

Original Article

Serum metabolic changes in rats of acute paraquat poisoning treated by pirfenidone

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Abstract: In this study, we developed a gas chromatography-mass spectrometry (GC-MS)-based serum metabolomic analysis method to evaluate the effects of pirfenidone in rats with acute paraquat poisoning. The rats were divided into four groups, acute paraquat poisoning group, low pirfenidone group, high pirfenidone group, and control group. The acute paraquat poisoning group rats were given 20 mg/kg of paraquat by intraperitoneal (ip) administration. In low and high pirfenidone groups, the rats were ip administrated 20 mg/kg of paraquat to induce acute paraquat poisoning, then treated by intragastric (ig) administration of pirfenidone (20 mg/kg and 40 mg/kg, respectively). Compared to acute paraquat poisoning group on the fourth day, pentanedioic acid increased in both low and high pirfenidone group. In addition, d-ribose increased in low pirfenidone group, while ethanedioic acid increased in high pirfenidone group, with ð-D-glucopyranoside and glycerol decreased in high pirfenidone group. Compared to acute paraquat poisoning group on the seventh day, no significant differences of metabolite levels were found between acute paraquat poisoning group and pirfenidone groups. The separation between the control group, paraquat poisoning group, and pirfenidone groups in PLS-DA scores were clear. Pathological changes of liver from different groups showed that pirfenidone intervention for paraquat poisonings reduced liver damage. These results indicate that GC-MS based metabolomics analysis method would be useful to elucidate the effect of pirfenidone in rats with acute paraquat poisoning.

Keywords: Metabolomics, GC-MS, pirfenidone, paraquat, poisoning, rat

Introduction

Paraquat (1, 1'-dimethyl-4,4'-bipyridinium dichloride, PQ) is a very effective herbicide that has been widely used throughout the world [1], and is also well-known for its fatal toxicity to human and animals [2]. Super-oxide anion formation and consequent reactive oxygen species (ROS) production are the basis of paraquat toxicity [3]. Paraquat poisoning from oral ingestion was first reported in 1966 [4]. The symptoms of paraquat poisoning largely depend on the amount of compound consumed [5], with lung damage as the most common characteristic. The subsequent severe inflammation and pulmonary fibrosis become the primary cause of death [6, 7]. Up to now, the rate of mortality in

paraquat poisoned patients remains very high owing to the lack of effective treatment [5].

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone), which was initially developed to treat inflammation, is an orally available broad-spectrum anti-fibrotic drug to suppress TGF-β1, TNF-α, PDGF and COL1A1 expression [8]. In addition, this compound exhibits excellent antioxidant properties *in vitro* [9]. It has been reported that treatment with pirfenidone appears to decrease pulmonary fibrosis and increase the survival rate in rats with paraquat poisoning [10].

Metabolomics is the scientific study of chemical processes involving metabolites in biological systems, and has been employed to study acute paraquat poisoned rats and patients

