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ORIGINAL ARTICLE

Astragali Radix Reduces Ischemia-Induced Brain Injury by Inhibiting Edema and Expression of Aquaporin-4 in Mice

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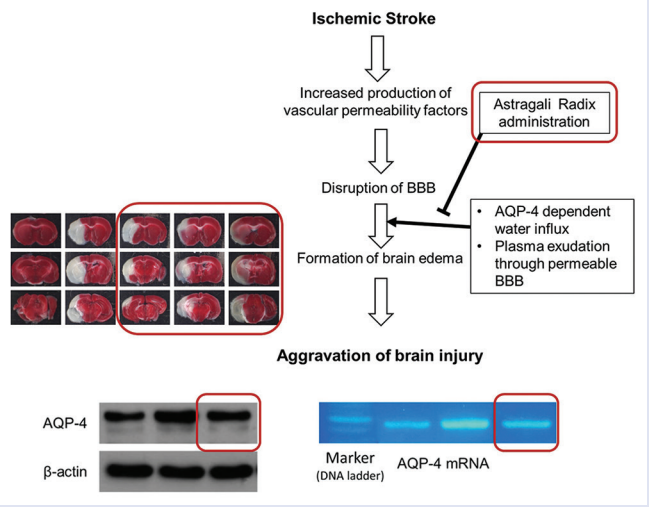
ABSTRACT

Background: Astragali Radix, the dried root of *Astragalus membranaceus* Bunge, is a widely used herbal and traditional medicine and is viewed as a primary energizing medicinal herb that invigorates vital energy. **Objective:** We investigated the effects of Astragali Radix administered on ischemia-induced brain injury in a mouse model. **Materials and Methods:** Ischemic brain injury was induced by middle cerebral artery occlusion (MCAO) for 2 h. Methanol extracts of Astragali Radix (Amex) were then orally administered daily for 3 days. Relative cerebral blood flow was measured under ischemic conditions. Infarct volumes were measured by triphenyltetrazolium chloride staining. Changes in brain edema, brain water content, and neurological deficit (ND) scores were also measured. Aquaporin-4 (AQP-4) protein and mRNA expression were evaluated and histological changes visualized by staining with hematoxylin and eosin or cresyl violet. **Results:** Oral administration of Amex for 3 days after MCAO significantly reduced brain infarct volumes, edema indices, and water contents and suppressed the expression of AQP-4 at the protein and mRNA levels. However, MCAO-induced increases in ND scores were not ameliorated by Amex. **Conclusion:** Oral administration of Amex following onset of brain injury reduced infarct volume and brain edema. Our results suggest that reduction of AQP-4 protein and mRNA expression is a possible mechanism for these effects.

Key words: Aquaporin-4, Astragali Radix, blood–brain barrier, brain edema, ischemic injury, middle cerebral artery occlusion

SUMMARY

- Brain edema is the result of several medical conditions, such as ischemic stroke, traumatic brain injury, and hyponatremia
- Induction of stroke in mice by ischemic injury resulted in brain damage and brain edema, and administration of methanol extracts of Astragali Radix (Amex) suppressed brain tissue damage and brain edema
- Amex treatment inhibited aquaporin-4 overexpression after reperfusion, suppressing water accumulation in the brain caused by degranulation of aquaporin-4
- Further studies should be conducted to examine the long-term effects of Amex.



Abbreviations used: r-tPA: Recombinant tissue plasminogen activator; BBB: Blood–brain barrier; Nrf2: Nuclear factor erythroid 2-related factor 2; Foxp3: Forkhead box P3; VEGF: Vascular endothelial growth factor; MMP-9: Matrix metallopeptidase-9; TRPC6: Transient receptor potential cation channel subfamily C member 6; p-CREB: Phospho-cyclic AMP response element binding; JNK3: C-Jun N-terminal kinase 3; SPF: Specific-pathogen-free; DMSO: Dimethyl sulfoxide; TTC: 2,3,5-triphenyltetrazolium chloride; PBS: Phosphate-buffered saline; PFA: Paraformaldehyde; OCT: Optimum cutting temperature; AQP-4: Aquaporin-4; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gels by electrophoresis; PVDF: Polyvinylidene difluoride; RT-PCR: Reverse transcription polymerase chain reaction.

