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L-3-n-butylphthalide protects against vascular dementia *via* activation of the Akt kinase pathway

Yaping Huai[1], Yanhong Dong[2], Jing Xu[1], Nan Meng[1], Chunfeng Song[3], Wenbin Li[4], Peiyuan Lv[1, 2]

1 *Department of Neurology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China*
2 *Department of Neurology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China*
3 *Electron Microscope Center, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China*
4 *Department of Pathophysiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China*

Research Highlights

(1) 3-N-butylphthalide, a green botanical medicine, is a successfully synthesized and stable chemical drug used for the treatment of ischemic stroke that has independent intellectual property rights in China. Three different stereo-isomers have been identified: l-, dl-, and d-3-n-butylphthalide. The first L-isomer, originally extracted from celery seed, was artificially synthesized from racemic acid, also known as butylphthalide. L-3-n-butylphthalide has been shown to reduce β-amylase-induced neuronal apoptosis and improve cognitive function in Alzheimer's disease animal models.
(2) As a neuroprotective drug for cerebrovascular disease, 3-n-butylphthalide has been confirmed to protect nerve cells in animal experiments of stroke. Because of the significant effects of l-3-n-butylphthalide in the clinical treatment of acute ischemic stroke, this study also adopted l-3-n-butylphthalide for the treatment of vascular dementia.
(3) This is the first report that shows pretreatment with l-3-n-butylphthalide can improve cognitive deficits and neuronal loss in the hippocampus of cerebral repetitive ischemia/reperfusion mice.
(4) L-3-n-butylphthalide may be a potentially beneficial and promising drug for the treatment and prevention of vascular dementia through upregulation of Akt expression in the hippocampus.

Abstract

As a neuroprotective drug for the treatment of ischemic stroke, 3-n-butylphthalide, a celery seed extract, has been approved by the State Food and Drug Administration of China as a clinical therapeutic drug for ischemic stroke patients. L-3-n-butylphthalide possesses significant efficacy in the treatment of acute ischemic stroke. The activated Akt kinase pathway can prevent the death of nerve cells and exhibit neuroprotective effects in the brain after stroke. This study provides the hypothesis that l-3-n-butylphthalide has a certain therapeutic effect on vascular dementia, and its mechanism depends on the activation of the Akt kinase pathway. A vascular dementia mouse model was established by cerebral repetitive ischemia/reperfusion, and intragastrically administered l-3-n-butylphthalide daily for 28 consecutive days after ischemia/reperfusion, or 7 consecutive days before ischemia/reperfusion. The Morris water maze test showed significant impairment of spatial learning and memory at 4 weeks after operation, but intragastric administration of l-3-n-butylphthalide, especially pretreatment with l-3-n-butylphthalide, significantly reversed these changes. Thionine staining and western blot analylsis showed that preventive and therapeutic application of l-3-n-butylphthalide can reduce loss of pyramidal neurons in the hippocampal CA1 region and alleviate nerve damage in mice with vascular dementia. In addition, phosphorylated Akt expression in hippocampal tissue increased significantly after l-3-n-butylphthalide treatment. Experimental findings demonstrate that l-3-n-butylphthalide has preventive and therapeutic effects on vascular dementia, and its mechanism may be mediated by upregulation of phosphorylated Akt in the hippocampus.

Yaping Huai, M.D., Associate professor, Associate chief physician.

Corresponding author: Peiyuan Lv, M.D., Professor, Chief physician, Doctoral supervisor, Department of Neurology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China; Department of Neurology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China, peiyuanlu@163.com.

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Key Words

neural regeneration; brain injury; ischemia/reperfusion; Akt; phosphorylated Akt; Morris water maze; cognitive function; 3-n-butylphthalide; hippocampus; learning; memory; dementia; grants-supported paper; neuroregeneration

INTRODUCTION

Vascular dementia, the second most common cause of dementia after Alzheimer's disease in the elderly[1], has attracted increasing attention. Recently, emerging epidemiological evidence indicates that vascular dementia is likely to be the leading cause of dementia in the Asian population[2-4]. Although the pathogenesis of vascular dementia remains unclear, increasing evidence suggests that vascular dementia is associated with a group of diverse pathologies affecting the cerebrovascular circulation[5]. One of the important mechanisms underlying vascular dementia is the pathophysiology after ischemia/reperfusion, including excitotoxicity, oxidative stress and inflammatory reactions[6-8]. Currently, there is no specific drug available that can prevent or cure vascular dementia.

