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Toxicity analysis of doxorubicin using plasma metabolomics technology based on rapid resolution liquid chromatography coupled with quadruple-time-of-flight mass spectrometry

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Abstract

Doxorubicin is a highly efficient antitumor drug, but it can induce toxicity, largely affecting people's life. Metabolomics technology, a part of systems biology, can offer information on the change on metabolic profiles of biofluids upon drug administration. Meanwhile, the plasma metabolomics study on doxorubicin toxicity using liquid chromatography-mass spectrometry technology is not very clear. In this study, plasma metabolomics approach using rapid resolution liquid chromatography coupled with quadruple-time-of-flight mass spectrometry technology was used to investigate the toxic mechanism of doxorubicin from metabolic view. The biochemical analysis and histopathological examination result showed that toxicity model can be built by intraperitoneal injection of doxorubicin with the dose of 15 mg/kg in male Wistar rats. Metabolomics results revealed fifteen biomarkers were changed due to doxorubicin-induced toxicity. Besides, arachidonic acid metabolism, valine, leucine and isoleucine biosynthesis, sphingolipid metabolism, glycerophospholipid metabolism and primary bile acid biosynthesis were mainly responsible for the toxicity of doxorubicin. The changed metabolites and interrupted pathways found in this study are meaningful and the result can lay the foundation for further research on the toxicity mechanism of doxorubicin.

Abbreviations: DOX, doxorubicin; GC, gas chromatography; LC, liquid chromatography; GC-MS, gas chromatography-mass spectrometry; LC-MS, Liquid

chromatography-mass spectrometry; RRLC, rapid resolution liquid chromatography; Q-TOF-MS, quadruple-time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; RRLC-Q-TOF-MS, rapid-resolution liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; AST, aspartate aminotransferase; CK, creatine kinase; BUN, blood urea nitrogen; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; VIP, variable importance plot; TIC, total ion current; LPC, lysophosphatidylcholine

Introduction

Doxorubicin (DOX), an anthracycline antibiotic and a derivative of *Streptomyces* bacteria, is a highly efficient antitumor drug, but its organotoxic potential (cardio-, hepato-, and nephrotoxicity) limits its clinical use. DOX is mainly metabolised in the liver, with doxorubicinol, doxorubicinone, and 7-deoxydoxorubicinone as its major metabolites.[1-2] Some mechanism of DOX-induced toxicity has been proposed, including the inhibition of nucleic acid and protein synthesis,[3] the generation of free radical,[4] induction of apoptosis,[5] but the exact metabolic causal mechanism of toxicity has not yet been fully elucidated. Thus, a simple and accurate method for revealing the mechanism of DOX is urgently needed.

Metabolomics, is now recognised as a widely used technique to advance the toxicology, disease diagnosis, and therapeutic efficacy.[6,7] Metabolomics aims to gain global information of metabolite profile in systems including cell, tissue and organism under a specific condition, such as pathophysiological stimuli, drug administration, environment, or other factors.[7-10] Nowadays, metabolomics has become a new and powerful method in the study of mechanism of drug-induced toxicity. In addition, metabolomics pays more attention to small and low weight molecules. It is these metabolites that are the final product in biological metabolic pathway and play an important role in metabolism.[11-13] Therefore, the changed level of specific metabolites in biofluid which can be identified by metabolomics technology can reflect physiological state in some extent and can reveal the

mechanism of toxicity in metabolic level.

Data acquisition is firstly performed to do further data analysis and find useful information in metabolomics study. Nowadays, mass spectrum analytical method is widely used to do data acquisition in metabolomics study. Because biofluid is complex and mixture sample, mass detector is usually coupled with a separation chromatography system, including gas chromatography (GC) and liquid chromatography (LC).[14-16] When analyzed with gas chromatography-mass spectrometry (GC-MS) technology, sample must be volatile or made volatile by derivatization. Liquid chromatography-mass spectrometry (LC-MS) does not have this problem and the sample preparation is relatively simple. What's more, rapid resolution liquid chromatography (RRLC) coupled with quadruple-time-of-flight mass spectrometry (Q-TOF-MS) has the advantage of high sensitivity and high resolution, becoming a powerful technology in data acquisition of metabolomics.

Until now, few metabolomics study had been applied to find the toxic effect of doxorubicin on heart issue[17] or cell *in vitro*[18] using GC-MS technology or nuclear magnetic resonance (NMR) technology. Besides, Wang *et al* adopted metabolomics technology coupled with LC-MS analysis to reveal the profiling of rat urinary metabolites interrupted by doxorubicin administration, finding six small weight molecular metabolites responsible for doxorubicin toxicity in urine.[19] It is a fact that the metabolites in different kinds of biofluid have some distinction, meanwhile, the endogenous metabolites detected by different technology (LC-MS, GC-MS or NMR)

also varied. Though some study had been performed to observe the toxic characteristic of doxorubicin, the plasma metabolomics investigation on doxorubicin toxicity using LC-MS technology is not very clear and still need to be further studied. In this study, metabolomics approach based on rapid-resolution liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (RRLC-Q-TOF-MS) technique was applied to investigate the effect of doxorubicin on plasma metabolites, determine the possible interrupted pathways related to doxorubicin-induced toxicity, aim for reveal the toxic mechanism of doxorubicin and provide important information for further research on doxorubicin toxicity.

