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Abstract

Liquid chromatography/mass spectrometry-based metabolomics analyses have been used in the evaluation of drug toxicity and finding detoxification methods. Yunnan Baiyao (YNBY) is a famous prescription for its effects on traumatic blood stasis in China. However, the side effects of YNBY including anaphylactic shock, arrhythmia and renal failure hindered its further development due to Aconitum kusnezoffii Radix. (caowu) in YNBY. The purpose of our research is to study the safety of YNBY compatibility of caowu for 4 weeks on SD rats by oral administration. In this study, we determined the toxic effects on rats by conducting examinations of histopathological and clinical physiological and biochemical indicators. Next, we prospectively analyzed known metabolites using an untargeted approach in serum of SD rats administrated with YNBY and NCY (caowu replaced by excipient from YNBY) respectively for four weeks by mass spectrometry analysis. As a result, we found caowu in YNBY had no toxicity on rats according to general toxicological evaluations. It was no obvious difference between administration and control groups on AKP, GOT, GPT, BUN, CK, MDA and LDH, except Na+/K+-ATPase (ATP) and SOD index. NCY had a greater influence on rats than YNBY according to metabolism. In this study, we found 77 biomarkers in YNBY and 110 biomarker of NCY, involved 12 and 20 metabolic pathways respectively. L-arginine, palmitic acid, oleic acid and stearic acid both in YNBY and NCY could participated in liver necrosis/cell death including apoptosis of hepatocytes, cell death of hepatocytes and lipoapoptosis of hepatocytes, which predicted by Ingenuity pathway Analysis (IPA). Of note, betaine produced by YNBY and cholic acid produced by NCY merely contributed to cell death of hepatocytes by prediction. Our findings based on metabolite profiles were that, caowu is safe existed in YNBY during four weeks. Caowu promoted the function of energy metabolism in heart and protecting the heart from oxygen free radicals. Betaine produced by YNBY and cholic acid produced by NCY may play a role in cell death of hepatocytes.

Introduction

Yunnan Baiyao (YNBY), a famous Chinese patent drug for its excellent efficacy clinically and special production process, formulated in 1902, has been designated as class one protected prescription by Chinese government. The major of traditional Chinese medicine are Panax notoginseng, Aconite kusnezoffii Radix. and so on according to the prescription compositions. The reputation of YNBY is characterized with the effects on hemostasis, promoting blood circulation, detumescence, analgesia and antibacterial [3] for more than one hundred years. Historically, YNBY made a great contribution to the Chinese soldiers in word II and later by the Viet Cong for control of hemorrhage. YNBY has been suggested that effect of hemostasis achieved by improving the release of alpha granules in plasma, facilitating platelet activation and wound sealing. In addition, YNBY is used for relieving the pain and inflammation caused by kinds of trauma even rheumatoid arthritis [4].

However, a few adverse drug reaction of anaphylactic shock, arrhythmia, renal failure and cardiac toxicity from YNBY in clinical limited its application and further development due to caowu included in recent years. Caowu, one ofaconitum herbs, has been widely applied to clinical for anti-inflammatory, cardiotonic, antipyretic and analgesic effects about 2000 years. Studies have shown that, intrathecal processed Aconitum jaluense showed antiallodynic activity on neuropathic pain through inhibiting P2X7R production and expression and decreasing microglial activation in the spinal cord. In addition, aconitum carmichaelii had the protective effect against acetaminophen
induced by hepatotoxicity was probably through inhibiting CYP2E1 activity, GSH depletion, and mitochondrial dysfunction. Of note, acoline extracted from the aconitum plant is both toxic and active ingredient [5–10]. The main chemical constituents of caowu are alkaloids including C20-, C19-, C18-diterpenoid alkaloids. Among them, C19-diterpenoid alkaloids are the most toxic on cardiovascular and nervous system, especially cardiotoxicity by inducing cardiovascular symptoms both atrial and ventricular dysrhythmias. Studies have shown that the toxicity of the diester alkaloid decreases to monoester and alcohol amine alkaloid. [11–15]. Clinically, caowu is usually processed to products [16,17] or used in combination with other drugs [18] to reduce its toxic side effects on the heart, liver, kidneys and nerves.

