Protective Effect of Ginsenoside-Re against Cerebral Ischemia/Reperfusion Damage in Rats

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To investigate the protective effect of ginsenoside Re (Re) against cerebral ischemia–reperfusion injury, adult male Wistar rats weighing 250–300 g were subjected to either sham surgery or middle cerebral artery occlusion (MCAO) for 2 h of brain ischemia and 2 h reperfusion. A fluorescence polarization assay was carried out for membrane fluidity of brain mitochondria. Lipid peroxidation [malondialdehyde (MDA) formation], superoxide dismutase (SOD) and glutathion peroxidase (GSH-Px) of rat brain were estimated by fluorometric methods. It was observed that Re (5, 10, 20 mg kg⁻¹ *p.o.* pretreatment for 7 d, once a day) significantly improved the fluidity of mitochondrial membranes as demonstrated by a reduction of average microviscosity, ameliorated lipid peroxidation by raising the activities of SOD and GSH-Px, and reduced the content of MDA in rat brain. This study demonstrated a direct protective effect of Re against cerebral ischemia–reperfusion injury.

Key words ginsenoside Re; ischemia; reperfusion; membrane fluidity; lipid peroxidation

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 radicals, acidosis,¹⁾ and so on. These changes are associated with mitochondrial dysfunction and rapid decreases in ATP.

The fluidity of mitochondrial membranes is crucial to their function, especially the production of energy. The structure of neuron mitochondria membrane is destroyed and the fluidity of membrane decreased when there is 2 h of brain ischemia and 2 h reperfusion in rat. Free radicals directly damage neuron mitochondria membranes during reperfusion from cerebral ischemia, resulting in lipid peroxidation.²⁾

Panax ginseng is widely used in traditional Chinese medicine to enhance stamina and capacity to cope with fatigue as well as physical stress. The mechanisms of action of ginseng remain largely unclear. Nevertheless, the therapeutic effects of ginseng have been attributed to its active ingredients, ginsenosides, sugar conjugates of dihydroxyl or trihydroxyl-dammarane triterpenes. Ginsenosides are normally fractionated into two groups based on the types of aglycone, namely the panaxadiol group (*e.g.*, Rb1 and Rc) and the panaxatriol group (*e.g.*, Rg1 and Re). Re (Fig. 1) is one of the ingredients with the highest content (0.15%) in *Panax ginseng*³⁾ and is responsible for many pharmacological effects of this plant, but protection of Re against cerebral ischemia–reperfusion injury has not been reported. In an attempt to elucidate the effect that Re prevents cerebral ischemia–reperfusion injury in the rat brain, we studied the membrane fluidity of brain mitochondria, MDA level, SOD and GSH-Px activities in rat brain.

MATERIAL AND METHODS

Animals and Pretreatments Male Wistar rats weighing 250–300 g were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Animals were housed in a room with temperature of 21–23 °C, relative humidity of 30–70%, and a 12-h light/12-h dark cycle (lights on at 08:00 h). They had free ac-

cess to food and water. All experimental procedures carried out in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Shenyang Pharmaceutical University in Shenyang.

Animals were randomly divided into five groups. Rats in ginsenoside-Re pretreated groups (ischemia 2 h–reperfusion 2 h+Re) were given *p.o.* ginsenoside-Re (5, 10, 20 mg kg⁻¹) once a day, for 7 d prior to ischemia (IR+Re). Rats in sham-operated group (SAM) and ischemia 2 h–reperfusion 2 h group (IR) were given *p.o.* 0.5% CMC–saline. Re was suspended in a 0.5% carboxymethylcellulose (CMC)–saline reaching solution.

Extraction of Ginsenoside Re Roots of *Panax ginseng* were collected from Fusong, Jilin province. The raw materials were ground into powder and passed through a 200 square mesh screen, then extracted by refluxing with MeOH. The MeOH extract was evaporated under vacuum to yield syrup that was suspended in H₂O. Total saponin was acquired by butanol extracted from a water solution and loaded on the silica column eluted by CHCl₃:MeOH:H₂O (65:25:10). The ginsenoside Re (Fig. 1) was enriched in fraction IV and purified by HPLC column Waters Prep Nova-Pak HR C18 (6 μm, 7.8×300 mm) with a linear gradient of CH₃CN/H₂O from 30:70 to 50:50 at a flow rate of 1.0 ml/min. The peak of Re was collected (refer to standard Re) and concentrated by evaporation under vacuum.

