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# Dl-3-n-butylphthalide protects the blood brain barrier of cerebral infarction by activating the Nrf-2/HO-1 signaling pathway in mice

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**Abstract. – OBJECTIVE: The aim of this study was to explore whether Dl-3-n-butylphthalide (DBT) could protect blood-brain barrier (BBB) of mice with experimental cerebral infarction and the relevant mechanism.**

**MATERIALS AND METHODS: Adult male CD-1 mice were selected as the study objects. The permanent middle cerebral artery occlusion (MCAO) model was prepared by Longa's modified suture-occluded method. The mice were randomly divided into 3 groups: the sham operation group (Sham group), the cerebral infarction model group (CI group) and the DBT (120 mg/kg) intervention group (DBT group). Neurologic function deficits were evaluated by Longa's modified scoring method after 24 h of permanent MCAO. The wet and dry weight method was used for measuring water content in brain tissues. 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining method was applied to determine the volume of cerebral infarction. Changes in the protein and messenger ribonucleic acid (mRNA) expression levels of matrix metallopeptidase 9 (MMP-9), claudin-5, vascular endothelial growth factor (VEGF), glial fibrillary acidic protein (GFAP), NF-E2 related factor 2 (Nrf-2) and heme oxygenase 1 (HO-1) in ischemic brain tissues were detected using immunohistochemistry, Western blotting and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Ultrastructure changes in BBBs were observed under an electron microscope.**

**RESULTS: DBT improved the neurologic function deficits of mice and reduced the infarction volume of mice with cerebral infarction. DBT alleviated edema and decreased the permeability of BBBs of mice with cerebral infarction. DBT down-regulated the expression of MMP-9 and up-regulated the expression of claudin-5 in brain tissues of mice with cerebral infarction. DBT increased the expressions of VEGF and GFAP. DBT improved the ultrastructure in capillary endothelial cells of BBBs and increased the expressions of Nrf-2 and HO-1.**

**CONCLUSIONS: DBT may protect BBB by activating the Nrf-2/HO-1 signaling pathway, thus achieving its protective effect on the brain.**

*Key Words:*
Dl-3-n-butylphthalide, Blood brain barrier, Nrf-2, HO-1.

## Introduction

Ischemic stroke is the most common type of stroke worldwide. Assessments of disease burden, injury, and risk factors have shown that cerebral infarction is the second most common cause of death and the third most common cause of disability[1]. Intravenous thrombolysis is considered to be the most effective treatment for acute cerebral infarction, but the rate of venous thrombolysis after cerebral infarction is not high due to the rigorous therapeutic time window and post-treatment bleeding complications[2,3]. Therefore, it is particularly important looking for a new drug target to provide a reliable basis for the therapeutic regimen of cerebral infarction.

The blood-brain barrier (BBB) is a special barrier existing between cerebral blood circulation and nerve tissues. Its main function is to maintain the steady state of brain tissues, regulates the balance of substance exchange in the brain, and protects the brain from infringement. The pathophysiology of ischemic stroke involves the BBB dysfunction, primary and secondary cell destructions, and death[4,5]. As the BBB plays a central role in the pathogenesis of cerebrovascular disease, it is considered as a new target for the treatment of cerebral infarction. BBB studies provide bases for clinical drug research. The nuclear transcription

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factor, NF-E2-related factor 2 (Nrf-2) regulates the expressions of various downstream detoxification enzymes and antioxidant enzymes by binding to cellular stress signals[6]. Nrf-2/heme oxygenase1 (HO-1) signaling pathway is the most important cell defense mechanism[7]. Nrf-2, as a brain protective factor, plays a vital role in regulating the inflammatory reaction and oxidative stress response after cerebral infarction. Studies[8] have shown that in the experimental brain injury model, the application of Nrf-2 agonist sulforaphane to activate the Nrf-2 signaling pathway improves the integrity and stability of BBB. Therefore, the Nrf-2 signaling pathway and downstream protective factors are key targets for the treatment of stroke-induced BBBs.

