Rehmannia and Rhubarb Decoction Exerts Neuroprotective Effects on the Blood-brain barrier during Acute Intracerebral Hemorrhage by Downregulating the HMGB1/TLR4/MMP9 signaling pathway

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Research

Keywords: Intracerebral hemorrhage, Rehmannia and Rhubarb Decoction, Blood brain barrier, HMGB1/TLR4/MMP9 signaling pathway

DOI: https://doi.org/10.21203/rs.3.rs-41479/v1

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Abstract

Background

Blood-brain barrier (BBB) is a gate-keeping system with selective permeability that serves to protect the central nervous system. The underlying neuroprotective mechanism of the BBB during acute intracerebral hemorrhage (ICH) remains poorly understood. Rehmannia and rhubarb decoction (RRD) is a classic traditional Chinese medicine formula that has been extensively applied for hemorrhagic diseases in China. In the present study, the potential protective effects of RRD on the BBB during acute ICH and the underlying mechanism were investigated.

Methods

The ICH model was established by injection of autologous blood (100 µl) into spontaneously hypertensive rats, which were randomly divided into the following groups: i) Sham; ii) ICH; iii) RRD; iv) TAK-242; v) TAK-242 + RRD; vi) high mobility group box 1 protein (HMGB1) inhibitor ethyl pyruvate (EP); and vii) EP + RRD. Neurological deficits, pathological examination, brain water content, Evans blue (EB) extravasation, immunofluorescence staining and the expression levels of HMGB1, toll-like receptor 4 (TLR4), matrix metalloproteinase 9 (MMP-9), Claudin-5, Occludin and zona occludens – 1 (Zo-1) were subsequently examined in each group on day 3 following operation. In addition, MRI and transmission electron microscopy were also performed to observe the BBB structure.

Results

RRD treatment markedly improved neurological functions, reduced brain water content and Evans blue extravasation, ameliorated the disruption of BBB and downregulated HMGB1, TLR4 and MMP-9 expression whilst upregulating the expression of Claudin-5, Occludin and Zo-1.

Conclusion

These results demonstrate that RRD has a protective effect on the BBB in rats during ICH and this protective effect is related to the down-regulation of HMGB1/TLR4/MMP9 signaling pathway.

Introduction

ICH is a common and potentially fatal cerebrovascular event in clinic that accounts for 10–15% of all stroke cases. The high rates of disability and mortality that is associated with ICH poses a serious threat to the quality of life and survival index [1, 2]. During ICH, the hematoma causes mechanical compression on the surrounding brain tissue, causing primary brain injury. Instantly, complications arising from the hematoma can induce cytotoxicity, excitotoxicity, oxidative stress and inflammation, resulting in BBB
disruption, brain edema and neurological impairment, characteristics of secondary brain injury [3, 4]. Disruption of the BBB, considered as one of the critical contributors of secondary brain injury, causes vasogenic brain edema highly that is associated with the prognosis of patients [5, 6].

MMP is a large family of zinc-containing enzymes that directly disrupt the BBB by degrading extracellular matrix proteins, including laminin, type IV collagen and fibronectin, in addition to tight junctions between endothelial cells [7, 8]. In particular, MMP-9 has been previously reported to be the key protein in mediating damage to BBB integrity in ICH [9, 10], suggesting that MMP-9 may serve as a potential therapeutic target. HMGB1 is a non-histone chromosomal binding protein that is widely expressed in eukaryotic cells, which regulates DNA replication, transcription, repair and cell movement. In the early stages of ICH, HMGB1 is released and induces the inflammation response by stimulating the TLR2, TLR4 receptor or receptor for advanced glycation end products (RAGE) [11, 12]. However, it remains unclear if these receptors aforementioned serve a role in ICH-induced secondary brain injury. It was found recently that the HMGB1/TLR4/MMP-9 signaling pathway served an important role in the destruction of the BBB during acute ischemic stroke. During acute intracerebral infarction, MMP-9 expression is upregulated by HMGB1 via the TLR4 receptor, leading to the destruction of the BBB, confirming MMP-9 to be the downstream signaling target of TLR4 [13]. However, the role of this signaling pathway in ICH has not been fully elucidated.

RRD is a classic traditional Chinese medicine formula that are comprised Rehmannia and rhubarb. It has been extensively applied for treating a variety of hemorrhagic diseases in China for a long period of time. Rehmannia is a sweet medicinal and edible herb that belongs to the Scrophulariaceae family, the root of which is generally taken for use. Active components that can be found in Rehmannia in sufficient quantities include catalpol, ajugol and acetoside [14], which have reported antioxidant, anti-apoptotic and anti-inflammatory properties [15–18]. By contrast, rhubarb is herb that is part of the Polygonaceae family. It is a laxative that contains a number of biologically and pharmacodynamically active ingredients, including anthraquinones and their glycosides, stilbenes, butyrophenones and chromones, tannins and saccharides [19]. Similarly, Rhubarb has also been widely used for medicinal purposes in China. The U.S. Food and Drug Administration classified rhubarb as "generally recognized as safe". A number of studies have previously found that rhubarb exerted neuroprotective, antioxidative and anti-inflammatory effects in treating stroke and brain injury [20–24]. In addition, RRD has been applied as an adjuvant therapy for patients with ICH in our department of neurology to obtain better therapeutic outcomes. However, the neuroprotective effects and exact mechanism mediated by RRD remain poorly understood.

