Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 77 (2014) 142–149

www.jcma-online.com

Original Article

Neuroprotective effects of ginsenoside Rg1 against oxygen–glucose deprivation in cultured hippocampal neurons

Qing He [a], Jianguo Sun [b], Qin Wang [b], Wei Wang [b], Bin He [c,*]

[a] *Department of Neurology, The First Hospital of Xuzhou, Xuzhou, Jiangsu, China*
[b] *Department of Neurology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China*
[c] *Emergency Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China*

Received January 20, 2013; accepted September 13, 2013

Abstract

Background: Ginsenoside Rg1 (Rg1) is believed to be one of the main active principles in ginseng, a traditional Chinese medicine extensively used to enhance stamina and deal with fatigue as well as physical stress. It has been reported that Rg1 performs multiple biological activities, including neuroprotective activity. In this study, we investigated the efficacy of ginsenoside Rg1 on ischemia–reperfusion injury in cultured hippocampal cells and also probed its possible mechanisms.

Methods: To establish a model of oxygen–glucose deprivation (OGD) and reperfusion, cultured hippocampal neurons were exposed to OGD for 2.5 hours, followed by a 24-hour reoxygenation. Cultured hippocampal neurons were randomly divided into control group, model group (vehicle), and ginsenoside Rg1 treatment groups (5μM, 20μM, 60μM). At 24 hours post-OGD, the intracellular free calcium concentration was detected using Furo-3/AM-loaded hippocampal neurons deprived of oxygen and glucose. Neuronal nitric oxide synthase (nNOS) activity was measured by chemical colorimetry. Cell apoptosis was evaluated by Hoechst staining, and the neuron viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Results: Excitotoxic neuronal injury of OGD was demonstrated by the increase of intracellular free calcium concentrations and elevated nNOS activity in the model group compared with the control group. The intracellular free calcium concentrations and the nNOS activity in the groups receiving intermediate and high dose of ginsenoside Rg1 were significantly lower than those of the control group ($p < 0.05$). In addition, intermediate and high dose of ginsenoside Rg1 administration could also attenuate the cell viability loss ($p < 0.05$) and cell apoptosis induced by OGD.

Conclusion: Ginsenoside Rg1 has neuroprotective effect on ischemia–reperfusion injury in cultured hippocampal cells mediated by blocking calcium over-influx into neuronal cells and decreasing the nNOS activity after OGD exposure. We infer that ginsenoside Rg1 may serve as a potential therapeutic agent for cerebral ischemia injury.

Keywords: calcium overload; cerebral ischemia; ginsenoside Rg1; hippocampal neurons; nitric oxide synthase

1. Introduction

Ischemic cerebral stroke is a devastating event that has become the third greatest cause of mortality and the leading cause of disability worldwide, and results in many serious physical, psychological, and social difficulties. However, the treatment of stroke still remains highly unsatisfactory. A variety of clinical trials of pharmacological neuroprotective strategies in stroke have been demonstrated to be

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Bin He, Emergency Center, The First Affiliated Hospital of Nanjing Medical University, 300, Guangzhou Road, Nanjing 210029, Jiangsu, China.

E-mail address: hbhbwywy34@sina.com (B. He).

1726-4901/$ - see front matter
http://dx.doi.org/10.1016/j.jcma.2014.01.001

disappointing.[1] Therefore, seeking new neuroprotective agents for cerebral stroke management in order to improve clinical prognoses in patients with brain ischemic injury is urgent.

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), a well-known traditional Chinese medicine, has been widely used to enhance stamina and deal with fatigue as well as physical stress for thousands of years in oriental countries.[2] Ginsenosides, or ginseng saponins, are widely considered to among the principal bioactive constituents of ginseng, and provide diverse benefits due to their structural isomerism and stereoisomerism.[3] Up to now, over 30 different ginsenosides have been isolated and identified from ginseng, and they are classified into two major groups: protopanaxadiol and protopanaxatriol saponins, according to their chemical structures.[3] Recently, the effects of different kinds of ginsenosides have been extensively studied, and a great spectrum of pharmacological responses has been reported, including effects on the central nervous system, cardiovascular system, endocrine system, immune system, and cancer.[3,4] Neuronal cell damage, including apoptosis, is the principal event occurring in the acute and subacute phases of cerebral ischemia. Ginsenoside Rg1 (Fig. 1),[5] belonging to the protopanaxatriol group, is an important active component of ginseng. The neuroprotective effects of ginsenoside Rg1 have been extensively investigated recently in several cell-damaging models, such as glutamate and kainic acid-induced excitotoxicity insults in spinal neurons,[6] rotenone-induced degeneration of cells in the substantial nigra,[7] glutamate-induced excitotoxic effect in primary hippocampal cultures,[8] serum withdrawal-induced apoptosis in primary cultured cortical neurons,[9] glutamate induced in dopaminergic cell loss[10] and 1-methyl-4-phenyl-pyridinium^{+}-induced apoptosis in human SHSY5Y cells.[11] However, little work has been done in hypoxic−ischemic conditions. Furthermore, the detailed mechanisms underlying the neuroprotective efficacy of ginsenoside Rg1 still await investigation.

