

3-N-butylphthalide improves neuronal morphology after chronic cerebral ischemia

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Abstract

3-N-butylphthalide is an effective drug for acute ischemic stroke. However, its effects on chronic cerebral ischemia-induced neuronal injury remain poorly understood. Therefore, this study ligated bilateral carotid arteries in 15-month-old rats to simulate chronic cerebral ischemia in aged humans. Aged rats were then intragastrically administered 3-n-butylphthalide. 3-N-butylphthalide administration improved the neuronal morphology in the cerebral cortex and hippocampus of rats with chronic cerebral ischemia, increased choline acetyltransferase activity, and decreased malondialdehyde and amyloid beta levels, and greatly improved cognitive function. These findings suggest that 3-n-butylphthalide alleviates oxidative stress caused by chronic cerebral ischemia, improves cholinergic function, and inhibits amyloid beta accumulation, thereby improving cerebral neuronal injury and cognitive deficits.

Key Words: nerve regeneration; depression; functional MRI; graph theory; complex networks; brain network; classification; feature selection; NSFC grant; neural regeneration

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Introduction

3-N-butylphthalide is a new drug treatment for ischemic stroke and has been independently researched and developed in China. Previous studies have found that NBP reduces focal cerebral ischemia volume in rats and improves local cerebral ischemia brain-induced edema, brain energy metabolism disorder, and apoptotic neuronal cell death (Peng et al., 2005; Dong and Feng, 2000). NBP has a variety of protective effects on brain tissues because it is a multi-target drug.

Vascular dementia, induced by chronic cerebral ischemia, such as cerebral arteriosclerosis and cerebral infarction, is a common disease in older people. The pathogenesis of vascular dementia is complex and is involved in energy metabolism disorder, oxidative stress injury, neuronal apoptotic cell death, and cholinergic nerve dysfunction (Cechetti et al., 2012; Feng et al., 2012; Du et al., 2013; Zhao et al., 2013). The anti-oxidative enzyme superoxide dismutase (SOD), and a lipid peroxidation product, malondialdehyde (MDA), may indicate the extent of oxidative stress injury. Acetylcholine (ACh) correlates with the function of cholinergic neurons. ACh content can be evaluated indirectly by the activity of the synthetase of ACh, choline acetyltransferase (ChAT), and the hydratase of ACh, true cholinesterase. Therefore, the activity of ChAT and true cholinesterase may indicate the function of cholinergic neurons. Furthermore, senile plaques are one of the characteristic pathological changes in brain

tissue of dementia patients, and β -amyloid (A β) peptide is the main component of senile plaques. Therefore, the level of A β in brain tissue may indicate the state of dementia.

Existing medications for improving cerebral circulation and promoting brain metabolism, as well as cholinesterase inhibitors (such as tacrine, donepezil, and rivastigmine), have therapeutic effects but are not ideal for improving symptoms of dementia (Schwarz et al., 2012; Gorelick and Nyenhuis, 2013).

Chronic cerebral ischemia is the most commonly used method to simulate vascular dementia, and the common method for causing chronic cerebral ischemia in rats is permanent ligation of the bilateral common carotid arteries. Tsuchiya et al. (1993) showed that blood flow in 15 brain regions (including regions important for learning and memory: encephalic region, cortex, hippocampus) decreased 1 week after ligation of the bilateral common carotid arteries of rats. This method has been used to simulate a disturbance of cerebral circulation induced by severe stenosis of cerebral vascular disease in addition to hypoperfusion. This effect can cause cerebral ischemia injury to different degrees, decrease learning and memory, induce white matter damage, and cause spatial cognitive deficits (Farkas et al., 2007; Zhang et al., 2011; He et al., 2012).

In the present study, a dementia model was prepared by permanent ligation of the bilateral common carotid arteries

in aged rats. We examined the effect of NBP on cognitive dysfunction in rats with dementia. Furthermore, oxidative stress, cholinergic function, A β accumulation, and neuronal morphology in brain tissue were explored to provide an experimental basis for the clinical application of NBP in vascular dementia.