In the present study, the investigate potential neuroprotective effect of RRD on the BBB in acute intracerebral hemorrhage was investigated. Additionally, the hypothesis that these effects are mediated by the HMGB1/TLR4/MMP9 signaling pathway was tested.

**Materials And Methods**

**Drugs and Reagents**
The TLR4 antagonist TAK-242 (MedChemExpress). HMGB1 inhibitor ethyl pyruvate (Sigma-Aldrich; Merck KGaA). Anti-gliial fibrillary acidic protein (cat. no. M00213-7; Boster Biological Technology). Anti-Claudin-5 (cat. no. 34-1600; Thermo Fisher Scientific, Inc.). FITC conjugated anti-Rabbit IgG (cat. no. BM2012; Boster Biological Technology). Anti-HMGB1 (cat. no. ab18256; Abcam). Anti-TLR4 (cat. no. ab13556; Abcam). Anti-MMP-9 (cat. no. ab58803; Abcam). Anti-Occludin (cat. no. ab222691; Abcam). Anti-Claudin-5 (cat. no. ab15106; Abcam). Anti-Zo-1 (cat. no. ab216880; Abcam). Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (cat. no. ab97057; Abcam). DAPI (Boster Biological Technology). In Situ Cell Apoptosis Detection Kit FITC (cat. no. MK1023; Boster Biological Technology).

RRD is comprised of two components, Rehmannia and Rhubarb (Traditional Chinese Medicine Pharmacy, Shuguang Hospital Affiliated to Shanghai University of TCM, Shanghai, China). To obtain the RRD, Rehmannia and Rhubarb (dose ratio, 3:1) were diluted in distilled water at a 1:10 ratio and incubated for 12 h before the whole filtered suspensions from every decoction for 1 h of three times were collected and centrifuged at 2,000 x g for 20 min at room temperature. Dehydrated alcohol was then added until the concentration of the alcohol reached 75% (v/v). The liquid was stirred overnight, which was evaporated to a final concentration of 2 g/ml (w/v) and the precipitate was discarded. Finally, the liquid was autoclaved and stored at -20˚C until further use.

**Animals and treatments**

Male spontaneous hypertensive rats (Beijing vitalriver laboratory animal technology co., LTD.; certificate: SCXK (Beijing) 2016-0006), 3-4 months old weighing 280-320 g and systolic pressure ≤180-200 mmHg as determined using the tail set method, were used for all experiments (n=182). All experimental procedures were conducted in accordance with the guidelines for animal experiments of Shanghai University of Traditional Chinese Medicine and approved by the university’s committee on animal experimentation (ethical code, PZSHUTCM170721001).

The animal study was divided into two steps. The first step aimed to investigate neuroprotective effects of RRD on the BBB following acute ICH, where the rats were divided into the following three groups: i) Sham group (Sham); ii) ICH group; and iii) RRD group. After confirming the neuroprotective effect of RRD, further experiments were conducted to verify if the neuroprotective effects of RRD on the BBB following acute ICH was mediated via the HMGB1/TLR4/MMP9 signaling pathway. Rats were randomly divided into the following 7 groups: i) Sham group (Sham); ii) ICH group; iii) RRD group (RRD); iv) TAK-242 group; v) TAK-242 + RRD group; vi) EP group; and vii) EP + RRD group. The administration of RRD was given at a dosage of 18.51 g/kg/d by intragastric administration for 3 days after ICH induction based on observations from previous studies. Rats in the TAK-242 group were administered with TAK-242 by intraperitoneal injection at 3 mg/kg [25] whilst those in the EP group were treated with an intraperitoneal injection of 80 mg/kg EP, once daily for 3 days after ICH induction [26].
ICH Model

ICH was induced by the autologous blood injection method. Briefly, rats were deprived of food except water before 12h of ICH modeling to prevent peritonitis. And on the following day, they were first anesthetized with 10% chloral hydrate (350 mg/kg) by intraperitoneal injection and then placed in a stereotaxic frame (David Kopf Instruments). When the rats were monitored to lose pain reflex meant deep anesthesia, a 26-gauge needle was then inserted through a burr hole into the caudate nucleus (3.0 mm lateral to the midline, 0.2 mm anterior to bregma, 6.0 mm in depth below the skull). A total of 100μl autologous whole blood taken from the tail vein was injected at a rate of 10μl/min. Following the completion of the injection, the needle was withdrawn slowly after standing 10 mins, paraffin was placed around the burr hole and the skin incision was closed with sutures. For rats in the sham group, only drilling was performed.

