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ORIGINAL PAPER

Serum metabolomics in mice after paraquat posioning

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Abstract

Backgrounds: Paraquat is toxic to humans and there is no antidote for paraquat poisoning. Moreover, the mechanisms of paraquat toxicity have not been clearly defined.

Methods: In this study, we developed a serum metabolomic method using gas chromatography- mass spectrometry (GC-MS) to evaluate the effects of paraquat poisoning on mice. Pattern recognition analysis, including both principal component analysis and cross orthogonal partial least squares-discriminate analysis revealed that paraquat poisoning induced energy/redox cycle and metabolic perturbations.

Results: Compared with the control group, the levels of creatinine, citric acid, succinic acid, fumaric acid and glycine in the sub acute paraquat poisoning group increased ($P<0.05$), while the levels of alpha-Tocopherol, 6-phosphogluconic acid and palmitoleic acid decreased ($P<0.05$).

Conclusion: These findings provide an overview of systematic responses to paraquat exposure and metabolomic insight into the toxicological mechanism of paraquat. Our results demonstrate that the metabolomic method based on GC-MS can be a useful tool to elucidate the mechanism of PQ toxicity, and provide a new direction for its clinical diagnosis and treatment.

Keywords: Paraquat, Toxicology, Metabolomics, Gas chromatography-mass spectrometry

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Introduction

The herbicide paraquat (PQ, 1,1′-dimethyl-4,4′-bipyridylium) has been widely used in agriculture, as it is a fast acting and non-selective compound. PQ is highly toxic for humans and animals. Exposure of high levels of paraquat cause toxicity in mammals[1] and accidental as well as intentional poisoning in humans[2] have been reported. There is no antidote to its effects or any effective therapy, producing a mortality rate of 50%-90% in affected individuals[3]. In the acute toxicity of paraquat poisoning, mainly involves pulmonary edema and hemorrhage, cardiac failure, renal failure and liver failure[4,5]. While in the sub acute toxicity, pulmonary functions are damaged, such as a hyaline membrane, pulmonary edema, fibrosis and hemorrhage[5]. The mechanism of paraquat toxicity is very complex, as it is known to generate superoxide anions in mitochondria and cytosol of mammalian cells leading to the formation of several reactive oxygen species[5]. This reaction sequence could lead to cell damage from NADPH depletion, free radical generation, and lipid peroxidation[6].

Metabolomics is a useful tool to study toxicity as it provides a unique mechanistic perspective on response to toxic insult[7]. By assessing the dynamic changes of metabolites in the biological system, the metabolic profiling (mainly small endogenous substance with low molecular weight below 1000) has been widely applied to uncover biomarkers and can be used as a potential technique for early diagnosis of PQ poisoning[3].

Because the mechanisms of paraquat toxicity have not been clearly defined[8], we developed a serum metabolomic method based on gas chromatography - mass spectrometry (GC-MS) to observe the metabolic profile, found out the interesting biomarkers between PQ poisoning group and the control group in order to reveal the mechanism of paraquat poisoning and provided for the potential drug treatments for PQ poisoning.

Materials & Methods

Chemicals and reagents

HPLC-grade Methanol were purchased from CNW technology company (Beijing, China). Pyridine and methylhydroxylamine hydrochloride were purchased form Adamas Industrial, Inc. (Shanghai, China). l-2-chlorphenylalanine (purity > 98%) was purchased from Hengbai biotechnology company (Shanghai, China). BSTFA (with 1% TMCS, v/v) were purchased from Regis technology (Shanghai, China).

Instrumentation and conditions

Agilent 7890 gas chromatography (Agilent Technologies, USA) coupled Pegasus HT time-of-flight mass spectrometer (LECO Corporation, USA). DB-5MS (30 m × 0.25 mm, 0.25 μm film thickness) were from J&W Scientific (Folsom, CA, USA). A 1 μL aliquot of the analyte was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL/min, and the gas flow rate through the column was 1 mL/min. The initial temperature was kept at 50°C for 1 min, then raised to 310°C at a rate of 20°C/min, then kept for 6 min at 310°C. The injection, transfer line, and ion source temperatures were 280°C, 280°C, and 250°C, respectively. The energy was 70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 12.5 spectra per second after a solvent delay of 4.8 min.

