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Neuroprotective effects of Huang-Lian-Jie-Du-Decoction on ischemic stroke rats revealed by ^{1}H NMR metabolomics approach

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ABSTRACT

Huang-Lian-Jie-Du-Decoction (HLJDD) is a representative antipyretic and detoxifying recipe in traditional Chinese medicine (TCM). This formula and its component herbs like Radix Scutellariae, Fructus Gardeniae show a variety of neuroprotective activities and have been used for the treatment of nervous system diseases including stroke. To comprehensively and holistically assess its therapeutic effect on ischemic stroke, a novel integrative metabolomics approach was applied. A rat ischemic stroke model was established by introduction of transient middle cerebral artery occlusion (MCAO) followed by reperfusion. The neurological deficit, cerebral infarct size and morphological abnormality were evaluated. An NMR technique combined with appropriate statistical analyses was then performed to explore the metabonomic profiles of serum and brain tissue extracts. Pattern analysis of the ^{1}H NMR data disclosed that HLJDD could relieve stroke rats suffering from the ischemia/reperfusion (I/R) injury by ameliorating the disturbance in energy metabolism, membrane and mitochondrial metabolism, neurotransmitter and amino acid metabolism, alleviating the oxidative stress from reactive oxygen species (ROS) and the inflammatory damage, and recovering the destructed osmoregulation.

1. Introduction

Stroke is the third most common cause of mortality, the leading cause of early disability worldwide, bringing a big burden to the healthcare system and society [1]. Considering its prevalence and severe consequences, and the fact that no well-accepted therapeutics are available, it's in urgent need to develop agents with definite efficacy and low side-effects to relieve people from the physical and economical burden brought by stroke.

Traditional Chinese medicine (TCM) has been widely used to treat many complex and refractory diseases for thousands of years. Firstly described in Wang Tao's "Wai Tai Mi Yao" two thousand years ago, Huang-Lian-Jie-Du-Decoction (HLJDD) is a representative antipyretic and detoxifying prescription. It is composed of four famous traditional Chinese medicinal herbs, namely Rhizoma Coptidis, Radix Scutellariae, Cortex Phellodendri and Fructus Gardeniae in a ratio of 3:2:2:3 (w/w/w/w). It is well documented that HLJDD along with its component herbs and their principles showed therapeutic effects on neurodegenerative diseases and could protect neurons from damage induced by acute or chronic cerebral ischemia due to a variety of functions they possessing: anti-oxidation, anti-inflammation, anti-apoptosis, and so on [2,3].

Metabolomics, by monitoring perturbations in the concentrations of endogenous low molecular weight metabolites in cell, tissue or biofluid, provides a new viewpoint to pharmacology, pathology, toxicology and genetics, which enabled its wide use in disease diagnosis, toxicity screening, drug safety and efficacy assessment [4–6]. The multi-component and multi-target characteristics of TCM make the clarification of its efficacy difficult by conventional pharmacological methods. Metabolomics could comprehensively and holistically detected the metabolite change in a biological matrix to evaluate the body status [7], and efficacy of therapy [8], which made it especially suitable for the evaluation of the holistic and synergistic effects of TCM formula [9].

Nuclear magnetic resonance (NMR) is an easy and convenient tool for high throughput metabolomic profiling due to many advantages associated with it, such as relatively little sample preparation, nondestructive analysis nature, easy reference-free quantification, short analysis time, and the powerful ability to elucidate a diverse metabolites simultaneously [10]. Multivariate statistical analysis methods, such as principal component analysis (PCA) and partial

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least squares discriminant analysis (PLS-DA), are commonly applied to analyze large-volume high-density data structures generated by NMR spectroscopy, detecting differential metabolite alterations and providing useful information on pathophysiological changes [11]. NMR-based metabolomics analysis of serum, brain tissue extracts, cerebrospinal fluid and/or urine from stroke victims or animal models have also been conducted successfully, providing useful information on the diagnosis and prognosis of stroke [6,12].

In this study, a middle cerebral artery occlusion (MCAO) rat model was established to imitate human ischemic stroke. ^{1}H NMR-based metabolomics approach was adopted for the first time to investigate the altered metabolic pathways and networks of ischemic stroke rats with ischemia/reperfusion (I/R) injury and the intervention effect of HLJDD.

