Abstract. Cerebral hypoperfusion is a common feature of vascular dementia and has recently been recognized to contribute to the progression of cognitive decline. The present study aimed to investigate the effects of Bushen Huoxue decoction (BHD), a two-herb Chinese Medicine, on cognitive impairment in a rat model of cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid arteries (2VO). The results demonstrated that BHD significantly attenuated learning and spatial memory deficits in the Morris water maze test in a dose-dependent manner. Transmission electron microscopy observation revealed that the reduction of synapse density in hippocampal CA1 and cortex parietal isolated from rats with 2VO was partially restored by BHD treatment. In addition, the expression levels of a number of antioxidants, including superoxide dismutase, catalase (CAT), glutathione and glutathione peroxidase-1 (GPx-1) increased, whereas malondialdehyde decreased in the hippocampi of rats with 2VO following BHD treatment. Polymerase chain reaction and western blot analysis further confirmed that the GPx-1 and CAT expression increased in the BHD treatment group. In conclusion the results suggested that BHD has therapeutic potential to treat vascular dementia, which may be associated with synapse density and anti-oxidant activities in the hippocampus.

Introduction

Vascular cognitive impairment (VCI) is the phenotypic outcome of a cascade of events, involving vascular risk factors that lead to vascular disease, which causes vascular brain injury in networks important for cognitive functioning (1-4). VCI encompasses numerous conditions from vascular dementia (VaD) to mild cognitive impairment of vascular origin (5). Clinical studies have reported that cerebral hypoperfusion associated with cognitive dysfunctions has been identified in Alzheimer's disease (AD) and in patients with VaD (6,7).

The pathological mechanisms that underlie chronic cerebral hypoperfusion-induced dementia are yet to be identified. Reactive oxidative stress and inflammatory cytokines have important roles in brain impairment and dementia progression (8). In addition, experimental evidence has indicated that synapse loss occurs in AD and VaD patients and studies revealed a strong correlation between synapse reduction and the severity of dementia (9). Although an increasing number of mechanistic pathological studies focused on VaD and hypoperfusion, no effective pharmacological intervention has been approved by the Food and Drug Administration (Silver Spring, MD, USA) and used clinically.

Herbal medicines, including Traditional Chinese Medicines, have been used for a long time as an alternative method to treat cerebral vascular diseases and dementia-like symptoms (10). A Chinese Medicine named Bushen Huoxue decoction (BHD) was prepared, which has a good clinical efficacy in treating patients with VaD. Two herbs are included in BHD, namely Epimedium (Epimedium Davidii Franch, Yin Yang Huo) and Salvia Miltiorrhiza (Salvia Miltiorrhiza Bunge, Dan Shen). BHD has the efficacy of nourishing kidney and activating blood (Bu Shen Huo Xue), which may be associated with synapse density and anti-oxidant activities in the hippocampus.

Key words: Bushen Huoxue decoction, vascular dementia, synapse density, antioxidant enzymes
Materials and methods

Plant materials and herbs water extraction. BHD consists of *Epimedium* and *Salvia miltiorrhiza*. The herbs were purchased from Fujian Pharmaceutical Co. (Fuzhou, Fujian, China) and were identified as eligible and pure medicinal material. These herbs were decocted at boiling temperature and extracted with water. The decoction was filtrated, concentrated and stored in a sterile bottle at 4°C until use. An extract of *Ginkgo biloba* (GB) leaves was purchased from Shenyang Green Pharmaceutical Co. (Shenyang, Liaoning, China).

Animals and 2VO surgery. The current study was approved by the ethics committee of Fujian University of Traditional Chinese Medicine, Fuzhou, China. Wistar rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China), weighing 250±20 g, were allowed to acclimatize for three days prior to experimentation and were housed in 25±1°C, 65±5% humidity and a 12-h light-dark cycle. Food and water were freely available. All experiments were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China. Efforts were made to minimize the suffering and number of animals used.

Cerebral hypoperfusion in rats was performed according to the previously described procedures (12). Each rat was anesthetized with ketamine hydrochloride (0.3 ml/100 g, i.p.). Both of the common carotid arteries were carefully separated from the cervical sympathetic and vagal nerves through a ventral cervical incision. The arteries were then ligated with 5.0 silk sutures. The sham-operated animals were treated similarly to the 2VO rats except for the carotid artery ligations.

