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Metabonomics study of urine from Sprague–Dawley rats exposed to Huang-yao-zi using ^{1}H NMR spectroscopy

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ABSTRACT

Urinary metabolic perturbations associated with liver toxicity induced by Huang-yao-zi (root of *Dioscorea bulifera* L.) were studied using nuclear magnetic resonance spectroscopy (^{1}H NMR) to determine the correlations between metabonomic profiling and histopathologic/biochemical observations and to discover biomarkers for liver toxicity. Huang-yao-zi with a maximal tolerance dose (MTD) was given to male Sprague–Dawley rats for 72 h followed by metabonomic analysis of urine samples collected at 24 and 72 h. The results revealed that the levels of taurine, creatine, betaine, dimethylglycine (DMG), acetate, glycine were elevated, whereas, the levels of succinate, 2-oxoglutarate, citrate, hippurate and urea were reduced. Partial least square (PLS)-discrimination analysis (DA) of NMR spectra revealed two apparent clusters between control groups and treatment groups, indicating metabolic changes observed in urine samples in response to Huang-yao-zi treatment. In addition, mechanism associated with oxidative injury of hepatic mitochondria was investigated. These results indicated that ^{1}H NMR-based metabonomics analysis in urine samples may be useful for predicting hepatotoxicity induced by Huang-yao-zi.

1. Introduction

Huang-yao-zi, the root of *Dioscorea bulbifera* L. (Dioscoreaceae), has been wildly used in Traditional Chinese Medicine (TCM). It has been known to have many functions, e.g., anti-tumor, anti-bacterial, anti-feedant, anti-fungal, anti-salmonella and hallucinogenic [1–4]. Much attention has been given to its primary preventive role against cancers [5,6]. It is reported that dioscin and diosbulbins B, which derived from Huang-yao-zi, are liver toxicities. So, this constrains the application of Huang-yao-zi in clinical therapy (LD50/ED50) [7]. Clinical reports have shown that the symptoms of Huang-yao-zi intoxication include nausea, abdominal pain, coma, and even death [8]. The mechanisms related to Huang-yao-zi induced liver toxicity have not been fully studied. The proposed mechanism is that Huang-yao-zi directly damages mitochondria and endoplasmic reticulum membrane, which in turn causes decreased activities of metabolizing enzymes and antioxidation enzymes [9]. Therefore, it is critical to investigate the toxicities of Huang-yao-zi.

The potentials of metabonomics have expanded to many fields including studies of TCM toxicity in recent years [10]. As an alternative approach, it has the advantage of being able to measure samples continuously over a long period of time and being able to collect data from multiple sites [11]. Metabonomics set whole biological systems by studying metabolic profiles, generally use biofluids, which gave a window on whole metabolism process and then provided crucial information on TCM toxicity. Furthermore, NMR combined with pattern recognition (PR) has provided a rapid, non-destructive, and high-throughput method that requires minimal sample preparation [12]. ^{1}H NMR spectrum is an inherently quantitative technique that can report on hundreds of compounds in a single measurement. Individual signals are dispersed depending on the chemical environment of the source nuclei. Thus, NMR spectra of a biofluid or tissue are rich in structural information that extremely complex, consisting of information relating to the concentration of these metabolites in the sample. Apparently, metabonomic approach plays an important role in effective preclinical screen and safety assessment during natural medicine discovery [13]. Indeed, metabonomic approach has been applied to the studies of toxicities related to the uses of natural medicines such as Hei-Shun-Pian and its toxic component aconitum alkaloids and Guan-mu-tong (*Aristolochia manshuriensis*) which contains aristolochic acid [14].

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Table 1
The effect of Huang-yao-zi on weight and (mean ± S.D., $n = 5$).

Treatment	Body weight (g)				Liver weight (g)	Organ/body coefficients (%)
	pre0–24 h	0–24 h	24–48 h	48–72 h		
Control	231.6 ± 13.4	239.2 ± 14.56	247.2 ± 13.34	254.3 ± 13.55	9.16 ± 1.01	3.59 ± 0.22
400 g/kg	234.4 ± 13.14	220.3 ± 15.15	216.4 ± 13.56[a]	209.2 ± 9.79[a]	19.68 ± 0.49[a]	9.43 ± 0.54[a]

[a] Statistics: $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to control.