Materials and Methods

Major reagents and equipment

NBP was purchased from Beijing University of Chemical Technology, China. SOD, MDA, ChAT, true cholinesterase, rat A β_{1-42} enzyme-linked immunosorbent assay (ELISA) and the Coomassie brilliant blue kit were purchased from Nanjing Jiancheng Biological Engineering Institute, Nanjing, China. The MT-200 water maze integrated tracking system was purchased from Chengdu Taimeng Science and Technology Limited, Chengdu, Sichuan Province, China. The Thermo Scientific MK3 type microplate reader was purchased from Thermo Fisher Scientific, New York, NY, USA. The IR2135 type paraffin slicing machine (Leica) was purchased from Solms, Wetzlar, Germany.

Animals

Eighty male Wistar rats, 15 months old and weighing 550–650 g, were used in the experiments. These animals were housed in a room maintained at $23 \pm 1^\circ\text{C}$ with a 12 hour light/dark cycle, and were allowed free access to water and food. All experiments were approved by the Experimental Animal Center of Hubei University of Medicine, China.

Ten rats were selected in the sham-operated group. Forty-five days after modeling, 27 rats survived, and those with equal cognitive levels were selected and randomly divided into three groups ($n = 9$ per group): (1) the model or (2) 30 or (3) 120 mg/kg NBP.

The chronic cerebral ischemia rat model and drug treatment

Rats (280 mg/kg) were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate (Xi'an Yuelai Medicine Technology Co. Ltd., Shaanxi Province, China). A neck operation was conducted to separate the bilateral common carotid arteries, which were then ligated permanently with 5-0 operation silk suture (Zhao et al., 2013). We injected 200,000 units of penicillin (i.p.) per day after the operation for 3 successive days. The bilateral common carotid arteries were separated without ligation in the sham-operated group. Chronic cerebral ischemia rat models were established in all three groups. Forty-five days after the operation, rats in the sham-operated and model groups were intragastrically administered peanut oil (2 mL/kg), and rats in the 30 or 120 mg/kg NBP groups were intragastrically administered 30 or 120 mg/kg NBP, respectively, dissolved in peanut oil for 45 successive days.

Morris water maze (MWM) task

Ten rats of the sham-operated group and nine rats of each remaining group were used for the MWM task (one rat of the sham-operated group died during the test). The MWM task

is a classical method for detecting recent memory and spatial discrimination ability in animals. It is mainly composed of a metal cylindrical tank, an automatic displaying, monitoring, and recording device, and a safety platform (Peng et al., 2007). In the experiment, the swimming activity of each rat was observed and recorded by a monitor, which was directly connected to a computer for processing and analysis.

Place navigation tests were used to detect spatial learning and memory ability during the water maze test for rats. The route map used by rats to find and climb onto a security platform as well as the time required were observed and recorded. The escape latency and swimming speed were recorded and the search strategy was analyzed. First, the memory training experiments were conducted for 5 consecutive days. The time taken to find the platform (latency), swimming speed, search strategy and other indicators were recorded, in which the latency was the main observation parameter. If a rat did not find the platform in 60 seconds, the latency was calculated as 60 seconds. The search strategy was divided into four categories: (1) edge mode (where rats moved along the edge of the water without a motivation for searching), (2) random search mode (where there was no clear direction in searching for the safety platform), (3) tendency mode (where rats remembered the possible location of the safety platform, turning less than four times before getting to the platform), and (4) straight line type (where rats clearly remembered the safety platform position and swam directly to it).

Memory retention tests were conducted on the first day after completion of the place navigation tests. The platform was removed, and rats were allowed to swim freely for 30 seconds to search for the platform. Movement times of rats in each quadrant were recorded, and the percentage of movement time in the target quadrant was calculated (Hall et al., 2013; Raghavendra et al., 2013).

Measurement of SOD, ChAT, true cholinesterase, and MDA content in brain tissues

Rats ($n = 8$ per group) were anesthetized with chloral hydrate (1 g/kg, i.p.). After decapitation, the skull was opened to obtain brain tissues. The cortex and hippocampus were separated on ice to prepare brain tissue homogenate. SOD, ChAT, true cholinesterase, and MDA contents were measured with specific reagent kits and their content in each unit tissue were calculated (He et al., 2013). The protein concentration was determined by the Coomassie brilliant blue method (Marshall and Williams, 1992).

