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ORIGINAL ARTICLE

# Rhubarb Attenuates Cerebral Edema via Inhibition of the Extracellular Signal-regulated Kinase Pathway Following Traumatic Brain Injury in Rats

**Zhaoyu Yang, Rong Fan, Peng Sun[1], Hanjin Cui, Weijun Peng[2], Jiekun Luo, Chunhu Zhang, Xingui Xiong, Wei Huang, Wei Liu[3]**

Department of Integrated Traditional Chinese Medicine, Xiangya Hospital, Central South University, Changsha, 410008, [1]Department of Key Laboratory of Traditional Chinese Medicine for Classical Theory, Ministry of Education, Shangdong University of Traditional Chinese Medicine, Jinan 250355, [2]Department of Integrated Traditional Chinese and Western Medicine, 2nd Xiangya Hospital, Central South University, 410011 Changsha, [3]Department of Cardiovascular Surgery, Xiangya Hosipital, Central South University, 410008 Changsha, China

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## ABSTRACT

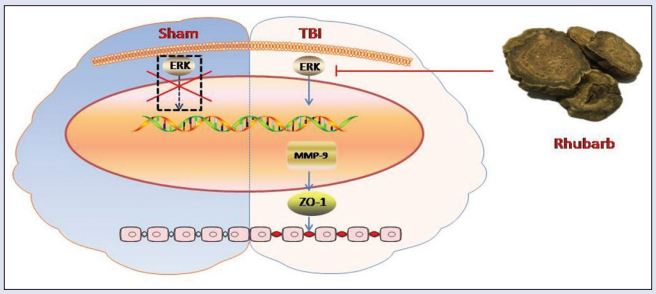
**Background:** Rhubarb is a traditional Chinese medicine for treating traumatic brain injury (TBI). **Purpose:** The purpose of this study is to elucidate the potential mechanism of rhubarb by suppressing extracellular signal-regulated kinase (ERK) to ameliorate brain edema. **Materials and Methods:** Sprague-Dawley rats were separated into four groups at random. One group received 3 g/kg rhubarb, and another group received 12 g/kg rhubarb, and the vehicle group and sham group were administered the same dose of saline solution. The blood–brain barrier disruption and edema were detected by Evans blue extravasation and water content, respectively. ERK, Matrix metalloproteinase 9 (MMP-9), and zonula occluden-1 (ZO-1) in the damaged tissue were measured by western blot analysis and quantitative real-time polymerase chain reaction. **Results:** Rhubarb attenuated the brain edema after TBI, especially at the dose of 12 g/kg. Rhubarb significantly suppressed ERK, down-regulated MMP-9, and up-regulated ZO-1. Rhubarb might be a prospective therapeutic regimen to decrease edema in TBI. **Conclusions:** Rhubarb alleviates the edema by restraining the ERK signaling pathway. Our results contribute to the validation of the traditional use of rhubarb in the treatment of TBI and its mechanism.

**Key words:** Brain edema, extracellular signal-regulated kinase, rhubarb, traditional Chinese medicine, traumatic brain injury

## SUMMARY

- The aim of this study was to explore the potential mechanism of rhubarb by suppressing extracellular signal-regulated kinase to ameliorate brain edema. Results: Rhubarb ameliorates edema caused by traumatic brain injury by inhibiting the ERK/Matrix metalloproteinase 9/zonula occluden-1 signaling pathway.

**Abbreviations used:** TBI: Traumatic brain injury, ERK: Extracellular signal-regulated kinase pathway, MMP-9: Matrix metalloproteinase 9, ZO-1: Zonula occluden-1, BBB: Blood-brain barrier, PCR: Polymerase chain reaction, TCM: Traditional Chinese medicine, MAPKs: Mitogen-activated protein kinases, CCI: Controlled cortical impact, DL: Rhubarb 3 g/kg in distilled water, DH: Rhubarb 12 g/kg in distilled water, EB: Evans blue, IOD: Integral optical density, MEK: Mitogen extracellular kinase, MMPs: Matrix metalloproteinases, NADPH: Nicotinamide adenine dinucleotide phosphate: ROS, reactive oxygen species.




