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Scutellaria flavonoid reduced memory dysfunction and neuronal injury caused by permanent global ischemia in rats☆

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Abstract

The purpose of this study is to investigate the effects of flavonoid, isolated from aerial parts of *Scutellaria baicalensis* Georgi (SSF), on memory deficits, neuronal degeneration and abnormal energy metabolism induced by permanent global ischemia in rats. The global ischemia was produced in female Sprague–Dawley rats by permanent occlusion of the bilateral common carotid arteries. The permanent global ischemia in rats resulted in a significantly increased latency of the rat to find the hidden platform and a decreased swimming distance from the target quadrant in the Morris water maze task. The pathological changes in the neurons of ischemic rats, observed in the hippocampus and cerebral cortex, included neuron loss, neuron swelling, nuclear shrinkage or disappearance, neuronophagia and reduced density of Nissl bodies in the neuron. Moreover, the levels of lactate and ATPase activity in ischemic rats were notably increased and decreased, respectively, in the hippocampus and cerebral cortex as compared with sham-operated rats. Daily oral administration of SSF (35 mg/kg, 19–20 days) dramatically reduced the decrease in learning and memory, attenuated neuronal injury and improved abnormality of energy metabolites in rats induced by global ischemia. These findings suggest that SSF may be beneficial for the treatment of vascular dementia.

Keywords: Flavonoid; *Scutellaria baicalensis* Georgi; Morris water maze; Global ischemia; Memory dysfunction; Neuronal injury; Energy metabolism

1. Introduction

With an increasing elderly population, various aging-related diseases such as hypertension, arteriosclerosis and different forms of dementia are also increasing. According to clinical observation, the patients who are characterized by intellectual declines often suffer from global ischemia, global ischemia–hypoxia, or cerebral hemorrhage-induced vascular disorder (Diehl and Kurz, 2002; Roman, 2002). It is reported that chronic global ischemia induces neuronal damage in selective, vulnerable regions of the brain, especially the hippocampus and cerebral cortex (Wang et al., 2000; Ni et al., 1995). Further, abnormal levels in the brain of a series of metabolites such as acetylcholinesterase, glucose, lactate, ATPase, cytochrome oxidase, NOS and free radicals resulted from the neuronal damage and these chronically impaired neuronal productions can lead to deficits in learning and memory. These neuropathological events underlie vascular dementia (Borbely, 2002; de la Torre et al., 1997; Tohgi et al., 1998; Alonso et al., 2002). The permanent occlusion of the bilateral common carotid arteries in rats is used as a chronic global ischemic model to study vascular dementia and the pharmacological effects of drug. Although the global ischemic model in rats cannot completely mimic the state of clinical patients with vascular dementia, it can partially reproduce neuronal and metabolic lesions along with learning and memory deficits (Tsuchiya et al., 1993; Zhou et al., 2001; Wang et al., 2000). Any improvements from the abnormal state of the chronic global ischemic model can help predict the possibility for a vascular dementia treatment.

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SSF, the novel flavonoids isolated from aerial parts of *Scutellaria baicalensis* Georgi, has proven to be a promising agent for palliative treatment of dementia. In previous studies, SSF has shown improvements in brain hypoxia, memory impairment and chemical neuronal damage (Shang et al., 2001, 2002, 2005). These findings inspired us to investigate whether SSF could manifest potential developments in the global ischemic model. The effects of chronic treatment with SSF on learning deficits, neuronal injury and cerebral metabolic disruption in rats induced by permanent bilateral occlusion of common carotid arteries are reported.

2. Methods

2.1. Animals

Sprague–Dawley rats (♀, 220–250 g, Clean grade, Certification No. 04057) were purchased from the Medical Administration Committee of Experimental Animals, Hebei Province, China. Rats were subjected to permanent global ischemia by permanent bilateral occlusion of common carotid arteries and were housed in-group (three or four per cage) at a temperature of 23±1 °C with a 12-h light–dark cycle and were allowed free access to food and water. 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 on November 14, 1988.

