Effects of Amelioration of Total Flavonoids from Stems and Leaves of *Scutellaria baicalensis Georgi* on Cognitive Deficits, Neuronal Damage and Free Radicals Disorder Induced by Cerebral Ischemia in Rats

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Received June 27, 2005; accepted October 17, 2005

Previous studies reported that the total flavonoids from the stems and leaves of *Scutellaria baicalensis Georgi* (TFSS) could enhance and improve learning and memory abilities in experimental animals, and reduce the neuronal pathologic alterations induced by some reagents in mice. The present study examined whether TFSS can improve memory dysfunction, neuronal damage, and abnormal free radicals induced by permanent cerebral ischemia in rats. The permanent cerebral ischemic model in rats was produced by bilateral ligation of the common carotid arteries. The influence of permanent cerebral ischemia on learning and memory was determined in the Morris water maze. The neuronal damage in the hippocampus and cerebral cortex was assessed by the neuronal morphologic observations. The contents of malondialdehyde (MDA) and nitric oxide (NO), and the activities of superoxide dismutase (SOD) and catalase (CAT) in the hippocampus and cerebral cortex were measured using thiobarbituric acid, nitrate reductase, xanthine–xanthine oxidase, and ammonium molybdate spectrophotometric methods, respectively. In learning and memory performance tests, cerebral ischemic rats always required a longer latency time to find the hidden platform and spent a shorter time in the target quadrant in the Morris water maze. TFSS 17.5—70 mg·kg⁻¹ daily orally administered to ischemic rats for 20 d, from day 16—35 after operation differently reduced the prolonged latency and increased swimming time spent in the target quadrant. In neuronal morphologic observations, daily oral TFSS 17.5—70 mg·kg⁻¹ for 21 d, from day 16—36 after operation markedly inhibited the ischemia-induced neuronal damage. In addition, the increased contents of MDA and NO, and SOD activity, and the decreased activity of CAT in the hippocampus and cerebral cortex induced by cerebral ischemia were differently reversed. The reference drug piracetam (140 mg·kg⁻¹ per day for 20—21 d) similarly improved impaired memory and neuronal damage but had no significant effects on free radicals in ligated rats. TFSS can improve memory deficits and neuronal damage in rats after permanent cerebral ischemia, which may be beneficial in the treatment of cerebrovascular dementia.

Key words *Scutellaria baicalensis Georgi*; cerebral ischemia; rat; Morris water maze; neuropathology; free radical

It is well known that vascular disorders in the brain are important causes of dementia, which are second only to Alzheimer’s disease in the elderly.1 Based on clinical observations, patients with vascular dementia often show a series of mental disorders, such as personality alterations, memory deficits, and thinking variations. The main pathophysiologic changes in the disorders include neuronal damage, hippocampal sclerosis, abnormal glial cell activation and proliferation, and abnormal changes in some metabolites.2—4 The permanent ligation of the bilateral common carotid arteries in rats is a common cerebral ischemic model to study vascular dementia and drug effects on the disorder.5—7 Because of the increasing numbers of patients with vascular dementia world wide, research on the protection from and treatment of vascular dementia is becoming a hot topic. However, effective medical and biological measures for treatment the disorder in clinical practice are not well established.1

The total flavonoids isolated from stems and leaves of *Scutellaria baicalensis Georgi* (TFSS) have been shown to have many important pharmacologic effects on vascular activities,5—7 The current study is designed to explore the actions of TFSS on cerebral ischemia in rats induced by permanent ligation of the common carotid arteries.

MATERIALS AND METHODS

**Animals** Male Sprague–Dawley rats (270—300 g, clean grade, certification no. 04057) were supplied by the Experimental Animal Center of Hebei Medical University. The rats were housed in groups of 4 or 5 per cage at a constant temperature (23—25 °C) in a light-controlled (12-h light–dark cycle) room. Food and water were freely available.

**Surgery** The rats were anesthetized with pentobarbital sodium (30 mg·kg⁻¹ i.p.) and allowed spontaneous respiration throughout the surgical procedure. Through a midline cervical incision, the bilateral common carotid arteries were exposed and ligated with no. 1 thread simultaneously in the permanent cerebral ischemia group. Rats that underwent the same surgery without carotid artery ligation served as the sham controls. The rats were placed on a heating pad after surgery until they had recovered from anesthesia.

