Systematic Evaluations of Doxorubicin-Induced Toxicity in Rats Based on Metabolomics

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ABSTRACT: Doxorubicin (DOX) is widely used to treat solid tumors, but its use is limited by its severe cardiotoxicity, nephrotoxicity, hepatotoxicity, and neurotoxicity. Metabolomic studies on DOX-induced toxicity are mainly focused on alterations in the heart and kidney, but systematic research on multiple matrices (serum, heart, liver, brain, and kidney) is rare. Thus, in our study, gas chromatography−mass spectrometry analysis of main targeted tissues (serum, heart, liver, brain, and kidney) was used to systemically evaluate the toxicity of DOX. Multivariate analyses, including orthogonal projections to the latent structure and t-test, revealed 21 metabolites in the serum, including cholesterol, D-glucose, D-lactic acid, glycine, L-alanine, L-glutamic acid, L-isoleucine, L-leucine, L-proline, L-serine, L-tryptophan, L-tyrosine, L-valine, MG (0:0/18:0/0:0), MG (16:0/0:0/0:0), N-methylphenylethanolamine, oleamide, palmitic acid, pyroglutamic acid, stearic acid, and urea. In the heart, perturbed metabolites included 3-methyl-1-pentanol, cholesterol, D-glucose, D-lactic acid, glycerol, glycine, L-alanine, L-valine, MG (16:0/0:0/0:0), palmitic acid, phenol, propanoic acid, and stearic acid. For the liver, DOX exposure caused alterations of acetamide, acetic acid, D-glucose, glycerol, L-threonine, palmitic acid, palmitoleic acid, stearic acid, and urea. In the brain, metabolic changes involved 2-butanol, carbamic acid, cholesterol, desmosterol, D-lactic acid, L-valine, MG (16:0/0:0/0:0), palmitic acid, and stearic acid. In the kidney, disturbed metabolites were involved in cholesterol, glycerol, glycine, L-alanine, MG (0:0/18:0/0:0), MG (16:0/0:0/0:0), and squalene. Complementary evidence by multiple matrices revealed disturbed pathways concerning amino acid metabolism, energy metabolism, and lipid metabolism. Our results may help to systematically elucidate the metabolic changes of DOX-induced toxicity and clarify the underlying mechanisms.

1. INTRODUCTION

Doxorubicin (DOX), a broad-spectrum antitumor antibiotic, is commonly used to treat various solid tumors and also used as a model drug.1 However, its use is limited because of its severe side effects like cardiotoxicity,2−4 hepatic lesion,5,6 kidney injury,7 and neuron damage in the brain,8 which involve the whole body. Although dozens of studies have been done on DOX and many hypotheses have been proposed for the mechanisms, including oxidative stress and neuroinflammatory response,8 the potential mechanism of DOX-induced toxicity remains unclear.

Metabolomics, an emerging “-omics” technology, could provide global metabolic profiling parameters and is a powerful tool for the discovery of biomarkers.9 Our previous metabolomic studies based on gas chromatography−mass spectrometry (GC−MS) could identify lots of metabolites and reveal the changes of metabolites from a global perspective, which may help explain some underlying mechanisms.10,11 Therefore, we think it may be a good fit to study the toxic side effects of DOX. Earlier studies on the toxicity of DOX were performed by analyzing the metabolic perturbations in the serum, urine, heart, liver, and kidney.12−14 However, a comprehensive understanding of DOX-induced toxicity in multiple biological matrices remains to be achieved, which is vital to account for the pathogenic process and toxicological mechanism of DOX.

In our study, we aimed to investigate the toxicity of DOX on the metabolic alterations of rat serum, heart, liver, kidney, and the whole brain. To this end, a GC−MS-based metabolomic profiling technique coupled with univariate and multivariate analyses was conducted to discover metabolic biomarkers in rat serum, heart, liver, kidney, and the whole brain in order to
Figure 1. Representative GC–MS total ion current chromatograms of the serum (A), heart tissue (B), liver tissue (C), brain tissue (D), and kidney tissue (E) samples from a mixture of the control and DOX-treated rats.
Figure 2. OPLS scores and 200 permutation tests for the OPLS-DA models: serum (A), heart tissue (B), liver tissue (C), brain tissue (D), and kidney tissue (E).
provide new insights into the metabolic pathogenesis on the DOX-induced toxicity.