2.2. Drug and administration

SSF was prepared at the Phytochemistry Laboratory, Institute of Materia Medica, Chengde Medical College, China. The dried aerial part of *S. baicalensis* (1 kg) was boiled with distilled water for 1 h then filtered. The residue was processed in the same manner and the filtrates were combined. The filtrate was concentrated at 60 °C under reduced pressure and the residue was dried at 80 °C and yielded 200 g of extract. The extract was then dissolved in saturated sodium bicarbonate solution, and filtered and the filtrate adjusted to pH 2 by adding HCl solution (1 N). This solution was kept at room temperature for 24 h and filtered. The residue was treated in the same manner to obtain the SSF, in which the total flavonoid is not less than 80% and scutellarein is the major ingredient. The reagent kits for measurement of lactate and ATPase were purchased from Nanjing Jiancheng Institute of Biological Engineering (China). Other reagents were AR grade.

SSF was dissolved in distilled water and the solution adjusted to pH 7.2–7.4 with saturated sodium bicarbonate solution prior to administration. All the rats recovered for 14 days after the operation. From Day 15 to Day 34, the rats were administered SSF (35 mg/kg ig, once a day), or distilled water (its vehicle) according to the design. The rats were trained in the water maze test 60 min after administration of SSF or distilled water.

2.3. Surgery procedure

Female Sprague–Dawley rats were subjected to permanent global ischemia (Ni et al., 1994). Under choral hydrate (350 mg/kg ip) anesthesia, bilateral common carotid arteries of the rats were exposed and carefully separated from the carotid sheath, cervical sympathetic and vagus nerves through a ventral cervical incision. The bilateral common carotid arteries of each global ischemic group were ligated with $1^{\#}$ thread. The rats which received the same operation without carotid arteries ligation served as the sham-operated control. The rats were placed on a heating pad during recovery from anesthesia to maintain the body temperature at 37.0±0.5 °C after surgery.

2.4. Morris water maze

The rats' learning and memory performance were evaluated by the Morris water maze (Morris, 1984). The maze apparatus consisted of a circular pool and a circular hidden platform (10 cm in diameter). The pool was 120 cm in diameter and 50 cm in depth and was filled with water to depth of 31.5 cm, while the platform was supported by a base resting on the bottom of the pool placing it 1.5 cm below the water surface. The pool water was kept at a temperature of 23±1 °C and made white opaque with non-fat milk powder. Every spatial sign around the maze was held constant for the whole test. For descriptive data collection, the pool was subdivided into four equal quadrants formed by imaginary lines, which intersected in the center of the pool at right angles called north, south, east and west. The hidden platform resided in the center of the southwest quadrant (Q3) for the entire training period. The swimming activity of each rat was monitored by a video camera linked to a computer through an image analyzer. Behavioral tasks were tested for 5 consecutive days and each rat received 4 trials a day. Within each block of 4 trials, one rat started at each of the starting location randomly. The time taken to find the hidden platform (latency) was measured and calculated for an average over 4 trials a day to evaluate the rats' learning performance. On the first day of rat training, each rat was allowed to swim in the maze to get accustomed to swimming for 120 s. From the second day, the rats were trained to find the hidden platform. If 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 by hand and remained there for 20 s. There was 10 s of recovery periods between two trials. The spatial probe trial was assessed immediately following the rats removal from the hidden platform, allowing the rats to swim for 60 s to search for the target location. The swimming distance from

the target quadrant (Q3) of the pool over 60 s was used to evaluate the rats' memory.

2.5. Neuronal morphology

All the rats were decapitated 60 min after last administration of SSF or distilled water on Day 34 after the operation. The hippocampus and cerebral cortex of the right hemisphere from four rats in each group were separated on ice. The brain was then fixed with 4% formalin and embedded in paraffin. Coronal sections were cut at approximately 6 μm and stained with thionine as described previously (Feng et al., 2003). Thionine-stained cells were visualized and photographed at a magnification of 20× and 40×. The number of living neurons per 0.0352 mm^2 in the cerebral cortex and 0.125 mm field in the middle CA_1 of the hippocampus was counted under a light microscope (×600) by a person without knowledge of the experimental conditions. The counts of cerebral cortex neurons were made in four different fields. The mean number of living neurons was determined such that the value obtained for each rat represents an average number of living neuron counted/600 × field. Neurons were scored as undamaged if they were Nissl-possive with a round- to oval-shaped cell body that exhibited no evidence of cell shrinkage (Duan et al., 2001).

