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Deciphering the Differential Toxic Responses of *Radix aconiti lateralis praeparata* in Healthy and Hydrocortisone-Pretreated Rats Based on Serum Metabolic Profiles

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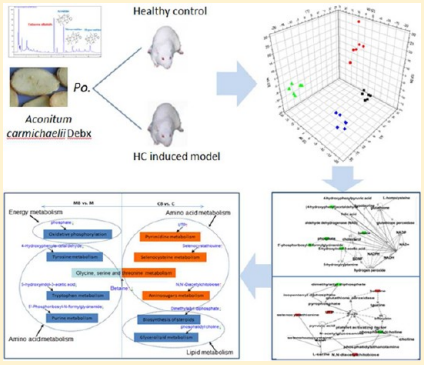
Supporting Information

ABSTRACT: *Radix aconiti lateralis praeparata* (Baifupian) has received great attention because of its excellent therapeutic effects as well as the associated adverse drug reactions. According to the traditional Chinese medicine (TCM) principle, Baifupian should only be used in patients with TCM "*kidney-yang*" deficiency pattern, a clinical state that can be mimicked by hydrocortisone induction in rats. This study aimed to decipher the differential toxic responses of Baifupian in healthy and hydrocortisone-pretreated rats based on serum metabolic profiles. Drug-treated rats received Baifupian intragastrically at the dose of 1.28 g/kg/day for 15 days. Serum metabolic profiles were obtained by using the LC-Q-TOF-MS technique. Our results show that Baifupian could induce severe toxicity in the heart, liver, and kidneys of healthy rats. These drug-induced toxic reactions were largely alleviated in hydrocortisone-pretreated animals. Changes of metabolic profiles in drug-treated healthy and hydrocortisone-pretreated rats were demonstrated, involving oxidative phosphorylation, amino acid and lipid metabolism as characterized by altered phosphate, betaine, and phosphatidyl choline. These metabolic alterations could be responsible at least in part for the differential toxic responses of Baifupian under various health conditions. This study provides a new paradigm for better understanding of the risks and limitations when using potentially toxic herbs in clinical applications.



KEYWORDS: *Baifupian (Radix aconiti lateralis praeparata), hydrocortisone pretreatment, TCM "kidney-yang" deficiency pattern, toxic responses, metabolic profiles*

INTRODUCTION

Use of herbal medicines has currently become more common worldwide not only due to their proven clinical efficacy and general nontoxic nature but also because of the comparatively good tonifying property in the human body and low cost when compared with conventional drugs.[1,2] However, there had been occasionally a few reports on the adverse reactions associated with herbal consumption.[3,4] *Radix aconiti lateralis praeparata* (*Zhi-Fuzi*) has been extensively used in various traditional Chinese medicine (TCM) decoctions based on its superb therapeutic value. *Zhi-Fuzi* is the processed product of the daughter or lateral roots of *Aconitum carmichaelii* Debx. (Figure 1A) that possesses beneficial effects in the treatment of various diseases such as rheumatic fever, painful joints, etc.[5] It must be emphasized that only processed *Fuzi* is allowed to be taken orally.[6] On the basis of the processing methods, there are three forms of commonly used *Zhi-Fuzi*: salted daughter root (*Yanfuzi*, Figure 1B), black slices (Heishunpian, Figure 1C), and white slices (Baifupian, Figure 1D). Among these, Baifupian has been most commonly used in clinics and was therefore tested in the present study. Although traditional processing procedures can largely reduce the toxic effects of herbal drugs,[5,6] there are still clinical cases of *Zhi-Fuzi* poisoning being reported in China and other parts of the world.[7,8] Despite the known toxicity of the herbs, lack of knowledge on the underlying toxicological mechanisms remains a major obstacle in the rational clinical applications of herbal medicines.[9,10]

According to the principle of TCM, a herb should only be used in patients with specific TCM pattern based on their

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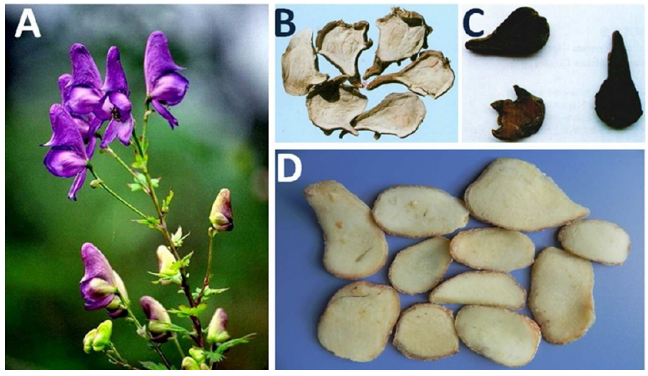

Figure 1. Pictures of the plant and different forms of *Zhi-Fuzi* for decoction. (A) plant of *Aconitum carmichaelii* Debx. (B) Salted daughter root (*Yanfuzi*). (C) Black slices (Heishunpian). (D) White slices (Baifupian).

differential body characteristics and conditions.[11] Baifupian is commonly used to treat patients with TCM "*kidney-yang*" deficiency pattern, characterized by the intolerance of cold temperature, faint respiration, icy extremities, diarrhea with undigested food, weak pulse, etc.[12−15] We hypothesize that the toxic reactions of Baifupian could be different in healthy subjects when compared to those being observed in individuals having the TCM *kidney-yang* deficiency pattern. Previous studies indicated that the TCM *kidney-yang* deficiency pattern is mainly characterized by the functional disorders in the hypothalamic-pituitary axis (involving the adrenals, thyroid, and gonads). Hydrocortisone can be used to induce a pathophysiological condition in experimental animals that mimics the TCM *kidney-yang* deficiency.[16,17] In this study, the TCM *kidney-yang* deficiency animal model was established by injecting a high dose of hydrocortisone in rats.[18]

In the elucidation of the potential toxicological mechanisms of Baifupian in rats, use of conventional research techniques such as histological and biochemical analyses may have certain disadvantages because: [1] multiple targets might be involved in its general toxic reactions;[19] [2] the herb contains multiple chemical components, such as aconitine (Supporting Information, Figure 1A), mesaconitine (Supporting Information, Figure 1B), and hypaconitine (Supporting Information, Figure 1C);[20] [3] there are biological variations in the absorption, distribution, metabolism, and excretion (ADME) of Baifupian;[21] and [4] there is an existing polymorphism of drug metabolism enzymes.[22] Alternatively, the metabolic profiling strategy enables us to identify the varying metabolites and related metabolic pathways in the complex regulatory network by monitoring many endogenous low-molecular-weight metabolites using liquid chromatography/mass spectrometry (LC−MS), followed by a combination of multivariate statistical techniques and pattern recognition techniques, such as principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA).[23−25] Metabolomics has brought enormous opportunities for improved detection of toxicity and biomarker discovery.[26] In particular, highly sensitive and specific biomarkers in biological fluids (serum, urine, and so on) are very useful for a comprehensive study of the efficacy and/or toxicity of raw and processed herbs.[27] In the present study, we compared the toxic reactions of Baifupian in healthy and hydrocortisone-pretreated rats and aimed to investigate their differential metabolic profiles. This metabolomic approach by using the liquid chromatography quadruple time-of-flight mass spectrometry (LC-Q-TOF-MS) technique could help us unveil the mechanism of adverse responses of Baifupian under different physiological states and facilitate a safer drug administration rationale in clinical practice.

