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Original Article

Neuroprotective effect of riluzole in rat model of subarachnoid hemorrhage

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Abstract: Riluzole, an anti-glutamate drug and sodium channel blocker with neuroprotective roles, which has been approved by US Food and Drug Administration (FDA) for amyotrophic lateral sclerosis. The purpose of this study was to explore whether Riluzole provides neuroprotection in early brain injury (EBI) after subarachnoid hemorrhage (SAH). Intraperitoneal injection of Riluzole (6 mg/kg) significantly improved neurological deficit at 24, 48 and 72 h. Riluzole alleviated the blood brain barrier (BBB) permeability of cortex and brain edema, also reduced the glutamate concentration of cerebrospinal fluid, increased the GLT-1 expression of cortex, increased GSH content and attenuated MDA content and neuronal apoptosis of cortex at 72 h after SAH. These results suggested that Riluzole treatment may ameliorate early brain injury in experimental SAH model.

Keywords: Subarachnoid hemorrhage, riluzole, neuroprotection

Introduction

Subarachnoid hemorrhage (SAH) is a scary cerebrovascular disease that implies bleeding into the subarachnoid space, which is a hemorrhagic stroke subtype with high morbidity and mortality. Early brain injury (EBI) is the most important pathophysiological mechanism for poor outcomes after SAH, which refers to the acute brain injury within the 72 h following SAH, contains the change of intracranial pressure (ICP) and cerebral perfusion pressure (CPP), reduction of cerebral blood flow (CBF), disturbed cerebral autoregulation, excitotoxicity, oxidative stress, inflammation, cell apoptosis and cerebral edema [1]. In particular, excitotoxicity damage is considered as important mechanism of early brain injury and has been proposed to play a major role in the cell death after SAH. Glutamate content of cerebrospinal fluid is higher after experimental SAH [2, 3], and causes excitotoxicity that induces calcium overloading and subsequent cell apoptosis [4]. Neuronal apoptosis is related to the development of neurological deficits and brain edema formation [5]. Hence, anti-glutamate and anti-apoptosis treatment is an effective therapy against SAH.

Riluzole, an FDA-approved neuroprotective compound, is a non-inactivating voltage dependent sodium channel inhibitor, and used in clinics for the treatment of amyotrophic lateral sclerosis [6]. Previous studies indicated three reasons for the neuroprotective of Riluzole: (i) block of persistent Na^+ currents that has been associated with degeneration of neural tissue [7, 8]; (ii) inhibition of glutamatergic neurotransmission through attenuating glutamate release and promotes reuptake, act as the anti-glutamate drug [9]; (iii) stimulation of neurotrophic factor expression [10]. Riluzole plays a neuroprotective effect in several experimental model of neurological diseases, such as in the treatment of Parkinson's disease [11], mood and anxiety disorders [12], Huntington's disease [13], multiple sclerosis [14], cerebral ischemia [15, 16]. However, it is not yet known whether Riluzole affects brain injury after SAH.

In the present study, we aim to assess the hypotheses: Riluzole treatment attenuates glutamate excitotoxicity, oxidative stress, and neuronal apoptosis after experimental SAH in rats.

Materials and methods

Experimental animals and design

Animal procedures are approved by the Institutional Animal Care and Use Committee of Taian Central Hospital that is in accordance