In the recent years, a wide range of metabolomic technologies as a systems biology approach are widely used in the modern research of TCM [19–25] with the most prevailing methods such as UPLC–MS with instrument analysis and data processing [26–33]. Metabolomics monitor the endogenous metabolites [34] of biofluid including serum, plasma [35], urine [36,37] and organism for evaluating the drug efficacy and safety, identifying new mechanisms [38] and targets of diseases development [39,40] and drugs treatment [41] via biomarker discovery and metabolic pathway research [42,43], discovering functional biomarkers for clinically accurate diagnosis and treatment. For TCM, UPLC–MS can characterize the fingerprint [44,45] and full-component analysis of single-Chinese medicines [46–53], formula compatibility [54–61] and absorbed bioactive components [62–67], which help us to understand the “black box” more clearly. On this basis, chinnmedomics [68–72] as a powerful approach integrating metabolomics with serum pharmacochemistry to evaluate the chinmedomics [68–72] as a powerful approach integrating metabolomics with serum pharmacochemistry to evaluate the role of caowu in YNB compatibility, we analyzed the survival status of rats after administrating with YNB and NCY by histopathological and clinical physiological and biochemical indicators respectively. In addition, we investigated the effects of YNB and NCY on serum metabolism of rats through non-target metabolomics techniques, so as to look for the effects of caowu in YNB and predict the toxicity by IPA.

Methods

Drugs and chemical reagents

YNBY pulvis and NCY pulvis were provided by Yunnan Baiyao Group Limited by Share Ltd. (Kunming, China). Carboxy methyl cellulose sodium (CMCNa) come from Kermel Chemical Reagent Co., Ltd (Tianjin, China). Acetonitrile and methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma-Aldrich (MO, USA). GOT, GPT, AKP, BUN, CK, ATP, LDH, SOD and MDA kits were charged from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and study designs

SD rats, 120 ± 10g, from Liaoning changsheng biotechnology co. Ltd. (Benxi, China), were housed in Heilongjiang Traditional Chinese Medicine University with SPF conditions. The center with a controlled environmental conditions: a 12:12 -h lighted–dark cycle with free access to food and water, a temperature with 24 ± 2 °C and the relative humidity with 55 ± 5%. All rats were randomly split into 3 groups of 12 rats each, control group, YNB group and NCY group, sex in half. The dose of medication administration teams was 205.8 mg/kg/day keeping feeding for four weeks, rats of control group were administered with 0.5% CMCNa solution according to body weight at the same time. The body weight of the rats and the fodder given to rats was recorded during the experiment. All experiments were performed in accordance with approved animal protocols and guidelines established by Heilongjiang University of Chinese Medicine.

Serum preparation

Blood of rats was collected from the hepatic portal vein with vacuum negative pressure tubes after the rats anaesthetized by injecting of 3% pentobarbital sodium in abdomen (0.3mL/100g body weight) when the treatment finished. Then, serum were obtained by means of centrifugation at 4000 rpm, 4 °C retaining 15min. The obtained serum was dispensed and frozen at -80 °C immediately. Frozen serum samples were placed into ice water for reconstitution and mixed by vortex mixer before use. We added 800μL methanol into 200μL serum sample and mixed by vortex mixer for 30s, then the suspensions centrifugated at 13,000 rpm, 4 °C retaining 15min for removing macromolecular compounds such proteins and extracting metabolites. After that, the supernate liquid acquired was transferred into a new tube and dried by nitrogen gas at 40 °C. The dried samples were added with 200μL methanol for ultrasonic dissolution and centrifuging at 13,000 rpm, 4 °C retaining 15min. The supernatant was filtrated by 0.22μm filter membrane before transferred into glass bottles for UPLC–MS analysis. In addition, 50μL of each serum sample was mixed as QC sample with the same method for processing. The QC sample contained entire information of all the samples that would be prepared to evaluate the stability of instrument and method. Analysis of a QC sample interspersed with every six sample using the same method.

