L-3-n-butylphthalide improves cognitive impairment induced by chronic cerebral hypoperfusion in rats

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Running title page

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Nonstandard abbreviations:
AD: Alzheimer’s disease; NBP: 3-n-butylphthalide; BCCAO: bilateral permanent occlusion of the common carotid arteries; VaD: Vascular dementia; CBF: cerebral blood flow; MCAO: middle cerebral artery occlusion; WM: white matter; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde, AChE: acetylcholinesterase; GFAP: glial fibrillary
acidic protein; ChAT: Choline acetyltransferase; DAB: 3,3'-diaminobenzidine tetrahydrochloride;
K-B: Klüver-Barrere.

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Abstract

3-n-butylphthalide (NBP) may be beneficial for the treatment of ischemic stroke with multiple actions on different pathophysiological processes. In the present study, we investigated the effect of NBP isomers on learning and memory impairment induced by chronic cerebral hypoperfusion in rats. Male Wister rats were orally administered 10 and 30 mg/kg l, d or dl-NBP daily for 23 days after bilateral permanent occlusion of the common carotid arteries (BCCAO). Rats receiving 10mg/kg l-NBP performed significantly better in tests for spatial learning and memory and had attenuated cerebral pathology, including neuronal damage, white matter rarefaction, and glial activation compared to controls. Furthermore, 10mg/kg l-NBP treated rats had significantly higher choline acetyltransferase (ChAT) activity, decreased cortical lipid peroxide and reduced hippocampal superoxide dismutase (SOD) activity, compared to vehicle controls. However, d- and dl-NBP did not show significant beneficial effects. The present findings demonstrate that the beneficial effects of l-NBP on hypoperfusion-induced cognitive deficits may be due to preventing neuropathological alterations, inhibiting oxidative damage and increasing Ach synthesis. Our results strongly suggest that l-NBP has therapeutic potential for the treatment of dementia caused by decreased cerebral blood flow.
Introduction

Senile dementia, a progressive aging-related disease, has become an important medical and social problem due to the increase in the number of elderly. Vascular dementia (VaD), as the second most common form of dementia in the elderly, accounts for approximately 20-30% of dementia cases (Giacobini, 2004). VaD is a syndrome presenting with both cognitive and non-cognitive symptoms. Cognitive deficits of VaD include memory deficits, executive function damage, slow processing of information and behavioral and mood abnormalities (Micieli, 2006). The underlying basis of VaD is complicated, as cerebral multi-infarct, cerebrovascular diseases, arterial hypotension, cardiac arrest and haemorrhagic may all result in VaD (Micieli, 2006). At present, no specific drug exists to prevent, delay or cure VaD.

It is well known that a decrease in cerebral blood flow (CBF) precedes the onset of VaD (Roman et al., 1993), and chronic cerebral hypoperfusion may be a trigger for VaD and the accompanying cognitive deficits (Kasparova et al., 2005). As well, the decrease in CBF relates to the cognitive impairment seen in AD (Kasparova et al., 2005). Bilateral permanent occlusion of the common carotid arteries (BCCAO) in rats results in a significant reduction in cerebral blood flow therefore it is a useful model of chronic cerebral hypoperfusion (Tsuchiya et al., 1993). This animal model exhibits learning and memory impairments resembling those found in AD and VaD, accompanied by neuronal degeneration and microvascular abnormalities (Farkas et al., 2004).

It has been widely accepted that chronic cerebral hypoperfusion induces oxidative stress damage and brain energy failure in neuronal tissues and cells, at least partially due to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aliev et al., 2003; de la Torre and Aliev,
2005). ROS are directly toxic to neurons and initiate a free-radical-mediated chain reactions resulting in neuronal system damage.

Some clinical symptoms and pathological changes seen in VaD are similar to AD, with cholinergic function abnormalities found in both diseases. In recent clinical reports, acetylcholinesterase (AChE) inhibitors, including rivastigmine, galantamine and donepezil which are used to treat AD patients, showed a therapeutic effect on both cognitive and non-cognitive abilities in VaD patients (Erkinjuntti, et al., 2002; Giacobini, 2004), suggesting that an increase in endogenous acetylcholine (Ach) is beneficial.

Cerebral white matter (WM) lesions are the main pathological hallmarks of Binswanger’s disease, a type of VaD, and are the result of chronic cerebral hypoperfusion (Pantoni and Garcia, 1997; Wakita et al., 2002). The typical neuropathological changes of WM lesions are diffuse demyelination and axon loss (Fisher, 1989; Wakita et al., 2002). The rat model of BCCAO shows marked white matter rarefaction and glial cell activation (Wakita et al., 1994; Wakita et al., 1998). WM lesions are thought to contribute to cognitive impairment and have a strong relationship with oxidative stress, apoptosis and inflammatory damage (Wakita et al., 1994; Tanaka et al., 2001; Watanabe et al., 2006).