**Correspondence:**

Dr. Wei Liu,
Department of Cardiovascular Surgery,
Xiangya Hospital, Central South University,
Changsha, China.
E-mail: lwei2001@tom.com
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## INTRODUCTION

Traumatic brain injury (TBI) is a disastrous condition related to substantial mortality and lifelong disability.[1] TBI has tremendous effects on both public health and the economic burden, and it carries high rates of mortality and disability in the United States,[2] with emergency treatments at approximately 1.4 million, and more than 235,000 require hospitalization.[3] The economic burden is approximately up to more than $37.8 billion.[3] Thus, the discovery of preventive and effective drugs remains to be drastically explored.

Brain edema is a crucial factor of brain injuries to the central nervous system.[4] Following TBI, the initial mechanical insult of TBI involves breaking down the blood-brain barrier (BBB). The breakdown of the BBB leads to leakage of plasma substances and proteins out of the bloodstream and their deposition in the subendothelial matrix and interstitium. The above alterations cause the accumulation of plasma proteins in the subendothelial matrix, further resulting in brain edema. Cerebral edema may be responsible for as many as half of the mortalities in TBI.[5] Hence, alleviating cerebral edema is a contributing factor to neurologic recovery.[6]



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Several medicines, including ethanol,[7] carbamylated erythropoietin[8] and astaxanthin,[9] are used to attenuate cerebral edema after TBI. Current medical approaches to effectively alleviate cerebral edema after TBI is not available.[10] The reason is that due to the complex pathogenesis of TBI, "one-compound, one-disease" based drugs fail to alleviate brain edema after TBI. Scientists and doctors tend to focus on traditional Chinese medicine (TCM) for the treatment of TBI because of its multiple-targets.

The TCM herb Rheum officinale Baill (Rhubarb, a member of the Polygonaceae family), the dried roots and rhizomes, has been used for thousands of years of ancient medicine. It is recorded in Shennong's Classic of Material Medical (Shen Nong Ben Cao Jing). Rhubarb has several pharmacological effects, including antiinflammatory, antibacterial, purgative, and anticancer properties.[11] Furthermore, a previous study demonstrates that rhubarb is highly efficient in treating patients with TBI by its multitargets effects.[12-14] However, the molecular mechanism of rhubarb in the alleviation of brain edema is still unknown.

Extracellular signal-regulated kinase (ERK) is a member of mitogen-activated protein kinases (MAPKs), which is activated after TBI.[15-17] Matrix metalloproteinase 9 (MMP-9) is downstream of ERK[15] and functions to degrade the major components of the basal lamina, such as the tight junctions, which cause BBB disruption after TBI, leading to edema.[18,19] Tight junction proteins, such as zonula occluden-1 (ZO-1), are a symbol of the integrity of the BBB that essentially contribute to its structural inviolacy. A change in tight junction protein assembly may contribute to the loss of the BBB integrity and BBB breakdown.[20] A study showed that the prohibition of the ERK pathway ameliorates cortical damage after cerebral trauma.[21] Therefore, inhibiting the ERK signaling pathway is a key to alleviating cerebral edema. Whether rhubarb suppresses the ERK signaling pathways to ameliorate the cerebral edema remains unclear.

The present study aimed to elucidate whether rhubarb relieved brain edema through the downregulation of MMP-9 and the upregulation of ZO-1 by inhibiting the ERK signaling pathway in a rat model of TBI. This research helps to provide a promising herbal drug to treat TBI.

## MATERIALS AND METHODS

### Experimental animals

Adult male Sprague-Dawley rats, weighing 220–300 g, were used in this study and were purchased from the Laboratory Animal Center of Central South University. The animals were housed under controlled conditions (temperature at 25°C, 65% ±5% relatively humidity, and 12 h: 12 h light/dark cycle). All the animal experimental protocols conducted in this study were approved by the Xiangya Hospital, Central South University (Changsha, China) and were put into effect according to Institutional Guidelines of the Animal Care and Use Committee.