Tissue weighting and histopathological examination

Extract the heart, liver, spleen, lungs and kidneys of rats immediately after anesthesia and blood collection. After rinsing the organs with physiological saline, the water was sucked with filter paper and weighed. About 200mg of myocardium was removed from the left ventricle of the heart and weighed accurate for the determination of heart tissue index. The remaining tissues were fixed by putting into formalin solution immediately for HE staining.
Biochemical assay test

Part of the collected serum was used to determine AKP, GOT, GPT, BUN and CK. Before the rats’ heart were fixed, approximately 200 mg of myocardium was removed from the left ventricle of the heart, added with 9 fold physiological saline, homogenized at a low temperature of 13,000rpm for 1 min. A part of the obtained homogenate was centrifuged at 2500rpm, 4°C retaining 10min, then the supernate was drawn for activity test of Na-K-ATPase assay. The remaining myocardial homogenate was centrifuged 5000rpm, 4°C for 20min, and the supernatant was collected for activity of SOD, MDA and LDH. SOD, MDA, BUN, CK and Na-K-ATPase were detected by UV spectrophotometer, while AKP, GOT, GPT and LDH were detected by microplate reader.

Chromatography and mass conditions

An ACQUITY UPLC system from Waters (Milford, MA, USA) combined with an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7μm, Waters Corporation, Milford, USA) were used for chromatographic separation. We used optimized conditions including the velocity was 0.4 mL min⁻¹, while the column temperature was 40°C, we adopt the optimal mobile phase containing 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The UPLC performed method for instrument analysis with a linear gradient elution procedure as follows: 0–2min, 1–32% A; 2–5min, 20–80% A; 5–10min, 80–99%; 10–11min, 99% A; 11–12min, 99–1%; 12–15.5min, 1% A.

ASYNAPT-G2-Si high definition mass spectrometer (HDMS) (Waters Milford, MA, USA) processed with a mass spectrometer system with an assembly of electrospray ionization source (ESI) were used for acquiring accurate mass data with ion voltages measured by MassLynx software on version 4.1 (Waters Milford, MA, USA). We adopted the MS parameters including an ion voltage including 3.0 KV in ESI⁺ and ESI⁻ mode, the flow rate of cone gas and desolvation gas were 50 L h⁻¹ and 800 L h⁻¹ respectively, the temperature of desolvation gas and ion sourcat at 350°C and 110°C respectively. The rate of MS data acquired was set at 0.2s per scan with a delay of 0.1s between scans from 50 to 1200Da in centroid mode. A lock mass of leucine enkephalin with a concentration of 4 ng μL⁻¹ was utilized for acquiring accurate mass data with a flow rate of 10μL min⁻¹ in ESI⁺ and ESI⁻ mode ( [M+H]⁺ = 556.2771, [M–H]⁻ = 554.2615).

Statistical analysis

The raw MS-data files acquired by UPLC-MS of serum samples from all rats were uploaded on to Progenesis QI software (Nonlinear Dynamics, version 2.0) for peak detection, normalization, visualization and multi-dimensional processing and analysis. The results of the above process were imported into Ezinfo 3.0 software for unsupervised principal components analysis (PCA) combined with orthogonal partial least square–discriminant analysis (OPLS-DA) and verified through analysis of variance (ANOVA) simultaneously. On this basis, we collected MS/MS information of metabolite peaks via the Mss FragmentTM (Waters corp., Milford, USA), ChemSpider (http://www.chemspider.com/) and Human Metabolism Database (HMDB) (http://www.hmdb.ca/) by chemically intelligent peak–matching algorithms for determining the compound structure preliminary. In addition, we combined KEGG (http://www.genome.jp/kegg) and MetPA (http://metpa.metabolomics.ca./MetPa/faces/Home.Jsp) for the analysis of biological pathways for determined the potential biomarkers meet the requirements of p-value of 0.05 or less by t-test and P-value threshold set at 1.0. All the identified biomarkers of YNBY and NCY were introduced to IPA (http://www.ingenuity.com) to focus on metabolic pathways that have been obtained resulting in more specific functional and disease-associated metabolic pathways. Simultaneously, IPA help us predict the toxic biomarkers and targets produced by drug.

