Para-hydroxybenzaldehyde against transient focal cerebral ischemia in rats via mitochondrial preservation

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Received May 24, 2022; Accepted August 22, 2022

DOI: 10.3892/etm.2022.11652

Abstract. Gastrodia elata (GE) Blume has been widely used for thousands of years to treat various central and peripheral nervous disorders. P-hydroxybenzaldehyde (PHBA) is a chemical component of GE. However, its role and mechanism in transient focal cerebral ischemia remain unclear. The present study aimed to investigate the protective effect of PHBA on middle cerebral artery occlusion (MCAO) rats. A total of 56 Sprague-Dawley male rats were randomly divided into control, model, PHBA-high dose (PHBA-H) and PHBA-low dose (PHBA-L) groups. The MCAO injury model was replicated in all rats except for the control group. In the control group, only the right common carotid artery was isolated without embolization. After treatment with PHBA, the protective effects (neurological deficit score, cerebral index, weight and cerebral infarct) were analyzed. Western blotting was performed to estimate the protein levels of Bcl-2, Bax and Caspase-3. Apoptotic cells were detected using hematoxylin-eosin staining and TUNEL immunofluorescence assay. Mitochondrial oxidative stress indicators, including reactive oxygen species (ROS), malondialdehyde (MDA) and total superoxide dismutase (T-SOD), while dysfunction indicators, including mitochondrial permeability transition pore (MPTP), ATP and cytochrome C oxidase, were measured using commercial kits. The ultrastructure of mitochondria was observed under an electron microscope. Once the model was successful established, the rats in the MCAO group suffered neurological damage (P<0.001), increased cerebral index (P<0.001), decreased body weight (P<0.001) and had severe cerebral infarction (P<0.001). Moreover, the number of apoptotic cells and the levels of ROS (P<0.001) and MDA (P<0.05)

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in mitochondria and the protein levels of Bax (P<0.001) and cleaved caspase-3 (P<0.001) were increased. The activities of T-SOD (P<0.001) and cytochrome C oxidase (P<0.001) in the mitochondria, ATP content (P<0.05) and Bcl-2 protein level (P<0.001) decreased, MPTP was stimulated to open and mitochondrial structures were damaged (P<0.001). PHBA treatment resulted in a decrease of the neurological deficit score (PHBA-H 24 h, P<0.001; PHBA-H 6 h and PHBA-L 24 h, P<0.01; PHBA-L 6 h, P<0.05), apoptotic cell number (P<0.001), mitochondrial ROS (P<0.001) and MPTP opening (P<0.001), Bax (P<0.01, P<0.001) and cleaved caspase-3 protein expression (P<0.001) in rats. And the expression of Bcl-2 protein (P<0.001) was increased. In addition, the cerebral index (P<0.05), weight loss (P<0.05), infarction rate (P<0.01) and MDA content (P<0.001) were decrease in the PHBA-H group. The level of ATP (P<0.05) and cytochrome C oxidase (P<0.05) and T-SOD activity (P<0.05) of PHBA-H group rats increased, but no significant difference was observed in the PHBA-L group. Overall, PHBA had a protective effect on transient focal cerebral ischemia in normal rats, regulated the expression of Bcl-2, Bax and cleaved caspase-3 proteins and improved the oxidative stress and dysfunction of mitochondria.

Introduction

Ischemic stroke is a clinically common and dangerous disease with the second highest morbidity, mortality and disability rates worldwide (1). The most serious hazard of ischemic stroke is neuronal apoptosis (2). Mitochondria are important organelles that can generate oxidative stress and dysfunction in response to ischemia and hypoxia events (3-6). This series of pathological processes decreases the anti-apoptotic protein Bcl-2 level, increases the pro-apoptotic protein Bax level and enhances the expression of cleaved caspase-3 (7-10). Neuronal cells with high energy depletion undergo large-scale apoptosis (11). While it takes a long period for cells to undergo apoptosis, drug treatment of transient focal cerebral ischemia is possible (12).