3-N-butylphthalide, a green botanical medicine, has a simple and stable chemical structure that has been successfully synthesized for the national treatment of ischemic stroke with independent intellectual property rights in China. 3-N-butylphthalide has three different stereo-isomers known as l-, dl-, and d-3-n-butylphthalide[9]. The l-isomer, originally extracted from celery seed, was artificially synthesized from racemic acid and is also known as butylphthalide. dl-3-n-butylphthalide is a neuroprotective drug that has been shown to protect against ischemic stroke through multiple mechanisms, for example improving energy metabolism, reducing oxidative damage[10-13], improving microcirculation in arterioles[13], decreasing neuronal apoptosis[14-15], improving mitochondrial function, and inhibiting inflammation[16]. Furthermore, l-3-n-butylphthalide reduces cell death induced by β-amyloid in neuronal cell cultures[17], and improves cognitive performance in an Alzheimer's disease animal model[18-20]. Although the positive effects of 3-n-butylphthalide on cerebral infarct and Alzheimer's disease have been confirmed in clinical studies and animal experiments, few studies have investigated if 3-n-butylphthalide is advantageous as a therapy for vascular dementia. A previous study discovered that only l-3-n- butylphthalide ameliorated cognitive disorders induced by chronic cerebral hypoperfusion while d- and dl-3-n-butylphthalide did not show a marked advantageous effect[21]. Moreover, the effects of l-3-n-butylphthalide in this area are still not clear.

Recently, accumulating evidence has shown that the phosphatidylinositol-3 kinase/Akt cell signaling pathway participates in the apoptotic pathway after ischemia/reperfusion. There are two obvious types of cell death: necrosis and apoptosis in post- ischemia/reperfusion neurons[22]. There may be a continuum between apoptosis and necrosis[23]. Therefore, the extent of apoptosis may be associated with the pathological process[24]. The Akt/protein kinase B signaling pathway regulates cell survival and is important in the pathogenesis of degenerative diseases[25]. Activating the Akt/protein kinase B pathway may be neuroprotective through blocking neuronal death after stroke[26-27]. Activated Akt blocks apoptosis by phosphorylating its substrates, for example forkhead transcription factor, Bcl-2-associated death protein, and glycogen synthase kinase 3β[28].

Therefore, this study aimed to examine whether l-3-n-butylphthalide treatment and pretreatment have the ability to improve cognitive impairment in a mouse model of vascular dementia induced by cerebral repetitive ischemia/reperfusion. Furthermore, this study also explored whether activation

of Akt was involved in the neuroprotective mechanism of l-3-n-butylphthalide.

RESULTS

Quantitative analysis of experimental animals

Forty-eight C57BL/5 mice were equally and randomly divided into four groups with identical mean body weights: sham group (carotid artery was exposed without ligation), ischemia/reperfusion group (intragastric administration of vegetable oil 6 mL/kg was given daily for 28 consecutive days after ischemia/reperfusion models were established), pre-l-3-n-butylphthalide group (intragastric administration of l-3-n-butylphthalide 30 mg/kg was given daily, 7 days before ischemia/reperfusion models were established), l-3-n-butylphthalide group (intragastric administration of l-3-n-butylphthalide 30 mg/kg was given daily for 28 consecutive days after ischemia/reperfusion models were established). Four mice (one in ischemia/reperfusion group, two in pre-l-3-n-butylphthalide and one in l-3-n-butylphthalide group) died during the ischemia/reperfusion modeling process and no new mice were supplemented. The remaining 44 mice were involved in the final analysis.

L-3-n-butylphthalide ameliorated learning and memory impairment induced by cerebral repetitive ischemia/reperfusion

Spatial learning and memory in mice was evaluated using the Morris water maze test at 4 weeks after operation[29]. Escape latencies gradually reduced in all groups in a time-dependent manner [$F_{(4, 160)}$ = 11.518, P < 0.01]. This showed that all mice effectively improved their spatial learning ability after 5 days of training. In addition, there was a marked therapeutic effect on escape latency after treatment [$F_{(3, 40)}$ = 9.065, P < 0.01], revealing that l-3-n-butylphthalide was protective against spatial learning impairment in cerebral repetitive ischemia/reperfu- sion mice. Furthermore, there was no marked treatment-by-day interaction effect [$F_{(12, 160)}$ = 1.494, P = 0.391].

