

## Original Article

# Serum metabonomic analysis of rats with cisplatin-induced nephrotoxicity and *panax notoginseng* saponins treatment

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**Abstract:** Previously, we reported that the *Panax Notoginseng Saponins* (PNS) had protection against cisplatin-induced nephrotoxicity. Here, we aimed to metabonomically analyze the serum samples from rats with early stage of cisplatin-induced nephrotoxicity and PNS treatment. The rats underwent intraperitoneal injection with a single dose of cisplatin, and a subset of rats underwent intraperitoneal injection with PNS once a day. After 24 hours, the renal function was evaluated, the pathological changes of renal tissue were examined, and the serum samples were analyzed using  $^1\text{H}$  nuclear magnetic resonance-based metabonomics technology. The results showed that PNS protected against cisplatin-induced nephrotoxicity. In addition, PCA, PLS-DA and OPLS-DA scores plots of serum samples showed that the normal control group was clearly separated from the cisplatin-only group and the cisplatin-PNS group, while there was a part overlap between the cisplatin-only group and the cisplatin-PNS group. Moreover, the expression of acetone, n-acetyl glycoprotein signals, pyruvate, lipid and VLDL was lower, while the expression of glucose, valine and tyrosine was higher in the cisplatin-only group and the cisplatin-PNS group than that in the normal control group. Furthermore, lactate expression was higher in the cisplatin-only group than that in the cisplatin-PNS group. Phenylalanine expression was higher while tyrosine expression was lower in the cisplatin-PNS group and the normal control group when compared with the cisplatin-only group. Taken together, these results confirmed that the metabolism of cisplatin-induced nephrotoxicity had a huge change and PNS would be an effective medicine. Its protective mechanism may be associated with correcting the disturbed metabolism of cisplatin-induced nephrotoxicity. And here is the first demonstration about it.

**Keywords:** Cisplatin, nephrotoxicity, *panax notoginseng* saponins, metabonomic, nuclear magnetic resonance, multivariate analysis

## Introduction

Cisplatin is one of the most widely used chemotherapeutic agents. However, nephrotoxicity is one of the major side effects of cisplatin and limits its clinical application. The mechanisms of cisplatin-induced nephrotoxicity have not yet been fully elucidated. Previous studies showed that it might be involving multiple mechanisms such as DNA damage, oxidative stress, inflammatory response [1], and apoptosis, etc [2-4]. Moreover, effective drug to protect against cisplatin-induced nephrotoxicity is lack at present.

*Panax Notoginseng Saponins* (PNS) is extracted from traditional Chinese medicine herb *Panax Notoginseng*, which contains a variety of

active components including notoginseng saponin R1, and ginseng saponin Rg1, Rb1, Re, etc. Thus, PNS has a variety of pharmacological effects [5, 6] such as antioxidant, anti-apoptotic [7] and anti-proliferative, etc. Studies have shown that PNS can increase the survival ratio of renal tubular epithelial cells [8], reduce the serum creatinine (Scr) and blood urea nitrogen (BUN) levels in mice with cisplatin-induced nephrotoxicity, and protect against renal disease [9, 10]. It has been reported recently that PNS can improve immune function, inhibit tumor growth [11] and enhance the antitumor effect of cisplatin [12]. In addition, our previous research has found that PNS can protect against cisplatin-induced renal damage by reducing the oxidative damage [13]. Therefore, it is necessary to system-

atically study the protective effect of PNS on cisplatin-induced nephrotoxicity through high throughput technology.

Metabolomics is a kind of high throughput technology, which can systematically study the metabolites in tissues or biofluids. The concentration and combination of metabolites have contributed to predict disease progression. Metabonomics has been widely used in the fields of life science, including disease diagnosis [14, 15], studying the effect and mechanism of medication treatment [16], etc.  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy is one technology of metabolomics, it can provide the detailed information on molecular structure, and the absolute or relative concentration of metabolites. There have been many successful applications of  $^1\text{H}$  NMR spectroscopy to urine, plasma and other biological specimens for studying altered metabolism in disease conditions [17].