L-3-n-butylphthalide (l-NBP) was extracted as a pure component from seeds of *Apium graveolens Linn*. Afterwards, dl-3-n-butylphthalide (dl-NBP) was synthesized and received approval by the State Food and Drug Administration (SFDA) of China for clinical use in stroke patients in 2002. NBP is a chiral compound, and it contains both l- and d- isomers which have been recently synthesized (Fig.1). Previous studies showed that NBP was beneficial as a treatment for stroke and involved multiple actions including improving microcirculation in pial arterioles in rats which had...
undergone middle cerebral artery occlusion (MCAO) (Xu and Feng, 1999), decreasing the area of cerebral infarct in focal cerebral ischemic rats (Liu and Feng, 1995; Peng et al., 2005) and improving energy metabolism in mice with complete brain ischemia (Feng et al., 1995). Additionally, NBP has been shown to decrease oxidative damage (Dong and Feng, 2002), reduce neuronal apoptosis (Chang and Wang, 2003) and inhibit the inflammatory response (Xu and Feng, 2000) in rats that experienced focal cerebral ischemia. Although the positive effects of NBP on cerebral ischemia and cerebral infarct have been verified in ischemic patients and animal models, no study has investigated if NBP could be beneficial as a treatment for VaD. In addition, the effects of l- and d-isomers of NBP in this area were still not clear.

The purposes of this study were, therefore, to evaluate whether the long-term administration of NBP isomers could attenuate the learning and memory deficits induced by chronic cerebral hypoperfusion. Furthermore, the possible mechanisms underlying its effect, including those associated with white matter lesions, oxidative stress, Ach synthesis and neuronal loss were examined.
Methods

Chemicals and Materials

Racemic NBP (purity >98%) and the enantiomers of NBP (l- and d-) were synthesized and separated (purity >98%) by the Department of Medical Synthetic Chemistry, Institute of Materia Medica (Beijing, China). The racemic n-butylphtalide was synthesized at first and then was isolated by chemistry into the l-3-n-butylphtalide and d-3-n-butylphtalide. The isomer, l-3-n-butylphtalide, was used in this study with the properties of [α]D>66.49°, optically purity>98% and chemical purity>98%, measured by chiral gas chromatogram and chiral HTLC as well as NMR, MS. They were diluted with vegetable oil. The superoxide dismutase (SOD) activity kit, the catalase (CAT) activity kit, glutathione peroxidase (GSH-Px) activity kit, malondialdehyde (MDA) kit, and acetylcholinesterase (AChE) activity kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The Polyclonal antibody GFAP was obtained from DakoCytomation (Glostrup, Denmark). Klüver-Barrera Luxol fast blue was bought from Sigma (Saint Louis, MO).

Animals

Male Wistar rats (280-300 g) were subjected to surgery. The animals used in this study were group-housed in our animal room maintained at 23±1°C with a 12h light/dark cycle and were allowed free access to water and food. All experiments were performed in accordance with institutional guidelines of the Experimental Animal Center of the Chinese Academy of Medical Sciences.

Surgery

Rats were anesthetized using pentobarbital-Na (50mg/kg, i.p.). A ventral mid-line incision was
made and the bilateral common carotid arteries were exposed and gently separated from the carotid sheath and vagus nerve. In the rats assigned to the ischemic groups, each artery was double ligated with 5-0 silk suture. During the surgical procedure, the animal’s body temperature was kept stable at 36.5±0.5°C using a heating pad. As sham-operated controls, another group of rats received the same operation without ligation.

**Drug Administration and Experimental Design**

Nine days after surgery, the hypoperfused rats were randomly divided into six groups. Each group consisted of 12-14 animals with identical mean body weights. Daily oral administration of l-NBP (10 and 30mg/kg), dl-NBP (10 and 30mg/kg), d-NBP 30 mg/kg or vehicle (vegetable oil) started on day 10 post-surgery and lasted for 23 days. (until the termination of the experiment on day 33). During the last five days of drug administration, spatial learning and memory was assessed in all rats. During the behavioral test, drugs were administered 40 minutes before the water maze training. The experimental design is shown in Figure 2.