### Controlled cortical impact model

The rats were anesthetized with 3% pentobarbital sodium (50 mg/kg), and the rats were mounted in a stereotaxic frame. Following a midline incision, the skin and temporal muscles were reflected to expose the skull. A craniotomy, with the diameter of 4.0 mm, was performed using dental bur at the left. Briefly, a controlled cortical impact (CCI) device (TBI0310, precision systems and instrumentation, Fairfax Station, VA) was used to impact the brain (tip diameter, 4.0 mm; cortical contusion depth, 5.0 mm; impact velocity, 6.0 m/s; dwell time, 50.0 ms). After injury, the skin incision was closed with nylon sutures. The sham group was only subject to craniotomy. The rats were placed on a heating pad during surgery. At the end of the procedure, the animals were removed from the stereotaxic frame, went back to their cages and were closely monitored until the recovery from anesthesia was complete.

### Experimental groups and the administration of drugs

Eighty rats were randomly divided into four groups in a blinded manner, including the sham (rats underwent the CCI procedure without impacting the cortex and were gavaged with 0.9% saline, $n$ = 20), vehicle (rats underwent the CCI procedure and received the same dose of saline, $n$ = 20), rhubarb 3 g/kg in distilled water (CCI rats and received rhubarb 3 g/kg, $n$ = 20), and rhubarb 12 g/kg in distilled water (CCI rats and received rhubarb 12 g/kg, $n$ = 20). The drugs were administered by gavage after post-TBI recovery. The investigators assessed all of the study outcomes, performed the calculations, and conducted the analyses. All the rats were calculated in the final blind data analysis expect for those that died before the end of the study.

### Preparation of rhubarb

The Rheum officinale Baill (dried roots and rhizomes, voucher specimen No. 20140624, Gansu, China) was purchased from the pharmacy of the Xiangya Hospital, Central South University. An herbal medicine botanist, Professor Suiyu Hu (Department of Herbal Medicine of Central South University in China), authenticated the plant. The rhubarb was boiled twice in distilled water (1:12, w/v) for 30 min.

### Brain water content

The brain water content was used on behalf of edema, which forms as a consequence of the BBB breakdown. After decapitating the rats ($n$ = 5 in each group), the brains were removed. The injured hemisphere was weighed (wet weight), dried at 100°C for 24 h, and reweighed (dry weight). The water content was determined by the equation ([wet weight – dry weight]/wet weight) ×100%.

### Evaluation of the blood–brain barrier permeability

Evans blue (EB) extravasation was used to investigate the BBB disruption. In short, the EB dye (2 ml/kg in 20% saline) was administered through the tail vein and was allowed to circulate for 2 h. Two hours later, the animals were anesthetized and perfused with saline to remove intravascular EB dye. The rats ($n$ = 5 in each group) were then decapitated, and the injured cortical tissues were harvested. Each tissue sample was weighed, homogenized in a 50% trichloroacetic acid solution and centrifuged at 10,000 rpm for 10 min. The supernatant was diluted with ethanol (1:3). The absorbance of each supernatant for the EB dye was measured at 620 nm using a spectrophotometer.

### Immunohistochemistry

The rats ($n$ = 5 in each group) were anesthetized with 3% pentobarbital sodium (50 mg/kg), and then, the rats were decapitated, and the brain was removed and stored in 4% paraformaldehyde until processing. Frozen areas (10 μm) of the injured cortex were brought to room temperature and were then incubated in 3% $H_2O_2$ for 10 min. After washing three times in phosphate buffer saline (PBS) for 5 min each at room temperature, 5% normal donkey serum was used to block the nonspecific binding. Immunostaining was performed using primary antibodies (1:400, Abcam ab59720, USA) specific for ZO-1 and antibodies for MMP-9 (1:400, Epitomics 2551-1, USA) at 4°C overnight. Next, staining with biotin-labeled secondary antibodies for 120 min was performed. Then, the avidin-biotin-peroxidase complex (1:100, Sigma, USA) was used at 37°C for 1 h. Diaminobenzidine (Boster Biotech Co. Wuhan, China) was applied to visualize the immunoreactivity. Under the light microscope, positive staining (brown yellow) was located for ZO-1 and MMP-9. The images were under a magnification of 400 × by randomly choosing 10 microscopic fields from each group, and the

digital software Image-ProPlus 5.0 (Media Cybernetics, USA) was used to automatically detect the integral optical density of each group.

