Journal of Ethnopharmacology 173 (2015) 266–272

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

The antithrombotic effect of RSNK in blood-stasis model rats

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ARTICLE INFO

Article history:
Received 22 November 2014
Received in revised form
18 May 2015
Accepted 16 June 2015
Available online 26 July 2015

Chemical compounds studied in this article:
tanshinone IIA (PubChem CID: 164676)
astragaloside (PubChem CID: 45006101)
ferulic acid (PubChem CID: 445858)
puerarin (PubChem CID: 5281807)
3,4-Dihydroxybenzaldehyde (PubChem CID: 8768)
Danshensu (PubChem CID: 11600642)
paeoniflorin (PubChem CID: 425990)

Keywords:
Reduction of Sheng-Nao-Kang decoction (RSNK)
Antithrombotic
Anticoagulation

ABSTRACT

Ethnopharmacological relevance: Reduction of Sheng-Nao-Kang decoction (RSNK), composed of Salvia miltiorrhiza Bge., Ligusticum chuanxiong Hort., Astragalus membranaceus (Fisch.) Bunge., Pueraria lobata (Willd.) Ohwi., Paeonia lactiflora Pall. and Panax notoginseng (Burk.) F. H. Chen., is a modified traditional Chinese medicinal formula of Sheng-Nao-Kang pill preparation, which has been investigated its protective effect on focal cerebral ischemia-reperfusion injury in rat in our previous report.
Aim of the study: To evaluate the antithrombotic effect of RSNK in blood stasis model rats and explore the potential mechanisms.
Materials and methods: Subcutaneous injection of norepinephrine and bovine serum albumin combined with ice water bath was used to establish the acute blood stasis rat model. The anticoagulant activities were investigated by measuring activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), and the content of fibrinogen (FIB). Meanwhile, the levels of thromboxane A_2 (TXA_2), prostaglandins I_2 (PGI_2), endothelial nitric oxide synthase (eNOS) and endothelin (ET) were detected.
Results: The treatment of RSNK was able to prolong APTT, TT and PT, and decrease FIB content obviously. Furthermore, it markedly suppressed TXB_2 level and up-regulated 6-keto-$PGF_{1\alpha}$ level of the blood-stasis model rats, accompanied with the decrease of T/K. The level of ET and TXA_2 in plasma was down-regulated and the levels of eNOS in plasma and PGI_2 in serum was up-regulated in RSNK-treated rats compared with model rats ($P < 0.05$).
Conclusion: The present study suggested that RSNK possessed remarkable antithrombotic property in blood stasis model rats induced by ice water bath and subcutaneous injection of norepinephrine and bovine serum albumin. This property could be associated with its anticoagulation activity, the regulation of active substances in vascular endothelium and maintaining the balance of TXA_2 and PGI_2.

1. Introduction

Thrombosis, the formation or presence of the thrombus in a blood vessel, is a multifactorial disease induced by promoting a combination of stasis and hypercoagulability (Berry et al., 1994). It can result in various cardiovascular and cerebrovascular diseases (CVD), such as coronary heart disease (CHD), stroke, embolism, atherosclerotic plaque rupture and deep vein thrombosis, etc., which have become primary causes of death, and the incidence has been increasing (Bao et al., 2009; Couture et al., 2013). Blood stasis syndrome is one of the common syndromes in the course of CVD in traditional Chinese medicine (TCM) theory. One of the most widely used animal models to assess arterial antithrombotic properties of new TCM is the blood stasis rat model.

There are various antithrombotic chemical drugs such as aspirin, ticlopidine, clopidogrel, abciximab, and so on. Most of them have side effects more or less, including gastrointestinal symptoms and hemorrhage (Cannon et al., 2010; Johansen, 2006). Developing antithrombotic agent with little side effects from medicinal herbs has attracted much interest recently (Ballabeni et al., 2007). Sheng-Nao-Kang pill preparation, containing fifteen

Abbreviations: AA, Arachidonic acid; ANOVA, one-way analysis of variance; APTT, activated partial thromboplastin time; CVD, cardiovascular and cerebrovascular diseases; ET, endothelin; eNOS, endothelial nitric oxide synthase; ELSD, Evaporative Light Scattering Detector; FIB, fibrinogen; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography; PGI_2, prostaglandins I_2; PT, prothrombin time; RSNK, Reduction of Sheng-Nao-Kang decoction; SD, Sprague-Dawley; S.E.M., standard error of mean; TCM, traditional Chinese medicine; TT, thrombin time; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; UV, Ultraviolet; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$.

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http://dx.doi.org/10.1016/j.jep.2015.06.030
0378-8741/

traditional Chinese medicines, has been used clinically over 10 years for the treatment of acute and chronic cerebrovascular relevant diseases because of its activities of activating blood circulation, dissipating blood stasis, dredging meridians and collaterals (Song et al., 2011). Its protective effect on focal cerebral ischemia-reperfusion injury in rat was reported in our previous paper (Chen et al., 2014). Sheng-Nao-Kang pill preparation was modified to RSNK according to TCM theory.

Therefore, the purpose of the present study was to investigate the antithrombotic effect of RSNK in blood-stasis model rats and explain its mechanism for the better clinical use in cardiovascular disease treatment.

