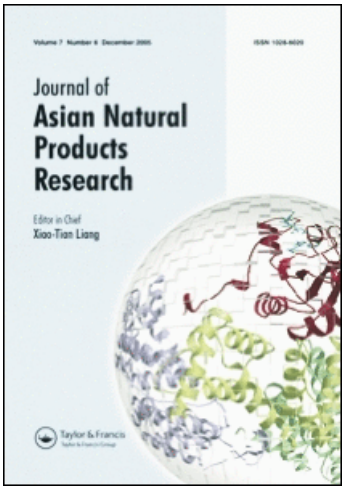

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Neuroprotection of ginsenoside Re in cerebral ischemia–reperfusion injury in rats

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In the present study, we have investigated the neuroprotective potential of ginsenoside Re (Re) in the middle cerebral artery occlusion model in Sprague–Dawley rats. Adult male Sprague–Dawley rats were treated with Re (5, 10 or 20 mg kg^{-1}, P.O. for 7 days, once a day) prior to occlusion. There was a significant increase in the neurological symptoms in ischemic animals as compared with the sham group animals. These effects were attenuated by 10 and 20 mg kg^{-1} Re, P.O. There was a significant increase in the level of malondialdehyde (MDA) in ischemic animals indicating oxidative stress. An elevated level of MDA in ischemic animals was reduced by 10 and 20 mg kg^{-1} Re, P.O., respectively. It was observed that Re significantly decreased mitochondrial swelling, thereby preventing the reduction of H^+-ATPase activity. This study demonstrates the neuroprotective potential of Re in cerebral ischemia–reperfusion injury in rats.

Keywords: ginsenoside Re; cerebral ischemia; reperfusion injury; H^+-ATPase; mitochondrial swelling

1. Introduction

The principal pathophysiological processes in cerebral ischemia are energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity, and free-radical-mediated toxicity. The reperfusion of ischemic tissue produces an influx of inflammatory cells and oxygen that can cause increases in oxygen-derived free radicals. Free radicals are also important in prolonged ischemia.[1] The pathophysiology of cerebral ischemia has been studied extensively in rats using various methods. The models of cerebral ischemia in rats have normally been produced by the occlusion of the middle cerebral artery (MCA).

Mitochondria, producing adenosine triphosphate by virtue of electron flow, have been shown to be both the sites of superoxide anion production and the target of free-radical attacks. Free radicals directly damage neuron mitochondria membranes during reperfusion from cerebral ischemia, resulting in lipid peroxidation. These changes are associated with mitochondrial dysfunction and rapid decreases in ATP. In this study, we evaluated these mechanisms in an *in vivo* cerebral ischemia model.

Panax ginseng is one of the precious traditional Chinese medicines. It has a long history of safe use in China for more than 2000 years as a tonic to combat stress agents. Ginseng has demonstrated pharmacological effects in the central nervous, cardiovascular, endocrine, and immune systems.[2] Ginsenosides are the most important active constituents

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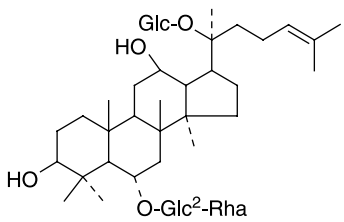

Figure 1. Structure of ginsenoside Re.

identified in all species of ginseng. Re (Figure 1) is one of the important active principles of ginseng and shares many pharmacological effects of this plant. It protects the heart against ischemia–reperfusion injury by shortening the action potential duration (APD), thereby prohibiting influx of excessive Ca^{2+}. Re enhances the slowly activating component of the delayed rectifier K^+ current and suppresses the L-type Ca^{2+} current, which may account for APD shortening.[3] We have investigated the role of Re in preventing MPTP-induced apoptosis of the substantia nigra neurons in the mouse model of Parkinson's disease[4] and the protection of Re against cerebral ischemia–reperfusion injury.[5] In this study, we investigated the level of brain mitochondria swelling, H^+-ATPase activity, malondialdehyde (MDA) level, and neurological symptoms in rat brain (Figure 2).

