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Research Paper

Neuroprotection, learning and memory improvement of a standardized extract from Renshen Shouwu against neuronal injury and vascular dementia in rats with brain ischemia

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ABSTRACT

Ethnopharmacological relevance: The Renshen Shouwu capsule (RSSW) is a patented Traditional Chinese Medicine (TCM), that has been proven to improve memory and is widely used in China to apoplexy syndrome and memory deficits. To investigate the neuroprotective and therapeutic effect of the Renshen Shouwu standardized extract (RSSW) on ischemic brain neuronal injury and impairment of learning and memory related to Vascular Dementia (VD) induced by a focal and global cerebral ischemia-reperfusion injury in rats.
Material and methods: Using *in vivo* rat models of both focal ischemia/reperfusion (I/R) injuries induced by a middle cerebral artery occlusion (MCAO), and VD with transient global brain I/R neuronal injuries induced by a four-vessel occlusion (4-VO) in Sprague–Dawley (SD) rats, RSSW (50,100, and 200 mg kg^{-1} body weights) and Egb761® (80 mg kg^{-1}) were administered orally for 20 days (preventively 6 days+therapeutically 14 days) in 4-VO rats, and for 7 days (3 days preventively+4 days therapeutically) in MCAO rats. Learning and memory behavioral performance was assayed using a Morris water maze test including a place navigation trial and a spatial probe trial. Brain histochemical morphology and hippocampal neuron survival was quantified using microscope assay of a puffin brain/hippocampus slice with cresyl violet staining.
Results: MCAO ischemia/reperfusion caused infarct damage in rat brain tissue. 4-VO ischemia/reperfusion caused a hippocampal neuronal lesion and learning and memory deficits in rats. Administration of RSSW (50, 100, and 200 mg/kg) or EGb761 significantly reduced the size of the insulted brain hemisphere lesion and improved the neurological behavior of MCAO rats. In addition, RSSW markedly reduced an increase in the brain infarct volume from an I/R-induced MCAO and reduced the cerebral water content in a dose-dependent way. Administration of RSSW also increased the pyramidal neuronal density in the hippocampus of surviving rats after transient global brain ischemia and improved the learning and memory ability of rats with 4-VO induced vascular dementia in a dose-dependent manner.
Conclusions: The *in vivo* results suggested that RSSW has significant neuroprotective effects against MCAO and 4-VO I/R injury and a therapeutic effect on cognitive disorders in VD rats. RSSW also improved the learning and memory ability of VD rats. These results convincingly demonstrated that RSSW may be useful to prevent and treat ischemia/reperfusion injury and vascular dementia disease.

1. Introduction

Ischemic stroke accounts for 88% of all strokes and is associated with a high incidence of morbidity and mortality. Ischemic stroke is characterized by rapidly developing clinical signs of a focal or global disturbance in the cerebral function. These symptoms may last from 24 h to weeks and can lead to death with no apparent cause other than avascular origin (Silasi and Murphy, 2014).

Vascular dementia (VD) refers to a group of clinical syndromes of a cognitive dysfunction resulting from brain tissue damage caused by

Abbreviations: RSSW, a standardized extract of RenSheng ShouWu; CNS, central nervous system; MCAO, middle cerebral artery occlusion; 4-VO, four-vessel occlusion; I/R, ischemia and reperfusion; AD, Alzheimer's disease; ROS, reactive oxygen species; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; p.o., per os.; TCM, Traditional Chinese Medicine; SBG, stilbene glucoside

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ischemic, hemorrhagic and acute and chronic hypoxic ischemic cerebrovascular disease. Vascular cognitive impairment is defined by alterations in cognition, ranging from subtle deficits to full-blown dementia, which are attributed to cerebrovascular causes. Often coexisting with Alzheimer's disease, mixed vascular and neurodegenerative dementia has emerged as the leading cause of age-related cognitive impairment (Savva et al., 2009; Costantino, 2013).

Patients with vascular dementia (VD) in Asian accounted for approximately half of all patients with dementia because of the higher incidence of stroke. In recent years, the morbidity and mortality of VD has been increasing yearly and has become the third most fatal disease in the world after heart disease and cancer (Myron and Ginsberg, 2008; Ginsberg, 2009). Because it leads to serious damage and there is no effective drug for its treatment, VD destroys the quality of life of the elderly and becomes a heavily burdensome to society and the family. It is our urgent hope to develop a new effective agent to prevent and treat VD (Barone, 2009).

Chinese medicine has a long history of preventing and treating cardiovascular and cerebrovascular diseases as well as other related brain diseases. These medicines have obvious curative effects and are deemed to be safe due to low toxicity and few side effects (Huang, 2009). The Renshen Shouwu (RSSW) capsule is a potent, patented, and Chinese Pharmacopoeia-approved Traditional Chinese Medicine (TCM). RSSW is composed of Renshen (Root of Ginseng-Panax ginseng C.A. Mey) and fleece-flower root (Radix Polygoni Multiflori -Polygonum multiflorum Thunb) and has the ability to strengthen the brain, pacify the nerves, tone the liver and kidney, and replenish qi and the blood. RSSW is recommended for Qi and blood deficiency, whitening beard and hair at early ages, neurasthenia, forgetfulness, insomnia, loss of appetite, fatigue and other symptoms. RSSW is also widely used to treat several diseases, including the following: diseases of cerebrovascular nervous system, amnesia, insomnia, and mental decline (Pharmacopoeia Committee of the Ministry of Health of the People's Republic of China, 2010).

