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Original Article

Study on the Intervention Effects of Pinggan Prescription (平肝方) on Spontaneously Hypertensive Rats Based on Metabonomic and Pharmacodynamic Methods*

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ABSTRACT **Objective**: To investigate the effects of Pinggan Prescription (平肝方, PGP) on hypertension by the associated methods of metabonomic and pharmacodynamic. **Methods**: A total of 32 male spontaneously hypertensive rats (SHRs) were randomly divided into two groups by using the random number table method: a treatment group (*n*=18) and a model group (*n*=14). The Wistar rats (*n*=14) were used as the normal group. Different prescription were used to intervene three groups: the treatment group in which PGP extract was administered orally at a dose of 18.336 g/kg (PGP/body weight), and the model group in which physiological saline was administered at the equivalent dose. The same treatment was applied to the normal group as the model group. The blood pressure was measured by tail-cuff method, and pharmacodynamic indexes including cyclic adenosine monophosphate (cAMP) and angiotensin Ⅱ (Ang Ⅱ) were tested by enzyme-linked immunosorbent assay. The plasma samples from three groups were detected by gas chromatography-mass spectrometry (GC-MS). **Results**: Compared with the model group, blood pressure of treatment group was obviously reduced after continuous curing with PGP (*P*<0.01). The pharmacodynamic results illustrated that the content of Ang Ⅱ increased with the raised blood pressure and the cAMP expressed the converse trend. After curing with PGP, the content of Ang Ⅱ decreased, the difference between model group and treatment group was significant (*P*<0.01), and the cAMP expressed the converse trend. Five potential biomarkers were identified, including arachidonic acid, hexadecanoic acid, elaidic acid, octadecanedioic acid and 9,12-octadecadienoic acid. These metabolites had shown significantly changes as followed: arachidonic acid, hexadecanoic acid and elaidic acid were significantly higher and octadecanedioic acid and 9,12-octadecadienoic acid were lowered in the model group than those in the normal group. After the treatment of PGP, the metabolites had the trends of returning to normal along with the reduced blood pressure. **Conclusions**: PGP intervention for hypertension played a major role in the metabolism of arachidonic acid and linoleic acid. Metabonomic with pharmacodynamic methods could be potentially powerful tools to investigate the mechanism of Chinese medicine.

KEYWORDS hypertension, Pinggan Prescription, Chinese medicine, metabonomic, pharmacodynamic

As a clinical syndrome, hypertension manifests arterial pathological change and various degrees of metabolic disorders. Some changes in the concentrations of the metabolites could be detected in blood and urine even when the blood pressure was well controlled. Therefore, these alterations may be associated with the disease itself.[1] Western medicine is still the main treatment for hypertension, while Chinese medicine (CM) also reflects good effect for the different mechanism. The efficacy evaluation of treatment for CM on hypertension is still a summary derived from clinical experience and lacks adequate scientific basis. Therefore, it is very important to set up the evaluation model for antihypertension in CM treatment, which was consistent with the characteristics of hypertension and could scientifically characterize their overall efficacy.[2]

Metabolomics is a speedy and sensitive method to characterize the overall functional status of organism in a variety stimulated of external factors. Thus it is also known as "biochemical phenotypes" which reflects the organisms overall functional

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status.[3,4] That is why metabolomics could express the outstanding advantages in the evaluation of CM prescription. In this study, the application of gas chromatography-mass spectrometry (GC-MS) metabolomics was combined with pharmacodynamics to explore the role of evaluation research in CM overall efficacy.

Pinggan Prescription (平肝方, PGP) was extracted based on the clinical experience of Prof. LI Yun-lun for treating hypertension. PGP had been widely used for hypertension in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine and expressed a good effect. Numerous pharmacodynamics studies[5,6] showed a closely relation between the blood pressure and the concentration of angiotensin Ⅱ (Ang Ⅱ) and cyclic adenosine monophosphate (cAMP). Meanwhile, the detailed analysis of metabolomic data with pattern recognition would reveal valuable information of essential hypertension (EH). However, the research in the correspondence between pharmacodynamics and metabolomics was rare. Therefore, the combined study was performed to analyze the intervention effects of PGP.

