Antihypertension effect of astragaloside IV during cerebral ischemia reperfusion in rats

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Received September 5, 2022; Accepted October 19, 2022

DOI: 10.3892/mmr.2022.12890

Abstract. Stroke is one of the leading causes of death from diseases. When the blood supply to the brain tissue is interrupted, neuronal core death occurs due to the lack of glucose and oxygen in min. Blood pressure lowering after ischemic stroke was proven to be an effective strategy to achieve neurovascular protection and reduce the risk of recurrent stroke. Astragaloside IV is a pure small molecular compound isolated from *Radix Astragali*, and it is well documented that astragaloside IV has neuroprotective effect on cerebral ischemia reperfusion (CIR) injury through many mechanisms, including antioxidant, anti-inflammatory and anti-apoptotic. The present study adopted mean arterial pressure (MAP) monitoring, neurological scoring, 2,3,5-triphenyltetrazolium chloride staining, enzyme-linked immuno-sorbent assay, western blotting and other experimental methods to investigate the effect of astragaloside IV on systemic blood pressure during CIR in a middle cerebral artery occlusion animal model. It was demonstrated that astragaloside IV pretreatment significantly alleviated CIR injury as previously reported. In addition, the elevation of MAP during CIR was significantly inhibited by astragaloside IV administration. Moreover, it was revealed that the expression of Na^+-K^+-$2Cl^-$ cotransporter isoform 1 in the hypothalamus was inhibited and the subsequent synthesis of vasopressin was reduced by astragaloside IV pretreatment in the CIR animal model. In conclusion, astragaloside IV may alleviate CIR injury partially by lowering systemic blood pressure.

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Key words: cerebral ischemia reperfusion, blood pressure, hypothalamus, vasopressin, Na^+-K^+-$2Cl^-$ cotransporter isoform 1, astragaloside IV

Introduction

Stroke is one of the leading causes of death from diseases. According to the Heart Disease and Stroke Statistics report from the American Heart Association (2019 updated), more than 10 million people are affected by stroke every year (1). A stroke occurs due to cerebrovascular abnormalities, of which 87% are ischemic stroke (2). When the blood supply to the brain tissue is interrupted, neuronal core death occurs due to the lack of glucose and oxygen in min (3).

Thrombolytic therapy is a standard strategy for clinical treatment for ischemic stroke (4). However, brain damage is always inevitable, even if the blood flow is restored, and it may leave a long-term disability (5). Previous studies revealed that the severity of cerebral edema predicted long-term functional outcomes (6,7). Cerebral edema occurs immediately after ischemia, reaches its peak at 15 min and continues with the vascular obstruction (8). With prolonged ischemia status, the blood-brain barrier (BBB) will collapse, and the fluid from the blood will enter the cerebral interstitial space, forming a later phase of edema that is well known as vasogenic edema. Cerebral edema cannot be relieved or even worsened after blood flow recovery (9). Therefore, controlling the sudden increase in cerebral blood flow may relieve cerebral edema in addition to reperfusion-induced hemorrhage (10). Since systemic blood pressure regulates blood flow distribution in the body, it is also important to control systemic blood pressure in the treatment of cerebral ischemia. A recent meta-analysis has shown that receiving blood pressure medicine, such as angiotensin-converting enzyme inhibitors or diuretics, will reduce the risk of recurrent stroke from transient ischemic attack (11). An animal study also proved that blood pressure lowering after reperfusion in acute ischemia may protect against neurovascular damage (12).

Astragaloside IV is a pure small molecular compound isolated from *Radix Astragali*, and it is well documented that astragaloside IV has neuroprotective effect on cerebral

ischemia reperfusion (CIR) injury through numerous mechanisms, including antioxidant, anti-inflammatory and anti-apoptotic (13). Moreover, the antihypertension effect of astragaloside IV has been proven in different hypertension animal models (14,15). However, such an effect during CIR remains unclear. Thus, the aim of the present study was to investigate the effect of astragaloside IV on systemic blood pressure during CIR in a middle cerebral artery occlusion (MCAO) animal model.

Materials and methods

Animals and group assignments. The experimental procedures in the present study were in accordance with the ARRIVE (Animals in Research: Reporting *In Vivo* Experiments) guidelines and were approved (approval no. 20210304037) by the Animal Care and Use Committee of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Male Sprague-Dawley rats (8-10 weeks old; 250-300 g; Experimental Animal Center affiliated with Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) were kept in a clean animal room, with free access to food and water (12 h:12 h light/dark cycle, 22±1˚C, 50% humidity). The rats were randomly assigned to 5 groups: Sham, the group of rats with sham operation; CIR, the group of rats with MCAO; Saline, the group of rats with MCAO and saline pretreatment; Ast, the group of rats with MCAO and astragaloside IV pretreatment; Bum, the group of rats with MCAO and bumetanide pretreatment. The animals with tissue sampling were anesthetized by sodium pentobarbital (20 mg/ml, i.p.), and the rest were sacrificed by carbon dioxide asphyxiation (flow rate=70% of the cage volume per min). Finally, the valid data was produced from a total of 86 rats. All possible efforts were made to minimize the number of animals and their suffering.