**Reagents** TFSS (purity 80.11%) was prepared by the Department of Phytochemistry in Institute of Chinese Materia Medica, Chengde Medical College, China. TFSS was produced in a standard fashion, which was extracted repeatedly from the milled stems and leaves of *S. baicalensis Georgi*, using the alkali-acid method. The final total flavonoid content of this extract was not less than 80%. Scutellarein was the major ingredient, comprising about 20% of total flavonoids as shown by HPLC analysis. The reference drug piracetam was supplied by Tianjin Jinshi Pharmaceutical Co. The reagent kits for measurement of malondialdehyde (MDA) and nitric oxide (NO) contents superoxide dismutase (SOD) and catalase (CAT) activities were purchased from the Nanjing Institute of Jiancheng Biological Engineering. Other reagents were of analytical grade.

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Morris Water Maze Task  The learning and memory performance of rats was evaluated using the Morris water maze. The maze apparatus consists of a circular pool 120 cm in diameter and 50 cm deep. The pool is filled with water at approximately 24 °C to a height of 31.5 cm and made opaquely white with no-fat milk powder. A transparent acrylic platform (diameter 10 cm) is located at a constant position in the middle of one quadrant 1.5 cm below the surface of the water. Every spatial cue external to the maze is held constant throughout the testing period. The task began on day 31 after surgery and the rats were tested for 5 consecutive days with two trials per day. The time taken to climb onto the hidden platform was recorded at latency and calculated for an average over two trials. When the rats found the hidden platform within 60 s, they were allowed to remain there for 20 s. If the rats failed to find the hidden platform within 60 s, they were placed on the platform and remained there for 20 s. Recovery periods of 10 s were allowed between the two trials. Following the last learning trial, each rat was immediately subjected to a 60 s probe trial, in which the platform was removed and the time spent in the target quadrant was recorded. Swimming activity was monitored by a video camera linked to a computer-based image analyzer.

Experimental Procedure  The entire experiment took 36 d as based on the previous reports. All rats were allowed 15 d for recovery from the surgery. TFSS was dissolved in distilled water prior to administration, and the solution pH was adjusted to 7.2—7.4 with saturated sodium bicarbonate. TFSS (17.5, 35, 70 mg·kg⁻¹), or piracetam (140 mg·kg⁻¹), or distilled water (10 ml·kg⁻¹) was administered orally daily for 21 d, from day 16—36 after surgery. The Morris water maze task began on day 31 after surgery and the rats were tested for consecutive 5 d. During the learning performance test, the drug was administered 60 min before the trial. Following the probe trial, all rats were decapitated on day 36 after surgery and the hippocampus and cerebral cortex were separated for morphologic observations and MDA, NO, SOD, and CAT examinations.

Measurements of Neuromorphology, Contents of MDA and NO, and Activities of SOD and CAT  Under ether anesthesia, the rats were decapitated 60 min following 21 d of drug administration on day 36 after surgery. The rat brains were rapidly placed on ice and the hippocampus and cerebral cortex of one hemisphere from 3 or 4 rats in each group were dissected. The two sections were then fixed with 4% formalin and embedded in paraffin. Coronal sections were cut at approximately 6 μm and stained with hematoxylin and eosin (HE) as described previously. HE-stained cells in CA 1 of the hippocampus and in the external pyramidal lamina of the cerebral cortex were visualized and photographed at magnification of 20× and 40× using an Olympus VANOX microscope. The measurement of neuromorphology was conducted by an investigator blinded to the experimental conditions.

Except for morphologic observations, the hippocampus and cerebral cortex were separated and homogenized in cold saline to obtain 10% homogenates to assay MDA and NO contents, and 1% homogenates for SOD and CAT activity measurement using thiobarbituric acid, nitrate reductase, xanthine–xanthine oxidase, and ammonium molybdate spectrophotometric methods, respectively. The SOD activity was expressed as the difference in nitrite content between the control in the test kit and sample. The nitrite content was calculated according to the standard curve, \( Y = 0.06683X - 0.009373 \), where \( Y \) refers to absorbance value, and \( X \) refers to the concentration of nitrite (mmol·l⁻¹). The whole process of measurement was in an ice-cold environment. The protein content was determined using the Coomassie blue protein-binding method, using bovine serum albumin as a standard.

Statistical Analysis  The data were expressed as \( \bar{x} \pm s \). Group differences in latency in the Morris water maze were analyzed using two-way analysis of variance (ANOVA) with repeated measures. Group differences in the probe trial and biochemical assays were evaluated using one-way ANOVA followed by Duncan’s multiple-range test.