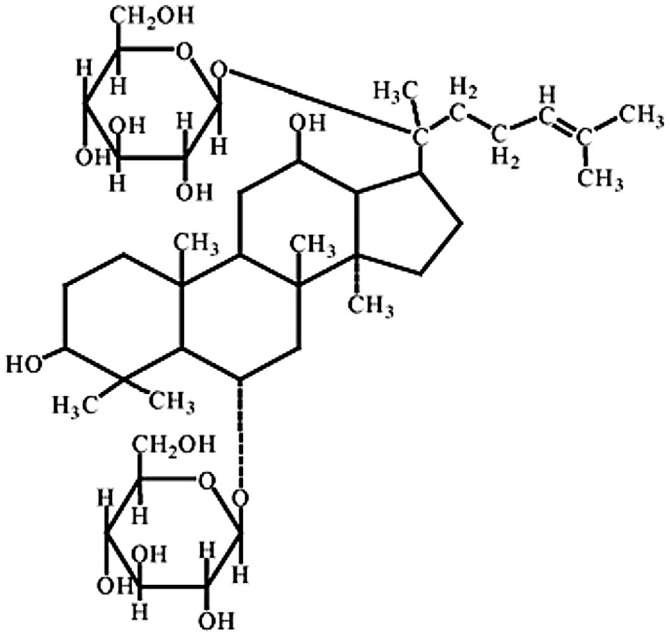


Fig. 1. The chemical structure of ginsenoside Rg1. The molecular formula of ginsenoside Rg1 is $C_{42}H_{72}O_{14}$, and the molecular weight is 801.01.

The maintenance of Ca^{2+} homeostasis is important for cell function and survival, conversely, intracellular calcium ($[Ca^{2+}]i$) overload is believed to be one of the first events triggered by brain ischemia.[12,13] Nitric oxide (NO) is a ubiquitous messenger molecule that also acts as a neuronal messenger molecule in the central nervous system. NO has been demonstrated to be produced enzymatically in response to activation of N-methyl-D-aspartate (NMDA) receptors.[14] This activation results in an elevated influx of Ca^{2+} ions into the postsynaptic structure, which in turn activates NO-synthase (NOS) under physiological conditions.[14−16] Prior studies have demonstrated that NOS mediates the ischemia-induced neuronal damage.[17] The present investigation was designed mainly to evaluate the role of Rg1 on Ca^{2+} influx and the activity of neuronal NOS (nNOS) in experimental stroke models induced by oxygen−glucose deprivation (OGD).

2. Methods

2.1. Reagents

Ginsenoside Rg1 was isolated and purified at China Pharmaceutical University (Nanjing, China). Neuronal NOS detection kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Poly-L-lysine, glutamine, trypsin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal horse serum, Dulbecco's modified Eagle medium (DMEM), Neurobasal, and B27 were obtained from Invitrogen Gibco (Carlsbad, CA, USA). Fetal bovine serum was provided by Sijiqing Biological Engineering Institute (Hangzhou, China). Hoechst 33258 fluorescent dye kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were of the purest grade available from regular commercial sources.

2.2. Primary rat hippocampal cell culture

Protocols were in accordance with the International Guidelines for Animal Research and were approved by the Animal Care and Experimental Committee of Nanjing Medical University (Jiangsu, China). Hippocampi were aseptically dissected out from embryonic Day 18 Sprague−Dawley rat embryos acquired from the Experimental Animal Center of Nanjing Medical University. Hippocampal neurons were dissociated from the tissues as previously described elsewhere.[18] After trituration and trypsinization, hippocampal cells were resuspended in DMEM supplemented with 10% fetal bovine serum, 10% horse serum, glutamine 100 μg/mL, and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively). The single-cell suspension was seeded in 35 mm petri dishes or 96-well plates (Corning Inc., Corning, NY, USA) coated with poly-L-lysine at a density of (2−5) × 10^5/mL. After 24 hours, cells were maintained in Neurobasal medium supplemented with 2% B27, penicillin/streptomycin

(100 U/mL and 100 μg/mL, respectively) in a humidified atmosphere of 5% CO_2 at 37°C.

2.3. OGD model establishment

Primary hippocampal neuronal cultures were incubated in deoxygenated, glucose-free medium to mimic the interruption of the supply of oxygen and nutrients to the brain parenchyma. Hippocampal neurons were cultured *in vitro* for 8−10 days prior to being randomly divided into control group, model group (vehicle), ginsenoside Rg1 intervention group (low-, intermediate-, and high-dose). The number of cells in each group was counted in four 20× fields, and all of them were equal (about 120−150 cells/20× fields). Ginsenoside Rg1 of different concentrations (5μM, 20μM, and 60μM) was added to the culture medium 30 minutes prior to OGD. The culture medium was replaced by prewarmed DMEM with low glucose (1 g/L) in the model group and ginsenoside Rg1 intervention group, whereas the control group received high-glucose (4.5 g/L) DMEM. The cultures were then transferred to a hypoxia chamber containing gas mixture of equilibrated 95%N_2/5%CO_2 for 2.5 hours at 37°C. OGD was terminated by placing the cultures back into the normoxic incubator with normal culture medium. Sham OGD cultures were exposed in normoxic conditions during the same period of time as the OGD cultures. Twenty-four hours after OGD procedures, neuron cultures were terminated to run tests.