Dl-3-n-butylphthalidle (DBT) is a kind of new drug that has been successfully developed in China for the treatment of cerebral ischemia. It can act on multiple links of injury after cerebral ischemia-reperfusion and reduce the degree of nerve damage. Animal experiments and clinical trials have confirmed that DBT exerts affirmative therapeutic effects on ischemic stroke[9-11]. However, there is no study on the effect of DBT on BBB after cerebral infarction. Therefore, a model of experimental cerebral infarction in middle cerebral artery occlusion (MCAO) mice was established using Longa's modified suture-occluded method. Whether DBT could protect BBB of mice with experimental cerebral infarction was observed, and the relevant mechanism of DBT was explored, thus providing a theoretical and experimental basis for the clinical application of DBT.

## Materials and Methods

### Experimental Animals and Models

Healthy adult male CD-1 mice (aged 10-12 weeks, weighing 27-30 g) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The right-side permanent MCAO (pMCAO) animal model was established using Longa's modified suture-occluded method[12]. The mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (35 mg/g), and fixed on the operating table in the supine position. After disinfection, the middle of the neck was longitudinally incised, and right common carotid artery (CCA), external carotid artery (ECA) and right internal carotid artery (ICA) were exposed. Sutures were inserted and used for ligation at the distal end of ECA 3-4 mm away from the bifurcation site of the CCA, and then branches at the distal end of ECA were separated. The separation was deepened 2-3 mm to the basis crania along the ICA, and the internal carotid artery was carefully occluded using a micro-artery clip. A 3.0-cm-long nylon occlusion was prepared, whose diameter was 0.1518 mm, and the head end was spherical. An about 0.2-mm incision in the stump of the ECA was cut, and the occlusion was inserted into the ECA. At this point, the micro-artery clip was removed, and the occlusion was reversed and inserted into the encephalic segment of the internal carotid. When the occlusion was smoothly inserted (9.5±0.5) mm in depth, slight resistance could be felt, and at this time, the spherical dot on the occlusion head was located in the anterior cerebral artery of the cranium, blocking the opening of the MCA. After the occlusion successfully entered the cranium, ligation was conducted in the internal carotid, and then the occlusion was cut off. Subcutaneous tissues and skin were sutured. Operations in the sham operation group were the same as those in the model group except that the occlusion was not inserted. This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

### Experimental Grouping

CD-1 mice were randomly divided into 3 groups: the sham operation group (Sham group), the cerebral infarction model group (CI group) and the DBT (120 mg/kg) intervention group (DBT group).

### Neurologic Function Deficit Scores

Based on Longa's scoring method for neurologic function deficits, the animals were scored at 24 h after they awaked from anesthetization, and symptoms of the neurologic function deficits were recorded. Scoring criteria: 0 points for no nerve injury symptom, 1 point for inability to extend the contralateral forelimb, 2 points for contralateral forelimb flexion, 3 points for mild rotation to the opposite side, 4 points for severe rotation to the opposite side, and 5 points for falling to the opposite side.

### Determination of the Volume of Cerebral Infarction

At 24 h after operation, the mice were sacrificed after anesthesia, and their brains were

quickly and completely removed. The olfactory bulbs and cerebellums were excised, and the brains were continuously and equidistantly cut into 5 coronal sections. The brain slices were completely immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution and incubated at 37°C for 15 min. TTC was discarded after staining. Then, the brain slices were fixed in 4% paraformaldehyde for 24 h, and placed in order. After that, pictures were taken at the same focal length, the images were imported into the computer for image analysis, and cerebral infarction area and volume were determined.

### Determination of Water Content in Brain Tissues

Water content in the brain was measured at 24 h after cerebral infarction using the wet and dry weight method. About 4-mm-thick brain tissues were taken after the removal of the forehead for the determination of water content in the brain.

The brain tissues were put into a piece of tinfoil weighed in advance (A), and then they were immediately weighed (B). Wet weight = B-A. Afterwards, the brain tissues were wrapped with tinfoil and dried in an oven at 100°C for 24 h before being removed and returned to room temperature for weighing (C). Dry weight = C-A. Water content of brain tissues were calculated in the formula: (wet weight of brain tissues - dry weight of brain tissue)/wet weight × 100%, namely, (B-C)/(B-A) × 100%.

