

L-butyl phthalein improves neural function of vascular dementia mice by regulating the PI3K/AKT signaling pathway

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Abstract. – **OBJECTIVE:** L-3-n-butylphthalide (L-NBP) is a type of anti-ischemic cranial nerve protective drug that may act on vascular dementia (VD). Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway can up-regulate B-cell lymphoma 2 (Bcl-2) expression, reduce reactive oxygen species (ROS) production, and alleviate cell apoptosis. This study aimed at investigating the role of L-NBP on neurological function and cell apoptosis in VD mouse through regulating PI3K/AKT signaling pathway.

MATERIALS AND METHODS: The mice were divided into four groups, including Sham, VD, VD + solvent, and VD + L-NBP. HT22 cells were cultured in vitro and treated by ischemia/reperfusion (I/R). HT22 cells were divided into four groups, including I/R, VD + solvent, VD + L-NBP, and VD + L-NBP + LY294002 groups. Phosphorylated AKT (p-AKT) and Bcl-2 expressions were tested. ROS content in hippocampus tissue was detected by flow cytometry. Cell apoptosis was evaluated by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay.

RESULTS: ROS content and cell apoptosis increased, while p-AKT and Bcl-2 expressions reduced in hippocampus tissue from VD group compared with sham group. L-NBP significantly up-regulated p-AKT and Bcl-2 expressions and decreased ROS content and cell apoptosis in hippocampus tissue. I/R treatment markedly induced HT22 cell apoptosis and ROS production, and reduced p-AKT and Bcl-2 expressions. L-NBP treatment markedly up-regulated p-AKT and Bcl-2 levels, restrained cell apoptosis, and reduced ROS content in HT22 cells intervened by I/R. LY294002 apparently attenuated the protective effect of L-NBP on HT22 cells.

CONCLUSIONS: L-NBP protects VD by up-regulating PI3K/AKT signaling pathway, elevating Bcl-2 expression, reducing nerve cell apoptosis, and restraining ROS production.

Key Words:

Vascular dementia, L-3-n-butylphthalide, PI3K/AKT, HT22 cells.

Introduction

Vascular dementia (VD) is a kind of brain circulation dysfunction and brain damage syndrome because of cerebrovascular factors, resulting in learning and memory impairment, accompanied by language, visual space, directional force, abstract thinking, and personality disorder. VD is the second largest dementia encephalopathy after Alzheimer's disease (AD)¹⁻³. In recent years, VD shows rising and younger trend, leading to severe impact on quality of life, health, and huge burden to society and family^{4,5}.

L-3-n-butylphthalide (L-NBP) is a type of anti-ischemic cranial nerve protective drug commonly used in clinic^{6,7}. It plays its effect through increasing the blood supply, improving microcirculation in ischemia area, alleviating cerebral edema, perfecting energy metabolism, protecting mitochondrial function, inhibiting glutamate release, preventing platelet accumulation and thrombosis^{8,9}. Phosphatidylinositol-3 kinase (PI3K) is an important member of growth factor receptor superfamily that can be activated by the stimulus of cytokines and mitogen.

It participates in the regulation of cell proliferation, cell cycle, and apoptosis by activating downstream protein kinase B (PKB/AKT)^{10,11}. PI3K/AKT signaling pathway is a classical pathway in antagonizing apoptosis and promoting survival that exists in various tissues and cells. Scholars¹²⁻¹⁴ revealed that PI3K/AKT signaling pathway plays an important regulatory role in up-regulating B-cell lymphoma-2 (Bcl-2) expression, alleviating cell injury after ischemia and hypoxia, antagonizing apoptosis, and facilitating cell survival. However, it is still unclear about the role of L-NBP on VD mouse model and related mechanism. This study established VD mouse model and observed the impact of L-NBP on neurological function, cell apoptosis, and PI3K/AKT signaling pathway to evaluate the potential mechanism.

