Propofol protects against blood-spinal cord barrier disruption induced by ischemia/reperfusion injury

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Graphical Abstract

Propofol attenuates disruption of the blood-spinal cord barrier following ischemia/reperfusion injury

Propofol treatment

A spinal cord ischemia/reperfusion injury rabbit model

Nuclear factor-kB (p65) ↓ → Matrix metalloprotease-9 ↓ → Claudin-5 ↓ → Loss reduction → Ocludin ↑

Abstract

Propofol has been shown to exert neuroprotective effects on the injured spinal cord. However, the effect of propofol on the blood-spinal cord barrier (BSCB) after ischemia/reperfusion injury (IRI) is poorly understood. Therefore, we investigated whether propofol could maintain the integrity of the BSCB. Spinal cord IRI (SCIRI) was induced in rabbits by infrarenal aortic occlusion for 30 minutes. Propofol, 30 mg/kg, was intravenously infused 10 minutes before aortic clamping as well as at the onset of reperfusion. Then, 48 hours later, we performed histological and mRNA/protein analyses of the spinal cord. Propofol decreased histological damage to the spinal cord, attenuated the reduction in BSCB permeability, downregulated the mRNA and protein expression levels of matrix metalloprotease-9 (MMP-9) and nuclear factor-XB (NF-kB), and upregulated the protein expression levels of occludin and claudin-5. Our findings suggest that propofol helps maintain BSCB integrity after SCIRI by reducing MMP-9 expression, by inhibiting the NF-kB signaling pathway, and by maintaining expression of tight junction proteins.

Key Words: nerve regeneration; spinal cord ischemia/reperfusion injury; blood–spinal cord barrier; propofol; matrix metalloprotease-9; nuclear factor-kB; tight junction proteins; neural regeneration

Introduction

Spinal cord ischemia/reperfusion injury (SCIRI) is a devastating complication of thoracoabdominal aortic interventions, such as thoracoabdominal aortic aneurysm surgery and thoracoabdominal aortic repair surgery (LeMaire et al., 2012; Panthee and Ono, 2015). Several strategies have been used to improve spinal cord ischemic tolerance; however, the incidence of paraplegia remains high and poses a persistent threat to patients (Bischoff et al., 2011). Recent studies demonstrate that disruption of the blood-spinal cord barrier (BSCB) plays an important role in SCIRI, and that maintaining its integrity can attenuate tissue damage (Lee et al., 2012; Li et al., 2014a). Barrier disruption leads to an increase in permeability, which may cause spinal cord edema, resulting in neuronal apoptosis and death (Sharma, 2005; Li et al., 2016). Thus, maintaining the integrity of the BSCB may improve the prognosis for patients with SCIRI.

Propofol, a short-acting intravenous anesthetic, widely used in clinical anesthesia, exerts neuroprotective effects, and can protect the brain and spinal cord following ischemia/reperfusion injury (IRI) and improve neuronal function (Zhou et al., 2013; Sahin et al., 2015). The anesthetic
possesses antioxidant, anti-apoptotic and anti-inflammatory actions (Fan et al., 2015). However, the effects of propofol on BSCB disruption in IRI are unclear.

Matrix metalloproteinase-9 (MMP-9) degrades the extracellular matrix during vascular remodeling, cell migration, reproduction and embryonic development (Chaturvedi and Kaczmarek, 2014). MMP-9 overexpression has been found in spinal cord injury and ischemic stroke (Kurzpea et al., 2014; Piao et al., 2014; Cai et al., 2015). MMP-9 plays an important role in blood-brain barrier (BBB) and BSCB damage following IRI (Lee et al., 2015; Ren et al., 2015). Nobel et al. (2002) demonstrated that MMP-9 regulates BSCB permeability and inflammation early after spinal cord injury, and that blocking MMP-9 activity decreases vascular permeability. Fang et al. (2013a, 2015) showed that MMP-9 plays a role in the disruption of the BSCB, and that bone marrow stromal cells and dexmedetomidine can suppress the expression of MMP-9. Nuclear factor-kappa B (NF-κB) is a transcription factor widely expressed in the nervous system. NF-κB participates in the regulation of BBB and BSCB permeability (Lee et al., 2014; Li et al., 2014c; Liu et al., 2014), and its overexpression leads to an increase in BBB and BSCB permeability (Lee et al., 2014; Li et al., 2014c; Liu et al., 2014). Inhibiting NF-κB expression reduces MMP-2 and MMP-9 expression (Li et al., 2012; Nguyen et al., 2014).

