

Rhubarb attenuates blood-brain barrier disruption via increased zonula occludens-1 expression in a rat model of intracerebral hemorrhage

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Abstract. Blood-brain barrier (BBB) disruption is a key pathophysiological factor of intracerebral hemorrhage (ICH). The level of zonula occludens-1 (ZO-1) has been closely associated with the degree of BBB damage, and is an indicator of BBB destruction. The aim of the present study was to evaluate the effects of rhubarb on BBB function in a rat model of ICH. ICH was induced in rats by treatment with type VII collagenase. Sham-operated rats were administered with an equal volume of saline. Following the administration of rhubarb decoction (20 g/kg), neurobehavioral function evaluation and Evans blue extravasation assays were performed at days 1, 3 and 5 after ICH. ZO-1 expression in the brain of ICH-induced rats were analyzed via reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analyses. The results suggested that rhubarb significantly ameliorated neurological symptoms and attenuated BBB permeability. The results of immunohistochemistry and RT-PCR studies indicated that the expression of ZO-1 expression was robust in the sham-operated group and was weak in the vehicle-treated group at day 3. The present data indicated that rhubarb effectively attenuated ICH-induced BBB damage in rats, raising the possibility that rhubarb or its active components may be considered useful as neuroprotective drugs for ICH. The protective mechanisms appeared to involve

the preservation of BBB integrity and elevation of ZO-1 protein expression levels.

Introduction

Intracerebral hemorrhage (ICH) was the cause of 10% of strokes in the US in 2014, leading to disability, morbidity and mortality (1). ICH remains the most severe form of stroke, with limited options to improve survival (2). In China, ICH accounts for a larger, more variable proportion of strokes in community-based Chinese compared with white populations (3). At present, China faces an increasingly heavy burden of stroke across a variety of health care settings (4).

Following the acute phase during ICH, injury to the blood-brain barrier (BBB) is a key pathophysiological factor and may contribute to perihematomal cell injury (5). BBB disruption increases the permeability of the brain microvasculature and may result in the development of vasogenic brain edema (6). Morbidity and mortality after ICH are largely determined with edema surrounding the hematoma (7). Vasogenic brain edema formation is believed to be a strong predictor of unfavorable functional outcome (8). Hence, BBB protection to ameliorate brain edema is a key point for treating ICH. Prevention or reduction of BBB disruption represents a potential target for therapeutic intervention.

The principal structures that serve the function of the BBB are the tight junction (TJ) proteins (9). TJ proteins form the first defense in the endothelial barrier disruption between blood and brain that leads to vasogenic edema and cell death (10,11). During ICH injury, the cascade of molecular events ends in the final common pathway for BBB disruption, which degrades the TJ proteins in endothelial cells (12). Zonula occludens-1 (ZO-1) is one of the main TJ proteins, changes in its levels are closely associated with the degree of BBB damage and it becomes a symbol of the BBB destruction (13). ZO-1 reduces the permeability of cerebral vessels by restricting the free molecular exchange between blood and brain tissues (14). Decreased expression and disarrangement of ZO-1 indicates reduced BBB integrity (11). Therefore, the normalization of ZO-1 expression may be able to promote BBB function repair.

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Abbreviations: ICH, intracerebral hemorrhage; BBB, blood-brain barrier; TJ, tight junction; ZO-1, zonula occludens-1; TCM, traditional Chinese medicine; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry; EB, Evans blue

Key words: rhubarb, intracerebral hemorrhage, blood-brain barrier, zonula occludens-1, traditional Chinese medicine

Numerous therapeutic strategies have been employed in the prevention of ICH, including intravenous hemodiluting agents, neuroprotectants and antihypertensives for secondary prevention (15). However, the case fatality and long-term mortality of patients with ICH has remained unchanged over recent decades (16,17), and there is no effective neuroprotective treatment option for BBB improvement after ICH (18). Traditional Chinese medicines (TCMs), such as rhubarb, have been shown to exert multi-target effects (19). It has been suggested that TCMs may improve ICH symptoms and reduce the risk of fatality, although further outcomes-based research is required (20).

