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Article

Metabonomic phenotype and identification of "heart blood stasis obstruction pattern" and "qi and yin deficiency pattern" of myocardial ischemia rat models

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The traditional Chinese medicine concepts of "Xinxueyuzuzheng (heart blood stasis obstruction pattern)" and "Qiyinliangxuzheng (qi and yin deficiency pattern)" for myocardial ischemia rat models were constructed in the present study. Endogenous metabolites in rat plasma were analyzed using the GC/TOF-MS-based metabonomic method. Significant metabolic differences were observed between the control and two model groups, and the three groups were distinguished clearly by pattern recognition. Compared with those of the control, the levels of hydroxyproline, threonic acid, glutamine and citric acid were strikingly up- or down-regulated in model rats. The metabolites contributing most to the classification between the two "pattern" rats were identified, such as valine, serine, threonine, ornithine, hydroxyproline, lysine, 2-hydroxybutanoic acid, 3-hydroxybutanoic acid, galactofuranose and inositol. These compounds were indicated as the potential biomarkers. The results suggested that the two "patterns" are involved in dysfunction in oxidative stress, energy metabolism and amino acid metabolism. These findings also provided the substantial foundation for exploring the scientific connotation of these two "Zhengxing (pattern types)" of myocardial ischemia, and "Bianzheng (pattern identification)".

Metabonomics, GC/TOF-MS, heart blood stasis obstruction pattern, qi and yin deficiency pattern, myocardial ischemia, rat model

In traditional Chinese medicine (TCM), "Bianzheng (pattern identification)" is used for recognizing, diagnosing and curing diseases, because only when the "Zheng (pattern)" is identified, is "Shizhi (treatment administration)" carried out. "Pattern" represents a specific stage of the disease, which is the term used by traditional Chinese physicians for certain pathoconditions after long-term clinical practice. "Pattern identification" is to recognize the essence of disease from humans' responses to disease stimuli, and to speculate concerning internal relationships among different clinical manifestations[1-2]. To date the conclusions obtained from "pattern identification" were all qualitative and heavily depended on personal experience, necessitating inves tigation of TCM "patterns" utilizing modern technology and thereby attempting to establish an objective and quantitative methodology for the "pattern identification" in TCM.

Metabonomics, like "genomics" and "proteomics", provides a platform for the study of "pattern identification" in TCM. In recent years, rapid progress of meta bonomics has been made in biomedical research[3−10]. Metabono-

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mics may be defined as "the quantitative measurement of metabolic responses to pathophysi-ological stimuli or genetic modification". It involves the comprehensive analysis of the entire metabolome to elucidate the global functional status of the organism, which agrees well with the holism of TCM. Traditional Chinese physicians recognize any organism's functional status through "Sizhen (four examinations)" including "Wang (inspection)", "Wen (audio-olfactory examination)", "Wen (inquiry)" and "Qie (palpation)", while in metabonomics an organism's functional status is examined by determining the endogenous metabolome using modern analytical techniques. TCM theory is intrinsically correlated with metabonomics, so using metabonomics facilitates TCM modernization, such as the essence of "patterns", "pattern identification", and "Bianzhengshizhi (identify patterns and administer treatment)". Preliminary NMR-based metabonomic studies of "patterns" in TCM[11,12] aimed directly at selected "patterns" with few studies published concerning the application of metabonomics to "pattern identification".

Coronary artery disease belongs to the category of "Xintong (heart pain)", "Juexintong (reversal heart pain)" or "Xiongbi (chest impediment)" in TCM, and is classified into eight kinds of "patterns" according to "Guidance for new drugs of traditional Chinese material medica (on trial)" [13]. "Xinxueyuzuzheng (heart blood stasis obstruction pattern)" and "Qiyinliangxuzheng (qi and yin deficiency pattern)" are the main clinical "Zhengxing (pattern types)". Patients with "heart blood stasis obstruction" were characterized by severe chest pain, fixed location pain, increasing in severity at night, chest distress, prolonged inability to recover, frequent agitation without calmness, dried and desiccated hair, dull purple tongue with or without stasis macule, and rough pulse or bound pulse. Patients with "qi and yin deficiency" have the symptoms of intermittent dull pain in the heart and chest, chest distress, shortness of breath, heart palpitation, dizziness and weakness, dry mouth and throat, vexation and sleeplessness, red tongue bearing dental impressions, thin tongue fur, and sunken, fine and weak pulse. TCM modernization has been restricted due to the shortage of TCM disease and "pattern" integrated animal models considering the lack of scientific methods for evaluating these animal models. Various pathophysiological indices used today are fairly segmentary and it is therefore difficult to standardize the indices of the "four examinations". Therefore, we attempted to construct myocardial ischemia and "heart blood stasis obstruction (SO) pattern" or "qi and yin deficiency (QY) pattern" integrated animal models and evaluate them utilizing metabonomics.

