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Prophylactic effect and mechanism of *p*-coumaric acid against hypoxic cerebral edema in mice

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ABSTRACT

Our previous study found that the anti-hypoxia effect of Tibetan turnip (*Brassica rapa ssp*. rapa) is directly related to its *p*-Coumaric acid (CA) and glucoside (*p*Coumaric acid-beta-D-glucopyranoside, CAG) contents. The present study aimed to investigate the role and mechanism of CA against hypoxic cerebral edema. Male mice were randomly divided into one normoxia group and three hypoxia groups, which were gavaged with sterilized water, CA, or dexamethasone, respectively, once daily for 4 days. The mice were then exposed to normoxia or hypoxia (9.5% O_2) for 24 h. The results showed that the brain water content (BWC) and blood-brain-barrier permeability were significantly lower in the CA treatment group than in the hypoxia vehicle group. Mice in the CA treatment group showed good blood-brain-barrier integrity; increased Na^+-K^+-ATPase activity and mitochondrial membrane potential; decreased oxidative stress and inflammation; and increased occludin protein levels. Prophylactic administration of CA and dexamethasone exerted similar effects against hypoxic cerebral edema. The mechanism involved improving the integrity of the blood-brain-barrier, and inhibiting oxidative stress and inflammation.

1. Introduction

High altitude cerebral edema (HACE) is a severe acute high altitude illness with a high mortality rate, which frequently occurs in plateau regions. In clinical practice, there is a lack of effective treatment and intervention measures for HACE. HACE generally occurs in people who ascend to an altitude above 3000 m quickly and the lowest altitude reported to induce HACE was 2100 m (Dickinson, 1979). The prevalence of HACE is about 0.5–1.0% among persons at 4000–5000 m (Bärtsch and Swenson, 2013). HACE is often regarded as the end-stage of acute mountain sickness (AMS) and is characterized by truncal ataxia and reduced consciousness. If appropriate and timely measures are not adopted, such symptoms can evolve into coma and even death (Zhou et al., 2017). HACE and AMS are mainly caused by the low oxygen content at high altitude. A previous study revealed that the severity of AMS is not significantly different between hypobaric and normobaric hypoxia at the same ambient O_2 content (Schommer et al., 2012). Besides, two previously published MRI studies (1.5–3 T) provided convincing evidence for mild vasogenic edematous brain swelling following 6–18 h passive exposure to normobaric hypoxia in a simulated high altitude chamber (about 12% O_2) (Kallenberg et al., 2007; Schoonman et al., 2008). Thus, normobaric hypoxia condition could be used to simulate high altitude climate and study HACE and AMS. To date, the pathophysiological mechanisms of HACE remain unclear, but are possibly caused by vasogenic edema and cytotoxic edema (Sagoo et al., 2016). Vasogenic cerebral edema is induced by an increase in the permeability of the blood-brain-barrier, which is directly related to increased cerebral blood pressure and cerebral blood flow, impaired cerebrovascular autoregulation function, and overexpression of vascular endothelial growth factor (VEGF) (Wilson et al., 2009; Schoch et al., 2002; Wilson et al., 2011). In addition, oxidative stress and inflammation may also induce vasogenic cerebral edema (Himadri et al., 2010). Cytotoxic edema is mainly induced by energy metabolism dysfunction and reduced Na^+-K^+-ATPase activity (Sarada et al., 2015).

Treatments for HACE include oxygen inhalation, administration of dexamethasone, immediate descent to a low altitude area, or entering a portable hyperbaric oxygen chamber (Davis and Hackett, 2017). Currently, effective measures to prevent HACE include administration of acetazolamide or dexamethasone a few days before ascending to high altitude. By inhibiting the activity of carbonic anhydrase, acetazolamide may cause metabolic acidosis and an increased respiratory rate, thus accelerating the rate of acclimatization to the hypoxic environment (Parati et al., 2013). Dexamethasone can prevent HACE by reducing the release of cytokines and decreasing the permeability of the blood-brainbarrier (Milledge et al., 2000). However, acetazolamide and dexamethasone have obvious side effects on the human body, which

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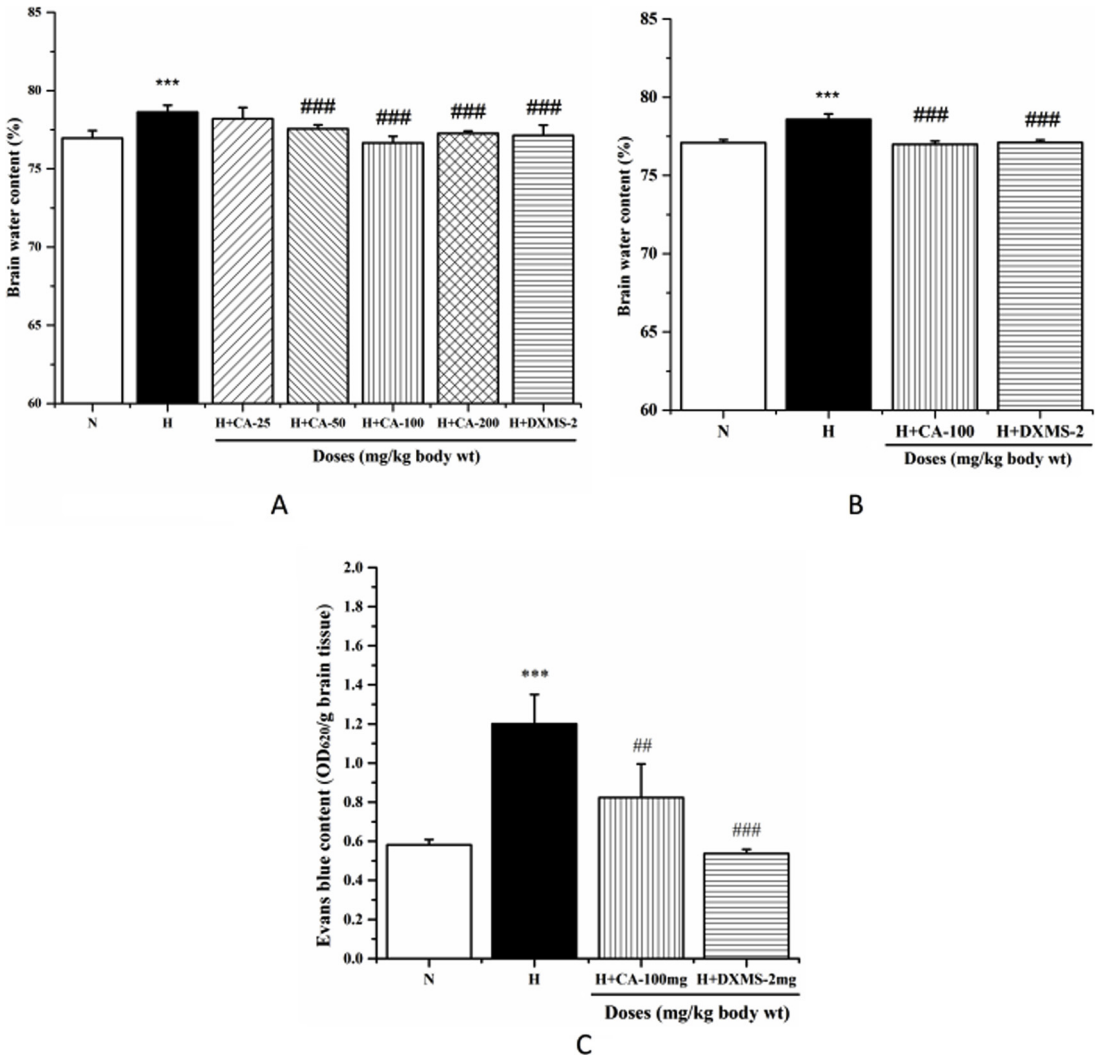

