

# XingNaoJing injections protect against cerebral ischemia/reperfusion injury and alleviate blood-brain barrier disruption in rats, through an underlying mechanism of NLRP3 inflammasomes suppression

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**[ABSTRACT]** The aim of this study was to explore the neuroprotective effect and mechanism of XingNaoJing injections (XNJ) on cerebral ischemia injury and blood-brain barrier (BBB) disruption. Middle cerebral artery occlusion (MCAO) method was applied to establish the model of cerebral ischemia/reperfusion (I/R) injury in rats. BBB permeability after I/R injury was assessed with the leaking amount of Evans Blue and the expression of occludin and ZO-1. The expression of NOD-like receptor family, pyrin domain containing (NLRP3) was checked to explore the inhibition of inflammation by XNJ. The results showed that XNJ could significantly increase the survival percent, decrease the infarct area and ameliorate neurological deficits and brain damage after I/R injury. Leaking amount of Evans Blue was reduced by XNJ, and the expression of tight junction protein, occludin and ZO-1 was also up-regulated by XNJ, which showed a role of protection on BBB disruption. The expression of NLRP3 was inhibited after exposure of XNJ, which was associated with inhibition of the inflammatory response. In summary, XNJ could suppress NLRP3 inflammasomes and improve BBB disruption and brain damage in rats after cerebral I/R injury, which provided a beneficial insight to further explore XNJ.

**[KEY WORDS]** XingNaoJing injections; Cerebral ischemia/reperfusion injury; Blood-brain barrier; NLRP3 inflammasomes

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## Introduction

Blood-brain barrier (BBB) is a monolayer of microvascular endothelial cells joined together by tight junction protein (TJP), such as occludin and ZO-1<sup>[1]</sup>, which block the passage of blood substrates into the central nervous system (CNS)<sup>[2-3]</sup>. Therefore, damage of the BBB can aggravate the CNS injury and brain dysfunction. BBB breakdown can be

found under many nervous system diseases, such as ischemic stroke, subarachnoid hemorrhage, neurotrauma and brain tumor<sup>[4-6]</sup>. Stroke is one of the most dangerous causes of death all over the world. Ischemic stroke leads to focal brain infarction and sudden neurologic deficits of the patients who undergo longer than one hour ischemia<sup>[7]</sup>. Update, more and more evidences prove that neuroinflammatory response plays a central role in the pathogenesis of cerebral ischemia, and the release of numerous inflammatory cytokines into ischemic regions, such as interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ )<sup>[8]</sup>. Most important, release of pro-inflammatory cytokines is a major inducer of BBB disruption that further aggravates brain injury and dysfunction<sup>[9]</sup>. Thus, inhibition of inflammation plays an important role in improving the dysfunction of BBB and relieving cerebral ischemia injury.

NOD-like receptor family, pyrin domain containing (NLRP3) inflammasome is a multi-protein structure in the cytoplasm which assembly with NLRP3, adaptor apoptosis-

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associated speck-like protein (ASC) and pro-caspase-1 [10]. The assembly of NLRP3 inflammasome is rarely in the resting condition but can be triggered by many environmental factors. When NLRP3 inflammasome assembling, pro-caspase-1 is activated and auto-cleaved into caspase-1. Active caspase-1 is released and cleaved pro-IL-1 $\beta$  and pro-IL-18 into mature IL-1 $\beta$ , IL-18, and then these pro-inflammatory cytokines leaked and contributed to the inflammatory response [11-12]. Increasing evidence suggested that NLRP3 inflammasome was activated and induced inflammatory response in cerebral ischemia condition [13-14], which was also associated with BBB disruption [15-16]. Thus, inhibition of NLRP3 inflammasome is a new thought in protection of BBB and therapy of ischemia stroke.