Gastrodia elata (GE) Blume is a common and valuable traditional Chinese medicine, mainly produced in central and south China (13). It has been used to treat lumbago, headache, epilepsy, paralysis, rheumatism and other diseases for thousands of years and has been recorded in the Chinese pharmacopoeia (14). GE has been widely studied in the central

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Key words: P-hydroxybenzaldehyde, *Gastrodia elata* Blume, ischemic stroke, middle cerebral artery occlusion, Bcl-2/Bax protein, caspase-3 protein, oxidative stress, dysfunction of mitochondria

nervous system (CNS) because it improves ischemia in several *in vitro* and *in vivo* models (15-19). It has been reported that GE extract can protect the dopaminergic cell toxicity induced by 1-methyl-4-phenylpyridinium through antioxidation and anti-apoptosis activities (20). In addition, several compounds present in GE, such as 4-hydroxybenzyl alcohol, vanillin, parisonoside C and 3,4-dihydroxybenzaldehyde, have been shown to have strong neuroprotective effects in cerebral ischemia (21-23). Therefore, several studies have focused on the effect of different components of GE on the CNS (24,25).

P-hydroxybenzaldehyde (PHBA) is one of the compounds isolated from GE and has various pharmacological effects, such as antioxidant, anti-inflammatory and vasodilation effects (26-28). Due to its lipid solubility and small molecule characteristics, PHBA is likely to pass through the blood-brain barrier (BBB) to exert a therapeutic role on the CNS (29,30). Simultaneously, the structure of its C-4-aldehyde and hydroxyl groups can regulate the neurotransmission function in the brain (31). Zhu *et al* (30) replicated the model of oxidative damage to the BBB by co-culturing mouse brain microvascular endothelial cells and astrocytes, and confirmed that PHBA has a protective effect on oxidative stress-induced BBB damage. Moreover, PHBA increases the expression of antioxidant-related proteins and improves the endogenous antioxidant capacity of cells (30).

Kim *et al* (23) revealed that PHBA increases neuronal survival by 43.21% in the reperfusion model of the common carotid artery replicated in Mongolian gerbils, although the study did not clarify the relevant mechanism underlying its neuroprotective activity. *In vitro* experiments showed that in the oxygen-glucose deprivation/reperfusion model, PHBA helps regain the lost neurons in the ischemic area by promoting the reprogramming conversion of astrocytes to neurons (32).

Therefore, PHBA has an important potential in the neuroprotection of cerebral ischemia, although its mechanism of action remains clear. Mitochondria play a critical role in the course of transient focal cerebral ischemia (33). Mitochondria-targeted therapy has shown promising results in the treatment of cerebral ischemia (34). In contrast to previous studies, the present study aimed to explore the protective effect of PHBA on middle cerebral artery occlusion (MCAO) model in rats based on mitochondrial function.

Materials and methods

Animals. A total of 56 male Sprague-Dawley rats (5-8-weeks-old; 250-350 g weight), were purchased from the Sichuan Central Animal Experimental Base, China [Laboratory animal qualification certificate: scxk (Chuan) 2018-24]. The rats were given free access to food and water; the rearing environment was maintained in a 12-h light/dark cycle with temperature (18-25°C) and relative humidity (40-60%). All animal experiments were approved by the Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine, Yunnan, China (approval no. R-062019039) and the care and use of experimental animals were carried out in accordance with the guidelines of the American National Institutes of Health (35). All efforts are made to reduce the number and the suffering of the animals.

Experimental schedule and MCAO model in rats. Rats were equally divided into four groups: Control (sham operation group), MCAO, PHBA-high dose (PHBA-H; 11.1 mg/kg PHBA) and PHBA-low dose (PHBA-L; 0.74 mg/kg PHBA) groups by random number table method. PHBA (cat no. DRE-C14228740; CAS:123-08-0; J&K Scientific, Ltd.) was administered intragastrically for 5 days (once daily) before the experiment on the MCAO rat model. Moreover, control and MCAO groups were given an equivalent volume of normal saline. Then, the cortex area in the ipsilateral hemisphere was chosen for experimental procedures.

In the present study, the operation method of MCAO was selected to establish the model, which resulted in cerebral ischemia injury in rats. Rats were anesthetized using 10% chloral hydrate intraperitoneal injection (cat. no. 20141012; Damao Chemical Reagent Factory) at a dose of 300 mg/kg weight (36). After routine disinfection, the following steps were undertaken: i) The skin was cut along the midline of the neck; ii) the muscle and fascia were separated layer by layer; iii) the vagus nerve was separated from the common carotid artery on the right; iv) the external carotid artery was ligated with nylon filament; and v) the middle cerebral artery was blocked by gently introducing 0.26-mm nylon filament (Ruibo Biotechnology Co., Ltd.) through the right common carotid artery. After 2 h of occlusion, the blood flow of the anterior cerebral artery was affected, resulting in local middle artery ischemia. After removing the occlusion, the blood flow of the middle cerebral artery was restored and the MCAO model was established in normal rats. In the sham group, only the right internal and external carotid branches were separated after anesthesia and the middle cerebral artery was not blocked. The temperature of the rats was maintained at 37.0±0.5°C during and after the operation. The MCAO model was assessed by laser speckle imaging (RWD Life Science Co., Ltd.). The state of the rats was closely observed during and after the modeling operation. During this period, none of the animals developed humane endpoint indications, such as non-feeding, dyspnea, convulsion and hypothermia, or died prematurely. At the time of sampling, one rat in each group was randomly selected to dissect the abdomen and no symptoms of peritonitis were observed.

