

# Protective effect of ginsenoside Rb1 on integrity of blood–brain barrier following cerebral ischemia

Wei Chen<sup>1</sup> · Yijun Guo<sup>1</sup> · Wenjin Yang<sup>1</sup> · Ping Zheng<sup>1</sup> · Jinsong Zeng<sup>1</sup> · Wusong Tong<sup>1</sup>

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**Abstract** Ginsenosides, the major bioactive compounds in ginseng root, have been found to have antioxidant, immunomodulatory, and anti-inflammatory activities. In the present study, we sought to investigate whether and how ginsenoside Rb1 (GS-Rb1), the most abundant ginsenoside, can protect blood–brain barrier (BBB) integrity following cerebral ischemia in middle cerebral artery occlusion (MCAO) animal model. ICR mice underwent MCAO and received GS-Rb1 by intraperitoneal injection at 3 h after reperfusion. We evaluated infarction, neurological scores, brain edema, Evans blue (EB) extravasation, and tight junction protein expression at 48 h after MCAO. We further examined whether GS-Rb1 protected BBB integrity by suppressing post-ischemic inflammation-induced activity of matrix metalloproteinase-9 (MMP-9) and nicotinamide adenine dinucleotide phosphate oxidase (NOX). First, GS-Rb1 decreased infarction and improved neurological deficits in MCAO animals. In addition, GS-Rb1 reduced EB extravasation and brain edema and preserved expression of tight junction proteins in the ischemic brain. Moreover, GS-Rb1 inhibited expression of pro-inflammatory factors including nitric oxide synthase and IL-1 $\beta$ , but increased expression of anti-inflammatory markers arginase 1 and IL-10 in the ischemic brain. Consistently, GS-Rb1 attenuated ischemia-induced expression and activity of

MMP9. Finally, GS-Rb1 reduced NOX-4 mRNA expression and NOX activity in ischemic brain. These results suggest that GS-Rb1 protects loss of BBB integrity in ischemic stroke by suppressing neuroinflammation induction of MMP-9 and NOX4-derived free radicals, and indicate its potential for treating brain injuries, such as ischemia and stroke.

**Keywords** Ginsenoside Rb1 · Blood–brain barrier · Cerebral ischemia · Cerebral artery occlusion

## Introduction

Physiologically, blood–brain barrier (BBB) exemplifies a diffusion barrier, which consists of an interdependent network to segregate the central nervous system (CNS) from the systemic circulation, and rigorously regulates paracellular permeability (Sandoval and Witt 2008). BBB has long been described as the gatekeeper of the CNS, acting to maintain the delicate homeostasis of the brain. In this respect, the endothelial tight junctions (TJs) of the capillary function as the primary mediators, limiting paracellular movement of water, solutes, and ions. These TJs are tightly regulated by highly specialized molecules, which can be modulated by various intracellular and extracellular signaling pathways. Despite the fact that physiological characteristics of the TJs have been intensely investigated in the past decades, less is known with regard to their regulation under pathological conditions.

The two major mechanisms causing brain damages in stroke are ischemia and hemorrhage. In ischemic stroke, which represents about 80 % of total (Meyers et al. 2011), the integrity of BBB TJs can be severely impaired following reduced or blocked circulating blood. As a

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✉ Wusong Tong  
wusongtong@163.com

<sup>1</sup> The People's Hospital of Pu Dong New Area, 490 South Chuanhuan Road, Chuansha New Town, Shanghai 201299, People's Republic of China

consequence, the disruption of BBB allows the harmful substances, such as inflammatory molecules entering into the brain, resulting in brain damage. Provided the complexity of events surrounding the different phases of TJ permeability, loss of BBB integrity is believed to be a consequence of activation of multiple independent molecular signaling pathways, which include ionic dysregulation, inflammation, oxidative and nitrosative stresses, enzymatic activity, and angiogenesis (Sandoval and Witt 2008).

Therapeutic strategies aimed at prevention of disruption of BBB and brain damage have been a major focus of academic and industrial research for the past few decades (Moroni and Chiarugi 2009). Understanding of the interrelation of the mechanisms addressed above is extremely helpful for the development of new therapies. Ginsenosides, a special group of triterpenoid saponins, have been a target of research in treatment of a variety of diseases associated with dysregulation of inflammatory signaling pathways. Ginsenosides are often classified into two groups: the Rb1 group characterized by the protopanaxadiol presence including Rb1, Rb2, Rc, and Rd and the Rg1 group with protopanaxatriol such as Rg1, Re, Rf, and Rg2 (Christensen 2009). As the most abundant bioactive form extracted from ginseng, ginsenoside Rb1 (GS-Rb1) can effectively inhibit inflammation in *in vivo* and *in vitro* models, including brain tissues in cerebral ischemia animal model (Zhu et al. 2012). The aim of the current study is to further investigate whether GS-Rb1 could suppress activation of the molecular signaling cascades preceding disruption of BBB, and therefore preventing brain damage resulting from loss of BBB integrity in the middle cerebral artery occlusion (MCAO) animal model.

## Materials and methods

### Middle cerebral artery occlusion (MCAO)

Male ICR mice, weighing 25–30 g, were provided by The People's Hospital of Pu Dong New Area. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of The People's Hospital of Pu Dong New Area. The IACUC committee members at The People's Hospital of Pu Dong New Area approved this study. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. We conducted MCAO with use of an intraluminal monofilament technique as previously described (Zhu et al. 2010; Wang et al. 2014). First of all, we inserted a heat-blunted and silicon-coated tip into the right internal carotid artery

of anesthetized mice with a 6–0 thread, which subsequently reached the original site of MCA. The abrupt drop in cortical perfusion (<25 % of the baseline) was monitored by the laser Doppler flowmetry (PRIMED Inc., Stockholm, Sweden). After 60 min of MCAO, the filament was removed to permit for reperfusion (>75 % of the baseline).