### Evans Blue Staining and Content Determination

Mice received tail intravenous injection of 2% Evans blue (4 mL/kg) at 22 h after operation. 2 h later, mice were anesthetized, and then normal saline was used for cardiac perfusion, followed by decollation and brain collection. After the forehead and cerebellum were removed from the brain tissues using mouse abrasives, the tissues were cut into 5 slices on average, and placed in order for photographing. Then, the brains were divided into two parts: the infarcted hemisphere and the non-infarcted hemisphere, which were collected in an Eppendorf (EP) tube, respectively, and they were prepared into homogenate with 1 mL trichloroacetic acid, followed by centrifugation for 20 min. Finally, the optical density of supernatant was measured using a microplate reader.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After 100 mg cortical brain tissues on the infarcted side of mice were taken, the total ribonucleic acid (RNA) in brain tissues was extracted using TRIzol method, and the purity and concentration of the extracted RNA were measured. According to the product instructions, complementary deoxyribonucleic acid (cDNA) reverse transcription was conducted, followed by PCR amplification. After amplification, β-actin was used as an internal reference gene to obtain the Ct value of the target gene expression compared with that of the control group, and the relative quantitative analysis of the data was carried out using the $2^{-\Delta\Delta Ct}$ method.

### Western Blotting

After 50 mg brain tissues on the infarcted side of mice were weighed, the total protein was extracted according to the instructions of the protein extraction kit. BCA protein detection kit was used to determine protein concentration. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted, and the primary and secondary antibodies of the target protein were incubated, respectively, after membrane transfer, which were then observed under exposing conditions.

### Immunohistochemistry

The brain tissues were fixed in 4% paraformaldehyde solution and received gradient dehydration using ethanol. Tissue blocks were embedded in paraffin. Paraffin-embedded brain tissues were made into serial sections with the thickness of 5 μm, which were attached to glass slides. The sections were dewaxed, put into water, and blocked using goat serum. Primary antibodies of the target protein were incubated overnight, secondary antibodies were incubated, followed by hematoxylin restaining the cell nucleus for 1 min on the next day, and finally neutral resins were used for mounting. Observation under a microscope: The positive and negative tissues of the experimental group and the control group were selected for photomicrography.

### Procedures of Electron Microscopy

At 24 h after operation, the mice were anesthetized, and the thoracic cavity was opened for perfusion. 400 mL stationary solution was added at the end of perfusion. The first 1/3 of the stationary solution was rapidly perfused, while the left 2/3 were slowly perfused. The brain was tak-

en from the forehead and put into 4% glutaraldehyde fixed vial at 4°C overnight. After the sample preparation, photographing was conducted using an electron microscope.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Comparisons of data among multiple groups were conducted using one-way analysis of variance (ANOVA). When the variances were homogeneous and significantly different, pairwise comparisons were further performed using the Student-Newman-Keuls (SNK) test. When the variances were heterogeneous, nonparametric rank sum test was performed. Pairwise comparisons of neurologic function deficit scores were carried out using the Mann-Whitney U test, and all the data were expressed as mean ±standard error of measurement (SEM) except neurologic function deficit scores. $p<0.05$ represented that the difference was statistically significant.

## Results

### DBT Improved the Neurologic Function Deficits of Mice With Cerebral Infarction

Longa's modified scoring method was used to determine the neurologic function of mice. The results showed that the neurologic function deficit score in CI group was significantly higher than that in sham group, indicating that pMCAO model causes obvious neurologic function deficits. Compared with that in CI group, neurologic function deficit score in DBT group was significantly decreased, and the difference was statistically significant ($p<0.05$) (Figure 1A).

### DBT Reduced the Infarction Volume of Mice With Cerebral Infarction

The volume of cerebral infarction was measured by TTC staining. The results showed that TTC staining in brain tissues of mice in sham group was uniform and red, whereas TTC staining manifested a large pale infarction area in ischemic brain tissues of mice in CI group. Compared with that in CI group, the volume of cerebral infarction in DBT group was notably reduced, and the difference was statistically significant ($p<0.05$) (Figure 1B).

### DBT Alleviated Edema in Mice With Cerebral Infarction

The water content of brain tissues was determined by the wet and dry weight method. The results revealed that the water content of brain tissues on the infarcted side in CI group was significantly increased. Compared with that in CI group, the water content of brain tissues in DBT group was markedly decreased at 24 h after operation, and the difference was statistically significant ($p<0.05$) (Figure 1C).

### DBT Decreased the Permeability of BBB of Mice With Cerebral Infarction

The effect of DBT on the permeability of BBB was evaluated by detecting the exudation rate of Evans Blue. The results showed that in sham group, there was no Evans blue exudation, and BBB were intact. At 24 h after operation, the Evans blue exudation was relatively severe in CI group. Compared with that in CI group, the Evans blue exudation was significantly decreased in DBT group at 24 h after operation ($p<0.05$) (Figure 2A).