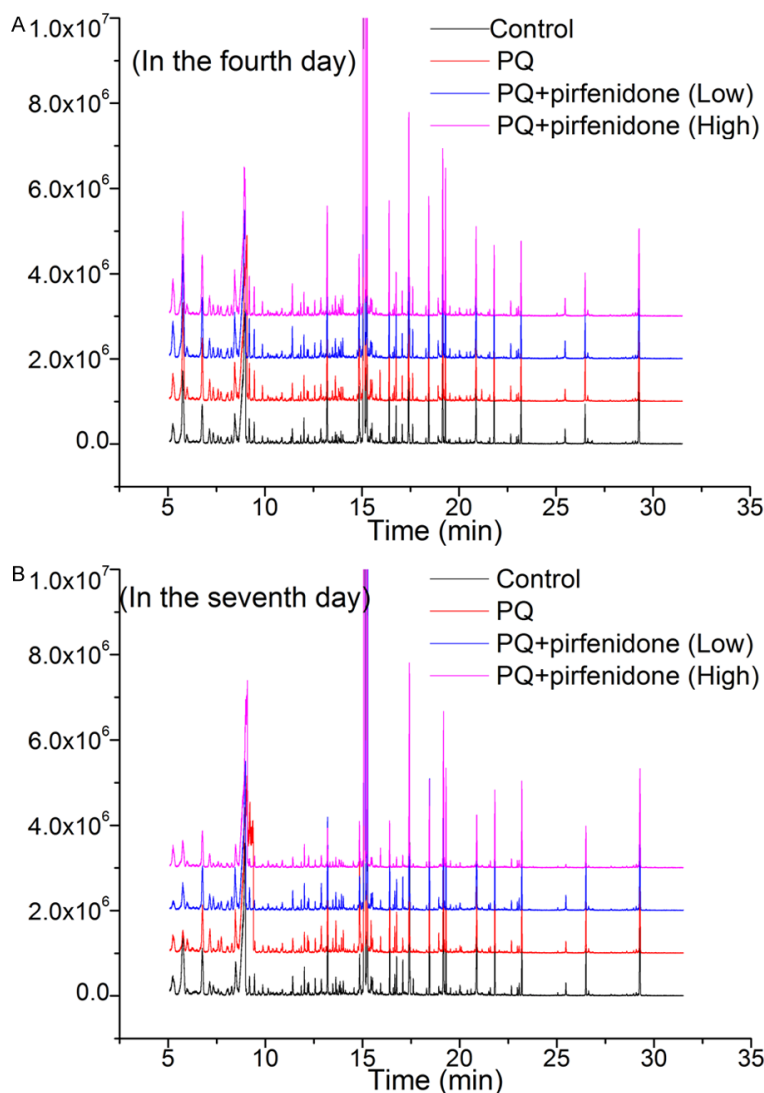


Figure 1. Typical GC-MS total ion chromatogram of rat serum on the fourth day (A) and the seventh day (B). PQ, acute paraquat poisoning group; PQ + pirfenidone (Low), low pirfenidone group; PQ + pirfenidone (High), high pirfenidone group.

based on their urinary and serum metabolite profiles [11-13]. By employing gas chromatography-mass spectrometry (GC-MS), this study elucidated the serum metabolomics changes caused by the intervention of pirfenidone in acute paraquat poisoned rats.

Material and methods

Chemicals and animals

N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and Trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich (Shanghai, China). Methylhydroxylamine hydrochloride and pyridine were purchased from Aladdin Indu-

strial, Inc. (Shanghai, China). HPLC-grade acetonitrile and n-heptane were purchased from Tedia Reagent Company (Shanghai, China). Sprague-Dawley rats (male, 220 ± 20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China).

Instrumentation and conditions

Agilent 6890N-5975B GC/MS, HP-5MS (0.25 mm×30 mm×0.25 mm), were from Agilent Company (Santa Clara, California, USA). Mass detection was conducted first in EI mode with electron energy of 70 eV, then in full-scan mode with m/z 50-550, and finally by splitless mode injection [11, 14]. The GC oven was initially set at 80°C and was kept at this temperature for 5 min. The temperature was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 min.

Sample preparation

250 µL of acetonitrile was added to 100 µL of serum, kept in ice-bath for 15 min, and then were centrifuged at 10,000 g for 10 min at 4°C. 150 µL of the supernatant was transferred to a GC vial

and evaporated to dryness under a stream of nitrogen gas. Methoximation was carried out at 70°C for 24 h after addition of 50 µL methylhydroxylamine hydrochloride (15 mg/mL in pyridine). 50 µL MSTFA (with 1% TMCS as catalyst) was added and kept at 70°C for another one hour, and then vortexed after adding 150 µL n-heptane [14].

