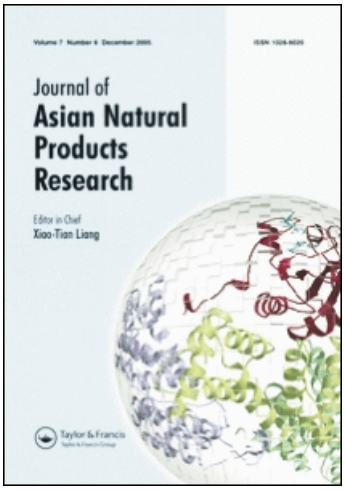

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Neuroprotective effect of liquiritin against focal cerebral ischemia/reperfusion in mice via its antioxidant and antiapoptosis properties

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Our present study was conducted to investigate whether liquiritin (7-hydroxy-2-[4-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyphenyl]-chroman-4-one, **1**), an active component of *Glycyrrhiza uralensis* Fisch., exerts a neuroprotective effect against focal cerebral ischemia/reperfusion (I/R) in male Institute of Cancer Research (ICR) mice. On the establishment of mice with middle cerebral artery occlusion (MCAO) for 2 h and reperfusion for 22 h, liquiritin at the doses of 40, 20, and 10 mg/kg was administered before MCAO once a day intragastrically for a subsequent 3 days. Neurological deficits and infarct volume were measured, respectively. The levels of malondialdehyde (MDA) and carbonyl, activities of superoxide anion (SOD), catalase (CAT) and glutathion peroxidase (GSH-Px) and reduced glutathione/oxidized disulfide (GSH/GSSG) ratio in brain were estimated spectrophotometrically. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) and terminal deoxynucleotidyl transferase-mediated DuTP-biotin nick end labeling (TUNEL)-positive cells were detected by immunohistochemical analysis. Our results showed that the neurological deficits, infarct volume, and the levels of MDA and carbonyl decreased, the ratio of GSH/GSSG and the activities of SOD, CAT, and GSH-Px were compensatorily up-regulated, and 8-OHdG and TUNEL-positive cells decreased after 22 h of reperfusion in liquiritin-treated groups. These findings suggest that liquiritin might be a potential agent against cerebral I/R injury in mice by its antioxidant and antiapoptosis properties.

Keywords: liquiritin; mice; focal cerebral ischemia/reperfusion; antioxidant; antiapoptosis

1. Introduction

Stroke is a disease with serious consequences in disability, morbidity, mortality, and cost to human, family, and society in the world. Ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage, three types of strokes, are generally seen in clinical patients. Ischemic stroke which is the most common type of stroke accounts.

Ischemic stroke initiates a complex and interconnected cascade of cellular or molecular events that lead to cell repair or

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destruction. Such as oxidative stress, excitotoxicity, and calcium overload are early events. Later events include apoptosis and neuroinflammation [1,2]. Oxidative stress is defined as an imbalance between the oxidant and antioxidant systems in favor of the oxidants and reactive oxygen species (ROS) of which molecules contain one or more unpaired electrons in their outer orbit and are highly reactive and accumulate during oxidative stress. Although ROS are key regulatory molecules vital for life, ROS may cause oxidative damage to lipid, protein, and deoxyribonucleic acid (DNA), and lead to the oxidative injury in brain [3]. Neuronal apoptosis has been indicated to participate in ischemia/reperfusion (I/R) injury in the brain [4]. Apoptosis, triggered by oxidative stress, excitotoxicity, inflammation, mitochondrial and DNA damage, and so on, is believed to be irreversible. Cerebral (I/R) is able to potentiate apoptosis by restoring cellular energy [3].

There is a continual improvement in the understanding of the pathophysiology of cerebral I/R, but the pharmacological approach of preventing or treating cerebral I/R injury has been limited in clinic. Flavonoids and isoflavones, extracted from medicinal plants, have antioxidant function and are proved to have protective effects on cerebral I/R-induced injury.

Licorice root has been widely used in China as a herbal medicine and its major bioactive component is flavonoid such as liquiritin (**1**, as shown in Figure 1), glabridin, and isoliquiritigenin [5]. Yu *et al.* [6] have demonstrated that glabridin had neuroprotective effect via modulation of multiple pathways associated with apoptosis. Isoliquiritigenin was also suggested to have protective effect against cerebral ischemia injury which might be due to the amelioration of cerebral energy metabolism and antioxidant activity [7]. It was reported that **1** produced significant antidepressant-like effects in the forced swimming test and tail-suspension test in mice [8]. Yang *et al.* [9] have established rat hippocampal neuronal damage model and demonstrated that **1** had neuroprotection and neurotrophism effects on primary cultured hippocampal cells. And Chen *et al.* [10] indicated that **1** might be a good candidate for treating various neurodegenerative diseases including Alzheimer's disease or Parkinson's disease. However, up to now, there are not any studies on the effect of **1** against brain injury caused by cerebral I/R.