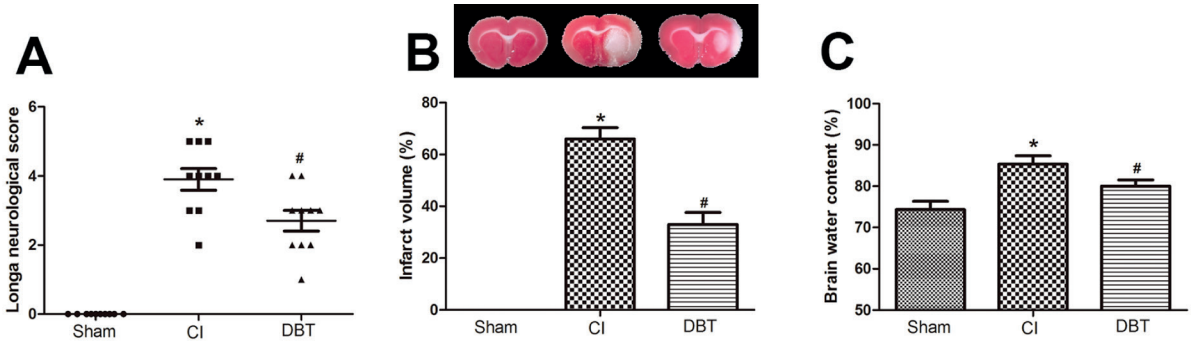


**Figure 1.** The effect of Dl-3-n-butylphthalide on cerebral infarction in mice. ***(A)*** Analysis of neurological deficit scores by Longa's modified scoring method. ***(B)*** Analysis of cerebral infarct volume by TCC. ***(C)*** Analysis of brain water content by the wet and dry weight method. *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* CI group.