Determination of A β_{1-42} content in brain tissues of rats by ELISA

The cortex and hippocampus brain tissue homogenates ($n = 8$ rats per group) were prepared. The A β_{1-42} content was measured using the rat A β_{1-42} ELISA kit, according to the manufacturer's instruction.

Hematoxylin-eosin (HE) staining

After anesthesia and decapitation, the skull was opened to

Table 1 Effect of NBP on SOD (U/mg protein) activity and MDA (nmol/mg protein) level of brain tissue from chronic cerebral ischemia rats

Group	Cortex		Hippocampus	
	SOD	MDA	SOD	MDA
Sham-operated	118.9±10.4	2.24±0.71	66.4±14.0	0.89±0.15
Model	134.6±13.9 ^b	3.51±0.81 ^b	84.1±27.0 ^a	1.21±0.26 ^b
NBP 30 mg/kg	123.8±12.9	2.39±0.31 ^c	76.3±7.2	0.71±0.10 ^c
NBP 120 mg/kg	112.3±7.6 ^c	1.56±0.19 ^c	69.1±5.0	0.83±0.05 ^c

Eight rats from each group were euthanized at 3 months after modeling (*i.e.*, at 1.5 months after administration). Their cortexes and hippocampi were separated on ice to prepare brain tissue homogenate. SOD activity and MDA content were measured with specific reagent kits. At the same time, the protein concentration was determined by Coomassie brilliant blue method. SOD activity and MDA content in each unit tissue were calculated. Data are expressed as mean ± SD. ^a $P < 0.05$, ^b $P < 0.01$, vs. sham-operated group; ^c $P < 0.01$, vs. model group. Difference among groups was compared using one-way analysis of variance, and significant differences between the two groups were analyzed using the least significant difference test. NBP: 3-N-butylphthalide; SOD: superoxide dismutase; MDA: malondialdehyde.

Table 2 Effect of NBP on ChAT (U/g protein) and TChE (U/mg protein) activity in brain tissue from chronic cerebral ischemia rats

Group	Cortex		Hippocampus	
	ChAT	TChE	ChAT	TChE
Sham-operated	1,608±224	0.15±0.06	1,807±401	0.25±0.05
Model	1,406±94 ^a	0.15±0.07	1,334±280 ^b	0.32±0.17
NBP 30 mg/kg	1,412±228	0.08±0.04	1,746±204 ^c	0.34±0.14
NBP 120 mg/kg	1,615±100 ^c	0.15±0.07	1,697±117 ^c	0.28±0.06

Eight rats from each group were euthanized at 3 months after modeling (*i.e.*, at 1.5 months after administration). Their cortexes and hippocampi were separated on ice to prepare brain tissue homogenate. Activities of ChAT and TChE were measured with specific reagent kits. At the same time, the protein concentration was determined by Coomassie brilliant blue method. Activities of ChAT and TChE in each unit tissue were calculated. Data are expressed as mean ± SD. ^a $P < 0.05$, ^b $P < 0.01$, vs. sham-operated group; ^c $P < 0.05$, vs. model group. Difference among groups was compared using one-way analysis of variance, and significant differences between the two groups were analyzed using the least significant difference (LSD) test. NBP: 3-N-butylphthalide; ChAT: choline acetyltransferase; TChE: true choline esterase.

Table 3 Effect of 3-n-butylphthalide (NBP) on the content of amyloid-beta (A β) peptide (μ g/g protein) in brain tissue from chronic cerebral ischemia rats

Group	Cortex	Hippocampus
Sham-operated	1.13±0.08	1.03±0.29
Model	1.49±0.20 ^b	1.36±0.24 ^a
NBP 30 mg/kg	1.24±0.18 ^c	1.07±0.30 ^c
NBP 120 mg/kg	1.06±0.08 ^d	0.99±0.18 ^d