CIR modelling. Rats were first subjected to 5% isoflurane (RWD Life Science) in 30% O_2 balanced with N_2O, then with 1.5% isoflurane for anesthesia maintenance. Cerebral blood flow was monitored in the territory of the middle cerebral artery using a laser doppler flowmetry (RWD Life Science). Thereafter, the right common carotid artery, internal carotid artery and external carotid artery of individual rats were exposed. Focal ischemia was induced by inserting a monofilament nylon suture with a round tip (Yuyan Instruments Co., Ltd.) inserted into the internal carotid artery through the external carotid artery stump and gently advanced to the middle cerebral artery. After 30 min of ischemia, the filament was removed to restore blood flow (reperfusion) and the skin incision was sutured. Sham-operated control rats received the same surgical procedure without insertion of a filament. Rats with intracranial hemorrhage and those that did not show a reduction in cerebral blood flow >80% during MCAO were excluded.

Drug administration. Astragaloside IV (Shanghai Yuanye Bio-Technology Co., Ltd.) was diluted in corn oil. Rats were treated with astragaloside IV (7.5 mg/ml) or saline via intraperitoneal injection 15 min before surgery. Bumetanide (0.18 mg/ml, 2 μl, 0.25 μl/min; cat. no. B3023; MilliporeSigma) was microinjected into bilateral paraventricular [AP 1.5 mm, ML 0.4 mm, DV 7.7 mm (16)] with a microinjection pump (Yuyan Instruments Co. Ltd.).

Mean arterial pressure (MAP) monitoring. Tail cuff blood pressure system (IITC Life Science Inc.) was used for the measurement of MAP. First, the rats were sufficiently acclimated to the restraint holder, and each rat was separated by an opaque partition. Then the tail was warmed with a warming pad for 15-20 min (until the rat was no longer irritable) before each cycle of blood pressure measurements. The final data was obtained from the average of 10 valid data. The data of animals those could not cooperate well in the experiment were eliminated.

Neurologic score. To estimate the degree of neurological impairment, a 48-point scoring system was used. This scoring system composes of general status (spontaneous activity, body symmetry, gait; 0-12), simple motor deficit (forelimb asymmetry, circling, hind-limb placement; 0-14), complex motor deficit (vertical screen climbing, beam walking; 0-8), and sensory deficit (hind limb, trunk, vibrissae and face touch; 0-14). The total score was the sum of the 4 individual scores, with 0=no deficit and 48=maximal deficit.

2,3,5-triphenyltetrazolium chloride (TTC) staining. Rats were euthanized on indicated time point. The brain sections were placed in 1% TTC (cat. no. 298-96-4; MilliporeSigma) at 37˚C for 30 min. The slices were flipped once every 5 min and then washed 3 times with ddH_2O. Images of the sections were captured with a digital camera. The area of infarct of each section (1 mm) was measured by subtracting the non-infarcted area in the ipsilateral hemisphere from the total area of the contralateral hemisphere, and then the final infarct volume was calculated by summing the infarct areas in all sections and multiplying by the section thickness (ImageJ v1.53e; National Institutes of Health).

Enzyme-linked immuno-sorbent assay (ELISA). To detect the level of arginine vasopressin (AVP), rat pituitary tissue was isolated, and dissolved with homogenizing medium (1:9). The sample was centrifuged at 3,500 x g for 10 min, and the supernatant was harvested. Diluted supernatant was added to the AVP ELISA kit (Rat) (cat. no. OKCD08532; Aviva Systems Biology, Corp.). Then, the plate was sealed with sealing film and incubated at 37˚C for 30 min. The sealing plate film was carefully removed and the liquid was discarded. The plate was washed for 5 times. Then, labeling reagents (50 μl) were added into each well (except blank). The incubation and washing process were repeated. Chromogenic agent A and B (50 μl) was added into each well in sequence. After 10 min coloration at 37˚C, 50 μl termination solution was added to each well. The absorbance of each well was measured at the wavelength of 450 nm.

