

Original Article

GC-TOF/MS-based metabolomic profiling of estrogen deficiency-induced obesity in ovariectomized rats

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Aim: To explore the alteration of endogenous metabolites and identify potential biomarkers using metabolomic profiling with gas chromatography coupled a time-of-flight mass analyzer (GC/TOF-MS) in a rat model of estrogen-deficiency-induced obesity.

Methods: Twelve female Sprague-Dawley rats six month of age were either sham-operated or ovariectomized (OVX). Rat blood was collected, and serum was analyzed for biomarkers using standard colorimetric methods with commercial assay kits and a metabolomic approach with GC/TOF-MS. The data were analyzed using multivariate statistical techniques.

Results: A high body weight and body mass index inversely correlated with serum estradiol (E2) in the OVX rats compared to the sham rats. Estrogen deficiency also significantly increased serum total cholesterol, triglycerides, and low-density lipoprotein cholesterol. Utilizing GC/TOF-MS-based metabolomic analysis and the partial least-squares discriminant analysis, the OVX samples were discriminated from the shams. Elevated levels of cholesterol, glycerol, glucose, arachidonic acid, glutamic acid, glycine, and cystine and reduced alanine levels were observed. Serum glucose metabolism, energy metabolism, lipid metabolism, and amino acid metabolism were involved in estrogen-deficiency-induced obesity in OVX rats.

Conclusion: The series of potential biomarkers identified in the present study provided fingerprints of rat metabolomic changes during obesity and an overview of multiple metabolic pathways during the progression of obesity involving glucose metabolism, lipid metabolism, and amino acid metabolism.

Keywords: metabolomics; obesity; gas chromatography coupled a time-of-flight mass analyzer; estrogen deficiency

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Introduction

Obesity is a medical condition in which excess body fat has accumulated to such an extent^[1] that it becomes linked to several common diseases, including insulin resistance^[2], cardiovascular disease^[3], stroke^[4], and certain cancers^[5–8]. Apart from the genetic, environmental, and nutritional factors involved in the occurrence of obesity, endogenous estrogens play an important role in the regulation of body weight and obesity^[9, 10]. The decrease in estrogen levels experienced by women after menopause could be associated with an increased risk of obesity and a series of devastating diseases^[11, 12]. The relationship between obesity and endog-

enous estrogen remains unclear. Similar to leptin, estrogens regulate body weight and energy metabolism^[13]. Moreover, ovariectomy-induced adiposity is associated with decreased energy expenditure, adipose tissue expansion, and hepatic steatosis^[14]. Estrogen receptors alpha and beta play essential roles in controlling the energy metabolism pathways, body fat distribution, and normal body weight.

Estrogen replacement therapy (ERT), the medical administration of estrogen, prevents obesity and estrogen-deficiency-induced complications^[15, 16]. In addition, estrogen has favorable effects on the lipid profile, significantly increasing high-density lipoprotein cholesterol (HDL-C) and reducing low-density lipoprotein cholesterol (LDL-C). ERT helps to relieve the cardiovascular diseases caused by obesity by decreasing cardiac mortality, improving endothelial function, and reducing LDL accumulation^[17, 18]. However, the safety of ERT is controversial, although appropriate amounts of estrogen

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moderate estrogen-deficiency-induced obesity in postmenopausal women. The extensive use of estrogens results in a significant increase in the incidence of breast cancer^[19] and ovarian cancer^[20] in postmenopausal women. In addition, an increased risk of deep vein thrombosis (DVT)^[21] and dementia has been reported when estrogen alone is used in postmenopausal women aged 65 years or older based on data from the Women's Health Initiative (WHI)^[22]. Therefore, an understanding of the comprehensive metabolic disorders and metabolic syndromes of estrogen-deficiency-induced obesity has become a topic of interest.

Metabonomics/metabolomics is defined as "the quantitative measurement of the dynamic multiparametric responses of a living system to pathophysiological stimuli or genetic modification"^[23]. It is a novel "-omics" discipline that monitors alterations in the levels of endogenous metabolites in response to biological stimuli using modern spectroscopic techniques and statistical approaches^[24]. Compared with genomics, transcriptomics, and proteomics, metabolomics profiling can provide a holistic overview and time-dependent snapshots of the physiologic process of endogenous metabolites in cells, tissues, organs, or organisms^[25]. As such, metabolomics has been comprehensively applied in physiology, disease, and toxicology research fields^[26]. Nuclear magnetic resonance (NMR) spectroscopy^[27], liquid chromatography coupled mass spectrometry (LC-MS)^[28], gas chromatography coupled mass spectrometry (GC-MS)^[29], and Fourier transform mass spectrometry^[30] have been successfully used to obtain metabolomic data. GC coupled to a time-of-flight mass analyzer (GC-TOF/MS) provides an improvement over conventional GC-MS analysis due to its high scan rates and the availability of mathematic algorithms to deconvolute closely overlapping peaks. Recent metabolomic approaches utilizing GC-TOF/MS for high-throughput analysis have been successfully applied to the study of hypertension^[31], hyperlipemia^[32], colon carcinoma, and professional athletes^[33] in serum or urine from rat and humans^[34]. Therefore, for accuracy and thoroughness, a GC/TOF-MS-based metabolomics approach was implemented to study the biochemical changes in the serum of estrogen-deficiency-induced obesity in female rats in our study. A bilateral ovariectomy was applied to induce estrogen deficiency in female rats. A GC/TOF-MS spectrogram of rat serum was analyzed using a multivariate statistical analysis (MVSA) to differentiate the stages of obesity in OVX rats. The alterations of endogenous metabolites were examined under the conditions of estrogen-deficiency-induced obesity.