### Drug administration and grouping of subjects

After 3-h reperfusion, mice were randomly subjected to receiving vehicle (normal saline or GS-Rb1 with concentrations at 5, 20 or 40 mg/kg) via intraperitoneal injection. Mice were then killed at 24 or 48 h after MCAO for further analysis.

### Behavior evaluation

Neurological behavior was assessed according to a five-point scale system at 48 h after MCAO, as previously described (Uchida et al. 2009): 0, no obvious neurological deficits; 1, unable to extend the contralateral forelimb fully; 2, failing to walk straight or circling to the ipsilateral side; 3, learning to the affected side; 4, no spontaneous locomotor activity; and 5, being dead.

### Analysis of infarct volume

The brains were harvested at 48 h after MCAO, and subsequently, processed to four 2-mm-thick slices. Slices were incubated in 1 % 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, USA) at 37 °C for 10 min and then fixed in 10 % buffered formaldehyde solution for 24 h. Slices were imaged with the infarct volumes analyzed by the software (SigmaScan Pro, USA).

### Assessment of Evans blue extravasation following MCAO

The assay of Evans blue (EB) leakage was used to evaluate BBB disruption following the experimental stroke, as described previously (Huang et al. 2013). Briefly, 2 % EB (4 ml/kg) was injected via the tail vein at 46 h after reperfusion. At 2 h after EB injection, mice were perfused under anesthesia. Tissue samples were collected from the cortex and striatum, which were then homogenized by 50 % trichloroacetic acid and centrifuged. The absorbance of supernatants was measured at 620 nm with infinite M200 PRO (TECAN, Austria).

### Real-time quantitative PCR

The tissue sample was collected from the ipsilateral cortex. The total RNAs were extracted with use of the

TRIzol reagent (Invitrogen, USA). Reverse transcription was performed using a reverse transcription kit (Applied Biosystems, USA). PCR reactions were performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems) as recently described (Wang et al. 2014). The sequence of primers is as follows: IL-1 $\beta$  (forward: 5'-TGGAAGCGGTTTGTCTTC-3', reverse: 5'-TAC CAGTTGGGGAAGCTCTGC-3'), MMP-9 (forward: 5'-ACG ACATAGACGGCATCCA-3', reverse: 5'-GCTGTGGTT CAGTTGTGGTG-3'), iNOS (forward: 5'-CAGGAGGAG AGAGATCCGATTTA-3'; reverse: 5'-GCATTAGCATGGA AGCAAAGA-3'), NOX-1 (forward: 5'-CATCCAGTCT CCAAACATGACA-3', reverse: 5'-GCTGTGGTTCAGTT-GTGGTG-3'), NOX-2 (forward: 5'-CCAAGTGGGATAA CGAGTTCA-3', reverse: 5'-GAGAGTTTCAGCCAAGGC TTC-3'), NOX-4 (forward: 5'-TGAAGTACAGTGAAG ATTCCTTGAAC-3', reverse: 5'-GACACCCGTCAGA CCAGGAA-3'), Arginase 1 (forward: 5'-GAACACGGC AGTGGCTTTAAC-3', reverse: 5'-TGCTTAGCTCTGTC TGCTTTGC-3'), and 18S (forward: 5'-GTAACCCGTT GAACCCATT-3', reverse: 5'-CCATCCAATCGGTAGTA GCG-3'). All test results were normalized with 18S rRNAs.

#### Western blot assay

The tissue sample was collected from the ipsilateral cortex. Proteins samples (30  $\mu$ g) were separated on SDS-12 % PAGE and then transferred onto PVDF membranes. The blots were incubated with appropriate primary antibody, anti-occludin (1:200; Invitrogen) or ZO-1 (1:500; Invitrogen) for overnight at 4 °C. Then membranes were washed and incubated with HRP-conjugated secondary antibodies (1:1000; Cell Signaling Technology, USA). Protein bands were visualized with an enhanced luminescence reagent (Millipore, USA) and photographed with ChemiDoc XRS+ (Bio-Rad, USA).

#### Edema assessment

The brains were harvested at 48 h after MCAO and dissected into the left and right hemispheres. The dissected hemispheres were weighed and placed into an oven at 80 °C for 48 h, followed by measurement of dried tissue weight. We assessed edema based on formula: (wet weight-dry weight)/wet weight.

#### MPO activity assay

Tissue samples were collected from the ipsilateral and contralateral cortex at 48 h after MCAO. The myeloperoxidase (MPO) activity was measured with use of the kit (Jiancheng Company, China).

#### ELISA

ELISA was used to measure IL-10 activity according to the manufacturer's instructions (Wuhan Boster Biological Technology LT, China).

#### NADPH oxidase activity assay

The activity of NOX activity was tested as previously described (Liu et al. 2008). Tissue samples were homogenized in PBS. After centrifugation at 12,000g for 10 min, 20  $\mu$ l of supernatant was transferred to a 96-well luminescence plate. After adding 80  $\mu$ l phosphate-buffered saline and 6.25  $\mu$ l 1 M lucigenin, the reaction was started by adding 100  $\mu$ M NADPH (Beyotime, China). Photoemission was measured by at absorbance at 340 nm every 30 s for 5 min with Infinite M200 PRO (TECAN).

#### Statistical analysis

All experiments were repeated at least three times. Data were expressed as mean  $\pm$  SEM with two-way ANOVA followed by Tukey's test.  $p < 0.05$  is considered significant.