Materials and Methods

Main Reagents and Materials

Male C57BL/6 mice at 8 weeks old and weighted 22-25 g were purchased from Victor River (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco BRL Co. Ltd. (Grand Island, NY, USA). Dispase was derived from Roche Pharmaceutical (Basel, Switzerland). Rabbit anti-mouse Bcl-2 antibody was got from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-mouse AKT, phosphorylated AKT (p-AKT), β -actin, and horseradish peroxidase (HRP) conjugated secondary antibodies were provided by Abcam Biotechnology (Cambridge, MA, USA). radioimmunoprecipitation assay (RIPA), transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) apoptosis detection kit, and Annexin V/PI kit were provided by Beyotime (Haimen, China). DCFH-DA probe was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse hippocampus neuron HT22 cells were bought from Yanyu Chemical Reagent co., Ltd (Shanghai, China). PI3K/AKT specific inhibitor LY294002 was synthesized by MedChem-Express (Monmouth Junction, NJ, USA).

Mice were used for all the experiments, and all procedures were approved by the Animal Ethics Committee of The Affiliated Longyan First Hospital of Fujian Medical University (Fujian, China).

VD Mouse Model Establishment

The mice were raised at free eating and drinking, 12 h day/night cycle, temperature 22-25°C, and relative humidity 50%-60%. The experiment began after 1 week's adaptive breeding. The mouse was anesthetized by 10% chloral hydrate abdominal injection and fixed on operating table. Then, the neck skin was disinfected and the incision was made on neck midcourt line. The muscle and connective tissue were separated to isolate bilateral common carotid artery for ligation. The vagus nerve was protected to avoid damage. A small incision was made on the common carotid artery to put the occlusion into the starting of the middle cerebral artery. Next, the occlusion was fixed and the skin was closed. After 2 h, the occlusion was removed upon diethyl ether rapid anesthesia. Antibiotics were used to prevent infection. The mice in the sham group received the same treatment without bilateral common carotid artery ligation. A total of 10 mice were selected as control.

VD was assessed by Zea Longa scoring. Score 0, normal activity without neurologic deficits; score 1, the left forepaw cannot be fully extended; score 2, turn left during walking; score 3, left-leaning during walking; score 4, loss of consciousness without spontaneous walking. The mice in score 1-3 were considered as successful modeling. The mice in score 0 and 4 were excluded from the experiment. A total of 30 mice were successfully established as VD model.

On the 21st day after modeling, the mice were killed and the hippocampus tissue was collected. The sample was embedded by paraffin, used for protein extraction, and digested for reactive oxygen species (ROS) content detection.

Experimental Animal Grouping and Intervention

The VD mice were randomly equally divided into three groups, including single VD group with normal feeding without other treatment after modeling, solvent group with corn oil gavage after modeling at 15 mg/kg/d for 20 days, and L-NBP group with L-NBP gavage after modeling at 15 mg/kg/d for 20 days.

Morris Water Maze Test

On the 21st day after modeling, the mice received Morris water maze test. Morris water maze device is a cylindrical tank at diameter 120 cm and height 50 cm. The tank was divided into four quadrants on the bottom. A transparent

organic glass cylindrical platform at diameter 12 cm and height 30 cm was put at the centre of a quadrant. The video camera was used to record the movement locus and search the incubation period of the platform. The water was added to the tank and stained by ink for non-transparent. The water surface was about 2 cm over the platform surface and the water temperature was maintained at 25°C. The mice swam for 2 min on the day before the experiment to adapt to the environment. The mouse was trained for 4 times each day from the first day. The roadmap and time needed for the mouse to search and climb up to the platform were recorded. The mouse was guided to the platform once it cannot find the platform within 120 s. The time interval between each time of training was 60 s. The experiment was repeated after 24 h to reflect the memory retention.

TUNEL Assay

The hippocampus tissue slice was dewaxed by xylene for 5-10 min, absolute ethyl alcohol for 5 min, 90% ethyl alcohol for 2 min, 70% ethyl alcohol for 2 min, and distilled water for 2 min. Then, the slice was added with 20 µg/ml protease K without DNase and incubated at 37°C for 20 min. Next, the slice was treated by 50 µl TUNEL detection liquid composed of 5 µl TdT enzyme and 45 µl fluorescence liquid at 37°C avoid of light for 60 min. After washed by phosphate buffered saline (PBS) three times and sealed, the slice was observed under the microscope to calculate the apoptotic cell ratio.

Hippocampal Neuron Cell Ischemia-Reperfusion (I/R) Treatment In Vitro

HT22 cells were cultured in low glucose DMEM without FBS to stimulate ischemia condition and maintained at 5% CO₂ and 95% N₂ to stimulate an anaerobic environment *in vivo*. After 12 h, the cells were changed to routine medium containing FBS and cultured at 5% CO₂ and 95% air for 12 h to stimulate reperfusion.