**The Morris Water Maze Task**

The Morris water maze (MWM) task (Morris, 1984) was carried out from day 29 after surgery to day 33. The apparatus consisted of a circular water tank. The tank is 120cm in diameter and 50cm high. To make the water opaque, 1 kg of powdered milk was added and its temperature was kept at 23±1°C. A translucent acrylic platform (10cm in diameter) was used and located in the center of the NE or SW quadrant during training. The top of the platform was approximately 1.5 cm below the surface of water. Spatial training of the hidden platform in the water maze was performed for 5 consecutive days. Each rat received two trials per day for 5 days with the intertrial interval being 1
min. The starting position (East, West, South or North) for each trial was pseudo-randomly chosen and counterbalanced across all experimental groups. Half of the animals were trained using the NE platform position and the other half for the SW position. The experimenter conducting the MWM was blind to the treatment groups.

The rats were gently placed into the water, facing the side-walls of the maze from one of the four pre-planned starting position (East, West, South or North). Swimming paths of the rats were monitored by a video camera linked to a computer through an image analyzer. For each training trial, the latency to escape onto the hidden platform and pathlength were recorded. The rats were given a maximum of 60 sec to find the hidden platform. If the rat failed to find the platform within 60 sec, the training was terminated and a maximum score of 60 sec was assigned, and it was guided to the hidden platform by hand. The rat was allowed to stay on the platform for 10 sec before it was removed from the water.

A probe test, in which the hidden platform was removed, was conducted immediately after the last trial on training day 5 (or 33 days after surgery). The rats were released into the water from the opposite quadrant with respect to the training quadrant. The rats were allowed to swim for 60 sec in the pool before they were removed from water by hand.

**Biochemical Examinations**

Following the behavioral testing, eight to nine animals of each group were decapitated under anesthesia, the brains removed and the cortex and hippocampus were dissected on ice. The tissues were rapidly frozen and stored at -80°C until assayed. Each brain region was weighed and homogenized with homogenizer in 9 volumes of ice-cold saline and the homogenate was further
diluted with an appropriate buffer solution for the determination of the relevant biochemical index. Choline acetyltransferase (ChAT) activity was determined using the spectrometric method of Wolfgram (Wolfgram, 1972). The activities of AChE, SOD, CAT, GSH-Px, and the MDA (a product of lipid peroxidation) level, were determined using specific kits.

**Histological Analysis**

Following the behavioral experiments, four or five rats from each group, were randomly chosen, anesthetized with pentobarbital-Na (50 mg/kg, i.p.), perfused with saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were rapidly removed, fixed in 4% paraformaldehyde for 4 hr, and embedded in paraffin following standard methods. Coronal sections (5µm) were cut. Sections were stained with Hematoxylin-eosin and Klüver-Barrera (K-B). The severity of white matter changes was graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and disappearance of myelinated fibers (grade 3) by two independent investigators blind to the treatment group (Wakita et al., 1994). Immunohistochemistry was performed according to the avidin-biotin-peroxidase complex (ABC) method, using a polyclonal anti-GFAP, antibody (Masumura et al., 2001). Briefly, the dewaxed and rehydrated sections were treated with 3% H2O2 to block endogenous peroxidase. After rinsing for 15 min in PBS, the sections were incubated with primary antibody for 1 hour at room temperature. Sections were washed in PBS for 15 min and incubated with Envision™ at room temperature for 30 min, followed by rinsing in PBS for 15 min. Immunoreactivity was detected using 3,3’-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. The sections were counterstained with hematoxylin. An Olympus microscope with a video camera system linked to a
computer was used to obtain digitized images. The person evaluating the histology was blinded to
the treatment group of the rats.

**Statistical Analysis**

The results are expressed as mean±SEM. The main treatment effect on the escape latency in the
water maze was analyzed using analysis of variance (ANOVA) with repeated measures. The Fishers
LSD *post hoc* test was used to test the differences between two groups. One way ANOVA was used
to analyze the probe trial data and the biochemical data. Performance in probe trials was analyzed
using a one sample t-test. Statistical significance was accepted at p<0.05.
Results

L-NBP significantly improved learning impairment induced by BCCAO

Figure 3 shows the results of the time required to find the hidden platform (escape latency) of all rats during water maze acquisition training. Firstly, the ANOVA revealed a significant day effect on escape latency \([F(4,352)=38.18, p<0.001]\) within the groups, suggesting that all the rats improved their spatial learning effectively across the 5-day training period. Secondly, we found a significant main treatment effect \([F(6,88)=6.33, p<0.001]\) on the escape latency data, demonstrating that the drugs are effective in improving learning in the hypoperfused rats. Interestingly, the ANOVA also revealed a significant treatment by day interaction effect \([F=2.01, p<0.05]\).

