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Baicalein improves cognitive deficits induced by chronic cerebral hypoperfusion in rats

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Abstract

The aim of this study is to investigate the effects of baicalein on cognitive impairment and neuronal degeneration in a rat model of chronic cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid arteries (2VO). It was found that baicalein (2 or 4 mg/kg/day, i.p.) significantly improved 2VO-induced cognitive deficits and neuropathological changes. Biochemical and histological examinations revealed that baicalein reduced the increased activities of superoxide dismutase (SOD) and malondialdehyde (MDA), and attenuated the decreased activities of glutathione peroxidase (GPx) and catalase in 2VO rats. The results of the present observation suggest that baicalein has therapeutic potential for the treatment of vascular dementia, which is most likely related, at least in part, to its antioxidant action.

Keywords: Baicalein; Learning; Memory; Free radical; Vascular dementia

1. Introduction

Dementia has already become a major public issue with the increasing elderly population. Vascular dementia (VD), which is characterized by progressive intellectual decline produced by ischemia hypoxia or hemorrhage brain lesion (Diehl and Kurz, 2002; Roman, 2002a,b), represents the second most common dementia accounting for about a quarter to a half of all cases of dementia in developed countries (Rockwood et al., 2000; Roman, 2002a,b). Previous studies have revealed that oxidative injury plays a key role in the pathogenesis of numerous neurodegenerative diseases including stroke, Alzheimer's disease, and VD, etc (Coyle and Puttfarcken, 1993; Markesbery, 1997; Chong et al., 2005). Oxygen free radicals and lipid peroxidation may have an aetiological role in the development of lesions in the central nervous system in patients with VD. Therefore, antioxidant therapy may be important for managing VD.

The dried root of *Scutellaria baicalensis* Georgi (Huang-qin), a Traditional Chinese Medicinal herb, has been widely used for several centuries in China as a "Qinghuo" agent (i.e. antibacterial and antiviral agent). Recently, flavonoids extracted from *S. baicalensis* have been demonstrated to improve experimental brain hypoxia, chemical neuronal damage, and cognitive deficits (Shang et al., 2001, 2002, 2005a,b). Baicalein (Bai) is one of the flavonoids, and has been proved to be a superior free radical scavenger and xanthine oxidase inhibitor (Hamada et al., 1993; Chang et al., 1993; Chen et al., 2000; Gao et al., 1999; Shieh et al., 2000). Recent studies showed that baicalein had neuron-protection against amnesia induced by β-amyloid peptide-(25–35) (Lebeau et al., 2001; Wang et al., 2004) and neuronal injury secondary to ischemia insult (Lee et al., 2003; Hwang et al., 2002). In addition, baicalein

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effectively prevented dopaminergic dysfunction induced by 6-OHDA (Im et al., 2005) and showed an effect of anxiolytic by binding to the $GABA_A$ receptor (Liao et al., 2003). Moreover, baicalein was found to be a promising agent for the treatment of arteriosclerosis and hypertension by protecting endothelial and vascular cells (Machha and Mustafa, 2005; Huang et al., 2005). These findings attract our interest to explore whether baicalein has beneficial effects on VD.

Brain hypoperfusion is believed to be a critical factor on the occurrence of VD (Roman, 2004). Permanent occlusion of the bilateral common carotid arteries (2VO) induced a state of chronic and moderate ischemia associated with cognitive alterations and neuronal degeneration in rats (Ni et al., 1994; Sarti et al., 2002), and this animal model allowed us to understand pathophysiology of chronic cerebrovascular disorders. In this study, we investigated possible beneficial effects of baicalein on cognitive function and brain neuronal damage in hypoperfused rats, and the related mechanisms.

2. Material and methods

2.1. Animals

Sprague–Dawley rats aged nine weeks (230±20 g) were housed in groups of three to four per cage at a temperature of 23± 1 °C with a 12 h light–dark cycle (light on 7 a.m.–7 p.m.), and had free access to the food and water. The animals were cared in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China on November 14, 1988. Behavioral testing was carried out between 9.00 and 17.00.