Materials and methods

Chemicals and reagents

Myristic-1,2-¹³C₂ acid, 99 atom %¹³C was used as an internal standard (IS). Methoxyamine hydrochloride (purity 98%), alkane standard solution (C8-C40), and pyridine (>99.8% GC grade) were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

N-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) plus 1% trimethyl chlorosilane (TMCS) were obtained from Fluka

(Buchs, Switzerland). Methanol (Merck, Darmstadt, Germany) and *n*-heptane (Tedia, Fairfield, OH, USA) were of high pressure liquid chromatography (HPLC) grade.

Purified water was produced by a Milli-Q system (Millipore, Milford, MA, USA). Kits for total cholesterol (TC), triglyceride (TG), HDL-C and LDL-C were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). An ELISA kit for estradiol (E2) was purchased from R&D Systems (Minneapolis, MN, USA).

Animals, surgery and sample preparation

Twelve virgin specific pathogen-free Sprague-Dawley female rats (6 months old, body weight 250±10.0 g) were obtained from the Animal Center of Nanjing Medical University. Rats were housed under controlled conditions (room temperature 22±1°C) with standard solid food and water provided *ad libitum* during the experiment. This study was reviewed and approved by the Animal Ethical Committee of China Pharmaceutical University.

Bilateral ovariectomy (OVX, *n*=6) or sham operations (sham, *n*=6) were performed after a seven-day acclimatization. A bilateral (left and right) incision (1–2 cm) that included the skin, muscle, and peritoneum was performed 2 cm below the last rib, and the left and right ovaries were extirpated. For the sham operation, only a small amount of fat around each ovary was extirpated. The rats were provided with standard solid food and deionized water following surgery. Body weights and body mass indexes^[36] (BMI, a measurement comparing weight and the height squared) of rats were measured weekly and blood samples were collected on day 0 prior to the operation and at the 3rd and 6th week after bilateral ovariectomy. Serum was immediately separated by centrifugation at 2000×*g* for 10 min at 4 °C after whole blood was placed for 20 min, and was aliquoted into two parts. One aliquot was used to assay the levels of serum E2 by ELISA and TC, TG, HDL-C, and LDL-C using standard colorimetric methods with commercial kits. The other aliquot was used for GC/TOF-MS analysis. All samples were stored at -80 °C until analysis. All rats were weighed, anesthetized, and bled from the carotid arteries at the end of the experiments 6 weeks post-surgery. Uterine tissues were removed and weighed, and uterus indexes were calculated by dividing the uterus weight by the body weight.

Sample preparation for GC-TOF/MS analysis

Two hundred microliters of methanol containing the internal standard myristic-1,2-¹³C₂ acid (12.5 µg/mL) was added to deproteinize a 50-µL serum sample. After vortex mixing for 5 min and centrifugation at 20000×*g* for 10 min at 4 °C, 100 µL of supernatant was injected directly into a GC vial and then evaporated to dryness under vacuum. Subsequently, 30 µL of methoxyamine in pyridine (15 mg/mL) was added to the residue and vortexed for 2 min. A methoximation reaction was performed at room temperature for 16 h before trimethylsilylation was performed by the addition of 30 µL MSTFA with 1% TMCS as catalyst for 1 h. Finally, the solution was vortexed for 30 s after methyl myristate in heptane (30 µg/mL),

the external standard, was added to a GC vial for GC-TOF/MS analysis^[33–35].