Tight junction proteins, including occludin and claudin-5, play a vital role in maintaining the permeability of the central nervous system capillary barrier (Na et al., 2015). Yang et al. (2015) reported that occludin and claudin-5 expression levels are reduced after acute stroke, along with an increase in permeability of the BBB. Similarly, disruption of tight junctions increases the permeability of the BSCB after spinal cord ischemic injury, concomitant with a reduction in occludin and claudin-5 levels (Lee et al., 2012; Fang et al., 2013a; Yu et al., 2015). Excessively high levels of MMPs, in particular MMP-9, significantly perturb BSCB integrity by digesting basement membranes and tight junction proteins in endothelial cells (Rosenberg et al., 1998; Yang et al., 2007). Boroujerdi et al. (2015) recently found that MMP-9 can degrade laminin and claudin-5. Therefore, decreasing MMP-9 expression should alleviate the disruption of tight junction proteins and help maintain the integrity of central nervous system capillaries. Interestingly, propofol inhibits the expression of MMP-9 after ischemic injury (Ji et al., 2015). Therefore, propofol might attenuate the loss of BSCB integrity by inhibiting MMP-9 expression. In the present study, we investigated the effects of propofol on the BSCB in a rabbit model of SCIRI, and we examined the levels of occludin, claudin-5, MMP-9 and NF-κB.

Materials and Methods

Animals

This protocol was approved by the Ethics Committee of Huazhong University of Science & Technology, China and was in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health publication No. 85-23, National Academy Press, Washington DC, revised 1996). Thirty-six healthy male Japanese white rabbits, 3 months of age, weighing 2.0–2.5 kg, were provided by the Experimental Animal Institute of Wuhan University, Wuhan, China (license No. SYXK (E) 2009-0027). All rabbits were neurologically intact before anesthesia and housed in standard cages with free access to food and water and separately housed after surgery. Rabbits were randomly assigned to three groups (n = 12 per group) by means of the random number table, including sham, ischemia/reperfusion (I/R only) and propofol (I/R + P) groups.

Establishment of SCIRI model

The SCIRI model was induced by occluding the aorta 0.5–1 cm below the left renal artery for 30 minutes, as reported previously (Fang et al., 2013b). All animals were anesthetized with 3% pentobarbital at an initial dose of 30 mg/kg. Animals were allowed to breathe room air spontaneously. Body temperature was continuously monitored with a rectal probe and maintained at 38.5 ± 0.5°C with the aid of a heated operating table. Two catheters were inserted into the ear and femoral artery to measure the proximal and distal blood pressure. Lidocaine (0.5%) was administered into the site of skin incision as a local anesthetic. Briefly, the abdominal aorta was carefully exposed following a midline laparotomy. Heparin, 200 U/kg, was administered 5 minutes before occlusion. A bulldog clamp was then placed across the aorta, 0.5–1 cm below the left renal artery. The ischemia lasted for 30 minutes, after which the clamp was removed, followed by 48 hours of reperfusion. Rabbits in the sham group underwent the same procedure, but the aorta was not occluded. All rabbits were allowed to recover in a plastic box at 28°C for 4 hours, and subsequently placed in their cages with free access to food and water.

Propofol intervention

The propofol group was intravenously infused with propofol (AstraZeneca, Shanghai, China) at 30 mg/kg in 30 mL of 0.9% sodium chloride at a rate of 3 mL/min 10 minutes before aortic clamping and at the onset of reperfusion. The sham and I/R groups were given 30 mL of 0.9% sodium chloride at the same time.

Neurological assessment

The motor function of the rabbits was assessed 48 hours after reperfusion. Scoring was performed using the modified Tarlov scale, as follows (Fang et al., 2013b): 0, paraplegic with no evident lower extremity motor function; 1, poor lower extremity motor function, weak antigravity movement only; 2, moderate lower extremity function with good antigravity strength but inability to draw legs under body; 3, excellent motor function with the ability to draw legs under body and hop, but not normally; and 4, normal motor function.

