

## Original Research Article

# Protective effect of S-allyl cysteine against cerebral ischemia/reperfusion injury in experimental rats

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### Abstract

**Purpose:** To investigate the protective effect of S-allyl cysteine (SAC) against cerebral ischemia reperfusion injury (CRI) in rats.

**Methods:** The protective effect of SAC was determined in a male Wistar rat model of middle cerebral artery occlusion (MCAO)-stimulated transient focal ischemia, followed by reperfusion. Cerebral ischemia reperfusion injury was induced via 90 min of MCAO, followed by 24-h reperfusion. The cerebral infarct size was determined by staining with 2,3,5- triphenyl tetrazolium chloride. The onset of cellular derangement, neurological deficit score and neuronal oedema were determined. In addition, the expressions of CRI markers and inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA).

**Results:** Rats subjected to CRI showed marked increases in cellular oxidative stress, as evidenced by significant increase in the levels of inflammatory markers, including MDA ( $p < 0.05$ ), MPO ( $p < 0.05$ ) and nitric oxide ( $p < 0.01$ ). In addition, CRI increased the mRNA expression levels of the marker genes TLR4, syndecan-1, CSF, aquaporin-1, OCT3, and RFX1. In contrast, rats pre-treated with SAC prior to CRI displayed reduced levels of inflammatory cytokines, with a near-normal cellular arrangement. SAC treatment significantly reduced the mRNA expression levels of the marker genes in CRI rats.

**Conclusion:** These findings suggest that SAC may protect the brain of rats from cerebral ischemia-reperfusion injury caused by amplification of oxidative stress and inflammatory signaling. Thus, S-allyl cysteine is a potential therapy for the management of CRI.

**Keywords:** S-allyl cysteine, Cerebral Ischemia, Reperfusion, Asthma, Inflammation

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## INTRODUCTION

Insufficient blood flow to the brain due to certain conditions is known as cerebral ischemia. The lack of blood supply causes a lack of oxygen in the brain cells, leading to brain tissue death or ischemic stroke [1]. This may lead to changes in brain metabolism and a decline in its functions.

Stroke is a major cause of morbidity and mortality worldwide. The restoration of brain blood supply as part of the treatment process induces more damage to the brain tissues after a long ischemic period. This injury is known as cerebral reperfusion injury or cerebral ischemia-reperfusion injury [2]. It is the result of the deterioration of the ischemic condition of the

brain. Injury caused by reperfusion of infarcted brain tissues may be due to thrombolysis and embolectomy, and may therefore be safely treated with thrombolytic therapy via intravenous administration of recombinant tissue plasminogen activator (r-tPA) which is the only available therapy approved by FDA for ischemic stroke [3].

Garlic has been traditionally used in the human diet, and it has been known to have a positive effect against many types of cancer and neurodegenerative diseases [4]. Studies have shown that garlic is effective against hyperlipidemia, high blood pressure, pro-inflammatory cytokines and platelet activation [5]. However, the pharmacological effect of garlic intake has not been investigated with respect to its anti-cancer, anti-oxidant, antiaging, immunomodulatory, cardioprotective and anti-allergic properties [6]. In this study, S-allyl cysteine (SAC) known for its antioxidant effects, was used in CRI rat model to protect brain tissues from reperfusion injuries. The suggestion that neuroprotective influence of SAC is based on the decrease of oxidative stress was investigated through the evaluation of various parameters of oxidation such as MDA, NO and inflammatory cytokines.

## EXPERIMENTAL

### Cerebral ischemia-reperfusion injury (CRI) model

The MCAO method was adopted for the establishment of CRI in overnight-fasted rats which were permitted unlimited access to drinkable H<sub>2</sub>O prior to the MCAO procedure. CRI was induced in rats under ketamine and xylazine anesthesia (75 mg/kg) via intraperitoneal injection. The animals were kept on a warming pad to keep their body heat at 37° C in the course of the surgical procedure. The surgical area was prepared by sterilization with 75% ethyl alcohol. A small incision was made on the skin, and left external carotid artery was removed from the vagal nerves. Left external carotid artery was ligated after isolating the left internal carotid artery. The carotid artery was occluded with 3-0 silk thread, while the middle cerebral artery was occluded for 90 minutes. Thereafter, the external carotid artery was permanently occluded, and the occlusion on the common carotid artery was removed to allow reperfusion for 24 h.