In this study, we used ^{1}H NMR and urine samples of rats exposed to Huang-yao-zi to investigate Huang-yao-zi induced liver toxicity. The changes of endogenous metabolites were determined by PLS-DA. The mechanism associated with Huang-yao-zi induced liver toxicity was also investigated.

2. Materials and methods

2.1. Decoction method of Huang-yao-zi

Huang-yao-zi was purchased from AnGuo ZhenYuanTang Chinese Herbal Pieces Ltd. (AnGuo, China). Half kilogram of the medicinal substances were immersed in 5 l distilled water for 1 h, and then boiled for 1 h. After filtering, the supernatant was collected and 4 l distilled water was added in the residue and boiled again for 45 min. The same procedure was repeated once and the supernatants were put together, filtered and concentrated to a volume equivalent to 5 g/ml raw material per milliliter, then stored at 4 °C until use.

2.2. Animals

Male Sprague–Dawley (SD) rats were obtained from National Beijing Animal Center and were housed individually in metabolism cages with a 12 h light/dark cycle (light 600–1800 h), temperature at 23 ± 2 °C, 50 ± 10% relative humidity [15]. Food and tap water were provided ad libitum. Before treatment, the rats were acclimatized for 3 d in standard group rat laboratory cages and for 2 d in metabolism cages. Body weights were recorded daily. Animal handling and husbandry were conducted in accordance with guidelines from A Guide for Care and Use of Laboratory Animals [16].

2.3. Drug administration and sample collection

Rats ($n = 10$) were allocated randomly to treatment group and control group. 400 g/kg of Huang-yao-zi was administrated to treatment group ($n = 5$) by oral treatment. The action of each animal was observed daily to detect the toxicity symptoms. Urine samples were collected in glass bottle containing 1 ml 0.1% sodium azide (Alfa Aesar, Lancashire, England) over ice packs for the 24 h pre-dose, post-dosing 0–24 h, 24–48 h and 48–72 h. Urine samples were processed by centrifuging at 3000 rpm for 10 min and the supernatants were collected and stored at −20 °C until analysis.

After 72 h treatment, all animals were sacrificed and blood was drawn from inferior vena cava. Blood samples were allowed to clot and serum was obtained by centrifugation at 14,000 rpm for 10 min. All serum were sterile and kept at −80 °C prior to biochemical assays.

Livers were collected immediately after blood was drawn and the tissues were washed with saline buffer. Livers were weighed to determine the organ coefficients (organ coefficient = organ weight/terminal body weight × 100%).

2.4. Biochemical assay and histopathology

Biochemical evaluation of liver function was determined by measuring the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by SABA-18 auto-biochemistry analyzer (Analyzer Medical System, Roma, Italy).

A portion of liver tissue was immersed in 10% neutral buffered formaldehyde solution (Sigma, St. Louis, MO, USA), the tissues were dehydrated, embedded in paraffin, cut at 4 μm thickness and stained with hematoxylin and eosin (H + E) (Genmed, Boston, USA) for histopathological examination [17].

2.5. ^{1}H NMR spectroscopic analysis

300 μl of urine sample was mixed with equal volume of phosphate buffer (pH 7.4, 0.2 M Na_2HPO_4/0.2 M NaH_2PO_4, Sigma, St. Louis, MO, USA), centrifuged at 14,000 rpm for 10 min to remove insoluble material. 500 μl of supernatants were placed in a 5 mm NMR tube containing 30 μl of sodium-3-trimethylsily-[2,2,3,3-$^2H_4$]-1-propionate (TSP, 0.1%, w/v, Alfa Aesar, Lancashire, England), which acted as internal standard reference (δ0.00) and 60 μl D_2O (CIL, Andover, USA) also transferred into the tube (internal lock signal).

