บทความวิจัย (Research Article)

สารสกัดกระชายลดการตายของเซลล์ประสาทและการกระตุ้นของเซลล์แอสโทรไซต์ในสมองส่วนฮิพโพแคมพัสของหนูจำลองโรคหลอดเลือดสมองชนิดขาดเลือด

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The extract of *Boesenbergia rotunda* attenuates neuronal loss and the activation of astrocytes within the hippocampus of rats following ischemic stroke

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Naresuan Phayao J. 2019;12(3):38-46.

Received: 18 October 2019; Revised: 20 December 2019; Accepted: 25 December 2019

บทคัดย่อ

โรคหลอดเลือดสมองชนิดขาดเลือด (Ischemic stroke) มีความสำคัญต่อการเกิดการบาดเจ็บของสมอง ซึ่งส่งผลต่อทั้งเซลล์ประสาท (Neuron) เซลล์เกลีย (Glial cell) รวมทั้งหลอดเลือด โดยโรคหลอดเลือดสมองเป็นโรคที่มีความสำคัญและเป็นปัญหาที่พบได้บ่อยของประชากรทั่วโลก โดยเฉพาะในกลุ่มผู้สูงอายุ และเนื่องด้วยข้อจำกัดทางด้านการรักษา ดังนั้นจึงมีความจำเป็นและตระหนักอย่างยิ่งในการหาแนวทางป้องกันและรักษา ซึ่งการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดกระชาย (*Boesenbergia rotunda*) ต่อการป้องกันการตายของเซลล์ประสาทและการเปลี่ยนแปลงของเซลล์แอสโทรไซต์ (Astrocytes) ในหนูที่ถูกจำลองโรคหลอดเลือดสมอง ในการทดลองครั้งนี้หนูสายพันธุ์ Wistar เพศผู้จะได้รับการป้อนด้วยสารสกัดกระชาย ขนาด 200 มก./กก. น้ำหนักตัว ทุกวัน เป็นเวลา 14 วัน ก่อนที่จะเหนี่ยวนำให้เป็นโรคหลอดเลือดสมองด้วยวิธีการอุดกั้นหลอดเลือด Middle cerebral artery ด้านขวา จากนั้นหนูจะได้รับการป้อนสารสกัดต่อเนื่องอีก 7 วันหลังจากที่เป็นโรคหลอดเลือดสมองแล้ว ผลการทดลองพบว่าสารสกัดกระชายขนาด 200 มก./กก. น้ำหนักตัว สามารถลดการตายของเซลล์ประสาทในสมองส่วน CA1 ของฮิพโพแคมพัสในหนูจำลองโรคหลอดสมองชนิดขาดเลือด และนอกจากนี้เรายังพบว่าสารสกัดกระชาย ขนาด 200 มก./กก. น้ำหนักตัว สามารถลดการแสดงออกของโปรตีน GFAP ในเซลล์ Astrocytes ในสมองบริเวณดังกล่าวนี้ด้วย ดังนั้นจากผลการศึกษา สรุปได้ว่าสารสกัดกระชายขนาด 200 มก./กก. น้ำหนักตัว สามารถลดการตายของเซลล์ประสาทผ่านการลดการกระตุ้นของเซลล์ Astrocytes ดังนั้นกระชายจึงเป็นสมุนไพรที่มีความสำคัญ ซึ่งอาจจะมีฤทธิ์ในการป้องกันหรือรักษาการบาดเจ็บของเซลล์สมองจากโรคหลอดเลือดสมองได้ แต่อย่างไรก็ตาม ข้อมูลจากการศึกษาครั้งนี้เป็นเพียงข้อมูลพื้นฐานเพื่อนำไปศึกษาในเชิงลึกของสารออกฤทธิ์ของกระชายต่อไป

คำสำคัญ: กระชาย, โรคหลอดเลือดสมองชนิดขาดเลือด, แอสโทรไซต์, ฮิพโพแคมพัส

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Abstract

Ischemic stroke significantly provoked the induction of brain damage, in which neurons, glial cells and the vasculature are affected. The defensive strategy of ischemic stroke, one of the most challenging problems in adult, especially in the elderly, has greatly received attention due to the limitation of therapeutic efficacy. In this study, we aimed to investigate the protective effect of *Boesenbergia rotunda* extract on neuronal cell and the alterations of astrocytes in animal model of ischemic stroke. Adult male Wistar rats were orally given *B. rotunda* extract at a dose of 200 mg/kg body weight once daily for 14 days before and 7 days after middle cerebral artery occlusion (MCAO). We found that stroke rats with *B. rotunda* extract showed a significant increase of neuronal density in CA1 of hippocampus. In addition, decreased expression of glial fibrillary acidic protein (GFAP) was observed in CA1 in stroke plus *B. rotunda* group. The results indicated that *B. rotunda* extract at a dose of 200 mg/kg could suppress the neuronal loss associated with lower activation of astrocytes. Therefore, *B. rotunda* is the potential medicinal plant that might help in prevention of the brain damage. However, further researches are essential to elucidate the possible active ingredient.