Reagents 1,6-Diphenylhexa-1,3,5-triene (DPH) was pur-

Fig. 1. Chemical Structure of Ginsenoside-Re

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chased from Sigma (U.S.A.). malondialdehyde (MDA), superoxide dismutase (SOD) and glutathion peroxidase (GSH-Px) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Chloral hydrate and silicone from No. 2 Pharmacy Factory (Beijing, China). Other chemicals were of analytical-reagent grade and were purchased locally.

Middle Cerebral Artery Occlusion Procedure to Induce Cerebral Ischemia Rats were anesthetized with chloral hydrate at the dose of 350 mg kg⁻¹, intraperitoneally. Rectal temperature was recorded and maintained at 37 °C throughout the surgical procedure and up to 2 h after reperfusion. A polyethylene tubing was inserted into the left femoral artery for continuous monitoring of blood pressure using a 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 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)^{4,5} at the distal is then introduced into the internal carotid artery and advanced 20 mm. In the sham-operated group, the external carotid artery was surgically prepared for insertion of the thread, but the thread was not inserted.⁶ Two hours after the induction of ischemia, the thread was withdrawn until the tip reached the external carotid artery. Animals were then closely monitored for 2 h. After 2 h of postischemic reperfusion, brains were quickly removed and stored at 4 °C.

Isolation of Mitochondria Rat brain mitochondria were isolated in a medium of 250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.4, by differential centrifugation of brain homogenates essentially as described previously.^{7,8} Mitochondria were resuspended in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and stored in ice.

Membrane Fluidity Determination A Hitachi 650-60 fluorescence spectrophotometer equipped with a 390 nm cut off filter was used to measure the fluorescence polarization.^{9,10} The fluorescence probe molecule, DPH, was incorporated into cells to monitor the dynamic behavior of mitochondria membrane lipids. DPH (10⁻⁶ M) was mixed with mitochondria and the mixture was incubated at 30 °C for 30 min in the dark. Excitation and emission wavelengths were set at 360 and 430 nm, respectively and the experiments were carried out at 25 °C. Steady-state fluorescence polarization (*P*) was calculated by using $P = (I_{vv} - gI_{vh}) / (I_{vv} + gI_{vh})$,¹¹ where *g* is a grating correction factor, equal to I_{hv} / I_{hh} . *I* is the intensity of light, and *v* and *h* denote polarizers vertically and horizontally oriented.¹² The first subscript refers to the exciting light and the second to the emitting light. Each data (*P*) represented an average of 10 measurements. Microviscosity was calculated using $\eta = 2P / (0.46 - P)$.¹³ A high microviscosity value correlates with low membrane fluidity and *vice versa*.

Lipid Peroxidation Determination Lipid peroxidation (LP) was assessed by measuring the concentration of malondialdehyde (MDA) in brain samples.¹⁴ To assess the level of oxidative stress, the MDA, an indicator of lipid peroxidation, was estimated 2 h following reperfusion after ischemia. The brain was frozen at -80 °C for biochemical analysis. Briefly, the ischemic brain tissues were homogenized with 0.1 M

sodium phosphate buffers (pH 7.4) to make 10% homogenates. The homogenates were centrifuged at 3000×*g* for 15 min at 4 °C. The supernatant was used for bioassays. The content of MDA was measured with a modified TBA test as described by Ohkawa *et al.*¹⁵ and a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at 532 nm. The results are expressed as nanomoles of MDA per milligram of protein (nmol mg⁻¹ protein).

Antioxidant Enzymes Determination For assaying the activities of antioxidant enzymes SOD, GSH-Px, the ischemic brain tissues were weighed and homogenized with a buffer consisting of 10 mM sucrose, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.4), and then centrifuged at 3000×*g* for 15 min at 4 °C. The supernatant was used for bioassays. The activity of SOD was determined using a xanthine/xanthine oxidase system for production of superoxide radical and subsequent measurement of cytochrome *c* as a scavenger of the radicals.¹⁵ Optical density was determined by spectrometer (UV-1601, Shimadzu) at 550 nm. One unit of enzyme activity was defined as the quantity of SOD required to inhibit the rate of reduction of cytochrome *c* by 50%. SOD activity is presented as units per milligram of protein (U mg⁻¹ protein). GSH-Px was determined using *t*-butylhydroperoxide as a substrate.¹⁶ The optical density was spectrophotometrically recorded at 340 nm. One unit of the enzyme is defined as micromoles (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute. The GSH-Px activity is expressed as U mg⁻¹ protein.

