Protective effects of dl-3n-butylphthalide against diffuse brain injury

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Research Highlights
(1) Dl-3n-butylphthalide can effectively treat cerebral ischemia; however, the clinical mechanism remains unclear. This study was designed to explore the mechanisms underlying the neuroprotective effects of dl-3n-butylphthalide against diffuse brain injury through changes in blood vessels.
(2) The protective effects of dl-3n-butylphthalide are associated with improvements in microcirculation disturbances and vascular permeability. Therefore, dl-3n-butylphthalide is a neuroprotective drug involving multiple pathological links (multiple targets), and it can improve cerebral blood flow.

Abstract
Dl-3n-butylphthalide can effectively treat cerebral ischemia; however, the mechanisms underlying the effects of dl-3n-butylphthalide on microcirculation disorders following diffuse brain injury remain unclear. In this study, models of diffuse brain injury were established in Sprague-Dawley rats with the vertical impact method. Dl-3n-butylphthalide at 80 and 160 mg/kg was given via intraperitoneal injection immediately after diffuse brain injury. Ultrastructural changes in the cerebral cortex were observed using electron microscopy. Cerebral blood flow was measured by laser Doppler flowmetry, vascular density was marked by tannic acid-ferric chloride staining, vascular permeability was estimated by the Evans blue method, brain water content was measured using the dry-wet method, and rat behavior was measured by motor function and sensory function tests. At 6, 24, 48, and 72 hours after administration of dl-3n-butylphthalide, reduced cerebral ultrastructure damage, increased vascular density and cerebral blood flow, and improved motor and sensory functions were observed. Our findings demonstrate that dl-3n-butylphthalide may have protective effects against diffuse brain injury by ameliorating microcirculation disorder and reducing blood-brain barrier damage and cerebral edema.

Key Words
neural regeneration; brain injury; diffuse brain injury; blood-brain barrier; brain edema; vascular density; cerebral blood flow; vascular permeability; brain water content; grants-supported paper; neuroregeneration
INTRODUCTION

With the rapid development of vehicular transportation, the prevalence of diffuse brain injury is rising, and it has become the main cause of serious disability, vegetative states, and death in people under 45 years old\(^1\). Because most diffuse brain injury patients are not suitable for surgery, the development of neuroprotective agents is of great importance. Dl-3n-butylphthalide is a widely used traditional Chinese medicine that is new to the clinic and is a national independent property with the same structure as natural butylphthalide\(^2\). Based on searches of PubMed and CNKI using the search term dl-3n-butylphthalide, it appears that studies have documented that dl-3n-butylphthalide is effective in ischemic brain injury by blocking various pathological links, such as improving cerebral energy metabolism and mitochondrial function; inhibiting the release or expression of oxygen free radicals, inflammatory factors, and amyloid-β; decreasing ischemic apoptosis of nerve cells; and reducing cerebral infarct areas\(^3-9\). Nonetheless, its role in diffuse brain injury remains unclear. Microcirculation disorders after diffuse brain injury include decreased cerebral blood flow, increased vascular permeability, damage to vascular integrity, and so other symptoms. Low perfusion caused by microcirculation disorders after diffuse brain injury may lead to energy metabolism disorders, stimulant toxicity, lactate accumulation, calcium ion accumulation in cells, massive catecholamine release, inflammatory reactions, and programmed cell death in nerve cells. These effects initiate and aggravate cerebral edema and are key targets for diffuse brain injury treatment\(^10-13\). Previous studies of butylphthalide mainly focused on ischemic cerebrovascular disease, but support a role for butylphthalide in inhibiting nerve cell apoptosis in rats with focal cerebral infarcts. In the present study, we aimed to determine the influence of dl-3n-butylphthalide on microcirculation, blood-brain barrier integrity, and cerebral edema in rats after diffuse brain injury, and to explore the protective role of dl-3n-butylphthalide in diffuse brain injury.

RESULTS

Quantitative analysis of experimental animals

A total of 211 Sprague-Dawley rats were randomly divided into four groups: a control group \((n = 40)\), injury group \((n = 58)\), low-dose dl-3n-butylphthalide group \((n = 57, 80 \text{ mg/kg})\), and high-dose dl-3n-butylphthalide group \((n = 56, 160 \text{ mg/kg})\). Rat models of diffuse brain injury were established in the latter three groups. Dl-3n-butylphthalide was given via intraperitoneal injection immediately after diffuse brain injury. Subsequently, each group was further assigned into five subgroups according to different time periods (3, 6, 24, 48, and 72 hours after injury). Eighteen rats in the injury group, 17 in the low-dose group, and 16 in the high-dose group died during the model establishment process. A total of 160 rats were involved in the final data analyses with 40 rats in each group.