Eight rats from each group were euthanized 3 months after modeling (*i.e.*, 45 days after administration). Their cortexes and hippocampi were separated on ice to prepare brain tissue homogenate. A β_{1-42} level was measured with rat A β_{1-42} enzyme-linked immunosorbent assay. At the same time, the protein concentration was determined by Coomassie brilliant blue method. The level of A β_{1-42} in each unit tissue was calculated. Data are expressed as mean ± SD. ^a $P < 0.05$, ^b $P < 0.01$, vs. sham-operated group; ^c $P < 0.05$, ^d $P < 0.01$, vs. model group. Difference among groups was compared using one-way analysis of variance, and significant differences between the two groups were analyzed using the least significant difference (LSD) test.

obtain brain tissues. Brains were fixed with 4% paraformaldehyde to make paraffin sections. The cortex and hippocampus tissue sections were stained with HE and sections were observed with a Caikang XDS-300C microscope (Shanghai Caikang optical instrument, China).

Statistical analysis

All data are expressed as mean ± SD and were analyzed by

two-way analysis of variance followed by the least significant difference test. Statistical analysis was performed using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). Significance was reached at a value of $P < 0.05$; $P < 0.01$ was considered highly significant.

Results

NBP improved cognitive function in rats with chronic cerebral ischemia

In the place navigation tests, the time taken to find the platform (escape latency) for sham-operated rats decreased gradually as the training days progressed. On the 4th and 5th training days, latency times decreased from approximately 60 seconds to about 20 seconds. The model group displayed close to no change in the latency time during the whole training period, with a latency time of approximately 60 seconds on the 5th day. A decrease in learning and memory was evident 3 months after permanent ligation of the bilateral common carotid arteries. The latency times gradually reduced in rats exposed to 30 mg/kg or 120 mg/kg NBP over the training days. The latency time on the 4th and 5th training day was dose-dependently reduced and was significantly ($P < 0.05$ or $P < 0.01$) different to that of the model group (Figure 1A).

As the training days progressed, the search modes of most animals in the sham-operated group transformed gradually from the edge and random types to the tendency and straight line types. However, the search types for the major-

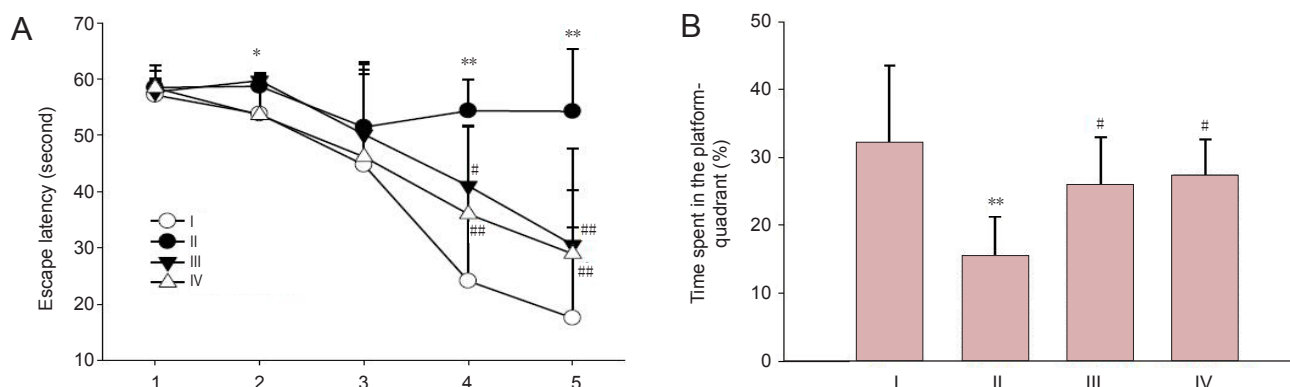


Figure 1 Effect of 3-n-butylphthalide (NBP) on chronic cerebral ischemia-mediated learning and memory impairment in rats.