Histological observation

All animals were euthanized by injection of pentobarbital (200 mg/kg) 48 hours after IRI, and the spinal cord (L5–6) was
quickly removed. The spinal cord samples were fixed with a 10% formalin solution for 24 hours, embedded in paraffin, and serial transverse sections (4-μm-thick) at the lumbosacral level were obtained. The sections were dehydrated in ethanol and stained with hematoxylin and eosin to observe the histological changes. Neuronal injury was evaluated at 400× magnification by light microscopy (Olympus, Tokyo, Japan). When the cytoplasm was diffusely eosinophilic and the structure was intact, the large motor neurons were considered to be necrotic or dead. When the cells demonstrated basophilic stippling (containing Nissl substance), the motor neurons were considered to be viable or alive.

Measurement of BSCB permeability

BSCB integrity was assessed by measuring the extravasation of vascular tracers into the spinal cord parenchyma (Fang et al., 2013b). Briefly, 48 hours after IRI, Evans blue (EB) fluorescence and content were used for the qualitative and quantitative examination of extravasation induced by BSCB disruption after SCIIRI, as previously described (Fang et al., 2015). 2% EB dye (10 mL/kg; Sigma-Aldrich, St. Louis, MO, USA) was slowly iv intravenously administered. The rabbits were anesthetized and perfused with 500 mL/kg saline through the left ventricle after the EB circulated for 1 hour, and the spinal cord (L4-L5) was removed. The spinal cord tissue was fixed in 4% paraformaldehyde, sectioned at 10 mm thickness, and frozen and sealed in a dark container before measurement of EB fluorescence. EB staining was visualized using an Eclipse Ci fluorescence microscope (Nikon, Tokyo, Japan). Thereafter, the spinal cord tissue was weighed and soaked in formamide for 24 hours (60°C), and then centrifuged. The absorbance of the supernatant was measured at 632 nm with a microplate reader (Bio Tek, Winooski, VT, USA). The content of EB in the spinal cord was determined (quantified as μg/g) using a standard curve.

Real-time PCR

Rabbits were anesthetized with an overdose of pentobarbital, and the L4-L5 segments were rapidly collected for real-time PCR 48 hours after surgery. Total RNA was extracted using the Trizol Kit (Life Technologies, Carlsbad, California, USA) and converted to first-strand complementary DNA according to the manufacturer’s instructions. Quantitative real-time PCR was performed using an ABI 7000 instrument (Applied Biosystems, Foster City, USA) and the SYBR Green real-time PCR was performed using an ABI 7000 instrument according to the manufacturer’s instructions. Quantitative PCR 48 hours after surgery to measure the expression levels of MMP-9, NF-κB (p65), claudin-5 and occludin. Briefly, the spinal cord tissues were homogenized, and total proteins were purified using cell and tissue protein extraction reagents according to the manufacturer’s instructions (Wuhan GuGe, Wuhan, Hubei Province, China). The samples were subjected to SDS-PAGE, and the proteins were blotted onto a membrane and probed with the following antibodies: mouse anti-NF-κB (p65) polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-MMP-9 polyclonal antibody (1:1,000); Affinity, Zhenjiang, Jiangsu, China), goat anti-claudin-5 polyclonal antibody (1:500; Santa Cruz Biotechnology), rabbit anti-occludin polyclonal antibody (1:1,000; Abcam, Cambridge, UK) and horseradish peroxidase (HRP)-conjugated secondary antibodies (goat and donkey; 1:3,000; Wuhan GuGe). The membranes were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 30 minutes at room temperature, blocked and visualized by enhanced chemiluminescence. Semi-quantitation of scanned films was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are expressed as the mean ± SD or median values (for neurologic assessments) and analyzed with SPSS software (version 19.0, IBM, Armonk, NY, USA). All variables were normally distributed. Groups were compared (except neurological assessment score) with one-way analysis of variance (ANOVA), followed by Tukey post hoc analysis. Neurological assessment scores were analyzed with the Kruskal-Wallis test. P values < 0.05 were considered statistically significant.