2. Materials and methods

2.1. Reagents and chemicals

Standards of tanshinol (110855-200506), protocatechuic aldehyde (110810-200205), paeoniflorin (0736-9608), puerarin (1111-081021), ferulic acid (110773-200611), tanshinone IIA (0766-200213) and astragaloside (0781-200510) were purchased from the National Institute for the Control of Pharmaceutical and Biology Products (Beijing, China). Asprin enteric-coated tablet (J20080078) as a positive control drug, was manufactured by Bayer Healthcare S.r.l. (Leverkusen, Germany) and dissolved in saline to 3 mg/mL before using. Fufang Danshen tablet (Z44023372), as another positive control drug, was produced by Baiyun Mountain Hutchison Whampoa Chinese medicine Co., LTD (Guangzhou, China), and the solution of Fufang Danshen (0.27 g/mL) was prepared by dissolving it in saline before using. Norepinephrine bitartrate injection (H42021301) was manufactured by Yuanda (China) pharmaceutical co., LTD and diluted to 0.01 mg/mL in saline before using. Bovine serum albumin was purchased from Sigma-Aldrich Co. (St. Louis, USA). Endothelial nitric oxide synthase (eNOS) ELISA kit (CK-E30338R) and endothelin (ET) ELISA kit (CK-E90917R) were purchased from Quanzhou LanTu Bio-Tech Co., Ltd. (Quanzhou, China). Thromboxane B_2 (TXB_2) radioimmunoassay kit (HY-10042) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) radioimmunoassay kit (HY-10049) were made by Beijing Sino-uk Institute of Biological Technology (Beijing, China). The other chemicals were all of analytical grade.

2.2. Experimental rats

Male Sprague-Dawley (SD) rats (Certificate no. SCXK 2012-0007), weighting 220–250 g were provided by Medical Laboratory Animal Center, The Fourth Military Medical University, Xi'an, Shaanxi, China. They were maintained in animal laboratory (Certificate no. SYXK 2010-004) of specific pathogen free controlled at constant temperature of 22 ± 2 °C with a relative humidity of 50 ± 5%. All animal procedures were approved by the Institutional Animal Care and Use Ethics Committee of the Northwest University, Xi'an, China, the approval number was 2013-010.

2.3. Preparation of RSNK

The medicinal formula of RSNK was shown in Table 1. Dried crude medicinal materials were purchased from Wanshou Road Chinese medicinal materials market (Xi'an, China) and were identified by Professor Wenji Sun of Northwest University (Xi'an, China). Dried *Salvia miltiorrhiza* Bge. and *Ligusticum chuanxiong* Hort. were mixed in certain ratio, refluxed with 70% ethanol solution for 2 times, 2 h every time, to get the filtrates. The residue was mixed with certainly proportion dried *Astragalus membranaceus* (Fisch.) Bge., *Pueraria lobata* (Willd.) Ohwi and *Paeonia lactiflora* Pall. The mixture was soaked in distilled water and boiled 2 h twice to get the filtrates. Combined all filtrates and condensed. Then it was freeze-dried under vacuum to make into drug powder eventually. Dissolve the drug powder in saline to obtain three different concentration solutions at 70, 140 and 280 mg/mL before using, respectively.

2.4. HPLC analysis

High performance liquid chromatography (HPLC) was used to identify the active compounds of RSNK to ensure its quality and stability. HPLC system was consisted of Waters 2695 HPLC System and UV System as well as ELSD System. The analytes were isolated on a Waters Symmetry Shield^{TM} C_{18} column(4.6 mm × 250 mm, with 3.5 μm particle size) and the column temperature was controlled at 30 ± 2 °C. UV detector was employed to determine the content of tanshinol, protocatechuic aldehyde, paeoniflorin, puerarin, ferulic acid and tanshinone IIA at 280 nm. The binary gradient elution system, composed of methanol as solvent A and 0.25% solution of acetic acid in water as solvent B, was set as follows: 0–5 min, 10–20% A; 5–10 min, 20–25% A; 10–20 min, 25–30% A; 20–30 min, 30–80% A; 30–35 min, 80% A; 35–40 min, 80–100% A at 1.0 mL/min flow rate. The content of astragaloside was detected by ELSD. The temperature of drift tube was 90 °C and the gas flow was 2.8 L/min (compressed air). All solvents were filtered through a 0.45 μm filter membrane before using.

2.5. Quantification of active compounds in RSNK

The contents of tanshinol (Danshensu), protocatechuic aldehyde (3,4-Dihydroxybenzaldehyde), paeoniflorin, puerarin, ferulic acid, tanshinone IIA and astragaloside in RSNK were quantitatively analyzed. The extract powder was dissolved in 80% methanol by ultra-sonic treatment for 1 h. The suspension was diluted to 100 times with 80% methanol, and then was centrifuged at 15,000 rpm for 10 min to get the supernatant before analyzing. HPLC chromatograms of RSNK extracts were shown in Fig.1. The contents of tanshinol, protocatechuic aldehyde, paeoniflorin, puerarin, ferulic acid, tanshinone IIA and astragaloside in RSNK extract were 1.15, 0.13, 4.48, 0.80, 0.72, 0.31, 3.12 mg/g, respectively, as shown in our previous study (Chen et al. 2013).

Table 1
The medicinal formula of RSNK.

Latin name (Chinese name)	Medicinal parts	Place of production	Voucher numbers	Weight (g)
Salvia miltiorrhiza Bge. (Dan Sen)	Root	Sichuan	20110010101	4
Ligusticum chuanxiong Hort. (Chuan Xiong)	Root	Shaanxi	20110010102	4
Astragalus membranaceus (Fisch.) Bge. (Huang Qi)	Root	Shanxi	20110010103	4
Pueraria lobata (Willd.) Ohwi (Ge Gen)	Root	Henan	20110010104	4
Paeonia lactiflora Pall. (Chi Shao)	Root	Shaanxi	20110010105	3
Panax notoginseng (Burk.) F.H. Chen (San Qi)	Root	Yunnan	20110010110	1