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Salvia miltiorrhiza Bunge (Danshen) extract attenuates permanent cerebral ischemia through inhibiting platelet activation in rats

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ARTICLE INFO

Chemical compounds studied in this article:
1. Tanshinone IIA (PubChem CID: 164676)
2. Tanshinone I (PubChem CID: 114917)
3. Cryptotanshinone (PubChem CID: 160254)

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ABSTRACT

Ethnopharmacological relevance: Danshen is a crude herbal drug isolated from dried roots of *Salvia miltiorrhiza Bunge*. This plant is widely used in oriental medicine for the treatment of cardiovascular and cerebrovascular diseases. The supercritical CO_2 extract from Danshen (SCED) (57.85%, 5.67% and 4.55% for tanshinone IIA, tanshinone I and cryptotanshinone respectively) was studied in this article, whose potential molecular mechanism remains unclear, especially in anti-thrombosis.

Aim of the study: The present study was designed to observe the protective effect of SCED on ischemic stroke in rats and to explore the underlying anti-thrombosis mechanism.

Materials and methods: Following induction of cerebral ischemia in rats by permanent middle cerebral artery occlusion (pMCAO). Neurological defect score, cerebral blood flow, infarct size, and brain edema were measured to evaluate the injury. Arteriovenous shunt thrombosis model and adenosine 5′-diphosphate (ADP) induced acute pulmonary embolism model were conducted to estimate the antithrombotic effect of SCED. In order to investigate the effects of SCED on platelet aggregation, rat platelet-rich-plasma (PRP) were incubated with SCED prior to the addition of the stimuli (ADP or 9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin F2α (U46619)). Aggregation was monitored in a light transmission aggregometer. Inhibitory effect of SCED on thromboxane A2 (TXA_2) release was detected by ELISA kit. Phospholipase C (PLC)/ Protein kinase C (PKC) signaling pathway was analyzed by a Western blot technique. The effect of the SCED was also studied in vivo on bleeding time in mice.

Results: SCED improved the neurological defect score, increased cerebral blood flow, reduced infarct size and alleviated brain edema in rats exposed to pMCAO. After administration of SCED, thrombosis formation in arteriovenous shunt was inhibited and recovery time in pulmonary embolism was shortened. The inhibitory effect of SCED on platelet activation was further confirmed by TXB_2 ELISA kit and Western blot analysis of PLC/PKC signaling pathway.

Conclusions: SCED attenuates cerebral ischemic injury. The possible mechanism is that SCED inhibits thrombosis formation, platelet aggregation and activation of PLC/PKC pathway. On this basis, this new extract could be a promising agent to inhibit thrombosis formation and protect against cerebral ischemia injury.

1. Introduction

Stroke is the first leading cause of death in China and fifth in the United States (Kochanek et al., 2014; Mozaffarian et al., 2015). Ischemic stroke, which is mainly caused by cardio-embolism and arterial occlusion, accounts for approximately 80% of deaths (Thrift

Abbreviations: ADP, adenosine 5′-diphosphate; CMC-Na, sodium carboxymethyl cellulose; DAG, diacylglycerol; ELISA, enzyme-linked immunosorbent assay; IP_3, inositol 1, 4, 5-trisphosphate; MAR, maximal aggregation rate; NS, normal saline; PAI, Platelet aggregation inhibition rate; PBS, phosphate buffered saline; PIP_2, phosphatidylinositol 4, 5-bisphosphate; pMCAO, permanent middle cerebral artery occlusion; p-PKC, phosphorylated-protein kinase C; p-PLCβ3, phosphorylated-phospholipase Cβ3; PPP, Platelet-poor-plasma; PRP, Platelet-rich-plasma; rCBF, regional cerebral blood flow; SCED, supercritical CO_2 extract from Danshen; TTC, 2,3,5-triphenyl-tetrazolium chloride; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; U46619, 9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$

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et al., 2001). Under pathological circumstances, the abnormal activation of platelets may result in the formation of thrombosis in cycle, which will block cerebral arteries and induce stroke (Hansson, 2005).

Platelets will be activated during ischemic stroke (Davì and Patrono, 2007; Grau et al., 1998). On platelet stimulation, activated PLCβ will hydrolyze phosphatidylinositol 4, 5-bisphosphate (PIP_2) to generate inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 results in increased cytoplasmic Ca^{2+} level. In the presence of suitable phospholipids, DAG activates PKC at basal intracellular Ca^{2+} levels. PKC activation is considered to play a major role in platelet secretion and in the expression of platelet surface fibrinogen binding sites, which is necessary for platelet aggregation (Rao, 1998). Besides, activation of PLCβ contributes to platelet shape change and granule secretion and promotes thrombosis formation. Therefore, activation of the PLCβ and PKC contributes to the platelet aggregation and thrombosis formation, which will exacerbate ischemic injury after stroke.

Danshen, the dried root of *Salvia miltiorrhiza Bunge*, is a commonly used traditional Chinese medicine and has been used widely for the treatment of vascular diseases including hypertension, stroke, hyperlipidemia, and atherosclerosis in Asia, America, and Europe (Han et al., 2008; Lee et al., 2012; Zhou et al., 2005). The main lipid soluble active ingredients in Danshen are tanshinones, including cryptotanshinone, dihydrotanshinone I, tanshinone I, tanshinone IIA, and tanshinone IIB (Gu et al., 2004). Tanshinones have been reported to exert antioxidant and anti-inflammatory effects in preventing ischemic injury in animal models (Dong et al., 2009; Lam et al., 2003). Drug in this study is extracted by supercritical carbon dioxide from Danshen and composed of tanshinone IIA (57.85%), tanshinone I (5.67%), cryptotanshinone (4.55%) and other components (31.93%), of which the effect in cerebral ischemia injury is still unknown.