Animal treatments and sample collection

Female Kunming mice (Changsheng biotechnology co., Ltd. Shenyang, China) with weights of 45 ± 5 g. The 10 female mice were randomly divided into two groups, the control group (n = 5) and the PQ poisoning group (n = 5). Feed according to the breeding regulations and adapt to the environment for 1 week. All mice were housed at Laboratory Animal Research Center of China Medical University under the controlled conditions of a temperature of 22°C and a natural light-dark cycle. All experimental procedures were conducted according to the Institutional Animal Care Guidelines and were approved as ethical by the Administration Committee of Experimental Animals at the Laboratory Animal Center of China Medical University.

The PQ poisoning group received a single intragastric administration (i.g.) of paraquat dissolved in 500 μL saline (30 mg/kg). At the same time, the control group received the same amount of saline solution. Serum samples were collected from the mice from the control group and the PQ poisoning group after 24 hours. The blood samples were collected and then centrifuged for 10 min under 4°C. The serum was stored at −80°C until measurement.

Sample preparation

Take 50 μL sample into the 1.5 mL EP tubes, extracted with 200 μL methanol as extraction liquid, add 5 μL of 2-Chloro-L-phenylalanine (0.5 mg/mL stock in dH2O) as internal standard, vortex mixing for 30 s; Keep at −20°C for 10 min; Centrifuge for 15 min at 12000 rpm, 4°C; Transfer the supernatant 200 μL into a fresh 1.5 mL EP tubes. Dry completely in a vacuum concentrator without heating; Add 30 μL Methyldroxylamine hydrochloride (20 mg/mL in pyridine) incubated for 30 min at 80°C; Add 40 μL of the BSTFA regent (1% TMCS, v/v) to the sample aliquots, incubated for 1.5 h at 70°C; All samples were analyzed by gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer (GC-TOF-MS).

Data preprocessing and annotation

Chroma TOF 4.3X software of LECO Corporation and LECO-Fiehn Rtx5 database were used for raw peaks exacting, the data baselines filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak identification and integration of the peak area[9]. Both of mass spectrum match and retention index match were considered in metabolites identification. Principle component analysis (PCA) was adopted to observe the overall distribution of samples in each group. Orthogonal partial least squares (OPLS) analysis was adopted to evaluate the accuracy of the model, and differential metabolites of the variable importance in the projection (VIP) value > 1 were selected. In this study, Independent samples t-test was applied in order to detect significant differences in all metabolites between the two groups. A *P*-value of < 0.05 was considered statistically significant.

Results

Analytical characteristic of global profiling methods

Representative GC-MS spectra of the serum samples obtained from the PQ poisoning and the control group are shown in Figure 1. As shown in the Figure 1, compared with the control group, the alterations of the serum profiles of the PQ poisoning group were apparent in the GC-MS spectra by visual examination. Principal component analysis of the results of sub acute paraquat poisoning provided a satisfactory separation of data between the PQ poisoning group and the control group as shown in Figure 2A. The potential constituents