2.2. Drugs

Baicalein was purchased from Fluka BioChemika (Buchs, Switzerland) and dissolved in saline in which pH was adjusted to 10 with NaOH. Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine HCl), a reference drug, was purchased from Sigma (Saintlouis, USA) and dissolved in physiological saline. From Day 7 to Day 24 and Day 56 to Day 74 post-surgery, baicalein (2 or 4 mg/kg) and tacrine (3 mg/kg) were daily administered (intraperitoneally, i.p.) at a volume of 0.2 ml per 100 g body weight. The sham-operated animals and 2VO animals were injected equal-volume of saline. After the first series of behavior tests performed during Day 21 to Day 24, the tacrine- and Bai-administration had been stopped for one month (from Day 25 to Day 55 post-surgery), then the second behavior tests were conducted. Behavior tests were conducted 60 min after drug administration. The reagent kits for determining MDA, SOD, GPx, and catalase were purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Other reagents were AR grade.

2.3. Surgery procedure

After rats were anesthetized with choral hydrate (350 mg/kg, i.p.), the bilateral common carotid arteries of the animal were exposed and carefully separated from carotid sheath, cervical sympathetic, and vagal nerves through a ventral cervical incision. The bilateral common carotid arteries were ligated with 4–0 type surgical silk in ischemia rats, whereas not ligated in sham-operated rats. The operation was performed on a heating pad to maintain body temperature at 37.5±0.5 °C, and the animal was kept on the pad until recovery from anesthesia.

2.4. Morris water maze test

Learning and memory performance were assessed at room temperature (23±1 °C) by the Morris water maze with a modified procedure as described in our previous studies (Wemmie et al., 2002; Xiong et al., 2006). The water maze pool consisted of a circular water tank (diameter 120 cm, depth 50 cm) and a circular transparent platform (diameter 10 cm). Four poles along the perimeter of the pool conceptually divided the maze into four equal quadrants. The platform was hidden at the center of the pool under 1.5 cm of the water surface. Every spatial sign around the maze was kept constant during the testing period. At the beginning of each test session a random sequence was generated of four starting quadrant points, and all rats followed this sequence. The rat was placed in the water facing the wall at one of four starting quadrant points and given 120 s to find the platform, and was allowed to rest on it for 20 s. The animals failed to find the location within the given time were gently guided to the platform and were allowed to stay on it for 20 s. Each rat was given four trials daily for four consecutive days, with an inter-trial interval of about 40 s. The water temperature and inter-trial interval were monitored to minimize the contribution of physical fatigue to the learning behavior. In each test, the escape latency to locate the platform, the swimming distance, and mean swimming speed were recorded. The mean data from daily test were used for statistical analysis. To determine whether the animal would take a spatial learning strategy to locate the platform, a single spatial probe trial was assessed following the last learning trial of each testing period by removing the platform from the water tank, and the rats were allowed to swim freely for 60 s. The time spent in the target quadrant (where the platform was located) was recorded. All movement in the whole trials was recorded and analyzed by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). These observations were conducted on the Day 21, 52, 70 post-surgery. The time course for each series of tests always lasted 4 days. New platform locations were selected for each test period.

2.5. Biochemical analysis

The oxidant–antioxidant status of the rat brains subjected to chronic cerebral ischemia was assessed by determining levels of lipid peroxidation, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, and catalase activity. Lipid peroxidation was determined by measuring levels of malondialdehyde (MDA), a by-product of lipid peroxidation (Halliwell, 1991). Rats were decapitated 60 min after the last administration at 74 days post-surgery. The cerebral cortex and hippocampus