General state of rats. After 6 and 24 h of MCAO model reperfusion, the neurological deficit score (Table I) was evaluated using the improved method of Bederson (37,38).

After 24 h of MCAO, anesthesia was induced by 5% isoflurane inhalation (RWD Life Science Co., Ltd.) and maintained with 3% isoflurane (39). Subsequently, rats entered deep anesthesia and had no response to tail pinching. The animals were rapidly sacrificed using a decapitation device. Brain tissue samples were collected and weighed to calculate the cerebral index as follows: Cerebral index=wet weight of the brain/weight of the body. The body weight before MCAO and 24 h after MCAO were recorded. The weight loss percentage=[(weight before modeling-weight after modeling)/weight before modeling] x100.

To calculate the volume of cerebral infarction, brain tissue was removed and cut into four coronal sections (2-mm thick) 24 h after successful modeling and stained with 0.5% triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA)

Parameters	Score			
	0	1	2	
Flexion degree of forelimb	No flexion	Wrist flexion	Wrist and elbow flexion	
Floor walking	Straight path	Curvilinear path	Walking in circles only	
Response to vibration	Sensitive response	Weak response	No response	
Body rotation	No rotation	Seldom rotation	Frequent rotation	

Table I. Neuro	logical	deficit	score	parameters

at 37°C for 30 min (40). A digital camera (Canon 600D; Canon, Inc.) was used to record the staining image and Motic image plus 2.0 (Motica China Group Co., Ltd.) was used for quantification analysis. Furthermore, brain edema may greatly affect the volume calculation of cerebral infarction as follows: The volume of infarcted=total volume of infarcted x left hemisphere volume/right hemisphere volume. The infarction rate was calculated as follows: Infarction (%)=(volume of infarcted/bilateral hemisphere volume) x100 (41).

Cerebral neuronal cell apoptosis assay. H&E staining was performed using a H&E kit (cat. no. KGA224; KeyGEN Biotech Co., Ltd.). The brain tissue was fixed in 4% paraformaldehyde at room temperature overnight, embedded in paraffin and cut into 5- μ m paraffin sections of brain tissue. The paraffin sections were placed in an electric heating, constant temperature, drying oven and baked at 60°C for 3 h. The dried paraffin sections were dewaxed with conventional xylene at room temperature, hydrated with a descending gradient of ethanol and washed with distilled water. The nuclei were stained with hematoxylin for 2 min at room temperature, differentiated with 1% hydrochloric acid alcohol for several seconds, washed with water and returned to blue. Then, the slides were stained with eosin staining solution for 1 min at room temperature, and the residual staining solution was washed away with water. Subsequently, the sections were dehydrated with graded alcohol, made transparent in xylene and sealed with neutral gum. A field of view was randomly selected using a phase contrast microscope (Olympus Corporation) and stained specimens were visualized at 400x magnification.

For the TUNEL immunofluorescence assay, the brain tissue was fixed in 4% paraformaldehyde at room temperature overnight, embedded in paraffin and cut into 5- μ m paraffin sections of brain tissue. The paraffin sections were placed in an electric heating, constant temperature, drying oven and baked at 60°C for 60 min. Then the sections were dewaxed twice with xylene, hydrated with descending ethanol series (100, 95, 80 and 75% ethanol) for 5 min each and rinsed three times in 1X PBS for 5 min each. Then, 100 μ l ready-to-use Proteinase K solution (10 μ l of 10X Proteinase K in 90 μ l 1X PBS per sample; Takara Biotechnology Co., Ltd.) was added dropwise to each tissue section and left to react at 37°C for 30 min. The sections were rinsed three times in 1X PBS for 5 min each and 50 µl TUNEL detection solution (cat. no. C1090; Beyotime Institute of Biotechnology), prepared in a ratio of 5 μ l terminal deoxynucleotidyl transferase enzyme and 45 μ l fluorescent labeling solution, was added to each sample and incubated at 37°C for 60 min in the dark. After rinsing with PBS for 5 min three times, the sections were counterstained with DAPI. The sections were rinsed 3 times with PBS again, and dehydrated with graded alcohol, made transparent in xylene and sealed with neutral gum. The images were observed and captured under a laser confocal microscope at 400x magnification (Zeiss LSM; Zeiss AG).