## Results

### GS-Rb1 reduces infarction and improves neurological deficits

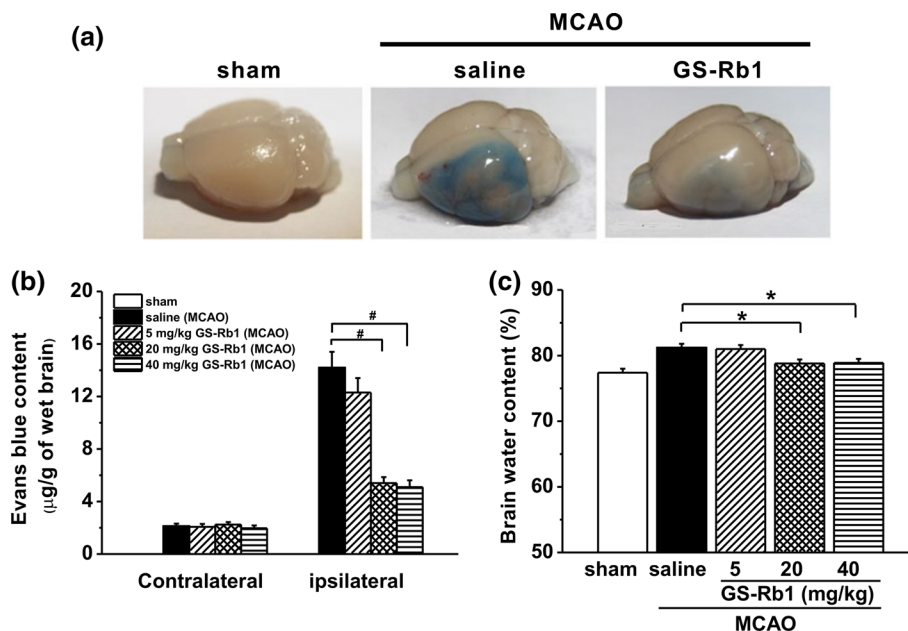
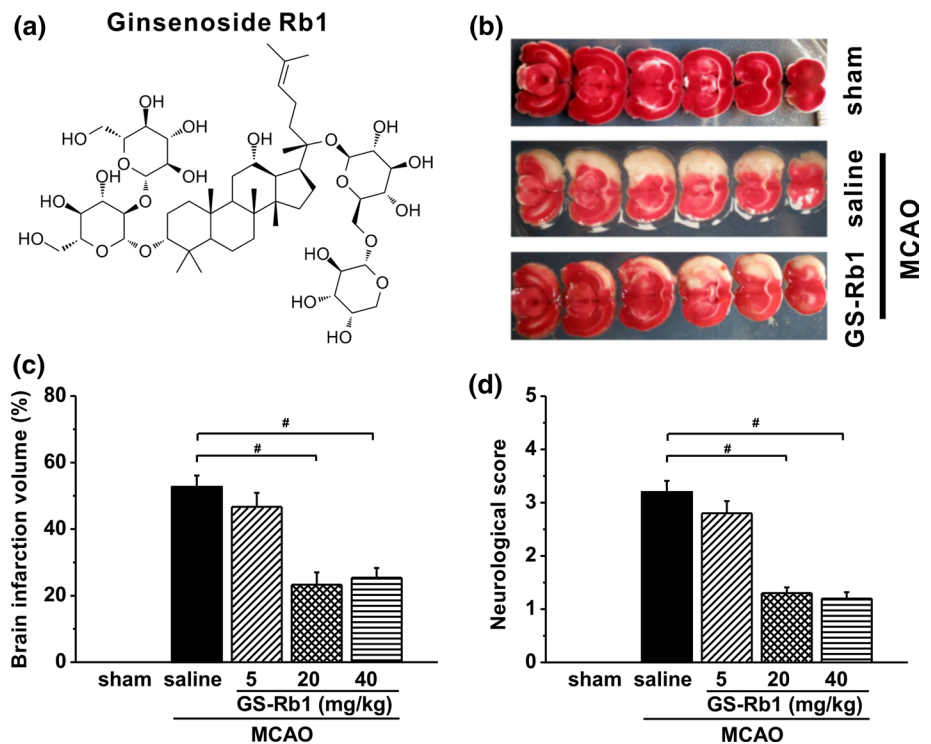
We first assessed the effects of GS-Rb1 at different concentrations (5, 20, and 40 mg/kg, intraperitoneal injection) on infarction and neurological deficits. Figure 1a illustrates the chemical structure of GS-Rb1. Treatment of GS-Rb1 (20 and 40 mg/kg) led to a significant decrease in cortical and hemispheric infarction at 48 h following MCAO compared with the vehicle group (Fig. 1b, c). Consistent with these data, a significant decrease in neurological score has also been observed in 20 and 40 mg/kg GS-Rb1 groups compared with the vehicle one (Fig. 1d), indicating the potential of GS-Rb1 in rescuing the neurological deficits after MCAO.

### GS-Rb1 inhibits disruption of BBB following MCAO

We determined whether ischemia can induce BBB disruption with use of EB leakage assay. At 48 h after MCAO, EB leakage into the ipsilateral hemispheres was significantly inhibited in GS-Rb1-treated mice at the doses of 20 and 40 mg/kg than in vehicle-treated group (Fig. 2a, b). To examine effects of GS-Rb1 effects on post-ischemic edema formation, we evaluated brain water

**Fig. 1** Post-ischemic administration of GS-Rb1 attenuated stroke severity at 48 h after middle cerebral artery occlusion (MCAO). **a** Chemical structure of GS-Rb1. **b** Representative images of TTC staining of sham, saline, and 20 mg/kg GS-Rb1-treated groups.

**c** The brain infarct volume for sham-operated rats, and for rats subjected to MCAO and then administered with saline, 5, 20, or 40 mg/kg of GS-Rb1 ( $n = 8$  for each group). **d** Neurological Bederson scores of the experimental groups at 48 h after MCAO ( $n = 8$  for each group). Data were presented as mean  $\pm$  SEM and analyzed by two-way ANOVA followed by Tukey's test. # $p < 0.01$