2. Results and discussion

2.1 *Effect of Re on mitochondrial swelling*

The decrease in OD at 540 nm indicates mitochondrial swelling. The mitochondrial swelling was high in the vehicle group. Pretreatment with Re (5, 10, or 20 mg kg^{-1}, P.O. for 7 days, once a day) decreased the level of mitochondrial swelling (Figure 3).

2.2 *Effect of Re on lipid peroxidation*

The MDA was instinctly increased in the vehicle group (27.16 ± 4.07 nmol/mg protein) ($P < 0.01$ compared with the sham group). The elevated level of MDA was reduced (20.45 ± 3.06 and 18.30 ± 2.93 nmol/mg protein) by 10 and 20 mg kg^{-1} Re, P.O., respectively. When compared with the vehicle group, pretreatment with Re significantly decreased ($P < 0.01$) the MDA content in a dose-dependent manner (Figure 4).

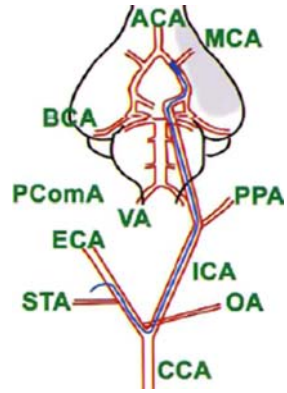

Figure 2. Schematic of MCAO by the suture method in rats. The left ECA was exposed and its branches electrocoagulated. A 4-0 suture was then introduced into the ICA via the ECA stump, advanced 20 mm distal to the carotid bifurcation, and the MCA occluded. The gray area is that subjected to ischemia.

2.3 *Effect of Re on neurological functions*

Cerebral ischemia–reperfusion injury produced a significant increase in the neurological score, indicating neurological dysfunction. The neurological score was instinctly increased in the vehicle group [(4.20 ± 0.42), $^{+++}P < 0.001$] when compared with the sham group. The elevated neurological score was significantly reduced (3.50 ± 0.53 and 1.70 ± 0.48) by 10 and 20 mg kg^{-1} Re, P.O., respectively, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the vehicle group. However, 5 mg kg^{-1} Re did not produce significant protection (Figure 5).

2.4 *Effect of Re on H^+-ATPase activity*

The activity of H^+-ATPase was decreased significantly ($P < 0.01$) in the vehicle group (1.80 ± 0.30 μmol Pi/mg protein min) as compared with the sham group (2.28 ± 0.24 μmol Pi/mg protein min). When compared with the vehicle group, pretreatment with Re (10 and 20 mg kg^{-1}, P.O. for 7 days,

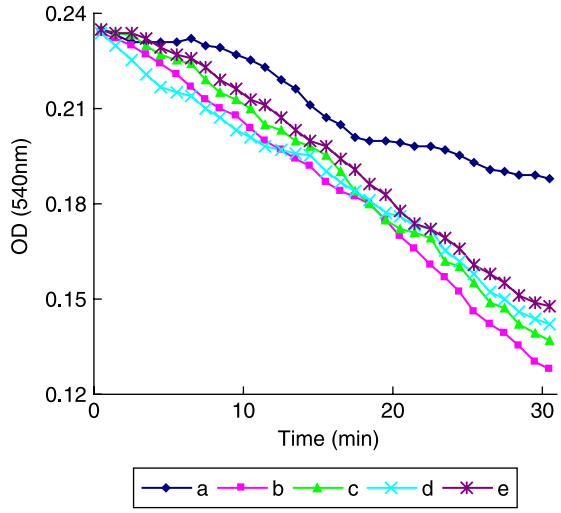

Figure 3. The effect of Re on mitochondrial swelling. Mitochondrial swelling was increased in b when compared with that in a. When compared with b, pretreatment with Re (5, 10, 20 mg kg^{-1}, P.O. for 7 days, once a day) significantly decreased the level of mitochondrial swelling in 'a'.

once a day) significantly increased the activity of H^+-ATPase (2.07 ± 0.23 μmol Pi/mg protein min and 2.22 ± 0.34 μmol Pi/mg protein min), respectively ($P < 0.05$ and 0.01; Figure 6).