Western blotting analysis. Western blotting was performed to measure the protein levels of Bc1-2, Bax and caspase-3 in brain tissue. Total protein was extracted from brain tissue samples using RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology). The lysate was centrifugated at 4°C and 14,300 x g for 10 min and the supernatant were collected. After determining and adjusting protein concentration using the Enhanced BCA Protein Assay kit (cat. no. P0010; Beyotime Institute of Biotechnology), the different mass fractions of protein (50 µg/lane) were separated via SDS-PAGE on a 10% gel (Criterion[™]; Trans-Blot[®]; Bio-Rad Laboratories, Ltd.). Then, the proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories, Ltd.) and the membrane was blocked in 5% skimmed milk at room temperature for 2 h. After blocking, the membrane was washed two/three times with TBST buffer (0.1% Tween; cat. no. QN1236; Beijing Biolab Technology Co., Ltd.). Subsequently, the membrane was incubated with the following primary antibodies at 4°C overnight: Anti-Bax (1:5,000; cat. no. 50599-2-Ig; Proteintech Group, Inc.), anti-Bcl-2 (1:1,000; cat. no. 26593-1-AP; Proteintech Group, Inc.) and anti-caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat. no. 4967; Cell Signaling Technology, Inc.). Following primary antibody incubation, a volume of 200 μ l horseradish peroxidase (HRP) was added to the surface of the membrane to make it uniformly adhere to the substrate. The membrane was incubated with the goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Finally, the enhanced chemiluminescence (ECL; cat. no. A38555; Thermo Fisher Scientific, Inc.) was added to visualize the immune response bands in the Bio-Rad ChemiDoc[™] XRS gel imaging system (Bio-Rad Laboratories, Ltd.). Image LabTM V4.0 software (Bio-Rad Laboratories, Ltd.) was used to detect the optical signal and to quantify the protein levels. All the experimental groups were divided into three parallel groups and the experiment was repeated three times and the data were averaged.

Measurement of mitochondrial oxidative stress indicators. High-purity mitochondria were extracted according to the instructions of the mitochondrial extraction kit (cat. no. SM0020; Beijing Solarbio Science & Technology Co., Ltd.), and assayed using reactive oxygen species (ROS) detection kits (cat. no. E004-1-1, Nanjing Jiancheng Bioengineering Institute) with an *in situ* loading probe. The mitochondrial pellet was resuspended in diluted (1:1,000) 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA; cat. no. CA1410, Beijing Solarbio Science & Technology Co., Ltd.) and incubated at 37°C for 25 min. The supernatant from probe-labeled mitochondrial suspension was collected by centrifugation at 1,000 x g for 5-10 min and then discarded, while the pellet was collected. The mitochondria were washed one/two times with PBS to adequately remove the excess of DCFH-DA Fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelength of 525 nm in each well using on a microplate reader (Thermo Fisher Scientific, Inc.).

Subsequently, the content of peroxidated lipids was estimated by colorimetry at 532 nm using the micro malondialdehyde (MDA) assay kit (cat. no. BC0025; Beijing Solarbio Science & Technology Co., Ltd.).

The right ischemic cerebral tissue was collected 24 h after reperfusion and then homogenized. After centrifugation at 1,000 x g and 4°C for 10 min, total superoxide dismutase (T-SOD) activity was measured in the supernatant using the total superoxide dismutase assay kit (hydroxylamine method; cat. no. A001-1-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Estimation of mitochondrial dysfunction indicators. The protein concentration of the freshly prepared mitochondrial suspension was adjusted to 10 mg/ml using the BCA method (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). The mitochondrial swelling assay was performed using a purified mitochondrial swelling colorimetric assay kit (cat. no. GMS10101; Shanghai Genmed Technology Co., Ltd.) according to the manufacturer's instructions. The 20 μ l of fresh mitochondrial suspension and 170 μ l of GENMED buffer from the kit were proportionally added to a 96-well plate and measured at 520 nm for 0 min absorbance. After 1 min, 10 μ l of GENMED expansion solution was added and measured again after 10 min to obtain 10 min absorbance.