Neurobehavioral function evaluation

Neurological function deficit were evaluated using the Longa five-point scale detailed as follows: i) 0, no neurological deficit; ii) 1, a slight neurologic deficit (unable to stretch the left forepaw fully); iii) 2, a moderate neurological deficit (hemiplegia or circling to the left); iv) 3, a severe neurological deficit (unable to stand or roll), and iv) 4, a critical neurological deficit (no spontaneous locomotor activity and disturbance of consciousness). On day 3 after operation, rats scoring 1-3 were selected for further study, while rats scoring 4 were so severely impaired in neurological function that they were unable to continue the next stage of the experiment.

Measurement of BBB structure

Transmission electron microscopy

On day 3 after operation, the rats were anesthetized with 10% chloral hydrate (350 mg/kg) by intraperitoneal injection. When the rats were monitored to lose pain reflex meant deep anesthesia, following which they were and perfused with saline that was pre-cooled to 4°C through the left ventricle until clear. The rat brain was then rapidly removed and placed on an ice box. The tissue around the hematoma of ICH was harvested quickly and soaked in a pre-cooled 2.5% glutaraldehyde specimen bottle, which was then cut to the size of 1 mm³ for full pre-fixation. After 1% osmium acid fixation and dehydration using an ascending alcohol gradient, the tissues were embedded in epoxy resin to make ultrathin sections. The sections were then mounted onto copper grids, stained with uranyl acetate and lead citrate and then observed under a transmission electron microscope (FEI Tecnai G2 Spirit TEM; Thermo Fisher Scientific, Inc.) to observe the ultrastructural changes of the BBB around the hematoma. Three rats were selected from each group for this examination.
Immunofluorescence staining

Immunofluorescence staining for Glial fibrillary acidic protein (GFAP) and Cladudin-5 was performed to observe the gross structure of the BBB. On day 3 after operation, the rats were anesthetized with 10% chloral hydrate (350 mg/kg), following which they were perfused with normal saline through the left ventricle with until clear and then with 250 ml 4% paraformaldehyde. The brain was quickly removed and soaked in 4% paraformaldehyde for >24 h. All brain tissues were embedded in paraffin and then sliced into 5-μm thick sections. The primary antibodies of GFAP (diluted 1:100; Boster Biological Technology) and Cladudin-5 (diluted 1:100; Thermo Fisher Scientific, Inc.) were used to incubate the sections overnight at 4˚C after permeabilization with 0.3 % Triton X-100 for 25 min. The next day, sections were incubated with the fluorescent-labeled secondary antibody FITC-conjugated anti-Rabbit IgG (1:100; Boster Biological Technology) at 37˚C for 1 h, followed by DAPI for 30 min after washing in PBS. The sections were then imaged by fluorescent microscopy, following which fluorescence intensity was determined using Image J 1.52a (National Institutes of health). Three rats were selected from each group for this experiment.

Measurement of BBB function

Evans blue assay

On day 3 after operation, the rats were first anesthetized with 10% chloral hydrate (350 mg/kg) before 2% EB dye (5 ml/kg) was injected into the blood circulation through the tail vein. After 2 h, rats were perfused with pre-cooled saline through the left ventricle until clear. The rat brain was then rapidly removed and weighed. Each sample was homogenized using 50% trichloroacetic acid and centrifuged at 15,000 x g for 30 min at 4˚C to obtain the supernatant, following which anhydrous ethanol was then added and incubated overnight at 4˚C. The samples were again centrifuged at 15,000 x g for 30 min at 4˚C and the optical density value was measured using a fluorescence spectrophotometer (excitation wavelength, 620 nm; emission wavelength, 680 nm) to calculate the sample EB concentration. EB extravasation was calculated according to the measured concentration and was defined as the amount of EB (μg) per g wet-weight brain tissue. (standard curve, y=0.23+6.58x10-3x, R2=0.984). Three rats were selected from each group for this experiment.

Brain water content

On day 3 after operation, the rats were anesthetized with 10% chloral hydrate (350 mg/kg) and perfused with pre-cooled 4˚C saline through the left ventricle until clear. The wet brain was then quickly removed and weighed using an electronic analytical balance. After drying in an electrothermostat for >24 h at 100˚C overnight, the dry brain was weighed again. Brain water content (%) was calculated using the
following formula: (Wet weight - dry weight)/wet weight x100%. Five rats were selected from each group or this experiment.

**MRI Examination**

Rats were anesthetized with 10% chloral hydrate (350 mg/kg) 3 days after operation and placed on the rat coil (channel 4) to perform MRI (Philips Achieva 1.5T; Philips Healthcare) using the following parameters: i) T1, TR/TE (repetition time/ echo time) =550/15; ii) T2, TR/TE=4000/112; iii) SL (slice thickness), 2; and iv) FOV (field of view), 100. During MRI, a series of imaging data were obtained. Areas of hyperintensity and the mixed regions of hyperintensity-hypointensity were measured from the MRI images and used to calculate brain edema volume using DicomGov1.0.0.8(Shenyang Link Imaging Technology Co., Ltd). Three rats were selected from each group for this experiment.