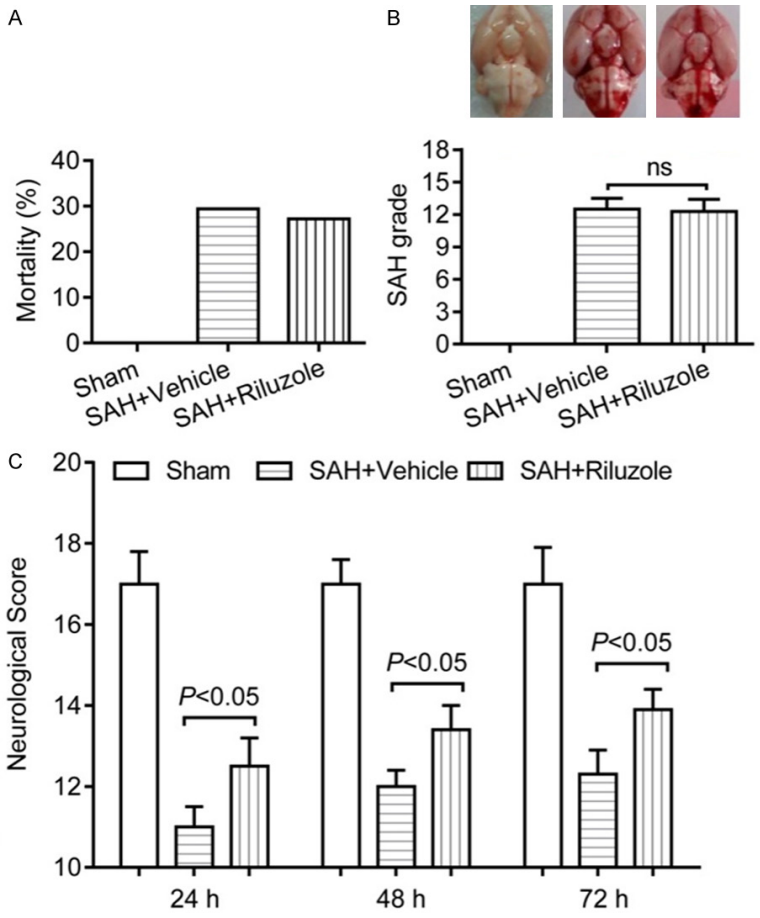


Figure 1. Riluzole improved neurological deficit after SAH. (A) The mortality, (B) The SAH grade, and (C) Neurological scores in the sham group, SAH + vehicle group, and SAH + Riluzole group. Data were represented as mean ± SD. ($P < 0.05$, no significant (ns), one-way ANOVA followed Bonferroni's multiple comparisons test, n = 6 per group).

with the guideline of the National Institutes of Health (NIH) on the use and care of experimental animals. Male Sprague-Dawley (SD) rats from the Experimental Animal Center of Shandong University are housed in cage at a constant humidity (55%-60%) and temperature (23-25°C) with the 12 h-12 h light/dark cycle.

91 rats were divided into three groups: the sham group (n = 24), the SAH + vehicle group (n = 34), and the SAH + Riluzole group (n = 33). Rat received Riluzole (0.5 ml, 6 mg/kg) or vehicle (0.5 ml, saline) via intraperitoneal injection at 2 h after surgery, and then twice daily. Dosage and time of Riluzole administration was based upon prior investigation in spinal cord injury model [17]. After the neurological assessment at 72 h after SAH, all rats were killed. Six rats in each group were for Evans blue assay, and six rats were for detecting brain water content. Six rats in each group were for western-blots and biochemical experiments. Six rats per group were for immunofluorescence staining.

Rat SAH model and SAH grade

Rat SAH endovascular perforation experimental model and the SAH grade were performed as previously described [18]. Briefly, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), then its left external carotid artery (ECA) was transected and reflected in line with the internal carotid artery (ICA). A blunted 4-0 monofilament nylon suture was inserted into the ECA stump and advanced into the ICA, penetrated the bifurcation of the anterior and the middle cerebral artery (MCA), then withdrawn and to allow reperfusion of the ICA. The sham group underwent the same procedure without the endovascular perforation. Experimental SAH severity was quantified according to the picture of the taken brain, which was based on the amount blood of six segments of the basal cistern. Each segment was scored from 0 to 3: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood in visible arteries; grade 3, blood clot covering all visible arteries. Total score (0-18) was calculated from the score of six segments.

Neurological scores

The neurological score was evaluated at 24, 48, and 72 h using the modified Garcia scoring system [19], which contains six test (spontaneous activity, climbing, forepaw, outstretching symmetry in the movement of all four limbs, body proprioception, and response to whisker stimulation) that were scored form 0-3 or 1-3. The neurological score was calculated from six tests by two 'blinded' investigators, while the sequence of testing was randomized. Higher score indicates better test performance.