Middle cerebral artery occlusion (MCAO) results in a cascade of events leading to a number of important cellular changes. These include calcium release from intracellular stores, the product of excessive free radical, acidosis, and so on. Free radicals directly damage neuron mitochondria membranes during reperfusion from cerebral ischemia, resulting in lipid peroxidation. These changes are associated with mitochondrial dysfunction and rapid decreases in ATP.

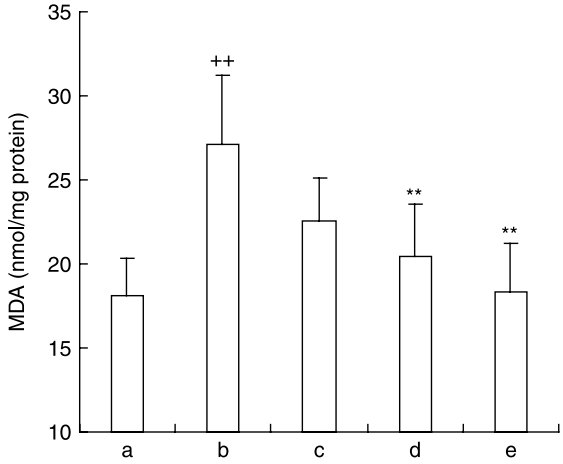

Figure 4. The effect of Re on lipid peroxidation (MDA). MDA was increased in b when compared with that in a. When compared with b, pretreatment with Re (5, 10, 20 mg kg^{-1}, P.O. for 7 days, once a day) significantly decreased the MDA content in 'a' in a dose-dependent manner. Data are means ± SD ($n = 10$). $^{**}p < 0.01$ *vs.* b, $^{++}p < 0.01$ *vs.* a.

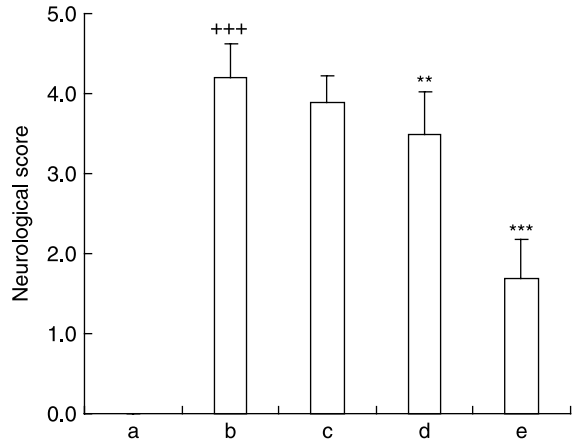

Figure 5. The effect of Re on neurological functions. These functions were assessed 24 h after ischemia and reperfusion. They were decreased significantly in b when compared with those in a, $^{+++}p < 0.001$. When compared with b, pretreatment with Re (10 and 20 mg kg^{-1}, P.O. for 7 days, once a day) significantly increased the neurological functions in 'a', $^{**}p < 0.01$, $^{***}p < 0.001$. Data are means ± SD ($n = 10$).

Because of its high energy demand for cellular function and its relatively modest defense against secondary oxidative stress, brain is one of the most vulnerable organs to ischemia. Under normal physiological conditions, brain mitochondria generate ATP by virtue of effective electron transport in their respiratory chain for cellular maintenance and integrity.

The biochemical function of mitochondria strongly depends on the membrane, which is one of the oxygen radical target sites. A disturbance in the mitochondrial membrane integrity causes mitochondrial dysfunction, leading to energy failure of brain cells. Therefore, energy failure because of mitochondrial dysfunction is likely to be an important factor in ischemic brain injury.