2.4. $[Ca^{2+}]i$ measurement using Fluo-3-AM-based microfluorimetry

To observe the possible influence of Rg1 on the elevated $[Ca^{2+}]i$ induced by OGD, the fluorescence intensity was detected 24 hours after OGD. The procedures of OGD model establishment and Rg1 treatment have been described above. At 24 hours post-OGD, the existing maintenance medium was carefully discarded and the hippocampal cells were washed with PBS three times. For loading with Fluo-3-AM, the hippocampal cells were incubated without light for 30 minutes at 37°C with a final concentration of 10μM Fluo-3-AM. Once dye-loaded, the cells were washed thoroughly with the assay buffer to remove any unincorporated dye. The intensity of fluorescence was recorded with the excitation wavelength at 488 nm and emission wavelength at 525 nm using a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The $[Ca^{2+}]i$ was estimated from the fluorescence signal, accounting for the binding of calcium to the dye. All the fluorescence image data were collected and analyzed with the LSM510, version 2.3 software (Carl Zeiss).

2.5. Neuronal NOS determination

Hippocampal cells were collected when cell culture was terminated 24 hours after OGD as mentioned above. After being washed with PBS, hippocampal cells were lysed with cell lysis buffer, followed by which supernatant was discarded after centrifugation at 4°C, 16,000*g* for 10 minutes. The nNOS activity was measured by chemical colorimetry according to the manufacturer's instructions. There were no endothelial cells and inflammatory cells involved in the cultured hippocampal cells, suggesting that determined NOS activity was totally attributed to nNOS.

2.6. Cell viability assay

Cell viability was evaluated by the MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to form a purple formazan product as described previously.[19] In brief, at 24 hours post-OGD, 20 μL of MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37°C for another 4 hours. The medium was then replaced by 100 μL DMSO and the amount of formed purple formazan dye was determined by measuring the absorbance value at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed using six replicated wells for each group and carried out independently three times.

2.7. Fluorescence analysis of apoptosis

Hippocampal cells were stained with Hoechst 33258 according to the manufacturer's instructions for the Hoechst Staining Kit (Beyotime Institute of Biotechnology) to detect the characteristic features of apoptotic nuclei. The cultured hippocampal neurons were fixed in ice-cold 4% phosphate-buffered paraformaldehyde overnight and then incubated at room temperature with 1 g/mL Hoechst 33258 dye (Beyotime Institute of Biotechnology) for 5 minutes. Cells were analyzed under an inverted phase-contrast fluorescence microscope at 400× magnification and counted by a blinded investigator from three independent experiments. The cultured hippocampal cells with fragmented DNA, condensed DNA, or normal DNA were counted. The ratio of apoptotic neurons to total neurons was calculated. In each experiment, 200 cells were examined in random fields from three culture wells for each condition to estimate the percentage of apoptotic hippocampal cells.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were statistically evaluated by one-way analysis of variance (ANOVA) to determine differences among the groups. A probability level of $p < 0.05$ was accepted as statistically significant. All statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA) software.

3. Results

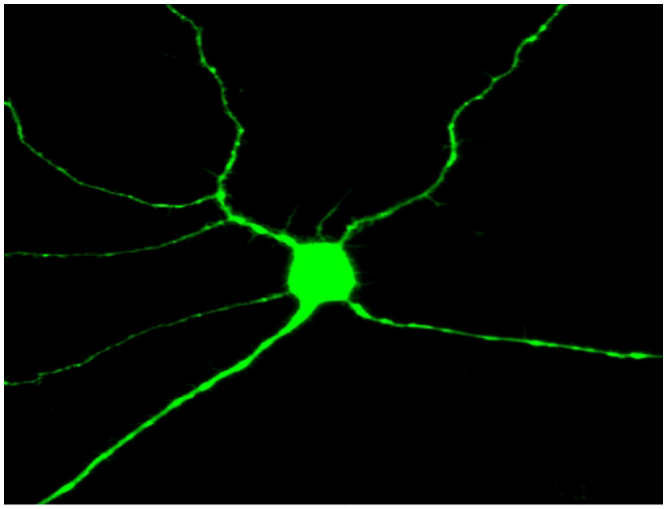
3.1. Effect of ginsenoside Rg1 on OGD-induced $[Ca^{2+}]i$ increase

After being stained with Fluo-3 AM, hippocampal neurons emitted fluorescence excited by an argon laser of 488 nm wavelength (Fig. 2). The effect of ginsenoside Rg1 on $[Ca^{2+}]i$ in hippocampal cells after OGD is shown in Fig. 3. $[Ca^{2+}]i$ of

