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Contribution of ginsenosides Rg1, Rb1 to the neuroprotective effect of *Panax notoginseng* in mouse organotypic hippocampal slice cultures exposed to oxygen and glucose deprivation

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Abstract:

We previously demonstrated that *Panax notoginseng* (PNG) root extract treatments exerted neuroprotective effects on brain injuries using middle cerebral artery occlusion in mice. The present study aims to investigate the neuroprotective effects of PNG extract and its ginsenosides Rg1 and Rb1 on ischemic neuronal damage caused by oxygen and glucose deprivation (OGD) in mouse organotypic hippocampal slice cultures (OHSCs). Before the experiments, hippocampal slices collected from 7-day-old *Swiss* mice were cultured for 7 days. OGD was triggered in OHSCs for 30, 60, or 90 min with the aim of finding the optimal period of OGD for drug testing. PNG extract (10, 30 µg/ml), ginsenosides Rg1 and Rb1 (5, 25 µM), or MK-801 25 µM, a reference drug, was added to the culture medium 24 h before OGD and these treatments were continued for 24 h after the optimum 60-min period of OGD. After 24 h of OGD exposure, the measurement of propidium iodide uptake was analysed in OHSCs to evaluate neuronal cell damage. The results showed that OGD time-dependently increased PI uptake of the OHSCs. PNG 30 µg/ml treatment reduced the OGD-induced neuronal cell damage in OHSCs. Ginsenosides Rg1 25 µM, Rb1 (5, 25 µM), as well as MK-801 (25 µM) significantly inhibited PI uptake 24 h after OGD exposure. However, ginsenoside Rg1 5 µM did not show any significant effects on the OGD-induced neuronal cell damage. These findings indicated that ginsenosides Rg1 and Rb1 contributed to the neuroprotective effects of PNG against ischemic damage in OHSCs and the neuroprotective effect of ginsenoside Rb1 was stronger than that of ginsenoside Rg1.

Keywords: **ginsenoside *Rb1*, ginsenoside *Rg1*, organotypic hippocampal slice cultures, oxygen and glucose deprivation, *Panax notoginseng*.**

Classification number: **3.3**

Introduction

Stroke, an acute cerebrovascular disease, is the second largest cause of death [1]. This disease remains the leading cause of disabilities globally and has a rapidly increasing rate in developing countries. Because of an arterial occlusion, ischemic stroke is responsible for the majority of strokes. However, there are only a few effective remedies for this disease. Management of the disease focuses on reperfusion therapy using intravenous thrombolysis. In addition, an endovascular thrombectomy is commonly performed for ischemic stroke. While both therapies reduce disabilities, they are time-critical [1]. Therefore, seeking new natural products that have beneficial effects for the prevention/therapy of ischemic stroke without causing adverse reactions are greatly needed.

The roots of *Panax notoginseng* (PNG) are widely used in Vietnamese traditional medicine for the treatment of blood disorders including blood stasis, bleeding, and blood deficiency [2]. It is reported that PNG with an intraperitoneal route of administration displays strong neuroprotective effects in rats against global cerebral ischemia-reperfusion [3]. We previously demonstrated that the ethanolic extract of PNG root (150 mg/kg) with oral administration exerted neuroprotective effects on the brain following ischemic injury in middle cerebral artery

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occlusions (MCAO) of subjected mice by reducing the infarct volume and improving neurofunctional recovery [4]. However, in this article, the question of which components in PNG are responsible for producing these neuroprotective effects is answered.

More than 20 major active components in *P. notoginseng* root are ginsenosides, which are a group of saponins that are identified chemically [5]. Because of their steroidal structure, ginsenosides possess diverse pharmacological activities. They enable interaction with cell membranes, ion channels, as well as extra- or intra-cellular receptors resulting in altering the transcriptional level. Ginsenosides Rb1 and Rg1 (Fig. 1) are the most abundant ginsenosides presenting in PNG roots [5]. Xian-Si Zeng, et al. demonstrated that treatments with ginsenosides Rg1 and Rb1 show roles in protecting the brain from ischemic damage by significantly reducing infarction volume and alleviating neurological deficits caused by cerebral ischemia-reperfusion [6]. Together, these findings raise the possibility that ginsenosides Rg1 and Rb1 may play a role on the neuroprotective effect of *P. notoginseng* root against cerebral ischemia.

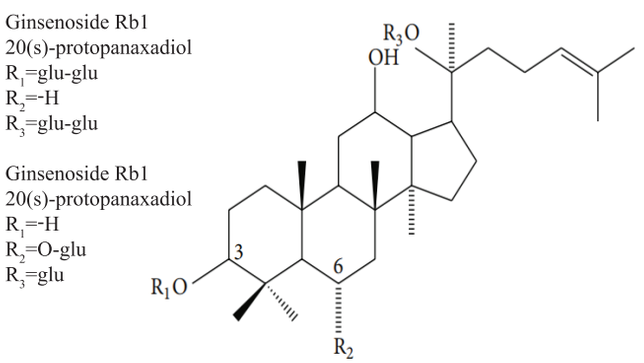

Fig. 1. Chemical structure of ginsenosides Rg1 and Rb1 [7].

