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# Dl-3-n-butylphthalide regulates cholinergic dysfunction in chronic cerebral hypoperfusion rats

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## Abstract

**Objectives:** To investigate whether dl-3-n-butylphthalide (NBP) affects cholinergic system function and ameliorates cognitive decline in a rat model of vascular dementia (VaD).

**Methods:** The VaD rat model was established by bilateral common carotid artery ligation (two-vessel occlusion, 2VO). Rats were divided into five groups: control, sham, 2VO, 2VO+NBP (80 mg/kg; intragastric), and 2VO+donepezil (1 mg/kg; intragastric). Treatments were administered once daily for 2 weeks from day 21 post-surgery. Spatial learning and memory were evaluated by Morris water maze performance. Hippocampal choline acetyltransferase (ChAT), acetylcholinesterase (AChE), vesicular acetylcholine transporter (VAChT), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF) expressions were detected using immunohistochemistry, immunofluorescence, and real-time polymerase chain reaction methods.

**Results:** The daily escape latency was significantly longer in 2VO rats than in the sham or control groups, while the time spent in the target quadrant was significantly shorter. The daily escape latency of the 2VO+NBP group was significantly shorter compared with the 2VO group. Following NBP treatment, ChAT, AChE, VAChT, and BDNF expressions were significantly upregulated in the hippocampus.

**Conclusions:** Central cholinergic dysfunction may be involved in VaD pathogenesis. NBP treatment significantly improved spatial learning and memory in VaD rats, and may enhance cholinergic system function via BDNF-mediated neuroprotection.

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## Introduction

Vascular dementia (VaD) is the second most common cause of dementia, after Alzheimer's disease (AD).[1] With the aging of the global population and the increasing incidence of cerebrovascular disease, VaD incidence is also increasing rapidly.[2] The pathogenesis and treatment of VaD have gained research attention in the past decades. Dl-3-n-butylphthalide (NBP) is a compound that is extracted from the seeds of *Apium graveolens* Linn, or Chinese celery, and is widely used in the treatment of cerebrovascular disease. Some evidence suggests that NBP is a potential drug candidate for VaD treatment.

Many studies have suggested that AD and VaD have common risk factors, including cerebrovascular disease, hypertension, diabetes mellitus, coronary heart disease, and smoking.[3] Deficits in cholinergic neurotransmission have also been considered as a common pathogenic factor of AD and VaD.[4] Studies have reported that patients with VaD exhibit cholinergic deficiency,[5,6] including decreased levels of acetylcholine (ACh) and cholinergic markers, such as choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and vesicular acetylcholine transporter (VAChT), in the brain. One study reported a loss of cholinergic neurons in 40% of VaD patients, which was accompanied by reduced ACh in the cortex, hippocampus, striatum, and cerebrospinal fluid. Furthermore, cholinergic reductions are correlated with cognitive impairment in VaD.[7] The key enzymes in the cholinergic system maintain the dynamic balance of ACh, and they also maintain normal learning and memory in mammals. For example, ChAT is an important enzyme for the synthesis of ACh, and is also a marker of cholinergic neurons.[8] The distribution of ChAT is almost identical to that of ACh, so it can be used as an indirect indicator of cholinergic neurons. AChE is a biological enzyme that breaks down ACh released into the synapse, thereby inhibiting ACh function. The function of AChE, targeting ACh, is essential for the normal functioning of the nervous system.[9] A previous study reported that AChE-positive cells in the rat striatum were cholinergic neurons, while AChE-positive fiber terminals in the hippocampus were cholinergic terminals.[10] Because ACh is degraded rapidly by AChE, its direct detection is very difficult. However, most studies have shown that damage and repair of the cholinergic system can be indirectly evaluated by detecting the expression and activity of ChAT and AChE. Central cholinergic neurons are mainly distributed in regions sensitive to cerebral hypoperfusion, such as the hippocampus, striatum, and cortex. Neurons in the hippocampus and cerebral cortex, and especially in the hippocampal CA1 area, play an important role in learning and memory processes.[11]