2. RESULTS

2.1. GC–MS Chromatograms of Serum and Tissue Samples. The representative chromatograms of the quality control (QC) serum and tissue samples (heart, liver, brain, and kidney) from a mixture of the DOX-treated and control rats all showed strong signals and good RT reproducibility, which can be seen in Figure 1.

2.2. Multivariate Statistics of the Metabolomics Data. The parameters obtained indicated efficient modeling that clearly separated the DOX and control groups (serum: \( R^2_X = 0.827, R^2_Y = 0.967, Q^2 = 0.873 \); heart tissue: \( R^2_X = 0.984, R^2_Y = 0.995, Q^2 = 0.982 \); liver tissue: \( R^2_X = 0.968, R^2_Y = 0.987, Q^2 = 0.964 \); brain tissue: \( R^2_X = 0.851, R^2_Y = 0.943, Q^2 = 0.889 \); and kidney tissue: \( R^2_X = 0.982, R^2_Y = 0.755, Q^2 = 0.825 \)). Values of these parameters approaching 1.0 indicate a stable model with predictive reliability. The statistical validation using permutation tests to assess the significant orthogonal projections to latent structures discriminant analysis (OPLS-DA) models revealed no overfitting, as the blue regression line of the \( Q^2 \)-points intersects the vertical axis (on the left) below zero, as shown in Figure 2. In addition, the OPLS-DA with variable importance in the projection (VIP) (VIP > 0.5) and the \( p \) value of \( t \)-test (\( p < 0.05 \)) compared with the controls revealed that the variations in the serum after DOX treatment showed increased cholesterol, \( \Delta \)-glucose, glycine, \( L \)-glutamic acid, \( L \)-isoleucine, \( L \)-leucine, \( L \)-proline, \( L \)-serine, \( L \)-tryptophan, \( L \)-tyrosine, \( L \)-valine, pyroglutamic acid, oleamide, \( N \)-methylphenylethanolamine, and urea, together with decreased \( D \)-lactic acid, \( L \)-alanine, \( MG (0:0/18:0/0:0) \), \( MG (16:0/0:0/0:0) \), palmitic acid, and stearic acid. In the heart, perturbed metabolites include elevation of cholesterol, \( \Delta \)-glucose, glycine, \( L \)-valine, and phenol with decline of \( D \)-lactic acid, \( L \)-alanine, 3-methyl-1-pentanol, glycerol, \( MG (16:0/0:0/0:0) \), palmitic acid, propanoic acid, and stearic acid. For the liver, DOX exposure caused accumulation of acetamide, \( \Delta \)-glucose, and urea with reduction of acetic acid, glycerol, \( L \)-threonine, palmitic acid, palmitoleic acid, and stearic acid. For the brain, metabolic changes showed that 2-butanol, carbamic acid, cholesterol, and desmosterol were increased, and \( \Delta \)-lactic acid, \( L \)-valine, \( MG (16:0/0:0/0:0) \), palmitic acid, and stearic acid were decreased. In the kidney, disturbed metabolites were involved in the elevation of cholesterol, \( \Delta \)-glucose, glycine, and urea with reduction of acetic acid, glycerol, \( L \)-threonine, palmitic acid, palmitoleic acid, and stearic acid. For the brain, metabolic changes showed that 2-butanol, carbamic acid, cholesterol, and desmosterol were increased, and \( \Delta \)-lactic acid, \( L \)-valine, \( MG (16:0/0:0/0:0) \), palmitic acid, and stearic acid were decreased. In the kidney, disturbed metabolites were involved in the elevation of cholesterol, \( \Delta \)-glucose, glycine, and squalene as well as the decline of \( L \)-alanine, \( MG (0:0/18:0/0:0) \), and \( MG (16:0/0:0/0:0) \). The detailed results of the metabolites are shown in Table 1, and the comparison of the distribution of biomarkers in each tissue are shown in Figure 3.