NMR spectra were recorded on a JEOL ECA 400 NMR spectrometer (JEOL, Tokyo, Japan) operating at 400.13 MHz for ^{1}H for at 298 K. Urine samples operated using a ^{1}D NOESY. A new pulse sequence (Multiple Pulse Filed Gradient Echo, MPFGE) to suppress the large residual water resonance was used. In brief, MPFGE sequence had two selective RF pulses: P1 pulse was 90° to make water signal trans to y-axis; P2 pulse was 180° to produce echoes after each pulse using a z gradient pulse made a good water suppression. ^{1}H NMR spectra were acquired using the following parameters: spectra width 4800 Hz, 64 K complex data points, 64 transients, mix time 0.3 s, relaxation delay time 1.2 s, temperature 298 K. The data were zero-filled by a factor of 2 and the free induction decays (FIDs) were multiplied by an exponential weighting function equivalent to a line boarding of 0.2 Hz prior to Fourier transformation (FT). Through Delta software, the ^{1}H NMR spectra were manually phased; baseline was corrected and referenced to TSP (δ0.0). The chemical shift range δ0.0–10.0 of NMR spectra was reduced into 250 segments corresponding to 0.04 ppm per intervals by MestRe Nova 5.3.1 (Mestrelab Research S.L., Santiago de Compostela, Spain). The regions δ4.60–5.20 were removed to prevent variation in water signal. The regions contributed to urea (δ5.64–5.96) were combined into single peaks (δ5.78) in order to avoid pH related peak shifts. Also, the citrate (δ2.76–2.66 and δ2.58–2.52) was combined into two single peaks (δ2.72, δ2.56) for that all the regions were scaled to the total integrated area of the spectra to reduce any significant concentration differences. The data were transferred into .xsl formation (Microsoft Excel 2003, Microsoft, Redmond, WA, USA) and the integrated data were normalized to the total integrals of each spectrum for pattern recognition analysis [18].

2.6. Pattern recognition analysis

^{1}H NMR spectra data were analyzed using SIMCA-P11.5 software package (Umetris AB, Umea, Sweden) for multivariate statistical analysis, all the variables were mean-centered and Pareto-scaled prior to PLS-DA.

