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Metabolomics Study of Protective Effects of Shexiang Baoxin Pill and its Bioactive Constitutes Combination in Treating the Early Period of Acute Myocardial Infarction in Rats

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Abstract

Ginsenoside Re, ginsenoside Rb1, muscone, cinnamic acid, bufalin, cholic acid and borneol are the main active ingredients of Shexiang Baoxin Pill (SBP) for protective effects on acute myocardial infarction (AMI). In this study, the protective effects of SBP, single component treatments and combination treatment of 7 bioactive ingredients of SBP (SFSBP) were investigated by metabolomics technology. Liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS) has been used to analyze the plasma samples of AMI rats. Partial least squares discriminate analysis (PLS-DA) models were built to evaluate the protective effects of different treatments at whole level. Reverse effect on mean levels of 19 identified biomarkers were also carried out to investigate the protective effects of different treatments. The results showed that the protective effect of multi-component medicines (SBP and SFSBP) was superior to 7 single-component treatments, demonstrated the multi-effective advantages of multi-component treatments (SBP and SFSBP). Meanwhile, the reverse effects of SFSBP were inferior to SBP, indicating that there might be other bioactive components in SBP taking responsibility for the protection effect on AMI rats, and the simplified formula of SBP should be further optimized, while also revealed the rationality of TCM formula, and it cannot be simply substituted by a combination of bioactive ingredients.

Keywords: Shexiang Baoxin Pill (SBP); Metabolomics; Traditional Chinese medicine (TCM); Simplified formula of SBP (SFSBP); LC-Q-TOF/MS; Acute Myocardial Infarction (AMI)

Introduction

TCM is a complex medical science. There are hundreds of compounds in a TCM. It reflects traditional Chinese culture and philosophical principles, and places the human body into a holistic system for observation and regulates humans to remain in a healthy status [1]. TCM has been adopted by patients as a superior and unique valuable property in many Asian countries for thousands of years. However, as a result of insufficient modern scientific research, TCM is facing severe challenges in its development abroad [2,3]. The traditional methodology used in the TCM research basically through partitioned reductive analysis, which is unable to capture the holistic and dynamic nature of diseases, will lead to difficult understanding of the features and advantages of TCM and prevent the actual value of TCM from fully understand.

Shexiang Baoxin Pill (SBP), which consists of seven herbal materials, including *Radix Ginseng*, *Moschus*, *Styrax*, *Cortex cinnamomi*, *Venenum Bufonis*, *Calculus Bovis* and *Borneolum Syntheticum*, has been widely used in treating coronary heart disease (CHD) in China for many years [4-8]. There are hundreds of compounds in SBP [9,10], but not all of them take responsibility for the therapeutic effect of CHD. Compounds, such as muscone, cinnamic acid, bufalin, ginsenoside Re, ginsenoside Rb1, cholic acid and borneol, are the main active components in each composition material (*Moschus*, *Cortex cinnamomi*, *Styrax*, *Venenum Bufonis*, *Radix Ginseng*, *Calculus Bovis* and *Borneolum Syntheticum*), which may also take major responsibility for the therapeutic effectiveness of SBP [11]. Early metabolomics studies [12-14] have demonstrated that the long term therapeutic mechanism on acute myocardial infarction (AMI) rat was focusing on inhibiting oxidative injury, inflammation and dysfunction of energy metabolism. The potential mechanism of protective effects of SBP has also been studied

precisely in our lab [15], but the comparative protective effects of SBP and its 7 bioactive components combination has not been investigated.

Metabolomics was originally an emerging subject of the post-genome era, which, together with genomics, transcriptomics and proteomics, jointly constitutes the 'Systems Biology' [16,17]. The metabolomics was defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' [17]. Metabolomics holds the promise of a comprehensive, non-invasive analysis of metabolic biomarkers that could detect early-stage disease, and help to monitor treatment response [18-23], which is reasonably coincident with the systematic and integrity character of TCM.

In this study, 7 bioactive components of SBP have been selected out and rebuilt as a simplified formula of Shexiang Baoxin Pill (SFSBP) according to their proportion in SBP [9-11]. As an ongoing study, the comparative protective effects of SBP, SFSBP and 7 single bioactive constituents on AMI rats have been investigated to reveal the advantages of multi-component and multi-effective character of SBP by using metabolomics strategy.

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Material and Methods

Chemicals

Acetonitrile and methanol (HPLC grade) were obtained from Honeywell (NJ, USA). Leucine enkephalin and formic acid (MS grade) were purchased from Sigma/Aldrich (St. Louis, MO, USA). A Milli-Q water purification system (MA, USA) was used to produce HPLC grade water. The assay kits used for lactate dehydrogenase (LDH) and creatine kinases (CK) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). SBP was offered by Shanghai Hutchison Pharmaceuticals (Shanghai, China). Cinnamic acid, muscone, bufalin, ginsenoside Rb1, ginsenoside Re, cholic acid and borneol were purchased from Shanghai Ronghe Biopharmaceutical Co. (Shanghai, China). Commercial standards used for identification of biomarkers were purchased from Sigma/Aldrich (MO, USA).