METHODS

Chemicals

N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), docosane, methylhydroxylamine hydrochloride and pyridine were of analytical grade and purchased from Fluka (Buchs, Switzerland). cAMP and Ang Ⅱ were the enzyme-linked immunosorbent assay (ELISA) and purchased from R&D SYSTEMS (R&D, USA)

Preparation of Ethanol Extracts of PGP

PGP was composed of *Coptidis Rhizoma* 60 g, *Ramulus Uncariae cum Uncis* 150 g, *Rhizoma Alismatis* 60 g and *Aloe vera* 5 g, which were all purchased from Jianlian Traditional Chinese Drug Store (Jinan, China) and identified by Prof. Zhou FQ (Shandong University of Traditional Chinese Medicine). Four herbs were weighed, powdered and mixed sufficiently. Then the mixture was extracted with 70% ethanol (600 mL, 3×) under thermal reflux for 1.5 h. After filtration, the ethanol extract was concentrated under reduced pressure. The residue was dissolved in water to give an extract with a concentration of 18.336 g crude drug/kg.

High Performance Liquid Chromatography Finger Print of PGP Extract

As the main herb of the PGP, *Coptidis Rhizoma* had significant reduction effect for blood pressure. Berberine hydrochloride was the major component of quality control. Therefore, *Coptidis Rhizoma* could be controlled by determining the content of berberine hydrochloride and the antihypertensive efficacy was guaranteed. An high performance liquid chromatography (HPLC) technique was applied to characterize the chromatograms of PGP extract. The mobile phases were composed of water. Elution was performed at a solvent flow rate of 1.0 mL/min. The column compartment was kept at the temperature of 25 ℃. The injection is 20 μL loop.

Animal Study and Sample Collection

A total of 32 male spontaneously hypertensive rats (SHRs, 200–250 g) and 14 male Wistar rats were commercially obtained from Experimental Animal Center of Shandong University of Traditional Chinese medicine [China, SCXL (Lu) 2008002]. The rats were maintained at 22 ℃ with a 12-h light/dark cycle, relative humidity of 45%–65% and had free access to standard chow and tap water. After 1-week habituation, all animals were housed individually in metabolism cages and allowed to acclimatize for a further 24 h. Two groups were randomly obtained from 32 SHRs by using the random number table method: a treatment group (n=18) in which PGP extract was administered orally at a dose of 18.336 g/kg (prescription/body weight), and a model group (n=14) in which physiological saline was administered at the equivalent dose. The same dose was also applied to a normal group (n=14) and the model group. Each group was given once daily between 8:00 and 10:00 a.m for the following 15 days. Rats were anesthetized with 3% pentobarbital sodium (40 mg/kg), the blood was collected from the abdominal aortic at day 15 of treatment into heparinized tubes and immediately centrifuged at 11,200×g for 10 min. The plasma was transferred into clean tubes and stored at –80 ℃.

Sample Preparation for GC-MS Detection

The plasma samples were processed as following. Prior to analysis, the rat plasma samples were thawed at room temperature. The plasma was diluted at a ratio of 1:2 with acetonitrile for protein-precipitation, then followed by centrifugation. The

150 μL of the supernatant was transferred to a gas chromatography (GC) vial and evaporated under a stream of nitrogen gas to dryness. Methoxymation was carried out at 70 ℃ for 1 h after ethylhydroxylamine hydrochloride was added. MSTFA was added and the trimethylsilylation was performed at 70 ℃ for 1 h. The derivatized sample was transferred to a GC microvial after filtration.[7]

Pharmacodynamic Index Detection

SHR tail artery blood pressure was measured before and after 14-day treatment during a totally normal waking state. The ELISA was applied to test the contents of Ang Ⅱ and cAMP in SHRs plasma.

Data Processing and Pattern Recognition

The collected plasma samples were represented by a GC-MS total ion chromatogram (TIC). Seventy-five peaks exceeding a signalto-noise (S/N) of 10 were selected. Their peak areas were analyzed by the software program SIMCA-P (version 11.5, Umea, Sweden). The data were exported to Metabo Analyst software. The data were subjected to principal components analysis (PCA) and partial least squares discriminate analysis (PLS-DA) with auto-scaling pretreated before.

Structural Identification of the Potential Biomarkers and Analysis of Related Metabolism Pathway

After PCA and PLS-DA, variable importance for the projection (VIP) was used to describe the variable significance for classification. There were significant features distinguished by the variable of VIP>1.[8] Potential biomarkers were identified based on National Institute of Standards and Technology (NIST) mass spectra library 2005 and metabolism pathways were researched by KEGG database.[9]

Statistical Analysis

The results are expressed as mean ± standard deviation ($\bar{x} \pm s$). The comparisons between groups were assessed by one- or two way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. A *P* value <0.05 was considered statistically significant.