Results

Effects of YNBY and NCY on weight and food intake in rats

Using weight and food intake assessment to evaluate the effects of YNBY and NCY. The body weight of YNBY and NCY group had little difference with the control group. There were no significant fluctuations in feed intake between the YNBY group and NCY group compared to the control group (Figures 1,2).

Effects of YNBY and NCY on histopathology and clinical biochemical index

There were no significant difference in the coefficient of heart, liver, spleen, lungs and kidneys both YNBY and NCY compared with control group (Figure 3). Cardiomyocytes are morphologically regular with clear horizontal stripes, a nuclear center, abundant interstitial capillaries and a small amount of connective tissue. Liver cells are polygonal, centered on the central vein and regularly arranged into a single row of plate-like structures with hepatic sinusoids between hepatocytes (Figure 4A). There were no significant difference both YNBY group and NCY group compared to control group in AKP, GOT,
GPT, BUN, CK, MDA and LDH index. The ATP index of YNBY group increased than control group with $P < 0.05$. Of note, there was no such result in NCY group. The SOD index of YNBY group and NCY increased than control group with $P < 0.01$. ATP and SOD indicators from cardiac tissue differ in groups (Figure 4B).

**Effects of YNBY and NCY on serum metabolic profiling**

In this study, we obtained the fingerprints (Figure 5A,B) of all the serum samples analyzed by UPLC-MS with the optimal conditions in positive and negative ionization modes. After QI software processing and analysis, we detected about 22631 peaks in positive ions and 24525 peaks in negative ions. The PLS-DA plots in positive ionization mode (Figure 5C) and negative ionization mode (Figure 5D) can reflect a clear distinction between YNBY and NCY group, which suggest that caowu made a great contribution to other medicines from YNBY by interfering body metabolism.

**Identification and relative intensity of metabolites influenced by YNBY and NCY**

VIP plots (Figure 5E,F) from PLS-DA help us analyze feature ions. The potential biomarkers met requirements used to pick out and identify chemical structure further. The candidate potential biomarkers used MassFragment™ application manager combined with ChemSpider to match the MSMS information carefully, for example, the compound of butyryl carnitine with chemical structure and cracked fragments information in positive ion mode (Figure 6). In the present study, we identified 77 potential biomarkers in serum of YNBY group, while 110 potential biomarkers in serum of NCY group.

The information of the identified results of the potential biomarkers of YNBY and NCY in ESI+ and ESI- mode in Table S1 and S2 including retention time, molecular weight, name and MSMS fragments, etc. The results of comparison of relative intensity of peaks after standardization of potential biomarkers produced by YNBY and NCY in figures 7,8 respectively.

**Metabolic pathways influenced by YNBY and NCY**

To gain insight to the role of caowu in YNBY, metabolic pathways of YNBY (Figure 9A) and NCY (Figure 9B) were constructed respectively by importing biomarkers identified to MetaboAnalyst website and the KEGG. It turned out that the most relevant pathways (impact value $> 0$) involved by YNBY mainly involved five classes of pathway for lipid, nucleotide, amino acid, carbohydrate metabolism and glycogen biosynthesis and metabolism. However, NCY group added metabolism of...
cofactors and vitamins than YNBY group for retinol metabolism and vitamin B6 metabolism due to the lack of caowu. Based on this results, we constructed the network of pathways produced by YNBY (Figure 9C) and NCY (Figure 9D), which indicate that caowu made a great importance to the integrity of metabolic network on rats for four weeks administration. The details comparison information of MetPA pathways produced by YNBY and NCY in Table S3. Then we known that the number of YNBY influenced the metabolic pathways was 10 and 20 metabolic pathways influenced by NCY was more than YNBY attached by 10 metabolic pathways, which indicated YNBY may be safer than NCY due to lower degree of influence (Figure 10).