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INTRODUCTION

Ischemic stroke results in disability and increased risk of morbidity, resulting in a massive burden on patients and caregivers. Currently, recombinant tissue plasminogen activator (r-tPA) is the only therapeutic agent approved by the US Food and Drug Administration for the treatment of acute ischemic stroke. However, due to side effects, which include hemorrhage and the limited time frame in which r-tPA administration is effective, its clinical applications are

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limited.[1-3] Therefore, safer and more effective therapeutic strategies are required.

Astragali Radix, the dried root of *Astragalus membranaceus* (Fisch.) Bunge, is one of the most widely used herbal medicines and is commercially cultivated in China and Korea.[4,5] Astragali Radix (known as Hwang-gi in Korean) is a primary energizing medicinal herb that is used to invigorate vital energy. It is frequently used to treat various age-related symptoms, such as immune system disorders, menopause, neuropathy, nephropathy, fatigue, hepatic injury, and cancer.[5-10]

The chemical constituents of Astragali Radix include astragalosides, calycosin, kumatakenin, formononetin, and their respective glycosides and malonates.[11] Astragaloside IV, a major active triterpenoid in Astragali Radix, has anticancer and neuroprotective effects.[12,13] Li *et al.* reported that astragaloside IV protected blood–brain barrier (BBB) integrity by activating the nuclear factor erythroid 2-related factor 2 signaling pathway.[14] Astragaloside IV has also been reported to alleviate behavioral deficiencies in an animal model of Parkinson's disease.[15] Calycosin is known to exert anticancer effects which are thought to occur by targeting the forkhead box P3 (Foxp3)-mediated expression of vascular endothelial growth factor and matrix metallopeptidase-9 (MMP-9).[16] Recently, calycosin was reported to exhibit neuroprotective effects against ischemia-induced cerebral injury by increasing the expression of transient receptor potential cation channel subfamily C member 6 and phospho-cyclic AMP response element-binding protein and by inhibiting calpain activation.[3] Song *et al.* reported that calycosin improved cognitive function by mitigating oxidative stress and inflammatory responses in a mouse model of Alzheimer's disease.[17] Fei *et al.* reported that formononetin ameliorated learning and memory deficits by suppressing the amyloid-beta clearance pathway.[18]

Liu *et al.* reported that Astragali Radix extract administered intraperitoneally to rats with ischemic injury suppressed c-Jun N-terminal kinase 3 gene expression, resulting in reduced infarct volumes and less neurobehavioral deficits.[19] Thus, Astragali Radix has therapeutic potential for the treatment of ischemic stroke. However, mechanisms responsible for the therapeutic effects of Amex are not fully explained by its neuroprotective properties. Further evaluation of the protective mechanisms of orally administered Amex is required.

In our preliminary study, pretreatment with methanol extracts of Astragali Radix (Amex) protected mice against ischemia-induced brain damage and brain edema. Thus, this study was conducted to investigate the therapeutic effects of Astragali Radix administered after ischemia-induced brain injury in a mouse model.

MATERIALS AND METHODS

Ischemic stroke model and Astragali Radix treatment

Male adult specific-pathogen-free C57BL/6 mice (22–25 g) were obtained from Daehan Biolink Co. (Chungbuk, Korea). Animals were housed in a temperature/humidity-controlled animal facility under a 12-h light/dark cycle and provided food and water *ad libitum*. All animal experiments were approved and regulated by the Ethics Committee of Pusan National University (PNU) (approval number PNU-2016-1087; date of approval, February 26, 2016).

Ischemic stroke was induced by middle cerebral artery occlusion (MCAO) as previously described.[20] Briefly, mice were anesthetized with 2% isoflurane and maintained using 1.5% isoflurane in a 70% N_2O and 30% O_2 mixture. The left common carotid artery was isolated, and an 11-mm-long 8-0 monofilament nylon suture (Ethicon Inc., NJ, USA) was inserted and advanced into the internal carotid artery (ICA) to occlude the MCA. The suture was removed after 2 h of MCAO to allow for reperfusion [Figure 1S]. After surgery, animals were transferred to a cage and maintained at 37°C for 30 min.