Effects of dl-3n-butylphthalide on the ultrastructure of cortical nerve cells and blood capillaries in diffuse brain injury rats

Under transmission electron microscopy, the shape of nerve cells and karyon were regular, mitochondria in the cytoplasm were abundant with a round or oval shape and orderly and clear cristae arrangements, karyotheca was complete, chromat in was evenly distributed, and electronic transparency was high in the control group. However, the nerve cells were markedly damaged in the injury group with the cytoplasm obviously dissolving, chromatin concentrating, karyotheca continuity interrupted, mitochondria showing a flask shape or vacuolation, cristae fracturing and disappearing, and matrix granules shedding and disappearing. In contrast, damage in the dl-3n-butylphthalide groups was extensively alleviated with cells having a clear nucleolus, smooth karyotheca, better continuity, regular mitochondria shape, mostly tightly and regularly arranged complete cristae, and less shedding matrix granules.
The structures of vascular endothelial cells were normal, and the basilemma thickness was even in the control group. In the injury group, vascular endothelial cells were swollen, lumens were compressed and narrow, vascular walls were rough, gap junctions were broken, and basilemma were loose, fuzzy, or even separated. In the dl-3n-butylphthalide groups, vascular endothelial cell agglutination was scarcely seen, membrane structures were complete and clear, and the basilemma thickness was even (Figure 1).

Effects of dl-3n-butylphthalide on cortical cerebral blood flow in diffuse brain injury rats
Cerebral blood flow in the control group was high and stable. In the injury group, in contrast, it decreased significantly and maintained a low level 3 hours after injury, continuously decreased from 6 and 24 hours. Cerebral blood flow decrease slowed between 48 and 72 hours in the injury group, but was still clearly lower than that in the control group. After treatment with dl-3n-butylphthalide for 6, 24, 48, and 72 hours, cerebral blood flow increased significantly in a dose-dependent manner \((P < 0.05)\) as it maintained a higher level in the high-dose dl-3n-butylphthalide group (Table 1).

Effects of dl-3n-butylphthalide on cortical microvessel density and microvessel area density in diffuse brain injury rats
Cortical arteries entered vertically into the brain with tree-like subbranches, and in the deep or end areas of the cortex, fewer arteries were sent out at acute angles. These arteries anastomosed into nets in the brain with round, oval, or irregular meshes in the control group. However, in the injury group, the amount of capillaries was reduced distinctly, the walls of the remaining capillaries were harder, and the microvessel density and microvessel area density were clearly lower than in the control group \((P < 0.05)\). Microvessel density and microvessel area density clearly decreased at 3 and 6 hours after injury and maintained a low level. Although the degree of reduction became slower, microvessel density and microvessel area density were still significantly lower than those in the control group. Microvessel density and microvessel area density increased markedly at 6, 24, 48 and 72 hours after treatment with dl-3n-butylphthalide in a dose-dependent manner \((P < 0.05)\) and these low lev-
els were not observed in the high-dose dl-3n-butylphthalide group (Tables 2, 3).

**Effects of dl-3n-butylphthalide on blood-brain barrier permeability and cerebral edema in diffuse brain injury rats**

Evans blue content was stable and low in the cortex of rats in the control group, whereas it clearly increased 3 hours after injury and maintained a high level. Although it slightly decreased at 6 and 24 hours, it was still markedly higher than that in the control group. Moreover, it increased again between 48 and 72 hours and then maintained a high level. Evans blue content decreased significantly at 3, 6, 24, 48 and 72 hours after treatment with dl-3n-butylphthalide in a dose-dependent manner ($P < 0.05$; Table 4).

The brain water content was distinctively higher at each time point in the injury group compared with that in the control group ($P < 0.05$), and reached a peak at 72 hours. Compared with the injury group, it was dose-dependently and significantly lower at 6, 24, 48, and 72 hours in the dl-3n-butylphthalide groups ($P < 0.05$; Table 5).

**Effects of dl-3n-butylphthalide on the behavior of diffuse brain injury rats**

Motor function was evaluated with open field activity, body balance, screen climbing and balance, which were graded as scores of 0–3 on each measure with a total possible cumulative score of 12. Sensory function was classified into neck touching, antenna touching, and tactile senses, which were graded as scores of 0–3 on each measure with a total possible cumulative score of 9.