Metabolomics study

Rats were housed under a natural light-dark cycle condition with controlled temperature (22°C). All rats were housed at Laboratory Animal Research Center of Wenzhou Medical

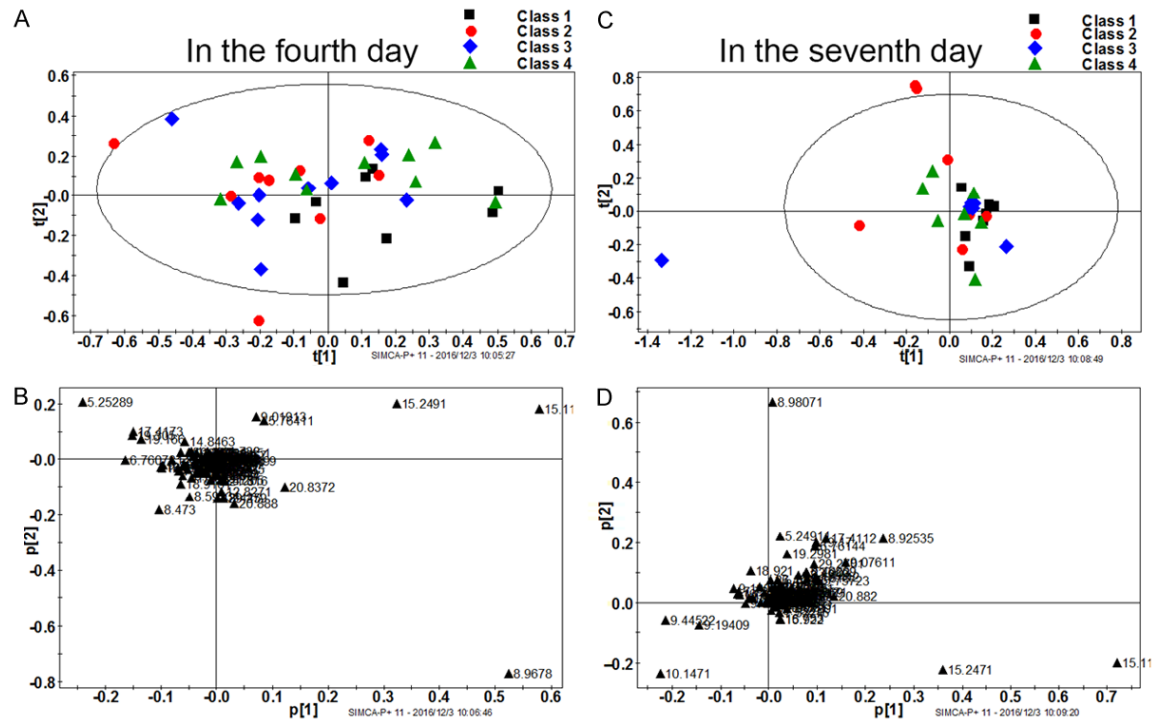


Figure 2. PCA scores of rat serum samples on the fourth (A) and the seventh day (C). Class 1, control group; Class 2, acute paraquat poisoning group; Class 3, low pirfenidone group; Class 4, high pirfenidone group (Class 4). The corresponding load diagram on the fourth (B) and the seventh day (D).

University. All experimental procedures were approved ethically by the Administration Committee of Experimental Animals of Wenzhou Medical University.

Thirty-eight rats (220 ± 20 g) were randomly divided to control group (8 rats), acute paraquat poisoning group (10 rats), low pirfenidone (20 mg/kg, ig) group (10 rats), and high pirfenidone (40 mg/kg, ig) group (10 rats). Rats in acute paraquat poisoning group were given paraquat by intraperitoneal (ip) administration (20 mg/kg), then were given saline by continuous intragastric (ig) administration for 7 days; Rats in low pirfenidone group were give paraquat by ip administration (20 mg/kg), followed by continuous ig administration of pirfenidone (20 mg/kg) for 7 days; Rats in high pirfenidone group were given paraquat by ip administration (20 mg/kg), followed by continuous ig administration of pirfenidone (40 mg/kg) for 7 days; Control group were given saline by continuous ig administration for 7 days.