Table 1. List of Perturbed Metabolites in the Serum, Heart, Liver, Brain, and Kidney

| metabolites               | serum VS | heart VS | liver VS | brain VS | kidney VS | pathway                           |
|---------------------------|----------|----------|----------|----------|-----------|-----------------------------------|
| acetamide                 | 5.72     |          |          |          |           | pyruvate metabolism               |
| acetic acid               | 1.12     |          |          |          |           |                                   |
| cholesterol               | 1.10     | 1.43     | 1.46     | 2.33     |           | steroid biosynthesis              |
| desmosterol               | 2.25     |          |          |          |           |                                   |
| squalene                  |          |          |          |          | 2.54      |                                   |
| \( \Delta \)-glucose      | 1.63     | 1.05     | 1.42     |          |           | glycolysis                         |
| \( \Delta \)-lactic acid  | 3.37     | 1.11     |          | 2.14     |           |                                   |
| glycine                   | 1.70     | 1.02     |          | 0.53     | 0.53      | amino acid metabolism             |
| \( L \)-alanine           | 2.36     | 1.11     |          | 0.53     |           |                                   |
| \( L \)-glutamic acid     | 0.90     |          |          |          |           |                                   |
| \( L \)-isoleucine        | 0.79     |          |          |          |           |                                   |
| \( L \)-leucine           | 1.44     |          |          |          |           |                                   |
| \( L \)-proline           | 0.93     |          |          |          |           |                                   |
| \( L \)-serine            | 0.86     |          |          |          |           |                                   |
| \( L \)-threonine         |          |          |          | 2.33     |           |                                   |
| \( L \)-tryptophan        | 0.95     |          |          |          |           |                                   |
| \( L \)-tyrosine          | 0.92     |          |          |          |           |                                   |
| \( L \)-valine            | 0.85     | 3.49     | 4.79     |          |           |                                   |
| pyroglutamic acid         | 0.94     |          |          |          |           |                                   |
| \( MG (0:0/18:0/0:0) \)   | 0.92     |          |          |          | 2.65      | lipid metabolism                  |
| \( MG (16:0/0:0/0:0) \)   | 1.48     | 1.19     | 0.82     | 2.72     |           |                                   |
| oleamide                  | 1.68     |          |          |          |           |                                   |
| palmitic acid             | 1.85     | 1.39     | 1.35     | 0.86     |           |                                   |
| palmitoleic acid          |          | 1.35     |          |          |           |                                   |
| stearic acid              | 1.30     | 1.30     | 1.21     | 0.81     | 1.05      | energy metabolism                 |
| glycerol                  | 3.50     | 2.48     |          |          |           | urea cycle                        |
| \( N \)-methylphenylethanolamine | 1.10 |          |          |          |           | others                            |
| urea                      | 4.40     | 1.30     |          | 1.51     |           |                                   |
| 2-butanol                 |          |          |          | 1.51     |           |                                   |
| 3-methyl-1-pentanol       | 1.27     |          |          | 2.18     |           |                                   |
| carbamic acid             |          |          |          |          |           |                                   |
| phenol                    | 5.26     |          |          |          |           |                                   |
| propanoic acid            | 1.99     |          |          |          |           |                                   |

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metabolism; the brain tissue: (i) steroid biosynthesis and the kidney tissue: (i) steroid biosynthesis, (g) primary bile acid biosynthesis, and (j) glycerolipid metabolism. The detailed results of the pathway analysis are shown in Table 2, with a summary shown in Figure 4.

3. DISCUSSION

Our study represents a metabolomic profiling of systemic alterations in the main targeted tissues (serum, heart, liver, brain, and kidney) following the DOX treatment. Our study revealed that there were 21, 13, 9, 9, and 7 identified metabolites between the DOX and control group in the serum, heart, liver, brain, and kidney, respectively. These perturbed metabolites in multiple biological matrices could provide some new insights into the pathophysiologic mechanism of DOX toxicity. The disturbances of the identified metabolites were mainly involved in amino acid, lipid, energy, and carbohydrate metabolism.

3.1. Cardiotoxicity-Related Metabolic Changes. DOX-induced dose-dependent cardiotoxicity is a major concern in clinical applications in anticancer therapy.\textsuperscript{15} The DOX-induced cardiotoxicity is associated with the elevated status of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase, and creatine kinase in previous studies.\textsuperscript{16,17} However, the mechanism of DOX-mediated cardiotoxicity is still not fully elucidated. Therefore, the discovery of early cardiotoxicity biomarkers has become more important for the identification of toxicity before cardiac tissues are pathologically damaged.