Because of the important *in vivo*-like organization of nervous tissues in organotypic hippocampal slice cultures (OHSCs), they are commonly used as a model for screening neuroprotective agents for many neurodegenerative diseases and ischemia [8]. It is well known that neurons need oxygen and glucose to maintain survival function. Although brain tissues constitute only 2% of one's total body weight, their metabolic demands are extremely high. Moreover, the lack of anaerobic metabolism and glycogen storage can decrease the blood flow thus lowering oxygen and glucose levels. During an ischemic stroke, the interruption of blood flow and oxygen-glucose deprivation (OGD) can cause neuronal death [9]. Thus, oxygen-glucose deprivation (OGD) in OHSCs are widely used as an *in vitro* model of ischemia to evaluate the neuroprotective effects of potential drugs.

In ischemia, glutamate excitotoxicity occurs when too much glutamate has been released into the synapse. This excess glutamate overstimulates postsynaptic glutamate receptors, especially N-methyl-d-aspartate (NMDA) receptors. Consequently, calcium overloads and activated cytotoxic enzymes proceed to the injured neurons [10]. An NMDA antagonist can protect many neurons against glutamate neurotoxicity. Hence, in this model, MK-801, a specific NMDA receptor antagonist, was used as the positive control.

This study aimed to investigate the anti-ischemia effects of *Panax notoginseng* root extract and its ginsenosides Rg1 and Rb1 on ischemic neuronal damage caused by oxygen and glucose deprivation in mouse organotypic hippocampal slice cultures.

Materials and methods

Preparation of the Panax notoginseng root extract

The root of *Panax notoginseng* (PNG) was identified and provided by Dr. Pham Thanh Huyen (Department of Medicinal Plant Resources, National Institute of Medicinal Materials, Hanoi, Vietnam). The extraction procedure of *Panax notoginseng root* extract has been described in our previous report [4]. Briefly, the root of PNG was sliced and dried in a hot-air oven at 50°C and ground to a powder. The powder (256.6 g) was extracted with ethanol 70% (1:7 w/v) at reflux for 2 h. The extraction process was repeated 2 times. Then, the extracts were concentrated under reduced pressure at 50°C and dried in a vacuum oven at 50°C to obtain the dry PNG extract (71.9 g). The extract was stored at 4°C until use. Based on high performance liquid chromatography (HPLC) analysis, the PNG extract was estimated to contain 15.86% ginsenoside Rg1 and 12.04% ginsenoside Rb1.

Animals

Seven-day-old *Swiss* mice (National Institute of Hygiene and Epidemiology, Hanoi, Vietnam) were housed in the laboratory animal room (25±1°C, 65±5% humidity, 12 h light/dark cycle). The animals were given food and water *ad libitum*.

Ischemia caused by oxygen and glucose deprivation in mouse organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures (OHSCs) were prepared and cultivated according to the method previously described in [8, 10]. Briefly, the 7-day-old pups were decapitated and the hippocampi were rapidly dissected in ice cold media. The hippocampus was cut transversely (450 µm thick) by using a McIlwain™ Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK) and

transferred onto a membrane filter (Omnipore™ Membrane Filters, JHWP02500; Merck Millipore, Bedford, MA). Organotypic hippocampal slices were laid on an O-shaped plastic doughnut plate in 6-well plates with a culture medium. The culture medium for the hippocampal slices included 50% minimum essential medium, 25% Hank's balanced salt solution, 23% heat-inactivated horse serum, and 2% B-27 (GIBCO, Rockville, MD, USA) supplemented with 6.5 g/l glucose, 50 units/ml penicillin-G, and 50 µg/ml streptomycin sulfate (GIBCO, Rockville, MD, USA). The slices were then kept in an incubator that maintained 37°C and 95% moist air/5% CO_2 for 7 d before the experiments. The culture medium was replaced by a fresh one two times a week.

Oxygen- and glucose-deprivation (OGD) of OHSCs: according to previous studies [8, 10], OGD was used as an *in vitro* model of ischemia-reperfusion injury with a slight modification. The culture medium was deprived of D-glucose and then equilibrated with 95% N_2-5% CO_2 for 90 min (OGD medium). The OHSCs were exposed to the OGD media and were then quickly placed in an airtight chamber with 95% N_2-5% CO_2 gas flow for 60 min, except in a specially stated case. After the exposure of OGD, the slices were returned to conventional media and maintained in the incubator for 24 h before imaging for cell death.