Fig. 1. Prophylactic effect of CA and dexamethasone against hypoxia induced cerebral edema.
(A) BWC of mice treated with different doses of CA and dexamethasone (n = 10); (B) BWC of mice treated with 100-mg/kg body wt CA and dexamethasone (n = 10); (C) Effect of CA and dexamethasone on blood-brain-barrier permeability (n = 5). Values were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with normoxia control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with hypoxia vehicle group. N, normoxia; H, hypoxia; H + CA-25, hypoxia + 25 mg CA; H + CA-50, hypoxia + 50 mg CA; H + CA-100, hypoxia + 100 mg CA; H + CA-200, hypoxia + 200 mg CA; H + DXMS-2, hypoxia + 2 mg dexamethasone.

limit their application. Acetazolamide can cause finger paresthesias and ruin the taste of carbonate beverages; dexamethasone can trigger symptoms such as mood disorders and high blood sugar (Zafren, 2014; Lee et al., 2013). Therefore, there is an urgent need to find safer and better natural products to prevent HACE.

The Tibetan turnip (*Brassica rapa* ssp. rapa) belongs to *Cruciferae Brassica*, and has been used traditionally to relieve hypoxia and alleviate fatigue (Chu et al., 2017). Our research group found that *p*-coumaric acid (CA) and *p*-coumaric acid-β-D-glucopyranoside (CAG) were the bioactive anti-hypoxia components of Tibetan turnip, which may also exert prophylactic effect against hypoxic cerebral edema (Chu, 2017). Under physiological conditions, CAG is converted to CA after ingestion. Recently, we have proved that CA could exert preventive effects against normobaric hypoxic pulmonary edema in mice (Li et al., 2018). In the present study, we aimed to explore the effect and mechanism of CA in preventing hypoxic cerebral edema and improving blood-brain-barrier function in an acute normobaric hypoxia mouse model.

2. Materials and methods

2.1. Experimental animals

One hundred and fifty healthy Institute of Cancer Research (ICR) male mice weighing 25–30 g (Super-B&K Laboratory Animal Corp., Shanghai, China) were used for the experiments. All animal procedures were performed strictly according to international ethical guidelines and were approved by the Committee on the Ethics of Animal Experiments of the Laboratory Animal Research Center, Zhejiang Chinese Medical University (Hangzhou, China) (Authorization No: ZSLL-2017-059). The animals were maintained in the Center's animal house and exposed to 12:12 h light-to-dark cycles. The mice were provided with food and water *ad libitum*.

2.2. Optimization of CA dose

After 3–4 days of adaptation, a total of 70 male ICR mice were divided into 7 groups of 10 mice each: (I) normoxia (N), (II) hypoxia (H), (III) hypoxia + 25 mg/kg body weight (wt) CA (H + CA-25), (IV) hypoxia + 50 mg/kg body wt CA (H + CA-50), (V) hypoxia + 100 mg/kg

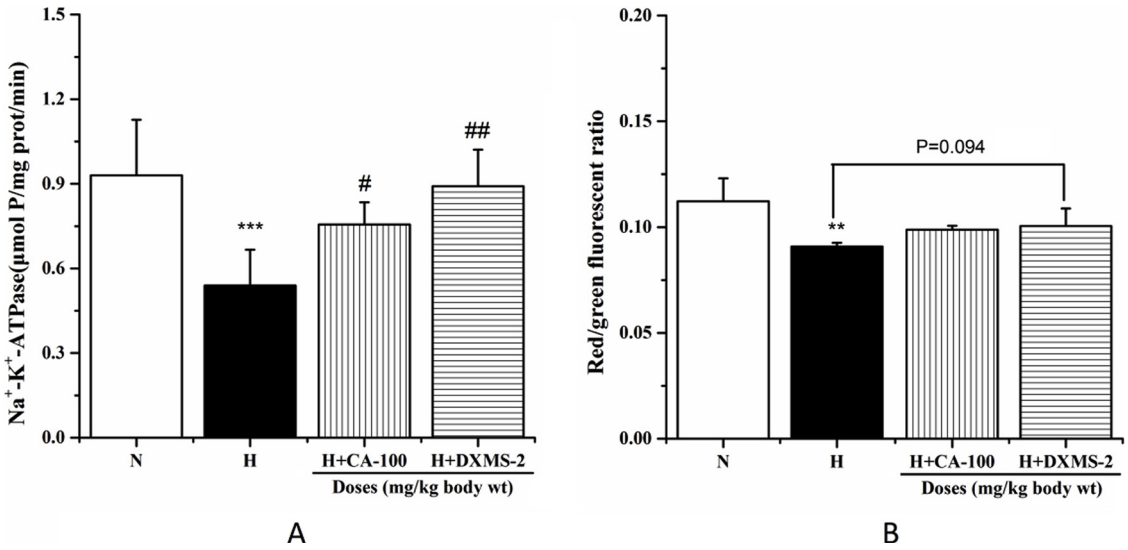

Fig. 2. Preventative effect of CA and dexamethasone on Na^+-K^+-ATPase activity (A, n = 10) and mitochondrial membrane potential (B, n = 5) in brain tissue.