**Nissl staining**

On day 3 after operation, rats were anesthetized with 10% chloral hydrate (350 mg/kg) and perfused with saline through the left ventricle until clear, followed by 250 ml 4 % paraformaldehyde. The brain was then quickly removed and soaked in 4% paraformaldehyde for >24 h. All brain tissues were embedded in paraffin and then sliced into 5-μm thick sections. After deparaffinization and rehydration, the slides were stained with cresyl violet (Nissl Staining Solution, Beyotime Institute of Biotechnology) for 10 min at 37-50˚C. The stained slides were then dehydrated by incubation with 100% alcohol twice at 5 min each followed by incubation with xylene twice at 5 min each. After the sections were mounted using a permanent mounting medium, the numbers of Nissl body in the tissue around the hematoma were counted per image (magnification, x400). Three rats were selected from each group for this experiment.

**TUNEL staining**

Apoptotic cells in the tissue around the hematoma were observed by TUNEL staining according to manufacturer’s protocol (In Situ Cell Apoptosis Detection Kit, FITC). After deparaffinization and rehydration of the coronal sections, the slides were labeled with the TUNEL reaction mix and converter-peroxidase. Following washing with TBS, the slides were incubated with streptavidin-biotin complex-FITC to induce a chromogenic reaction, where apoptotic nuclei were stained brown. The number of TUNEL-positive cells were counted in each image using a fluorescence microscope (magnification, x400). Three rats were selected from each group for this experiment.

Western blotting. On day 3 after operation, the rats were anesthetized with 10% chloral hydrate (350 mg/kg) and perfused with saline through the left ventricle until clear. The rat brain tissues were then
quickly removed, flash-frozen in liquid nitrogen and then preserved at -80°C. In total, 20 mg brain tissues around the hematoma were homogenized and lysed separately in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology), which were then centrifuged for 20 min at 14,000 x g at 4°C. The supernatants were collected and protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μg per lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The following primary antibodies were used for western blotting: Anti-HMGB1 antibody (1:1,000), anti-TLR4 antibody (1:1000), anti-MMP-9 (1:1,000), anti-Claudin-5 antibody (1:1,000), anti-Occludin antibody (1:1,000) and anti-ZO-1 antibody (1:500). The membranes were then incubated with the primary antibodies with gentle agitation at 4°C overnight. After washing with TBS-T, the membranes were incubated with the HRP-conjugated secondary antibody (Abcam 1:2,000) for 2 h. The relative density of the blot bands was determined using Image J 1.52a (National Institutes of Health). Three rats were selected from each group for this experiment.

**Statistical Analysis**

All data were presented as the mean ± S.E.M. The neurobehavioral deficit scores were used Repeated Measures of General Linear Model, whilst all other data were analyzed using after one-way ANOVA. The differences among multiple groups were compared by LSD as a post hoc tests. SPSS 21.0 (IBM Corp.) software was used for all statistical analyzes. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**RRD ameliorates neurological deficit induced by ICH**

The Longa score was used to evaluate neurological deficits in rats from the different groups at 3 h and on days 1 and day 3 after operation. Significant neurological deficits were observed in the different groups after surgery, whilst rats in the RRD group exhibited markedly reduced neurological deficit scores compared with the ICH group. (Sham vs. ICH vs. RRD, 0.00±0.00 vs. 2.40±0.55 vs. 2.40±0.55 at 3 h, 0.00±0.00 vs. 2.20±0.45 vs. 2.00±0.00 at 1 day and 0.00±0.00 vs 2.40±0.55 vs. 1.00±0.00 at 3 days; Fig. 1).

**RRD exerts protective effects against ICH-induced BBB structure destruction**

To examine the BBB structures in the tissues isolated from rats in the different groups directly, transmission electron microscopy was used 3 days after operation. The BBB structure was demonstrated to be damaged following ICH induction compared with that in the sham group, whilst the extent of damage was found to be relatively mild in RRD group. (Fig. 2A). In the sham group, the BBB basal lamina was complete, where the thickness was consistent. Tight junctions between the endothelial cells were clearly observed, where the surrounding tissues were tightly connected. By contrast, in the ICH group, the
BBB basal lamina was found to be severely damaged with an irregularly discontinuous appearance. The nuclei of the endothelial cells were sparse and the membrane was separated from the basal lamina. No tight junctions were found, the surrounding tissue was loose and filled with edema fluid. Following treatment with RRD, structural damage to the BBB was observed to be significantly reduced. The BBB basal lamina was relatively complete with regular thickness although slightly swollen. Pinocytotic vesicles were observed in the cytoplasm of endothelial cells, which were connected by tight junctions and the surrounding tissue was partially edematous.