The myocardial ischemia rat model was established by repeated subcutaneous injections of isoproterenol into rats[14], which were subcutaneously injected with adrenaline and soaked in ice water for modeling the "SO pattern" induced by "Ganqiyujie (binding depression of liver qi)"and "Hanxie (cold pathogen)"[15], while the "QY pattern" model was built through sleep deprivation which made the rats tense, anxious, tired and terrified[16]. The GC/TOF-MS-based metabonomic technology[17,18] was used to identify these two models and explore the metabonomic variation of "Tongbingyizheng (unlike pattern of like disease)". This study provided an opportunity for constructing the quantitative evaluation system of TCM "pattern types", for "Fangzhengduiying (formula corresponding to pattern types)", and global pharmacodynamic evaluation of traditional Chinese material media.

1 Materials and methods

1.1 Materials, reagents and animals

Isoproterenol hydrochloride injection (ISO, 1mg/2mL, Shanghai Hefeng Pharmaceutical Co. Ltd., China); adrenaline hydrochloride injection (ADR, Tianjin Jinyao Pharmaceuticals Group Corporation, China); 1,2-^{13}C2-myristic acid (the stable-isotope-labeled internal standard compound, IS, Isotec, USA); methoxyamine hydrochloride (purity 98%, Sigma-Aldrich, Germany); alkane standard solution (C_8-C_{40}, Sigma-Aldrich, Switzerland); pyridine (≥99.8% GC, Sigma-Aldrich, India); MSTFA plus 1% TMCS (MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; TMCS, trimethylchlorosilane, Pierce, USA); Methanol (HPLC grade, Tedia, USA); n-Heptane (HPLC grade, Merck, Germany); Purified water, produced by a Milli-Q system (Millipore, USA).

The separation and detection system was GC/TOF-MS in which GC was an Agilent 6980 GC equipped with an Agilent 7683 Series autosampler and a fused-silica capillary column chemically bonded with 0.18 μm DB-5 stationary phase (10 m×0.18 mm i.d., J&W Scientific, USA), and TOF-MS was a Pegasus III TOFMS (Leco, USA). SORVALL Biofuge Stratos Centrifuge

(Sollentum, Germany) was used to centrifuge; SPD2010-230 SpeedVac Concentrator (Thermo, USA) was used for evaporating the extracted organic solvents to dryness; the LG-R-80 blood rheology system and E & H64 ESR/HCT system (Beijing Shidi Scientific Instrument Co. Ltd., China) were used to analyze blood rheology and hematocrit.

Twenty-five male Sprague-Dawley rats (140—160 g, SPF) were provided by the Laboratory Animal Center of the Academy of Military Medical Science (licence NO.: SCXK-(Military) 2002-001, China) and were cared for under controlled conditions ((20 ± 2) ℃, RH (50 ± 20) %, 12 h/12 h light-dark cycle). The studies were approved by the Animal Ethics Committee of the China Pharmaceutical University.

1.2 Modeling method and sample collection

The rats were randomly divided into three groups: the control group (n=7), the "SO pattern" of the myocardial ischemia group (SO group, n=9) and the "QY pattern" of the myocardial ischemia group (QY group, n=9). The rats were acclimated for 2 weeks before the experiment started. Normal saline (NS), ISO (2 mg/kg) and ISO (2 mg/kg) were subcutaneously injected for 10 d in the control, SO and QY group (slight modification according to the report[14]). The control group was raised under normal conditions. After recovering for 3 d, the rats in the SO group were subcutaneously injected with ADR (0.6 mL/kg) twice at 4 h intervals, and were soaked in 4 ℃ ice water for 5 min in the middle of this period for 2 d. The rats recovered for 2 d and were exposed to the same stress again for 2 d. Blood samples were drawn in the morning of the following day. After recovering for 2 d, rats in the QY group were deprived of sleep for 72 h using the platform-over-water method. After 2 d of rest, the rats were deprived of sleep for 72 h again and their blood was sampled in the morning of the following day. The rats fasted for 12 h before sampling and were allowed access to fresh drinking water. Repeated additional stress mimicked the chronic development of disease in clinical settings. The ethological changes of rats were recorded during the experiment.

