

## Protective Effect of Flavonoids from *Scutellaria baicalensis* Georgi on Cerebral Ischemia Injury

By: Yongyu Zhang, Xiaoyan Wang, Xiaorong Wang, Zhaohui Xu, Zhe Liu, Qian Ni, Xiangping Chu, Mingfeng Qiu, Aihua Zhao, and Wei Jia

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### **Abstract:**

The protective effect of flavonoids extracted from *Scutellaria baicalensis* Georgi on cerebral ischemia injury has been explored in experimental animals. *Scutellaria* flavonoid (SF) could significantly prolong gasping time (prolonged ratio, 23.79%) and survival time after carotid artery occlusion, and decrease attenuate malondialdehyde (MDA) content in damaged brain tissues from  $118.56 \pm 47.95$  nmol/g in untreated to  $199.29 \pm 24.24$  nmol/g. SF could also increase the content of superoxide dismutase (SOD) in brain tissues after ischemic mice from  $1486 \pm 94$  NU/g in untreated to  $1168 \pm 76$  NU/g, and showed significant protective effect on cerebral hypoxia and reperfusion brain tissues in middle cerebral artery occlusion (MCAO) procedure. Additionally, SF has inhibitory effect on platelet aggregation, with the average inhibition rate of 45.52%, while the aspirin group was 54.96%. These results suggest that SF has a significant protective effect on cerebral ischemia and ischemia-reperfusion induced brain injury.

### **Article:**

#### **INTRODUCTION**

*Radix Scutellaria baicalensis* Georgi (Labiatae), Chinese name, 'Huang-qin', has been routinely used in the treatment of bronchitis, hepatitis, allergy, inflammation, arteriosclerosis and has been listed in the Pharmacopoeia of the People's Republic of China. Flavonoids such as baicalein, wogonin and their glycosides are the major and effective components in *Radix Scutellaria* (Xiao, 2002).

Many flavonoids, such as quercetin, luteolin and catechins, are better antioxidants than the antioxidant nutrients such as Vitamins C, E and l-carotene on a mole for mole basis (Rice-Evans et al., 1995). In *Radix Scutellaria*, Flavonoids with *o*-di-hydroxyl group or *o*-three-hydroxyl group or in ring A, such as baicalin and baicalein, etc., could be good free-radical-scavengers and used to cure head injury associated with free radical assault (Gao et al., 1999). Studies have

reported that *Scutellaria baicalensis* could inhibit lipid peroxidation in rat liver (Kimura et al., 1981 and Kimura et al., 1982) and attenuate oxidant stress in cardiomyocytes (Shao et al., 1999).

Cerebral ischemia or stroke, one of the leading causes of death and long-term disability in aged populations, often results in irreversible brain damage and subsequent loss of neuronal function. Many herbal drugs and prescriptions including *Ginkgo biloba* extract (GBE) (Lee et al., 2003), gypenosides (Cao et al., 2002), green tea extract (Hong et al., 2001) and puerariae (Yan et al., 2004) and garlic extracts (Numagami et al., 1996) have been used clinically for the treatment of this condition.

In the previous paper (Zhang et al., 1994a, Zhang et al., 1994b, Zhang et al., 1997, Zhang et al., 1998a and Zhang et al., 1998b), we have reported the study and quantitative analysis of the flavonoids extracted from the roots of *Scutellaria baicalensis* Georgi. The aim of this paper, therefore, is to explore the protective effect of the same flavonoids on cerebral ischemia injury.

## MATERIALS AND METHODS

### *Animals*

Kunming mice weighing 18–24 g, male Wistar rats weighing 260–280 g, male rabbits 2–3 kg were purchased from the Institute of Clinical Research, China Medical University (Certificate No. SCXK 2003-0009, China). The rats were housed 4 or 5 per cage, allowed free access to water and food, and maintained under constant temperature ( $23 \pm 1$  °C) and humidity ( $60 \pm 10\%$ ) under a 12 h light/dark cycle (light on 07.30–19.30 h). All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Shanghai Jiaotong University and were approved by the university committee for animal experiments.