MATERIALS AND METHODS

Chemicals and Reagents

Hydrocortisone was purchased from Tianjin Biochemistry Pharmaceutical Company (Tianjin, China). LC−MS grade acetonitrile was purchased from Honeywell Burdick and Jackson (MI, U.S.A). Mass spectroscopic grade formic acid was purchased from Fluka (Buchs, Switzerland). Formic acid (spectroscopic grade), leucine enkephalin (spectroscopic grade), and all chemical standards were purchased from Sigma-Aldrich (MO, U.S.A) unless specified otherwise.

Preparation of the Ethanol Extract of Baifupian

Baifupian (Cat no. 081117) was purchased from Yanjing Drug Store (Beijing, China) and authenticated by a specialist in pharmacognosy. Powdered Baifupian (50 g) was extracted with 75% ethanol (600 mL for 3 times) under thermal reflux for 1.5 h. After filtration, the ethanol extract was concentrated under reduced pressure. The resulting residue was dissolved in 0.5% sodium carboxyl methyl cellulose to give an extract with the concentration of 2 g/mL (expressed as the weight of raw materials). We had performed a quality control test on the Baifupian ethanol extract using high-performance liquid chromatography (HPLC) and AAS-ICP and found no trace of heavy metals, organic solvents, or other contaminants.

Animal Model

A total of 48 male Sprague−Dawley (SD) rats (230 ± 20 g, license no. SCXK 2009−004) were obtained from the Experimental Animal Center of Beijing Capital University of Medical Sciences (China). They were reared under standard laboratory conditions. The TCM *kidney-yang* deficiency condition was induced by intraperitoneal (ip) injection of hydrocortisone at a dose of 10 mg/kg of body weight once daily for 15 days.[18] The use of this high dose of hydrocortisone intervention has been proven to put animals into a state of "hyperfunction", facilitating a series of metabolic changes such as activated hypothalamic monoamine transmitters and accelerated energy metabolism. The resulting "overconsumption" of the energetic and immune systems of the animals could lead to a state of "exhaustion" as evidenced by the signs of fatigue, weight loss, and reduced activity. These pathophysiologic conditions mimic the state of the TCM *kidney-yang* deficiency syndromes, which make the hydrocortisone induction animal model a widely accepted method.[12−14] Experimental groups were established as follows: [C] healthy control rats, [CB] healthy rats with administration of Baifupian, [M] hydrocortisone-pretreated rats, and [MB] hydrocortisone-pretreated rats with administration of Baifupian. All animal experiments were performed under the Prevention of Cruelty to Animals Act (1986) of China and the NIH Guidelines for Care and Use of Laboratory Animals (U.S.A) and had also obtained approval by the Animal Ethics Committee of the China Academy of Chinese Medical Sciences under the project "TCM disease syndrome classification research" (date of approval: June 18, 2010).

Baifupian Administration and Sample Collection/Preparation

Rats in the CB and MB treatment groups were administrated orally by gavage with Baifupian extract at the dose of 1.28 g/kg of body weight once daily for 15 days. The dosage being used in mice is equivalent to the clinically relevant human adult dose based on an established formula for human−mice drug conversion.[28]

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Rats in the C and M groups received an equal volume of the vehicle orally. Whole blood was collected from the abdominal vein of the rats on day 15 and centrifuged at 3500*g* for 15 min after standing for two hours at 4 °C. The serum was then transferred into new tubes and stored at −80 °C for further analysis. A portion of the collected serum was used for routine laboratory analysis of urea nitrogen (BUN), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) according to the manufacturer's instructions of respective commercial test kits. Another portion of 100 μL of serum was added to 200 μL of acetonitrile, and the mixture was vortexed for 30 s. After centrifugation at 9560*g* for 10 min at 4 °C, the supernatant was stored at −80 °C for LC−MS analysis. All experimental rats were sacrificed following blood collection. Fresh cardiac, hepatic, and renal tissues were obtained and fixed in 10% neutral buffered formaldehyde at 4 °C for paraffin embedment. Organ samples (4 μm) were sectioned and stained with H&E.

LC-Q-TOF-MS Analysis

The use of high and ultrahigh resolution mass analyzers (e.g., time-of-flight, TOF) is capable of obtaining accurate mass measurements for the determination of elemental compositions of metabolites and to carry out tentative identification based on metabolites databases (such as the KEGG Pathway Database). Combining this technique with conventional MS/MS will provide useful additional structural information for the identification of metabolites. The rapid, sensitive performance and versatility of LC-Q-TOF-MS accelerates drug discovery and development,[29] including the screening and active mechanism research of herbal drugs.[30]

In this study, LC-Q-TOF-MS analysis was performed by using an Agilent-1200 LC system coupled with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) and an Agilent-6520 Q-TOF mass spectrometry. Separation of all samples was performed on an Eclipse plus C18 column (1.8 μm, 3.6 mm × 100 mm, Agilent) with a column temperature set to 45 °C. The flow rate was 0.25 mL/min, and the mobile phase consisted of ultrapure water with 0.1% formic acid and acetonitrile. The following gradient program was used: 2% acetonitrile for 0−1.5 min; 2−100% acetonitrile for 1.5−13 min; washed with 100% acetonitrile for 13−16 min; re-equilibration step for 5 min. The sample injection volume was 5 μL.

Mass detection was operated in both positive and negative ion modes with the following setting: drying gas (N2) flow rate, 8 L/min; gas temperature, 330 °C; pressure of nebulizer gas, 35 psig; Vcap, 4000 V; fragmentor, 160 V; skimmer, 65 V; scan range, m/z 80−1000. All analyses were acquired using the instrument mass spray to ensure accuracy and reproducibility. Leucine enkephalin was used as the instrument reference mass (m/z 556.2771) at a concentration of 50 fmol/μL with the flow rate 40 μL/min. The MS/MS analysis was acquired in targeted MS/MS mode with collision energy from 10 to 40 V.