## Quantitative real-time polymerase chain reaction analysis

The rats ($n$ = 5 in each group) were anesthetized with 3% pentobarbital sodium (50 mg/kg), underwent cardiac perfusion with ice-cold saline. Cortex was dissected from the brain tissue using a fine-straight and a fine-angled dissecting forceps and immediately transferred to liquid nitrogen. For total RNA isolation from the ipsilateral cerebral cortexes that had been treated with PBS using the Trizol reagent (Invitrogen, USA), the cortexes were homogenized in 1 ml of Trizol (Invitrogen, USA), and the total RNA was isolated using the manufacturer's instructions. Spectrophotometry was used to confirm the purity. The green polymerase chain reaction (PCR) kit (Fermentas, USA) and gene-specific primers were to quantify the mRNA on a Bio-Rad C × 96 Detection System (Bio-Rad, USA). cDNA served as a template for quantitative real-time RT-PCR. β-actin was used as an internal control to normalize the of gene expression levels. ZO-1 and MMP-9 were amplified with the specific primers presented in Table 1. The thermal cycling began with a 2 min incubation at 50°C followed by a 10 min denaturation step at 95°C and 40 cycles at 95°C for 10 sand 59°C for 50 s. The comparative threshold cycle (Ct) method was used to determine the relative quantities of the candidate genes and β-actin mRNA.

## Western blot analysis

The rats ($n$ = 5 in each group) were anesthetized with 3% pentobarbital sodium (50 mg/kg), and perfused transcardially with ice-cold saline. Cortex was carefully dissected from the brain tissue and immediately transferred to liquid nitrogen. The protein samples obtained from the frozen rats brain tissues with the various treatments were subjected to a Western blot. The samples were lysed in 500 μl of precooled RIPA buffer (Applygen Technologies Inc., Beijing) and were homogenized by hand for 15 rounds and then were transferred to a 1.5 ml centrifuge tube. The homogenate was centrifuged at 12,000 rpm at 4°C for 5 min to collect the supernatant. The protein concentration was measured before the detection of specific proteins by Western blotting. Primary antibodies against p-ERK (#4307S; rabbit; 1:2000), ERK (#4695S; rabbit; 1:1000), ZO-1 (ab59720; rabbit; 1:50), and MMP9 (ab38898; rabbit; 1:1000) were purchased from Santa Cruz Biotechnology.

**Table 1:** Reverse transcription polymerase chain reaction primer sequences

| Gene | Primers | Sequences | Length |
|---|---|---|---|
| MMP-9 | Forward | 5'-GCAAACCCTGCGTATTTCCAT-3' | 76 bp |
| | Reverse | 5'-CCATCCGAGCGACCTTTAGTG-3' | |
| ZO-1 | Forward | 5'-CGTTTATCGCCGCATTG-3' | 201 bp |
| | Reverse | 5'-CCTCGCTCTACCTCCTTGTG-3' | |
| Actin | Forward | 5'-CATCCTGCGTCTGGACCTGG-3' | 107 bp |
| | Reverse | 5'-TAATGTCACGCACGATTTCC-3' | |

MMP-9: Matrix metalloproteinase 9; ZO-1: Zonula occludens-1

## Statistical analysis

The values are represented as the mean ± standard deviation for each group. For analyses involving two groups, Student's $t$-test or Satterthwaite was performed using a one-way ANOVA to analyze three groups or more, and $P < 0.05$ was considered statistically significant. The analyses were performed in GraphPad Prim 5.0.