Portilla D [18] studied the metabolic change of urine from mice exposed to cisplatin using  $^1\text{H}$  NMR metabolomics, the findings showed that this method could be useful for studying cisplatin-induced acute renal failure. Kwon HN predicted the idiopathic toxicity of cisplatin-induced renal damage by animal urine NMR spectra, and found that marker metabolites could explain the idiopathic toxicity mechanism [19]. So far no metabolomics studies of serum from rat with cisplatin-induced nephrotoxicity and the protective effect of PNS have been undertaken. In this context, the  $^1\text{H}$  NMR metabonomics method was used to study the serum metabolites in rats with early stage of cisplatin-induced nephrotoxicity and intervened by PNS.

## Materials and methods

### Chemicals

PNS (Batch number 20110115), a commercial drug, was purchased from Guangxi Wuzhou Pharmaceutical Co., Ltd. (Wuzhou, China). Cisplatin powder for injection (Batch number 0070152DB) was obtained from Qilu Pharmaceutical Co., Ltd. (Jinan, China). BUN, Scr and N-acetyl- $\beta$ -D-Glucosaminidase (NAG) kits were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Deuterium oxide ( $\text{D}_2\text{O}$ ), chloroform- $d$  ( $\text{CDCl}_3$ ) and sodium trimethylsilylpropionate (TSP) were purchased from Cambridge Isotope Laboratories, Inc (USA).

### Equipment

An INOVA 600 MHz NMR spectrometer was obtained from Varian Unity (Varian, Inc. USA). Nuclear magnetic resonance (NMR) tube (diameter 5 mm) was obtained from Wilmad-LabGlass (USA).

### Animals

Male Sprague-Dawley (SD) rats weighing  $200 \pm 20$  g were taken from the Experimental Animal Center of Guangxi Medical University (Guangxi, China). The experiment was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University (approval no. 12061726).

### Experimental design and drugs treatment

The animals were housed four per cage. Water and food were provided *ad libitum*. After one week of acclimation period, the animals were divided randomly into three groups ( $n=15/\text{group}$ ) as follows:

The normal control group (Group I): rats received the same volume of saline as that of cisplatin and PNS.

The cisplatin-only group (Group II): rats received a single dose of cisplatin (5 mg/kg), and then received the same volume of saline as that of PNS.

The cisplatin-PNS group (Group III): rats received a single of cisplatin (5 mg/kg), and then received PNS (31.35 mg/kg).

The rats in Group II and Group III were administered cisplatin to induce kidney damage. The doses of cisplatin and PNS were adopted according to the rat dosages which were converted from the clinical dosages of adult and the previous studies [13, 20]. All drugs were administered by intraperitoneal injection.

24 hours later, rat blood samples and urine samples were collected, centrifuged at  $4^\circ\text{C}$ , 3000 rpm, stored at  $-80^\circ\text{C}$  for evaluating the renal function and metabonomic analysis. Then, rats were anesthetized with pentobarbital sodium (30 mg/kg, i.v.). After lavaged *in situ* with ice-cold saline through the abdominal aorta, the kidneys of rat were dissected, washed immediately with ice-cold saline, and fixed for 2 hours in 3% glutaraldehyde for histopathological analysis using H&E-staining.

**Table 1.** The NMR instrument detection parameters of serum samples

Parameter r	Value	Paramete	Value
Origin	Varian	Pulse width	15.4
Spectrometer	vnmrs	Acquisition time	0.9984
Solvent	D <sub>2</sub> O	Spectrometer frequency	599.91
Temperature	25°C	Spectral width	8012.8
Pulse sequence	cpmgt2pr	Lowest frequency	-1125.3
Experiment	1 D	Nucleus	<sup>1</sup> H
Number of scans	128	Acquired size	8000
Receiver gain	30	Spectral size	16384
Relaxation delay	2 s		

After the renal function was evaluated, the levels of serum BUN, Scr and urinary NAG were found to significantly increase in rats exposed to cisplatin [20, 21]. Moreover, PNS significantly decreased the levels of serum Cr, BUN and urinary NAG [20, 21]. The pathological examination, using H&E-staining, showed that PNS obviously attenuated the pathological damages of renal tissues induced by cisplatin [20, 21].

#### *Sample preparation and NMR data acquisition*

Plasma buffer (600 mM PB solution, PH=7.4): 2.2130 g K<sub>2</sub>HPO<sub>4</sub>•3H<sub>2</sub>O and 0.3782 g NaH<sub>2</sub>PO<sub>4</sub>•2H<sub>2</sub>O were taken into volumetric flask, dissolved and diluted to 20 mL with D<sub>2</sub>O.