High-purity mitochondria were thawed from -80° C, 400 μ l of the extract (a salt-containing buffer) from the kit below was added to the purified cerebral mitochondria, followed by ultrasonication (ice bath, 200 W, ultrasonication for 5 sec with an interval of 10 sec, fifteen times) to measure the enzyme activity assay of complex IV (cytochrome C oxidase) using a Mito Check complex IV activity assay kit (cat. no. BC0945, Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions.

To measure the ATP content, 5 μ l of high-purity cerebral mitochondria extract was mixed with 80 μ l boiling double distilled water and boiled for 10 min for mitochondria lysis. The lysate was centrifugated at 3,500 x g and 4°C for 10 min, the supernatant was collected and the absorbance was recorded at 570 nm on a microplate reader to measure the ATP content using an ATP assay kit (cat. no. A016-1, Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Transmission electron microscopy. Rat brains were removed quickly following euthanasia and cut into 1-mm³ sections before fixing via immersion in 4% pre-cooled glutaraldehyde at 4°C for 4 h. Then, the specimens were fixed with 1% osmic anhydride (cat. no. GP18456; Beijing Zhongjingkeyi Technology Co., Ltd.) at 4°C for 2 h, followed by stepwise dehydration with gradient alcohol and acetone: 50% Alcohol for 10 min, 70% alcohol for 10 min, 80% acetone for 10 min two times, 90% acetone for 10 min two times and anhydrous acetone for 10 min two times. After dehydration, the sections were embedded in epoxy resin and polymerized at 60°C incubator for 48 h. Ultra-thin sections were cut at a thickness of 100-nm using a microtome and stained with uranium acetate and lead citrate (cat. nos. GZ02625 and GA1070; Beijing Zhongjingkeyi Technology Co., Ltd.) for 15 min each at room temperature. Images of the ultrastructure of the mitochondria were observed and captured using transmission electron microscopy.

Statistical analysis. Data were analyzed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). Data are presented as mean \pm standard deviation. Data conform to the normal distribution, if the variance is homogeneous, multiple comparisons were performed using the ANOVA test followed by Bonferroni's correction. Multiple comparisons among data with unequal variance were performed using the Welch's ANOVA test followed by the Dunnett's T3 post hoc test. Multiple comparisons among abnormally distributed data were performed using the Kruskal-Wallis test followed by the Dunn's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PHBA reduces infarct volume. Compared with the sham group, the MCAO group showed high neurological deficit scores at 6 and 24 h after reperfusion, as well as high cerebral index, weight loss rate and infarction rate (all P<0.001; Fig. 1). The neurological deficit scores at 6 (P<0.01; Fig. 1A) and 24 h (P<0.001; Fig. 1B) after reperfusion, cerebral index (P<0.05; Fig. 1C), weight loss rate (P<0.05, Fig. 1D) and infarction rate (P<0.01; Fig. 1F) of in the PHBA-H group were lower compared with those in the MCAO group. With the exception of the neurological deficit score at 6 (P<0.01 vs. MCAO; Fig. 1A) and 24 h after reperfusion (P<0.01 vs. MCAO; Fig. 1B), no significant differences were observed between PHBA-L and MCAO groups in all the remaining indicators.

PHBA reduces neuronal apoptosis induced by MCAO. The results of H&E staining showed that the brain tissue of the rats in the sham group was intact, the cells were arranged orderly, the shape was normal and the nuclei were clear and intact (Fig. 2A). The brain tissue in the MCAO group had edema, the structure gap was widened and the cells showed pathological conditions, such as cell body dissolution, nuclear pyknosis and fragmentation and disordered arrangement of nerve fibers (Fig. 2A). The pathological conditions of the brain tissue in the PHBA-H and PHBA-L groups were improved and the changes in cell morphology were alleviated (Fig. 2A).



Figure 1. PHBA has a protective effect on transient focal cerebral ischemia. Neurological score after (A) 6 h and (B) 24 h reperfusion. (C) Cerebral index and (D) weight loss rate were monitored. (E) TTC staining was performed and (F) quantified to detect the extent of cerebral infarction. Data are presented as mean ± standard deviation. ***P<0.001 vs. sham. *P<0.05, **P<0.001 vs. MCAO. PHBA, *P*-hydroxybenzaldehyde; MCAO, middle cerebral artery occlusion; PHBA-H, PHBA-high dose; PHBA-L, PHBA-low dose.