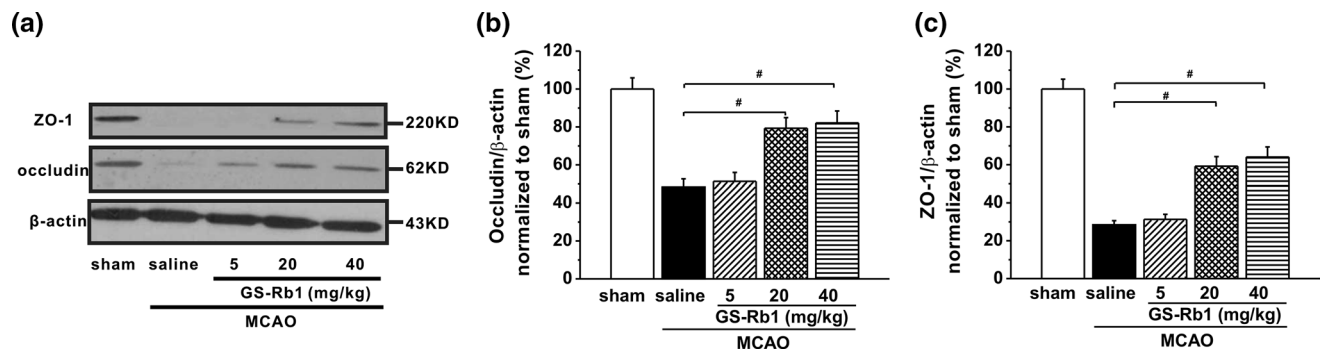


**Fig. 2** GS-Rb1 protected blood–brain barrier (BBB) integrity following MCAO. **a** Representative images of Evans blue leakage into the ischemic brain at 48 h after MCAO in the sham (without MCAO), saline and 20 mg/kg GS-Rb1-treated groups. **b** The concentrations of Evans blue leaking into the ipsilateral and contralateral brains in the experimental groups. Note that the concentrations of Evans blue leak-

ing in 20 and 40 mg/kg GS-Rb1 treatment groups were significantly lower than that in saline group.  $n = 8$  in each group. **c** Brain water content in the experimental groups. 20 and 40 mg/kg GS-Rb1 markedly alleviated brain edema of MCAO rats at 48 h. Data were presented as mean  $\pm$  SEM and analyzed by two-way ANOVA followed by Tukey test. \* $p < 0.05$ ; # $p < 0.01$

content at 48-h reperfusion after MCAO. Consistent with EB extravasation results, 20 and 40 mg/kg GS-Rb1-treated groups displayed a reduction in water content of

the ischemic hemispheres compared with vehicle-treated mice (Fig. 2c), suggesting that GS-Rb1 inhibits edema following MCAO.



**Fig. 3** GS-Rb1 treatment prevented stroke-induced loss of tight junction proteins in the ischemic brain. **a** Representative images of Western blot analysis of occludin and ZO-1 in the ipsilateral cortex of the experimental groups at 48 h after MCAO. Histogram of occludin/ $\beta$ -actin (**b**) and ZO-1 (**c**) in the experimental groups, normalized to

that of sham. Note that 20 and 40 mg/kg GS-Rb1 significantly alleviated MCAO-induced occludin and ZO-1 loss in the ipsilateral cortex compared with saline-operated group. Data were presented as mean  $\pm$  SEM and analyzed by two-way ANOVA followed by Tukey's test. # $p < 0.01$

### GS-Rb1 prevents ischemia-induced loss of tight junction proteins

In order to further evaluate BBB disruption following MCAO, we assessed MCAO-induced loss of tight junction proteins in the ischemic cortex. As shown in Fig. 3, despite the fact that non-treated mice displayed significantly decreased levels of two tight junction proteins, occludin and ZO-1, in the ipsilateral cortex at 48 h after MCAO, the loss of occludin and ZO-1 proteins in the ipsilateral cortex was attenuated in 20 and 40 mg/kg GS-Rb1-treated groups.

### GS-Rb1 suppresses inflammation in the ischemic brain

BBB disruption may result from local inflammatory responses in the ischemic brain (Huang et al. 2013). We profiled gene expression of pro- and anti-inflammatory cytokines in the ischemic cortex at 24 h after reperfusion. Compared to sham operation, MCAO resulted in a significant increase in mRNA levels of the pro-inflammatory genes such as iNOS and IL-1 $\beta$ . In contrast, 20 and 40 mg/kg GS-Rb1 remarkably diminished MCAO-induced iNOS and IL-1 $\beta$  expression (Fig. 4a, b).

In addition, 20 and 40 mg/kg GS-Rb1 considerably increased mRNA expression of the anti-inflammatory marker arginase 1 (Fig. 4c) (Hu et al. 2012). ELISA results further confirmed that the protein levels of IL-10, an important anti-inflammatory cytokine, were also increased in protective doses of GS-Rb1-treated groups (Fig. 4d). Increased MPO activity in the brain suggests that cerebral inflammatory responses are associated with peripherally infiltrated leukocytes or locally activated microglia (Chen et al. 2008). Here we show that MCAO induced MPO activity in the ipsilateral cortex, and 20 and 40 mg/kg GS-Rb1 treatment blocked ischemia-induced MPO activity in the ipsilateral cortex (Fig. 4e). Post-ischemic inflammation-induced

activity of MMP-9 typically promotes BBB injury. Consistent with the findings that GS-Rb1 suppressed pro-inflammatory gene expression but boosted anti-inflammatory activity, GS-Rb1 significantly reduced MMP-9 mRNA levels at 24 h after MCAO (Fig. 4f).