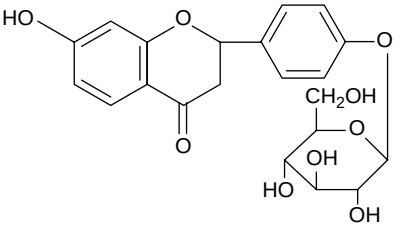

Figure 1. Chemical structure of liquiritin.

The present study therefore sought to confirm the protective effect of **1** against cerebral I/R-induced brain damage, and investigated whether **1** can improve cerebral I/R-induced brain damage with the use of the middle cerebral artery occlusion (MCAO) model in mice.

2. Results and discussion

2.1 Effect of 1 on the neurological deficits and infarct volume

The results showed that neurological deficits and infarct volume of cerebral I/R mice were significantly higher than those of the sham group after 22 h of reperfusion. Treatment with **1** (40 mg/kg) significantly decreased neurological deficit scores and reduced the percentage of infarction as well (Table 1 and Figure 2). These results demonstrated that **1** was able to protect mouse brain from I/R injury.

Table 1. Effect of **1** on the neurological deficits and infarct volume in mice subjected to cerebral I/R.

Group	Dose (mg/kg)	Neurological deficit scores	Infarct volume (%)
Model	/	2.38 ± 0.74	29.20 ± 1.71
Sham	/	0.00 ± 0.00**	0.00 ± 0.00**
1	40	1.63 ± 0.52**	20.11 ± 1.70**
1	20	1.75 ± 0.71	23.63 ± 1.92**
1	10	2.00 ± 0.76	27.88 ± 1.76
Edaravone	3	1.50 ± 0.76**	16.10 ± 1.81**

Notes: All values given in Table 1 are expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

2.2 *Effect of 1 on the redox status*

As shown in Figure 3, cerebral I/R induced the acute decrease in reduced glutathione/oxidized disulfide (GSH/GSSG) ratio in brain tissue. Compared with model group, treatment with **1** increased the ratio of GSH/GSSG. The value of the high concentration of treatment with **1** (40 and 20 mg/kg) was more obvious than those of middle and low concentrations.

2.3 *Effect of 1 on the levels of malondialdehyde (MDA) and carbonyl*

When subjected to cerebral I/R, mice showed a significant elevation in the levels of malondialdehyde (MDA) and carbonyl, as compared with the sham group. Administration of **1** showed a significant decrease in the MDA and carbonyl levels (Figure 4).

2.4 *Effect of 1 on the activities of antioxidant enzyme*

Table 2 showed the activities of superoxide anion (SOD), catalase (CAT), and glutathion peroxidase (GSH-Px) in the brain of vehicle- and drug-treated mice. In model group, the activities of these enzymes declined significantly as compared to sham group. Pretreatment with **1** dose-dependently increased the activities of these enzymes.

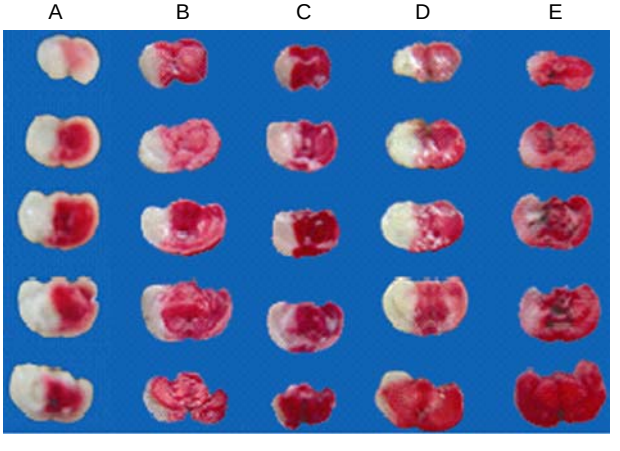

Figure 2. Representative coronal brain sections (1.5 mm thick) stained with 0.5% TTC after 22 h of reperfusion showing infarction. Red-colored region in the TTC-stained sections indicates no-ischemic portion of brain and pale-colored region indicates ischemic portion. (A) Model group; (B) **1**-treated group (40 mg/kg); (C) **1**-treated group (20 mg/kg); (D) **1**-treated group (10 mg/kg); and (E) Edaravone-treated group (3 mg/kg).