RESULTS

Effects of TFSS on Water Maze Learning  In naive rats, the mean latency in finding the hidden platform declined progressively during the training period of 5 consecutive days. The model group consistently took longer latency to find the platform than the sham group in the Morris water maze. Two-way ANOVA (group × days, 6 × 5) with repeated measures revealed a significant main effect of groups \( [F(1,7)=38.8, p<0.01] \) and significant interaction \( [F(4,28)=11.8, p<0.01] \). The prolonged latency could be shortened by TFSS. Two-way ANOVA with repeated measures showed the main effect of groups \( [F(1,7)=1.1, p>0.05] \) and interaction \( [F(4,28)=8.1, p<0.05] \) for TFSS 17.5 mg·kg⁻¹; \( [F(1,7)=21.6, p<0.01] \) and \( [F(4,28)=13.4, p<0.01] \) for TFSS 35 mg·kg⁻¹; as well as \( [F(1,7)=5.8, p<0.05] \) and \( [F(4,28)=12.3, p<0.01] \) for TFSS 70 mg·kg⁻¹. The reference drug piracetam also showed similar improvements \( [F(1,7)=15.3, p<0.01] \) and \( [F(4,28)=10.4, p<0.01] \) (Fig. 1A).

In the probe trial, the swimming time spent in the target quadrant of the model group was 41.3% lower compared with the sham group. The time was increased by 8.5% \( (p>0.05) \), 54.0% \( (p<0.01) \), and 36.9% \( (p<0.05) \) by TFSS 17.5, 35 and 70 mg·kg⁻¹, respectively, compared with the model group. A similar result was also observed in ligated rats administrated piracetam 140 mg·kg⁻¹ (Fig. 1B).

Effects of TFSS on Neuronal Damage  All rats were decapitated 60 min after following 21 d drug administration on day 36 after surgery. The brains were immediately placed on ice and the cerebral histologic changes were observed with the naked eye. Compared with the sham group, the model group brain exhibited severe neuronal damage. The brain became soft and shapeless; the cerebral cortex thinned and appeared light yellow. Consecutive daily oral administration of TFSS (17.5, 35, 70 mg·kg⁻¹) or piracetam (140 mg·kg⁻¹) for 21 d reduced the damage. Light microscopic observations assessed the neuron morphology. Compared with the sham group (Fig. 2A, A 1), the neurons of hippocampus and cerebral cortex showed typical colliquative necrosis, including reticular softening lesions, nucleus shrinkage or disappearance, cell membrane breaks, and cytoplasm dissolution and dense inflammatory cell infiltration in necrotic regions were found in the model group (Fig. 2B, B 1). The neuronophagia, satellitosis, neuroglial cell proliferation, and glial node formation were also found from the observation of other im-
Daily and orally administered TFSS (17.5, 35, 70 mg·kg⁻¹) for 20 d, from day 16—35 after bilateral common carotid arteries were ligated. Each rat was subjected to two trials daily for 5 d and begun on day 31 after surgery. A: Mean latency to find the hidden platform. The data were analyzed using two-way ANOVA (group × days, 6×5) with repeated measures. B: Swimming time spent in target quadrant in 60s probe trial (no platform). Each rat was immediately subjected to 60s observation following the last learning performance on day 35 after surgery. Figs. 1C, 1D, *p<0.05, **p<0.01, compared with the sham group; #p<0.05, ##p<0.01, compared with the model group.

Fig. 1. Effects of Total Flavonoids Obtained from Stems and Leaves of Scutellaria baicalensis Georgi (TFSS) on Morris Water Maze Performance Deficits Induced by Permanent Cerebral Ischemia in Rats (n=8). Daily and orally administered TFSS (17.5, 35, 70 mg·kg⁻¹) or piracetam (Pir, 140 mg·kg⁻¹) for 20 d, from day 16—35 after bilateral common carotid arteries were ligated. Each rat was subjected to two trials daily for 5 d and begun on day 31 after surgery in the water maze. A: Mean latency to find the hidden platform. The data were analyzed using two-way ANOVA (group × days, 6×5) with repeated measures. B: Swimming time spent in target quadrant in 60s probe trial (no platform). Each rat was immediately subjected to 60s observation following the last learning performance on day 35 after surgery. ¶¶, n=8, *p<0.01, compared with the sham group; #p<0.05, **p<0.01, compared with the model group.

Aged rats. These types of neural damage were prevented by TFSS 17.5 mg·kg⁻¹ (Fig. 2C, C₁), 35 mg·kg⁻¹ (Fig. 2D, D₁), 70 mg·kg⁻¹ (Fig. 2E, E₁) and piracetam 140 mg·kg⁻¹ (Fig. 2F, F₁).

