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Original article

Capture of anti-coagulant active ingredients from Moutan Cortex by platelet immobilized chromatography and evaluation of anticoagulant activity in rats

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A B S T R A C T

Moutan Cortex (MC) is a well-known Chinese medicine for promoting blood circulation and relieving blood stasis. The intent of this study was to evaluate the anticoagulant activity of MC and capture the bioactive compounds by platelet immobilized chromatography. Sprague Dawley (SD) rats were randomly divided into the control group, aspirin group and MC group (1.25, 2.5, 5 g/kg/d). Coagulation system and platelet activity were investigated to evaluate the anti-coagulation effect of MC. The effective components of MC were captured by platelet immobilized chromatography. High performance liquid chromatography-diode array detection (HPLC-DAD) and liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) analysis were used to identify the binding ingredients. Meanwhile, the efficacy of active ingredients was assessed through inhibiting platelet adhesion and regulating the expression of platelet related proteins. Principal findings showed that 2.5 g/kg/d MC significantly prolonged thrombin time (TT) and 5 g/kg/d MC significantly prolonged TT and prothrombin time (PT). MC exhibited an inhibitory potency on adenosine diphosphate-induced platelet aggregation. Four active compounds were found by platelet immobilized chromatography including oxypaeoniflorin, tetragalloylglucose, pentagalloyl glucose and benzoylpaeoniflorin; these active ingredients significantly up-regulated the expression of hsp-70 and coronin-1B, reduced the ratio of adhesion platelets. These results suggest that MC markedly promoted blood circulation and relieved blood stasis by inhibiting platelet activation, as an anti-coagulant, elucidating its potential capacity to treat cardiovascular diseases.

1. Introduction

Cardiovascular diseases (CVDs) remain a major healthcare problem, with a significant human and economic toll. In many countries, CVDs, such as stroke, myocardial infarction and atherosclerosis, have a responsibility for huge proportion of human mortality [1]. According to the American Heart Association statistic, about $297.7 billion were spent on CVDs in the United States in 2008 [2].

Previous studies have showed that reduced or modified dietary fat and aspirin therapy all contribute to weaken cardiovascular disease [3,4]. Sensible diet system could prevent the occurrence of CVDs without therapeutic means. Aspirin is well-accepted for the primary and secondary prevention of cardiovascular events and the significant clinical effects that cannot be denied. However, at the same time, there are major adverse reactions including anti-platelet resistance and increased risk of bleeding in the digestive and nervous systems [5–7]. Other side effects, such as hypertension or renal toxicity, are usually dose-related [8].

Coagulation system and platelet function play important roles in the pathogenesis of CVDs, such as thrombotic diseases, coronary artery diseases and ischemic diseases [9–11]. It was reported that many CVDs were related to platelet abnormalities, excessive activation and

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hyperactivity [12]. Prothrombin time (PT), activated partial thromboplatin time (APTT), thrombin time (TT), fibrinogen (FIB) were associated with coagulation, which are frequently used to examine thrombotic diseases [13,14]. Thus, it has a great potential to treat CVDs with the inhibition of platelet function and improvement of the coagulation system. Traditional Chinese Medicines (TCMs), which are mostly based on multi-components, multi-channels, multi-targets to treat CVDs, have attracted more and more researchers [15–17].

Moutan Cortex (MC, the root bark of *Paeonia suffruticosa* Andr.) is a well-known medicinal herb. It has been widely investigated in diabetic nephropathy, acute hepatotoxicity, inflammation and other diseases recently [18–20]. According to traditional Chinese medicine, MC can promote blood circulation and relieve blood stasis. Paeonol, one of the major components of MC, improves blood circulation by inhibiting platelet aggregation and blood coagulation [21,22]. MC, as a member of complex and orderly traditional Chinese medicines (TCMs) which have a better efficacy through multi-components synergism, contains a variety of ingredients such as oxypaeoniflorin, paeoniflorin, trigalloyl glucose, benzoylpaeoniflorin, etc [23]. However, little is clear about the components contributing to improve blood circulation in MC prior to our research.

Recently, methods for capture of active ingredients from TCMs including molecularly imprinted polymers and high-throughput methodology have been developed increasingly [24,25]. Cell membrane immobilized chromatography is based on the interaction of drug molecules with cell membranes by receptors, channels and enzymes targets [26–28]. Cell membrane immobilized chromatography technology consists of two parts: one is the biologic membrane used for immobilizing active substances in TCMs; the other is the HPLC–MS/MS to ascertain the structures of active substances [29]. Platelet has a complete cell membrane structure to be utilized as the solid phase to select active components from MC.

The present study was conducted to evaluate the efficacy of anticoagulant, anti-platelet aggregation, and the regulatory effect of MC on platelet proteins and to establish the platelet immobilized chromatography for capturing the potential active components in MC which interacted with platelets.