Immunofluorescence assay was subsequently performed to analyze Claudin-5 expression and astrocyte presence within the BBB structure. Tissues in the sham group showed strong positive green fluorescence staining for GFAP and Claudin-5, which was weaker in ICH group, where the green fluorescence was occasionally detected (Fig. 2B). Tissues in the RRD group showed stronger fluorescence intensity representing increased protein expression compared with that in the ICH group. In addition, the fluorescence intensity was found to be significantly reduced after ICH induction compared with that in the sham group, whilst that in the RRD group demonstrated marked improvements compared with that in the ICH group (Sham vs. ICH vs. RRD, Claudin-5 IOD: 69.57±2.81 vs. 39.36±4.17 vs. 53.42±4.09; Fig. 2B(a); GFAP IOD: 70.72±0.59 vs. 43.66±1.78 vs. 62.12±1.98; Fig. 2B (b)).

**RRD reduces the permeability of BBB, brain water content and brain edema**

On day 3 after operation, EB extravasation was used to examine BBB permeability in tissues isolated from rats from the different groups. Evans blue leakage rate was found to be significantly increased after ICH induction compared with that in the sham group, whilst the leakage rate in the RRD group showed marked improvement compared with that in the ICH group (Sham vs. ICH vs. RRD, 1.46±0.15 vs. 5.15±0.13 vs. 2.89±0.39 μg/g; Fig. 3A). Brain water content assay was subsequently examined to evaluate BBB function in the different groups on day 3 after operation. Brain water content was found to be significantly increased after ICH-induction compared with that in the sham group whereas RRD treatment markedly reduced brain edema compared with that in the ICH group (Sham vs. ICH vs. RRD, 78.62±0.38 vs. 80.89±0.28 vs. 79.64±0.11%; Fig. 3B). In addition, MRI was performed to measure the brain edema volume in rats from the different groups 3 days after operation. Results from the MRI images were revealed to be consistent with those of brain water content (Sham vs. ICH vs. RRD, 0.00±0.00 vs. 123.33±22.34 vs. 78.31±3.18 mm³; Fig. 3C).

**RRD treatment protects neuronal function and inhibits apoptosis**

The numbers of Nissl body represent neuronal function. The number of Nissl bodies was found to be significantly reduced after ICH induction compared with that in the sham group whereas RRD group showed marked improvements compared with that in the ICH group (Sham vs. ICH vs. RRD, 167.33±4.67 vs. 53.00±4.16 vs. 88.67±3.18 numbers/HP(high powered image); Fig. 4A). In addition, TUNEL assay was performed to measure apoptosis in the different groups. Apoptosis was significantly increased after ICH induction compared with that in the sham group whereas the extent of apoptosis in
the RRD group demonstrated marked reductions compared with that ICH group. (Sham vs. ICH vs. RRD, 4.33±0.88 vs. 145.33±10.71 vs. 116.67±1.20 numbers/HP; Fig. 4B)

**RRD downregulates HMGB1, TLR4 and MMP-9 expression**

Western blotting was performed to measure the expression levels of HMGB1, TLR4 and MMP-9 around the peri-hematoma tissue isolated from rats in the different groups. The expression of HMGB1, TLR4 and MMP-9 was all revealed to be increased after ICH induction compared with that in the sham group, which was found to be reversed in the RRD group, demonstrating the marked improvements compared with those in the ICH group (Fig. 5).

**RRD upregulates Cladudin-5, Occludin and Zo-1 expression**

Western blotting was performed to examine the expression of tight junction proteins, Cladudin-5, Occludin and Zo-1, around the peri-hematoma tissue isolated from rats in the different groups. Cladudin-5, Occludin and Zo-1 expression were all found to be decreased following ICH induction compared with those in the sham group (Fig. 6). By contrast, RRD treatment upregulated the expression levels of Cladudin-5, Occludin and Zo-1 by varying degrees, showing the marked improvements compared with those in the ICH group (Fig. 6).

