Bushenhuoxue improves cognitive function and activates brain-derived neurotrophic factor-mediated signaling in a rat model of vascular dementia

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OBJECTIVE: To explore the protective mechanisms of the Traditional Chinese Medicine Bushenhuoxue (BSHX) in a rat model of vascular dementia (VD).

METHODS: A rat model of VD was developed using bilateral common carotid artery occlusion (BCCAO). Rats were administered BSHX (10.14 or 5.07 g/kg), nimodipine (11.06 mg/kg; positive control), or saline (control) by gavage daily for 30 d post-surgery. Learning and memory abilities were assessed using the Morris water maze. Morphological changes in the hippocampus were observed using light microscopy (hematoxylin and eosin staining) and transmission electron microscopy (TEM). The mRNA and protein expression levels of brain-derived neurotrophic factor (BDNF), tyrosine receptor kinase B (TrkB), phosphatidyl inositol 3-kinase (PI3K), serine/threonine kinase (AKT), and cAMP response element binding protein (CREB) were measured by real-time polymerase chain reaction (RT-PCR) and Western blot, respectively.

RESULTS: Compared with the sham group, rats with BCCAO exhibited impaired learning and memory abilities (Morris water maze) and showed abnormalities in neuronal morphology (light microscopy) and ultrastructure (TEM) in the hippocampus. They also had decreased mRNA and protein expressions of BDNF, TrkB, PI3K, AKT, and CREB in hippocampal tissue (all \( P < 0.05 \)). In rats with BCCAO, administration of BSHX attenuated deficits in learning and memory, improved the morphology and ultrastructure of hippocampal neurons, and enhanced mRNA and protein expression levels of BDNF, TrkB, PI3K, AKT, and CREB (all \( P < 0.05 \)).

CONCLUSION: BSHX may protect hippocampal...
neurons and improve learning and memory abilities, at least in part via the activation of BDNF/TrkB/PI3K/AKT/CREB signaling.

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**Keywords:** Dementia, vascular; Brain-derived neurotrophic factor; Phosphatidylinositol 3-kinase; Protein serine-threonine kinases; Bushenhuoxue prescription

**INTRODUCTION**

Vascular dementia (VD) is a clinical syndrome associated with advanced neurocognitive dysfunction caused by brain damage. VD is characterized by cognitive and behavioral deterioration; in this disease, neuronal injury in brain regions, particularly the hippocampus, is accompanied by progressive learning and memory deficits. Dementia is recognized as a major public health problem. In Europe and the USA, VD accounts for 15%-20% of all dementia cases, second to Alzheimer’s disease (AD). In contrast, in Asia and many developing countries, VD is a more common cause of dementia than AD, and accounts for 30%-60% of cases. Currently, the treatment of VD mainly involves the use of calcium antagonists and agents that enhance brain circulation or improve cerebral metabolism. However, these drugs do not guarantee optimal outcomes, and there is ongoing research into novel agents that might prevent or reverse VD.

Bushenhuoxue (BSHX) is a Traditional Chinese Medicine that has various beneficial actions in patients with Parkinson’s disease. BSHX has also been reported to improve learning and memory abilities in rodents with experimental VD and diabetes. The beneficial effects of BSHX on cognitive decline in mice VD models may involve the mRNA expression of brain-derived neurotrophic factor (BDNF), a critical member of the neurotrophic factor family. BDNF is thought to play an important role in the pathogenesis of depression, AD, stroke, and VD. Several studies have revealed that BDNF is reduced in patients with VD and AD, indicating that serum BDNF could potentially be used as a biomarker for the early diagnosis of dementia and the assessment of its severity. BDNF promotes neuronal survival by binding to tyrosine receptor kinase B (TrkB) and activating the phosphoinositide 3-kinase (PI3K)/AKT (serine-threonine kinase, also known as protein kinase B) signaling pathway. Several investigations have reported that activation of PI3K/AKT signaling can improve neuronal survival and attenuate declines in cognitive function. In addition, cAMP response element binding protein (CREB) is a transcription factor known to regulate BDNF expression, and BDNF can in turn regulate the phosphorylation and activity of CREB via extracellular signal-regulated kinase (ERK).

