PROTECTIVE EFFECTS OF ETHYL ACETATE EXTRACTION FROM GASTRODIA ELATA BLUME ON BLOOD-BRAIN BARRIER IN FOCAL CEREBRAL ISCHEMIA REPERFUSION

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Abstract

Background: Damage of the blood brain barrier (BBB) during the process of cerebral ischemic injury is a key factor which influences the therapeutic efficacy to the cerebral ischemic injury. The present study was designed to verify the mechanisms underlying the protective effects of the ethyl acetate (EtOAc) extraction from Gastrodia elata Blume (GEB) on the BBB by developing a model of cerebral ischemia-reperfusion in rats.

Material and methods: MCAO/R model in rats was developed through a thread embolism method. The neurological scales, the moisture and the evans blue (EB) contents of brains were detected. Meanwhile, the release of nitric oxide (NO) and activities of NO synthase (NOS) in brain tissues were measured. Western blotting analyses were also performed to assess the protein expressions of AQP-4, Occludin and Claudin-5 in brain tissue.

Results: After rats were pretreated with different concentrations of EtOAc extractions from GEB, the neurologic scores, the EB contents in the brain tissues and the moisture of the brains were significantly decreased. Meanwhile, the release of NO, the activities of nNOS and iNOS were notably inhibited. Furthermore, the protein expression of AQP-4 was markedly decreased, but the protein expressions of -5 and Occludin were significantly increased.

Conclusion: the EtOAc extracts of GEB may decrease the permeability of BBB when focal cerebral ischemia occurs. The inhibition of the NOS pathways, the attenuation of the protein expression of AQP-4 and the enhancement of the expressions of the tight junction proteins may contribute to the protective effects of the EtOAc extracts from GEB on BBB.

Key words: Gastrodia elata Blume.; blood-brain barrier; TJ; AQP-4

Introduction

As one kind of traditional Chinese medicine, Gastrodia elata Blume (GEB) is documented to treat many symptoms in nervous system, such as language paralysis, trance, headache and dizzy spin, which commonly occur in cerebral ischemic injury (CIRI). Investigations also suggest that GEB and its compounds reduce the clinical neural function defect assessment and increase the ability of daily life (An et al., 2010; Hayashi et al., 2002; Jung et al., 2007). Meanwhile, the anti-ischemic stroke effect of GEB and its compounds were also observed (Descamps et al., 2009; Yu et al., 2010). However, the mechanisms of the protective effects of GEB on the nervous system are largely unknown.
Our previous studies have shown that the ethyl acetate (EtOAc) extracts of GEB significantly protected the cerebrum from the ischemic injury in a cerebral ischemia-reperfusion animal model (MCAO/R) (LI, 2013). Further studies found that EtOAc extracts of GEB also significantly inhibited the aggregation of platelets, prevented the hippocampal CA1 cell death and attenuated the generation of nitric oxide (NO) in the brain (DUAN, 2013; LI 2011; Qing LIN., 2006; Qing LIN., 2002; Xiaohua Duan., 2013; Ye, 2008). All the findings suggest that GEB may exert protective effect on the blood-brain barrier (BBB).

BBB is a physical barrier between the blood and brain tissue. Disruption of BBB is a critical event during CIRI, followed by passive diffusion of water leading to vasogenic edema and secondary brain injury (Al Ahmad et al., 2010; Ballabh et al., 2004). After CIRI, BBB appears damage on the structure and function level, including the endothelial cells pyknosis, capillary basement membrane rupture and blood capillary lumen narrow deformation (Ballabh et al., 2004). Consequently, the increase of BBB permeability eventually leads to brain edema and increases the nerve damage, which is often considered as the secondary brain injury (Bigdeli et al., 2008; Fang et al., 2010). Reduction of cerebral edema is one of the most important remedies for reducing subsequent chronic neural damage in stroke, molecular water transport has generated interest in new targets for edema therapy (Yiu et al., 2011). Therefore, effective control of the pathophysiological process of BBB damage may play a critical role in the treatment and prognosis of CIRI. Several studies have reported that the dysfunction of BBB after ischemic stroke was attributed to the attenuation of the vascular endothelial cell tight junction, degradation of the major structural protein (occluding and claudin-5), over expression of aquaporin-4 (AQP4), inflammation reaction and the excessive accumulation (Cucullo et al., 2002; Cui et al., 2010; Gu et al., 2012; Haj-Yasein et al., 2011). Therefore, the present study was designed to verify the mechanisms underlying the protective effects of the EtOAc extraction from GEB on the BBB by developing a model of cerebral ischemia-reperfusion in rats.