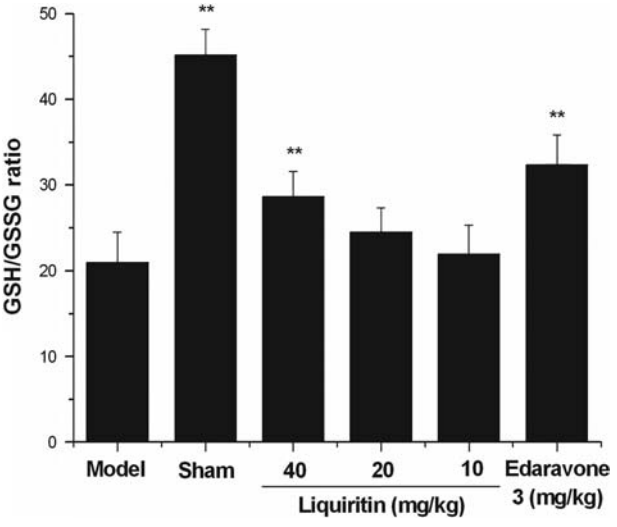

Figure 3. Effect of **1** on the GSH/GSSG ratio in mice subjected to cerebral I/R. All values given in Figure 3 are expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

2.5 Effect of 1 on the DNA oxidative damage

Figure 5 shows that very weak 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunoreactivity was found in the sham group, and that 8-OHdG immunoreactivity in the model group was obviously increased after I/R, whereas 8-OHdG immunoreactivity was slightly elevated in the **1**-treated groups.

2.6 Effect of 1 on apoptosis

Very few terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were seen in mouse brain tissue of sham group. There was a significant increase in TUNEL-positive cells in model group as compared to the sham group. Moreover, TUNEL-positive cells were markedly diminished in

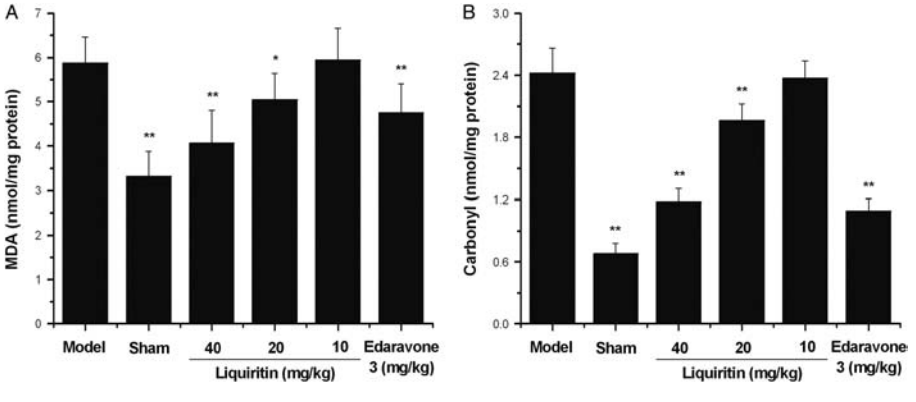

Figure 4. Effect of **1** on MDA level (A) and carbonyl level (B) in mice subjected to cerebral I/R. All values given in Figure 4 are expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

Table 2. Effect of **1** on the activities of antioxidant enzymes in mice subjected to cerebral I/R.

Group	Dose (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)
Model	/	62.02 ± 6.65	6.18 ± 0.59	681.42 ± 45.88
Sham	/	82.50 ± 4.65**	8.27 ± 0.45**	1086.5 ± 60.52**
1	40	75.11 ± 5.80**	7.62 ± 0.48**	887.24 ± 79.23**
1	20	69.13 ± 4.68*	6.92 ± 0.59*	766.45 ± 71.88*
1	10	63.68 ± 6.02	6.65 ± 0.56	693.92 ± 62.16
Edaravone	3	79.97 ± 5.22**	7.75 ± 0.53**	891.30 ± 58.13**

Notes: All values given in Table 2 are expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

the **1** group compared with those in the model group (Figure 6).