Values were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with normoxia control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with hypoxia vehicle group. N, normoxia; H, hypoxia; H + CA-100, hypoxia + 100 mg CA; H + DXMS-2, hypoxia + 2 mg dexamethasone.

Table 1
Inflammatory cytokines in brain tissues of mice in different groups (n = 10, mean ± SD).

Group	IL-2 (ng/L)	IL-10 (ng/L)
N	25.61 ± 2.70[d]	16.79 ± 8.01[cf]
H	49.07 ± 5.06[bf]	41.15 ± 14.54[af]
H + CA-100	32.51 ± 4.19[d]	73.63 ± 15.87[bd]
H + DXMS-2	37.33 ± 2.56[d]	78.94 ± 28.49[bd]

$^{a}P < 0.05$, $^{b}P < 0.01$ compared with normoxia control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with hypoxia vehicle group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with dexamethasone group. N, normoxia; H, hypoxia; H + CA-100, hypoxia + 100-mg/kg body wt CA; H + DXMS-2, hypoxia + 2-mg/kg body wt dexamethasone.

body wt CA (H + CA-100), (VI) hypoxia + 200 mg/kg body wt CA (H + CA-200), and (VII) hypoxia + 2 mg/kg body wt dexamethasone (H + DXMS-2). The mice in the N and H groups were gavage administered with sterile water (1.0 ml/100 g); mice in other groups were gavage administrated with the corresponding drugs. The gavage administration was performed once daily for 4 consecutive days. One hour after the final gavage on the fourth day, mice in the hypoxia exposure groups were put into a normobaric hypoxia chamber (containing 9.5 ± 0.2% O_2 and 90.0 ± 0.2% N_2) for 24 h, but not the mice in normoxia control group (N group). Within 24 min, the oxygen content was slowly decreased from 21.0 to 9.5%, and the nitrogen content was slowly increased from 78.0 to 90.0%. The temperature and humidity in the chamber were maintained at 20 ± 2 °C and 50–60%, respectively. After hypoxia exposure for 24 h, the mice were anesthetized using sodium pentobarbital intraperitoneally (50 mg/kg body wt) and sacrificed. Their brains were dissected for BWC (brain water content) analysis to confirm the appropriate dosage of CA against hypoxic cerebral edema. To determine the BWC, brain tissues were quickly weighed (wet weight) and dried at 80 °C for 72 h to a constant weight. Calculation of BWC percentage was performed according to a previously published method (Chen et al., 2014). The dose of 100-mg/kg body wt of CA led to significantly reduced BWC compared with that in the hypoxia vehicle group and was found to be the lowest compared with that of the other groups of mice receiving different doses ofCA. Its effect was comparable to that of 2-mg/kg body wt dexamethasone. Therefore, 100-mg/kg body wt CA was selected for subsequent experiments.

2.3. Determination of cerebral edema

The experiment was performed in three steps:

Step I. Forty male ICR mice were randomly divided into 4 groups of 10 mice, each with following conditions: (I) normoxia (N); (II) hypoxia (H); (III) hypoxia + 100 mg/kg body wt CA (H + CA-100); (IV) hypoxia + 2 mg/kg body wt dexamethasone (H + DXMS-2). The mice in the N and H groups were gavage administered with sterile water (1.0 ml/100 g); mice in the other groups were gavage administered with the corresponding drugs. The gavage administration was performed once daily for 4 consecutive days. One hour after the final gavage, mice in the hypoxia groups were put in the normobaric hypoxia chamber for 24 h (9.5 ± 0.2% O_2) but not the mice in group N. Within 24 min, the oxygen content was slowly decreased from 21.0 to 9.5% and the nitrogen content was slowly increased from 78.0 to 90.0%. The temperature and humidity in the chamber were maintained at 20 ± 2 °C and 50–60% respectively. After hypoxia exposure, mice were anesthetized and plasma was collected. The mice were sacrificed and the whole brains were dissected to determine BWC and relevant biochemical indicators, including Na^+-K^+-ATPase activity, antioxidant enzymes activities, and inflammatory factors.

Step II. Twenty healthy male ICR mice were divided into four groups, gavaged and normoxia/hypoxia treated for 24 h according to step I, with five mice in each group. With reference to previously described methods, the blood-brain-barrier permeability determination was performed (Sarada et al., 2015; Lian et al., 2008). Briefly, mice in the normoxia and hypoxia exposure groups were inoculated intravenously with 200 μL of a 2% (w/v) solution of Evans blue dye *via* the caudal vein, 1 h before the predetermined time of hypoxia exposure. Immediately after the injection, the mice in the hypoxia exposure groups were put back into the hypoxia chamber up to the determined time. After hypoxia exposure, the mice were anesthetized with sodium pentobarbital and perfused with 5 mL of normal saline (0.9% NaCl). The whole brain was then removed, washed with cold saline, and dissected into two equal parts. One part of the brain was packaged in tin foil and dried at 80 °C for 72 h to a constant weight. The other part of the brain was homogenized with phosphate-buffered saline (PBS) and incubated with formamide at 60 °C for 24 h followed by centrifugation at 4 °C for 15 min at 6000 × *g*. The optical density (OD) of the supernatant was measured at 620 nm. The results of blood-brain-barrier permeability were presented as OD per gram (OD_{620}/g) dry weight.