Keywords: *Boesenbergia rotunda*, middle cerebral artery occlusion, ischemic stroke, astrocytes, hippocampus

Introduction

Stroke is recently reported as the second most common cause of mortality worldwide, with almost 6 million deaths from stroke every year. [1] It is also the third biggest cause of disability, demonstrating a high economic and social burden for communities. [2] Ischemic stroke results from the blockage of blood supply to the brain, which can cause pathological changes of the brain leading to memory deficit. [3] Furthermore, the changes within the brain after stroke are associated with energy failure, excessive glutamate release, increased intracellular Ca^{2+} levels, production of free radicals, inflammation, and neuronal cell death via necrosis and apoptosis. [4] The hippocampus is the main region responsible for memory formation [5] and is also recognized as a major sensitive site in response to cerebral ischemic injury. [6] Previous studies have demonstrated that loss of memory is an important cause of dementia after stroke associating with neuronal damage and death, [4, 7, 8] the alterations of glial cells [9, 10], and the vasculature. [11] Neurons are very highly sensitive to the lack of glucose and ATP and are the first brain cells to die in the area directly affected by the lack of blood flow. [12] Moreover, under ischemia condition, the hyperreactivity of astrocytes can disrupt neuronal function, leading to cell death. [13]

Boesenbergia rotunda is a ginger species belonging to the family of Zingiberaceae. This herbal plant is also used as a traditional medicine to treat various illnesses including rheumatism, muscle pain, and peptic ulcer. [14] Importantly, *B. rotunda* extract also possesses antioxidant activity [15, 16] and anti-inflammatory activity. [17] Because *B. rotunda* could scavenge free radicals and its anti-inflammatory activity that play as crucial factors in causing the brain damage induced by cerebral ischemia. We hypothesized that the *B. rotunda* extract might be able to protect brain damage induced by ischemic stroke via the reduction of neuronal loss and the activation of astrocytes. In the study, we aimed to determine possible effects of *B. rotunda* extract on neuronal cell loss that may be associated with the alterations of astrocytes within the hippocampus after the occlusion of the right middle cerebral artery.

Material and Method

Animals

Adult male Wistar rats aged 8 weeks were obtained from the Nomura Siam International Co., Ltd. (Bangkok, Thailand) and were allowed a week for acclimatization prior to the commencement of experimentation. All animals were housed and maintained on a 12:12h light-dark cycle in a constant temperature (21 ± 1 °C) and humidity room with food and water available ad libitum. All procedures were approved by the Ethics Committee of the Laboratory Animal Research Center, University of Phayao (approval no. 58 01 04 0010).

Plant materials and preparation

Plant sample, the roots of *B. rotunda*, were bought from local market in Muang District, Phayao Province from November 2016-March 2017. The samples were cleaned and cut to small pieces then dried with hot air oven at 60°C for 48 hours. Dried samples (3 kg of *B. rotunda*) were ground and extracted with 95% v/v ethanol by maceration. The ethanol was removed from the extracts under vacuum condition to yield ethanol crude extracts.

Middle cerebral artery occlusion (MCAO) model

The middle cerebral artery occlusion stroke model was generated according to modified method of Longa et al. [18] Briefly, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). In anesthetized rats, the right side of the common carotid artery and the external carotid artery were exposed and ligated proximally. The middle cerebral artery (MCA) was occluded by inserting a 4-0 monofilament nylon suture (USS DGTM sutures; Tyco Healthcare group LP, Connecticut, USA) with a heat-rounded tip into the internal carotid artery, which approximately 17-18 mm distal to the carotid bifurcation until a mild resistance was felt. Then the wound was sutured, and the animals were returned to their home cages. The incision sites were infiltrated with 10% povidone-iodine solution for anti-septic postoperative care.