Astragali Radix was purchased from Naemomedah (Ulsan, Korea) and authenticated by Dr. Cho (PNU School of Korean Medicine). A voucher specimen (No. AM16-0509) was deposited at the low-temperature (4°C) room of a laboratory at the PNU School of Korean Medicine.

Amex were prepared by adding 500 g of the dried ground roots of Astragali Radix to methanol (2,000 mL). This mixture was left at room temperature (25°C) for 3 days, and supernatant was collected. This process was repeated three times. The combined supernatants were vacuum evaporated and freeze-dried yielding 64.2 g (12.8% recovery) of Amex, which was stored in microtubes at −20°C until use. To validate the quality of Amex, fingerprinting data of Amex were obtained using high-performance liquid chromatography [Figure 2S].

Amex were prepared for administration to mice by dissolution in dimethyl sulfoxide (DMSO). This solution was diluted with 0.9% normal saline, passed through a 0.45-μm pore size syringe filter, and adjusted to concentrations of 100, 300, and 1000 mg/kg.

Mice were divided into five groups of six mice: sham-operated (normal) group, MCAO-operated (control) group, and three MCAO-operated Amex-treated groups with Amex concentrations of 100, 300, and 1000 mg/kg (titled Amex 100, 300, and 1000, respectively). Mice in the Amex-treated groups received oral Amex at the designated concentrations at 2, 26, and 50 h after reperfusion [Figure 1S]. Mice in the normal group and the control group were administered the same volume of normal saline.

Body weight and physiological parameter measurements

Mice were weighed daily during the experimental period, and blood was collected by cardiac puncture under deep anesthesia 3 days after MCAO. To obtain serum, blood samples were centrifuged at 1500 ×g for 15 min at 4°C. Serum concentration of electrolytes, such as sodium (Na^+), potassium (K^+), and chloride (Cl^-), was measured using an electrolyte analyzer (Dri-Chem 3500i, Fuji, Japan) to monitor and exclude animals with potential electrolyte imbalances that might affect results.

Infarct volume measurement and brain edema calculation

After ischemia for 2 h and reperfusion for 24 h, the brains were removed, sliced into ten coronal sections (1 mm thick), stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 17 min at room temperature (25°C), and immersed in 10% neutral buffered formalin for 2 h. Total infarct volumes were calculated using infarct areas in these sections as previously described[20] using a digital camera (Canon 20D, Canon Korea, Korea) and ImageJ software.

Brain edema indices were calculated by dividing injured brain hemisphere areas by normal brain hemisphere areas using TTC-stained brain sections. Whole-brain water content was determined using a previously described vacuum drying method[21] to evaluate edema formation and was calculated by dividing brain wet weights by dry weights.

Neurological deficit scores

Neurological deficit (ND) scores were quantified at 3, 24, 48, and 72 h after reperfusion in a blinded manner using a five-point scale.

- Grade 0: No ND
- Grade 1: Failure or incomplete extension of right forepaw
- Grade 2: Reduced voluntary movement in all directions and circling to the right when the tail was pulled

- Grade 3: Walking or circling to the right and highly sensitive response to pain when the tail was stimulated
- Grade 4: No response to stimulation or brain injury-related death.

Hematoxylin and eosin staining and cresyl violet staining of histological sections

To investigate neuroprotective effects of Amex, histological integrity and tissue damage were observed after MCAO in brain sections using hematoxylin and eosin (H and E) and cresyl violet staining.[20]

Experimental mice were euthanized by CO_2 inhalation 72 h after reperfusion and transcardially perfused with phosphate-buffered saline (PBS), then with 4% paraformaldehyde, and finally by 30% sucrose. The brains were isolated, immediately immersed in liquid nitrogen, embedded in optimum cutting temperature compound, and coronally sliced into 15-μm-thick sections using a cryostat (HM 550, Zeiss, Germany). Sections were placed on glass slides and stained with H and E or cresyl violet. A microscope (ZEISS AXIO, Zeiss, Germany) was used to observe histological changes after MCAO-induced brain injury. Staining densities were used to estimate cell viabilities.