Rhubarb rhizome and root (*Rhizoma rhei*) is one of the most commonly used and investigated TCMs (21), and is listed in the Chinese Pharmacopoeia (22). Rhubarb is thought to exert brain protection via the action of anthraquinones, including aloe-emodin, rhein, emodin and chrysophanol (23), which are appointed as the bioactive compounds and quality control standard of rhubarb in the Chinese Pharmacopoeia (22). The traditional primary usage of rhubarb has been for its neuroprotection. Pharmacological studies demonstrated that rhubarb exhibited multiple biological activities which may be of relevance to BBB function, including anti-oxidation, anti-inflammation and inhibition of aquaporin expression (24-26). However, whether or not rhubarb is able to increase the expression of TJ proteins such as ZO-1, and thus improve BBB function, remains unclear.

In the present study, the effect of rhubarb was evaluated in a rat model of ICH. Specifically, we investigated the hypothesis that rhubarb would improve BBB function via the upregulation of ZO-1 expression.

Materials and methods

Plant materials and chemicals. The raw plant material of dried rhubarb was purchased from the Pharmacy of Xiangya Hospital, Central South University (Changsha, China). The voucher specimen was deposited in the Laboratory of Ethnopharmacology of Xiangya Hospital. The crude plant material was also authenticated by the herbal medicine botanist Professor Hu SY of the Department of Traditional Chinese Medicine of Central South University. Marker compounds, including aloe-emodin, rhein, emodin and chrysophanol (purity, >98%), were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical grade methanol was purchased from Tedia Company, Inc. (Fairfield, OH, USA) and acetic acid from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Triple-distilled water was prepared using silica glass equipment (Colton Water Department, Colton, CA, USA), and was used for preparation of the mobile phase. All other reagents were of analytical grade.

Dried rhubarb was crushed into powder and twice soaked in distilled water (1:12 w/v) at 100°C for 10 min. The rhubarb was extracted twice by reflux in boiling water for 10 min, according to a previous study (27). Following centrifugation at 4,000 × g for 20 min at 4°C, the supernatant was concentrated and lyophilized using the ZG-1 Vacuum Freeze Dryer (Shanghai Cinyaa Group, Co., Ltd., Shanghai, China). The lyophilized powder was resolved to the scale with distilled

water according to the standard of 1 g/ml (w/v) prior to the experiment.

Determination of anthraquinones in rhubarb by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). As a quality control for rhubarb, the marker compounds in rhubarb were determined using UPLC-MS/MS. UPLC-MS/MS analysis was performed using a Waters Acquity UPLC™ system coupled to Waters TQD triple quadrupole tandem mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was performed on an Acquity UPLC BEH 2.1×50 mm, 1.7-μm C₁₈ column (Waters Corporation) using an Acquity ultra performance liquid chromatography system equipped with an Acquity photo-diode array detector (Waters Corporation). The mobile phase consisted of methanol-deionized water containing 0.1% formic acid with gradient elution (0 min, 45:55; 15 min, 75:25). The wavelength in the UV spectrum was 254 nm. The flow rate was kept constant at 0.25 ml/min during the analysis, the operating temperature was maintained at 35°C and the sample volume injected was 5 μl.

For operation in the MS/MS mode, the Waters Acquity™ TQD triple quadrupole tandem mass spectrometer equipped with electrospray ionization interface was connected to the UPLC system. Masslynx™ software, version 4.1 (Waters Corporation) was used for data acquisition and processing. For mass spectrometry, the electrospray ionization source was operated in negative mode with the capillary voltage set at 2.5 KV. The desolvation temperature was fixed at 365°C and the source temperature was set at 110°C. Nitrogen was used as the desolvation gas flow (650 l/h) and cone gas flow (50 l/h). For collision-induced dissociation, argon was used as the collision gas at a flow rate of 0.2 ml/min. The multiple reaction monitoring mode was used for quantification.

As shown in Fig. 1, the over all intra- and inter-day variations were <5% for all the four analytes. The method was reproducible with good precision. The accuracy tests were conducted using a recovery test. Recovery of all four tested compounds were >90%. The result showed that the contents of anthraquinones in rhubarb decoction were as follows: Aloe-emodin, 1.87±0.17 mg/g; rhein, 0.96±0.07 mg/g; emodin, 1.55±0.12 mg/g; and chrysophanol, 0.79±0.06 mg/g.