Results

Propofol improved neurological function after SCIRI

All rabbits showed normal motor function of the hind limbs before the induction of ischemia. The sham-operated rabbits retained normal hind limb motor function throughout the study. The 30-minute infrarenal aortic occlusion resulted in severe lower extremity neurologic deficits in the I/R and propofol groups. In the I/R group, most rabbits had low neurological scores, while neurological scores in the propofol groups were significantly higher than those in the I/R group (Figure 1).

Propofol improved the morphology of the spinal cord after I/R

Hematoxylin-eosin staining showed that in the sham
Figure 1 Propofol improved motor function after SCIRI. Motor function was evaluated using the Tarlov scale 48 hours after ischemia/reperfusion. The higher the Tarlov score, the better the motor function. Data are presented as individual values for each animal as well as median values for each group ($n = 6$/group). $^* P < 0.05$, vs. sham group; $^# P < 0.05$, vs. I/R group the Kruskal-Wallis test. Sham: Sham group; I/R: ischemia/reperfusion group; P: propofol group (I/R + P); SCIRI: spinal cord ischemia/reperfusion injury.

Figure 2 Effect of propofol on the morphology of the ischemia/reperfusion-injured spinal cord (hematoxylin-eosin staining, original magnification, $\times 400$).

Figure 3 Propofol attenuated BSCB damage after SCIRI. (A–C) BSCB permeability was assessed using EB dye 48 hours after ischemia/reperfusion, which was visualized as red fluorescence under the fluorescence microscope (original magnification, $\times 40$). (A) Sham group; (B) I/R group; (C) P group. (D) Quantification of the Evans blue dye content in the spinal cord. Data are expressed as the mean $\pm$ SD ($n = 6$/group). $^* P < 0.05$, vs. sham group. $^# P < 0.05$, vs. I/R group (one-way analysis of variance followed by Tukey post hoc analysis). BSCB: Blood-spinal cord barrier; SCIRI: spinal cord ischemia/reperfusion injury; I/R: ischemia/reperfusion group; P: propofol group (I/R + P).

Figure 4 Propofol suppressed the upregulation of NF-κB (p65) protein (A) and mRNA (B).

(A) NF-κB (p65) protein expression, which is expressed as the optical density ratio of NF-κB (p65) to GAPDH. (B) NF-κB (p65) mRNA expression. NF-κB (p65) mRNA expression is expressed as $2^{-\Delta\Delta CT}$. All data are given as the mean $\pm$ SD ($n = 6$/group). $^* P < 0.05$, vs. sham group; $^# P < 0.05$, vs. I/R (one-way analysis of variance followed by Tukey post hoc analysis). Sham: Sham group I/R: ischemia/reperfusion group; P: propofol group (I/R + P). NF-κB: Nuclear factor-kB; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

group, neurons had a normal morphology. In contrast, in the I/R group, there were no normal-looking neurons, and dead neurons with cytosolic vacuoles were apparent. In the propofol group, some normal neurons were visible (Figure 2).

Propofol decreased BSCB permeability after IRI
As shown in Figure 3, compared with the sham group, there was a significant increase in EB dye extravasation in the I/R group. Propofol treatment reduced extravasation following IRI, indicating that it helped maintain BSCB integrity.
Figure 6 Propofol inhibited the reduction in claudin-5 (A) and occludin (B) protein levels after ischemia/reperfusion injury, as assessed by western blot assay.

The protein levels of claudin-5 and occludin are expressed as the optical density ratio of claudin-5 or occludin to GAPDH. Data are presented as the mean ± SD (n = 6/group). *P < 0.05, vs. sham group; #P < 0.05, vs. I/R group (one-way analysis of variance followed by Tukey post hoc analysis). Sham: Sham group; I/R: ischemia/reperfusion group; P: propofol group (I/R + P). MMP-9: Matrix metalloprotease-9; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 6 Propofol inhibited the reduction in claudin-5 (A) and occludin (B) protein levels after ischemia/reperfusion injury, as assessed by western blot assay.