Animals and administration

Eighty-eight male Sprague-Dawley rats (200 ± 15 g) were obtained from the Slac Laboratory Animal Co., Ltd. (Shanghai, China) and were housed under standard conditions with free access to food and tap water. The animals were acclimatized to the facilities for 2 weeks. All animals were treated with human care during the experiment. This experiment was performed under the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Shanghai, China, and the study was approved by the Animal Care and Use Committee of the Second Military Medical University.

Animals were divided into 10 groups, and orally dosed once daily consecutively for 7 days according to different administrations, including SBP group (28 mg/kg/day, n=10), SFSBP group (10 mg/kg/day, n=9), cinnamic acid group (10 mg/kg/day, n=8), muscone group (10 mg/kg/day, n=8), bufalin group (10 mg/kg/day, n=8), ginsenoside Rb1 group (10 mg/kg/day, n=8), ginsenoside Re group (10 mg/kg/day, n=8), cholic acid group (10 mg/kg/day, n=8), and borneol group (10 mg/kg/day, n=8). SBP was grounded into a fine powder and dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution. SFSBP was consisted of cinnamic acid, muscone, bufalin, ginsenoside Rb1, ginsenoside Re, cholic acid and borneol according to the percentage content of SBP [18].

AMI model on rats

An AMI model was performed by left anterior descending coronary artery (LADCA) ligation [24,25] on the seventh day of consecutively administration. The experiment was carried out under sterile conditions. The animals were anesthetized with ether, heart was then rapidly exteriorized after the chest was opened, a 4-0 black silk ligature was securely placed under the LADCA to form the occlusion. The sham group was performed as above process except ligation step. In order to ascertain that the MI model is successful, electrocardiograms (ECGs) were recorded by MPA 2000 bio-signal analysis system (Alcott Biotech Co. Ltd., Shanghai, China) for at least 5 min to obtain an abnormal Q wave (less than 0.3 mV) after five hours of the AMI operation. At last, 58 AMI rats (SBP group, n=8; SFSBP group n=8; cinnamic acid group, n=6; muscone group, n=6; bufalin group, n=6; ginsenoside Rb1 group, n=6; ginsenoside Re group, n=6; cholic acid group, n=6; and borneol group, n=6) and 8 sham rats were survived. Serum was collected immediately from the ophthalmic venous plexus after recording ECGs. Hearts were also collected for the infarction size of the myocardial infarction.

Preparation of histopathologic samples

Blood samples were centrifuged (3500 rpm) for 10 min after standing for 30 min. the supernatant serum was collected and divided into two parts, one part were used for the measurement of serum concentrations of CK and LDH, and the other part was used for the metabolomics analysis. The concentrations of CK and LDH in serum were detected by UV 1100 ultraviolet spectrophotometer (Beijing Rayleigh Analytical Instrument Corp., Beijing, China).

In order to investigate the infarction size of AMI, each heart was dissected into five transverse slices from apex to base. Those slices were then incubated in triphenylterazolium chloride solution (phosphate buffer, pH 7.4) and put in the water bath at 37°C for 15 min. At last, the stained tissue was removed, and the unstained tissue was weighed. The results of the infarct size of MI were expressed as the percentage of the infarcted heart weight (unstained tissue) to total heart weight.

Preparation of metabolomics samples

100 μ L aliquots of serum were diluted with 1000 μ L of methanol, centrifuged (12000 rpm) for 10 min after vortex-mixing for 1 min, and then the supernatant was transferred to autosampler vials. All the samples were prepared in the same working day and stored at -80° until analysis.

Quality control (QC) sample was prepared through pooling the same volume (20 μ L) of each serum sample. The QC sample was analyzed randomly throughout the analytical batch to monitor the stability. Additionally, a random serum sample was selected and prepared in the same process as aforementioned sample preparation method for 6 times to investigate the repeatability of sequence analysis.

Blank sample (acetonitrile) was prepared for needle wash following every 2 sample injections to minimize the carry-over throughout the sequence analysis.

LC-Q-TOF/MS analysis

Experiments were carried out on an Agilent 1200 LC system coupled with electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) and equipped with an Agilent-6520 accurate-mass Q-TOF mass spectrometer. All the samples were performed on an Eclipse plus C18 column (1.8 mm, 3.0 mm \times 100 mm, Agilent). The mobile phases were consisted of ultrapure water (A, with 0.1% formic acid) and acetonitrile (B), and the flow rate was 0.25 ml/min. The column temperature was maintained at 45°. The gradient was set as follows: 0-1.5 min, 2% B; 1.5-5 min, 15% B; 5-10 min, 80% B; 10-11 min, 100% B; 11-14 min, 100% B; 14-17 min, 2% B in positive mode. 0-1.5 min, 2% B; 1.5-3 min, 30% B; 3-15 min, 100% B; 15-18 min, 100% B; 18-21 min, 2% B in negative mode. The injection volume was 5 μ L.

The parameters of mass detection was set as follows: the gas temperature was 330°; the drying gas (N₂) flow rate was 8 L/min; the capillary voltage was 4 KV, and the pressure of nebulizer gas was 35 psig; the scan range was *m/z* 50-1000; the MS/MS analysis was performed in targeted MS/MS mode with three collision energy 10 ev, 20 ev and 40 ev respectively.