According to the results of TUNEL immunofluorescence assay, the cells showed red fluorescence, nuclei showed blue fluorescence and the positive cells showed rose-red fluorescence after the images were merged (Fig. 2B). Compared with the sham group, the number of positive cells/mm² in the brain tissue in the MCAO group was significantly increased (P<0.001 vs. sham; Fig. 2C). The number of positive cells/mm² in the PHBA-H and PHBA-L rats was significantly lower compared with in the MCAO group (P<0.001; Fig. 2C).

Altered expression of Bcl-2, Bax and cleaved Caspase-3 in brain mitochondria. Compared with the sham group, the

expression levels of cleaved caspase-3 and Bax were significantly higher in the MCAO group, while the levels of Bcl-2 were significantly lower (P<0.001 for all; Fig. 3). PHBA-H and PHBA-L groups showed significantly higher expression of Bcl-2 and lower expression of cleaved caspase-3 and Bax compared with the that in the MCAO group (P<0.001 for all; Fig. 3), while no significant difference was observed in the protein expression of Caspase-3 between these groups (Fig. 3D).

Effects of PHBA on indicators of mitochondrial oxidative stress and dysfunction. Compared with the brain mitochondria



Figure 2. PHBA reduces apoptotic cells after MCAO injury. (A) H&E staining (scale bar, 50 μ m). The red arrows point to the pathological state of the cell. (B) TUNEL immunofluorescence assays were performed and (C) quantified to measure the effect of PHBA on apoptosis. Data are presented as mean ± standard deviation. Magnification, x400. ***P<0.001 vs. sham; *##*P<0.001 vs. MCAO. PHBA, *P*-hydroxybenzaldehyde; MCAO, middle cerebral artery occlusion; PHBA-H, PHBA-high dose; PHBA-L, PHBA-low dose.

in the sham group, the activities of T-SOD (P<0.001; Fig. 4A), cytochrome C oxidase (P<0.001; Fig. 4F) and the content of ATP (P<0.05; Fig. 4E) were significantly decreased in the MCAO group, while the levels of ROS (P<0.001; Fig. 4B) and MDA (P<0.05; Fig. 4C), as well as the opening degree of mitochondrial permeability transition pore (MPTP; P<0.001; Fig. 4D) were significantly increased. The levels of ROS (P<0.001; Fig. 4B) and MDA (P<0.001; Fig. 4C) and the degree of MPTP opening (P<0.001; Fig. 4D) in the PHBA-H group were significantly lower compared with those in the MCAO group, while the activities of T-SOD (P<0.05; Fig. 4A) and cytochrome C oxidase (P<0.05; Fig. 4F), as well as the content

of ATP (P<0.05; Fig. 4E) were significantly higher compared with those in the MCAO group. Compared with the MCAO group, the activity of T-SOD and cytochrome C oxidase, as well as the content of ATP and MDA of PHBA-L, were not significantly different, while the levels of ROS (P<0.001; Fig. 4B) and the degree of MPTP opening (P<0.001; Fig. 4D) were significantly improved.

PHBA improves the ultrastructural changes in the mitochondria. The electron microscopy results (Fig. 5) showed that the morphology of the mitochondria in the sham group was normal, with intact membranes, dense mitochondrial cristae



Figure 3. PHBA regulates the expression of apoptosis-related proteins in MCAO rats. (A) Representative western blots of Bcl-2, Bax, caspase-3, cleaved caspase-3. Expression levels of Bcl-2 (B), Bax (C), Caspase-3 (D) and cleaved caspase-3 (E) were measured through band density analysis. Data are presented as mean ± standard deviation. ***P<0.001 vs. sham. #*P<0.01 and ###P<0.001 vs. model. PHBA, *P*-hydroxybenzaldehyde; MCAO, middle cerebral artery occlusion; PHBA-H, PHBA-high dose; PHBA-L, PHBA-low dose.

and uniform staining of the matrix pattern. By contrast, the mitochondrial morphology of the MCAO group was severely impaired. The mitochondrial membrane shrunk, the cristae were sparse and fractured and the matrix was electron-lucent. Interestingly, the PHBA-H and PHBA-L groups improved the ultrastructure of the mitochondria. The mitochondrial morphology and membrane integrity were restored, the density of the matrix decreased minimally and the majority of mitochondrial cristae were intact, especially in the PHBA-H group.