The experimental timeline and tissue processing

All animals were randomly divided to one of three treatment conditions (n = 5-7/group) as described following. (1) sham operated group, (2) stroke + vehicle, rats were orally given 1% of carboxymethylcellulose (CMC) which was used as vehicle to dissolve the tested substance, (3) stroke + *B. rotunda*, rats were orally given the extract of *B. rotunda* at a dose of 200 mg/kg BW. The dose of the extract used in this study was selected base on the previous study. [19] The animals in all groups were given the extract orally once daily for 14 days before and 7 days after the occlusion of right middle cerebral artery (MCAO). Animals were deeply anesthetized via intraperitoneal injection of sodium pentobarbital (70 mg/kg) and transcardially perfused with ice cold 0.1M PBS for 5 min followed by ice cold 4% paraformaldehyde (pH 7.4) for 15 min. Brains were removed and post-fixed in the same fixative. Then, brains were embedded in paraffin and sectioned into 5 μm thick coronal sections.

Immunohistochemistry

For immunoperoxidase labelling, all reactions were run at the same time, with the same reagents, at the same concentrations. Serial 5-μm-thick paraffin sections were deparaffinized in xylene and hydrated in a series of graded alcohols and distilled water. Antigen retrieval was performed by boiling the samples with a 10-mM sodium citrate buffer (pH 6.0) for 20 min by using microwave (750 W) and then treating them with 3% hydrogen peroxide (H_2O_2). After blocking in 10% normal horse serum for 1 h, the sections were incubated with primary antibodies at 4 °C overnight. Primary antibodies used were mouse anti-glial fibrillary acidic protein (GFAP, 1:100; Millipore, catalog #MAB5628), rabbit anti-vascular endothelial growth factor (VEGF, 1:100; Merck, catalog #ABS82). Sections were rinsed by 0.1 M PBS for 30 min and incubated for 2 h at room temperature with biotinylated donkey anti-mouse secondary antibody (1:500; Jackson Immunoresearch; catalog #715-065-150), or anti-rabbit secondary antibody (1:500; Jackson Immunoresearch; catalog #711-005-152). Sections were rinsed, incubated in 0.1% extravidin peroxidase for 1 h, and then rinsed again. Immunolabelling was developed using a nickel-enhanced 3,3′-diaminobenzidine (DAB) reaction. Tissues of three groups were performed simultaneously and the DAB reactions were developed for exactly the same length of time following the addition of glucose oxidase (1:1000). For cresyl violet staining, paraffin sections of the brains were stained with 0.2% cresyl violet, dehydrated through graded alcohols (70, 95, 100% 2x), cleared in xylene, and coverslipped using DPX mountant.

Image acquisition, thresholding and cell count analysis

Hippocampal images (+3 mm from Bregma) were captured at 10× using a bright-field microscope (Olympus). The immunoreactive signal was determined using Image J software. The images were cropped into 4 subregions of the hippocampus including cornu ammonis one (CA1), two (CA2) and three (CA3), and the dentate gyrus (DG). These cropped regions were thresholded and the data was presented as the percentage of thresholded area. The term '% of thresholded area' refers to the percentage of pixels that were captured to the total number of pixels in each image. To determine the density of neuron, 40x images of CA1, CA2, CA3, and DG were exhaustive manual counts by using SXView program.

Data analysis

All data were analyzed using GraphPad Prism 7 (Graph-Pad Software, USA). Statistical analysis was performed using one-way ANOVA, followed by Tukey post hoc test for multiple comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Data were presented as mean ± SEM.

Results

Effect of *B. rotunda* extract on neuronal density in the hippocampus

The effect of *B. rotunda* extract on neuronal density in the hippocampus was shown in **Figure 1.** Compared to sham animals, stroke alone induced a significant reduction of neuronal density in CA1, CA2 and CA3 of the hippocampus. While, the extract of *B. rotunda* at dose of 200 mg/kg BW particularly extended the neuronal density in only CA1. Thus, this finding suggested that the extract can improve neuronal damage induced by stroke in CA1 region.

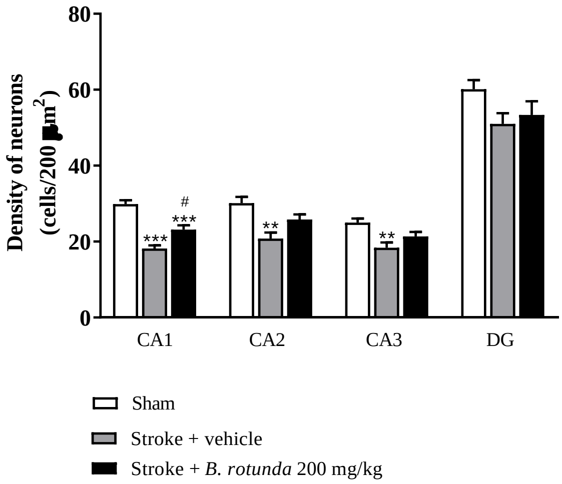

Figure 1 Effect of *B. rotunda* extract on neuronal density in various subregions of the hippocampus. Data expressed as mean ± SEM for sham (n = 7), stroke (n = 5) and stroke + *B. rotunda* 200 mg/kg BW (n = 6). **p* <0.05, ***p*<0.01, ****p*<0.001 compared to the value of the sham group; $^{\#}p$<0.05 compared to stroke + vehicle group.

