Antithrombotic effects of Huanglian Jiedu decoction in a rat model of ischaemia-reperfusion-induced cerebral stroke

Huan Liu, Xiaoyan Chen, Yanling Liu, Chunjuan Fang and Shaofen Chen

Jiangxi University of Technology, Nanchang, Jiangxi, China

ABSTRACT

Context: Huanglian Jiedu Decoction (HLJJD) has a variety of pharmacological activities, such as anti-inflammatory and neuroprotection against ischaemic brain injury.

Objectives: This ex vivo study explores its antithrombosis activity and inhibition of platelet aggregation.

Material and methods: To study the antithrombosis activity of HLJJD ex vivo, saline, or HLJJD (100, 200, and 500 mg/kg) was treated prophylactically by gavage for 3 days in Wistar rats (n = 4). Based on the rat model of transient middle cerebral artery infarction (MCAO) or normal rats (n = 4), the antithrombotic activity in the normal group and HLJDD subgroups on prothrombin time, thrombus weight, platelet aggregation, and others was evaluated, followed by the antiplatelet aggregation of its main components (n = 4).

Results: The weight of the thrombus increased significantly at 24 h after MCAO onset. HLJJD did not influence the change of PT, but significantly inhibited thrombosis by 12.5, 20.0, and 20.5% in reducing the dry weight of thrombus, and blocked collagen-induced platelet aggregation by 25.5, 39.0, and 42.7% and adhesion of blood platelet by 17.3, 26.2, and 27.3%. The IC50 value of HLJJD on collagen-induced platelet aggregation was 670 mg/kg. Geniposide only facilitated antiplatelet aggregation induced by collagen, but not AA or ADP. Both baicalin and berberine showed markedly antiplatelet aggregation induced by all activators. The antithrombotic activity of baicalin was relatively higher than that of berberine (35.0–47.8% vs. 20.6–33.5%).

Conclusion: Our results indicated that HLJDD regulated blood circulation by inhibiting platelet aggregation and thrombosis, which might also extensively contribute to the clinical prevention and treatment of cerebrovascular diseases.

Introduction

Antithrombosis is a critical treatment with anticoagulants to prevent stroke prevention, deep-vein thrombosis, heart attack, and pulmonary embolism. Many ingredients in herbal medicines have antithrombotic activities, such as afzeloside extracted from Malus halliana Koehne (Rosaceae) flowers (Cui et al. 2018), myricitrin isolated from Cercis chinensis Bunge (Leguminosae) leaves (He et al. 2019), compounds from Radix Paeoniae Rubra (Ranunculaceae) (Xie et al. 2017), and the procoagulant effects of constituents from Cordyceps militaris L. ex Fr. (Clavicipitaceae) link (Zhang et al. 2018).

As a famous prescription in China, Huanglian Jiedu Decoction (HLJJD) was found to perform neuroprotective effects with its major active compounds (containing 42.12% baicalin and 31.17% berberine), and possess cardioprotective effects via preventing pathological cardiac hypertrophy and regulating lipid metabolism (Fang et al. 2017; Chen et al. 2021). As an ancient prescription originated during the Tang Dynasty in China (618–906 A.D.), HLJJD consists of four Chinese medicines: the roots of Captis chinensis Franch. (Ranunculaceae), roots of Scutellaria baicalensis Georgi. (Lamiaceae), dry bark of Phellodendron chinense C.K. Schneid. (Rutaceae), and ripe fruits of Gardenia jasminoides J. Ellis (Rubiaceae). This prescription was applied extensively in clinics for the treatment of cerebrovascular diseases, transient cerebral ischaemia, and Alzheimer’s disease in China and Japan (Zhao et al. 2014; Gu et al. 2018; Chen et al. 2021). However, its antithrombotic effects against thrombosis and platelet aggregation were uncertain, which might contribute to the effect of HLJDD in treating stroke sequelae.

To study the mechanism of anti-ischaemic cerebrovascular disease in the aspect of the antithrombotic effect, we conducted a series of thrombotic experiments to study the antiplatelet aggregation of HLJDD and its potential active ingredients.

Materials and methods

Animals and chemical agents

Healthy male Wistar rats (8–10 weeks old, bodyweight 220 ± 25 g) were obtained from the Experimental Animal Centre of Jiangxi University of Traditional Chinese Medicine (License No.: SCXK2015-0378). The rats were fed with rodent food and water in a temperature- and humidity-controlled environment on a 12 h light/dark cycle, for at least 3 days before the experiments. The animal protocols were approved by the Ethics Committee of Jiangxi University of Traditional Chinese Medicine.
Committee of Jiangxi University of Science and Technology and followed the regulatory animal care guidelines of the United States National Institute of Health (Bethesda, MD, USA).