Since there is a highly significant drug-treatment effect, we performed post-hoc analysis on the escape latency data. Compared to the sham rats, the hypoperfused vehicle group was significantly impaired with regards to escape latency \((p<0.001)\), suggesting that hypoperfusion successfully induces learning deficit in our rat BCCAO model. Additionally, the 10mg/kg l-NBP treated rat group did not differ from the sham rats \((p>0.05)\), but differed significantly from the hypoperfused group \((p<0.01)\) on the escape latency. These results indicated that daily administration of 10mg/kg l-NBP significantly rescues learning impairment caused by BCCAO. We observed that, although the therapeutic effect of 30mg/kg l-NBP was very close to that of 10mg/kg \((p=0.079)\), they did not differ significantly from the hypoperfused group \((p>0.05)\), suggesting that the higher dose of l-NBP has some beneficial effects on learning impairment, but can not fully rescue it. Neither d-NBP nor dl-NBP (10mg/kg or 30mg/kg) had any effects on spatial learning of the ischemia rats \((p > 0.05)\).

Furthermore, performance during the search for the hidden platform in all groups from days 3-5...
was analyzed, with the results showing a marked treatment effect $[F(6,88)=8.66, p<0.001]$, and the performance of the sham treated animals ($p<0.001$) and 10mg/kg l-NBP treated animals ($p<0.05$) was superior to the performance of the hypoperfused vehicle animals. However, the groups treated with l-NBP 30mg/kg, dl-NBP 10 or 30 mg/kg, d-NBP 30mg/kg did not significantly differ from the control group (data not shown).

**L-NBP significantly rescued memory deficits caused by BCCAO**

To investigate the effects of L-NBP on memory deficits induced by BCCAO, we conducted a probe test immediately after the 5 days of training in the water maze. As shown in Figure 4, we plotted the performance of different treatment groups during the probe trial by analyzing percentage time spent in the target quadrant where the hidden platform had previously been available. First of all, the analysis of variance indicated a significant treatment effect on the quadrant occupancy $[F(6,88)=2.83, p<0.05]$, suggesting that the drugs we administered can significantly affect memory performance of BCCAO-treated rats. We thus performed a number of *post-hoc* analyses.

Firstly, we found that chronic cerebral hypoperfusion successfully induces a memory deficit in the BCCAO model ($p<0.05$). Although this impairment is not highly striking, i.e. the sham group showing 29.5% and the hypoperfusion vehicle group showing 23.8% quadrant occupancy, we believe that this is due to the weak training protocol (2 trials/day, 5 days). Nonetheless, our sham treated rats were still able to show significant preference for the target quadrant, relative to chance performance ($p<0.05$, one sample t-test) Secondly, 10mg/kg l-NBP attenuated the memory deficit in BCCAO-treated rats ($p<0.05$). We found that the 10mg/kg l-NBP treated rats spent significantly more time in the target quadrant than chance performance ($p<0.05$, one sample t-test). Thirdly,
although the 30mg/kg l-NBP treated group also showed better-than-chance performance (28.4%, p=0.07, one sample t-test), it did not differ from the hypoperfused vehicle rats (p>0.05). Again, this suggests that high dose l-NBP has beneficial effects but it can’t fully rescue memory impairment. Finally, we found that different doses of dl-NBP and d-NBP showed no significant effects on the ischemia rats (p > 0.05). In order to exclude the effect of the surgery and chronic hypoperfusion on the motor function, the swimming speed was observed. There was no difference amongst the groups (data not shown).

**L-NBP significantly increased ChAT activity in BCCAO-treated rats**

The central cholinergic systems play an important role in cognitive function. It was demonstrated that BCCAO reduced the level of ACh in rat brain (Ni et al., 1995). After behavioral testing, the rats were sacrificed and the activities of ChAT and AChE in the cortex and hippocampus were measured. The results are shown in Table.1. We found that BCCAO caused a significant decrease of ChAT activity in the cortex (34.4% compared to sham group animal, p<0.05). This indicates that cholinergic function was impaired by chronic cerebral hypoperfusion. However, l-NBP at 10mg/kg significantly alleviated the decrease of ChAT activity induced by cerebral hypoperfusion (compared to vehicle hypoperfused animals, p<0.05). 30mg/kg l-NBP, d- or dl-NBP had no affect on ChAT activity. These data support the behavioral test results as only 10mg/kg l-NBP improved the learning and memory impairment. ChAT activities in the hippocampus did not differ among the sham, vehicle or NBP-treated rats, suggesting that hypoperfusion caused less damage to the hippocampus. Furthermore, no changes in AChE activities were observed in the cortex or hippocampus in any groups (the data of d- and dl-NBP treatment are not shown).
L-NBP attenuated the oxidative damage induced by BCCAO