Western blot analysis

Expression levels of aquaporin-4 (AQP-4) in mouse brains were evaluated by Western blotting. In brief, mice were euthanized 3 days after MCAO by CO_2 inhalation, and the brains were carefully isolated and placed on ice-cold glass. Ischemic left hemispheres were dissected, homogenized, and incubated in modified PBS containing 150-mM NaCl, 1-mM EDTA, 50-mM Tris, and 1:100 (v/v) of proteinase inhibitor. Residues were removed from lysates by centrifugation (13,250 ×g) at 4°C for 10 min, and total proteins were obtained using a protein extraction solution (PRO-PREP, iNtRON, Gyeonggi-do, Korea). Total protein contents of lysates were measured using a protein assay kit (Quick Start, Bio-Rad, USA). In addition, equal amounts of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST, Sigma-Aldrich, USA) for 1 h at 25°C. Following blocking, membranes were incubated overnight at 4°C with primary antibodies (anti-AQP-4, 1:500; anti-β-actin, 1:1000) (Cell Signaling, MA, USA), washed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:5000; Enzo Life Sciences, NY, USA) for 2 h at 25°C. After three washes with TBST, membranes were treated with an enhanced chemiluminescence kit (GenDEPOT, TX, USA) to develop protein bands, which were detected using a photosensitive luminescent analyzer system (Amersham™ Imager 600, UK). Signal intensities of the blots were analyzed using ImageJ software, and relative protein quantities were determined versus β-actin.

Reverse transcription polymerase chain reaction assay

Reverse transcription polymerase chain reaction was used to assess messenger RNA (mRNA) expression in mouse brains. Total RNA was extracted from brain tissues using TRI reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized using a cDNA synthesis kit (Roche, IN, USA), and amplification was performed in a thermal cycler (Biometra, Germany). Products were identified by electrophoresis in a 1% agarose gel. The primer sequences used for measurement of AQP-4 expression were GAGTCACCACGGTTCATGGA (forward) and CGTTTGGAATCACAGCTGGC (reverse).

Statistical analysis

One-way analysis of variance followed by Tukey's *post hoc* analysis was used to determine significant intergroup differences. Dunn's test was used for multiple group comparisons when data were nonnormally distributed. Analysis was performed using Sigmaplot v12.0 software (Systat Software Inc., IL, USA), and results are presented as means ± standard deviations. Results were considered statistically significant for $P < 0.05$.

RESULTS

Effects of middle cerebral artery occlusion operation and Amex treatment on body weight change and physiological parameters in mice

After MCAO, body weights tended to decrease but not significantly [Figure 1a]. This was attributed to dehydration and general recovery at 2 days after surgery. Treatment with Amex did not affect body weights.

Serum electrolyte levels in the control group and Amex-treated groups were similar to those of the normal group [Figure 1b], which indicated no physiological changes had occurred that may have affected the results of between-group comparisons.

Effects of Amex on total infarct volume, brain edema, and behavioral deficit

Representative TTC-stained brain sections are shown in Figure 2a. MCAO for 2 h caused damage to left hemispheres (mean infarct volume,

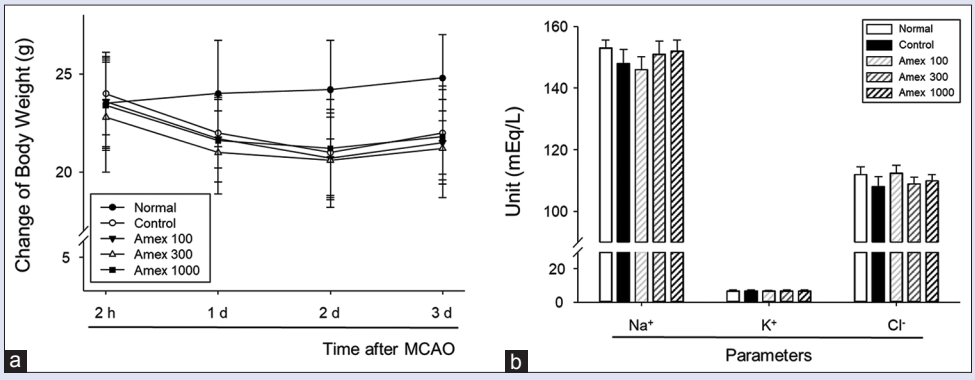

Figure 1: Influence on middle cerebral artery occlusion-induced brain injury and effect of treatment with methanol extracts of Astragali Radix (Amex) on body weight changes (a) and physiological parameters (b). Mice were weighed daily during the 4-day experimental period. Serum samples were obtained and concentrations of sodium (Na^+), potassium (K^+), and chloride (Cl^-) were measured

