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Neuroprotective Effects of Scutellarin against Hypoxic-Ischemic-Induced Cerebral Injury *via* Augmentation of Antioxidant Defense Capacity

Hong Guo, Li-Min Hu, Shao-Xia Wang, Yu-Lin Wang, Fang Shi, Hui Li, Yang Liu, Li-Yuan Kang, and Xiu-Mei Gao

Institute of Traditional Chinese Medicine
Tianjin University of Traditional Chinese Medicine
Tianjin Key Laboratory of Traditional Chinese Medicine Pharmacology
Tianjin 300193, People's Republic of China

Abstract

An increasing number of studies has indicated that hypoxic-ischemic-induced cerebral injury is partly mediated *via* oxidative stress. Recent researches have focused on searching for drug and herbal manipulations to protect against hypoxic-ischemic-induced oxidative cell damage. Scutellarin is a flavonoid derived from the *Erigeron breviscapus* (vant.) and has been reported to exhibit neuroprotective properties. However, its precise mechanism, particularly its antioxidation mechanism, remains elusive. In the present study, we investigated the neuroprotective effects of scutellarin on middle cerebral artery occlusion (MCAO)-induced brain damage in rats, and oxygen-glucose deprivation (OGD)-induced toxicity in primary culture of rat cortical neurons. *In vivo*, intraperitoneal injections of scutellarin (20 and 60 mg/kg) improved the neurological score and diminished the percentage of brain infarct volume. At the same time, scutellarin significantly increased superoxide dismutase (SOD), catalase (CAT) activities and glutathione (GSH) level in ischemic brain tissues, enhancing endogenous antioxidant activity. Moreover, pretreatment of scutellarin (25, 50 and 100 μM) protected neurons against lethal stimuli, decreased the percentage of apoptotic cells and inhibited reactive oxygen species (ROS) generation in OGD-induced primary cortical neurons *in vitro*. These results suggest that the preventive and therapeutic potential of scutellarin in cerebral injury patients is, at least in part, ascribed to augmentation of cellular antioxidant defense capacity.

Key Words: scutellarin, neuroprotective effects, antioxidant capacity

Introduction

Hypoxic-ischemic brain injury produces neurological, cognitive and other neurobehavioral impairments as well as substantial functional disability. The neurotoxic effects of hypoxic-ischemia are at least in part mediated by the generation of reactive oxygen species (ROS) and depletion in the endogenous antioxidant system including certain antioxidants and antioxidant enzymes (4, 9). It is considered to be one of the primary risk factors that exacerbate the damage caused by cerebral ischemia. Under normal conditions, a natural defense system provided by several enzymes and nonenzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) performs a vital role in the detoxification of free radicals. When there is an imbalance between free radical production and the available antioxidant defense capacity, antioxidant enzymes cannot efficiently eliminate ROS, and much ROS is stored in cells causing oxidative damage to brain biomembrane, lipids, proteins and DNA, which leads to brain dysfunction and cell death

Corresponding author: Li-Min Hu, M.D., Professor, Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, #312 Anshan xi road, Nankai district, Tianjin 300193, People's Republic of China. Tel: (+86) 22-23051120, Fax: (+86) 22-23050129, E-mail: huliminth@126.com
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(1, 21). Therefore, increasing antioxidant activity of tissues may be an important factor in providing protection from neurological damage caused by hypoxic-ischemic-associated oxidative stress.

Despite an increasing number of studies focusing on the mechanisms of ischemia, few effective therapeutic drugs have been used in clinical practice. Plant and herbal extracts have been reported to inhibit free radical generation and oxidative stress. Some of these have been used in the management of ischemia and found to attenuate reperfusion injury following cerebral ischemia (22). In China, extensive experience and abundant clinical data on the treatment of stroke have been documented with traditional Chinese medicine, which has been developed over a period of thousands of years. *Erigeron breviscapus* (vant.) has a long history of medicinal use in Chinese medicine. Its numerous preparations have been extensively used in clinical settings to treat ischemic coronary and cerebrovascular diseases for a considerable period (6, 8). Fleabane injection has been used to treat cerebral ischemia injury in China. However, its efficacy and mechanism require further studies.

Scutellarin (Fig. 1) is a flavonoid derived from *Erigeron breviscapus*, which is the capital chemical of fleabane injection. In the present study, we examined whether scutellarin could protect against hypoxic-ischemic brain injury *in vivo* and *in vitro* by enhancing the antioxidant defense capacity.