Western blotting. The paraventricular nucleus (PVN) tissue was homogenized in the lysis buffer (10 mM Tris-HCl and 320 mM sucrose) containing 1% protease inhibitor mixture (cat. no. 36978; Thermo Fisher Scientific) and then centrifuged (Eppendorf 5810) at 8,000 x g for 5 min. Then the supernatant was centrifuged (Eppendorf 5810) at 40,000 x g for 30 min to obtain crude membrane in the pellet. The cellular membranes were resuspended in lysis buffer with protease inhibitor mixture. Protein concentrations were determined using Bio-Rad protein reagent (Bio-Rad Laboratories, Inc.). Samples were heated at 95˚C for 10 min in loading buffer before 20 μg

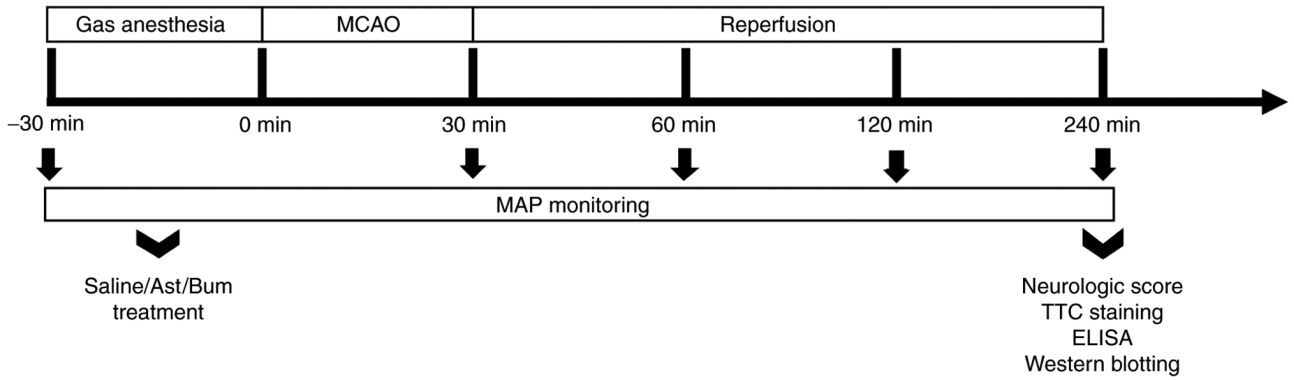

Figure 1. Schematic of experimental process. MCAO, middle cerebral artery occlusion; MAP, mean arterial pressure; Saline, the group of rats with MCAO and saline pretreatment; Ast, the group of rats with MCAO and Astragaloside IV pretreatment; Bum, the group of rats with MCAO and bumetanide pretreatment. TTC, 2,3,5-triphenyltetrazolium chloride; ELISA, enzyme linked immunosorbent assay.

protein was loaded for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically separated, and then transferred to polyvinylidene difluoride membranes at 4˚C. After blocking with 5% non-fat milk for 2 h at room temperature, membranes were incubated overnight at 4˚C with a primary antibody [rabbit anti-Na^+-K^+-$2Cl^-$ cotransporter isoform 1 (NKCC1) (1:1,000; cat. no. 4828S; Cell Signaling Technology, Inc.)]. Membranes were washed with PBS buffer, incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG (1:2,000; cat. no. ab6721; Abcam) for 2 h at room temperature, and the bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0050; MilliporeSigma). The relative levels of the target protein were determined by performing a densitometry analysis using ImageJ v1.53e software (National Institutes of Health).

Statistical analysis. SigmaStat 3.5 (Jandel Scientific Software) was used to perform the statistical analyses. The data were expressed as the mean ± SD. Student's t-test (independent t-test) was used when two groups were compared. The MAP data were analyzed by using one-way repeated-measures ANOVA or two-way repeated-measures ANOVA followed by the Bonferroni post hoc test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

CIR induces brain damage and MAP elevation in rats. To reduce the mortality of the animals, the middle cerebral artery of the rat was occluded for only 30 min, and the subsequent reperfusion lasted 210 min in the present study (Fig. 1). The results showed significant neurologic dysfunction (Sham vs. CIR=0 vs. 22.60±5.07, n=5 per group, Student's t-test, P<0.001, Fig. 2A) and increased infarct volume (Sham vs. CIR=0 mm^3 vs. 130.13±27.08 mm^3, n=5 per group, Student's t-test, P<0.001, Fig. 2B and C) after CIR. Meanwhile, the MAP significantly elevated, and prolonged at least for 210 min (n=10 per group, two-way repeated-measures ANOVA followed by the Bonferroni post hoc test, P<0.001, Fig. 2D, Table I). These results indicated that CIR induces a long-term MAP elevation.