Analytical method assessment

The stability and repeatability of this experiment were measured for assessment of the newly developed LC-Q-TOF/MS approach. According to different polarities and mass (*m/z*), five ions have been extracted for validation according to the variation of their retention times and peak intensities.

Six parallel samples which were extracted from a random serum sample were injected continuously to evaluate the repeatability. The data of QC samples were used for the evaluation of stability of the large-scale sequence analysis. The retention time and peak intensity of the five extracted ions were then involved to analyzing the variation.

Data processing

The MS spectra were first processed using the Agilent Mass Hunter Qualitative Analysis Software (Agilent Technologies, Palo Alto, CA, USA). Agilent Mass Hunter Qualitative Analysis Software allows detection and retention time alignment of the peaks eluting in each chromatogram. All the data were combined into a matrix by aligning peaks with the extract mass/retention time pair, together from each data file of the data set and along with their associated intensities. A table was then generated including the information of retention time, *m/z* and ion intensity. The ion intensities of detected each peak were then normalized to total area and all the data were imported into the software of SIMCA-P (Ver. 11, Umetrics, Umea, Sweden) for multivariate analyses, and partial least squares-discriminate analysis (PLS-DA) were used for calculation. One-way analysis of variance (ANOVAs) was used for significance analyses and significant was considered when *p* value was less than 0.05.

Results and Discussion

Pharmacology studies

In the present study, ECG has been recorded to ascertain AMI model. The results of ECGs showed that T wave in AMI model group have been clearly inversed, indicating that the AMI model in the experiment was successful.

As shown in Table 1, levels of LDH and CK [26,27] in AMI model group were significant increased (*p*>0.01, compare with sham group) comparing with sham group, which also confirmed that the AMI model was successful. Additionally, SBP and SFSBP treated groups got significant intervention effects (*p*<0.05, compare with AMI model group) on the dysfunction of concentrations of serum enzymes. The results of serums enzymes indicated that the AMI model was reliable and the two treatments (SBP and SFSBP) were positively effective. Only the level of CK has been significantly reversed to sham level in ginsenoside Rb1 and borneol treated groups, none of the other single-component treatments got significant reverse effects on the two myocardial enzymes.

Table 1: Results of concentration of serum enzymes (LDH and CK) and infarct size of MI

Groups of administration	LDH (U/L)	CK (U/L)	Total heart weight/infarcted heart weight
Sham	673 ± 119	1339 ± 207	
AMI model	2275 ± 122 ^{##}	3882 ± 676 ^{##}	28.4 ± 3.14
SBP	1685 ± 261 [*]	2779 ± 155 [*]	24.4 ± 3.08
SFSBP	1725 ± 270 [*]	2852 ± 348 [*]	21.69 ± 4.98 [*]
Ginsenoside Re	2054 ± 733	3152 ± 727	23.0 ± 4.21
Ginsenoside Rb1	2038 ± 358	1522 ± 169 ^{**}	27.3 ± 0.52
Borneol	2219 ± 255	2027 ± 380 [*]	25.6 ± 5.20
Cinnamic acid	1985 ± 175	3962 ± 617	27.8 ± 3.20
Bufoalin	1878 ± 230	3804 ± 527	27.6 ± 5.16
Muscovone	2983 ± 212	3608 ± 475	24.2 ± 5.50
Cholic acid	2078 ± 245	2985 ± 380	26.0 ± 4.94

Note: ##represents *P* value <0.01 when compared sham group with AMI model group; *represents *P* value <0.05 when with AMI model group; **represents *P* value <0.01 when with AMI model group.

Furthermore, cardiac infarction size was also tested to validate the AMI model and evaluate the effect of different pretreatments. As shown in Table 1, only SFSBP got significant intervention effect on cardiac infarction size induced by AMI. None of the single-component treatments got significant reverse effects on cardiac infarction size.

Method validation of LC-Q-TOF/MS

For the validation of system repeatability, the relative standard derivations (RSDs) of these five peaks were less than 0.5% for retention times and 15% for peak areas in positive mode, while 1% for retention times and 15% for peak areas in negative mode. For the validation of system stability, the RSDs of these five peaks were less than 1% for retention times and 10% for peak areas in positive mode, while 0.5% for retention times and 16% for peak areas in negative mode.

The data demonstrated that the repeatability and stability of this proposed method was satisfactory for the large-scale sample sequence analysis.

Metabolomic study

Multivariate statistical analysis of LC-Q-TOF/MS data: A large number of ion signals were obtained from the serum samples. In this study, a PLS-DA approach was established and applied to identify biomarkers that related to the development of AMI. *R*²*Y* and *Q*²*Y* are important parameters for evaluating the model of PLS-DA. To acquire highly predictive ability of the model and ascertain the model is not over-fitted, the values of *R*²*Y* and *Q*²*Y* should be closed to 1. As shown in Figure 1, the PLS-DA score plots of AMI model and sham groups exhibited satisfactory classification both in positive and negative modes. The value of *R*²*Y* and *Q*²*Y* were 0.546 and 0.983 in positive and 0.503 and 0.992 in negative modes, respectively, indicating that the model had an acceptable prediction character.