Drug administration. The adult normal dosage of BHD was defined as a moderate dose (M), 1/2 of the moderate dose as the low dose (L) and two times the moderate dose as the high dose (H). On the day 18 following surgery, the 2VO rats were randomly divided into five groups with n=12/group. The rats in each group were intragastrically administered twice daily with saline (vehicle control, 2VO), 2.75 g/kg (BHD-L), 5.5 g/kg (BHD-M), 11 g/kg (BHD-H) BHD and 5.85 mg/kg GB, respectively. GB, a well-known antioxidant and a good anti-dementia drug, was used as a positive control (13,14). The sham-operated and vehicle groups were administered an equal volume of physiological saline solution. The administration was performed for 14 consecutive days. The animals were subjected to behavioral tests prior to the surgery, post-surgery and on days 7 and 14 following administration, respectively, to observe the learning and memory ability. The time scale of the drug treatment regimen is illustrated in Fig. 1.

Morris water maze tests (MWM). Prior to the surgery, post-surgery and on days 7 and 14 following treatment, respectively, the rats were subjected to spatial learning and memory tests as examined using the MWM as previously described (15). The MWM consisted of a black circular pool of water and a hidden platform submerged 2 cm below the water surface, with the water temperature maintained at 21±1°C. Four points around the edge of the pool were designated as North (N), South (S), East (E) and West (W). A video camera was mounted above the center of the pool and all performance was recorded and then analyzed by a computerized video imaging analysis system (Institute of Material Medical, Chinese Academy of Medical Sciences, Beijing, China). At the beginning of the trials, the rats were free to swim for 2 min and the swimming distances were recorded. At first, the rats were provided an escape latency trial once per day for four days. The rats were placed from E, S, W and N one by one every day. If the rat did not find the escape platform within the allotted time (2 min), the record was designated as 120 sec. On day 15, the platforms were removed and the rats were placed into the maze as the escape latency trial. A 120 sec probe trial was performed and the number of crossings over the target quadrant was recorded. Each test was repeated four times for each rat at every time-point. The time scale of the MWM test is illustrated in Fig. 1.

Synapse density observed by transmission electron microscopy. The synapse density in hippocampal CA1 and cortex parietal was observed with a transmission electron microscope (H7650, Hitachi, Ltd., Tokyo, Japan). Briefly, following regular fixation, embedding and ultra-thin section, the brain sections of each rat were placed onto three copper grids. Each grid was scanned to capture 4-5 images, with a total of 10-15 pictures for each rat brain. The numerical density of synapses per unit volume, $N_v$, was calculated using the following formula: $N_v = \frac{Q}{V_{dis}}$, where $Q$ is the mean number of synapses counted in each sector and $V_{dis}$ is the mean sector volume. The total number of synapses, $N_{syn}$, was calculated for each case using the following formula: $N_{syn} = N_v \cdot V_{ref}$.
The hippocampus homogenates were isolated and stored at -80˚C until use. Superoxide dismutase (SOD) and catalase (CAT) levels were determined with respected ELISA kits (Hufeng Tech, Shanghai, China) according to manufacturer's instructions.

Western blot analysis. The denatured protein samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Following blocking, the membranes were incubated overnight at 4°C with primary antibodies [β-actin and CAT monoclonal rabbit antibodies, 1:1,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA; polyclonal rabbit glutathione peroxidase-1 (GPx-1) antibody, 1:1,000 dilution, Abcam, Cambridge, UK] followed by incubation with the goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Chemiluminescence detection was performed using enhanced chemiluminescence advance western blotting detection reagents (BD Biosciences, San Jose, CA, USA). Band intensities were measured with the SX-300 image analysis system (Bio-Rad Laboratories, Hercules, CA, USA).

Table I. Polymerase chain reaction primers, fragment length and annealing time of GPx-1, CAT and β-actin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
<th>Annealing temperature (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>F: 5'-CTATCTGACACTCACGGCCAT-3'</td>
<td>372</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TTTCTTGACGGCTTTTTCTTGGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPx-1</td>
<td>F: 5'-CTC GGTTCCCGTGCAATCAG-3'</td>
<td>431</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GTGCAGCCAGTAATCAACAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-CTGACCGAGCCTTGACTAC-3'</td>
<td>505</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCTGCTTTGCTGATCCACA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Polymerase chain reaction primers, fragment length and annealing time of GPx-1, CAT and β-actin.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the hippocampi with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using the AMV reverse transcription system (Fermentas, Burlington, Canada). Amplification of cDNA fragments of CAT, GPx-1 and β-actin was performed using the Green PCR Master Mix kit (Fermentas). DNA was amplified immediately with a single cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, annealing temperature of each gene for 30 sec and 72°C for 30 sec followed by a final extension step 72°C for 10 min. Ethidium bromide-stained gels were scanned and qualified using Tanon Image software (Science & Technology Co. Ltd., Shanghai, China). The
intensity of each band was normalized against the intensity of β-actin. The PCR primers, fragment length and annealing time of GPX-1, CAT and β-actin are summarized in Table I.