Discussion

A previous study has shown that GE extract has significant antioxidant and anti-apoptotic properties (42). To date, the majority of studies have focused on the pharmacological activity and effects of gastrodin on the CNS (43). Previous studies, performed on >10 phenolic compounds isolated from GE, have shown that the abundant phenolic compounds in GE can enhance the endogenous antioxidants and improve disease prognosis in transient focal cerebral ischemia (44-46). The majority of studies have focused on the pharmacological effects of gastrodin in GE on CNS-related mechanisms (47-49). However, the effect of the large number of phenolic compounds in GE on the nervous system remains unclear. Although a previous pharmacokinetic study showed that PHBA in GE has brain targeting activity during drug distribution (50), its effect and mechanism on transient focal cerebral ischemia remains unclear. At present, PHBA can already be synthesized artificially (51). The preliminary experiment of our research group confirmed that the commercially available PHBA is consistent with the PHBA isolated from GE (52). The MCAO model is consistent with the course of transient focal cerebral ischemia and is often used to evaluate the efficacy of the treatment of stroke and its mechanism (53,54). Therefore, the present study aimed to use the MCAO model to study the effect of PHBA on transient focal cerebral ischemia, its correlation with mito-chondrial dysfunction and oxidative stress, as well as the effect on the protein expression of Bcl-2, Bax and cleaved caspase-3.

Cerebral edema in ischemic penumbra leads to severe consequences and adverse clinical events post-ischemic brain injury (55). In the present study the cerebral index was used to show the severity degree of encephaledema. CNS injury was assessed by neurological deficit score and infarct area using TTC staining (56). H&E staining revealed the morphological changes of brain cells during apoptosis after transient focal cerebral ischemia. TUNEL immunofluorescence was used to evaluate cell apoptosis during cerebral ischemia.

Mitochondria are the major organelles involved in the cellular respiration, controlling the oxidation of substances to generate the energy needed for cell survival (32). Cerebral ischemia triggers mitochondrial dysfunction leading to oxidative stress, which stimulates O_2 to lose electrons to form a large amount of ROS (57). Under normal physiological conditions, ROS regulates the operation of the mitochondrial redox signaling pathway (58). However, when transient focal cerebral ischemia occurs, T-SOD, a key antioxidant enzyme, cannot normally scavenge excess oxygen free radicals due to its reduced activity, resulting in excessive ROS-oxidized lipids in the mitochondria (59). MDA is a product of lipid peroxidation that directly reflects the level of oxidative stress (60-62). This change causes oxidative stress, alters mitochondrial membrane permeability and releases pro-apoptotic factors into the cytoplasm that in turn promote neuronal apoptosis (63). In addition, excess O₂ is consumed to destroy the supply of the respiratory chain, ATP content drops sharply and energy



Figure 4. PHBA alleviates oxidative stress and dysfunction in mitochondria in the MCAO model. Changes in the (A) T-SOD activity, (B) level of ROS and (C) MDA, (D) the opening degree of MPTP, (E) ATP level and (F) cytochrome C oxidase activity after PHBA treatment were detected with the corresponding kits. Data are presented as indicated by mean ± standard deviation. *P<0.05 and ***P<0.001 vs. sham; *P<0.001 vs. model. PHBA, *P*-hydroxybenzaldehyde; MCAO, middle cerebral artery occlusion; PHBA-H, PHBA-high dose; PHBA-L, PHBA-low dose; T-SOD, total superoxide dismutase; ROS, reactive oxygen species; MDA, malondialdehyde; MPTP, mitochondrial permeability transition pore.

metabolism is abnormal. It affects the structural integrity and function of the mitochondria, prompting the organelle to function appropriately (64).

MPTP reflects the structure and function of the mitochondria and it is located between the inner and outer membranes of the organelle (65). The lipid oxidative stress damage to the mitochondrial membrane leads to the abnormal opening of MPTP, which cannot maintain the normal potential difference of the inner membrane, while the oxidative phosphorylation on the inner membrane cannot proceed smoothly (66,67). The cytochrome C oxidase is also known as respiratory chain complex IV and it is embedded in the bilayer lipid membrane of the inner mitochondrial membrane (68). As a key proton pump for energy generation in the respiratory chain, MPTP transfers the H⁺ of cytochrome C to O_2 and reduces it to H₂O (69,70). In the MCAO model established in the present study, MPTP was opened and cytochrome C oxidase activity was reduced. These two changes resulted in the loss of the H⁺ gradient in the inner membrane, disruption of the oxidative phosphorylation of the respiratory chain and impairment of the normal synthesis and supply of ATP. Because of the lack of energy supply, neuronal cells reached apoptosis rapidly.