Because of the marked drug effect, we implemented *post hoc* analysis on escape latency data. The mice in the sham group were able to reach the platform within 30 seconds on day 7, suggesting that mice had learnt where the platform was located. The escape latency of the ischemia/reperfusion group was evidently longer than that of the sham group (P < 0.01; Figure 1). This is evidence that cerebral repetitive ischemia/reperfusion successfully induced learning impairment. Compared with the ischemia/reperfusion group, the escape latency significantly decreased after treatment with 30 mg/kg l-3-n-butylphthalide for 3 and 5 days (P < 0.05), and also decreased after pretreatment with 30 mg/kg l-3-n-butylphthalide for 2–5 days (P < 0.01, P < 0.05; Figure 1). These findings indicate that intragastric administration of 30 mg/kg l-3-n-butylphthalide, especially pretreatment with 30 mg/kg l-3-n-butylphthalide, significantly rescued learning impairment caused by cerebral repetitive ischemia/reperfusion.

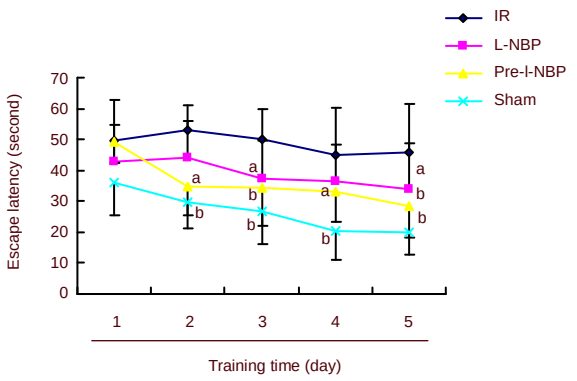

Figure 1 Effects of l-3-n-butylphthalide (L-NBP) on spatial memory impairment induced by cerebral repetitive ischemia/reperfusion (IR) in mice.

Mean escape latency (second) was calculated for each day in the sham (n = 12), IR (n = 11), L-NBP (n = 11) and pre-L-NBP (n = 10) groups in the place navigation phase of Morris water maze test. Mean latency (second) refers to the time taken to find the hidden platform during 5 consecutive days of training. After 5 days of training, the escape latency in the L-NBP group and pre-L-NBP group was significantly reduced compared with the IR group. Data were expressed as mean ± SD and were evaluated by repeated measure analysis of variance. ^aP < 0.05, ^bP < 0.01, *vs*. IR group. sham: Sham surgery.

L-3-n-butylphthalide significantly rescued memory deficits induced by cerebral repetitive ischemia/ reperfusion

To investigate the effect of l-3-n-butylphthalide on memory impairment caused by cerebral repetitive ischemia/reperfusion, we conducted a spatial probe test after the Morris water maze. There was a significant effect of l-3-n-butylphthalide treatment. During the probe trial, we plotted the performance of different groups by analyzing the percentage of time in the target quadrant where the hidden platform had previously been available (Figure 2). Results found that the l-3-n-butylphthalide pretreated mice showed a significant preference for the target quadrant compared with the ischemia/reperfusion group (P < 0.01). Although mice treated with 30 mg/kg l-3-n-butylphthalide spent more time in the target quadrant than mice in the is-

chemia/reperfusion group (33.54 ± 14.38% *vs*. 22.03 ± 17.68%), there was no marked difference between the l-3-n-butylphthalide group and the ischemia/reperfusion group ($P > 0.05$). This evidence suggests that treatment with 30 mg/kg l-3-n-butylphthalide has an advantageous effect, but it cannot fully inhibit the memory impairment.

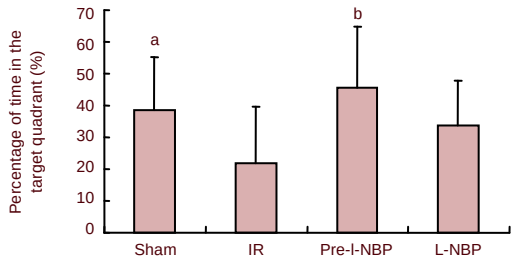

Figure 2 Effects of l-3-n-butylphthalide (L-NBP) on memory deficits induced by cerebral repetitive ischemia/reperfusion (IR) in mice.

Mean percentage of time in the target quadrant was calculated in the sham ($n = 12$), IR ($n = 11$), l-NBP ($n = 11$) and pre-l-NBP ($n = 10$) groups in the spatial probe phase. Data are expressed as mean ± SD and evaluated by one-way analysis of variance and least significant difference-*t* test. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. IR group. sham: Sham surgery.