XingNaoJing injections (XNJ) are derived and prepared with modern technology according to a famous and classic traditional Chinese prescription named An-Gong-Niu-Huang pill [17]. XNJ are composed by four Chinese herbs including Moschus, Radix Curcumae, borneol and Fructus gardeniae, and have been approved by the China Food and Drug Administration (CFDA) in the treatment of stroke in clinical [18]. Evidence from randomized controlled trials and system reviews suggests that XNJ may be a beneficial therapeutic drug for the treatment of hypoxic ischemic encephalopathy, cerebral infarction and stroke [18-21]. However, the underlying mechanism in the treatment of cerebrovascular diseases is reported rarely.

In the present study, we aimed to investigate the effects of XNJ on cerebral ischemia/reperfusion (I/R) injury and permeability of BBB. Furthermore, we want to explore whether NLRP3 inflammasomes were involved in XNJ inhibiting inflammation and improving the disruption of BBB.

## Materials and Methods

### Animals

Male Sprague-Dawley rats (250–300 g) were purchased from Liaoning Changsheng biotechnology Co., Ltd. (Benxi, China, permit #SCXK-Liao 2015-0001) and housed in a controlled environment (20–22 °C, 50%–55% humidity, 12 h light/dark cycles) and fed with water and chow diet.

### Drugs and reagents

XingNaoJing injections (batch No. 20160608) were produced by Henan Tiandi Pharmaceutical Co., Ltd. (Kaifeng, China) with a CFDA permit #z41020664. Content of curzerenone in XNJ would not be lower than 0.1 mg·mL<sup>-1</sup> according to drug quality standard and the drugs used in this research were all met this criterion. Triphenyltetrazolium chloride (TTC) was purchased from Sigma (St. Louis, MO, USA). Evans Blue was purchased from Biosharp Life Sciences (Hefei, China). RIPA buffer was purchased from Beyotime Biotechnology (Shanghai, China). The primary antibodies used in our study were rabbit anti-ZO-1 and mouse anti-IL-1 beta (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-occludin and rabbit anti-NLRP3 (Bioss

Antibodies, Beijing, China), rabbit anti-caspase-1 (Abcam, Cambridge, MA, USA) and rabbit anti- $\beta$ -actin (Cell Signaling Technology, Beverly, MA, USA).

### Treatment of rats

All experimental animal procedures were approved by the Animal Experiments Committee of the First Hospital of Jilin University under approval No. 2018-057 on Feb 22th, 2018. Thirty-six rats were randomly divided into three groups and 12 rats in each group: Sham group (sham- operated), I/R group (I/R model, vehicle with saline) and XNJ group (treated with 15 mL·kg<sup>-1</sup> XingNaoJing for Injection) [22].

Focal cerebral I/R model was established by middle cerebral artery occlusion (MCAO) method [23]. Briefly, rats were anesthetized by 10% chloral hydrate and laid on a heating pad at 37 °C during the surgery. The right external carotid artery and the common carotid artery were isolated and the internal carotid artery was dissected. A 4-0 nylon filament of 2 cm long was inserted to the middle cerebral artery to occlude it for 2 h. After ischemia, the filament was withdrawn to accomplish with reperfusion. Rats in sham group received the same operations but without the ischemia and reperfusion process. XNJ (15 mL·kg<sup>-1</sup>) or vehicle (in sham and I/R group) was administered (i.p.) firstly at 24 h before operation and secondly at the time of reperfusion. Twenty-four hours after reperfusion, the neurological scores were evaluated. At last, the rats were euthanized and brains were collected and stored at -80 °C for further steps. The overall process was shown in Fig. 1 to clear the treatment of rats.

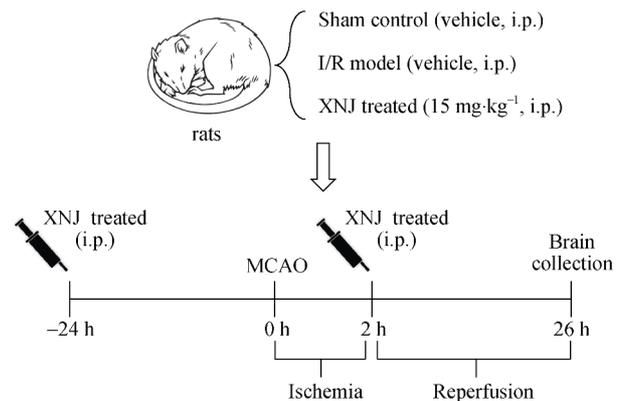


Fig. 1 The diagram of the experimental protocol

### Neurological deficits

Neurological scores were used to evaluate the neurological deficits of experimental rats after I/R injury. Estimation of neurological scores was proceeded blindly by a new researcher according to Longa's method [15]. The score criterion was listed as follows: 0, no deficit; 1, forelimb weakness and failed to entirely extend; 2, circling to the contralateral side; 3, failed to bear weight on the injury side; 4, no spontaneous locomotor activity or barrel rolling.