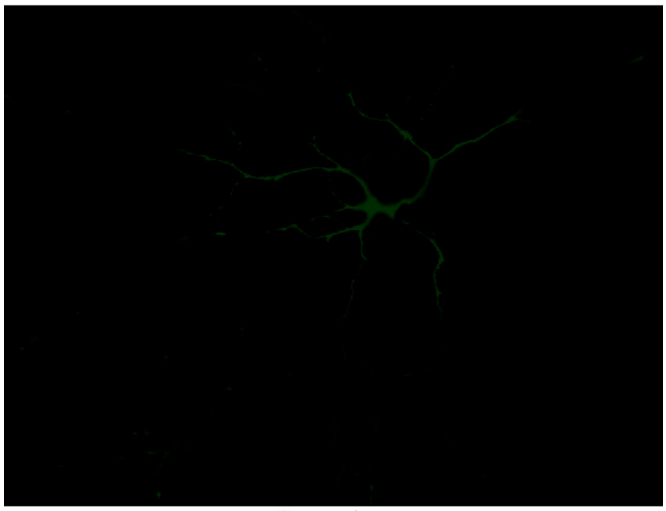
the model group increased significantly ($p < 0.05$) compared with the control group. However, compared with the model group, [Ca^{2+}]i of intermediate and high dose (20μM, 60μM, respectively) ginsenoside Rg1 intervention groups of decreased significantly ($p < 0.05$), whereas such variation did not occur in the low-dose (5μM) ginsenoside Rg1 intervention group. Pretreatment with relatively high concentration (20μM, 60μM) of ginsenoside Rg1 inhibited OGD-induced [Ca^{2+}]i increase; however, the amplitude of decline was not statistically different between the two groups. In addition, pretreatment with a low concentration of ginsenoside Rg1 (5μM) did not affect the OGD-induced [Ca^{2+}]i response.

3.2. Effect of ginsenoside Rg1 on OGD-induced nNOS activity increase

The nNOS activity of hippocampal cells in the OGD group was significantly elevated ($p < 0.05$) compared with that in the control group. Compared with the model group, the nNOS activity of the intermediate and high dose (20μM, 60μM, respectively) ginsenoside Rg1 intervention groups of decreased significantly ($p < 0.05$); however, the low-dose (5μM) ginsenoside Rg1 intervention group did not reach statistical significance compared with the model group. Pretreatment with a relatively high concentration (20μM, 60μM) of ginsenoside Rg1 inhibited OGD-induced elevated nNOS activity. However, pretreatment with a low concentration of ginsenoside Rg1 (5μM) did not significantly affect the OGD-induced elevated nNOS activity (Fig. 4).



Model group



Control group

Fig. 2. Representative microphotographs of hippocampal neurons that emitted fluorescence when excited by an argon laser of 488 nm wavelength after being stained with Fluo-3 AM (original magnification 400×) by using a Zeiss LSM-510 laser scanning confocal microscope.

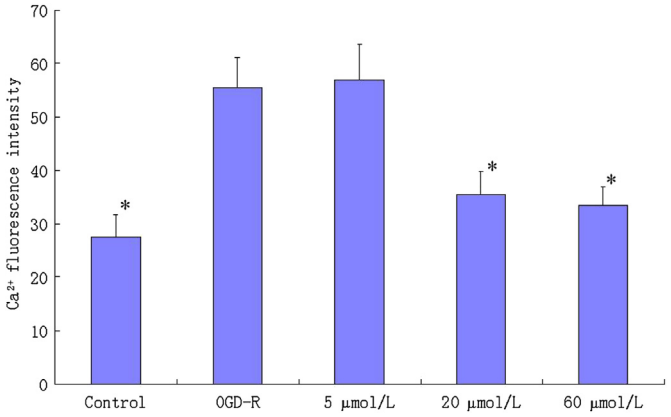


Fig. 3. Effect of ginsenoside Rg1 on oxygen–glucose deprivation (OGD)-induced [Ca^{2+}]i increase. To observe the possible influence of Rg1 on the elevated [Ca^{2+}]i induced by OGD, Rg1 was added 30 minutes prior to the procedure of OGD, and the fluorescence intensity was detected 24 hours after OGD procedures. The data are means ± standard error of the mean from three independent experiments. Statistical significance was evaluated by one-way analysis of variance. $p < 0.05$ was regarded as statistically significant. * $p < 0.05$ versus model group (OGD).

3.3. Effect of ginsenoside Rg1 against OGD-induced cytotoxicity

The possibility that ginsenoside Rg1 can have a direct protective effect on hippocampal neurons against ischemia/reperfusion injury was detected by analyzing the effect of ginsenoside Rg1 on OGD-induced neuronal death by MTT assay. As shown in Fig. 5, exposure of hippocampal neurons to 150 minutes of OGD induced significant cell death at 24 hours after reperfusion. The cell survival in the control group was standardized to 100%. Pretreatment with a relatively high concentration (20μM, 60μM) of ginsenoside Rg1 inhibited OGD-induced neuronal death with a significant elevation of neuronal survival rate ($p < 0.05$), whereas a low dose (5μM) of ginsenoside Rg1 slightly increased hippocampal neuronal viability compared with the model group, but did not reach statistical significance.