Statistical analysis. Statistical analysis was performed with SPSS 12.0 (SPSS, Inc, Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Analysis of variance was used to determine statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

BHD improves spatial learning and memory of rats with 2VO in MWM. To observe whether BHD improves cognitive deficits, the classical MWM test was used to examine spatial learning and memory, as described in materials and methods above. As demonstrated in Fig. 2A, the escape latency to find the hidden platform in the sham group decreased gradually, while the 2VO rats spent more time in the training trials (P<0.01, compared with the model group). These observations are consistent with the results of behavioral tests reported elsewhere (16). The rats in the BHD-M, BHD-H dosage of BHD and positive control (GB) groups demonstrated improved cognitive abilities compared with the 2VO rats from days 7 and 14 of the tests (P<0.05 or P<0.01, compared with the model group), whereas low-dose BHD did not induce any significant improvements.

The number of crossings over the target quadrant in the probe trials was also examined in each group. A large number of times of crossing over the target quadrant where the hidden platform was placed demonstrated good spatial learning and memory. Fig. 2B demonstrated that the rats in the 2VO group required less time than the sham group (P<0.01), and the number of crossings over the target quadrant increased following treatment with BHD as well as GB. The results suggested that rats with 2VO treated with BHD-M or BHD-H exhibited a significant increase in the number crossings over the target quadrant (P<0.05, compared with the model group), particularly at a high dose (P<0.01, compared with the model group). Overall, these results demonstrated that BHD dose-dependently improved cognitive deficits.

BHD increases the number of synapses in hippocampal CA1 regions and the parietal cortex in rats with 2VO. Cognitive impairment is strongly associated with a reduction in the number of synapses. (17) To detect whether the resultant loss in the number of synapses due to cerebral hypoperfusion could be alleviated by BHD administration, the synapses in the hippocampal CA1 region and parietal cortex in rats with 2VO were observed using transmission electron microscopy, counting the number of synapses in each specified area. The results in Fig. 3 revealed that the synapse density in both areas was reduced in the 2VO model, which is consistent with a previous study (18). Notably, both the high dosage of BHD and GB treatment reversed the synaptic impairments observed in the parietal cortex and hippocampal CA1 regions.

Expression of antioxidants increases following BHD treatment in the hippocampi of rats with 2VO. To further study the molecular mechanism underlying the BHD-induced cognitive
improvement, antioxidant levels and activities were examined. Increased levels of malondialdehyde (MDA) and a decrease in the activity of SOD, CAT and glutathione (GSH) in cerebral hypoperfusion induced by 2VO was observed (P<0.01, compared with the sham group; Table II). Both the GB and BHD groups demonstrated a significant reduction in the MDA levels in the hippocampus, with the GB, BHD-M and BHD-H groups exhibiting significantly decreased MDA levels (P<0.01, compared with the model group). In addition, compared with the model group, both GB and BHD increased the activities of SOD, CAT and GSH (P<0.01).

2VO markedly downregulated the mRNA and protein expression levels of CAT and GPx-1 in the hippocampus (P<0.01, compared with the sham group; Fig. 4). Both GPx-1 and CAT expression increased at the mRNA and protein levels in a dose-dependent manner following BHD treatment, which is consistent with the ELISA data.

Discussion

Cognitive impairment of all types, from mild to severe associated with cerebrovascular damage, is defined as vascular cognitive impairment, as first proposed by O'Brien et al (19) which is characterized as the prodromal stages of ischemic VaD (20). Accumulating evidence has implicated VCI as a prominent cause of cognitive decline in the elderly (21,22).

The MWM test is a classic method to examine spatial learning and memory function (23). In the present study, to exclude the possibility that the 2VO model and drug administra-

---

Table II. Effects of BHD on the content of SOD, CAT, GSH and MDA in the 2VO rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/mg)</th>
<th>SOD (U/mg)</th>
<th>CAT (U/mg)</th>
<th>GSH (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8.32±0.52b</td>
<td>115.03±6.77b</td>
<td>6.94±0.69b</td>
<td>104.90±13.33b</td>
</tr>
<tr>
<td>2VO</td>
<td>11.49±0.54</td>
<td>100.19±8.53</td>
<td>5.20±0.35</td>
<td>79.54±9.85</td>
</tr>
<tr>
<td>BHD-L</td>
<td>10.79±1.45</td>
<td>109.16±2.83</td>
<td>6.01±0.63a</td>
<td>88.97±7.99a</td>
</tr>
<tr>
<td>BHD-M</td>
<td>9.81±0.67b</td>
<td>110.68±12.43a</td>
<td>6.51±0.79b</td>
<td>94.41±16.66a</td>
</tr>
<tr>
<td>BHD-H</td>
<td>8.49±0.49b</td>
<td>130.60±9.13b</td>
<td>6.65±0.43b</td>
<td>98.47±10.16b</td>
</tr>
<tr>
<td>GB</td>
<td>8.90±0.53b</td>
<td>110.84±7.11a</td>
<td>6.05±0.63a</td>
<td>92.63±7.36a</td>
</tr>
</tbody>
</table>