Sequence Analysis

The pooled QC sample was analyzed at the beginning, the end, and randomly through the analytical run to monitor the stability of sequence analysis. The typical batch sequence of serum samples consisted of the consecutive analysis of 1 QC serum sample (at the beginning of the study), followed by 6 unknown serum samples and 1 QC serum sample, before running another 6 unknown serum samples, etc. Meanwhile, samples were analyzed in a random order for a normal good practice. An identical sequence was repeated to complete the total set of injections (n = 29, including QCs) analyzed in less than 1 day per mode.[31,32]

Data Processing and Statistical Analysis

The LC−MS raw data were exported by Agilent Mass Hunter Qualitative Analysis Software (Agilent Technologies, Palo Alto, CA, USA). The data of each sample were normalized to the total area to correct for the MS response shift between injections due to any possible intra- and interday variations. The sum of the ion peak areas within each sample was normalized to 10 000. Partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares (OPLS) were used for metabolite profile analysis. Multivariate analysis was performed by the SIMCA-P version 11 software (Umetrics AB, Umeå, Sweden). The data obtained show a normal distribution. In all cases, two-way ANOVA, the least significant difference (LSD) test, and the independent sample t-test were used for comparison between multiple groups and the two groups, respectively. $P < 0.05$ was considered as statistically significant.

IPA Analysis

Ingenuity pathway analysis (IPA, www.ingenuity.com) was performed based on database sources including KEGG (http://www.genome.jp/kegg) and METLIN (http://metlin.scripps.edu) to identify the affected metabolic pathways.

RESULTS

Main Constituents of the Baifupian Extract

HPLC analysis of the ethanol extract of Baifupian indicates that the three major constituents are aconitine (0.0169 mg/g), mesaconitine (0.5056 mg/g), and hypaconitine (0.0253 mg/g), respectively (Supporting Information, Figure 2). The Baifupian herbal extract also includes a collection of alkaloids that share a common C19-norditerpenoid skeleton, which are responsible for both its therapeutic and toxic properties.[33]

Identification of Biochemical and Histopathological Changes

The serum levels of CK (representing the severity of myocardial injury), ALT (representing hepatic damage), as well as BUN and CRE (representing the severity of renal damage) were all significantly elevated in healthy rats following Baifupian treatments (CB vs C). In contrast, there was generally no significant difference being detected between levels of these pathological biomarkers in complementary groups of hydrocortisone-pretreated animals (MB vs M). In the Baifupian-treated rats, all the above detrimental biochemical changes were ameliorated significantly when hydrocortisone was pretreated (MB vsCB), with a drastic drop of CK and AST levels (Table 1). The histopathological changes of the heart, liver, and kidneys were further examined in rats. Among these, severe morphological damages were shown in the heart (Figure 2) with inflammatory infiltration, edema, and rupture of the cardiomyocytes being observed in Baifupian-treated (CB) rats (Figure 2B). On the other hand, the histopathological damages in MB rats (with hydrocortisone pretreatment) were relatively mild (Figure 2D). These results demonstrate that Baifupian extract would induce more severe adverse reactions manifested as internal organ injury in healthy rats when compared to those in animals acquired with the TCM *kidney-yang* deficiency pattern.

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Table 1. Effects on Biochemical Parameters in the Serum of Healthy and Hydrocortisone-Pretreated Rats with or without Administration of Baifupian (mean ± SD, n = 12)[a,b,c,d]

group	CK (U/L)	LDH (U/L)	AST (U/L)	ALT (U/L)	BUN (mmol/L)	CRE (mmol/L)
C	202.74 ± 35.80	197.14 ± 49.50	212.38 ± 32.04	57.63 ± 6.91	5.26 ± 1.43	41.75 ± 1.39
CB	302.10 ± 81.29$^{###}$	242.55 ± 63.02	248.25 ± 61.78	74.75 ± 14.65$^{##}$	6.34 ± 0.98$^{#}$	46.63 ± 4.50$^{##}$
M	236.14 ± 45.20	133.21 ± 74.81	183.38 ± 37.24	56.13 ± 8.11	6.63 ± 0.95	44.25 ± 4.67
MB	214.41 ± 38.71*	226.02 ± 97.84†	167.71 ± 38.46**	67.29 ± 17.26	6.34 ± 1.61	43.14 ± 3.80

[a]Note: All serum samples were collected from the rats at the end of the experiments. [b]CB vs C. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$. [c]MB vs CB: $^{*}p < 0.05$, $^{**}p < 0.01$. [d]MB vs M: $^{†}p < 0.05$.

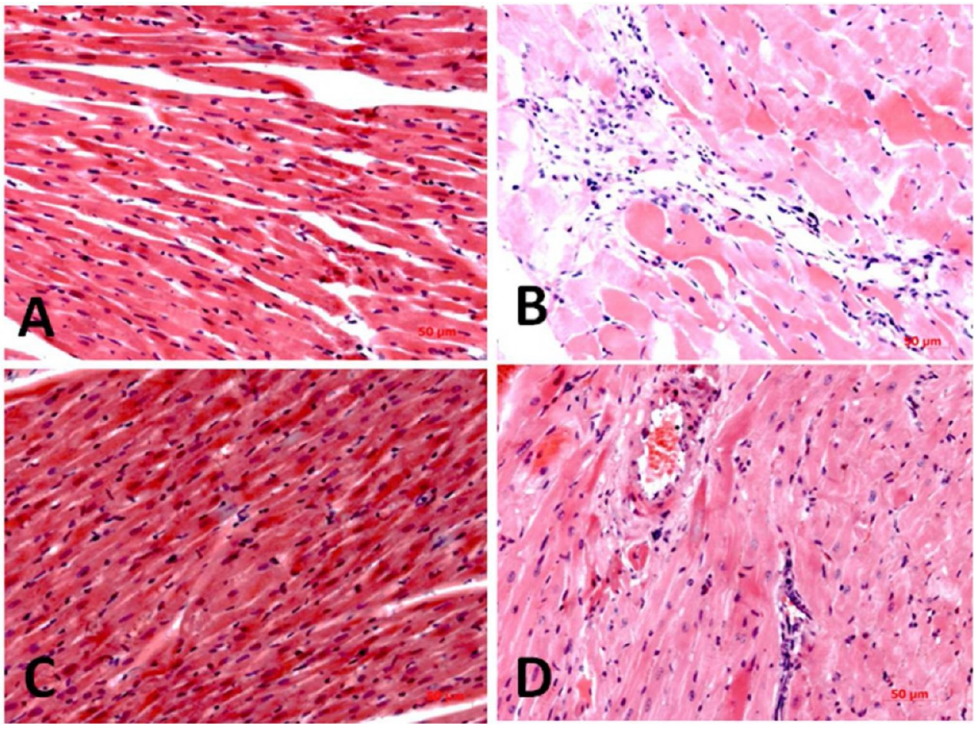

Figure 2. Heart histopathology, H & E staining, 200×. (A) Healthy control [C]: myocardial fibers in longitudinal section and normal the central nuclei and the syncytial arrangement of the fibers. (B) Healthy control exposed to Baifupian [CB]: myocardial fibers with losing cross striations and the nuclei not clearly visible, inflammatory infiltration. (C) Hydrocortisone-induced model control [M]. (D) Model control exposed to Baifupian [MB]: the histopathological changes were milder than in part B.

