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Short communication

Clinical metabolomics analysis of therapeutic mechanism of *Tongmai Yangxin* Pill on stable angina

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ABSTRACT

Tongmai Yangxin Pill (*TMYX*) is a traditional Chinese medicine for the treatment of angina and arrhythmia. Although its clinical application is extensive, and the curative effect is significant, little information is available on the molecular biological basis and therapeutic mechanism of *TMYX* for the treatment of stable angina. In this study, we analyzed serum samples of clinical patients collected from seven different clinical units in China after oral administration of *TMYX* using ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS). Multiple statistical analysis including principal component analysis (PCA) and partial least square discrimination analysis (PLS-DA), were used to examine metabolite profile changes in serum samples. After *TMYX* treatment, 10 biomarkers were reversed to the normal conditions. The above biomarkers were mainly involved in energy metabolism, amino acid metabolism, oxidative stress and inflammation. These results suggested that *TMYX* exerted therapeutic effects by improving myocardial energy supply disorder and amino acid dysfunction, and attenuating oxidative stress and inflammation. The present study, as the first multicenter clinical study which reveals the molecular biological basis and therapeutic mechanism of *TMYX* on stable angina, can provide objective indicators for efficacy evaluation of *TMYX* on stable angina. And it also lays a foundation for the use of *TMYX* clinically.

1. Introduction

TMYX, a commonly used clinical drug, has been marketed in China for the treatment of CHD, angina, palpitation and irregular heartbeat [1]. It is collected in the *Chinese Pharmacopoeia 2015* and is composed of eleven Chinese medicinal herbs: *Rehmannia glutinosa*, *Caulis Spatholobi*, *Ophiopogon japonicus*, *Glycyrrhiza uralensis*, Radix Polygonum Multiflorum, Ejiao, *Schisandra chinensis*, *Codonopsis*, tortoise, Jujubae and Cassia twig [2,3]. Wang et al. [4,5] demonstrated that *TMYX* has antioxidant and anti-inflammatory effects through animal experiments and cell experiments in vitro. Sun Lanjun et al. [6] carried out the clinical observation of *TMYX* and observed its clinical effectiveness through the related signs, myocardial enzyme detection, blood lipid detection, electrocardiogram, and cardiac ultrasound. However, up till now, no study especially clinical metabolomics study, has been able to systematically explain the molecular biological basis and mechanism of *TMYX*.

Metabolomics, an important component of systems biology, aims to monitor changes of endogenous metabolites with small molecular weight in physiological or pathological states. As a mature technique, it has become widely used in many medical related fields, such as disease diagnosis, pharmacodynamic mechanisms, and drug toxicology [7–9].

In this study, we selected study subjects from seven clinical units in China. UPLC/Q-TOF-MS was used to perform metabolomics analysis of serum samples of healthy people, stable angina patients and patients after treatment. Then combined with conventional biochemical detections, the molecular biological basis and therapeutic mechanism of *TMYX* in treating stable angina was analyzed.

2. Materials and methods

2.1. Clinical trial registration and ethics statement

The trial was registered with the China Clinical Trial Registration

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Center and the America Clinical Trial Registration Center. The registration numbers were ChiCTR-OOC-15006765 and NCT02526381 respectively. The registration unit was Tianjin University of Traditional Chinese Medicine. This study has been reviewed by the Ethics Committee of Tianjin University of Traditional Chinese Medicine, and the approval number TJUTCM-EC20150001 has been examined. This experimental study follows the Declaration of Helsinki.

2.2. Apparatus and reagents

ALLLEGRATM-64R High Speed Centrifuge (Beckman USA), KQ-300DV NC Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co., Ltd., Jiangsu Province), XK96-A Quick Mixer (Jiangyan Xinkang Medical Instrument Co., Ltd.), Waters ACQUITY UPLC Liquid Chromatograph (Waters, Milford, USA), Waters Xevo G2 Q-Tof/MS Mass Spectrometer (Waters, USA), ACQUITY UPLC BEH C18 Column (2.1 × 100 mm, 1.7 m) (Waters Company, Milford, USA), High pressure liquid chromatography (HPLC)-grade acetonitrile and formic acid were provided by Oceanpak (Gothenburg, Sweden) and ROE SCIENTIFIC INC (Beijing, China), respectively [10]. Distilled water was purchased from Wahaha (Hangzhou, China). *TMYX* was obtained from Tianjin Zhongxin Pharmaceutical Group Corporation Limited. (Biochemical testing reagents were placed in Supplementary material).