GC-TOF/MS analysis

An Agilent 6980 GC was equipped with an Agilent 7683 Series autosampler (Agilent, Atlanta, GA). Chromatographic separation was performed on a fused-silica capillary column (10 m×0.18 mm ID) chemically bonded with a 0.18 μm DB5-MS stationary phase (J&W Scientific, Folsom, CA, USA). Mass spectra were obtained using a Pegasus III TOF/MS (Leco). The injector temperature and the constant flow rate of carrier gas were controlled at 250 °C at a rate of 1 mL/min with the following parameters: a gradient temperature programming of 70 °C (2.0 min), 70–310 °C linear (35 °C/min), 310 °C (2.0 min); a purge time and flow rate of 60 s at 20 mL/min for 1 min; transfer line temperature and ion source temperature set at 250 °C and 200 °C, respectively; ion source voltage and current of 70 eV and 3.0 mA. The mass spectrometric data were acquired in scan mode over a range of *m/z* 50–800 at a rate of 20 spectra/s, and the acceleration voltage was turned on at -1650 V with a solvent delay of 170 s.

Data analysis

Automatic peak detection and calculation of the peak area of specific compounds and IS were analyzed by ChromaTOF 2.00 software (Leco). Peaks with signal-to-noise (S/N) ratios lower than 30 were rejected^[35]. The retention index for each peak or compound was obtained by comparing its retention time against those of the alkane series (C8–C40). Identification of all of the compounds was performed by comparing their mass spectra and retention indexes with the authentic reference standards and the standards listed in the NIST 2.0 (2005), the Wiley library, and the in-house mass spectra library database established in the Key Laboratory of Drug Metabolism and Pharmacokinetics at China Pharmaceutical University. In addition, potential metabolites were searched using the Human Metabolome Database (<http://www.hmdb.ca>).

Multivariate statistical analysis (MVSA) was performed using SIMCA-P 11 software (Umetrics, Umeå, Sweden). The data matrix was constructed by GC-TOF/MS responses of each peak as variables with the sample names/IDs as observations in columns and each of the peaks in rows. Subsequently, a principal component analysis (PCA) and a partial least-squares-discriminant analysis (PLS-DA) were used to process the acquired data from the GC-TOF/MS analysis^[33].

All data are expressed as means±SD, and statistical analyses were performed using Student's *t*-test for independent two-sample and one-way ANOVA for the comparison of multiple means. *P*<0.05 indicated statistical significance.

Results

Body weight, body mass, and uterine index

Body weight, BMI, and the uterine index were determined; the results are listed in Figure 1A–1C). A significant increase (*P*<0.05) in body weight from the third week post-operation was observed in the OVX group (Figure 1A). Body weight

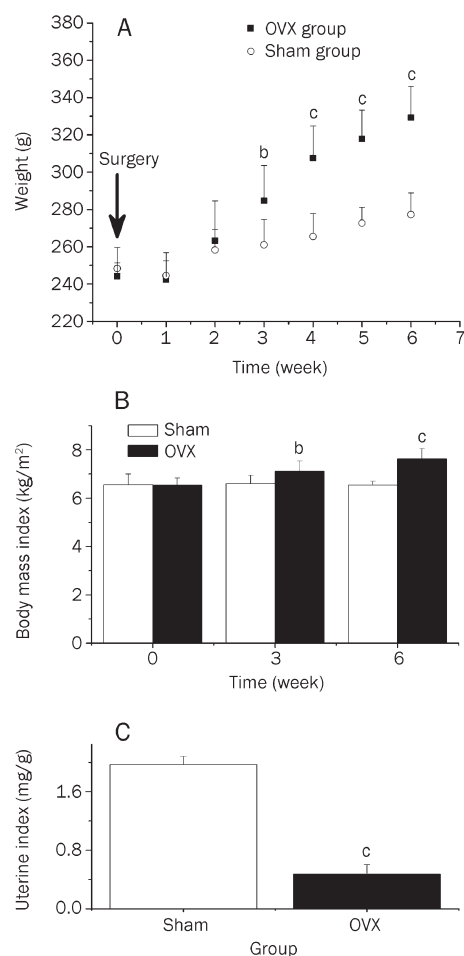


Figure 1. Body weight, body mass index, and uterus index from OVX and the sham rats. (A) Body weight of the rats was recorded weekly during the experiment. (B) Body mass index observed in the pre-surgery and weeks 3 and 6 post-surgery groups. (C) Uterus index. Values with a superscript are significantly different from the sham group (^b*P*<0.05, ^c*P*<0.01).

was significantly elevated (*P*<0.001) in the OVX group compared to the sham group in the 4th, 5th, and 6th weeks after surgery. In addition, BMI was significantly increased in the OVX group in the 3rd (*P*<0.05) and 6th (*P*<0.001) weeks compared to the sham group (Figure 1B). Week 6 post-operation, the level of the uterine index was significantly lower (*P*<0.001) than those in the sham group (Figure 1C).