RESULTS

Chromatograms of PGP

The content of berberine hydrochloride was 38.56 mg/g as determined by HPLC.

Change of Pharmacodynamic Index from Different Groups

Compared with the model group, the blood pressure of treatment group was obviously reduced after continuous curing with PGP (*P*<0.01), and the PGP treatment showed the better antihypertensive effect (Figure 1). The similar trend of Ang Ⅱ could also be observed and cAMP had the converse trend (Figure 2).

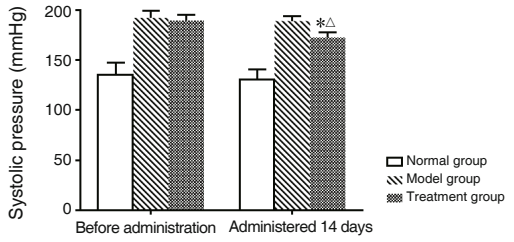

Figure 1. Changes in Systolic Blood Pressure of Rats in Each Group before and after Continuous Administration of PGP for 14 Days

Notes: *$^{*}P$<0.01 *vs*. the normal group; $^{\triangle}P$<0.01 *vs*. the model group; the same in Table 2

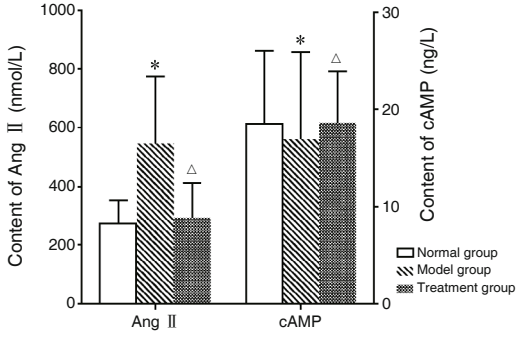

Figure 2. Changes of Pharmacodynamic Index of Each Group before and after Continuous Administration of PGP for 14 Days

GC-MS Metabolic Profiling of Plasma

Typical GC-MS TIC of plasma samples from the model group was shown in Figure 3. Seventy-five selected peaks were aligned mainly based on their retention time similarity, which were used to construct a 75-dimensional vector to characterize the metabolic pattern of rat plasma. Metabolic profile of rat plasma was illustrated as the TIC chromatogram in GC/MS analysis.

PCA and PLS-DA

There was obvious separation between the treatment group, model group and normal group from PCA score plots (Figure 4A). PLS-DA (Figure 4B) was employed for further analysis of potential biomarkers, because PLS-DA can find the information correlated with

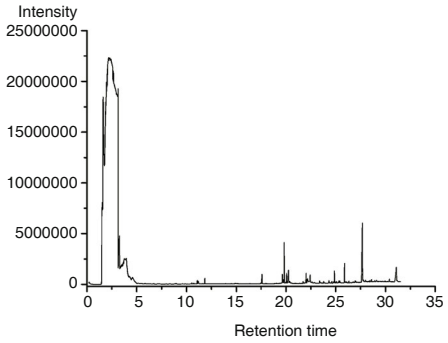

Figure 3. Metabolic Profile of Rat Serum Illustrated as TIC Chromatogram in GC-MS Analysis

the sample classification and make the most correlative, so it was the powerful tool to search the biomarkers. The PLS-DA results showed that R2Y=0.953, which indicated the principal component from the model can explain 95.3% of total variable, Q2(cum)=0.888, which indicated the prediction ability of model is 88.8%. The importance of variable to classification was analyzed by VIP in the analysis of PLS-DA.