### *Materials*

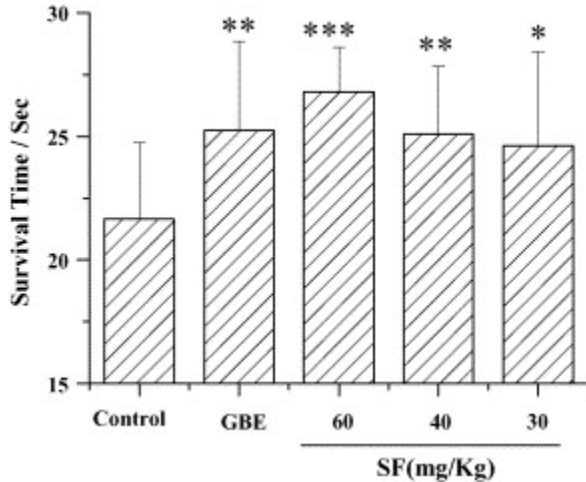
*Scutellaria baicalensis* Georgi was collected in September 2003 in Lingyuan, Liaoning Province, China, and identified by Prof. Yunzhen Guo of Shenyang Pharmaceutical University. The sample material of the same batch was kept in our laboratory as the voucher specimen (030807) for future reference.

Preparation of *Scutellaria* extract. Crushed roots of *Scutellaria baicalensis* Georgi were incubated with a four-fold volume of water at  $37 \pm 0.5$  °C for 8 h and then filtrated and dried. The dried roots were extracted with ethyl acetate ( $2 \times 10$  l, then  $1 \times 6$  l, for 1 h each) under reflux. After concentration of the ethyl acetate extract, the *Scutellaria* flavonoid (SF) containing 65% flavonoid was obtained as assayed by UV (Shimadzu 2201, at 274 nm) with Baicalein as control.

*Ginkgo biloba* extract (GBE, containing Ginkgo Leaf Flavonoid 24%, Lactone 5%) was obtained from Shenyang Jiqi Pharmaceutical Factory. Potassium cyanide (KCN) and dimethyl sulfoxide (DMSO) were purchased from Shenyang Chemical Reagent Factory. Thiobarbituric acid (TBA) was purchased from Shanghai Reagent Company. Catalase (CAT) Detection Kit, Superoxide Dismutase (SOD) Detection Kit and Malondialdehyde (MDA) Detection Kit were purchased from Nanjing Jiangcheng Bioengineering Institute.

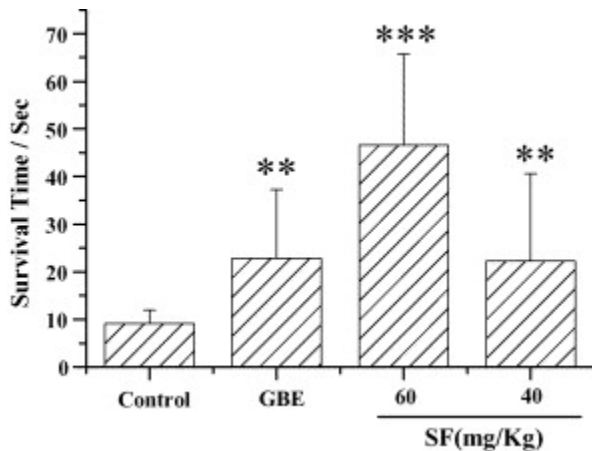
### *Hypoxia-resistant experiments*

**Hyperventilation test.** Fifty Kunming mice of both sexes were randomly divided into five groups (10 mice each group) for the hyperventilation test. The groups were treated with the following agents by intraperitoneal injection (i.p.). Group 1 received SF (dissolved and dispersed with 20% DMSO) at 60 mg/kg; Group 2 received SF (with 20% DMSO) at 40 mg/kg; Group 3 received SF (with 20% DMSO) at 30 mg/kg; Group 4 received GBE at 60 mg/kg; Group 5 (control) received 20% DMSO in the same volume as other groups. Sixty minutes after the administration, the mice were sacrificed using the decollation apparatus and the hyperventilation time was recorded and expressed in Fig. 1.