Materials and Methods

Middle Cerebral Artery Occlusion (MCAO)-Induced Focal Cerebral Ischemia in Rats

Protocols involving the use of animals complied with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee. Male Wistar rats (250~280 g) were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). Body temperature was monitored and maintained at 37.0 ± 0.5°C during surgery. Transient focal cerebral ischemia was induced by a modified method as described previously (14). Briefly, after the midline of the ventral cervical skin incision, the left common carotid, external carotid and internal carotid were exposed. A nylon monofilament (diameter 0.34 mm) coated with polylysine was introduced into internal carotid artery through the common carotid artery to occlude the origin of the middle cerebral artery. After 1 h of ischemia, the monofilament was removed. In sham-operated rats, the left carotid artery was exposed without introducing the filament into the internal carotid artery.

Drug Administration

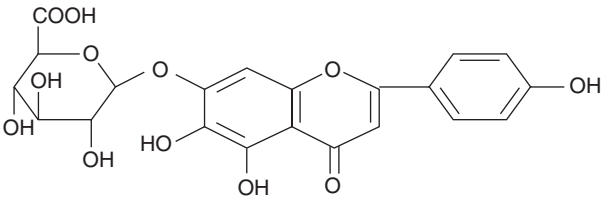

Fig. 1. Chemical structure of scutellarin.

Scutellarin (purity > 98%, HPLC grade) was provided by Biovalley Pharm., Inc. (Yunnan, PRC). We converted a dosage of scutellarin commonly used in clinical practice as the chosen dose (20, 60 mg/kg) in our study. Scutellarin was administered intraperitoneally to the animals 30 min before ischemia and immediately at the onset of reperfusion. Animals were randomized and assigned to one of four treatment groups: [1] sham-operated group; [2] vehicle-treated group; [3] 20 mg/kg scutellarin-treated group; and [4] 60 mg/kg scutellarin-treated group. In the case of the sham-operated and vehicle-treated groups, an equal volume of solvent was administered in the same manner.

Neurological Score and Percentage of Brain Infarct Volume

Twenty-four hours after the MCAO and reperfusion, the neurological score was determined by an examiner blinded to the treatment protocols, and determined using the method previously described (11) with the following scale system: [1] reflex folding of contralateral paw over chest; [2] weakened grip to cage top; [3] circles to the right or left when placed on floor; [4] moves only when stimulated; and [5] dead.

The rats were then sacrificed, their brains were quickly removed and coronal slices were made at 2 mm from the frontal tips. Slices were stained with 2% 2,3, 5-tripenyltetrazolium chloride (TTC, Sigma Chemical Co, St. Louis, MO, USA) at 37°C for 10 min. The infarct area in each slice was photographed and evaluated using the ImageJ program. The infarct volumes were calculated directly by adding the infarct volume of each section. The infarct volume percentage was calculated thus: (contralateral hemisphere volume–volume of nonischemic ipsilateral hemisphere)/ contralateral hemisphere volume × 100%.

Monitor of SOD, CAT Activities and GSH Level

To determine SOD, CAT activities and GSH level, the ischemic brain cortices were collected at 24 h after MCAO. A 10% (w/v) homogenate was prepared in ice-cold saline. The homogenates were

centrifuged at 3,000 *g* for 15 min at 4°C. Supernatant was used for bioassays, as the methods provided by the assay kits (Jiancheng, Nanjing, Jiangsu, PRC). Protein content was measured by the BCA method using bovine serum albumin as a reference standard. The assay results were normalized by protein concentration in each sample and expressed as U/mg protein or mg/g protein.

Primary Cortical Neuron Culture

Primary cortical neurons were prepared by dissecting cortices from fetal rat brains on embryonic day 17 as previously described with modifications (15). The cortices were minced, treated with 0.125% trypsin at 37°C for 5 min. Then, the cells were filtered through a 75-μm cell strainer, and centrifuged briefly. The pellet was resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). The neurons were plated at a density of 1×10^6/ml on plates coated with poly-L-lysine (10 μg/ml), and incubated at 37°C in a humidified incubator with 5% CO_2. Four hours after plating, the medium was replaced with DMEM/F12 containing 2% B27 (Gibco, Calsbad, CA, USA), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cytosine arabinoside (2.5 μM) was added within 48 h of plating to prevent the growth of non-neuronal cells. The purity of neuronal cultures was > 95% as determined by microtubule-associated protein 2 (MAP2) immunocytochemical staining (Millipore, Temecula, CA, USA).