A study of post-mortem brains revealed a significant reduction in ChAT activity in patients with VaD.[12] Similarly, significant reductions in AChE and ChAT activity were also found in the hippocampus of VaD animal models, and accompanied abnormalities in cholinergic neurons.[13]

VAChT regulates the storage and packaging of ACh in synaptic vesicles and plays an important role in learning, memory, and attention in VaD rats.[14] Kolisnyk et al.[15] demonstrated that a selective deletion of VAChT in the forebrain impairs hippocampal synaptic plasticity and induces deficits in executive function. Moreover, Prado et al.[16] reported that VAChT-deficient animals exhibit impairments in the acquisition and extinction of spatial memory in high-attention tasks. Furthermore, a previous study has revealed increased hippocampal expression of VAChT in AChE-inhibitor-treated spontaneously hypertensive rats (a VaD model), possibly because a more efficient storage mechanism was required for the increased level of acetylcholine at the synaptic cleft.[17] This previous study also demonstrated increased VAChT expression in the hippocampus, frontal cortex, and striatum of rivastigmine-treated VaD rats.

The most common pathogenesis of VaD is caused by cerebral ischemia. In addition, energy failure and subsequent events, including inflammation, calcium overload, glutamate-mediated excitotoxicity, oxidative stress, and structural or functional changes, can also cause VaD.[18] These factors might interact with one another to contribute to cellular damage, involving a cholinergic deficit, which finally causes cognitive impairment. Cholinergic agents, including AChE inhibitors, have shown considerable benefits as VaD therapies.[19] For example, donepezil is an AChE inhibitor, and was used as a positive control in our study. However, the effect of cholinesterase inhibitors is reduced when cholinergic neurons are seriously damaged, and enzymes and other precursors of synthetic acetylcholine are reduced. Based on the pathogenesis of VaD, we chose to evaluate NBP in the present study as a possible treatment for VaD. NBP has been reported to effectively attenuate cognitive impairments and reduce neuronal loss in multiple animal models of dementia. The beneficial effects of NBP include the prevention of neuropathological alterations, improvement of cerebral blood perfusion and microcirculation, protection of mitochondria, regulation of energy metabolism, inhibition of inflammatory injury and oxidative damage, reduction of neuronal apoptosis, and increase of acetylcholine synthesis.[20,21] However, the mechanisms of NBP in the treatment of VaD remain unknown. To investigate the cholinergic pathogenesis of VaD and explore new targets for VaD treatment, we examined the effects of NBP on regulating cholinergic dysfunction and learning and memory ability in VaD rats.

## Materials and methods

### Experimental animals

Male Sprague Dawley rats were purchased from the Vital River Corporation (BioRiver Co., Ltd., Beijing, China). The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All experimental protocols were approved by the Animal Ethics Committee of Tianjin Medical University.

### VaD model

The rats were randomly divided into a control group, an operated group, and a sham-operated group. The methods used to establish the two-vessel occlusion (2VO)-operated model have been described previously.[31] Briefly, both carotid arteries were gently separated from the vagus nerve, and the arteries were permanently occluded with silk thread. Sham-operated rats received the same surgical procedure without the double ligation occlusion.

### Treatments and grouping

After verifying that the 2VO was performed successfully (described in the following section), the rats in the 2VO group were randomly divided into three groups: the 2VO group (2VO); donepezil group (2VO+donepezil; 1 mg/kg of donepezil; intragastric); and NBP group (2VO+NBP; 80 mg/kg of NBP; intragastric). Daily administration began on day 21 post-surgery, and treatments were administered once daily for 2 weeks.

### Morris water maze (MWM) test

The MWM was performed as previously described[22] to evaluate the cognitive deficit and motor ability of each rat. Spatial learning and memory was evaluated by each rat's performance in the MWM, which was conducted for 5 days. Each trial lasted until the rat located the hidden escape platform, or until 2 minutes had passed. If the rat did not find the platform within 2 minutes, it was guided by an experimenter to the hidden platform and left there for 2 s. The escape latency (the time taken to find the platform) was recorded to assess spatial memory. After 5 days of learning trials, a probe trial was conducted on day 6 to evaluate spatial memory. The platform was removed, and each rat was allowed to swim freely for 60 s. The percentage of time spent in the target quadrant was recorded to assess spatial memory.