**Figure 1:** Effect of SF on gasping time by decapitation in mice.

**Bilateral common carotid artery occlusion.** Fifty Kunming mice of both sexes were randomly divided into five groups (10 mice per group). Five groups received the same dosage of drugs as in Section 2.3.1 once a day for 7 days. Forty minutes after the last administration, all mice were anesthetized by urethane (at 1.3 g/kg). After 60 min, all groups underwent the operation for common carotid artery and vagus nerves ligation. Then the survival time of mice were recorded and expressed in Fig. 2.



**Figure 2:** Effect of SF on survival time of mice subjected to bilateral carotid artery ligation.

**Brain injury of mice by KCN.** Forty Kunming mice of both sexes were randomly divided into four groups (10 mice per group). The mice were treated with the following drugs by i.p. Group 1 received SF (with 20% DMSO) at 60 mg/kg; Group 2 received SF (with 20% DMSO) at 40 mg/kg; Group 3 received GBE at 60 mg/kg; Group 4 (control) received 20% DMSO in the same volume as other groups. One hour after the last administration, all groups received KCN (4.5 mg/kg, i.p.). The reactions of mice were observed and recorded. After 1 h, all mice were sacrificed. Brain tissues were obtained and homogenized by saline. The content of malonic aldehyde (MDA) were determined (Table 1) by thiobarbituric acid (TBA) method.

**Table 1:** Effect of SF on MDA in brain of brain injury mice

Group	Dose (mg/kg)	MDA (nmol/g brain tissue)	Inhibition ratio (%)
Control	–	199.29 ± 24.24	–
GBE	60	160.16 ± 31.35*	19.63
SF	60	118.56 ± 47.85***	40.51
SF	40	139.29 ± 65.10**	30.11

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  in comparison with control.

**The contents of SOD and MDA in brain tissue after hypoxia.** The content of MDA and the activity of SOD were determined respectively using a TBA method (Ohkawa et al., 1979). Fifty Kunming mice of both sexes were randomly divided into five groups (10 mice per group). The first four groups were treated with the same drugs as in Section 2.3.3, and the sham operation group received DMSO administration as the control group. Forty minutes after administration, all mice were anesthetized by ether. Bilateral common carotid artery were drawn off and ligated. The sham operation group did not undergo ligation. After 5 min, all mice were sacrificed. The brain tissues were obtained and quickly frozen. The content of MDA and the activity of SOD were determined by TBA method in Table 2.

**Table 2:** Effect of SF on SOD activity and MDA in brain of ischemic mice ( $\bar{X} \pm s$ ,  $n = 10$ )

Group	Dose (mg/kg)	SOD (NU/g)	MDA (nmol/g brain tissue)
Control	–	1168 ± 76	250.46 ± 29.28
Sham-operated	–	2178 ± 156	177.18 ± 13.30
GBE	60	1312 ± 86** $\Delta\Delta$	191.35 ± 28.73*** $\Delta\Delta$
SF	60	1486 ± 94** $\Delta\Delta$	193.34 ± 18.98*** $\Delta\Delta$
SF	40	1376 ± 91** $\Delta\Delta$	187.27 ± 29.10*** $\Delta$

\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  in comparison with control.  $\Delta$   $p < 0.05$ ,  $\Delta\Delta$   $p < 0.01$  in comparison with sham-operated.

### *Focal cerebral ischemia experiments*

**Middle cerebral artery occlusion (MCAO).** The MCAO was produced using an intraluminal filament model (Longa et al., 1989). The rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). Briefly after making a median incision in the neck skin, the right common carotid artery, external and internal carotid artery were exposed. External carotid artery and its arborization, pinna palatine process artery were all ligated in order to block the collateral circulatory blood flow from the neck. A 4/0-nylon thread with a rounded tip (coated with silicon) was inserted from the bifurcation to the MCAO until a slight resistance was felt. Such resistance indicated that the filament had passed beyond the proximal segment of the anterior cerebral artery. The filament would be withdrawn for recanalization. Twenty-four hours after the induction of ischemia, the filament was slowly withdrawn until tip reached external carotid artery. In sham-operated rats, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted.