Blood samples were collected from the rats in all groups at 8:00 am on the fourth and the seventh day. After collection, the blood samples

were centrifuged at 8,000 g for 10 min at 4°C to obtain serum samples. The serum samples were stored at -80°C until measurement.

Histopathology

After metabolomics experiment, rats were deeply anesthetized with 10% chloral hydrate (20 mg/kg) by ip injection. The livers were rapidly isolated and immersed in freshly prepared 4% w/v formaldehyde (0.1 M phosphate buffers, pH 7.2) for 48 h and then embedded in paraffin. 4- μ m-thick histologic sections were prepared and stained with hematoxylin and eosin by routine hematoxylin-eosin staining method. The liver morphological changes were observed under light microscope.

Data analysis

The GC-MS data were exported into Microsoft Excel, with the peaks normalized to the total sum of spectrum prior to multivariate analyses. The resulting data were processed by principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using

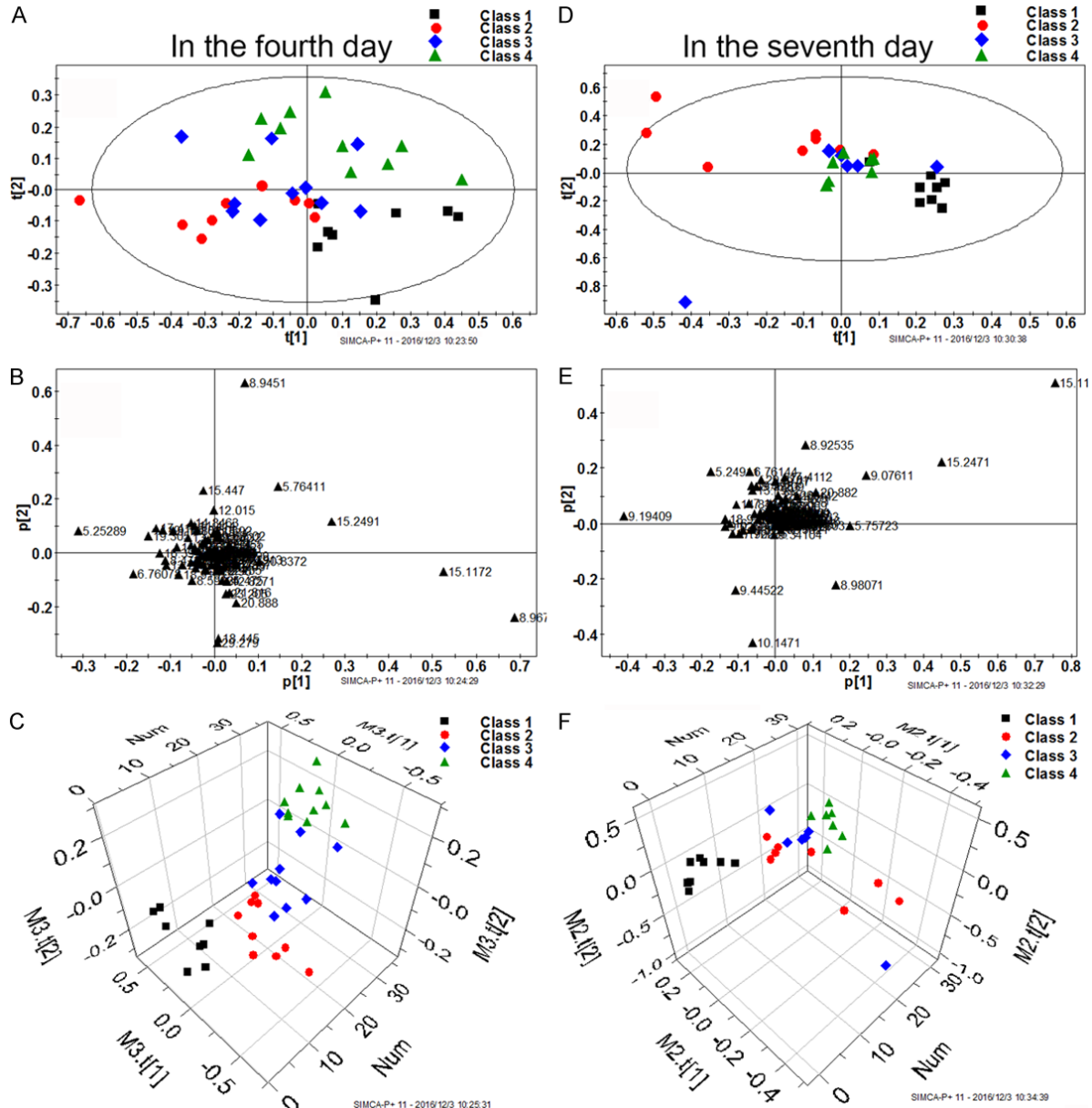


Figure 3. PLS-DA score results of rat serum samples on the fourth (A) and the seventh day (D). Class 1, control group; Class 2, acute paraquat poisoning group; Class 3, low pirfenidone group; Class 4, high pirfenidone group (Class 4). The corresponding load diagram on the fourth (B) and the seventh day (E). PLS-3D score results on the fourth (C) and the seventh day (F).

SIMCA-P 11.5 software (Umetrics, Umea, Sweden).

Statistical analysis

Statistical analysis was carried out using SPSS software (Version 18.0, SPSS). Independent samples T-test was applied to detect significant differences in all metabolites between every two groups. A P value of <0.05 was considered statistically significant.

Results and discussion

Metabolomics study

Figure 1 shows the typical metabolic profiles of serum samples acquired from GC-MS. Metabolic profile data pretreatment resulted in a final dataset of metabolic features from GC-MS analyses. NIST 2005 mass spectrometry database was used to identify the endogenous metabolites in the serum.