L-3-n-butylphthalide protected pyramidal neurons from injury in the hippocampal CA1 region 35 days after cerebral repetitive ischemia/reperfusion

To determine whether the protective effect of l-3-n-butylphthalide on the progression of learning impairment and memory deficits was due to delayed pyramidal neuron loss in the hippocampal CA1 region, histological grade and neuronal density[30] of pyramidal neurons in the hippocampus was evaluated (Figure 3).

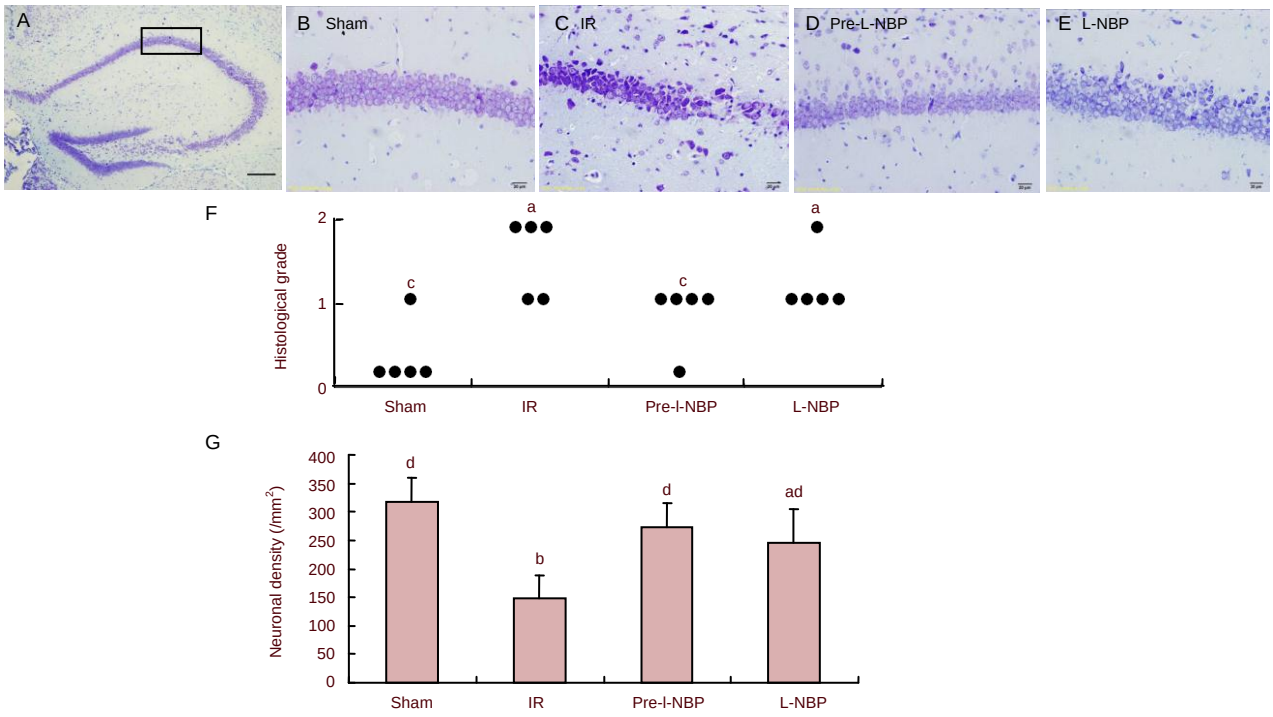

Figure 3 Histological grade changes in the hippocampal CA1 region in mice treated with l-3-n-butylphthalide (L-NBP).

(A) Image of the hippocampus. Black frame represents the position photographed in the following four images (thionine staining, × 100).

(B–E) L-NBP reduces the histological grade changes in the hippocampal CA1 region in each group. The upper panels are representative of thionine-stained photomicrographs (thionine staining, × 400).

The lower dot and bar graphs are quantitative presentations of the histological changes, with histological grades (F) and neuronal density (G) at 35 days after ischemia/reperfusion (IR). Each dot indicates one animal was assigned that grade. Data are expressed as mean ± SD ($n = 5$). $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. sham group; $^{c}P < 0.05$, $^{d}P < 0.01$, *vs*. IR group. A Kruskal-Wallis analysis of variance on ranks with Dunn's multiple comparisons test was performed to test differences in histological grades between groups. The neuronal density was tested by one-way analysis of variance and least significant difference *t*-test. Histological grades were divided into four grades as follows: grade 0: no neuronal death; grade I: scattered single neuronal death; grade II: mass neuronal death; grade III: almost complete neuronal death. sham: Sham surgery.