Throughout the procedure, the room temperature was maintained at  $25 \pm 0.5$  °C and body temperature was maintained at  $37 \pm 0.5$  °C.

**Grouping.** Eighty rats were evenly divided into 10 groups. Group 1 was served as the sham operation group. Group 2 was the control which received the same volume of solvent as other groups. Group 3 was prepared MCAO, which was induced for 24 h followed by 2 h of reperfusion, and treated with SF (at 80 mg/kg, i.p.) at 10 min and 24 h after MCAO. Group 4 was the same as Group 3, except for SF treatment at 60 mg/kg. Group 5 was the same as Group 3, except for SF treatment at 40 mg/kg. Group 6 was induced MCAO for 24 h followed by 24 h of reperfusion. After 10 min, 24 h and 36 h, rats were treated with SF at 80 mg/kg, i.p. Group 7 was the same as Group 6, except for SF treatment at 60 mg/kg. Group 8 was the same as Group 6, except for SF treatment at 40 mg/kg. Groups 9 and 10 served as the sham and control group for Groups 6–8.

**Neurological evaluation, morphometric measurement of infarct volume and water content assay.** Animals were tested for neurological deficits either 2 or 24 h after reperfusion by an observer naive to the treatment groups as described ([Zhang et al., 1994a] and [Zhang et al., 1994b]). Referring to the literature, neurological deficit was scored with an eight-point [from 0 (no deficit) to 7 (severe)].

Then rats were sacrificed by decapitation. The brain tissues were removed and sectioned at 2 mm intervals in the coronal plane without olfactory bulb, cerebellum and low-set brain stem (Kondoh et al., 2002). Sections were incubated for 30 min at 37 °C in a solution containing phosphate buffered saline and 2% 2,3,5-triphenyltetrazolium chloride (TTC). Then the sections were fixed in 10% phosphate-buffered formalin. Normal tissues were stained and infarct tissues were not stained. Photographs of coronal sections were analyzed by luxex-F image analyzer. The infarct areas were calculated from the whole area (%).

To evaluate the degree of edema formation, the water contents were determined by a dry-humid weight method (Numagami et al., 1996). The rats were decapitated under anesthesia after MCAO. The brain tissues were obtained without olfactory bulb, cerebellum and low-set brain stem. The wet tissues were immediately weighed to obtain the wet weight (*W*). Then the wet tissues were dried in an oven at 110 °C for 24 h, and reweighed to obtain the dry tissue weight (*D*). The water content (%) was calculated ( Table 3 and Table 4) as  $(W - D)/W \times 100$ .

**Table 3:** Effect of SF on protection of brain in rats with 24 h MCAO followed by 2 h reperfusion ( $\bar{X} \pm s, n = 8$ )

Group	Dose (mg/kg)	Neurological evaluation	Infarction area (%)	Water content (%)
Sham-operated	–	1.1 ± 0.2	0	78.6 ± 0.8
Control	–	5.3 ± 0.8 <sup>*</sup>	28.6 ± 3.1 <sup>*</sup>	84.5 ± 0.7 <sup>*</sup>
SF	80	3.3 ± 0.6 <sup>**</sup>	16.2 ± 2.0 <sup>**</sup>	81.1 ± 0.6 <sup>***</sup>
SF	60	3.8 ± 0.7 <sup>**</sup>	20.2 ± 1.8 <sup>**</sup>	82.1 ± 0.7 <sup>Δ</sup>
SF	40	4.1 ± 0.6 <sup>***</sup>	22.2 ± 1.8 <sup>**</sup>	83.2 ± 0.8 <sup>Δ</sup>