# RESULTS

## Cerebral edema and cortical lesion volumes are reduced by rhubarb

The brains from a CCI model were used to evaluate the brain water content and BBB permeability. In the vehicle-treated groups, compared with sham, the water content increased after TBI [Figure 1a]. Brain edema was attenuated in a dose-dependent manner with the rhubarb treatment. Moreover, the cerebral edema was dramatically attenuated in the 12 g/kg rhubarb-treated rats compared to the vehicle-treated rats [Figure 1a]. In the CCI model, EB extravasation was used to investigate the BBB disruption. Similarly, rats treated with rhubarb showed a significantly decreased BBB disruption in a dose-dependent manner. We observed a significant difference in the 3 g/kg and 12 g/kg rhubarb-treated groups compared with the vehicle-treated group [Figure 1b].

## Rhubarb alleviates trauma-induced phospho-extracellular signal-regulated kinase and extracellular signal-regulated kinase levels

The expression of p-ERK protein was dramatically increased in the CCI rat model [Figure 2a]. Furthermore, the ERK protein expression was upregulated in the brains of the CCI rats [Figure 2a]. The rhubarb

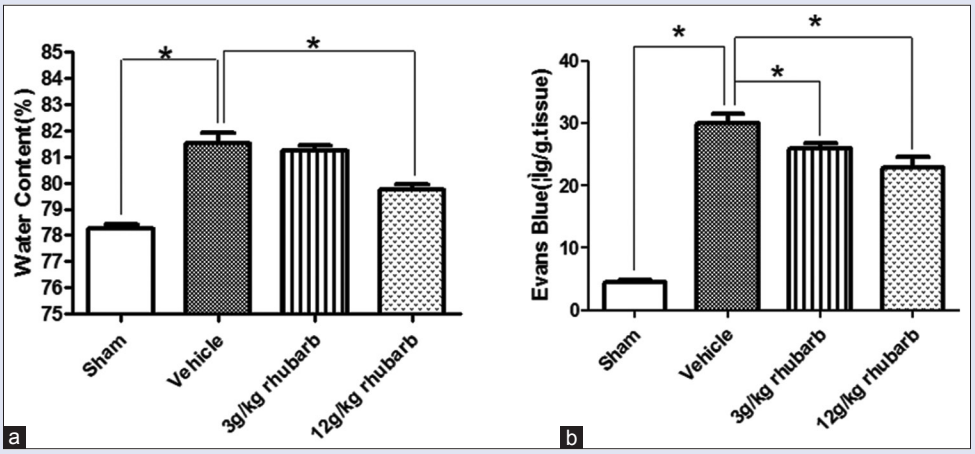


**Figure 1:** (a) Compared the brain water content (%) after traumatic brain injury in rats. The water content increased after traumatic brain injury and treated with rhubarb alleviated it. (b) Evans Blue is used to detect the blood–brain barrier permeability. Posttraumatic brain injury leads to blood–brain barrier disruption, the uptake of rhubarb remits the blood–brain barrier permeability. The data are represented as the mean ± standard deviation. *$P < 0.05$, $n$ = 5 in each group