### Neuropathological analyses

Rats from each group were anesthetized. After pericardial perfusion with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA), brains were removed and post-fixed in 4% PFA overnight. The brains were then dehydrated in 30% sucrose until the tissue had sunk. Next, the brains were frozen in Optimal Cutting Temperature (O.C.T.) compound, and 8-μm axial sections at the site of the hippocampus were cut using a cryostat (Leica Microsystems LM3050S, Wetzlar, Germany) and mounted onto poly-L-lysine-coated slides. Hematoxylin and eosin (H&E) staining was performed using a kit (Solarbio, Beijing, China) according to the manufacturer's instructions. The morphology was assessed by image analysis using a Nikon Coolscope (Nikon, Dusseldorf, Germany).

Immunohistochemistry was used to evaluate the expression of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). Briefly, hippocampal sections were fixed in 3% hydrogen peroxide for 10 minutes at room temperature and exposed to bovine serum albumin (BSA) for 30 minutes. Next, the slides were incubated with either rabbit anti-rat BDNF (1:600; Abcam, Cambridge, UK) or rabbit anti-VEGF (1:400; Millipore, Billerica, MA, USA) antibodies overnight at 4°C, and then incubated with goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 40 minutes at 37°C.

Immunofluorescent staining was performed to evaluate central cholinergic activity. After rewarming the slides to room temperature, they were fixed in 4% PFA for 10 minutes and permeabilized in 0.5% Triton X-100 for 5 minutes, washing with ice-cold PBS after each step. After blocking with 3% BSA for 30 minutes at 37°C, the slides were incubated overnight at 4°C with the following primary antibodies diluted in 3% BSA: goat anti-rat ChAT (1:100; Millipore), rabbit anti-VAChT (1:200; Abcam), and rabbit anti-AChE (1:100; Abcam). After washing in PBS, the slides were then incubated with the appropriate secondary antibodies for 60 minutes at room temperature. The following secondary antibodies were used: goat anti-rabbit (1:200; Jackson ImmunoResearch Inc.) and donkey anti-goat (1: 200;

Invitrogen Corp., Carlsbad, CA, USA). The nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI).

### *Quantitative real-time polymerase chain reaction (qPCR)*

Total RNA was extracted from the hippocampus using Trizol reagent (Invitrogen) and qPCR was performed using SureScript™ First-Strand cDNA Synthesis Kit, All-in-One™ qPCR Mix, and All-in-One™ qPCR Primer (Genecopoeia, Inc., Rockville, MD, USA) according to the manufacturer's instructions. All qPCR reactions were performed in triplicate on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the comparative cross threshold (Ct) method.

### *Statistics*

SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA) was used to compare the differences between groups. Data are expressed as the mean ± standard error of the mean (SEM). The average escape latency data were analyzed using two-way repeated ANOVA, while other data were analyzed using one-way ANOVA. A result was considered to be significant at $P < 0.05$.

## Results

A total of 115 rats, each weighing 280 to 300 g, were used in this study. Rats were randomly divided into a control group (30 rats), a 2VO-operated group (55 rats), and a sham-operated group (30 rats). Eighteen rats in the 2VO group were randomly assigned into three treatment groups ($n = 6$ per group): no treatment (2VO), donepezil (2VO+donepezil), or NBP (2VO+NBP). These rats were compared with six rats in each of the sham and control groups.