SOD, GSH-Px, and catalase are important antioxidant enzymes, and are responsible for protecting brain tissue from oxidative stress injury (Sozmen et al., 1994). The brain tissue of the rats that had completed the water maze test was examined for the activities of these antioxidant enzymes. The results are shown in Table 2. Hippocampal SOD activity in the vehicle group was increased by 40.2% as compared to the sham group (p<0.05), but l-NBP 10 mg/kg markedly attenuated the changes in SOD activity induced by hypoperfusion (compared to the vehicle group, p<0.05). L-NBP (30mg/kg), dl- and d-NBP had no effect on BCCAO-induced SOD activity in the hippocampus. In the cortex, the SOD activity among all groups did not differ, this may be because the basal level of SOD in the cortex of the sham rats is relatively high, and the hypoperfusion only caused a small increase.

After BCCAO, the GSH-Px activity in the hippocampus of vehicle-treated rats was decreased by 11% compared to the sham-treated group, and l-NBP at 10mg/kg dose reversed this decrease. However, significance was not reached. The activities of catalase in hippocampus and cortex homogenates, and the activities of GSH-Px in the cortex did not differ among the groups.

Toxicity caused by hypoperfusion is accompanied by increased lipid peroxides. MDA, a product of lipid peroxidation, was used as an index of membrane oxidative injury. After BCCAO, the MDA concentration was significantly increased by 24.9% in the cortex of the vehicle group compared to the sham group (p<0.001). However, this increase in MDA level was significantly attenuated when rats received treatment of either 10 or 30 mg/kg l-NBP (p<0.001 and p<0.01). Dl- and d-NBP treatment did not inhibit the increased MDA level. In the hippocampus, although MDA levels did not
differ among groups (Table 2), there was a moderate increase after the hypoperfusion. We found that 10 mg/kg L-NBP only showed a small effect on reducing MDA level in the hypoperfused rats. The dl-and d-NBP data are not shown.

**L-NBP inhibited neuronal loss, white matter lesions and astrocyte activation**

Typical neuropathological changes were observed in the cortex and hippocampus five weeks following BCCAO. Neuronal loss, shrinkage and dark staining of neurons were observed in the CA1 area of the hippocampus as well as in the cortex of vehicle treated rats. In the sham group, there no pathological abnormalities were seen. Administration of L-NBP at 10 mg/kg attenuated the chronic hypoperfusion-induced neuronal damage seen in vehicle treated rats (Fig. 5).

The different areas of white matter have differing vulnerabilities to chronic cerebral hypoperfusion. The optic nerve and optic tract exhibited the most severe rarefaction, with less intense changes being observed in the medial part of the corpus callosum adjoining the lateral ventricle, the anterior commissure, the internal capsule and the fiber bundles of the caudoputamen (Wakita et al., 1998). This same pattern was also observed in the current experiments. Vehicle-treated rats showed severe rarefaction in the optic tract. In addition, there was an increase in the crookedness and disarrangement of myelin fibers in the corpus callosum of the vehicle group. Chronic treatment of 10mg/kg L-NBP after BCCAO resolved the lesions in optic tract and corpus callosum (Fig. 6).

GFAP-positive astrocytes were rare in the hippocampus and white matter of the sham-treated rats. However, in vehicle-treated rats which had undergone BCCAO, activated astrocytes were markedly increased in the hippocampus and caudate-putamen. Chronic administration of 10mg/kg
l-NBP substantially reduced the number of GFAP immunolabeled astrocytes in these areas (Fig. 7).

The other treated groups did not have any observable neuropathological improvements (data not shown).
Discussion

The rate of cerebral perfusion and the morphological integrity of the circulatory network of the brain play a critical role in the maintenance of proper neuronal function and related memory capacity (Farkas et al., 2002). In addition to VaD, growing evidence has demonstrated that some of the cognitive decline seen in AD patients may be due to hypoperfusion (de la Torre JC, 2004). The most frequently used model of chronic cerebral hypoperfusion is permanent bilateral ligation of the common carotid arteries of rats. Decreased cerebral blood flow has been observed in 15 brain regions after 1 week of BCCAO (Tsuchiya et al., 1993). These regions include the cortex and hippocampus, which amongst other regions play important roles in learning and memory (Tsuchiya et al., 1993). In chronic cerebral hypoperfusion, the white matter is preferentially damaged, accompanied by an increase in reactive astroglia and activated microglia (Wakita et al., 1994). It has also been shown that damage is severe in the optic tract, but moderate in the medial part of the corpus callosum, anterior commisure, internal capsule and caudate-putamen (Wakita et al., 1998). Microglia activation may play a role in the pathogenesis of white matter lesions through the release of cytokines and cytotoxins such as proteases, reactive oxygen radicals and nitrogen intermediates (Guastadisegni et al., 1997). Further, reactive astrocytes are known to express inducible nitric oxide synthase in transient global ischemia (Endoh et al., 1994).