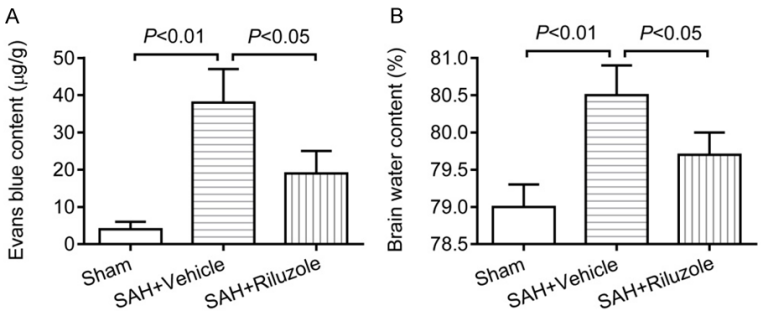


Figure 2. Riluzole attenuated the BBB permeability and brain edema after SAH. (A) The Evans blue content as indices of BBB permeability and (B) brain water content were determined at 72 hours in the sham group, SAH + vehicle group, and SAH + Riluzole group. Data were represented as mean ± SD. ($P < 0.05$ or 0.01, one-way ANOVA followed Bonferroni's multiple comparisons test, n = 6 per group).

Blood brain barrier permeability and brain water content

At 72 h after SAH, the BBB permeability was assessed by Evans blue extravasation method according to our previous study [20]. In brief, 0.5 ml of 2% (w/v) Evans blue dye (Sigma) was injected intravenously and circulated for 1 h. Then rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), and preformed left ventricular perfusion with PBS buffer. The brain was quickly taken and separated into the left and right hemisphere. The cortex of left hemisphere was weighed and homogenized in PBS, and incubated with an equal volume of trichloroacetic acid with ethanol (1:3) for 12 h. The supernatant of sample and standards were measured at 610 nm using a microplate reader. The Evans blue content of each group was calculated from the standard curve.

At 72 h after SAH, brain water content was determined using the wet/dry method (brain water content = [(wet weight - dry weight)/wet weight] × 100%). In brief, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), the brain was quickly taken and separated into the left and right hemisphere. Then the cortex of left hemisphere was weighed to obtain the wet weight and dried at 80°C for 72 h before determining the dry weight.

Measurement of glutamate concentration in cerebrospinal fluid

At 72 h after SAH, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), and approximate 50 μl cerebrospinal fluid was extracted with a 1 ml syringe. The 50 μl CSF was mixed with 100 μl Glutamate Assay Buffer in a 1.5 ml microfuge tube. Centrifuge the samples at 13000 rpm for 10 min and the 100 μl supernatant was measured using glutamate assay kit (BioVision) following the manufacturer's instructions. Glutamate concentration of CSF each group represented as micromoles per liter.

Immunofluorescence and TUNEL staining

At 72 hours after SAH, immunofluorescence and TUNEL staining were performed as described previously [18]. In briefly, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), and preformed left ventricular perfusion with PBS buffer and followed by 4% paraformaldehyde/PBS solution. The brain was quickly taken and embed with optimal cutting temperature compound (OCT). The coronal section was got from the Leica cryostat, and permeabilized with 0.5% Triton X-100 and blocked in 5% goat serum. The section was incubated with GLT-1 antibody (1:100, ab41621, Abcam) or NeuN antibody (1:100, MABN140, Millipore) and then incubated anti-rabbit IgG-FITC (F9887, 1:200, Sigma) or anti-mouse IgG-TRITC antibody (T5393, 1:200, Sigma). For TUNEL staining, the coronal section incubated with NeuN staining and then used In Situ Cell Death Detection Kit with Fluorescein (Roche, Germany) following the manufacturer's instructions. Three microscope fields (20×) of GLT-1 or TUNEL/NeuN positive cells in basal cortex were chosen and imaged from a fluorescent microscope (Olympus, Japan) under the constant parameters. The number of GLT-1 or TUNEL/NeuN positive cells of basal cortex was counted in six pictures by two 'blinded' investigators, and the mean per group was calculated.

Western-blots analysis

At 72 hours after SAH, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg), and the cortex of left hemisphere was dissected out and homogenized in RIPA lysis buffer (Beyotime, China) and clarified by centrifuging, and then