In sham mice, pyramidal neurons in the hippocampal CA1 region were arranged in sequence with 4–5 cell layers, full nuclei and clear nucleoli. The histological grade values were 0–1, and neuronal density was 316.8/mm^2. Some neurons died at 35 days after cerebral repetitive ischemia/reperfusion, which were represented by increases in histological grade ($P < 0.05$) and remarkable decreases in neuronal density ($P < 0.01$) when compared with the sham group. Whereas, there was no significant elevation in histological grade or reduction of neuronal density between the pre-l-3-n-butylphthalide group and sham group ($P > 0.05$). Compared with the ischemia/reperfusion group, there was a marked reduction in histological grade ($P < 0.05$) and an increase in neuronal density ($P < 0.01$) in the pre-l-3-n-butylphthalide group, and a reduction in histological grade ($P > 0.05$) and a marked increase in neuronal density ($P < 0.01$) in the l-3-n-butylphthalide group. These findings indicate that pretreatment with l-3-n-butylphthalide protected pyramidal neurons in the hippocampal CA1 region against injury induced by cerebral repetitive ischemia/reperfusion.

L-3-n-butylphthalide promoted the expression of phosphorylated Akt in the hippocampus of mice after cerebral repetitive ischemia/reperfusion mice

As shown in Figure 4, total Akt and phosphorylated Akt expression levels were detected in the mouse hippocampus. Western blot analyses were performed to determine whether treatment or pretreatment with l-3-n-butylphthalide was able to induce expression and phosphorylation of Akt in the hippocampus. There was no marked difference in total Akt expression among the four groups. However, phosphorylated Akt expression was significantly increased in the ischemia/reperfusion group when compared with the sham group ($P < 0.01$). Moreover, l-3-n-butylphthalide treatment and pre-l-3-n-butylphthalide led to strong expression of phosphorylated Akt ($P < 0.01$) when compared with the ischemia/reperfusion group.

DISCUSSION

Repetitive cerebral ischemia/reperfusion is generally involved in the pathogenesis of vascular dementia. Therefore, we used male C57BL/6 mice injured by cerebral repetitive transient ischemia/reperfusion as the vascular dementia model to mimic vascular dementia. Because the cross-sectional diameters of the posterior communicating arteries, which deliver blood to the brain, of C57BL/6 mice are smaller than those of other mouse strains[31], transient bilateral carotid occlusion has a more severe outcome[32-33]. In this study, the ischemia/reperfusion model mice showed lower learning and memory function in the Morris water maze test and more neuronal damage in the hippocampus CA1 region than sham animals at 4 weeks post-surgery, which was consistent with a previous study[34]. Hence, the models presented here can imitate the onset of human vascular dementia *via* a repeatable surgical procedure, and can provide an appropriate model of vascular dementia.

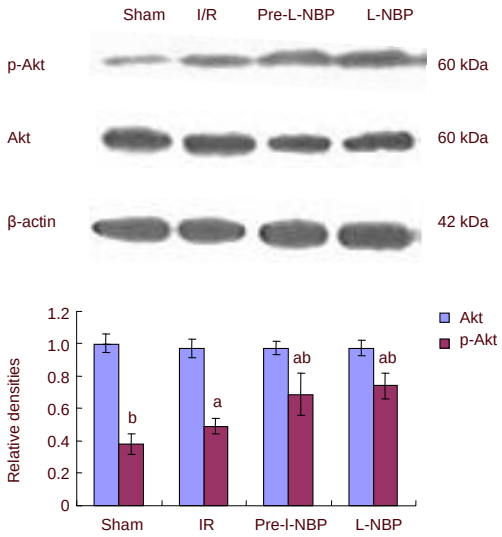

Figure 4 Western blot analysis of Akt and phosphorylated Akt (p-Akt) expression in the hippocampus of mice after cerebral repetitive ischemia/reperfusion (IR).

The upper figure shows bands of the immunoblot, and the lower figure shows the quantitative presentation of the immunoblots with ratios of integrated absorbance values for the bands corresponding to Akt and β-actin or p-Akt and β-actin. Data are expressed as mean ± SD ($n = 9$) and were evaluated by one-way analysis of variance and least significant difference *t*-test. [a]$P < 0.01$, *vs*. sham group; [b]$P < 0.01$, *vs*. IR group. L-NBP: L-3-n-butylphthalide; sham: sham surgery.