We hypothesized that BSHX may inhibit cognitive impairment in VD, and that changes in expression levels of proteins in the BDNF signaling pathway may be involved in this effect. Therefore, this study investigated whether BSHX protected hippocampal neurons and altered the expressions of BDNF, TrkB, PI3K, AKT, and CREB in a rat model of VD.

**MATERIALS AND METHODS**

**Experimental drugs**

BSHX consists of Renshen (Radix Ginseng, 10 g), Heshouwu (Radix Polygoni Multiflori, 15 g), Yizhi (Fructus Alpiniae Oxyphyllae, 15 g), Hutaorou (Nuts Juglans Regia, 15 g), Chuanxiong (Rhizoma Chuanxiong, 15 g), Danshen (Radix Salviae Miltiorrhizae, 15 g), Danggui (Radix Angelicae Sinensis, 15 g), and Yujin (Radix Curcumae Wenyujin, 10 g). Each of the different components were provided by Shijiazhuang YongXin Traditional Chinese Medicine Co., Ltd., Shijiazhuang, China. These Traditional Chinese Medicine elements were weighed, placed into beakers, and 500 mL of distilled water was added to them. The elements were then soaked for 30 min and decoked for 30 min in a conventional manner under an alcohol lamp, and the filtered liquid was heated to concentrate it to a solution containing the raw drug at 1.014 or 0.507 g/mL, respectively. After cooling, the concentrate was stored at 4 °C.

We used nimodipine produced by Hebei Yongfeng Pharmaceutical Co., Ltd., Shijiazhuang, China (approved number: Chinese medicine quasi-word H13021882. Batch number: 20160106, Specification: 20 mg/tablet). Tablets were crushed before use, and were dissolved in the appropriate quantity of distilled water to obtain a solution with a concentration of 1.106 mg/mL.

**Reagents and equipment**

The reagents used were as follows: PrimeScript RT Reagent (Perfect Real Time, TAKARA-RR037A, Takara Bio, Japan); SYBR Premix EX Taq™ (Perfect Real Time, TAKARA-RR820A, Takara Bio, Japan); RNAiso Plus Reagent (TAKARA-9108, Takara Bio); EASY Dilution (for Real Time PCR, TAKARA-9160, Takara Bio); DL2000 DNA Marker (TAKARA-3427A, Takara Bio, Japan); BDNF antibody (sc-546, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); TrkB antibody (sc-12, Santa Cruz Biotechnology, Inc.); PI3-K antibody (sc-602, Santa Cruz Biotechnology, Inc.,); AKT antibody (sc-8312, Santa Cruz Biotechnology, Inc.); CREB antibody (sc-186, Santa Cruz Biotechnology, Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (sc-25778, Santa Cruz Biotechnology, Inc.).

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The water maze was purchased from Beijing ZhongShi-DiChuang Technology Development Co., Ltd., Beijing, China; the epoxy resin mixture was purchased from Head Biotechnology Co., Ltd., Beijing, China; the H-7500 transmission electron microscope was purchased from Hitachi, Tokyo, Japan; light microscopy was purchased from Olympus Corporation, Tokyo, Japan; the Furi Bio-Electrophoresis Image Analysis System was purchased from Shanghai Furi Technology Co., Ltd., Shanghai, China; the Quantity One 1-D Analysis Software was purchased from Bio-Rad, Hercules, CA, USA; and the ABI 7500 Real-Time PCR System was purchased from Applied Biosystems, Foster City, CA, USA; SPSS 16.0 statistical software was purchased from SPSS Inc., Chicago, IL, USA.