Animal model preparation. The protocol was approved by the Medical Ethics Committee of Xiangya Hospital of Central South University. Animal experiments were performed according to the guidelines for the care and use of animals, as established by the Central South University. A total of 270 adult male Sprague-Dawley rats (age, 8-10 weeks; weight, 180-220 g) were obtained from the Animal Center of Central South University (SCXK2006-0002; Changsha, China). Rats were housed at 25°C under a 12-h light/dark cycle, with *ad libitum* access to food and water. The rats were randomly divided into three groups with 90 rats per group, as follows: i) Sham-operated group; ii) saline vehicle-treated group; and iii) rhubarb-treated group (20 g/kg). Each group was further divided into three subgroups that were treated with rhubarb or vehicle for 1, 3 or 5 days.

Collagenase-induced ICH was performed according to the protocol described in a previous study (28). Briefly, the

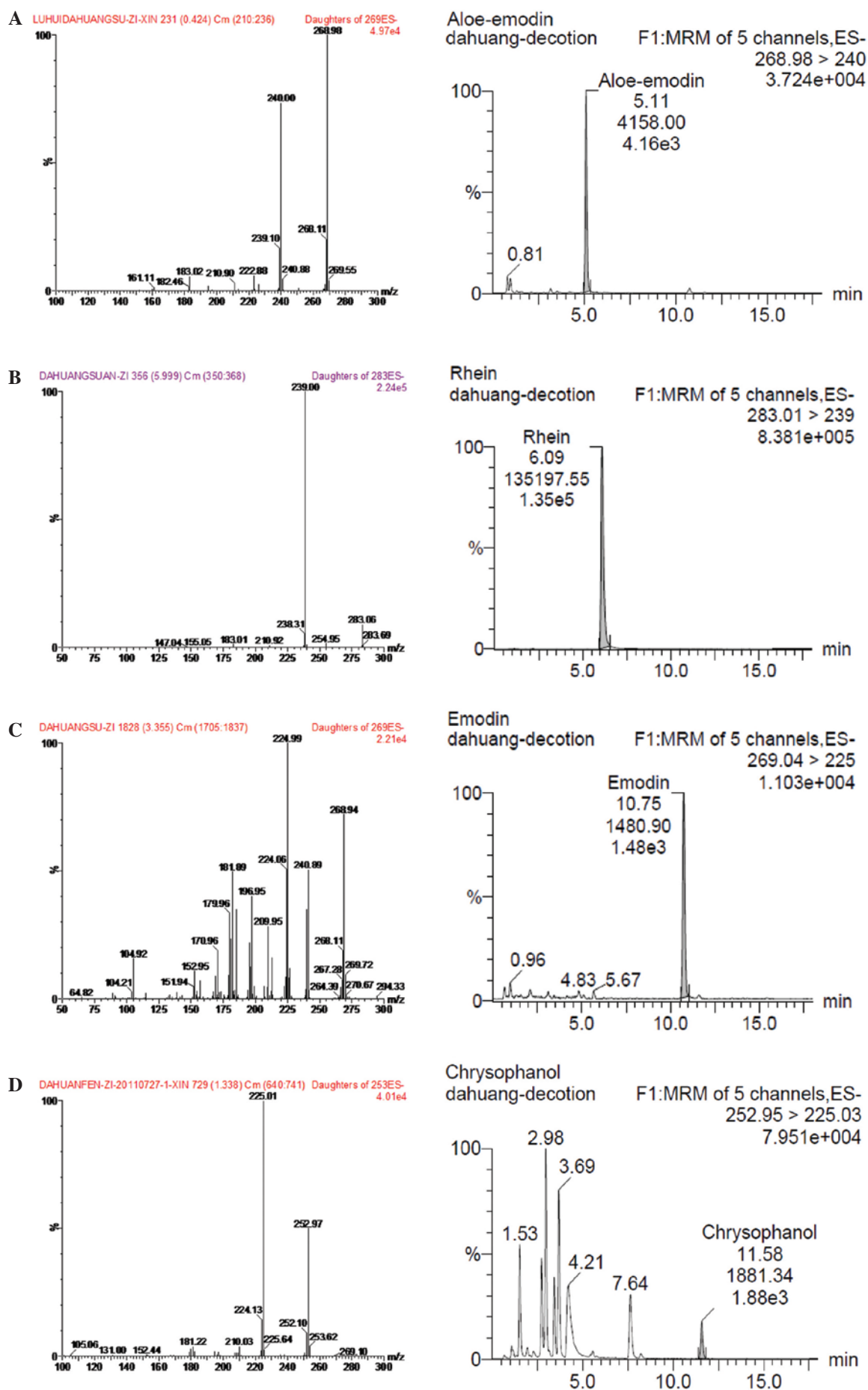


Figure 1. Full-scan negative product ion mass spectra and each corresponding representative multiple reaction monitoring chromatograms (E-H) of anthraquinones in rhubarb decoction: (A) Aloe-emodin; (B) rhein; (C) emodin; and (D) chrysophanol.

Table I. Specific primers for ZO-1 and β -actin.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Size (bp)
ZO-1	GGAAACCCGAACTGATGC	TTGGACAGAGGCGGAACT	124
β -actin	CGTTGACATCCGTAAAGAC	TGGAAGGTGGACAGTGAG	201

ZO-1, zonula occludens-1.

rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (400 mg/kg; from the Pharmacy of Xiangya Hospital) and fixed in a prone position on a stereotactic frame (Stoelting Co., Wood Dale, IL, USA). Following a scalp incision, a small cranial burr was drilled near the right coronal suture 3.2 mm lateral to the midline. Bacterial type VII collagenase (0.5 U) in 2.5 μ l sterile saline (0.9%; Sigma-Aldrich, St. Louis, MO, USA) was slowly injected into the right globus pallidus (1.4 mm posterior and 3.2 mm lateral to the bregma and 5.6 mm ventral to the cortical surface) with a 5- μ l Hamilton syringe (The Gaoe Company, Shanghai, China) over 5 min, with the needle left in place for a further 5 min. The bone hole was sealed with bone wax, and the wound was sutured. Each animal was placed in a warm box to recover individually. Sham-operated rats were administered an equal volume of saline without collagenase. The rats with ICH were randomly divided into two groups, as follows: The vehicle-treated and rhubarb-treated (20 g/kg) groups. Rectal temperatures were monitored during all operations and maintained at 37.5°C with a feedback controlled heating pad (Huaibei Zhenghua Biology Instrument Equipment Co., Ltd., Huaibei, China).