Assessment of the Repeatability and Stability of the LC-Q-TOF-MS Method

Extracts from six aliquots of a random blood sample were continuously injected to evaluate the repeatability. Five common extracted ion chromatograms (EICs) shared by these injections were selected according to their different chemical polarities and m/z values. The relative standard derivations (RSDs) of these peaks were 4.34−14.21% for peak areas and 0.03−0.99% for retention times.

The LC−MS system stability for the large-scale sample analysis was demonstrated by the test of pooled QC samples. The principal components analysis (PCA) result shows the QC samples are tight clustered. Moreover, peak areas, retention times, and mass accuracies of five selected EICs in five QC samples also showed good system stability. RSDs of the five peaks were 5.14−13.89% for peak areas, 0.03−1.04% for retention times, and 0.13 × 10^{-04}%−0.88 × 10^{-04}% for mass accuracies. The result indicated the large-scale sample analysis had hardly any effect on the reliability of data.

Examination of MS Spectra and Identification of the Differential Metabolites

Typical total ion current (TIC) chromatograms of serum samples were obtained from both healthy and hydrocortisone-pretreated rats, whether or not treated with Baifupian (Supporting Information, Figure 3). The top 200 significant ions were selected for metabolite identification. A total of 42 metabolites were identified from the serum samples, while 18 metabolomic metabolites were found to be most significant among the groups (Table 2). On the basis of the metabolic changes in M and MB rats (rats with hydrocortisone pretreatment) as revealed by TIC chromatography, we adopted the multiple pattern recognition methods PLS-DA (Figure 3) and OPLS (Figure 4). These approaches facilitate classification of the metabolic phenotypes and enable us to further identify the differential metabolites. Score plots from PLS-DA have shown obvious separation between the C and M (effect of hydrocortisone pretreatment), C and CB, as well as M and MB (effects of Baifupian under healthy or TCM *kidney-yang* deficient condition) groups of rats as illustrated in Figure 3. The separation of the groups could be achieved with the model parameters R^2Y = 0.958 and Q^2 = 0.665. Q^2Y obtained from cross-validation procedure represents the predictive accuracy of the model, and R^2Y shows how well the model fits to the data. These parameters indicate that the two models can accurately describe the data. Moreover, the results from permutation tests have shown that the two models are not

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Table 2. Identified Differential Metabolites in the Serum of Healthy and Hydrocortisone-Pretreated Rats with or without Administration of Baifupian[a]

n	t_R (min)	extract mass	formula	ID	compound	M vs C	MB vs M	CB vs C	pathway
1	2.2232	97.9769	H3O4P	C00009	phosphate	↑	↓		oxidative phosphorylation
2	2.1455	117.0790	C5H11NO2	C00719	betaine	↑	↓	↑	glycine, serine, and threonine metabolism
3	6.7978	136.0524	C8H8O2	C03765	4-hydroxyphenyl acetaldehyde	↑	↓		tyrosine metabolism
4	5.3171	191.0582	C10H9NO3	C05635	5-hydroxyindol-3-acetic acid	↑	↓		tryptophan metabolism
5	6.4323	219.1107	C9H17NO5	C00864	D-pantothenic acid	↑			pantothenate and CoA biosynthesis
6	1.6536	226.1066	C9H14N4O3	C00386	carnosine	↑			alanine and aspartate metabolism
7	1.7229	240.1222	C10H16N4O3	C00884	homocarnosine	↑			arginine and proline metabolism
8	7.2862	314.0427	C16H10O7	C04376	5′-phosphoribosyl-*N*-formylglycinamide	↑	↓		purine metabolism
9	14.8504	382.2719	C22H38O5	C04741	prostaglandin E1	↑			arachidonic acid metabolism
10	12.8678	103.0997	C5H13NO	C00114	choline	↓			phospholipid metabolism
11	8.0597	139.9875	C2H5O5P	C00227	acetyl phosphate	↓			taurine and hypotaurine metabolism
12	14.7515	304.2412	C20H32O2	C00219	arachidonic acid	↓			arachidonic acid metabolism
13	10.6948	427.2934	C23H41NO6	C00639	PGF2α	↓			arachidonic acid metabolism
14	2.7716	246.0058	C5H12O7P2	C00235	dimethylallyl diphosphate			↓	biosynthesis of steroids
15	15.2393	483.9685	C9H15N2O15P3	C00075	UTP			↑	pyrimidine metabolism
16	7.1714	753.5309	C42H76NO8P	C00157	phosphatidyl choline			↓	glycerolipid metabolism
17	8.9805	424.1693	C16H28N2O11	C01674	*N*,*N*-diacetylchitobiose			↑	aminosugars metabolism
18	7.2601	270.0119	C7H14N2O4Se	C05699	selenocystathionine			↑	selenocysteine metabolism

[a]Note: ↑ shows up-regulated metabolite; ↓ shows down-regulated metabolite.

overfitting but rather reflect the metabolic changes incurred (intercepts: $R^2 = 0.878$, $Q^2 = -0.214$).

To fully differentiate between the metabolites in the M (hydrocortisone-pretreated) and C (healthy control) groups, OPLS was conducted. OPLS is an efficient method for identifying ions that contribute to the clustering of samples. It also helps to eliminate noncorrelated variations contained within spectra. As shown in Figure 4A, there was a distinct clustering between M and C groups. Q^2Y and R^2Y in the OPLS models indicate that the class prediction ability of all models was high and that there was an authentic difference between the two groups. The corresponding S-plot (Figure 4B) in turn shows the contribution of different variables for the differentiation between M and C groups. Each triangle in the S-plot represents an ion. Ions far away from the origin are potential biomarkers.

Among the 13 identified metabolites in the hydrocortisone-pretreated (M) rats, 9 were up-regulated, while the other 4 were down-regulated (Table 2). Alternatively, if Baifupian was treated [MB rats], 5 of the originally up-regulated metabolites (as in the M group) now became down-regulated. In addition, 6 identified metabolites were perturbed in healthy [C] rats after Baifupian administration. Among the 6 metabolites being modulated by Baifupian, only betaine was altered in both healthy [CB] and hydrocortisone-pretreated [MB] rats, of which there was an up-regulation in the former group and a down-regulation in the latter group.

Metabolic Pathway Analysis with IPA

To further understand the correlation between the candidate biomarkers, bioinformatics analyses were performed using the IPA software, leading to the identification of biological association networks. As shown in Figure 5, the network was built based on the 13 differentiated metabolites between the hydrocortisone-pretreated [M] and healthy control [C] rats. The established network function in hydrocortisone-pretreated rats includes energy production, amino acid metabolism, lipid metabolism, molecular transport, organismal injury, and abnormalities.