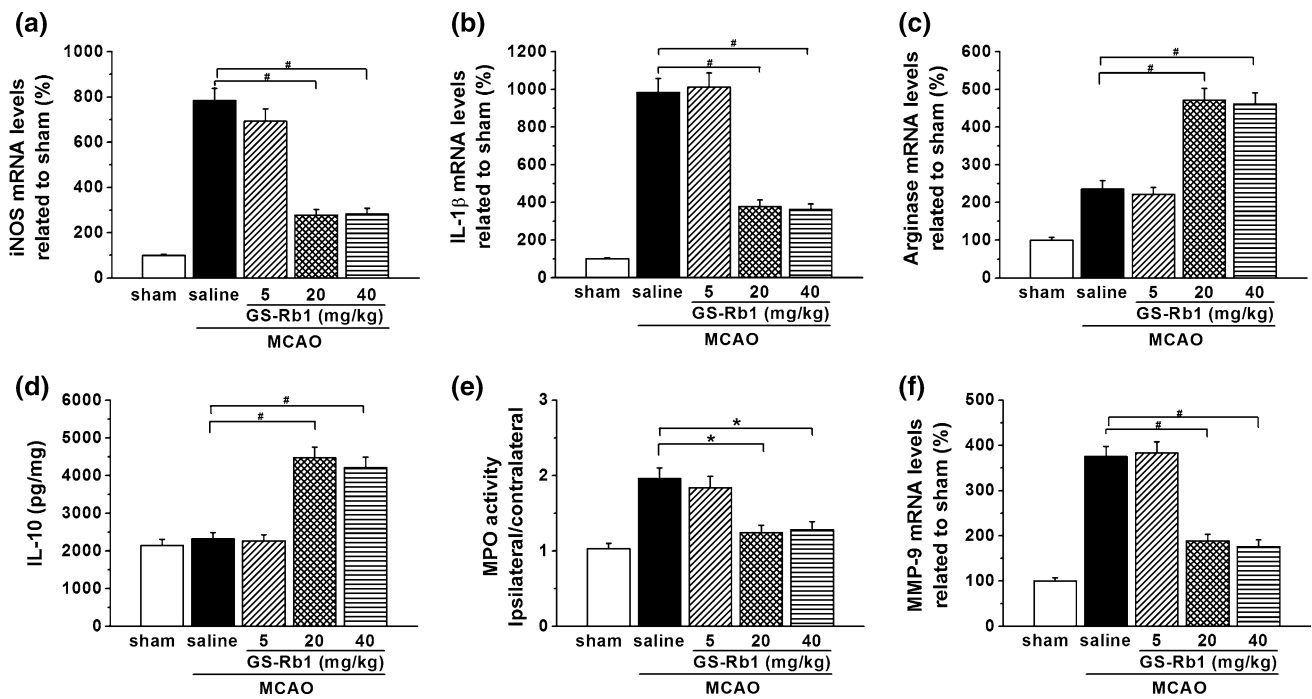
### GS-Rb1 represses NADPH oxidase expression and activity following MCAO

Inducible production of reactive oxygen species (ROS) constitutes one of major mechanism underlying BBB disruption following stroke. NOXs are important sources of ROS following cerebral ischemia and thereby play a critical role in post-stroke BBB disruption. Three types of NOXs have been identified in CNS: NOX1, NOX2, and NOX4 (Infanger et al. 2006). Here we found that 20 and 40 mg/kg GS-Rb1 inhibited expression of NOX1 mRNA (Fig. 5a). However, only ischemia-induced NOX4 mRNA expression, but not NOX2 mRNA, was attenuated by GS-Rb1 in the ipsilateral cortex (Fig. 5b, c). We further tested NOX activity in the ischemic cortex at 24 h after MCAO. As expected, post-ischemic induction of NOS activity in the ipsilateral cortex was significantly inhibited by 20 and 40 mg/kg GS-Rb1 treatment.

## Discussion

We demonstrated in the present study that GS-Rb1 confers a robust vaso- and neuro-protection against ischemia-induced BBB disruption. Our results further suggest the potential of GS-Rb1 in prospective therapy for ischemic stroke.

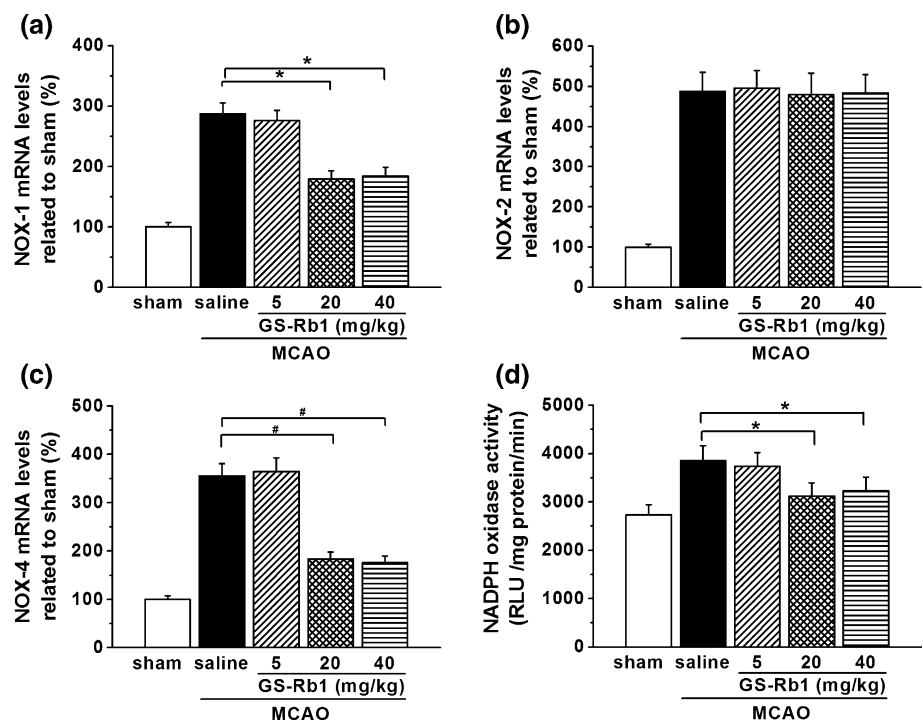
It has been well documented that disruption of BBB is preceded by activation of multiple independent molecular pathways consisting of ionic dysregulation, inflammation,