3.4. Effect of ginsenoside Rg1 on OGD-induced apoptosis

To better characterize the effects of ginsenoside Rg1 on OGD-induced neuronal cell death, Hoechst 33258 (Beyotime

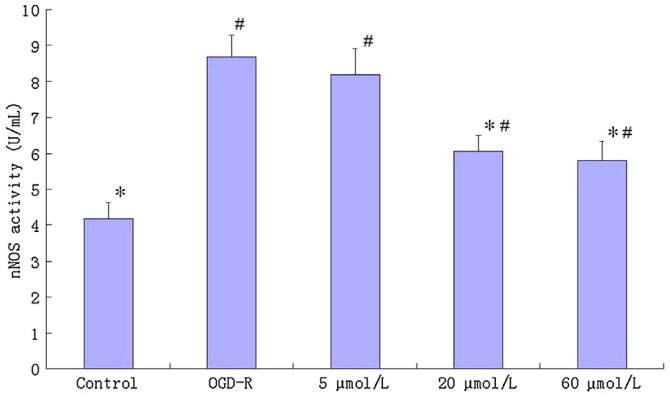


Fig. 4. Effect of ginsenoside Rg1 on oxygen–glucose deprivation (OGD)-induced neuronal nitric oxide synthase (nNOS) activity increase. The neuronal NOS activity was measured by chemical colorimetry according to the manufacturer's instructions in the assay kits. The data are mean ± standard error of the mean from three independent experiments. Statistical significance was evaluated by one-way analysis of variance. $p < 0.05$ was regarded as statistically significant. * $p < 0.05$ versus model group (OGD). ** $p < 0.05$ versus control group.

Institute of Biotechnology) was added to cultured cells after OGD insult in the presence or absence of ginsenoside Rg1. Exposure to OGD also resulted in apoptotic death of hippocampal neurons, as revealed by the appearance of condensed (bright blue) nuclei in Hoechst-stained cultures (Fig. 6A). A total of 200 cells spotted on glass slides from randomly chosen microscopic fields were examined and counted by fluorescence microscopy, and the percentage of cells exhibiting apoptotic degeneration relative to the total number was calculated as the apoptotic index from three independent experiments. Through cell counting, it was determined that 46.94 ± 3.14% of cultured neurons were apoptotic at 24 hours post-OGD, as compared to 5.24 ± 0.92% of neurons apoptotic in the total population of the control group. However, treatment with ginsenoside Rg1 (5μM, 20μM, 60μM) significantly decreased OGD-induced apoptotic cell death, with the percentage of apoptotic neurons being 44.24 ± 4.27%, 36.24 ± 2.75%, and 34.32 ± 2.18%, respectively. Pretreatment with relatively high concentrations (20μM, 60μM) of ginsenoside Rg1 inhibited OGD-induced elevated hippocampal neuronal apoptotic index ($p < 0.05$), whereas a low dose (5μM) of ginsenoside Rg1 slightly decreased OGD-induced hippocampal neuronal apoptotic index, but did not reach statistical significance (Fig. 6).

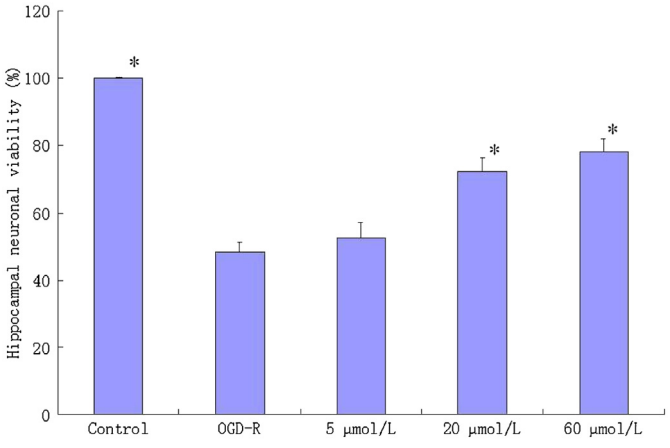


Fig. 5. Effect of ginsenoside Rg1 on oxygen–glucose deprivation (OGD)-induced cytotoxicity. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to evaluate the protective efficacy of ginsenoside Rg1 on hippocampal neurons against ischemia/reperfusion injury. The data are mean ± standard error of the mean shown as percentages of the viability of the control group from three independent experiments. Each experiment was performed using six replicated wells for each group. Statistical significance was evaluated by one-way analysis of variance. $p < 0.05$ was regarded as statistically significant. * $p < 0.05$ versus model group (OGD).

4. Discussion

In the present study, we investigated whether treatment of primary cultures of rat hippocampal cells with ginsenoside Rg1 was able to prevent neurotoxicity resulting from OGD. The present data in our cell-damaging models highlight that ginsenoside Rg1, one of the active ginsenosides, exhibited protective effects against neuronal damage induced by hypoxic–ischemic insults. We found that Rg1 improved neuronal viability and ameliorated apoptotic rate in cultured hippocampal neurons after OGD. Moreover, with Rg1 treatment, elevated intracellular Ca^{2+} concentrations and nNOS activity induced by OGD were markedly inhibited, suggesting that the neuroprotective activities of Rg1 were mediated by blocking calcium over-influx into neuronal cells and attenuating nNOS activity. These results suggest that Rg1 could prove to be a novel therapeutic candidate for the treatment of ischemic cerebral stroke.