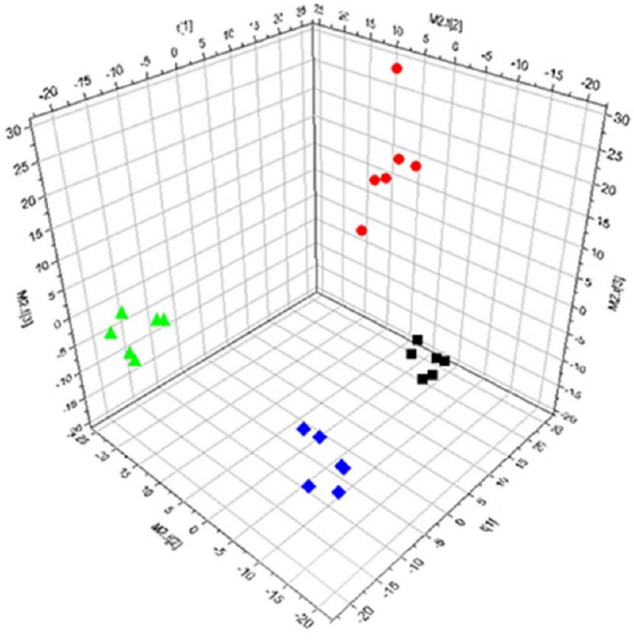

Figure 3. Results of multiple pattern recognition of serum metabolites impacted by different groups with or without exposure to Baifupian. PLS-DA score plot ($n = 6$, $R^2Y = 0.983$, $R^2X = 0.302$, $Q^2 = 0.744$). (blue ♦) Hydrocortisone-pretreated group. (green ▲) Healthy control group. (black ■) Healthy rats exposed to Baifupian. (red ●) Hydrocortisone-pretreated rats exposed to Baifupian.

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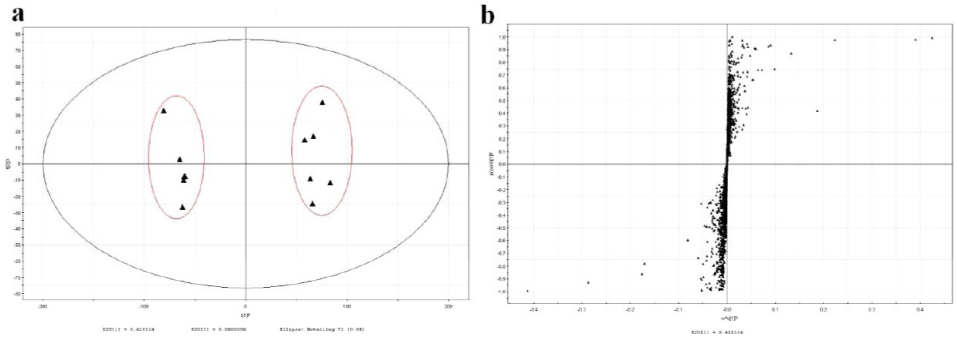

Figure 4. Results of multiple pattern recognition of serum biomarkers between the healthy control and hydrocortisone-pretreated group. (A) OPLS score plot (n = 6, R^2Y = 0.999, R^2X = 0.496, Q^2 = 0.967) of (left ▲) healthy control and (right ▲) hydrocortisone-pretreated group. (B) OPLS S-plot. Each triangle in the S-plot represents an ion. Ions far away from the origin were responsible for potential biomarkers.

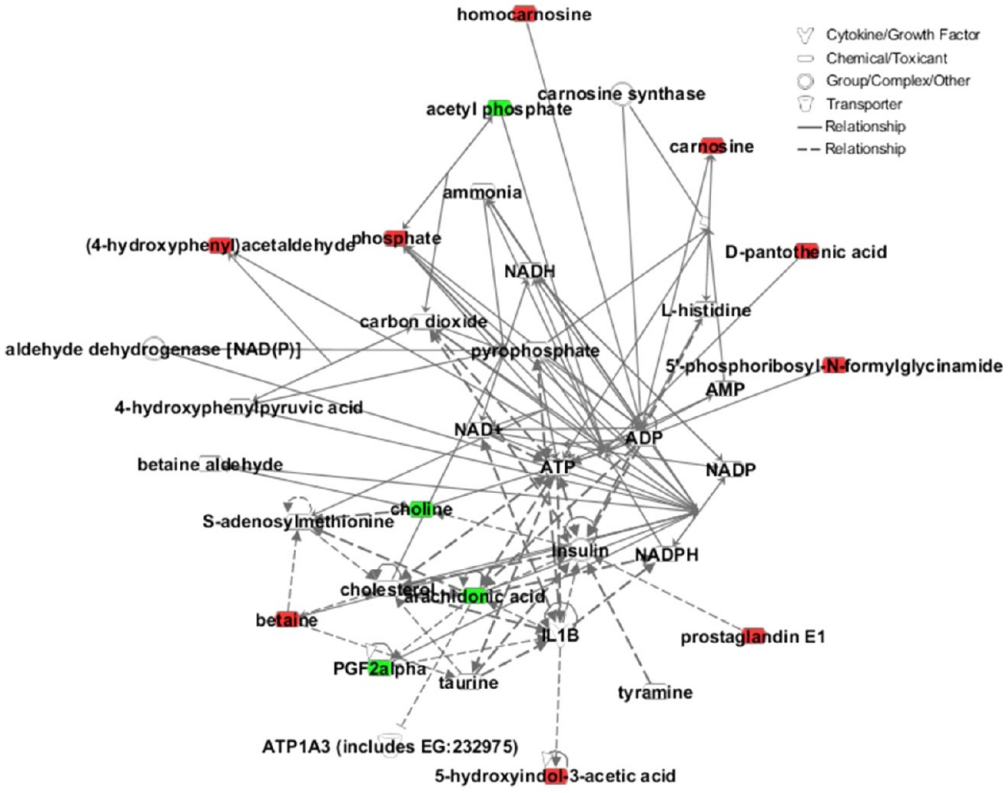

Figure 5. Hydrocortisone-perturbed molecular network. The network was gained by overlapping hydrocortisone-pretreated group's data to healthy group's data. Metabolites are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent metabolites that occur in the tested data, while the transparent entries are molecules from the Ingenuity Knowledge Database. Red symbols represent up-regulated metabolites; green symbols represent down-regulated metabolites. Solid lines between molecules indicate a direct physical relationship between molecules; dotted lines indicate indirect functional relationships.

Among these, the five top canonical pathways include glycine, serine, and threonine metabolism, tryptophan metabolism, taurine and hypotaurine metabolism, oxidative phosphorylation, as well as pantothenate and CoA biosynthesis.