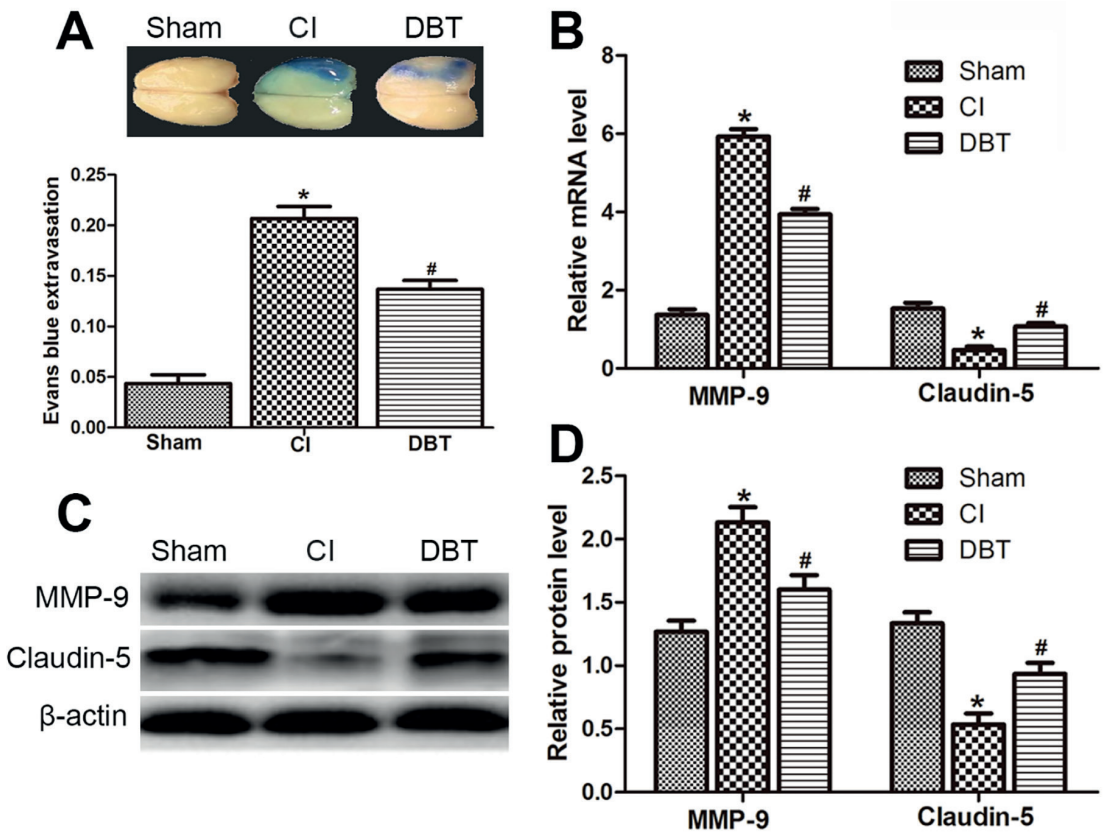


**Figure 2.** The effect of Dl-3-n-butylphthalide on blood brain barrier after cerebral infarction in mice. ***(A)*** DBT decreased the permeability of BBBs of mice. ***(B)*** Analysis of mRNA level of MMP-9 and claudin-5. ***(C)*** Western blotting showed that DBT down-regulated the expression of MMP-9 and up-regulated the expression of claudin-5. ***(D)*** Analysis of protein level of MMP-9 and Claudin-5. *$p$<0.05 *vs.* Sham group, #$p$<0.05 *vs.* CI group.

### *DBT Down-Regulated the Expression of Matrix Metallopeptidase 9 (MMP-9) and Up-Regulated the Expression of Claudin-5 in Brain Tissues of Mice with Cerebral Infarction*

Western blotting and quantitative RT-PCR (qRT-PCR) demonstrated that compared with those in CI group, the protein and messenger ribonucleic acid (mRNA) expression levels of MMP-9 in DBT group were significantly decreased at 24 h after operation, and the differences were statistically significant ($p$<0.05). The protein and mRNA expression levels of claudin-5 were overtly increased at 24 h after operation, and the differences were statistically significant ($p$<0.05) (Figure 2B-D).

### *DBT Increased the Expressions of Vascular Endothelial Growth Factor (VEGF), Glial Fibrillary Acidic Protein (GFAP)*

Western blotting and qRT-PCR indicated that compared with those in CI group, the protein and mRNA expression levels of VEGF and GFAP in DBT group were significantly increased at 24 h after cerebral infarction, and the differences were statistically significant ($p$<0.05) (Figure 3A-C).

### *DBT Improved the Ultrastructure in Capillary Endothelial Cells of BBB*

In sham group, the endothelial cell structure was normal with a small amount of pinocytotic vesicles, while in CI group, cytoplasmic edema appeared in endothelial cells of BBB with increased pinocytotic vesicles at 24 h after operation. Compared with that in CI group, edema of endothelial cells in DBT group was alleviated with decreased pinocytotic vesicles (Figure 3D).

### *DBT Increased the Expressions of Nrf-2 and HO-1*

It was found from Western blotting and qRT-PCR that compared with those in CI group, the