Astragaloside IV relieves CIR-induced brain damage and MAP elevation. It is consistent with most of previous findings that astragaloside IV pretreatment relieved the neurologic dysfunction of rats induced by CIR (Saline vs. Ast=23.67±4.55 vs. 14.33±4.46, n=6 per group, Student's t-test, P=0.005, Fig. 3A) and reduced infarct volume (Saline vs. Ast=173.46±60.97 mm^3 vs. 74.97±35.05 mm^3, n=6 per group, Student's t-test, P=0.006, Fig. 3B and C). Moreover, the rats treated with astragaloside IV showed a milder fluctuation of MAP (n=10, one-way repeated-measures ANOVA followed by the Holm-Sidak method, P<0.001 compared with the baseline before modeling, Fig. 3D, Table I). These results indicated that astragaloside IV relieves CIR-induced MAP elevation.

Upregulation of NKCC1 in hypothalamus-mediated MAP elevation during CIR. AVP, also called antidiuretic hormone, is synthesized in the PVN of the hypothalamus and stored in the pituitary gland. It is released into the blood circulatory system to regulate systemic blood pressure when needed (17). It has been demonstrated that the upregulation of NKCC1 in AVP neurons contributes to the generation of sodium-dependent hypertension by increasing AVP release (18). One of the main functions of NKCC1 is to transport Cl^- into cells, increasing the concentration of intracellular Cl^-, thereby weakening GABAergic inhibition and even turning it into depolarization (19,20). Therefore, the upregulation of NKCC1 in hypothalamus may promote the excitability of hypothalamic neurons, thus increasing the synthesis and secretion of AVP.

The present results showed that CIR significantly increased the AVP level (ng) in the pituitary gland (g) (Sham vs. CIR=18.12±2.25 ng/g vs. 48.64±9.06 ng/g, n=6 per group, Student's t-test, P<0.001, Fig. 4A) and the expression level of NKCC1 in the PVN (Sham vs. CIR=1.00±0.19 vs. 3.61±1.30, n=6 per group, Student's t-test, P<0.001, Fig. 4B). To investigate the contribution of NKCC1 to generating MAP elevation, bumetanide, an NKCC1 inhibitor, was delivered by paraventricular microinjection. It was found that bumetanide pretreatment significantly reduced the MAP during CIR (n=10, one-way repeated-measures ANOVA followed by the Holm-Sidak method, P<0.001 compared with the baseline before modeling, Fig. 4C, Table I). These results indicated