By using a similar method, we have also mapped the metabolic network by means of five identified metabolites in MB rats when compared to those in rats without Baifupian treatment [M] (Figure 6). The established network functions of these metabolite changes following hydrocortisone induction include energy production, amino acid metabolism, cardiovascular disease, molecular transport, and free radical scavenging, while the five top canonical pathways are the protein ubiquitination pathway, oxidative phosphorylation, glycine, serine, and threonine metabolism, tryptophan metabolism, as well as purine metabolism, respectively. In the CB group of rats (when compared with healthy control rats in the C group), the established network was intervened with both up-regulated (betaine, uridine triphosphate (UTP), *N*,*N*-diacetylchitobiose, and selenocystathionine) and down-regulated (dimethylallyl diphosphate and phosphatidyl choline) metabolites (Figure 7). The established network functions include amino acid metabolism, lipid metabolism, small molecule biochemistry, and drug metabolism, whereas the top five canonical pathways are glycine, serine, and threonine metabolism, aminosugars metabolism, pyrimidine metabolism, purine metabolism, and biosynthesis of steroids (Figure 8).

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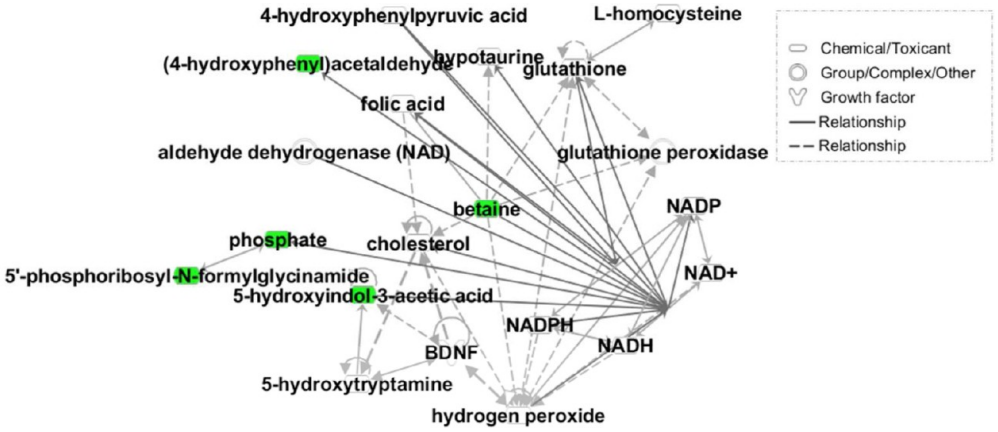

Figure 6. Molecular network of hydrocortisone-pretreated rats exposed to Baifupian. The network was overlapped by hydrocortisone-pretreated rats with or without exposure to Baifupian. Metabolites are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent metabolites that occur in our data, while the transparent entries are molecules from the Ingenuity Knowledge Database. Green symbols represent down-regulated metabolites. Solid lines between molecules indicate a direct physical relationship between molecules, and dotted lines indicate indirect functional relationships.

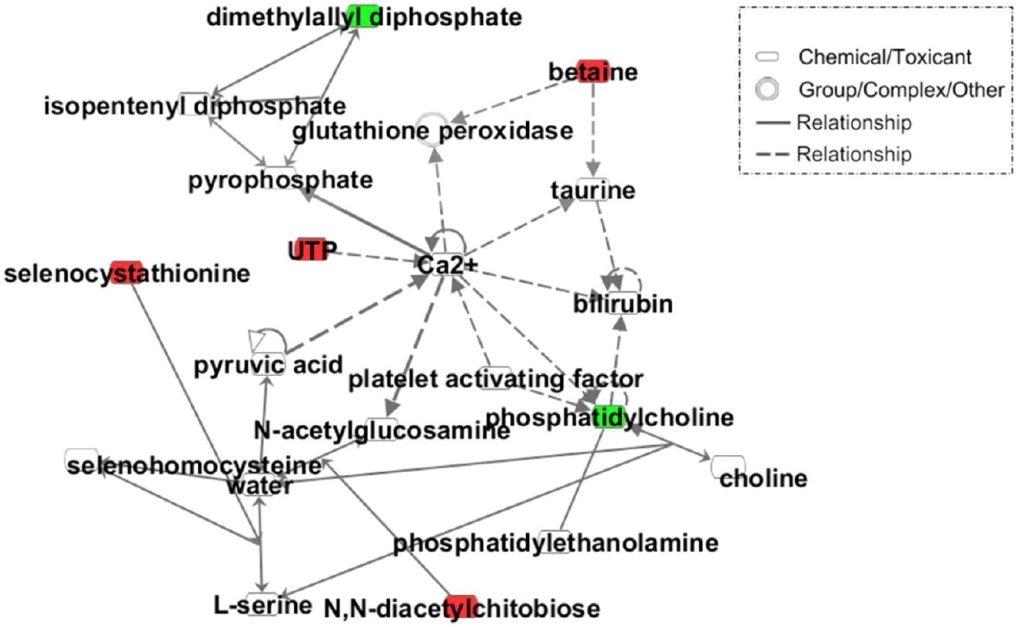

Figure 7. Molecular network of healthy rats exposed to Baifupian. The network was overlapped by healthy rats with or without exposure to Baifupian. Metabolites are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent metabolites that occur in our data, while the transparent entries are molecules from the Ingenuity Knowledge Database. Red symbols represent up-regulated metabolites; green symbols represent down-regulated metabolites. Solid lines between molecules indicate a direct physical relationship between molecules, and dotted lines indicate indirect functional relationships.

DISCUSSION

We are the first group to report that Baifupian administration induced differential toxic reactions in healthy and hydrocortisone-pretreated rats (with the TCM *kidney-yang* deficiency condition). The altered energy metabolism, amino acid metabolism, and lipid metabolism should be at least partly responsible for the systemic toxicity being brought forth by the herbal drug. This in fact confirms the use of Baifupian only in subjects with a particular body condition.

Zhi-Fuzi is commonly prescribed by TCM practitioners. Its clinical use was first recorded around 200 B.C. in *Shennong's Materia Medica* ("*Sheng Nong Ben Cao Jing*" in Chinese), one of the earliest Chinese *materia medica* classics. Contemporary published works have shown that *Zhi-Fuzi* is good at preventing congestive heart failure and portal hypertension.[34,35] Nevertheless, it has been suggested that the alkaloids in *Fuzi* are responsible for the toxicity in the heart, liver, and other vital organs.[36−41] In the present study, the differential toxic responses of Baifupian (most commonly used *Zhi-Fuzi*) in healthy and hydrocortisone-pretreated rats were investigated. The steroid hormone hydrocortisone plays a complex role in regulating diversified body functions. An unique pathophysiologic state can be established by injecting a high dose of hydrocortisone into rats, which consequently show signs of exhaustion such as weight loss, tendency to cluster with dropped appetite, reduced motor activity and response to external stimuli, cold limbs and back, painful waists and knees, tinnitus, impairment of hearing, and looseness of teeth.[12−15] All these body states resemble

