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# Postischemic administration of Z-Ligustilide ameliorates cognitive dysfunction and brain damage induced by permanent forebrain ischemia in rats

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**Abstract**

Previous studies have demonstrated that Z-Ligustilide (LIG), a characterized phthalide constituent present in numerous medical Umbelliferae plants, has significant neuroprotective effects in transient forebrain ischemia and permanent cerebral focal ischemia. The present study further investigated the effect of LIG on chronic cerebral hypoperfusion. Male Wistar rats were subjected to permanent ligation of both common carotid arteries (2VO). On Days 8–12 postsurgery, rat cognition was assessed in the Morris water maze. Rats with significantly impaired acquisition of spatial information were randomly allocated to three groups and orally administered LIG (10 or 40 mg/kg/day) or volume-matched vehicle on Days 13–40 post-2VO surgery. The sham-operated group served as controls. After long-term treatment with LIG, the impaired animals' behavioral, biochemical, and histopathological features were examined. Compared to the sham-operated group, significant cognitive impairment was observed in the vehicle-treated group 40 days after 2VO. Shortened mean escape latency was detected in the Morris water maze in rats treated with LIG ($p<0.01$ *vs.* vehicle-treated group) during the same trial days. Chronic 2VO-induced pathological changes included neuronal loss and an increase of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the hippocampus. These effects were prevented with LIG treatment ($p<0.01$ *vs.* vehicle-treated group). LIG also significantly reduced malondialdehyde levels and increased superoxide dismutase activity in ischemic brain tissue ($p<0.05$ and $p<0.01$ *vs.* vehicle-treated group). In addition, LIG significantly increased choline acetyltransferase activity and inhibited acetylcholinesterase activity in ischemic brain tissues ($p<0.05$ and $p<0.01$ *vs.* vehicle-treated group). The present data demonstrate that LIG significantly prevented chronically hypoperfused cognitive deficits and brain damage at least partly through an antioxidant effect and improved cholinergic activity. The present findings suggest that LIG may have therapeutic potential in treating vascular dementia and cerebrovascular insufficiency.


*Keywords:* Z-Ligustilide (LIG); Chronic cerebral hypoperfusion; Spatial learning/memory dysfunction; Cholinergic system; Oxidative stress

## 1. Introduction

Vascular dementia is the second most common cause of dementia associated with Alzheimer's disease and accounts for 10–50% of all dementias (Rockwood et al., 2000). Vascular dementia is not a singular disease, but rather a group of conditions with different pathological and pathophysiological mechanisms (Desmond, 2004; Wallin and Blennow, 1993). Cerebral circulation disturbances have been associated with a decline in cognitive function in elderly subjects and development of vascular dementia (Kalaria, 1996; Sekhon et al., 1997). Cerebral ischemia produces abnormal levels of glucose, cholinergic substances, reactive oxygen species, and other metabolic substrates, which may initiate and sustain the cascade of neuropathological events underlying vascular dementia (Beal et al., 1993; Chan, 2001; Love, 1999; Ni et al., 1995; Tsuchiya et al., 1993). Permanent bilateral ligation of the common carotid arteries (2VO) in rats is a chronic cerebral hypoperfusion model that results in significant reduction of cerebral blood flow (Ni et al., 1994; Tsuchiya et al., 1991) and can cause learning and memory impairment and neuronal damage resembling the effects observed in vascular dementia (Ni et al., 1995; Sarti et al., 2002). 2VO rats provide a useful model to understand the pathophysiology of chronic

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cerebrovascular disorders and to screen drugs with potential therapeutic value in vascular dementia.

Z-Ligustilide (3-butylidene-4,5-dihydrophthalide, LIG) (Fig. 1) is a volatile oil and characteristic phthalide component of numerous Umbelliferae plants. LIG has been considered to be the main active ingredient of many medicinal plants, such as *Radix Angelicae sinensis* (Lin et al., 1979) and *Ligusticum chuangxiong* (Naito et al., 1996). Numerous studies from our group and others indicate that LIG has diverse biological activities, including smooth muscle relaxation (Du et al., 2006; Ko, 1980), improved microcirculation (Shi et al., 1995), anti-asthmatic and analgesic effects (Du et al., 2007; Tao et al., 1984), and antiproliferative effects on smooth muscle cells (Kobayashi et al., 1992, 1993). Previous studies have shown that LIG has significant neuroprotective effects on transient forebrain ischemia in mice (Kuang et al., 2006) and focal cerebral ischemia in rats (Peng et al., 2007) through antioxidant and antiapoptotic mechanisms. However, the effect of LIG on chronic cerebral hypoperfusion has not been reported. The present study investigated the cognition-enhancing and brain damage ameliorative effects of LIG in 2VO rats using the Morris water maze, histopathological examination, and analysis of antioxidant defense and the cholinergic system.

## 2. Materials and methods

### 2.1. Animals and surgery

A total of 60 male SPF Wistar rats weighing 300–350 g (certificate: SCXK2004-16) were purchased from the Center of Experimental Animals, Sichuan, P.R. China. Rats were housed in groups of four per cage at 22±1 °C with a 12-h light–dark cycle and were allowed free access to food and water. Food was withdrawn 12 h prior to surgery. All rats were used 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 (November 14, 1988). Fifty rats were subjected to 2VO surgery as described previously (Ni et al., 1994). Briefly, under choral hydrate (350 mg/kg, i.p.) anesthesia, the bilateral common carotid arteries of rats were exposed and carefully separated from the carotid sheath and cervical sympathetic and vagus nerves through a ventral cervical incision, and ligated with silk thread. Body temperature was maintained at about 37 °C during recovery from anesthesia with a feedback-regulated heating pad. The other 10 animals that received the same surgical operation without ligation of the carotid arteries served as sham-operated controls.

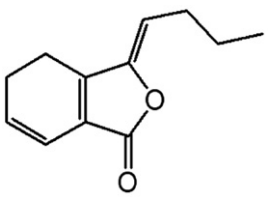

Fig. 1. Chemical structure of Z-Ligustilide (LIG).