As a popular, widely used behavioral test, the Morris water maze was used to estimate spatial learning and memory of rodents[35-36]. The Morris water maze, an important behavioral test in neuroscience research, is usually performed to assess cognitive ability of animal models, or potency of drugs and/or other treatments. In 1984, Morris described the basic procedures and subsequently added details and procedures for evaluating related forms of learning and memory[37-40]. The Morris water maze has several advantages, including the lack of required pretraining, cross-species utility (mice, rats and humans in a virtual maze[41]), specificity as a survey of place learning, and plentiful evidence of its validity as a measure of hippocampus-dependent spatial navigation

and reference memory. For example, learning impairments in the Morris water maze are independent of locomotor effects because land-based locomotor reductions do not affect swimming speed. Morris water maze performance has been related to long-term potentiation and N-methyl-D-aspartic acid receptor function[42-43], which make it a key method in hippocampal circuitry research.

It was approved by the State Food and Drug Administration of China. As a pure component, the L-isomer of 3-n-butylphthalide was extracted from seeds of Apium graveolens Linn. Vascular dementia includes various subtypes of cerebrovascular diseases based on different vascular pathologies: cortical infarction, subcortical infarction, hemodynamic (hypotensive) infarction, and white matter lesions[44]. A subsequent vascular event can cause a sudden and 'stepwise' deterioration in cognitive function. In this study, treatment with l-3-n-butylphthalide after ischemia/reperfusion significantly reversed the deterioration in cognitive function and neuronal damage, which is in agreement with a previous study[45]. Furthermore, this study is the first to demonstrate that pretreatment with l-3-n-butylphthalide significantly ameliorates cognitive impairment and delays pyramidal neuron loss in the hippocampal CA1 region of mice following ischemia/reperfusion. Evidence suggests that l-3-n-butylphthalide may be a potentially beneficial and promising drug for the treatment and prevention of vascular dementia. However, the protective molecular mechanisms of l-3-n-butylphthalide remain elusive.

For potential clinical applications, it is necessary to verify the effects of 3-n-butylphthalide on vascular dementia models. Damage induced by cerebral repetitive ischemia/reperfusion causes an inflammatory reaction, further activates signaling pathways and eventually leads to neural cell apoptosis or necrosis, which induces vascular dementia. The neuroprotective role of phosphatidy- linositol-3 kinase/Akt in cerebral ischemia has been widely studied[46-48]. The phosphatidylinositol-3 kinase/ Akt pathway plays a major role in the process of cell growth, proliferation and survival under physiologic and pathophysiologic conditions[49]. The Akt/mTOR signal pathway induces activation of the nuclear factor kappa B pathway and adjusts the balance of Bcl-2 and Bax, which play an important role in the nerve cell inflammatory response and apoptosis during ischemic cerebrovascular disease, respectively[50]. Akt inhibits the Bax-dependent apoptosis pathway through forkhead box transcription factor, and then protects cells from apoptosis[51]. The anti-apoptotic activity of Akt is mediated by the activation of the phosphatidylinositol-3 kinase/Akt pathway[52]. The mechanism may be as follows: First, phosphatidylinositol-3 kinase is activated by membrane-bound receptor tyrosine kinase phosphorylation, which generates phosphatidylinositol-3, 4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate. Second, phosphatidylinositol-3,4,5-trisphosphate combines with cells containing the PH domain of the signaling protein Akt and phosphoinositide dependent kinase, which changes the protein structure of Akt and allows Akt translocation to the cell membrane. Third, phosphoinositide dependent kinase 1 and 2 protein phosphorylation on the membrane induces Akt phosphorylation on Thr-308 and leads to Akt activation. Following Thr308 phosphorylation, either Akt autophosphorylation or a yet unidentified phosphoinositide-dependent protein kinase-2 phosphorylates Ser473[53]. Once Ser473 is phosphorylated, Akt is completely activated regardless of the phosphorylation state of Thr-308[54]. Activated Akt blocks apoptosis by inactivating several targets, including Bad, caspase-9, glycogen synthase kinase-3β and forkhead transcription factors[55].