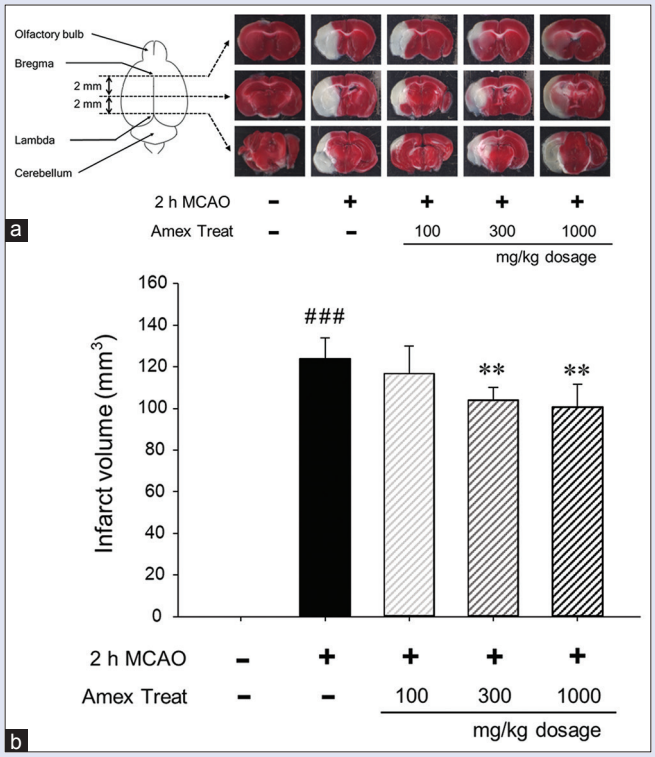

Figure 2: Representative images of brain sections (a) and the effects of Amex treatment postmiddle cerebral artery occlusion on brain infarct volumes (b). Treatment with 300 or 1000 mg/kg of Amex for 3 days after middle cerebral artery occlusion significantly suppressed infarct volumes. Results are presented as mean ± standard deviations. ###$P < 0.001$ versus normal group, **$P < 0.01$ versus control group; $n = 6$ per group

123.83 ± 9.99 mm^3), while sham surgery did not induce damage. However, treatment with 300 or 1,000 mg/kg Amex for 3 days significantly reduced infarct volumes (300 mg/kg, 104.17 ± 5.85 mm^3; 1,000 mg/kg, 100.67 ± 11.09 mm^3) compared to the control group [Figure 2b]. Although the extent of damage in the Amex 1000 group was similar to that in the Amex 300 group, the pink area stained by TTC was markedly larger, suggesting that high-dose Amex might inhibit damage in the penumbral region.

Brain edema indices were calculated by dividing the brains into three parts [Figure 3a]. Mean brain edema index was significantly lower in the Amex 1000 group than in the control group [Figure 3b]. This result agrees with total infarct volume results, which suggests that severity of damage after cerebral ischemia correlates with the degree of brain edema. As such, brain edema was assumed to be an important therapeutic target in treating brain damage. Mean brain water content was significantly lower in the Amex 1000 group than in the control group [Figure 3c], which concurred with the observed brain edema index changes.

MCAO significantly increased ND scores, and no improvement was observed over the experimental period. Amex treatment did not affect ND scores [Figure 4].

Effects of Amex on histological changes

Histological changes were investigated by staining ischemic brain sections with H and E or cresyl violet. H and E stain provides information regarding cell structure and function,[22] and cresyl violet staining is routinely used to estimate the total number of hippocampal neurons.[23] Thus, we used H and E or cresyl violet intensity, as measured using ImageJ software (NIH, MD, USA), as indices of cell survival [Figure 5a]. H and E and cresyl violet staining intensities were significantly less in the control group than in the normal group [Figure 5b and c]. The Amex 1000 group showed greater histological integrity, implying less neuronal cell death, than the control group [Figure 5c]. These