### Animal groups

Male Wistar rats (mean weight = 120 ± 10 g) were used in this study. The rats were housed in

a cage in an environment with a 12-h light/ 12-h dark cycle, with temperature and humidity maintained at 25 ± 2°C and 55 ± 5%, respectively, in a temperature-controlled room. The animals received free access to regular standard rat chow and reverse-osmosis water. The preclinical animal procedure was done after receiving prior authorisation from the Institutional Animal Ethics Committee, Tianjin, China (approval no. TJCH1907). The experiments were carried out in accordance with the guidelines of the Association for the Evaluation and Accreditation of Laboratory Animal Care International [7]. The rats were randomly assigned to 4 groups (8 rats per group). Group 1 served as a vehicle control group, while group 2 was an untreated CRI-induced group. In group 3, the CRI-induced rats received SAC intraperitoneally at a dose of 20 mg/kg, starting 2 weeks prior to CRI induction, while group 4 rats were given SAC alone (drug control group). The animals were sacrificed at the end of the experiment and the brains were rapidly separated and freezing for 2 h at -80° C. Subsequently, the frozen brain tissues were sectioned into 2-mm coronal slices and instantly kept in 2 % 2,3,5-triphenyl tetrazolium chloride (TTC) at 37° C for 10 minutes for CRI assessment. Usually, healthy brain tissue subjected to TTC staining shows dark red color, while infarcted tissues are unstained. Further to TTC staining, the tissues were fixed in 4 % of paraformaldehyde for 24 h and scanned with a digital camera. In the calculation of the percentage infarct area relative to the whole brain area, the size of the infarct was determined via the NIH Image J software. In addition, brain oedema was measured by rinsing parts of the brain tissue of control and experimental groups, and then quickly weighing to determine the wet mass (WM). Then, the dissected brain tissue was heated in an oven at 100° C for 24 hours and measured to obtain dry mass (DM). Brain water content (B) was calculated as shown in Equation 1:

$$\text{Brain water content (\%)} = (\text{WM} - \text{DM})/\text{WM} \times 100 \dots\dots\dots (1)$$

Furthermore, the neurological deficit score was determined as described earlier. Following reperfusion, neurological assessments were done by scientists blinded to the treatments used in the experimental groups. The neurological scores were classified in the following way: 0 (no deficits); 1 (discomfort completely extending the forelimb of the contralateral); 2 (inability to extend the forelimb of the contralateral); 3 (moderate circumference to the contralateral); 4 (extreme circumference to

the contralateral, and 5 (dropping to the contralateral side). In addition, the blood marker enzymes of CRI, i.e, clusterin, neuron-specific enolase, and occludin were assayed using commercial assays kits from Abcam (USA), while KCC-positive cells were elucidated as described earlier [8]. Furthermore, the expression levels of CRI markers were determined using Clusterin ELISA kits (Abcam Inc, Cambridge, Massachusetts, USA). Neuron-specific enolase was assayed using kits from Elabscience (USA), while occludin was assayed with an ELISA kit (Cusabio Technology LLC., Houston, Texas, USA).

### Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expressions of onset marker genes of CRI and other biomarker genes were assayed using qRT-PCR. Total RNA was extracted from each brain tissue from the control and tested animals using TRIzol reagent (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The concentration of the RNA was quantified spectrophotometrically. Then, the RNA was reverse-transcribed to cDNA employing reverse transcriptase (Promega) in line with the manufacturer's instructions. This was followed by qRT-PCR using SYBY green/ROX master mix for specific genes and amplification using real-time PCR system (Bio-Rad, Hercules, California, USA). The forward (F) and reverse (R) primers used for the specific genes are indicated in Table 1. They were purchased from Eurofins Genomics Germany GmbH (Operon Inc., Huntsville, Alabama, USA). The mRNA expression levels of the genes were calculated from the CT values, and the fold-increases were determined using the  $2^{\Delta\Delta CT}$  method, with the mRNA expression value of GAPDH as the endogenous control.

### Cytokine assay

The expression levels of various cytokines and pro-inflammatory markers (IL-2, IL-6, IL-9, TNF- $\alpha$ , CXCR4, and MCP-1) were assayed using their respective assay kits (Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China), as per the manufacturer's instructions.