HT22 cells were divided into four groups, including single I/R group treated by I/R; L-NBP group treated by I/R after 12 h pre-treatment of 10 µg/ml L-NBP; solvent group treated by I/R after 12 h pre-treatment of corn oil; and combined treatment group treated by I/R after 12 h pre-treatment of 10 µg/ml L-NBP and 10 µM LY294002. The cells were collected for protein, apoptosis, and ROS content detection.

Cell Apoptosis Detection

The cells were re-suspended in 500 µl binding buffer and incubated in 5 µl Annexin V-FITC avoid of light for 15 min. Next, the cells were added with 5 µl propidium iodide (PI) and tested on Beckmann CytoFLEX flow cytometry to evaluate cell apoptosis.

ROS Content Detection

The cells were digested by trypsin and incubated in 0.1% DCFH-DA probe diluted in serum-free medium at 37°C for 20 min. After washed by serum-free medium for three times, the cells were detected on Beckmann CytoFLEX to assess ROS content.

Western Blot

Total protein was extracted by RIPA from cells or tissues for 20 min and centrifuged at 10000 ×g for 10 min. A total of 40 µg protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Next, the membrane was blocked and incubated in primary antibody at 4°C overnight (AKT, p-AKT, Bcl-2, and β-actin at 1:3000, 1:1000, 1:2000, and 1:10000, respectively). Then, the membrane was incubated in HRP labeled secondary antibody (1:30000) for 60 min after washed by PBST for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among the groups. *p* < 0.05 was considered statistically significant.

Results

Mouse Learning and Memory Functions Deteriorated in VD Model

The mice in sham group exhibited good mental state, normal activity, food, and drink, and rapid movement. The mice in VD model group presented lack of mental state, lethargy, drowsiness, decreased activity, feeding, and drinking, accompa-

Table I. Mouse learning and memory functions comparison.

Group	Cases	Learning ability		Memory ability	
		Escape latent time (s)	Mistake times	Platform stay time (s)	Swimming speed (cm/s)
Sham	10	46.3 ± 3.9	14.2 ± 1.3	34.6 ± 2.9	19.1 ± 1.7
VD	10	87.6 ± 5.8*	37.2 ± 2.9*	18.2 ± 1.4*	10.3 ± 1.2*
VD + solvent	10	89.2 ± 6.1*	36.9 ± 2.7*	17.3 ± 1.5*	9.6 ± 1.2*
VD + L-NBP	10	73.3 ± 5.5*#	22.2 ± 1.8*#	28.3 ± 2.3*#	14.8 ± 1.8*#

* $p < 0.05$, compared with Sham group, # $p < 0.05$, compared with VD + solvent.

nied by weight loss, irritability, excitement, and ataxia symptoms. Compared with sham group, the escape latent time significantly prolonged and mistake times evidently increased, while platform stay time and swimming speed markedly reduced in VD model group ($p < 0.05$). L-NBP gavage apparently improved mouse learning and memory functions, resulting in the decrease of escape latent time and mistake times, and elevated platform stay time and swimming speed ($p < 0.05$). The corn oil gavage presented no statistical impact on mouse learning and memory functions (Table I).

L-NBP Reduced Hippocampal Cell Apoptosis Through Up-Regulating PI3K/AKT Signaling Pathway Activity and Bcl-2 Expression

Flow cytometry detection showed that ROS content significantly increased in hippocampus tissue from VD group compared with sham group, while L-NBP gavage markedly declined ROS content. Solvent gavage failed to affect ROS

content compared with VD group (Figure 1A). TUNEL assay revealed that cell apoptosis markedly enhanced in hippocampus tissue from VD compared with sham group, whereas L-NBP gavage apparently alleviated cell apoptosis. Solvent gavage exhibited no significant influence on cell apoptosis compared with VD group (Figure 1B). Western blot detection demonstrated that p-AKT and Bcl-2 protein significantly down-regulated in hippocampus tissue in VD group than that in Sham group, while L-NBP gavage markedly enhanced p-AKT and Bcl-2 protein levels. Solvent gavage did not impact p-AKT and Bcl-2 protein expressions in hippocampus tissue (Figure 1C).