### 2.2. Z-Ligustilide preparation

*Radix Angelicae sinensis* was purchased from its cultivating base at the Good Agricultural Practice in Min Xian County, Gansu Province, P.R. China. Its identity was confirmed by comparison with descriptions of characteristics and the appropriate monograph in the Chinese Pharmacopoeia 2000. LIG was prepared by a well-established procedure in our laboratory. Briefly, *Angelica sinensis* essential oil was extracted using supercritical-$CO_2$ fluid. LIG was isolated from the oil by silica–gel column chromatography and identified by electron impact ionization mass spectrometry, [$^1$H]nuclear magnetic resonance, and [$^{13}$C]nuclear magnetic resonance spectrometry. Purity was determined to be >98% based on the percentage of total peak area by high-performance liquid chromatography. LIG was prepared with 3% Tween-80 before use.

### 2.3. Pharmacological treatment

On the eighth day following 2VO (Day 8), all rats were subjected to a hidden platform screening trial twice daily for 5 consecutive days (Yu et al., 2005). The mean of the two escape latency values for each 2VO-operated rat (value 1) and the total mean escape latency for the sham-operated rats (value 2) on Day 12 were calculated. *Screen ratio* was selected as the index for evaluating cognitive deficits for each 2VO-operated rat, where *Screen Ratio = (value 1 − value 2)/value 2*. The rat was considered to have cognitive impairment if its *Screen Ratio* was greater than 0.2. The 2VO-operated rats with cognitive deficit then were randomly allocated to three groups based on escape latency and treated with LIG (10 or 40 mg/kg, i.g.) or volume-matched vehicle. Meanwhile, vehicle was administered to the sham-operated group. Daily oral administration of LIG or its vehicle was started on Day 13 and terminated on Day 40 following 2VO. At the end of the experiment, behavioral tests were performed 1 h after LIG or vehicle treatment.

### 2.4. Morris water maze

Cognitive function was tested in the Morris water maze (Morris, 1984; Yu et al., 2005). The maze consisted of a black circular pool (1.2 m diameter, 40 cm height) filled to a depth of 20 cm with water (22±1 °C). At the beginning of each day, the water was made opaque by adding 2 kg of milk to prevent the animals from seeing the submerged platform. A circular platform (14 cm diameter) was submerged 2 cm below the surface of the water and hidden from the rat's view. Four points, equally spaced along the circumference of the pool, were arbitrarily assigned as North, South, East, and West. The pool, therefore, was divided into four quadrants (Northeast, Southeast, Southwest, and Northwest). These points served as the starting positions for the rat being gently lowered into the water, with its head facing the wall of the water maze. The water maze test consisted of five stages: Days 8–12 for hidden platform training before treatment, Days 34–35 for a "post-treatment" latency test, Day 36 for a probe test, Days 37–39 for a reversal trial, and Day 40 for a visible platform trial.

#### 2.4.1. Hidden platform trial

Rats were given two trials per day with a submerged platform that they could climb onto to escape from the water. The location of the escape platform was fixed throughout training in the middle of the NE quadrant, 30 cm from the wall. Two starting points, equidistant from the platform location, were used. The starting points were different for consecutive trials, but were counterbalanced to prevent order effects. At the beginning of each trial, the rat was gently placed into the water at the start location, always facing the side of the tank. A trial ended when the rat escaped onto the platform, and the escape latency for each trial was recorded. If a rat failed to escape within 60 s, an escape latency of 60 s was recorded. Each rat was allowed to spend 10 s on the escape platform and then was placed for an additional 20 s in a holding cage before the next trial, resulting in an intertrial interval of 35 s. The mean escape latency of each daily trial then was calculated.

#### 2.4.2. Probe trial

In this 1-day test, each rat was subjected to a probe trial (60 s) in which there was no platform present on which to escape. One of two starting positions in the hidden platform trial was used; this position was consistent for all rats. For the probe trial, two measurements were made: (1) the time spent in the quadrant of the former platform position; (2) the number of crossings of the exact location where the platform had been located previously.

#### 2.4.3. Reversal trial

Rats were given a reversal trial similar to that described above for the hidden platform trial, except the platform was located in a novel position opposite to the location used in the hidden platform trial.

#### 2.4.4. Visible platform trial

To exclude the effects of motivational or sensorimotor factors on learning performance, the visible platform trial for all experimental animals was performed on Day 40. Rats were given two trials per day similar to those described above for the hidden platform trial, but the escape platform was elevated 2 cm above the water surface.

### 2.5. Histopathological examination

At the end of behavioral testing performed on Day 40, four rats in each group were deeply anaesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde, and brains were removed and embedded in paraffin. Coronal sections were cut into approximately 5 μm sections and stained with hematoxylin and eosin. In each CA1 subregion of the hippocampus, the number of intact-appearing pyramidal cells showing a distinct nucleus and nucleolus was counted along a 1.35 mm transection (×40 magnification). The number of pyramidal cells per mm in each rat was expressed as the mean of the three coronal sections.

GFAP antibody was immunostained with streptoavidin–biotin complex kit (Zhongshan Golden Bridge Biotechnology Corporation, Beijing, P.R. China). Brain sections were incubated with primary GFAP antibody (1:200, Zhongshan Golden Bridge Biotechnology Corporation, Beijing, P.R. China) at 4 °C overnight. Following washing and incubation with biotinylated secondary antibody, staining was visualized by diaminobenzidine. Blank staining was performed similarly as above, with the exception of eliminating the primary antibody. The numerical density of GFAP-immunoreactive cells in a unit area (×40 magnification) was counted in the dentate gyrus of the hippocampus. The number of astrocytes per $mm^2$ in each rat was expressed as the mean of the three coronal sections.

### 2.6. Biochemical analysis

At the end of behavioral testing performed on Day 40, six rats in each group were deeply anaesthetized with chloral hydrate and perfused transcardially with 100 ml saline. Brains were removed quickly and stored at −80 °C until assayed. For biochemical analysis, whole brain tissues were homogenized in 0.1 M sodium phosphate buffer (pH 7.4).