400 µL serums were taken into 1.5 mL centrifuge tube, 30 µL plasma buffers and 170 µL D<sub>2</sub>O were added, and vortex mixing for 1 minute. After the sample was centrifuged at 4°C, 12000 rpm for 10 minutes, the supernatant was placed in 500 µL NMR tube for the detection.

NMR spectra were recorded at 25°C using a Varian 600 spectrometer, the <sup>1</sup>H resonant frequency was 599.91 Hz. The weighted experiment of the transverse relaxation was performed using a Carr-Purcell-Meiboom-Gill (CPMG) sequence of water peak suppression. The NMR instrument detection parameters of serum samples were showed in **Table 1**.

#### *NMR spectra data processing and multivariate statistical analysis*

All the 1 D NMR spectra were referenced to lactate's methyl group (at δ1.33), manually phased, Fourier transformation, baseline corrected, phase-adjustment and scaled using MestReNova software (Version 7.1, Mestrelab

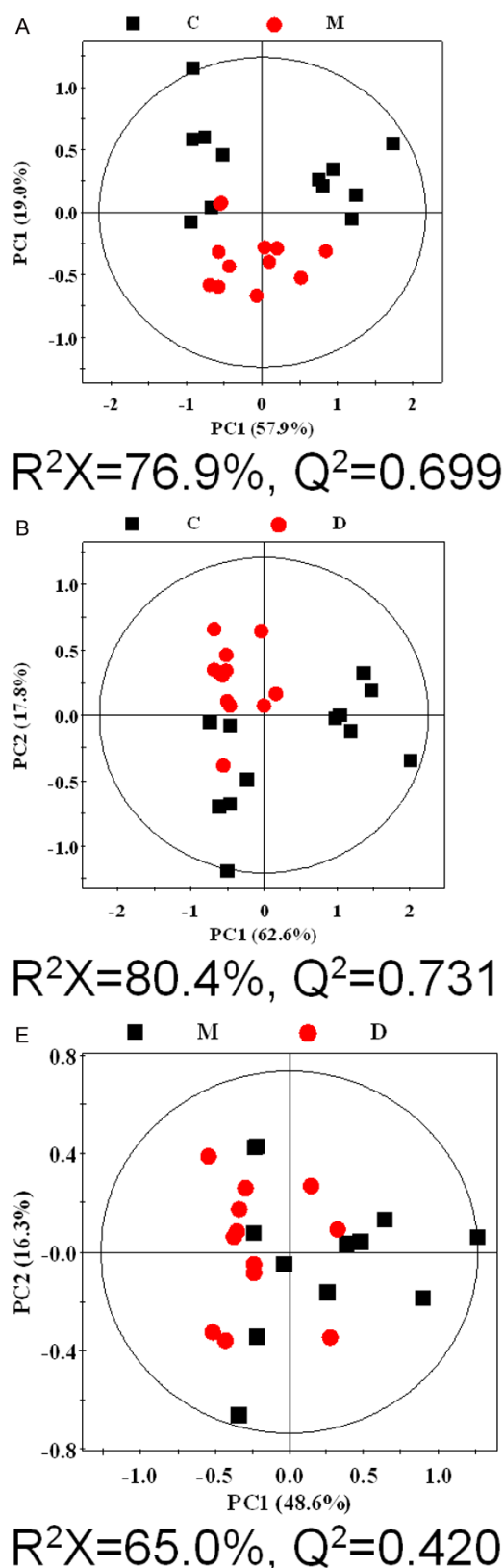
Research SL). In order to improve the signal-to-noise ratio, all 1 D free induction decays were multiplied by an exponential weighting factor of 1 Hz line-broadening factor before they were Fourier transformation. Those peaks which had no overlap for each metabolite in the 1 D NMR were integrated. The resulting unambiguous metabolites in serum samples were normalized to the sum of the spectral intensity to make up for differences in sample concentration. Spectra in the region of δ0.5-9.0 were subdivided into integrated regions of 0.002 ppm width, and the spectra in the δ5.90-5.50 ppm (urea) as well as 5.22-4.30 ppm (water) were excluded from data reduction.