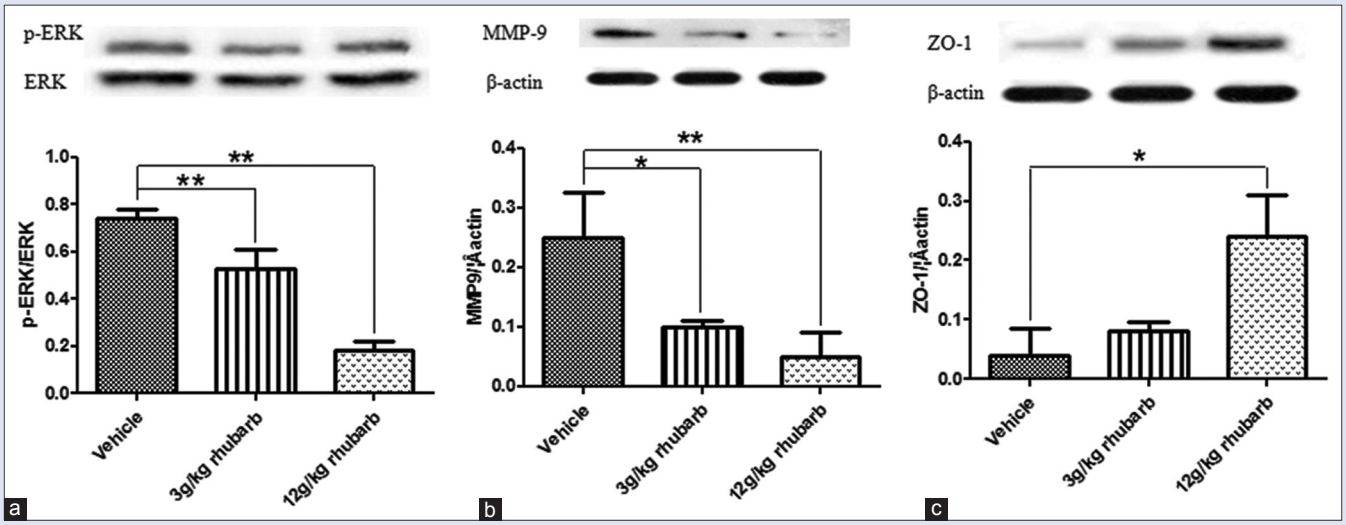


**Figure 2:** Expression of the protein in the brain tissue ipsilateral to the injury after treatment ($n$ = 5). (a) p-extracellular signal-regulated kinase and extracellular signal-regulated kinase. (b) Matrix metalloproteinase 9. (c) zonula occluden-1. The data are represented as the mean ± standard deviation. *: $P < 0.05$, **$P < 0.01$, $n = 5$ in each group

