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3-n-butylphthalide inhibits the apoptosis of nerve cells in rats with cerebral small vessel disease *via* the PI3K/Akt pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of 3-n-butylphthalide (NBP) on the apoptosis of nerve cells in vascular dementia (VaD) model rats caused by cerebral small vessel disease (CSVD), and to explore its regulatory mechanism.

MATERIALS AND METHODS: The model of VaD was successfully established in rats by carotid artery ligation. All rats were randomly divided into three groups, including the sham operation group, model group and NBP group. The neurobehavioral score was used to verify whether the model was successfully established. The changes in learning and memory abilities of rats were detected via water maze experiment. The levels of Bcl-2-associated X protein (Bax) and cysteinyl aspartate specific proteinase-3 (Caspase-3) in the serum of rats was detected by enzyme-linked immunosorbent assay (ELISA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was adopted to detect the apoptosis of nerve cells in brain tissues of rats. Moreover, the protein levels of phosphorylated phosphatidylinositol-3-kinase (PI3K) and phosphorylated protein kinase B (Akt) in brain tissues of rats were measured using Western blotting.

RESULTS: Compared with the sham operation group, the neurobehavioral score of rats increased significantly, whereas learning and memory abilities decreased markedly in the model group. The levels of Bax and Caspase-3 in rat serum were remarkably up-regulated, and the apoptosis rate of nerve cells in brain tissues of rats increased significantly in the model group as well. Meanwhile, the levels of phosphorylated PI3K and phosphorylated Akt were notably declined. Compared with the model group, the neurobehavioral score decreased markedly, while learning and memory abilities were remarkably improved in the NBP group. The levels of Bax and Caspase-3 in rat serum were significantly down-regulated, and the apoptosis rate of nerve cells in brain tissues of rats were reduced in the NBP group. Furthermore, the protein levels of phosphorylated PI3K and phosphorylated Akt were remarkably elevated in the NBP group.

CONCLUSIONS: NBP can improve the morphology of brain tissue cells and the learning and memory abilities, and inhibit the apoptosis of nerve cells in VaD model rats with CSVD. The possible underlying mechanism may be related to the activation of the PI3K/Akt signaling pathway.

Key Words:
Cerebral small vessel disease (CSVD), 3-n-butylphthalide, PI3K/Akt signaling pathway, Nerve cell, Apoptosis.

Introduction

Cerebral small vessels (CSVs) refer to small perforating arteries and arterioles, capillaries and venules of the brain. Previous studies have found that they play important roles in maintaining brain function and morphology. CSV disease (CSVD) refers to various pathological changes in CSVs, which is one of the important types of cerebrovascular diseases[1]. The pathological manifestations of CSVD include cellulose degeneration, amyloidosis, hemorrhage, occlusion and other changes. Clinically, it is characterized by stroke, cognitive dysfunction, affective dysfunction, etc. Meanwhile, the Imaging manifestations of CSVD are lacunar infarction, white matter lesions, perivascular space enlargement and intracerebral hemorrhage[2,3]. With the improvement of modern scientific and technological means, researchers have formed a better understanding of the possi-

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ble pathogenesis of CSVD. However, the pathogenesis of CSVD has not been fully elucidated. Currently, its main pathogenesis theory involves endothelial dysfunction, blood-brain barrier injury and ischemia and hypoperfusion injury[4]. The clinical manifestations of patients with CSVD are decreased consciousness function, sluggish mobility and decreased or lost self-care ability. This may eventually bring inconvenience to patients' daily life, exert economic pressure to patients' families, and impose serious burdens to the society[5]. According to different pathological conditions of blood vessels, CSVD can be divided into six categories, namely, arteriosclerotic disease, amyloid angiopathy, white matter disease, SVD with inflammation and immune dysfunction, collagen vascular disease and other SVDs[6].