Neurobehavioral function evaluation. Neurobehavioral function was evaluated using a previously-described scoring system (29). The system consists of three individual tests, each with a score range of 0-4 (0=best, 4=worst), with a maximum total score of 12. The tests included: i) Spontaneous ipsilateral circling behavior; ii) contra-lateral forelimb and hindlimb retraction capability; and iii) ability to walk a 70x32.4-cm wood beam. The evaluation was conducted by a masked observer at days 1, 3 and 5 after the induction of ICH.

BBB permeability to Evans blue. BBB integrity was quantitatively evaluated by assessing Evans blue (EB) dye (Sigma-Aldrich) extravasation. Briefly, 30 rats (10/group) were intravenously infused with EB dye solution (2% in saline, 2 ml/kg) over 1 min, which was allowed to circulate for 2 h. Subsequently, the rats were anesthetized with 10% chloral hydrate, and then sacrificed by decapitation. The brain was transcardially perfused with 250 ml saline until colorless perfusion fluid was obtained. The injured hemisphere was rapidly extracted and weighed. The samples were then placed in formamide (1 ml/100 mg; Sigma-Aldrich) for 48 h at 60°C. The absorbance of the supernatant solution was measured using a spectrophotometer (cat. no. 722-2000; Shandong Gaomi Caihong Analytical Instruments, Co., Ltd., Gaomi, China) at 620 nm. The quantitative calculation of the dye content in the brain was quantified from a standard curve derived from known quantities of the dye and was expressed per gram of tissue.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cerebral cortices obtained from the ischemic penumbra of 30 rats (10/group) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA purity was confirmed by measuring the ratio of absorbance at 260 and 280 nm using a spectrophotometer. All RNA samples were treated with DNase I prior to RT-qPCR to remove contaminating genomic DNA. RNA (2 μ l) was reverse transcribed into cDNA using MuLV Reverse Transcriptase (cat. no. EP0352; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using 2 μ l cDNA, an Absolute Blue QPCR Mix, SYBR Green, low ROX kit (cat. no. AB4323A; Applied Biosystems; Thermo Fisher Scientific, Inc.) and gene-specific primers (Takara Bio, Inc., Otsu, Japan) (Table I). The PCR cycling conditions were as follows: Initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. qPCR reactions were performed three times for each sample and a negative control group without the template DNA was used to confirm the absence of non-specific amplification. The relative mRNA expression levels of ZO-1 were calculated using the $2^{-\Delta\Delta C_q}$ method (30), following normalization to β -actin.