### Infarct area

Rat brains collected after 24 h reperfusion were cut into

coronal slices in 2 mm thickness, and then stained with 2% TTC at 37 °C for 30 min. After reaction, the slices were photographed and the area ratio of infarction in slices was analyzed by Image J software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA).

**Histopathology**

Rat brains in each group were fixed in 4% formaldehyde for 7 days and embedded in paraffin. Brain sections in 5 μm thickness were stained with hematoxylin & eosin (H&E) and examined by a BX51 light microscope with a magnification of 400 times (OLYMPUS, Japan). An independent researcher blindly assessed the degree of brain damage for each section.

**BBB permeability**

BBB permeability was measured by determining Evans Blue (EB) extravasations in the brains. EB was soluted in saline at a concentration of 2% and injected into the tail vein immediately after reperfusion at a dose of 2 mL·kg<sup>-1</sup>. After 24 h reperfusion, rats were anesthetized and transcardially perfused with saline until the effusion was clear. Then the brains were collected and cut into slices after weighting. Each brain was homogenized with equal volume PBS and then centrifuged at a speed of 15 000 r·min<sup>-1</sup> for 30 min. The supernatant was diluted with 3 times volume 50% trichloroacetic acid solution, and incubated at room temperature for 2 h. Then, the solutions were centrifuged at a speed of 15 000 r·min<sup>-1</sup> for 20 min and the supernatants were removed for measuring with a 160A spectrophotometer (Shimadzu, Kyoto, Japan) at 610 nm. The concentration of EB was calculated with the EB reference solution and presented as micrograms in per gram tissue.

**Western blot**

Rat brains were homogenized in RIPA buffer and centrifuged at a speed of 12 000 r·min<sup>-1</sup> for 10 min at 4 °C. Protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China), and 50 μg of protein was loaded onto precast SDS-PAGE gels then transferred to PVDF membranes (Merck Millipore Ltd., Tullagreen, IRL). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for

1 h at room temperature. The membranes were then incubated overnight at 4 °C with rabbit anti-ZO-1 (1 : 1000), rabbit anti-occludin (1 : 1000), rabbit anti-NLRP3 (1 : 500), mouse anti-IL-1β (1 : 1000), rabbit anti-caspase-1 (1 : 1000) and rabbit anti-β-actin (1 : 2000). The membranes were washed for 10 min three times with TBST and incubated with secondary antibodies (1 : 3000) for 1 h at room temperature. Immunoreactive proteins were visualized using an ECL kit (Yeasen Biotech, Shanghai, China) and the density of the bands was analyzed using Image J software (version 1.8.0). All bands were normalized to β-actin as an internal standard.

**Immunohistochemistry**

Rat brain sections were deparaffinized, rehydrated, and immunohistochemically stained using the DAB method as the instruction of manufacturer. The sections were incubated overnight at 4 °C with rabbit anti-ZO-1 (1 : 200), occludin (1 : 500) and NLRP3 (1 : 400), then labeled with streptavidin-peroxidase (SP). The immunoreactive proteins were viewed with a BX51 light microscope (OLYMPUS, Japan) at a magnification of 400 times, and the quantification was measured by density and blindly analyzed with Image-Pro Plus (version 6.0, National Institutes of Health, Bethesda, MD, USA).