2.3. Participants collection and clinical information

A total of 38 stable angina patients and 40 healthy participants were enrolled from seven clinical medical units from April 2016 to April 2017, with the informed consent of each participant. According to the relevant guidelines for diagnosis of angina in American College of Cardiology (ACC)/American Heart Association (AHA) revised edition, and guideline for diagnosis and treatment of patients with chronic stable angina published in 2007 [11], the study candidates all comply with any one or more of the following: 1) who can be diagnosed with clear history of myocardial infarction. 2) Who underwent coronary angiography or coronary angiography (CTA) examination showed at least one of the main branches of coronary artery lumen diameter stenosis 50%. 3) Who underwent coronary artery revascularization [percutaneous coronary intervention (PCI) or coronary artery bypass grafts (CABGs)] treatments. All patients underwent differential diagnoses to exclude diseases that may cause chest pain, such as unstable angina, hyperthyroidism, or gastro-esophageal reflux disease. The age-matched healthy participants were aged between 35 and 65, and they adhered to strict standards for inclusion [12]. We established strict subject inclusion and exclusion criterions to reduce the impact of confounding factors such as gender, age, and location on experimental outcomes. During this study, patients with stable angina can maintain the original regimen before admission, and the introduction period was 2 weeks, so that the condition of patients was stable, in line with the criteria of the group. But the use of beta blockers, calcium channel blockers, energy metabolism drugs, nitrates in the 4 do not allow > 3 kinds of drugs. In addition, it is forbidden to add any other western medicines that can be used to treat stable angina. *TMYX* specifications: 10 pills weight 1 g, and patients with stable angina underwent oral administration 40 pills each time, 2 times daily for 8 weeks. The clinical characteristics of stable angina and control subjects are listed in Table 1. (Details of clinical trial were placed in Supplementary material).

2.4. Samples collection and preparation

We established strict sample collection standards at the beginning of the program. All candidates were notified that smoking, alcohol, coffee, tea, cheese, chocolate and other caffeinated foods or beverages were forbidden during the week prior to the experiment. After a light diet for one week, fasting blood samples were collected and centrifuged at 3000 rpm for 15 min at 4 °C within 2 h. And the supernatant was centrifuged at 3500 rpm for 8 min at 4 °C. The obtained supernatant is serum, and it was stored in a −80 °C refrigerator for metabolomics research. Samples should be handled gently to avoid turbidity.

The serum samples were removed from refrigerator and thawed at room temperature. 100 μL serum was mixed with 300 μL acetonitrile for the protein precipitation. The resultant mixture was ultrasonicated in ice water for 10 min, vortexed for 1 min, and then centrifuged at 13000 rpm for 15 min at 4 °C. The supernatants were used for the metabolomic analysis.

We singled out the serum samples from each group, and mixed them together to make quality control (QC) samples. Containing the biological information of all samples, QC samples were used for method validation to monitor the reproducibility and stability of the system. QC results were shown in supplementary material.

Table 1
Demographic and clinical characteristics of subjects.

Characteristics	Healthy people	Stable angina
n	40	38
Sex (male/female)	21/19	29/9
BMI (kg/m^2)	23.97 ± 2.29	25.49 ± 3.06
Blood pressure, mm Hg		
Systolic	118.25 ± 10.22	129.63 ± 13.68
Diastolic	74.13 ± 6.12	83.63 ± 8.53
Waist circumference	84.39 ± 9.52	90.51 ± 10.09
Hip circumference	97.38 ± 7.87	101.00 ± 7.46
Combined disease		Frequency (%)
High blood pressure		18 (75%)
Diabetes		4 (16.67%)
Gastritis		5 (20.83%)
Thyroid dysfunction		1 (4.17%)
Cerebral infarction		1 (4.17%)
Cervical spondylosis		2 (8.33%)
Degenerative knee disease		1 (4.17%)
Benign prostatic hyperplasia		1 (4.17%)
Lumbar disc herniation		1 (4.17%)
Sleep disorder		1 (4.17%)

Biochemical indicators	Healthy people	Stable angina group	*TMYX* treatment group
TG	1.21 (1.93, 0.86)	1.35 (0.90, 2.25)	1.29 (0.93, 1.75)
LDL-c	3.16 (3.71, 2.42)	2.24 (1.62, 2.63)	2.05 (1.66, 2.54)
ApoA1	1.40 (1.59, 1.28)	1.28 (1.19, 1.43)	1.36 (1.22, 1.51)
HDL-c	1.21 (1.53, 1.01)	1.01 (0.89, 1.24)	1.04 (0.92, 1.23)
sCD40L	11,235.75 (12,826.75, 9072.50)	11,437.75 (9533.50, 12,433.75)	10,215.75 (8295.75, 11,953.00)
IL-1	44.50 (66.00, 31.00)	39.00 (28.00, 60.25)	28.00 (26.25, 41.25)
TNF-α	181.50 (232.00, 129.25)	155.50 (116.50, 204.00)	125.00 (82.50, 159.00)
GMCSF	24.00 (36.00, 18.00)	21.25 (18.00, 25.00)	18.00 (15.00, 21.00)

BMI, body mass index; TG, triglyceride; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; ApoA1, Apolipoprotein A1; sCD40L, soluble CD40 ligand; NF-κB, nuclear factor-κb; MCP1, monocyte chemoattractant protein-1; GMCSF, granulocyte macrophage colony stimulating factor; Ang2, angiopoietin-2.

2.5. UPLC/Q-TOF-MS analysis

Analysis was performed by a Waters UPLC/Q-TOF-MS system (Waters, Milford, USA). All samples were randomly injected into ACQUITY UPLC C18 column (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, USA) at 45 °C using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The parameters were as follows: the flow rate was 0.3 mL/min, the injection volume was 5 μL and the gradient elution program utilized began with 99% A, then 99% A at 0–0.5 min, 99%–50% A at 0.5–2 min, 50%–1% A at 2–9 min, 1%–1% A at