In each group, about 250 μL of blood was drawn from the fossa orbitalis and mixed with EDTA-2Na, then centrifuged at 1600×g for 5 min at 4℃. The supernatant plasma was transferred and stored at −80℃ for analysis.

Additionally, 5 mL of blood was sampled from the common carotid artery, and heparin anticoagulant blood was used to determine blood viscosity under the shear rates of 200, 100, 30, and 1 S^{-1}, plasma viscosity and hematocrit. Rats were sacrificed, the hearts were immediately removed, washed by cold saline (4℃), sopped up by filter paper. The hearts (HW) were weighed for calculating the heart index. Finally, they were fixed in 10% formalin solution for pathological sectioning.

1.3 Sample preparation

100 μL of plasma was added to 400 μL of methanol containing IS (4 μg). After being vigorously vortex-mixed for 5 min, the solution was placed in 4℃ for 1 h, then centrifuged at 20000×g for 10 min at 4 ℃. 100 μL of the supernatant was transferred to a GC vial, and then evaporated to dryness under vacuum. 30 μL of methoxyamine in pyridine (10 mg/mL) was added to the GC vial, vortex-mixed for 3 min, and the methoximation reaction was carried out for 16 h at room temperature, then 30 μL of MSTFA plus 1% TMCS was added to the samples for trimethylsilylation for another 1 h. At last, 30 μL of heptane containing external standard methyl-myristate was added to the GC vial, and the solution was evaluated utilizing GC/TOF-MS.

1.4 Data acquisition and processing of GC/TOF-MS

The GC/TOF-MS operating conditions were as follows: sample volume: 1 μL, splitless mode; carrier gas: helium, 1 mL/min; gradient temperature programming: 70℃ (2.0 min), 70—310℃ linear (35℃/min), 310℃ (2.0 min); the injector temperature: 250℃; purge time and flow rate: 1 min, 20 mL/min; the transfer line temperature: 250℃; ion source temperature: 200℃; ion source voltage and current: 70 eV, 3.0 mA. The MS data was acquired in scan mode over the range m/z 50—800 at a rate of 20 spectra/s. Following a solvent delay of 170 s, the detecting voltage was set at −1650v.

Automatic peak detection and calculation of peak areas of IS and detected compounds were made using the ChromaTOF 2.00 software of the Leco Corporation (USA)[17]. Peak width in automatic peak detection and mass spectrum deconvolution were all set to 2 s. Peaks whose signal-to-noise (S/N) ratios were lower than 20 were rejected. The retention index of each peak was calculated by comparison of its retention time against those of the alkane series C_8-C_{40}. Identification of compounds was achieved through comparing the mass spectra and retention index of all detected compounds with authentic reference standards and those in the NIST

library 2.0 (2005).

1.5 Multivariate data analysis

Multivariate data analysis and modeling was fulfilled by SIMCA-P 11 software (Umetrics, Umeå, Sweden)[19]. The data matrix was constructed with the sample names as observations and the peak areas normalized by IS as the response variables. Then dimension reduction of the GC/TOF-MS data was implemented using principal component analysis (PCA) and partial least squares projection to latent structures & discriminant analysis (PLS-DA) with most differences between groups being described by several principal components (PCs). PCA was used to observe the cluster and scatter of samples and outliers. The scores plot of PLS-DA was applied to showing the differences among groups, herein, separating control, SO and QY groups. The loading plot of PLS-DA was utilized to look for the endogenous metabolites contributing to the classification. Crossvalidation was used throughout to determine the number of PCs[20]. R^2X, R^2Y and Q^2Y in cross-validation were the indices to evaluate the mathematical model. R^2Y and Q^2Y were the percent of all observation/sample variables explained and predicted by a model, and R^2X was the percent of all GC/TOF-MS response variables explained by a model. The range of these parameters was 0-1, and the more they approached 1, the better they were able to explain or predict. Further statistical analysis was made using one-way ANOVA, and Bonferroni was used for the multiple comparison among groups (the mean difference is significant at 0.05 or 0.01 level). The variables with significant deviation may be the biomarkers and their structures were identified using authentic reference standards and the NIST library 2.0 (2005).

2 Results

2.1 Pathological section and blood rheology

As shown in Figure 1, in the control group, cardiocyte transverse striations were clear, nuclei were centrally located, no angioectasia or inflammatory cell infiltration were observed, and there were no abnormalities in the endocardium and epicardium. Histological sections of the SO group showed widespread subendocardial necrosis where the structure of the myocardial cells had disappeared, replaced by hyperplastic fibroblasts, and new vessel neogenesis was observed. The pathological section results of the QY group were similar to those of the SO group and there was no significant differences between them. Compared with the control group, the heart index of the two model groups notably increased, being significantly higher in the SO group than in the YO group. These findings indicated that myocardial damage accompanied with myocardial hypertrophy occurred in the model groups.