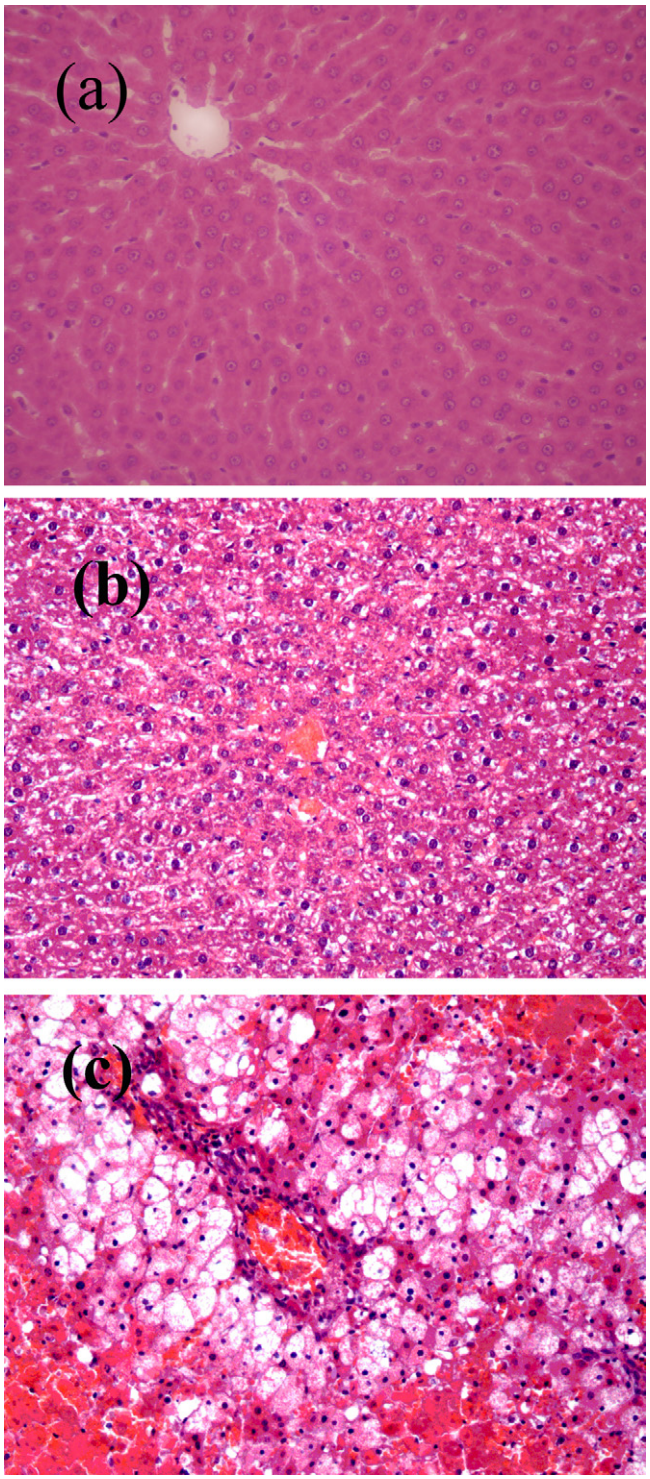

Fig. 1. Hepatic histopathology of rats insult by Huang-yao-zi. (a) Representative control group of hepar showed normal condition after 72 h distilled water treatment to rats. (b) Representative control renal group showed normal condition after 72 h distilled water treatment to rats. (c) Showed dose treatment induced severe diffuse hepatocyte degeneration, apoptosis and necrosis. *All the histopathologic results were expressed based on a light microscopic examination.

2.7. Statistical analysis

All the statistical analyses for body weight, organ coefficients and serum parameters were performed using the Statistical Package for Social Science program (SPSS 11.5, SPSS, Chicago). $P < 0.05$ from Student's t-test was considered the statistical significance of apparent difference between treatment and control group.

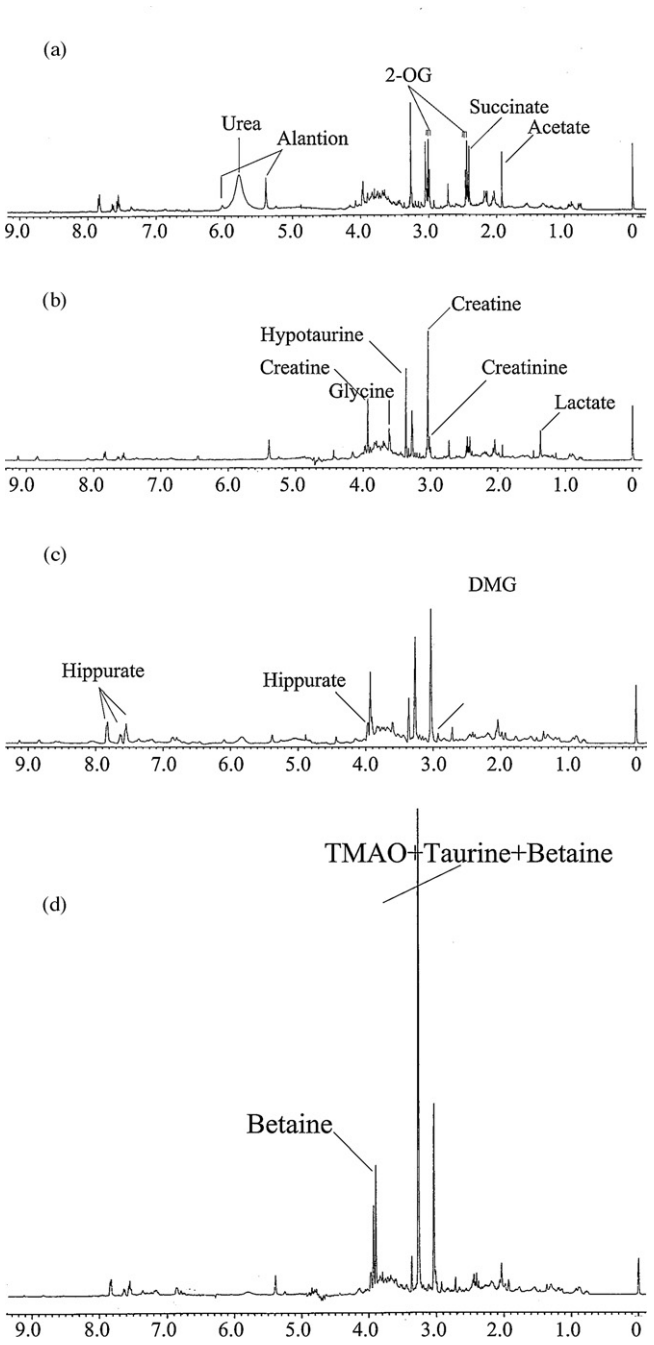

Fig. 2. ^{1}H NMR spectra δ0.0–9.0 at 400 MHz of urine from SD rats control, 0–24 h, 24–48 h and 48–72 h. (a) Control, (b) 0–24 h, (c) 24–48 h, and (d) 48–72 h.