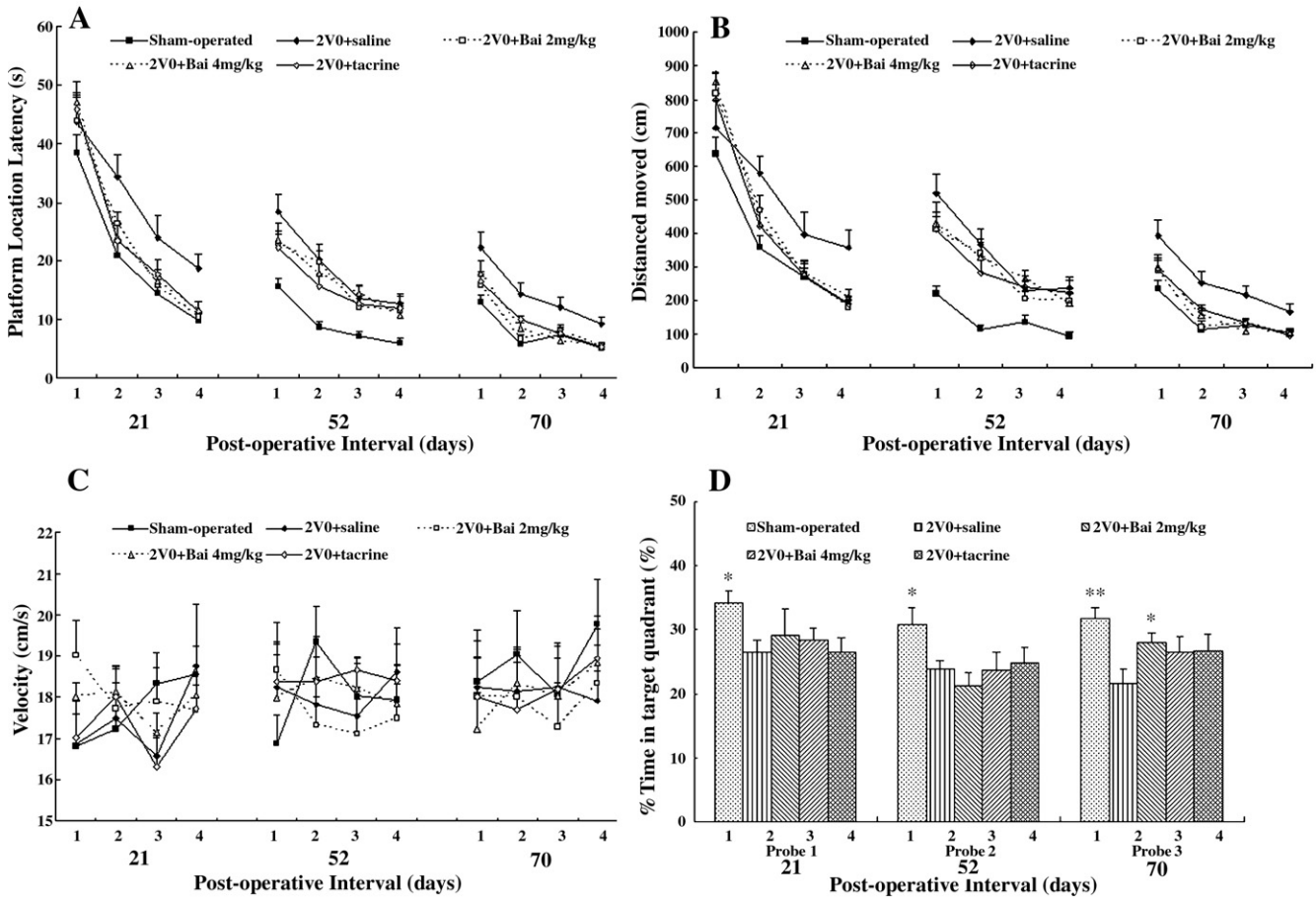

Fig. 1. Effects of baicalein (Bai) on learning and memory deficits of rats induced by permanent occlusion of bilateral common carotid arteries. Morris water maze tests were repeated on the Day 21, 52 and 70 post-surgery. Each rat was subjected to four trails daily for four consecutive days at each post-operative date. Baicalein (2 or, 4 mg/kg/day) and tacrine (3 mg/kg/day) were administered intraperitoneally from Day 7 to Day 24 and Day 56 to Day 74 post-surgery. Rats in the sham-operated group and 2VO group injected the same volume of saline. During the behavior tests, drugs or saline were administered 60 min before the trials. (A) Escape latency to locate the hidden platform. (B) Swimming distance moved in the hidden platform test. (C) Swimming velocity in the hidden platform test. (D) Percent of time spent in target quadrant within 60 s in the probe trials (no platform). Each datum represents the mean ± S.E.M. for 12 animals. $^{**}P<0.01$, $^{*}P<0.05$ compared with saline-treated 2VO group.