In the present study, we observed that NBP isomers ameliorated many of the damaging changes seen following chronic cerebral hypoperfusion in rats. The main findings were that l-NBP at a lower dose improved the cognitive deficits, white matter lesions and neuronal damage induced by hypoperfusion. The neuroprotective action of l-NBP may be due to inhibiting oxidative damage,
reducing the inflammatory reaction and enhancing cholinergic functions.

Previous animal experiments demonstrated that chronic cerebral hypoperfusion could induce learning and memory deficits (Pappas et al., 1996). NBP has exhibited various neuroprotective effects in vitro and in vivo models, therefore, we chronically administered NBP isomers to hypoperfused rats. Interesting, only l-NBP ameliorated the impairment seen in the reference memory and probe trials. At a dose of 10mg/kg, l-NBP showed a significant reversal effect, while at a higher concentration of 30mg/kg, there was improvement but it did not reach significant levels. In beta-amyloid (Aβ) intracerebroventricular infused rats, we also found that the lower dose of l-NBP showed stronger effects to improve learning and memory deficits (data not shown). Dl-NBP and d-NBP had no significant effects on the cognitive deficits.

Hypoperfused rats treated with vehicle alone showed significant neuronal cell damage and white matter rarefaction, such as vacuolation in the optic tract and disarrangement of the myelin fibers in the corpus callosum. Furthermore, many reactive astrocytes were apparent in the hippocampus, caudate-putamen and corpus callosum. Chronic treatment with l-NBP 10mg/kg ameliorated the white matter damage and neuronal cell death in the cortex and hippocampus. Moreover, GFAP-positive astrocytes were also reduced. These results are consistent with the notion that histological abnormalities are indicative of the decreases in intellectual function such as learning, memory and spatial discrimination.

There is evidence showing that free radicals are capable of mediating neuron degeneration and death, and are possibly involved in the pathogenesis of neuron death in neurodegenerative disease such as AD and VaD (Makesbery, 1997). Free radicals are normal products of cellular aerobic
metabolism. However, when the production of free radicals increases or the defense mechanisms of the body are decreased, these radicals cause cellular dysfunction by attacking the polyunsaturated sites found in biological membranes leading to lipid peroxidation. In the hypoperfused rats, the level of MDA in the cortex was significantly increased, but that in the hippocampus was not significantly increased. It may be due to that the hypoperfusion has caused more severe damage to the cortex than to the hippocampus. We found that daily treatment of l-NBP 10mg/kg significantly reduced the MDA level in the cortex. Meanwhile, we observed that 10mg/kg l-NBP also showed small improving effects on the MDA levels in the hippocampus, although not significant. This may be because the hippocampus is less damaged by the hypoperfusion.

In this study, we have reported that the activity of SOD was significantly elevated in the hippocampus of the hypoperfused rats. However, there was only a small change in the SOD activity in the cortex. It is possible that a compensatory rise in antioxidant activity occurs in response to increased free radical generation. Treatment of the hypoperfused rats with l-NBP at 10 mg/kg showed significant effects on reversing abnormal SOD activities in the hippocampus. Since cortical SOD activity was relatively unimpaired, this is why 10 mg/kg l-NBP only showed a modest improvement.

Central cholinergic functions are known to be closely linked to intellectual abilities such as learning and memory. Like the pathology of AD, VaD patients usually show cholinergic abnormalities and serious cognitive disturbances (Blokland, 1995). The acetylcholine (Ach) concentration in the cerebrospinal fluid of VaD patients has been shown to be significantly lower than that in controls, and significantly correlated with dementia scale scores (Tohgi et al., 1996).
Postmortem studies have shown that brain ChAT activities in VaD patients were decreased in the cortex, hippocampus and striatum. In this study, we found that the ChAT activity was reduced by 34.4% in the cortex of hypoperfused rats compared to the sham group. Treatment with 10mg/kg l-NBP significantly improved the decrease of ChAT activity in the cortex. Since the ChAT activity in the hippocampus is not significantly reduced in the hypoperfused group, we did not see a significant improvement. This may suggest that l-NBP significantly improves impaired ChAT levels, but does not affect normal ChAT level. Interestingly, a previous report also showed that hypoperfusion causes a significant decrease in the cerebral levels of Ach (Murakami et al., 2005). The beneficial effects of l-NBP on the ischemia-induced cognitive deficits may be due to its enhancing effects on central cholinergic tone to supplement impaired acetylcholine synthesis.