Serum biochemical analysis

As shown in Table 1, serum TC levels in the OVX group in weeks 3 and 6 increased significantly after surgery compared to the sham group (*P*<0.05 and *P*<0.01, respectively). Compared with pre-surgery levels, serum TC (*P*<0.001) and TG (*P*<0.05 and *P*<0.001, respectively) levels were significantly increased in weeks 3 and 6 post-bilateral OVX. Similar to TC and TG, serum LDL-C levels in the OVX group in week 6 post-operation were significantly elevated (*P*<0.001) compared to the sham group and the OVX group prior to surgery. In addition, HDL-C levels in the OVX group observed at weeks 3 and

Table 1. Serum biochemical parameters from the sham group and the OVX group prior to and week 3 and 6 post bilateral ovariectomy. The values represent the means±SD. *n*=6. ^b*P*<0.05, ^c*P*<0.01 vs Sham; ^e*P*<0.05, ^f*P*<0.01 vs the OVX group prior to surgery.

Parameter	Group					
	Pre-surgery	Sham		OVX		
		Week 3 postsurgery	Week 6 postsurgery	Pre-surgery	Week 3 postsurgery	Week 6 postsurgery
TC (mmol/L)	1.48±0.207	1.59±0.252	1.59±0.296	1.51±0.222	2.38±0.347 ^{cf}	3.09±0.460 ^{cf}
TG (mmol/L)	1.06±0.240	1.08±0.232	1.21±0.159	1.02±0.130	1.39±0.162 ^{bf}	1.59±0.216 ^{cf}
HDL-C (mmol/L)	0.85±0.275	0.93±0.227	0.95±0.217	0.86±0.248	0.86±0.190	0.79±0.176
LDL-C (mmol/L)	1.06±0.237	1.14±0.219	1.08±0.230	1.07±0.279	1.34±0.262	2.15±0.499 ^{cf}
E2 (pg/mL)	40.21±10.27	39.32±9.47	38.77±8.32	37.88±8.75	10.72±4.51 ^{cf}	8.65±3.21 ^{cf}

6 post-surgery were slightly reduced but showed no statistical difference compared with the OVX group prior to surgery or with the sham group.

Metabolomic analysis of plasma samples in rats with estrogen-deficiency-induced obesity

The total ion current chromatograms produced by GC-TOF/MS analysis are shown in Figure 2. More than 110 compounds, including amino acids, organic acids, saccharides, and fatty acids, were identified in the metabolomic profiling. In our experiment, the relative standard deviations of the internal standard and the external standard samples were 4.38% and 3.47%, respectively.

PLS-DA was performed on the acquired metabolomic dataset. The PLS-DA score plot for the whole data set categorized serum samples into pre-surgery and weeks 3 and 6 post-surgery in the OVX group (Figure 3A). All of the serum samples in the metabolomic profile of the OVX group (pre-surgery and weeks 3 and 6) were calculated using PLS-DA. Subsequently, a PLS-DA model of two principal components could explain 59.8% of the GC-TOF/MS response variables and predict 72.5% and explain 95.3% according to R^2X , R^2Y , and Q^2Y parameters (R^2X is the percentage of all GC/TOF-MS response variables explained by the model. R^2Y is the percentage of all observation or sample variables explained by the model. Q^2Y is the percentage of all observation or sample variables predicted by the model^[33]). The scores are plotted in Figure 3A, in which the x-axis and y-axis are labeled PC1 (the first prin-

cipal component) and PC2 (the second principal component), respectively. Time-dependent changes of the scores plot in the serum metabolomic profile are obvious in the 2D plane graph. The scores plot shows the samples from rats prior to surgery clustered in the right area of the plot. However, the samples from rats 3 weeks post-surgery are gathered in the top left quadrant of the plot. The samples from rats in week 6 are in the bottom left quadrant of the plot. Our analysis indicated that the stages of estrogen-deficiency-induced obesity could be differentiated clearly between pre-surgery and weeks 3 and 6 post-surgery. Fold changes of endogenous metabolites were further studied by the loadings plot (not shown); the results are shown in Table 2. Cholesterol, glycerol, octadecadienoic acid, 3-hydroxy-butanoic acid, glucose, isoleucine, valine, leucine, and glycine were notably increased in plasma at weeks 3 and 6 post-surgery compared to levels in rats prior to surgery, and glyceraldehyde 3-phosphate, alanine, and arabinofuranose were significantly decreased at weeks 3 and 6 after surgery.