Statistical Analysis All data are expressed as mean ± S.D. Statistical significance between groups was determined by one-way analysis of variance (ANOVA). Data analysis was performed using the SPSS 10.0 (SPSS Inc., Chicago, IL, U.S.A.) statistical package programme. *p* < 0.05 was considered as statistically significant.

RESULTS

Effect of Re on Mitochondria Membrane Fluidity Effect of Re on membrane fluidity was demonstrated by a reduction of average microviscosity (*η*). As shown in Table 1, Re significantly decreased (*p* < 0.05) average microviscosity of the brain mitochondria membrane at doses from 5 to 20 mg kg⁻¹ in a dose-dependent manner in ischemia-reperfusion rats.

Effect of Re on Lipid Peroxidation Effect of Re on

Table 1. Effect of Re on Mitochondria Membrane Fluidity

	Average microviscosity (<i>η</i>)
SAM	3.015 ± 0.381
IR	6.582 ± 0.821 ⁺
(IR+Re)	
5 mg kg ⁻¹	4.875 ± 0.102*
10 mg kg ⁻¹	4.523 ± 0.508*
20 mg kg ⁻¹	3.902 ± 0.406*

Rats in IR+Re were given Re (*p.o.* 5, 10, 20 mg kg⁻¹ for 7 d, once a day) prior to ischemia-reperfusion. Rats in SAM and IR (ischemia-reperfusion) were given 0.5% CMC-saline (*p.o.*). Mitochondria membrane fluidity was demonstrated by the microviscosity (*η*). Fluorescence polarization (*P*) was calculated by using $P = (I_{vv} - gI_{vh}) / (I_{vv} + gI_{vh})$. Microviscosity (*η*) was calculated by using $\eta = 2P / (0.46 - P)$. A high microviscosity value correlates with low membrane fluidity and *vice versa*. Each data represented an average of 10 measurements. **p* < 0.05 vs. IR group. +*p* < 0.05 vs. SAM group.

Fig. 2. Effect of Re on Lipid Peroxidation (MDA)

The MDA was instantly increased in IR (b) vs. SAM (a). When compared with IR (b), pretreatment with Re (5, 10, 20 mg kg⁻¹, *p.o.* for 7 d, once a day) significantly decreased the MDA content in a dose-dependent manner. Data are means $\pm$ S.D. (*n*=10). **p*<0.05 vs. IR (b). +*p*<0.05 vs. SAM (a). a. SAM represents sham-operated group, b. IR represents ischemia 2 h–reperfusion 2 h, c. IR+5 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 5 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, d. IR+10 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 10 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, e. IR+20 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 20 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d.

Fig. 3. Effect of Re on Antioxidant Enzymes (SOD)

The activities of SOD was decreased significantly in IR (b) vs. SAM (a). When compared with IR (b), pretreatment with Re (10, 20 mg kg⁻¹, *p.o.* for 7 d, once a day) significantly increased the activities of SOD in a dose-dependent manner. Data are means $\pm$ S.D. (*n*=10). **p*<0.05 vs. IR (b). +*p*<0.05 vs. SAM (a). a. SAM represents sham-operated group, b. IR represents ischemia 2 h–reperfusion 2 h, c. IR+5 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 5 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, d. IR+10 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 10 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, e. IR+20 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 20 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d.

lipid peroxidation was demonstrated by brain MDA content. Figure 2 indicates that the MDA was instantly increased in the vehicle-pretreated group. When compared with the IR group, pretreatment with Re significantly decreased (*p*<0.05) the MDA content (*p*<0.05) in a dose-dependent manner.

Effect of Re on Antioxidant Enzymes SOD, GSH-Px activities in MCAO occlusion–reperfusion rats are shown in Figs. 3 and 4. The activities of SOD and GSH-Px were decreased significantly (*p*<0.05) in the vehicle-pretreated group as compared to the sham group. When compared with the IR group, pretreatment with Re (5, 10, 20 mg kg⁻¹, *p.o.* for 7 d, once a day) significantly increased the activities of SOD (*p*<0.05) and GSH-Px (*p*<0.05).