Vascular dementia (VaD) caused by CVSD is a progressive cognitive dysfunction syndrome resulted from CVS injury. The incidence rate of VaD is as high as about 20% in European and American countries. Meanwhile, the incidence of VaD is significantly higher than that of Alzheimer's disease in developing countries[7]. In China, due to the extremely accelerated aging, the prevalence rate of VaD in middle-aged and elderly people has risen rapidly, showing a younger trend. Clinically, VaD is mainly manifested as memory and cognitive dysfunction. In current years, there is no effective drug to cure the disease. It is worth noting that VaD, different from Alzheimer's disease, is a disease that can be prevented, delayed or reversed. Therefore, researchers should focus more on the prevention and treatment of VaD[8,9].

3-n-butylphthalide (NBP), also known as apigenin, has a chemical structural formula of $C_{12}H_{14}O_2$. The relative molecular mass of NBP is 190, with three structures. It was developed by the Institute of Medicine, Chinese Academy of Medical Sciences. Previous studies have demonstrated that it exerts a significant therapeutic effect on acute ischemic stroke. Clinical data have shown that NBP is safe and effective for treatment[10]. Numerous studies have revealed that NBP has a multi-target anti-cerebral ischemia effect. It can improve local cerebral blood flow in ischemic areas, reduce cerebral infarction area, enhance mitochondrial function, inhibit the generation of free radicals, inhibit inflammatory reactions, reduce cerebral edema caused by cerebral ischemia, and resist thrombosis and platelet aggregation. In addition, studies have proved that NBP can inhibit nerve cell apoptosis, as well as protect nerve cells from injury, neurological deficits and cerebral ischemia and memory impairment caused by ischemia and hypoxia[11]. However, few reports have elucidated the role of NBP in the treatment of VaD model rats. Therefore, we first established the model of VaD in rats using common carotid artery ligation method. The aim of this study was to investigate the regulatory effect of NBP on nerve cell apoptosis and its mechanism.

Materials and Methods

Reagents

Enzyme-linked immunosorbent assay (ELISA) kit was purchased from Nanjing KeyGEN BioTech Co., Ltd. (Nanjing, China), radioimmunoprecipitation assay (RIPA) lysate and the terminal deoxynucleotidyl transferase dUTP nick end the labeling (TUNEL) kit from Beijing Solarbio Life Science Co., Ltd. (Beijing, China), and phosphorylated phosphatidylinositol-3-kinase (PI3K), phosphorylated protein kinase B (Akt) and β-actin primary antibodies, and horseradish peroxidase (HRP) secondary antibodies from Cell Signaling Technology (Danvers, MA, USA).

Instruments

Microplate reader and electrophoresis instrument were purchased from Bio-Rad (Hercules, CA, USA), low-speed centrifuge and pipette from Eppendorf (Hamburg, Germany), ultraviolet spectrophotometer from Beckman (Miami, FL, USA), inverted fluorescence microscope from Nikon (Tokyo, Japan), ultra-low temperature refrigerator from Qingdao Haier Co., Ltd. (Qingdao, China), ultraviolet spectrophotometer from Shanghai Metash Instrument Co., Ltd. (Shanghai, China), and water maze from Beijing ZS Dichuang Technology Development Co., Ltd. (Beijing, China).

Animals

Clean Sprague Dawley (SD) male rats, weighing (200 ± 10) g, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). This study was approved by the Animal Ethics Committee of Tangshan Gongren Hospital Animal Center. All rats were raised in daylight for 12 h, with free access to water and food.

Preparation of the Model of VaD in Rats

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate, and fixed on

a fixed plate in the supine position. After shaving the neck hair, the middle cervical was cut with scissors. Then bilateral common carotid arteries were separated and clamped with arterial clamps. Subsequently, the blood flow was blocked for 20 min, followed by bloodletting from the tail. The arterial clamps were released, and the blood flow was restored for 10 min. Next, the arterials were clamped with arterial clamps again to block the blood flow for 20 min. The whole process was repeated 3 times, and the respiration and heartbeat of rats were observed. If the above indexes were normal, the skin was sutured after disinfection with gentamicin, and the temperature was preserved. After fully recovering, the rats were put back into cages for feeding.