**Materials and methods**

**Development of a model of MCAO/R in rat**

Adult male Sprague-Dawley rats (weighing 250 to 300 g) were provided by the Laboratory Animal Center of Experimental Animal Center (Sichuan, China) and housed under diurnal lighting conditions (12h light/dark cycle). Rats were allowed free access to food and water before surgery. Rats were allowed free access to food and water before surgery. All experimental protocols and animal handling procedures were performed in accordance with the experimental animal ethics committee of Yunnan University of Traditional Chinese Medicine (TCM).

The MCAO/R model was performed as described previously (Longa EZ, 1989; Rabb, 1995). Briefly, the animals were anaesthetized with intraperitoneal injections of 10% chloral hydrate (0.3 mL/100g). The left common carotid artery (CCA) and the external carotid artery (ECA) were exposed. Then, a 3-0 surgical monofilament nylon suture (Prodo, Japan) was carefully inserted from the CCA into the internal carotid artery (ICA) and was advanced towards to occlude the root of the left middle cerebral artery (MCA) until a light resistance was felt (18-22 mm from CCA bifurcation). After 2 h of MCAO, the nylon suture was removed to restore blood flow (reperfusion), followed by 24 h of reperfusion. The rats were placed into cages to recover after incision closure, with free access to food and water. Sham-operated rats underwent identical surgery except that the suture was not inserted. Core body temperature was maintained at 37.0±0.5°C using a heating pad and Air conditioner during the whole procedure. After 2 h of MCAO, the nylon suture was withdrawn, followed by 24h of reperfusion.

**Drug extraction, animal grouping and drug delivery**

The EtOAc extraction was obtained from GEB. The original medicinal herbs were extracted by 95% ethanol at 10.62% of the crude drug extract rate. Then the ethanol extraction was treated by ethyl acetate (at 1.41% of the crude drug extract rate). All the extraction compounds are stored at -40°C.

Animals were divided into the following 4 groups: 1) High dose group: rats were treated with 102.57mg/kg EtOAc extraction from GEB; 2) Low dose group: rats were treated with 11.397mg/kg EtOAc extraction from GEB; The EtOAc extraction were...
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pre-treated for 5 days (q.d.) before right middle cerebral artery was occluded; 3) Sham-operated group; and 4) Control group: rats were treated with 1mL/100g distilled water.

Neurological score recording

Neurological score recording method was described previously (Bederson JB PL, 1986; Choi et al.). The severity of neurological deficits was evaluated 24 h after the ischemic insult using a five-point deficit score (0 = normal motor function; 1 = flexion of torso and of contralateral forelimb upon lifting of the animal by tail; 2 = circling to the contralateral side but normal posture at rest; 3 = leaning to contralateral side at rest; and 4 = no spontaneous motor activity).

BBB permeability determine

To determine the BBB permeability, evans blue (EB, Sigma, USA) exudation was assessed as previously described (Lee et al., 2012; Liu et al., 2011; Zehendner et al.). Briefly, 2% EB solution was injected (0.4 mL/100 g, i.p.) 15 min after surgery ischemia, followed by MCAO/R for 24h. After rats were anesthetized with intraperitoneal injections of 10% chloral hydrate (0.3 mL/100g), the thoracic cavity was opened and perfused with normal saline. The brain tissue was removed and hemispheres were separated and weighed. The hemispheres were then dried at 105°C for 24 h for measuring their dry weights (DW). In addition, the hemispheres were incubated in formamide solution (Sigma, USA, 1 mL/each hemisphere brain tissue) at 50°C for 24 h. Then, OD values were measured at 610 nm using an Enzyme standard instrument (Infinite M200 PRO, Tecan, Swiss). Results were expressed as μg/g brain tissue calculated according a standard curve.