I/R Treatment Induced Hippocampal Neuron Apoptosis and Downregulated PI3K/AKT Signaling Pathway Activity and Bcl-2 Expression

Flow cytometry demonstrated that ROS content significantly increased and cell apoptosis enhanced in HT22 cells treated by I/R (Figure 2A and B). Western blot showed that p-AKT and Bcl-

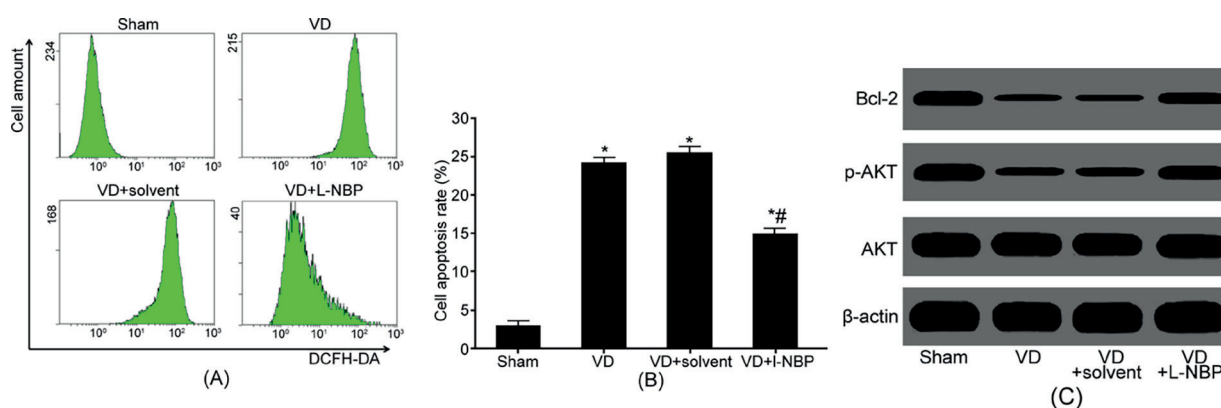


Figure 1. L-NBP reduced hippocampal cell apoptosis through upregulating PI3K/AKT signaling pathway activity and Bcl-2 expression. **A**, Flow cytometry detection of ROS content. **B**, TUNEL assay detection of cell apoptosis. **C**, Western blot detection of protein expression. * $p < 0.05$, compared with Sham group, # $p < 0.05$, compared with VD + solvent.

2 protein contents significantly reduced in HT22 cells treated by I/R (Figure 2C).

L-NBP Antagonized TH22 Cell Apoptosis Induced by I/R Through Enhancing PI3K/AKT Signaling Pathway

Flow cytometry presented that L-NBP pretreatment markedly declined ROS production and cell apoptosis in HT22 cells induced by I/R, while the solvent control failed to show the statistical impact (Figure 3A, B). The LY294002 intervention significantly increased ROS content and cell apoptosis in HT22 cells compared with L-NBP pretreatment group (Figure 3A, B). Western blot showed that L-NBP pretreatment significantly up-regulated p-AKT and Bcl-2 protein contents in HT22 cells induced by I/R, whereas LY294002 intervention markedly alleviated the influence of L-NBP on p-AKT and Bcl-2 expressions (Figure 3C).

Discussion

The major pathogenesis of VD is a cerebrovascular lesion, of which ischemic cerebrovascular disease accounts for the leading morbidity¹⁵. At present, the incidence of VD is about 2-7%, while it is up to 6-12% in the population older than 70. There are more than 18 million VD patients around the world, which is estimated at over 30 million till 2020⁴.

L-NBP is a yellow oily liquid monomers compound extracted from celery seed by the Institute of Chinese Academy of Medical Sciences. Its optical isomers include L-NBP, dextral butylphthalide (D-NBP), and despun butylphthalide (LD-NBP), of which L-NBP shows the strongest efficacy. The existence of D-NBP exhibits an antagonism effect on the pharmacological effect of L-NBP⁸. As an anti-ischemic cranial nerve protective drug commonly used in clinic, L-NBP is a type of new drug with independent intellectual property rights in China¹⁶. L-NBP plays its impact on protecting cranial nerve function after ischemia by increasing blood supply, improving the microcirculation in ischemic area, shrinking cerebral infarction area, alleviating cerebral edema, improving energy metabolism, reducing cell apoptosis, promoting cell survival, decreasing intracellular calcium concentration, protecting mitochondrial function, inhibiting oxygen free radical generation, elevating anti-oxidant enzyme activity, suppressing glutamate

release, and preventing platelet aggregation and thrombosis^{8,9}.