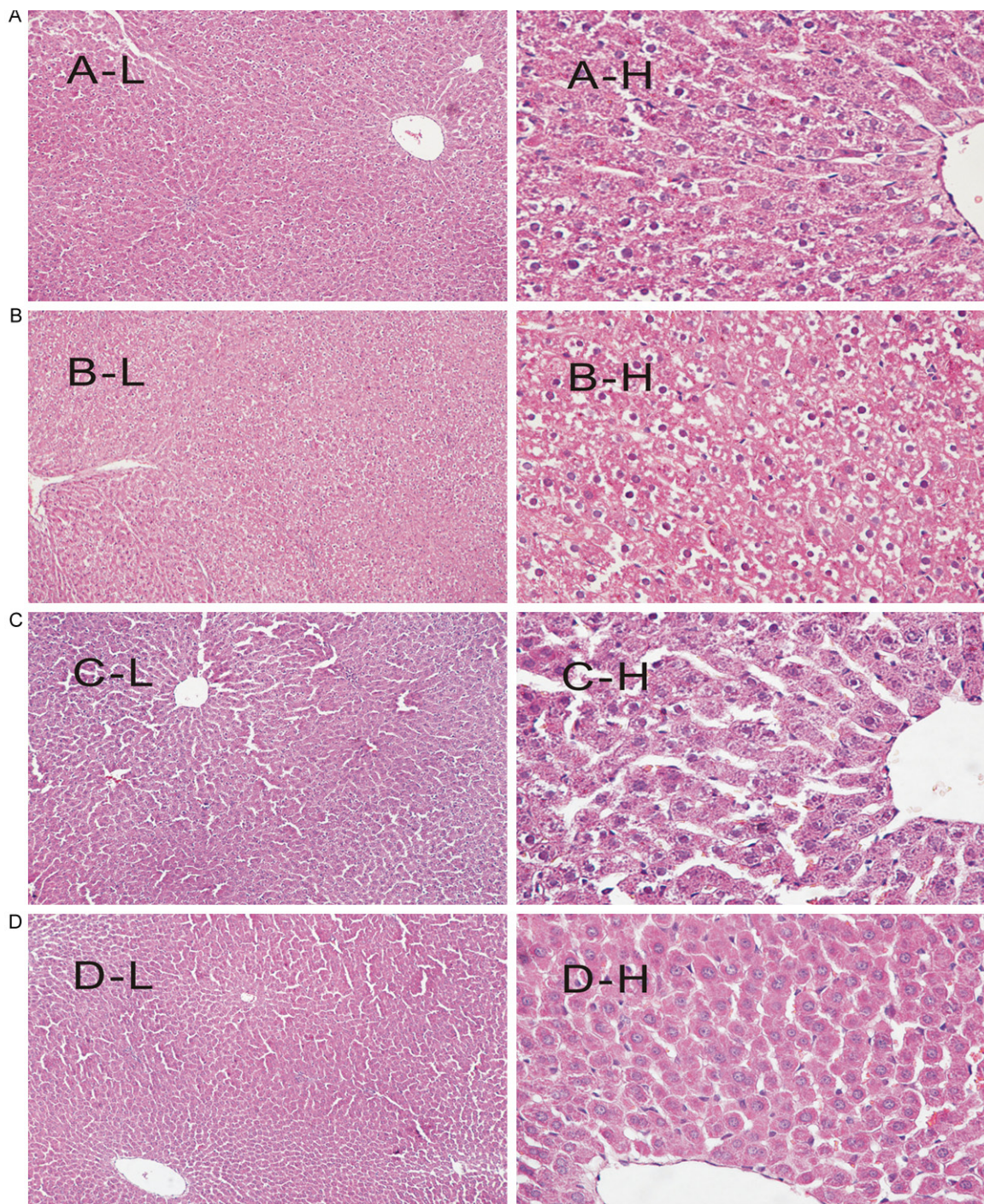


Figure 4. Rat liver pathologic changes after intervention with pirfenidone. Control group (A); Acute paraquat poisoning group (B); Low pirfenidone group (C); High pirfenidone group (D). L×100, H×400.

To explore the effect of pirfenidone on metabolic profile in rats with acute paraquat poisoning, we carried out principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) on the control group (Class 1), acute paraquat poisoning group (Class 2), low pirfenidone group (Class 3), and high pir-

fenidone group (Class 4). The PCA scores of rat serum samples in all groups on the fourth day are shown in **Figure 2A**, while the corresponding load diagram is shown in **Figure 2B**. The PLS-DA scores of rat serum samples on the fourth day are shown in **Figure 3A**, the corresponding load diagram is shown in **Figure 3B**,

Metabolomics in rats with paraquat poisoning treated by pirfenidone

Table 1. Relative levels of metabolite changes in rat serum in the fourth day