### *NBP treatment improves spatial learning and memory*

The MWM is a validated behavioral method to evaluate spatial learning and memory. Compared with the normal and sham groups, the daily escape latency in the 2VO group was significantly longer ($P < 0.05$). The percentage of time spent in the target quadrant was significantly lower in the 2VO group than in the normal and sham groups ($P < 0.05$). As shown in Figure 1a, the navigation assay indicated that all rats in each group established spatial memory after 5 days of training ($P < 0.001$). During the training period, the escape latency in each group became gradually shorter as the number of training days increased; this trend was more pronounced in the drug treatment groups, the normal group, and the sham group. The rats from the 2VO group and the 2VO+NBP group had longer daily escape latencies compared with the normal and sham groups. Of these, the difference between the 2VO group and the normal or sham group was the most significant. There was no significant difference between the 2VO+donepezil group and the normal or sham group. The daily escape latency of the 2VO+NBP group was significantly shorter compared with that of the 2VO group ($P < 0.05$). Although the daily escape latency of the NBP group tended to be longer than that of the 2VO+donepezil group, the difference was not significant. The spatial exploration experiments revealed that the percentage of time spent in the target quadrant was significantly higher in the 2VO+donepezil and 2VO+NBP groups than in the 2VO group ($P < 0.05$, $P = 0.018$), but there was no difference between the 2VO+donepezil and 2VO+NBP groups (Figure 1b).

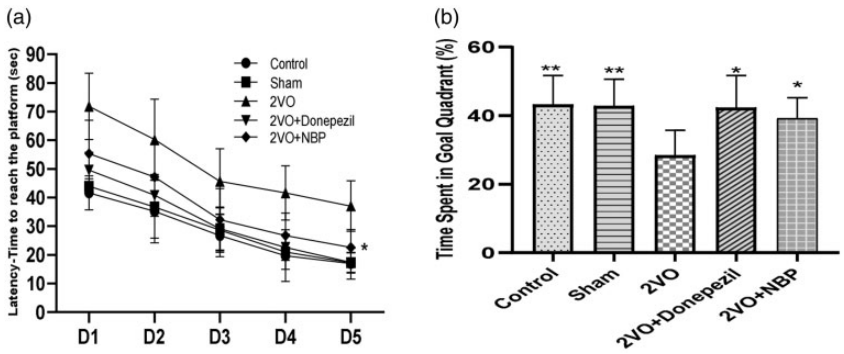


**Figure 1.** Effects of dl-3-n-butylphthalide (NBP) treatment on cognitive deficits induced by chronic cerebral hypoperfusion in rats. (a) Average escape latency to find the target platform in the Morris water maze test. *$P < 0.05$ compared with the normal group and sham groups. (b) The percentage of time spent in the target quadrant within 60 s and the number of times crossing the platform in the probe trial. *$P < 0.05$ compared with the two-vessel occlusion (2VO) group.

## *NBP treatment reduces pathological changes and alleviates cell loss*

Because the hippocampal CA1 area, which is involved in learning and memory, is sensitive to hypoxia–ischemia conditions, we focused our analysis on this limbic area. The H&E stain is an important method to evaluate neuronal loss and morphology. As shown in Figure 2, intact neurons could be seen in the CA1 area of the hippocampus in the normal and sham groups. The cells in the hippocampus had clear boundaries. Pyramidal cells were polygonal or triangle-shaped, with many layers, and were neatly arranged. The cytoplasm was red-tinged, while the nucleus was blue. The protrusions were neatly arranged in the radiation layer, and part of the nucleolus could be seen under high magnification. In contrast, many neuronal changes were observed in this area in the 2VO group. In the 2VO group, the number of cells and cell layers in the hippocampal CA1 area were reduced, and the cells had a disordered arrangement. We also observed a loss of neuronal cells. Furthermore, cell volume was reduced or cells were swollen. Some cells were vacuolated and a large number of cells had died. Shrunken nuclei and darkly stained neurons were observed. The structure of some cells was unclear and sometimes even nonexistent. Glial cell hyperplasia and a large amount of inflammatory cell infiltration were seen around the area, in contrast to what was observed in the normal group. Compared with the 2VO group, rats in the 2VO+NBP and 2VO+donepezil groups showed reduced pathological changes and alleviated cell loss induced by chronic cerebral hypoperfusion.

