Neuroprotective effect of ethyl acetate extract from gastrodia elata against transient focal cerebral ischemia in rats induced by middle cerebral artery occlusion

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Abstract

OBJECTIVE: To investigate the protective effect of neuroprotection against transient focal cerebral ischemia of the extract from Tianma (Rhizoma Gastrodiae) and the possible mechanisms underlying the action.

METHODS: Cerebral ischemia-reperfusion injury was induced through middle cerebral artery occlusion (MCAO). Adult male Sprague-Dawley rats were randomly divided into four groups: sham-operated, ischemia-reperfusion model, 102.6 mg/kg extract treated and 11.4 mg/kg extract treated groups. The extract was prepared from gastrodia elata with ethyl acetate. The effect of the extract tested on rat neurological deficits and Cerebral index, cerebral infarct volume, brain injury, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and B-cell lymphoma-2 (Bcl-2) positive cells.

RESULTS: The extract was able to reduce neurological scores, cerebral index and cerebral infarction rate. The brain injury was also relieved by the extract. The results of immunofluorescence analysis indicated that the extract increased the expression of Bcl-2 and reduced TUNEL-positive cells significantly in the extract treated groups.

CONCLUSION: These results suggested that the extract relieved ischemic injury induced by transient focal cerebral ischemia in rats, and this neuroprotective effect might be partially due to the attenuated apoptosis pathway.

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Key words: Ethyl acetate; Gastrodia; Neuroprotective agents; Reperfusion injury; Apoptosis

INTRODUCTION

In China, 1.5 million people die from stroke every year, and in developed countries, stroke is the third leading cause of death after cancer and heart diseases. Among all stroke cases, ischemic strokes account for approximately 87%, and it has produced negative im-
MATERIALS AND METHODS

Animals
Adult male Sprague-Dawley rats of Specific Pathogen Free (SPF) grade (five-month-old, weighing 300 ± 50 g), were used in the experiments. The rats were purchased from the Central Animal Facility of sichuang Chinese Laboratory animal certificate: scxk (chuang) 2008-24, Sichuan, China) and fed in the Laboratory Animal Center of Yunnan University of Traditional Chinese Medicine. The study was approved by the experimental animal ethics committee of Yunnan University of Traditional Chinese Medicine. The study was approved by the experimental animal ethics committee of Yunnan University of Traditional Chinese Medicine.

Preparation of EEGE
Dried and ground rhizomes of GE (Xiaoacaoba, Zhaotong, China) 10.0 kg were extracted with 70% Ethanol (EtOH) (Kemiou Chemical Reagent Co., Ltd., Tianjin, China) (25L x 3) at room temperature. The EtOH extract, after removal of the solvent by evaporation, was suspended in H2O and partitioned with ethyl acetate (EtOAc). The resulting fractions is a portion of the EtOAc-soluble fraction (EEGE, 114 g). The maximum tolerated dose (MTD) of EEGE is 634 mg/kg, equivalent to 205 times the clinical dose. EEGE-treated groups including 102.6 mg/kg EEGE and 11.4mg/kg EEGE, were safe.

EEGE administration
Thirty-two adult male Sprague-Dawley rats were randomly divided into A, B, C and D groups (n = 8 per group) by random number table method: group A, sham-operated; group B, schema-reperfusion models; group C, treated with 102.6 mg/kg of EEGE, and group D treated with 11.4 mg/kg of EEGE. The EEGE was administered for 5 days (q.d.) before right middle cerebral artery was occluded. Sham-operated and modeled animals were given equal volumes of same vehicle.

Establishment of transient cerebral ischemia models in rats
Transient cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) in rats as previously described.11,13 Briefly, rats were anesthetized by 10% chloral hydrate (3 mL/kg, intraperitoneally). Body temperature was maintained at (37.0 ± 0.5) °C during and shortly after surgery by a heating lamp. After a midline neck incision, the right common carotid artery was carefully isolated from the vagal nerve, and external carotid artery was ligated. A nylon filament (diameter 0.26 mm) (Prodo Co., Ltd., Tokyo, Japan) was gently introduced into the right internal carotid artery through the common carotid artery, so as to occlude the origin of the middle cerebral artery. After 2 h ischemia, the nylon filament was carefully removed to establish reperfusion. Sham-operated animals underwent exposure of vessels without occlusion of common carotid arteries.

Measurement
Focal cerebral ischemia in the rats was induced by MCAO. After 6 h and 24 h reperfusion, the neurological deficits were evaluated by Bederson’s improved method.14,15 The neurological evaluation parameters are described in Table 1.

