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. However, there had been occasionally a few reports on the adverse reactions associated with herbal consumption. *Radix aconiti lateralis praeparata* (Zhi-Fu) has been extensively used in various traditional Chinese medicine (TCM) decoctions based on its superb therapeutic value. Zhi-Fu is the processed product of the daughter or lateral roots of *Aconitum carmichaelii* Del. (Figure 1A) that possesses beneficial effects in the treatment of various diseases such as rheumatic fever, painful joints, etc. It must be emphasized that only processed *Fuzi* is allowed to be taken orally. On the basis of the processing methods, there are three forms of commonly used Zhi-Fuzi: salted daughter root (*Yan-Fuzi*, 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, there are still clinical cases of Zhi-Fu poisoning being reported in China and other parts of the world. 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.

According to the principle of TCM, a herb should only be used in patients with specific TCM pattern based on their health status and treatment purposes. Without adequate caution, the toxicity of herbal medicines may occur in patients with TCM “kidney-yang” deficiency pattern, a clinical condition characterized by phosphate, betaine, and phosphatidyl choline metabolic changes. Our results indicate that Baifupian could induce toxicity in healthy rats and this toxicity was largely alleviated in hydrocortisone-pretreated animals. These differential toxic responses of Baifupian in healthy and hydrocortisone-pretreated rats suggest a new paradigm for the better understanding of the risks and limitations when using potentially toxic herbs in clinical applications.

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differential body characteristics and conditions.\textsuperscript{11} Baifupian is commonly used to treat patients with TCM "kidney-yang" deficiency, fever, palpitation, tachycardia, hypertension, steep pulse, etc.\textsuperscript{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.\textsuperscript{16,17} In this study, the TCM kidney-yang deficiency animal model was established by injecting a high dose of hydrocortisone in rats.\textsuperscript{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;\textsuperscript{19} [2] the herb contains multiple chemical components, such as aconitine (Supporting Information, Figure 1A), mesaconitine (Supporting Information, Figure 1B), and hyponaconitine (Supporting Information, Figure 1C);\textsuperscript{20} [3] there are biological variations in the absorption, distribution, metabolism, and excretion (ADME) of Baifupian;\textsuperscript{21} and [4] there is an existing polymorphism of drug metabolism enzymes.\textsuperscript{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).\textsuperscript{23–25} Metabolomics has brought enormous opportunities for improved detection of toxicity and biomarker discovery.\textsuperscript{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.\textsuperscript{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 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 material). 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{28}
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 3500g 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 9560g 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 embedding. 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, including the screening and active mechanism research of herbal drugs.

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, 10 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 and sprayed to ensure accuracy and reproducibility.

The typical batch sequence of sequence analysis. The batch consists of a total of 228 injections (n = 29, including QCs) analyzed in less than 1 day per mode.

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 inter-day 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) t-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 hyaconitine (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.

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 treatment (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 vs CB), 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 demonstrated that Baifupian extract would induce more severe reverse reactions manifested as internal organ injury in healthy rats when compared to those in animals acquired with the TCM kidney-yang deficiency pattern.
304 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 $R^2 = 0.958$ and $Q^2 = 0.665$. $Q^2$ obtained from cross-validation procedure represents the predictive accuracy of the model, and $R^2$ 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 significantly different.

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.

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

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

Note: All serum samples were collected from the rats at the end of the experiments. $^a$CB vs C. $^b$CB vs CB vs M. $^c$CB vs C. $^d$MB vs CB. $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$. $^\ddagger$MB vs C. $^\$MB vs M: $^p < 0.05$. $^\dagger$MB vs M: $^p < 0.05$.
overfitting but rather reflect the metabolite changes incurred 
(92.09, 0.17). 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 identi-
fying 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. Q²Y and R²Y 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 
later 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
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), \(N_2N\)-diacetylchitobiose, and seleno-cystathionine) 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).

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.
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. Nevertheless, it has been suggested that the alkaloids in Fuzi are responsible for the toxicity in the heart, liver, and other vital organs. 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 waist and knees, tinnitus, impairment of hearing, and looseness of teeth. All these body states resemble

![Figure 6](image1). 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](image2). 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.
Traditionally, Baifupian should only be used for treatment of patients with the TCM kidney-yang deficiency pattern. 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.

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. 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.

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These results are consistent with previous studies on the TCM kidney-yang deficiency pattern. 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. Through arachidonic acid conversion to active components such as PGF2α, the repair and growth of skeletal muscle tissue will be facilitated; down-regulation of those active components may cause weight loss and body fatigue. To summarize, 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.
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, whereas tryptophan depletion eventually affects pacemaker activity and thus heart rate stability. Besides, purine metabolism that can be regulated by 5′-phosphoribosyl- N-formylglycinamide plays an important role in heart failure.

Cardiac ischemia-reperfusion could also produce remarkable reduction in the release of purine catabolites. Purine metabolism in liver cells is also important in maintaining normal liver functions. 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, while hepatomas are characterized by enzymic imbalance in serine metabolism since a majority of the threonine oxidation occurs in the hepatocytes. 4-Hydroxyphenyl acetaldehyde is involved with tyrosine metabolism, of which its increased metabolism could be related to nephrotoxicity since tyrosin in plasma is reduced substantially in chronic renal impairment. 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, 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, Baifupian 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. 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. Besides, betaine might influence liver functions by perturbing glycine, serine, and threonine metabolism (as explained earlier).

While it also contributes to the osmoregulation of various renal cells. 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. 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. UTP also inhibits ATP-sensitive and voltage-dependent K⁺ currents while having no effect on inwardly rectifying and Ca2+-activated K⁺ channels.

Aconitine in Baifupian could interact with the voltage-dependent sodium-ion channels. 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.

In fact, cardiac toxicity induced by aconite (from other toxic plants such as Aconitum species) has been correlated with polynsaturated fatty acid metabolic disorders 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-diacylchitobiose is capable of reducing the release of inflammatory mediators. The anti-inflammatory activity of Aconitum, as shown in a previous study, might be due to an increased N,N-diacylchitobiose level. Taken together, the facilitation of glycine, serine, threonine, and pyrimidine metabolism as well as disruption of glycero lipid 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. 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 75,76 malaria in certain tropical and southern Asia populations with the inherited trait of G-6-PD deficiency. Indeed, a drug having differential toxicities in subjects with distinctive phenotypes, as discussed earlier, is not uncommon in contemporary clinical practice.

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.
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Notes

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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 F2α.

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