## *NBP treatment regulates cholinergic activity*

The immunofluorescence assays revealed that the expressions of ChAT, AChE, and VAChT were decreased in the 2VO group compared with the control and sham groups. Compared with the 2VO group, more immunofluorescence staining of positive cells was detected in the 2VO+NBP group. Rats in the 2VO+donepezil group had less AChE-positive expression compared with the 2VO group, but more ChAT- and VAChT-positive expression. The mRNA levels of ChAT, AChE, and VAChT in the hippocampus were significantly lower in the

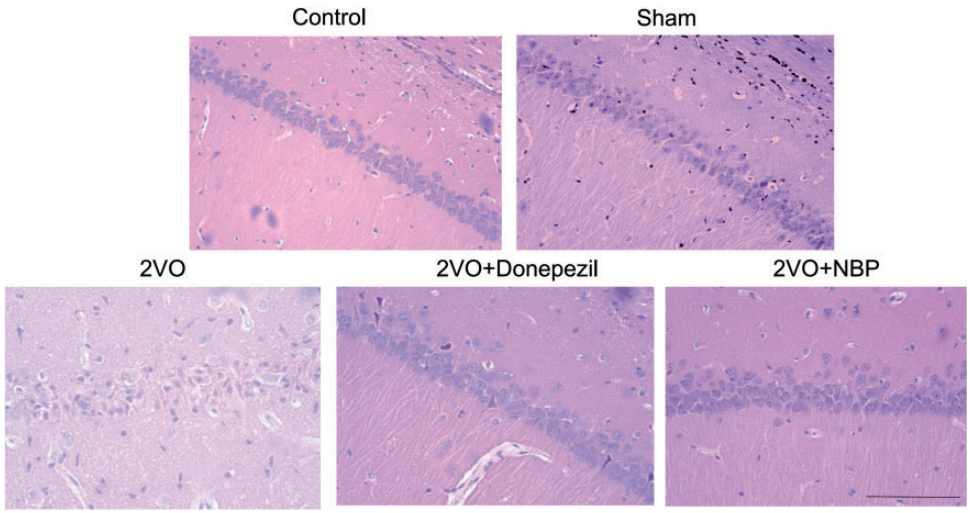


**Figure 2.** Representative photographs of tissue sections stained with hematoxylin and eosin in the hippocampal CA1 area (magnification: 200×). A section from the sham-operated group shows intact neurons and well-preserved cell density. A section from the two-vessel occlusion (2VO) group shows neuronal loss with ischemic changes (neuronal cell loss, nuclei shrinkage, and dark staining of neurons). A section from the 2VO+NBP (dl-3-n-butylphthalide) group shows relatively intact neurons with intact chromatin. Scale bar = 50 μm, n = 4.

2VO group than in the sham group ($P < 0.001$), and the mRNA levels of ChAT and VAChT in the 2VO+donepezil and 2VO+NBP groups were significantly higher than those of the 2VO group. There were no significant differences in the mRNA levels of ChAT and VAChT between the 2VO+NBP and 2VO+donepezil groups. AChE mRNA levels appeared lower in the 2VO+donepezil group than in the 2VO group, but this apparent difference was not significant. Compared with the 2VPO+donepezil and 2VO groups, AChE mRNA levels were higher in the 2VO+NBP group ($P < 0.001$, Figure 3).

## *NBP treatment increases BDNF and VEGF expression*

Immunohistochemical staining revealed that BDNF-positive cells were mainly concentrated in the hippocampus: in the CA1 region, CA3 pyramidal cells, and dentate gyrus granule cells (Figure 4). The immunopositive reaction was observed as deep brown staining in the cytoplasm. BDNF expression in the 2VO group was slightly lower than in the control and sham groups. Rats in the 2VO+NBP group had markedly more BDNF staining compared with the 2VO group. No differences were observed between the 2VO and 2VO+donepezil groups. Hippocampal BDNF mRNA expression appeared slightly downregulated in the 2VO group compared with the sham group, but there was no statistical difference between the two groups. The mRNA levels of BDNF were higher in the 2VO+donepezil and 2VO+NBP groups than in the 2VO group, but this difference was only significant in the 2VO+NBP group ($P < 0.01$). In addition, BDNF mRNA levels were significantly higher in the 2VO+NBP group than in the