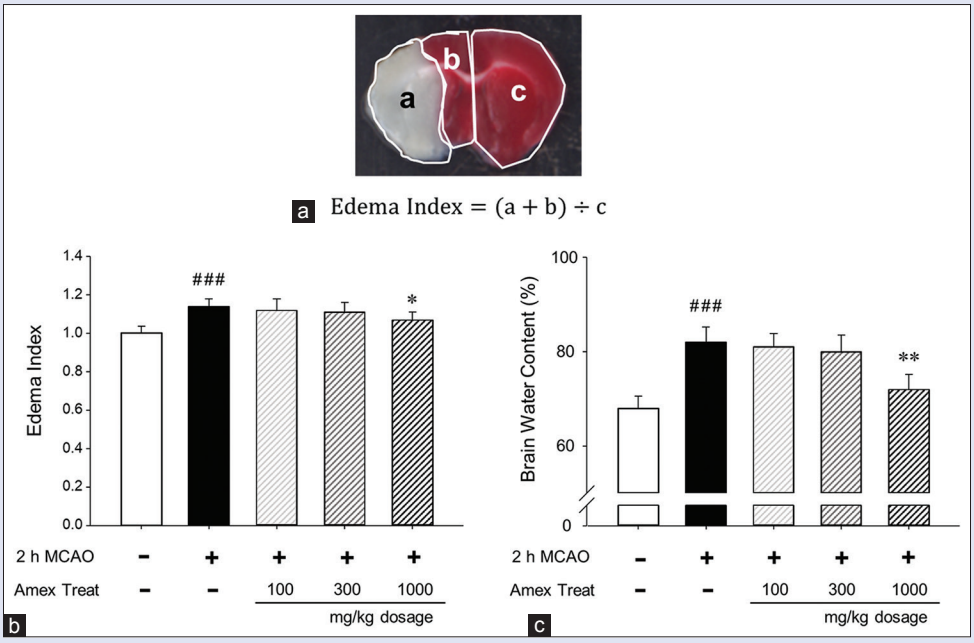

Figure 3: Brain edema indices (a) and the effects of Amex posttreatment on these indices (b) and on brain water content (c) in middle cerebral artery occlusion-injured mice. Treatment with 1000 mg/kg Amex postmiddle cerebral artery occlusion significantly suppressed brain edema index increases (b) and increases in whole brain water content (c). Results are presented as mean ± standard deviations ###$P < 0.001$ versus normal group, *$P < 0.05$, **$P < 0.01$ versus control group; $n = 6$ per group

results indicated that Amex had potent neuroprotective effects after ischemia-induced brain injury.

Effects of Amex on aquaporin-4 protein and gene expression

To evaluate the role of AQP-4 in ischemic brain injury, we measured changes in its protein and gene expression after ischemia-induced brain injury. Ischemic brain injury resulted in upregulation of AQP-4 mRNA and protein expression. However, 1000 mg/kg Amex treatment significantly reduced AQP-4 protein and mRNA expression compared with the control group [Figure 6].

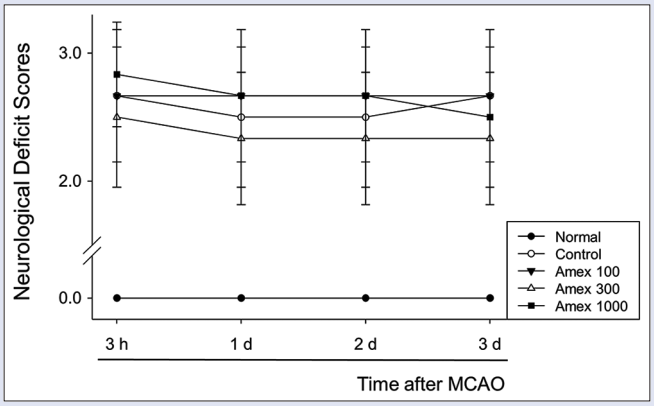

Figure 4: Effects of Amex postmiddle cerebral artery occlusion on neurological deficit scores in middle cerebral artery occlusion-injured mice. Amex treatment postmiddle cerebral artery occlusion did not improve ND scores. Results are presented as mean ± standard deviations; $n = 6$ per group