In a previous study, the expression of phosphorylated Akt Ser473 protein dramatically increased at 2 and 4 weeks after cerebral ischemia/reperfusion, then returned to basal levels at 6 weeks while total Akt had no significant change at 2, 4 and 6 weeks (data not shown). Ischemic preconditioning protected the brain by induction of ischemic tolerance resulting from a sublethal ischemic insult accompanied by phosphatidylinositol-3 kinase/Akt pathway activation[56]. This study also found there was no significant change in the levels of total Akt expression in sham and model groups at 5 weeks after cerebral ischemia/reperfusion. In contrast, phosphorylated Akt Ser473 expression in hippocampal tissue markedly increased in the model groups when compared with the sham group. Many neuroprotectants, including propofol[47], estradiol[57-58], melatonin[59], isoflurane[60], geranyl- geranylacetone[46], humanin[48], atorvastatin and tissue-type plasminogen activator[61], exert their protective effects through the phosphatidylinositol-3 kinase/Akt pathway. The present study also showed that while behavioral and morphologic damage in mice improved dramatically, phosphorylated Akt Ser473 expression in the hippocampus significantly increased in the l-3-n-butylphthalide and pre-l-3-n-butylphthalide groups when compared with the sham and model groups. Given the neuroprotective role of the phosphatidylinositol-3 kinase/ Akt pathway in cerebral ischemia, we hypothesized that the neuroprotective effect of l-3-n-butylphthalide may partly be due to the activation of the phosphatidylinositol-3 kinase/Akt pathway after cerebral ischemia/reper

fusion injury.

In summary, l-3-n-butylphthalide significantly attenuated learning and memory impairments induced by cerebral repetitive ischemia/reperfusion and thus was protective in vascular dementia mice. Therefore, l-3-n-butylphthalide is regarded as an effective treatment and preventative measure for vascular dementia. Further studies to identify the molecular targets of l-3-n-butylphthalide are underway.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This experiment was conducted at the Department of Pathophysiology, Hebei Medical University, China from January 2012 to November 2012.

Materials

Animals

Forty-eight C57BL/6 mice weighing 22–26 g, of clean grade, aged 10–12 weeks, were purchased from Beijing Vitalriver Laboratory Animal Technology Limited Company, China (license No. SCXK (Jing) 2012-0001). The experimental animals were group-housed in an animal room maintained at 23 ± 1°C with a 12-hour light/dark cycle, and allowed free access to food and water. 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[62].

Drugs

L-3-n-butylphthalide, with the formula of $C_{12}H_{14}O_2$, was extracted from celery seed. The extracted liquid was yellow and oil-like with a purity of 99.6%, and was provided by Shijiazhuang Pharmaceutical Group NBP Pharmaceutical Co., Ltd (Shijiazhuang, Hebei Province, China). The extract was directly diluted with vegetable oil.

Methods

Establishment of repetitive ischemia/reperfusion model

Mice were anesthetized using 1% (v/v) pentobarbital-sodium (Kebaiao Biological Reagent Co., Ltd., Beijing, China; 50 mg/kg). In the ischemia/reperfusion, and l-3-n-butylphthalide and pre-l-3-n-butylphthalide groups, repetitive ischemia/reperfusion was performed *via* bilateral common carotid artery occlusion according to the previously described method with some modifications[63]. Bilateral common carotids were exposed and isolated from nearby tissues, then occluded with 4-0 silk for 20 minutes, followed by reperfusion for 10 minutes. This pattern was repeated three times. In the sham group, protocols were the same as the model group but bilateral common carotids were only exposed. All the operations were performed on a heating station and the rectal temperature was maintained at 37.0 ± 0.5°C.

Drug administration

At 7 days before operation, mice from the pretreatment group were given 30 mg/kg l-3-n-butylphthalide by gavage once a day[20]. While the remaining mice were given a daily administration of l-3-n-butylphthalide (30 mg/kg) or vehicle (vegetable oil) for 28 days after cerebral ischemia/reperfusion. From day 29 to day 35 after surgery, spatial learning and memory was assessed in all mice. The experimental design is shown in Figure 5.

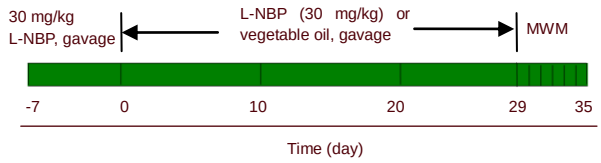

Figure 5 Experimental design.

L-NBP: L-3-n-butylphthalide; MWM: Morris water maze.