After the PLS-DA processing, variable ions that significantly contributed to the clustering and discrimination were selected and identified according to the threshold of variable importance in the projection (VIP) values (VIP>1.5). Finally, 19 of them (7 in positive and 12 in negative) were identified by searching MS and MS/MS fragments data in Human Metabolome Database (HMDB) (<http://www.hmdb.ca>) and Biofluid Metabolites Database (<http://metlin.scripps.edu>), and part of them were confirmed by commercial standards. The details of the identified biomarkers were shown in Table 2.

Identification of biomarkers related to AMI and their functions:

As shown in Table 3, 14 out of 19 identified biomarkers were up-regulated and the other 5 were depressed in AMI group when compared with sham group. The relative pathways of these biomarkers were obtained by searching HMDB (<http://www.hmdb.ca>) and KEGG PATHWAY Database (<http://www.genome.jp/kegg/>), and a network of 18 identified biomarkers which might related to AMI has been established. As shown in Figure 2, these biomarkers were principally involved in tryptophan metabolism, pyrimidine metabolism, and amino acid metabolism, which related to 3 main pathological processes including oxidative injury, dysfunction of energy metabolism and inflammation the development of early period of AMI.

As shown in Figure 2, six biomarkers including 6-hydroxymelatonin, acetylcarnitine, L-tryptophan, kynurenic acid, N-acetyl-DL-tryptophan and tryptamine were involved in tryptophan metabolism. Among these biomarkers, essential amino acid tryptophan is considered as an important mechanism in immunological responses. The kynurenine (Kyn) pathway is the main route for non-protein metabolism of

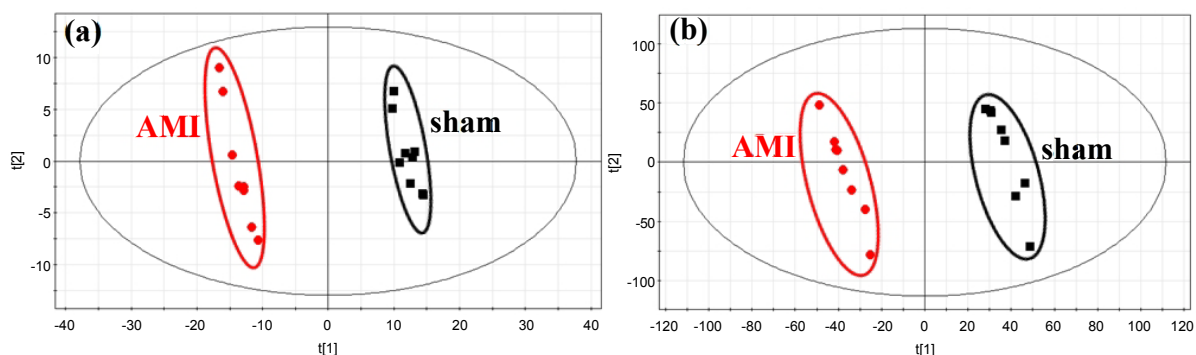


Figure 1: PLS-DA score plots of AMI model and sham groups. (a) PLS-DA score plot in positive mode; (b) PLS-DA score plot in negative mode.

Table 2: Identified 19 biomarker and related information

Mode	No.	Mass	R.T. (min)	Compound ^b	Formula	Trend ^a	Related pathway
ESI (+)	1	129.07	1.6	L-Pipecolic acid	C ₆ H ₁₁ NO ₂	↑	Not known
	2	248.11	2.19	6-Hydroxymelatonin	C ₁₃ H ₁₆ N ₂ O ₃	↑	Tryptophan metabolism
	3	243.09	2.68	Cytidine	C ₉ H ₁₃ N ₃ O ₅	↑	Pyrimidine metabolism
	4	203.12	2.75	Acetylcarnitine	C ₉ H ₁₇ NO ₄	↑	Tryptophan metabolism
	5	204.1	8.2	L-Tryptophan ^b	C ₁₁ H ₁₂ N ₂ O ₂	↑	Tryptophan metabolism
	6	242.09	6.55	Thymidine ^b	C ₁₀ H ₁₂ N ₂ O ₅	↓	Pyrimidine metabolism
	7	179.05	9.58	Hippuric acid ^b	C ₉ H ₁₄ NO ₃	↓	Phenylalanine metabolism
ESI (-)	8	117.04	1.87	Guanidineacetic acid	C ₃ H ₇ N ₃ O ₂	↑	Glycine, serine and threonine metabolism
	9	105.04	1.7	L-Serine ^b	C ₃ H ₇ NO ₃	↑	Glycine, serine and threonine metabolism
	10	308.04	2.42	dUMP	C ₉ H ₁₃ N ₂ O ₈ P	↓	Pyrimidine metabolism
	11	288.03	3.01	Orotidine	C ₁₀ H ₁₂ N ₂ O ₆	↑	Pyrimidine metabolism
	12	324.02	1.9	UMP ^b	C ₉ H ₁₃ N ₂ O ₉ P	↑	Pyrimidine metabolism
	13	189.04	6.3	Kynurenic acid ^b	C ₁₀ H ₇ NO ₃	↑	Tryptophan metabolism
	14	246.12	10.07	N-Acetyl-DL-tryptophan ^b	C ₁₃ H ₁₄ N ₂ O ₃	↑	Tryptophan metabolism
	15	219.12	5.97	Pantothenic Acid	C ₉ H ₁₇ NO ₅	↑	β-alanine metabolism
	16	160.11	8.02	Tryptamine ^b	C ₁₀ H ₁₂ N ₂	↓	Tryptophan metabolism
	17	189.08	8.89	3-Indolepropionic acid	C ₁₁ H ₁₁ NO ₂	↓	Not known
	18	280.2	12.8	12S-HHTrE	C ₁₇ H ₂₈ O ₃	↑	Archidonic acid metabolism
	19	320.24	14.32	12 (S)-HETE	C ₂₀ H ₃₂ O ₃	↑	Archidonic acid metabolism

Note: "↑" represents up-regulate compared with sham group, "↓" down-regulate compared with sham group. ^bmetabolites that have validated with authentic standards.