The heart, a highly energy-demanding organ, depends on a steady supply of glucose, lipids, and amino acids to produce ATP to maintain a normal beating heart rhythm.\textsuperscript{18,19} p-glucose-based energy supply is an important source for heart beating; our results showed that the increased level of p-glucose in DOX-treated rats in the heart, liver tissue, and serum may be due to inhibition in the energy supply, which was in agreement with an earlier metabolomic study based on 1H NMR to assess systematic alterations in a DOX-induced rat model.\textsuperscript{12} However, another metabolomic study based on GC/MS and ultraperformance liquid chromatography/tandem mass spectrometry revealed that the level of p-glucose in myocardial samples was decreased; they think, in the context of DOX treatment, cardiac energy metabolism undergoes remodeling, leading to inhibition of the oxidation of fatty acids, and thus, the utilization of glucose was increased.\textsuperscript{2}

Inconsistency of these results needs further study. Lipid metabolism is perturbed in the DOX group. Our data showed that cholesterol, glycerol, MG (16:0/0:0/0:0), palmitic acid, propanoic acid, and stearic acid were significantly changed. Among them, cholesterol was elevated, in line with the previous study,\textsuperscript{2} which may be the result of a lipolysis blockade caused by DOX.\textsuperscript{20,21} Amino acid metabolism was also disturbed, which suggested that amino acids played important roles in the progression of cardiotoxicity.\textsuperscript{15,22} Alterations in glycine, l-valine, and l-alanine were found in the heart tissue; additionally, l-glutamic acid, l-isoleucine, l-leucine, l-proline, l-serine, l-tryptophan, l-tyrosine, and pyroglutamic acid were changed in the serum in the DOX group. The level of l-alanine is controversial, but as an amino acid, it is an important energy metabolism precursor and can be transformed into some biomolecules, and that is certain. l-glutamic acid plays a key role in inhibiting the myocardial oxidative damage.\textsuperscript{23} l-valine, l-isoleucine, and l-leucine were branched chain amino acids.

Table 2. Pathway Analysis by MetaboAnalyst 4.0

| pathway name                  | raw p  | impact |
|-------------------------------|--------|--------|
| aminocyt-tRNA biosynthesis    | 2.991 × 10^{-11} | 0.167  |
| glutathione metabolism        | 4.548 × 10^{-3}  | 0.115  |
| glyoxylate and dicarboxylate metabolism | 6.669 × 10^{-3}  | 0.148  |
| arginine biosynthesis         | 1.250 × 10^{-2}  | 0.117  |
| alanine, aspartate, and glutamate metabolism | 4.674 × 10^{-2}  | 0.197  |
| phenylalanine, tyrosine, and tryptophan biosynthesis | 4.947 × 10^{-2}  | 0.500  |
| galactose metabolism         | 1.536 × 10^{-3}  | 0.035  |
| primary bile acid biosynthesis | 4.198 × 10^{-2}  | 0.056  |
| galactose metabolism         | 8.082 × 10^{-3}  | 0.035  |
| steroid biosynthesis         | 1.454 × 10^{-2}  | 0.028  |
| steroid biosynthesis         | 7.173 × 10^{-3}  | 0.056  |
| primary bile acid biosynthesis | 8.577 × 10^{-3}  | 0.056  |
| glycerolipid metabolism      | 4.970 × 10^{-2}  | 0.237  |
(BCAA) involved in the progression of cardiotoxicity, which was associated with the dysfunction of the energy metabolism. L-tyrosine has been reported to be associated with cardiac hypertrophy. In all, altered amino acid metabolism, lipid metabolism, and energy metabolism were involved in the pathophysiologic process of DOX-induced cardiotoxicity.

3.2. Hepatic Lesion-Related Metabolic Changes. The liver is a crucial metabolic organ that plays a key role in the storage of glycogen and detoxification and synthesis of protein. There is mounting evidence that the application of even lower doses of DOX (1 mg/kg) in rats could cause irreversible liver damage and an elevation of the apoptotic processes in the hepatic tissue. As we all know, ALT, alkaline phosphatase, and total bilirubin are known indicators of hepatic lesion. In our work, we observed accumulation of acetamide, D-glucose, and urea with reduction of acetic acid, glycerol, L-threonine, palmitic acid, palmitoleic acid, and stearic acid in the liver tissue because of DOX treatments involving galactose, lipid, energy, and amino acid metabolism. The liver is the main metabolic site of aromatic amino acids (AAAs); weakening of amino acid metabolism and the increase of hepatocyte necrosis and protein decomposition will lead to the increase of AAAs. L-tryptophan and L-tyrosine were elevated in the serum in the DOX group in our study, which further showed that DOX caused the hepatic lesion and is in accordance with previous studies.