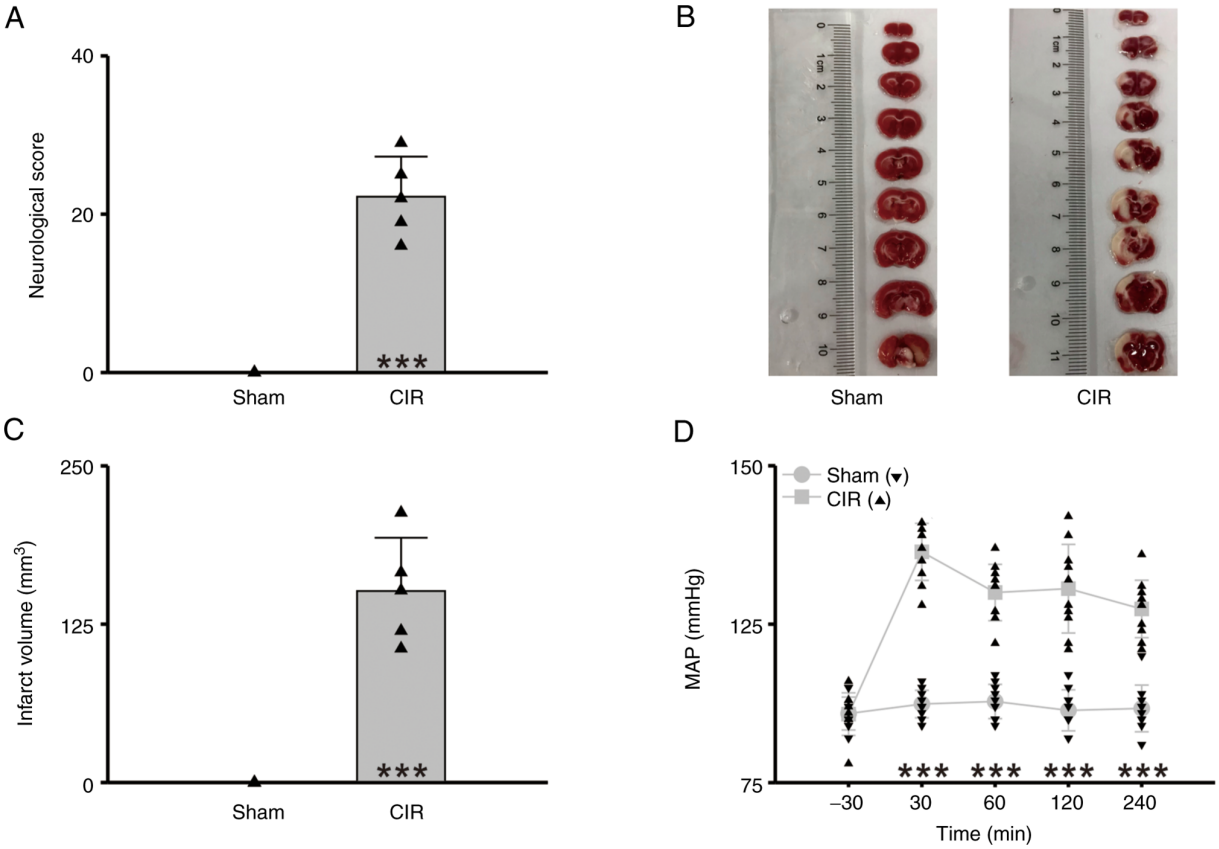

Figure 2. CIR-induced brain damage and hypertension. (A) Statistical chart of neurologic score (n=5 per group, Student's t-test, ***P<0.001). (B) 2,3,5-triphenyltetrazolium chloride-stained brain tissue specimens. (C) Statistical chart of infarct volume (n=5 per group, Student's t-test, ***P<0.001). (D) Statistical chart of MAP (n=10 per group, two-way repeated-measures ANOVA followed by the Bonferroni post hoc test, ***P<0.001, Sham vs. CIR at the same time point). Sham, the group of rats with sham operation; CIR, the group of rats with CIR. Triangles indicate the individual data obtained from each group. CIR, cerebral ischemia reperfusion; MAP, mean arterial pressure.

that upregulation of NKCC1 in hypothalamus mediates MAP elevation during CIR.

Astragaloside IV inhibits NKCC1 expression in hypothalamus of MCAO rats. The results of the present study showed that astragaloside IV intraperitoneal injection significantly inhibited the NKCC1 expression in PVN of MCAO rats (Saline vs. Ast=1.00±0.32 vs. 0.43±0.15, n=6 per group, Student's t-test, P=0.002, Fig. 5A). Moreover, the AVP level in pituitary gland was also reduced. (Saline vs. Ast=49.05±9.59 ng/g vs. 31.89±6.45 ng/g, n=6 per group, Student's t-test, P=0.005, Fig. 5B). These results indicated that astragaloside IV inhibits NKCC1 expression in hypothalamus of MCAO rats.

Discussion

The human brain accounts for 15-20% of the cardiac output and is extremely sensitive to ischemia. Blood pressure is one of the important vital signs of the human body. As a driving force to promote the blood flow, blood pressure ensures the blood supply of important organs. When ischemia occurs in tissues or organs, the human body may activate the autoregulation system (heart rate, cardiac output, blood pressure) to maintain normal hemodynamics (21). Thus, the elevation of blood pressure after cerebral ischemia is very common, and blood pressure lowering is an important strategy to improve functional outcomes after ischemic stroke (22,23). It has been reported that the MAP increased immediately upon MCAO and remained elevated after 24 h of reperfusion (12). Prolonged elevation of MAP in the MCAO model was also revealed in the present study. Such prolonged hypertensive state may worsen the cerebral edema, thereby aggravating the reperfusion injury (24).

AVP elevates blood pressure mainly by promoting water reabsorption, blood volume and the contraction of vascular smooth muscle (25,26). It has been reported that cerebral ischemia may increase the AVP level of plasma (27-29). The current study showed that ischemia reperfusion led to the increase of AVP in rat pituitary. Meanwhile, the expression of NKCC1 was upregulated in PVN. The results of the present study also showed that the increase of MAP during CIR could be significantly reduced when NKCC1 is interfered by pretreatment of bumetanide. These results indicated that CIR drives the occurrence of central hypertension.

In traditional Chinese medicine, the stroke is described as a series of symptoms (abrupt coma, paraesthesia, hemiplegia, facial paralysis, speech disorder) induced by obstruction of cerebral meridians (ischemic stroke) or blood spill over from cerebral meridians (hemorrhagic stroke). The most important pathogenesis of ischemic stroke is the decline of vital Qi (energy) with aging or prolonged illness. *Radix Astragali* (Huang Qi) is a commonly used herbal

Table I. Mean arterial pressure (Sham vs. CIR).