The protein levels of claudin-5 and occludin are expressed as the optical density ratio of claudin-5 or occludin to GAPDH. Data are presented as the mean ± SD (n = 6/group). *P < 0.05, vs. sham group; #P < 0.05, vs. I/R group (one-way analysis of variance followed by Tukey post hoc analysis). Sham: Sham group; I/R: ischemia/reperfusion group; P: propofol group (I/R + P). MMP-9: Matrix metalloprotease-9; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Propofol inhibited the expression of NK-κB (p65) in the I/R-injured spinal cord

Western blot analysis demonstrated that NF-κB (p65) expression increased 48 hours after injury, and propofol significantly inhibited this upregulation (P < 0.05; Figure 4A). Real-time PCR showed that propofol treatment significantly decreased NF-κB (p65) mRNA expression after SCIRI (P < 0.05; Figure 4B).

Propofol inhibited the upregulation of MMP-9 after SCIRI

The expression of MMP-9 increased 48 hours after reperfusion, as assessed by western blot analysis, while propofol treatment significantly depressed the upregulation of MMP-9 (P < 0.05). As shown in Figure 5A, in the I/R group, the expression level of MMP-9 was remarkably increased. Similarly, real-time PCR showed that propofol treatment significantly decreased mRNA expression of MMP-9 (P < 0.05; Figure 5B).

Propofol suppressed the reduction in occludin and claudin-5 expression in the I/R-injured spinal cord

Western blot analysis indicated that the levels of occludin and claudin-5 were decreased 48 hours after I/R injury in the I/R group (Figure 6A), while propofol suppressed this decrease in expression of occludin and claudin-5 (P < 0.05; Figure 6B).

Discussion

Propofol is widely used in clinical anesthesia, as well as seda-
tion (Hutchens et al., 2006). Propofol has protective effects on some organs, such as the heart, kidney, lung and liver (Vasileiou et al., 2012; Shirakawa et al., 2014; Ge et al., 2015; Bellanti et al., 2016). Furthermore, the neuroprotection provided by propofol has been demonstrated to be efficacious in a number of studies (Fang et al., 2015; Yue et al., 2015; Zhou et al., 2015; Hou et al., 2016). Neuroprotective effects of propofol have been reported in a spinal cord ischemia model and in a cerebral ischemia model (Zhou et al., 2013; Sahin et al., 2015; Yu and Yang, 2016). Propofol has antioxidant, anti-apoptotic and anti-inflammatory actions.

The physical barriers between the blood and the central nervous system (BBB and BSCB) protect the central nervous system from toxic and pathogenic agents in the blood. Indeed, disruption of the barrier aggravates damage to the central nervous system (Palmer, 2010). A study has shown that propofol treatment helps maintain BBB integrity in a rat model of focal cerebral IRI (Ji et al., 2015).

Our results demonstrate that propofol pre- and post-treatment helps maintain BSCB integrity, and significantly improves both neurological and histological outcomes following ischemia/reperfusion. The expression levels of MMP-9 and NF-κB (p65) were increased in the IRI group, and claudin-5 and occludin expression levels were decreased. Our findings suggest that propofol might attenuate disruption of the BSCB by inhibiting the expression of MMP-9 and NF-κB (p65) and by suppressing the reduction in claudin-5 and occludin levels. Our study is of great significance for ischemia-induced spinal cord injury in which disruption of BSCB integrity plays a major pathogenetic role.

NF-κB, a key transcription factor, plays an important role in inflammatory responses and oxidative stress induced by I/R (Yu et al., 2016). Propofol has been found to inhibit the inflammatory reaction by inhibiting NF-κB activation during focal I/R, which may be one of the mechanisms of neuroprotection (Feng et al., 2004). Indeed, overexpression of NF-κB leads to BBB and BSCB dysfunction, including increased permeability (Li et al., 2014b; Liu et al., 2014). Ulbrich et al. (2016) found that propofol inhibits the NF-κB signaling pathway. In the present study, we found that NF-κB expression is significantly elevated after ischemia/reperfusion, and that propofol treatment inhibited this increase. The high levels of NF-κB likely increased BSCB permeability. Therefore, inhibition of the NF-κB signaling pathway by propofol may help maintain BSCB integrity after IRI.