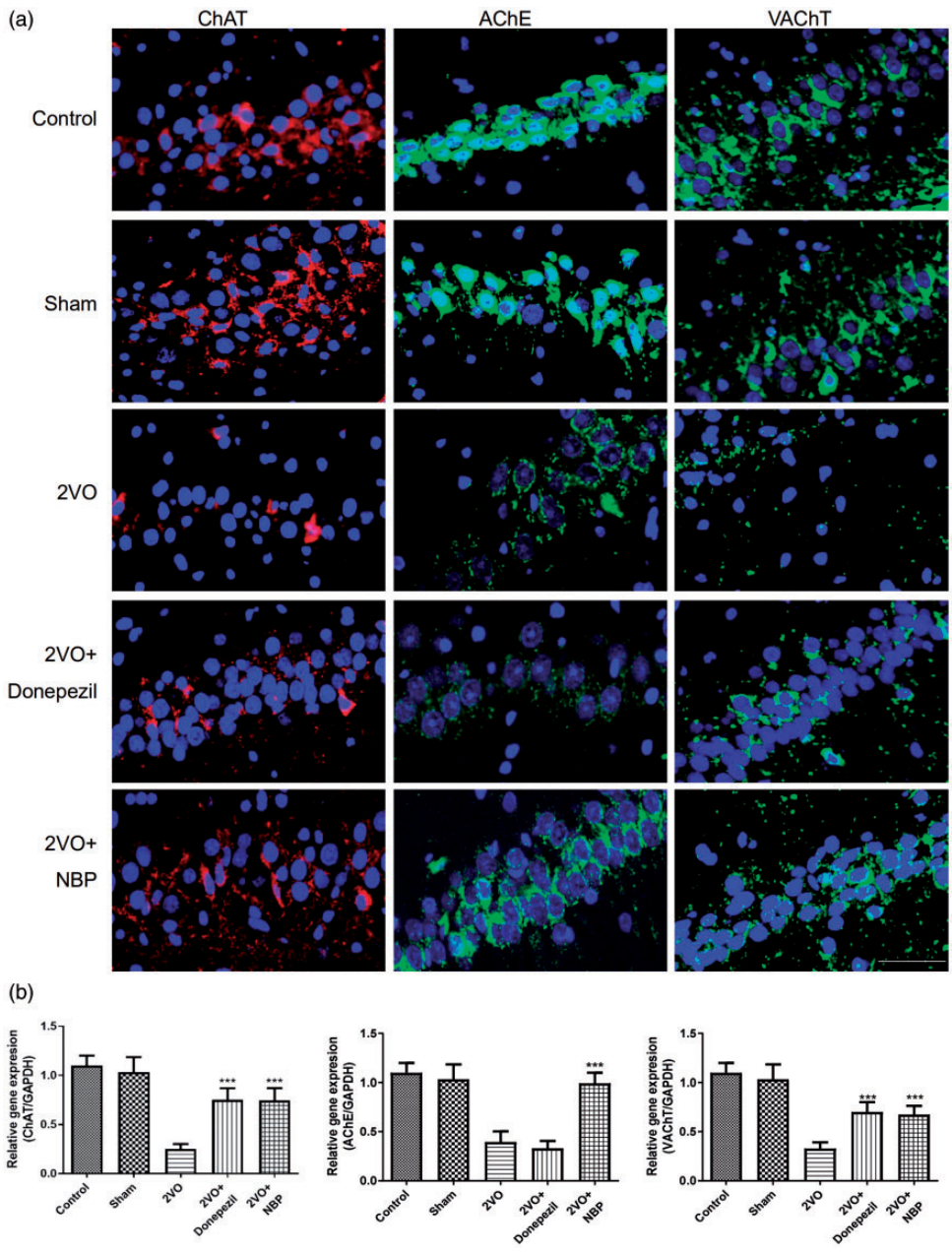


**Figure 3.** DI-3-n-butylphthalide (NBP) treatment regulated the expression of acetylcholinesterase (AChE), choline acetyltransferase (ChAT), and vesicular acetylcholine transporter (VAChT) in the hippocampus of two-vessel occlusion (2VO) rats. (a) Immunofluorescence staining in each group; AChE- or VAChT-positive cells were detected using green fluorescence (FITC-labeled) under excitation at 488 nm. ChAT-positive cells were detected using red fluorescence (Alexa Fluor®-labeled). Nuclei were stained using DAPI, which is shown in blue. A merged image is provided. Scale bar = 50 μm. (b) Relative mRNA expression of ChAT, AChE, or VAChT in each group. ***$P < 0.001$ vs. 2VO group.