Likewise, a two principal components PLS-DA model was performed between the sham group and the OVX group at week 6 post-surgery. Figure 3B shows the metabolomic movement of both the sham and the OVX groups 6 weeks after bilateral OVX, which could be classified clearly with R^2X : 51.2%, R^2Y : 99.9%, and Q^2Y : 90.7%. In addition, estrogen-deficiency-related modifications of metabolites at different time points are listed in Table 2. The results suggested that estrogen-deficiency-induced obesity greatly affects endogenous

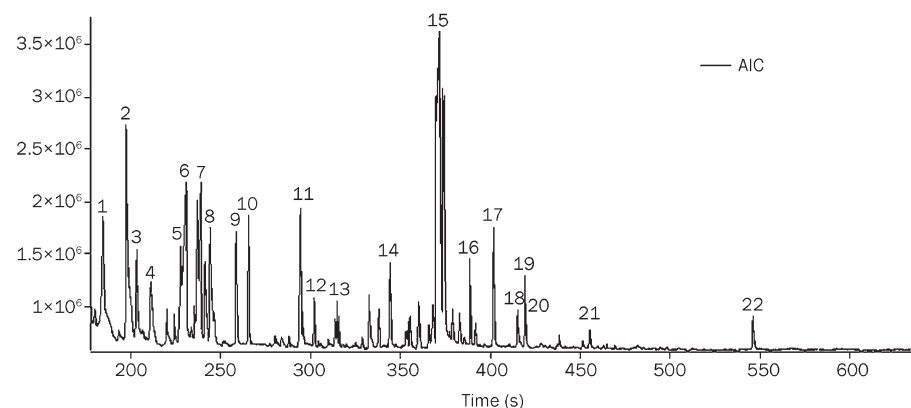


Figure 2. GC-TOF/MS chromatograph of a serum sample obtained from a sham-operated rat. 1, Alanine; 2, Lactic acid; 3, β -Hydroxybutyric acid; 4, Valine; 5, Leucine; 6, Glycerol; 7, Phosphoric acid; 8, Glycine; 9, Isoleucine; 10, Cysteine; 11, Glyceraldehyde 3-phosphate; 12, Creatinine; 13, Glutamic acid; 14, Glutamine; 15, Glucose; 16, Hexadecanoic acid; 17, Uric acid; 18, Oleic acid; 19, Octadecadienoic acid; 20, Octadecanoic acid; 21, Arachidonic acid; 22, Cholesterol.

Table 2. Fold changes of endogenous metabolites detected by GC/TOF-MS. The values represent the means±SD. $n=6$. ^b $P<0.05$, ^c $P<0.01$ vs Sham; ^e $P<0.05$, ^f $P<0.01$ vs in the OVX group prior to surgery.

Metabolites	Retention index	Biological role	Fold change		
			A	B	C
Cholesterol	3170.2	Biomarker of hyperlipidemia	2.10 ^c	2.77 ^c	2.45 ^f
Glycerol	1290.6	An important component of triglycerides and of phospholipids, Galactose metabolism	1.67 ^c	2.02 ^c	1.34 ^f
Octadecadienoic acid	2216.4	An essential fatty acid	2.02 ^c	2.02 ^c	1.65 ^f
Arachidonic acid	2375.2	Precursor of prostaglandins, thromboxanes, and leukotrienes	1.49 ^c	1.47 ^c	1.25 ^f
Oleic acid	2215.3	Saturated fatty acids	2.78 ^c	2.33 ^c	1.83 ^f
Glucose	1935.6	Primary source of energy	1.53 ^b	1.24 ^b	1.27 ^e
Glyceraldehyde-3-phosphate	1547.4	An important metabolic intermediate in both glycolysis and gluconeogenesis	0.57 ^b	0.55 ^b	0.84 ^e
3-Hydroxy-butanoic acid	1172.8	Ketone body, fatty acid biosynthesis	6.38 ^c	5.14 ^c	2.73 ^f
Alanine	1107.5	An important participant and regulator in glucose metabolism	0.89	0.10 ^c	0.06 ^f
Isoleucine	1305.8	Essential amino acids, stress, energy and muscle metabolism	3.96 ^c	4.99 ^c	2.20 ^f
Valine	1230.8		1.84 ^c	2.05 ^c	1.56 ^f
Leucine	1285.2		1.43 ^c	1.87 ^c	1.75 ^f
Glycine	1347.6	γ -glutamyl cycle	1.46 ^b	1.67 ^c	1.40 ^e
Glutamic acid	1633.5	A key molecule in cellular metabolism, γ -glutamyl cycle	1.12	1.40 ^c	1.46 ^e

A: Ratio of abundance of endogenous metabolites in serum samples between the week 3 post operation to pre-surgery in OVX group

B: Ratio of abundance of endogenous metabolites in serum samples between the week 6 after operation to pre-surgery in OVX group

C: Ratio of abundance of endogenous metabolites in serum samples between the OVX to the sham group at week 6.