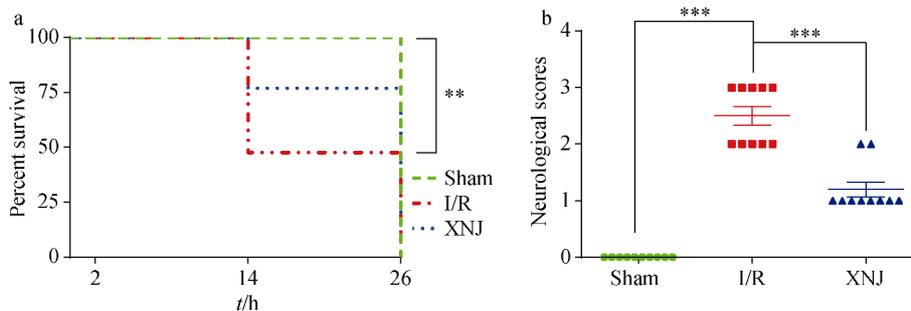
**Statistical analysis**

All data are presented as means ± standard deviation (SD) and were analyzed by SPSS 18.0 software (IBM, Armonk, NY, USA). Differences between groups were determined using one-way ANOVA followed by the Tukey test when equal variances were assumed. Differences between groups were considered to be statistically significant when *P* < 0.05.

**Results**

**XNJ increased survival and ameliorated neurological deficits**

In our study, survival percent after reperfusion for 24 h in I/R group was 47.62% (10/21), and significantly lower than 100% (10/10) in the Sham group (*P* < 0.01). After treated with XNJ, survival percent was increased to 76.92% (10/13) and without significant difference compared with the Sham group (Fig. 2a). The data suggested that XNJ could alleviate the brain I/R injury and increase the survival.



**Fig. 2 The general pharmacological effects of XNJ on cerebral I/R injury in rats. (a) Overall survival percent after reperfusion, (b) neurological scores. All data are presented as means ± SD, n = 10. \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001**

Rats in the Sham group showed a normal neurological function and without deficit. However, the mean neurological

score in I/R group was (2.5 ± 0.52) and significantly increased than Sham group with (0 ± 0) point (*P* < 0.001),

which indicated a severe destruction of neurological function. Neurological function of XNJ group was obviously improved with the results of significantly decreasing the score ( $1.2 \pm 0.42$ ) than I/R group (Fig. 2b,  $P < 0.001$ ).

*XNJ decreased infarct area*

Unstained sections (white color) were defined as infarction in Fig. 3. According to data from the Image J software, the ratio of unstained area in I/R group was  $27.74\% \pm 3.26\%$ , significantly higher than  $0.92\% \pm 0.27\%$  in the Sham group ( $P < 0.001$ ). The ratio in XNJ group was  $16.30\% \pm 3.29\%$  and significantly improved than that in I/R group ( $P < 0.001$ ).

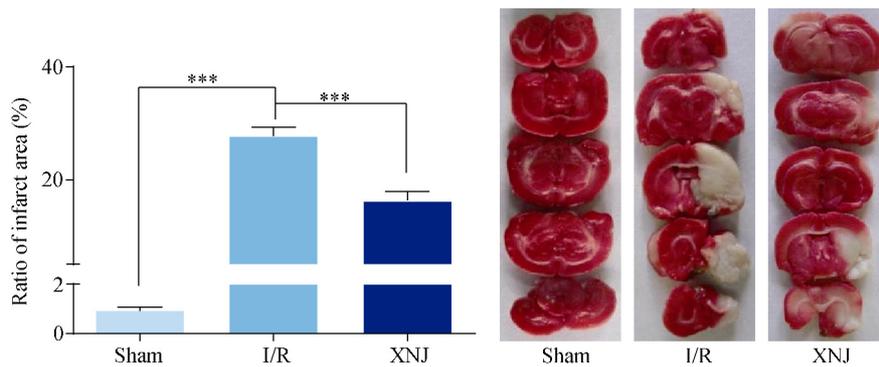
*XNJ attenuated cerebral I/R injury*

Using histopathological analysis in Fig. 4, we observed severe brain damage occurred after I/R of exposure to vehicle both in the cerebral cortex and white matter. Disordered and unconsolidated arrangement of neurocyte was seen, and cyto-