Table 3: Regulation effects of SFSBP and 7 single component treated groups.

Compounds	SBP	SFSBP	GRb1	GRe	CiA	Buf	ChA	Bro	Mus
L-Pipecolic acid	++		++						
6-Hydroxymelatonin		++							++
Cytidine	++								
Acetylcarnitine	++								
L-Tryptophan		++		++					++
Thymidine									
Hippuric acid	++				++	++			
Guanidineacetic acid	++	++	++	++	++	++	++	++	++
L-Serine	++		++			++		++	
dUMP	++	++	++	++	++	++	++		++
Orotidine	++	++	++	++	++	++	++	++	++
UMP	++								
Kynurenic acid	++	++							
N-Acetyl-DL-tryptophan									
Pantothenic Acid	++	++	++		++	++		++	++
Tryptamine	++	++		++		++		++	
3-Indolepropionic acid	++	++	++	++	++	++	++	++	
12S-HHTrE	+	+						+	
12 (S)-HETE	+	++			+				

Note: "+" represents P value <0.05, there is significant difference when compared with AMI model group. "++" represents P value <0.01, there is significant difference when compared with AMI model group. GRb1: Ginsenoside Rb1; GRe: Ginsenoside Re; CiA: Cinnamic acid; Buf: Bufalin; ChA: cholic acid; Bro: Borneol; Mus: Muscone.

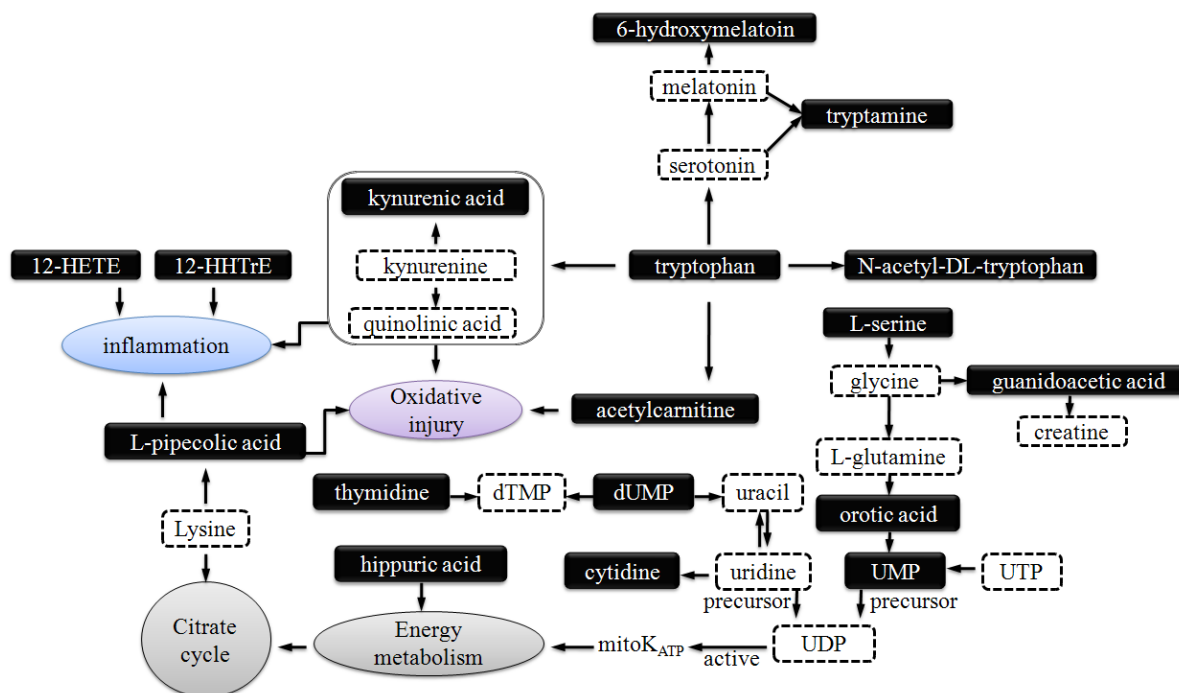


Figure 2: Network of identified 18 biomarkers. The dashed area denotes represent identified biomarkers.