After the data were normalized, the obtained data were invariably scaled for principal components analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares discriminate analysis (OPLS-DA) using SIMCA-P<sup>+</sup> V11.0 software package (Umetrics, Umea, Sweden). The data scale conversion method of mean center scaling was used in PCA, and the data scale conversion method of unit variance scaling was used in both PLS-DA and OPLS-DA. The score plots of PCA and PLS-DA were visualized with the first principal component (t[1]) and the second principal component (t[2]). The OPLS-DA was calculated with the first predictive (t[1]P) and an orthogonal component (t[2]O). R<sup>2</sup>X (cum), R<sup>2</sup>Y (cum) and Q<sup>2</sup> (cum) parameters used to test the goodness of fit and model validity were calculated by the cross-validation procedure. The parameters R<sup>2</sup>X (cum) and R<sup>2</sup>Y (cum) are respectively the fraction of the sum total of the squares of the whole X's and Y's explained by the model. The parameter Q<sup>2</sup> (cum) stands for the cross-validated explained variation. Cross validation (12-round) and permutation tests (300 cycles) were implemented to assess the robustness of the models.

#### *Screening and identification of the differences metabolites*

The statistically significant metabolites were further inductively analyzed through the analysis of OPLS-DA and the analysis of the corresponding correlation coefficients of each metabolite. The correlation coefficients of the variables relative to the first predictive component in the OPLS-DA model were extracted from

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**Figure 2.** 2D PCA scores plot based on  $^1\text{H}$  CPMG NMR spectra of serum obtained from groups C, M and D. C: the normal control group, M: the cisplatin-only group, D: the cisplatin-PNS group.

mainly distributed on the left side of the ordinate while those from the cisplatin-only group mainly distributed on the right side of the ordinate (**Figure 2E**).

To further explore the differences in the metabolic profiles of these groups, PLS-DA was employed. The PLS-DA models of the cisplatin-only group and the cisplatin-PNS group both indicated a clear difference from the normal control group, with a good authenticity and predictability of the separation between the normal control group and the other two groups (**Figure 3A, 3B**). Additionally, there was a good authenticity between the cisplatin-only group and the cisplatin-PNS group, but the predictability was slightly low (**Figure 3E**).

**Figure 4** showed the OPLS-DA scores plots (**Figure 4**, left panel) and corresponding coefficient loading plots (**Figure 4**, middle and right panels) derived from serum  $^1\text{H}$  CPMG NMR spectra of different groups. The score plots showed that there was a clear difference between the normal control group and the cisplatin-only group (**Figure 4A**:  $R^2X=17.3\%$ ,  $Q^2=0.749$ ). Explained variation  $R^2$  and predictive capability  $Q^2$  were indicated that the rat model of cisplatin-induced nephrotoxicity was well established. The OPLS-DA score plots showed that there was a clear difference between the normal control group and the cisplatin-PNS group, too (**Figure 4B**:  $R^2X=20.3\%$ ,  $Q^2=0.659$ ). The score plots between the cisplatin-only group and the cisplatin-PNS group were shown in **Figure 4E** ( $R^2X=21.6\%$ ,  $Q^2=0.129$ ), its predictive capability  $Q^2$  was low.