### Statistical analysis

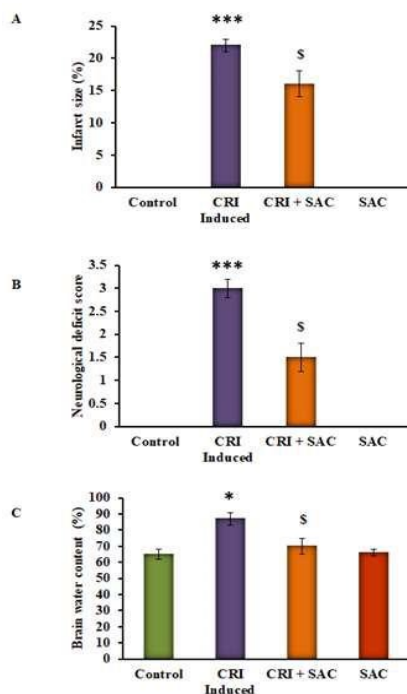
The results are presented as mean  $\pm$  standard error (n = 8). One-way analysis of variance was used. The study was done with GraphPad Prism program (GraphPad Software, San Diego, CA). Statistical significance was set at  $p < 0.05$ .

## RESULTS

The neurological deficit score of rats subjected to MCAO was considerably greater after 48 h of reperfusion than that of control rats. The results from TTC staining showed that CRI was effectively established. Furthermore, evaluation of the neurological deficit score revealed a defect in neurological performance. There was a high level of cerebral oedema in the CRI rats ( $p < 0.001$ ). On the other hand, rats pre-treated with SAC before CRI had significantly reduced infarct area and markedly low oedema, when compared with CRI rats ( $p < 0.001$ ). Moreover, the neurological deficit score of SAC-treated rats was significantly lower than that of rats subjected to MCAO ( $p < 0.05$ ). These findings indicate that SAC may improve neurological function following brain ischemia-reperfusion injury in rats. These results are shown in Figures 1 A - C.

**Table 1:** Sequences of primers used

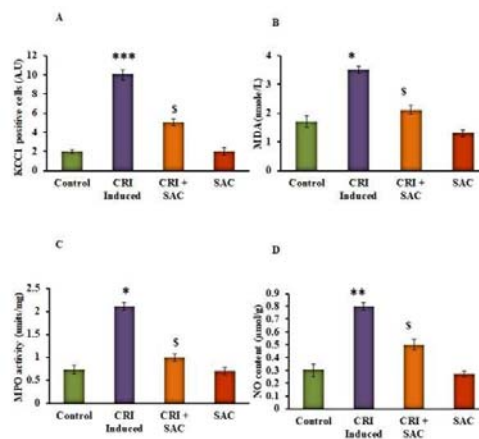
Primer	Forward (F) and reverse (R) (5' - 3')	Primer sequence	Annealing temperature (°C)
TLR4	F	ACCTCGAGTGGGAGGACAAT	59
	R	ATGGGTTTTAGGCGCAGAGT	
Syndecan-1	F	TTGAGTATGGCTGGCACTGG	59
	R	AGAGGCTGGCTACGTCTGTA	
CSF	F	ACTTTCTGCTCAAGGACGCA	57
	R	TGGGGATACCCAGAGAGTGG	
Aquaporin-1	F	ATTCATTGGGAGTGCCCTGG	56
	R	ATTACTGCCCCAGACAAGGC	
OCT3	F	CCTGTGCTCCCCTTAACCAG	58
	R	ACTAGAGGCCTCAGAGGTGG	
RFX1	F	GCCGCCTATACCTACAACCC	59
	R	CAGAAGCCACTGGAGCGTG	
GAPDH	F	AGTGCCAGCCTCGTCTCATA	59
	R	GATGGTGATGGGTTTCCCGT	