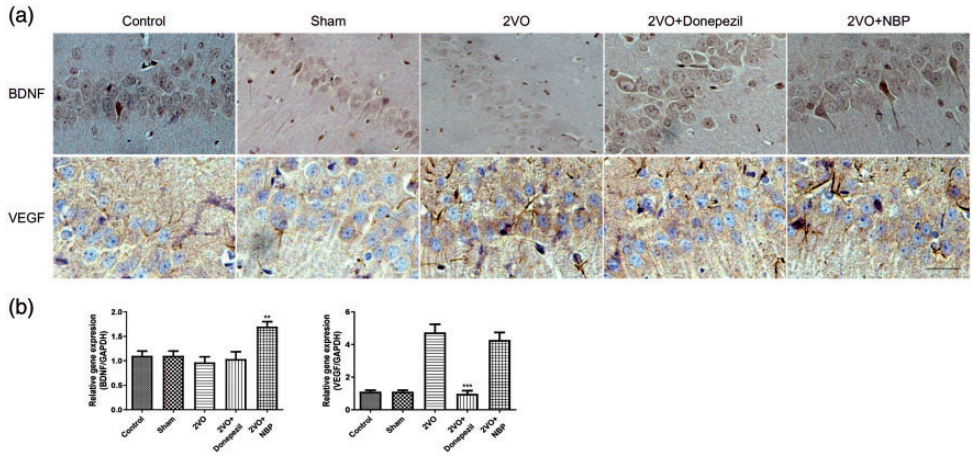


**Figure 4.** Dl-3-n-butylphthalide (NBP) treatment increased the expression levels of brain-derived neurotrophic factor (BDNF) and of vascular endothelial growth factor (VEGF) in the hippocampus of two-vessel occlusion (2VO) rats. (a) BDNF- and VEGF-positive cells were mainly concentrated in the hippocampus, and positive immunohistochemical reactions were observed in the cytoplasm and cell membrane (brown staining). Magnification: 200×; scale bar = 50 μm. (b) The mRNA levels of BDNF or VEGF in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. 2VO group.

2VO+donepezil group ($P < 0.01$). VEGF-positive cells were detected in the hippocampal CA1 region in all of the groups, and the positive staining was mainly concentrated in the cytoplasm and cell membrane. Hippocampal VEGF expression in the 2VO group was increased compared with that of the control and sham groups, and VEGF-positive astrocytes were also observed. Rats in the 2VO+donepezil group were observed to have the least VEGF expression compared with the 2VO group, and there were also fewer astrocytes in this group. No differences were observed between the 2VO+NBP and 2VO groups. Similar to the immunohistochemical results, hippocampal VEGF mRNA expression was significantly higher in the 2VO group than in the sham group ($P < 0.001$), and VEGF levels were significantly lower in the 2VO+donepezil group compared with the 2VO group ($P < 0.001$).There was no significant difference in VEGF expression between the 2VO+NBP and 2VO groups.

## Discussion

There is a significant reduction in neurotransmitters in the cortex, hypothalamus, striatum, and hippocampus during VaD onset.[23] ChAT has been reported as significantly reduced in the cerebrospinal fluid of patients with multi-infarct dementia, and this is consistent with the finding that the number of cholinergic neurons in the nucleus basalis of Meynert is reduced in this disease.[6,10] The relationship between AChE, VAChT, and VaD remains controversial.[17] Our study revealed that hippocampal CA1 expressions of ChAT, AChE, and VAChT were significantly lower in our VaD rat model compared with rats in the sham group. This result was consistent with behavioral findings demonstrating that the spatial learning and memory ability of these VaD rats was significantly lower than in the sham group. In general, our results were consistent with those of previous studies,[24] indicating that cholinergic dysfunction is involved in VaD.