MMP-9, a potent regulator of inflammation, has an important role in acute inflammation (Liu et al., 2015). Overexpression of MMP-9 impairs the integrity of the BSCB after IRI, likely by degrading tight junction proteins and basement membranes of endothelial cells (Yang et al., 2007). Numerous studies have shown that BBB and BSCB dysfunction induced by stroke or I/R is associated with the upregulation of MMP-9 (Noble et al., 2002; Xiang et al., 2012; Chaturvedi and Kaczmarek, 2014; Fang et al., 2015). MMP-9 inhibitors, such as tissue inhibitor of matrix metalloproteinases (TIMPs) and BB-1101, appear to help maintain BBB integrity (Cunningham et al., 2005). Therefore, therapies that inhibit MMP-9 expression might attenuate the disruption of the BBB and BSCB. Ji et al. (2015) found that propofol post-treatment improves neurobehavioral dysfunction and attenuates BBB damage by decreasing MMP-9 and aquaporin-4 expression in a rat model of I/R-induced focal cerebral injury. NF-κB upregulates expression of matrix metalloproteinases, including MMP-2 and MMP-9 (Yang et al., 2016). In our current study, MMP-9 expression was substantially increased after IRI, and propofol reduced its expression. Concomitant with the increase in MMP-9 expression, NF-κB levels were increased in the I/R group. These increases in MMP-9 and NF-κB expression were suppressed in the propofol group, indicating that propofol reduces MMP-9 expression by inhibiting the expression of NF-κB.

Tight junctions between cerebral microvascular endothelial cells are the functional and structural basis of the BBB and are responsible for blocking the free movement of compounds from the blood into the parenchyma (Zlokovic, 2008). Cerebral IRI leads to tight junction disruption, allowing numerous compounds to cross the BBB (Jiao et al., 2011). Occludin and claudin-5, the major tight junction-associated proteins, play a critical role in connecting the membrane with cytoskeletal proteins. The expression of occludin and claudin-5 are significantly decreased after SCIRI (Fang et al., 2013a, 2015; Yu et al., 2015), which increases the permeability of capillaries and aggravates spinal cord injury. MMP-9 proteolytically digests occludin and claudin-5, resulting in barrier dysfunction (Fang et al., 2013a; Li et al., 2014b; Boroujerdi et al., 2015; Yu et al., 2015). In our present study, the reduction in occludin and claudin-5 levels paralleled the increase in MMP-9 expression in the I/R group, consistent with previous studies showing that MMP-9 degrades claudin-5 and occludin after SCIRI.

There are a number of limitations of our study. First, we assessed BSCB integrity at only one time point—48 hours after reperfusion. Previous studies demonstrate that BSCB dysfunction worsens 48 hours after SCIRI (Li et al., 2014c). Hence, we selected this time window for this study. However, in future studies, a wider observation window would help better evaluate the protective effects of propofol on the BSCB after SCIRI. Second, we used a rabbit model. Experiments using other animal models are necessary to confirm the findings. In addition, the high dose of propofol used in the current study might impair the respiratory and circulatory systems (Simons et al., 2013; Meng et al., 2016). Indeed, clinically, propofol is not suitable for patients suffering from heart diseases. At present, although the protective effects of propofol against SCIRI have been clarified in animal models (Zhou et al., 2013; Fan et al., 2015; Yang et al., 2015), no clinical study has examined whether propofol protects against BSCB dysfunction in humans.

Our findings show that intravenous infusion of propofol maintains the integrity of the BSCB after SCIRI, laying the foundation for further research. Future studies should examine the effectiveness of a combination of drugs and treatment...
strategies. Recently, propofol injection combined with bone marrow mesenchymal stem cell transplantation was shown to improve electrophysiological function of the hind limb and attenuate spinal cord injury in a rat model (Wang et al., 2015). Moreover, a more detailed examination of the cellular and molecular mechanisms is also warranted (Ning et al., 2014; Li et al., 2015).

In summary, propofol pre- and post-treatment attenuates BSCB damage induced by I/R and ameliorates neurobehavioral deficits by maintaining levels of occludin and claudin-5, downregulating MMP-9, and inhibiting the NF-κB signaling pathway. Our findings should assist in the development of novel therapeutic strategies for SCI/R.

Author contributions: LJX, JXH, JY and SSZ participated in the experimental procedures and data process; LJX, JXH and FY were in charge of western blot assay and statistics; LJX and SSZ were in charge of real-time PCR and statistics; JY and QYF were in charge of neurological and histological assessment. LJX and JH were responsible for data analysis and experimental results. LJX, JXH and JH designed the study and wrote the paper. All authors approved the final version of this paper.

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