Immunohistochemistry detection for ZO-1. A total of 30 rats (10/group) were anesthetized with 10% chloral hydrate (400 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (from the Pharmacy of Xiangya Hospital). Following sacrificed by decapitation, the brains were removed and stored in 4% paraformaldehyde until processing.

Frozen sections (30- μ m) of brain tissue were brought to room temperature and incubated in 3% H₂O₂ for 15 min. After washing three times in phosphate-buffered saline for 5 min each, nonspecific binding was blocked with 5% bovine serum albumin (Sigma Aldrich) for 1 h at 37°C. Immunostaining was performed using rabbit anti-ZO-1 polyclonal antibody (1:400; cat. no. sc-5562; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, followed by staining with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:200; cat. no. BA1054; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 120 min. Immunoreactivity was visualized using 3,3'-diaminobenzidine (Wuhan Boster Biological Technology, Ltd.) under a light microscope (Zeiss Primo Star; Carl Zeiss AG, Oberkochen, Germany). For the image analysis, 10 microscopic fields were selected randomly from each group and the integrated optical densities (IODs) were measured

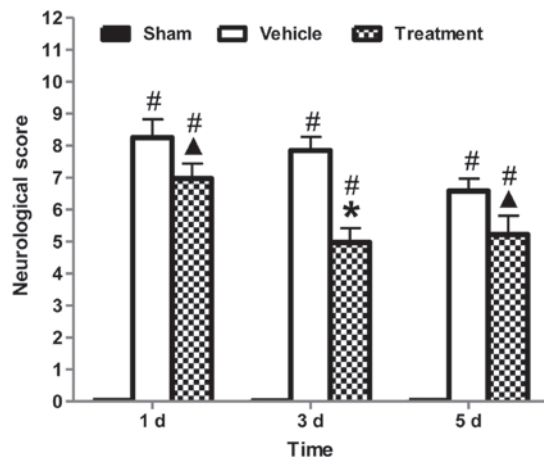


Figure 2. Neurological score. Aggregate score represents the average neurobehavioral function (n=10 per group). $\Delta P < 0.05$ vs. vehicle; $*P < 0.01$ vs. vehicle; $\#P < 0.01$ vs. sham.

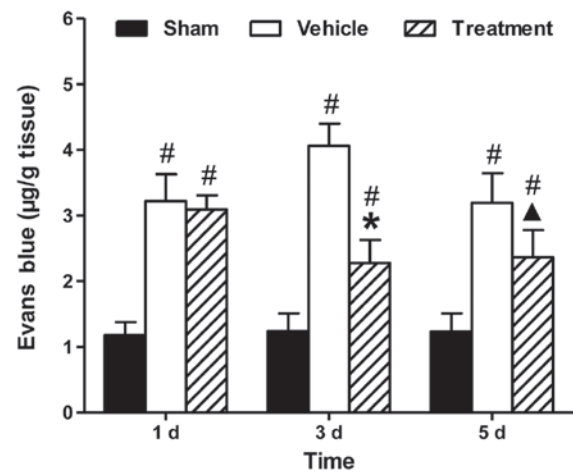


Figure 3. Evans blue extravasation assays. Evans blue leakage was represented as $\mu\text{g/g}$ brain weight, which represents the average blood-brain barrier permeability (n=10 per group). $\Delta P < 0.05$ vs. vehicle; $*P < 0.01$ vs. vehicle; $\#P < 0.01$ vs. sham.

using Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation and were analyzed using Student's t-test and one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

Effect of rhubarb on neurobehavioral functions. Neurobehavioral deficits were evaluated using an established scoring system (29). Fig. 2 indicated that after the induction of ICH, all ICH groups showed neurobehavioral deficits as compared with the sham-operated group. The neurobehavioral deficits attenuated over time in each group, suggestive of self-recovery ability of the rats. At each time point, rhubarb treatment significantly ameliorated neurofunctional deficits compared with the vehicle group ($P < 0.05$).