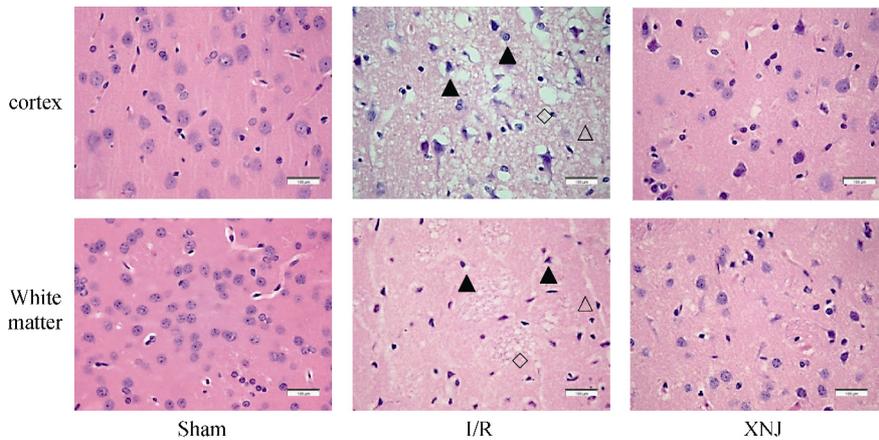
plasm vacuolation and nucleus condensation was also seen in the cortex and white matter of I/R group. On the contrary, sham group showed a normal morphology with ordered and consolidated arrangement of full neurocyte in cortex and white matter. After treated-XNJ, the improved morphology changes were observed compared with I/R group both in the cerebral cortex and white matter, which showed a protective effect of XNJ on cerebral I/R injury.

*XNJ reduced BBB leakage after I/R*

The protective effect of XNJ on BBB integrity was assessed with the leaking amount of EB in Fig. 5. EB staining was much easier observed in I/R group than Sham group, but was remarkably reduced in XNJ group. The determination of EB amount in brain also showed that, leaking amount of EB in I/R group was significantly increased compared with Sham and XNJ group ( $P < 0.001$ ).



**Fig. 3** The effect of XNJ on ratio of infarct area after cerebral I/R injury in rats. All data are presented as means  $\pm$  SD,  $n = 4$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$



**Fig. 4** Light microscopy of histopathology sections of rat brains after cerebral I/R injury ( $\times 400$ ) ( $n = 4$ ).  $\blacktriangle$ nucleus condensation,  $\triangle$ unconsolidated arrangement of neurocyte,  $\diamond$ cytoplasm vacuolation, Scale bar = 100  $\mu$ m

*XNJ increased the expression of TJPs after I/R*

Occludin and ZO-1 were important TJPs which contributing to BBB integrity. Data from Western blot in Figs. 6a and 6b showed that occludin and ZO-1 were significantly reduced in I/R group compared with the Sham group ( $P < 0.001$ ) after cerebral I/R injury. But rats treated with XNJ were associated with increased expression of occludin and ZO-1 compared

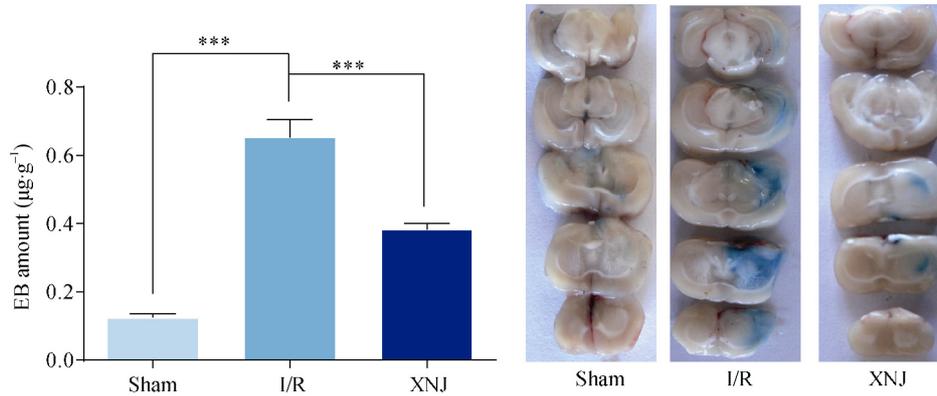
with I/R group ( $P < 0.05$ ). Expressions of occludin and ZO-1 were also shown by immunohistochemistry. The positive expressions of occludin and ZO-1 in Fig. 6c were stained by gray to brown color, and apparently seen in Sham group. Positive expressions were rare seen in I/R group, but reversed by XNJ after cerebral I/R injury. Thus, according to the data, XNJ could increase the expression of TJPs, such as occludin