Studies have shown that the RSSW compound ginsenoside Rb1 (an active component of Renshen, 10–40 mg/kg intravenously *i.v.* 30 min before MCAO or a single dose of intranasal) protected the brain from ischemic and reperfusion injuries by decreasing infarct size, and neurologic deficit. However, its bioavailability is low after oral administration due to poor absorption (Zhang et al., 1996; Lu et al., 2011). Despite its wide clinical uses, the neuroprotective effects of oral administration of RSSW on cerebral ischemia injury and its improvement of learning and memory impairment have not yet been reported. In this study, we examined the neuroprotective effects of oral administration of RSSW on a focal I/R injury induced by a middle cerebral artery occlusion (MCAO), on a transient global brain I/R injury induced by a modified Pulsinelli four-vessel occlusion (4-VO), and on learning and memory improvement in Sprague–Dawley rats.

2. Materials and methods

2.1. Plant materials

Hongshen (Processed Ginseng Root of Ginseng [Panax ginseng C.A. Mey] and ZhiShouwu (Processed Radix Polygoni Multiflori [Polygonum multiflorum Thunb] were purchased from the Guangzhou Zhixin TCM herbs Medicine Lit Co. and were authenticated by Professor Shuyang Li, Herbarium, School of TCM, Guangdong Pharmaceutical University; with a voucher specimen of GDPUTCM 121003, 121004.

2.2. Preparation of a standardized extract from Renshen Shouwu

Preparation of a standardized extract from Renshen Shouwu (RSSW) was prepared according to the Renshen Shouwu Capsule from thePharmacopoeia of the People's Republic of China, 2010, Vol. 1 (for Traditional Chinese Medicine) with the following protocol. One kilogram of Hongshen (Processed Radix Gingshen)and 1.5 kg of Zhiheshouwu (Polygoni Multiflori Radix Praeparta) were grinded into powders (filtered through a 2 mm-steel sieve), and refluxed in 10 L of a hydroethanolic solution (30% v/v), at 85 °C for 2 h two times. The 30% ethanol extract solution was filtered through a Whatman paper filter no. 12, and was evaporated under reduced pressure (Buchi R-114, Switzerland). Finally the extract was dried under vacuum below 60 °C. In total, 502.5 g of RSSW was retrieved.

2.3. HPLC fingerprint of RSSW

The analyses were performed using Waters high performance liquid chromatograph Alliance e 2695 equipment (Singapore) coupled with a photodiodearray detector (PDA) 2998 (and 2424 ELS Detector), controlled by LC-Solution Multi-PDA software. Chromatographic separation of the analytes was achieved on a Ultimate-XBC18 column (Phenomenex, 250 mm × 4.6 mm; 5 μm particle size) using acetonitrile (A) – deionized and HPLC grade water (B) as a mobile phase in a gradient elution program using the following method: 0–20.0 min, B 19%; 20.0–40.0 min, B 19–26%; 40.0–60.0 min, B 26–38%; and 60–65 min, B 38%. The flow rate was set to 0.8 ml/min and 10 μL of sample was injected. A C18 guard column (Phenomenex Technologies) was used to prevent column degradation.

Calibration curves were prepared by plotting the peak area ratios of the various concentrations of analytes followed by linear regression. A correlation coefficient (r) of $r^2 \geq 0.99$ was considered to be acceptable. Linearity was determined using the correlation coefficients from the calibration curves using nine concentrations of each analytes. Separately, ginsenoside and stilbene glucoside (SBG) standard curves were preparedby dissolving accurately weighed samples in methanol to obtain concentrations ranging from 0.2 to 500 μg/mL. RSSW samples were prepared in the same way to adjust the ginsenoside and stilbene glucoside (SBG) concentration within the linearity range. The total ginsenoside Rb1, Rg1, Re, and Rd contents were expressed as the sum of the peak areas, which were previously identified as the main ginsenoside found in RSSW. Each result represented the mean value of three determinations.

The ginsenoside Rb1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rd, and 2,3,5,4-tetrahydroxy-stillbene-a-O-3-D-glucoside (stilbene glucoside, SBG, purity > 98.0%) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

HPLC grade acetonitrile and methanol were obtained from Merck KGaA (Darmstadt, Germany). Deionized and HPLC grade water were prepared using a Millipore water purification system (Millipore, Bedford, MA, USA). Formic acid was HPLC grade and all other reagents were analytical grade.

2.4. Animals

Adult male healthy Sprague–Dawley (SD) rats (weighing 180–200 g) were obtained from the Guangdong Medicinal Laboratory Animal Center, Guangzhou, China. All animals were maintained according to the Chinese government guidelines for care and use of laboratory animals with a controlled environment at room temperature (RT) of 25 ± 1.0 °C, with relative humidity of 40–60%, an automatic day–night rhythm (12 h cycle) and 10–15 air changes per hour. The rats had free access to standard lab chow and filtered tap water. Procedures involving animals and their care were approved by the Institutional Animal Ethics Committee of Guangdong Pharmaceutical (GDPUIAEC no. 20130513) which is in a compliance with NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1985). All of the surgical procedures were conducted using aseptic and anesthetic techniques in special purpose operating suites.