Oxygen and Glucose Deprivation (OGD) Treatment

The neuronal injury during ischemia is due to a reduction in the supplement of oxygen and glucose. The OGD insult *in vitro* is thought to mimic the pathological conditions of ischemia. In the present study, neurons were treated by OGD as described previously (3). Briefly, neurons were rinsed twice with an OGD solution (pH 7.4) containing (in mM): 20 $NaHCO_3$, 120 NaCl, 5.36 KCl, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1.27 $CaCl_2$, 0.81 $MgSO_4$. Neurons were incubated in OGD solution in a hypoxic incubator (model 3131, Thermo Forma, San Jose, CA, USA) containing 94% N_2, 1% O_2 and 5% CO_2 for 3 h. For reoxygenation (Reox), the neurons were incubated in OGD solution containing 5.5 mM glucose at 37°C in a humidified 5% CO_2 atmosphere. Scutellarin (25, 50 and 100 μM) was continuously administrated from 2 days before OGD to the end of the experiment. Apart from the OGD treatment, the same procedure was carried out with the control neurons.

Lactate Dehydrogenase (LDH) Release and DAPI Staining

Cell viability was determined by LDH (Zhongsheng, Beijing, PRC) assay. LDH is a stable cytoplasmic enzyme present in normal cells. When cells are damaged, LDH would be rapidly released into the cell culture supernatant. The release of LDH from cells was used to evaluate cytotoxicity. Briefly, neurons were treated with OGD for 3 h in the presence or absence of various concentrations of scutellarin. The supernatants of the cells were then collected and measured by a semi-automatic biochemistry analyzer (microlab300, vital, Spankeren, Netherlands) at 340 nm. Data were normalized against the total LDH release from full-kill (FK) control cultures that had been lysed for 10 min with 0.2% Triton X-100, a reagent that damages the plasma membrane resulting in total release of LDH.

The specific DNA stain, DAPI (Sigma), was used to assess changes in chromatin and nuclear structures. Briefly, after OGD 3 h/Reox 21 h, neurons on coverslips were fixed in 4% paraformaldehyde and stained with 1 μg/ml DAPI for 15 min. Cells were then rinsed and visualized under UV light by fluorescence microscopy (DM3000, Leica, Wetzlar, Germany).

Analysis of Neuronal ROS Levels

To verify possible involvement of oxidative stress as an alternative mechanism for hypoxic-ischemic-induced neuron damage, the formation of ROS was evaluated using cell-permeable probe 2, 3-dichlorofluorscein diacetate (DCFH-DA, Beyotime, Nantong, Jiangsu, PRC). After 3 h for OGD, neurons were washed three times and incubated with 10 μM DCFH-DA at 37°C for 20 min. The fluorescence was measured by a fluorescent microplate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA) (λfex = 488 nm, λfem = 525 nm).

Statistical Analysis

Data were expressed as means ± S.D. (n = 6 per group for the *in vivo* and *in vitro* experiments). Statistical comparison between different treatments was done either by Student's *t*-test or one-way analysis of variance. Differences were considered statistically significant for $P < 0.05$.

Results

Effects of Scutellarin on Neurological Score and Percentage of Brain Infarct Volume in MCAO Rats