At present, dl-NBP is being used in clinical practice in China for the treatment of ischemic patients. Our previous studies also showed that l-NBP significantly improved microcirculation in pial arterioles (Xu and Feng, 1999), reduced the area of cerebral infarct (Peng et al., 2005), improved mitochondrial function (Xiong and Feng, 2000; Dong and Feng, 2002), decreased oxidative damage (Dong and Feng, 2002), reduced neuronal apoptosis (Chang and Wang, 2003), inhibited the inflammatory response (Xu and Feng, 2000) in experimental ischemic animal models. However, further studies showed that d-NBP may antagonize the beneficial effects of l-NBP on reducing the release of cytochrome C, decreasing caspase-3 activation, and inhibiting DNA fragmentation induced by transient focal cerebral ischemia (Chang and Wang, 2003). In addition, we also found that l-NBP is the most potent form in decreasing cerebral infarct volume in MCAO rats and inhibiting platelet aggregation and thrombus formation (Peng et al., 2004; Peng et al., 2005). The
mechanism of actions of the different chiral isomers is still unclear. The existence of stereoselectivity between biological macromolecules and small drug molecules may be one answer.

In the present study, we found that only l-NBP improved the learning and memory deficits in hypoperfused rats. We did not find any behavioral improvement in dl-NBP or d-NBP treated hypoperfused rats. These results strongly suggest that l-NBP has therapeutic potential for dementia caused by decreased cerebral blood flow and may be a potential new anti-dementia agent. The multi-targets action might involve in the neuroprotective effects of l-NBP. Besides inhibiting oxidative stress and inflammatory reaction discussed in the present research, we consider that increasing cerebral blood flow and enhancing energy metabolism, ameliorating mitochondrial failure and inhibiting neuronal apoptosis might be the related mechanisms in the neuroprotective effect of l-NBP. In this study, treatment with 10mg/kg l-NBP ameliorated the learning and memory deficits after BCCAO in rats. However, 30mg/kg l-NBP had no significant effects. Thus, we deduce that the neuroprotective dose-response curve for l-NBP might be U-shaped, similar to those of other cognitive enhancers (Sakakibara et al., 2000). Although the reasons underlying these findings are not yet clear, it suggests that it cannot be assumed that increasing the dosage of a putative neuroprotective drug will lead to improved neuroprotection. This may explain why certain drugs, shown to be protective in preclinical studies, are not found to be protective in clinical trials and underscores the need for optimal dosing regimens, including dosage and duration, from preclinical trial data.

In conclusion, our findings suggest that l-NBP attenuates the learning and memory deficits induced by chronic cerebral hypoperfusion, mainly due to preventing white matter lesions,
decreasing oxidative damage, improving cholinergic function and inhibiting inflammatory responses.
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Legends for Figures

Figure 1. Chemical structures of 3-n-butylphthalide (NBP).

Figure 2. Experimental design.

Figure 3. Effects of l-NBP on water maze performance deficits induced by bilateral permanent occlusion of the common carotid arteries (BCCAO) in rats. (A) The vehicle group spent more time to find the hidden platform in the training phase than the sham group, indicating the cerebral hypoperfusion significantly impaired spatial learning in rats. (Two way ANOVA, p<0.001 sham group vs. vehicle group). (B) After 5 days training, l-NBP significantly decreased the latency to escape to the platform compared to the vehicle group (Two way ANOVA, p<0.01 10mg/kg l-NBP treated group vs. vehicle group). (C) L-NBP at the dose of 30mg/kg shortened the escape latency faster than vehicle, but there was no statistically significant difference. (D) Other treatment groups exhibited no difference in finding the hidden platform compared to the vehicle group. *p<0.05, **p<0.01 vs vehicle group. All values are means±SEM (n=12-14).

Figure 4. Effects of l-NBP on the spatial memory impairments induced by bilateral permanent occlusion of the common carotid arteries (BCCAO) in rats. (A) showed the time that sham, vehicle and 10mg/kg l-NBP treated groups spent in the target quadrant (in which the platform had been placed during the training phase) and the mean time for the other three quadrants during the probe trial. The sham treated animals and 10mg/kg l-NBP treated animals showed preferential searching in the target quadrant relative to chance (25%) performance (p<0.05). After BCCAO, the vehicle group spent less time in the target quadrant than the sham group, indicating the spatial memory was significantly impaired in hypoperfused rats (p<0.05). 10mg/kg l-NBP treated rats spent more time in
the target quadrant than did the vehicle rats (p<0.05). (B) showed that the other groups didn’t show better than chance performance (n.s). All values are means±SEM (n=12-14). *p<0.05 vs vehicle group.