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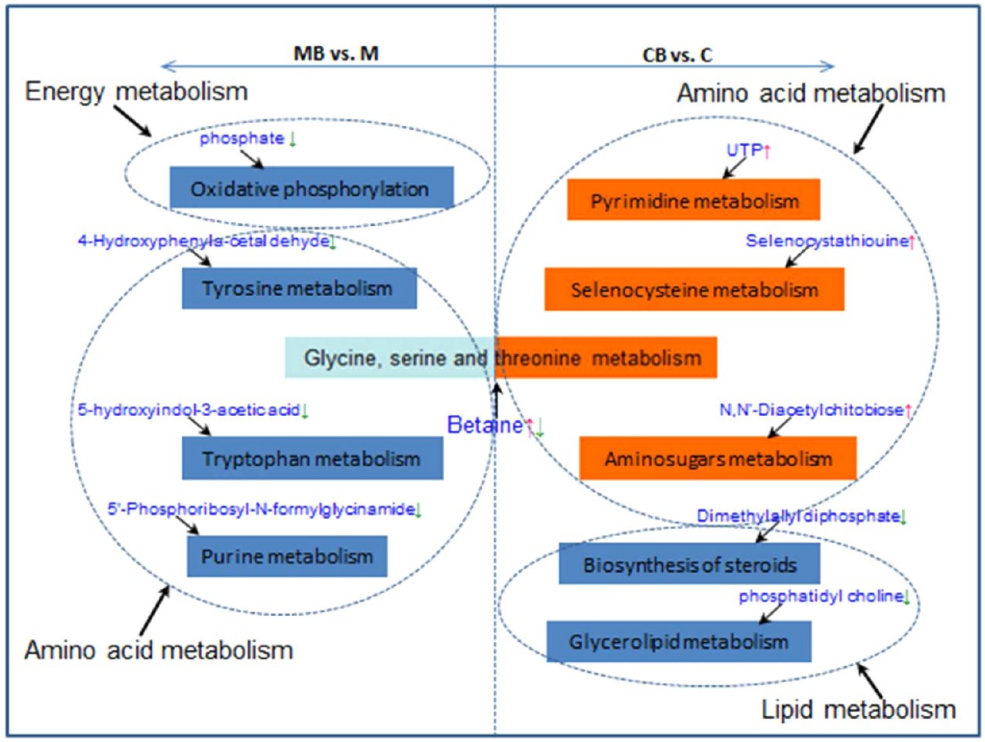

Figure 8. Different metabolites and corresponding pathways in hydrocortisone-pretreated rats or healthy rats with or without Baifupian administration. The green text box represents downregulated metabolic pathways, and the red text box represents upregulated metabolic pathways. "↑" and "↓" represent that the metabolite is up- or down-regulated. In hydrocortisone-pretreated rats with Baifupian administration [MB], oxidative phosphorylation, glycine, serine, and threonine metabolism, tyrosine metabolism, tryptophan metabolism, and purine metabolism were down-regulated when compared with the corresponding group without drug treatment [M]. In healthy rats with Baifupian administration [CB], glycine, serine, and threonine metabolism, pyrimidine metabolism, aminosugars metabolism, and selenocysteine metabolism were up-regulated; however, biosynthesis of steroids and glycerolipid metabolism were down-regulated, all being compared with the corresponding group without drug treatment [C].

TCM *kidney-yang* deficiency in humans.[16,17] Our histopathological and biochemical findings both indicate that Baifupian could lead to severe cardiac, hepatic, and renal damages in healthy control rats but exerted a comparatively mild detrimental effect in hydrocortisone-pretreated rats (with the TCM *kidney-yang* deficiency pattern). To further unveil the precise mechanisms of the differential toxic responses to Baifupian in healthy and hydrocortisone-pretreated rats, a metabolomics approach was employed to determine the metabolic profiles, whereas the metabolic networks and pathways involved had been analyzed.

Traditionally, Baifupian should only be used for treatment of patients with the TCM *kidney-yang* deficiency pattern.[16,17] Our findings using the hydrocortisone rat model have indicated that energy production would be the first most important network function being perturbed, such as the enhancement of oxidative phosphorylation by up-regulating phosphate. Oxidative phosphorylation is a metabolic pathway that involves oxidation of nutrients to produce adenosine triphosphate (ATP), a pervasive pathway that efficiently generates energy.[42] In addition, the up-regulated D-pantothenic acid as shown in our study further accelerates energy metabolism. Pantothenic acid participates in a wide array of key biological roles, which is essential to all forms of life.[43] It is particularly important in the synthesis of coenzyme A (CoA), an acyl group carrier that forms acetyl-CoA and other related compounds.[44,45] Other than oxidative phosphorylation and CoA biosynthesis, hydrocortisone-pretreated rats were also characterized by alteration of amino acid metabolism, with up-regulated glycine, serine, and threonine metabolism, tyrosine metabolism, tryptophan metabolism, alanine and aspartate metabolism, arginine, proline metabolism, purine metabolism, as well as down-regulated taurine and hypotaurine metabolism. These results are consistent with previous studies on the TCM *kidney-yang* deficiency pattern.[12,16] Besides, phospholipid and arachidonic acid metabolism was also perturbed in hydrocortisone-pretreated rats with decreased levels of choline and prostaglandin F2alpha (PGF2α). Choline, the basic constituent of lecithin being found in animal organs, is essential as a methyl donor in phospholipid metabolism; insufficient choline can cause bone abnormalities.[46] Through arachidonic acid conversion to active components such as PGF2α, the repair and growth of skeletal muscle tissue will be facilitated;[47] down-regulation of those active components may cause weight loss and body fatigue.[48,49] To summerize, the accelerated energy metabolism, down-regulated phospholipid metabolism, and perturbed amino acid metabolism all reflect the metabolic characteristics in the hydrocortisone-pretreated rats, a representation of the TCM *kidney-yang* deficiency pattern.

Most of the up-regulated metabolites in hydrocortisone-pretreated rats became down-regulated after Baifupian treatment, including phosphate, betaine, 4-hydroxyphenyl acetaldehyde, 5-hydroxyindol-3-acetic acid, and 5′-phosphoribosyl-*N*-formylglycinamide, which participate mainly in energy metabolism and amino acid metabolism. We have analyzed the metabolites and corresponding pathways that could lead to possible toxic response of Baifupian in hydrocortisone-pretreated rats. Disruption of oxidative phosphorylation attributed to down-regulated phosphate is linked to energy deficiency in the ischemic heart[50] and also influences calcium-activated cascades that result in arrhythmia.[51]