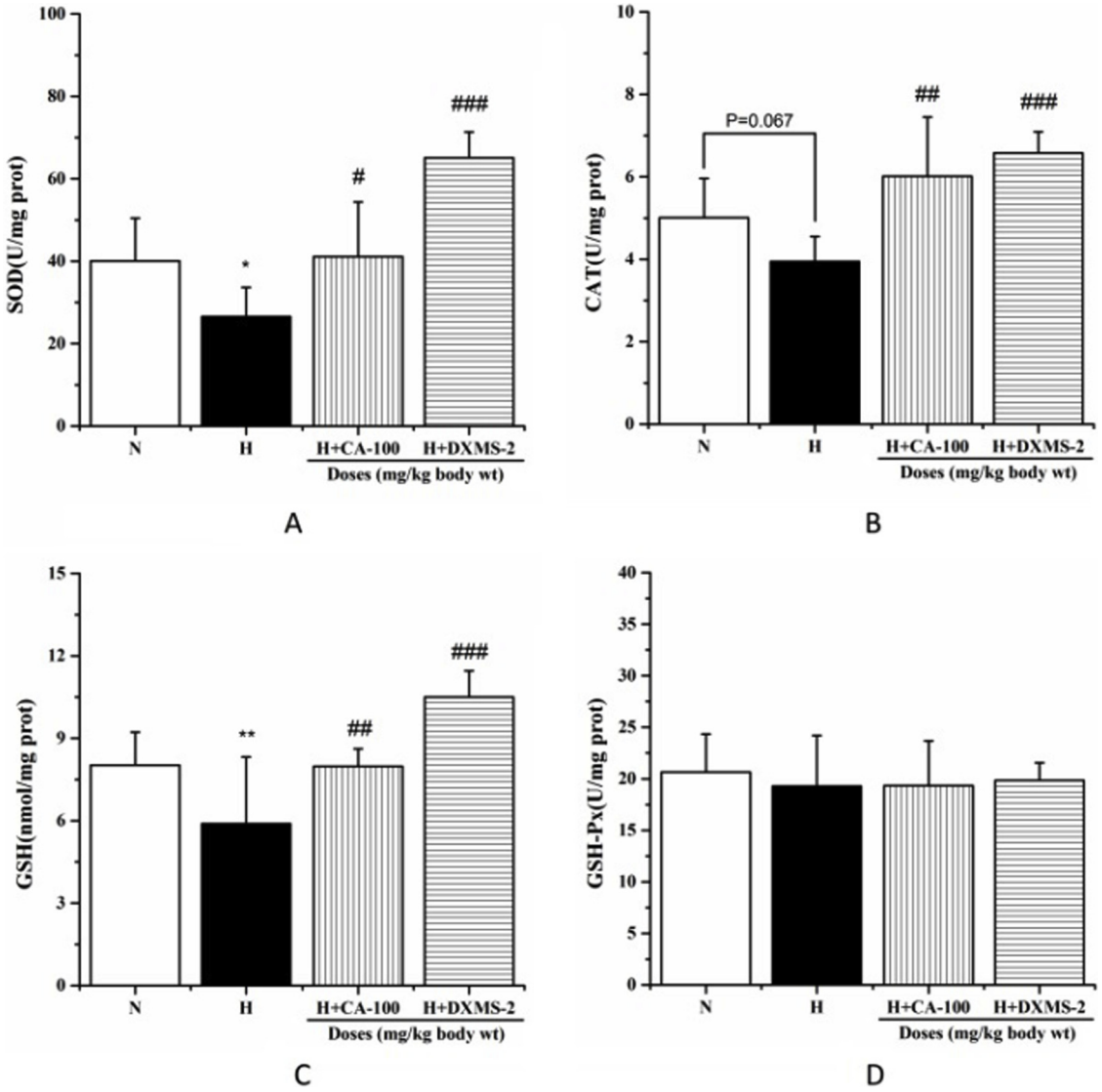

Fig. 3. Preventative effect of CA and dexamethasone on antioxidant enzymes (n = 10): (A) SOD activity; (B) CAT activity; (C) concentration of GSH; (D) concentration of GSSG in brain tissues.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with normoxia control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with hypoxia vehicle group. N, normoxia; H, hypoxia; H + CA-100, hypoxia + 100 mg CA; H + DXMS-2, hypoxia + 2 mg dexamethasone.

Step III. Twenty healthy male ICR mice were divided into groups, gavaged and normoxia/hypoxia treated according to step I, with five mice in each group. After hypoxia exposure, the mice were sacrificed and their brain tissues were obtained. About 100 mg of brain tissue was used for mitochondria isolation and mitochondrial membrane potential assay, which were performed strictly in accordance with the manuals supplied with the kits (Beyotime, Shanghai, China) and determined using a fluorescence microplate reader (Tecan Group Ltd., Männedorf, Switzerland). When detecting the JC-1 monomer, the excitation wavelength was set to 490 nm and the emission wavelength was set to 530 nm. When testing J-aggregates, the excitation wavelength was set to 525 nm and emission wavelength was set to 590 nm. The results were expressed as an increase in the green to red fluorescence ratio reflecting the transformation of JC-1 aggregates into monomers when the mitochondrial membrane becomes depolarized (Zhu et al., 2009). The remaining brain tissues were used to detect occludin expression level and to do the histological and ultrastructural analysis.

2.4. Analytical procedures for relevant biochemical indicators

Na^{+}-K^{+}-ATPase, glutathione (GSH), and glutathione peroxidase (GSH-Px) in brain tissues were determined using detection kits (Nanjing Jiancheng, Nanjing, China). Superoxide dismutase (SOD) and catalase (CAT) in brain tissues were measured using commercial kits (Beyotime, Shanghai, China). In addition, the protein concentration in the brain tissue homogenate was determined using a bicinchoninic acid (BCA) protein concentration determination kit (Chengdu Lilai Biotechnology, Chengdu, China).

Inflammatory factors in the brain tissue, including interleukin (IL)-2 and IL-10 were determined using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng), and the results were expressed as ng/L.

2.5. Histological and ultrastructural analysis of the cerebral cortex

Histological and ultrastructural analyses of the cerebral cortex were carried out according to previous studies, with slight modifications (West et al., 1995). The histological observation was done as follows:

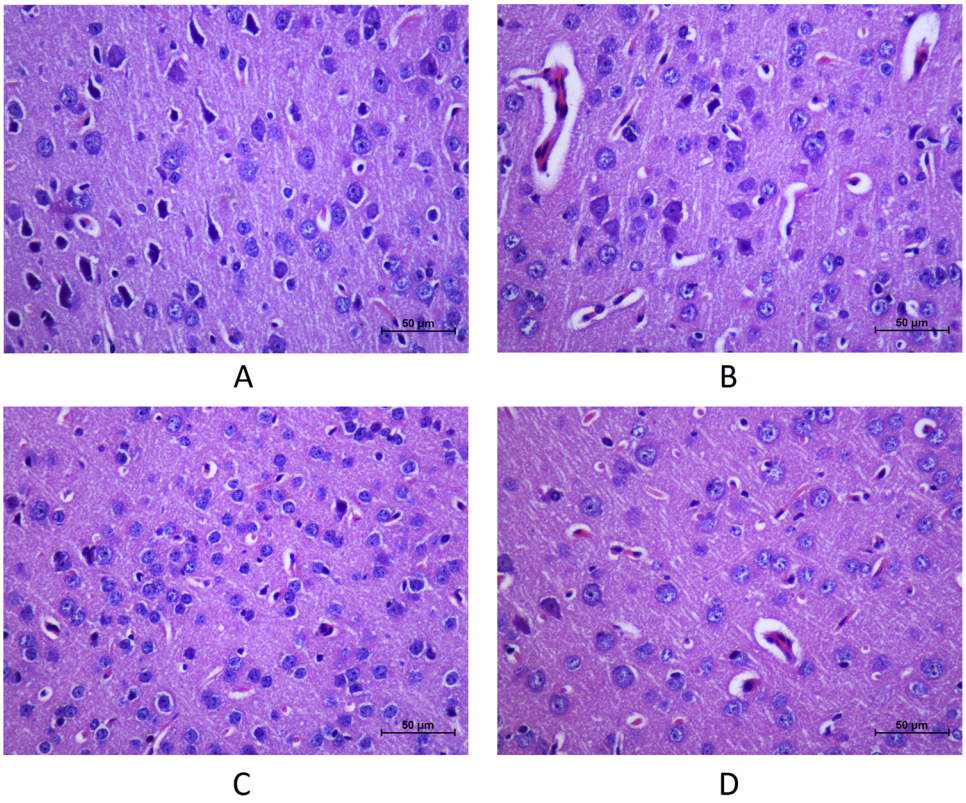

Fig. 4. Observation results of cerebral cortex histopathology (n = 5).
(A) normoxia control group mice, (B) hypoxia vehicle group mice, (C) 100-mg/kg body wt CA treatment group mice, (D) 2-mg/kg body wt dexamethasone treatment group mice. Scale bar indicates 50 micron (400 ×).

the cerebral cortex was cut into small pieces and put into 10% freshly prepared neutral formalin solution, paraffin embedded, sectioned, and stained using hematoxylin-eosin. Ultrastructural analysis was performed as follows: the cerebral cortex was cut into small pieces and fixed overnight in freshly prepared 2.5% glutaraldehyde and fixed with 1.0% osmium tetroxide. After rinsing with PBS three times, dehydration, embedding, and slicing, the ultrastructure of the cerebral cortex was observed using a Hitachi H-7650 transmission electron microscope (Hitachi H-7650, Hitachi, Japan) after staining with lead citrate solution and uranium acetate.

2.6. Detection of occludin in the cerebral cortex

Cerebral cortices (n = 5) from each group were used to detect the expression of the tight junction protein occludin. It was determined by immunohistochemistry using an anti-occludin mouse monoclonal antibody (Abcam, Cambridge, UK) (Liu et al., 2014). After incubation with the anti-occludin antibody overnight at 4 °C, the cerebral cortex slices were washed with PBS and incubated with goat anti-rabbit secondary antibody for 30 min. The slices were then stained with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Image pro plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used to analyze the Integrated Optical Density (IOD) of the positive cells in each slice to determine the occludin protein expression.

2.7. Data processing and statistical analysis

Measurement data were expressed as mean ± SD. Comparisons between normoxia and hypoxia-exposed mice were assessed by using unpaired Student's *t* test, and differences between hypoxia and CA/dexamethasone administered hypoxia-exposed groups of animals were assessed by using one-way analysis of variance (ANOVA) with the least significant difference (LSD) post-hoc test for multiple comparisons among groups. $P < 0.05$ were considered statistically significant. All statistical tests were performed with the SPSS statistical software, version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Prophylactic effect of CA and dexamethasone against hypoxic cerebral edema

The BWC is an important index of cerebral edema. As shown in Fig. 1A, after acute hypoxia exposure, the BWC level was significantly increased in the H group (78.62 ± 0.44% in the H group *vs.* 76.96 ± 0.48% in the N group), which indicated that the hypoxic cerebral edema mouse model was constructed successfully. The results of the BWC levels suggested that CA exerted a protective effect against hypoxic cerebral edema in a dose-response manner. Mice in the 100-mg/kg body wt CA group showed the lowest level of BWC (76.65 ± 0.43%), which was comparable to that of the dexamethasone group (77.13 ± 0.66%), therefore 100-mg/kg body wt was chosen for further experimentation. Similarly, as shown in Fig. 1B, the BWC level increased significantly in the H group (78.58 ± 0.32%) compared with that in the N group (77.09 ± 0.19%). Thus, 100-mg/kg body wt of CA can produce a good preventive effect against hypoxic cerebral edema (BWC, 76.98 ± 0.21%).

As shown in Fig. 1C, compared with that of the N group, the blood-brain-barrier permeability of mice in the H group increased significantly (by 106.5%). However, mice prophylactically administered with 100 mg/kg body wt CA and 2 mg/kg body wt dexamethasone showed a significant reduction of blood-brain-barrier permeability (decreased of 31.44% and 55.26%, respectively, compared with that in the H group). These results revealed that CA could improve the blood-