In order to analysis the difference of the 53 common biomarkers form YNBY group and NCY group, a clustering heat map (Figure 11) was constructed. Based on the above, we analyzed 13 biomarkers from YNBY and NCY on ROC curve and quantitative analysis (Figure 12), the significant difference of these biomarkers included LysoPC (20:5(5Z,8Z,11Z,14Z,17Z)), oleic acid, 9,10,13-TriHOME, eicosenoic acid, PE(14:0/14:1(9Z)), PI(16:0/22:4(7Z,10Z,13Z,16Z)), PE(20:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), pelargonic acid, eicosadienoic acid, indoleacetaldehyde, N-acetylhistidine, PC(18:2(9Z,12Z)/P-18:1(11Z)), LysoPC(22:5(4Z,7Z,10Z,13Z,16Z)) between YNBY and NCY group indicate that caowu may act on other medicines of YNBY to influence the metabolism.

The canonical pathways and GO enrichment of biological processes of YNBY and NCY

In order to gain the most important message of canonical pathways, IPA software was used to help us analyze the network interactions between dysregulated biomarkers identified in metabolism analysis by IPA database. The analysis of canonical pathway showed that related three pathways in YNBY and four related pathways in NCY (Figure 13). The four pathways in YNBY comprised glycine degradation, stearate biosynthesis and salvage pathways of pyrimidine ribonucleotides and iNOS signaling. The three pathways in NCY comprised tryptophan degradation, FXR/RXR activation and stearate biosynthesis.

The biomarkers produced by YNBY including 1D-myoinositol 1,3,4,5,6-pentakisphosphate, 2-methoxyestriolone, betamuricholic acid, cytidine, deoxycoformycin, dihydrosphingosine 1-phosphate, glycolithocholic acid, pelargonic acid, pentadecanoic acid, prostaglandin E3, stearic acid participated in DNA replication, recombination and repair, nucleic acid metabolism, small molecule biochemistry (Figure 14A). The biomarkers produced by YNBY including 15:0/20:4(5Z,8Z,11Z,14Z) phosphatidylcholine, betaine, dihydrosphingosine 1-phosphate, elaidic acid, L-arginine, oleic acid, palmitic acid, stearic acid participated in cell death and survival, immunological disease, inflammatory disease (Figure 14B). The biomarkers produced by YNBY including betaine, myristic acid, S-adenosylhomocysteine, sn-glycero-3-phosphocholine, suberic acid, taurodeoxycholic acid, uridine participated in lipid metabolism, molecular transport, small molecule biochemistry (Figure 14C). SOD, AKT and ALT were the key predictors by YNBY from IPA results.

The biomarkers produced by NCY including corticosterone, dihydrosphingosine 1-phosphate, dinoprost, elaidic acid, indole, L-arginine, L-carnitine, linolenic acid, oleic acid, palmitic acid, phytanic acid, thromboxane B2, uric acid, 1-16:0 lysophosphatidylcholine, 1-16:1(9Z) lysophosphatidylcholine, 1-18:2(9Z,12Z) lysophosphatidylcholine, 1-oleoyl lysophosphatidylcholine, 13-hydroxyoctadecadienoic acid, 15:0/20:4(5Z,8Z,11Z,14Z) phosphatidylcholine, 16:0/20:4(5Z,8Z,11Z,14Z) phosphatidylcholine, dihydrosphingosine 1-phosphate, icosapent, L-alpha-lysophosphatidylcholine, stearoyl, linolenic acid, pyridoxamine, taurodeoxy-
cholic acid, tauroliothocholic acid, participated in lipid metabolism, molecular transport, small molecule biochemistry (Figure 14D,E). The biomarkers produced by NCY including beta–muricholic acid, butyrylcarnitine, cytidine, deoxyctydine, glycocholic acid, indole–3–acetaldehyde, myristic acid, omega–muricholic acid, pelargonic acid, pentadecanoic acid, retinaldehyde, thromboxane B2, tryptamine participated in cell death and survival, cellular compromise, nucleic acid metabolism (Figure 14F). The biomarkers produced by NCY including 6–epoxyeicosatrienoic acid, cholic acid, D–glucose, indole, L–tyrosine, phenylpyruvic acid, stearic acid participated in carbohydrate metabolism, energy production, small molecule biochemistry (Figure 14G). LDH, SOD and AKT were the key predictors by NCY from IPA results.