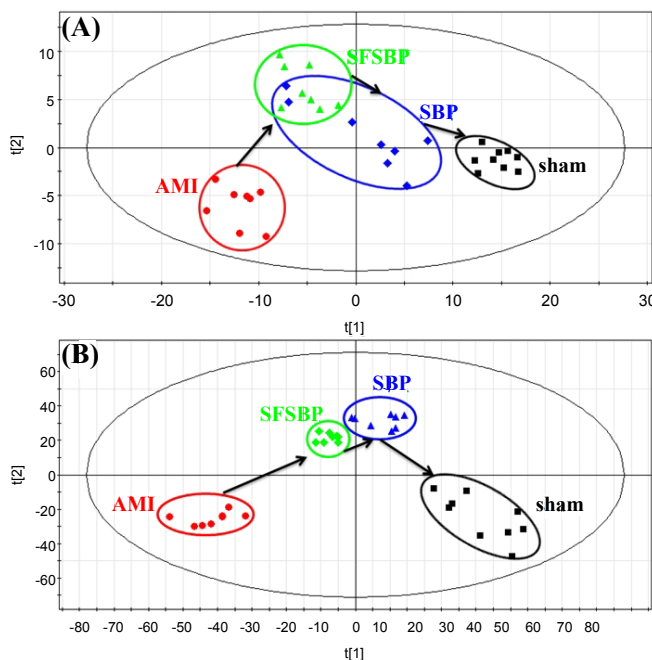
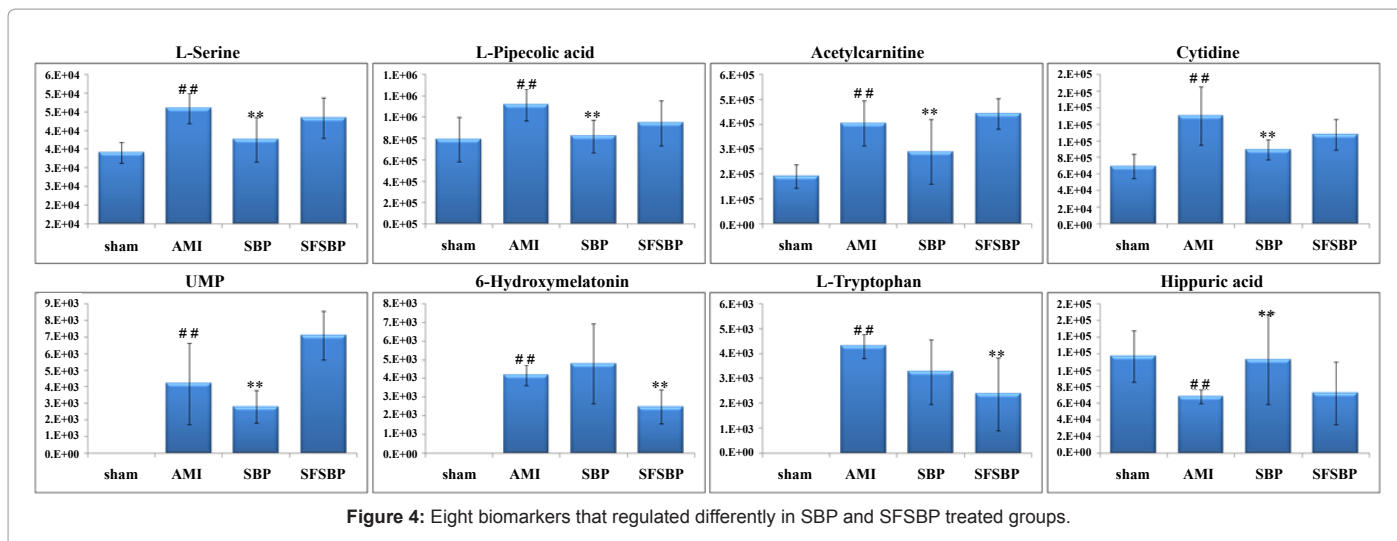


Figure 3: PLS-DA score plots of SBP, SFSBP, AMI and sham groups both in positive (A) and negative (B) modes. (A) $R^2X_{(cum)}=0.546$, $R^2Y_{(cum)}=0.988$, $Q^2Y_{(cum)}=0.729$; (B) $R^2X_{(cum)}=0.253$, $R^2Y_{(cum)}=1$, $Q^2Y_{(cum)}=0.666$.

tryptophan. Kynurenic acid is a product of oxidative tryptophan metabolism along the kynurenine (Kyn) pathway [28,29]. In addition, it is also involved in inflammation when tryptophan metabolic pathway is activated during inflammatory conditions [30-34]. Acetylcarnitine is an acetic acid ester of carnitine during the oxidation of fatty acid. When AMI occurs, acetylcarnitine significantly accumulated as

the intermediate of oxidative metabolism of carnitine increased [35]. Melatonin is an important biomarker involved in circadian dysregulation in the metabolism pathway of tryptophan [36,37], the concentration of 6-Hydroxymelatonin significantly increases when the circadian rhythm is disturbed. We have not found direct connection changes of melatonin in AMI rats, further studies will be needed to



find out the relationship between melatonin and AMI. Four biomarkers including thymidine, deoxyuridine monophosphate (dUMP), orotidine and uridine monophosphate (UMP) were involved in pyrimidine metabolism. Among these biomarkers, UMP and orotidine are involved in energy metabolism when AMI occurs. Two biomarkers including 12S-HHTrE and 12(S)-HETE which are in the pathway of Arachidonic acid metabolism are involved in inflammation [38,39].