**Animals and grouping**

A total of 75 male, specific pathogen-free Sprague-Dawley rats (body weight 250-300 g) were provided by the Laboratory Animal Center of Hebei Medical University (Shijiazhuang, China). Rats were housed at (23 ± 1) °C with a 12/12 h light/dark cycle and allowed free access to water and food. After 1 week of adaptive feeding, five rats were excluded from further study due to failure in the Morris water maze test. The remaining rats were allocated randomly at a ratio of 1:1:1:1 to sham, VD, high-dose (HD)-BSHX, low-dose (LD)-BSHX, and nimodipine groups. Rats in the VD, HD-BSHX, LD-BSHX, and nimodipine groups were subjected to bilateral common carotid artery occlusion (BCCAO) as a model of VD, while animals in the sham group received sham surgery. Following surgery, all rats received medication by gavage once daily for 30 d, as follows: 10.14 g/kg BSHX (HD-BSHX group), 5.07 g/kg BSHX (LD-BSHX group), 11.06 mg/kg nimodipine (nimodipine group, used as a positive control), or 10 mL/kg physiological saline solution (sham and VD groups). The drug dosages and treatment duration were chosen based on a previous study. All animal experiments were approved by the Animal Care and Use Committee of Hebei College of Traditional Chinese Medicine (Shijiazhuang, China) and performed in accordance with the internationally recognized National Institutes of Health Guide for the Care and Use of Laboratory Animals (2010).

**Establishment of the VD model**

The rat model of VD was developed using the two- vessel occlusion method. In brief, each rat was fasted for 12 h (food) and 4 h (water) before surgery and anesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg). Rats were then immobilized in the dorsal position and the neck was shaved and disinfected with 75% ethanol. A neck median incision was made, and blunt separation of the skin, muscle and connective tissue was conducted to expose and separate the bilateral common carotid arteries, which were then ligated with silk thread (in all groups except the sham group). Subsequently, the muscle and skin were sutured, and gentamicin was injected subcutaneously in the region surrounding the incision to prevent wound infection. Exposure and separation of the bilateral common carotid arteries was also carried out in the sham group, but ligation was not performed.

**Morris water maze test**

After 30 d of treatment with the appropriate medication, three rats from each group were selected randomly to investigate changes in hippocampal structure and the remaining rats were assessed using the Morris water maze task to monitor spatial learning and memory. The water maze consisted of a large circular pool (120 cm in diameter, 60 cm in height, partially filled to a depth of 35 cm with water at 22 °C) that was located in a dimly lit, quiet room. The maze was divided into four equal quadrants. A transparent escape platform was submerged approximately 1 cm below the surface of the water in the center of one quadrant during training (the first 5 d of the test). The position of the rat was tracked by a digital camera, and images were analyzed using software provided with the water maze analysis system.

Spatial learning was assessed during the first 5 d of the Morris water maze test. Each rat was gently placed in the water at one of four preplanned starting positions, and the time to find the hidden platform (escape latency) was recorded for each 2 min training trial. The rat was given a maximum of 2 min to find the hidden platform; a score of 120 s was assigned if the platform was not found within this time. The rat was then guided to the hidden platform and allowed to remain there for 15 s before being removed from the water. The experiment was performed four times per day and the average value was calculated. Animals with very low swim speeds (< 10 cm/s) were excluded from further analysis.

The hidden platform was removed on day 6, and spatial memory was assessed with the spatial probe test, which measures time spent in the quadrant where the hidden platform was previously located. Each rat was placed into the water and allowed to swim freely for 2 min. The percentage of time spent in the target quadrant (where the platform had been located) and the number of crossings of the original platform location were recorded.

**Structural changes in the hippocampus**

Rats were sacrificed by decapitation and brains were excised and placed on ice. The right hemisphere was immersed in 4% paraformaldehyde for 12 h, gradually dehydrated with ethanol, and embedded in paraffin. Paraffin sections were prepared using a microtome and stained with hematoxylin and eosin (HE). Histopathological changes in the right hippocampus were observed using light microscopy. The hippocampal region (approximately 1 mm ×
1 mm × 1 mm) was excised on ice from the left hemisphere and pre-fixed in 4% glutaraldehyde for 2 h at 4 °C. The hippocampus was then rinsed three times for 10 min each with 0.1 M phosphate-buffered saline (PBS) and fixed in 1% osmic acid for 1 h at 4 °C. After repeated rinsing in PBS, specimens were subjected to gradient ethanol dehydration and 100% acetone dehydration. Samples were then sequentially immersed for 15 min each in acetone: resin mixtures at 3:1, 1:1, and 1:3 ratios. After a final immersion in pure resin for 30 min, specimens were embedded and aggregated in an epoxy resin mixture, sliced at a thickness of 50 nM on an ultramicrotome, and stained with uranyl acetate for 30-40 min and lead citrate for 30 min. The ultrastructure of hippocampal neurons was observed and photographed using an H-7500 transmission electron microscope.