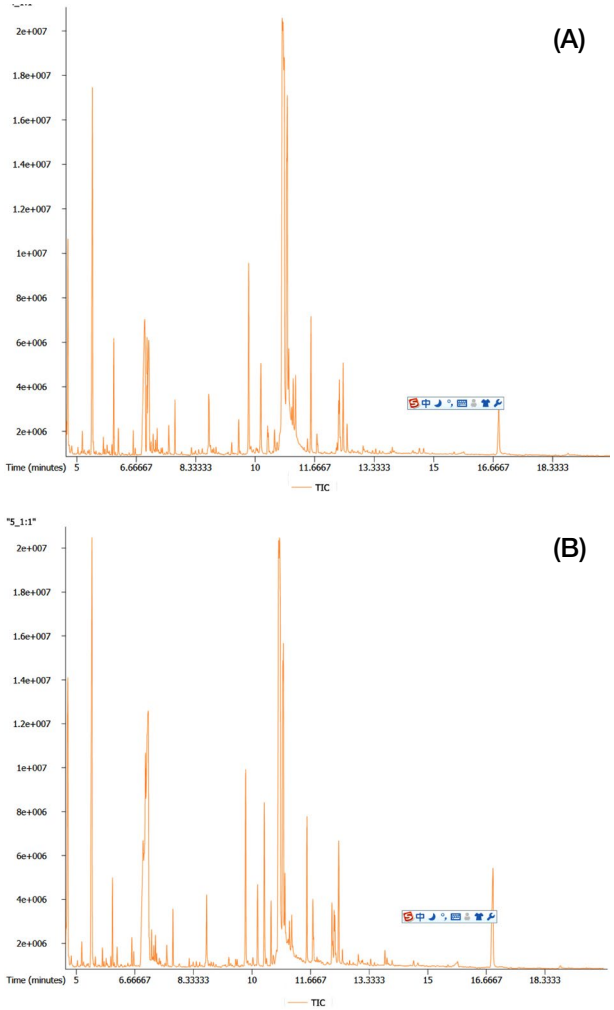

Figure 1. Representative GC-MS Spectra of Serum Sample Obtained from the Control Group and the PQ Poisoning Group (A the control group; B the PQ poisoning group).

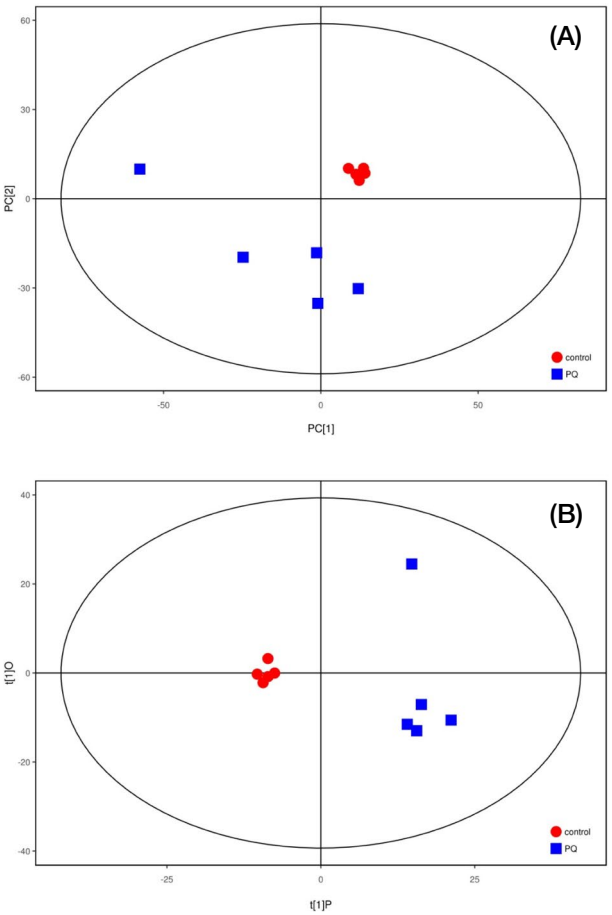

Figure 2. PCA Score Results of Mice serum samples (A), OPLS-DA score results of mice serum samples (B).

were screened by cross orthogonal partial least-squares discriminant analysis (OPLS-DA), which was used to reveal the differences in serum composition of two different groups[10,11]. In order to evaluate the metabolic profile changes of the PQ poisoning group, we compared the GC-MS chromatogram of OPLS-DA ($R^2X=0.518$, $R^2Y=0.995$, $Q^2Y=0.69$) of each serum samples from the PQ poisoning group (n=5) and the control group (n=5) (Figure 2B). The OPLS-DA scores plot of the first and second principal components (t [1] and t [2]) showed a very good discrimination between the PQ poisoning group and the control group (Figure 2B). A total of 591 peaks were detected in this experiment.

Quality control

Whether there is the residues disturbance or not in the process of detecting samples can be examined using blank samples. It can be seen from the Figure 3 that there is no significant peak detection in the blank sample, indicating no contamination between samples.

Metabolomics study

Metabolomics focus on the low molcecular weight endogenous metabolites in biological samples[11], as a promising tool to identity novel biomarkers that could help elucidate the mechanisms of paraquat toxicity by investigating the changes in metabolic signatures induced by drug exposure[12,13]. As shown in Figure 4, the gray plot means there is no difference between the control group and the paraquat poisoning group. The red plots mean up-regulated endogenous metabolites in the paraquat poisoning group, while the blue plots mean down-regulated endogenous metabolites in the paraquat poisoning group. We concluded the eight distinguished difference metabolites as shown in Table 1.