DISCUSSION

Brain edema is the result of several medical conditions, such as ischemic stroke, traumatic brain injury, and hyponatremia.[24,25] In addition, ischemic stroke is the leading cause of morbidity and mortality in stroke patients. Furthermore, ischemic stroke causes edema, which increases intracranial pressure and can cause permanent brain injury or death.[26,27] Substantial translocation of sodium (Na^+), chloride (Cl^-), and water across the BBB causes brain swelling, and BBB disruption associated with ischemic stroke, Alzheimer's disease, or traumatic brain injury is known to exacerbate disease progression.[28] Furthermore, Nicaise *et al.* reported that BBB breakdown correlated with AQP-4 induction in perivascular astrocytes.[29]

AQP-4 is one of 13 AQP subtypes and facilitates movement of water through plasma membranes.[30,31] Deregulation of these proteins may lead to water accumulation in organs, and AQP-4 is known to be important for the maintenance of water balance in the brain.[24,25] Moreover, brain edema is a primary target of ischemic stroke treatment in humans.

The chemical constituents of Astragali Radix, which include astragalosides, calycosin, kumatakenin, and formononetin, have neuroprotective properties[3,12,13,15,17,18] and have potential beneficial effects on ischemic stroke,[3] PD,[15] and Alzheimer's disease.[17] However, few studies have reported the effects of Astragali Radix on ischemic brain injury.[19] Furthermore, no studies have investigated its effects when administered after injury.[3]

In a preliminary study, we found ischemia-induced brain edema in mice was suppressed to a greater degree by Amex than by other herbal medicines. We observed pharmacological activity of Amex after a single pretreatment with Amex. The dose used was relatively high (1000 mg/kg), as protective activity was not observed at low concentrations. Potential toxicity associated with this dose was of concern. Therefore, the dose was decreased to 300 mg/kg, with three doses administered, resulting in

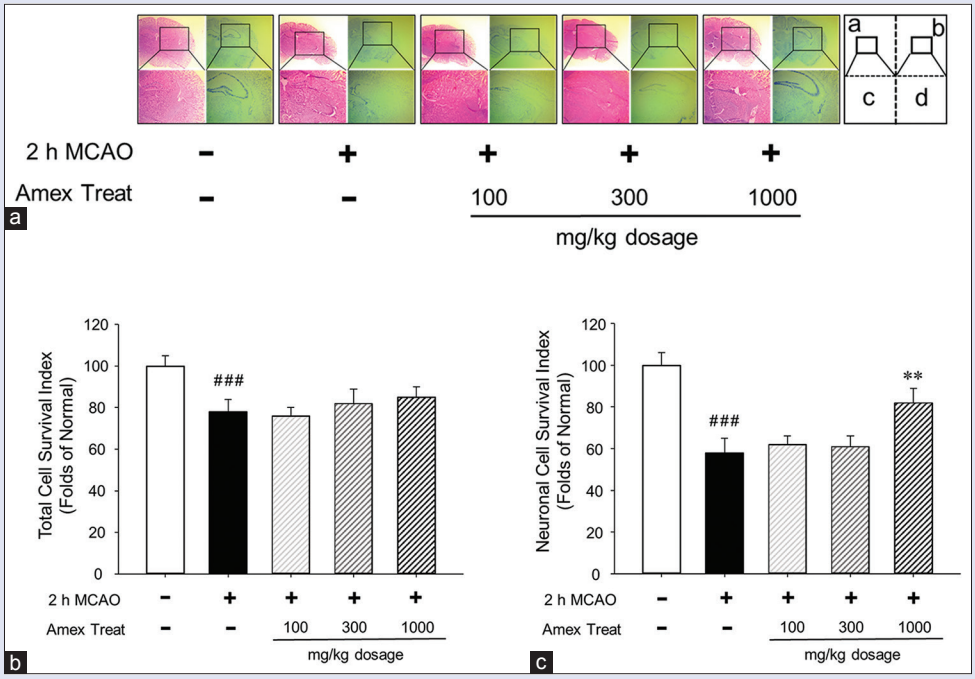

Figure 5: Representative images of H and E and cresyl violet-stained brain sections (a) and color intensities (b and c). Tissue damage in mouse brains was assessed by H and E (b) or cresyl violet staining (c) at 3 days postmiddle cerebral artery occlusion. The Amex 1000 group showed better histological integrity than the control group, showing less neuronal cell death. (a) H and E stained; (b) cresyl violet stained; (c and d) enlargements of (a and b), respectively. Results are presented as mean ± standard deviations. ###$P < 0.001$ versus normal group, **$P < 0.01$ versus control group; $n = 6$ per group