HLJDD contained 300 g roots of *Coptis chinensis*, 200 g roots of *Scutellaria baicalensis*, 200 g dry bark of *Phellodendron chinense*, and 300 g ripe fruits of *Gardenia jasminoides*. The water-soluble extract was obtained by two reflux extractions with the 10-times volume of water; the yield was 2.6%. The powder of the whole prescription was prepared by concentration under reduced pressure and spray drying.

Aspirin (Yangzhou Second Pharmaceutical Factory, Jiangsu, China); arachidonic acid (AA), ADP and collagen (Aldrich-Sigma, St. Louis, MO, USA); geniposide (>95%, Sichuan Shuangzi Bio Sci. Co., Ltd., Chengdu, China); baicalin and berberine (>90%, Nanjing Zelang Medical Technology Co. Ltd., Nanjing, China). Prothrombin time (PT) reagents (Thermo Scientific Co. Ltd., Waltham, MA, USA); the chemicals or organic reagents for HPLC were of chromatographically pure grade.

**Rats MCAO modelling and antithrombotic studies**

Adult rats were subjected to MCAO surgery according to the modified Longa suture method (Wang et al. 2019). After cerebral vascular blockage for 90 min, the filament was withdrawn to achieve reperfusion. The wound was treated with penicillin powder against microbial infection. Saline or HLJDD were treated by gavage for 3 days. The last administration was exerted at 60 min before antithrombotic studies. In addition, geniposide, berberine, and baicalin were treated by intravenous injection with last administration at 30 min before subsequent experiments. For the normal group, normal rats were subjected to antithrombotic studies directly (n = 4).

Twenty-four hours after MCAO surgery for the model control group and treatment subgroups (n = 4), arteriovenous shunt (silk thread model) was performed according to the previous report (Lorrain et al. 2003). Briefly, after anaesthetization with pentobarbital sodium (i.p.), the right carotid artery and the left jugular vein of rats were inserted separately with 10 cm long polyethylene tubes (1 mm i.d., linked by a central part (8 cm long; 2 mm i.d.) containing a 6 cm silk thread in 200 U/mL heparin). This central part of the shunt was removed after 15 min of blood circulation and the silk thread with thrombus attached was collected and weighted after dried at 80°C for 1 h.

Twenty-four hours after MCAO surgery for the model control group and treatment subgroups (n = 4), orbital blood was collected in a citrated tube. PT was determined using a platelet-rich plasma (PRP) was prepared by centrifugation of citrated rat plasma at 1000 rpm for 5 min and further centrifuged at 3000 rpm for 10 min to prepare platelet-poor plasma. Platelet aggregation was measured by a turbidimetric method using a whole-blood aggregometer (WBA analyzer; Mebanix, Tokyo, Japan). Briefly, 200 μL of rat PRP was incubated at 37°C and stirred at 1000 rpm for 3 min in the aggregometer. Subsequently, 2 μg/mL of collagen, 1.0 mM arachidonic acid, or 1.0 μM ADP was added to stimulate platelet aggregation in phosphate buffer solution (PBS). Changes in light transmission were tested for 5 min and the maximal aggregation rate was recorded (Day et al. 2006).

The adhesion of blood platelet to collagen type I was determined with Tuszynski’s and Murphy’s method (Olas et al. 2017). After a 96-well microtiter dish incubated with 50 μL of fibrinogen for 2 h and washed with PBS, the wells were supplemented with 50 μL of 10 μM ADP. Then 100 μL of platelet suspension was added to each well and the plate was incubated at 37°C for 1 h. Non-adherent cells were removed by aspiration and the wells were washed with PBS three times. The total cell-associated protein was determined by dissolving the attached blood platelets directly in the microtiter wells with 200 μL of working solution of bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA), and incubated at 37°C for 60 min. The absorbance of each well was determined at 560 nm with a microtiter plate reader (Thermo Labsystems, Vantaa, Finland).

**The main components of HLJDD in HPLC test**

The platform of Agilent 1100 High-Performance Liquid Chromatograph (Beijing Keyi Hengda Technology Co., Ltd., Beijing, China) was applied to study the main components of HLJDD. Chromatographic column: Agilent TC-C18 (4.6 × 250 mm, 5 μm); mobile phase: acetonitrile (A)-0.2% phosphoric acid water (B); linear-gradient elution program: 0-10 min, 10–22% A; 10–40 min, 22–23% A; 40–45 min, 23–24% A; 45–60 min, 24% A; detection wavelength: 260 nm; flow rate: 1.0 mL/min; sample volume: 10 μL; column temperature: 25°C. The sample for determination was prepared by resolving 10 mg powder in 5 mL of 30% methanol and filtered with 0.45 μm microporous membrane.