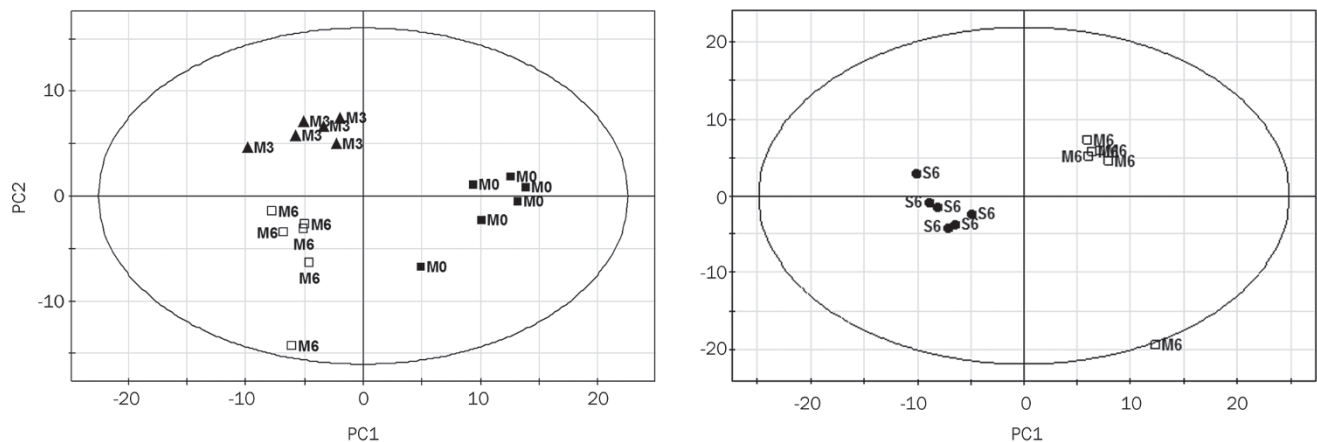


Figure 3. PLS-DA score plots of serum samples. (A) PLS-DA score plots of OVX rats over the whole experiment (pre-surgery, weeks 3 and 6 post-surgery). (B) PLS-DA score plots of OVX rats and sham rats at 6 weeks post-surgery. The x-axis and y-axis are labeled as PC1 (the first principal component) and PC2 (the second principal component), respectively. One data point stands for one subject: ■, OVX rats prior to surgery; □, OVX rats at week 6 post-surgery; ●, sham rats at week 6 post-surgery; ▲, OVX rats at week 3 post-surgery.

metabolite levels compared to the sham group, including a significant increase in the levels of cholesterol, octadecadienoic acid, arachidonic acid, 3-hydroxy-butanoic acid, glutamic acid, glucose, and glycerol and a significant decrease in the levels of glyceraldehyde 3-phosphate, alanine, galactopyranose, and arabinofuranose.

Discussion

Women now, on average, spend more than a third of their life post-menopause. The decline of endogenous estrogen levels,

together with physical inactivity, could account for obesity post-menopause, which could lead to an increased risk of cardiovascular diseases (eg, high blood pressure, high blood cholesterol, and high triglyceride levels), diabetes, stroke, and depression in postmenopausal women. Bilateral ovariectomy is the gold standard widely used to simulate postmenopausal obesity in female virgin rats^[37, 38]. Our results showed that rat weight was significantly elevated in the OVX group compared to the sham group. A higher BMI was observed in estrogen-deficient rats beginning the third week post-surgery despite

similar food consumption between the sham and OVX groups. Body weight and BMI were negatively correlated with levels of estradiol (E2), indicating that the rat model of estrogen-deficiency-induced obesity was successfully established.

However, the mechanisms of increases in body weight and fat content caused by estrogen deficiency remain unclear. It is generally recognized that rapid decrease in E2 secretion can bring about a hypothalamic-pituitary-ovarian (HPO) axis imbalance, which affects insulin, adrenal cortical hormone, growth hormone, and other hormone metabolism and leads to *in vivo* glucose and lipid metabolism disorders. These disorders are likely responsible for the pathogenesis of obesity among postmenopausal women. In addition, significantly elevated levels of TC, TG, and LDL-C and slightly reduced levels of HDL-C in the OVX group caused by estrogen-deficiency-induced obesity could indicate high risk for a series of complications.

Estrogen-deficiency-induced obesity is associated with fat accumulation and excessive intake of energy that break down the balance of energy metabolism (Figure 4). Fats are triesters of glycerol and fatty acids. Using the metabolomic approach, glycerol and fatty acid levels (*eg*, hexadecanoic acid, octadecadienoic acid, oleic acid, and arachidonic acid) were significantly increased in rats with estrogen-deficiency-induced obesity. We presumed that estrogen had a key modulator role in lipid metabolism. Herrero *et al* reported that myocardial fatty acid utilization was higher in women taking estrogens compared to those not receiving estrogens, which might be attributable to the regulation of β -oxidation of fatty acids^[39]. It has been reported that mRNA expression and the activity of enzymes including medium-chain acyl coenzyme A dehy-