The place navigation test (A) and the memory retention test (B) of Morris water maze were performed 3 months after establishing the model. (A) In the place navigation test, a shorter escape latency indicates stronger spatial learning and memory abilities. (B) In the memory retention test, a longer time spent in the platform-quadrant indicates stronger retention memory. Data are expressed as mean \pm SD. There are nine rats in each group (at each time point). * $P < 0.05$, ** $P < 0.01$, vs. sham-operated (sham) group; # $P < 0.05$, ## $P < 0.01$, vs. model group. Differences among the groups were compared using two-way analysis of variance, and significant differences between the two groups were compared using the least significant difference (LSD) test. I-IV: Sham, model, NBP 30 mg/kg, NBP 120 mg/kg groups, respectively.

ity of animals in the model group remained as random and edge types at the end of the training. As the training days progressed, searching types from animals of the two doses of NBP changed from the edge and random type to tendency and straight line type gradually, with particularly increased change in the higher dose group.

In the memory retention experiment, animal movement time in the target quadrant in the sham-operated group was more than 30%, and that of the model group was significantly ($P < 0.01$) reduced to approximately 15%, indicating that 3 months after permanent ligation of the bilateral common carotid arteries in rats, memory retention markedly decreased. However, compared with the model group, the percentage movement times of rats in the target quadrant significantly ($P < 0.05$) increased 45 days after continuous administration of 30 mg/kg NBP or 120 mg/kg NBP (Figure 1B).

In the place navigation and memory retention experiments, no significant differences were observed in the swimming speeds of rats between all groups. Furthermore, no change in swimming speed of animals from the same group during the 6 days of the experiment was evident.

NBP reduced oxidative stress in brain tissue of rats with chronic cerebral ischemia

Levels of SOD and MDA indirectly reflect oxidative stress damage (Marshall and Williams, 1992; He et al., 2013). Compared with the sham-operated group, cortical SOD activity in the model group significantly ($P < 0.01$) increased three months after chronic cerebral ischemia. SOD activity in the 30 and 120 mg/kg NBP groups were lower compared with the model group and a significant ($P < 0.01$) difference existed in the high dose group, indicating a dose-dependent effect. Chronic cerebral ischemia significantly ($P < 0.05$) increased SOD activity in the hippocampus. The two doses of NBP also decreased SOD activity, but the differences were not statistically significant (Table 1). MDA content in the cortex and hippocampus in the model group was significant-

ly ($P < 0.01$) increased compared with the sham-operated group. The 30 and 120 mg/kg NBP groups significantly ($P < 0.01$) decreased MDA content in the cortex and hippocampus 45 days after drugs were administered compared with the model group (Table 1).

NBP enhanced cholinergic function in rats with chronic cerebral ischemia

Compared with the sham-operated group, ChAT activity in the cortex and hippocampus markedly ($P < 0.05$) decreased 3 months after chronic cerebral ischemia in rats. Compared with the model group, ChAT activity in the cortex and hippocampus significantly ($P < 0.05$) increased 45 days after administration of 30 and 120 mg/kg NBP, and these effects were dose-dependent. However, no significant differences were found between the two drug dose groups. Compared with the sham-operated group, there was no change in the activity of true cholinesterase in brain tissue of the model group. True cholinesterase activity did not change between the 30 mg/kg or 120 mg/kg NBP group and the model group (Table 2).

NBP reduced $A\beta_{1-42}$ in brain tissue of rats with chronic cerebral ischemia

Cortical and hippocampal $A\beta_{1-42}$ markedly ($P < 0.01$) increased after cerebral ischemia in rats. However, its content in both brain regions significantly ($P < 0.05$) reduced with 30 mg/kg and 120 mg/kg NBP in a dose-dependent manner (Table 3).

NBP improved neuronal morphology in rats with chronic cerebral ischemia

Compared with the sham-operated group, cortical neurons in the model group shrunk and were deeply stained. The nuclei were also not clear. The CA1 and CA3 regions of the hippocampus showed similar changes but to a lesser degree. No change was evident in the hippocampal CA2 region. Abnormal neuronal morphology in the cortex and hippocam-