The results of blood rheology are shown in Table 1. Compared with the control group, blood viscosity under

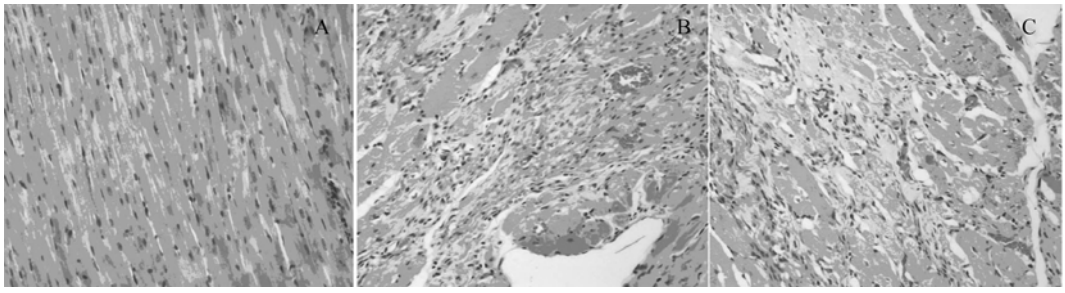

Figure 1 Myocardium pathological section（×200）
A, Control group; B, SO group; C, QY group

Table 1 Blood viscosity, plasma viscosity, hematocrit and heart index of "heart blood stasis obstruction pattern" and "qi and yin deficiency pattern" myocardial ischemia rat models（mean ± SE）[a)]

Group	Blood viscosity /s^{-1}				Plasma viscosity /s^{-1}	Hematocrit /%	Heart index /HW·BW^{-1}, mg·g^{-1})
	200	100	30	1			
Control(n=7)	3.61±0.23	3.90±0.25	4.77±0.33	18.16±1.72	2.02±0.47	55±1	3.11±0.06
SO (n=9)	4.39±0.20*	4.80±0.23*	6.06±0.35**	26.90±3.11*	2.58±0.33	52±1††	4.38±0.08**†
QY(n=9)	5.13±0.18**‡	5.48±0.19**‡	6.64±0.24**	24.27±1.61	2.59±0.47	47±1**	4.09±0.09**

a) *, **: significantly different from the control group at 0.05 and 0.01 levels; †, ††: significantly higher than QY group at 0.05 and 0.01 levels; ‡: significantly higher than the SO group at 0.05 level (One-way ANOVA and Bonferroni).

the 200, 100, 30, and 1 S^{-1} shear rate in the SO group was notably elevated, indicating that the "heart blood stasis obstruction pattern" formed. The QY group had a significantly higher blood viscosity under the 200, 100 and 30 1 S^{-1} shear rate and a lower hematocrit than the control and SO group had, suggesting that the "qi and yin deficiency pattern" induced by sleep deprivation also had the symptom of increased blood viscosity. In clinical observations, declined hematocrit was often caused by anaemia, so "Xu (deficiency)" induced by "Lao (fatigue)" may be reflected as the decreased hematocrit in the QY group. In addition, sleep deprived rats have the symptoms of low spirits, decreased activities, dry hair, vertical hair and arched back, which all indicated that the "qi and yin deficiency pattern" model offered a degree of relevance.

2.2 GC-TOF/MS plasma profile

Figure 2 showed the total ion current (TIC) chromatogram of rat plasma. After analysis by SIMCA-P 11 software, each TIC chromatogram contained about 200