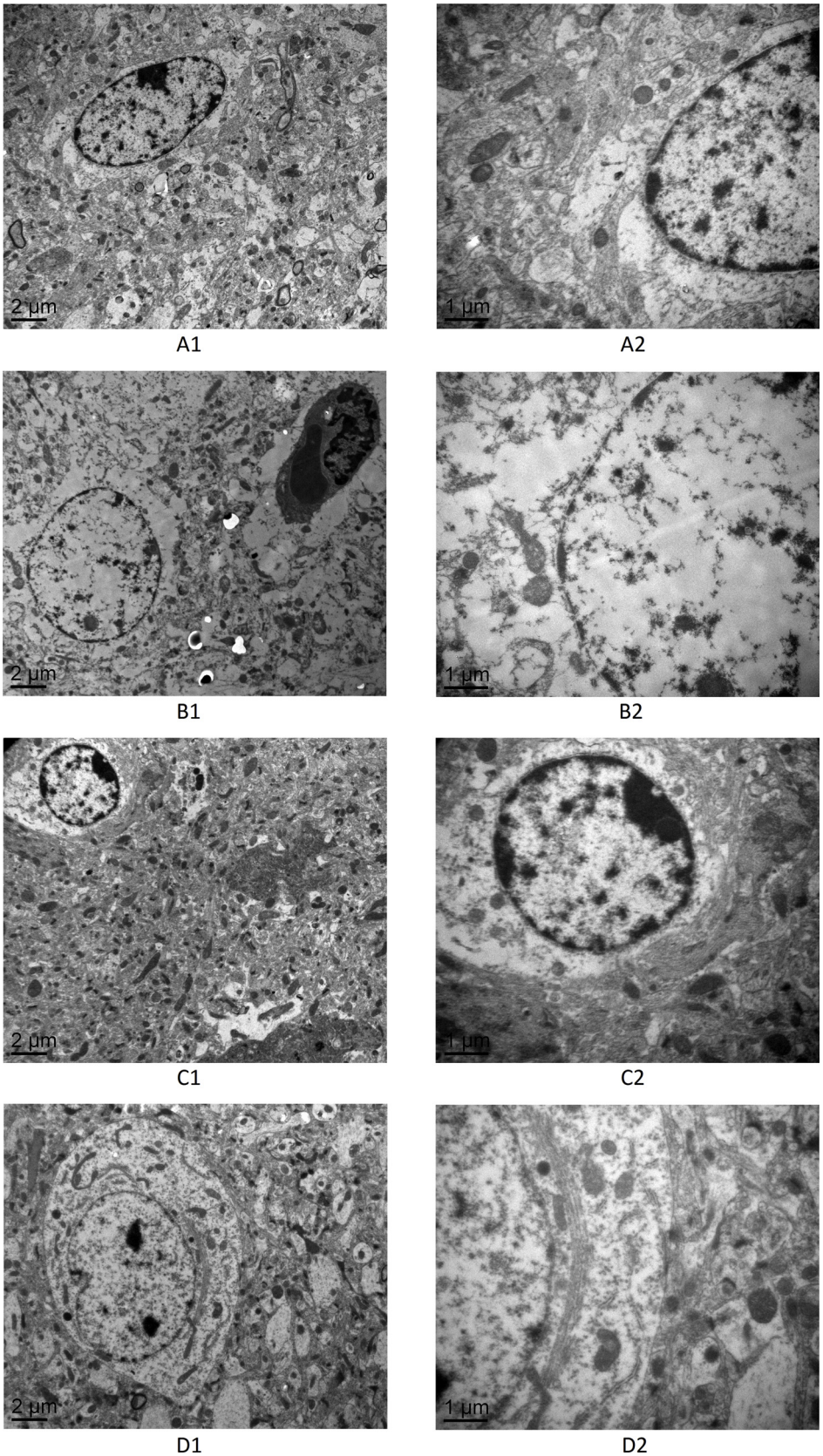

Fig. 5. Observation results of cerebral cortex ultrastructure (n = 5).
(A) normoxia control group mice, (B) hypoxia vehicle group mice, (C) 100-mg/kg body wt CA treatment group mice, (D) 2-mg/kg body wt dexamethasone treatment group mice. Scale bar in A1, B1, C1, D1 indicates 2 micron (8000 ×); Scale bar in A2, B2, C2, D2 indicates 1 micron (15,000 ×).

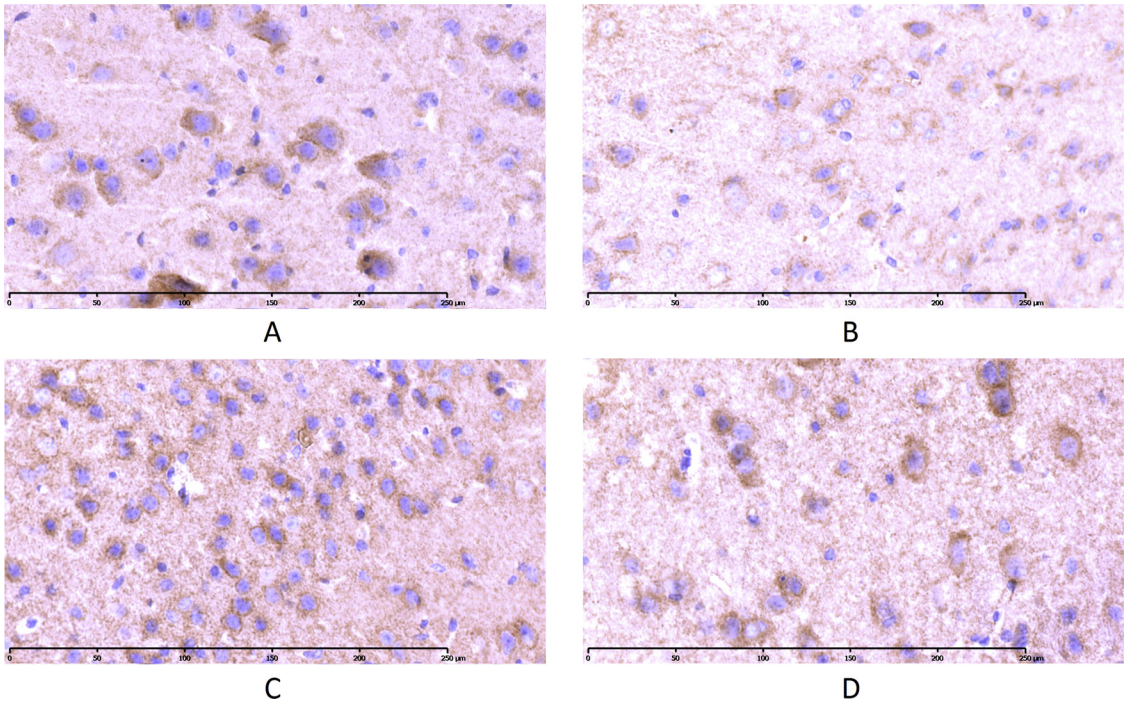

Fig. 6. Immunohistochemical visualization of occludin expression in brain tissue of mice (n = 5).
(A) normoxia control group mice, (B) hypoxia vehicle group mice, (C) 100-mg/kg body wt CA treatment group mice, (D) 2-mg/kg body wt dexamethasone treatment group mice. Scale bar indicates 250 micron (400 ×).

Table 2
Expression level of occludin in brain tissues of mice in different groups (n = 5, mean ± SD).

Group	Occludin abundance (IOD)
N	2107.63 ± 530.83[d]
H	266.08 ± 85.53[bf]
H + CA-100	2672.40 ± 887.43[d]
H + DXMS-2	2279.88 ± 974.36[d]

[a]$P < 0.05$, [b]$P < 0.01$ compared with normoxia control group; [c]$P < 0.05$, [d]$P < 0.01$ compared with hypoxia vehicle group; [e]$P < 0.05$, [f]$P < 0.01$ compared with dexamethasone group. N, normoxia; H, hypoxia; H + CA-100, hypoxia + 100-mg/kg body wt CA; H + DXMS-2, hypoxia + 2-mg/kg body wt dexamethasone.

brain-barrier integrity of hypoxic mice.