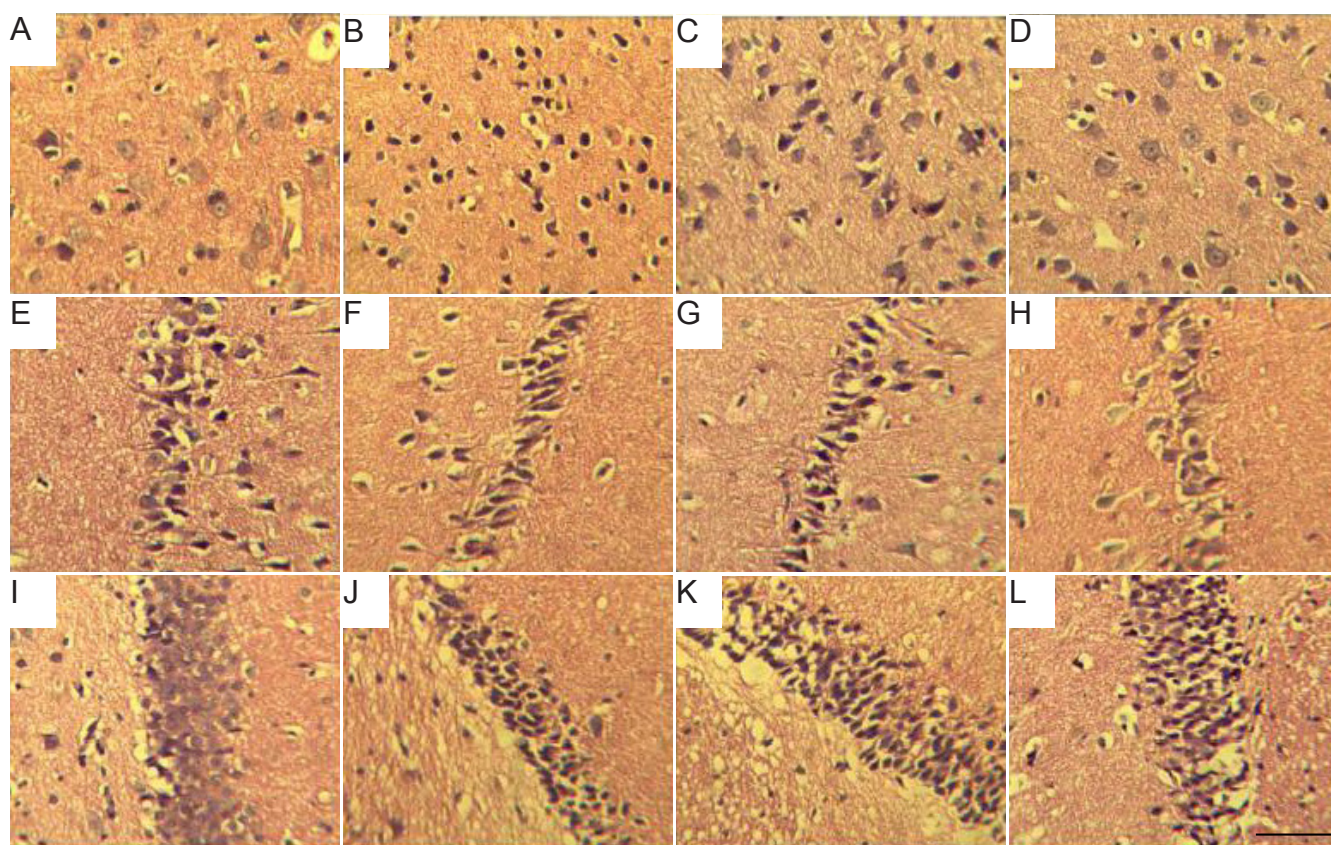


Figure 2 Effect of 3-n-butylphthalide (NBP) on neuronal morphology in the cortex and hippocampus of chronic cerebral ischemia rats. Hematoxylin and eosin of: (A–D) cortex, (E–H) CA1 hippocampal region, and (I–L) CA3 hippocampal region. (A, E and I) Sham-operated group. (B, F and J) model group. (C, G and K) 30 mg/kg NBP group. (D, H and L) 120 mg/kg NBP group. Three rats in each group were euthanized 3 months after modeling. Whole-brain coronal sections were stained with hematoxylin and eosin, and the morphology of the cortex and hippocampus was observed under an optical microscope. Cortical neurons in the model group shrank and nuclei were not clear, compared with the sham-operated group. The hippocampal areas of CA1 and CA3 showed similar changes. 3-N-butylphthalide improved the abnormal morphology of neurons in the cortex and hippocampal CA1 and CA3 regions dose-dependently. Scale bar: 50 μ m.

pal CA1 and CA3 regions were reversed by 30 mg/kg and 120 mg/kg NBP (Figure 2).