Measurement of water content of the brain

The water content of the brain was determined using a method as previously described (Dai, 2015; Huang et al., 2012). Rats were killed 24 h after MCAO/R and the hemispheres were separated. The left and right cerebral hemispheres were weighed to obtain their wet weight (WW), respectively. Then, the hemispheres were dried at 110°C for 24 h to determine their DW. The water content was expressed by using the following formula: (WW-DW)/WW×100%.

BBB ultrastructure detection

After anesthetization, rats were intracardiovascularily perfused with 2.5% glutaraldehyde-4% paraformaldehyde after 24 h of MCAO/R. The right parietal lobe cortex (1x1 mm³) was submerged into 2.5% glutaraldehyde-2% paraformaldehyde. After washed three times with PBS, the samples were post-fixed in 4% osmium acid at room temperature. After fixation, the samples were dehydrated in gradient alcohol and embedded in Epon 618. The ultrathin sections were negatively stained with uranyl acetate and lead citrate and examined with a JEM-1200EX electron microscope to observe the changes of the ultrastructure of BBB.

Measurements of the release of NO and the activity of NOS

Rats were killed 24 h after MCAO/R. After decapitation, the forebrains were rapidly dissected out, cleaned by rinsing with chilled saline and divided into two sets. Forebrains were divided sagitally through the midline into two halves and was stored at -80°C for biochemical and the protein expression analysis. The forebrain tissue was minced into small pieces, homogenized in cold PBS buffer saline (0.05 mol/L, pH 7.4) and centrifuged at 5000 g for 15 min at 4°C. The supernatants were obtained and the NO contents were detected using assay kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to them manufacturer’s
The activities of NOS, including the endothelial NOS (eNOS), neuronal NOS (nNOS) and the inducible NOS (iNOS) were measured using the ELISA kits (Beyotime, China). Total protein concentration was determined by a method of BCA (Beyotime, China).

**Western blot analysis**

Right parietal lobe cortex was homogenized in 10 mmol/L Tris homogenization buffer (pH 7.4) with protease inhibitors (1 tablet in 50 ml, Solarbio, USA). The samples were centrifuged at 14,000 rpm for 3-5 min and the supernatant was collected for western blotting analysis. After determining the protein concentrations of the supernatants (BCA method, Beyotime, China), 50-120 μg protein samples were loaded onto the 8% -15% SDS gel and separated by electrophoresis and transferred to PVDF membrane. Then, the membranes were incubated with a rabbit anti-AQP-4 polyclonal IgG (1:2000, Proteintech Biotechnology, USA), a rabbit anti-Occludin polyclonal IgG (1:300, Proteintech Biotechnology, USA) and a rabbit anti-Claudin-5 polyclonal IgG (1:200, Santa Cruz Biotechnology, USA), respectively. After washed three times with TBS, the membranes were incubated with a goat anti-rabbit HRP-IgG (1:1500, Proteintech Biotechnology, USA). The immunoreactive bands were visualized by an ECL Western blotting detection kit (BI, USA) on light sensitive film. Imaged and analyzed by a western blot imaging system (UVP) using ImageJ for analyzing image grey value.

**Statistical analysis**

In western blotting assay, changes in protein levels were described as the ratio to β-actin. Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data were presented as means ± SD. Comparison between two groups was analyzed using Student’s t-test. Comparison among three or more groups was analyzed using one-way ANOVA. Values of P<0.05 were accepted to indicate statistically significant differences.

