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Decichine enhances hemostasis of activated platelets via AMPA receptors

Ling-Fang Huang [a,b,1], Hai-Lian Shi [a,1], Bo Gao [a,b], Hui Wu [a], Li Yang [a], Xiao-Jun Wu [a,*], Zheng-Tao Wang [a,b,c,*]

[a] The Ministry of Education (MOE) Key Laboratory for Standardization of Chinese Medicines, Shanghai Key Laboratory of Complex Prescription, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China
[b] Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China
[c] Shanghai R&D Center for Standardization of Traditional Chinese Medicines, Shanghai, China

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ABSTRACT

Introduction: Decichine, one of the non-protein amino acids present in the roots of Panax notoginseng, has been found to shorten bleeding time of mice and increase the number of platelets. However, the exact underlying mechanisms have not been elucidated yet. This study was aimed to identify the hemostatic effect of decichine and uncover its mechanisms.
Materials and methods: Hemostatic effect was assessed by measuring tail bleeding time and coagulation indices of rats. PT, APTT, TT and FIB concentration were measured using a Sysmex CA-1500 plasma coagulation analyzer. Platelet aggregation rate was determined by using a platelet aggregometer. Concentration of cyotosolic calcium was evaluated by Fluo-3 and levels of cyclic adenosine monophosphate (cAMP) and thromboxane A_2 (TXA_2) were measured by ELISA method.
Results and conclusion: Decichine administered orally shortened tail bleeding time, reduced APTT and TT but increased the concentration of FIB in plasma in a dose-dependent manner. When induced with trap, decichine could elevate the cytoplasmic concentration of calcium, and secretion of TXA_2 as well as the ratio of TXA_2 to PGI_2 from platelets. Meanwhile, it decreased the level of intracellular cAMP. However, CNQX could block the enhanced hemostatic effect of decichine. These results suggested that decichine exerted hemostatic function via AMPA receptors on platelets, therefore, facilitated coagulation cascade in a paracrine fashion by control of platelet cytosolic calcium influx, cAMP production and TXA_2 release. Current study may contribute to its clinical use in therapy of hemorrhage.

Introduction

Panax notoginseng (Burk) F.H. Chen, namely Tianqi or Sanqi, is a well-known traditional Chinese medicine in Asia. The roots of the herb are used extensively in clinic to facilitate hemostasis and promote blood circulation in addition to relieve swelling, alleviate pain and nourish the blood [1,2].

β-*N*-oxalyl-L-α,β-diaminopropionic acid (decichine), a non-protein amino acid in roots of *P. notoginseng*, has been reported widely to induce neurological disorders. Chicken treated with high dose of decichine showed balance problems [3]. Human beings often displayed neurotoxic symptoms such as astasia, head retraction, neck stiffness and extensor paralysis of the legs after excessive consumption of *Lathyrus sativus* (grass pea seeds) rich in decichine [4,5]. However, study also disclosed that decichine contributed to stop bleeding by increasing the number of platelets and thus reduced bleeding time [3]. And the dose of decichine to induce hemostasis was much less than that to elicit neurotoxicity [6]. But its exact hemostatic mechanism has not been elucidated yet.

Blood platelets play an important role in hemostasis by involving in the formation of thrombi to prevent blood loss and maintain vascular integrity. Platelets in a non-adherent rest state are activated after exposure to immobilized adhesive proteins or soluble platelet agonists, such as von Willebrand factor, collagen, ADP, thrombin, and thromboxane A2 (TXA_2). These agonists induce signal transduction via respective receptors and converge into common signaling events that ultimately induce the "inside-out" signaling process. This leads to the activation of integrin αIIbβ3, which mediates platelet adhesion and aggregation, and triggers "outside-in" signaling transduction. Consequently, the process results in platelet spreading, additional granule secretion, stabilization of platelet adhesion and aggregation, and clot cohesion. Uses of all of these

Abbreviations: TA, Tranexamic acid; CMC, Carboxymethylcellulose; ADP, adenosine diphosphate; PT, prothrombin time; APTT, activated partial thromboplastin time; FIB, fibrinogen concentration; TT, thrombin time; PBS, phosphate buffer saline; TXB_2, thromboxane B_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; cAMP, cyclic adenosine monophosphate; PRP, platelet-rich plasma; PPP, platelet-poor plasma; EDTA, Ethylenediaminetetraacetic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Trap, thrombin receptor-activating peptide.

* Corresponding authors at: Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Pudong District, Shanghai 201203, PR China. Tel.: +86 21 51322507; fax: +86 21 51322519.
E-mail addresses: xiaojunwu320@126.com (X.-J. Wu), wangzht@hotmail.com (Z.-T. Wang).
[1] These two authors contributed equally to this work.

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agonists contribute significantly to the hemostasis after surgery or bleeding disorders [7–10].

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, is one of the glutamate receptors in neuronal cells. Previous studies indicated that dencichine could facilitate and/or prolong glutamatergic transmission through activating AMPA receptors to inhibit glutamate transport [11]. As a result, it altered cellular Ca^{2+} homeostasis that resulted in excitotoxic attack, thus, led to extensive neuronal cell loss [12]. AMPA receptor has also been found to be expressed on platelets and involved in their activation [13]. However, whether the hemostatic effect of dencichine is mediated by platelet AMPA receptors or not has not been demonstrated yet. Current study elaborated the role of AMPA receptor on platelets in the hemostatic process of dencichine for the first time. The results may contribute to the clinical use of dencichine in therapy of hemorrhage.