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Down-regulated 5-hydroxyindol-3-acetic acid is involved in tryptophan metabolism. An increased rate of tryptophan degradation and thereby lowered tryptophan level are associated with coronary heart disease,[52] whereas tryptophan depletion eventually affects pacemaker activity and thus heart rate stability.[53] Besides, purine metabolism that can be regulated by 5′-phosphoribosyl-*N*-formylglycinamide plays an important role in heart failure.[54] Cardiac ischemia-reperfusion could also produce remarkable reduction in the release of purine catabolites.[55] Purine metabolism in liver cells is also important in maintaining normal liver functions.[56] Despite this, glycine, serine, and threonine metabolism could be perturbed by betaine. A previous study indicated that the kinetics of glycine are substantially altered in severe cirrhosis,[57] while hepatomas are characterized by enzymic imbalance in serine metabolism[58] since a majority of the threonine oxidation occurs in the hepatocytes.[59] 4-Hydroxyphenyl acetaldehyde is involved with tyrosine metabolism, of which its increased metabolism could be related to nephrotoxicity,[60] since tyrosine in plasma is reduced substantially in chronic renal impairment.[61] It is remarkable that prolonged intervention by hydrocortisone is likely to result in a worsened body state in the experimental animals, involving physical changes of the immune system and associated organs as other investigators reported,[62] although the duration of our hydrocortisone-induced experiment was too short to demonstrate such changes. However, possible subsequent conditions such as diabetes and other cardiovascular disorders are expected to gradually develop, which can be reflected by the altered metabolites and associated pathways. Among these, Baifupian only caused down-regulation of the elevated parameters in the hydrocortisone-pretreated rats (MB vs M), while in healthy rats (CB vs C), most of these metabolites remained unaltered following Baifupian administration. The only concern should be about the up-regulated betaine level after drug treatment in healthy rats, which implicates a possibility that Baifupian may produce toxicity in healthy subjects through interference of glycine, serine, and threonine metabolism, a risk that is less essential in individuals who possess the TCM *kidney-yang* deficiency pattern.

Perturbed metabolites and altered metabolic pathways in healthy individuals after exposure to Baifupian could well explain the toxic responses of the drug being reported in recent years. As discussed earlier, Baifupain will down-regulate betaine levels in healthy rats. Betaine is an essential osmolyte and methyl group donor, and its metabolism links several metabolites that together play an important role in preserving normal cardiac functions.[63] Elevated plasma betaine promotes up-regulation of multiple macrophage scavenger receptors that are linked to an increased risk of secondary heart failure and acute myocardial infarction.[64] Besides, betaine might influence liver functions by perturbing glycine, serine, and threonine metabolism (as explained earlier),[57−59] while it also contributes to the osmoregulation of various renal cells.[65] Collectively, these toxic responses of Baifupian in the heart, liver, and kidneys of healthy individuals might be partially caused by the elevated betaine level. Other than betaine, dimethylallyl pyrophosphate was found to be down-regulated by Baifupian in healthy rats. This compound is a novel pain-producing molecule, which can enhance acute inflammation.[66] Down-regulation of dimethylallyl pyrophosphate in turn suggests an antinociceptive potential of the drug. UTP being up-regulated in Baifupian-administered healthy rats has the role as a body energy provider and substrates activator during metabolic reactions, and an elevated UTP level is commonly observed during myocardial infarction.[67] UTP also inhibits ATP-sensitive and voltage-dependent K^+ currents while having no effect on inwardly rectifying and Ca^{2+}-activated K^+ channels.[68] Aconitine in Baifupian could interact with the voltage-dependent sodium-ion channels.[39] Thus, up-regulated UTP might be involved in the potential cardiac toxicity being induced by Baifupian in healthy subjects. Alternatively, the major constituent of cell membranes, phosphatidylcholine, was down-regulated by Baifupian in healthy rats. Such down-regulation could contribute to fulminant and subacute hepatic failure.[69] In fact, cardiac toxicity induced by aconite (from other toxic plants such as *Aconitum* species) has been correlated with polyunsaturated fatty acid metabolic disorders,[70,71] and it is of interest to have further investigations on phosphatidylcholine as a potential target of Baifupian's toxicity. As an inhibitor of lysozyme *c*, *N*,*N*-diacetylchitobiose is capable of reducing the release of inflammatory mediators.[72] The anti-inflammatory activity of *Aconitum*, as shown in a previous study,[73] might be due to an increased *N*,*N*-diacetylchitobiose level. Taken together, the facilitation of glycine, serine, threonine, and pyrimidine metabolism as well as disruption of glycerolipid metabolism by Baifupian could be responsible for its toxic responses in healthy individuals. However, the beneficial antinociceptive and anti-inflammatory properties of the drug due to its alteration of the biosynthesis of steroids and aminosugar metabolism could explain why Baifupian is still actively used in many TCM formulations.

Our results demonstrated that Baifupian would induce more severe toxic reactions in the heart, liver, and kidneys in healthy rats than in hydrocortisone-induced rats. This phenomenon supports the TCM theory of "*You Gu Wu Yun*" (translated as "a toxic herb may exhibit maximal therapeutic effects when it is prescribed to patients with a complementary TCM pattern"). This theory had been established some 2000 years ago and is still regarded as one of the most important guidelines in contemporary TCM clinical practices when using toxic herbs. In fact, this report provides a basis for a better understanding and explanation of the *You Gu Wu Yun* principle in metabolic and molecular levels. If we attempt to compare this idea with modern pharmacological principles, we could quote the example of G-6-PD deficiency and malaria. It has been proposed that there is a low correlation between the degree of malarial endemicity and the frequency of G-6-PD deficiency.[74] This is because the malaria parasites are microaerophilic and sensitive to the state of oxidative stress, which is the condition of individuals acquired with G-6-PD deficiency. This in turn creates a higher degree resistance to malaria in certain tropical and southern Asia populations with the inherited trait of G-6-PD deficiency.[75,76] Indeed, a drug having differential toxicities in subjects with distinctive phenotypes (e.g., acetylator) is not uncommon in contemporary clinical practice.[77]

In conclusion, the differential toxic responses observed after Baifupian administration in healthy and hydrocortisone-induced rats had been verified in the present study. An altered metabolic profile involving oxidative phosphorylation, amino acid, and lipid metabolism as characterized by altered phosphate, betaine, and phosphatidyl choline may be associated with a differential toxic response profile. Results from this investigation provide a new paradigm for assessing the risks of potentially toxic herbs to facilitate their rational and safer clinical applications.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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▽These authors contributed equally to this work. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

TCM, traditional Chinese medicine; HPLC, high-performance liquid chromatography; LC−MS, high-pressure liquid chromatography combined mass spectrometry; LC-Q-TOF-MS, liquid chromatography quadruple time-of-flight mass spectrometry; EIC, extracted ion chromatograms; ESI, electrospray ionization; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; CK, creatine kinase; LDH, lactate dehydrogenase; PCA, principal components analysis; PLS-DA, partial least-squares discriminate analysis; OPLS, orthogonal partial least-squares; LSD, least significant difference test; IPA, ingenuity pathway analysis; ANOVA, analysis of variance; UTP, uridine triphosphate; PGF2α, prostaglandin F2alpha

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