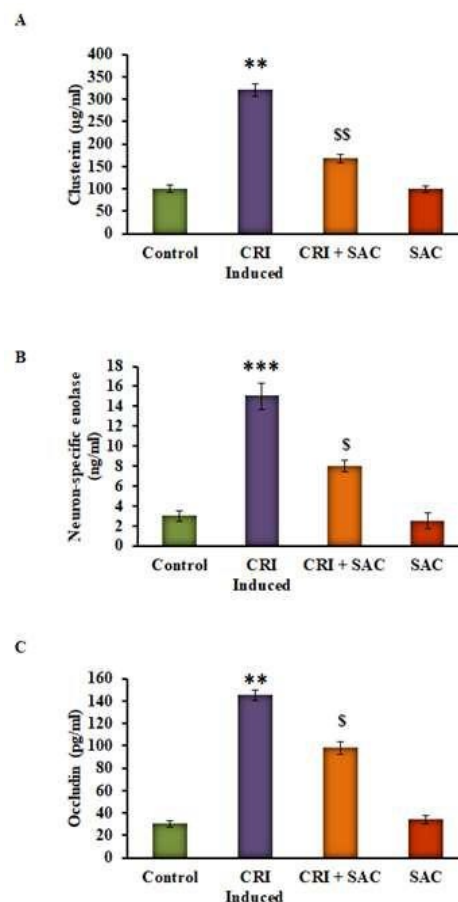
**Figure 1:** Infarct size (A), neuronal deficit score (B) and oedema level (C) of rats in the various groups. Data is stated as mean  $\pm$  SE. \* $P < 0.05$ , \*\*\* $p < 0.001$  (CRI compared to sham-controls); \$ $p < 0.05$  (SAC-pre-treated CRI rats compared with untreated CRI rats)

Figure 2 shows the protein expressions of KCC1 in control and CRI rats. Compared to control rats, the MACO rats had significant upregulations in the expressions of KCC1 in the plasma membrane and dendrites of neurons. In contrast, the expression of KCC1 was significantly downregulated in the SAC pre-treated rats. However, SAC did not change the level of protein expression of KCC1 in control rats. Furthermore, there were significant increases in the levels of MDA ( $p < 0.05$ ), MPO ( $p < 0.05$ ) and nitric oxide ( $p < 0.01$ ) in the CRI rats, when compared to control values. However, these increases were attenuated by SAC pre-treatment, resulting in significant mitigation of CRI-induced damage (Figure 2 A - D).

The expression levels of marker genes of CRI and related neuron responsive genes were assayed with ELISA, and the results are shown in Figure 3. The expressions of clusterin, neuron-specific enolase and occludin were significantly increased in CRI rats, relative to control. However, these increases were markedly reversed by SAC ( $p < 0.01$ ; Figure 3 A - C).



**Figure 2:** KCC1-positive cells (A), MDA levels (B), MPO activity (C), and nitric oxide (NO) levels (D) in the various rat groups. Data is stated as mean  $\pm$  SE. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (CRI compared to sham-control); \$ $p < 0.05$  (CRI rats pre-treated with SAC compared with untreated CRI rats)

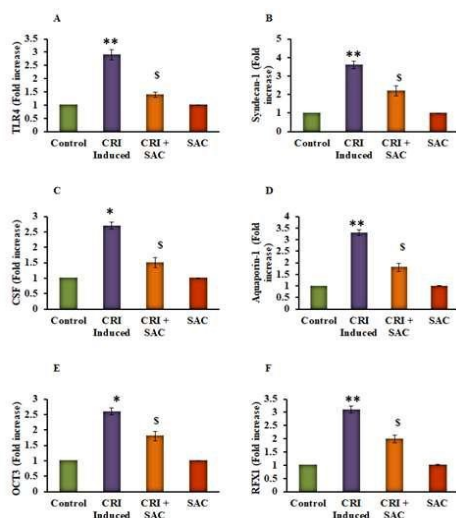


**Figure 3:** Expression levels of CRI markers in the various rat groups. A: clusterin; B: neuron-specific enolase, and (C): occludin. Data is stated as mean  $\pm$  SE. \*\* $P < 0.01$ , \*\*\* $p < 0.001$  (CRI compared to sham-control); \$ $p < 0.05$ , \$\$ $p < 0.01$  (CRI rats pre-treated with SAC compared with untreated CRI rats)