Cerebral index
The cerebral index was defined as: cerebral index = brain wet weight / body weight. The brains were removed and weighed 24 h after MCAO. The preoperative weight and postoperative weight (24 h after MCAO) were weighed. Rate of decreased weight = (preoperative weight – postoperative weight) / preoperative weight × 100%
and observed at 400× amplification times of light microscopic. Brown granules represented positive staining, and the number of positive cells in six randomly chosen fields was counted. 

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay**

To observe DNA strand breaks in nuclei, the TUNEL assay was performed according to the manufacturer’s instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany). Briefly, the sections were treated with Proteinase K (TaKaRa) and 0.3% H2O2. Then the sections were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37 °C for 60 min. Further incubation with peroxidase-conjugated anti-body was performed for 30 min at 37 °C. 3’,3’-Diaminobenzenidine (DAB) was used for the coloration of apoptotic cells. The photographs were taken at a magnification of 10 × 40.

**HSP-70 expression**

Immunohistochemistry for HSP-70 were carried out on formalin-fixed, paraffin-embedded sections. After deparaffinized with xylene and dehydrated, the 3 μm sections were quenched with 3% hydrogen peroxide (H2O2) in absolute methanol and treated with sodium citrate buffer (pH = 6.0) in a 1000 W microwave oven for 15 min for antigen retrieval. Then the primary monoclonal antibody of HSP-70 (rabbit-anti-rabbit, 1: 100) (Proteintech, Chicago, USA) was applied overnight at 4 °C after nonspecific antigen was blocked with normal goat serum. The sections were washed and then incubated with secondary antibodies (goat-anti-rabbit, 1: 500) (Proteintech, Chicago, IL, USA), and color reaction was developed by using diaminobenzidine as the chromagen. The slides were then counterstained with hematoxylin, dehydrated by graded alcohols and xylene, and observed at 400 amplification times of light microscopy. Brown granules represented positive staining, and the number of positive cells in six randomly chosen fields was counted. 

**Statistical analysis**

Data are presented as mean ± standard deviation. The differences between the groups were analyzed with a one-way analysis of variance using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significant level was P < 0.05.
RESULTS

Neurological deficit scores
The neurological deficit scores in model was significantly higher than sham-operated group \( (P < 0.05) \). And the cerebral infarction rate of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE were significantly lower than model group \( (P < 0.05) \), Figure 1.

Cerebral index
The mean cerebral index in model was significantly higher compared to sham-operated group \( (P < 0.05) \), while 102.6 mg/kg EEGE was significantly lower than that of the model group \( (P < 0.05) \). The group of 11.4 mg/kg EEGE had no obvious difference compared to MCAO group (Figure 2).

The neurological scores (Points)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>Model</th>
<th>102.6 mg/kg EEGE</th>
<th>11.4 mg/kg EEGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurological scores</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

![Figure 1](image1.png)

The rate of decreased weight in MCAO was significantly higher compared to sham-operated group \( (P < 0.05) \), while the rate of 102.6 mg/kg EEGE group was significantly lower than that of the model group \( (P < 0.05) \). The group of 11.4 mg/kg EEGE had no significant difference compared to MCAO group (Figure 3).

Cerebral infarction
The cerebral infarction rate in model was significantly higher than that of sham-operated group \( (P < 0.01) \). And the cerebral infarction rates of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE groups were significantly lower than that of the model group \( (P < 0.01) \), Figure 4.

HE staining
Microscopic examination of histological sections revealed regions of affected areas, which were clearly distinguished from normal brain parenchyma (Figure 5).

Bcl-2 expression
The expressions of Bcl-2 in the rat brain were defined and quantified. Positive staining particles were brown in color and mainly located in the cyto-plasm. The expressions of Bcl-2 positive cell in the groups of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE were significantly higher than that of the model group \( (P < 0.01) \), Figure 6.

In situ labeling of DNA fragmentation
The apoptotic cell death was determined by terminal deox-y-nucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL staining). TUNEL-positive cells (exhibiting shrunken cell bodies and condensed nuclei with numerous apoptotic bodies.) were detected in the injured cerebral hemispheres.
Cerebral infarction (%)

Figure 4 The impact of cerebral infarction rate after oral administration EEGE
A-D: The TTC staining of sham, model, 102.6 mg/kg EEGE and 11.4 mg/kg EEGE groups. Sham group and model group treated with normal saline. 102.6 and 11.4 mg/kg EEGE group treated with 102.6, 11.4 mg/kg EEGE. EEGE: ethyl acetate extract of Gastrodia elata. E: cerebral infarction. Data are presented as mean ± standard deviation (n = 8). The significant differences between the model group and the sham-operated group were designated as *P < 0.01. The significant differences between the groups of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE, and with the model group designated as **P < 0.01.