**Statistical analysis**

All data were expressed in the form of mean ± SEM. Data were analyzed by one-way ANOVA, followed by Student’s two-tailed t-test for comparison between two groups. p < 0.05 was considered as a significant difference between each group.

**Results**

**Antithrombotic activity of HLJDD in MCAO rats**

Many indicators were studied to evaluate the antithrombotic activity of HLJJD in MCAO rats, such as thrombosis, PT, adhesion of blood platelet, and platelet aggregation. HLJDD did not influence the change of PT, but significantly inhibited thrombosis by 12.8–20.6% in reducing the dry weight of thrombus, and blocked collagen-induced platelet aggregation by 25.6–42.7% and adhesion of blood platelet by 17.3–27.3% in a dose-dependent manner (Figures 1(A–D)). The IC50 value of HLJJD on anti-platelet aggregation was 670 mg/kg.

**HPLC assay of main components in HLJDD**

High-performance liquid chromatography was applied to analyze the main ingredients in HLJDD. Due to the complexity of components, the selection of detection wavelengths greatly impacted the confirmation of different compounds. It was confirmed that the peaks of the main components geniposide, baicalin, and...
berberine could be completely separated when detected at the wavelength of 260 nm, with high detection sensitivity and large peaks (Figures 2(A,B)).

**Antithrombotic effects of main ingredients on platelet aggregation**

The antithrombotic effects of geniposide, baicalin, and berberine were tested on platelet aggregation induced by AA, ADP, and collagen, respectively (Figure 3). Our results indicated that geniposide only performed antiplatelet aggregation induced by collagen, but not AA or ADP. Both baicalin and berberine showed markedly antiplatelet aggregation induced by all activators. The antithrombotic activity of baicalin was relatively higher than that of berberine (35.0–47.8% vs. 20.6–33.5%).

**Discussion and conclusions**

Ischaemic stroke can induce the blood to be in a state of high concentration, high viscosity, and high degree of aggregation (Grau et al. 1994, Hovhannesyan and Hovhannisyan 2019). In our study, the weight of thrombus increased significantly at 24 h after MCAO onset. HLJDD could significantly inhibit thrombosis by improving platelet adhesion and platelet aggregation but had no effect on the clotting time of PT. It was assumed that the active ingredients in HLJDD might play an antithrombotic effect mainly by inhibiting the activation of platelet function. A review of the literature revealed the antithrombotic activities of many other decoctions were reported, such as Sheng Hua decoction, Buyang Huangwu decoction, Yiqi Huoxue decoction, etc. (Qian and Yu 2011; Liao et al. 2018; Wu et al. 2019). Their activities were relatively moderate with antiplatelet aggregation of 20–40%, similar to HLJDD.

In subsequent antiplatelet experiments, it was found that at 24 h after MCAO surgery, platelet adhesion increased obviously and the rates of platelet aggregation induced by ADP, collagen, and AA were significantly enhanced. Geniposide exhibited the strongest inhibitory effect on platelet aggregation induced by collagen with high specificity. The collagen-induced platelet aggregation is closely related to the release and metabolism of AA, which suggests that the anti-platelet aggregation of geniposide may be related to its adjustment to the metabolism of AA. Our result on geniposide is consistent with previous related research (Suzuki et al. 2001; Zhang et al. 2013). Baicalin was studied only on ADP-induced platelet aggregation in a former study (Lee et al. 2015). In addition, there was no related report on the anticoagulant activity of berberine.

In conclusion, the extract of HLJDD possessed the effect of promoting blood circulation, which was consistent with antiplatelet aggregation of baicalin and berberine, as the main components of the prescription. The activation of HLJDD on blood circulation will become one of its important pharmacological mechanisms for the clinical prevention and treatment of cerebrovascular diseases.
Disclosure statement

The authors declare no competing interests in this study.

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Data availability statement

Additional information and requests for data should be directed to the corresponding author X. Y. Chen (chenxyan5@126.com).

References

Chen QQ, Wang FX, Cai YY, Zhang YK, Fang JK, Qi LW, Zhang L, Huang FQ. 2021. Untargeted metabolomics and lipidomics uncovering the cardioprotective effects of Huanglian Jiedu decoction on pathological cardiac hypertrophy and remodeling. J Ethnopharmacol. 270:113646.

Cui LL, Xing MM, Xu LT, Wang JY, Zhang XF, Ma CY, Kang WY. 2018. Antithrombotic components of *Malus halliana* Koehne flowers. Food Chem Toxicol. 119:326–333.