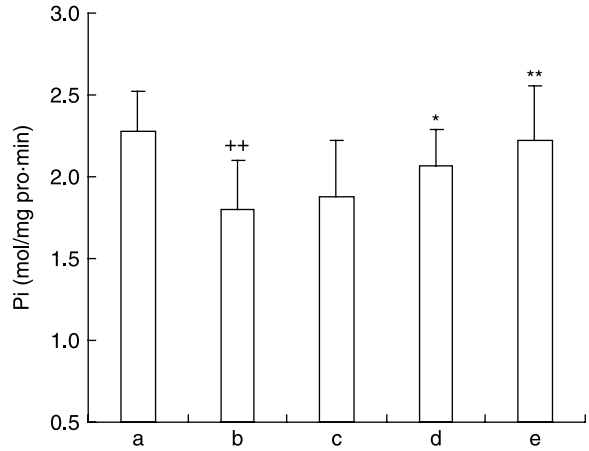

Figure 6. The effect of Re on H^+-ATPase activity. The activity of H^+-ATPase was decreased significantly in b when compared with that in a. When compared with b, pretreatment with Re (5, 10, 20 mg kg^{-1}, P.O. for 7 days, once a day) significantly increased the activity of H^+-ATPase in 'a' in a dose-dependent manner. Data are means ± SD ($n = 10$). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* b, $^{++}p < 0.01$ *vs.* a.

Mitochondrial swelling is one of the most striking and initial ultrastructural changes after brain ischemia.[6] Our results show that the protective effect of Re against rat cerebral ischemia–reperfusion injury in that Re decreases the level of mitochondrial swelling and prevents the reduction of H^+-ATPase activity.

For years, scientists used lipid peroxidation as an index of oxidative stress in biological systems.[7] Lipid peroxidation is one of the most important organic expressions of oxidative stress, where unsaturated lipids, such as arachidonic acid, undergo a reaction with oxygen free radicals to yield lipid hydroperoxides. The consequence of lipid peroxidation is the degradation of the membrane's polyunsaturated fatty acids, with a subsequent disorganization of membrane structure and disturbance in membrane function.

Ischemia-induced brain damages are accompanied by biochemical alterations and neurological sequelae.[8] There are substantial experimental evidences that reactive oxygen species (ROS) are produced in the brain during ischemia and reperfusion injury. ROS such as superoxide radical, hydroxyl radical, and hydrogen peroxide contribute to ischemic brain damage. It is very vulnerable to ROS that cause oxidative damage to brain biomembrane, lipids, proteins, and DNA, leading to brain dysfunction and cell death.[9] During reperfusion, a sudden supply of molecular oxygen, which serves as a substrate for xanthine oxidase for nucleotide metabolism, results in the increased generation of hydrogen peroxide and superoxide by-products. It is conceivable that oxygen radical generation and subsequent impairment of mitochondrial function. The present study showed that pretreatment with Re significantly reduced the post-ischemic-enhanced MDA level as compared with the vehicle group. These results suggested that pretreatment with Re improved the cerebral ischemia–reperfusion injury in rats by its antioxidant property.

In conclusion, Re showed a significant protection in MCAO model of cerebral ischemia in rats. It is definite that Re decreased the product (MDA) of lipid peroxidation and the level of mitochondrial swelling, prevented the reduction of H^+-ATPase activity, and attenuated the neurological deficits. This study suggests the beneficial effects of Re in cerebral ischemia–reperfusion injury, but the mechanism needs to be studied in future.

3. Experimental

3.1 Materials and methods

3.1.1 Animals

All experiments conducted were in accordance with the National Institutes of Health's *Guiding Principles for Research Involving Animals and Human Beings*. Male Sprague–Dawley rats weighing 250–300 g were purchased from the Experimental Animal Center of Chinese Academy of Medical Sciences (Beijing, China). The rats were maintained under standard laboratory conditions at 22 ± 2°C, relative humidity $50 \pm 15\%$, and a 12 h:12 h light:darkness cycle. The animals were provided with standard diet and water *ad libitum*.

3.1.2 Reagents

Re was purchased from Shenyang Pharmaceutical University; chloral hydrate, carboxymenthylcellulose (CMC)-saline and silicone were purchased from No. 2 Pharmacy Factory (Beijing, China); MDA assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); all other chemicals were reagents of molecular biology grade obtained from standard commercial sources.

3.1.3 Treatment schedule

Rats were randomly divided into five groups, each consisting of 10 animals: (a) Sham group, the rats were given 0.5% CMC-saline, (b) vehicle group (ischemia 2 h reperfusion 2 h group (IR) + 0.5% CMC-saline); Re-treated groups (IR + Re), in which the rats

were subdivided into (c) 5 mg kg^{-1} Re group, (d) 10 mg kg^{-1} Re group, and (e) 20 mg kg^{-1} Re group, P.O., once a day, for 7 days prior to ischemia. On day 7, 1 h after the above treatment, the rats were subjected to the following evaluation tests.