Group	-30 min	30 min	60 min	120 min	240 min
Sham (mmHg)	85.90±2.60	87.40±2.17	87.80±2.70	85.80±3.36	86.70±3.68
CIR (mmHg)	86.40±3.24	111.40±4.50	105.00±4.45	105.60±7.00	102.40±4.53
Ast (mmHg)	86.80±3.08	102.10±6.23	88.60±4.60	87.80±4.59	89.30±3.40
Bum (mmHg)	86.10±3.48	98.50±6.29	88.50±4.12	85.70±4.50	87.30±6.00

CIR, cerebral ischemia reperfusion; Ast, the group of rats with MCAO and Astragaloside IV pretreatment; Bum, the group of rats with MCAO and bumetanide pretreatment.

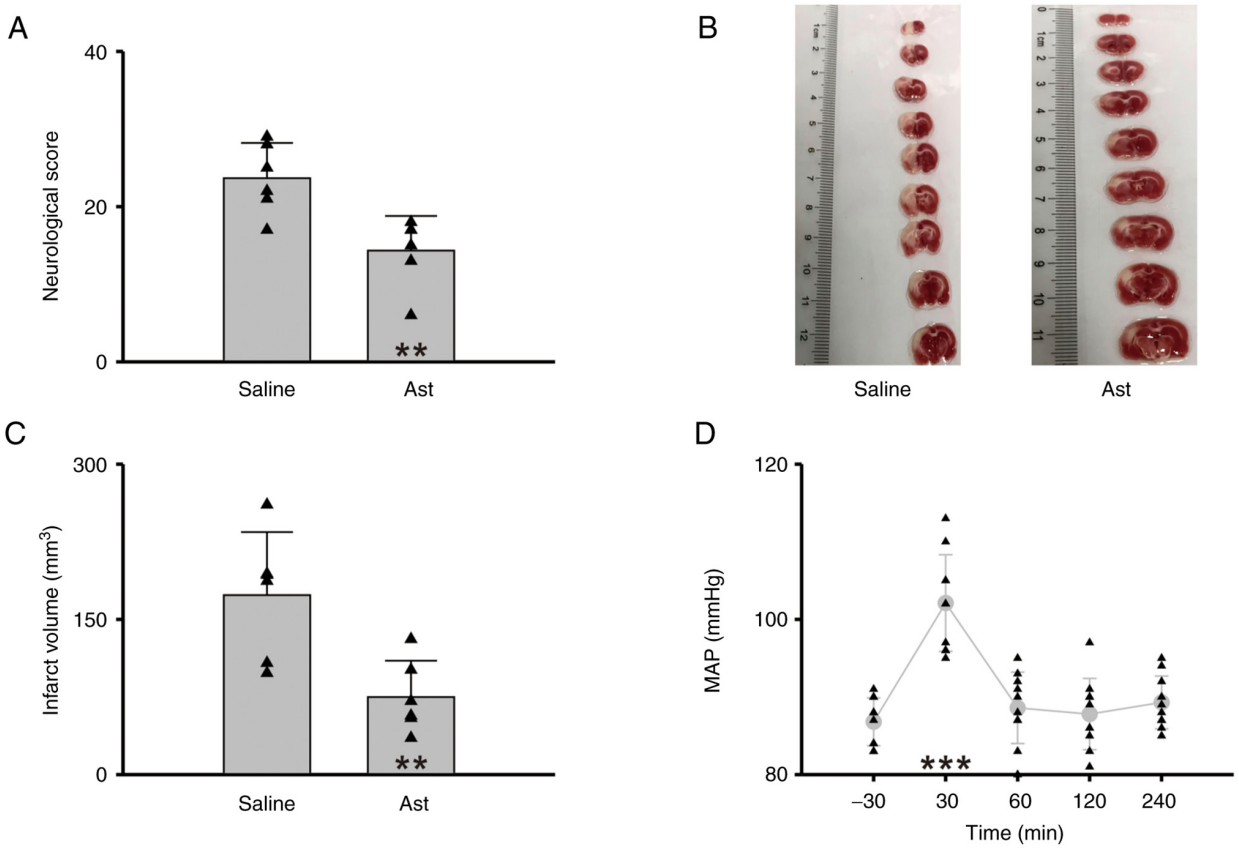

Figure 3. Astragaloside IV relieves CIR-induced brain damage and hypertension. (A) Statistical chart of neurologic score (n=6 per group, Student's t-test, **P<0.01). (B) 2,3,5-triphenyltetrazolium chloride-stained brain tissue specimens. (C) Statistical chart of infarct volume (n=6 per group, Student's t-test, **P<0.01). (D) Statistical chart of MAP (n=10, one-way repeated-measures ANOVA followed by the Holm-Sidak method, ***P<0.001, compared with the baseline before modeling). Saline, the group of rats with CIR and saline pretreatment; Ast, the group of rats with CIR and Astragaloside IV pretreatment. Triangles indicate the individual data obtained from each group. CIR, cerebral ischemia reperfusion; MAP, mean arterial pressure.