Effect of *B. rotunda* extract on the suppression of GFAP activation in CA1 of the hippocampus

In this study, we determined the effect of *B. rotunda* extract on the alterations of astrocytes. It was found that stroke induced the activation of astrocytes characterized by changing their morphology from resting state to reactive state as presented in **Figure 2.** The immunoreactivity of GFAP was outstandingly increased after stroke. While, stroke-treated with *B. rotunda* at a dose of 200 mg/kg showed significantly lower expression of astrocytes.

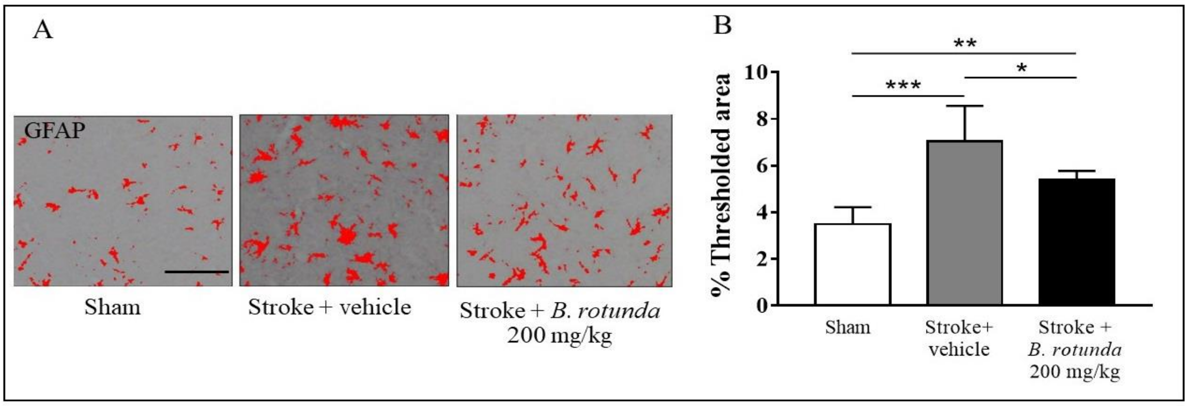

Figure 2 Immunohistochemical labelling of GFAP within CA1 of the hippocampus. (A) Images of the thresholding analysis by using Image J software. (B) Graphs showed the percentage of material thresholded of GFAP. Stroke + vehicle treated group demonstrated a significant increase in thresholded materials of GFAP. Stroke-treated with *B. rotunda* extract, the expression of GFAP was declined. Data expressed as mean ± SEM for sham (n = 7), stroke (n = 5) and stroke + *B. rotunda* 200 mg/kg BW (n = 6). **p*<0.05, ***p*<0.01, ****p*<0.001. Scale bar = 100 µm.

Effect of *B. rotunda* extract on the expression of VEGF within CA1 of the hippocampus

We also examined the effect of *B. rotunda* extract on VEGF labelling in CA1 of the hippocampus. There was a remarkable decrease in levels of VEGF expression in stroke + vehicle treated animals compared to sham operated animals, but no significant difference was observed between stroke + vehicle treated group and stroke + *B. rotunda* 200 mg/kg group **(Figure 3).**