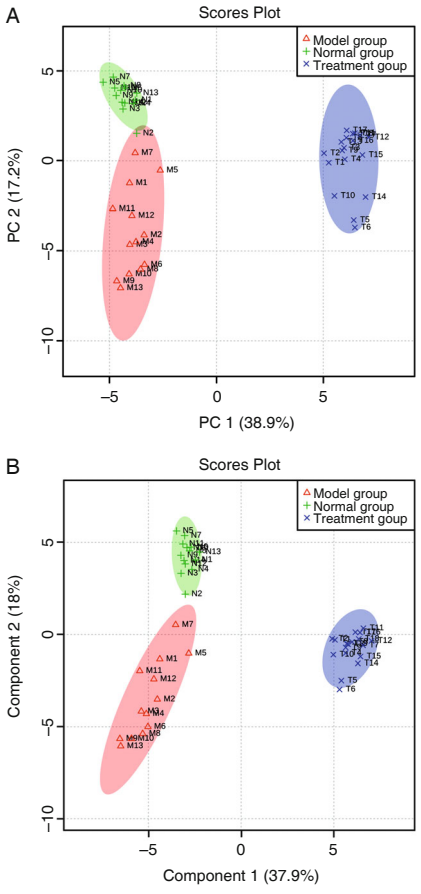

Figure 4. Score Scattering Plot for Rat Serum Sample
Note: A: PCA; B: PLS-DA

The structure of biomarker identified is shown in Table 1 and the related metabolism pathways are analyzed in Table 2. In the present work, five potential biomarkers were identified, including arachidonic acid (AA), hexadecanoic acid, elaidic acid, octadecanedioic acid and 9,12-octadecadienoic acid. The biomarkers trends are shown in Figure 5.

Table 1. VIP (>1) of Rats Potential Biomarker

No.	VIP	Retention time	Compound
1	1.5446	25.092	Elaidic acid
2	1.3493	19.727	Hexadecanoic acid
3	1.3420	26.939	Octadecanedioic acid
4	1.3072	23.372	AA
5	1.2977	22.140	9,12-Octadecadienoic acid

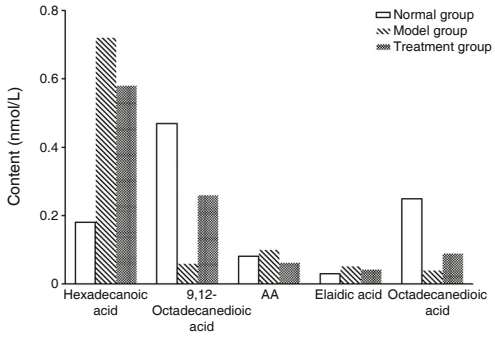

Figure 5. Changed Metabolic Trend of Potential Biomarkers in Different Groups

DISCUSSION

Obvious changes in blood pressure were observed in rats after the treatment of PGP, which suggested that PGP had a significant antihypertensive effect. Synchronized with the blood pressure, pharmacodynamic illustrated that the concentration of Ang Ⅱ and cAMP changed appropriately. The changed metabolic biomarkers were the terminal behavior of changed metabolic pattern induced by hypertension.

Prostaglandins (PG), leukotrienes (LTs) and thromboxanes (TX) were the major metabolites of AA. AA regulated blood pressure through the impact on content and ratio of TXA_2 and PGI_2. TXA_2 could inhibit adenylate cyclase and reduce cAMP formation, which promoting vasoconstriction. While PGI_2 was the stimulant of adenylate cyclase and it had opposite effect.[10] Studies had shown that large doses of AA could elevate TXA_2 but no significant increase in PGI_2.[11,12] Therefore, the rise of TXA_2 inhibited adenylate cyclase and cAMP concentration decreased. Numerous studies confirmed that cAMP could decrease blood pressure through reducing peripheral resistance

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Table 2. Hypertension Potential Biomarkers and Metabolic Pathway

KEGG No.	Compound	Metabolic pathway	Molecular weight
C00249	Hexadecanoic acid	Fatty acid biosynthesis and metabolism	$C_{16}H_{32}O_2$
256.2402	1.3493	19.727	Hexadecanoic acid
C01595	9,12-Octadecadienoic acid	Linoleic acid metabolism	Octadecanedioic acid
Biosynthesis of unsaturated fatty acids	$C_{18}H_{32}O_2$	23.372	AA
280.2402	1.2977	22.140	9,12-Octadecadienoic acid
C00219	AA	Arachidonic acid metabolism	Linoleic acid
Biosynthesis of unsaturated fatty acids			
Vascular smooth muscle contraction	$C_{20}H_{32}O_2$		
304.2402			
C01595	Linoleic acid	Linoleic acid metabolism	
Biosynthesis of unsaturated fatty acids	$C_{18}H_{32}O_2$		
280.2402			

and dilating blood vessels.[13-16] The pathways of AA and cAMP affecting blood pressure were shown in Figure 6. In our study, PGP also had the similar pharmacodynamics expression. The concentration of cAMP and blood pressure was negatively correlated and synchronized with AA. Through the intervention of PGP, the content of AA reduced to normal level. So AA could be validated as a biomarker and AA-cAMP metabolism was the characteristic metabolic pathway of hypertension.