Figure 5. L-NBP attenuated the morphologic alterations in the hippocampus CA1 subfield (A, B and C) and cortex (D, E and F) after bilateral permanent occlusion of the common carotid (BCCAO). No remarkable neuronal abnormalities were observed in brains from the sham group (A and D). But after BCCAO, all brains of the vehicle group (B and E) showed neuronal degeneration, such as neuronal loss, shrinkage and dark staining of neurons in cerebral cortex and hippocampus. Treatment with l-NBP 10mg/kg (C and F) markedly attenuated pathological damage. Magnification x 400.

Figure 6. Klüver-Barrera Luxol fast blue staining of the white matter in the corpus callosum (A, B and C) and the optic tract (D, E and F) of rats on day 33 after bilateral permanent occlusion of the common carotid arteries (BCCAO). In sham-operated (A and D) animals, no cell damage was observed. After BCCAO, the vehicle group (B and E) showed significant vacuolation in the optic tract and disarrangement of myelin in the corpus callosum. The extent of the white matter rarefaction was profoundly reduced in the l-NBP 10mg/kg treated group (C and F). Magnification x 400.

Figure 7. GFAP-positive astrocytes in 10mg/kg l-NBP treated rats were reduced compared to vehicle-treated rats. In the sham group, astrocytes were rarely detected. After bilateral permanent occlusion of the common carotid arteries (BCCAO), astrocytes significantly increased in the hippocampus and caudate-putamen. In the l-NBP treated group, the GFAP-positive astrocytes were much fewer in number. Immunohistochemical staining for GFAP is shown in the hippocampus (A, B
and C) and caudate-putamen (D, E and F). (A and D): Sham-operated group; (B and E): Vehicle group; (C and F): l-NBP 10mg/kg treated group. Magnification x 200.
Table 1. Effects of l-NBP on choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities in rats on Day 33 after permanent bilateral ligation of the common carotid arteries (BCCAO). Rats were decapitated 40 min after the last administration. From Day 10 to Day 33, different doses of l-NBP (10 and 30mg/kg) were administered orally once per day.

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<th>Group</th>
<th>Choline acetyltransferase</th>
<th>Acetylcholinesterase</th>
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<tbody>
<tr>
<td></td>
<td>(% of Sham )</td>
<td>(nmol/min/mg protein )</td>
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<tr>
<td></td>
<td>Cortex</td>
<td>Hippocampus</td>
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<tr>
<td>Sham</td>
<td>100.0±13.3</td>
<td>100.0±7.2</td>
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<tr>
<td>Vehicle</td>
<td>65.6±15.1#</td>
<td>102.6±15.1</td>
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<tr>
<td>l-NBP(10mg/kg)</td>
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<td>110.0±13.5</td>
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<td>l-NBP(30mg/kg)</td>
<td>66.3±5.7</td>
<td>96.8±12.3</td>
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All values are means±SEM (n=8-9). #p<0.05 vs Sham group. *p<0.05 vs Vehicle group.
Table 2. Effects of l-NBP on the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), and lipid peroxide (MDA) levels of cortex and hippocampus (Hippo) in rats on Day 33 after bilateral permanent occlusion of the common carotid arteries (BCCAO). Rats were decapitated 40 min after the last administration. From Day 10 to Day 33, different doses of l-NBP (10 and 30mg/kg) were administered orally once per day.

<table>
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<tr>
<th></th>
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<th>l-NBP (10mg/kg)</th>
<th>l-NBP (30mg/kg)</th>
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<tr>
<td></td>
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<td>3.10±0.09***</td>
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<td>3.42±0.12</td>
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<td>CAT (U/mg protein)</td>
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<td>12.44±0.69</td>
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<tr>
<td></td>
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<td>2.86±0.19</td>
<td>3.02±0.22</td>
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</table>

All values are means±SEM (n=8-9). #p<0.05, ###p<0.001 vs Sham group; *p<0.05, **p<0.01, ***p<0.001 vs Vehicle group.
Bilateral permanent occlusion of common carotid arteries of rats

\[ \text{l-, d- and dl-NBP p.o. (10 or 30mg/kg)} \]

Water maze test
Probe trial
Sacrifice

Figure 2
Figure 3
Figure 4
Figure 6