were separated on ice, and were homogenized with ice-cold saline to be 10% (w/v) homogenates. MDA formation was determined by measuring thiobarbituric-acid reacting substances (Agar et al, 1999). SOD was determined based on its ability to inhibit the oxidation of oxymine by O^{2-} produced from the xanthine/xanthine oxidase system (McCord and Fridovich, 1988). GPx activity was determined using the procedure described by Armstrong and Browne (1994), and catalase activity was measured by employing hydrogen peroxide to generate H_2O and O_2 (Chance and Maehly, 1995). Protein concentration was determined by the Coomassie blue protein-binding (Bradford, 1995) using bovine serum albumin (BSA) as a standard. The detailed procedures of measurements followed the manufacture instruction in different reagent kits (Nanjing Jiancheng Institute of Biological Engineering, China).

2.6. Histopathological examination

After the last administration at 74 days post-surgery, rats from different groups were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.), then perfused transcardially with 100 ml saline followed by 400 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following decapitation, the brains were taken out, postfixed in the same fixative at 4 °C, dehydrated, and then embedded in paraffin blocks. Coronal sections of 6 μm were stained with hematoxylin–eosin. Cell counts in stratum pyramidale of cerebral cortex and hippocampal CA1 were carried out at 20× magnification using a light microscope (Olympus, Japan). The cell with a round- or oval-shaped nuclei exhibiting no evidence of shrinkage or edema was scored as undamaged.

Cells on bilateral dorsal hippocampus were counted from three sections at −3.3, −3.8, −4.8 mm from bregma, and cell numbers from overlying cerebral cortex and hippocampal CA1 were summarized in six different fields of each section by a blinded observer. The mean values from the three sections were used for statistical analysis.

2.7. Statistical analysis

Results were presented as mean ± S.E.M. Group differences in the escape latency in the Morris water maze training task

Table 1
Effects of Baicalein (Bai) on MDA levels and SOD activities caused by permanent occlusion of the bilateral common carotid arteries (2VO) in rats

Group	MDA (nM/mg protein)		SOD (nU/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus
Sham-operated	0.38±0.02***	0.33±0.04***	134.37±7.71**	139.34±4.33***
2VO+saline	0.77±0.03	0.68±0.04	162.99±5.48	173.65±3.15
2VO+Bai 2 mg/kg	0.63±0.04**	0.45±0.03***	126.99±4.97***	162.05±4.22*
2VO+Bai 4 mg/kg	0.66±0.04*	0.50±0.04**	121.00±7.27***	159.05±4.12*

All values are means±S.E.M. (n=8–9). Sham-operated group and Bai-treated groups were compared with the saline-treated 2VO group. *P<0.05; **P<0.01; ***P<0.001.

were analyzed with two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups. The other data were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. A value of P<0.05 was considered statistically significant.