Ginsenosides are amphiphilic and have the ability to intercalate into the plasma membrane, which leads to alterations in membrane fluidity and affects membrane function.[3] The effects of ginsenosides may be initiated at the plasma membrane by interacting with multireceptor systems,[20] as well as via intracellular protein binding to produce genomic effects. Accumulated evidence has been found to demonstrate the neuroprotective effects of ginsenosides. Ginsenosides may rescue neuronal cells by increasing cell survival, extending neurite growth, and protecting neurons from death either *in vivo* or *in vitro*.[10,21,22] Ginsenosides have been exhibited to act as antioxidants, or scavengers for free radicals, by increasing the activity of superoxide dismutase and inhibiting lipid peroxidation.[23] In addition, ginsenosides upregulate nerve growth factor expression, which in turn promotes neuron survival.[24] Furthermore, ginsenosides could protect cortical neurons from glutamate insults by blocking Ca^{2+} influx through glutamate receptors.[25] Thus, Rg1, a single ginsenoside, may also exhibit diverse functions by interacting with multireceptor systems.[3] Studies have demonstrated that Rg1 is able to increase dopamine content in the striatum.[26] Much work has been done to find Rg1 to be protective in some cell-damaging models in recent years. Liao et al[6] observed that Rg1 could protect spinal neurons from excitotoxicity induced by glutamate and kainic acid, as well as oxidative stress induced by H_2O_2 in a dose-dependent way. In a glutamate

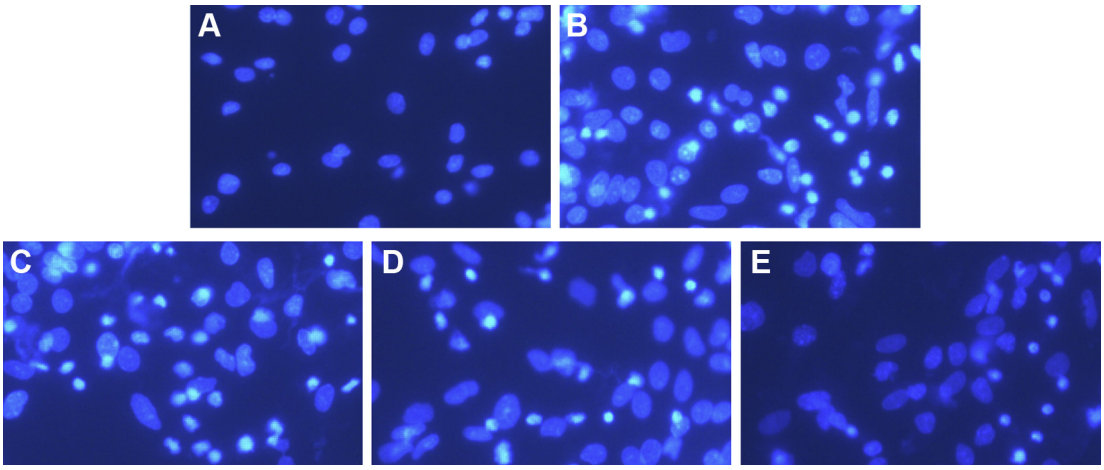


Fig. 6. Effect of ginsenoside Rg1 on oxygen–glucose deprivation (OGD)-induced apoptosis. The apoptosis of hippocampal cells in each group was examined by Hoechst staining. Cells were harvested and fixed in ice-cold 4% phosphate-buffered paraformaldehyde overnight and examined under an inverted phase-contrast fluorescence microscope at 400× magnification by staining with Hoechst 33258 dye. (A) Normal control group. (B) Model group: hippocampal neurons subjected to OGD. (C) Low dose of ginsenoside Rg1: hippocampal neurons subjected to OGD in the presence of 5μM Rg1. (D) Intermediate dose of ginsenoside Rg1: hippocampal neurons subjected to oxygen-glucose deprivation in the presence of 20μM Rg1. (E) High dose of ginsenoside Rg1: hippocampal neurons subjected to OGD in the presence of 60μM Rg1. The figure represents three experiments with similar results. Hoechst 33258 dye was used to stain the DNA of the shrunken nuclei and the chromatin condensation in nuclei in the fluorescence pictures.

excitotoxic model, Liu and Zhang[8] found that Rg1 increased the cell viability and reduced the LDH release of cortical neurons. Li et al[9] reported that Rg1 inhibited the apoptosis of the cultured cortical neurons with serum withdrawal. Leung et al[7] showed that Rg1 exerted a protective effect on nigral neurons against rotenone toxicity. Radad et al[10] documented that ginsenoside Rg1 had a partial neurotrophic and neuroprotective effect in dopaminergic cell culture stressed with glutamate.

The results of our present study are consistent with most of these findings. Physiological Ca^{2+} signals are indispensable for cell function and survival, whereas Ca^{2+} overload or perturbations of intracellular Ca^{2+} compartmentalization can activate or promote mechanisms leading to cell death. Alterations of intracellular Ca^{2+} storage can integrate with death signals to promote processing of cellular components and death by apoptosis or necrosis. Furthermore, Ca^{2+} can directly activate catabolic enzymes such as proteases, phospholipases, and nucleases that directly cause cell demise.[13] In neurons, the Na^{+}/Ca^{2+} exchanger and ATP-driven plasma membrane Ca^{2+} pump are involved in the plasma membrane Ca^{2+} extrusion systems to regulate the intracellular Ca^{2+} content so that Ca^{2+} fluctuations are kept within physiological level.[27] It is generally accepted that calcium overload is involved in ischemic brain injury.[12,13] In pathological conditions, including cerebral ischemia, a massive glutamate release leads to glutamate neurotoxicity. The neurotoxicity is mainly due to activation of NMDA receptors, which cause excessive elevation of $[Ca^{2+}]i$ and subsequent neuronal cell death.[28] Elevation of $[Ca^{2+}]i$ following NMDA receptor activation also stimulates NOS.