### Table 1 Effects of dl-3n-butylphthalide on cerebral blood flow (%) in the cortex of diffuse brain injury rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after drug administration (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>99.9±9.4</td>
</tr>
<tr>
<td>Injury</td>
<td>28.5±8.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low-dose dl-3n-butylphthalide</td>
<td>31.1±8.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High-dose dl-3n-butylphthalide</td>
<td>32.2±8.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as a ratio of the basic blood flow volume, mean ± SD, of five rats in each group at each time point. Differences between groups were compared with one-way analysis of variance and Student-Newman-Keuls test. $^aP < 0.05$, vs. control group; $^bP < 0.05$, vs. injury group; $^cP < 0.05$, vs. low-dose dl-3n-butylphthalide group. Rats in low- and high-dose dl-3n-butylphthalide groups were treated with intraperitoneal injection of 80 and 160 mg/kg dl-3n-butylphthalide, respectively.

### Table 2 Effects of dl-3n-butylphthalide on microvessel density in the cortex of diffuse brain injury rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after drug administration (hour)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>69.7±12.1</td>
</tr>
<tr>
<td>Injury</td>
<td>4.4±2.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low-dose dl-3n-butylphthalide</td>
<td>4.7±2.3</td>
</tr>
<tr>
<td>High-dose dl-3n-butylphthalide</td>
<td>5.1±2.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of five rats in each group at each time point. Differences between the groups were compared with one-way analysis of variance and Student-Newman-Keuls test. $^aP < 0.05$, vs. control group; $^bP < 0.05$, vs. injury group; $^cP < 0.05$, vs. low-dose dl-3n-butylphthalide group. Rats in low- and high-dose dl-3n-butylphthalide groups were treated with intraperitoneal injection of 80 and 160 mg/kg dl-3n-butylphthalide, respectively.

### Table 3 Effects of dl-3n-butylphthalide on microvessel area density in the cortex of diffuse brain injury rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after drug administration (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>11.1±2.3</td>
</tr>
<tr>
<td>Injury</td>
<td>2.9±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low-dose dl-3n-butylphthalide</td>
<td>3.7±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High-dose dl-3n-butylphthalide</td>
<td>3.6±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of five rats in each group at each time point. Differences between the groups were compared with one-way analysis of variance and the Student-Newman-Keuls test. $^aP < 0.05$, vs. control group; $^bP < 0.05$, vs. injury group; $^cP < 0.05$, vs. low-dose dl-3n-butylphthalide group. Rats in low- and high-dose dl-3n-butylphthalide groups were treated with intraperitoneal injection of 80 and 160 mg/kg dl-3n-butylphthalide, respectively.
Microcirculation disorder caused by traumatic brain injury includes changes in cerebral blood flow and microvascular permeability and damage to microvascular integrity. During diffuse brain injury, blood interruption occurs by vascular damage, rupture, or occlusion when the subject receives a blow to the head; blood flow decreases because of vascular endo-

DISCUSSION

This study showed that cerebral ultra-structure damage was lessened and behavior scores improved after treatment with dl-3n-butylphthalide, indicating a favorable effect of dl-3n-butylphthalide in diffuse brain injury. This was consistent with previous studies suggesting that dl-3n-butylphthalide has a positive effect on the prognosis of cerebrovascular diseases[14-17]. Neural functional recovery after cerebral injury is largely dependent on the survival of neurons within a therapeutic window[18]. Although the death of nerve cells in this study was not studied, nerve cell loss after cerebral injury could be estimated by observing the favorable effects of dl-3n-butylphthalide on neuron ultrastructure. It has been confirmed that dl-3n-butylphthalide reduces the expression of Caspase-8 and Smac, a proapoptotic protein, while increasing expression of Bcl-2, protecting mitochondrial functions, and reducing nerve cell apoptosis and death after ischemia[19-20].
thelium edema or deformation and lumen collapse and narrowing. After the injury, high blood viscosity leads to low blood flow and blood flow further decreases because of central and peripheral sympathetic nerve release of a large number of vasoconstrictive substances, such as 5-hydroxytryptamine. Besides, cerebral edema compressing brain tissue also results in a slowdown or decrease of blood flow. A previous study indicates that cerebral ischemia occurs 4 hours after severe brain injury with low cerebral blood flow, arterial oxygen tension differences, and cerebral oxygen metabolic rates[23]. Rafols et al [22] showed that cerebral blood flow decreased by 37% between 12 and 48 hours after brain injury. Wang et al [29] reported that microvascular density sharply decreased 30 minutes after injury and recovered to some extent at 7 days by observing changes in cerebral capillaries after acute contusion injury in rats based on an endogenous peroxidase histochemical method. In the present study, we chose 3 hours after injury as the initial observation time using a severe diffuse brain injury model in rats that died within 1–2 hours after damaging the brainstem and lost substantial amounts of blood. Compared with the control group, microvessel density and cerebral blood flow clearly decreased 3 hours after injury, and cerebral blood flow presented its lowest level after 6–48 hours but increased somewhat at 72 hours in the injury group. We contend that severe diffuse brain injury, often accompanied by subarachnoid hemorrhage, vasospasm, cerebral ischemia, anoxia, and cerebral edema, together with interactive actions of the above factors, brings about a continuous cerebral ischemia and persistent low cerebral blood flow.