3.1.4 MCAO procedure to induce cerebral ischemia

Rats were anesthetized intraperitoneally with chloral hydrate (400 mg kg^{-1}). The rectal temperature was recorded and maintained in a range between 36.5 and 37.5°C using a heating pad throughout the surgical procedure and up to 2 h after reperfusion. A polyethylene tube was inserted into the left femoral artery for continuous monitoring of blood pressure using RM-6240 polygraph. The carotid bifurcation in the neck is exposed, the common carotid artery is occluded, and the branches of the external carotid artery (ECA) are dissected and divided. The internal carotid artery is followed rostrally, and the pterygopalatine branch is identified and divided. The 4-0 surgical thread coated by 5 mm silicone (a diameter of 0.25 mm) at the distal is then introduced into the internal carotid artery (ICA) and advanced 20 mm (Figure 2).[10] In the sham group, the ECA was surgically prepared for insertion of the thread, but the thread was not inserted. Two hours after the induction of ischemia, the thread was withdrawn until the tip reached ECA. The animals were then closely monitored for 2 h. After 2 h of reperfusion, they were sacrificed and the brains were quickly removed and stored at 4°C.

3.1.5 Isolation of mitochondria

Rat brain mitochondria were isolated in a medium of 250 mmol/l sucrose, 10 mmol/l Tris–HCl, 1 mmol/l EGTA (pH 7.4) by differential centrifugation of brain homogenates essentially, as described previously.[11] The mitochondria were resuspended in 250 mmol/l sucrose, 10 mmol/l Tris–HCl buffer (pH 7.4) and stored in ice. The mitochondrial protein concentration was measured for each specimen by the Lowry method.

3.1.6 Mitochondrial swelling determination

Mitochondrial swelling changes were monitored at 540 nm using a spectrophotometer, within 4 h of isolation.[12] The measurements of mitochondrial swelling were carried out at 25°C in an incubation medium consisting of 250 mmol/l sucrose, 10 mmol/l EGTA, 10 mmol/l Hepes-Tris buffer (pH 7.4), and 0.2 mg mitochondrial protein. The duration of each measurement was 30 min.

3.1.7 Lipid peroxidation determination

MDA, an indicator of lipid peroxidation, was estimated 2 h following reperfusion after ischemia. Briefly, ischemic brain tissues were homogenized with 0.1 mol/l sodium phosphate buffers (pH 7.4) to make 10% homogenates; the homogenates were centrifuged at 3000*g* for 15 min at 4°C and the supernatant was used for bioassays. The MDA content was measured using a modified TBA test[13] and a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) reading at 532 nm. The results are expressed as nanomoles of MDA per milligram of protein (nmol/mg protein). All solutions were freshly prepared on the day of assaying.

3.1.8 Neurological symptoms

Twenty-four hours after ischemia and reperfusion, the rats were assessed for neurological symptoms,[14] which is as follows: no symptom = 0, hunched posture or hair roughed up = 1, ptosis = 2, circling behavior = 3, splayed-out hind limb = 4, and seizures = 5. The higher the score was, the higher was the ischemic insult.

3.1.9 H^+-ATPase activity determination

The reaction buffer consisting of 5 mmol/l ATP, 5 mmol/l $MgCl_2$, and 50 mmol/l Tris buffer (pH 7.95) was incubated at 25°C for 2 min; then, mitochondria protein was added into 100 μl reaction buffer at a final concentration of 0.2 mg ml^{-1} and incubated for 2 min at 25°C, and the reaction was stopped by the

addition of trichloroacetic acid.[15] The solution was centrifuged at 3000*g* for 2 min and the supernatant obtained was monitored at 690 nm using a spectrophotometer. The H^+-ATPase activity was defined as micromoles of Pi released per milligram of protein per minute (μmol Pi/mg protein min).

3.1.10 Statistical analysis

All data are expressed as means ± SD. The differences between groups were examined for statistical significance using one-way ANOVA with Student's *t*-test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

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