2.7 Discussion

To our knowledge, the present study is the first report on the effect of **1** on the focal I/R in mice. Our results showed that the treatment of **1** had the ability to reduce the neurological deficits, infarct volume and the levels of MDA and carbonyl, compensatorily up-regulate the ratio of GSH/GSSG and the activities of SOD, CAT, and GSH-Px, and to decrease 8-OHdG and TUNEL-positive cells as compared to the model group.

Combining neurological measurement with infarct volume assessment is commonly employed in neuroprotective drug study [11]. Many neurological deficits are difficult to assay in animals, while motor deficits are perhaps the easiest measures due to the fact that motor functions are available in mice. In the present study, the neurological deficits were determined using the four-point scale which described by Longa *et al.* [12]. The infarct volume is the chief outcome in focal I/R model and 2,3,5-triphenyltetrazolium chloride (TTC) staining which is rapid, easy, cheap, and is the most widely used. TTC is a lipid soluble and colorless chemical agent

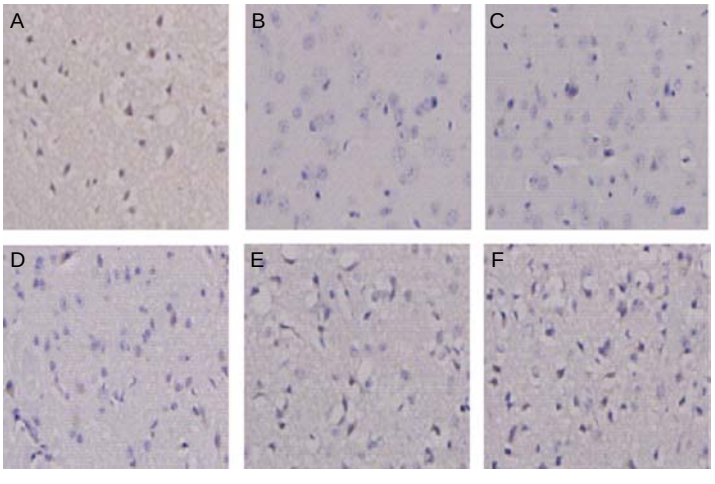

Figure 5. Immunohistochemical staining for 8-OHdG in mouse brain after 22 h of reperfusion. The nucleus of positive expressed cell is brown colored. (A) Model group; (B) sham group; (C) Edaravone-treated group (3 mg/kg); (D) **1**-treated group (40 mg/kg); (E) **1**-treated group (20 mg/kg); and (F) **1**-treated group (10 mg/kg).

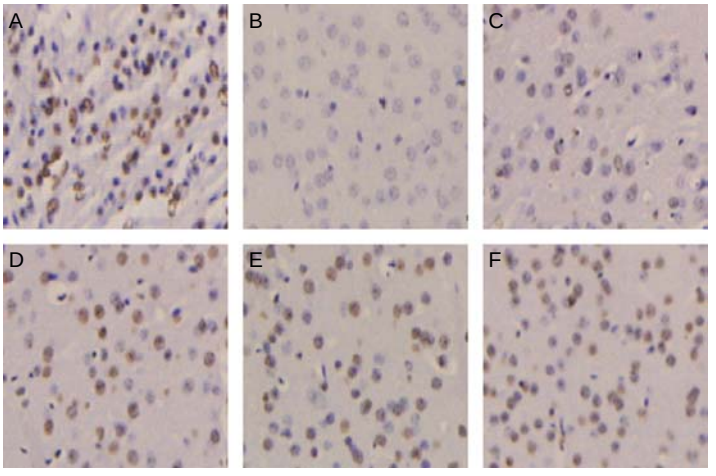

Figure 6. Immunohistochemical staining for apoptosis in mouse brain after 22 h of reperfusion. There were brown granules in the nucleus of positive cells. (A) Model group; (B) sham group; (C) Edaravone-treated group (3 mg/kg); (D) **1**-treated group (40 mg/kg); (E) **1**-treated group (20 mg/kg); and (F) **1**-treated group (10 mg/kg).

reduced by mitochondrial enzymes into a compound that stains normal brain regions red, whereas infarcted area remains white [13]. The results showed that neurological deficit scores and infarct volume obviously increased in model group as compared to sham group, and liquiritin could efficiently decrease neurological deficits and infarct volume, indicating that **1** had neuroprotective ability.

The GSH/GSSG ratio is a marker for oxidative stress in ischemic brain and is normally maintained at a high level [14]. There was a significant decrease in the ratio of GSH/GSSG in I/R mice. And **1** markedly inhibited the decrease in GSH/GSSG ratio as compared to the model group and relieved oxidative stress.