Assessment of spatial memory using Morris water maze

After drug administration was complete, all mice were trained and tested in the Morris water maze (JLBehv type, Shanghai, China) to monitor spatial learning and memory. The maze consisted of a large circular pool (120 cm in diameter, 60 cm in height, water depth of 45 cm, and water temperature 21 ± 1°C). The maze was divided into four equal quadrants. There was a 9-cm-diameter platform submerged 1.5 cm below the water surface in the center of quadrant N. On the first day of the training session, the mice were subjected to a single probe test for 60 seconds, in which the platform was removed from the maze. The swimming paths of the mice were recorded with a video camera placed above the maze. From the second day to the sixth day, the animals were trained with a hidden platform. Each mouse was placed in one of four quadrants (not containing the platform) in a random manner, with the head facing the wall. Timing of the latency to find the submerged platform started and ended by the experimenter. If the mouse could not find the es-

cape platform within 60 seconds, the experimenter gently assisted it onto the platform and allowed it to stay there for 15 seconds. The mouse was then dried and placed in a cage, and rested for at least 1 hour. Six trials were conducted each day, for 5 consecutive days. The platform was kept at the same position during the training period. The escape latency (swimming time to locate the hidden platform) was used to assess the performance of spatial learning and memory of the animals. On day 7, the retention of spatial memory was assessed using the spatial probe test. In the probe trial, the platform was removed, and the mice were tracked during a 60-second exploration of the pool. The time around the quadrant in which the platform was located and the number of crossings at the exact place where the platform was located (platform spans) were analyzed. The evaluator conducting the Morris water maze was blinded to the treatment groups.

Analysis of pyramidal neurons in the hippocampal CA1 region using thionine staining

At the given time points, mice were anesthetized with 1% (v/v) pentobarbital-sodium (50 mg/kg) and perfused through the ascending aorta with normal saline followed by 4% (w/v) paraformaldehyde. The unilateral brain was fixed in 4% (w/v) paraformaldehyde. Two days later, the brain was embedded in paraffin and sectioned into 6 μm thickness. The sections were stained with thionine (Sigma, St Louis, MO, USA). The delayed neuronal death of pyramidal neurons in the CA1 hippocampus was evaluated under a light microscope (Olympus, Tokyo, Japan) and scored based on histological grades and neuronal density. Histological grades were divided into four grades according to the following criteria[32]: grade 0: no neuron death; grade I: scattered single neuron death; grade II: mass neuron death; grade III: almost complete neuron death. Neuronal density of the hippocampal CA1 subfield (*i.e.*, the number of intact pyramidal cells per 1-mm linear length of CA1 stratum pyramidale) was determined with the method of Kirino *et al* [64]. The average numbers of pyramidal neurons in the hippocampal CA1 were calculated to establish the neuronal density value (/mm^2).

Akt and phosphorylated Akt expression in the mouse hippocampus by western blot analysis

Mice were decapitated at predetermined time points, and the hippocampus was quickly excised and homogenized in five volumes of lysis buffer. The homogenates were centrifuged at 11 600 × *g* for 15 minutes at 4°C, and the supernatants were analyzed. Fifty micrograms of protein (Coomassie assay) from each sample was loaded with loading buffer. The samples were electrophoresed on a 12% (w/v) sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. After the membranes were blocked in 5% (w/v) nonfat milk for 2 hours, they were incubated overnight at 4°C with rabbit-anti-mouse Akt monoclonal antibody (1:1 000; lot No. YE121003; Epitomics, Burlingame, CA, USA), rabbit-anti-mouse phosphorylated Akt Ser473 monoclonal antibody (1:1 000; lot No. YH111814D; Epitomics) or mouse-anti-β-actin polyclonal antibody (1:2 000; lot No. SC-47778; Proteintech Group, Chicago, IL, USA). Primary antibody binding was detected using the following secondary antibodies: goat-anti-rabbit IgG (1:10 000; lot No. SA00001-2; Proteintech Group) and goat-anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:10 000; lot No. SA00001-1; Proteintech Group). Detection was achieved with the enhanced chemiluminescence kit (Amersham Bioscience, Little Chalfont, U.K.) according to the protocol using X-ray films (Lucky, Hebei Province, China). Each of the membranes was probed with β-actin antibody to verify equal protein loading.

Statistical analysis

Measurement data were expressed as mean ± SD and analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). The data in the Morris water maze training task and the probe test were evaluated by repeated measure analysis of variance and one-way analysis of variance, respectively. Post *hoc* multiple comparisons test, combined with a least significant difference *t*-test as a multiple comparison method to test for differences between groups, was used to determine differences of means between each group. A Kruskal-Wallis analysis of variance on ranks with Dunn's multiple comparisons test was performed to test differences in histological grade between groups. Other data were presented as mean ± SD, and were tested by one-way analysis of variance, combined with a least significant difference *t*-test as a multiple comparison method to test differences between groups. A $P < 0.05$ was considered to be significant.

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