Western blot
Rats were sacrificed by decapitation and brain tissues were excised onto sterile plates on ice. Brain tissue was cut along the coronal plane between the optic chiasm and optic papilla with a sterile scalpel blade, and the intact bilateral hippocampus was isolated with blunt dissection. The left hippocampus was isolated and rapidly immersed in liquid nitrogen for 1 min before storage at −80 °C. For protein extraction, 100 mg tissue was homogenized in 1 mL extraction solution and centrifuged at 16099 × g for 5 min at 4 °C, and the supernatant was collected. A total of 30 mg of protein was centrifuged at 5 for 16099 g and the supernatant was used in 1% non-fat milk for 2 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies (1:250 in blocking solution) against BDNF, PI3K, AKT, TrkB, CREB, and GAPDH (used as a control). Membranes were then rinsed three times with PBS Tween-20 before incubating with horseradish peroxidase-labeled secondary antibody (1:15 000 in blocking solution) for 1 h at room temperature. Membranes were then immersed in 3,3'-diaminobenzidine solution for color development, rinsed with distilled water, and transferred to PBS for subsequent observation, photography, and analysis using Quantity One 1-D Analysis Software and the Furi Bio-Electrophoresis Image Analysis System.

Real-time polymerase chain reaction (RT-PCR)
RNA was extracted using the RNAiso Plus kit in accordance with the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using PrimeScript RT Reagent as per the manufacturer's instructions. Quantitative PCR was performed on an ABI 7500 RT-PCR System using SYBR Premix EX Taq™ with GAPDH as the control. PCR was performed using the following conditions: denaturation at 95 °C for 10 min followed by 40 cycles of amplification (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s). The melting curve profiles were analyzed to confirm the specificity of the amplification. The Ct value for each test gene was normalized to the average Ct value for GAPDH. The primers were as follows:
- GAPDH: F-5′-GGCAAGTTCAATGGGACACGT-3′, R-5′-TGGTGAAGACGGCATAGAC-3′;
- BDNF: F-5′-CAGGGGCTAGAGACAAAG-3′, R-5′-CTTCCCTTTTTAATGTGTC-3′;
- TrkB: F-5′-TCAGTTGCGAGACATTCC-3′, R-5′-CGAAGAAGCGGATGTGTC-3′;
- PI3-K: F-5′-TGGTTCTTCCGAATCTGATAG-3′, R-5′-CTGCTGGCTGAAGTCTGTA-3′;
- AKT: F-5′-TGGCATCCCTCTTTACAGGC-3′, R-5′-CGCTACGACAGATGGAGA-3′;
- CREB: F-5′-TCAGGCGGTATGACTCATTCC-3′, R-5′-CCCTCTCTTTCTGTCGCT-3′.

Statistical analysis
Data are expressed as the mean ± standard deviation and were analyzed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA), and inter-group comparisons were made using the least significant difference test. Two-tailed P < 0.05 was considered statistically significant.

RESULTS

BSHX attenuated deficits in spatial learning and memory associated with BCCAO
Escape latency was significantly prolonged in the VD group compared with the sham group (P < 0.01). Compared with the VD group, escape latency was significantly shorter in the nimodipine (P < 0.05), LD-BSHX (P < 0.05), and HD-BSHX (P < 0.01) groups (Figure 1A). The VD group also had significant reductions in the numbers of crossings of the initial platform location (P < 0.01) and time spent in the target quadrant (P < 0.01) compared with the sham group. Compared with the VD group, both of these parameters were significantly increased in the HD-BSHX (number of crossings, P < 0.01; time in the target quadrant, P < 0.01), LD-BSHX (number of crossings, P < 0.05; time in the target quadrant, P < 0.05), and nimodipine (number of crossings, P < 0.05; time in the target quadrant, P < 0.01) groups (Figure 1B-C).