and ZO-1, and improve the destruction of BBB after cerebral I/R injury.

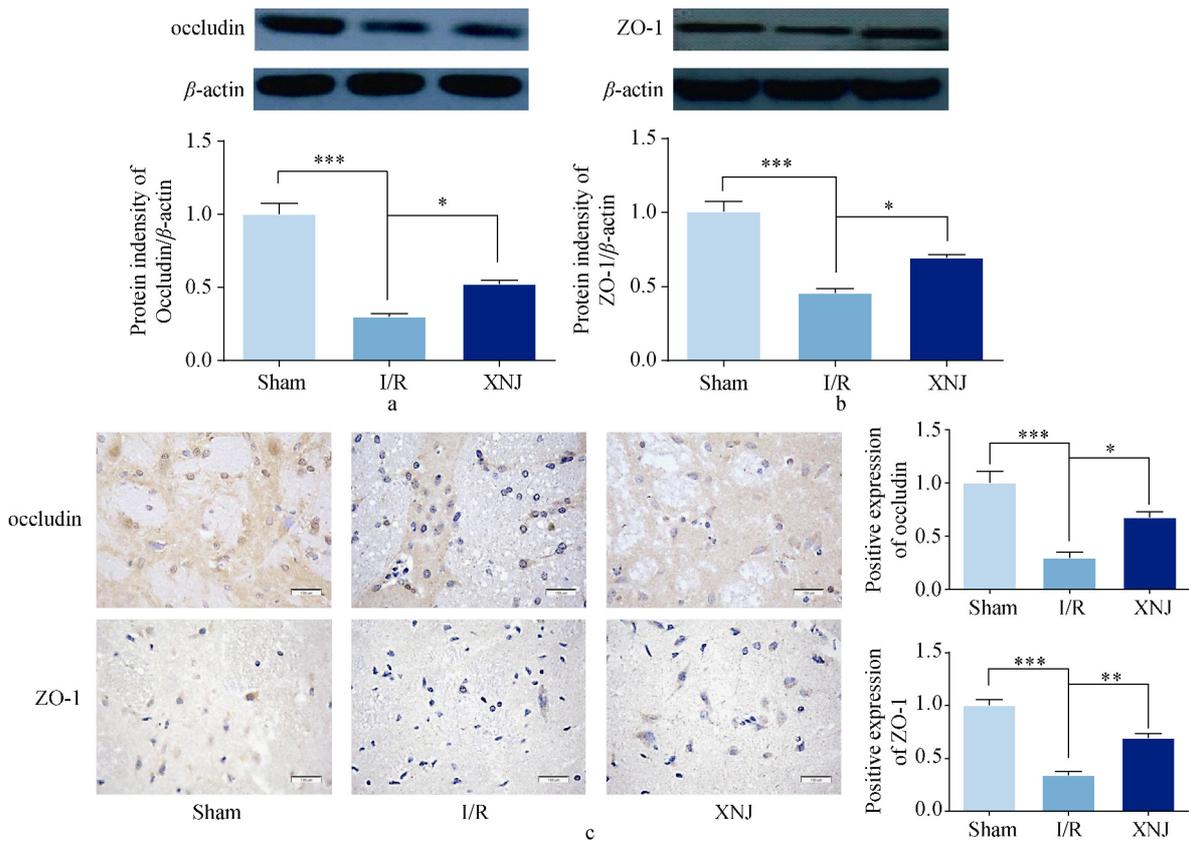
*XNJ inhibited the activation of NLRP3 inflammasome*