Experimental

Reagents and materials

Doxorubicin was obtained from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). The assay kits were obtained from the Biosino Bio-technology and Science Inc. (Beijing, China). High performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Oceanpak (Goteborg, Sweden). HPLC-grade formic acid was purchased from ROE (USA).

Animal experiment

This study was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine under permit number TCM-2011-065-F02. Male Wistar rats weighing 200 g to 220 g were kept in the same room at a temperature of 25 ± 1 °C and at humidity of 50 ± 5%, on a 12 h/12 h light/dark cycle. Before the

experiment, the rats were acclimated with free access to food and water for one week. The rats were randomly divided into 2 groups of 12 rats each: DOX group and control group. DOX was first dissolved in normal saline (0.9% w/v), and then the rats in DOX group were injected doxorubicin intraperitoneally with a single dose of 15 mg/kg. The control rats were injected intraperitoneally with 1 ml saline so that they were exposed to the same stress. At 24 h after administration, blood samples and liver, kidney, and heart tissues were collected. All of the procedures described above were conducted in accordance with the Chinese national legislation and local guidelines.

Clinical chemistry and histopathology

The serum samples were analyzed with an auto-chemistry analyzer (BIOSINO, Ltd.) for aspartate aminotransferase (AST), creatine kinase (CK), and blood urea nitrogen (BUN). The fixed tissues were processed, embedded in paraffin wax, cut (5 μm thickness), and stained with hematoxylin and eosin. Finally, the liver, kidney, and heart tissues were observed for histopathological changes using a light microscope at 200× magnification.

Metabolomics data acquisition

Plasma metabolites profiling was performed by RRLC-Q-TOF-MS (Agilent, USA). After plasma was thawed at room temperature, 300 μL acetonitrile was added to 100 μL of the plasma and ultrasonicated in cold water for 10 min, vortexed for one minute, then centrifuged at 15, 000 rpm for 15 min. 10 μL of supernatant was

injected onto ACQUITY UPLC HSS C_{18} column (2.1 mm × 100 mm, 1.8 μm particles; Waters) maintained at 40 °C at a flow rate of 0.3 ml/min. The gradient mobile phase condition was composed of phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile). The gradient started with 99% A, then at 0 min to 3 min, A: 99% to 48%; at 3 min to 7 min, A: 48% to 26%; at 7 min to 9 min, A: 26% to 20%; at 9 min to 10 min, A: 20% to 10%; at 10 min to 12 min, A: 10% to 1%; at 12 min to 16 min, A: 1% to 1%; at 16 min to 17 min, A: 1% to 99%; at 17 min to 20 min, A: 99% to 99%. MS system was operated using the ESI^+ mode and the mass range was set at 50-1000 m/z in the full scan mode. The optimal capillary voltage was set at 3.5 kV, and drying gas temperature was set at 325 °C, the drying gas flow was set at 10 ml/min and desolvation gas flow rate was 600 l/h, nebulizer pressure was 350 psi, the fragmentor voltage for MS was 175V and the collision energy for MS/MS was set at 70V. All samples were maintained at 4 °C during analysis. Before injecting samples collected in different groups, Quality control (QC) sample, a mixture of plasma gained from each group, was first applied to detect the instrument precision and stability. if the total chromatographic system was not stable, the injection of plasma can not be permitted and the chromatographic acquisition could start until the whole system was at a good and stable condition. Moreover, QC samples were injected to test for the stability of samples and system during the whole acquisition.

Metabolomics data processing

After data acquisition, a large amount of information was gained. To exclude useless information, all the MS raw files was extracted by Agilent MassHunter Qualitative Analysis B.04.00 software with noise elimination level 5, converting important and useful information into CDF format. Automated peak detection, peak alignment, and normalization were performed by Agilent MassHunter Mass Profiler software (version 4.0). The data was processed by normalisation by sum (analogous to total ion count normalization). The corresponding parameters were set as follows: Mass tolerance (intercept 2.0 mDA, slope 5.0 ppm), Retention Time (RT) tolerance (intercept 0.5 min, slope 0.5%), Result filters (min relative frequency 80% in at least group), special mass excluded, neutral losses ignored, isotope peaks removed. According to the former procedure, the data was processed and transformed to an excel format, containing whole information of the Mass, RT, peak area of the samples.