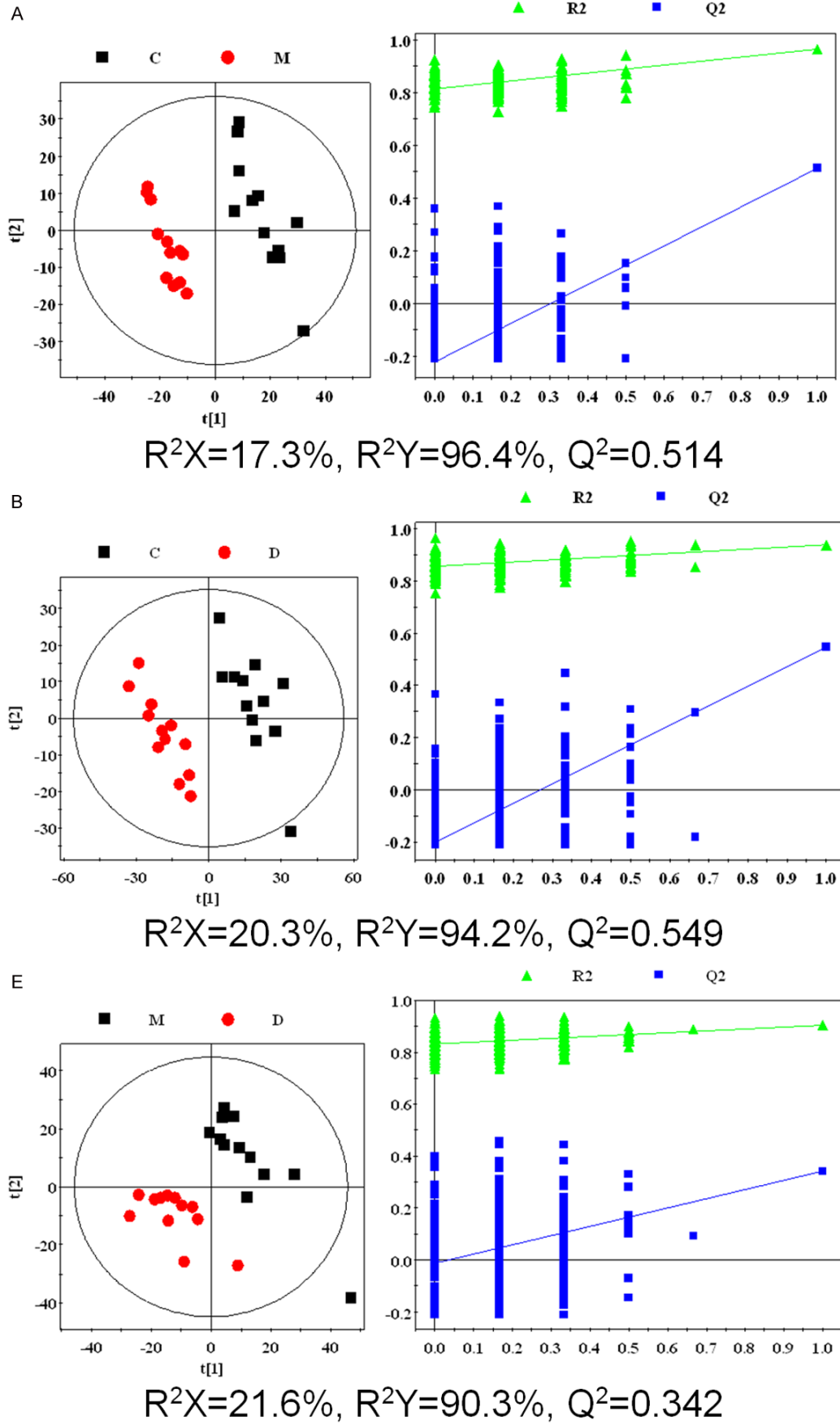
#### *Selection and identification of differences metabolites*

After the analysis of OPLS-DA and the analysis of the corresponding correlation coefficients of each metabolite, the differences metabolites were obtained (**Table 2**). These differences metabolites mainly included amino acids (isoleucine, leucine, tyrosine, valine and cytidine), lipids and substances related to the generation of energy (glucose, acetone, pyruvate and lactate).