Representative brain slices showed that normal

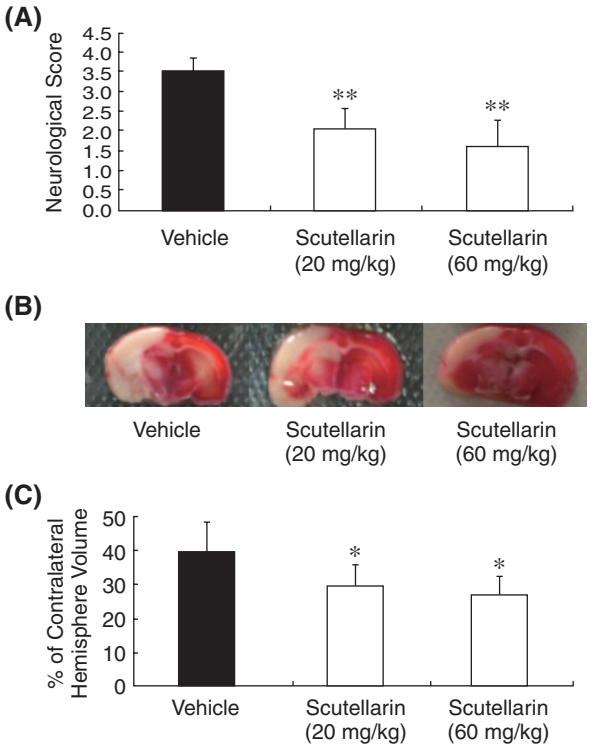

Fig. 2. Neuroprotective effects of scutellarin on neurological score and percentage of brain infarct volume in MCAO rats. (A) Neurological score was measured at 24 h after MCAO. Scutellarin of 20 and 60 mg/kg i.p. decreased the neurological score compared to the vehicle-treated group. (B) Representative coronal brain sections stained with 2% TTC from the vehicle-treated group, and groups treated with scutellarin 20 and 60 mg/kg, respectively. (C) Quantitative analysis of the percentage of brain infarct volume. Scutellarin of 20 and 60 mg/kg i.p. diminished the percentage of brain infarct volume in MCAO model. There is a statistical difference compared with the MCAO group. Data are expressed as means ± S.D., $*P < 0.05$, $**P < 0.01$ *vs*. vehicle-treated group, n = 6.

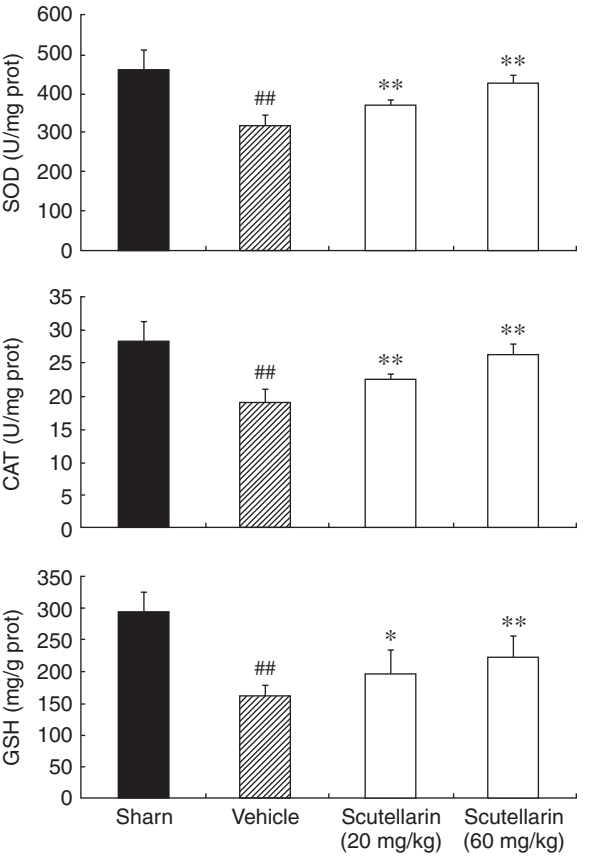

Fig. 3. Antioxidant effects of scutellarin in MCAO rats. After the MCAO 24 h, brain cortices were quickly removed for determination of SOD, CAT activities and GSH level with methods as described in the assay kits. The activities of SOD, CAT and the level of GSH decreased significantly in the MCAO group compared to the sham-operated group. When compared with the vehicle-treated group, treatment with scutellarin (20 and 60 mg/kg i.p.) significantly increased SOD, CAT activities and GSH level. Data are expressed as means ± S.D., $^{\#\#}P < 0.01$ *vs*. sham-operated group, $*P < 0.05$, $**P < 0.01$ *vs*. vehicle-treated group, n = 6.

brain tissues were stained deep red, whereas the infarct tissues were not stained by TTC. MCAO caused serious brain infarct, whereas scutellarin treatment resulted in a decrease in the percentage of brain infarct volume compared to the vehicle-treated group. Similarly, neurological score was also significantly reduced in scutellarin-treated rats (Fig. 2).

Antioxidant Effects of Scutellarin

MCAO-induced antioxidant enzyme changes are shown in Fig. 3. At 24 h after the MCAO, SOD, CAT activities and GSH level were much lower in the MCAO group than in the sham-operated group, which implied that oxidative stress had occurred. The treatments with scutellarin of 20 and 60 mg/kg increased SOD, CAT activities and GSH level compared with the MCAO group, which implied that scutellarin significantly suppressed oxidative stress in ischemic brain cortex.