Toxic targets produced by YNBY and NCY predicted by IPA

In addition, IPA help us to further analyze detailed functional changes and predict system toxicity by YNBY and NCY on rats. L–arginine, palmitic acid, oleic acid and stearic acid both in YNBY and NCY could participated in liver necrosis/cell death including apoptosis of hepatocytes, cell death of hepatocytes and lipoapoptosis of hepatocytes, which predicted by IPA. Of note, betaine produced by YNBY and cholic acid produced by NCY merely contributed to Cell death of hepatocytes.

Discussion

YNBY is famous for exact curative effect of hemostasis, blood circulation, anti–inflammatory and analgesia clinically, even without the prescription compositions. However, caowu participated in YNBY impeded its applications due to its cardiotoxicity and neurotoxicity. Recent work has highlighted the safety and effectiveness of caowu on active ingredients, toxic components, effect target and mechanism. Yet, it largely has remained unknown what is the role of caowu in YNBY on the influence on other medicines from YNBY and what is the aftereffects if remove caowu from YNBY. Thus, we designed experiment to explore the difference with untargeted serum metabolism between YNBY and NCY on rats after four administration. Experimental results showed that YNBY and NCY had no effect on intake, body weight and organ coefficient included heart, liver, spleen, lung and kidney of rats, which indicated that, YNBY and NCY had no significant effect on the above indicators during the administration period. The compatibility of caowu in YNBY would not have toxic effect on rats during the experiment. This indicate and liver functions that it is safety for rats with YNBY for four weeks with clinical equivalent dose even participated with caowu.

In addition, we analyzed the effects of compatibility with caowu in YNBY on the liver, heart and kidney with clinical biochemical indicators and hematological detections. The serum biochemical index and hematological detections are widely applied to diagnosis of the diseases, evaluation of the prognosis and the access and monitor the body metabolic status. As far as we, aconitum plants have a great influence on the functions of heart, liver and kidneys as target organs. Thus, we detected the levels of ATP, SOD, MDA and LDH from myocardium and CK from serum, which represent the severity of myocardial injury [85]. The serum level of AKP, GOT and GPT applied to our research, represented the severity of liver
injury [86], due to several enzymes leak out into the blood from the cytosol of hepatocytes [87]. The level of BUN from serum represented the severity of renal damage by YNBY participated with caowu [88]. Cardiac toxicity of aconitum plants performed with severe arrhythmias and even leading to death [89], which could be caused by inhibiting ATP activity remarkably, then increase Na+ influx and the Ca2+ concentration in intracellular, which leading to damage of the heart tissue [90]. Several studies proved that ATP been known as a membrane protein plays an important role for the active transport of Na+ and K+ across over the cell membrane by converting the energy of ATP [85,89,92]. The present study revealed YNBY increased the activity of ATP in the heart significantly, which was lack of NCY groups. SOD plays a crucial role on the balance of oxidative and antioxidant function in the body by scavenging superoxide anion free radicals and protecting cells from damage. The present studies showed that both YNBY and NCY could increase the level of SOD significantly. The studies showed that caowu could assist other drugs in YNBY to enhance the protection effect of the heart function. However, there was no difference between YNBY and NCY. Histopathological results show that, heart and liver were not observed for lesions and injuries after administration with YNBY and NCY, which implying that they were safe for rats during the experiments.