2. Materials and methods

2.1. Materials

Moutan Cortex, the root bark of *Paeonia suffruticosa* Andr. (batch number 140506), were purchased from Anhui Bozhou Wanzhen Chinese Medicine Technology Co., Ltd. The taxonomy of the medicinal material was identified by Prof. Dekang Wu from Nanjing University of Chinese Medicine. Aspirin was purchased from Bayer HealthCare Manufacturing S.r.l. (Beijing, China); HPLC-grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI); HPLC-grade water was obtained using a water purification system (Milli-Q Reagent Water System, MA, USA); standards of oxypaeoniflorin, pentagalloyl glucose and benzoylpaeoniflorin, with the purity of more than 98% were offered by National Institutes for Food and Drug Control. The other kits were obtained as follows: TXB_2 kit (Nanjing Jiancheng Bioengineering Institute); 6-keto-$PGF_{1\alpha}$ kit (Nanjing Jiancheng Bioengineering Institute); BCA kit (Nanjing KeyGEN Biotech. Co., Ltd.); coronin 1 B (Santa Cruz Biotechnology, California, CA, USA); p-hsp70 (Bioss, Beijing, China); hsp70 (Santa Cruz Biotechnology, California, CA, USA).

2.2. Preparation of MC extract

The roots of *Paeonia suffruticosa* Andr. (300 g) was extracted under reflux with 2400 mL ethanol/water (75:25, v/v) for 1.5 h. The procedure was repeated twice and all the extraction was combined together. The extract was evaporated to 300 mL (1 g/mL) concentrated solution.

2.3. Evaluation of MC anticoagulant activity in vivo

2.3.1. Animals experiment and drug administration

Male Sprague Dawley (SD) rats (n = 30) weighing 180–220 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and used for the study. All rats were given sterile water and kept at a temperature of 25 °C and a relative humidity at 45% for three days. The rats were randomly assigned to 5 groups (n = 6 for each group): control group rats were treated with normal saline (1 mL/100 g/d) by gavage; positive control group (treated with aspirin, 50 mg/kg/d); MC groups (1.25, 2.5, 5.0 g/kg/d). All rats were administered every day for one week. Rats were fasted for 12 h but given free access to drinking water before surgery.

2.3.2. The assessment of APTT, PT, TT, FIB in plasma

Blood samples were collected from the abdominal aorta at 12 h after the last gavage and mixed with 3.8% sodium citrate (citrate: blood = 1: 9, v/v). Plasma was separated from blood by centrifuging at 3000 rpm for 10 min for the detection of plasma anticoagulation [30]. APTT, PT, TT and FIB were assessed by automatic coagulation analyzer (Thrombolyzer XRM, Wuhan, China). These indicators were detected within 2 h after blood collection.

2.3.3. Effects of MC on levels of plasma 6-keto-PGF1α, TXB2 and ADP-induced platelet aggregation

Blood were collected from abdominal aorta and anti-coagulated with 3.8% sodium citrate (citrate:blood = 1:9, v/v). Platelet-rich plasma (PRP) was separated from blood by centrifuging at 800 rpm for 10 min. The PRP was carefully removed and the pellet was centrifuged again at 3000 rpm for 10 min to obtain platelet-poor plasma (PPP) [31]. PRP samples were diluted with PPP to a cell density of 3×10^9 cells/mL for the determination of platelet aggregation by automatic platelet aggregation instrument (LBY-NJ4A, Beijing China) [32]. Platelet aggregation rate were detected, recorded the maximum aggregation rate within 5 min. Levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ in serum were measured by ELISA kits according to manufacturer's instructions.

2.3.4. Western blotting assays

Platelets were prepared as the method described by Sokolova [33]. In brief, PRP were collected by centrifuging at 800 rpm for 10 min, and then the PRP were centrifuged at 3000 rpm for 10 min to collect platelets.

The platelets were sonicated and incubated on ice for 30 min and then the insoluble material was removed by centrifuging at 12000 rpm for 5 min at 4 °C. The protein in the supernatant was collected for Western blot analysis. The total protein concentrations were measured by the BCA protein assay kit (Nanjing Keygen Biotech. Co., Ltd.) according to the manufacturer's instructions. Equalized amounts of proteins from each sample were subjected to polyacrylamide-sodium dodecyl sulfate gel electrophoresis and then transferred to polyvinylidenedifluoride (PVDF) membranes. Nonspecific binding sites were blocked in TBST (NaCl: 8 g, Tris powder: 2.42 g, double-distilled water volume to 1 L, pH was adjusted to 7.4 with NaOH, then add Tween 20 1 mL to the solution) containing 5% nonfat milk and 0.1% Tween 20 for 2 h at room temperature. After being washed three times with TBST, the membranes were incubated with primary antibodies raised against coronin-1B (1:1000), hsp70 (1:1000), p-hsp70 (1:1000), and β-actin (1:1000) at 4 °C overnight. The membranes were washed with TBST three times and incubated with the secondary antibody (Goat-anti-Rabbit) conjugated with IgG-HRP for 1 h at room temperature. Immune complexes were detected using an enhanced chemiluminescence system. The densities of the bands were determined using image analysis software Gel-pro Analyzer 4.5.

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