Materials and Methods

Reagents

Raw roots of *P. notoginseng* were collected from a Good Agricultural Practice farm in Yunnan Province of China. Dencichine (purity > 98%) was purchased from Nanjing Aoduofuni Biotechnology Corporation (Nanjing, Jiangsu, China). Tranexamic acid (TA), trap-6, carboxymethylcellulose (CMC), adenosine diphosphate (ADP), apyrase and Fluo 3-AM were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reagents for determining prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB) concentration and thrombin time (TT) were obtained from Dade Behring Inc. (Deerfield, IL, USA). CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline-2,3-dione), enzyme linked immunoassay (ELISA) kits for thromboxane B_2 (TXB_2), 6-keto-prostaglandin F1α (6-keto-PGF1α), and cyclic adenosine monophosphate (cAMP) were all purchased from Shanghai Huayi Biotech Corporation (Shanghai, China). All the other reagents used were of analytical grade from commercial sources.

Animals

Male Wistar rats weighing 200–300 g were purchased from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine (TCM) (Shanghai, China). All animals were allowed to acclimatize for at least one week before experiments, and kept under the same laboratory condition of temperature (25 ± 2 °C), humidity (62 ± 2%) and lighting (12 h light:12 h dark cycle) with unrestricted access to food and water. All animal experiments were conducted according to the ethical guidelines of National Guide for the Care and Use of Laboratory Animals and were approved by the institutional Ethics Committee of Shanghai University of TCM.

Experimental Design

For long-term hemostatic-effect study of dencichine, twenty-four rats were randomly divided into four groups (n = 6 per group) and administered orally once daily with 0.5% CMC (control), dencichine (1.8 mg/kg), TA (140 mg/kg), and raw powder of *P. notoginseng* roots (310 mg/kg), respectively [14]. After treatment for 14 consecutive days, the rats were fasted for 12 h followed by the last treatment. Thirty minutes later, the rats were anaesthetized intraperitoneally (i.p.) with urethane (1 g/kg) and subjected to further bleeding time and plasma coagulation analysis.

For acute hemostatic-effect study of decichine, forty-two rats were randomly divided into seven groups (n = 6 per group) and given intragastrically with dencichine dissolved in PBS at doses of 0, 5, 10, 15, 20, and 40 mg/kg, respectively. Thirty minutes later, the rats were anaesthetized and used to determine bleeding time and platelet aggregation rate.

To determine thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) levels in plasma, sixty-four Wistar rats were randomly divided into eight groups (n = 8 per group), and were injected intravenously with either PBS or CNQX (0.036 mg/kg). Ten minutes later, the rats were given intragastrically with dencichine at doses of 0, 10, 15, and 20 mg/kg, respectively. After being treated for 30 min, the rats were anaesthetized and the blood was collected and used to determine TXA_2 and PGI_2 levels of plasma with respective ELISA kits.

Bleeding Time (BT) Assay

The tail bleeding model was conducted based on the previous methods with slight modifications [15]. Briefly, the tails of rats were transected with a sterile razor blade at the site that 5 mm apart from the tip. The resultant wound was gently blotted with filter paper every 30 s. The bleeding time was defined as the time from the start of transection to bleeding cessation. The time without outflow of blood for 30 s was considered as bleeding cessation. Pressure was applied to the wound to stop bleeding if the bleeding time was greater than 30 min.

Plasma Coagulation Assay

The blood collected from abdominal aorta of rats was placed in a test-tube pre-coated with 3.8% sodium citrate. After being centrifuged at 1000 rpm for 10 min at room temperature, the plasma (platelet-rich plasma, PRP) was subjected to prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB) concentration and thrombin time (TT) assays in accordance with the manufacturer's protocols. The coagulation experiments were performed using a Sysmex CA-1500 plasma coagulation analyzer supplied by Sysmex Corporation (Kobe, Japan) [16].

Platelet Aggregation Assay

The PRP obtained as described above was centrifuged at 3000 rpm for 3 min at room temperature to get platelets and platelet poor plasma (PPP). After being washed twice with a modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH7.4), the platelets were resuspended in the Tyrode-HEPES buffer containing 1 mM $CaCl_2$ at the concentration of 4.8×10^8 platelets/ml and kept at room temperature [17]. After being treated with dencichine, glutamate (150 μM), and PBS with or without CNQX (10 μM) at 37 °C for 3 min, 225 μl of washed platelets were mixed with 25 μl of trap (5 μM) and incubated at 37 °C for 5 min. The aggregation was monitored with a platelet aggregometer (Helena Laboratories, TX, USA), and the data were analyzed using a HemoRAM software.

Cytosolic Ca^{2+} Measurement in Platelets

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with Fluo 3-AM as previously reported [18]. Briefly, 2 ml of PRP (1.4×10^9 platelets/ml) was incubated with Fluo 3-AM (5 μM) at 37 °C for 45 min. After addition of 80 μl of acid citrate dextrose buffer, the plasma was centrifuged at 3000 rpm for 3 min. The precipitate was washed with HEPES buffer A (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, 0.2 U/ml Apyrase, 0.1% (w/v) BSA, pH6.6) to remove the unloaded dye. Afterwards, the platelet was resuspended in HEPES buffer B (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, 0.2 U/ml Apyrase, 0.1% (w/v) BSA, pH7.5) and adjusted to the final concentration of 2×10^8 platelets/ml. Probenecid (2.5 mM) was added to all the buffers in order to prevent the leakage of dye. After incubation with PBS, glutamate (150 μM), or dencichine