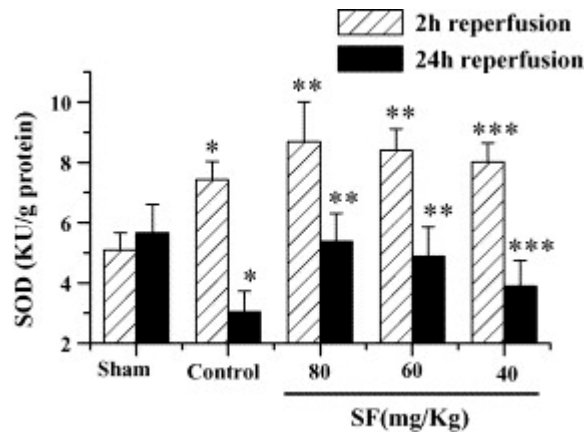
<sup>\*</sup>*p* < 0.01 vs. sham-operated. <sup>Δ</sup>*p* > 0.05, <sup>\*\*\*</sup>*p* < 0.05, <sup>\*\*</sup>*p* < 0.01 vs. control.

**Table 4:** Effect of SF on protection of brain in rats with 24 h MCAO followed by 24 h reperfusion ( $\bar{X} \pm s, n = 8$ )

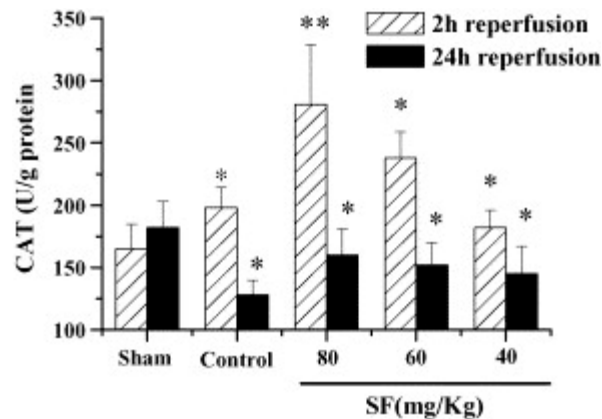
Group	Dose (mg/kg)	Neurological evaluation	Infarction area (%)	Water content (%)
Sham-operated		1.2 ± 0.2	0	78.8 ± 0.8
Control		5.8 ± 0.9*	31.1 ± 3.8*	84.3 ± 0.9*
SF	80	3.9 ± 0.4**	18.6 ± 2.5**	81.2 ± 0.5***
SF	60	4.5 ± 0.5***	24.3 ± 2.6***	82.0 ± 0.5 <sup>Δ</sup>
SF	40	4.8 ± 0.6***	26.6 ± 3.5***	81.4 ± 0.8***

\*  $p < 0.01$  vs. sham-operated. \*\*  $p < 0.01$ , \*\*\*  $p < 0.05$ , <sup>Δ</sup> $p > 0.05$  vs. control.

**The anti-oxidation effect of SF.** To estimate the antioxidant capacity, the activities of antioxidant enzymes CAT, and SOD were assayed at 2 or 24 h after reperfusion and ischemia (Sections 2.4.1 and 2.4.2). Protein concentrations were determined by the method of Lowry et al. (1951), and using bovine serum albumin as the standard. Rats were sacrificed by decapitation at 2 or 24 h after reperfusion. The brain tissues were obtained without olfactory bulb, cerebellum and low-set brain stem. The brain were weighed and homogenized in an ice-cold buffer consisting of 10 mmol/l sucrose, 10 mmol/l Tris-HCl, and 0.1 mmol/l EDTA (pH 7.4), and then centrifuged at 4000 rpm for 15 min (4 °C). The supernatant was used for bioassays. The activity of CAT and SOD was assayed by CAT Detection kit and SOD Detection kit (Fig. 3 and Fig. 4).



