Journal of Ethnopharmacology 138 (2011) 530–536

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

# Biomarkers in the early period of acute myocardial infarction in rat serum and protective effects of *Shexiang Baoxin Pill* using a metabolomic method

Peng Jiang[a], Weixing Dai[a], Shikai Yan[b], Zhongliang Chen[c], Ruilin Xu[c], Jianmi Ding[c], Li Xiang[a], Shuping Wang[a], Runhui Liu[a,*], Weidong Zhang[a,b,**]

[a] *School of Pharmacy, Second Military Medical University, Shanghai 200433, PR China*
[b] *School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200030, PR China*
[c] *Shanghai Hutchison Pharmaceuticals Company, Shanghai 200331, PR China*

ARTICLE INFO

*Article history:*
Received 24 February 2011
Received in revised form
12 September 2011
Accepted 18 September 2011
Available online 1 October 2011

*Keywords:*
Biomarkers
Serum
Early period
Myocardial infarction
Metabolomic
Traditional Chinese medicine

ABSTRACT

*Ethnopharmacological relevance:* To identify the biomarkers in early period of acute myocardial infarction (AMI) in rat serum and reveal the effective mechanism of a Traditional Chinese Medicine (TCM) named *Shexiang Baoxin Pill* (SBP).
*Material and method:* A metabolomic approach using reversed-phase liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) was developed.
*Results:* Fourteen biomarkers in the early period of acute myocardial infarction (AMI) in rat serum were identified. These biomarkers include 5-methylcytosine, cystathionine ketimine, 2-oxoadipic acid, thymidine, epinephrine, homocystine, uric acid, 12(S)-hydroperoxyeicosatetraenoic acid (12s-HPETE), 11-dehydrocorticosterone, 12(S)-hydroxyeicosatetraenoic acid (12s-HETE), deoxycorticosterone, corticosterone, aldosterone and cortisol. Through pathway analysis of these biomarkers, inflammation, hypertrophy and oxidative injury were considered the most relevant pathological changes in early period of AMI.
*Conclusion:* Identification of AMI biomarkers not only supplied a systematic view of the progression of AMI in the early period but also provided the theoretical basis for the prevention or treatment of AMI. The results demonstrated that SBP pretreatment could offer protective effects for AMI through regulating the pathway of steroid hormone biosynthesis.



## 1. Introduction

Cardiovascular diseases (CVDs) are the world's largest killers and will claim 23.6 million lives by 2030 according to the report of World Health Organization (http://www.who.int/mediacentre/factsheets/fs317/en/index.html). Most CVDs such as angina, arrhythmias and heart failure are associated with the formation of acute myocardial infarctions (AMIs). Though previous studies have identified some important biomarkers such as troponin I and T for the early diagnosis of AMI (Apple and Wu, 2001), the mortality induced by AMI is still high. Further investigation to achieve an understanding of the biological process of AMI is therefore still needed to find new biomarkers and illuminate the disease mechanism. Because the earlier detection and treatment of AMI can greatly decrease the mortality and morbidity of patients, the identification of the related biomarkers in the early period of AMI will be important.

Metabolomics is a newly developed method which can be used to monitor the changes of metabolites in biofluids. Through identification technology such as liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR), the metabolite profiles will be established. Then, by using multivariate analysis, screening the data from metabolite profiles will become possible and identification of potential biomarkers related to the

*Abbreviations:* SBP, *Shexiang Baoxin Pill*; AMI, acute myocardial infarction; TCM, traditional Chinese medicine; CVs, cardiovascular diseases; LC–MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; LADCA, left anterior descending coronary artery; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; CKs, creatine kinases; ECGs, electrocardiograms; CMC-Na, carboxymethyl cellulose sodium salt; QC, quality control; LC–Q-TOF-MS, liquid chromatography–quadrupole-time of flight-mass spectrometry; ESI, electrospray ionization; ANOVAs, one-way analyses of variance; RSD, relative standard deviation; PLS-DA, partial least squares-discriminate analysis; LVs, latent variables; VIP, variable importance plot; AA, arachidonic acid; 12(S)-HPETE, 12(S)-hydroperoxyeicosatetraenoic acid; 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid.

* Corresponding author. Fax: +86 21 81871245.
** Corresponding author at: School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China. Fax: +86 21 81871244.
*E-mail addresses:* lyliurh@126.com (R. Liu), wdzhang@hotmail.com (W. Zhang).

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.09.049

changes in the physiological state will also become possible. The identified metabolites may be successfully incorporated into clinical practice in the future. Metabolomics has been applied to the prediction of many diseases such as septemia and hepatotoxicity (Xu et al., 2008; Wang et al., 2008).

Though some biomarkers related to AMI have been identified using a metabolomics method in recent years (Zhang et al., 2009; Lv et al., 2010; Yao et al., 2010), the metabolites that changed in the early period of AMI had still not been investigated. To achieve a complete understanding of the metabolite profile associated with AMI in the early period, an AMI model using the left anterior descending coronary artery (LADCA) ligation method was adopted, and then the serum biomarkers were analyzed after 5 h of the heart operation using a metabolomic method.