BSHX reduced hippocampal tissue loss associated with BCCAO
In comparison with the sham group, the VD group had fewer pyramidal cell layers in the hippocampal CA1 region (because of cell loss), a looser arrangement of cells, cell structural abnormalities, nuclear shrinkage, and nuclear pyknosis (Figure 2B). In contrast, in the hippocampal CA1 region of the BSHX groups,
there was only a small amount of pyramidal cell loss, nerve fibers were arranged neatly, and nuclear pyknosis was limited to only a few cells (Figure 2C, D). In the nimodipine group, histological features of the hippocampal CA1 region were similar to those in the BSHX group, with partial pyramidal cell loss and triangular or polygonal nuclear morphology caused by shrinkage in a portion of cells (Figure 2E).

**BSHX attenuated ultrastructural abnormalities associated with BCCAO in hippocampal neurons**

Compared with the sham group, in the VD group, there was severe swelling of hippocampal neurons, a marked reduction in the number of organelles, fusion of most mitochondrial cristae with the mitochondrial membrane (resulting in a blurred appearance), fracture or absence of mitochondrial cristae (arrow 1; Figure 3B2), and fusion or degranulation of particles on the rough endoplasmic reticulum (RER; arrow 2; Figure 3B2). In the HD-BSHX group, there was only mild swelling of neurons, a more limited fusion of mitochondrial cristae with the mitochondrial membrane (arrow 1; Figure 3C2), and less particle fusion or degranulation on the RER (arrow 2; Figure 3C2); however, there was also a decrease in the number of small synap-
tic vesicles (ssv) and the presence of synaptic gap fusion (Figure 3C1, C2). Ultrastructural features in the LD-BSHX and nimodipine groups were similar: neuronal swelling, reduced numbers of organelles, fusion of most mitochondrial cristae with the mitochondrial membrane (and blurring of their appearance), fracture or absence of cristae (arrow 1, Figure 3D2, E1), and particle fusion or degranulation on the RER (arrow 2; Figure 3D2, E2).

**BSHX increased hippocampal expression of BDNF, TrkB, PI3K, AKT, and CREB**

As shown in Figure 4A-B, the expression levels of BDNF, TrkB, PI3K, AKT, and CREB proteins were significantly lower in the VD group than in the sham group ($P < 0.05$ or $P < 0.01$). Compared with the VD group, protein levels of BDNF, TrkB, PI3K, AKT, and CREB were significantly higher in the HD-BSHX, LD-BSHX, and nimodipine groups ($P < 0.05$ or $P < 0.01$). In addition, mRNA expression levels of TrkB, PI3K, AKT, and CREB were significantly lower in the VD group than in the sham group ($P < 0.05$ or $P < 0.01$; Figure 4C), and compared with the VD group, the mRNA expressions of BDNF, TrkB, PI3K, AKT, and CREB were significantly higher in the HD-BSHX, LD-BSHX, and nimodipine groups ($P < 0.05$ or $P < 0.01$). These mRNA results were consistent with the changes that were observed in protein levels (Figure 4C).

**DISCUSSION**

The primary findings of the present study indicated that BSHX administration in a VD rat model attenuated deficits in learning and memory, improved the morphology and ultrastructure of hippocampal neurons,
and enhanced mRNA and protein expressions of BDNF, TrkB, PI3-K, AKT, and CREB. These observations suggest that the BDNF/TrkB/PI3-K/AKT/CREB signaling pathway may contribute to the mechanisms by which BSHX protects hippocampal neurons and improves learning and memory in a rat model of VD.