Detection of Learning and Memory Abilities of the Rats via Water Maze Experiments

The rats were placed into water with their heads toward the pool wall, and the placement position was random. The time (s) when the rats found the underwater platform was recorded. In previous trainings, if the time to find the platform exceeded 60 s, the rats were trained to stay on the platform for 10 s. After the last training, the platform was removed and the rats were placed in the opposite quadrant of the original platform. The number of times crossing the original platform and the residence time was recorded.

Detection of the Levels of Bcl-2-Associated X Protein (Bax) and Caspase-3 in Rat Serum via ELISA

According to the instructions of the kit, 100 μL of sample diluent and reference substance were added to each well, followed by incubation in an incubator at 37°C for 1.5 h. Subsequently, 100 μL biotin-labeled antibody working solution was added to each well for 1 h of incubation at 37°C. After that, 100 μL of the avidin-HRP-labeled working solution was added to each well for incubation at 37°C for 30 min. After washing with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) three times, TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution (Thermo Fisher Scientific, Waltham, MA, USA) was added to terminate the reaction. The absorbance at λ=450 nm was measured using a microplate reader. Finally, the levels of Bax and Caspase-3 were calculated according to the calculation formula.

Detection of Nerve Cell Apoptosis in the Brain Tissues of Rats via TUNEL Assay

Brain tissue sections were fixed with 4% paraformaldehyde, washed with PBS 3 times and added with 50 μL TdT enzyme reaction solution dropwise. Subsequently, 50 μL streptavidin-TRITC labeling solution was added dropwise for 30 min of incubation in the dark. After washing with PBS 3 times, the nucleus was restained with 4',6-diamidino-2-phenylindole (DAPI) staining solution (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation at room temperature for 15 min. Finally, the staining was observed under a microscope.

Detection of the Levels of Phosphorylated PI3K and Phosphorylated Akt in Brain Tissues of Rats via Western Blotting

Brain tissues of rats were first extracted and lysed with 1× RIPA lysate. After centrifugation, the supernatant was collected, and the concentration of extracted protein was determined by the bicinchoninic acid (BCA) assay (Pierce, Waltham, MA, USA). 20 μg of sample proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under 100 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 5% bovine serum albumin (BSA) solution for 1 h, the membranes were incubated with primary antibodies of Bax, Caspase-3 and β-actin at 4°C overnight. On the next day, the membranes were washed with Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) solution and incubated with corresponding secondary antibodies. Diaminobenzidine (DAB) color developing solution (Solarbio, Beijing, China) was used for color development. Finally, the gray values of bands were statistically analyzed using Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used in all statistical analysis. Experimental data were expressed as mean ± standard deviation. The homogeneity of variance was firstly conducted, followed by *t*-test to compare the difference between the two groups. One-way ANOVA test was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Table I. Comparison of the neurobehavioral score of the rats in each group.

Group	Neurobehavioral score (point)
Sham operation group	0.00 ± 0.00
Model group	2.98 ± 0.65*
NBP group	2.14 ± 0.35#

Note: *p<0.05: model group *vs.* sham operation group. *p<0.05: NBP group *vs.* model group.

Results

Successful Establishment of VaD Model in Rats

On the 5th day after model preparation, the neurobehavioral score of rats in each group was compared, and the results were shown in Table I. Compared with the sham operation group, the neurobehavioral score of rats in the model group increased significantly ($^{*}p$<0.05). Compared with that in the model group, the neurobehavioral score of rats in the NBP group decreased markedly ($^{\#}p$<0.05). The above results indicated that the model of VaD was successfully established in rats, and NBP could reduce its score.