3.3. Neurotoxicity-Related Metabolic Changes. In our previous studies, we found that DOX treatment could cause depression-like behaviors in rats, and oxidative stress, neuroinflammation, and cell death in the brain tissue induced by DOX treatment were also confirmed, which collectively revealed that DOX caused neurotoxicity. Metabolic studies on the whole brain of DOX-induced rats have rarely been done. Thus, seeking potential metabolic changes in the whole brain to understand the neurotoxic effects of DOX is of great importance. In our study, DOX rats exhibited changes of 2-butanol, carbamic acid, desmosterol d-lactic acid, L-valine, MG (16:0/0:0/0:0), palmitic acid, and stearic acid, involved in steroid biosynthesis. Cholesterol was also elevated, in line with the serum and heart. Disorders of d-lactic acid indicated that both aerobic and anaerobic processes were impaired in DOX rats, which means energy metabolism was affected. L-valine is a
branched-chain amino acid and linked solely to carbohydrates; additionally, L-valine deficiency is marked by neurological defects in the brain. The abovementioned evidence demonstrated that L-valine was associated with brain injury, which was further confirmed by the decreased L-valine in the DOX treatment in our study. Further study is needed to uncover more metabolic changes linked to the neurotoxicity of DOX.

3.4. Nephrotoxicity-Related Metabolic Changes. Renal damage often occurs during the course of DOX therapy, and additionally, DOX administration could be used as an animal model of nephropathy. In our study, disturbed metabolites were involved in the elevation of cholesterol, glycerol, glycine, and squalene as well as the decline of L-α-amino acids are basic units for the synthesis of protein in an organism, and increasing evidence has suggested that renal injury is closely linked to protein expression abnormality and amino acid reabsorption. Among amino acids, glycine is the simplest amino acid, which is involved in the synthesis of creatine, heme, purines, and other biomolecules, and could also act as the precursor of glutathione, a primary antioxidant in the human body. A previous study confirmed that glycine could ameliorate renal damage, and the protective effects of glycine on the kidney might be associated with oxidative stress. Furthermore, glycine also participates in the biosynthesis of primary bile acid, and its concentration is correlated with the microbial activity, which is also demonstrated in an earlier report. Therefore, the above evidence indicated that steroid biosynthesis, primary bile acid biosynthesis, and glycerolipid metabolism were altered in the kidney in the DOX group.

The abovementioned results suggested that the DOX exposure caused cardiotoxicity, neurotoxicity, nephrotoxicity, and hepatic lesion, which results in alterations of amino acid, lipid, and energy metabolism. However, there are still some limitations that should be mentioned. First, a single metabolomics approach based on GC–MS was used, and other technologies (e.g., LC–MS) are needed to confirm our findings. Second, sex difference is a verified factor to affect the metabolic profile, and only male rats were studied in our study. Third, metabolic changes of other organs such as the spleen and lung should also be studied to completely understand the systematic toxicity of DOX.

4. CONCLUSIONS

In the current study, a GC–MS-based profiling of main targeted tissues (serum, heart, liver, brain, and kidney) was employed to systematically assess the toxicity of DOX. Our present study provided a panoramic and systematic view of metabolic alterations in DOX-treated rats, correlated with amino acid, lipid, and energy metabolism, which provided some predictive information for DOX-induced toxicity and helped us understand the toxicological mechanism of DOX.

5. MATERIALS AND METHODS

5.1. Animals. Eight week old Sprague-Dawley rats (male, 180–240 g, Beijing Vital River Laboratory Animal Technology Co., Ltd.) were initially housed in a temperature-controlled (24 ± 1 °C) environment under a day–night reversal (12 h/12 h) with free access to food and water. The study protocol was approved by the Medical Ethics Committee of the Jining No 1 People’s Hospital (protocol number 20170026). All animal procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (Chinese Council).

Animals were randomly divided into the control and DOX groups (n = 8). Rats in the DOX group were given DOX every two days via intraperitoneal injection at a dose of 2.5 mg/kg for each injection for a total of seven injections. The untreated control group was injected with the same volume of normal saline. The dose of DOX was chosen based on our previous studies.11,32

5.2. Reagents. Heptadecanoic acid (purity: ≥98%; lot: SLBX4162), an internal standard (IS), and N,0-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS; v/v; lot: BCBZ4865) were from Sigma-Aldrich (Saint Louis, MO, USA). Pyridine (lot: C10551455) was purchased from Shanghai Macklin Biochemical (Shanghai, China). o-Methyl hydroxylamine hydrochloride (purity: 98.0%; lot: LG10T16) was obtained from J&K Scientific Ltd. (Beijing, China). Chromatographic-grade methanol was from Thermo Fisher Scientific (Waltham, MA, USA). Water was purchased from Hangzhou Wahaha Company (Hangzhou, China).

