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Integrating candidate metabolites and biochemical factors to elucidate the action mechanism of Xue-sai-tong injection based on ^{1}H NMR metabolomics

Miaomiao Jiang[a], Xiaoping Zhao[b,*], Linli Wang[c], Lei Xu[a], Yan Zhang[a], Zheng Li[a,*]

[a] Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, PR China
[b] College of Preclinical Medicine, Zhejiang Chinese Medical University, Hangzhou 310053, PR China
[c] Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, PR China

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ABSTRACT

A strategy of integrating candidate metabolites with crucial biochemical factors was proposed in this study to discover relevant biological functions for interpreting the action mechanism of Traditional Chinese Medicines (TCM). This approach was applied to Xue-Sai-Tong injection (XST) to reveal the action mechanism based on the metabolic response in an ischemia/reperfusion (I/R) rat model by analyzing NMR profile. Partial least squares discriminate analysis (PLS-DA) was used to compare metabolic profiles of serum samples and revealed nine metabolites altered by I/R injury could be restored to normal status (sham-operated group) under the therapy of XST. The pathway enrichment analysis suggested the metabolic changes were mainly involved in pyruvate metabolism, glycolysis, and citrate cycle. The functional roles of the candidate metabolites were further identified by Pearson correlation analysis with the key biochemical factors in serum. The results indicated pyruvate, succinate, acetate and lysine showed significant associations with the oxidative stress factors. Elevated level of pyruvate was found as an essential metabolic response for the major effect of XST against I/R injury by enhancing glycolysis and overcoming the induced reactive oxygen species (ROS). This metabolomics approach provides a better understanding of the mechanisms of TCM and helps to develop a holistic view of TCM efficacy.

1. Introduction

Traditional Chinese Medicines (TCM) treats diseases with multiple components acting upon multiple targets through multiple pathways. It is thus important to elucidate the complex action mechanism of TCM to improve its clinical efficacy and safety. The emergence of omics technologies provides a global exploration to systematically elucidate the action mechanism [1]. Metabolomics in particular enables a comprehensive analysis of the metabolic responses in an organism to pathophysiological stimuli or genetic modification [2]. By measuring and mathematically modeling changes of endogenous metabolites in biofluids, metabolomics exhibits significant potential to offer a unique perspective for elucidating the action mechanism of TCM [3–6].

Nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–tandem mass spectrometry (GC–MS), and liquid chromatography–tandem mass spectrometry (LC–MS) are three major analytical techniques for metabolomics [7,8]. We summarized in Table 1 the advantages and drawbacks of these techniques for the analysis of biological samples such as blood serum and urine. In general GC–MS and LC–MS yield a higher sensitivity and specificity, but need more time-consuming sample preparation and chromatographic separation. In a given sample, GC–MS mainly restricts the detection to volatilizable and stable metabolites, while LC–MS can cover polar and non-polar metabolites only through hyphenating with different chromatographic columns instead of in a one-step process [9,10]. On the other hand, the inherent properties of NMR allow it to be a universal and unbiased technique with the capability to detect all metabolites that bear hydrogen atoms [11,12]. To prevent NMR from low sensitivity and poor resolution, cryoprobe can be used to equip with NMR instrument and optimize with two-dimensional NMR (2D NMR) experiments. NMR thus appears to be a more suitable method for performing non-targeted metabolic profiling of a biofluid sample to reflect the holistic effect of TCM.

In the present study, a strategy was proposed to investigate the related biological functions of the pathways linked with candidate metabolites and discover the potential action mechanism

* Corresponding authors. Fax: +86 22 5959 6536.
E-mail addresses: zhaoxiaoping@zcmu.edu.cn (X. Zhao), lizheng1@gmail.com (Z. Li).

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Table 1
Comparison of the major analytical techniques used in metabolomics studies.