3. Results

3.1. Bai improves spatial learning and memory of 2VO rats in Morris water maze

Fig. 1A shows the latency of the animal to locate the hidden platform tested on the Day 21, 52, 70 post-surgery. The animals from saline-treated 2VO group took longer to find the hidden platform than the sham-operated group. Significant differences were observed between sham-operated group and 2VO group at post-surgery tests on Days 21, 52 and 70 (all P<0.01 in the three comparisons). Baicalein (2 or 4 mg/kg/day) and tacrine (3 mg/kg/day) were administered from Day 7 to Day 24 and Day 56 to Day 74 post-surgery. Significant effects were observed in terms of groups tested on the Day 21 and Day 70 post-surgery [ANOVA, F (4, 44)=5.25, P<0.01; F (4, 44)=5.73, P<0.01, respectively]. As expected, tacrine (3 mg/kg/day, i.p.) markedly improves 2VO-induced spatial memory impairment in rats (both P<0.01 at the Day 21 and Day 70 tests). The prolonged escape latency in 2VO rats was significantly reduced by long-term administration of Bai at 2 mg/kg and 4 mg/kg. No difference was observed between Bai groups and tacrine treated groups (both P>0.05 at the Day 21 and Day 70 tests). At the test conducted on the Day 52 post-surgery (after a pause of drug-treatment), 2VO rats showed an impaired maze performance to locate the hidden platform. No difference was seen between the 2VO group and the three drug-pretreatment groups (P>0.05 at the Day 52 test).

Fig. 1B shows the swimming distance in the three testing periods closely paralleled to the difference in escape latency. Long-term treatment with Bai at 2 mg/kg/day and 4 mg/kg/day from Day 7 to Day 24 and Day 56 to Day 74 post-surgery substantially shortened the swimming distance prolonged by 2VO (both P<0.05 at the tests on Day 21 and Day 70). In order to determine whether the group difference in platform location latency and swimming distance were due to the difference in swimming ability, the swimming velocity was also evaluated (Fig. 1C). No significant differences were observed between each group (all P>0.05 at the Day 21, Day 52 and Day 70 tests).

The results of spatial probe trials are shown in Fig. 1D. The sham-operated rats spent more time in searching in the target quadrant where the platform was located than the saline 2VO animals did in the three post-surgery test periods (both P<0.05 at the Day 21 and Day 56 tests, P<0.01 at the Day 70 day test). The drug-treatment groups spent as much time in searching the platform in the target quadrant as the sham group, and significant difference was observed between 2VO group and Bai 2 mg/kg group (P<0.05) in the test on Day 70 post-surgery. The groups did not differ at any other test date.

3.2. The antioxidant effects of Bai in vitro

Table 1 shows the results of effects of Bai on MDA levels and SOD activities in the cortex and hippocampus. MDA levels and SOD activities in saline-treated 2VO rats increased by 100.8% and 21.3% in cortex, and by 106.1% and 24.6% in hippocampus when compared with those in sham-operated group. Bai significantly attenuated the increase in MDA level and SOD activity induced by 2VO in the two brain regions.

Table 2 shows the effects of Bai on GPx and catalase activities in cortex and hippocampus. GPx and catalase activities in saline-treated 2VO rats were decreased by 9.4% and 38.4% in cortex, and by 17.4% and 51.7%, in hippocampus, respectively, when compared with those in sham-operated group. Bai significantly attenuated the reduction of GPx and catalase activities by 2VO in the two brain regions.

Table 2
Effects of Baicalein (Bai) on the activities of GSH-px and catalase caused by permanent occlusion of the bilateral common carotid arteries (2VO) in rats

Group	GPx (nU/mg protein)		Catalase (U/g protein)	
	Cortex	Hippocampus	Cortex	Hippocampus
Sham-operated	8.72±0.24*	3.84±0.23	51.44±5.58**	153.14±13.81***
2VO+Saline	7.90±0.17	3.17±0.18	31.67±4.04	73.90±7.36
2VO+Bai 2 mg/kg	8.59±0.31	4.50±0.68**	46.15±5.14*	123.20±10.72**
2VO+Bai 4 mg/kg	8.63±0.24*	4.29±0.30**	44.27±3.00	151.20±7.75***

All values are means±S.E.M. (n=8–9). Sham-operated group and Bai-treated groups were compared with the saline-treated 2VO group. *P<0.05; **P<0.01; ***P<0.001.