To find the changed metabolites, multivariate data analysis should be firstly performed to select the potential biomarkers of doxorubicin-induced toxicity. Multivariate data analysis was performed with SIMCA-P+ 12.0 software (Umetrics AB, Umea, Sweden). The data was mean centered and scaled prior to data analysis. We used pareto scaling in performing further analysis. Principal component analysis (PCA), a widely used multivariate data analysis, was used to gain an overall insight of samples from DOX group and control group. We can also remove outlier beyond 95% confidence level by PCA. Partial least squares-discriminant analysis (PLS-DA),

a supervised method, was subsequently applied to determine changed plasma metabolites among control group and DOX group. The established model was validated by cross validation and permutation tests.[20] The validation statistics, R^2 (cum) and Q^2 (cum), indicated the fitness and prediction of the model. These two parameters were below 1 and can show a proper and good model when close to 1. In this study, score plot was used to visualize the established model. In the score plot, every point represented one sample and every sample included various variables. The changed endogenous metabolites disturbed by drug administration were also observed from the S-plot, loading plot and variable importance plot (VIP). In the S-plot and loading plot, changed metabolites were always located far away from the cluster. In this study, metabolites with VIP >1 and variations far away from the S-plot and the loading plot were firstly chosen as potential biomarkers. Then, an independent sample t-test was performed on the metabolites with VIP>1 using SPSS 17.0 software, and only significantly changed metabolites ($p<0.05$) were summarized for further study.

Metabolite identification

The significantly changed m/z value of the metabolites was determined, which was the relatively accurate provided by the Q-TOF-MS analysis platform. Identification of the candidate biomarkers was based on retention behavior, mass assignment, and online database query. The accurate mass and structure information of candidate metabolites were matched with those of metabolites obtained from HMDB

(www.hmdb.ca), METLIN (metlin.scripps.edu/) and KEGG (www.genome.jp/kegg/) databases. Some biomarkers were identified based on their MS/MS fragment information, which was supplied by RRLC-Q-TOF-MS analysis coupled with available database information from MassBank (http://www.massbank.jp/). Besides, other endogenesis were identified by comparing mass spectra and retention time with the available reference standards.

Disturbed metabolic pathways and metabolic network visualization

In this study, MetPA (http ://metpa.metabolomics.ca./MetPA/faces/Home.jsp) was used to find the disturbed pathways related to doxorubicin toxicity. MetPA was a web-based tool that combined the result from powerful pathway enrichment analysis with the pathway topology analysis. MetPA can researchers identify the most relevant pathways involved in the conditions under study. It had become an important tool in metabolomics study.[21] Besides, The selected corresponding pathways were imported into Cytoscape software (http://www.cytoscape.org/) for visualization of metabolomics result. These pathways were considered to have close relationship with the mechanism of doxorubicin-induced toxicity.

Results and discussion

Clinical chemistry and histopathological assessment

Various clinical parameters were measured in plasma to monitor the toxic effect of DOX. In the DOX group, the concentration of AST, BUN, and CK were significantly increased in blood at 24 h after administration (Fig. 1). As shown in Fig. 2,

toxicity-related alterations including eosinophilic degeneration in the liver cell, irregularly arranged cells and cytoplasmic condensation in the stripe region of heart. The swelling of kidney tubules was also observed in the DOX group at 24 h after administration. Combined clinical chemistry analysis with histopathological examination, it can be concluded that single intraperitoneally injection of DOX with the dose of 15 mg/kg actually caused liver, kidney, and heart injury in rats.

Metabolic profiling analysis

The typical total ion current (TIC) chromatograms of plasma (Fig. 3) in the control and DOX group in positive mode were gained based on the RRLC-Q-TOF-MS platform. About 4500 features were detected after MS data preprocessed. The preprocessed metabolomics data was used to perform PCA, aiming to discover principal components that accounted for the majority of the differences in the data. In a PCA score plot, similar samples were grouped together and different samples were dispersed.[22,23] Fig. 4a showed distinctions among control group and DOX group. The plasma samples in the DOX group were located far from those of the control group, indicating that the plasma endogenous substances changed after administration, which was concordant with our clinical chemistry analysis and histopathological observations. PLS-DA was applied to determine the differences in the metabolite profiles among different groups. The PLS-DA results was shown in Fig. 4b, the rat plasma profiles showed changes due to DOX. In the S-plot and loading plot, some metabolites in the plasma were distant from the cluster and thus

treated as potential biomarkers for further study (Fig. 4c and Fig. 4d).

The $R^2X(cum)$, $R^2Y(cum)$ and $Q^2(cum)$ value of the established PLS-DA model were 0.55, 0.986 and 0.961, respectively. The high value of Q^2 and R^2 indicated the rationale of the model built in this study. What's more, the PLS-DA assessment was validated by cross validation and permutation tests and the permutation statistics R^2 and Q^2 intercepts were 0.548 and -0.141, respectively. Besides, In the PLS-DA model used for the selection of biomarkers, there were 153 variables with VIP>1 which contributed to the separation of plasma endogenous metabolites between control group and DOX group. Among these 153 variables, 96 variables exhibited a significant change ($p < 0.05$), which will be performed to further biomarker identification.

Identification of potential biomarkers

Multivariate statistical analysis facilitated the identification of biomarkers and the m/z value of the significantly changed biomarkers were selected from a large amount of plasma endogenous metabolites, which was the relatively accurate provided by the Q-TOF-MS analysis platform. We searched the molecular of metabolites in online database such as HMDB using available m/z value. The final identification of biomarkers were based on MS/MS information, former reported research and available commercial standard.