Effect of rhubarb on BBB permeability after ICH. In order to assess BBB permeability, the assay of EB dye extravasation was performed at days 1, 3 and 5 after ICH (n=10 per group). Fig. 3 showed that significantly more extravasated dye was measured in the ICH groups, as compared with the sham-operated group. Treatment with rhubarb significantly reduced dye extravasation at days 3 and 5 compared with vehicle ($P < 0.05$).

Effect of rhubarb on ZO-1 expression in the brain. ZO-1 has been shown to have an important role in maintaining the integrity of the BBB (31). The mRNA expression levels of ZO-1 were analyzed by RT-qPCR at day 3, since neurobehavioral function and BBB permeability assays (Figs. 2 and 3) had indicated that rhubarb significantly exerted brain protective effects that peaked on day 3. As shown in Fig. 4, ICH induced a significant reduction in the expression levels of ZO-1 mRNA, as compared with the sham-operated group. Conversely, rhubarb treatment resulted in significantly upregulated ZO-1

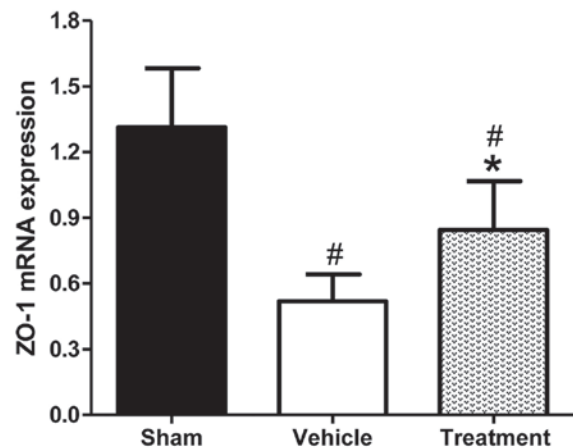


Figure 4. Rhubarb induced upregulation of ZO-1 mRNA expression following intracerebral hemorrhage at day 3. ZO-1 mRNA expression was determined by reverse transcription-quantitative polymerase chain reaction. Quantitation represents the average relative ratio of ZO-1 mRNA to β -actin per rat (n=10 per group). $*P < 0.01$ vs. vehicle; $\#P < 0.01$ vs. sham. ZO-1, zonula occludens-1.

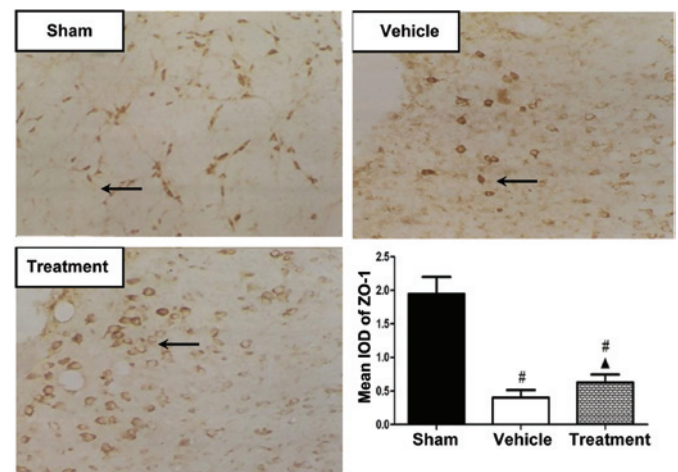


Figure 5. Immunohistochemical examination for ZO-1 at day 3. ZO-1 expression assessment as integrated optical density scores. Arrows indicate positive deposition (n=10 per group). $\Delta P < 0.05$ vs. vehicle at day 3; $\#P < 0.01$ vs. sham at day 3. ZO-1, zonula occludens-1.

mRNA expression levels on day 3, as compared with the vehicle-treated group ($P<0.01$).