medicine in China, that enriches the primordial Qi, nourishes the heart and dredges the pulse, fortifies the spleen and disinhibits the dampness according to the theory of traditional Chinese medicine. The main active components of *Radix Astragali* are polysaccharides and astragalosides (30). Astragaloside contains four subtypes (I-IV), among which astragaloside IV has the best biological activity (31,32). Due to the multiple target advantages of astragaloside IV, its preparation is widely used in the adjuvant treatment of cardiovascular and cerebrovascular diseases (33,34). It has the effects of improving immunity, reducing stress reaction, antivirus, anti-inflammation, analgesia, organ protection and promoting metabolism (31,32). In addition, astragaloside IV has the effect of regulating blood pressure (35,36).

It is consistent with previous findings that astragaloside IV had an obvious preventive effect on CIR injury. Meanwhile, the rats treated with astragaloside IV showed a milder fluctuation of MAP. Those results indicated that the inhibitory effect of astragaloside IV on blood pressure may contribute to alleviate ischemia reperfusion injury to a certain extent. In addition, pretreatment with astragaloside IV inhibited the expression of NKCC1 in PVN nuclei and the level of AVP in pituitary of CIR rats. However, in the present study, it was not clear whether the antihypertensive effect of astragaloside IV directly acts on the central system or peripheral.

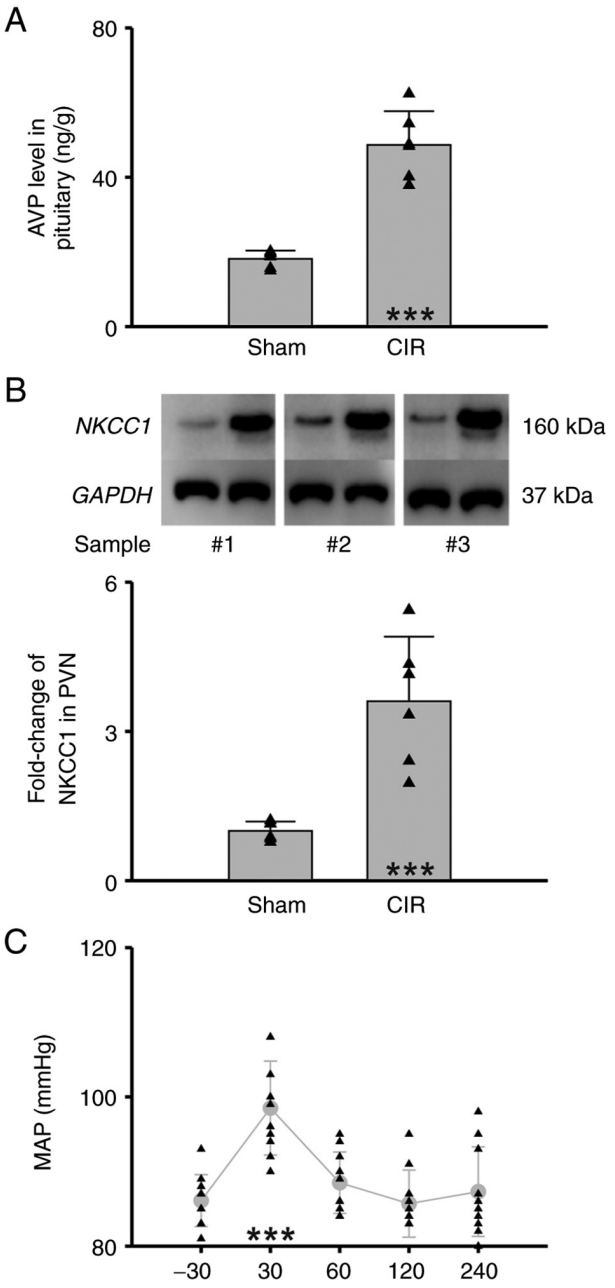

Figure 4. Upregulation of NKCC1 in hypothalamus-mediated hypertension during CIR. (A) Statistical chart of AVP level in pituitary (n=6 per group, Student's t-test, ***P<0.001). (B) Statistical chart of NKCC1 level in hypothalamus. Upper panel represents three pairs of western blot samples (n=6 per group, Student's t-test, ***P<0.001). (C) Statistical chart of MAP with bumetanide pretreatment (n=10, one-way repeated-measures ANOVA followed by the Holm-Sidak method, ***P<0.001, compared with the baseline before modeling). Sham, the group of rats with sham operation; CIR, the group of rats with CIR. Triangles indicate the individual data obtained from each group. NKCC1, Na^+-K^+-$2Cl^-$ cotransporter isoform 1; CIR, cerebral ischemia reperfusion; AVP, arginine vasopressin; MAP, mean arterial pressure.