In this study, we chose 15 significantly changed metabolites in plasma among control group and DOX group. Among these selected biomarkers, thirteen

metabolites were identified by their MS/MS information. To better understand this identification, we took the ions at (t_R = 9.56 min, m/z 494.3236) as an example to be described below. The molecular formula was supposed to be $C_{24}H_{48}NO_7P$ searched by HMDB database using m/z 494.3236. In addition, the main fragment ions in positive MS/MS spectrum were found at m/z 476.3, 184.0, 125.0 and 104.0, which could be the ions formed by the $[M+H]^+$ of lost $-H_2O$, $-C_{19}H_{35}NO_2$ $-C_{22}H_{42}NO_3$ and $-C_{20}H_{40}NO_4P$ respectively. Based on the MS/MS information, the metabolite was finally identified as lysophosphatidylcholine (16:1) [LPC (16:1)]. Some biomarkers were identified by their standards, such as valine and arachidonic acid. These two biomarkers were identified by comparing mass spectra and retention time with the available reference standards. Details of our analyses was shown in Table 1.

Visualization of disturbed metabolic pathways

The identified biomarkers responsible for DOX-induced toxicity played an important role in specific metabolic pathways. Therefore, finding the disturbed metabolic pathways can help us better understand the mechanism of DOX-induced toxicity. In this study, the disturbed pathway was analyzed by MetPA. In MetPA analysis, we selected the pathways with impact value above 0 as the affected pathways. As shown in Fig. 5, arachidonic acid metabolism (a), valine, leucine and isoleucine biosynthesis (b), sphingolipid metabolism (c), glycerophospholipid metabolism (d) and primary bile acid biosynthesis (e) were disturbed in the rats of

DOX group. The visualization of identified biomarkers and corresponding pathways can be seen in Fig. 6.

Effects of doxorubicin toxicity on plasma metabolites

Valine is one of amino acid in proteins. It belongs to the branched chain amino acids which may play an important role in myocardial ischemia as energy substrates for the heart.[24] It had been proved that valine was an potential biomarkers in myocardial ischemia rat models.[25] The decreased level of valine was also observed in myocardial tissue due to doxorubicin administration[17], indicating the existing of the relationship between valine and DOX-induced toxicity. Arachidonic acid, which takes part in arachidonic acid metabolism, belongs to unsaturated fatty acids. It was found that doxorubicin toxicity can result in altered P450-mediated arachidonic acid metabolism.[26] Besides, Arachidonic acid had been previously shown to mediate inflammation.[27] Inflammation reaction can lead to kidney parenchymal cell death and acute kidney injury.[28] It also had been proved that inflammation can lead to fibrosis of kidney, finally inducing nephrotoxicity.[29] DOX had been reported to induce inflammatory response in the model of DOX cardiotoxicity.[30] Other metabolic profile analysis on heart tissue induced by doxorubicin also selected arachidonic acid as potential biomarker,[17] supporting that arachidonic acid was related to DOX-induced toxicity and DOX may induce toxicity through active regulation of the inflammatory pathway. Sphingosine and sphinganine are both sphingolipids, participating in sphingolipid metabolism. Phytosphingosine is a kind

of phospholipid that took part in sphingolipid metabolism. The changed level of sphingosine, sphinganine and phytosphingosine indicated that DOX may induce toxicity by regulating sphingolipid metabolism. Glycocholic acid, a bile acid, had proved to be a biomarkers of liver injury.[31] Lysophosphatidylcholine (LPC) is a relevant component of human plasma originating from lecithin-cholesterol acyltransferase, hepatic secretion[32, 33] or action of phospholipase A2,[34] which had been suggested to play a functional role in the pathogenesis of various diseases, including vasodilation,[35] inflammation[36] and atherosclerosis.[37] The change in LPC content may be related to DOX-induced toxicity. The changed level of these biomarkers may reveal the mechanism of DOX-induced toxicity in some content.

Conclusion

In this study, plasma metabolomics technology was used to identify toxic metabolites and metabolic pathways related to doxorubicin-induced toxicity, providing information for the causal mechanism of system toxicity induced by DOX. Male Wistar rats were treated with DOX with the dose of 15 mg/kg. Clinical chemistry analysis and histopathological examination showed that DOX caused liver, kidney, and heart injury in rats. The metabolomics result showed that fifteen metabolites were changed due to drug administration.Besides, Network analysis proposed probable pathways of DOX related to toxicity, and imported into Cytoscape for visualisation of the metabolomics result. The result showed that the pathways including arachidonic acid metabolism, valine, leucine and isoleucine

biosynthesis, sphingolipid metabolism, glycerophospholipid metabolism and primary bile acid biosynthesis mainly involved in the toxicity of DOX. The result indicated that the built metabolomics method can be applied to research on the mechanism of drug-induced toxicity, benefiting for later accurate predictions of drug toxicity.

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References

1. K. Sakai-Kato, E. Saito, K. Ishikura and T. Kawanishi, *J Chromatogr B Analyt Technol Biomed Life Sci.,* 2010, **878,** 1466-1470.

2. K. Sakai-Kato, K. Nanjo, T. Kawanishi and H. Okuda, *Chem Pharm Bull.,* 2012, **60,** 391-396.

3. M. B. Meliksetian, T. K. Daviyan, I. V. Chirkowa, T. Alexanyan Yu and T. N. Ignatova, *Cell Biol Int.,* 1997, **21,** 69-74.