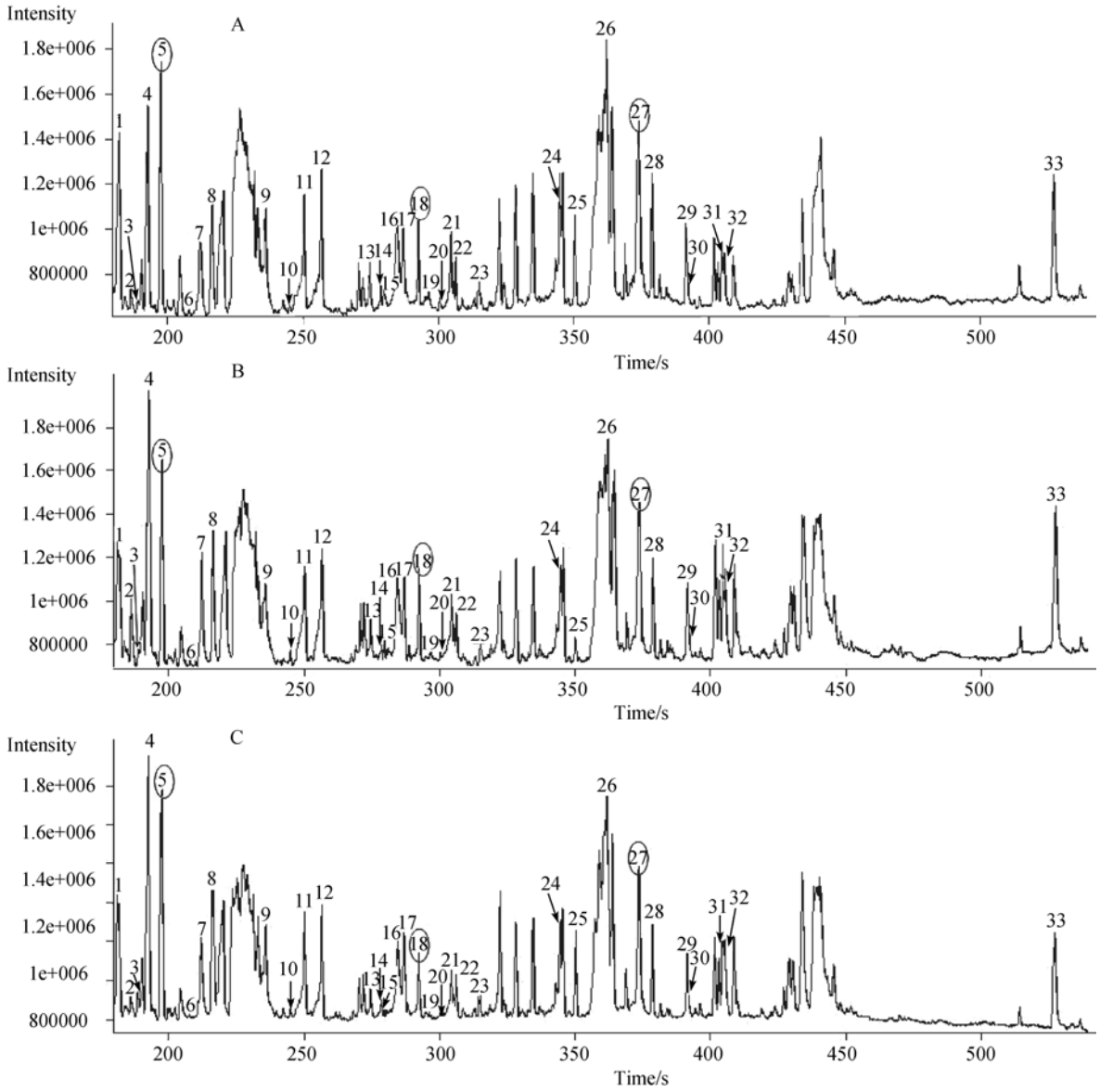

Figure 2 Typical GC/TOF-MS TIC chromatogram of rat plasma. A, Control group; B, SO group; C, QY group. 1, alanine; 2, glycine; 3, 2-hydroxybutanoic acid; 4, lactate; 5, 3-hydroxybutanoic acid; 6, 3-methyl-3-hydroxybutanoic acid; 7, valine; 8, ethanolamine; 9, succinic acid; 10, fumaric acid; 11, serine; 12, threonine; 13, aminomalonic acid; 14, malic acid; 15, 2,5-diaminovalerolactam; 16, methionine; 17, hydroxyproline; 18, creatinine; 19, threonic acid; 20, glutamine; 21, ornithine; 22, glutamic acid; 23, taurine; 24, citric acid; 25, galactofuranose; 26, glucose; 27. hyaluronic acid; 28, palmitic acid; 29. myo-inositol; 30. uric acid; 31. linoleic acid; 32. oleic acid; 33, cholesterol. Visual comparison of the TIC chromatogram of plasma reflected some variance among groups (labeled with circles).

compounds (S/N>20), in which about 80 compounds were identified using the NIST library 2.0 and authentic reference standards. The endogenous metabolites included amino acids, organic acids, amines, lipids, saccharide and fatty acids. In the TIC chromatogram, relative peak intensities of several compounds were significantly different among the three groups, such as 3-hydroxybutanoic acid, creatinine and hyaluronic acid (labeled with circles in Figure 2).