PI3K/AKT signaling pathway widely exists in multiple tissues and cells. Under the stimulus of growth factor or mitogen, PI3K can be activated through conformation changes and translate phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 can phosphorylate AKT protein at Ser473 and Thr308 loci under the assistant of 3-phospho-

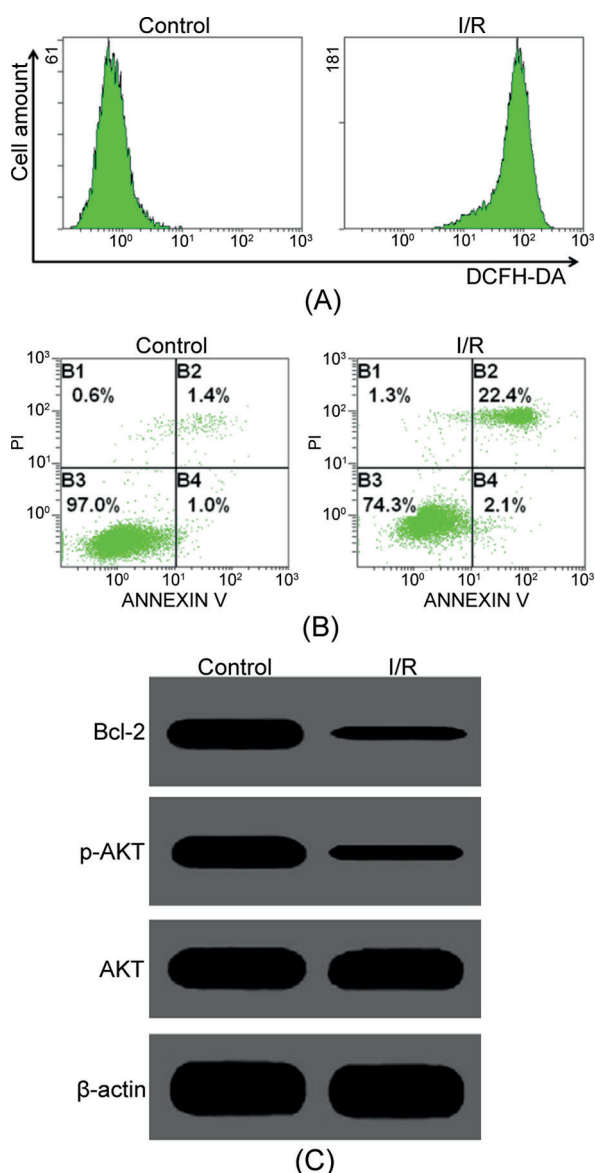


Figure 2. I/R treatment induced hippocampal neuron apoptosis and downregulated PI3K/AKT signaling pathway activity and Bcl-2 expression. **A**, Flow cytometry detection of ROS content. **B**, Flow cytometry detection of cell apoptosis. **C**, Western blot detection of protein expression.