4. J. H. Doroshow, *Cancer Res.,* 1983, **43,** 460-472.

5. G. Minotti, P. Menna, E. Salvatorelli, G. Cairo and L. Gianni, *Pharmacol Rev.,* 2004, **56,** 185-229.

6. R. D. Beger, J. Sun and L. K. Schnackenberg, *Toxicol Appl Pharmacol.,* 2010, **243,** 154-166.

7. J. Xuan, G. Pan, Y. Qiu, L. Yang, M. Su, Y. Liu, J. Chen, G. Feng, Y. Fang, W. Jia,

Q. Xing and L. He, *J Proteome Res.,* 2011, **10,** 5433-5443.

8. D. Portilla, L. Schnackenberg and R. D. Beger, *Semin Nephrol.,* 2007, **27,** 609-620.

9. X. Wang, B. Yang, H. Sun and A. Zhang, *Anal Chem.*, 2012, **84,** 428-439.

10. S. Rochfort, *J Nat Pro*d*.,* 2005, **68,** 1813-1820.

11. J. Xuan, G. Pan, Y. Qiu, L. Yang, M. Su, Y. Liu, J. Chen, G. Feng, Y. Fang, W. Jia, Q. Xing and L. He, *J Proteome Res.,* 2011, **10,** 5433–5443.

12. X. Wang, H. Wang, A. Zhang, X. Lu, H. Sun, H. Dong and P. Wang, *J Proteome Res.,* 2012, **11***,* 1284–1301.

13. E. Holmes, I. D. Wilson and J. K. Nicholson, *Cell.,* 2008**, 134,** 714–717.

14. E. J. Want, A. Nordström, H. Morita and G. Siuzdak, *J Proteome Res.,* 2007**, 6,** 459 -468.

15. H. Yoshida, J.Yamazaki, S. Ozawa, T. Mizukoshi and H. Miyano, *J Agric Food Chem.,* 2009**, 57,** 1119–1126.

16. R. S. Plumb, P. D. Rainville, W. B. Potts III, K. A. Johnson, E. Gika and I. D. Wilson, *J Proteome Res.,* 2009, **8,** 2495–2500.

17. G. Tan, Z. Lou, W. Liao, Z. Zhu, X. Dong, W. Zhang, W. Li and Y. Chai, *PLoS One.,* 2011, **6,** e27683.

18. M. N. Triba, A. Starzec, N. Bouchemal, E. Guenin, G. Y. Perret and L. Le Moyec, *NMR Biomed.,* 2010, **9,** 1009-1016.

19. J. Wang, T. Reijmers, L. Chen, R. Van Der Heijden, M. Wang, S. Peng, T.

Hankemeier, G. Xu and J. Van Der Greef, *Metabolomics.,* 2009, **5,** 407-418.

20. J. A. Westerhuis, H. C. J. Hoefsloot, S. Smit, D. J. Vis, A. K. Smilde, E. J. J. van Velzen, J. P. M. van Duijnhoven and F. A. van Dorsten, *Metabolomics.,* 2008, **4,** 81-89.

21. J. Xia and D. S. Wishart, *Bioinformatics.,* 2010, **26,** 2342-2344.

22. X. Wang, B. Yang, H. Sun and A. Zhang, *Anal Chem.*, 2012, **84**, 428-439.

23. WoId. S, K. Esbensen and P. Geladi, *Chemometrics and Intelligent Laboratory Systems.,* 1987, **2,** 37-52.

24. P. H. McNulty, R. Jacob, L. I. Deckelbaum and L. H. Young, *Metabolism.,* 2000, **49,** 1365-1369.

25. B. Yan, A. J, H. Hao, G. Wang, X. Zhu, W. Zha, L. Liu, E. Guan, Y. Zhang, Q. Huang and Y. Zheng, *Sci China C Life Sci.,*2009, **52,** 1081-1090.

26. B. N. Zordoky, A. Anwar-Mohamed, M. E. Aboutabl and A. O. El-Kadi, *Drug Metab Dispos.,* 2011, **39,** 1440-1450.

27. M. Shoeb, U. C, Yadav, S. K. Srivastava and K. V. Ramana, *Free Radic Biol Med.,* 2011, **51,** 1686-1696.

28. G. R. Kinsey, L. Li and M.D. Okusa, *Nephron Exp Nephrol.,* 2008, **109,** 102-107.

29. M. E. Clements, C. J. Chaber, S. R. Ledbetter and A. Zuk, *PLoS One.*, 2013, **8,** e70464.

30. R. Guo, K. Wu, J. Chen, L. Mo, X. Hua, D. Zheng, P. Chen, G. Chen, W. Xu and J. Feng, *Cell Physiol Biochem.,* 2013, **32,** 1668-1680.