Fig. 4. Effect of Re on Antioxidant Enzymes (GSH-Px)

The activities of GSH-Px was decreased significantly in IR (b) vs. SAM (a). When compared with IR (b), pretreatment with Re (5, 10, 20 mg kg⁻¹, *p.o.* for 7 d, once a day) significantly increased the activities of GSH-Px in a dose-dependent manner. Data are means $\pm$ S.D. (*n*=10). **p*<0.05 vs. IR (b). +*p*<0.05 vs. SAM (a). a. SAM represents sham-operated group, b. IR represents ischemia 2 h–reperfusion 2 h, c. IR+5 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 5 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, d. IR+10 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 10 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d, e. IR+20 mg kg⁻¹ Re represents ischemia 2 h–reperfusion 2 h pretreated with 20 mg kg⁻¹ ginsenoside Re *p.o.* for 7 d.

DISCUSSION

Many pharmacological actions of ginseng are attributed to its ginsenosides. Some pharmacological studies of the ginsenosides on ischemia have been reported. For example, ginsenoside Rg3 might provide neuroprotection against the cerebral ischemia-induced injury in rat brain through reducing lipid peroxides, scavenging free radicals and improving the energy metabolism¹⁷⁾; ginsenoside Rb1 protects the cerebral cortex and the hippocampal CA1 neurons against ischemic damage,^{18,19)} and so on.

To our knowledge, this is the first report providing evidence of the effectiveness of ginsenoside Re in a MCAO rat model. Our results show that the protective effect of Re against rat cerebral ischemia–reperfusion injury in a dose-dependent manner in Ginsenoside-Re significantly decreased the average microviscosity of the brain mitochondria membrane and the products (MDA) of lipid peroxidation, and increased the activities of antioxidative enzymes (SOD and GSH-Px).

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,¹⁾ and so on. It is generally acknowledged that the changes are associated with mitochondrial dysfunction and rapid decreases in ATP. The fluidity of mitochondrial membranes is crucial to its function, especially the production of energy. Brain mitochondrial membrane can be protected by Re in cerebral ischemia–reperfusion. In order to show the consequences on the mitochondrial membrane fluidity, a highly hydrophobic probe, DPH, was used to point out lipid phases of the mitochondrial membrane.²⁰⁾ The distribution of DPH is heterogeneous, with a significant fraction of molecules lying with their long axis perpendicular to the preferred direction of the acyl chains. Fluorescence anisotropy is directly proportional to membrane viscosity so that decreased mobility of DPH into the membrane is indicated by the increasing value of fluorescence polarization. Our study revealed that pretreatment with ginsenoside-Re in rats subjected to 2 h of MCAO

markedly decreased average microviscosity of the brain mitochondria membrane in a dose-dependent manner at 2 h of reperfusion. This may explain the protective effects of Re on brain injury at least partly due to brain energy metabolism.

Lipid peroxidation is considered a major mechanism of oxygen free radical attack. This process initiates with an oxygen radical attack to unsaturated double bonds. Biomembrane structure contains many unsaturated double bonds which are especially sensitive to free radical-induced lipid peroxidation, and relatively low activities of antioxidative enzymes including SOD, GSH-Px, and so on. The process is very vulnerable to reactive oxygen radicals (ROS) induced by ischemia-reperfusion, which cause oxidative damage to brain biomembrane, lipids, proteins, and DNA, leading to brain dysfunction and cell death.^{21,22} During reperfusion a sudden supply of molecular oxygen, which serves as a substrate for xanthine oxidase for nucleotide metabolism, results in increased generation of hydrogen peroxide and superoxide as by-products. It is conceivable that there is oxygen radical generation and subsequent impairment of the mitochondrial function.¹⁸ The present study showed that pretreatment with Re significantly reduced the postischemic-enhanced MDA level as compared to a vehicle-pretreated group. The activities of SOD and GSH-Px were decreased significantly in the vehicle-pretreated group compared with the sham-operated group, while the activities of these antioxidant enzymes were increased in the rats pretreated with Re. These results suggested that pretreatment with Re improved the cerebral ischemia-reperfusion injury in rats because of its antioxidant property.

In conclusion, our paper has shown that pretreatment is necessary for the antioxidant property of Re in ischemia-reperfusion, and also confirms the protective effect of Re against cerebral ischemia-reperfusion. Re clearly decreased the average microviscosity of the brain mitochondria membrane and the product (MDA) of lipid peroxidation, and increased the activities of the antioxidative enzymes (SOD and GSH-Px).

Whether or not the ginsenosides of ginseng extract have a synergistic effect and what the mechanism is remain to be

examined in future experiments.

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