NBP Could Improve the Learning and Memory Abilities of Rats with VaD

The water maze trajectory maps of rats in each group were shown in Figure 1A, and the data were analyzed. As shown in Figure 1B and 1C, compared with the sham operation group, the number of times the rats crossed the quadrant where the original platform was located and the residence time were significantly reduced in the model group ($^{*}p$<0.05, $^{*}p$<0.05). Meanwhile, the number of times the rats crossed the quadrant where the original platform was located and the residence time increased markedly in the NBP group when compared with the model group ($^{\#}p$<0.05, $^{\#}p$<0.05).

NBP Could Inhibit the Levels of Bax and Caspase-3 in Serum of Rats with VaD

Compared with the sham operation group, the levels of Bax and Caspase-3 in the serum of rats in the model group were significantly up-regulated ($^{*}p$<0.05, $^{*}p$<0.05). Meanwhile, the levels of Bax and Caspase-3 in the serum of rats in the NBP group were remarkably lower than those of the model group ($^{\#}p$<0.05, $^{\#}p$<0.05; Figure 2A and 2B). The above results suggested that NBP could inhibit the expression of pro-apoptotic factors.

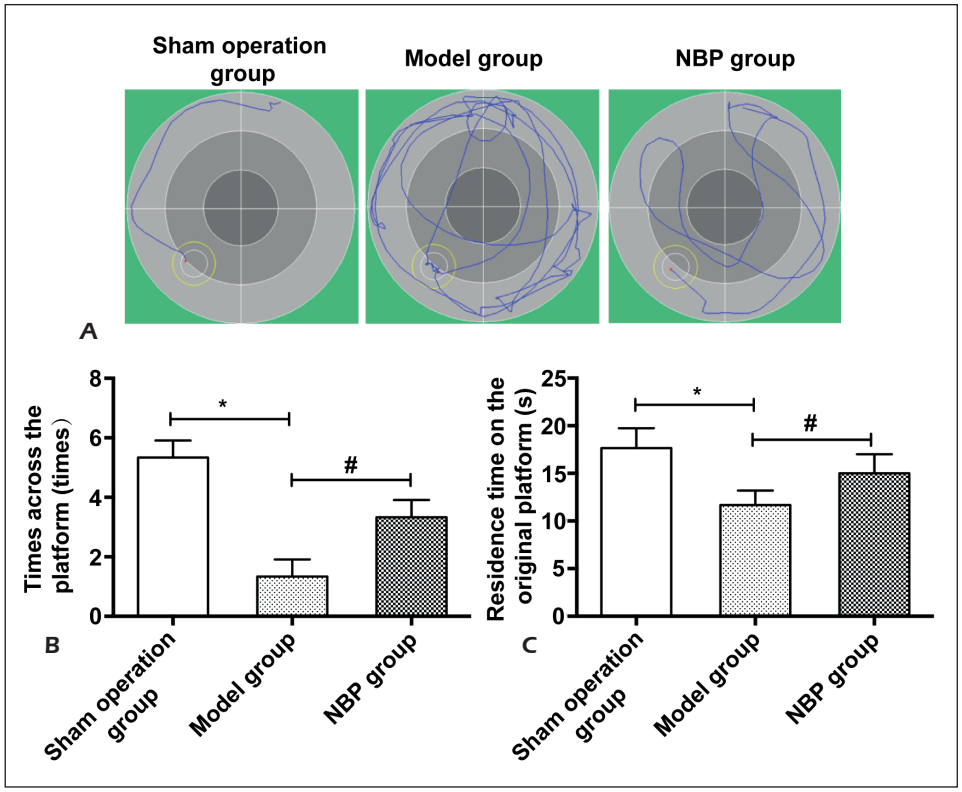

Figure 1. Water maze experimental results of rats in each group (*p<0.05, #p<0.05). **A**, Swimming trajectory maps of rats. **B**, Number of times the rats crossed the original platform. **C**, Residence time on the original platform.