Dl-3n-butylphthalide increases cerebral blood flow by changing hemorheology to make local blood speed faster and improves vascular regulation by regulating Prostaglandin I2/Thromboxane A2 levels and endothelial nitrogen monoxide production[23–25]. This study illustrated that capillary damages is lessened in an obvious and dose-dependent manner after dl-3n-butylphthalide treatment, microvascular density and cerebral blood flow increased greatly and the lowest phase of cerebral blood flow was not seen until 6 hours after the treatment. We believe that dl-3n-butylphthalide improves cerebral blood flow after injury by lessening vascular injury in brain tissue, protecting the integrity of vascular structure and function, and promoting the regeneration of vascular structure in damaged regions.

Damage to the blood-brain barrier and cerebral edema are main causes of patient death and disability after brain injury. Baskaya et al [27–30] suggested that the blood-brain barrier in the injured brain appears to open in two phases, and how open it becomes is not always in line with cerebral edema as early stage edema changes with the openness of the blood-brain barrier, yet delayed blood-brain barrier openness has little relationship with cerebral edema. However, Ikeda et al [31–32] considered that the formation of the cerebral edema is in line with changes to the blood-brain barrier, and most domestic studies support their ideas[33–38]. In this study, Evans blue staining increased significantly after 3 hours, was maintained at 6 hours, increased again at 24 hours, and reached its peak at 72 hours. Cerebral edema increased steeply and also reached its peak at 72 hours. This indicates that direct injury is caused by mechanical damage of cerebral capillaries, openness of tightness connections, increased blood-brain barrier permeability, and finally vasogenic cerebral edema. In the late stages, the structure and function of the blood-brain barrier are further damaged because of the aggravation of cerebral ischemia, anoxia, and edema. This study illustrated that vascular damage clearly lessened after dl-3n-butylphthalide treatment, and the extent of Evans blue staining and cerebral edema decreased in a distinct and dose-dependent manner 6–72 hours after injury. This suggests that dl-3n-butylphthalide may inhibit blood-brain barrier damage and alleviate cerebral edema. Gao et al [35–39] discovered that dl-3n-butylphthalide may reduce matrix metalloproteinase 9 expression 6–24 hours after ischemia reperfusion injury, decrease the permeability of the blood-brain barrier, and increase the expression of collagen IV of basilar membrane. Research into the application of dl-3n-butylphthalide in diffuse brain injury is rare. Feng et al [40] reported that dl-3n-butylphthalide could lessen the extent of edema in rats with closed cerebral injuries and improve their learning and memory functions. However, they did not elucidate the mechanism for this phenomenon. It is well known that low perfusion caused by microcirculation disorder in brain injury may result in energy metabolism disorders of nerve cells, stimulant toxicity, lactic acid and calcium ion build-up, massive release of catecholamines, inflammatory reactions, and programmed cell damage and death cascade reactions, which are indispensable to initiating and aggravating cerebral edema[40–43]. The neuroprotective functions of dl-3n-butylphthalide in ischemic brain injury involve blocking various pathological links, such as improving cerebral energy metabolism and mitochondrial function; inhibiting the release or expression of oxygen free radicals, inflammatory factors, and amyloid-β; decreasing ischemic
apoptosis of nerve cells; and reducing cerebral infarct areas. This study showed that dl-3n-butylphthalide improves microvascular density and cerebral blood flow and reduces Evans blue staining and cerebral water content, which indicated that the protective function of dl-3n-butylphthalide towards brain injury may be related to its function in improving cerebral blood flow after injury.