31. J. Collazos, *Clin Investig.,* 1993, **72,** 36-39.

32. P. V. Subbaiah, M. Liu, P. J. Bolan and F. *Paltauf, Biochim Biophys Acta.,* 1992, **1128,** 83-92.

33. G. Sekas, G. M. Patton, E. C. Lincoln and S. J. Robins, *J Lab Clin Med.,* 1985, **105,** 190-194.

34. T. Kita, N. Kume, K. Ishii, H. Horiuchi, H. Arai and M. Yokode, *Diabetes Res Clin Pract.*, 1999, **45,** 123-126.

35. T. D. Vuong ,S. de Kimpe, R de Roos, T. J. Rabelink, H. A. Koomans and J. A. Joles, *Kidney Int.,* 2001, **60,** *1088-1096.*

36. S. Koizumi, S. Yamamoto, T. Hayasaka, Y. Konishi, M. Yamaguchi-Okada, N.Goto-Inoue, Y. Suqiure, M. Setou and H. Namba, *Neuroscience., 2010,* **168,** 219-225.

37. A. J. Lusis, *Nature.,* 2000, **407,** 233-241.

Table 1 Identified potential biomarkers in the DOX group vs. control group

No.	t_R (min)	Metabolite	Obsd $[M+H]^+$	Calcd $[M+H]^+$	Error (ppm)	Molecular Formula	Content variance[a]	VIP value	MS/MS	P value [d]
1	0.89	Valine[b]	118.0861	118.0863	-1.7	$C_5H_{11}NO_2$	↓	4.72		**
2	13.41	Sphingosine[c]	300.2889	300.2897	-2.7	$C_{18}H_{37}NO_2$	↑	2.87	300.2 $[M+H]^+$ 282.2 $[M+H-H_2O]^+$ 172.0 $[M+H-C_7H_{11}O_2]^+$ 146.1 $[M+H-C_{10}H_{17}O]^+$	**
3	9.10	Sphinganine[c]	302.3050	302.3054	-1.3	$C_{18}H_{39}NO_2$	↓	5.77	302.3 $[M+H]^+$ 284.3 $[M+H-H_2O]^+$ 217.1 $[M+H-C_3H_5O_2]^+$ 146.1 $[M+H-C_{10}H_{19}O]^+$	**
4	13.69	Arachidonic acid[b]	305.2474	305.248	-2.0	$C_{20}H_{32}O_2$	↑	1.93		**
5	8.00	Phytosphingosine[c]	318.2995	318.3003	-2.5	$C_{18}H_{39}NO_3$	↑	3.78	318.2 $[M+H]^+$ 300.2 $[M+H-H_2O]^+$ 256.2 $[M+H-C_2H_5O_2]^+$ 146.1 $[M+H-C_{11}H_{23}O]^+$	**
6	7.17	Glycocholic acid[c]	466.3161	466.3163	-0.4	$C_{26}H_{43}NO_6$	↑	2.71	466.3 $[M+H]^+$ 448.3 $[M+H-H_2O]^+$ 432.3 $[M+H-H_2O_2]^+$	**
7	9.56	LPC(16:1)[c]	494.3236	494.3244	-1.6	$C_{24}H_{48}NO_7P$	↓	6.13	494.3 $[M+H]^+$ 476.3 $[M+H-H_2O]^+$	**

									184.0 $[M+H-C_{19}H_{35}NO_2]^+$ 125.0 $[M+H-C_{22}H_{42}NO_3]^+$ 104.0 $[M+H-C_{20}H_{40}NO_4P]^+$	
8	11.33	LPC(16:0)[c]	496.3444	496.3449	-1.0	$C_{24}H_{50}NO_7P$	↑	6.30	496.3 $[M+H]^+$ 478.3 $[M+H-H_2O]^+$ 419.2 $[M+H-C_3H_{10}NO]^+$ 313.2 $[M+H-C_5H_{13}NO_4P]^+$ 184.0 $[M+H-C_{19}H_{37}NO_2]^+$	*
9	9.50	LPC(18:3)[c]	518.3239	518.3241	-0.4	$C_{26}H_{48}NO_7P$	↓	2.10	518.3 $[M+H]^+$ 500.3 $[M+H-H_2O]^+$ 184.0 $[M+H-C_{16}H_{32}NO_4P]^+$, 125.0 $[M+H-C_{24}H_{42}NO_3]^+$	**
10	12.37	LPC(18:0)[c]	524.3697	524.3711	-2.7	$C_{26}H_{54}NO_7P$	↑	8.36	524.3 $[M+H]^+$ 506.3 $[M+H-H_2O]^+$ 184.0 $[M+H-C_{21}H_{41}O_2]^+$ 125.0 $[M+H-C_{24}H_{48}O_3]^+$	**
11	10.94	LPC(20:4)[c]	544.3384	544.3398	-2.6	$C_{28}H_{50}NO_7P$	↓	1.17	544.3 $[M+H]^+$ 526.3 $[M+H-H_2O]^+$ 184.0 $[M+H-C_{19}H_{38}NO_3P]^+$ 125.0 $[M+H-C_{26}H_{44}NO_3]^+$	**
12	12.37	LPC(20:3)[c]	546.3552	546.3554	-0.4	$C_{28}H_{52}NO_7P$	↑	2.92	546.4 $[M+H]^+$ 527.4 $[M+H-H_2O]^+$ 184.1 $[M+H-C_{18}H_{36}NO_4P]^+$ 125.1 $[M+H-C_{26}H_{46}NO_3]^+$	**