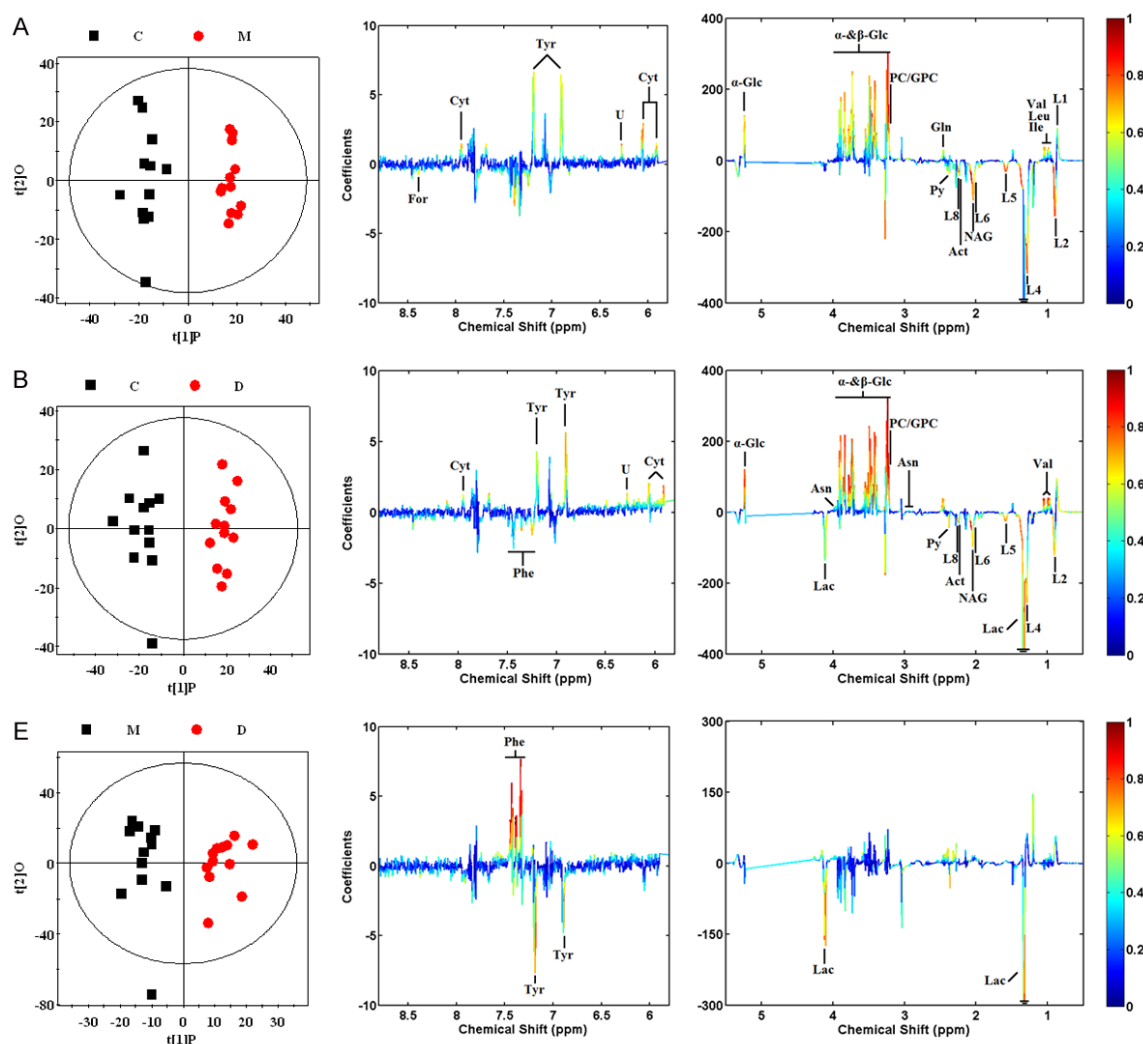
#### *Pathway analysis of differences metabolite*

The serum differences metabolites were connected to different metabolic pathways based on the KEGG database (**Table 3**), including glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate metabolism, and so on. Th-





**Figure 3.** Scores plots (left panel) and cross validation by permutation test (right panel) of PLS-DA derived from the  $^1\text{H}$  CPMG NMR spectra of serum obtained from different groups. C: the normal control group, M: the cisplatin-only group, D: the cisplatin-PNS group.



**Figure 4.** OPLS-DA scores plots (left panel) and corresponding coefficient loading plots (middle and right panels) derived from the  $^1\text{H}$  CPMG NMR spectra of serum from different groups. The color map shows the significance of metabolites variations between the two classes. Peaks in the positive direction indicate metabolites that are more abundant in the groups in the positive direction of first principal component. Consequently, metabolites that are more abundant in the groups in the negative direction of first primary component are presented as peaks in the negative direction. Keys of the assignments were shown in Figure 1. C: the normal control group, M: the cisplatin-only group, D: the cisplatin-PNS group.

ese metabolic pathways were mainly involved in energy metabolism, indicated that the energy metabolism is a major change in the early stage of cisplatin-induced nephrotoxicity and treated with PNS.

## Discussion

We investigated the changes of serum metabolites in early stage of rat intervened with cis-

platin and PNS using  $^1\text{H}$  NMR metabolite profiling approach. In this study, we found that serum BUN and Scr levels of rat significantly increased, and the renal tissues were damage after a single intraperitoneal injection of cisplatin [20, 21]. Moreover, PNS significantly reduced the serum BUN and Scr levels, and attenuated renal histopathological damages [20, 21]. These results suggest that the model of cisplatin-induced renal damage success and