Discussion

Compared with the control group, the level of 6-phosphogluconic acid, the intermediate metabolite in pentose phosphate pathway (PPP), significantly decreased in mice treated with PQ. The redox cycling properties of paraquat was considered to be the main mechanism to interfere with the cell metabolism. A disruption of the electron transport chain (ETC), tricarboxylic acid cycle (TCA cycle) has been found after paraquat poisoning[14-16]. Glucose is the obligatory energy substrate to maintain animal living. Glucose metabolism is primarily to generate NADPH through the pentose phosphate pathway (PPP) for support antioxidant defenses[16]. Paraquat "hijacks" the PPP to increase NADPH reducing equivalents and stimulate paraquat redox cycling, oxidative stress, and cell death[15]. Lei S *et al*.[17] reported that paraquat up-regulates the pentose phosphate pathway and glucose-6-phosphate dehydrogenase (G6PD) levels, the rate-limiting enzyme of the PPP, that was paralleled by down-regulation of the TCA cycle. But they did not mention the effects on the 6-phosphogluconic acid after PQ treatment. We suggested that though the levels of the glucose-6-phosphate and G6PD increased in order to generate NADPH, the levels of the 6-phophogluconic acid decreased, as it can generate the ribulose 5-phosphate to produce the NADPH to defense the attack of paraquat (Figure 5).

Paraquat interfere with the TCA cycle, resulting in the accumulation of citric acid, succinic acid and fumaric acid, which keep the consistent with the discovery that PQ induced an accumulation of citrate reported by Gantu D *et al*.[18]. We summarized the metabolites within PPP and TCA cycles significantly changed after PQ treatment.

Compared with the control group, the level of glycine increases in the paraquat poisoning group, which revealed that paraquat interferes with the metabolism of amino acids.

Compared with the control group, the contents of the palmitic acid and alpha-tocopherol decreased in the paraquat poisoning group, which is consistent with the

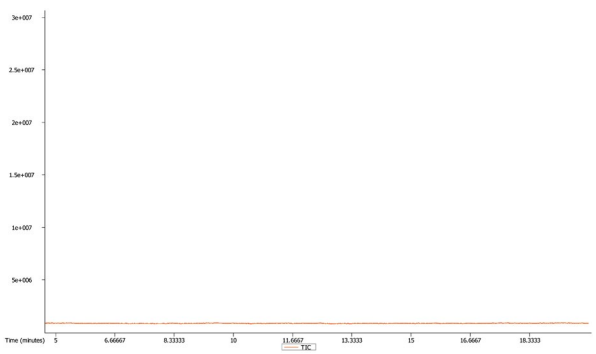

Figure 3. GC-MS spectra obtained from the blank sample using for quality control.

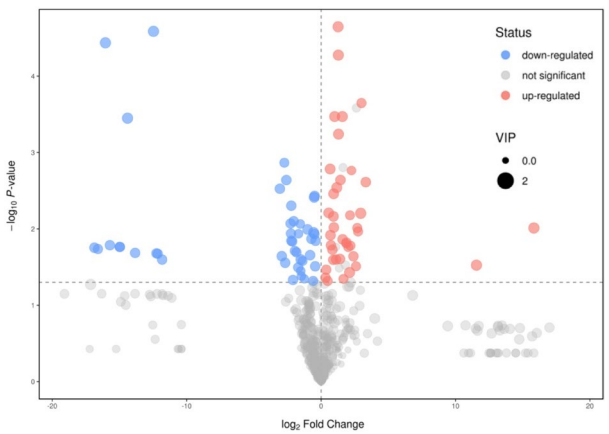

Figure 4. The volcano plot of the serum metabolomics in mice between the PQ poisoning group and the control group (the gray plots mean there is no significant difference between the control group and the paraquat poisoning group; The red plots mean up-regulated endogenous metabolites in the paraquat poisoning group; the blue plots mean down-regulated endogenous metabolites in the paraquat poisoning group).