2.6. Energy metabolite examinations

In order to measure the energy metabolites in the rats' brain, the rats were decapitated 60 min after the last administration on Day 34 following the operation. The brain was rapidly dissected on ice. The hippocampus and cerebral cortex were separated and homogenized in cold saline to obtain 10% homogenates for lactate examination, and 2% homogenates for ATPase activity measurement.

Lactate assay was based on the lactate dehydrogenase enzymatic method (Rosenberg and Rush, 1966). Lactate releases hydrogen, which is accepted by NAD^+, and finally NBT to produce a purple substance. The maximal absorbance (A) of the purple substance was at $\lambda = 530$ nm. The measurement of lactate was based on the reagent kit instructions (Nanjing Jiancheng Institute of Biological Engineering, China). The level of lactate was calculated as follows:

Lactate level in tissue protein $= (A_1 - A_3) \div (A_2 - A_3) \times C \div T$ nmol/mg protein, where A_1, A_2 and A_3 are the absorbances of the sample, standard and blank, respectively. C is the standard concentration, and T is the protein content of sample.

ATPase activity was determined by measuring the rate of formation of phosphoric acid from ATP (He et al., 1999). The process of ATPase activity measurement was determined according to the reagent kit instruction (Nanjing Jiancheng Institute of Biological Engineering, China). The ATPase activity was calculated as follows:

ATPase activity in tissue protein $= (A_1 - A_3) \div A_2 \times C \times D \times 6 \div T$ μmol P_i/mg protein, where A_1, A_2 and A_3 are the absorbances of sample, standard and control, respectively. C is the phosphoric content of standard. D is the dilute times of sample and T is the protein concentration of sample.

Protein content was determined according to the Coomassie blue protein-binding method, using bovine serum albumin as standard (Bradford, 1976).

2.7. Statistics

All results were expressed as mean ± S.E.M. The behavioral test data used one- or two-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. Others were compared by Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of SSF on memory impairment induced by global ischemia in rats

As shown in Fig. 1A, the mean latency in finding the hidden platform declined progressively in all groups during the 5-day water maze trial. The ligated rats treated with distilled water (model group) consistently exhibited a longer latency than sham-operated rats [Group × Time $F(4,30) = 13.22$, $P < 0.01$]. Compared with the model group, the prolonged latency in ligated rats was markedly shortened by SSF at a dose of 35 mg/kg [Group × Time $F(4,29) = 11.39$, $P < 0.01$]. The results in Fig. 1A were parallel to Fig. 1B. In the probe trial, the swimming distance from the target quadrant (Q3) was used to evaluate the rats' retention performance. Fig. 1C shows that sham-operated and 35 mg/kg SSF rats remained at a longer distance from Q3 within 60 s than model group rats [$F(2,25) = 20.39$, $P < 0.01$].

3.2. Effects of SSF on neuronal injury

Fig. 2 shows that the typical neuropathological changes in the hippocampus and cerebral cortex following permanent global ischemia in rats include neuron loss, neuron swelling, nucleus shrinkage or disappearance, neuronophagia and reduced density of Nissl body staining in the cytoplasm of pyramidal cells. Consecutive administration of SSF (35 mg/kg ig) for 20 days could significantly attenuate the global ischemia-induced neuropathological damages in the two brain areas.

The number of neurons in the brain of ischemic rats was also reduced significantly. Compared with the sham-operated group, the neuron number lowered 32.35%/0.125 mm ($P < 0.01$) in the hippocampus CA1 subfield and 38.42%/0.0352 mm^2 ($P < 0.01$) in the cerebral cortex. The decrease was suppressed 29.78% (CA1) and 34.78% (cerebral cortex) by SSF at dose of 35 mg/kg.