Previous studies have shown that Bcl-2/Bax mainly control the apoptosis of neuronal cells by acting on mitochondria and the protein dimer formed by Bcl-2/Bax is the main component of MPTP (71). When the expression of Bax is higher compared



Figure 5. PHBA improves the ultrastructural changes in mitochondria. The red arrows in the picture point to mitochondria. The ultrastructure of mitochondria in the Sham group was normal, while severe morphological changes were detected in the MCAO group. The changes in mitochondrial ultrastructure were improved in the PHBA-H and PHBA-L groups, especially in the PHBA-H group. Magnification, x400. PHBA, *P*-hydroxybenzaldehyde; MCAO, middle cerebral artery occlusion; PHBA-H, PHBA-high dose; PHBA-L, PHBA-low dose.

with that of Bcl-2 after transient focal cerebral ischemia, the MPTP is opened, facilitating the passage of apoptotic factors into the cytoplasm to promote apoptosis, as well as leading to mitochondrial dysfunction and abnormal transport of the ion channels (72,73). Bcl-2 family members are a members of the apoptosis regulatory proteins: Anti-apoptosis protein Bcl-2 and pro-apoptosis protein Bax (74). Bcl-2 is the most widely studied anti-apoptotic gene (75). Its high expression can directly alleviate to oxidative stress injury, inhibit ROS production and regulate mitochondrial membrane permeability to maintain mitochondrial homeostasis and prevent apoptosis (76). Finally, it improves ischemia-induced neuronal apoptosis (77). However, Bax can activate ion channels on the mitochondrial membrane and some small molecular substances, such as cytochrome C leak into the cytoplasm, causing cell damage (78). Under normal circumstances, the proportion of the Bcl-2/Bax is maintained in a certain range (79). An imbalance in the proportion of the two molecules leads to apoptosis (80). The high levels of Bax induce cytochrome C to enter the cytoplasm and activate the caspase cascade (81). The caspase protein family is important for apoptosis and Caspase-3 is an executory protein as it is the convergence point of multiple apoptosis signaling pathways (82). When Caspase-3 is stimulated by apoptosis, it is activated and cleaved into cleaved Caspase-3, an essential condition for the initiation of apoptosis (83). Subsequently, cleaved Caspase-3 will decompose the nuclear DNA repair enzymes, resulting in nuclear DNA chromatin condensation and damage, eventually leading to apoptosis (83-85).

The present study demonstrated that PHBA reduces cerebral index, neurological dysfunction, weight loss, infarction rate and the number of TUNEL-positive cells. On the other hand, PHBA reduces mitochondrial oxidative stress and dysfunction. Moreover, the expression levels of Bcl-2 protein were increased, while those of Bax and cleaved caspase-3 proteins were decreased. At present, single-targeted therapy can shorten the onset time of the drug, make it reach the lesion quickly and gain valuable time for disease treatment (86). PHBA can target the brain (87), and the present study demonstrated that prophylactic administration can significantly reduce the size of cerebral infarction and effectively improve neuronal damage caused by cerebral ischemia.

The present results suggested that PHBA could be used as a clinical candidate for the prevention and treatment of transient focal cerebral ischemia (87). However, due to the lack of research on PHBA, the specific brain targeting mechanism remains unclear. For clinical applications, additional experiments are required to address the issues pertaining PHBA. The present study is only a preliminary study on the effect of PHBA on mitochondria during the treatment of cerebral ischemia, while mitochondrial dysfunction also involves several other pathways, such as abnormal energy metabolism, nitrosative stress, mitophagy and so on (87-89). Therefore, *in vitro* experiments will be the focus of future investigations on mitochondrial energy metabolism to provide a pharmacological basis for PHBA-targeting mitochondria in the treatment of cerebral ischemia-related diseases.

Acknowledgements

Not applicable.

Funding

This study was supported by the National Natural Science Foundation of China (grant no. 81960733) and the Applied Basic Research Program of Yunnan Province (grant no. 2019FB120).

Availability of data and materials

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

Authors' contributions

XD and LY conceived and designed this study. TX and LY participated in the experiments. PC and TX analyzed the data. TX wrote the manuscript. PC and XD reviewed and revised the manuscript. All authors read and approved the final version of the manuscript. TX and XD confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine (approval no. R-062019039) and the care and use of experimental animals were performed in accordance with the guidelines of the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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