**Results**

**Evaluation of the MCAO/R model in rats**

Compared with the sham-operated group, obvious neurological deficits were observed in rats of the control group (Fig.1A and B). Meanwhile, the EB contents in the brain tissues were increased significantly (Fig.1C and D). In addition, the transmission electron microscope (TEM) results showed that the ultrastructural damages of BBB occurred in varying degrees (Fig.1E-H). The integrity of BBB was disrupted for the lesions of BBB were observed in the control group. The capillary lumen was shrunk and the edema was detected around the capillary. Astrocyte foot processes surrounding the capillaries appeared woolen. The capillaries consisted of severely vacuolated endothelial cells surrounded by damaged basement membrane. The tight junction between endothelial cells was unclear. Capillary endothelial cells showed endoplasmic reticulum swelling and formation of numerous electron lucent vacuoles in their cytoplasm (Fig.1F and H). However, all these traits were not found in rats underwent sham-operation.
The brain water content was used to evaluate cerebral edema, and EB extravasation was used as an indicator for the BBB breakdown. Compared with the Sham-operated group, the neurologic scores, moisture of the brains (right) and the EB contents in the brain tissues (right panels) were increased significantly in Control group (P<0.001). However, in rats pre-treated with the EtOAc extracts from GEB (10.257mg/kg), the neurologic scores (Fig.2A) (P<0.01), the moisture of the brains (Fig.2B) (P<0.05) and the EB contents (Fig.2C) in the brain tissues (P<0.01) were decreased significantly. Meanwhile, after rats were treated with the EtOAc extracts from GEB (11.397mg/kg), significant decrease of EB contents was also observed (Fig.2C) (P<0.01). However, the moisture of brains and EB levels in the contralateral hemisphere (left) were not significantly different among different groups (Fig.2).

**Figure 1:** The model of MCAO/R in rats. A, C, E and G: Sham-operated group. B, D, F and H: Control group. A and B: Neurological symptoms. C and D: EB permeability changes. E-H: BBB ultrastructural changes.

**Protective effects of the EtOAc extract from GEB on BBB**
Figure 2: Protective effect of the EtOAc extract from GEB on the BBB. A: Neurological score alterations, B: Water content alterations, C: EB content alterations of the rat were pre-treated with the EtOAc extracts from GEB. Data were represented as mean±SD (n=6). ▲▲▲P< 0.001 vs. Sham; *P<0.05 vs Control. **P<0.01 vs Control.

Mechanisms involved in the effects of EtOAc extracts from GEB on BBB

GEB EtOAc extracts decrease the release of NO and the activities of NOS Compared with the Sham-operated group, the release of NO (Fig.3A) were increased significantly (P<0.001) in Control group. In addition, the activities of nNOS (Fig.3B) and iNOS (Fig.3C) were decreased significantly (P<0.01 and P<0.05, respectively). After pre-treated with different concentrations (102.57mg/kg and 11.397mg/kg) of the EtOAc extracts from GEB, the release of NO (Fig.3A) were decreased significantly (P<0.001 and P<0.01, respectively). Whereas the activities of nNOS (Fig.3B) and iNOS (Fig.3C) were decreased significantly (P<0.01 and P<0.05, respectively), the activity of eNOS (Fig.3D) was not markedly changed among different groups.
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Figure 3: Effect of EtOAc extract from GEM on the release of NO and the activities of NOS. A: The release of NO; B: The activities of nNOS; C: The activities of iNOS; D: The activities of eNOS of the rat were pre-treated with the EtOAc extracts from GEB. Data were represented as mean±SD (n=6). ▲▲▲ P<0.001 vs Sham. * P<0.05 vs Control, ** P<0.01 vs Control, *** P<0.001 vs Control.

EtOAc extracts from GEB regulate BBB related protein expression

Compared with the Sham-operated group, the expression of AQP-4 (Fig.4) was significantly increased (P<0.001), in contrast, the expressions of Claudin-5 (Fig.5,A) and Occludin (Fig.5,B) were markedly decreased (P<0.001 and P<0.05, respectively) in Control group. After pre-treated with the EtOAc extracts from GEB (102.57mg/kg), the expression of AQP-4 (Fig.4) were decreased significantly (P<0.05), in contrast, the expressions of Claudin-5 (Fig.5,A) and Occludin (Fig.5,B) were markedly increased (P<0.001 and P<0.05, respectively). Meanwhile, after rats were pre-treated with the EtOAc extracts from GEB (11.397mg/kg), significant increase of the expression of Occludin was also observed (Fig.5,B) (P<0.05).