*Shexiang Baoxin Pill* (SBP), which consists of seven medicinal materials including *Moschus*, *Radix Ginseng*, *Calculus Bovis*, *Cortex cinnamomi*, *Styrax*, *Venenum Bufonis* and *Borneolum Syntheticum*, is a widely used traditional Chinese medicine (TCM) in clinical practice for the treatment of AMI (Song et al., 2002; Wu et al., 2005; Yang et al., 2005; Ye, 2006). SBP was also officially recorded in the 2010 edition of the Chinese pharmacopoeia (Editorial Committee of Pharmacopoeia of Ministry of Health PR China, 2010). To elucidate its intervention effects and the mechanism of AMI, the established metabolomic method was also adopted.

## 2. Experiment

### 2.1. Materials

High performance liquid chromatography (HPLC)-grade acetonitrile and formic acid were purchased from JT Baker (NJ, USA). Ultrapure water from a Milli-Q50 SP reagent water system (Millipore Corporation, MA, USA) was used for the preparation of samples and mobile phase. The assay kits for lactate dehydrogenase (LDH) and creatine kinase (CK) were purchased from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China). Commercial standards were purchased from Sigma/Aldrich (MO, USA). SBP was kindly offered by Shanghai Hutchison Pharmaceuticals Company (Shanghai, China).

### 2.2. Animal and MI model

Twenty male Sprague-Dawley rats ($200 \pm 15$ g) were purchased from the Slac Laboratory Animal Co., Ltd. (Shanghai, China). The animals were housed in stainless steel metabolic cages with free access to food and tap water under standard conditions of humidity ($50 \pm 10\%$), temperature ($25 \pm 2\,^{\circ}C$) and 12 h light–dark cycle. The animals were acclimatized to the facilities for 5 days. All animals were handled with humane care throughout the experiment.

The AMI model was established by LADCA ligation, as described previously (Wang et al., 2002). The chest was opened by a middle thoracotomy under sterile conditions. After pericardiotomy, the heart was rapidly exteriorized and a 4-0 black silk ligature was then placed under the LADCA to form an occlusion. Three rats died before collecting serum. Seventeen rats survived, including 11 AMI rats and 6 control rats (without ligation). All the rats were fixed on pad in a position on the back and two-lead electrocardiograms (ECGs) were recorded by the MPA 2000 biosignal analytical system (Shanghai Alcott Biotech Co., Ltd., Shanghai, China) after 5 h of the heart operation.

### 2.3. Drug administration and sample collection

According to the clinical dosage of SBP (2.25 mg/kg/d), 6 of 11 AMI rats were treated with SBP at a dosage of 14 mg/kg/d by oral gavage on 4 consecutive days before LADCA ligation (Wang et al., 2004; Hu et al., 2009). SBP was ground into a fine powder and dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution (2.8 mg/mL). Isocyatic CMC-Na aqueous solution was administered orally to control ($n = 6$) and AMI ($n = 5$) rats. Serum was immediately collected from the ophthalmic venous plexus after recording ECGs. The collected serum was divided into two parts. One part was used for the analysis of serum concentrations of LDH and CK, and the other part was used for the metabolomic analysis. Serum concentrations of LDH and CK were measured by UV 1100 ultraviolet spectrophotometer (Beijing Rayleigh Analytical Instrument Corp., Beijing, China).

The experiment was carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Shanghai, China. The study protocol was approved by the Animal Care and Use Committee of the Second Military Medical University.

**Table 1**
HPLC gradient elution programme.

| Time (min) | *A*% (0.1% formic acid in $H_2O$) | *B*% (acetonitrile) |
|---|---|---|
| 0 | 97 | 3 |
| 3 | 80 | 20 |
| 6 | 40 | 60 |
| 12 | 40 | 60 |
| 13 | 20 | 80 |
| 14 | 2 | 98 |
| 20 | 2 | 98 |

### 2.4. Sample preparation

For LC–MS analysis, 50 μL of rat serum was extracted with 150 μL acetonitrile. After vortex-mixing for 30 s, these samples were centrifuged at 12,000 rpm for 10 min to remove proteins. The supernatant was then transferred to autosampler vials.

To ensure the stability of sequence analysis, a quality control (QC) sample was prepared by pooling the same volume (10 μL) from each serum sample and then preparing the pooled QC sample in the same way as the samples. The pooled QC sample was analyzed randomly through the analytical batch. In addition, six aliquots of serum sample from the same rat were treated in the same process to validate the repeatability of the sample preparation method.

### 2.5. Conditions of liquid chromatography–quadrupole-time of flight-mass spectrometry (LC–Q-TOF-MS)

Metabolomic analysis was performed on an Agilent-1200 LC system which was coupled with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent-6520 accurate-mass Q-TOF mass spectrometer. The separation of all samples was performed on an Eclipse Plus C18 column (1.8 μm, 2.1 mm × 100 mm, Agilent) with a column temperature maintained at 40 °C. The flow rate was 0.3 mL/min and the mobile phase consisted of ultrapure water with 0.1% formic acid (A) and acetonitrile (B). The gradient programme is shown in Table 1. The sample injection volume was 3 μL.

The mass spectrometer was operated in both positive and negative ion modes with parameters set as follows: drying gas ($N_2$) flow rate, 8 L/min; gas temperature, 330 °C; pressure of nebulizer gas, 35 psig; $V_{cap}$, 4000 V; fragmentor, 160 V; skimmer, 65 V; scan range, $m/z$ 50–1000. The MS/MS analysis was acquired in the targeted MS/MS mode with collision energy ranging from 5 V to 20 V.