3.2. Effect of CA and dexamethasone on Na^+-K^+-ATPase activity and mitochondrial membrane potential

Na^+-K^+-ATPase is essential to maintain membrane electrochemical gradients in brain tissue, and it can catabolize approximately 70% of the ATP in the brain to produce energy. Thus, it plays an important role in maintaining membrane potential and energy supply in brain tissue (Rambo et al., 2013). In the present study, the Na^+-K^+-ATPase activity was significantly reduced in the H group compared with that in the N group (0.54 ± 0.13 μmol P/mg prot/min *vs.* 0.93 ± 0.20 μmol P/mg prot/min in N group), resulting in a significant reduction of the mitochondrial membrane potential (decreased by 19.08% compared with that in the N group), which suggested that cell apoptosis occurred in the brain tissue (Fig. 2) (Zhu et al., 2009). However, in the mice treated with CA (0.76 ± 0.08 μmol P/mg prot/min) and dexamethasone (0.89 ± 0.13 μmol P/mg prot/min), Na^+-K^+-ATPase activity was significantly increased and mitochondrial membrane potential was increased to some extent (increased 8.69% and 10.68%, respectively, compared with that in the H group). These results revealed that CA and dexamethasone could alleviate cytotoxic edema by enhancing Na^+-K^+-ATPase activity and improving mitochondrial function.

3.3. Effect of CA and dexamethasone on inflammatory cytokines in brain tissue

As shown in Table 1, in the H group, the IL-2 level was significantly elevated in brain tissue, followed by a significantly increased IL-10 level. However, as compared with the mice in H group, the IL-2 level was significantly decreased and IL-10 level was significantly elevated in the CA and dexamethasone treated mice. No significant difference was observed in these two intervention groups. These results indicated that inflammation occurred in brain tissue under hypoxic conditions, and CA supplementation prior to hypoxia exposure significantly reduced the levels of proinflammatory cytokines followed by enhanced anti-inflammatory cytokines.

3.4. Effect of CA and dexamethasone on antioxidant enzymes in brain tissue

As shown in Fig. 3, under hypoxic conditions, the SOD activity and GSH content were significantly decreased (by 33.66% and 26.54%, respectively, compared with those of the N group). The CAT and GSH-Px activities were also decreased (by 36.37% and 6.60%, respectively, compared with those of the N group). These results indicated that the oxidant/antioxidant balance was disrupted. In mice pretreated with CA and dexamethasone, the SOD activity (increased by 54.60% and 144.89%, respectively, compared with that in the H group), and CAT activity (increased by 52.35% and 66.71%, respectively, compared with that in the H group) were significantly increased, as was the GSH content (by 35.47% and 78.28%, respectively, compared with that in the H group). These results revealed that CA and dexamethasone pretreatment could significantly increase antioxidant enzyme activities and restore the oxidant/antioxidant balance.

3.5. Observation results of mice cerebral cortexes using hematoxylin-eosin staining

The results of hematoxylin-eosin staining are shown in Fig. 4. Cerebral cortex sections of mice in the N group (Fig. 4A) showed the normal structure of the cortical neurons and normally configured cerebral capillaries. However, sections from the mice in H group showed a pronounced widening of the pericellular and perivascular space (Fig. 4B). Sections of dexamethasone-treated mice showed moderate widening of the perivascular space and no widening of the pericellular space (Fig. 4D). CA-treated mice showed normal morphology in the cerebral cortex sections with no obvious widening of the pericellular or perivascular space (Fig. 4C).

3.6. Observation results of mice cerebral cortexes using electron microscopy

Electron microscopy observation (Fig. 5) revealed that the mice in the N group had a normal ultrastructure. The cortical neurons contained large oval nuclei with homogeneously distributed euchromatin, clear mitochondria, and the endoplasmic reticulum had plenty of ribosomes (Fig. 5 A1 and A2). For the mice in the H group, vacuolized mitochondria were observed around the nucleus in edematous neurons. In addition, the electronic density in the perinuclear cytoplasm was reduced, the number of organelles was reduced, and some organelles being degraded or missing (Fig. 5 B1 and B2). In the mice pretreated with dexamethasone, the chromatin was homogeneously distributed, some mitochondria were slightly swollen, and the ultrastructure of the nucleus was largely similar to that of the N group (Fig. 5 D1 and D2). In comparison with the H group and dexamethasone-treated group, the impairments of the CA-treated group were reduced. A few mitochondria were slightly swollen and most mitochondria, endoplasmic reticulum, and other organelles were in the normal state (Fig. 5C1, C2).

3.7. Impact of CA and dexamethasone on occludin expression in brain tissue

Immunochemical staining of occludin (Fig. 6) revealed that it was mainly expressed in the cerebral cortex, and appeared as brown granules. As shown in Table 2, compared with that in the N group, the level of occludin in the H group was significantly decreased, which suggested that the blood-brain-barrier was disrupted and permeability had increased. The levels of occludin in the CA and dexamethasone treatment groups were significantly increased compared with that in the H group. These results were in line with results of the blood-brain-barrier permeability.

4. Discussion

CA is a hydroxy derivative of cinnamic acid that is widely found in plants and plant-based foods, which possesses antioxidant, anti-inflammatory, antiulcer activities, improves memory impairment, and has neuroprotective effects (Kim et al., 2017, 2018). However, to date, there has been little research on its effect against hypoxic cerebral edema. In the present study, we built a hypoxic cerebral edema model in ICR mice, compared the prophylactic effect of CA and dexamethasone against hypoxic cerebral edema, and analyzed their impact on oxidative stress, inflammation, and blood-brain-barrier integrity. The results showed that, after normobaric hypoxia (9.5% O_2) exposure for 24 h, the mice in the H group exhibited pronounced cerebral edema. Pretreatment with CA and dexamethasone before hypoxia exposure resulted in significantly decreased inflammation and blood-brain-barrier permeability, thus they exerted similar protective effects against hypoxic cerebral edema.