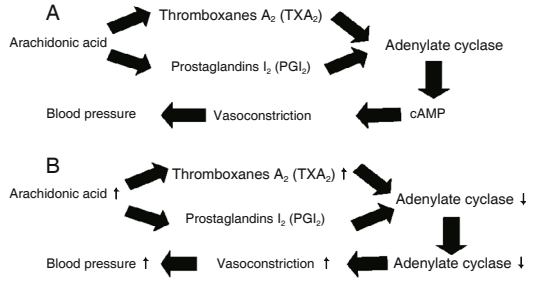

Figure 6. Pathway of Arachidonic Acid and cAMP Affecting Blood Pressure

Note: A: Wistar rats, B: SHRs

9,12-Octadecadienoic acid (linoleic acid) was the synthetic precursor of prostaglandins.[17] Prostaglandins could inhibit the norepinephrine releasing in vascular sympathetic nerve endings and weaken the vasoconstrictor effect of Ang Ⅱ and other vasoconstrictor substances.[18] Ang Ⅱ was the main effective material for RAAS and it played a key role in blood pressure regulation mechanism.[19] The pathway of linoleic acid and Ang Ⅱ affecting blood pressure was shown in Figure 7. As the pharmacodynamics shown, compared with the model group, the concentration of Ang Ⅱ in the treatment group decreased significantly. In the metabolism trends histogram, the change of linoleic acid was obviously reduced from normal group to the model group. And treatment group had a trend back to the normal group. Combined with pharmacodynamic, we inferred that PGP may decrease blood pressure through the way of linoleic acid-Ang Ⅱ-RAAS.

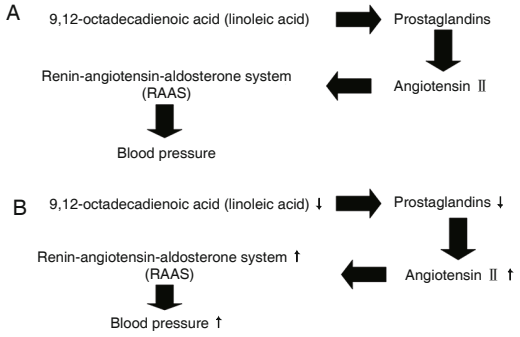

Figure 7. Pathway of Linoleic Acid and Ang Ⅱ Affecting Blood Pressure

Note: A: Wistar rats, B: SHRs

Insulin resistance was the pathophysiological basis of hypertension and expressed glucose transport barrier. Hexadecanoic acid could inhibit insulin-stimulated fat cells under the glucose transporter.[20] As shown in Figure 5, the concentration of hexadecanoic acid increased in model group and returned to normal after treatment of PGP, which may be attributed to several effects. Hexadecanoic acid could lead to insulin resistance.[21,22] Insulin resistance caused hyperactive sympathetic nervous system activity and the blood pressure elevated.[22] This suggested that the abnormal metabolism of hexadecanoic acid would occur through hypertension process and it may be served as the biomarker of hypertension.

In this study, we combined metabonomics with pharmacodynamic for the characterization of the perturbation of plasma metabonome in SHR and intervention effects of PGP. The results showed a significant difference in whole metabolism pattern between the model group and the treatment group. Five potential biomarkers were identified as fatty acid. Associated with pharmacodynamic results, we inferred that AA-cAMP and linoleic acid-Ang Ⅱ-RAAS may be two pathways of effecting blood pressure, while hexadecanoic acid increased blood pressure by insulin resistance. The research provided the experimental foundation that fatty acid influenced the blood pressure. Application of metabonomic combined with pharmacodynamic was introduced to study the essence of CM syndrome and intervention effects of PGP.

Conflict of Interest

The authors declared no conflict of interest.

Author Contributions

Li YL conceived and designed the experiment, contributed to discussion and drafted the manuscript. Xie J and Yang WQ performed the experiment, wrote the manuscript. Jiang HQ, Nei L and Zhou HL collected data and analyzed. All authors read and approved the final manuscript.

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