NO.	Retention time/min	Metabolite	VIP	Control	PQ	PQ+pirfenidone (Low)	PQ+pirfenidone (High)	Metabolic pathways
1	8.95	Urea	4.03	4.020	7.961*	8.761**	8.589*	Urea cycle
2	29.28	à-D-Glucopyranoside	2.52	1.685	1.473*	1.442*	1.114*,##	
3	15.12	d-Glucose	2.13	44.850	42.465	43.107	44.179	Galactose metabolism
4	5.76	Ethanedioic acid	2.03	4.037	3.305	4.065	4.297*	
5	15.45	Pentanedioic acid	1.97	0.249	0.232	0.440*	0.617*,##	
6	5.25	Butanoic acid	1.71	0.823	2.033*	1.677*	1.072	Carbohydrate digestion and absorption
7	6.76	glycerol	1.35	1.663	2.135**	2.084**	1.696**	Galactose metabolism
8	15.25	d-Galactose	1.28	12.111	11.983	11.978	12.629	Galactose metabolism
9	23.21	Octadecanoic acid	1.18	0.916	0.773**	0.778**	0.732**	Fatty acid biosynthesis
10	21.82	Hexadecanoic acid	1.17	0.845	0.677**	0.720*	0.657**	Fatty acid metabolism
11	12.83	d-Ribose	1.16	0.231	0.060	0.213*	0.125	Pentose phosphate pathway
12	16.40	Myo-Inositol	1.15	0.964	1.409**	1.273**	1.155**	Galactose metabolism