BSHX is the most frequently used Traditional Chinese Medicine for the treatment of VD in China. A previous clinical study reported that BSHX markedly improves learning and memory abilities in patients with VD, and that it also regulates lipid metabolism and reduces event-associated potential P300 (an electroencephalogram-detected wave thought to reflect information processing associated with attention and memory), with a total effective rate of 83.72%. In the present study, BSHX was observed to improve learning and memory abilities and attenuate ischemic injury to the hippocampus in a rat model of VD. Our data are consistent with those of previous studies showing that BSHX enhances learning and memory in rodent models of VD.

The pathogenesis of VD involves local or whole-brain ischemia and hypoxia due to cerebrovascular disease, causing damage to specific regions of the brain associated with cognition and memory. Although the underlying mechanisms are complex and not completely understood, the cascade of reactions caused by cerebral ischemia is thought to include energy exhaustion, toxicity by excitatory amino acids, peri-infarct depolarization, inflammatory responses, cell apoptosis, oxidative stress, dysregulation of neurotransmitter release and synaptic transmission, and neuronal degenerative inju-
Upon binding to its cognate receptor, BDNF, the MAPK/ERK/CREB and PI3K/AKT signaling pathways are activated. These pathways recruit numerous targets, including antioxidant actions (evidenced by enhanced levels of superoxide dismutase, catalase, glutathione, and glutathione peroxidase-1, and reduced levels of malondialdehyde), increased cortical levels of norepinephrine, and enhanced expression of basic fibroblast growth factor and BNDF. In addition, BSHX or its components were reported to have anti-inflammatory properties, increase hippocampal levels of glutamate and GABA, decrease activation of nuclear transcription factor kappa-B, and reduce levels of nitric oxide, tumor necrosis factor-α, and interferon-γ in the brain. Therefore, it has been suggested that BSHX attenuates ischemia-induced cognitive dysfunction via multiple actions on numerous targets. However, a potentially important mechanism of BSHX identified by both this study and a previous study is an increased expression of BNDF. Studies have revealed that BNDF can promote neuronal growth and differentiation after cerebral ischemia, maintain the functions of mature neurons, stimulate the sprouting of axons and dendrites in adult neurons, enhance the release of synaptic transmitters, strengthen synaptic connections, repair synaptic plasticity, and improve impaired learning and memory. The protective mechanism of BNDF in neurons relies on the high-affinity binding of BNDF to its receptor TrkB, which activates intracellular tyrosine kinases to induce self-phosphorylation of TrkB, and thus the transactivation of downstream signal pathways. In the present study, the expression levels of BNDF and its receptor, TrkB, were reduced in hippocampal tissue in a rat model of VD caused by cerebral ischemia. These changes were accompanied by pathological abnormalities of neurons in the hippocampal CA1 region, implying that neuronal injury by cerebral ischemia is a critical cause of dementia. Notably, in the VD rat model, BSHX enhanced both the protein and mRNA expression of BNDF and TrkB in the hippocampus. These findings raise the possibility that BSHX enhances BNDF/TrkB expression and thus promotes the repair of hippocampal neurons. The binding of BNDF to TrkB activates multiple intracellular signal transduction pathways, including mitogen-activated protein kinase (MAPK)/ERK, PI3K, and phospholipase C pathways that eventually activate CREB. Of the intracellular signaling pathways recruited by BDNF, the MAPK/ERK/CREB and PI3K/AKT pathways are known to be important for neuronal survival. PI3K is a critical member of the phospholipid kinase family. Upon binding to its cognate receptor, PI3K activates a series of downstream protein kinases, including AKT, which is phosphorylated on amino acid residues Ser473 and Thr308 by PI3K. Several studies have revealed that the PI3K/AKT signal transduction pathway plays an active role in the antagonism of cell apoptosis, and disorders of this pathway lead to the functional deficiency of cerebral microvessel endothelial cells. In addition, the PI3K/AKT/mTOR pathway regulates the activity and expression of hypoxia inducible factor-1, which improves glucose transportation, modulates cell apoptosis, and promotes angiogenesis.

In conclusion, activation of BDNF/TrkB/PI3K/AKT/CREB signaling may be an important mechanism by which BSHX protects hippocampal neurons and improves learning and memory in a VD rat model. However, further studies are warranted to extend these findings.

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