Day JR, Haskard DO, Taylor KM, Landis RC. 2006. Effect of aprotinin and recombinant variants on platelet protease-activated receptor 1 activation. Ann Thorac Surg. 81(2):619–624.

Fang K, Wu GR, Wang H, Zhao R, Zhang XY, Xue NN, Chen M, Guo WB, Chu FH, Xu B, et al. 2017. Study on chemical constituents of self-settling...
from Huanglian Jiedu decoction and effects on PC12 induced by CoCl2. Zhong Cao Yao. 48:3714–3719.

Grau AJ, Sigmund R, Hacke W. 1994. Modification of platelet aggregation by leukocytes in acute ischemic stroke. Stroke. 25(11):2149–2152.

Gu XR, Fang SY, Ren W, Wang HJ, Yang J, Si N, Bian BL, Zhao HY. 2018. Pharmacodynamics of Huanglian Jiedu decoction in Alzheimer’s disease (AD) model rats and effect on improvement of inflammation microenvironment in brain. Zhongguo Zhong Yao Za Zhi. 43:3006–3011.

He N, Wang PY, Niu YY, Chen JQ, Li CQ, Kang WY. 2019. Evaluation antithrombotic activity and action mechanism of myricitrin. Ind Crops Prod. 129:536–541.

Hovhannesyan RA, Hovhannisyan IG. 2019. Platelet aggregation and interleukins indicators impacting the outcomes of ischemic stroke. J Stroke Cerebrovasc Dis. 28(7):2038–2044.

Kim DC, Ku SK, Bae JS. 2012. Anticoagulant activities of curcumin and its derivative. BMB Rep. 45(4):221–226.

Lee W, Ku SK, Bae JS. 2015. Antiplatelet, anticoagulant, and profibrinolytic activities of baicalin. Arch Pharm Res. 38(5):893–903.

Liao FY, Yu AM, Yu JY, Wang D, Wu YN, Zheng HZ, Meng YJ, He DM, Shen X, Wang LS. 2018. Identification of active ingredients mediating anti-platelet aggregation effects of BuyangHuanwu decoction using a platelet binding assay, solid phase extraction, and HPLC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 1092:320–327.

Lorrain J, Millet L, Lechaire I, Lochot S, Ferrari P, Visconte C, Sainte-Marie M, Lunven C, Berry CN, Schaeffer P, et al. 2003. Antithrombotic properties of SSR182289A, a new, orally active thrombin inhibitor. J Pharmacol Exp Ther. 304(2):567–574.

Olas B, Kontek B, Szczesna M, Grabarczyk L, Stochmal A, Zuchowski J. 2017. Inhibition of blood platelet adhesion by phenolics’ rich fraction of Hippophae rhamnoides L. fruits. J Physiol Pharmacol. 68(2):223–229.

Qian X, Yu H. 2011. Effects of shenghua decoction on hemorheology, thrombosis and microcirculation. Zhongguo Zhong Yao Za Zhi. 36(4):514–518.

Suzuki Y, Kondo K, Ikeda Y, Umemura K. 2001. Antithrombotic effect of geniposide and genipin in the mouse thrombosis model. Planta Med. 67(9):807–810.

Wang BX, Xu J, Hu J, Hu ML, Huang JM, Zhu XD. 2019. Effects of miR-153 on angiogenesis in MCAO rats through Shh signaling pathway. Eur Rev Med Pharmacol Sci. 23(2):732–739.

Wu H, Lei Z, Gao SB, Dai LP, Han YJ, Gao HX, Wang XZ, Wang ZT, Han LH. 2019. YiqiHuoxue decoction and its ethanol precipitation show antiplatelet and antithrombotic effects by suppressing thromboxane B2 formation. Acta Cardiol Sin. 35(5):524–533.

Xie PY, Cui LL, Shan Y, Kang WY. 2017. Antithrombotic effect and mechanism of Radix Paeoniae Rubra. Biomed Res Int. 2017:9475074.

Zhang HY, Liu H, Yang M, Wei SF. 2013. Antithrombotic activities of aqueous extract from Gardenia jasminoides and its main constituent. Pharm Biol. 51(2):221–225.

Zhang JJ, Zhang W, Yin ZH, Li CQ, Kang WY. 2018. Procoagulant constituents from Cordyceps militaris. Food Sci Hum Well. 7(4):282–286.

Zhao H, Long JF, Zou HY, Zhang QX, Zhang J, Wang L, Zhang L, Bian BL, Zhao HY. 2014. Huanglian Jiedu decoction active fraction protects ipsilateral thalamus injury in MCAO rats through regulating astrocytes. Zhongguo Zhong Yao Za Zhi. 39:4405–4410.