NLRP3 inflammasomes play an important role in inflammatory response and the activation are found after cerebral I/R injury. According to the results of Western blot, we found that the expression of NLRP3 was significantly reduced in

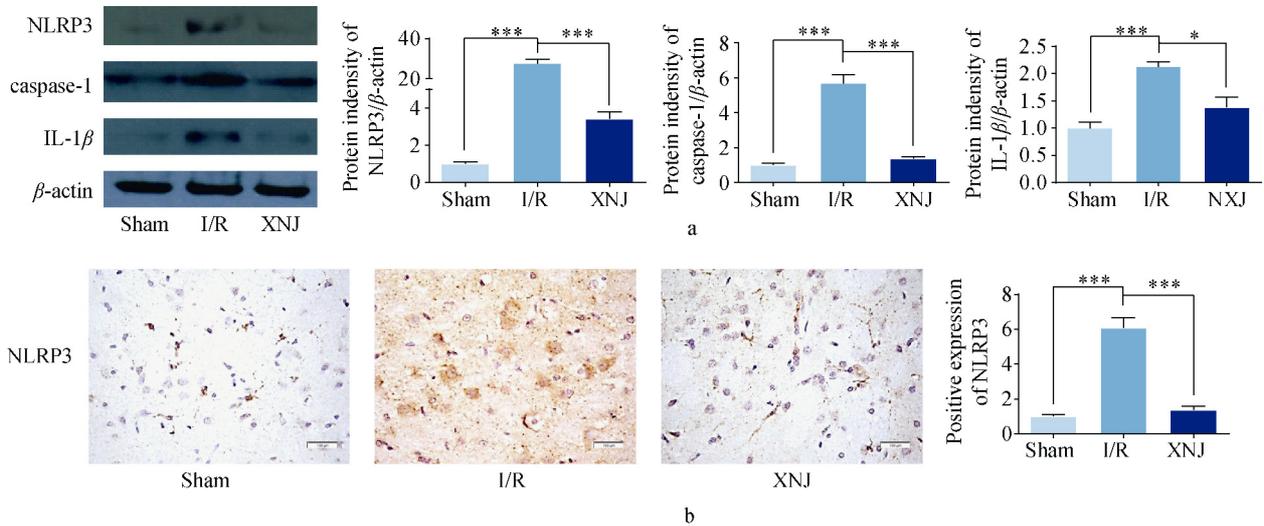
XNJ group ( $P < 0.001$ ), and the expressions of caspase-1 and IL-1 $\beta$  were also significantly reduced ( $P < 0.001$  and  $P < 0.05$ ) in Fig. 7a. The immunohistochemistry staining also showed that the positive expression of NLRP3 was reduced in XNJ group in Fig. 7b. The results suggested that XNJ could relieve the inflammatory response by inhibiting the expression of NLRP3.



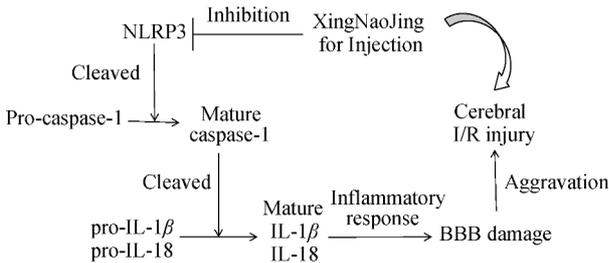
**Fig. 5** The effect of XNJ on alleviating BBB disruption *via* assessing leaking amount of EB after cerebral I/R injury. All data are presented as means  $\pm$  SD,  $n = 4$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$



**Fig. 6** Expressions of occludin and ZO-1 after cerebral I/R injury in rat brains. (a and b) Expressions of occludin and ZO-1 in rat brain by Western blot ( $n = 4$ ), relative expression of protein was calculated as intensity of the target protein normalized to  $\beta$ -actin. (c) Immunohistochemistry sections showing the expressions in rat brains ( $400\times$ ,  $n = 4$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . Scale bar = 100  $\mu$ m