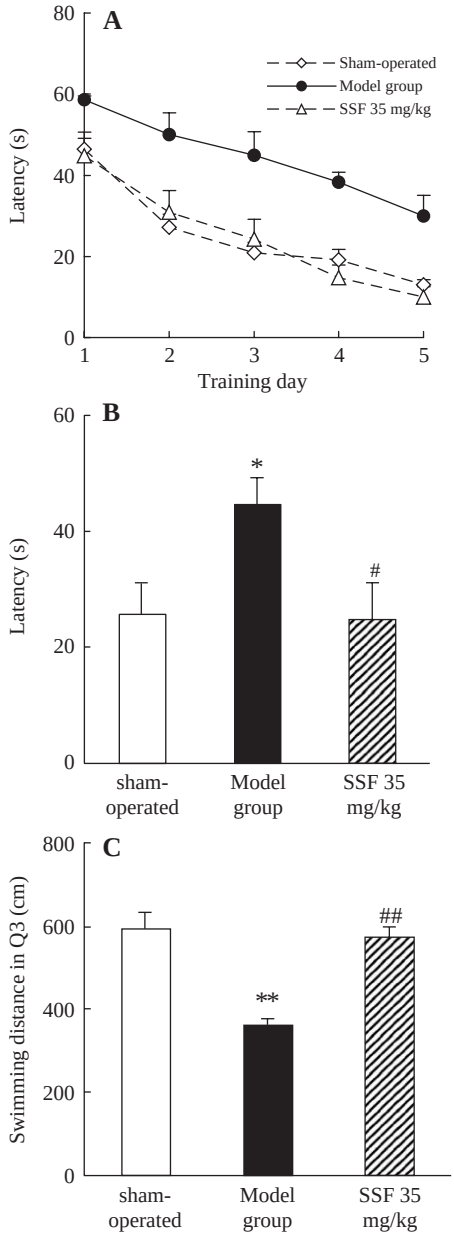

Fig. 1. Effects of SSF (35 mg/kg ig 19 days) on water maze performance deficit of rats induced by permanent occlusion of the bilateral common carotid arteries ($n=9-10$). Sham-operated or model or SSF 35 mg/kg rats were administered ig 60 min before testing. (A) Mean latency to find the hidden platform. Each rat was subjected to 4 trials daily for 5 consecutive days. (B) Mean latency for 5 days. (C) Swimming distance in target quadrant (Q3) within 60 s in the probe trial (no platform). Each column and bar represented mean ± S.E.M. $^{**}P<0.01$, $^{*}P<0.05$, compared with sham-operated group. $^{\#\#}P<0.01$, $^{\#}P<0.05$, compared with model group.

3.3. Effects of SSF on energy metabolites

Table 1 shows the lactate content and ATPase activity in the hippocampus and cerebral cortex. In the model rats, an increased lactate concentration and decreased ATPase activity were observed in the two brain regions. The abnormal levels of lactate and ATPase activity could be inhibited by SSF in dose of 35 mg/kg.

4. Discussion

Many animal models, including drug-, brain lesion-, or transient ischemia-induced amnesia, have been developed to study human dementia. The behavioral deficits and neuronal degenerations in these models often appear to be transient and have time-dependent recoveries to normal levels (Ni et al., 1995). However, according to clinical observation, the patients who suffer from vascular dementia manifest signs of insidious and progressive cognition impairments and neuronal pathological alterations (Barclay et al., 1985). Therefore, using the animal model with long-lasting and/or progressive cognitive deficits and neuronal damages coincides with the states of clinical vascular dementia. The permanent occlusion of the bilateral common carotid arteries in rats is considered the most common model to understand the pathophysiology of vascular dementia and to evaluate therapeutics of possible drugs. The permanent global ischemia produced progressive neuronal damage and abnormal changes in the free radicals and energy metabolites in the brain. These disruptions in the brain contributed to the global ischemia-related progressive and long-lasting cognitive dysfunctions (Borbely, 2002; de la Torre et al., 1997; Tohgi et al., 1998; Alonso et al., 2002). In the present behavioral trial, the learning performance in the water maze task was severely impaired in permanent bilateral ligation rats. This result corresponds well with the previous data that show the cerebral ischemia resulted in an increase in the time required finding the hidden platform and a decrease in the swimming time in target quadrant (Ohta et al., 1997; Wang et al., 2000). Many studies have demonstrated that the hippocampus and cerebral cortex play important roles in the processing of memories. Memory impairments observed in the present study were quite similar to those following structural lesions of neurons in the hippocampus and cerebral cortex (Zhou et al., 2001). An irregular nuclear shape, dense chromatin masses in the nucleus, extensive vacuolation, dilated organelles and flocculent mitochondrial densities within the cytoplasm were observed after cerebral ischemia through electric ultrastructural observation (Zhou et al., 2001). With the light microscope, the density of Nissl staining in neuronal cytoplasm is considered as a target to evaluate the damages of the neuron. The Nissl bodies stained by thionine are actually endoplasmic reticulum (ER) under light microscope observation. ER is as an important organelle in cytoplasm and the location for protein synthesis and transportation. Synthesized proteins such as enzymes, peptide hormones and antibodies directly interfere with the normal metabolism and functions of a cell. Thus, the state of Nissl staining in neurons can be utilized to assess the brain injury-induced neuropathological changes. The density of Nissl staining in present studies is significantly decreased in neurons in global ischemic rats. The results consistently supported the notion that the density of Nissl bodies indicates the neuronal status and will be lowered after neuronal injury or with aging. Both