NO is generated from the amino acid L-arginine by NOSs. Mammals contain three NOS isoforms: nNOS, inducible NOS, and endothelial NOS.[29] NO is a messenger molecule that has numerous molecular targets. Neuronal NOS is expressed in specific neurons of the brain, the activity of which is regulated by Ca^{2+} and calmodulin. Neuronal NOS has been implicated in modulating physiological functions, such as learning, memory, and neurogenesis. Abnormal NO signaling is very likely to be involved in a variety of neurodegenerative pathologies, including excitotoxicity following stroke.[30] Hyperactive nNOS, stimulated by massive Ca^{2+} influx into neuronal cells, has been implicated in NMDA receptor-mediated neuronal death in cerebrovascular stroke. Under these conditions, NO contributes to excitotoxicity, probably via peroxynitrite activation of poly adenosine diphosphate-ribose polymerase and mitochondrial permeability transition. High levels of NO can also exert energy depletion, owing to inhibition of mitochondrial respiration and glycolysis.[31,32]

In the present study, cultured hippocampal neurons were subjected to OGD to mimic the cerebral ischemia injury. After OGD insult, intracellular free Ca^{2+} concentration of hippocampal neurons markedly increased, probably via activating ionotropic and metabotropic glutamate receptors. We showed that Rg1 inhibits the increase of Ca^{2+} influx in hippocampal neurons subjected to OGD. Recently, Zhang et al[33] reported that inhibition of Rg1 on NMDA receptors and L-type voltage-dependent Ca^{2+} channels may be potentially involved. In addition, nNOS activity resulting from OGD was significantly elevated, which was attributed to stimulation of massive Ca^{2+} influx into hippocampal neurons.[17] The elevated nNOS activity was markedly inhibited by Rg1 treatment. We may hypothesize that it is partially attributable to inhibition of Ca^{2+} influx mediated by NMDA receptors and L-type voltage-dependent Ca^{2+} channels. However, whether other mechanisms are attributable to this phenomenon remain to be elucidated. Hence, ginsenoside Rg1 attenuates OGD-induced neuronal cell death by inhibition of both $[Ca^{2+}]i$ increase and Ca^{2+}-dependent nNOS activity. It is suggested that the neuroprotective effect of Rg1 is probably mediated by the

inhibition of Ca^{2+} influx to maintain the Ca^{2+} homeostasis and keep an appropriate nNOS activity. Given that the overall pharmacology of ginsenosides is complex, it is likely that other factors in addition to Ca^{2+} and nNOS activity stabilizing effects may also contribute to the actions of ginsenoside Rg1. Therefore, more studies on pharmacological mechanisms of neuroprotective actions and toxicity of ginsenoside Rg1 are required.

In conclusion, ginsenoside Rg1 exerted a neuroprotective effect on OGD hippocampal neurons, which was probably mediated by the inhibition of Ca^{2+} influx and nNOS activity, suggesting that, at least in part, the protective actions resulted from inhibition of Ca^{2+} signaling. As a result, neuronal viability was elevated and apoptotic neurons diminished in number. However, the molecular mechanisms that underlie the protective actions of ginsenosides are not fully understood. Even so, ginsenoside Rg1 may serve as a potential therapeutic agent for cerebral ischemia injury, and further *in vivo* studies on stroke should be encouraged to confirm the potential neuroprotective effects of Rg1.

Acknowledgments

This study was partly supported by grants from the Open Project Program of State Key Laboratory of Natural Medicines (China Pharmaceutical University, No. SKLNMKF201311), the National Natural Science Foundation of China (Nos. 30901578 and 81171853), the Jiangsu Provincial Personnel Department "the Great Six Talented man Peak" Project (No. 2011-WS-031), Qing Lan Project 2010 of the Jiangsu Province Department of Education (No. JX2161015003), the Medical Key Talented Person Foundation of the Jiangsu Provincial Developing Health Project (No. RC2011072), the Nature and Science project in Suzhou (No. SYSD2010136), the Natural Science Foundation of Jiangsu Province (No. BK2011850), the Open Foundation of the Health Department of Jiangsu Province (No. XK20200905), and was a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