Discussion

Vascular dementia is primarily caused by the decrease of cerebral blood flow, changes in cerebral multiple focal ischemia, and loss of specific cortical nerve conduction function. These changes result in a low supply of cerebral glucose and oxygen, which, in turn, causes mitochondrial dysfunction and decreased cognitive function, which may be a possible pathophysiological process leading to vascular dementia (Boussier and Chabriat, 2012; de la Torre, 2012; Akinyemi et al., 2013; Benisty, 2013).

In the present study using the MWM, we observed that recent memory and spatial discrimination decreased in aged rats with chronic cerebral ischemia. However, the two doses of NBP improved these deficits. Furthermore, no significant differences were observed in the swimming speed of rats between each group, suggesting that the search for the safety platform involved spatial cognitive abilities but not physical ability (Deng et al., 2010; Ito et al., 2012).

Oxidative stress is an important mechanism for ischemic

neuronal injury. Free radicals can regulate neurodegeneration and thus, may be involved in neuronal death (Makesbery, 1997; Zhang et al., 2012; Colín-González et al., 2013). Free radicals are products of aerobic metabolism in cells. When free radicals are produced in large amounts or the free radical scavenging mechanism is weakened, they attack membrane polyunsaturated fatty acids, leading to lipid peroxidation and cell dysfunction. SOD is an important antioxidant enzyme that can eliminate free radicals and relieve oxidative damage. MDA is the main oxidation product. Tissue antioxidant enzyme activity reflects tissue antioxidant levels, and MDA reflects tissue lipid peroxidation. In the present study, the level of MDA in brain tissues of rats with chronic cerebral ischemia significantly increased, suggesting that the production of free radicals increased. The activity of SOD increased simultaneously, thereby indicating a compensatory response of the body to the increased production of free radicals. Similar results have been reported in previous studies (Wang et al., 2000; Peng et al., 2007). Our results with long-term administration of NBP suggest that it restores the dynamic balance between oxidation and anti-oxidation in brain tissue. Therefore, the reduction of MDA may likely be

caused by decreased production of free radicals, indicating that NBP can reduce oxidative stress damage in brain tissue caused by chronic cerebral ischemia.

The central cholinergic system is an important pathway for learning and memory. ACh is a cholinergic neurotransmitter that participates in information transmission and processing. The synthesis of ACh is mainly conducted in the nerve endings by choline and acetyl coenzyme A in the presence of ChAT, which is mainly hydrolyzed by true cholinesterase. Cognitive processes of rats are associated with increased ACh levels and ChAT activity in the cortex and hippocampus (Ruan et al., 2010; Park et al., 2012; Weinstock et al., 2013). Numerous studies have confirmed that reduced function of the cholinergic pathway in new cortical or hippocampal parts is involved in the loss of learning and memory in senile dementia (Kelley et al., 2011; Allard et al., 2012; Haense et al., 2012). Other studies have found that enhancing ACh content and ChAT activity in the hippocampus can improve cognitive impairment in rats with dementia (Jiang et al., 2001; Witty et al., 2012). The present study showed that chronic cerebral ischemia attenuated the activity of ChAT in the cortex and hippocampus of rats. Furthermore, NBP increased the activity of this enzyme, indicating that it can up-regulate the synthesis of ACh thus enhance cholinergic function. This effect has also been observed by Shen et al. (2007). True cholinesterase controls information transmission of cholinergic synapses by hydrolysis of ACh. Studies have shown that true cholinesterase activity in the brain tissue of senile dementia patients increases significantly, and the increased activity enhances ACh decomposition, leading to a decline in cognitive ability (Allan et al., 2013; Zaganas et al., 2013). However, in the present study, no change in brain tissue true cholinesterase was evident in the model group compared with the sham-operated group. Moreover, NBP did not significantly affect the activity of true cholinesterase in brain tissue. Therefore, these results suggest that the effects of NBP on abnormal activity cannot be determined by true cholinesterase.