Based on the above findings, the significance of YNBY compatibility of caowu was reserved. Therefore, we detected the all serum metabolites of rats influenced by YNBY and NCY based on UPLC–MS method. We found the degree of NCY disturbed the normal serum metabolic profiles of rats than YNBY greatly according to the score of PCA, the number of biomarkers, metabolic pathways, classes of metabolism.

Lipid metabolism. Phospholipids consisted of LysoPCs, PCs and PEs is a kind of important ingredients of the lipid bilayer of cells in biosphere, involving in various metabolisms and signaling process. In the present studies, we found 13 LysoPCs, 5 PCs, 4 PEs and 3 PIs were the major biomarkers expressed with significant difference between administration groups to control group by serum metabolism, which involved in lipid metabolism including glycerophospholipid, ether lipid, glycerolipid metabolisms and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. The contents of LysoPCs both in YNBY and NCY were increased than control group, especially the levels of LysoPC (20:5(5Z,8Z,11Z,14Z,17Z)), LysoPC (22:5(4Z,7Z,10Z,13Z,16Z)) and oleic acid in NCY were higher than YNBY significantly. Many studies have shown that blood status is associated with LysoPCs, linoleate and alphanolenoic acid height participated in lipid metabolism [93], which affected the expression of choline substances [94]. Phosphatidylcholine produced LysoPCs with the action of phospholipase A2 attaching the effects on regulating vascular tension and producing endothelial dysfunction. The mechanism of the procedure is the expression of nitric oxide synthase which means NOS decreased with the level of mRNA to produce vascular endothelium injury due to the expression of NO disturbed by LysoPC [95,96]. The mechanism is consistent with the canonical pathways expressed by IPA. In addition, studies proved that LysoPC performs on KB binding sites to reduce the transcriptional activity and promote the level of cAMP in mononuclear cells in order to regulate the production of tissue factors participating in thrombosis syndrome during precipitation [97]. SM(d18:0/18:1(n2)), sphingosine 1-phosphate and sphinganine 1-phosphate both produced by YNBY and NCY involved sphingolipid metabolism from lipid metabolism class and had effects of potential biological activity in the sphingolipid metabolism, calcium signal transduction procedure and neuroactive ligand-receptor interaction. Cholic acid, glycocholic acid, 7 alpha-hydroxy-3-oxo 4-cholestenoate, urocholic acid, 3a,6a,7b-trihydroxy-5b-cholanoic acid and lithocholyltaurine induced by NCY, chenodeoxycholic acid glycine conjugate and lithocholic acid glycine conjugate induced by YNBY, and 3a,6b,7b-Trihydroxy-5b-cholanoic acid from administration groups involved primary bile acid biosynthesis. The abnormal secretion of cholic acid in the NCY has brought hidden troubles on the normal metabolism of rats, which explains that the disorder of cholic acid affects the vitamin metabolism only occurs in NCY. In abnormal, bile acids as a kind of hepatotoxic substance circulating through the liver repeatedly have proved the potent toxic properties if sustained accumulation in blood and tissues result of membrane disruption. Bile acids accumulated in liver may damage liver and lead to hepatocyte apoptosis and deterioration of other severe liver diseases such as cholestatic [98]. The mechanisms of liver apoptosis was induced the transcriptional activity of AP-1 (activation protein-1) by lithocholyltaurine [99,100]. Chenodeoxycholic acid glycine conjugate from YNBY and lithocholyltaurine from NCY increased the risks of liver apoptosis by drugs, which is consistent with the results of IPA for toxic prediction. Estrone sulfate and 2-methoxyestrone produced from YNBY involved steroid hormone and biosynthesis. Mestosterone form two administration groups is a potential doping agent in equine sports by modulating the autonomic innervation of the vesical musculature and has the effects of decreasing the cholesterol in human serum [101,102]. Prostaglandin E1 and prostaglandin E3 increased only from YNBY, as the cycloxygenase metabolism of eicosapentaenoic acid, are potent endogenous vasodilator agent, which can promote peripheral blood flow by inhibiting platelet aggregation for achieving various vascular protective functions. There are several mechanisms of PGEs hepatic cytoprotection: inhibiting cytotoxicity induced by T-cell, enhancing DNA replication, recombination, and repair of the injured liver after partial hepatectomy by stimulating cyclic AMP function which is consistent with GO enrichment of biological processes predicted by IPA, promoting the level of ATP in liver to accelerate the recovery of mitochondrial respiratory function after reperfusion, and maintaining membrane microviscosity [103,104].