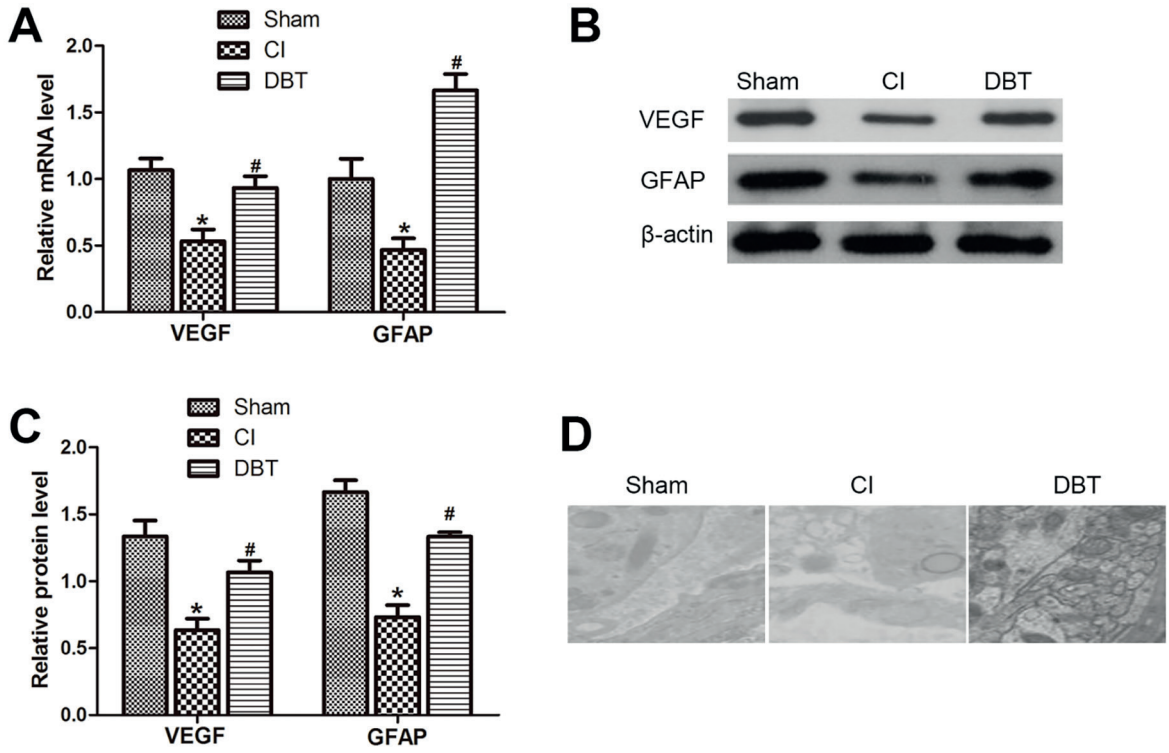


**Figure 3.** The effect of Dl-3-n-butylphthalide on the expression of VEGF/GFAP and ultrastructure of capillary endothelial cells. ***(A)*** Analysis of mRNA level of VEGF and GFAP. (B) Western blotting showed that DBT increased the expressions of VEGF and GFAP. ***(C)*** Analysis of protein level of VEGF and GFAP. ***(D)*** The representative images of ultrastructure of capillary endothelial cells of BBBs. *$p$<0.05 *vs.* Sham group, #$p$<0.05 *vs.* CI group.

protein and mRNA expression levels of Nrf-2 and HO-1 in DBT group were significantly increased at 24 h after operation, and the differences were statistically significant ($p$<0.05). Immunohistochemical staining results were consistent with the above results (Figures 4 and 5).

## Discussion

Cerebrovascular disease has become the major cause of disability and death worldwide. Although major research progress has been made in the pathophysiology of cerebral infarction, the treatment of cerebral infarction remains the focus of future scientific research. The pathological mechanism of secondary brain injury after cerebral infarction mainly includes oxidative stress response, inflammatory response, excitatory amino acid toxicity, apoptosis and calcium overload[13,14]. Oxidative stress aggravates the damage to BBB after cerebral infarction, and promotes neurons, endothelial cells and other cells to participate in the cascade injury reaction. Therefore, anti-oxidative stress response is becoming an important way to treat cerebral infarction. Mouse MCAO model is able to induce the effects of a series of pathological injury drugs. In this work, a pMCAO was used to better simulate the clinical experiment. Evaluating the neurologic function score of mice after cerebral infarction and determining the infarction volume and brain water content, are common research tools to assess the degree of brain injury and evaluate the efficacy of drugs. In the experiment, Longa's modified scoring method was used to assess the neurologic function of mice after cerebral infarction. After DBT intervention, the neurologic function deficit score of mice was significantly decreased, indicating that DBT can improve neurologic function deficits in mice with experimental cerebral infarction, and play a protective role in the brain. TTC staining was applied to determine the volume of cerebral infarction. TTC is a liposoluble photosensitized complex, which reacts with the dehydrogenase in normal tissues showing red. The infarcted tissues