**HMGB1 inhibitor EP and the TLR4 antagonist TAK-242 synergizes with RRD in mediating neuroprotective effects**

To verify that the neuroprotective effect of RRD was mediated by inhibiting MMP-9 expression and HMGB1/TLR4 signaling upstream, the HMGB1 inhibitor EP or the TLR4 antagonist TAK-242 were used following ICH induction. Both EP and TAK-242 were revealed to reduce neurological deficits in rats following ICH induction, effects that were similar to those observed following RRD administration (Fig. 7A: Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 0.00±0.00 vs. 2.40±0.55 vs. 2.40±0.55 vs. 2.60±0.55 vs. 2.60±0.55 vs. 2.60±0.55 at 3 h, 0.00±0.00 vs. 2.20±0.45 vs. 2.00±0.00 vs. 2.00±0.00 vs. 2.20±0.45 at 1 day and 0.00±0.00 vs. 2.40±0.55 vs. 1.00±0.00 vs. 1.20±0.45 vs. 1.20±0.45 vs. 1.00±0.00 vs. 1.20±0.45 at 3 days). Likewise, administration of RRD combined with TAK-242 or EP reduced destruction of the BBB structure induced by ICH (Fig. 7B), where the damage to the BBB basal lamina was observed to be less severe, the surrounding tissue was less edematous and higher expression levels of GFAP or Claudin-5 were found (Fig. 7B, Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, Claudin-5 (Fig. 7B(b-1)) IOD: 69.57±2.81 vs. 39.36±4.17 vs. 53.42±4.09; GFAP (Fig. 7B(b-2)) IOD: 70.72±0.59 vs. 43.66±1.78 vs. 62.12±1.98 vs. 54.96±0.57 vs. 57.46±2.04 vs. 58.86±3.28 vs. 57.05±4.28). In addition, administration of RRD combined with TAK-242 or EP were found to reduce BBB permeability (Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 1.46±0.15 vs. 5.15±0.13 vs. 2.89±0.39 vs. 2.78±0.48 vs. 2.90±0.86 vs. 2.88±0.11 vs. 2.86±0.14 μg/g; Fig. 7C), brain water content (Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 78.62±0.38 vs. 80.89±0.28 vs. 79.64±0.11 vs. 79.19±0.24 vs. 78.97±0.32 vs. 78.74±0.20 vs. 78.66±0.20%; Fig. 7D) and brain edema (Sham vs. ICH vs. RRD vs. TAK-
242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 0.00±0.00 vs. 123.33±22.34 vs. 78.31±3.18 vs. 79.47±1.29 vs. 74.80±3.72 vs. 1.72±3.43 vs. 64.36±2.29 mm³; Fig. 7E). However, there was no difference among these drugs. In addition, combination of EP or TAK-242 with RRD showed a synergistic effect in preserving neuronal function (Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 167.33±4.67 vs. 53.00±4.16 vs. 88.67±3.18 vs. 99.00±2.31 vs. 110.33±1.45 vs. 108.33±2.33 vs. 123.67±3.53 numbers/HP; Fig. 7F) and inhibiting apoptosis (Sham vs. ICH vs. RRD vs. TAK-242 vs. TAK-242+RRD vs. EP vs. EP+RRD, 4.33±0.88 vs. 145.33±10.71 vs. 116.67±1.20 vs. 83.67±3.71 vs. 72.00±4.58 vs. 61.00±7.10 vs. 51.67±0.33 numbers/HP; Fig. 7G) compared with that in rats treated with RRD alone.

**EP and TAK-242 alters the expression of HMGB1, TLR4 and MMP-9**

Western blotting was performed to further elucidate the potential effects of RRD on the HMGB1/TLR4/MMP-9 signaling pathway in rats following ICH induction. Compared with those in the ICH group, HMGB1, TLR4 and MMP-9 expression were revealed to be lower in the EP group, whilst the expression levels of TLR4 and MMP-9 expression were lower in the TAK-242 group (Fig. 8). In addition, the combination of EP or TAK-242 with RRD exhibited a synergistic effect in inhibiting the HMGB1/TLR4/MMP-9 signaling pathway following ICH induction.

**EP and TAK-242 synergizes with RRD in exerting protective effects on the expression of Claudin-5, Occludin and Zo-1, proteins associated with tight junction**

The expression levels of Claudin-5, Occludin and Zo-1 were all demonstrated to be higher in the EP and TAK-242 groups compared with those in the ICH group (Fig. 9). Additionally, combined treatment of EP or TAK-242 with RRD showed synergistic effects of increasing of Claudin-5, Occludin and Zo-1 expression following ICH induction.

**Discussion**

In the present study, the neuroprotective effects of RRD was first demonstrated, which ameliorated neurological deficits, protected the BBB from disruption, reduced BBB extravasation, brain water content and edema volume, whilst increasing the numbers of Nissl bodies and inhibiting apoptosis. It was subsequently demonstrated that the HMGB1/TLR4/MMP9 signaling pathway was underlying the neuroprotective effects of RRD on the BBB during acute ICH.

The BBB is an important protective structure in the central nervous system that can resist harmful substances present in the peripheral circulation. It consists of cerebrovascular endothelial cells, pericytes, astrocytes, extracellular matrix and tight junction proteins, including Claudin-5, Occludin and Zo-1[30, 31]. Cytotoxicity, excitotoxicity, oxidative stress and the inflammatory response, all of which can be induced following ICH, can lead to the destruction of the BBB [5]. One of the events following the disruption of BBB is brain edema, which adversely affect the outcome and prognosis of patients with ICH [6, 32]. Therefore, protecting the integrity of the BBB is crucial for attenuating secondary brain injury following ICH. The present study showed that administration of RRD protected the BBB in rats following ICH.
Compared with the ICH group, rats in the RRD group exhibited lower BBB extravasation, lower brain water content and brain edema volume in addition to a BBB structure that was better preserved.