Our research group has previously investigated the mechanisms of oxidative stress damage and reduced cholinergic function in rats (Zhao et al., 2008). The present results have shown that reducing oxidative stress is an important mechanism by which NBP improves spatial cognitive ability in rats with cerebral ischemia. Under physiological conditions, the cyclin dependent protein kinase5/p35 (CDK5/p35) complex is close to the cytoplasmic membrane substrate (tau protein). In the presence of A β , intracellular Ca²⁺ increases, activating calpain and resulting in the degradation of p35 into p25. p25 is a strong activator of CDK5. p25/CDK5 complexes leave the cytoplasmic membrane resulting in tau protein phosphorylation near microtubules. Abnormal phosphorylation of tau protein leads to paired helical filament/neurofibrillary tangles (PHF/NFT), microtubule dissociation, and neuronal dystrophy, causing neuronal death. A β is generally recognized as the most important factor in the pathogenesis of Alzheimer's disease and is neurotoxic *via* its disruption of cellular Ca²⁺ homeostasis, induction of oxidative stress,

increase in tau protein phosphorylation, and upregulation of the pro-apoptotic gene, BAX (Song et al., 2011; Arora et al., 2013; Hannula et al., 2013; Sutherland et al., 2013). A number of studies have demonstrated that A β induces tau protein phosphorylation *in vitro*, thereby destroying microtubule stability and causing injury, damaging axonal transport, and inducing neuronal death (Cancino et al., 2008; Marwarha et al., 2010). Previous studies on amyloid precursor protein transgenic mice and Ganges river monkeys injected with A β into the cerebral cortex have demonstrated that the phosphorylation of tau protein and/or neuronal death is increased (Calhoun et al., 1998; Masliah et al., 2001). These results suggest that the deregulated level of A β may be the initiation factor for the pathogenesis of vascular dementia in the present study. The present results showed that NBP stabilized the level of A β and NBP inhibited the over-expression of A β in rat brains with chronic cerebral ischemia. Therefore, by inhibiting over-expressed A β in rat brains with chronic cerebral ischemia, NBP may have subsequently reduced the phosphorylation of tau protein, stabilized tubulin, reduced axonal injury. This thereby improved neuronal injury and brain function, and hence learning and memory. The improvement in learning and memory by NBP in chronic cerebral ischemia rats warrants further investigation on its effect on tau phosphorylation, tubulin, and axonal injury as potential mechanisms.

The cerebral cortex is the center of learning and memory. The hippocampus plays a major role in the storage of new memories. Numerous studies have demonstrated the importance and specificity of the hippocampus in spatial learning using the MWM task (Yao et al., 2004; Rosi et al., 2008; Lui et al., 2011). Spatial learning defects are evident after delayed neuronal death in the CA1 hippocampal region (Hara et al., 2007; Kumaran et al., 2008). Therefore, hippocampal cells in place navigation tests are considered to be the original basis of spatial memory ability and the key in acquiring and extracting spatial information, followed by consolidation and storage of memory (Bernasconi-Guastalla et al., 1994; Yao et al., 2004; Vasconcellos et al., 2005). In the present study, chronic cerebral ischemia induced abnormal neuronal morphology in the cortex and hippocampus, and this response was inhibited by NBP. Therefore, these results suggest that NBP improves spatial cognitive function by preventing abnormal cerebral and hippocampal neuronal morphology in rats with cerebral ischemia.

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Author contributions: Zhao WH and Wang HQ were responsible for the study concept and design, acquired the supporting funds, supervised the experiments, wrote the manuscript, and reviewed the study. Luo C performed the experiments and analyzed experimental data. Wang J and Gong J performed the experiments. Li B performed the pathological experiments and

analyzed the pathological slices. Gong YX provided technical and information support. Wang J partially performed the experiments and data analysis. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

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