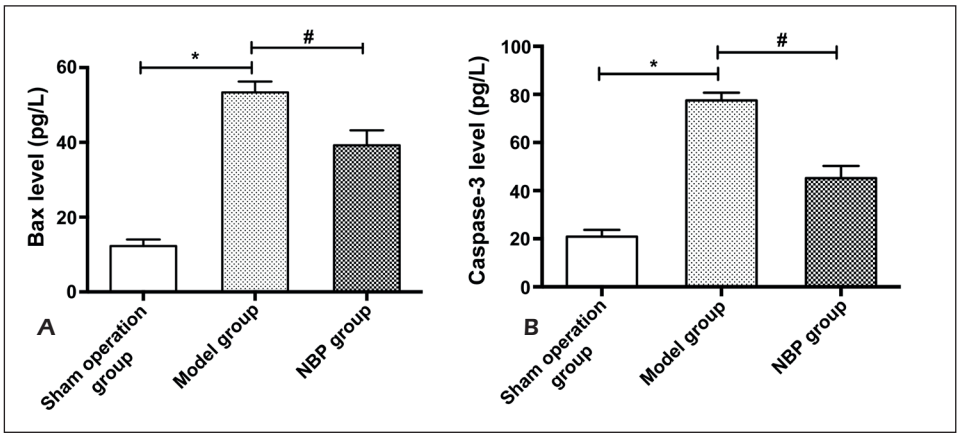

Figure 2. Levels of Bax and Caspase-3 in rat serum (*p<0.05, #p<0.05). **A**, Bax level in rat serum. **B**, Caspase-3 level in rat serum.

NBP Could Suppress Nerve Cell Apoptosis in Brain Tissues of Rats with VaD

TUNEL staining manifested that the apoptosis of nerve cells in brain tissues of rats in the model group increased significantly when compared with the sham operation group (*p<0.05; Figure 3A). Compared with the model group, the apoptosis of nerve cells in brain tissues of rats in the NBP group was evidently reduced (#p<0.05; Figure 3B). The above results indicated that NBP was capable of suppressing the apoptosis of nerve cells in brain tissues of rats with VaD.

NBP Could Increase the Levels of Phosphorylated PI3K and Phosphorylated Akt in Brain Tissues of Rats

According to the results of Western blotting, the protein levels of phosphorylated PI3K and phosphorylated Akt in brain tissues of rats in the model group were significantly down-regulated than the sham operation group, (*p<0.05; Figure 4A). Meanwhile, compared with the model group, the protein levels of phosphorylated PI3K and phosphorylated Akt in brain tissues of rats were significantly up-regulated in the NBP group (#p<0.05; Figure 4B). The above results suggested that NBP could inhibit the apoptosis of nerve cells in brain tissues of rats with VaD, and that its mechanism might be related to the activation of the PI3K/Akt signaling pathway.

Discussion

CSVD refers to the pathological changes in intracranial small vessels, including arterioles, arterioles, capillaries and venules. This may eventually lead to cerebral ischemia, stroke or hemorrhagic damage, as well as pathological changes such as cellulose degeneration, amyloidosis and hemorrhagic occlusion[12,13]. In China, the incidence rate of CSVD in the elderly over 60