**Table 2.** Serum differences metabolites obtained from different pair-wise groups

Metabolites	$r^a$		
	C-M*	C-D	M-D
Acetone: 2.23 (s) <sup>b</sup>	-0.625	-0.569	-
Asparagine: 2.86 (dd), 2.92 (dd), 3.97 (dd)	0.613	-	-
Cytidine: 5.91 (d), 6.06 (d), 7.83 (d)	0.736	0.725	-
DMG: 2.93 (s)	0.635	-	-
Formate: 8.46 (s)	-	-0.572	-
Glutamine: 2.14 (m), 2.45 (m), 3.78 (m)	-	0.660	-
Isoleucine: 0.94 (t), 1.01 (d)	-	0.562	-
L1: LDL, CH <sub>3</sub> -(CH <sub>2</sub> ) <sub>n</sub> :- 0.87 (br)	-	0.600	-
L2: VLDL, CH <sub>3</sub> -(CH <sub>2</sub> ) <sub>n</sub> :- 0.89 (br)	-0.735	-0.618	-
L4: VLDL, CH <sub>3</sub> -(CH <sub>2</sub> ) <sub>n</sub> :- 1.30 (br)	-0.713	-0.711	-
L5: Lipid, -CH <sub>2</sub> -CH <sub>2</sub> -C=O: 1.58 (br)	-0.698	-0.655	-
L6: Lipid, -CH <sub>2</sub> -CH=CH:- 1.96 (br)	-0.593	-0.575	-
L8: Lipid, CH <sub>2</sub> -C=O: 2.23 (br)	-0.662	-0.652	-
Lactate: 1.33 (d), 4.11 (q)	-	-	-0.658
Leucine: 0.96 (t)	-	0.713	-
NAG:N-acetyl glycoprotein signals: 2.04 (s)	-0.576	-0.778	-
PC/GPC: 3.22 (s)	0.681	0.793	-
Phenylalanine: 7.33 (d), 7.37 (t), 7.43 (dd)	-0.632	-	0.792
Pyruvate: 2.37 (s)	-0.565	-0.610	-
Tyrosine: 6.90 (d), 7.20 (d)	0.583	0.634	-0.654
Unknown: 6.28 (t)	0.658	0.639	-
Valine: 0.99 (d), 1.04 (d)	0.631	0.789	-
α-Glucose: 3.42 (t), 3.54 (dd), 3.71 (t), 3.73 (m), 3.84 (m), 5.24 (d)	0.734	0.780	-
β-Glucose: 3.25 (dd), 3.41 (t), 3.46 (m), 3.49 (t), 3.90 (dd), 4.65 (d)	0.798	0.886	-

Note: <sup>a</sup>Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of  $|r| > 0.553$  was used as the cutoff value for the statistical significance based on the discrimination significance at the level of  $P=0.05$  and  $df$  (degree of freedom)=11. “-” means the correlation coefficient  $|r|$  is less than 0.553. <sup>b</sup>Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet; br, broad resonance. \*C: the normal control group, M: the cisplatin-only group, D: the cisplatin-PNS group.

PNS can protect against cisplatin-induced nephrotoxicity.

Multivariate pattern recognition analysis (**Figures 2-4**) indicated a clear discrimination between the normal control group and the cisplatin-only group. As shown in the OPLS-DA coefficient loading plot (**Figure 4A**) and **Table 2**, compared with the normal control group, the cisplatin-only group was characterized by decreased levels of acetone, pyruvate, phenylalanine, N-acetyl glycoprotein signals, LDL, VLDL and Lipid, as well as characterized by increased levels of phosphocholine/glycerolphosphocholine, asparagine, cytidine, tyrosine, valine, α-glucose and β-glucose. These data indicated that the metabolic patterns altered, such as the lipid metabolism was suppressed, gly-

colysis and sugar dysplasia was abnormal in the cisplatin-only group.

There was a clear discrimination between the normal control group and the cisplatin-PNS group (**Figures 2, 3**). Although there was a small part overlap between the cisplatin-only group and the cisplatin-PNS group (**Figure 2**), PLS-DA scores plots (**Figure 3**) showed that the cisplatin-only group and the cisplatin-PNS group were significantly divided in different quadrants. In addition, OPLS-DA analysis showed that there were a series of serum differences metabolites between the normal control group and the cisplatin-PNS group, while there were only some serum differences metabolites between the cisplatin-only group and the cisplatin-PNS group rats (**Table 2**). These results suggest that