Amino acid metabolism. L-arginine used to create NO, creatine, glutamate and proline, even glucose and glycogen if necessary for body decreased in YNBY and NCY. L-arginine plays a important role in amino and energy metabolisms due to promote the secretion of insulin by stimulating skeletal muscle consuming glucose with the help of AMP kinase activated by L-arginine [105]. Betaine decreased in YNBY plays
an important role in protecting normal cardiac and kidneys functions from damage [105]. In addition, several studies showed that betaine had the effect of inhibiting the release of NO in activated microglial cells which may benefit to control neurological disorders [106,107]. In addition, researches have shown that it is associated with amino acid and lipid metabolism, which may have a role in cardiovascular [108-111]. Of note, the level of betaine increased may damage heart, liver, and kidneys of healthy individuals [112]. Thus, we confirmed that caowu promote the protective functions of rats through decreasing the level of betaine. The present study results indicated caowu participated in the YNBY would not have a toxic effect on heart.

Nucleotide metabolism. Cytidine increased and deoxycytidine decreased were included both in YNBY and NCY. A study proved that cytidine may have a effect on neurodegenerative diseases [113]. Simultaneously, uric acid increased only coming from NCY, is a heterocyclic purine derivative, which may lead to a type of arthritis known due to gout with excess accumulation. The pathways we obtained showed that cytidine effected uric acid and deoxycytidine at the same time, which involved purine and pyrimidine metabolism.

Energy Metabolism. L-acetylcarnitine decreased in YNBY and NCY, which may promote the process of acetyl-CoA into the mammalian mitochondria matrix during the fatty acids oxidation process. In addition, L-acetylcarnitine plays a role in neuroprotective, neuromodulatory, and neurotrophic properties for against numerous disease processes [114].

Conclusions

To our knowledge, this is the first study using metabolism approach for the comprehensive assessment of endogenous metabolites, which provide a method to solve the phenomenon and mechanism that clinical physiological and biochemical indicators and histopathology cannot. On the basis of intake, body weight and organ coefficient results, we can conclude that The compatibility of caowu in YNBY would not have toxic effects on rats during the four weeks experiment with clinical equivalent dose. In addition, 77 biomarkers produced by YNBY, which participated in 12 metabolic pathways, 110 biomarkers produced by NCY, which participated in 22 metabolic pathways, which indicates that YNBY may be safer than NCY due to lower degree of influence. In addition, 13 biomarkers from YNBY and NCY on ROC curve and quantitative analysis results suggest that caowu may act on other medicines of YNBY to influence the metabolism. The results of canonical pathways and GO enrichment of biological processes of YNBY and NCY illustrate that four pathways in YNBY comprised glycine degradation, stearate biosynthesis and salvage pathways of pyrimidine ribonucleotides and iNOS signaling, while three pathways in NCY comprised tryptophan degradation, FXR/RXR activation and stearate biosynthesis. Of note, rats administrated YNBY and NCY for longer may produce liver necrosis/cell death including apoptosis of hepatocytes, cell death of hepatocytes and lipoapoptosis of hepatocytes.

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