Properties	GC–MS	LC–MS	NMR
Sample preparation	Weight/dilution/derivatization Destroying samples	Weight/dilution/filtration Destroying samples	Weight/dilution Non-destructive and recovered samples
Detection	Restriction of volatile and stability Sample bias	Restriction of volatile and stability Sample bias Matrix effect	Universal detection Sample unbiased
Metabolites identification	Standards and data base dependent	Standards and data base dependent	Standards and data base independent 2D NMR methods for identification
Reproducibility	Instrument dependent	Instrument dependent	Instrument independent
Sensitivity	High nM–pM	High nM–pM	Low μM
Specificity	High	High	Low Resonance overlapping

(Fig. 1). From the metabolic profiles of biofluids, the significantly altered metabolites were subsequently selected by classification procedures including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) [13]. As a result, about a quarter to a third of identified metabolites can be extracted as differential candidates for indicating metabolic responses under a therapeutic intervention of TCM [14–17]. The next important issue is how to take advantage of these candidate metabolites for elucidating the mechanism of TCM, however, which has not been effectively resolved through a well-established way. A new framework was proposed to investigate the related biological functions of the pathways linked with candidate metabolites through relational biochemical factor analysis (Fig. 1). The testing biochemical factors in biofluids were carefully chosen according to the "gold standards" as well as the generally accepted views in the physiological and pathological processes of diseases. Visualization of the relationships between the candidate metabolites and the crucial factors are further established to extract the direct information that could be transformed to biological knowledge to interpret the action mechanism of TCM.

As a case study, we applied ^{1}H NMR-based metabolomics approach in combination with the above mentioned strategy to interpret the protective mechanisms of XST against myocardial Ischemia/reperfusion (I/R) injury. I/R injury is the most serious adverse event after thrombolytic therapy and cardiac surgery, such as percutaneous coronary intervention, on the patients with myocardial infarctions (MI) [18–21]. The currently known mechanisms of myocardial I/R injury include energy metabolism, free radicals, calcium overload, and endothelial dysfunction [22]. Despite of the substantial progress in understanding the mechanisms of I/R, the results of translating these findings to patient care have been largely disappointing [23]. Recent studies on the MI models reveal that TCMs have the potential to ameliorate I/R injury by pre- or post-conditioning during reperfusion [24,25]. *Panax notoginseng* (Burk) F. H. Chen, also known as Sanqi, is a highly valued medicinal plant used to treat cardiovascular diseases in East Asia. Xue-Sai-Tong injection (XST), a drug prepared with the total saponins of Sanqi [26], has been widely used in the clinical treatment of myocardial infarction, cerebral infarction, thrombosis, and coronary heart disease. Notoginsenoside R1, ginsenoside Rg1, Rb1, Rd and Re have been identified as the main active ingredients of XST [27–29], and the individual saponin displayed protective effect on myocardial I/R injury with different mechanisms [30–34]. Our main objective is to provide valuable metabolic clues to elucidate the holistic action mechanism of XST on ameliorating myocardial dysfunction.

As a result, the differential metabolites extracted by statistical analysis were obtained as candidates and the relationship between the crucial biochemical factors and the candidate metabolites of serum samples were further established by Pearson correlation analysis. The metabolites with significant correlations with the biochemical factors were recognized as the key ones with potential biological functions. XST treatment was found to induce a series of changes in energy metabolism including glycolysis, pyruvate metabolism, and citrate cycle by significantly up-regulating the levels of pyruvate, succinate, acetate and lysine which were in close relationship with the elevations of the oxidative stress factors, including T-SOD, GSH and GSH-P*x*. The involvement of these metabolites such as succinate accumulation in I/R injury through ROS related pathways has been validated in literature [35]. Therefore, integrating ^{1}H NMR metabolomics with biochemical factors analysis is an effective approach to elucidate the action mechanism of TCM.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade sodium chloride, sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Phosphate buffer was prepared with 0.1 M Na_2HPO_4–NaH_2PO_4 and stored at −20 °C. Deuterium oxide (D_2O, 99.9% in D) was bought from Cambridge Isotope Laboratories, Inc. (MA, USA). Chloral hydrate was obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). 2,3,5-Triphenyltetrazolium chloride (TTC) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Rat interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) ELISA Kits, and creatine kinase-MB (CK-MB) assay kits were from Bio-Swamp Immunoassay R&D Center (Shanghai, China). Total superoxide dismutase (T-SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-P*x*), glutathione (GSH), and lactate dehydrogenase (LDH) were purchased from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). The XST lyophilized powder for injection in clinical practices (Batch no. A20130203) was manufactured by Heilongjiang Zhenbaodao pharmaceutical Co., Ltd. (Heilongjiang, China).

2.2. Animals

Male Sprague-Dawley (SD) rats (220–260 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a certified SPF facility at the centre for drug safety assessment and research of Tianjin Institute of Pharmaceutical Research (Tianjin, China). All procedures were approved by Institutional Animal Ethical Committee of Tianjin Centre for Drug Safety Assessment (Tianjin, China) in accordance with the national