MMP-9 is an extensively studied protein of the zinc-enzyme family that directly disrupts BBB by degrading the extracellular matrix and tight junction proteins between brain vascular endothelial cells, leading to brain edema [9]. The HMGB1/TLR4 pathway has been previously reported as a inducer of inflammatory cytokine expression and inflammation in response to stroke [13, 33, 34]. Considering the homodromous nature of MMP-9, the HMGB1/TLR4 pathway and brain edema following ICH induction, it was hypothesized that MMP-9 was the downstream signaling target of the HMGB1/TLR4 pathway. Interestingly, the present study found MMP-9 to be a target protein of the HMGB1/TLR4 pathway. TAK-242 is a novel small molecule compound that selectively inhibits TLR4 signaling, where it was previously found to inhibit the production of inflammatory mediators in response to lipopolysaccharide in vitro by binding to the intracellular domain of TLR4 [35]. TAK-242 selectively binds TLR4, disrupting the interaction between TLR4 and adaptor proteins, in turn inhibiting signal transduction downstream. In the present study, treatment with TAK-242 demonstrated a number of neuroprotective effects, including ameliorating neurological deficits, protecting the BBB from disruption, reducing BBB extravasation, brain water content and brain edema volume, increasing Nissl body numbers, inhibiting neuronal apoptosis, reducing the expression levels of MMP-9, TLR4 and HMGB1 and upregulating the expression of tight junction proteins Claudin-5, Occludin and Zo-1. All these observations aforementioned suggest that inhibition of TLR4 by TAK-242 is a key mechanism in protecting the BBB during ICH. EP is a HMGB1 inhibitor that has been widely used for studying diseases in the central nervous system. Similar to TAK-242, EP treatment also demonstrated protective effects on the BBB in the present study. The present study showed that TAK-242 or EP treatment can act via the same downstream target to protect the BBB, where rats in the ICH group that were treated with TAK-242 or EP exhibited lower MMP-9 expression, reduced functional and structural damage to the BBB and improved neurological deficits. These observations suggested that the HMGB1/TLR4 pathway is a key regulatory mechanism upstream of MMP-9. The HMGB1/TLR4/MMP-9 signaling pathway serves an important role in mediating BBB damage during ICH. Rats in the ICH group that were treated with RRD, including the RRD, TAK-242 + RRD and EP + RRD groups, exhibited marked preservation of the BBB. Although it remains unclear which of the RRD, EP and TAK-242 treatments exerted the most favorable effects, the neuroprotective effects of RRD on the BBB during acute ICH was less ambiguous. Collectively, the HMGB1/TLR4 pathway was induced following ICH, which then activates MMP-9 to degrade extracellular matrix and tight junction proteins, including Claudin-5, Occludin and Zo-1, causing BBB disruption. This pathological effect was found to be reversed by RRD treatment.

The present study verified the neuroprotective mechanism of RRD on the BBB following acute ICH. However, there were some limitations. The neuroprotective effects of RRD on the BBB was assessed within only 3 days. The long-term outcomes of RRD treatment on neurological function should be evaluated in future studies. Since RRD also possesses antioxidant and anti-cytotoxicity properties, the effects of these characteristics on BBB protection also warrant further study. To conclude, RRD was
found to protect BBB during acute ICH by downregulating the HMGB1/TLR4/MMP9 signaling pathway, which provides a promising treatment target for ICH.

**Conclusion**

In conclusion, our results demonstrate that RRD has a protective effect on the BBB in rats during ICH and this protective effect is related to the down-regulation of HMGB1/TLR4/MMP9 signaling pathway.

**Abbreviations**

BBB: Blood-brain barrier; ICH: Intracerebral hemorrhage; RRD: Rehmannia and rhubarb decoction; HMGB1: high mobility group box 1 protein; EP: Ethyl pyruvate; TLR4: toll-like receptor 4; MMP-9: Matrix metalloproteinase 9; Zo-1: Zona occludens -1; RAGE: Receptor for advanced glycation end products; GFAP: Glial fibrillary acidic protein; EB: Evans blue.

**Declarations**

**Acknowledgements**

Not applicable.

**Authors’ contributions**

XFY conceived and designed the experiments. SJ and CML performed the experiments. SJ, CML, JSZ and XFY analyzed the experimental data. SJ and JSZ wrote the manuscript. All authors read and approved the final manuscript.

**Funding**

This study was supported by the grant from the National Natural Science Foundation of China (no.81503555).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.
Ethics approval and consent to participate

All experimental procedures were conducted in accordance with the guidelines for animal experiments of Shanghai University of Traditional Chinese Medicine and approved by the university's committee on animal experimentation (ethical code, PZSHUTCM170721001).

Consent for publication

All the co-authors and participants have given their consent for publication in Journal of neuroinflammation.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References


Figures
Figure 1

Neurological deficit score of rats in each group. Data are presented as the mean ± SEM. n=5 rats per group. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. ICH, intracerebral hemorrhage.