Scutellarin Protected Cortical Neurons against OGD-Induced Nerve Injury

The protective effect of scutellarin on cell viability was confirmed using the LDH leakage assay. As shown in Fig. 4A, a significant increase in LDH release was observed after cortical neurons were exposed to OGD for 3 h. Scutellarin (25, 50 and 100 μM) significantly attenuated this increase in LDH release. Therefore, 100 μM scutellarin showed the greatest protective effect.

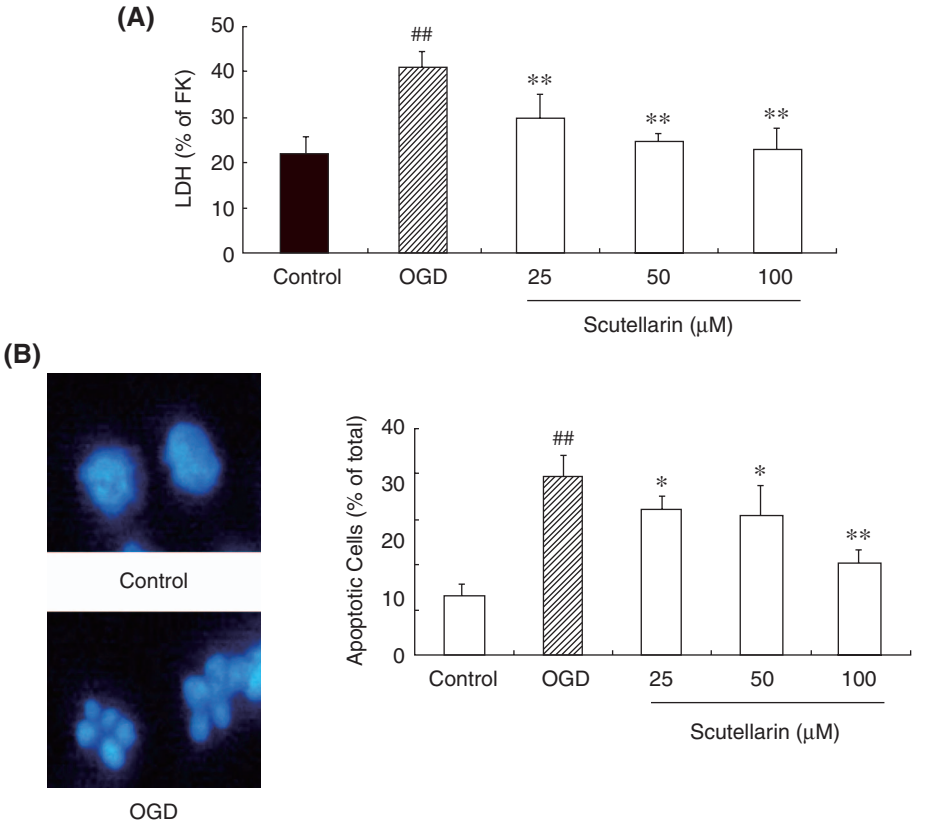

Fig. 4. Scutellarin protected cortical neurons against OGD-induced nerve injury. (A) Scutellarin reduced LDH release from rat cortical neurons after OGD. Data were expressed as the percentage (%) of full kill (FK). (B) Quantification of apoptotic neurons by DAPI stains. The nuclei of normal cells appeared intact whereas those exposed to OGD cells showed fragmented chromatin. Treatment with scutellarin could attenuate cellular damage and decrease the percentage of apoptotic cells. $^{\#\#}P < 0.01$ *vs*. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. OGD group, n = 6. The experiments were repeated at least three times with similar results.

The results from DAPI staining are shown in Fig. 4B. Almost 30% of cortical neurons in the OGD group showed apoptotic characteristics, including highly condensed and fragmented chromatin. Pretreatment with scutellarin (25, 50 and 100 μM) could attenuate cellular damage and decrease the percentage of apoptotic cells.

Scutellarin Inhibited the OGD-Induced Intracellular Accumulation of ROS

Intracellular ROS accumulation caused by OGD treatment was markedly reduced by scutellarin pretreatment. As shown in Fig. 5, compared with normoxic neurons, neurons treated with OGD for 3 h showed increased ROS production. In contrast, treatment with scutellarin (25, 50 and 100 μM) significantly reduced the increases in ROS generation.