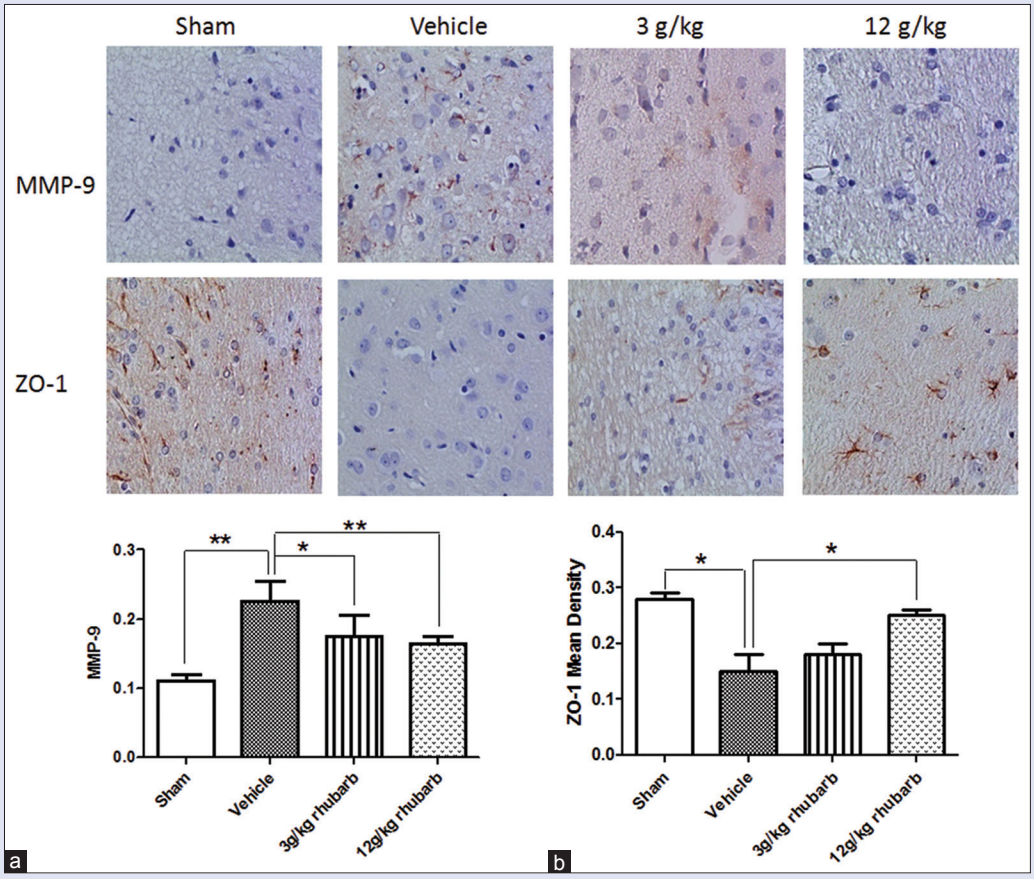


**Figure 3:** Comparison of (a) matrix metalloproteinase 9 and (b) zonula occluden-1 expressions after traumatic brain injury. (a) Separate treatments with 3 g/kg rhubarb and 12 g/kg rhubarb significantly reduce the matrix metalloproteinase 9. (b) Compared with the vehicle, treating with 12 g/kg rhubarb significantly increased the zonula occluden-1 expression. The data are represented as the mean ± standard deviation. ** $P < 0.01$, *: $P < 0.05$, $n = 5$ in each group

treatment (3 g/kg and 12 g/kg) inhibited the TBI-induced activation of the p-ERK and ERK [Figure 2a]. We observed a significant difference in the 3 g/kg and 12 g/kg rhubarb-treated groups compared with the vehicle-treated group ($P < 0.05$), [Figure 2a].

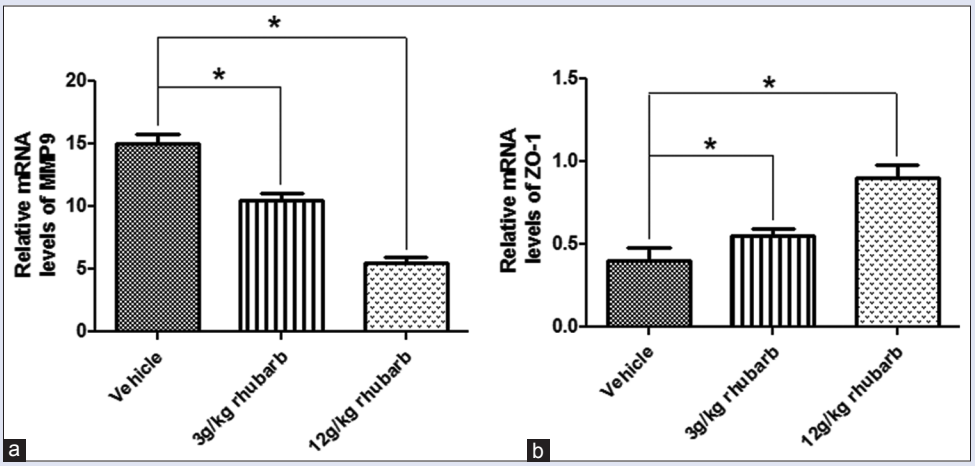


**Figure 4:** Treating with rhubarb changes the expression of matrix metalloproteinase 9 and zonula occluden-1. (a) The rats ($n$ = 5) feed with rhubarb had decreased Matrix metalloproteinase 9 and (b) increase the zonula occluden-1. The data are represented as the mean ± standard deviation. *$P$ < 0.05, $n$ = 5 in each group

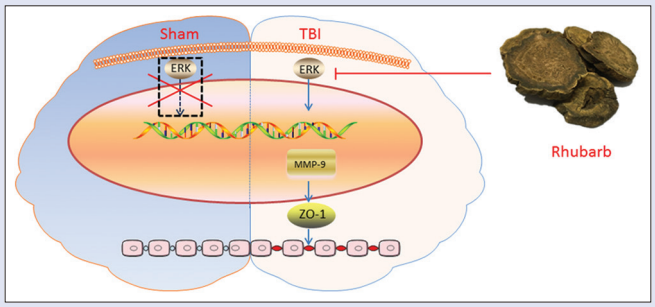


**Figure 5:** Traumatic brain injury induced activation of extracellular signal-regulated kinase. To attenuate the edema, rhubarb significantly inhibited the extracellular signal-regulated kinase signaling pathway to downregulate matrix metalloproteinase 9 and upregulate zonula occluden-1