2.3 Multivariate data analysis results

A PLS-DA model with three principal components was calculated among the control, SO and QY groups. It explained 89.6% and predicted 51.2% of the sample types, and explained 33.3% of the GC/TOF-MS response variables according to the R^2Y, Q^2Y and R^2X parameters. In the scores plot (Figure 3), the three groups could be separated clearly from each other.

The loading plot was made to display the specific compounds contributing to the classification. Considering the scores and loading bi-plot of the control and SO groups, it displayed not only the differentiation between the two groups, but also the specific compounds resulting in the separation (Figure 4). Direct comparison of the data by One-way ANOVA suggested the same results. After searching in the NIST library 2.0 and using reference standards for authentication, most of the compounds were identified which might be the biomarkers (Table 2).

Compared with the control, the SO model rats showed significant elevation of valine, creatinine and an unidentified compound 1, and a reduced level of an unidentified compound 4. While QY model rats exhibited increased levels of 2-hydroxybutanoic acid and 2-amino-

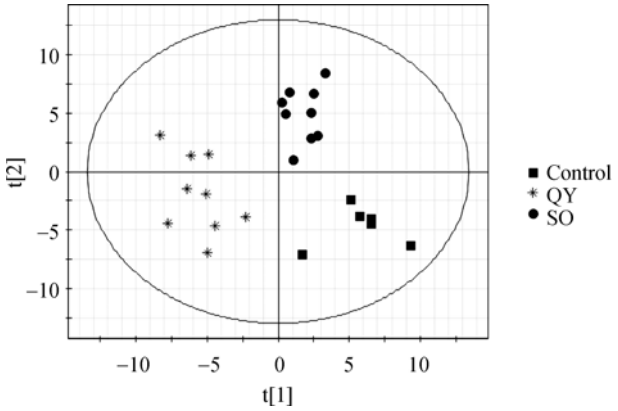

Figure 3 PLS_DA scores plot (t[1]/t[2]) of plasma metabolome of the control, SO and QY groups.

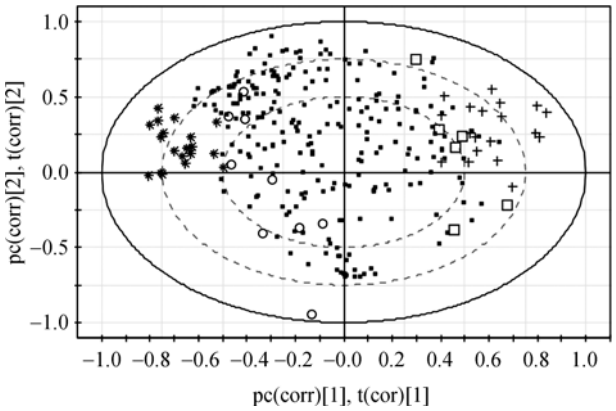

Figure 4 PLS-DA scores and loadings bi-plot (pc(corr)[1], t(corr)[1] / pc(corr)[2], t(corr)[2]) of plasma metabolome of the control and the SO group. □: control group; ○: SO group; + and * stood for the metabolites whose levels were relatively high in the control and SO groups.

butyric acid, and decreased levels of pyroglutamic acid, glutamic acid, hyaluronic acid, glycine, serine, threonine, 2,5-diaminovalerolactam and ornithine. The two model rats had significantly higher levels of an unidentified compound 2, and lower levels of hydroxyproline, threonic acid, glutamine, citric acid and an unidentified compound 3.

The main endogenous metabolites contributing to the separation between the two "pattern" models included valine, 2-aminobutyric acid, glycine, serine, threonine, 2,5-diaminovalerolactam, ornithine, hydroxyproline, lysine, 2-hydroxybutanoic acid, 3-hydroxybutanoic acid, galactofuranose, myo-inositol and unidentified compound 4. Additionally, the previous nine metabolites were higher and the rest were lower in the SO group than in the QY group, so they were the potential biomarkers for distinguishing these two "pattern types".

3 Discussion

Results from pathological inspection, blood rheology determination and ethology observation all suggested that the "heart blood stasis obstruction pattern" and the "qi and yin deficiency pattern" of myocardial ischemia rat models were relevant. By means of metabonomics, the variation between the control, SO and QY model rats were well differentiated. Biomarkers contributing to the classification were identified and they suggested perturbed metabolism pathways in model rats.