**Table 3.** Results of metabolic pathways analysis from serum differences metabolites

Pathway	Compounds
rno01100 Metabolic pathways-Rattus norvegicus (rat) (7)	cpd: C00022 Pyruvate cpd: C00058 Formate cpd: C00207 Acetone cpd: C00221 beta-D-Glucose cpd: C00267 alpha-D-Glucose cpd: C00475 Cytidine cpd: C01026 N, N-Dimethylglycine
rno01200 Carbon metabolism-Rattus norvegicus (rat) (4)	cpd: C00022 Pyruvate cpd: C00058 Formate cpd: C00221 beta-D-Glucose cpd: C00267 alpha-D-Glucose
rno00010 Glycolysis/Gluconeogenesis-Rattus norvegicus (rat) (3)	cpd: C00022 Pyruvate cpd: C00221 beta-D-Glucose cpd: C00267 alpha-D-Glucose
rno00260 Glycine, serine and threonine metabolism-Rattus norvegicus (rat) (2)	cpd: C00022 Pyruvate cpd: C01026 N, N-Dimethylglycine
rno00030 Pentose phosphate pathway-Rattus norvegicus (rat) (2)	cpd: C00022 Pyruvate cpd: C00221 beta-D-Glucose
rno01220 Degradation of aromatic compounds-Rattus norvegicus (rat) (2)	cpd: C00022 Pyruvate cpd: C00058 Formate
rno00620 Pyruvate metabolism-Rattus norvegicus (rat) (2)	cpd: C00022 Pyruvate cpd: C00058 Formate
rno00630 Glyoxylate and dicarboxylate metabolism-Rattus norvegicus (rat) (2)	cpd: C00022 Pyruvate cpd: C00058 Formate
rno00500 Starch and sucrose metabolism-Rattus norvegicus (rat) (2)	cpd: C00221 beta-D-Glucose cpd: C00267 alpha-D-Glucose

the alteration of metabolic pattern in the cisplatin-PNS group is similar to that in the cisplatin-only group, and there are certain protective effect on cisplatin-induced nephrotoxicity, which effect can reflect in the changes of serum differences metabolites at 24 hours after injection PNS.

It is worth noting that among these differences metabolites, lactate expression was higher in the cisplatin-only group than that in the cisplatin-PNS group, it suggested that anaerobic respiration in cells was enhanced in the cisplatin-only group. In addition, phenylalanine expression was higher while tyrosine expression was lower in the cisplatin-PNS group and the normal control group than that in the cisplatin-only group. Moreover, phenylalanine expression was no difference in between the cisplatin-PNS group and the normal control group. Phenylalanine (an essential amino acid) is converted to tyrosine by phenylalanine hydroxylase [22], and the conversion reflects the activity of phenylalanine-hydroxylase and associated

with inflammation [23]. Our results indicated that PNS could improve the activity of phenylalanine-hydroxylase, and it might be related to the mechanism of PNS protection against cisplatin-induced nephrotoxicity.

Pyruvate is an important energy substrate and a physiological antioxidant. Pyruvate is converted from phosphoenolpyruvate by pyruvate kinase, and generated adenosine triphosphate (ATP) [24] through tricarboxylic acid cycle or glycolysis to maintain cell basic function. Pyruvate participates in the mutual transformation among sugar, fat and amino acids, so it plays a pivotal role in the metabolism of these three major nutrients. It has been shown that pyruvate was benefit cellular energy metabolism and reduced free radical formation. Previous studies have indicated that pyruvate is able to improve ischemia/reperfusion damage in various tissues [24-26]. Cells cultured with pyruvate could increase the ATP level, and sustain functional hepatocytes for a long period [27]. Pyruvate increased levels of hepatic lac-

tate, alanine and succinate, and maintained higher ATP levels in livers [25]. Pyruvate had antioxidant action, and could rapidly provide ATP and GTP, so it could improve liver function of patients with alcoholic liver disease [28]. Additionally, pyruvate and glucose could obviously be quite informative with regard to cell stress, cell function and organ function [29]. In this experiment, serum pyruvate expression was lower in the cisplatin-only group and the cisplatin-PNS group than that in the normal control group. It prompts that the energy metabolic disorders in rat exposed to cisplatin, especially the insufficient energy production. This may be one important mechanism of cisplatin-induced nephrotoxicity.

In summary, we demonstrated the serum metabolic characteristics in rats with early stage of cisplatin-induced nephrotoxicity and that of after PNS intervention using  $^1\text{H}$  NMR-based metabolomic technology, and our results show that it is feasible and meaningful.

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### Disclosure of conflict of interest

None.

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