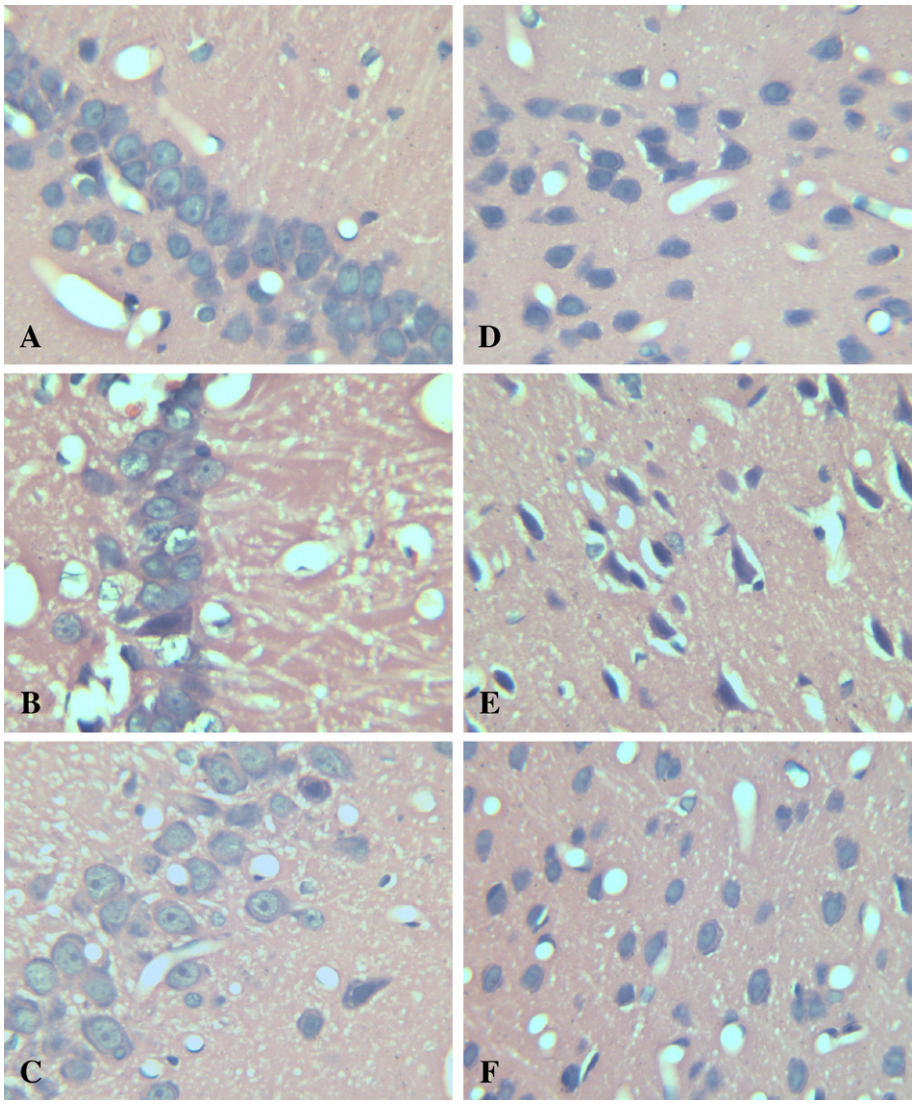

Fig. 2. Representative photomicrographs in the rat hippocampus (A, B and C) and cerebral cortex (D, E and F) at 74 days after permanent occlusion of the bilateral common carotid arteries. (A and D) Sham-operated group; (B and E) saline-treated group: showing neuronal loss, shrinkage and marked vacuolar changes in the CA1 areas of the hippocampus as well as some infarctions and glial proliferation in the cortex of saline-treated 2VO rats; (C and F) baicalein 2 mg/kg-treated group: showing long-term administration of Bai 2 mg/kg attenuated these morphological alterations. (Magnification=400×).