Effects of TFSS on MDA and NO Contents and SOD and CAT Activities Compared with the sham group, the contents of MDA and NO, and SOD activity showed a strong increase, while the activity of CAT markedly decreased in the hippocampus and cerebral cortex in the model group (Table 1). Consecutive administration of TFSS (17.5, 35, 70 mg·kg⁻¹ per day for 21 d) differently reversed the increases in MDA and NO contents, SOD activity, and the decrease in CAT activity in the two brain areas. However, piracetam (140 mg·kg⁻¹ per day for 21 d) had no marked effects on MDA, NO, SOD, and CAT changes in the two brain areas of ligated rats (p>0.05).

DISCUSSION

The Morris water maze is a common method used to evaluate cognitive performance in rodents. As a cognitive task requires the development of a spatial map, the Morris water maze is analogous to nonverbal tests of cognitive function which are especially sensitive in detecting senescent and dementing disorders in the clinical setting.11) The validity of the Morris water maze as a measure of cognitive function in dementia in rats is supported by the fact that among demented rats the decline in noncognitive functions such as motor, sensorimotor, and visual abilities is unrelated to their performance in the Morris water maze.12) In the Morris water maze test, the platform trial detects rat learning acquisition. Rats can use any one of several strategies (e.g., circling the tank at a certain distance from the edge) to locate the platform and form memory through repeated training. The probe trial tests the rat memory preservation and may allow some distinction between different strategies used to find the precise location of the platform. The probe trial is a more reliable measurement of the accuracy of memory.13) In addition, the cognitive impairment of aged/globally ischemic rats in the Morris water maze might be due to the loss of visual acuity. Aged rats or rats with global ischemia suffer from some loss of visual acuity; however, there is no evidence that the loss of visual acuity affects performance in the Morris water maze.14) Therefore the Morris water maze is effective in evaluating the cognitive performance of rats and cognitive drug screening.

Various types of animal models, such as drug-brain lesion, hypoxia, or transient ischemia-induced amnesia, have been developed to study human dementia. The model of permanent ligation of the bilateral common carotid arteries in rats is often used, because of its progressive and long-lasting cognitive deficits accompanied by progressive neuronal damage.14) Several papers also reported that the permanent surgery leads to high mortality rates in rats and the use of the model has been limited.15) In the present study, rats weighing 270—300 g rats (about 20 weeks old) were subjected to the surgery and the mortality rate was less than 20%. The data are consistent with the lower mortality rate in the operated rats 13 weeks old and over.16) The result indicates that rats 13 weeks old and over can be used to investigate the behavioral and histologic consequences after the chronic cerebral ischemia.

There is increasing evidence showing that cerebral ischemia results in severe neuronal damage and changes in free radicals and energy metabolites. These detrimental alterations in the brain may contribute to cerebral ischemia-related learning and memory impairment.5,17,18) In the present behavioral trial, permanent bilateral-ligated rats had severely impaired learning performance in the Morris water maze task, and the impairment developed over 4 weeks. This result agrees with previous reports showing that cerebral ischemia induces an increase in the time required to find the hidden platform and a shorter swimming time in the target quadrant.5,19) We also found cerebral tissue retrogression and collquiative necrosis in the brain of cerebral ischemic rats, which have been demonstrated to be correlated with impairment in learning and memory and also consistent with the notion that neural system integrity is involved in cognitive function and may initiate and sustain the cascades of neuropathologic events that underlie vascular dementia.2,20) In our studies, ischemic rats treated with TFSS performed better
Fig. 2. Representative Photomicrographs of Neurons in the Hippocampus and Cerebral Cortex of Rats with Permanent Cerebral Ischemia (n = 3—4)

Daily and orally administered TFSS (17.5, 35, 70 mg kg⁻¹) or piracetam (140 mg kg⁻¹) for 21 d, from day 16—36 after surgery. All rats were decapitated 60 min after last drug administration. A—F, hippocampus neurons; A₁—F₁, cerebral cortex neurons. (A and A₁), sham group; (B and B₁), model group. Typical colliquative necrosis, reticular softening lesions, nucleus shrinkage or disappearance, cell membrane breaks, and cytoplasmic dissolution and dense inflammatory cell infiltration are seen in the necrotic region. (C and C₁), TFSS 17.5 mg kg⁻¹ group; (D and D₁), TFSS 35 mg kg⁻¹ group; (E and E₁), TFSS 70 mg kg⁻¹ group; (F and F₁), piracetam 140 mg kg⁻¹ group. TFSS and piracetam protected against neuronal injury. Magnification in hippocampus and cerebral cortex was 200 and 400, respectively. HE staining; scale bar, 40 μm.
than model rats in acquiring and retaining maze performance. Oral administration of TFSS also reduced the extent of ischemic neuronal damage and had significant morphologic protection in the hippocampus and cerebral cortex. These data provide direct evidence that TFSS can confer marked behavioral and histopathologic protection against permanent cerebral ischemia. The reference drug piracetam also prevented cognitive impairment and neuronal damage induced by ischemic insult.