## Rhubarb decreases the upregulation of matrix metalloproteinase 9 and reduces the degradation of the tight junction protein ZO-1

The results of the immunohistochemical examination, real-time PCR, and western blot are shown in Figure 3a and b, Figure 4a and b, and Figure 2b and c. In the vehicle group compare to the sham group, we observed the activation of MMP-9 and the reduction of ZO-1. The rhubarb treatment decreased MMP-9 expression and was accompanied by increased ZO-1 expression. Furthermore, the Western blot and real-time PCR revealed that rhubarb downregulated the expression levels of the MMP-9 mRNA [Figure 4a] and protein [Figure 2b] and was accompanied by elevated the expression levels of the ZO-1 mRNA [Figure 4b] and protein [Figure 2c].

# DISCUSSION

This study revealed that rhubarb significantly decreased the extent of cerebral edema and the permeability of the BBB in the CCI model. Moreover, a suppression of ERK and MMP-9, to activate ZO-1, was observed. The results showed that rhubarb provided a significant neuroprotection to ameliorate brain edema formation through prohibiting the ERK signaling pathway. This research provided a potential molecular mechanism of rhubarb in the treatment of TBI.

The ERK pathway, which regulates the activity of many transcriptional factors associated with the proliferation of various cells, is activated in lesions in regions of selective vulnerability after TBI.[22] Raf/mitogen extracellular kinase (MEK)/ERK is the canonical pathway to activate ERK, in which the β-Raf activation of MAPK (MEK) that then phosphorylates and activates ERK.[16] MMP-9 is one of the inducible enzymes[23] of MMPs, which degrade tight junctions in the brain.[25] MMP-9 activity is significantly elevated in TBI.[24] Several studies demonstrate that MMP-9 proteolytic activity is associated with brain edema.[25-27] ZO-1 is present in brain endothelial tight junctions, which are the main structural barrier proteins.[23] Previously, studies showed that inhibit ERK reduces MMP-9 levels and increases ZO-1 levels, which then attenuates traumatic brain edema.[15] Until now, it was not reported that the TCM rhubarb, by restrained ERK, decreasing MMP-9 and increasing ZO-1, alleviates edema.

As presented in Figure 5, the brain is sensitive to brain edema following TBI, which may be successfully treated on the condition of a timely beginning. Our previous study indicated rhubarb protects the BBB following TBI through the NADPH oxidase/ROS/ERK/MMP-9/ZO-1 signaling pathway.[28] The figure showed that rhubarb lowed the MMP-9 level and raised ZO-1 expression. Moreover, it is necessary to analyze the mechanism of how the brain edema is alleviated after TBI through MMP-9 prohibition and ZO-1 elevation, especially for the ERK signaling pathway. Therefore, we assessed the influence of rhubarb on the ERK/MMP-9/ZO-1 signaling pathway. The data suggested that rhubarb stopped the effects of ERK, which subsequently prohibited MMP-9 and boosted ZO-1. The results presented that rhubarb might be used as a potential therapeutic agent to attenuate brain edema in the treatment of TBI.

This study has several limitations. Although rhubarb alleviate edemas, it is unclear that the absorbed bioactive compositions derived from rhubarb exert are protective functions in the brain. Furthermore, it is unknown the manner by which the activated ERK/MMP-9/ZO-1 signaling pathway was suppressed after TBI is unknown. More efforts are needed to elucidate the mechanisms underlying these symptoms.

# CONCLUSIONS

Rhubarb exerts neuroprotective effects by ameliorating edema caused by TBI through inhibiting the ERK/MMP-9/ZO-1 signaling pathway.[15] Therefore, rhubarb has potential as a promising neuroprotective candidate for TBI.

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## Conflicts of interest

There are no conflicts of interest.

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