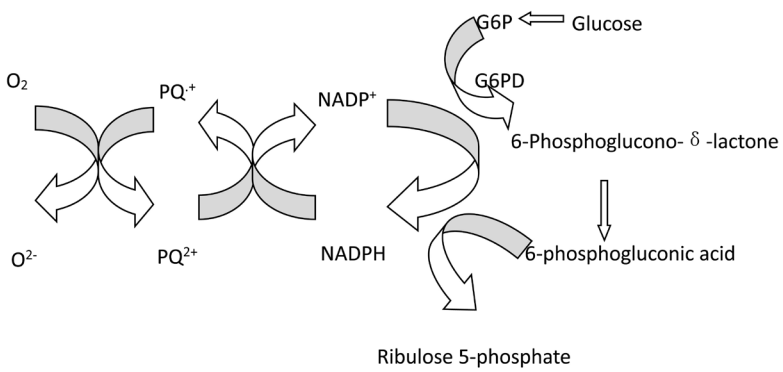

Figure 5. The effects of pentose phosphate pathway after PQ poisoning.

Table 1. The eight distinguished difference metabolites between the paraquat poisoning group and the control group.

No.	peak	Super Class	Class	Sub Class	Metabolic Pathway	vip	p	trend
1	creatine	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and alogues	Glycine, serine and threonine metabolism; Arginine and proline metabolism	1.50	0.025	↑
2	citric acid	Organic acids and derivatives	Carboxylic acids and derivatives	Tricarboxylic acids and derivatives	TCA cycle	1.95	0.006	↑
3	succinic acid	Organic acids and derivatives	Carboxylic acids and derivatives	Dicarboxylic acids and derivatives	TCA cycle	1.64	0.014	↑
4	fumaric acid	Organic acids and derivatives	Carboxylic acids and derivatives	Dicarboxylic acids and derivatives	TCA cycle	1.74	0.003	↑
5	alpha-Tocopherol	Lipids and lipid-like molecules	Prenol lipids	Quinone and hydroquinone lipids	Ubiquinone and other terpenoid-quinone iosynthesis	1.55	0.016	↓
6	glycine	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and alogues	Primary bile acid biosynthesis	1.58	0.002	↑
7	6-phosphogluconic acid	Organic compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Pentose phosphate pathway	1.56	0.023	↓
8	palmitoleic acid	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates	Fatty acid biosynthesis	1.10	0.036	↓

relationship between alpha-tocopherol and free radical elimination reported in literature[19-21]. There is delicate balance between antioxidant defense system and free radical production in the body. Oxidative stress results from an imbalance between the production of free oxygen radicals and elimination of these antioxidants[22]. These antioxidants can eliminate the free oxygen radicals generated in aerobic cells that can be sourced exogenous and endogenous[20]. In this study, alpha-tocopherol is endogenous antioxidant in female mice, which can prevent the formation of free oxygen radicals, increase clearance of these radicals, protect the damage from the free oxygen radicals.

Paraquat damage the kidney function accompanying with the rising of creatinine (in Table 1). PQ is mainly excreted from urine as a prototype. However, the damage of kidney induced by PQ poisoning reduces the clearance rate of creatinine, resulting in that PQ cannot be excreted efficiently from the body, which contribute significantly to paraquat toxicity and poor prognosis of PQ poisoning patients[23,24].

Conclusion

Compared with the control group, we found these biomarkers (creatinine, citric acid, succinic acid, fumaric acid, alpha-tocopherol, glycine, 6-phosphogluconic acid and palmitoleic acid) were the additional evidence in the PQ poisoning group. As we known that our study is the first using the female mice serum metabolomics to find out the biomarkers in PQ toxicity.

We demonstrated that PQ poisoning mechanism is complex including disturbing Energy/Redox Metabolism (i.e. the pentose phosphate pathway and Tricarboxylic acid cycle). Especially the decrease of the alpha-tocopherol and palmitoleic acid in PQ poisoning group demonstrate that the living organism has its own defense system for the free oxidative stress.

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Conflict of Interest Lina Gao, Guang Wang, Huiya Yuan, Enyu Xu , Guojie Liu & Junting Liu declare that they have no conflict of interest.

Human and animal rights All experimental procedures about animal treatment and sample collection were conducted according to the Institutional Animal Care Guidelines and were approved as ethical by the Administration Committee of Experimental Animals at the Laboratory Animal Center of China Medical University.

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