5.3. Sample Collection. Rats were euthanized with 1% sodium pentobarbital via intraperitoneal injection at a dose of 50 mg/kg. Blood samples were collected from the cardiac coronary artery after anesthesia, centrifuged (5000 rpm, 5 min) to obtain the supernatants (serum), and then stored at −80 °C before use. The brains were quickly removed, and all rats were dissected on an ice surface. The whole brain, heart, liver, and kidney samples were washed with phosphate-buffered saline (pH = 7.2), and then, all tissue samples were frozen at −80 °C until needed.

5.4. Sample Preparation. 100 μL serum samples were mixed with 350 μL methanol (containing 100 μg/mL IS), and after centrifugation (14,000 rpm, 4 °C, 10 min), the supernatants were transferred to 2 mL tubes and dried at 37 °C under a gentle stream of nitrogen. Then, the extracts were mixed with 80 μL of o-methyl hydroxylamine hydrochloride (15 mg/mL in pyridine) and incubated for 90 min at 70 °C. Then, 100 μL of BSTFA + 1% TMCS was added to each sample, followed by incubation for 1 h at 70 °C. The solution was then vortexed, centrifuged (14,000 rpm, 4 °C, 2 min), and filtered through a 0.22 μm filter membrane before GC–MS analysis.

50 μg tissue (heart, liver, brain, and kidney) was homogenized with 1 mL methanol (containing 1 mg/mL IS), transferred to a 2 mL tube, and centrifuged (14,000 rpm, 4 °C, 10 min). The supernatants were transferred into a 2 mL tube and dried at 37 °C under a gentle stream of nitrogen gas. Subsequently, the extracts were mixed with 80 μL of o-methyl hydroxylamine hydrochloride (15 mg/mL in pyridine) and incubated in a water bath (70 °C, 90 min), followed by addition of 100 μL of BSTFA + 1% TMCS and incubation for a further 1 h at 70 °C to create a derivatized solution. The solution was then vortexed, centrifuged (14,000 rpm, 4 °C, 2 min), and filtered through a 0.22 μm filter membrane before GC–MS analysis.

5.5. GC–MS Analysis. QC of the samples (serum, heart, liver, brain, and kidney) was defined as a mixture from the DOX and control rats. The stability of retention time (RT) was evaluated by the RT of IS. GC–MS analysis was conducted on a 7890B GC system with a 7000C mass spectrometer. Sample separation was conducted on an HP-
SMS fused-silica capillary column, and 1 μL aliquots of the derivatized solution was run in the split mode (50:1), with helium as the carrier gas and a front inlet purge flow of 3 mL/min; the gas flow rate was 1 mL/min. The GC temperature program began at 60 °C for 4 min, increased to 300 °C at 8 °C/min, and ended with a final 5 min maintenance at 300 °C. The temperatures associated with the injection, transfer line, and ion source were 280, 250, and 230 °C, respectively. Electron impact ionization (−70 eV) was used with an acquisition rate of 20 spectra/s in the MS setting. MS detection was performed by electrospray ionization in the full-scan mode, involving mass/charge (m/z) values of 50–800.

5.6. Multivariate Statistical Analysis. Metabolites were first explored using GC–MS, involving deconvolution, alignment, and data reduction to produce a list of m/z and RT pairs, with the corresponding intensities. The resulting table was exported into Excel and normalized. The sample names (observations) and normalized peak area percentages were imported into SIMCA-P 14.0 (Umetrics, Umeå, Sweden) for statistical analysis. Unsupervised principal component analysis (PCA) was employed to see the distribution of the DOX and control groups. The validity of the model was verified using permutation tests (200 permutations). Statistical analysis was performed using two-tailed Student’s t-test. A calculated p value <0.05 and VIP values > 0.5 were considered to be statistically significant in the present study. MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) and the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www. kegg.jp) were used in the pathway analysis, and raw p < 0.05 and impact > 0 were defined as significant. These common metabolomic analytical methods were also used in our previous studies.10,11 Venn diagram analysis was performed using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools), and it was also used in our previous study.11

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Notes

The authors declare no competing financial interest.

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