Note: PQ, acute paraquat poisoning group; PQ + pirfenidone (Low), low pirfenidone group; PQ + pirfenidone (High), high pirfenidone group. Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 1.0. Compared with control group, *P<0.05 and **P<0.01; Compared with acute paraquat poisoning group, *P<0.05 and **P<0.01.

Table 2. Relative levels of metabolites changes in rat serum in the seventh day

NO.	Retention time/min	Metabolite	VIP	Control	PQ	PQ+pirfenidone (Low)	PQ+pirfenidone (High)	Metabolic pathways
1	10.15	à-d-Riboside	4.60	0	0.074	2.296	0	
2	15.12	d-Glucose	4.25	45.084	41.159	41.485	42.724*	Galactose metabolism
3	9.19	L-threonine	3.09	0.115	0.764	0.474	0.205	Glycine, serine and threonine metabolism
4	5.76	Acetic acid	2.89	2.650	1.241**	1.238*	1.581*	
5	5.25	Butanoic acid	2.60	0.795	1.628	1.008	1.086*	Carbohydrate digestion and absorption
6	15.25	d-Galactose	2.23	12.791	11.620	12.032	12.340	Galactose metabolism
7	8.98	Urea	1.77	7.819	2.198*	1.865	1.906*	Urea cycle
8	14.84	d-Mannose	1.63	0.602	0.957	0.768	0.782*	Fructose and mannose metabolism
9	6.76	glycerol	1.43	1.730	2.132*	1.771	1.745	Galactose metabolism
10	29.27	à-D-Galactopyranoside	1.12	1.710	2.013	1.719	1.894	
11	16.39	Myo-Inositol	1.12	0.853	1.076	1.107	0.952	Galactose metabolism
12	9.45	Propanedioic acid	1.07	0.272	0.196*	0.660	0.227	
13	19.30	9,12-Octadecadienoic acid	1.04	1.010	1.288	0.799	1.089	
14	15.92	Glucopyranose	1.03	0.155	0.048	0.043	0.099	
15	13.20	L-Proline	1.03	1.284	1.537	1.442	1.303	Arginine and proline metabolism
16	18.92	Uric acid	1.02	0.127	0.386	0.257	0.195	

Note: PQ, acute paraquat poisoning group; PQ + pirfenidone (Low), low pirfenidone group; PQ + pirfenidone (High), high pirfenidone group. Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 1.0. Compared with control group, *P<0.05 and **P<0.01.

and PLS-3D scores are shown in **Figure 3C**. The PCA and PLS-DA scores of rat serum samples on the seventh day are shown in **Figures 2C** and **3D**, the corresponding load diagrams are shown in **Figures 2D** and **3E**, and PLS-3D score results is shown in **Figure 3F**. PLS-DA 3D (**Figure 3C** and **3F**) score chart indicates that the rats in acute paraquat poisoning group were distinguished from the rats in the control group or pirfenidone groups.

Figure 3C demonstrates that a clear separation between the control group, paraquat poisoning group, and pirfenidone groups was observed.

Scores from rats in acute paraquat poisoning changed dramatically after pirfenidone therapy compared to untreated rats, but high-dose pirfenidone treatment had a stronger effect than that of low-dose treatment. This result was consistent with the liver morphological changes (**Figure 4**).