drogenase and acetyl CoA oxidase, enzymes with fatty acid β -oxidation, are reduced under estrogen deficiency^[40]. Therefore, fatty acid levels may be increased because the estrogen deficiency interferes with the β -oxidation of fatty acids and leads to a reduced decomposition rate of fatty acids. Linoleic acid and essential fatty acids cannot be synthesized by humans and other animals and are only supplied from food components. In our research, linoleic acid was increased 1.4-fold and 1.2-fold compared to pre-surgery levels and to the sham group in the 6th week, respectively. Increased levels of fatty acids might be due to reduced β -oxidation of fatty acids. Moreover, levels of arachidonic acid, a precursor of prostaglandins, thromboxanes, and leukotrienes were elevated in the OVX rats, and arachidonic acid was biosynthesized by linoleic acid catalyzed by a series of enzymes. Therefore, estrogen deficiency might cause a lower rate of β -oxidation of fatty acids and greater biosynthesis of fatty acids. Furthermore, elevated levels of serum glycerol in OVX rats compared to sham rats might be a consequence of self-regulation because obese rats have higher basal lipolytic rates than normal subjects^[41].

Likewise, glycolysis is regulated by estrogen and estrogen receptors. Furman *et al* observed that estrogens stimulated the glycolysis process in MCF-7 cells, which could be inhibited by tamoxifen (TAM)^[42]. Using metabolomic analysis, the levels of glyceraldehyde 3-phosphate and alanine were significantly decreased in rats with estrogen deficiency. Glyceraldehyde 3-phosphate, an important metabolic intermediate in glycolysis, is bio-synthesized by phosphoglycerate kinase, which catalyzes the conversion of 1,3-bisphosphoglycerate into glyceraldehyde 3-phosphate, which is the first step of ATP production in glycolysis. A decrease in glyceraldehyde 3-phosphate

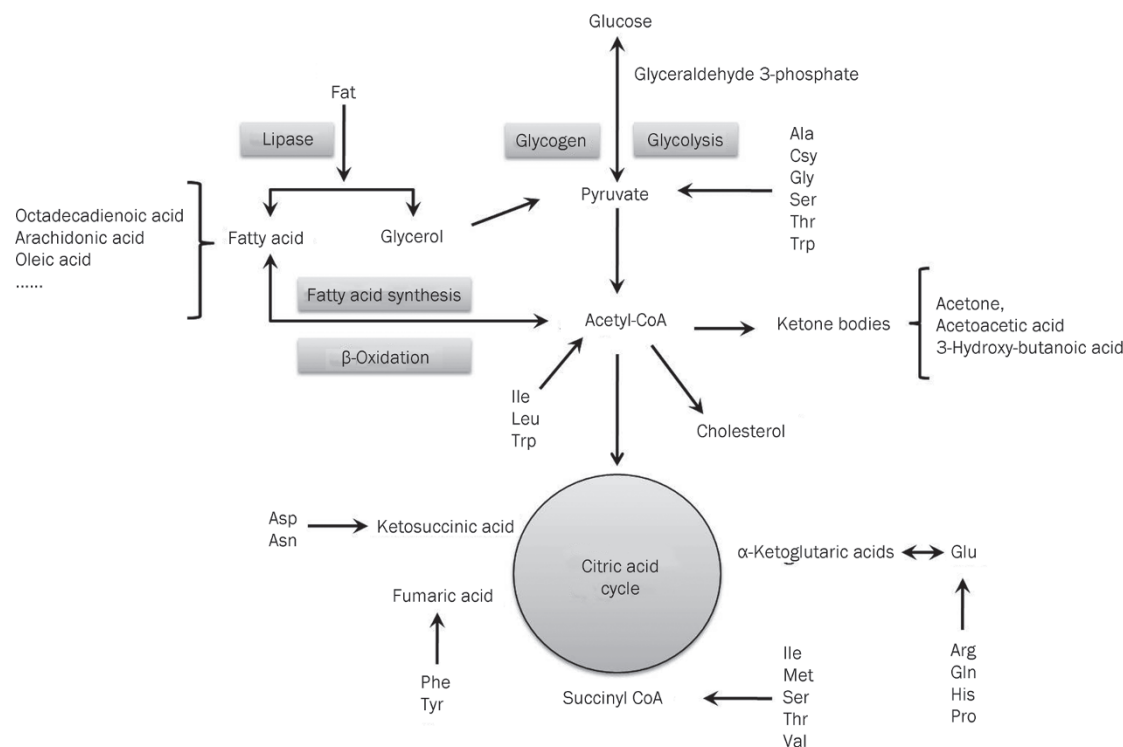


Figure 4. Regulation of glycometabolism, lipid metabolism, and amino acid metabolism by estrogen deficiency (significant difference from the endogenous metabolite is indicated in bold).

suggests that estrogen has an adjustment effect in glycolysis, and an estrogen deficiency could interfere with this process. Similar to glyceraldehyde 3-phosphate, the lower alanine levels found in rats with estrogen deficiency would be indirectly correlated with the glycolysis process because disruption of glycolysis leads to pyruvate insufficiency. Moreover, alanine plays a key role in the glucose-alanine cycle between muscle tissue and the liver; glycolysis in the muscle produces pyruvate, which is further converted to alanine. The results of our study are also suggestive of a modified alanine-glucose cycle. In addition, endogenous estrogen regulates skeletal muscle mass in developing rats. Therefore, estrogen loss can alter skeletal muscle recovery from disuse^[43, 44]. As a result, decreased muscle function may also be a reason for the reduction of alanine biosynthesis.