HACE is induced by vasogenic edema and cytotoxic edema. Cytotoxic cerebral edema results from intracellular accumulation of osmotic molecules, such as Na^+, Cl^-, and Ca^{2+}, concomitant with the shift of osmotically obligated water into the intracellular space (Rungta et al., 2015). In the central nervous system, Na^+-K^+-ATPase activity accounts significantly for the maintenance of the electrochemical gradient across the plasma membrane, and seems to be particularly sensitive to free radicals induced by oxidative stress (Lima et al., 2009). Under physiological conditions, Na^+-K^+-ATPase regulates Na_i^+ in the cell. However, under hypoxic conditions, low ATP levels would inactivate Na^+-K^+-ATPase, concomitant with an increased inorganic phosphate level, low pH, and generation of free radicals. This in turn increases Na_i^+. During this period, the Na^+/H^+ exchanger would be activated as a consequence of acidosis and/or through phosphoinositide hydrolysis, resulting in the activation of protein kinase C, which, in turn, stimulates the Na^+/H^+ exchange activity (Kuribayashi et al., 1999). Eventually, a marked increase of Na^+ may occur, leading to Ca^{2+} overload, and this can induce mitochondrial depolarization and cytotoxic cerebral edema (Rungta et al., 2015). Therefore, cytotoxic cerebral edema could be prevented by increasing Na^+-K^+-ATPase activity and improving mitochondrial energy metabolism. Curcumin and dexamethasone could improve the severity of cerebral edema through increasing Na^+-K^+-ATPase activity (Sarada et al., 2015; Rosa et al., 2016). Quercetin may ameliorate hypobaric hypoxia-induced memory impairment by maintaining mitochondrial cristae integrity and neuron function *via* the PGC-1α pathway (Liu et al., 2015). In the present study, after hypoxia exposure, Na^+-K^+-ATPase activity and the mitochondrial membrane potential decreased, which may lead to Na^+ and Ca^{2+} overload, inducing the appearance of large amounts of swollen and vacuolized mitochondria and the degradation of some organelles. CA pretreatment may exert a prophylactic effect against hypoxic cerebral edema by increasing Na^+-K^+-ATPase activity and improving the mitochondrial membrane potential.

Vasogenic cerebral edema is induced by disruption of the blood-brain-barrier and increasing blood-brain-barrier permeability. Tight junctions play a significant role in the blood-brain-barrier and the transmembrane protein occludin is an important component of tight junctions. Occludin is crucial for maintaining the structure and function of the blood-brain-barrier, and its degradation would lead to increased bloodbrain-barrier permeability (Han et al., 2014; Yao et al., 2014). Activation of protein kinase C (PKC) would lead to dephosphorylation of occludin at the Ser/Thr site, which would disrupt tight junctions and result in the destruction of blood-brain-barrier integrity (Shao and Bayraktutan, 2013). As stated above, under hypoxic conditions, Na^+-K^+-ATPase would be inactivated and might lead to the activation of PKC, which would result in the downregulation of occludin and disruption of the blood-brain-barrier. In addition, under hypoxia-reoxygenation conditions, oxidative stress would induce the movement of occludin away from the tight junctions, thus increasing blood-brain-barrier permeability (Lochhead et al., 2010). Thus, occludin could be upregulated by elevation of Na^+-K^+-ATPase activity or inhibition of oxidative stress to restore blood-brain-barrier integrity (Förster et al., 2005). Previous studies reported that onion extracts could improve blood-brainbarrier integrity and protect against traumatic cerebral edema by elevating the expression level of occludin (Hyun et al., 2013). In the present study, pretreatment with CA significantly upregulated the occludin level, which suggested that CA might exert a preventive effect against vasogenic cerebral edema by improving blood-brain-barrier integrity.

Inflammation also plays an important role in hypoxic cerebral edema (Himadri et al., 2010). Inflammation leads to downregulation of occludin and increased blood-brain barrier permeability (Huber et al., 2001; Brooks et al., 2005). Hypobaric hypoxia can enhance the levels of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 in the plasma of humans and animals, which positively correlated with AMS (Song et al., 2016). Systemic inflammation induced by lipopolysaccharide would disrupt the blood-brain-barrier under hypobaric hypoxic conditions, leading to vasogenic cerebral edema, thus many anti-inflammatory drugs could exert

prophylactic effect against HACE (Zhou et al., 2017; Gong et al., 2018; Patir et al., 2012; Wang et al., 2017). Ghrelin is a 28-amino acid hormone that is principally released from the stomach, which can reduce brain edema induced by acute systemic normobaric hypoxia (11% O_2, 48 h) and at least part of the anti-edematous effects of ghrelin is due to decrease of serum TNF-α levels (Hossienzadeh et al., 2013). Patir et al. studied the effect of quercetin against HACE by putting rats into a hypobaric hypoxic environment (25,000 ft, 24 h). The results showed that quercetin could protect against HACE by efficiently attenuating inflammation and cerebral edema formation (Patir et al., 2012). Puerarin could prevent cerebral edema of rats exposed to hypobaric hypoxia *via* inhibiting the release of inflammatory cytokines, and by down-regulating the NF-κB signaling pathway and AQP4 (Wang et al., 2017). These results indicated that hypoxia would cause inflammation in brain tissue and lead to cerebral edema. The results of the present study suggested that CA might prevent the elevation of blood-brain-barrier permeability by inhibiting oxidative stress and IL-2 expression, and promoting IL-10 expression.

Currently, acetazolamide and dexamethasone are effective drugs to prevent HACE. However, they both have many side effects. Besides, a high dosage of acetazolamide (500 mg/d) reduced exercise capacity following a 5-day ascent to 4559 m, which would be harmful for acclimatization (Bradwell et al., 2018). Similarly, dexamethasone in high doses can completely remove the symptoms of AMS and cerebral edema, but it does not help with acclimatization (Ling et al., 1981). Additionally, AMS symptoms seem to persist when the drug is stopped. To date, many traditional plant-derived medicines have been studied to prevent HACE. Plant-based formulations like *Ginko biloba* extract has been used to treat AMS and HACE; however, there is some controversy regarding its efficacy (Wilson et al., 2009). Therefore, it is urgent to find a safe, effective, and less expensive natural product to prevent HACE. In the present study, compared with dexamethasone, pretreatment with CA exerted similar effects in the mouse model of HACE and thus might provide similar protection against HACE in humans.

5. Conclusion

The results of BWC, histopathology, and ultrastructural observation demonstrated that we successfully constructed a normobaric hypoxic cerebral edema model in ICR mice. Pretreatment with CA could significantly reduce the BWC and blood-brain-barrier permeability, which suggested that it might have a good effect against hypoxic cerebral edema. The mechanisms of this effect included: 1) increased occludin expression, thereby improving the integrity of the blood-brain-barrier; 2) enhanced Na^+-K^+-ATPase activity in mice brain tissue, which partly restored the mitochondrial membrane potential and decreased the severity of mitochondrial swelling and vacuolization; and 3) maintained the oxidant/antioxidant balance, inhibited IL-2 expression, and enhanced IL-10 expression.

In future, there may be a need to modify *p*-coumaric acid to obtain high-efficiency compounds to treat hypoxic cerebral edema and analyze its molecular mechanisms. Furthermore, there is an urgent need to develop promising therapeutic agents to treat HACE or dietary intervention measures to exert prophylactic effects against HACE.

Conflict of interest

The authors of this manuscript have declared that no competing interests exist.

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