2. Experiment protocols

2.1. Materials and the preparation of HLJDD

The four component herbs of HLJDD, Rhizoma Coptidis from *Coptis chinensis* Franch, Radix Scutellariae from *Scutellaria baicalensis* Georgi, Cortex Phellodendri Chinensis from *Phellodendron chinensis* Schneid, Fructus Gardeniae from *Gardenia jasminoides* Ellis, were bought from Jiangsu Medicine Company (Nanjing, China), and authenticated by Professor Mian Zhang, Department of Medicinal Plants, China Pharmaceutical University, Nanjing, China. The voucher specimens, deposited at the herbarium of the Department of Natural Medicinal Chemistry, China Pharmaceutical University, were 2012066-RC, 2012067-RS, 2012068-CP and 2012069-FG for Rhizoma Coptidis, Radix Scutellariae, Cortex Phellodendri and Fructus Gardeniae, respectively. Deuterium oxide (D_2O, 99.9%) was bought from Sea Sky Bio Technology Co. Ltd. (Beijing, China). Ultra-pure distilled water was prepared by a Milli-Q purification system. TSP (3-trimethylsilyl-propionic acid) was purchased from Sigma (St. Louis, MO, USA). The kit of TTC (2,3,5-triphenyltetrazolium chloride) was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Rhizoma Coptidis, Radix Scutellariae, Cortex Phellodendri and Fructus Gardeniae in a ratio of 3:2:2:3, reaching a total weight of 500 g, were extracted with 70% ethanol (1:10, 1:10 and 1:5, w/v) under reflux for three times, 1 h each. The decoction was concentrated to dryness to afford 147.25 g HLJDD (yield: 29.45%) using a rotary vacuum evaporator, then stored in refrigerator at 4 °C and suspended in 0.5% CMC-Na (carboxymethyl cellulose sodium salt) before intragastric administration.

2.2. Construction of the middle cerebral artery occlusion (MCAO) model

MCAO model was employed to mimic ischemic stroke as substantial local cerebral blood flow and blood oxygen was supplied by middle cerebral artery (MCA) [3]. The intraluminal technique described by Longa et al. [13] was adopted to establish the MCAO model with little modification. Briefly, a short incision was made on rats anesthetized with chloral hydrate (350.00 mg kg^{-1}, i.p.) to facilitate the separation of the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) from adjacent nerves and tissue with caution. The CCA and ICA were temporarily clamped with microsurgical clips. At the distal part of the ECA, two closely spaced permanent knots were tied to prevent the backflow of blood and the ECA was cut between the knots.

A poly lysine coated nylon monofilament of 50 mm length with tip rounded by heating near glowing ember (final tip diameter of 0.38 ± 0.02 mm) (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was inserted into the arteriotomy hole made between the ECA stump and the carotid bifurcation, and impelled gently, nearly 18–20 mm, from ECA to block MCA, to achieve cerebral ischemia. Twenty-four hours reperfusion was accomplished by gently pulling out the filament, 2 h later. The sham-operated control group rats underwent the same surgical operation except for the insertion of nylon monofilament. Throughout the whole surgery, the environment temperature was kept constant to maintain the rectal temperature of rats at 37 ± 1 °C.

2.3. Experiment animals, group allocation and drug administration

Ten-week-old Sprague-Dawley male rats (280 ± 20 g), clear grade, were purchased from Comparative Medicine Center of Yangzhou University (Yangzhou, China). During the whole experiment procedure, all rats were reared on a 12/12-h light/dark cycle at 25 ± 2 °C and allowed free access to water and standard chow ad lib., cared and handled strictly according to obligations of the Animal Ethics Committee of China Pharmaceutical University and the standard guidelines for the Care and Use of laboratory animal from the National Institute of Health (NIH).

Rats were randomly divided into sham-operated (sham), model (I/R) and HLJDD treated (HT) groups (≥20 mice each) after 7-day accustomization. Rats in the latter two groups underwent I/R surgery, while rats in the sham group only underwent arteries separation without filament insertion. Rats in the sham and I/R groups were intragastrically administered with CMC-Na for 10 days, while those in the HT group were treated with 5.0 g kg^{-1} HLJDD (weight ratio between crude drug and rat). Behavioral changes were assessed 24 h after surgery to evaluate neural function, and blood serum and brains were collected for NMR recording and stored at −80 °C before use.

2.4. Neurobehavioral abnormality evaluation

Neurobehavioral dysfunction of rats in the three groups (n = 10 in each group) was estimated by observers blind to the experiment using Longa's [13] five-point scale: 0, normal (no neurobehavioral dysfunction); (1) slight (failure of flexing left forepaw fully); (2) moderate (circling counterclockwisely); (3) severe (leaning to the affected side) and (4) very serious (no autonomous activity and unconsciousness) [3].

2.5. Measurement of cerebral infracted area

After cerebellum removal, fresh rat brains were immediately frozen at −20 °C for 20 min and then were sliced into 5 uniform 2-mm sections. The tissue sections were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min, and fixed in 4% paraformaldehyde overnight away from light following the instruction of manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Dehydrogenase catalyze the reaction of TTC and NADH (β-Nicotinamide adenine dinucleotide), the viable cells are thus stained deep red, whereas infracted cells are still in gray or white [3]. Slices stained with TTC were photographed, and calculated for the infarct area using image analysis software (Image-Pro Plus 5.1).

2.6. Histopathological assessment by HE Staining

Fresh rat brains were immediately immersed in 10% phosphate-buffered formalin for 12 h fixation, and then embedded in paraffin. A series of adjacent 5-μm-thick sections were cut from the coronal plane of the brain, stained with HE (hematoxylin–eosin) to examine