**Fig. 4** GS-Rb1 treatment suppressed local neuroinflammation in the ischemic brain following MCAO. **a–c** qPCR analysis of mRNA expression of pro-inflammatory genes iNOS and IL-1 $\beta$  and anti-inflammation-associated gene arginase 1 in the ipsilateral cortex of sham-operated, saline-operated and different doses of GS-Rb1-treated mice at 24 h after MCAO. **d** The anti-inflammation cytokine IL-10 in

the ipsilateral cortex in the experimental groups at 48 h after MCAO by ELISA assay. **e** MPO activity in the experimental groups at 48 h after MCAO. **f** qPCR analysis of mRNA expression of MMP-9 in the ipsilateral cortex in the experimental groups at 24 h after MCAO. Data were presented as mean  $\pm$  SEM and analyzed by two-way ANOVA followed by Tukey's test. \* $p < 0.05$ ; # $p < 0.01$

**Fig. 5** GS-Rb1 inhibited NADPH oxidase (NOX) expression and activity in the ischemic brain. **a–c** mRNA expression of NOX-1, NOX-2, and NOX-4 in the ipsilateral cortex at 24 h after MCAO related to those in sham brain. **d** NOX activity in the ipsilateral cortex in the experimental groups. Data were presented as mean  $\pm$  SEM and analyzed by two-way ANOVA followed by Tukey's test. \* $p < 0.05$ ; # $p < 0.01$



oxidative and nitrosative stresses, enzymatic activity, and angiogenesis. The ginsenosides' potent anti-oxidative and anti-inflammation activities play important roles on the neuroprotective effects in insulted ischemic brain (Siddique et al. 2000; Zhu et al. 2012). Especially, GS-Rb1 functioned as antioxidants (Liu et al. 2003) and exerted efficaciousness in protecting neurons from oxidative damage (Kim et al. 1998). The cerebral vasculature is particularly susceptible to oxidative stress insult (Lehner et al. 2011), which in turn disrupts the maintenance of the BBB. From this view, GS-Rb1 is expected to improve the integrity of BBB against the MCAO-induced impairments, which is further proved by this study and another study (Zhou et al. 2014). Besides, we tested another model of thrombolysis to observe the effects of GS-Rb1 on BBB integrity, as thrombolysis is believed to induce BBB dysfunction. The results also suggest the capability of GS-Rb1 protecting BBB integrity against brain injuries, as evidenced by that recombinant tissue plasminogen activator (rt-PA) treatment after thrombolysis induced significant cerebral hemorrhage and Evans blue dye extravasation, while combination of rt-PA + 40 mg/kg GS-Rb1 treatment partially rescued the thrombolysis-induced impairments (Supplementary Figure S1).

Phosphorylation is a major regulatory mechanism of both transmembrane and accessory tight junction proteins. Constitutive phosphorylation of the myosin light chain (MLC) by MLC kinase (MLCK) in epithelial cells has been shown to increase tight junction permeability (Shen et al. 2006). Protein kinase C (PKC) may be key regulator of BBB permeability under ischemia/reperfusion (I/R)-associated stress conditions (Fleegal et al. 2005). Calcium overload correlates with neural excitotoxicity, and elevations of intracellular calcium lead to I/R-related BBB alterations. Inflammation associated with I/R involves the induction of cytokines and adhesion molecules, coupled with the activation and migration of neutrophils and microglia. TNF, IL-1, and IL-6 can increase paracellular permeability in microvascular endothelial cells (Candelario-Jalil et al. 2007). Cytokines induced production and release of chemokines such as MCP-1, which is a major determinant involved in leukocyte infiltration into brain parenchyma in ischemic stroke (Huang et al. 2006). IL-1 induced neutrophil adhesion, and increased BBB paracellular permeability has been shown to occur with loss of occluding and ZO-1 at the junctions (Bolton et al. 1998). Oxidative stress significantly contributes to BBB disruption and vasogenic edema, especially in reperfusion (Heo et al. 2005). The reactive oxygen species (ROS), including superoxide and hydroxyl radicals, can swiftly exceed capacity of the endogenous scavenging mechanisms, thus leading to damage of cellular macromolecules. ROS can also induce monocyte migration across the endothelial monolayer (Schreibelt et al. 2007).

NADPH oxidases (NOXs) and nitric oxide (NO) are important contributors to production of ROS (Bedard and Krause 2007). During ischemic stroke, many proteases including heparanases and cathepsins are activated as well. Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that have been shown as the major mediators of I/R outcomes associated with BBB tight junction disruption. MMP-2 and MMP-9 have been the focus of many studies of cerebral ischemia due to their substrate specificity for fibronectin, laminin, and collagen. Increased MMP-2 and MMP-9 have been identified with initiation of ischemia and vasogenic edema (Montaner et al. 2001a, b; Chang et al. 2003; Rosenberg and Yang 2007). Apart from the mechanisms addressed above, lots of studies have shown that angiogenic growth factors are involved in structural formation and stabilization of blood vessels, and mitigation of I/R-associated TJ permeability and edema (Weis and Cheresch 2005; Peters et al. 2007). Understanding the interrelation of these mechanisms is critical for development of new therapeutic approaches.

Ginseng root has been extensively used in traditional Chinese medicine, diet, or dietary supplement. Ginsenosides, a class of steroid glycosides and triterpene saponins, are the major bioactive compounds in the ginseng root. Lots of studies have shown that ginsenosides possess antioxidant, immunomodulatory, anti-inflammatory, and lipid-lowering effects in *in vitro* and *in vivo* animal models (Kimura et al. 2012; Hou et al. 2014; Li et al. 2014; Wu et al. 2014; Cho et al. 2015). Rb1 is considered as the most abundant ginsenoside among more than 30 ginsenosides. The present study shows that GS-Rb1 is capable of effectively inhibiting infarct, protecting BBB integrity, preventing loss of tight junction proteins, suppressing local inflammation, and reducing NADPH activity, presumably in turn, production of ROS, suggesting that GS-Rb1 can be used clinically in treatment of ischemic stroke.