3. Results and discussion

3.1. Action of animals

Throughout the observation period, Huang-yao-zi administrated rats became thin; yellow and clutter in furs; apathetic and burnout in spirit.

3.2. Weight and organ/body coefficients

In toxic evaluation, we determined the impact of toxicant to the organ according to the extent of weight difference from normal

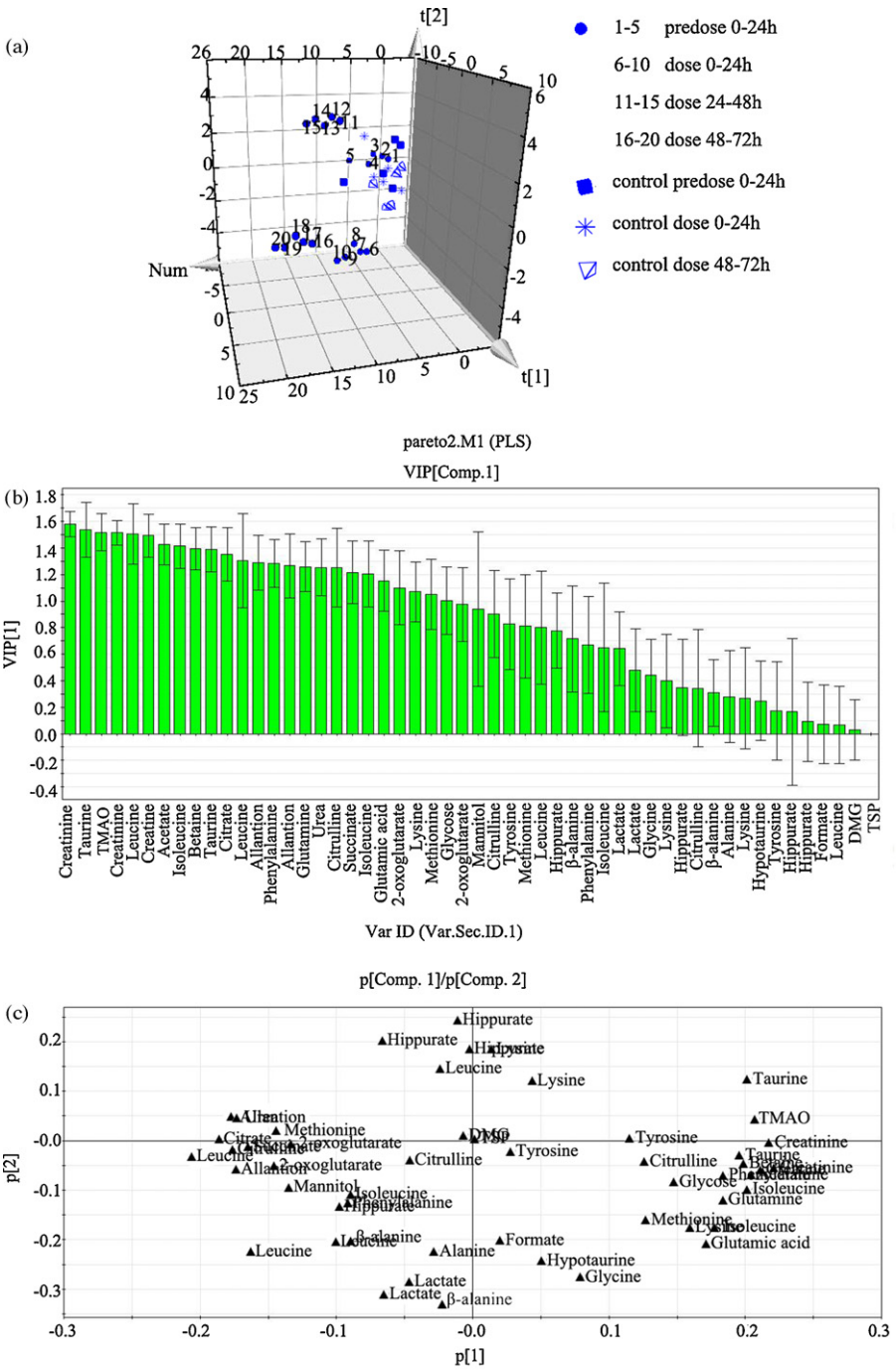

Fig. 3. PLS-DA analysis of ^{1}H NMR spectra of urine. (a) 3D scores plot ($R^2X = 0.542$, $R^2Y = 0.895$, $Q^2 = 0.981$), (b) variable important plot (VIP) and (c) P1 *vs* P2 loading plot.