Figure 5 Representative microscopic findings stained with hematoxylin and eosin 24 h after the induction of MCAO and photothrombosis (× 400)
A-D: The pathological changes of sham, model, 102.6 and 11.4 mg/kg EEGE groups. Sham group and model group treated with normal saline. 102.6 and 11.4 mg/kg EEGE group treated with 102.6, 11.4 mg/kg EEGE. EEGE: ethyl acetate extract of Gastrodia elata. Data are presented as mean ± standard deviation (n = 8). The 102.6 mg/kg EEGE reduced the pathological changes and increased the number of brain cells.

The TUNEL-positive cells in model was significantly higher compared to sham-operated group (*P < 0.01). All treated groups was significantly lower than that of the model group (**P < 0.01, Figure 7).

Figure 6 Impact of Bcl-2 positive cells number after oral administration EEGE
A-D: The Bcl-2 expressions of sham, model, 102.6 and 11.4 mg/kg EEGE groups (× 400). Dyeing method of all pictures are the Immunohistochemical SP method, PBS was used as negative control. Sham group and model group treated with normal saline. 102.6 and 11.4 mg/kg EEGE group treated with 102.6, 11.4 mg/kg EEGE. EEGE: ethyl acetate extract of Gastrodia elata. E: Bcl-2 positive cells. Data are presented as mean ± standard deviation (n = 8). The significant differences between the model group and the sham-operated group were designated as *P < 0.01. The significant differences between the groups of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE, and with the model group designated as **P < 0.01.

HSP-70 expression
The expressions of HSP-70 in the rat brain were defined and quantified. Positive staining particles were brown in color. The expressions of HSP-70 positive cell in the groups of 102.6 mg/kg EEGE and 11.4 mg/kg EEGE were significantly higher than that of the model group (**P < 0.05, Figure 8).

DISCUSSION
Stroke is a global health problem. It is the second commonest cause of death and a leading cause of adult disability worldwide. Moreover, the number of patients with stroke will increase in the future because of demographic changes and the inadequate control of major
terminal demise process, in order to maintain a stable internal environment. Therefore, inhibition of the IP area apoptosis becomes one of the biggest concerns in the world.

Cerebral ischemia is a pathological disorder caused by the interruption of blood supplying to the brain, which is usually due to the blockade by a clot or an embolus in a blood vessel of the brain. The consequent lack of glucose and oxygen, if prolonged, leads to permanent damage to the nervous tissue. The MCAO is one of the most commonly used models on cerebral ischemia. Its advantages are: (a) cerebral vascular anatomical char-
acertistics close to humans; (b) the experimental data are rich on rat physiological, biochemical, morphological and pharmacological, which is conductive to study and compare; (c) low price; (d) the varieties is relatively consistent, less variation; (e) brain size is relatively small, and is conductive to the histopathology. The original application of *Rhizoma Gastrodiae* was to treat brain diseases with good effect, such as epilepsy, convulsions, tetanus, limb numbness, and brain injury. Afterwards, its application was expanded to treat cardio-cerebrovascular diseases. Part of its mechanism is believed to be associated with hemorhology improvement including apoptosis activity.

Brain edema in ischemic penumbra is usually accompanied with brain ischemia and is the most common inducement in patients with ischemic brain damage, demonstrating harmful clinical outcome. Cerebral index directly reflects the extent of brain edema. The damage of the central nervous system was evaluated by the neurological deficit scores; the infarct size was analyzed with (TTC) staining, and the histopathological changes of brain were assessed by hematoxylin-eosin (HE) staining in the brain slices. Apoptosis is a major cell death pathway activated after cerebral ischemia/reperfusion. One of the important mechanisms for Bcl-2 is to block cell apoptosis. The early intervention of Bcl-2 to interrupt the apoptotic process and inhibit the development towards necrosis direction, which plays an important role in maintaining neuronal survival after MCAO. TUNEL can be used to identify cells in the last phase of apoptosis undergoing DNA degradation, reflecting final DNA damage. HSP-70 could confer protective effects on damaged tissue or organ during stress. Some previous reports have indicated that HSP-70 is extensively expressed in the brain during brain ischemia and was suggested to play an important role in cell survival and recovery after ischemic injury.

Our results revealed that the neurological scores, Cerebral index, rate of decreased weight and Cerebral infarction rate were reduced. The brain injuries were relieved by the extraction. Results of immunofluorescence staining analysis indicated that the extraction increased the expression of Bcl-2 and HSP-70. The number of TUNEL-positive cells is reduced significantly in the treated group.

In summary, our results suggested that EEGE reduced the ischemic injury induced by transient focal cerebral ischemia in rats, and this neuroprotective effect might be partly due to attenuated apoptosis pathway.

REFERENCES