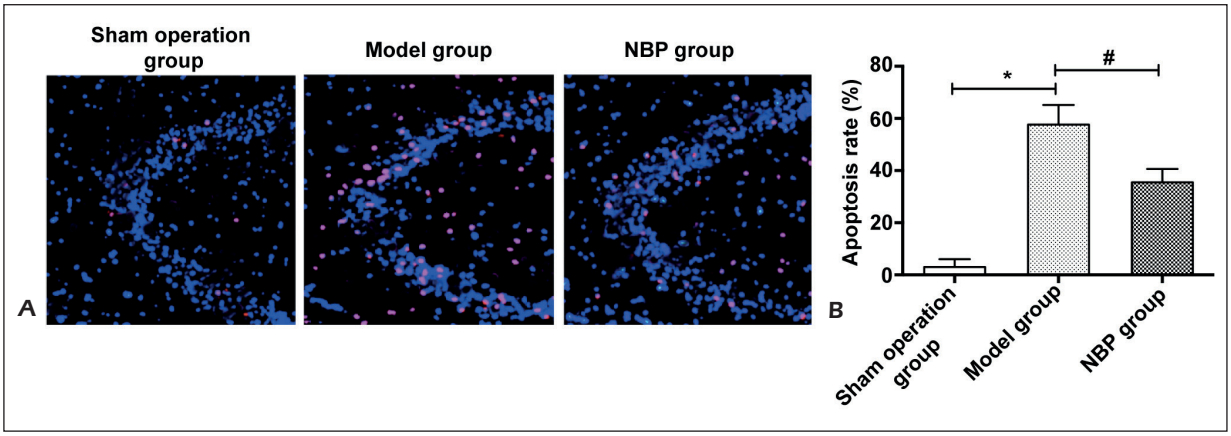

Figure 3. Apoptosis of nerve cells in brain tissues of rats in each group (*p<0.05, #p<0.05). **A**, TUNEL staining for apoptosis (Magnification × 20). **B**, Apoptosis rate in each group.

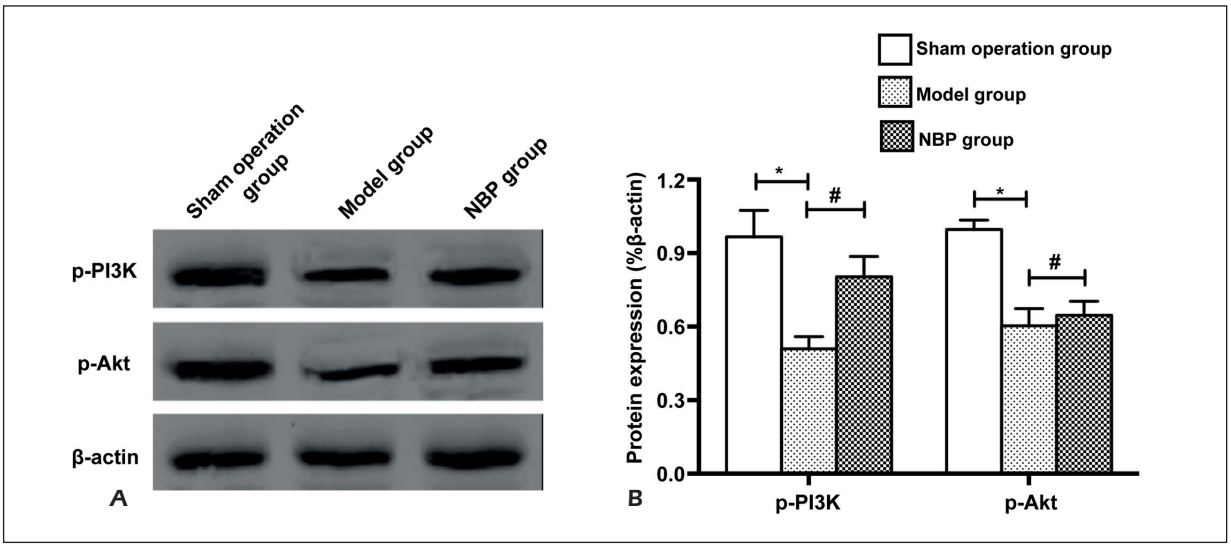

Figure 4. Levels of phosphorylated PI3K and phosphorylated Akt in the brain tissues of rats in each group (*p<0.05, #p<0.05). **A**, Western blotting, with β-actin as an internal reference. **B**, Statistical graphs of bands.

years old is as high as about 27%. Meanwhile, it has shown a straight upward trend compared with previous years. VaD is the only dementia disease that has been found preventable so far. Early detection and prevention of VaD can reduce social and family burdens and improve the life quality of patients[14]. Recent studies have found that nerve cell apoptosis plays an important role in the pathogenesis of VaD. Due to severe mitochondrial damage and free radical damage of nerve cells in brain tissues, a large number of neurons and glial cells in brain tissues rapidly die[15]. However, its pathogenesis still remains unclear.