[19]. Compared to control group, Huang-yao-zi has an apparent inhibitory effect on the body weight, and the organ coefficients of liver and kidney were significantly increased for the rats exposed to Huang-yao-zi (Table 1).

3.3. Biochemical parameters

To investigate whether Huang-yao-zi induces acute hepatic injury, serum levels of AST and ALT were measured. The AST (1127.8 ± 62.1 U/l, mean $\pm$ S.D., $n = 5$) was significantly elevated in the treatment group and the increased ALT (408.2 ± 21.7 U/l) was observed with P values less than 0.01 compared to control animals (AST: 83.5 ± 8.1 U/l, ALT: 39.6 ± 2.8 U/l). Elevated ALT and AST indicated that treatment of rats with Huang-yao-zi increase the hepatotoxicity. It is worth to mention that the activity of AST was less than 100 U/l, and ALT was less than 50 U/l in control rats, which both are within the range of normal values [20].

3.4. Histopathological observations

Histopathology of livers exposed to Huang Yao-zi for 72 h was examined. As demonstrated in Fig. 1, the main lesion showed diffuse hepatocyte degeneration, apoptosis, and necrosis (Fig. 1b). Significant swelling (ballooning degeneration) of hepatocytes was also observed. Hepatic sinusoidal and portal areas vascular were remarkably congested (Fig. 1c). These data implied that severe

Table 2
^{1}H NMR spectral data chemical shifts and changes of selective metabolites detected in rats urine.

Metabolites	^{1}H chemical shifts (δ) and multiplicity[a]	Levels of metabolites changes (0–72 h)
Acetate	1.93s	↑0–72 h
Succinate	2.43s	↓0–72 h
2-Oxoglutarate	2.47t, 3.01t	↓0–72 h
Citrate	2.72d, 2.56d	↓0–72 h
DMG	2.91s	↑0–72 h
Creatine	3.04s, 3.92s	↑0–72 h
Betaine	3.27t, 3.90s	↑0–72 h
Taurine	3.26t, 3.43t	↑0–72 h
Glycine	3.56d	↑0–72 h
Hippurate	3.97d,	↓0–48 h
	7.64t, 7.55t	↑48–72 h
Urea	5.78s	↓0–72 h

[a] S: singlet, d: doublet, dd: didoublet, t: triplet, q: quartet, and m: multiplet.

liver damage was induced by Huang Yao-zi. Taken together, both histopathological observations and elevated ALT/AST activities indicate liver toxicity occurred when rats were exposed to 400 g/kg Huang Yao-zi for 72 h.

3.5. ^{1}H NMR analysis of urine samples

^{1}H NMR spectra analysis was performed on urine samples for both control and treated samples which represent physiological states and pathological conditions. The representative ^{1}H NMR spectra of control and treated urine samples are shown in Fig. 2. Prior to biomarker identification and characterization, the raw data were mean-centered and pareto-scaled [21]. PLS-DA was employed for classification or discrimination analyses because PLS-DA calculation yielded a significant three-component model and was carried out on the ^{1}H NMR spectra to determine the distinct degree of biological variation. PLS-DA analysis showed distinct clusters and the samples clustered according to the control or treated samples and the clustering of dose group on pre0–24 h was positioned among control group, indicating dose treatment to the rats induced a substantial and characteristic change in the NMR profiles. The complete separation of two clusters between control and treated samples in three-dimensional scores plot (Fig. 3a) of the PLS-DA model is consistent with blood chemistry data in terms of elevation of ALT and AST, which were observed in treated samples but not in controls. Regardless, the experimental protocol used was unable to obtain clinical chemistry and histopathology between 0 and 48 h, but the data from ^{1}H NMR urinary spectra were able to evaluate metabolic parameters in the time interval. Variable importance parameters (VIP) plot (Fig. 3b) was used to identify the endogenous metabolites according to the orders of their contributions to the separation of clustering. Loadings plot (Fig. 3c) predicates the list of endogenous metabolites helping positioning the distance from different groups. Based on VIP and corresponding loadings plot, 11 endogenous metabolites were identified and were selected as the biomarkers. They are citrate (2.72d, 2.56d), 2-oxoglutarate (2.47t, 3.01), succinate (2.43s), acetate (1.92s), urea (5.78s), taurine (3.26t, 3.43t), creatine (3.04s, 3.92s), betaine (3.27t, 3.90s), DMG (2.91s), glycine (3.56d), and hippurate (3.97d, 7.64t, 7.55t).