3.3. Bai protects neuron from hypoperfusion-induced neuronal injury

Fig. 2 shows the representative photomicrographs of hematoxylin and eosin staining in cerebral cortex and hippocampus CA1 at 74 days after permanent bilateral ligation of the common carotid arteries. In sham-operated rats, no ischemia cell damage was seen in cortex and hippocampus. Neuronal loss, shrinkage and marked vacuolar changes were observed in CA1 areas of the hippocampus as well as some infarctions and glial proliferation in cortex of saline-treated 2VO rats. Long-term administration of Bai 2 mg/kg attenuated the chronic hypoperfusion-induced neuronal injury.

Table 3
Results of cell counts after 74 days of permanent occlusion of the bilateral common carotid arteries (2VO) in rats

	Cortex	Hippocampus
Sham-operated	60±6	51±4*
2VO+Saline	52±7	42±5
2VO+Bai 2 mg/kg	58±4	48±5*

All values are means±S.D. (n=7–8). Sham-operated group and Bai-treated group were compared with the saline-treated 2VO group. *P<0.05.

The results of cell counts are summarized in Table 3. On the Day 74 post-surgery, saline-treated 2VO rats showed a significant loss of hippocampus CA1 cells when compared to sham-operated rats (P<0.05). The reduction was attenuated by Bai treatment (P<0.05). No difference in cell numbers of stratum pyramidale was observed in cerebral cortex.

4. Discussion

The present study demonstrated that Bai improved cognitive deficits and neuronal injury induced by long-term cerebral

hypoperfusion in rats, and its antioxidant action is likely involved in the therapeutic effect.

It is believed that the permanent 2VO model mimics clinical conditions of the cerebrovascular hypoperfusion associated with aging in humans and exacerbated in victims of Alzheimer's disease and/or vascular dementia (de la Torre, 2000; Farkas and Luiten, 2001. This model is different from other animal models such as drug, brain lesion, or transient ischemia induced amnesia in rodents based on their progressive and long-lasting cognitive deficits accompanied by progressive histopathologic damage (Tsuchiya et al., 1992; de la Torre et al., 1992; Ni et al., 1994; Pappas et al., 1996). Therefore, the 2VO rat is a useful model for studying pathophysiology of learning and memory deficits in human dementia with cerebral circulation impairment, and for assessing therapeutic potential and/or exploring possible mechanisms of putative anti-dementia drugs (Sarti et al., 2002).

In the present study, we found that learning performance of permanent 2VO rats was progressively impaired in the water maze task and after surgical operation of 21, 52 and 70 days. This result is consistent with previous reports (Ni et al., 1994; Pappas et al., 1996). Long-term administration of Bai and/or tacrine effectively improved the impaired learning performance in 2VO rats. When the tacrine- and Bai-pretreated 2VO rats were paused for using the drugs for 31 days the animals showed significant learning and memory deficits in behavioral test on the Day 52 post-surgery. The cognitive deficits were improved when Bai and tacrine were administered again. This suggests that Bai, like tacrine, ameliorates the cognitive deficits in 2VO rats; however, the persistent effect requires a continuous administration (Murakami et al., 2000).

The histological examination is important for evaluating neuronal damage and drug action (Hunter et al., 1998). Neuronal degeneration was found to be correlated to deficits in spatial learning and memory (Ni et al., 1994). It was reported that 2VO caused an early-emerging impairment of Morris water maze acquisition and late-emerging CA1 cell loss (Pappas et al., 1996). Hippocampal pyramidal neurons degenerated after several weeks of reduced blood perfusion and this caused a progressive memory impairment (Pappas et al., 1996), indicating that the neurodegeneration of 2VO rats is a slow-onset and progressive process. In the present observation, we prudently selected to examine the histological change on the Day 74 after permanent 2VO. The surviving cell number significantly reduced in CA1 pyramidal area, but not in cortex. This discrepancy may be due to methodological factors and/or CA1 area is particularly susceptible to ischemia. Despite the cell number was not significantly altered, we found remarkable neuropathological alterations in both hippocampus CA1 and cortex. Bai attenuated chronic hypoperfusion-induced histological damage, consistent with the behavioral test results.