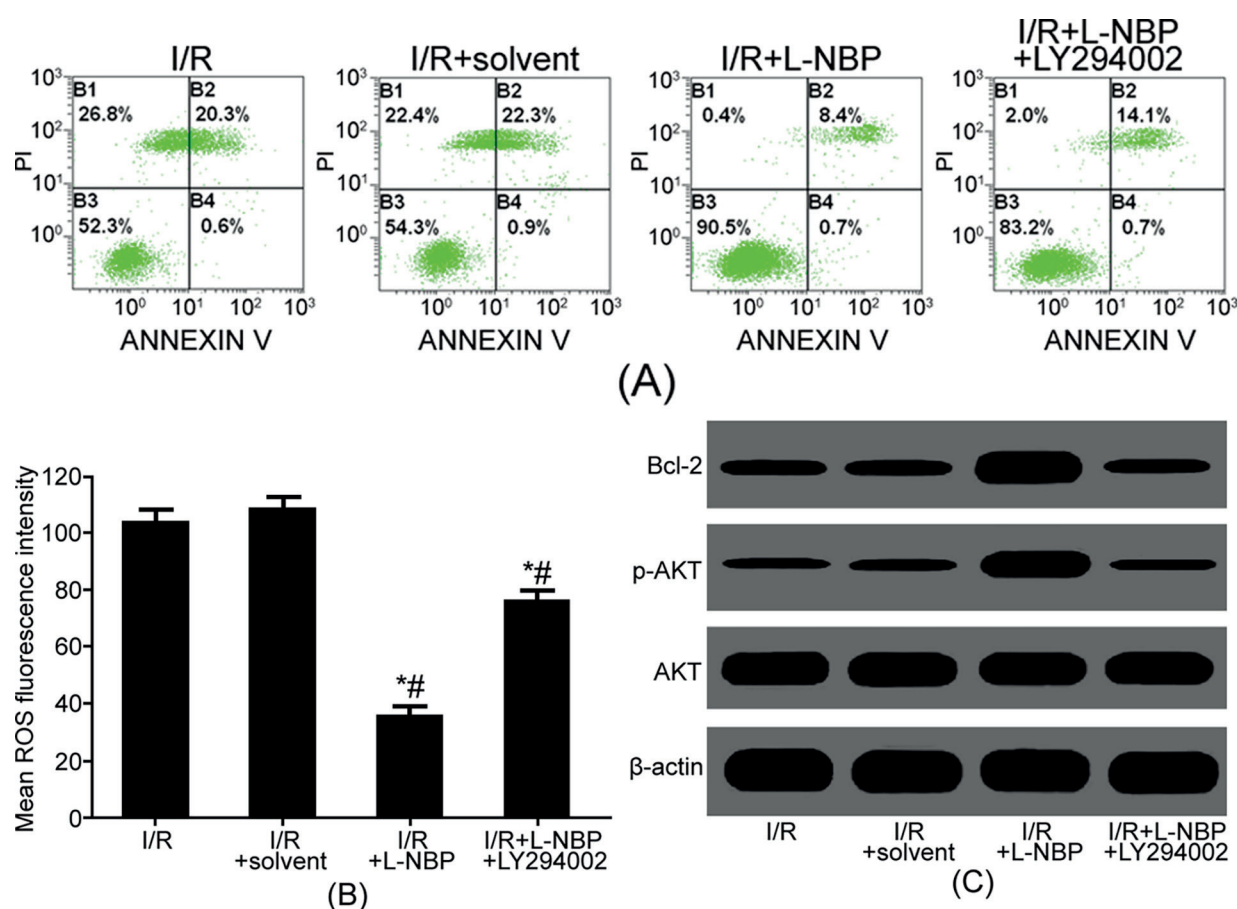


Figure 3. L-NBP antagonized TH22 cell apoptosis induced by I/R through enhancing PI3K/AKT signaling pathway. **A**, Flow cytometry detection of cell apoptosis. **B**, Flow cytometry detection of ROS content. **C**, Western blot detection of protein expression. ^{*} $p < 0.05$, compared with Sham group, [#] $p < 0.05$, compared with VD + solvent.

inositide-dependent protein kinase-1 (PDK1) and PDK2. Phosphorylated AKT controls various target genes transcription and translation, thus regulates cell survival, proliferation, and apoptosis. PI3K/AKT signaling pathway is a classic pathway in antagonizing cell apoptosis and promoting cell survival. PI3K/AKT signaling pathway plays a protective role against tissue and cell ischemia and anaerobic injury, including heart¹⁷, brain¹⁸, lung¹⁹, and kidney²⁰. Bcl-2 is an important anti-apoptotic factor affecting mitochondrial function, restraining Cyt C release, impacting calcium ion transmembrane transport, and inhibiting apoptotic protease activating factor-1 (Apaf-1) activation²¹. Bcl-2 also plays a crucial role in suppressing ROS production and anti-oxidative stress²². Numerous studies¹²⁻¹⁴ demonstrated that Bcl-2 is a key target gene of PI3K/AKT signaling pathway to play a role in anti-oxidation, antagonizing cell apoptosis, and promoting cell survival.

Currently, the role of L-NBP on VD mouse model and related mechanism is still unclear. This study established VD mouse model and observed the impact of L-NBP on neurological function, cell apoptosis, and PI3K/AKT signaling pathway to evaluate the potential mechanism.