Immunohistochemical studies were performed to investigate the protein expression of ZO-1 in the various groups, and the IODs were calculated to quantify the protein expression levels of ZO-1 (Fig. 5). On day 3, the protein expression levels of ZO-1 were significantly reduced in the rhubarb-treated group, as compared with the sham-operated group ($P<0.01$), and were significantly increased in the rhubarb-treated group, as compared with the vehicle-treated group ($P<0.05$).

Discussion

BBB is a key feature of ICH and may contribute to perihematomal cell injury (5). Although there are a number of ongoing clinical trials, there is currently no US Food and Drug Administration approved treatment for BBB protection following ICH (32). In the present study, the data showed that rhubarb (20 g/kg) significantly ameliorated the neurological symptoms and attenuated BBB permeability. Additionally, immunohistochemical and RT-qPCR analyses indicated that rhubarb exerted BBB improvement effects via the upregulation of ZO-1 expression. These results suggested that rhubarb has the potential to be utilized as a promising neuroprotective candidate for ICH.

The unique structure of the BBB maintains the normal physiological state of the central nervous system by inhibiting the passage of numerous large molecular weight, high polarity drugs and harmful substances from the bloodstream into the interior of the brain. Damage to the BBB is a factor in the pathogenesis of ICH, and may occur in the initial 24 h following the onset of bleeding and cellular damage (33). As a result of disruption to the BBB, cells in the central nervous system that survive the initial impact are subsequently exposed to marked changes in their neurochemical environment, which results in a period of secondary damage occurring in the subsequent hours and days (34). Hence, the prevention or reduction of BBB disruption represents a potential approach for therapeutic intervention.

The BBB is predominantly formed by endothelial cells with the complex TJs that serve the function of the BBB (9). TJs form the initial barrier at the endothelial cells between the blood and brain (11). TJs are governed by intracellular proteins, and are responsible for the restriction and control of the paracellular flux between epithelial and endothelial cells (35). TJ proteins form the first defense in the endothelial barrier disruption that leads to vasogenic edema and cell death (10). During ICH, the cascade of molecular events ends in the final common pathway for BBB disruption which degrades the TJ proteins in endothelial cells (12). Decreased expression and disarrangement of the TJ proteins indicate reduced BBB integrity and increased paracellular permeability (11).

ZO-1 is a well-characterized TJ protein and may accurately indicate the pathological changes of BBB, making it a valuable marker of endothelial barrier (36). It has been shown that absence of ZO-1 results in vascular leakage and the aggravated edema is likely due to a decreased level of the TJ protein ZO-1 (37). Therefore, increasing ZO-1 protein expression may promote repair of the BBB.

A previous study showed that rhubarb could improve BBB function via the inhibition of the expression of the

aquaporin-4 gene (*AQP4*) in rats with ICH (24). However, whether or not rhubarb was able to increase ZO-1 expression to improve BBB function was unclear. The present results showed that rhubarb (20 g/kg) significantly improved neurological outcome at days 1, 3 and 5 post-ICH induction compared with vehicle, which implied that rhubarb may be able to ameliorate ICH-induced neural behavioral defects. Collagenase induction notably increased the content of EB extravasation after ICH, reaching a peak at day 3. Rhubarb significantly alleviated ICH-induced BBB disruption compared with vehicle according to the assay of EB dye extravasation. In addition, immunochemistry and RT-qPCR analyses demonstrated that the decreased expression and disarrangement of ZO-1 protein after ICH were increased and rearranged by rhubarb.

Collectively, the aforementioned results suggest that rhubarb has the potential to be utilized as a neuroprotective drug for ICH. However, further investigation is required in order to quantitatively evaluate the changes in ZO-1 protein expression and to define the optimal dose of rhubarb for the treatment of ICH.

In conclusion, the present study indicated that rhubarb effectively attenuated BBB damage following ICH in a rat model, raising the possibility that rhubarb or its active components may have the potential to be utilized as a neuroprotective drug for ICH. The protective mechanisms may involve the preservation of the BBB integrity and elevation of ZO-1 protein expression levels.

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