#### 2.6.1. Antioxidant activity measurement

Estimation of lipid peroxidation was done by measuring the lipid peroxidation product malondialdehyde (MDA) (Ohkawa et al., 1979) using commercially available kits (Jianchen Bioengineering, Nanjing, Jiangshu Province, P.R. China). The concentration of lipid peroxides was expressed in nanomoles of MDA per mg of protein using 1,1,3,3-tetraethoxypropane as the external standard. Superoxide dismutase activity in ischemic brains was assessed by adopting the procedure of Kakkar et al. (1984) using commercially available kits (Jianchen Bioengineering, Nanjing, Jiangshu Province, P.R. China). One unit of superoxide dismutase activity was defined as the ability to reduce absorbance by 50% at 550 nm.

#### 2.6.2. Cholinergic enzyme analysis

Choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) activities were determined spectrophotometrically (Chao and Wolfgram, 1972; Ellman et al., 1961) using commercially available kits (Jianchen Bioengineering, Nanjing, Jiangshu Province, P.R. China). One unit of ChAT or AChE activity was defined as the number of hydrolyzed nanomoles of acetylcholine, or micromoles of acetylthiocholine iodide, per min per mg of protein. Brain tissue protein content was estimated by the method of Bradford (1976).

### 2.7. Statistics analysis

All experimentation was performed by observers blind to treatment and histological assessment. Data analyses were performed with SPSS v.10.0. Data obtained over training days from the hidden platform trial and reversal trial were analyzed by two-way analysis of variance (ANOVA). Mean escape latency was the dependent variable, day was the within-subjects variable, and the five groups were the between-subjects variables. When appropriate, *post hoc* comparisons were assessed using the Least Significant Difference (LSD) test (equal variances

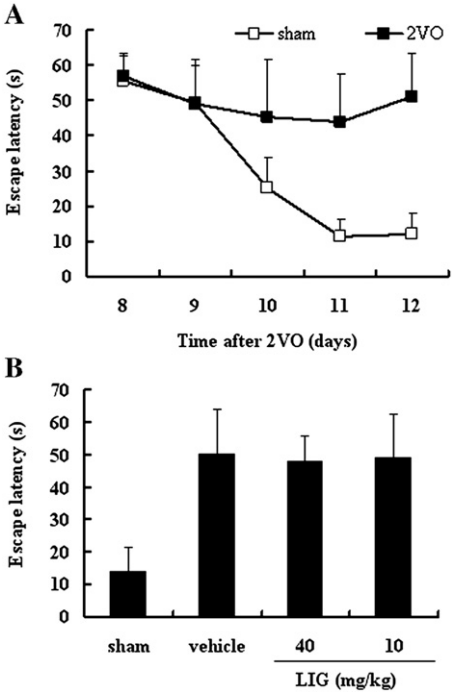


Fig. 2. Comparison of average escape latency times for each group in the hidden platform trial before treatment. (A) 2VO group ($n=36$) and sham-operated group ($n=10$). (B) Sham-operated group, vehicle-treated group, LIG 10 or 40 mg/kg-treated group ($n=10$ each group).

assumed) or Dunnett's T3 test (equal variances not assumed). Remaining data were analyzed by one-way ANOVA. All results are shown as mean±S.E.M. In all statistical comparisons, $p<0.05$ was used as the criterion for significance.

## 3. Results

### 3.1. Cognition function

#### 3.1.1. Hidden platform trial

On Day 8 postsurgery, the survival rate of the 2VO group was 72% (36 of 50). In the hidden platform trial, the escape latency for the sham-operated group ($n=10$) decreased in a day-dependent manner. Although the surgery group ($n=36$) followed a similar pattern (Fig. 2A), group comparisons revealed that it had a longer latency to find the platform than the sham-operated group across time ($F_{1,44}=39.691$, $p<0.001$; Fig. 2A). There were 30 operated rats exhibiting a longer latency (*screen ratio* >0.2), which were then randomly assigned to three groups ($n=10$) and used for the subsequent study. There was no significant difference among the 2VO groups in escape latency before treatment (Fig. 2B).

After long-term treatment, there was a significant difference among groups in escape latency ($F_{3,36}=46.685$, $p<0.001$). LIG (10, 40 mg/kg)-treated groups showed a significant decrease in escape latency compared to the vehicle group ($p<0.01$). No statistically significant differences were observed between the LIG-treated and sham-operated groups ($p>0.05$; Fig. 3, Days 34–35).

#### 3.1.2. Probe trial

Finding a hidden platform can be accomplished with some proficiency without animals employing a "spatial" strategy. Thus, the probe trial was employed to determine how well the rats located the platform in the maze. On Day 36 after 2VO, data were obtained from two measures of probe trial performance (Fig. 4A and B). As shown in Fig. 4A, the LIG (40 mg/kg)-treated group spent more time than the vehicle group in the training quadrant where the platform was previously located ($p<0.01$); the vehicle group visited all quadrants equally during 60 s of free swimming. Similar results were obtained from former platform crossings. The LIG (40 mg/kg)-treated group

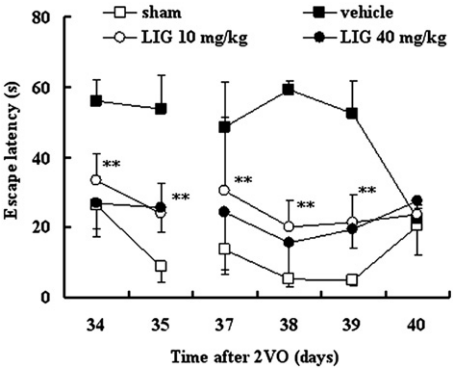


Fig. 3. Performance in the Morris water maze test for each group after LIG treatment. Data points represent the average latency to find the platform ($n=10$ each group). Days 34–35: hidden platform trial; Days 37–39: reversal trial; Day 40: visible platform trial. **$p<0.01$ *vs.* vehicle-treated group.