Figure 3F also shows a clear separation between the control group, paraquat poisoning group, and pirfenidone groups, but the effect was less stronger than that of in **Figure 3C**. Those results indicates that the therapeutic effects of pirfenidone remains in rat with acute

paraquat poisoning, but high-dose pirfenidone therapy was more effective than that of low dose.

Morphological changes of liver

Figure 4 shows the liver morphological changes in the four groups with hematoxylin-eosin (HE) pathological examination. In the control group, hepatic lobule structure was clear. Hepatocyte centered around the central vein, radiated out in all directions, formed liver cytokines. And there was no inflammatory cells in liver blood sinus. Liver cell pulp was fully dyed, the nucleus was clear, and no cavity or cell pycnosis necrosis was observed.

In acute paraquat poisoning group, hepatic lobule structure was observed. Hepatocyte centered around the central vein, radiated out in all directions. However, liver cell pulp was lightly dyed, the nuclei became pyknotic, cytoplasm presented cavitation. The boundary of liver blood sinus could not be distinguished, and there was no obvious inflammatory cell invasion.

In low pirfenidone group, hepatic lobule structure was observed. Hepatocytes centered around the central vein, radiated out in all directions. Compared to the paraquat group, the cytoplasm dyeing was darker and hepatic cells could be distinguished, but some liver cell cytoplasm staining remained very light with obvious cavitation.

In high pirfenidone group, most of the hepatic lobule structures were integrate. Hepatocytes centered around the central vein, radiated out in all directions, with no obvious difference compared to the control group. Hepatic cells were clearly distinguished, liver cells arranged closely, and liver blood sinus was clearly visible. The liver pathological changes from different groups indicated that pirfenidone intervention in paraquat poisonings would reduce liver damage.

Changes in metabolite

Metabolomics was a newly emerging omics approach for the investigation of metabolic phenotype changes induced by environmental or endogenous factors [12, 13, 15-19]. In this study, the metabolite changes in acute para-

quat poisoning group, pirfenidone treatment groups, and control group were shown in **Tables 1** and **2**.

Table 1 shows the metabolite changes on the fourth day. Compared to the control group, the level of urea, butanoic acid, glycerol, and myo-Inositol increased in acute paraquat poisoning group ($P<0.05$); While α -D-glucopyranoside, octadecanoic acid, and hexadecanoic acid decreased ($P<0.05$). After treating with pirfenidone for four days, pentanedioic acid increased in both low and high pirfenidone group compared to acute paraquat poisoning group. In addition, d-ribose also increased in the low pirfenidone group, while ethanedioic acid increased in the high pirfenidone group, with α -D-glucopyranoside and glycerol decreased in the high pirfenidone group. **Table 2** shows the metabolite changes on the seventh day. Compared to the control group, the level of glycerol ($P<0.05$) increased, while the level of acetic acid, urea, and propanedioic acid decreased ($P<0.05$) in acute paraquat poisoning group. After treating with pirfenidone for seven days, there was no significant difference in metabolite changes between acute paraquat poisoning group and pirfenidone groups. These finding provided new evidences on therapeutic effect of pirfenidone in rats with acute paraquat poisoning.

Conclusion

In this study, metabolites (pentanedioic acid, d-ribose, ethanedioic acid, α -D-glucopyranoside, and glycerol) changes provided the evidence on the therapeutic effects of pirfenidone in rats with acute paraquat poisoning. Meanwhile, pirfenidone intervention in paraquat poisonings could reduce liver damage. Additionally, there were clear separations between the control group, paraquat poisoning group, and pirfenidone groups in PLS-DA scores. Overall, we demonstrated that GC-MS-based metabolomic analysis method provided a useful tool to elucidate the effects of pirfenidone in paraquat poisoning study.

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Disclosure of conflict of interest

None.

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