Zhao et al[16] have found that ligustrazine exerts a neuroprotective effect on VaD model rats by regulating the expression of pro-apoptosis and anti-apoptosis factors. Yang et al[17] have indicated that the learning and memory abilities of rats with VaD after low-frequency repetitive cranial magnetic stimulation are significantly improved. Meanwhile, this stimulation can protect nerve cell synapse and their plasticity, and inhibit the apoptosis of nerve cells. The above results indicate that nerve cell apoptosis plays a key role in the pathogenesis and development of VaD. If nerve cell apoptosis is markedly inhibited, the development of VaD can be delayed or reversed. Xiang et al[18] have demonstrated that levo-NBP can improve the learning and memory abilities of APP/PS1 double transgenic mice, reduce the deposition of amyloid proteins in the brain tissues of mice, and reverse the memory damage. The possible underlying mechanism may be correlated with the activation of the BDNF/TrkB/PI3K/Akt signaling pathway.

NBP is a new drug indigenously developed in China for the treatment of cerebral ischemia injury. It can significantly promote the metabolism of brain tissues and reduce the area of brain death, showing a good protective effect on brain tissues. Meanwhile, NBP can inhibit the release of inflammatory factors, and has a regulatory effect on immune function. Compounds that modify the structure of NBP also exhibit good therapeutic effects in the treatment of CSVD. He et al[19] have indicated that DL-NBP shows neuroprotective and anti-inflammatory effects on traumatic spinal cord injury. Both *in vivo* and *in vitro* experiments have proved that DL-NBP can significantly enhance the activation of microglial cells and the release of inflammatory factors, improve the motor ability of spinal cord injury, and reduce the area of spinal cord injury in the lumen of model rats. Its mechanism may be related to the inhibition of the TLR4/NF-κB signaling pathway. Previous studies have revealed that DL-NBP has a broad therapeutic prospect in the treatment of stroke. Zhao et al[20] have discovered that DL-NBP shows a good therapeutic effect on traumatic brain injury. Meanwhile, experiments have verified that after treatment with DL-NBP, the level of apoptotic factors in the area around the injury is significantly reduced. The release of inflammatory factors such as interleukin-1β is markedly inhibited, and the apoptosis of injured peripheral nerve cells is suppressed. Moreover, DL-NBP can promote neurogenesis,

angiogenesis and cerebral artery generation, and up-regulate the generation of neurotrophic factors in the brain. Ultimately, this can significantly improve the motor function of acute traumatic brain injury. The above research results manifest that NBP has a good therapeutic effect in the treatment of CSVD. However, there are few reports on the effect of NBP in the treatment of VaD model rats.

In this study, we first established the rat model of VaD by common carotid artery ligation method. The results showed that NBP could reduce the neurobehavioral score. According to water maze experiments, NBP could remarkably increase the levels of Bax and Caspase-3 in the serum of VaD rats, and reduce the apoptosis rate of nerve cells in the brain tissues of rats. Further experiments found that NBP could notably increase the expressions of phosphorylated PI3K and phosphorylated Akt. The above results indicated that NBP was able to improve neurological function, enhance learning and memory abilities, reduce the expression of pro-apoptotic factors and inhibit nerve cell apoptosis in VaD rats. The possible underlying mechanism might be associated with the activation of the PI3K/Akt signaling pathway.

Conclusions

We revealed that NBP can improve brain tissue cell morphology and learning and memory abilities, and inhibit the apoptosis of nerve cells in VaD model rats with CSVD. In addition, its mechanism may be related to the activation of the PI3K/Akt signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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