**Fig. 7** NLRP3 expression in rat brains. (a) Expressions of NLRP3, caspase-1 and IL-1β in rat brains by Western blot ( $n = 4$ ), relative expression of protein was calculated as intensity of the target protein normalized to β-actin. (b) Immunohistochemistry sections showing the expression in rat brains (400 ×) ( $n = 4$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . Scale bar = 100 μm



**Fig. 8** Schematic illustration of underlying protective mechanism of XNJ on cerebral I/R injury in rats. XNJ could inhibit the activation of NLRP3, and then alleviate inflammatory response after cerebral I/R injury. This effect contributed to the improvement of BBB disruption, which was associated with the protective role on cerebral ischemia diseases

**Discussion**

Xingnaojing injections are used clinically in China for treatment of stroke more than a decade. Moschus, *Radix curcumae*, borneol and Fructus gardeniae are the Chinese herbs of XNJ prescription with the effects of clearing heat, sedation, resuscitation and detoxification. Clinical trials have demonstrated that XNJ can relieve brain injury and improve the functional recovery after ischemia stroke [17].

Disruption of the BBB can be induced as secondary damage by cerebral I/R injury [24]. Furthermore, BBB disruption has been found to contribute to a worse neuronal outcome in I/R injury [25-26]. Based on this evidence, the relationship between BBB disruption and cerebral I/R injury may play a key role in the onset and progression of ischemic cerebrovascular disease. Relieving damage to the BBB can improve brain injury and neurological function after I/R. Therefore, protection of BBB integrity is a beneficial therapeutic method in the treatment of cerebral ischemia diseases, such as

stroke [27]. In our study, the decrease in cerebral EB permeability suggested that treatment with XNJ may alleviate the disruption of BBB integrity after cerebral I/R injury and reverse the expression levels of TJPs (e.g., occludin and ZO-1) compared with those reported in the I/R group. Consequently, the integrity of the rat BBB improved the neurological function and brain damage. Hence, treatment with XNJ appears to restore BBB integrity and block the introduction of harmful substrates into regions of the brain, which may play a key role in protecting against cerebral I/R injury in rats. Besides, it has been shown that BBB disruption may be the consequence of many cerebrovascular conditions, such as stroke, ischemic and hemorrhagic injury, traumatic injury, brain tumors, and sclerosis [28]. This indicates that XNJ may be useful in the recovery from various cerebrovascular events – apart from stroke.

Inflammatory response is an important mechanism in BBB disruption, leading to secondary damage and delayed functional recovery in patients with ischemic cerebrovascular disease [5, 29]. The resultant BBB disruption also accelerates the leakage of proinflammatory-induced cytokines [15, 30]. Therefore, inhibition of inflammation, especially the assembly of NLRP3 inflammasomes, may be the mechanism underlying the action of XNJ in the protection of BBB integrity. Accumulating evidence has suggested that NLRP3 inflammasomes are activated after cerebral I/R injury, leading to an inflammatory response [13, 31-32]. In the present study, after cerebral I/R injury, the expression of NLRP3 was inhibited in the XNJ group, alleviating the inflammatory response. This process may represent a mechanism of protection against BBB damage, *via* treatment with XNJ. Furthermore, an increasing number of studies have reported that inhibition of NLRP3 was a key factor in mitigating BBB disruption in cerebral diseases [13, 15-16, 33]. Hence, inhibition of NLRP3

inflammasomes appears to be associated with protection against BBB disruption and cerebral I/R injury in rats, *via* treatment with XNJ. The underlying mechanism was shown in Fig. 8.

Currently, numerous preparations from traditional Chinese medicine prescriptions are widely used in clinical practice in China. However, most of these preparations are used empirically, without a theoretical basis. Thus, the use of these effective drugs is not applicable on a large scale. In summary, this study described the mechanism underlying the protective effect of XNJ against ischemic cerebrovascular disease, and provides useful additional information regarding the theoretical basis of treatment with XNJ. XNJ may mitigate cerebral I/R injury in rats and offer protection against BBB disruption following I/R injury, *via* suppression of NLRP3 inflammasomes.

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