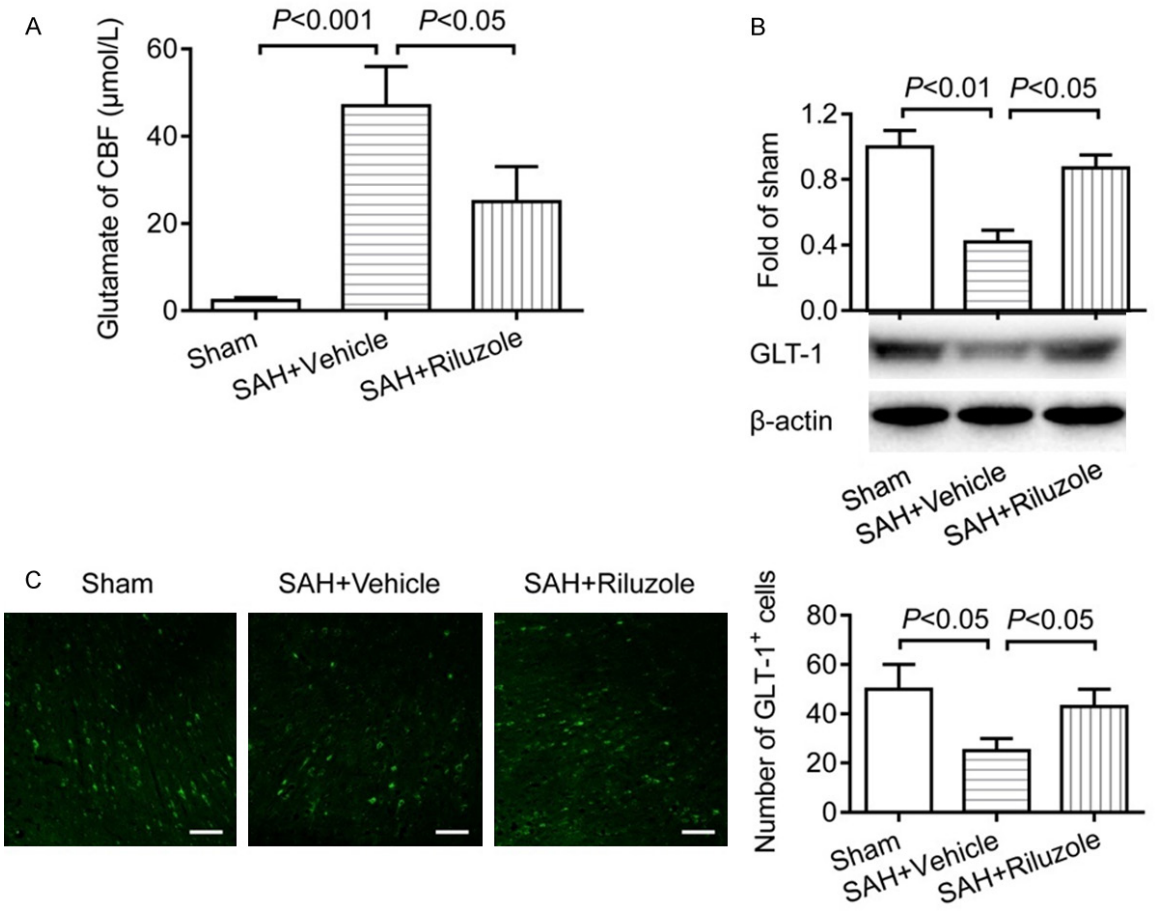


Figure 3. Riluzole reduced glutamate concentration of CBF and increased GLT-1 expression of cortex after SAH. A. The glutamate concentration of CSF. B. Representative western-blots showed that expression of GLT-1. C. Representative immunofluorescence staining slices of GLT-1 and quantitative analysis of GLT-1 staining positive cells were determined at 72 hours in the sham group, SAH + vehicle group, and SAH + Riluzole group. ($P < 0.05$, 0.01 or 0.001, one-way ANOVA followed Bonferroni's multiple comparisons test, n = 6 per group).

determined the protein concentration with the Bradford Protein Assay Kit (Beyotime, China). The supernatant containing 50 μg of protein was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Next, the membrane was blocked with 5% nonfat milk and then incubated with GLT-1 antibody (1:1000, ab41621, Abcam) or cleaved caspase-3 antibody (1:1000, 9507S, Cell Signaling Technology). After incubating with anti-rabbit IgG HRP-linked antibody (1:3000, 7074S, Cell Signaling Technology), the membrane were viewed using the chemiluminescence kit (Millipore, USA) under the ChemiDoc MP system (Bio-Rad).

Measurement of MDA and GSH content

At 72 hours after SAH, rat was anesthetized with 5% chloral hydrate (i.p, 400 mg/kg). The brain was quickly taken and separated into the left and right hemisphere. Then the cortex of left hemisphere was homogenized in saline clarified by centrifuging, and then determined the protein concentration with the Bradford Protein Assay Kit (Beyotime, China). The 100 μl supernatant was measured using MDA assay kit and GSH assay kit (Beyotime, China) following the manufacturer's instructions. The MDA and GSH content of each group represented as μmol/g or nmol/g protein.