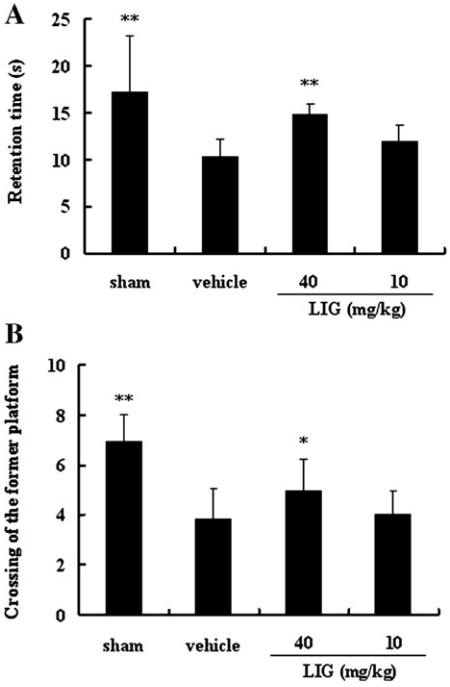


Fig. 4. Probe trial performance for each group after LIG treatment. Data represent the mean±S.E.M. ($n=10$ each group). (A) Time spent in the quadrant of the former platform position. (B) Crosses over the former platform location. *$p<0.05$, **$p<0.01$ *vs.* vehicle-treated group.

crossed over the platform location more frequently than the vehicle group ($p<0.05$, Fig. 4B).

### 3.1.3. Reversal trial

Fig. 3 shows the average latency to find the platform that was located in a different quadrant (SW) from the one used for the hidden platform (NE). In the successive three training days, there was a significant difference among groups ($F_{3,36}=134.837$, $p<0.001$). LIG (10 or 40 mg/kg)-treated groups spent significantly less time finding the platform than the vehicle-treated group ($p<0.01$).

### 3.1.4. Visible platform trial

Training in the visible platform trial took place after spatial training was completed. There was no significant difference in escape latency among the groups in the visible platform trial ($F_{3,36}=2.236$, $p>0.05$; Fig. 3, Day 40), indicating that motivation and sensory motor skills were intact.

## 3.2. Histopathological examination

Typical neuropathological changes occurred in rat hippocampus 40 days after 2VO. As shown in Fig. 5, neuronal

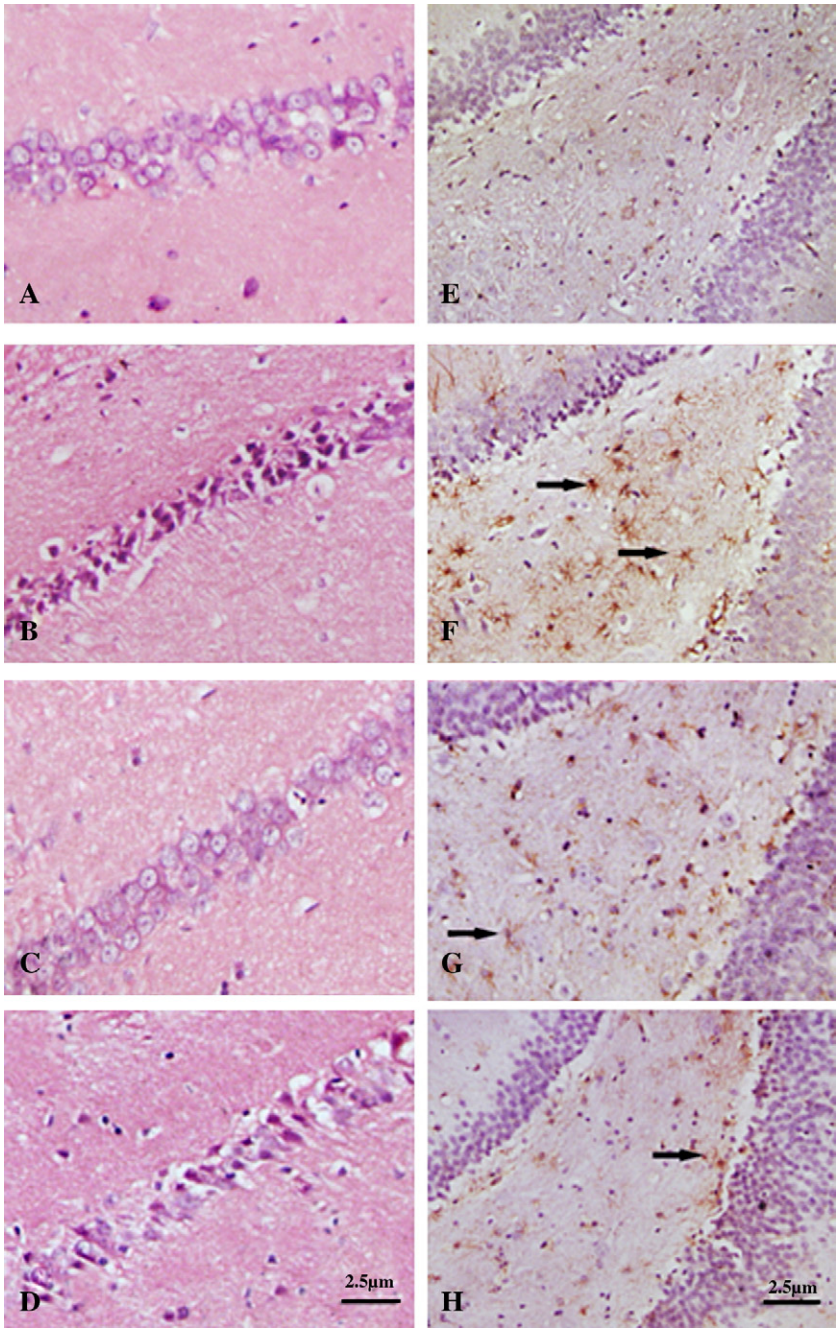


Fig. 5. Photomicrographs of histopathological changes in the rat hippocampus 40 days post-2VO. A–D, hippocampal CA1 subregion (HE, ×40 magnification); E–H, hippocampal dentate gyrus (immunohistochemistry of GFAP protein, ×40 magnification). A and E, sham-operated rat; B and F, 2VO rat treated with vehicle; C and G, 2VO rat treated with LIG 40 mg/kg; D and H, 2VO rat treated with LIG 10 mg/kg.