It is well known that the brain is particularly susceptible to oxidation by reactive oxygen species (ROS) because of its dependency on aerobic metabolism, large contents of polyunsaturated lipids in mitochondrial and cell plasma membranes, and low antioxidant defenses (Reiter, 1995). The permanent global ischemia produced progressive neuronal damage and abnormal changes in free radicals in the brain. The free radicals participate in mediating neuron degeneration and death, and are likely involved in pathogenesis of neurodegenerative diseases such as VD (Coyle and Putfarcken, 1993; Markesbery, 1997; Chong et al., 2005). ROS can highly damage neuronal cells due to the oxidation of essential cellular constituents such as lipids, proteins and DNA. Excessive generation of ROS results in lipid peroxidation of cell membrane, and subsequent damage is indicated by accumulation of MDA, a by-product of lipid peroxidation. We found that lipid peroxidation significantly increased in both cortex and hippocampus as a result of free radical generation induced by chronic hypoperfusion (Table 1). Meanwhile, the activity of SOD also elevated in the two regions. It is possible that a compensatory rise in antioxidant activity occurs in response to the increased free radical generation. The increased SOD activity could be considered an indication that the brain's antioxidant machinery is activated in the setting of overwhelming oxidative stress (Bannister et al., 1987). However, this activation of SOD seems not necessarily protective, because the experiments performed in rats with exogenous SOD (Imazuimi et al., 1990) showed that this enzyme had a protective effect against cerebral damage induced by ischemia, but reports on endogenous SOD level or activity in cerebrovascular ischemia are not consistent: SOD activity and concentration in brain tissue after ischemia/reperfusion was found to be either decreased (Tokuda et al., 1993) or increased (Sutherland et al., 1991). Enhanced SOD activity catalyzes the conversion of superoxide anions to hydrogen peroxide which is more toxic than the oxygen-derived free radicals and requires further scavenging by glutathione redox pathway and catalase (Bannister et al., 1987; Fridovich, 1995; Chong et al., 2005). The deleterious effect of enhanced SOD activity in the setting of oxidative stress has been recognized by a body of evidences in both in vivo and in vitro (Ratych et al., 1987; Omar and McCord, 1990). Free radicals can also directly damage antioxidant enzymes and reduce their activities (Escobar et al., 1996). In the present study we found that GPx and catalase activities significantly reduced in cortex and hippocampus in chronic cerebral ischemia. The diminished GPx and catalase activities account, at least in part, for the accumulation of lipid hydrogen peroxides, which can potentially turn on a chain reaction wherein more unsaturated lipids become targets for further peroxidative tissue injury. This is supported by concomitant increase in MDA (Table 1). Therefore, the increment in MDA level and SOD activity observed in 2VO group suggests a clear increased oxidative process, whereas the down-regulation of GPx and catalase activities represent deficiencies in endogenous antioxidant ability. Long-term treatment with Bai reversed the abnormality of free radical system, suggesting that this compound may attenuate the excessive formation of ROS and compensate antioxidant ability secondary to ischemia insult in vivo. This is consistent with the previous report that Bai possesses significant antioxidant activity (Hamada et al., 1993; Chang et al., 1993; Chen et al., 2000; Gao et al., 1999; Shieh et al., 2000). Recently numerous studies from other laboratories (Tabet et al., 2001; DeFeudis and Drieu, 2000; Liao et al., 2004) as well as our laboratory (Xiong et al., 2006) have demonstrated that agents possessing antioxidants and radical scavenger properties have

been available to treat vascular dementia in animal or in clinical trials. Our present observation showed that the dosages of Bai (2 mg/kg and 4 mg/kg) we selected could alleviate the cognitive deficits and neuronal impairment. These improvements were parallel to its antioxidant activity. However, the possibility of other mechanisms participating in the action of Bai would not be excluded, which remains to be studied in future.

To summarize, we have demonstrated that Bai significantly improves the cognitive deficits and neuropathological changes induced by chronic cerebral hypoperfusion in rats, and this effect is likely related, at least in part, to its antioxidant action. This information is important when the compound is considered for clinical trial in the treatment of vascular dementia.

Acknowledgments

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