It has been reported that NBP treatment can improve the learning and memory deficits induced by chronic cerebral hypoperfusion in rats.[25] However, the specific target of NBP remains unclear. It has been suggested that NBP may provide neuronal protection by interacting with multiple targets and improving cerebral blood flow. Most of the protective effects of NBP have been demonstrated in animal models and ischemic patients. NBP treatment has been shown to improve cognitive deficits, and this process might be mediated by preventing the decline of the central cholinergic system in the brain.[26] Previous studies have reported that treating rats with NBP significantly increased ChAT activity, and also decreased cortical lipid peroxidation and hippocampal superoxide dismutase activity compared with controls.[25] In the present study, NBP treatment significantly increased the hippocampal expressions of ChAT, AChE, and VAChT compared with the VaD model group, and also improved the spatial learning and memory of VaD rats. The improvement in cognitive impairment was not significantly different between rats who were treated with NBP and rats who were treated with donepezil. Donepezil is a cholinesterase inhibitor, and was used as the positive control drug. Our study also showed a reduction in AChE expression in the hippocampus of donepezil-treated VaD rats.

Neurotrophic factors play an important role in the regeneration and repair of the nervous system. Our study focused on VEGF and BDNF because these proteins are closely related to VaD. VEGF is involved in inflammatory reactions after cerebral ischemia and hypoxia. However, studies on the relationship between VEGF and cholinergic system have reported inconclusive results. One study demonstrated that NBP can improve spatial learning and memory in ischemic animals, and can also promote angiogenesis by increasing the expression of VEGF.[27] In our study, however, there was no significant change in VEGF in NBP-treated VaD rats. In contrast, BDNF is a key protein that promotes memory, growth, synaptic plasticity, and neuronal survival.[28] The spatial learning and memory ability of VaD rats may be related to changes in BDNF expression. Supporting this idea, a deficiency in BDNF was previously observed in VaD patients.[29] It has also been shown that BDNF has a protective effect against neuronal ischemic injury.[30] AChE-positive neurons and ChAT activity can be increased two- to three-fold in low-density cholinergic neurons treated with BDNF.[31]

It has been reported that NBP can promote adult hippocampal neurogenesis and neuroplasticity in rat models of dementia and cerebral ischemia via the BDNF/TrkB/CREB pathway. Yang et al.[32] demonstrated that NBP improved behavioral recovery and elevated hippocampal neurogenesis after cerebral ischemia in rats. Furthermore, Lei et al.[33] suggested that NBP stimulated the proliferation of adult hippocampal progenitor cells and alleviated cognitive impairment by promoting the release of neurotrophic factors and activating BDNF/CREB signaling. Additionally, Zhao et al.[34] reported that NBP treatment increased neurogenesis, angiogenesis, and arteriogenesis in the brain after traumatic brain injury, and that these changes were accompanied by the upregulation of regenerative genes such as BDNF. Our results revealed that NBP treatment increased hippocampal BDNF, ChAT, and VAChT expression in a rat model of VaD. NBP may have neuroprotective effects by increasing BDNF secretion, which can protect hippocampal cholinergic neurons, thereby improving cognitive function in VaD rats. A more in-depth study of the underlying mechanisms, such as BDNF pathways and related factors, will be performed in our future studies.

## Conclusion

Our study suggested that central cholinergic dysfunction may be involved in the pathogenesis of VaD, and indicated that NBP improves spatial learning and memory in VaD rats. NBP treatment increased the hippocampal expressions of ChAT, AChE, VAChT, and BDNF in 2VO rats. Together, these results suggest that, similar to the cholinesterase inhibitor donepezil, NBP can also protect cholinergic neurons and reduce damage by regulating BDNF mechanisms. Our study provides new targets and theoretical bases for drug therapies of VaD.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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