1. Minnerup J, Sutherland BA, Buchan AM, Kleinschnitz C. Neuroprotection for stroke: current status and future perspectives. *Int J Mol Sci* 2012;**13**:11753−72.
2. Qi LW, Wang CZ, Yuan CS. Isolation and analysis of ginseng: advances and challenges. *Nat Prod Rep* 2011;**28**:467−95.
3. Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. *Biochem Pharmacol* 1999;**58**:1685−93.
4. Qi LW, Wang CZ, Yuan CS. Ginsenosides from American ginseng: chemical and pharmacological diversity. *Phytochemistry* 2011;**72**: 689−99.
5. Liu ZJ, Zhao M, Zhang Y, Xue JF, Chen NH. Ginsenoside Rg1 promotes glutamate release via a calcium/calmodulin-dependent protein kinase II-dependent signaling pathway. *Brain Res* 2010;**1333**:1−8.
6. Liao B, Newmark H, Zhou R. Neuroprotective effects of ginseng total saponin and ginsenosides Rb1 and Rg1 on spinal cord neurons *in vitro*. *Exp Neurol* 2002;**173**:224−34.
7. Leung KW, Yung KK, Mak NK, Chan YS, Fan TP, Wong RN. Neuroprotective effects of ginsenoside-Rg1 in primary nigral neurons against rotenone toxicity. *Neuropharmacology* 2007;**52**:827−35.
8. Liu M, Zhang JT. Protective effects of ginsenoside Rb1 and Rg1 on cultured hippocampal neurons. *Acta Pharm Sin* 1999;**30**:674−8.
9. Li JQ, Zhang XG, Zhang JT. Study on the anti-apoptotic mechanism of ginsenoside Rg1 in cultured cortical neurons. *Acta Pharm Sin* 1997;**32**:406−10 [Article in Chinese].
10. Radad K, Gille G, Moldzio R, Saito H, Rausch WD. Ginsenosides Rb1 and Rg1 effects on mesencephalic dopaminergic cells stressed with glutamate. *Brain Res* 2004;**1021**:41−53.
11. Chen XC, Fang F, Zhu YG, Chen LM, Zhou YC, Chen Y. Protective effect of ginsenoside Rg1 on MPP+-induced apoptosis in SHSY5Y cells. *J Neural Transm* 2003;**110**:835−45.
12. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003;**4**:552−65.
13. Berliocchi L, Bano D, Nicotera P. Ca^{2+} signals and death programmes in neurons. *Philos Trans R Soc Lond B Biol Sci* 2005;**360**:2255−8.
14. Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci U S A* 1991;**88**:6368−71.
15. Buisson A, Plotkine M, Boulu RG. The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia. *Br J Pharmacol* 1992;**106**:766−7.
16. Yun HY, Dawson VL, Dawson TM. Glutamate-stimulated calcium activation of Ras/Erk pathway mediated by nitric oxide. *Diabetes Res Clin Pract* 1999;**45**:113−5.
17. Eliasson MJ, Huang Z, Ferrante RJ, Sasamata M, Molliver ME, Snyder SH, et al. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J Neurosci* 1999;**19**:5910−8.
18. Kira M, Tanaka J, Sobue K. Caldesmon and low Mr isoform of tropomyosin are localized in neuronal growth cones. *J Neurosci Res* 1995;**40**:294−305.
19. Schäbitz WR, Kollmar R, Schwainger M, Juettler E, Bardutzky J, Schölzke MN, et al. Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* 2003;**34**:745−51.
20. Tachikawa E, Kudo K, Kashimoto T, Takahashi E. Ginseng saponins reduce acetylcholine-evoked Na^{+} influx and catecholamine secretion in bovine adrenal chromaffin cells. *J Pharmacol Exp Ther* 1995;**273**:629−36.
21. Radad K, Gille G, Liu L, Rausch WD. Use of ginseng in medicine with emphasis on neurodegenerative disorders. *J Pharmacol Sci* 2006;**100**:175−86.
22. Rausch WD, Liu S, Gille G, Radad K. Neuroprotective effects of ginsenosides. *Acta Neurobiol Exp* 2006;**66**:369−75.
23. Chu GX, Chen X. Anti-lipid peroxidation and protection of ginsenosides against cerebral ischemia-reperfusion injuries in rats. *Zhongguo Yao Li Xue Bao* 1990;**11**:119−23.
24. Salim KN, McEwen BS, Chao HM. Ginsenoside Rb1 regulates ChAT, NGF and trkA mRNA expression in the rat brain. *Mol Brain Res* 1997;**47**:177−82.
25. Kim YC, Kim SR, Markelonis GJ, Oh TH. Ginsenosides Rb1 and Rg3 protect cultured rat cortical cells from glutamate-induced neurodegeneration. *J Neurosci Res* 1998;**53**:426−32.
26. Wang J, Xu HM, Yang HD, Du XX, Jiang H, Xie JX. Rg1 reduces nigral iron levels of MPTP-treated C57BL6 mice by regulating certain iron transport proteins. *Neurochem Int* 2009;**54**:43−8.
27. Carafoli E, Santella L, Branca D, Brini M. Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol* 2001;**36**:107−260.
28. Chen Z, Lu T, Yue X, Wei N, Jiang Y, Chen M, et al. Neuroprotective effect of ginsenoside Rb1 on glutamate-induced neurotoxicity: with emphasis on autophagy. *Neurosci Lett* 2010;**482**:264−8.
29. Moncada S, Higgs A, Furchgott R. International union of pharmacology nomenclature in nitric oxide research. *Pharmacol Rev* 1997;**49**:137−42.

30. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, et al. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 1993;**364**:626−32.
31. Brown GC. Nitric oxide and neuronal death. *Nitric Oxide* 2010;**23**: 153−65.
32. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012;**33**:829−37.
33. Zhang YF, Fan XJ, Li X, Peng LL, Wang GH, Ke KF, et al. Ginsenoside Rg1 protects neurons from hypoxic-ischemic injury possibly by inhibiting Ca^{2+} influx through NMDA receptors and L-type voltage-dependent Ca^{2+} channels. *Eur J Pharmacol* 2008;**586**:90−9.