Compared with model group *P<0.05, †P<0.01 and n=12. BHD, Bushen Huoxue decoction; GB, Ginkgo biloba; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; 2VO, occlusion of bilateral common carotid arteries; BHD-L/M/H, Bushen Huoxue decoction-low/moderate/high dose.

Figure 4. BHD increases the mRNA and protein expression levels of GPx-1 and CAT in the hippocampus. Protein levels of GPx-1 and CAT in the hippocampus were (A) assessed using western blot analysis and (B) quantified. mRNA levels of GPx-1 and CAT in hippocampus were (C) observed using quantitative polymerase chain reaction and (D) quantified. n=3 per group, *P<0.05 and †P<0.01 vs. the 2VO group. GB, Ginkgo biloba; GPx-1, glutathione peroxidase-1; CAT, catalase; 2VO, occlusion of bilateral common carotid arteries; BHD-L/M/H, Bushen Huoxue decoction-low/moderate/high dose.
tion affect motoric disability rather than cognitive decline, the swimming velocity was measured prior to the formal MWM test. There was no significant difference in the time to swim the distance (120 sec) between the groups (P>0.05, data not shown). This quality control ensured the equal basic physical and motoric ability in the water for each group. Next, escape latency and probe trials were conducted to determine the acquisition and retention ability of the experimental rats. The results demonstrated that in rats with cerebral hypoperfusion, treatment with BHD significantly increased the number of crossings over the target quadrant and decreased the escape latency to reach the platform, particularly at the mid and high dosages, indicating that BHD improved cognitive functioning in the rats with 2VO.

Synapse formation and plasticity are necessary for memory recall and consolidation. Previous studies have revealed that synapse loss is associated with cognitive impairment observed in various cognitive dysfunction models, including AD (24,25), middle cerebral artery occlusion (26) and 2VO (27). In the present study, BHD treatment evidently alleviated the synaptic loss induced by cerebral hypoperfusion. Reversing this pathological change may have largely contributed to the memory recall and improvement of cognitive impairment.

Increased levels of oxidative stress and antioxidant deficiencies may pose as risk factors for cognitive decline (28). Chronic vascular hypoperfusion induces oxidative stress and brain energy failure and leads to neuronal death. This is because the brain is particularly susceptible to free radical attack, which generates more toxins per gram of tissue than any other organ in the body (29). Therefore, anti-oxidative therapy is considered as a good way to improve cognitive deficits (30). MDA is a marker of lipid peroxidation and the levels of MDA indicate the degree of oxidative stress (31). In the antioxidant enzyme defense system, SOD catalyzes the formation of hydrogen peroxide from superoxide radicals, while CAT and GPx prevent or remove toxic hydroxyl radicals generated by hydrogen peroxide (32).

The most significant ingredient of BHD, Epimedium, an important Chinese Herb which nourishes the kidney and enhances brain function, has been proved to possess antioxidant activities (33,34). The ministerial drug Salvia Miltiorrhiza, which has an activating effect on the blood and is often used for cerebrovascular diseases, was also demonstrated to have anti-oxidant activities (35). The extract of GB leaves, the positive control used in this study, was found to possess cognitive enhancing effects associated with its antioxidant properties (36). In the present study, BHD reduced MDA levels and enhanced the activity of SOD, CAT and GSH in the hippocampi of rats with 2VO. BHD treatment also increased the expression of SOD and GPx at both the protein and mRNA levels. These results indicated that the role of BHD improving learning and memory is associated with the contents or activity of antioxidants and antioxidative enzymes.

In conclusion, the present study evidently demonstrated that BHD improved learning and memory deficits induced by cerebral hypoperfusion, and the restoration of elevated antioxidants and synapse density may have contributed to this improvement. The present study provided evidence that will facilitate developing BHD as a preventive or therapeutic for VaD.

Acknowledgements

This study was supported by the Developmental Fund of Chen Keji Integrative Medicine, no. CKJ2010025, and the Key Foundation of Society Development in Fujian province, no. 2013Y0059.

References