The present study suggested that dl-3n-butylphthalide can alleviate brain edema by reducing cortical blood-brain barrier damage in severe diffuse-brain-injury rats, and this is associated with improved cerebral blood flow and vascular permeability. Although these findings may provide a pharmacological foundation for dl-3n-butylphthalide treatment in diffuse brain injury, further study is needed to elucidate the mechanisms underlying these neuroprotective effects.

**MATERIALS AND METHODS**

**Design**

A randomized, controlled animal experiment.

**Time and setting**

This experiment was conducted at the Experimental Center of the Affiliated Hospital of Hebei United University, China from March 2009 to July 2010.

**Materials**

**Animals**

Specific pathogen-free male Sprague-Dawley rats, aged 2.5 months, weighing 250 ± 20 g, were purchased from the Experimental Center of Hebei United University, China (license No. SCXK (Jing) 2002-003). All rats were housed in a laboratory room at controlled temperature (22–26°C) and relative humidity (40–70%), and were allowed adequate light and diet. The protocols were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

**Drugs**

Di-3n-butylphthalide was provided by Shijiazhuang Pharmaceutical Co., Ltd., Shijiazhuang, Hebei Province, China. Synthesis of dl-3n-butylphthalide: Phthalic anhy-dride (I) was treated with butyric anhydride (II) and sodium butyrate (III) to obtain lactone (IV), which was hydrogenated to the target compound (chemical structural formula is shown in Figure 2). It was given as soft capsules and has the chemical formula C_{12}H_{16}O_{2}. It was made into an emulsion (concentration 8 mg/mL) in 0.5% Tween-80 before use.

**Methods**

**Establishment of diffuse brain injury models**

The diffuse brain injury model was established according to Marmarou’s method. In brief, rats were anesthetized with diethyl ether for 70–150 seconds, and the scalp was disinfected and opened using routine procedures. Then, the periosteum was stripped along the sagittal suture and transplanted with a stainless steel pad with a 10 mm diameter and 3 mm thickness. Rats were fixed in the prone position on a 10 cm × 10 cm × 20 cm sponge mat under a 2-meter tall Plexiglas tube, and then hit with a copper bar with a weight of 450 g and diameter of 18 mm that fell vertically from a plastic pipe at a height of 1.5 meters. After the strike, rats were quickly removed to avoid a secondary injury by the rebound of the copper bar. The rat’s scalp was disinfected routinely and sutured after injury. They were then returned to the cage and given water and food at room temperature. The control animals were anesthetized and their scalp was incised and sutured but not injured. Dl-3n-butylphthalide emulsion was injected into the abdominal cavity after injury in both the low- (80 mg/kg) and the high-dose dl-3n-butylphthalide groups (160 mg/kg) every 24 hours for 72 hours.

**Observation of cerebral ultrastructure**

One or two rats were selected at 3, 6, 24, 48, and 72 hours after injury, and were sacrificed after being anesthetized with 30 mg/kg of 3% pentobarbital sodium. Then, their hearts were opened and perfused with a mixed fixative liquid composed of 2.5% glutaraldehyde and 2% paraformaldehyde phosphate buffer. Tissue 0.2 cm away from cerebral coronal suture was harvested and cut into sections at 1 mm × 1 mm × 1 mm, and was fixed by 4% glutaraldehyde and washed twice with 0.1 mol/L cacodylic acid buffer solution, fixed again with 1% osmium tetroxide, and washed again with buffer solution. Afterwards, these tissue sections were dehydrated by acetone step by step, saturated by epoxy resin, em-
bedded, cut into ultrathin slices, stained with uranyl acetate and lead citrate, and then observed using transmission electron microscopy (H-7650 Hi-tachi; Days-US Science and Technology Co., Ltd., Beijing, China).

**Measurement of cerebral blood flow**

Five rats in each group were selected at 3, 6, 24, 48, and 72 hours after injury. Their heads were fixed horizontally by a stereotaxic apparatus (Narishige Company, Tokyo, Japan), and opened sagittally in the middle of the calvarium. A 1.5 mm diameter hole was drilled 2.0 mm posterior to the bregma and 2.3 mm right to the midline. A laser Doppler flowmeter probe (MP-150; BIOPAC, New York, NY, USA) was inserted 1 mm deep into the cerebral cortex and fixed to measure cerebral blood flow. The mean value of blood flow volume 10 minutes before ischemia was considered as basic blood flow volume, and its ratio was analyzed.