Figure 2

Effects of RRD on the BBB structure following ICH. (A) Transmission electron microscopy was used to observe the structure changes in rat brain tissues from the different groups. The BBB consists of
endothelial cells, pericytes, astrocytes and basal lamina. Representative images of the BBB structure in the sham, ICH and RRD groups are shown. n=3 rats per group. Scale bar, 2 μm. (B) Immunofluorescence was used to observe changes in astrocytes and tight junctions in the sham, ICH and RRD groups. Representative images of (a) Claudin-5 and (b) GFAP expression in the BBB structure in the sham, ICH and RRD groups are shown. n=3 rats per group. Scale bar, 50 μm. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; GFAP, glial fibrillary acidic protein.

Figure 3

Effects of RRD on BBB function following ICH induction. (A) Evans blue extravasation assay, (B) brain water content assay and (C) MRI were performed to evaluate BBB function in the sham, ICH and RRD groups 3 days after operation. Data are presented as the mean ± SEM. n=3 rats in each group for Evans blue extravasation assay and MRI. n=5 rats in each group for brain water content assay. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction.

Figure 4
Effects of RRD on brain injury and neuronal apoptosis following ICH induction. (A) Nissl and (B) TUNEL staining were performed to evaluate pathological injury in brain tissues surrounding the hematoma in the sham, ICH and RRD groups. Images were collected ≥3 times from each group. Scale bars, 50 μm. Data are presented as the mean ± SEM, n=3 rats in each group. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction.

![Graphs showing HMGB1, TLR4 and MMP-9 expression](image1)

**Figure 5**

Effects of RRD on the HMGB1/TLR4/MMP-9 pathway following ICH induction. Western blotting was performed to evaluate expression levels of (A) HMGB1, (B) TLR4 and (C) MMP-9 in brain tissues surrounding the hematoma in the sham, ICH and RRD groups. Data are presented as the mean ± SEM. n=3 rats in each group. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; HMGB1, high mobility group box 1 protein; TLR4, toll-like receptor 4; MMP-9, matrix metalloproteinase 9.

![Graphs showing Claudin-5, Occludin and Zn-1 expression](image2)

**Figure 6**

Effects of RRD on the expression of tight junction proteins following ICH induction. Western blotting was performed to evaluate the expression levels of tight junction proteins (A) Claudin-5, (B) Occludin and (C) Zn-1.
Zo-1 in brain tissues surrounding the hematoma in the sham, ICH and RRD groups. Data are presented as the mean ± SEM. n=3 rats in each group. #P<0.05 vs. Sham group and *P<0.05 vs. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; Zo-1, zona occludens-1.

Figure 7

Effects of RRD and/or TAK-242 or EP on rat neurological and BBB function following ICH induction. (A) Neurological deficits were evaluated at 3 h and on days 1 and 3 after operation. (B) (a) Transmission electron microscopy performed to observe the BBB structure 3 days following operation. (b) Immunofluorescence analysis was performed 3 days following operation to assess Claudin-5 and GFAP expression in the BBB. (C) Evan's blue extravasation assay, (D) brain water content and (E) MRI were performed to evaluate BBB function. (F) Nissl and (G) TUNEL staining were used to evaluate pathological injury to the BBB. Scale bars, 25 μm. Data are presented as the mean ± SEM. n=3 rats in each group. #P<0.05 vs. Sham group; *P<0.05 vs. ICH group; %P<0.05 vs. RRD group; &P<0.05 vs. TAK-242 group and $P<0.05 vs. TAK-242 + RRD group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; GFAP, glial fibrillary acidic protein; EP, ethyl pyruvate.
Figure 8

Effects of RRD and/or TAK-242 or EP on HMGB1, TLR4 and MMP-9 expression following ICH induction. Western blotting was performed to evaluate (A) HMGB1, (B) TLR4 and (C) MMP-9 expression in brain tissues surrounding the hematoma in each group. Data are presented as the mean ± SEM, n=3 rats in each group. #P<0.05 vs. Sham group; *P<0.05 vs. ICH group; %P<0.05 vs. RRD group; &P<0.05 vs. TAK-242 group and $P<0.05 vs. TAK-242 + RRD group. ICH group. BBB, blood-brain barrier; ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; HMGB1, high mobility group box 1 protein; TLR4, toll-like receptor 4; MMP-9, matrix metalloproteinase 9; EP, ethyl pyruvate.

Figure 9

Effects of RRD and/or TAK-242 or EP on the expression of tight junction proteins following ICH induction. Western blotting was performed to evaluate the expression of tight junction proteins, (A) Claudin-5, (B) Occludin, and (C) Zonula-1.
Occludin and (C) Zo-1 in brain tissues surrounding the hematoma in each group. Data are presented as the mean ± SEM. n=3 rats in each group. #P<0.05 vs. Sham group; *P<0.05 vs. ICH group; %P<0.05 vs. RRD group; &P<0.05 vs. TAK-242 group. ICH, intracerebral hemorrhage; RRD, Rehmannia and rhubarb decoction; EP, ethyl pyruvate; Zo-1, zona occludens-1.