13	10.23	LPC(22:5)[c]	570.3547	570.3554	-1.2	$C_{30}H_{52}NO_7P$	↓	1.64	570.3 $[M+H]^+$ 552.3 $[M+H-H_2O]^+$ 184.0 $[M+H-C_{21}H_{41}NO_3P]^+$ 125.0 $[M+H-C_{28}H_{47}NO_3]^+$ 104.0 $[M+H-C_{22}H_{45}NO_7P]^+$	**
14	11.38	LPC(22:4)[c]	572.3708	572.3711	-0.5	$C_{30}H_{54}NO_7P$	↓	1.35	572.4 $[M+H]^+$ 553.4 $[M+H-H_2O]^+$ 258.2 $[M+H-C_{13}H_{32}NO_5P]^+$ 184.1 $[M+H-C_{21}H_{42}NO_3P]^+$	**
15	13.43	LPC(22:2)[c]	576.4018	576.4024	-1.0	$C_{30}H_{58}NO_7P$	↑	2.92	576.4 $[M+H]^+$ 557.4 $[M+H-H_2O]^+$ 184.1 $[M+H-C_{25}H_{45}NO_2]^+$ 166.1 $[M+H-C_{25}H_{45}O_4]^+$	**

[a] ↑, content increased; ↓, content decreased. [b] confirmed by commercial standards. [c] confirmed by MS/MS information.

[d] the p-value for selected biomarkers, **, $p < 0.01$, compared with the control group; *, $p < 0.05$, compared with the control group.

Graphical Abstract LC-MS-based metabolomics study on the change of plasma metabolite and metabolic pathway induced by doxorubicin toxicity

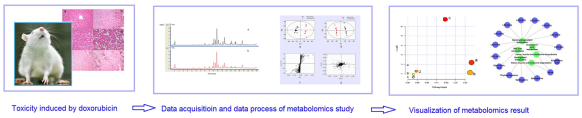


Fig. 1 The serum level of AST, BUN and CK in control group and DOX group. The serum samples were analyzed with an auto-chemistry analyzer, the concentration of AST, BUN, and CK were significantly increased in blood after administration. (a) Changes in AST levels. (b) Changes in BUN levels. (c) Changes in CK levels. Data represent mean ± SD (**p < 0.01).

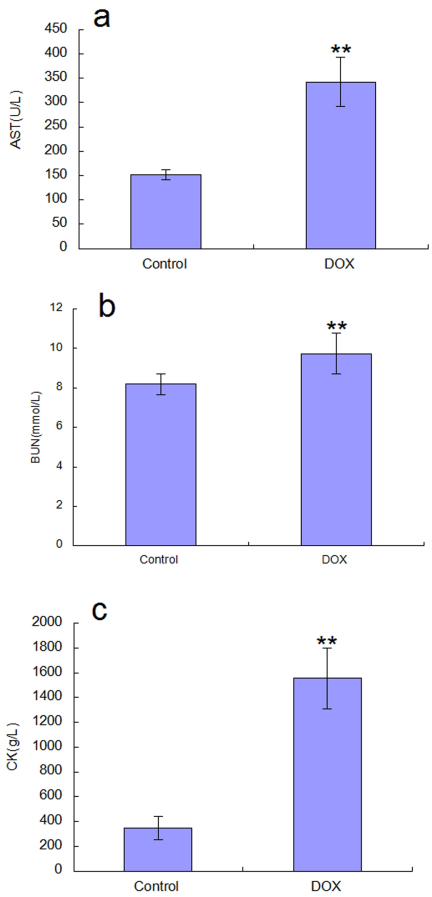


Fig. 2 Histopathological examination of organ lesions stained with hematoxylin and eosin. The fixed tissues were embedded in paraffin wax, cut (5 μm thickness), and stained with hematoxylin and eosin. the tissues were observed for histopathological changes using a light microscope at 200× magnification. Panels a and b, liver; panels c and d, kidney; panels e and f, heart. Panels a, c, and e were treated with saline, whereas panels b, d, and f were treated with DOX.

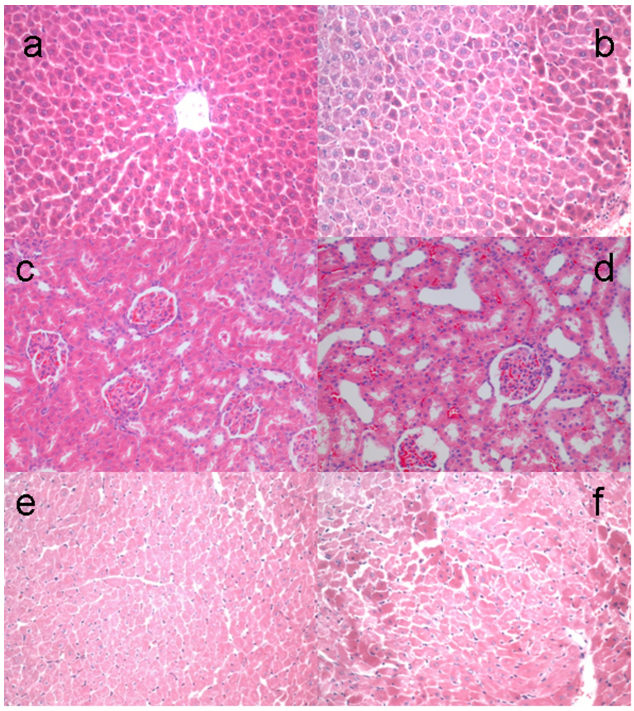


Fig. 3 Typical total ion current (TIC) chromatograms of plasma sample in control and DOX group obtained from RRLC-Q-TOF-MS in the positive ESI mode.