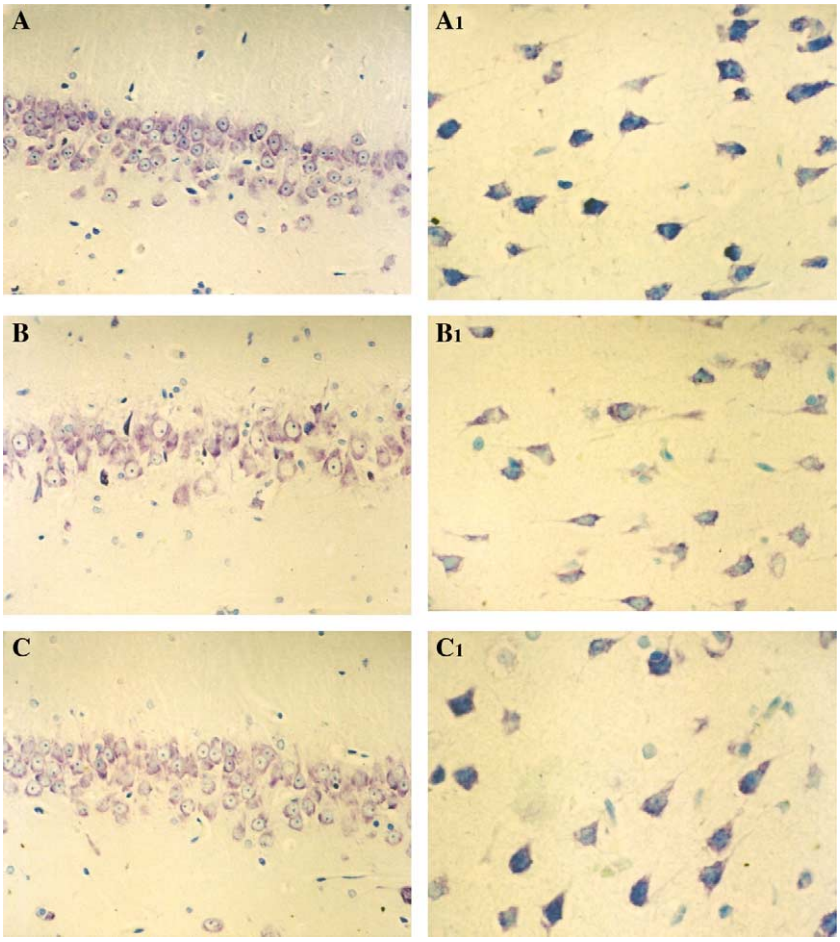

Fig. 2. Representative photomicrographs of pyramidal cell in the hippocampus (A, B and C) and cerebral cortex (A_1, B_1 and C_1) of rats after permanent occlusion of the bilateral common carotid arteries on day 33. (A and A_1) Sham-operated group; (B and B_1) model group; showing neuron loss, neuron swelling, nucleus shrinkage or disappearance, neuronophagia, and reduced densities of Nissl's bodies staining in the neuronal cytoplasm; (C and C_1) SSF (35 mg/kg)-treated group: showing intact neuronal number increase and fullness, and increased densities of Nissl's bodies in neuron cytoplasm with 35 mg/kg SSF. Magnification in hippocampus and cerebral cortex was 200 and 400, respectively. Thionine stain.

have been shown to correlate with protein synthesis capabilities and furthermore affect advanced functions of brain such as learning, memory and cognition (Amenta et al., 1994; Tan and Zhou, 1992; Tarabal et al., 2001). In our studies, chronic administration of SSF could markedly attenuate the water maze performance deficits and neuronal damage in the brain of rats induced by permanent bilateral ligation. The effect of SSF on cognitive improvement runs parallel with the effect on neuropathological attenuation. Thus, our results support that the beneficial effects of SSF on cognitive deficit, caused by permanent bilateral ligation, were due primarily to its improvements of neuronal survival.

