated with diabetes were significantly called back to the control group, and the aminomalonic acid associated with oxidative stress was significantly lower than that of the DM group, and L-ascorbic acid was significantly increased, indicating that exercise can regulate the oxidative stress status associated with diabetes, to a certain extent, may inhibit the occurrence and development of diabetic complications. The results of this study also show that metabolomics methods are an important means of studying the mechanisms of exercise intervention in disease.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

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**Author contribution statement**

Wang Chengji participated in the study design, analysis, report development, and interpretation of study findings, Li Jing participated in writing and analysis.

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