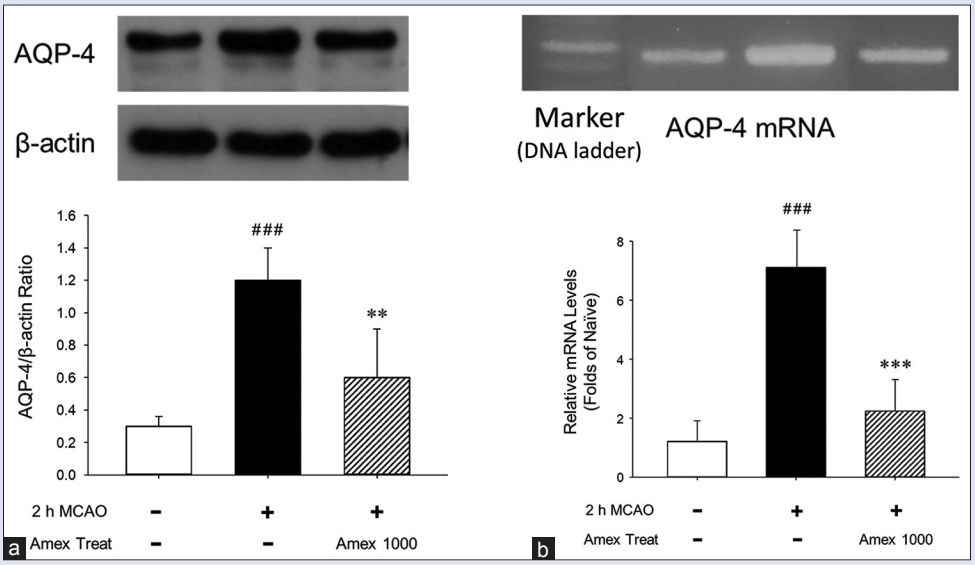

Figure 6: Effects of Amex administered after middle cerebral artery occlusion on protein (a) and mRNA (b) expression of Aquaporin-4 in the brains of middle cerebral artery occlusion-injured mice. Amex was found to significantly suppress Aquaporin-4 overexpression. Representative Western blots and quantitative analysis of Aquaporin-4 protein expression (a) demonstrating the suppressive effect of Amex in brain tissue. Reverse transcription polymerase chain reaction showed that Aquaporin-4 mRNA expression was also suppressed (b). Results are presented as mean ± standard deviations. $^{###}P < 0.001$ versus normal group, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group; $n = 6$ per group

a neuroprotective effect. This concentration is estimated to be relatively safe when administered to humans.

The main aims of the present study were to evaluate the effects of Amex posttreatment on ischemia-induced brain injury in mice and to determine whether these effects were related to amelioration of ischemia-induced edema.

Induction of stroke in mice by ischemic injury resulted in brain damage [Figure 2] and brain edema [Figure 3]. Amex administration suppressed brain tissue damage and brain edema [Figures 2 and 3]. However, no changes in serum electrolyte concentrations were observed [Figure 1b]. As such, we presumed that the observed suppression of brain edema by Amex was driven by mechanisms not related to electrolyte changes.

Amex may have exerted protective effects by inhibition of AQP-4 overexpression following reperfusion [Figure 6]. Decreased AQP-4 expression suppressed water accumulation. However, NDs were not ameliorated by Amex treatment [Figure 4], and thus, further studies should be conducted to examine the long-term effects of Amex.

CONCLUSION

In the present study, we found that Amex suppressed ischemia-induced brain injury in mice. Reduction in injury was likely due to reduced brain edema. Amex administered post-MCAO was found to reduce the mRNA and protein expression of AQP-4, which suggests that AQP-4 may be a target for the treatment of brain injury. Currently, we are examining the constituents of Astragali Radix with a view toward identifying the molecules responsible for this inhibition. Our findings highlight the importance of traditional herbal medicines for the treatment of brain injury and suggest that Astragali Radix should be considered as a potential agent for the treatment of ischemic brain injury. Our results have important clinical implications because they show that Amex, a traditional herbal medicine with a history of safe use, has the potential to suppress brain edema caused by brain injury.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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