Estrogens are involved in the regulation of not only fatty acid metabolism and glycolysis but also the TCA cycle. The activity of citrate synthase (CS) in skeletal muscle and cerebral blood vessels is enhanced by estrogen therapy in OVX rats^[45]. Raben *et al* found that β -hydroxyacyl-CoA dehydrogenase (HADH) and CS levels were 20% lower in formerly obese women^[46]. Moreover, pyruvate dehydrogenase, aconitase, and ATP-synthase are regulated by estrogen^[47]. Therefore, more precursor acetyl-CoA may enter into the fatty acid, ketone (*eg*, acetoacetate, 3-hydroxy-butanoic acid, and acetone), and cholesterol biosynthesis processes as a result of the TCA cycle being attenuated under estrogen deficiency. As a result of the affected metabolic pathway, high levels of cholesterol and fatty acids were observed in OVX rats, and a marked increase in the levels of 3-hydroxy-butanoic acid was observed in rats with bilateral ovariectomy-induced obesity. Furthermore, glycolysis, the TCA cycle, and insulin would decrease the levels of blood glucose in contrast to gluconeogenesis (the generation of glucose from non-carbohydrates), which could increase the levels of blood glucose. In our study, the levels of blood glucose were slightly elevated during obesity in OVX rats, which might be connected with the subdued glycolysis and TCA cycle caused by estrogen deficiency. Insulin resistance resulting from estrogen deficiency is a known risk factor for type 2 diabetes and leads to the high blood glucose levels observed.

Higher cholesterol levels were found in OVX rats compared to sham rats by metabolomic analysis, which was in agreement with the conventional measurements using commercial kits. High blood cholesterol levels in postmenopausal women are a biomarker of obesity due to ovarian hormone deficiency. Mataumoto *et al* reported that the level of cholesterol 7 α -hydroxylase activity, a key enzyme in cholesterol catabolism, was significantly reduced in the liver in OVX rats^[48]. Therefore, excessive acetyl-CoA is used for cholesterol synthesis in OVX rats, but the rate of cholesterol degradation is attenuated because of the decrease in 7 α -hydroxylase activity. Obviously, the elevated levels of serum cholesterol were a result of a combination of the above two factors.

The branched-chain aliphatic amino acids (BCAAs), including isoleucine, valine, and leucine, are markedly increased after estrogen deficiency. The BCAAs play important roles in

protein synthesis, neurotransmitter synthesis, and branched-chain fatty acid synthesis. They cannot be synthesized by the animal itself and must be supplied by the diet. In our study results, serum amino acid levels were elevated in obesity, which is consistent with previous research^[49-51]. She *et al*^[52] reported that obesity may play a potential role in the regulation of BCAA catabolism. They found that the activities of the BCATm and BCKD enzyme complex, the key enzymes in the first two steps of the BCAAs catabolic pathway, were depressed in obese *ob/ob* mice and Zucker rats. Therefore, we presumed that the alternations in BCAA metabolism may contribute to increase in BCAAs in obesity. The γ -glutamyl cycle plays a key role in glutathione synthesis, and the transport system for amino acids and glutamic acid, glycine, and cystine take part in the γ -glutamyl cycle. Higher glutamic acid, glycine, and cystine levels in estrogen-deficiency-induced obesity would influence the γ -glutamyl cycle.

Conclusions

Metabolomic profiling with multivariate statistical analysis combined with conventional measurement kit assays indicated a progression of experimental obesity induced by estrogen deficiency in rats. The series of potential biomarkers identified provide fingerprints of rat metabolomic changes during obesity and an overview of multiple metabolic pathways during the progression of obesity that involves glucose metabolism, lipid metabolism, and amino acid metabolism.

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Author contribution

Bo MA and Qi ZHANG have been involved in all phases of this project. Guang-ji WANG designed the research. Ji-ye A analyzed the data, and Di WU revised and wrote the paper. Bei CAO, Ying-ying HU, Lin-sheng LIU were involved in measurements. Ying LIU, Yong-lu WANG, and Ya-ya ZHENG were involved in the animal experiments of the project.

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