Figure 4: Effect of EtOAc extract from GEM on the expression of AQP-4. Data were represented as mean±SD (n=6). ▲ P<0.05 vs. Sham; * P<0.05 vs. Control.
Figure 5: Effect of EtOAc extract from GEM on the expression of TJ. A: Claudin-5 protein expression; B: Occludin protein expression of the rat were pre-treated with the EtOAc extracts from GEB. Data were represented as mean±SD (n=6). ▲▲P<0.01 vs Sham; ▲P<0.05 vs Sham. **P<0.01 vs. Control; *P<0.05 vs. Control.

Discussion

Damage of BBB during the process of cerebral ischemic injury is a key factor which significantly influences the therapeutic efficacy to the cerebral ischemic injury. Our results showed that after pre-treated with the GEB EtOAc extracts the neurologic scores of rats, the EB contents and moisture of the brains were significantly improved. All these evidences suggest that GEB EtOAc extracts may protect BBB when CIRI occurred.

Previous studies suggest that CIRI have been attributed to a number of mechanisms, including calcium overload and glutamate excitotoxicity. Meanwhile, many studies point out that the increased production of endogenous NO plays an important role in the pathogenesis of CIRI (Gu et al., 2012; Mohammadi et al., 2012). Increased NOS activities in the ischemic lesion sites suggest that the increased NO deteriorate the process of BBB breakdown and edema formation. The endothelial NOS (eNOS), neuronal NOS (nNOS) and the inducible NOS (iNOS) are three forms of NOS that play important but opposing roles in CIRI. NO derived from iNOS and nNOS and its oxidative effects by product peroxynitrite are thought to contribute to neuronal death (Wu Xiaohua 2014). In contrast, NO produced by eNOS has a physiologically protective role and regulates the paracrine homeostatic functions of the endothelium, including inhibition of leukocyte and platelet adhesion, control of vascular tone, scavenging of free radicals and maintenance of a thrombo resistant interface between the blood stream and the vessel wall. In the present study, the release of NO was decreased significantly in GEB EtOAc extracts pre-treated group. In addition, the activities of nNOS and iNOS were decreased significantly, while the activity of eNOS had no significant change. Our results suggest that the inhibitions of the NO and NOS contribute to the protective effects of the EtOAc extracts from GEB on the BBB.
Opening of BBB with an osmotic insult induces brain edema which represents a factor triggering axonal impairment (Haj-Yasein et al., 2011). Reduction of cerebral edema is one of the most important remedies for reducing subsequent chronic neural damage in stroke (He et al., 2015; Li et al., 2015; Stokum et al., 2015). Therefore, cellular water transport has generated interest in new targets for edema therapy. AQP-4, which is currently considered as a therapeutic gene, influences the brain extracellular space and plays an important role in maintaining brain water homeostasis under various neurological insults (He et al., 2015). In the present study, the expression of AQP-4 was significantly decreased in drug treated group. This result suggested that the protective effects of GEB extracts possibly involved in inhibiting AQP-4 overexpression.

It is well known that tight junction (TJ) between the epithelial cells could form a metabolic and physical barrier which maintained cerebral homeostasis and restricted macromolecule movement between the blood and brain (Gu et al., 2013; Ren et al., 2015). The tight junction barrier of BBB is formed by the interaction of membrane-associated accessory proteins including Occludin and Claudin-5 (Alluri et al., 2015; Gu et al., 2013; Ren et al., 2015; Zhang et al., 2014). It has been reported that the expressions of Occludin and Claudin-5 in the endothelial cells were decreased at the early period of embolism. In the present study, Occludin and Claudin-5 were markedly upregulated in drug groups, which implies the potential mechanism that the EtOAc extracts from GEB protects the BBB against ischemia are perfusion-induced disruption by up-regulating the expression of tight junctional proteins.

In conclusion, the GEB EtOAc extracts significantly decrease the permeability of BBB in a focal cerebral ischemia rat model. The inhibitions of the NOS and the enhancements of the expressions of the tight junction proteins, including Occludin, Claudin-5, and the attenuation of the protein expression of AQP-4 contribute the BBB protective effects of the GEB EtOAc extracts.

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