Statistical analysis

All values were expressed as mean ± SD (Std. Deviation) and analyzed with the GraphPad Software Prism. The comparison was made by one-way ANOVA followed Bonferroni's multiple comparisons test. P value < 0.05 represented statistical significance.

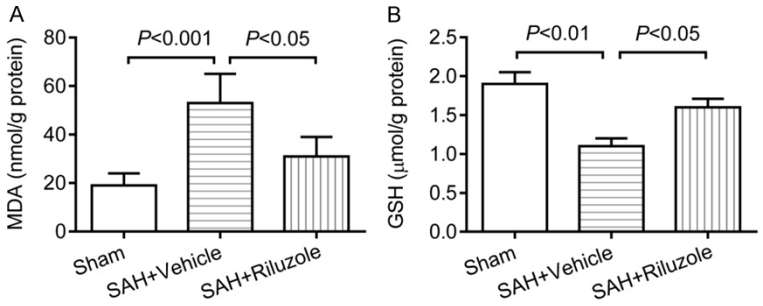


Figure 4. Riluzole attenuated the MDA and GSH content after SAH. (A) MDA content as indices of damage marker of lipid and (B) GSH content was determined at 72 hours in the sham group, SAH + vehicle group, and SAH + Riluzole group. Data were represented as mean ± SD. ($P < 0.05$ or 0.001, one-way ANOVA followed Bonferroni's multiple comparisons test, n = 6 per group).

Results

Riluzole treatment alleviates neurological deficit after SAH

No rats die in the sham group (0 of 24 rats). The mortality in the SAH + vehicle group reaches 29.4% (10 of 34 rats), 27.2% (9 of 33 rats) in the SAH + Riluzole group at (**Figure 1A**). The SAH grading scores are not significantly different between the SAH + vehicle group and the SAH + Riluzole group (**Figure 1B**). At 24, 48 and 72 h after SAH, the modified Garcia score method shows that the average neurological scores in the SAH + vehicle group is significantly lower than in the sham group, while the scores in the SAH + Riluzole group is significantly higher than in the SAH + vehicle group (**Figure 1C**). These results indicate that Riluzole improves the neurological deficit after SAH.

Riluzole treatment attenuates BBB permeability and brain edema after SAH

At 72 h after SAH, the Evans blue content of brain tissue is significantly increased in the SAH + vehicle group as compared to those of sham group. However, the Evans blue content in SAH + Riluzole group is significantly reduced as compared with the SAH + vehicle group (**Figure 2A**). Brain water content is significantly increased in the SAH + vehicle group as compared with that observed in the sham group at 72 h, while Riluzole administration decreases the brain water content compared to those of the SAH + vehicle group (**Figure 2B**). These results indicate that Riluzole attenuates BBB permeability and brain edema after SAH.

Riluzole treatment reduces the glutamate concentration of CSF and increases the GLT-1 expression after SAH