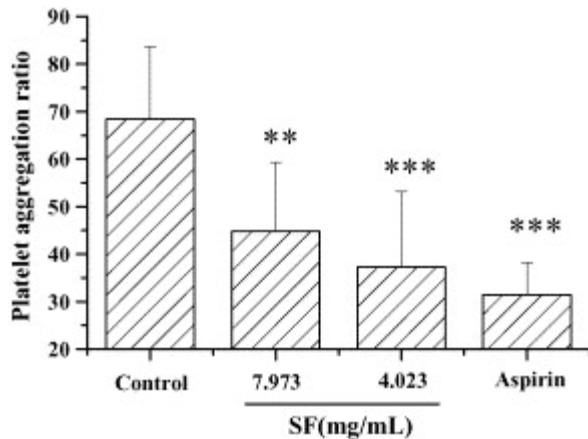
**Figure 3:** Effect of SF on SOD activity in brain of ischemic rats with reperfusion after MCAO 24 h.



**Figure 4:** Effect of SF on CAT in brain of ischemic rats with 24 h reperfusion after MCAO 24 h.

### *The detection of platelet aggregation of rabbits*

Blood was withdrawn from rabbit ear central artery and mixed with 3.8% trisodium citrate (9:1 v/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 500 rpm for 8 min at room temperature. The platelet-poor plasma (PPP) was prepared by centrifugation of the pellet at 2500 rpm 10 min at room temperature. Platelet aggregation was monitored by the turbidimetric method of Born and Cross in a Chrono-Log aggregometer. PRP (300  $\mu$ l) was incubated at 37 °C for 1 min with continuous stirring at 900 rpm (Born and Cross, 1963). Platelet aggregation was induced by ADP (5  $\mu$ M, 20  $\mu$ l/tube). SF (with 20% DMSO) were incubated with the PRP samples 5 min before addition of the aggregating agent. Aspirin was served as a positive control, and DMSO was used as blank (Fig. 5).



**Figure 5:** Inhibition effect of SF on platelet aggregation of rabbits by turbidimetry.

## RESULT

### *Effect of SF on gasping time by decapitation in mice*

As shown in Fig. 1, there was no significant difference ( $p > 0.05$ ) in the survival time between control and SF (30 mg/kg) group. The survival time of SF (40 mg/kg) group and GBE (60 mg/kg) group increased significantly as compared to the control group. SF (60 mg/kg) resulted in a significant 23.79% ( $p < 0.01$ ) prolonged time to the control group in mice.

### *Effect of SF on survival time of mice subjected to bilateral common carotid artery ligation*

As shown in Fig. 2, the SF treatment group at 60 mg/kg resulted in a significant difference in the survival time as compared to the control group. There was no significant difference ( $p > 0.05$ ) in the survival time between GBE (control) group at 60 mg/kg and SF group at 40 mg/kg.

### *Effect of SF on MDA in brain of brain injury mice*

As shown in Table 1, compared with control group, the SF treatment groups at 40 and 60 mg/kg significantly reduced the MDA content after brain injury induced by KCN.

### *Effect of SF on SOD activity and MDA in brain of ischemic mice*

Both SOD and MDA are indicators of lipid peroxidation to assess the level of oxidative stress in brain. As shown in Table 2, both 40 and 60 mg/kg SF significantly increased the activity of SOD and reduced the MDA content in brain of ischemic mice. The level of MDA was significantly reduced after ischemia in the 40 mg/kg group.

### *Neuroprotective effect of SF on protection of focal cerebral ischemia*

After the procedure of 24 h MCAO followed by 2 h reperfusion, all rats were found to have neurological impairment. Observations include adduction of left shoulder and left anterior limb when lifted by tails, muscle weakness and left-handed when walking. The symptoms in the control group were more significant. There were visible infarction and edema in brain tissues, as shown in Table 3. The neuroprotective effect of SF became significant in 2 h reperfusion.

As shown in Table 4, after 24 h MCAO followed by 24 h reperfusion, behavioral disturbance became more obvious and the infarction focus area expanded, the weight of brain edema increased. But there were remarkable improvement in the SF treatment groups, i.e., the neurological impairment was improved, infarction areas were decreased, and the water content in brain was decreased as well.