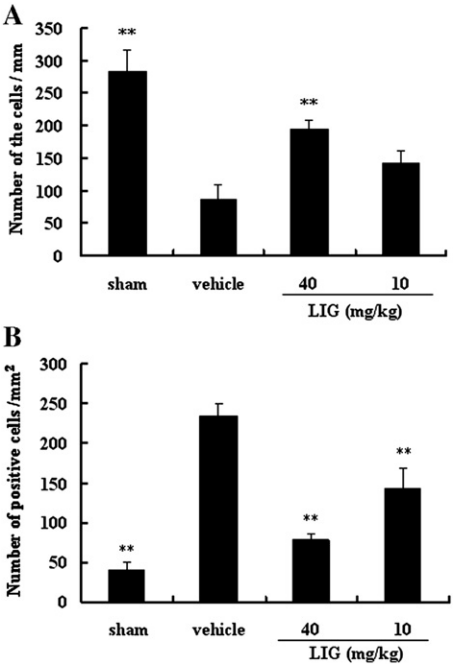


Fig. 6. Quantitatively histopathological changes of the hippocampus in 2VO rats 40 days post-2VO. (A) The pyramidal cells in CA1 regions were counted in the high power fields. (B) The GFAP-positive astrocytes in the hippocampal dentate gyrus were counted in the high power fields. Values are the mean±S.E.M. ($n=4$). **$p<0.01$ *vs.* vehicle-treated group.

loss, shrinkage, and dark staining of neurons were observed in the CA1 subregion of the hippocampus in the vehicle group. These changes were accompanied by a significant increase in the number of GFAP-immunoreactive astrocytes in the dentate gyrus of the hippocampus. LIG treatment greatly attenuated chronic hypoperfusion-induced morphologic changes. The effect of LIG was reflected by quantitative analysis of the number of CA1 neurons and the intensity of GFAP labeling in the dentate gyrus (Fig. 6). LIG dose-dependently inhibited hippocampal neuronal loss and astrocyte proliferation compared to the vehicle group ($p<0.01$).

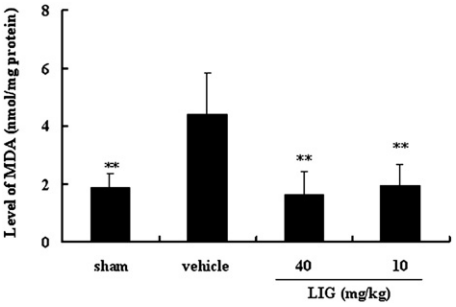


Fig. 7. Effects of LIG on MDA content in the brains of 2VO rats. Data represent the mean±S.E.M. ($n=6$ each group). MDA content significantly increased in the vehicle-treated group compared to the sham-operated group. Long-term treatment with LIG significantly decreased MDA content in brain tissue. **$p<0.01$ *vs.* vehicle-treated group.

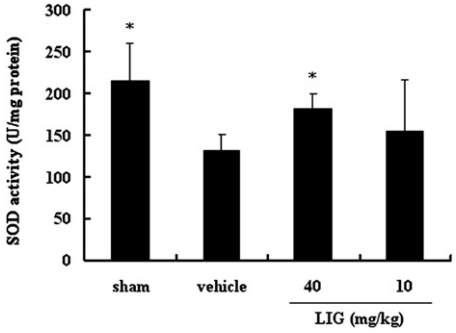


Fig. 8. Effects of LIG on superoxide dismutase activity in the brains of 2VO rats. Data represent the mean±S.E.M. ($n=6$ each group). Superoxide dismutase activity significantly decreased in the vehicle-treated group compared to the sham-operated group. Long-term treatment with LIG dose-depedently increased superoxide dismutase activity in brain tissue. *$p<0.05$ *vs.* vehicle-treated group.

### 3.3. Antioxidant activity

To study the mechanisms involved in the neuroprotective potential of LIG, its antioxidant effect was assessed by measuring MDA and superoxide dismutase activity in ischemic brain tissue. As shown in Fig. 7, MDA content significantly increased in the vehicle group (4.39±1.41 nmol/mg protein) compared to the sham-operated group (1.81±0.45 nmol/mg protein), whereas LIG treatment attenuated the increase in MDA content in 2VO rats. MDA content was significantly lower in the 10 mg/kg LIG group (1.94±0.74 nmol/mg protein) and 40 mg/kg LIG group (1.62±0.81 nmol/mg protein) compared to the vehicle group ($p<0.01$). In addition, the sham-operated group showed superoxide dismutase activity of 214±45 U/mg protein in brain tissues, which was decreased to 132±19 U/mg protein in the vehicle group ($p<0.05$). Consistent with this antioxidant effect, LIG treatment restored superoxide dismutase activity in ischemic brain tissue (Fig. 8). LIG dose-dependently increased superoxide dismutase activity at doses of 10 mg/kg (154±61 U/mg protein) and 40 mg/kg (182±17 U/mg protein, $p<0.05$ *vs*. vehicle group).

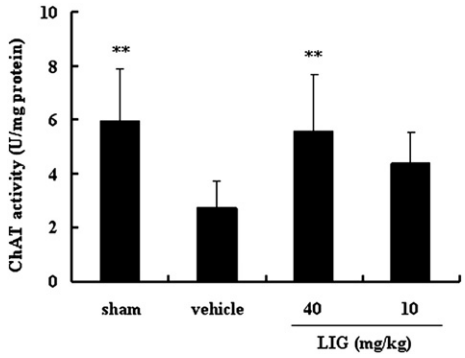


Fig. 9. Effects of LIG on ChAT activity in the brains of 2VO rats. Data represent the mean±S.E.M. ($n=6$ each group). ChAT activity significantly decreased in the vehicle-treated group compared to the sham-operated group. Long-term treatment with LIG dose-dependently increased ChAT activity in brain tissue. **$p<0.01$ *vs.* vehicle-treated group.