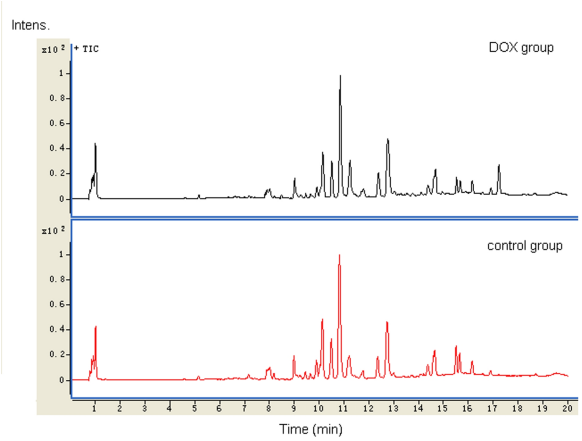


Fig. 4 Result of multivariate statistical analysis. (a) PCA score plot of control and DOX groups. (b) PLS-DA score plot of the control and DOX groups. (c) PLS-DA S-plot of control group and DOX group. (d) PLS-DA loading plot of control group and DOX group.

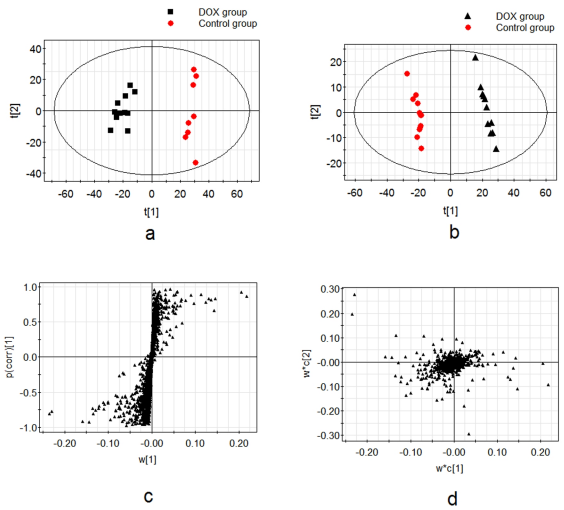


Fig. 5 The disturbed pathways in the rats of DOX group analyzed by MetPA. (a): arachidonic acid metabolism, (b): valine, leucine and isoleucine biosynthesis, (c): sphingolipid metabolism, (d): glycerophospholipid metabolism, (e): primary bile acid biosynthesis.

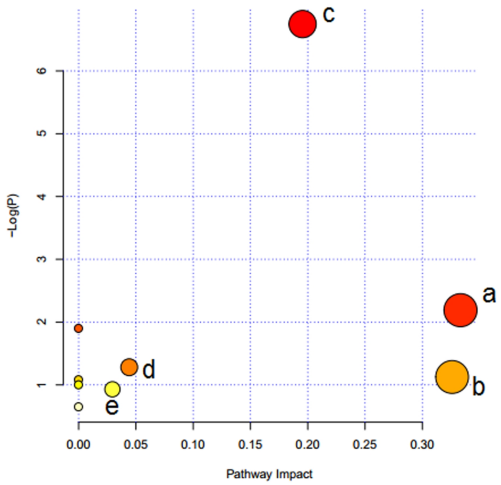
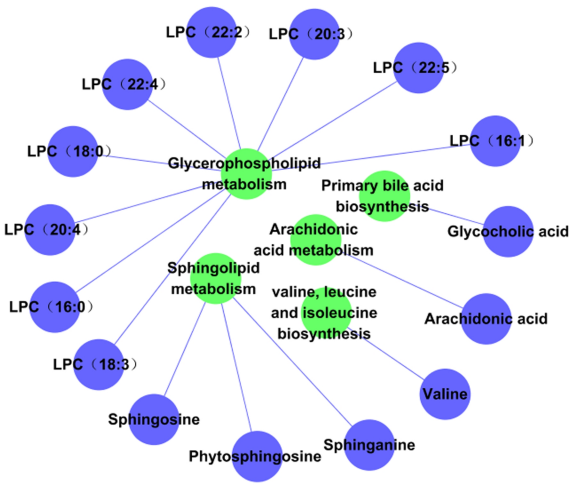


Fig. 6 Visualization of disturbed metabolic pathways. Green: disturbed metabolic pathways, Blue: changed plasma metabolites identified by metabolomics technology.



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