### *Anti-oxidation effect of SF*

After the procedure of 24 h MCAO followed by 2 h reperfusion, the activities of SOD and CAT significantly increased, while in the procedure followed by 24 h reperfusion, the activities of SOD and CAT significantly decreased. However, in those groups treated with SF (i.p.) at 80, 60, 40 mg/kg, the activity of SOD and CAT were continually increasing at 10 min, 24 h and 36 h after MCAO, suggesting that SF treatment inhibited the decrease of anti-oxidation activities (Fig. 3 and Fig. 4).

### *Effect of SF on platelet aggregation of rabbits*

As shown in Fig. 5, SF significantly inhibited the platelet aggregation ratio of rabbits induced by ADP.

## DISCUSSION

Stroke is one of the main fatal diseases in the world. There is no proven efficient treatment for such a condition primarily because the pathophysiology involved is not yet well understood (Read et al., 1999). It has been shown that a series of events, including massive release of excitatory amino acids, intracellular calcium overload, and free radical generation, has been involved in the pathogenesis of stroke-induced neuronal injury (Siesjö, 1992 and Parnham and Sies, 2000). Recently many evidences have suggested that the excessive generation of oxygen free radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide during reperfusion plays a major role in brain injury associated with stroke (Ikeda and Long, 1990 and Chan, 1996). Because of the brain's low concentrations of antioxidant substances glutathione (Sinet et al., 1980), antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase (CAT) (Mizuno and Ohta, 1986), the brain is exceptionally vulnerable to ischemia and reperfusion-induced oxygen free radicals, which cause oxidative damage to brain lipids, proteins, and nucleic acids, leading to brain dysfunction and cell death (Braugher and Hall, 1989 and Oliver et al., 1990).

A number of previous studies have suggested that flavonoid compounds possess antioxidant properties, although the precise mechanism of action is unknown. The antioxidant effectiveness of phenolic compounds may relate to their ability to enter cells and to their orientation in biomembranes (Thomas et al., 1992 and Kaneko et al., 1994). Flavonoids anchor to the polar heads of membrane phospholipids, forming reversible physicochemical complexes (Saija et al.,



1995). The degree of glycosylation affects various properties of some flavonoids, particularly their hydrophobicity (Manach et al., 1997).

The aglycones of flavones, being free of sugar moieties, are more lipid soluble and may readily penetrate membranes. It has been previously reported that total flavonoids from stem and leaves of *Scutellaria baicalensis* George exerted protective effects on cerebral hypoxia in mice (Shang and Cao, 2004), and that *Scutellaria baicalensis* George extracts and its constituent flavones such as baicalein could attenuate oxidant stress and protect cells from lethal oxidant damage in an ischemia reperfusion model (Shao et al., 1999).

This study indicated that *Scutellaria* flavonoid (SF) could significantly prolong gasping time after decapitation and survival time after carotid artery occlusion and attenuate MDA content in damaged brain tissues. It was also shown from the experiments that SF could increase the content of SOD in brain tissues after cerebral hypoxia. In MCAO procedure, SF showed the protective effect on cerebral hypoxia and reperfusion brain tissues. The neurological impairment was improved, infarction areas were reduced, and the water content in brain was decreased. Additionally, SF has inhibitory effect on platelet aggregation, with the average inhibition rate of 45.52%, while the aspirin group was 54.96%.

## CONCLUSION

In summary, the present study demonstrates that SF has a significant protective effect on cerebral ischemia and ischemia-reperfusion induced brain injury. Anti-free radical appears to be a basic and important mechanism of the protective effect of SF. Further studies are to be carried out to determine the mechanism of anticoagulation of SF, as well as other mechanisms of action on cerebral ischemia.

## ABBREVIATIONS

SF	<i>Scutellaria</i> flavonoid
MDA	malondialdehyde
SOD	superoxide dismutase
MCAO	middle cerebral artery occlusion
GBE	<i>Ginkgo biloba</i> extract
CAT	catalase
TBA	thiobarbituric acid

## ACKNOWLEDGEMENT

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