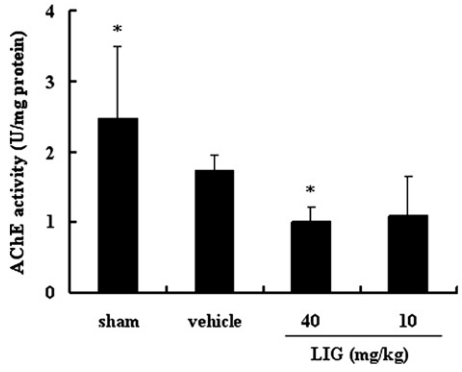


Fig. 10. Effects of LIG on AChE activity in the brains of 2VO rats. Data represent the mean±S.E.M. ($n=6$ each group). AChE activity significantly decreased in the vehicle-treated group compared to the sham-operated group. Long-term treatment with LIG dose-dependently decreased AChE activity in brain tissue. *$p<0.05$ *vs.* vehicle-treated group.

### 3.4. Cholinergic activity

To further study the mechanisms involved in the neuroprotective potential of LIG, the activities of the cholinergic enzymes ChAT and AChE were assessed. The basal activities of ChAT and AChE were 5.97±1.94 and 2.47±1.02 U/mg protein, respectively, in the brains of sham-operated animals. As shown in Figs. 9 and 10, 2VO caused a significant decrease in cerebral ChAT activity (2.71±0.98 U/mg protein) and AChE activity (1.73±0.21 U/mg protein) compared to the sham-operated group ($p<0.01$ and $p<0.05$, respectively). LIG induced a dose-dependent increase in ChAT activity together with a decrease in AChE activity in the ischemic brain tissues of 2VO rats. LIG (40 mg/kg) administration significantly recovered ChAT activity to 5.58±2.11 U/mg protein and reduced AChE activity to 1.00±0.20 U/mg protein ($p<0.01$ and $p<0.05$ *vs*. vehicle-treated group).

## 4. Discussion

Z-Ligustilide is the primary active component of many plants used in traditional Chinese medicines and has been found to facilitate blood circulation and remove blood stasis. Phytochemical studies indicate that LIG is a highly lipophilic compound with an oil/water partition coefficient (logP) of 2.87 and molecular weight of 190, and has been shown to penetrate the blood brain barrier (preliminary data, not shown). Previous studies have demonstrated that LIG ameliorated brain damage and neurobehavioral deficits caused by transient forebrain ischemia (Kuang et al., 2006) and permanent focal cerebral ischemia (Peng et al., 2007). The present study demonstrates that postischemic treatment with LIG also prevented both functional and structural abnormalities in rats subjected to chronic cerebral hypoperfusion.

Clinical observations indicate that vascular dementia manifests with severe, progressive cognitive impairment and neuropathological changes (Barclay et al., 1985; Wang et al., 2000). Therefore, the importance of functional and/or morphological measures of neuronal damage has been emphasized in preclinical studies on the efficacy of potential neuroprotective drugs (Stroke Therapy Academic Industry Roundtable, 1999). In general, cognition includes at least three primary processes: acquisition, consolidation, and retention. The Morris water maze is a hippocampus-dependent memory task (Moser et al., 1995) that has been used to assess cognitive deficits in the ischemic brain. In the present study, the hidden platform trial measured acquisition, the reversal trial measured relearning ability, and the probe trial measured retention (Kucukatay et al., 2002). Postischemic LIG administration (10 or 40 mg/kg/day, i.g.) significantly decreased the latency of 2VO rats to reach the platform in both the hidden platform and reversal trials compared to vehicle controls. LIG treatment also increased the time 2VO rats spent swimming in the former quadrant and across the former position of the platform. These observations suggest that postischemic LIG administration significantly improves cognitive dysfunction caused by chronic cerebral hypoperfusion. In addition, 2VO is hypothesized to induce visual dysfunction, which affects behavioral performance. However, studies have reported that retinal/optic nerve dysfunction in 2VO rats varies substantially in rats of different strains, of different ages, or from different suppliers (Davidson et al., 2000; De Butte et al., 2002), possibly due to differing propensities to establish retrograde flow to the internal carotid artery from the primary vertebral artery and subsequent branches. In the present visible platform trial conducted on Day 40 post-2VO, there was no significant difference in the time spent finding the visible platform in the water maze between sham-operated and 2VO rats (300–350 g), suggesting chronic 2-VO-induced visual dysfunction did not play a role in the present results. In addition, we observed neuropathological alterations in the hippocampus of rats 40 days after permanent 2VO, and found that LIG treatment attenuated the hippocampal damage. Counting of pyramidal cells in the CA1 regions confirmed that there were significantly increased numbers of intact CA1 cells in LIG-treated 2VO rats.

It is well known that cerebral ischemia can induce abnormal proliferation of astrocytes that divide and/or migrate to the lesioned area, go through hypertrophy, and subsequently form the bulk of the mature glial scar. Most adult mammalian brain areas, such as the CA1, cannot regenerate after lesion. This failure to regenerate is attributed to limited intrinsic neurogenic ability and the presence of dense glial scarring and other associated inhibitory molecules (Fawcett and Asher, 1999). The hippocampal dentate gyrus subgranular zone is one of two neurogenic regions in the adult mammalian brain (Alvarez-Buylla and Lim, 2004; Drapeau et al., 2003), and the production of new neurons in the dentate gyrus in mammals occurs continuously throughout life (van Praag et al., 1999). Therefore, the effect of LIG on the number GFAP-positive astrocytes in the dentate gyrus of 2VO rats was measured. The results demonstrated that LIG dose-dependently decreased dentate gyrus GFAP protein immunoreactivity, which may be beneficial for neuronal regeneration in the ischemic hippocampal dentate gyrus and contribute to improving, or maintaining, learning and memory function in 2VO rats.

Many studies have suggested that multiple mechanisms are involved in the development of vascular dementia (Farkas et al.,

2002). There is evidence showing that lipid peroxidation significantly damages membranes and generates neurotoxic secondary products (Bassett and Montine, 2003). Clinical research findings also indicate reduced antioxidant defense involved in the pathophysiology of vascular dementia (Floyd, 1999; Ryglewicz et al., 2002). A marked increase in lipid peroxidation in brain tissues, resulting from free-radical generation induced by chronic cerebral hypoperfusion, was observed in the present study. Activity of the antioxidant enzyme superoxide dismutase also was decreased in brain. Chronic LIG treatment reversed free radical system dysfunction induced by permanent 2VO. The present findings suggest that the palliative effect of LIG on cognitive deficits is associated with its antioxidant activity.