Apart from GSH/GSSG ratio, three diagnostic markers such as 8-OHdG, MDA, and carbonyl are present in brain and used as predictors for oxidative stress. DNA oxidative damage which is able to lead to DNA-protein cross linking, strand breaks, and modifications can be determined by measuring the expression of 8-OHdG. MDA is a well-known end product of lipid peroxidation and its level is usually determined by the thiobarbituric acid-reacting substances assay. Protein carbonyl is a commonly used marker for protein oxidation. The present study demonstrated that cerebral I/R-induced oxidative damage to DNA, lipid, and protein, and **1** could efficiently prevent these biomacromolecules from oxidative injury.

Brain itself also possesses an array of antioxidant defense systems including SOD, CAT, and GSH-Px to prevent injury induced by cerebral I/R. SOD catalyzes the dismutation of SOD to peroxide. CAT and GSH-Px dismutate peroxide into water and molecular oxygen. The present investigation showed that the activities of SOD, CAT, and GSH-Px decreased significantly in model group as compared with sham group, whereas the activities of these antioxidant enzymes increased in the mice treated with **1**.

Based on terminal deoxynucleotidyl TUNEL staining, it was suggested that brain tissue underwent apoptosis following cerebral I/R [15]. Sublethal injury to neurons favors the initiation of apoptosis in neurons during cerebral I/R [16]. The **1**-mediated protection was found to target

the area where cells underwent apoptosis and consequently reduced cell death as observed in the TUNEL assay. Hence, **1** appeared to possess the ability to protect mouse brain from I/R damage.

In conclusion, our findings clearly demonstrated that **1** provided obviously neuroprotective effect on MCAO-induced focal cerebral I/R. The effect of **1** is, at least in part, attributable to its antioxidant and antiapoptosis activities.

3. Materials and methods

3.1 Chemicals and reagents

Liquiritin (purity ≥ 98%) was purchased from JF-NATURAL Chemical Co. (Tianjin, China). TTC, hypoxanthine, xanthine oxidase, CAT, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), oxidized disulfide, and GSH were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1,1,3,3-tetramethoxypropane was obtained from Fluka Chemical Co. (Ronkonkoma, NY, USA). All other chemicals and reagents were of analytical grade.

3.2 Animals

Male ICR mice weighing 26 ± 2 g were obtained from Vital River Laboratories (Peking, China). Mice were allowed to acclimatize for at least 7 days prior to experimentation and housed at a room temperature of 24 ± 1°C and a relative humidity of 50 ± 5%. Standard food and water were made available *ad libitum.* Our experiments were approved by the Institutional Animal Care and Use Committee of National Institute of Pharmaceutical Education and Research.

3.3 Drug administration and model

ICR mice in our study were randomly divided into model group (0.5% sodium carboxymethylcellulose, 10 ml/kg, i.g.), sham group (0.5% sodium carboxymethylcellulose, 10 ml/kg, i.g.), **1** (40, 20, and 10 mg/kg, i.g.)-treated group, and edaravone (3 mg/kg, i.p. used as the positive control)-treated group. **1** was dissolved in 0.5% sodium carboxymethylcellulose. Drug or 0.5% sodium carboxymethylcellulose was administered once a day for 3 days before ischemia. After 3-day pretreatment with **1**, the operation was performed.

Focal cerebral I/R was produced by the occlusion of MCAO as reported by Longa *et al.* [12] and Ha *et al.* [17] with little modification. Briefly, mice were anesthetized with chloral hydrate (400 mg/kg, i.p.). Body temperature was maintained at 37 ± 0.5°C by a lamp. Midline incision was made on ventral side of the mouse neck. The left common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were carefully exposed and dissected away from adjacent muscles and nerves. Microvascular aneurysm clips were applied to the left CCA and the ICA. A coated filament was introduced into an arteriotomy hole, fed distally into the ICA, and advanced about 12 mm from the carotid bifurcation. The ICA clamp was removed and focal cerebral ischemia started. After ischemia for 2 h, the filament was gently pulled out. The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued, and the animals were returned to the prewarmed cages. Animals in sham group underwent a neck dissection and coagulation of the ECA, but no occlusion of middle cerebral artery.