The ligation of the bilateral common carotid arteries produces incomplete global ischemia. This type of cerebral ischemia in rats is characterized by decreased cerebral blood flow, the neurons may not completely die, and the brain maintains partial function. Therefore the model rats with the ligation of bilateral common carotid arteries still preserve some cognitive function in the Morris water maze. It is suggested that this delayed death of neurons provides a possibility for therapeutic intervention in cerebral postischemia.

There is a consensus of opinion that the brain ischemia-induced free radicals are capable mediating neuronal degeneration and death and are possibly involved in the pathogenesis of neuronal death in neurodegenerative diseases such as Alzheimer’s disease and vascular disease. The brain is liable to be peroxidized by free radicals as a result of its high oxygen consumption rate, abundant lipid content, and related paucity of antioxidant enzymes compared with other tissues. Lipid peroxidation readily decomposes to liberate carbonyl fragments, the most prominent being MDA, which are highly reactive and responsible for cytotoxic effects and neuronal death. There is evidence showing that NO plays a pivotal role in mediating oxidative stress. NO is involved in the diffusion-limited reaction between superoxide and nitric oxide, giving rise to peroxynitrite. The highly reactive peroxynitrite provides a mechanistic basis for oxidative stress derived from increased NO production caused by ischemia. Therefore the concentrations of MDA and NO indicate the extent of brain damage due to free radicals in cerebral ischemia. SOD is considered to be a key constituent in oxidant stress. The reported abnormal alteration in SOD activity in cerebral ischemia may further aggravate disorders of the brain. In the present study, the activity of SOD significantly decreased in the hippocampus and cerebral cortex. The result is agreement with previous observations and may be correlated with the ischemia-induced low synthetic ability of enzyme protein. The present study confirmed that TFSS has the potential to improve cognitive deficits and neuronal damage. The results likely suggest that the effects of TFSS in improving cognitive impairment are partially due to the reduction of neuronal damage and substantial deterioration of brain free radicals.

There were no marked changes in the free radicals examined with piracetam (140 mg · kg\(^{-1}\) per day for 21 d) could decrease MDA and NO contents, regulate SOD activity, and increase CAT activity in the two areas. The effects of TFSS on these substrates parallel the attenuation of cognitive deficits and neuronal damage. The results likely suggest that the effects of TFSS in improving cognitive impairment are partially due to the reduction of neuronal damage and substantial deterioration of brain free radicals.

### Table 1. Effects of TFSS on the Contents of Malondialdehyde (MDA) and Nitric Oxide (NO), and the Activities of Superoxide Dismutase (SOD) and Catalase (CAT) in Rats Induced by Cerebral Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg · kg(^{-1}))</th>
<th>MDA/µmol · g(^{-1}) protein</th>
<th>NO/µmol · g(^{-1}) protein</th>
<th>SOD/µmol · min(^{-1}) · g(^{-1}) wet tissue</th>
<th>CAT/µmol · s(^{-1}) · g(^{-1}) protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hippocampus</td>
<td>Cortex</td>
<td>Hippocampus</td>
<td>Cortex</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td>0.4±0.02</td>
<td>0.4±0.03</td>
<td>0.8±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Model</td>
<td></td>
<td>0.8±0.06**</td>
<td>0.5±0.04**</td>
<td>1.7±0.3**</td>
<td>1.3±0.2**</td>
</tr>
<tr>
<td>TFSS</td>
<td>17.5</td>
<td>0.5±0.04*</td>
<td>0.5±0.07</td>
<td>0.9±0.2*</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.3±0.03*</td>
<td>0.3±0.03*</td>
<td>0.7±0.2**</td>
<td>0.6±0.2**</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>0.4±0.06*</td>
<td>0.3±0.04**</td>
<td>0.8±0.1**</td>
<td>0.4±0.1**</td>
</tr>
<tr>
<td>Pir</td>
<td>140</td>
<td>0.8±0.07</td>
<td>0.5±0.05</td>
<td>1.5±0.3</td>
<td>1.4±0.0</td>
</tr>
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</table>

See legend of Fig. 2 for rat treatments. SOD activity is expressed as the difference of nitrite content between the control in the test kit and sample. \(±S.E., n=8\), \(∗∗p<0.01\), compared with the sham group; \(∗p<0.05\), \(∗∗∗p<0.001\), compared with the model group.

Acknowledgment This work was supported by grants...
REFERENCES