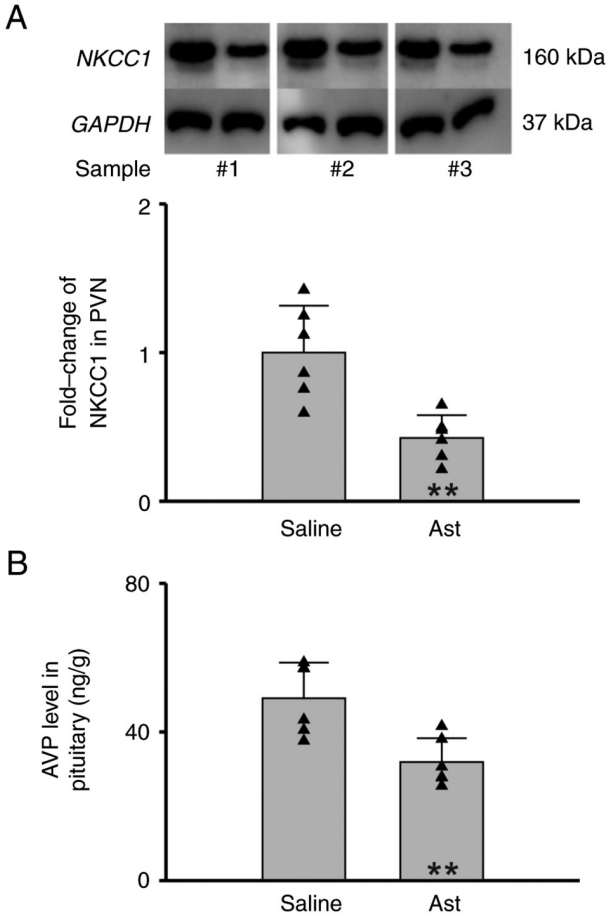

Figure 5. Astragaloside IV inhibits NKCC1 expression in hypothalamus during CIR. (A) Statistical chart of NKCC1 level in hypothalamus. Upper panel represents three pairs of western blot samples (n=6 per group, Student's t-test, **P<0.01). (B) Statistical chart of AVP level in pituitary (n=6 per group, Student's t-test, **P<0.01). Saline, the group of rats with CIR and saline pretreatment; Ast, the group of rats with CIR and Astragaloside IV pretreatment. Triangles indicate the individual data obtained from each group. NKCC1, Na^+-K^+-$2Cl^-$ cotransporter isoform 1; CIR, cerebral ischemia reperfusion; AVP, arginine vasopressin.

It was considered that there are two possible mechanisms for astragaloside IV regulating blood pressure during CIR. According to the data provided by Traditional Chinese Medicine Systems Pharmacology database and analysis platform, astragaloside IV is BBB non-penetrable, which may have limited effect on the central nervous system. It has been reported that astragaloside IV relieved hypertension via improving inflammation, pulmonary artery remodelling and oxidative stress (35,36). Then the decrease of NKCC1 expression may be due to the stable blood pressure after CIR that led to the reduction of AVP demand. Another possibility is that the permeability of BBB will increase since its structure is damaged after ischemic stroke (37,38). Then astragaloside IV is likely to have opportunity to act on the central nervous system. However, in any case, it will not affect the contribution of antihypertension effect to the alleviation of CIR by astragaloside IV. Therefore, a possible mechanism for astragaloside IV to reduce CIR injury was proposed in the present study. Further investigation is needed to explore the integrated mechanism of CIR-induced central hypertension and the specific target of astragaloside IV.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Foundation of Shanghai Municipal Science and Technology Commission

(grant no. 19ZR1407500) and the Shenzhen Traditional Chinese Medicine Hospital '3030 Program' Chinese Medicine Clinical Research Project (grant no. 3030202108).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FS, JH and LW designed the research. FS, YM and YH conducted the experiments. FS, YM and BH analyzed the data. JH and LW confirm the authenticity of all the raw data. FS, JH and LW prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental procedures in the present study were in accordance with the ARRIVE (Animals in Research: Reporting *In Vivo* Experiments) guidelines and were approved (approval no. 20210304037) by the Animal Care and Use Committee of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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