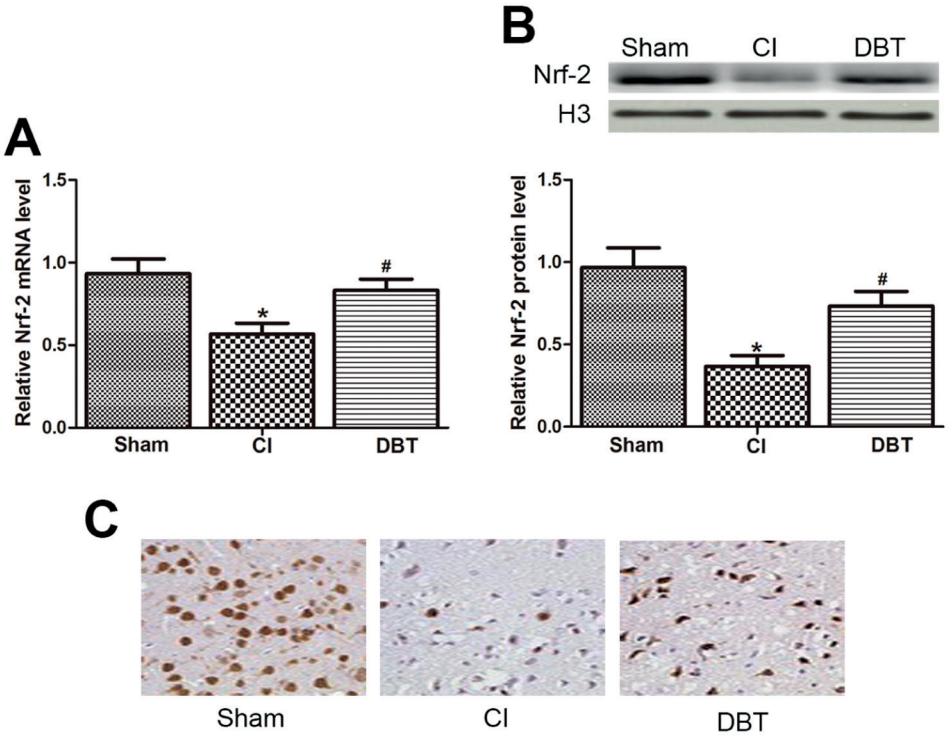


**Figure 4.** The effect of Dl-3-n-butylphthalide on the expression of Nrf-2. ***(A)*** Analysis of mRNA level of Nrf-2. ***(B)*** Analysis of protein level of Nrf-2 by Western blotting *in vivo*. ***(C)*** The representative images of immunohistochemistry. $p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* CI group.

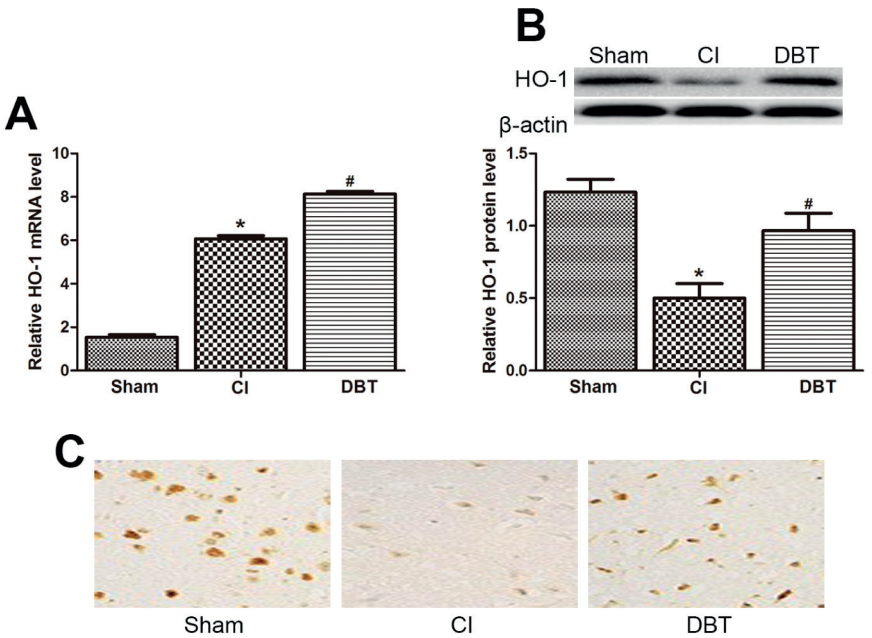


**Figure 5.** The effect of Dl-3-n-butylphthalide on the expression of HO-1. ***(A)*** Analysis of mRNA level of HO-1. ***(B)*** Analysis of protein level of HO-1 by Western blotting *in vivo*. ***(C)*** The representative images of immunohistochemistry. $p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* CI group.