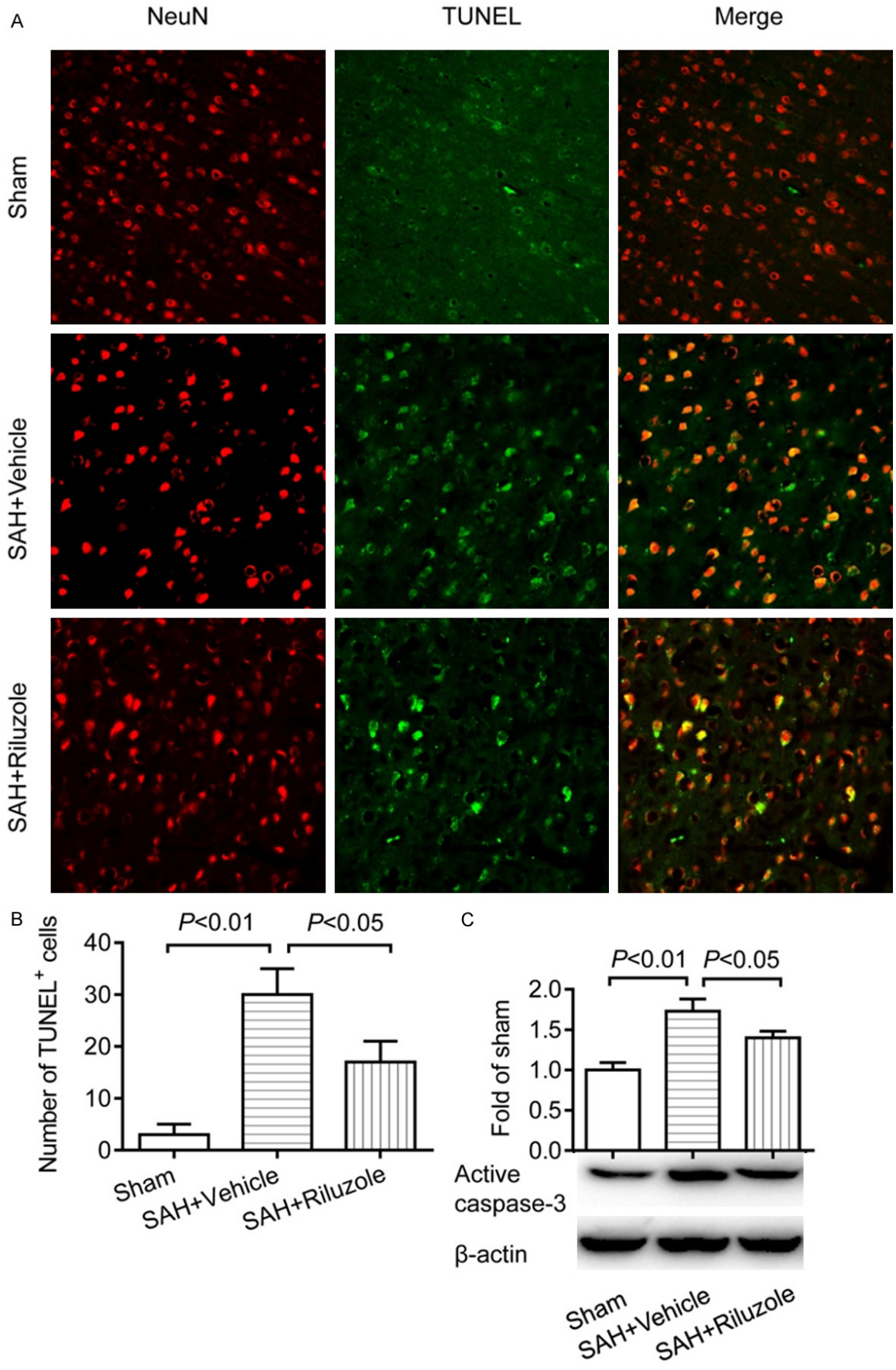
At 72 h after SAH, the glutamate concentration of cerebrospinal fluid (CSF) is significantly higher in the SAH + vehicle group as compared with that observed in the sham group, while Riluzole administration reduces the glutamate concentration of CSF compared to those of the SAH + vehicle group (**Figure 3A**). Next, we examine the effect of Riluzole on the expression of GLT-1, which is a predominant member of functional glutamate transporters. Western-blots analysis suggests that SAH significantly decreases the GLT-1 protein expression compared to the sham group at 72 h, while Riluzole treatment attenuates this decrease compared to those in SAH + vehicle group (**Figure 3B**). Meanwhile, SAH significantly decreases the GLT-1-positive cells of cortex compare with the sham group at 72 h, and Riluzole treatment significantly increases the GLT-positive cells when compared to the SAH + vehicle group (**Figure 3C**). These results indicate that Riluzole attenuates higher concentration of glutamate through up-regulating the GLT-1 expression after SAH.

Riluzole treatment reduced MDA content and increased GSH content after SAH

To gain insight into the effect of Riluzole in oxidative stress after SAH, the content of MDA that is oxidative damage marker of lipid, and the GSH content are determined at 72 h after SAH. As shown in **Figure 4**, the content of MDA obviously increases in the SAH + vehicle group when compared to the sham group, while Riluzole significantly attenuates the MDA increase induced by SAH (**Figure 4A**). The GSH content in the SAH + vehicle group significantly decreases at 72 h compared with the sham group, which was reversed by Riluzole treatment (**Figure 4B**).