The central cholinergic system is well known to play an important role in cognitive function, such as memory and attention, and is modulated by acetylcholine (ACh) (Muir et al., 1993; Whishaw and Tomie, 1987). ChAT and AChE are considered to be definitive markers of cholinergic function. It has been reported that oxidative stress enhances AChE activity, and exogenous antioxidants can prevent increased AChE activity via maintaining mitochondrial membrane integrity and controlling reactive oxygen species efflux (Jiang et al., 2007). In the present study, chronic cerebral hypoperfusion induced a significant decrease in ChAT and AChE activity in ischemic brain tissues, possibly due to loss of cholinergic neurons, which is consistent with 2VO-induced cholinergic dysfunction (Tanaka et al., 1996). Long-term treatment with LIG, however, possibly restored decreased ChAT activity to levels seen in sham-operated rats through cholinergic neuroprotection, and further inhibited decreased ischemic cerebral AChE activity via antioxidation. Thus, the current observations indicate that the beneficial effect of LIG on 2VO-induced cognitive dysfunction may be associated with its ameliorative effect on central cholinergic tone. Impaired ACh synthesis with 2VO may be supplemented by increased cholinergic tone through LIG-induced AChE inhibition as well as ChAT improvement.

Furthermore, central cholinergic systems play important roles in regulating cerebral circulation (Scremin et al., 1973). LIG has been reported to have potent vasodilatory effect and improve microcirculation (Shi et al., 1995). Therefore, it is likely that LIG helps to establish collaterals, improve cerebral perfusion, and improve microcirculation in permanent 2VO. However, further studies are needed to elucidate the effects of LIG on cerebral microcirculation in the ischemic brain.

The brain is easily damaged, compared to other tissues, by global ischemia as a result of high brain oxygen and energy consumption rates. Global ischemia causes serious brain injury through rapid energy depletion. Slow (or weak) excitotoxicity has been proposed as a mechanism of cerebral neuronal damage due to impaired cellular energetics (Doble, 1999). In addition, the importance of reactive oxygen species in mediating excitotoxicity is highlighted by the ability of antioxidants and nitric oxide inhibitors to attenuate excitotoxic damage (Nakao et al., 1996; Schulz et al., 1995). Thus, investigating the effects of LIG on cerebral ischemia-induced energy shortage and excitotoxicity may be crucial for fully understanding the mechanisms of its neuroprotective effects.

Taken together, the present results indicate that LIG significantly ameliorates cognitive deficits and histopathological disturbances, and improves abnormal changes in endogenous antioxidant defense and cholinergic activity due to chronic cerebral hypoperfusion in rats. The data suggest that LIG may be of therapeutic potential in treating vascular dementia and cerebrovascular insufficiency.

## References


Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. Neuron 2004;41:683–6.

Barclay LL, Zemcov A, Blass JP, Sansone J. Survival in Alzheimer's disease and vascular dementias. Neurology 1985;35:834–40.

Bassett CN, Montine TJ. Lipoproteins and lipid peroxidation in Alzheimer's disease. J Nutr Health Aging 2003;7:446–51.

Beal MF, Hyman BT, Koroshetz W. Do defects in mitochondrial metabolism underlie the pathology of neurodegenerative diseases? Trends Neurosci 1993;16:125–31.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cerebr Blood Flow Metab 2001;21:2-14.

Chao LP, Wolfgram F. Spectrophotometric assay for choline acetyltransferase. Anal Biochem 1972;46:114–6.

Davidson CM, Pappas BA, Stevens WD, Fortin T, Bennett SA. Chronic cerebral hypoperfusion: loss of pupillary reflex, visual impairment and retinal neurodegeneration. Brain Res 2000;859:96-103.

De Butte M, Fortin T, Pappas BA. Pinealectomy: behavioral and neuropathological consequences in a chronic cerebral hypoperfusion model. Neurobiol Aging 2002;23:309–17.

Desmond DW. Vascular dementia. Clin Neurosci Res 2004;3:437–48.

Doble A. The role of excitotoxicity in neurodegenerative disease: implications for therapy. Pharmacol Ther 1999;81:163–221.

Drapeau E, Mayo W, Aurousseau C, Le Moal M, Piazza PV, Abrous DN. Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. Proc Natl Acad Sci USA 2003;100:14385–90.

Du J, Bai B, Kuang X, Yu Y, Wang C, Ke Y, et al. Ligustilide inhibits spontaneous and agonists- or K+ depolarization-induced contraction of rat uterus. J Ethnopharmacol 2006;108:54–8.

Du J, Yu Y, Ke Y, Wang C, Zhu L, Qian ZM. Ligustilide attenuates pain behavior induced by acetic acid or formalin. J Ethnopharmacol 2007;112:211–4.

Ellman GL, Courtney KD, Andres Jr V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

Farkas E, de Wilde MC, Kiliaan AJ, Luiten PG. Chronic cerebral hypoperfusion-related neuropathologic changes and compromised cognitive status: window of treatment. Drugs Today (Barc) 2002;38:365–76.

Fawcett JW, Asher RA. The glial scar and central nervous system repair. Brain Res Bull 1999;49:377–91.

Floyd RA. Antioxidants, oxidative stress, and degenerative neurological disorders. Proc Soc Exp Biol Med 1999;222:236–45.

Jiang H, Zhang J, Zhu H, Li H, Zhang X. Nerve growth factor prevents the apoptosis-associated increase in acetylcholinesterase activity after hydrogen peroxide treatment by activating Akt. Acta Biochim Biophys Sin (Shanghai) 2007;39:46–56.

Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 1984;21:130–2.

Kalaria RN. Cerebral vessels in ageing and Alzheimer's disease. Pharmacol Ther 1996;72:193–214.