Of note, nearly all of the outcome parameters we observed in this study, including brain infarction, BBB integrity, brain edema and inflammatory markers, and ROS production, were affected by GS-Rb1 treatment, which appears to be unspecific model of actions. Considering the complexity of the *in vivo* study, it is quite difficult to observe one parameter without affecting other parameters in the whole tissue or organ, especially when these parameters are somehow connected; it may not be a surprise to see that these parameters were affected by GS-Rb1. For example, since the impairment of BBB integrity after MCAO was highly possibly connected to the loss of tight junction proteins, GS-Rb1 is expected to enhance the tight junction protein expression, given that it can rescue the BBB integrity insulted by MCAO. However, it is still possible that the observed effects in this study may not be very specific, which might require delicate *in vivo* or *in vitro* studies.

Pharmacologically, it is previously assumed that the ginsenosides must be metabolized by human intestinal microbes after oral use (Kim et al. 2013). Thus, GS-Rb1 needs to be metabolized to 20-*O*-D-glucopyranosyl-20(*S*)-protopanaxadiol (compound K) by human intestinal bacterial flora and absorbed into the blood. The resultant product of compound K is capable of efficiently inhibiting lipid peroxidation, TNF- $\alpha$ , NO, prostaglandin E2 (PGE2), intercellular adhesion molecule (ICAM)-1, and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (Li et al. 2014). The present study, however, shows that intraperitoneal injection of GS-Rb1 also led to potent anti-inflammatory actions in a dose-dependent manner. These results suggest that GR-Rb1 metabolized to compound K is not required for its action, and GS-Rb1 per se can directly and effectively protect integrity of BBB tight junctions and in turn prevent subsequent damage of the brain. Our study provides further insights into pharmacokinetics of GS-Rb1. As a result, other routes of GS-Rb1 administration apart from oral use in treatment of ischemic stroke are expected to result in.

## Conclusions

In summary, GS-Rb1 has been shown to vigorously prevent loss of BBB integrity following cerebral ischemia by negatively regulating local inflammation and NOX4-derived ROS production. Our study will facilitate discoveries of novel therapeutic approaches based on GS-Rb1 in treatment of ischemic stroke.

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**Ethical standards** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87:245–313. doi:10.1152/physrev.00044.2005
- Bolton SJ, Anthony DC, Perry VH (1998) Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood–brain barrier breakdown in vivo. *Neuroscience* 86:1245–1257
- Candelario-Jalil E, Taheri S, Yang Y et al (2007) Cyclooxygenase inhibition limits blood–brain barrier disruption following intracerebral injection of tumor necrosis factor- $\alpha$  in the rat. *J Pharmacol Exp Ther* 323:488–498. doi:10.1124/jpet.107.127035

- Chang DI, Hosomi N, Lucero J, Heo JH, Abumiya T, Mazar AP, del Zoppo GJ (2003) Activation systems for latent matrix metalloproteinase-2 are upregulated immediately after focal cerebral ischemia. *J Cereb Blood Flow Metab* 23:1408–1419. doi:10.1097/01.WCB.0000091765.61714.30
- Chen JW, Breckwoldt MO, Aikawa E, Chiang G, Weissleder R (2008) Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis. *Brain* 131:1123–1133. doi:10.1093/brain/awn004
- Cho YL, Hur SM, Kim JY et al (2015) Specific activation of insulin-like growth factor-1 receptor by ginsenoside Rg5 promotes angiogenesis and vasorelaxation. *J Biol Chem* 290:467–477. doi:10.1074/jbc.M114.603142
- Christensen LP (2009) Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. *Adv Food Nutr Res* 55:1–99. doi:10.1016/S1043-4526(08)00401-4
- Fleegal MA, Hom S, Borg LK, Davis TP (2005) Activation of PKC modulates blood–brain barrier endothelial cell permeability changes induced by hypoxia and posthypoxic reoxygenation. *Am J Physiol Heart Circ Physiol* 289:H2012–H2019. doi:10.1152/ajpheart.00495.2005
- Heo JH, Han SW, Lee SK (2005) Free radicals as triggers of brain edema formation after stroke. *Free Radic Biol Med* 39:51–70. doi:10.1016/j.freeradbiomed.2005.03.035
- Hou YL, Tsai YH, Lin YH, Chao JC (2014) Ginseng extract and ginsenoside Rb1 attenuate carbon tetrachloride-induced liver fibrosis in rats. *BMC Complement Altern Med* 14:415. doi:10.1186/1472-6882-14-415
- Hu X, Li P, Guo Y et al (2012) Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke* 43:3063–3070. doi:10.1161/STROKEAHA.112.659656
- Huang J, Upadhyay UM, Tamargo RJ (2006) Inflammation in stroke and focal cerebral ischemia. *Surg Neurol* 66:232–245. doi:10.1016/j.surneu.2005.12.028
- Huang J, Li Y, Tang Y, Tang G, Yang GY, Wang Y (2013) CXCR4 antagonist AMD3100 protects blood–brain barrier integrity and reduces inflammatory response after focal ischemia in mice. *Stroke* 44:190–197. doi:10.1161/STROKEAHA.112.670299
- Infanger DW, Sharma RV, Davissou RL (2006) NADPH oxidases of the brain: distribution, regulation, and function. *Antioxid Redox Signal* 8:1583–1596. doi:10.1089/ars.2006.8.1583
- Kim YC, Kim SR, Markelonis GJ, Oh TH (1998) Ginsenosides Rb1 and Rg3 protect cultured rat cortical cells from glutamate-induced neurodegeneration. *J Neurosci Res* 53:426–432
- Kim KA, Jung IH, Park SH, Ahn YT, Huh CS, Kim DH (2013) Comparative analysis of the gut microbiota in people with different levels of ginsenoside Rb1 degradation to compound K. *PLoS ONE* 8:e62409. doi:10.1371/journal.pone.0062409
- Kimura Y, Sumiyoshi M, Sakanaka M (2012) Effects of ginsenoside Rb(1) on skin changes. *J Biomed Biotechnol* 2012:946242. doi:10.1155/2012/946242
- Lehner C, Gehwolf R, Tempfer H, Krizbai I, Hennig B, Bauer HC, Bauer H (2011) Oxidative stress and blood–brain barrier dysfunction under particular consideration of matrix metalloproteinases. *Antioxid Redox Signal* 15:1305–1323. doi:10.1089/ars.2011.3923
- Li J, Zhong W, Wang W et al (2014) Ginsenoside metabolite compound K promotes recovery of dextran sulfate sodium-induced colitis and inhibits inflammatory responses by suppressing NF- $\kappa$ B activation. *PLoS ONE* 9:e87810. doi:10.1371/journal.pone.0087810
- Liu ZQ, Luo XY, Liu GZ, Chen YP, Wang ZC, Sun YX (2003) In vitro study of the relationship between the structure of ginsenoside and its antioxidative or prooxidative activity in free radical