3.6. Biomarkers selection and observation

NMR urinary analysis was used to detect abnormal metabolism, in the organism, the changes of endogenous metabolites that reflect the state of biological systems more accurately. Biomarkers serving the determination of exposure to harmful substances provide a platform for monitoring a toxicant, and to quantitatively assess harmful effects of a toxicant to organism. Data was collected from the ^{1}H NMR spectra of rat urine during the experiment period (0–72 h). As toxins can damage more than one target part and biomarkers are rarely totally specific, a single biomarker, in general, is rarely enough, so 14 endogenous metabolites were selected as biomarkers from VIP and loading plots together to get a complete picture. Table 2 lists the chemical shifts and changes of such endogenous metabolites from rat urine. With a careful analysis of these metabolites, we found many altered metabolites involved in mitochondrial functions. For instance, the levels of citrate, 2-oxoglutarate and succinate, the intermediate products of tricarboxylic acid cycle (TCA), were decreased in the 72 h treatment group. Decreasing of citrate, 2-oxoglutarate and succinate implies impaired energy metabolism in mitochondria. TCA cycle is a significant biological metabolic pathway in the body; it not only involves glucose aerobic oxidation, but it also involves the major pathways for fat and amino acid metabolisms, so that the inhibition of such cycle could cause organ degeneration. Acetate is the end product of fat metabolism and the increased level of it in urine demonstrated disorder of energy metabolism. The increase of glycine in 0–72 h also suggest the injury of hepatic mitochondria, which causes suppressed dynamic glycine cleavage system and thus glycine cannot generate N^5,N^{10}-methylene tetrahydrofolic acid. When the liver is damaged, the production of urea decreases resulting in very low level of urea in the urine [22]. Our data evidenced by the inhibition of ornithine cycle and the production of urea (Fig. 2), indicate that the damage may have possibly occurred in mitochondria because mitochondria serves as the ornithine delivery medium and is the site for citrulline synthesis. Previous publication has shown that elevated taurine in response to the depletion of glutathione is caused by oxidative hepatic damage [23,24]. The lack of ATP inhibits hippurate synthesis, which is dependent on the supply of ATP. Taken together; these changes due to the treatment of Huang Yao-zi imply hepatic mitochondrial injury [25]. Further study is needed for eliciting detailed mechanism of Huang Yao-zi induced mitochondrial dysfunction.

4. Conclusions

Evaluating the toxicity of natural products is a complex scientific problem. Toxic evaluations have been focused on acute, sub-acute, and long-term toxicity tests, and the indicators tend to be histological and biochemical changes. Although the contribution of these approaches to the phenomenon and characteristics of toxicity has been recognized, it has not clarified the changing discipline of toxic material basis. For instance, the influence and regular pattern of endogenous and exogenous metabolites from the body biochemical process induced by natural products on the toxicity were not clear. Therefore, it is not sufficient to only measure the toxicity in a single organ or tissue. The biofluids, such as urine reflect the changes in a variety of cells, tissues and organs, thus measuring changes in biofluids could serve as surrogates for risk assessment.

In addition, the potential mechanism of toxicity can be elucidated from the perspective of metabolites.

In our study, we investigated the hepatotoxic effects of Huang-yao-zi using metabonomic approach and by measuring the metabolic changes in urine and we found numerous endogenous molecules altered by the treatment of Huang-yao-zi. With molecular function analysis of these changed metabolites, it is apparently involved in the toxic mechanism of the mitochondrial dysfunction. Our study may provide evidence that ^{1}H NMR technique-based metabonomics is a powerful tool to investigate the toxicological effects of natural products, to elucidate toxic mechanisms, and to identify novel biomarkers.

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