3.1 Modification of energy metabolism

Injection of ADR to rats in the SO group increased energy consumption and heat production[21], besides, cold

Table 2 Endogenous metabolites statistically different between two "pattern types" of myocardial ischemia rat models [a)]

Metabolic pathway	Retention Index	Metabolites	Control	SO	QY
Energy metabolism	1153.6	2-Hydroxybutanoic acid	4.33e5±8.02e4	3.64e5±3.96e4	1.06e6±1.61e5**‡‡
	1181.9	3-Hydroxybutanoic acid	1.85e7±2.52e6	1.53e7±1.26e6	2.27e7±1.37e6‡‡
	1813.9	Citric acid	1.17e7±5.80e5	8.82e6±3.28e5**	8.92e6±3.76e5**
Oxidative stress	2018.1	Hyaluronic acid	2.05e6±2.68e5	1.48e6±1.94e5	1.27e6±1.95e5*
	1145.0	Glycine	2.96e6±7.06e5	2.54e6±2.77e5†	1.32e6±1.93e5**
Amino acid metabolism	1628.0	Glutamic acid	6.93e5±6.11e4	5.88e5±4.47e4	4.63e5±6.54e4*
	1528.1	Pyroglutamic acid	3.15e6±2.48e5	2.83e6±1.53e5	2.41e6±2.62e5*
	1579.7	Threonic acid	3.44e5±4.62e4	2.07e5±2.36e4**	2.40e5±2.47e4*
	1233.3	Valine	6.27e6±3.27e5	7.54e6±4.33e5*††	5.41e6±3.64e5
	1361.3	Serine	1.29e7±8.41e5	1.22e7±5.64e5††	9.04e6±6.40e5**
	1400.6	Threonine	1.67e7±1.12e6	1.43e7±7.07e5†	1.15e7±6.34e5**
	1602.5	Glutamine	8.29e6±8.32e5	6.42e6±4.63e5*	5.86e6±3.73e5**
	1714.2	Lysine	8.10e6±5.61e5	9.09e6±8.90e5†	7.13e6±4.92e5
Others	1537.2	Hydroxyproline	4.91e6±4.28e5	3.66e6±2.71e5**††	1.80e6±1.14e5**
	1546.0	Creatinine	4.76e6±3.70e5	6.32e6±4.45e5*	5.49e6±3.80e5
	2125.8	Myo-inositol	4.58e6±3.42e5	3.86e6±1.55e5	4.88e6±4.11e5‡
	1194.0	2-Aminobutyric acid	3.85e5±2.69e4	4.15e5±1.61e4	5.07e5±4.57e4*
	1504.3	2,5-Diaminovalerolactam	7.30e5±3.23e4	6.46e5±2.16e4††	5.32e5±2.98e4**
	1622.9	Ornithine	6.88e6±4.17e5	6.39e6±3.58e5††	4.40e6±2.49e5**
	1869.4	Galactofuranose	2.02e6±2.38e5	1.51e6±5.43e5	3.16e6±2.55e5‡‡
Unidentified	1206.9	Unidentified compound 1	1.11e5±9.98e3	1.60e5±7.96e3**	1.36e5±1.15e4
	1469.5	Unidentified compound 2	4.77e5±7.20e4	1.44e6±2.18e5**	1.18e6±2.55e5*
	1577.1	Unidentified compound 4	3.70e5±2.29e4	3.03e5±1.34e4**	2.90e5±1.18e4**
	1625.2	Unidentified compound 3	8.18e5±7.07e4	5.66e5±3.77e4**	7.47e5±6.34e4‡

a) Relative peaks areas of metabolites (normalized against internal standard) are expressed and the data are given as means ± SE. *, **: significantly from the control group at 0.05 and 0.01 levels; †, ††: significantly higher in the SO group than in the QY group at 0.05 and 0.01 levels; ‡, ‡‡: significantly higher in the QY group than in the SO group at 0.05 and 0.01 levels (one-way ANOVA and Bonferroni).

stress also induced elevated energy production. In the YQ group, sleep deprivation made rats tired, so raised their demand for energy, which activated glucose oxidization and fat mobilization and oxidization. Citric acid and hyaluronic acid were the intermediates of the tricarboxylic acid cycle (TCA) and free fatty acid (FFA) metabolism. However, the declined levels of citric acid in the model groups and hyaluronic acid in the QY group indicated inhibition of TCA, decreased fat oxidization and decreased energy production. The reasons for the seemingly contradictory phenomena were that TCA and fat metabolism were accelerated during ADR administration in addition to ice-water stress or in the initial stage of platform-standing, so excessive consumption of intermediates and accumulation of end products gave rats the symptoms of low spirits and decreased activities. Consequently, energy consumption decreased. High plasma levels of 2-hydroxybutanoic acid in the QY also had a relationship with energy deficiency[22]. Additionally, 3-hydroxybutanoic acid was the intermediate product of FFA oxygenolysis in the liver and was the energy source for tissues excluding the liver. It was reported[23,24] that 3-hydroxybutanoic acid had the ability to improve cerebral function and protect rat brains against ischemic damage resulting from permanent and transient focal cerebral ischemia. Therefore, increased 3-hydroxybu tanoic acid in the QY group in comparison with the SO group also indicated that this "pattern type" was related to disordered energy metabolism, and the elevation of 3-hydroxybutanoic acid reflected the self-regulation of rats. Dysfunction in energy metabolism may have resulted in "Qixue Yunxingbuchang (inhibited move of qi and blood)", which fit well with "Xiongbiertong (chestpain induce by