- induced hemolysis of human erythrocytes. *J Agric Food Chem* 51:2555–2558. doi:[10.1021/jf026228i](https://doi.org/10.1021/jf026228i)
- Liu W, Sood R, Chen Q et al (2008) Normobaric hyperoxia inhibits NADPH oxidase-mediated matrix metalloproteinase-9 induction in cerebral microvessels in experimental stroke. *J Neurochem* 107:1196–1205. doi:[10.1111/j.1471-4159.2008.05664.x](https://doi.org/10.1111/j.1471-4159.2008.05664.x)
- Meyers PM, Schumacher HC, Connolly ES Jr, Heyer EJ, Gray WA, Higashida RT (2011) Current status of endovascular stroke treatment. *Circulation* 123:2591–2601. doi:[10.1161/CIRCULATIONAHA.110.971564](https://doi.org/10.1161/CIRCULATIONAHA.110.971564)
- Montaner J, Alvarez-Sabin J, Molina C et al (2001a) Matrix metalloproteinase expression after human cardioembolic stroke: temporal profile and relation to neurological impairment. *Stroke* 32:1759–1766
- Montaner J, Alvarez-Sabin J, Molina CA, Angles A, Abilleira S, Arenillas J, Monasterio J (2001b) Matrix metalloproteinase expression is related to hemorrhagic transformation after cardioembolic stroke. *Stroke* 32:2762–2767
- Moroni F, Chiarugi A (2009) Post-ischemic brain damage: targeting PARP-1 within the ischemic neurovascular units as a realistic avenue to stroke treatment. *FEBS J* 276:36–45. doi:[10.1111/j.1742-4658.2008.06768.x](https://doi.org/10.1111/j.1742-4658.2008.06768.x)
- Peters S, Cree IA, Alexander R et al (2007) Angiopoietin modulation of vascular endothelial growth factor: effects on retinal endothelial cell permeability. *Cytokine* 40:144–150. doi:[10.1016/j.cyto.2007.09.001](https://doi.org/10.1016/j.cyto.2007.09.001)
- Rosenberg GA, Yang Y (2007) Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia. *Neurosurg Focus* 22:E4
- Sandoval KE, Witt KA (2008) Blood–brain barrier tight junction permeability and ischemic stroke. *Neurobiol Dis* 32:200–219. doi:[10.1016/j.nbd.2008.08.005](https://doi.org/10.1016/j.nbd.2008.08.005)
- Schreibelt G, Kooij G, Reijkerk A et al (2007) Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling. *FASEB J* 21:3666–3676. doi:[10.1096/fj.07-8329com](https://doi.org/10.1096/fj.07-8329com)
- Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE, Turner JR (2006) Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. *J Cell Sci* 119:2095–2106. doi:[10.1242/jcs.02915](https://doi.org/10.1242/jcs.02915)
- Siddique MS, Eddeb F, Mantle D, Mendelow AD (2000) Extracts of Ginkgo biloba and Panax ginseng protect brain proteins from free radical induced oxidative damage in vitro. *Acta Neurochir Suppl* 76:87–90
- Uchida M, Palmateer JM, Herson PS, DeVries AC, Cheng J, Hurn PD (2009) Dose-dependent effects of androgens on outcome after focal cerebral ischemia in adult male mice. *J Cereb Blood Flow Metab* 29:1454–1462. doi:[10.1038/jcbfm.2009.60](https://doi.org/10.1038/jcbfm.2009.60)
- Wang Y, Jia J, Ao G et al (2014) Hydrogen sulfide protects blood–brain barrier integrity following cerebral ischemia. *J Neurochem* 129:827–838. doi:[10.1111/jnc.12695](https://doi.org/10.1111/jnc.12695)
- Weis SM, Cheresch DA (2005) Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 437:497–504. doi:[10.1038/nature03987](https://doi.org/10.1038/nature03987)
- Wu Y, Yu Y, Szabo A, Han M, Huang XF (2014) Central inflammation and leptin resistance are attenuated by ginsenoside Rb1 treatment in obese mice fed a high-fat diet. *PLoS ONE* 9:e92618. doi:[10.1371/journal.pone.0092618](https://doi.org/10.1371/journal.pone.0092618)
- Zhou Y, Li HQ, Lu L, Fu DL, Liu AJ, Li JH, Zheng GQ (2014) Ginsenoside Rg1 provides neuroprotection against blood brain barrier disruption and neurological injury in a rat model of cerebral ischemia/reperfusion through downregulation of aquaporin 4 expression. *Phytomedicine* 21:998–1003. doi:[10.1016/j.phymed.2013.12.005](https://doi.org/10.1016/j.phymed.2013.12.005)
- Zhu W, Wang L, Zhang L et al (2010) Isoflurane preconditioning neuroprotection in experimental focal stroke is androgen-dependent in male mice. *Neuroscience* 169:758–769. doi:[10.1016/j.neuroscience.2010.05.038](https://doi.org/10.1016/j.neuroscience.2010.05.038)
- Zhu J, Jiang Y, Wu L, Lu T, Xu G, Liu X (2012) Suppression of local inflammation contributes to the neuroprotective effect of ginsenoside Rb1 in rats with cerebral ischemia. *Neuroscience* 202:342–351. doi:[10.1016/j.neuroscience.2011.11.070](https://doi.org/10.1016/j.neuroscience.2011.11.070)