Metabolomics study of protective effects of SBP and SFSBP: The pharmacology results of SBP and SFSBP shown in “3.1 pharmacology studies” demonstrated that there were positive reverse effects of SBP and SFSBP for treating AMI. To further evaluate the holistic intervention effects of SBP and SFSBP on AMI, PLS-DA score (Figure 4) plots both in positive and negative modes have been established. As shown in Figure 3, both treatments were far away from AMI model group and close to sham group in positive and negative modes. However, SBP treated group was located closer to sham group than SFSBP treated group, which indicated that the holistic reverse effects of SBP was superior to SFSBP group on AMI rats.

Additionally, reverse effects of identified biomarkers were also analyzed to evaluate the intervention efficacy of SBP and SFSBP treatments. Six biomarkers (L-serine, L-pipecolic acid, acetylcarnitine, cytidine, hippuric acid and UMP) were significantly regulated to sham level in SBP treated group but not in SFSBP treated group. Contrarily, two biomarkers (6-hydroxymelatonin and L-tryptophan) were positively regulated to sham level in SFSBP treated group but not in SBP treated group. Among these biomarkers, acetylcarnitine, 6-hydroxymelatonin and L-tryptophan were involved in dysfunction of tryptophan metabolism, indicating that both SBP and SFSBP treatments got significant regulation effects on the dysfunction of tryptophan metabolism. Furthermore, UMP and hippuric acid were involved in dysfunction of energy metabolism, and L-pipecolic acid was involved in inflammation and oxidative injury, indicating that SBP was superior in regulating the dysfunction of energy metabolism, inflammation and oxidative injury that induced by AMI.

All these results demonstrated that the holistic therapeutic effects of SBP were slightly superior to SFSBP on AMI rats. Considering the fact that the concentration of SFSBP treated group was much higher than SBP treated group, whereas, the regulation effect of SFSBP treated group on AMI rat was inferior to SBP treated group, indicating that

there might be other bioactive components which take responsibility for the protective effects of SBP. Furthermore, the regulation results of SFSBP, SBP and 7 mono-therapy treatments on AMI rats also indicating the rationality of the TCM formula, and TCM formula cannot be simply substituted by a single bioactive compound or a combination of bioactive compounds from the formula.

Comparison of regulation effects of SFSBP and its' 7 constitutes mono-therapy groups on AMI rats: According to PLS-DA score plots (Figure 5) that built to evaluate regulation effects of SFSBP and its 7 constitutes treated groups on AMI holistically both in negative and positive modes. As was shown in Figure 5, none of the mono-therapy groups located closer to sham group than SFSBP treated group in both modes, whereas there are overlap between mono-therapy and SFSBP treated groups in negative modes. However, both results indicate that the holistic regulation effect of SFSBP was superior than single component treated groups.

Mean levels of 19 identified biomarkers were also analyzed to evaluate the therapeutic effects of SFSBP and 7 mono-components. As was shown in Table 3, twelve biomarkers were significantly reversed by SFSBP, while there were 7, 6, 7, 8, 4, 7 and 6 biomarkers were significantly reversed by ginsenoside Rb1, ginsenoside Re, cinnamic acid, bufalin, cholic acid, borneol and muscone respectively. Two biomarkers including kynurenic acid and 12S-HHTrE were significantly reversed to sham level but not in any single component treated groups. The two biomarkers were involved in inflammation, indicating that SFSBP was superior in regulating inflammation than any other mono-therapy groups.

All these results demonstrated that SFSBP showed a stronger and more stable therapeutic effect on treating acute myocardial injury. Considering the fact that each mono-therapy treated groups was presented at a higher dose concentration than SFSBP treated group, SFSBP showed the highest degree of recovery among the 8 different treated groups. These results were consistent with pharmacology results indicating that SFSBP could amplify the therapeutic effects of each constitute of SFSBP and might exert synergistic therapeutic efficacies