Ko WC. A newly isolated antispasmodic-butylidenephthalide. Jpn J Pharmacol 1980;30:85–91.

Kobayashi S, Mimura Y, Notoya K, Kimura I, Kimura M. Antiproliferative effects of the traditional Chinese medicine Shimotsu-To, its component cnidium rhizome and derived compounds on primary cultures of mouse aorta smooth muscle cells. Jpn J Pharmacol 1992;60:397–401.

Kobayashi S, Mimura Y, Naitoh T, Kimura I, Kimura M. Chemical structure-activity of cnidium rhizome-derived phthalides for the competence inhibition of proliferation in primary cultures of mouse aorta smooth muscle cells. Jpn J Pharmacol 1993;63:353–9.

Kucukatay V, Balkan S, Yaras N, Yargicoglu P, Agar A. The effect of pergolide on cognitive performance of young and middle-aged rats. Int J Neurosci 2002;112:1027–36.

Kuang X, Yao Y, Du JR, Liu YX, Wang CY, Qian ZM. Neuroprotective role of Z-ligustilide against forebrain ischemic injury in ICR mice. Brian Res 2006;1102:145–53.

Lin M, Zhu GD, Sun QM, Fang QC. Chemical studies of *Angelica sinensis*. Acta Pharmceut Sin 1979;14:529–34.

Love S. Oxidative stress in brain ischemia. Brain Pathol 1999;9:119–31.

Morris R. Developments of a water maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.

Moser MB, Moser EI, Forrest E, Andersen P, Morris RG. Spatial learning with a minislab in the dorsal hippocampus. Proc Natl Acad Sci USA 1995;92:9697–701.

Muir JL, Page KJ, Sirinathsinghji DJS, Robbins TW, Everitt BJ. Excitotoxic lesions of basal forebrain cholinergic neurons: effects on learning, memory and attention. Behav Brain Res 1993;57:123–31.

Naito T, Ikeya Y, Okada M, Mistuhashi H, Maruno M. Two phthalides from Ligusticum chuanxiong. Phytochemistry 1996;41:233–6.

Nakao N, Grasbon-Frodl EM, Widner H, Brundin P. Antioxidant treatment protects striatal neurons against excitotoxic insults. Neuroscience 1996;73:185–200.

Ni J, Ohta H, Matsumoto K, Watanabe H. Progressive cognitive impairment following chronic cerebral hypoperfusion induced by permanent occlusion of bilateral carotid arteries in rats. Brain Res 1994;653:231–6.

Ni JW, Matsumoto K, Li HB, Murakami Y, Watanabe H. Neuronal damage and decrease of central acetylcholine level following permanent occlusion bilateral common carotid arteries in rats. Brain Res 1995;673:290–6.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.

Peng HY, Du JR, Zhang GY, Kuang X, Liu YX, Qian ZM, et al. Neuroprotective effect of Z-ligustilide against permanent focal ischemic damage in rats. Biol Pharm Bull 2007;30:309–12.

Rockwood K, Wentzel C, Hachinski V, Hogan DB, MacKnight C, McDowell I. Prevalence and outcomes of vascular cognitive impairment. Neurology 2000;54:447–51.

Ryglewicz D, Rodo M, Kunicki PK, Bednarska-Makaruk M, Graban A, Lojkowska W, et al. Plasma antioxidant activity and vascular dementia. J Neurol Sci 2002;203/204:195–7.

Sarti C, Pantoni L, Bartolini L, Inzitari D. Cognitive impairment and chronic cerebral hypoperfusion: what can be learned from experimental models. J Neurol Sci 2002;203/204:263–6.

Schulz JB, Matthews RT, Jenkins BG, Ferrante RJ, Siwek D, Henshaw DR, et al. Blockade of neuronal nitric oxide synthase protects against excitotoxicity in vivo. J Neurosci 1995;15:8419–29.

Scremin OU, Rovere AA, Raynald AC, Giardini A. Cholinergic control of blood flow in the cerebral cortex of the rat. Stroke 1973;4:233–9.

Sekhon LHS, Morgan MK, Spence I, Weber NC. Chronic cerebral hypoperfusion: pathological and behavioral consequences. Neurosurgery 1997;40: 548–56.

Shi LF, Zheng XM, Cai Z, Wu BS. Comparison of influence of essential oil from *Ligusticum chuanxiong* Hort. on microcirculation in rabbit conjunctiva bulbar before and after decomposition of ligustilide. Chin J Pharmcol Toxicol 1995;9:157–8.

Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999;30:2752–8.

Tao JY, Ruan YP, Mei QB, Liu S, Tian QL, Chen YZ, et al. Studies of antiasthmatic action of ligustilide of dang-gui, *Angelica sinensis* (Oliv.) Diels. Acta Pharmaceut Sin 1984;19:561–5.

Tanaka K, Ogawa N, Asanuma M, Kondo Y, Nomura M. Relationship between cholinergic dysfunction and discrimination learning disabilities in Wistar rats following chronic cerebral hypoperfusion. Brain Res 1996;729:55–65.

Tsuchiya M, Sako K, Yura S, Yonemasu Y. Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats. Exp Brain Res 1991;89:87–92.

Tsuchiya M, Sako K, Yura S, Yonemasu Y. Local cerebral glucose utilisation following acute and chronic bilateral carotid artery ligation in Wistar rats: relation to changes in local cerebral blood flow. Exp Brain Res 1993;95:1–7.

van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 1999;2:266–70.

Wallin A, Blennow K. Heterogeneity of vascular dementia: mechanisms and subgroups. J Geriatr Psychiatry Neurol 1993;6:177–88.

Wang LM, Han YF, Tang XC. Huperzine A improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. Eur J Pharmacol 2000;398:65–72.

Whishaw IQ, Tomie JA. Cholinergic receptor blockade produces impairments in a sensorimotor subsystem for place navigation in the rat: evidence from sensory, motor and acquisition tests in a swimming pool. Behav Neurosci 1987;101:603–16.

Yu J, Liu C, Zhang X, Han J. Acupuncture improved cognitive impairment caused by multi-infarct dementia in rats. Physiol Behav 2005;86:434–41.