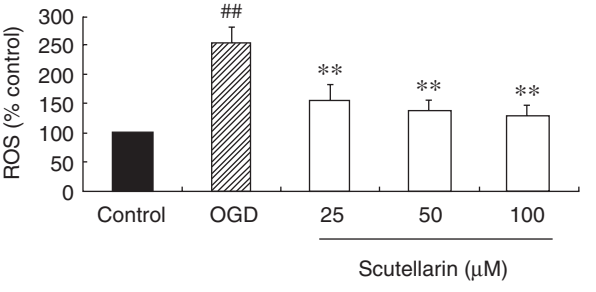

Fig. 5. Scutellarin inhibited OGD-induced ROS generation. Neurons were exposed to OGD for 3 h and ROS levels were evaluated by the measurement of oxidation of the fluorescent probe DCFH-DA. Scutellarin (25, 50 and 100 μM) inhibited OGD and elevated ROS generation. Data were expressed as the percentage (%) of control values. $^{\#\#}P < 0.01$ *vs*. control group, $^{**}P < 0.01$ *vs*. OGD group, n = 6. The experiments were repeated at least three times with similar results.

Discussion

Experimental models of hypoxic-ischemic-induced brain damage have been developed in animals in an attempt to mimic the events of human cerebral ischemia. In the present study, we used the temporary MCAO rat model to determine the actions of scutellarin. Scutellarin (20 and 60 mg/kg) was injected

twice at 0.5 h before ischemia and immediately at the onset of reperfusion, respectively. We proved that treatment with scutellarin decreased the percentage of brain infarct volume and improved the behavioral score. Our results agree with previous studies (10, 20). In addition, we found that scutellarin could significantly improve endogenous antioxidant function in molecular biology. Physiologically, the overproduction of free radicals can be detoxified by endogenous antioxidants. SOD, CAT and GSH play crucial roles as free radical scavengers (12). In the present study, cerebral ischemia was induced by MCAO for 1 h followed by 23 h reperfusion after which oxidative stress markers were estimated in the homogenate of rat brain cortices. A decrease in SOD, CAT activities and GSH level is consistent with earlier reports and suggests enhanced oxidative stress following ischemia (5). Treatment with scutellarin (20 and 60 mg/kg) significantly increased the post ischemic-reduced SOD, CAT activities and GSH level as compared to the MCAO rats. Our data suggested that treatment with scutellarin improved the cerebral ischemia–reperfusion injury in rats, at least in part, through enhancing the antioxidant potency.

OGD has become a well-established model for studying the neuropathology of oxidative stress in central nervous system disorders *in vitro*. It can augment the accumulation of ROS and result in neuron apoptosis and death (7). Our previous results confirmed that short-term treatment of scutellarin has no obvious effect on OGD-induced neurons damage (data not shown), so we decided to continuously administer it from 2 days before OGD to the end of the experiment in this paper. Although this is in contradiction to the animal experiment, it shows the variability *in vivo* and *in vitro*. We reported that the exposure of cortical neurons to OGD for 3 h notably increased LDH leakage and apoptotic percentage, consistent with previous findings which were ameliorated by pretreatment scutellarin (2, 16). Given neuronal apoptosis in late injury, we detected apoptosis at OGD 3 h / Reox 21 h. We found that pre-incubation with scutellarin (25, 50 and 100 μM) maintained the cell membrane intact and inhibited neuronal apoptosis.

Oxidative stress induces cell injury by a variety of proposed mechanisms. Accumulating evidence shows that an increase in ROS generation is a key element in neuronal cell death (19). Recent evidence has suggested that scutellarin could inhibit oxidative stress induced by superoxide in synaptosomes and by H_2O_2 in PC12 cells (13, 18). However, the authors believed it was a culture of primary neurons and the establishment of an OGD model *in vitro* that better simulate cerebral ischemic/hypoxic injury *in vivo*. In our present study, we investigated the effect of scutellarin on ROS generation-induced by OGD using rat primary cortical neurons. Our data demonstrated that ROS generation was significantly increased at OGD 3 h, which is consistent with previous reports (17), and pretreatment with scutellarin significantly decreased ROS generation.

In conclusion, the present study demonstrated that treatment with scutellarin could up-regulate certain antioxidant enzymes and relieve hypoxia-induced brain damage *in vivo*, and could also inhibit OGD-induced neuron death and decrease ROS formulation *in vitro*. These results suggest that scutellarin may protect against hypoxic-ischemic-induced cerebral injury, at least in part, through the augmentation of antioxidant defense capacity.

Acknowledgments

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