Conclusion

In the present study, metabolomics technology coupled with LC-Q-

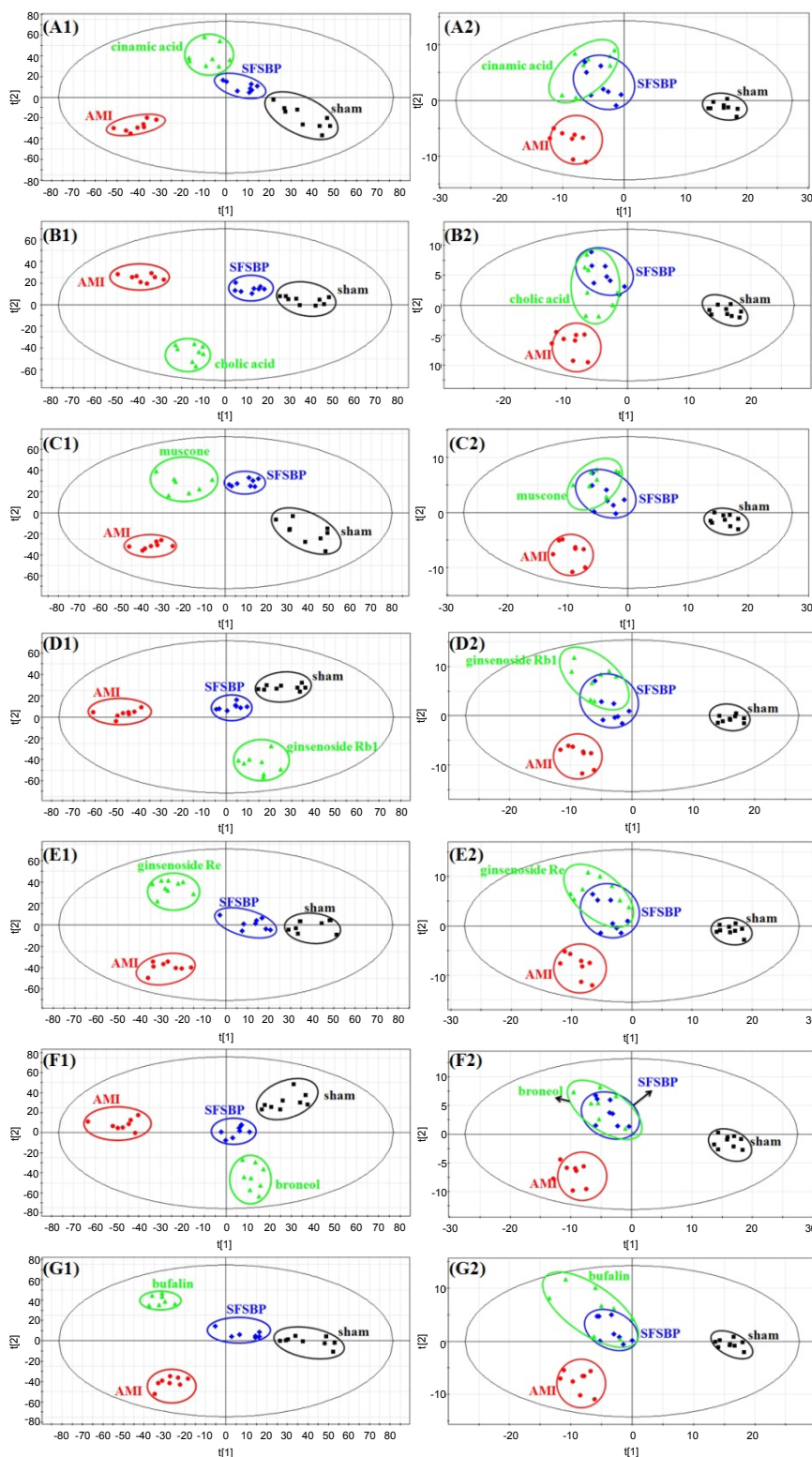


Figure 5: PLS-DA score plots of 7 mono-therapy groups and SFSBP treated group both in negative and positive modes. (A1) $R^2X_{(cum)}=0.553$, $R^2Y_{(cum)}=0.991$, $Q^2Y_{(cum)}=0.781$; (B1) $R^2X_{(cum)}=0.549$, $R^2Y_{(cum)}=0.985$, $Q^2Y_{(cum)}=0.671$; (C1) $R^2X_{(cum)}=0.534$, $R^2Y_{(cum)}=0.993$, $Q^2Y_{(cum)}=0.708$; (D1) $R^2X_{(cum)}=0.562$, $R^2Y_{(cum)}=0.993$, $Q^2Y_{(cum)}=0.714$; (E1) $R^2X_{(cum)}=0.56$, $R^2Y_{(cum)}=0.991$, $Q^2Y_{(cum)}=0.714$; (F1) $R^2X_{(cum)}=0.517$, $R^2Y_{(cum)}=0.985$, $Q^2Y_{(cum)}=0.775$; (G1) $R^2X_{(cum)}=0.539$, $R^2Y_{(cum)}=0.979$, $Q^2Y_{(cum)}=0.765$; (A2) $R^2X_{(cum)}=0.269$, $R^2Y_{(cum)}=0.999$, $Q^2Y_{(cum)}=0.606$; (B2) $R^2X_{(cum)}=0.261$, $R^2Y_{(cum)}=1$, $Q^2Y_{(cum)}=0.755$; (C2) $R^2X_{(cum)}=0.273$, $R^2Y_{(cum)}=0.999$, $Q^2Y_{(cum)}=0.696$; (D2) $R^2X_{(cum)}=0.261$, $R^2Y_{(cum)}=1$, $Q^2Y_{(cum)}=0.715$; (E2) $R^2X_{(cum)}=0.26$, $R^2Y_{(cum)}=0.999$, $Q^2Y_{(cum)}=0.701$; (F2) $R^2X_{(cum)}=0.231$, $R^2Y_{(cum)}=0.998$, $Q^2Y_{(cum)}=0.647$; (G2) $R^2X_{(cum)}=0.272$, $R^2Y_{(cum)}=0.999$, $Q^2Y_{(cum)}=0.723$. A1-G1 was in positive modes; A2-G2 was in negative modes.

TOF/MS has been used to study the protective effect of a TCM formula (SBP) on AMI rats. The results showed that the prevention effects of SFSBP is weaker than SBP, but much better than its' single component constitutes treatments, indicating the rationality of TCM formula, and TCM formula cannot be simply substituted by a single bioactive compound or a combination of bioactive compounds from it. Finally, we believe that such holistic study strategy is an efficacious approach for studying the advantages of multi-component and multi-effective character of TCM.

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