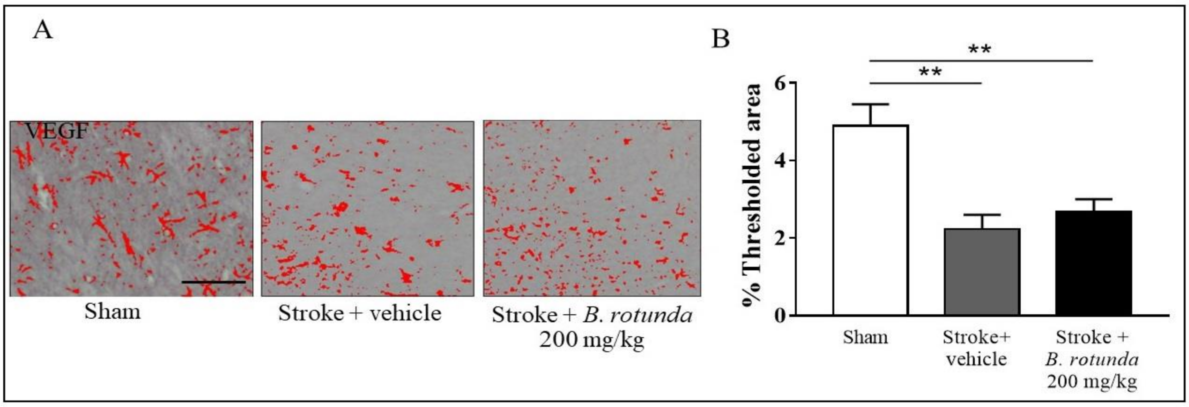

Figure 3 Immunohistochemical labelling of VEGF in CA1 of the hippocampus. (A) Images of the thresholding analysis by using Image J software. (B) Graphs showed that there was no significant effect of *B. rotunda* extract on the levels of VEGF compared to stroke + vehicle treated group. Data expressed as mean ± SEM for sham (n = 7), stroke (n = 5) and stroke + *B. rotunda* 200 mg/kg BW (n = 6). **p*<0.05, ***p*<0.01, ****p*<0.001. Scale bar = 100 μm.

Discussion

The findings advised for the first time that *B. rotunda* extract could prevent neuronal loss in a rat model of ischemic stroke. In addition, *B. rotunda* also induced suppression of GFAP within the hippocampus after vascular occlusion. Ischemic stroke is triggered by the blockage of blood supply can cause pathological changes of the brain leading to memory deficit. [3] Importantly, stroke affects all cellular elements of the brain such as neurons, glial cells; microglia, astrocytes, oligodendrocytes, and vascular cells. [7, 9, 11]

In the current study, we obviously demonstrated that the occlusion of middle cerebral artery exhibited significant neuronal loss in CA1, CA2, CA3 of the hippocampus. Stroke-treated with the extract of *B. rotunda* at a dose of 200 mg/kg BW showed the significant elevation of the neuronal density only in CA1. However, the mechanisms of this regional vulnerability of CA1 neuron are poorly understood. In the hippocampus neuronal populations that indicate differential vulnerability to ischemia. [20, 21] Previous studies demonstrated that the dentate gyrus and CA3 appear to be more resistant to ischemia. [22] In addition, there are many factors that are critical in neuronal death and survival such as glial cell proliferation, excess glutamate release and glutamate receptors. [23] Moreover, the neuronal death gradually occurred three to four days after transient ischemic injury which are proposed to involve oxidative stress, excitotoxicity, and inflammation. [9, 24]

In ischemic condition, astrocytes have been known to involve in negative response through their morphological changes which disturb neuronal function. [13] Several studies previously reported that the immunoreactivity of GFAP was significantly increased after stroke characterized by hypertrophied morphology. [25, 26] These changes have been reported to generate proinflammatory cytokines such as interleukin 1, 6 (IL-1, IL-6), tumor necrosis factor (TNF) and reactive oxygen species (ROS) associating with the disruption of neuronal function. [27] Therefore, we determined the effect of *B. rotunda* extract on the suppression of GFAP in the hippocampus. Similar to the results, ischemic stroke alone induced significant astrocytic activation in CA1 of the hippocampus. In addition, the extract of *B. rotunda* at a dose of 200 mg/kg also inhibited the hyperreactivity of astrocytes significantly. The possible mechanism of reduced astrocytic activation might be involved in the antioxidant and anti-inflammatory activities of the extract. These effects might suppress activated astrocytes-induced proinflammatory cytokines secretion.

According to neurons and astrocytes, VEGF also has a crucial role in neurogenesis and angiogenesis. [28] The overexpression of VEGF promoted angiogenesis following focal cerebral ischemia in mice. [29] So, we investigated the effect of *B. rotunda* extract on the alteration of VEGF labelling in CA1 of the hippocampus. However, there was a significant decrease in levels of VEGF expression in animals followed stroke compared to sham operated animals, but no significant difference was detected between stroke + vehicle treated group and stroke + *B. rotunda* 200 mg/kg treated group.

In conclusion, the results indicated that *B. rotunda* possessed the protective effect against focal cerebral ischemia. It could attenuate neuronal cell loss and the astrocytic activation especially in CA1 of the hippocampus. Therefore, *B. rotunda* could be a potential medicinal plant that might prevent brain damage. However, further studies are essential to clarify the possible active ingredient.

Acknowledgments

The current study was financially supported by the University of Phayao, Mueang, Phayao, Thailand (grant no. RD60075).

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