3.4 Neurological deficits

After 22 h of reperfusion, the neurological deficits were blindly examined by a single examiner. Performance was scored with use of a 4-point scale described by Longa *et al.* [12]. 0: no neurological deficit; 1: failure to extend the right forepaw fully; 2: circling to the right; 3: falling to the right; and 4: no spontaneous walking with a depressed level of consciousness.

3.5 Infarct volume

The TTC staining was used to evaluate the infarct volume. Animals were killed by decapitation after 22 h of reperfusion and their brains were quickly removed and cut into five 1.5-mm thick coronal sections. Brain sections were incubated in 0.5% TTC at 37°C for 20 min. The stained brain sections were fixed in 10% formalin solution and photographed with a digital camera, and the infarct areas of each section were determined with the analysis of pixel counting by a computer program of Photoshop 6.0.

3.6 Biochemical assay of brain homogenates

3.6.1 Tissue preparation

To determine redox status, oxidative product levels, and antioxidant enzyme activities, these mouse brains were homogenized in ice-cold normal saline after blotting and weighing and the 10% homogenate was centrifuged at 4624 *g* for 15 min. The supernatant was used for biochemical assay.

3.6.2 Redox status

Redox status in the brain tissue was determined in terms of GSH and GSSG ratios. The levels of total glutathione (T-GSH), GSH, and GSSG were estimated according to the method of Candelario-Jalil *et al.* [18]. In brief, T-GSH was determined by the DTNB-GSSG reductase recycling assay. GSSG was assayed by measuring 5-thio-2-nitrobenzoic acid (TNB) which was produced from the reaction of reduced GSH with DTNB. The GSH level in brain tissue was calculated as the difference between T-GSH and GSSG.

3.6.3 Determination of MDA level

MDA, an indicator of lipid peroxidation, was estimated according to the method of Cao *et al.* [19]. 1,1,3,3-tetramethoxypropane was used as a standard, and the level of MDA was expressed as nmol/mg protein.

3.6.4 Determination of carbonyl level

Carbonyl, an indicator of protein oxidation, was assayed following the method of Levine *et al.* [20]. Carbonyl level was calculated using the extinction coefficient of $22{,}000\,M^{-1}\,cm^{-1}$/mg protein and expressed as nmol/mg protein.

3.6.5 Determination of antioxidant enzyme activities

SOD activity was assayed by a previously described method [21]. One unit of SOD activity was defined as the amount that shows 50% inhibition. SOD activity was expressed as U/mg protein.

CAT activity was determined according to the method described by Campo *et al.* [22]. One unit of CAT activity was defined as the amount of CAT required to decompose 1 μmol/l of hydrogen peroxide per minute. CAT activity was expressed as U/mg protein.

GSH-Px activity was measured using the method described by Jagetia *et al.* [23]. One unit of GSH-Px activity was defined as the GSH-Px in 1 mg of protein that led to the decrease of 1 μmol/l of GSH in the reactive system per minute. GSH-Px activity was expressed as U/mg protein.

3.6.6 Protein assay

Protein content was determined by the method of Bradford [24], and bovine serum albumin was used as the protein standard.

3.7 Immunohistochemistry assay

After 22 h of reperfusion, mice were overdosed with an anesthetic and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH = 7.4). Brains were

removed and further fixed in 4% paraformaldehyde at 4°C for 24 h and then cut into equally spaced blocks. Paraffin-embedded blocks were cut into a series of 5-μm thick slices.

8-OHdG immunohistochemistry was used to identify oxidized DNA and performed according to the method described by Wang *et al.* [25]. In brief, deparaffinized brain sections were immunostained with 5 μg/ml of mouse anti-8-OHdG antibody followed by 0.5% goat anti-mouse IgG labeled with horseradish peroxidase.

Terminal deoxynucleotidyl transferase-mediated TUNEL assay was used to identify cells with nuclear DNA fragmentation in ischemic areas and was also performed using the method of Wang *et al.* [25]. Briefly, brain sections were deparaffinized and rehydrated followed by incubating with 20 μg/ml of proteinase K in 0.01 M Tris–HCl (pH = 7.4) and permeabilized in a solution which contains 0.1% Triton-X 100 and 0.1% sodium citrate. Then, these sections were incubated in TUNEL-reaction mixture containing terminal deoxynucleotidyl transferase.

3.8 Statistical analysis

Statistical analysis was performed with the use of the SPSS statistical program (SPSS Inc., Chicago, IL, USA). All values were expressed as mean ± S.M.E. Differences between groups were measured by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. *P* values less than 0.05 were considered statistically significant.

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