In the present research, we demonstrated that SCED could reduce brain damage induced by pMCAO, inhibit platelet aggregation through PLC/PKC pathway, and exert potent efficiency on restricting thrombosis.

2. Materials and methods

2.1. Animals

All animals were purchased from Qinglongshan Animal Farm of Nanjing, China. Male Sprague-Dawley rats of SPF level (250–300 g) were used for establishing model of cerebral ischemia. Male SD rats of SPF level (180–220 g) were used for arteriovenous shunt thrombosis model establishment, platelet aggregation function study and TXA_2 release assay. ICR mice of SPF level (half male and female, 18–22 g) were used for pulmonary embolism model establishment and bleeding time determination. Animals were housed under a normal 12 h/12 h light/dark schedule and housed at 24 ± 2 °C with relative humidity (55 ± 5%). Standard chow and water were supplied ad libitum. All animals were cared for in compliance with institutional guidelines of China Pharmaceutical University (Nanjing, China). All experiments were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (license number: SYXK (Su) 2016-0011). Furthermore, the European Community guidelines (EEC Directive of 1986: 86/609/EEC) were implemented on all the procedures and animal care. All efforts were made to minimize animal suffering and to reduce their number.

2.2. Chemicals and reagents

Reference standard of tanshinone IIA (purity > 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China, JVZP-967E). Reference standard of cryptotanshinone (purity > 98%) was from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China, C28M6Y1). Reference standard of tanshinone I (purity > 98%) was also from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China, C28M6Y1). ADP was bought from Biosharp (Hefei, China, Amresco0160). U46619 was the product of Enzo (New York, USA, 32BFC12G1). TXB_2 ELISA kit was bought from Shanghai Lengton Bioscience Co., Ltd. (Shanghai, China, 20150826NM). Ginaton (*Ginkgo biloba* extract) tablets used as positive control in experiments in vivo were bought from Dr. Willmar Schwabe GmbH & Co. KG (Karlsruhe, Germany, 7910715). Each Ginaton tablet contains 40 mg *Ginkgo biloba* extract and is standardized to contain 24% *Ginkgo* flavone glycosides and 6% terpene lactones. Ginaton injection used as positive control in experiments in vitro was bought from Chi Sheng Chemical Corporation (Taiwan, China, 10068). The Ginaton injection is an aqueous solution which contains 17.5 mg *Ginkgo biloba* extract for injection per 5 mL ampoule. The extract is standardized to 24% *Ginkgo* flavone glycosides. All other reagents were of analytical grade and commercially available.

2.3. Preparation of Danshen extract using supercritical fluid extraction

The dried roots of *Salvia miltiorrhiza Bunge* (Danshen) were collected from the province of Shandong, China, in the month of October 2015 and were authenticated by Professor Feng Feng (China Pharmaceutical University, Jiangsu, China) for genuine medicinal materials, according to the Pharmacopoeia of the People's Republic of China (2015). A voucher specimen (DS150401) was deposited at Herbarium of Department of Pharmaceutical analysis, China Pharmaceutical University, Jiangsu, China.

300 g of dried roots of *Salvia miltiorrhiza* were cleaned, dried in the shade, and ground into fine coarse powder and then extracted with the supercritical CO_2 extraction instrument at 60 °C under 30 MPa for 2 h at a flow of 20 L/h. Ethanol (95%) was used as a co-solvent at a flow of 0.8 g/min. Crude extract was obtained after the evaporation of ethanol in rotary evaporator. The crude extract was washed with 10-fold of ethanol (95%), dried in oven at 80 °C for 2 h, and then stored at 4 °C away from light. The yields of SCED was 0.8% (weight %). At the time of use, the extract was dissolved in distilled water containing 0.5% sodium carboxymethyl cellulose (CMC-Na).

2.4. Quantitative analysis by HPLC

Preparation of test solution: 10 mg of SCED was weighed accurately in a 50-mL volumetric flask. Acetonitrile was added to dissolve. The test solution was obtained after filtration.

Preparation of reference standard solution: The reference standard samples (Tanshinone IIA, tanshinone I, cryptotanshinone) were weighed accurately and dissolved in acetonitrile. After diluted, the reference standard solution containing tanshinone IIA (0.2 mg/mL) and the solution containing tanshinone I and cryptotanshinone (10 μg/mL, respectively) were obtained.

The major components of SCED (tanshinone IIA, tanshinone I and cryptotanshinone) were identified and quantified through high-performance liquid chromatography (HPLC). HPLC-ultraviolet (UV) analysis was conducted using an HPLC system (Shimadzu, LC-2010HT). An Inertsil C_{18} column (Shimadzu, 4.6 × 150 mm, 5 μm) was used as the analytical column. Acetonitrile-water (67:33) was used as mobile phase. The injection volume was 10 μL, the flow rate was 1 mL/min, and detection was performed at 270 nm. The external standard method is employed for quantitative analysis.

2.5. Induction of permanent focal cerebral ischemia injury by MCAO

2.5.1. Groups and drug administration

A total of 90 male Sprague-Dawley rats (250–300 g) were randomly divided into 6 groups: 1) Sham operation group; 2) Ischemia injury model group; 3) Positive control group of Ginaton (15 mg/kg); 4)