This investigation showed that compared with sham group, the escape latent time significantly prolonged and mistake times markedly increased, while platform stay time and swimming speed markedly reduced in VD model group. L-NBP gavage apparently improved mouse learning and memory functions, resulting in the decrease of escape latent time and mistake times, and elevated platform stay time and swimming speed. It suggested that L-NBP markedly improved the neurologic function of VD mouse and enhanced learning and memory abilities. Xiang et al¹⁶ reported that L-NBP treatment significantly improved the learning and cognitive function of

APP/PS1 dual-transgenic dementia mouse. Yang et al²³ revealed that L-NBP gavage remarkably improved the neurological function of cerebral ischemia mouse. This study also observed the improvement effect of L-NBP on the neurological function of dementia mouse, which was similar with Xiang et al¹⁶ and Yang et al²³. Cerebral ischemia can induce cerebral infarction or selectively cerebral neuron death. It may cause VD when the ischemia appeared in the region related to learning and memory. Hippocampus is the key for learning and memory, and is also sensitive to ischemia and anoxia. Therefore, this study investigated the impact of L-NBP on the hippocampus of the mouse model. It exhibited that L-NBP gavage significantly alleviated ROS production, reduced hippocampal cell apoptosis, and up-regulated p-AKT and Bcl-2 expressions in VD mouse. Yang et al²³ presented that L-NBP gavage significantly down-regulated cleaved caspase-3 and Bcl-2 associated X protein (Bax) expressions, promoted neuron regeneration, and improved neurological function in the cerebral ischemic rat. Xiang et al¹⁶ found that L-NBP treatment markedly enhanced PI3K/AKT pathway activity in cerebral tissue and improved cognitive and memory functions in APP/PS1 dual-transgenic dementia mouse. Huai et al²⁴ demonstrated that L-NBP apparently improved learning and memory functions, and elevated p-AKT expression in hippocampus tissue from VD mouse. We observed that PI3K/AKT pathway attenuation and Bcl-2 down-regulation play a regulatory role in hippocampal cell apoptosis, ROS production, and the pathogenesis of VD. L-NBP intervention alleviated cell apoptosis and ROS production through elevating PI3K/AKT signaling pathway activity and Bcl-2 expression, which was in accordance with the reports of Yang et al²³, Xiang et al¹⁶, and Huai et al²⁴. We also found that I/R treatment significantly induced HT22 cell apoptosis and ROS production, and reduced p-AKT and Bcl-2 expressions. L-NBP pretreatment markedly enhanced PI3K/AKT pathway activity and Bcl-2 expression, alleviated cell apoptosis, and antagonized ROS production. L-NBP combined PI3K/AKT specific inhibitor LY294002 significantly down-regulated p-AKT and Bcl-2 expressions and attenuated the apoptosis protective and anti-oxidative effects on hippocampal neuron cells. It suggested that L-NBP plays apoptosis protective and anti-oxidative effects on hippocampal neuron cells through affecting PI3K/AKT pathway activity

and Bcl-2 expression. Lei et al²⁵ demonstrated that L-NBP protected nerve cell apoptosis and injury induced by Alzheimer's disease pathogenic factor Abeta25-35 through upregulating Bcl-2 expression, which was in accordance with our results. Peng et al⁹ also reported that L-NBP has a protective effect on nerve cell apoptosis induced by Abeta25-35. Both the reports of Xiang et al¹⁶ and Huai et al²⁴ showed that L-NBP improved neurological function and upregulated PI3K/AKT signaling pathway in dementia mouse. This work showed that L-NBP regulates PI3K/AKT pathway activity and neuron cell apoptosis in HT22 cells induced by I/R, while LY294002 effectively attenuated the protective impact of L-NBP on hippocampus neuron cells, thus directly confirming the role of L-NBP on VD. Except for PI3K/AKT signaling pathway, other pathways, such as ERK/MAPK, also play crucial regulatory roles in cell proliferation, survival, and apoptosis, and are related to Alzheimer's disease and VD. However, whether L-NBP may protect neuron through affecting ERK/MAPK signaling pathway still needs further exploration.

Conclusions

We found that PI3K/AKT signaling pathway down-regulation plays a role in neuron cell apoptosis and VD pathogenesis. L-NBP protects VD by up-regulating PI3K/AKT signaling pathway, elevating Bcl-2 expression, reducing nerve cell apoptosis, and restraining ROS production.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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