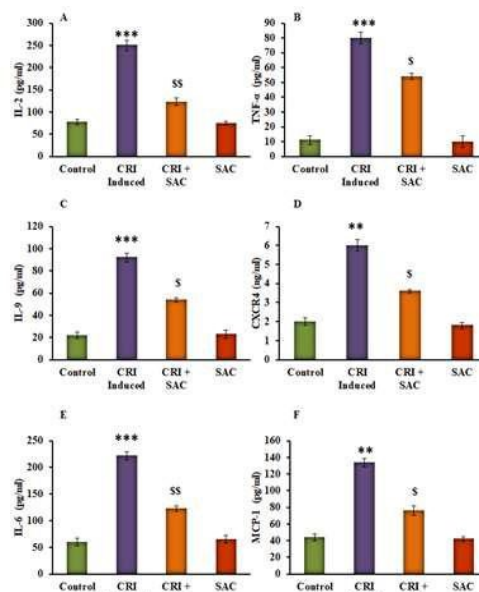
Figure 4 shows the mRNA expressions of genes inter-linked with CRI, as assayed using qRT-PCR. There were marked upregulations in mRNA expressions of TLR4, syndecan-1, CSF, aquaporin-1, OCT3, and RFX1 in CRI rats when compared to the corresponding expression levels in control rats. However, the mRNA expressions of these marker genes were downregulated by pre-treatment with SAC (Figure 4 A - F), indicating that the drug exerted a protective effect against CRI. Moreover, there were significant increases in the expression levels of the cytokines IL-2, TNF- $\alpha$ , IL-9, CXCR4, IL-6, and MCP-1 in CRI rats, when compared with control rats. However, the expressions of these inflammatory cytokines were decreased by SAC treatment (Figure 5 A - F). These results indicate that CRI occurs through inflammation-mediated cytokine recruitment, while SAC mitigated the CRI-induced inflammation.

## DISCUSSION

The influence of SAC on a rat model of cerebral ischemic reperfusion injury was determined. It was observed that SAC reduced oxidative damage and protected the rats from CRI-induced brain damage due to its antioxidant properties. Middle cerebral artery occlusion model is a perfect model of CRI because it is similar to a stroke in humans, and it affects behaviour and neuronal alterations in the brain through the generation of ROS.



**Figure 4:** mRNA expression level of CRI signaling genes normalized in the various groups. Data is stated as mean  $\pm$  SE. \* $P < 0.05$ , \*\* $p < 0.01$  (CRI rats compared with sham-controls); \$ $p < 0.05$  (CRI rats pre-treated with SAC compared with untreated CRI rats)



**Figure 5:** Expression levels of proinflammatory cytokines (IL-2, IL-6, IL-9, TNF- $\alpha$ , CXCR4, and MCP-1) in the control and experimental group of rats. Data is stated as mean  $\pm$  SE. \*\* $P < 0.01$ ,  $p < 0.001$  (CRI rats compared to sham-control); \$ $p < 0.05$ , \$\$ $p < 0.01$  (CRI rats pre-treated SAC compared with non-treated CRI rats)

However, treatment of the SCI rats with SAC resulted in attenuation of the reperfusion injury and improved recovery of the rats. Abnormalities in neurological function, brain oedema and morphological changes in brain tissue are features of an effective CRI model [9]. The infarct volume determines the severity of the ischemic stroke and subsequent brain lesions that result in neuronal impairment. Thus, neurologic symptom scores, brain water content, and cerebral infarction volume were measured in this study so as to ascertain that the CRI model was successfully established. The results confirmed that animals subjected to MCAO had increased neurological deficit scores and increased infarct volume.

An increase in brain water content may aggravate brain injury and exacerbate brain oedema arising from reperfusion [10]. Water accumulation in the brain and infiltration of inflammatory cytokines are due to vasogenic oedema which is as a result of disruption of blood brain barrier [11]. In this investigation, pre-treatment of rats with SAC prior to SCI resulted in a marked reduction in neurological score and volume of cerebral infarct. These results show that the CRI model is effective in animals. Moreover, the results demonstrate the protective effect of SAC against CRI.

When the supply of blood to the ischemic brain is restored, the oxygen supply is also enhanced, resulting in elevation of the concentrations of oxygen-free radicals. As a result, there is a build-up of oxidative stress which is a pathological event in CRI. This would increase the concentrations of superoxide anion, hydrogen peroxide and peroxynitrite [12]. These events might lead to increased cell damage and cell death through peroxidation of cell membrane lipids, elevated malondialdehyde concentration, increased activities of myeloperoxidases and raised nitric oxide levels, all of which are indicators of oxidative damage caused by the reperfusion process [11,13].