impediment)", "Shouzubuwen (lack of warmth in the extremities)" and "Sizhijueleng (reversal cold of the limbs)" induced by "cold pathogen", and "Qiduanfali (shortness of breath and strength)" reflected from "qi and yin deficiency pattern".

3.2 Occurrence of oxidative stress

Glutathione homeostasis is a component in the maintenance of prooxidant-antioxidant balance[25], and is synthesized in the γ-glutamyl cycle[26]. Compared with the control group, plasma levels of glycine, glutamic acid and pyroglutamic acid, the intermediates of the γ-glutamyl cycle, were lowered in the models, especially in the QY group indicating of decreased activity in the γ-glutamyl cycle- and an enhanced oxidative stress reaction. In addition, the declined level of threonic acid in models may also be related to oxidative stress[27,28].

3.3 Perturbation of amino acid metabolism

In comparison with the control, significantly decreased glutamine in model groups indicated attenuated immune function[29]. Hydroxyproline is a major component of collagen and elasin, and serves as an indicator to determine collagen, maintaining the stability of collagen, because it permits the sharp twisting of the collagen helix and forms hydrogen bonds which provide stability to the triple-helical structure of collagen. (http://hmd b.-ca/index.html). Low levels of hydroxyproline in model groups suggested collagen or elasin synthesis was inhibited, so that the amount of free hydroxyproline entering the circulation decreased, accordingly, reflecting the degree of aging[30]. Therefore, it is hypothesized that these two "pattern types" of myocardial ischemia models were related to aging, and the variance in levels of hydroxyproline between the two models reflected different levels of aging between them. In model groups, especially in the SO group, increased creatinine indicated that renal injury occurred[31,32], suggesting that these two "pattern types", especially the "heart blood stasis obstruction pattern" may be accompanied with renal function dysfunction. Myoinositol is a key ingredient of the phosphatidylinositol second messenger system (PI-cycle). Abnormal nerve cell myo-inositol levels and PI-cycle regulation may be involved in pathophysiology and such psychiatric disorders as depressive disorder, panic disorder and obsessivecompulsive disorder[33]. There was a significant deviation from the level of myo-inositol between the SO and QY groups, suggesting adjustment of the second messenger system was different between these two "pattern types". The ethological differences were observed between SO and QY rats. Abnormal glutamic acid metabolism is often involved in central pivot fatigue[34], so the "dizziness and weakness" in "qi and yin deficiency pattern" was probably related to disorders in glutamic acid metabolism.

In general, these two "pattern types" of myocardial ischemia had the same characteristics of disorders in several metabolic pathways, such as energy metabolism, oxidative stress and amino acid metabolism. The perturbation of these metabolic pathways reflected biochemical variation of multiple systemic functions. However, each "pattern" had its own features. All the findings in the present study showed that metabonomics profiles metabolic processes and distinguishes the specific diversity of the "heart blood stasis obstruction pattern" and the "qi and yin deficiency pattern" of myocardial ischemia. Thus, it has substantial significance for elucidating the nature of these "pattern types", and providing scientific indices for "identifying patterns and administering treatment" in Traditional Chinese clinical medicine.

Metabonomics is used to analyze and differentiate the endogenous metabolome in body fluid or in drug components *in vivo* and *in vitro* before and after patients are administered traditional Chinese materia medica or are treated with TCM. Application of metabonomics to TCM will enhance the interrelationship between TCM and western medicine, and facilitate the acceptance of TCM theory [35]. Additionally, it is an effective tool for investigating pharmacological material, therapeutic effects, and the rational usage of traditional Chinese materia medica[36]. Related studies have been carried out in many institutes in China[37−42]. Our study identified "pattern"-related biomarkers which will serve as a basis for further research on TCM treatment and "formula corresponding to pattern types". Nevertheless, it requires further investigation to conclusively determine whether "pattern"-related endogenous metabolites are the pharmacodynamic action-related biomarkers.

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