**Microvascular ultrastructure and quantitative analysis**

The rats were killed after being anesthetized by 3% pentobarbital sodium (30 mg/kg). A catheter was inserted into the ascending aorta from the left ventricle, and the vessels were washed rapidly with 100 mL normal saline at 37°C and then perfused with a mixed mordanting stationary liquid containing 0.1 mol/L phosphate buffer, 4% paraformaldehyde, and 10% tannic acid. Brain tissue was taken from the optic chiasma to the cerebral transverse fissure, and cut into continuous corona slices at 25 μm thickness. Afterwards, one out of every five slices (100 μm) was colorized with 2% ferric chloride. Finally, sections were observed by optical microscope (Olympus, Tokyo, Japan) and photos were taken after dehydration, vitrification, and slide mounting. The microvessel density and microvessel area density of the cortex in the frontal lobe were analyzed quantitatively using an MiVnt image analysis system (Bio-Rad Co., Ltd., California, CA, USA) by randomly selecting three slices in a cortical vertical plane (localized by the blood flow direction in the cerebral cortex, as opposed to the upper part of the hippocampal ring).

**Measurement of blood vascular permeability**

Rats were injected with 2 mL/kg 2% Evans blue into the caudal vein for 15 minutes before execution, and then anesthetized with 30 mg/kg 3% pentobarbital sodium. The heart was opened and perfused with saline, a tube was inserted into the aorta through the left ventricle, the right atrium was cut, and 150 mL physiologic saline was administered through the tube. Partial brain tissues were taken and the wet weight was measured precisely, and the tissues were placed into a medium-sized tube. Then, 3 mL of formamide was added into the tube, incubated for 48 hours in a 37°C water bath, and centrifuged for 15 minutes at 1 006.2 × g (centrifugal radius 10 cm). The absorbance of the supernatant was measured with a spectrophotometer (λ = 632 nm) (UNICO Co., Ltd., New York, NY, USA). The formamide method was used to detect Evans blue content in the cortex to assess damage severity in blood-brain barrier. The formula was as follows: Evans blue content in the cortex: B × formamide (mL)/wet weight (g), where B refers to the Evans blue content of the sample (μg/mL) given by the linear regression equation according to standard curve.

**Measurement of brain water content**

Rat were anesthetized as described above and sacrificed by removing the carotid artery. Then, the bilateral cerebral cortex was separated on ice, the wet weight was measured precisely, and the cortex was dried in 95°C oven. Finally, brain water content was calculated according to the formula: brain water content (%) = (wet weight – dry weight)/wet weight × 100%.

**Animal behavioral tests**

Open-field tests were conducted as previously described\(^{(8)}\).

Motor functions: (1) Open field activities: Normal rats walk along the walls of the chamber as natural behavior when they are put in an open field. After the operation, their activities were graded at four levels: no activities, moved but did not stay near the wall, walked and stayed near 1–2 walls, and walked and stayed near 3–4 walls. (2) Body balance: The rats were put on the ground to observe their body movement, which was graded at four levels: no activities of the left forelimb, slight activities, irregular activities, and symmetrical extension. (3) Climbing a sieve: We fixed a hanging sieve, helped the rats to grasp it, and then let the rats go and make observations. The rats’ reactions were divided into four levels: did not grasp, grasped for less than 4 seconds, grasped but did not move, and moved and passed through the sieve. (4) Balancing: We fixed hanging wooden rods, put the rats on them, and observed their behavior. The rats’ reactions were graded at four levels: falling down within less than 2 seconds, falling down after more than 2 seconds, grasped the rod but did not move, and moved and crossed through the wood. All rats were graded in each test with a value of 0–3.

Sensory functions: (1) Proprioception was graded at three levels: no head-turn reaction, asymmetric turns, and symmetric turns. (2) Tentacle testing was divided...
into no reaction, asymmetric turns, and symmetric turns. (3) Touching the neck was classified as no reaction, slower, and immediately. All rats were graded in each test with a value of 1–3.

**Statistical analysis**

Measurement data are expressed as mean ± SD, and are calculated using SPSS 17.0 software (SPSS, Chicago, IL, USA). Differences between groups were compared with one-way analysis of variance and Student-Newman-Keuls test. P values less than 0.05 were considered significant.

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