Another important mechanism that occurs after cerebral ischemia is the activation of KCC1, which is the K-Cl co-transporter that is expressed in brain tissues. This molecule transports Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> into cells. Due to ischemia and trauma, KCC1 expression in the brain cells are upregulated [14]. This results in increases in extracellular K<sup>+</sup> and intracellular Ca<sup>2+</sup> levels, and release of glutamate. Activation of KCC1 results in accumulation of Na<sup>+</sup> and Cl<sup>-</sup>, and K<sup>+</sup> uptake, leading to swelling of the brain astrocytes [15]. However, SAC decreased the activation of KCC1, thereby suppressing the accumulation of water in the brain and brain oedema. Consequently, the volume of cerebral infarct was reduced, thereby lowering CRI neurological deficit score.

In order to determine the extent of brain injury due to reperfusion, the levels of biomarkers expressed as a result of CRI were determined. These biomarkers increase the permeability of the brain to water and other inflammatory molecules. Neuron-specific enolase (NSE) which is specifically expressed during traumatic brain injury, has been linked to the severity of brain injury [16]. Positive expression of NSE in CRI animals could be an indication of neurodegeneration and neuronal cell death [17]. S-Allyl cysteine (SAC), a bioactive compound isolated from *Allium sativum*, has been shown to reduce neuronal cell death by reducing the expression of NSE in the brain. The significance of the protection exerted by SAC in reducing the expression of occludin in SAC-pre-treated cells is not due to its effect on the blood-brain barrier, but rather on its ability to decrease ROS arising from various processes as reported earlier [18].

Consistent with the observation in all CRI animal models, the CRI model in the present study also expressed high levels of clusterin in neuronal and astroglial cells [19]. This factor is produced as a result of high levels of ROS and they are

cytoprotective against neuronal injuries [20]. The reduced expression levels of clusterin and occludin in the SAC-pre-treated CRI rat model are indicators of neuronal cell recovery, as well as protective effect of SAC against CRI.

Toll-like receptor 4 (TLR4) is a critical molecule that is constitutively expressed in microglial cells during neuroinflammation and brain injury [21]. It acts through the nuclear translocation of NF-KB, and results in neuronal death and damage to the blood brain barrier. It has been demonstrated that SAC inhibits TLR4 and NF-KB, thereby protecting the cells from neuronal damage [18]. It is known that SAC reduces the expressions of MMP-2 and MMP-9 [22]. These two enzymes also disrupt the blood brain barrier. The formation of vasogenic oedema is a precursor to the reduction of aquaporin-1 expression which is associated with the movement of water through the blood brain barrier during the formation of cerebral oedema as a result of cerebral ischemia [11]. Aquaporin-1 expression is reduced by pre-treatment with SAC, probably through the NF-KB pathway, thereby resulting in cerebral oedema [23]. The level of regulatory factor X1 (RFX1) increases with the onset of ischemic injury after reperfusion, and it is crucial for neuronal survival, like other known transcription factors.

In this study, it was observed that SAC pre-treatment exerted an inhibitory role on CRI and activated the path of neuronal survival. Improvement in the treatment of cerebral ischemia occurred with SAC pre-treatment by effectively regulating the expression of OCT3, another transporter on the blood brain barrier. Histamine release is part of the regulation of anti-inflammatory properties. The expression of syndecan was down-regulated by SAC, indicating that the inhibitory effect of SAC on the inflammatory gene network involves the NF-KB pathway [24]. It has been speculated that SAC exerts its anti-inflammatory effect through a mechanism involving IL-2 and TNF- $\alpha$  which are part of the activating network of cyclooxygenase 2 (COX2). This would also have effects on the other pro-inflammatory mediators such as MCP-1, IL-6, IL-9 and CXCR4 in executing its neuroprotective effect against cerebral ischemia.

## CONCLUSION

This study has shown that S-allyl cysteine provides neuroprotection in CRI rats by attenuating oxidative stress and inhibiting the expressions of various NF-KB-controlled proinflammatory mediators. Thus, S-allyl cysteine is a therapeutic agent for CRI.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. XL, FW and LZ conducted the experiments, and QH designed the experiments and wrote the manuscript. All authors read and agree on the final MS.

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