cannot make responses due to the decrease of dehydrogenase activity caused by the ischemic reaction, so it does not change to become pale. We found that, compared with that in CI group, the volume of cerebral infarction was significantly reduced in DBT group, indicating that DBT exerts a protective effect in cerebral ischemic injury. The water content of brain tissues was measured by the standard wet and dry weight method, and then calculated by formula to determine the degree of brain edema in mice with experimental cerebral infarction. Compared with that in CI group, the water content of brain tissues in DBT group was significantly decreased at 24 h after cerebral infarction, suggesting that DBT plays a protective role in ischemic brain tissues of cerebral infarction. Neuronal damage and BBB permeability are important parts of the pathophysiology of cerebral infarction. Recent studies have confirmed that the damage to the BBB integrity will aggravate the physiopathological process of brain ischemia after the occurrence of cerebral infarction[15,16]. Maintaining BBB stability can reduce the damage to ischemic brain tissues and protect the brain. The tight junction protein between endothelial cells is an important part of the BBB, and its dysfunction is an important aspect of the pathogenesis of cerebral infarction. In MCAO rats, the expressions of tight junction proteins including claudin-5, occludin and zonula occludens-1 (ZO-1) were down-regulated, and BBB permeability was increased[17]. MMPs play special roles in the pathophysiological mechanism of BBB dysfunction[18,19]. They degrade the tight junction proteins including claudin-5. With the aggravation of brain injury, MMPs are activated, which will further aggravate the tissue damage, brain edema, micro-hemorrhage in brain tissues and cell death. The study confirmed that at 24 h after experimental cerebral infarction, BBB permeability in ischemic brain tissues was increased, and the integrity was impaired. However, DBT could significantly reduce BBB permeability and maintain BBB integrity, thereby reducing damages to brain tissues. After the application of DBT, the expression of MMP-9 in ischemic brain tissues was decreased, suggesting that DBT effectively inhibits the activity of MMP-9, inhibits MMP-9 to resolve tight junction proteins, and alleviates damages to brain tissues and cell death due to the activation of MMP-9, thus reliving cerebral edema and reducing cerebral infarction area. After the application of DBT, claudin-5 expression was up-regulated, and BBB tightness was increased, thus enhancing BBB stability. VEGF is a key factor to promote angiogenesis. Researches[20] have shown that the content of VEGF begins to increase at 3 h after cerebral infarction for 3 to 7 days. This work revealed that DBT could increase the content of VEGF in ischemic brain tissues, thus initiating the process of angiogenesis after infarction, promoting the repair of endothelial cells after infarction and maintaining the density of blood vessels in ischemic brain tissues. The application of DBT could also reduce endothelial cell swelling and reduce the number of pinocytotic vesicles of endothelial cells in the infarction area, suggesting that DBT can protect the morphology of endothelial cells, thus maintaining the capillary structural stability and increasing the BBB stability. Astrocytes are involved in the BBB cytoskeleton[21], and GFAP is a marker for their activation, which can maintain the astrocyte tightness. GFAP-deficient mice have significantly reduced tolerance to hypoxia after cerebral infarction[22]. We demonstrated that the expression of GFAP was increased after the application of DBT, suggesting that DBT can increase the tolerance of mice with cerebral infarction to hypoxia, stabilize the skeleton structure of astrocyte membrane, stabilize the BBB structure, thus strengthening the BBB function and increasing the BBB stability. HOs play important roles in endogenous anti-oxidative damage during cerebral ischemia and inflammation[23,24]. HO-1 is a free radical scavenger *in vivo*. It is induced by various factors such as oxidative stress, cytokines and ischemia-reperfusion, and plays an important role in the resistance to oxidative tissue damages. A large number of studies have shown that Nrf-2 is the most important transcription factor of HO-1, and it is a key factor in cellular oxidative stress[25,26]. The expression of Nrf-2 is significantly up-regulated in the parts relatively sensitive to ischemia and hypoxia in brain tissues. The activation of Nrf2 can enhance the expression activities of a variety of antioxidant and detoxifying enzymes, and greatly improve the antioxidant and detoxification abilities of cells. In the experimental brain injury model, the Nrf-2 agonist sulforaphane activates the Nrf-2 signaling pathway, increasing the integrity and stability of BBBs. We found that DBT could increase the mRNA and protein expression levels of Nrf-2 and HO-1, and increase the number of positive cells expressing Nrf-2 and HO-1, indicating that after the experimental cerebral infarction, DBT can activate the Nrf-2/HO-1 signaling pathway in ischemic brain tissues.

## Conclusions

We showed that DBT may protect BBBs by activating the Nrf-2/HO-1 signaling pathway, thus achieving its protective effect on the brain.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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