The brain is critically dependent on a continuous supply of energy largely generated through oxidative phosphorylation. The global ischemia caused serious brain injury through rapid energy depletion (Ames, 2000; Suzuki et al., 2001; Sztark et al., 1999). The brain is easily damaged by hypoxia, anoxia and ischemia as a result of the high brain oxygen and energy consumption rates as compared with

Table 1
Effects of SSF on abnormal changes of lactate concentration and ATPase activity caused by permanent occlusion of the bilateral common carotid arteries in rats

Group	Lactate (nmol/mg protein)		ATPase (μmol p_i/mg protein/hour)	
	Hippocampus	Cortex	Hippocampus	Cortex
Sham-operated	29.58±4.35	22.21±8.65	0.58±0.10	0.54±0.08
Model group	83.18±5.94**	52.92±11.26**	0.36±0.06**	0.40±0.15*
SSF 35 mg/kg	26.98±3.60$^{\#\#}$	32.42±11.01$^{\#\#}$	0.35±0.05	0.52±0.04$^{\#}$

Data were expressed as mean±S.E.M. (n=9–10).
**P<0.01, *P<0.05, compared with sham-operated group.
$^{\#\#}P$<0.01, $^{\#}P$<0.05, compared with saline-treated group.

other tissues (Ames, 2000; Markesbery, 1997). Under normal conditions, the brain mainly utilizes glucose as its primary source of energy, which is then metabolized completely through the glycolytic pathway to finally produce the high-energy substrate ATP. However, with global ischemia, the brain does not produce energy via efficient oxidative metabolism but via inefficient anaerobic metabolism. This type of glycolysis causes the lowered ATP level and excess lactate accumulation in the brain; the latter attributes to the resulting increased intracellular H^+ concentration. The high H^+ content of a cell may be harmful to the brain structure and furthermore may affect advanced functions such as learning, memory and cognition (Natale et al., 1990; Siesjo, 1988; Suzuki et al., 2001). The great reduction of Na^+–K^+-ATPase activity in the brain is also characterized by the onset of global ischemia due to the decline in intracellular ATP. The decline in Na^+–K^+-ATPase activity allows for the accumulation of intracellular sodium, which upsets the sodium gradient normally used to drive calcium out of the cell via the Na^+/Ca^+-exchanger. Inhibition of the Na^+/Ca^+-exchanger allows calcium to accumulate inside the cell. This calcium overload in cerebral tissues partly results in irreversible brain injury (Stys, 1998; Palmer et al., 1988). In the present studies, the permanent bilateral ligation in rats caused lactate accumulation and a decrease in Na^+–K^+-ATPase activity in the hippocampus and cerebral cortex. Treatment with SSF could significantly reverse the accumulation of lactate and reduced Na^+–K^+-ATPase activity in the two areas of ischemic rats. The effects of SSF on the altered lactate and Na^+–K^+-APTase levels, induced by global ischemia, are one mechanism of SSF to brain protection. The effects of SSF on lactate and Na^+–K^+-ATPase are also parallel with the improvement of cognitive deficit and the attenuation of neuronal damage. The results suggest that the palliative effects of SSF on the cognitive impairment and neuronal damage are partially if not fully due to reduction of substantial deterioration of brain energy metabolites.

In the present studies, the dose selected for SSF, 35 mg/kg, can produce cognitive improvement and neuropathological attenuation. These improvements run parallel with the effect on brain energy metabolites disruptions. The selection of SSF dosing is basis on previous reports using other paradigms, which is consistent with small effective dose in mice experiment (Shang et al., 2001, 2005).

Recent studies demonstrated that numerous agents such as acetylcholinesterase inhibitors, antioxidants, radical scavengers and calcium antagonists have been available to treat vascular dementia in animal or in clinical trials (Tabet et al., 2001; DeFeudis et al., 2000; Frishman, 2002; Moretti et al., 2002). Multiple mechanisms underlie the therapeutic potential of these agents. The present study demonstrates that SSF is beneficial for ameliorating cognitive deficit, protecting neuronal injury and modulating abnormal changes in energy metabolites in rats after the onset of global ischemia. These findings suggest that the therapeutic effects of SSF against global ischemia-induced disturbances may slow down or block the pathogenic process in vascular dementia.

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