Riluzole treatment attenuated the neuronal apoptosis after SAH

At 72 h after SAH, the presence of neuronal apoptosis in cortex is determined using the immunofluorescence staining slices of NeuN (a


A
NeuN
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B
Number of TUNEL+ cells
P<0.01
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40
30
20
10
0
Sham
SAH+Vehicle
SAH+Riluzole
C
Fold of sham
P<0.01
P<0.05
2.0
1.5
1.0
0.5
0.0
Active caspase-3
β-actin
Sham
SAH+Vehicle
SAH+Riluzole

Figure 5. Riluzole attenuated the neuronal apoptosis after SAH. A. Representative immunofluorescence staining slices of NeuN plus TUNEL staining. B. Quantitative analysis of TUNEL/NeuN staining positive cells. C. Representative western-blots showed that expression of active caspase-3 was determined at 72 hours in the sham group, SAH + vehicle group, and SAH + Riluzole group. Data were represented as mean ± SD. ($P < 0.05$ or 0.01, one-way ANOVA followed Bonferroni's multiple comparisons test, n = 6 per group).

neuronal marker) plus TUNEL staining. SAH obviously increases the TUNEL/NeuN-positive cells compare with the sham group, while Riluzole treatment significantly reduces the TUNEL/NeuN-positive cells when compared to the SAH + vehicle group (**Figure 5A** and **5B**). Additionally, western-blots analysis shows that active caspase-3 expression is significantly higher in the SAH + vehicle group compared with the sham group, while active caspase-3 expression is lower in the SAH + Riluzole group compared to the SAH + vehicle group (**Figure 5C**). These results indicate that Riluzole treatment attenuates the neuronal apoptosis after SAH.

Discussion

In this study, we show that intraperitoneal injection of Riluzole (6 mg/kg) ameliorated BBB permeability and brain edema, decreased glutamate level of CSF, increased the cortical GLT-1 expression and GSH content, reduced cortical MDA content and neuronal apoptosis in rat SAH model. These results suggest that Riluzole is neuroprotective during early brain injury after SAH through an anti-glutamate and anti-apoptosis mechanism.

Neurological deficit is common and important features in early brain injury and important indicator for assessing the outcome after SAH. Our findings suggest that Riluzole improved neurological deficit following SAH, which is in accordance with previous studies in ischemia stroke [21, 22]. Accumulated evidences show that BBB disruption occurs very earlier and facilitates the infiltration of serum into brain parenchyma that leads to brain edema formation and neuroinflammation [23]. The reduction of glutamate concentration of CSF and inhibition of oxidative stress has shown to ameliorate BBB disruption after SAH [3, 24]. Oxidative stress always induce DNA damage, lipid peroxidation and protein breakdown and mediates the blood brain barrier disruption and neuroinflammation [25]. Riluzole significant decreases the glutamate content of CSF, and the content of MDA that is oxidative damage marker of lipid. Therefore, we speculate that Riluzole prevent SAH-induced BBB disruption which maybe involve the inhibition of glutamate neurotoxicity and oxidative stress.

The mechanisms of neuroprotection of Riluzole have recently been reviewed [26], which is a non-inactivating voltage dependent sodium channel inhibitor and is anti-glutamatetergic via inhibition of glutamate release and increase of glutamate uptake. Our results show that Riluzole attenuated post-SAH elevated the TUNEL/NeuN-positive cells of brain cortex in rat, indicate that the anti-apoptotic effect of Riluzole as a potential mechanism against SAH-induced early brain injury. Ionic distribution and ion channel expression in the brain is rapidly and severely impaired, and Na^+ influx through voltage gated Na^+ channel is critical step in axon injury after SAH [1]. Excessive glutamate exists in the CSF after SAH, and caused neurotoxicity, play an essential role in the pathophysiology of ictus [27]. Therefore, we speculate that Riluzole prevent SAH-induced neuronal apoptosis which maybe involve the inhibition of Na^+ influx and glutamate release. Even with the limitations of this study, our results provide information about the neuroprotective effect of Riluzole against SAH-induced cerebral injury. Further investigations would be necessary to evaluate the mechanism of Riluzole on brain injury after SAH.

In conclusion, the present study showed for the first times that Riluzole against early brain injury in experimental SAH. Intraperitoneal injection of Riluzole (6 mg/kg) significantly improved neurological deficit, reduced blood brain barrier permeability and brain edema, decreased the glutamate level of CSF, increased GSH content, reduced MDA content and neuronal apoptosis in rat SAH model. Our findings indicate that Riluzole may provide neuroprotection after SAH.

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Disclosure of conflict of interest

None.

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