(+)-MK-801 hydrogen maleate (MK-801; Sigma Chemicals, St. Louis, MO, USA) and ginsenosides Rg1 and Rb1 (Chendu Biopurify Phytochemical Ltd., China) were dissolved in dimethyl sulfoxide (DMSO) to form a 10 mM test drug stock solution. The stock solutions were unopened and stored at -20°C until use. The OHSCs were incubated with the culture media supplemented with PNG extract (10 and 30 µg/ml), ginsenosides Rg1 and Rb1 (5 and 25 µM), or MK-801(25 µM) for 24 h before OGD exposure and these treatments were continued for 24 h after a period of OGD.

Evaluation of the neuronal cell damage in OHSCs

The neuronal cell damage in OHSCs was quantified 24 h after exposure to OGD by using propidium iodide (PI) staining as done in previous studies [8, 10]. OHSCs were stained with PI (2.5 µg/ml, Sigma Chemicals, St. Louis, MO, USA) for 30 min. Then, the hippocampal cell damage was evaluated using a microscope (Olympus IX73, Olympus Inc., Tokyo, Japan). Image-J software (ver. 1.41, NIH; Bethesda, MD, USA) was employed to analyse the average intensities of the slices. The value obtained from the control slices exposed to 100 µM N-methyl-D-aspartic acid (NMDA; ACROS Organics™, India) for 24 h was used as 100% PI-fluorescence.

Data analysis

SigmaPlot 12.0 (SYSTA Software Inc, Richmond, CA, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-test were assessed to compare the differences between groups. Statistically significant differences were considered when the p value was less than 0.05.

Results

Determination the optimum period of OGD

With the aim to find the optimal period of OGD, OHSCs were exposed to OGD for 30, 60, and 90 min. The results in Fig. 2 show that exposure of OHSCs to OGD for 30, 60, and 90 min significantly increased the percentage of total PI fluorescence. The measurement of % PI-fluorescence 24 h after OGD demonstrated that OGD caused hippocampal neuronal cell damage in a manner depending on the duration of OGD exposure. The most suitable OGD time is 60 min. Thus, in the following experiments, we applied the 60-min optimum duration of OGD to induce ischemia in OHSCs.

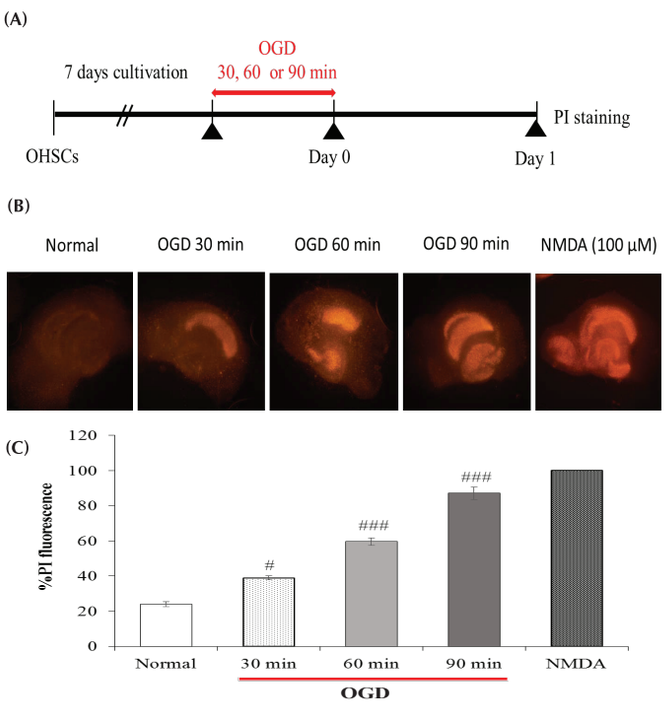

Fig. 2. Oxygen and glucose deprivation (OGD) induced neuronal cell damage in the organotypic hippocampal slice culture (OHSCs). (A) Experimental protocol: scheduling time of OGD. The neuronal cell damage was determined by measuring the PI uptake signal at 24 h after OGD treated. **(B)** Images of hippocampal neuronal damage caused by 30, 60, 90 min OGD treatment. **(C)** % of total PI fluorescence. Data represents Mean±S.E.M. (n=4). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to normal OHSCs.

Preventive effects of PNG against OGD-induced neuronal cell damage in OHSCs

Using OGD induced ischemia in the OHSC model, we evaluated the neuroprotective effect of PNG. The results in Fig. 3 show that the PI uptake signal of the vehicle-treated OHSC markedly increased. The treatment with 10-µg/ml PNG extract insignificantly reduced the percentage of PI fluorescence in the OGD-induced OHSCs. In contrast, 30 µg/ml PNG extract and MK-801 (25 µM) significantly attenuated OGD-induced neuronal cell damage.

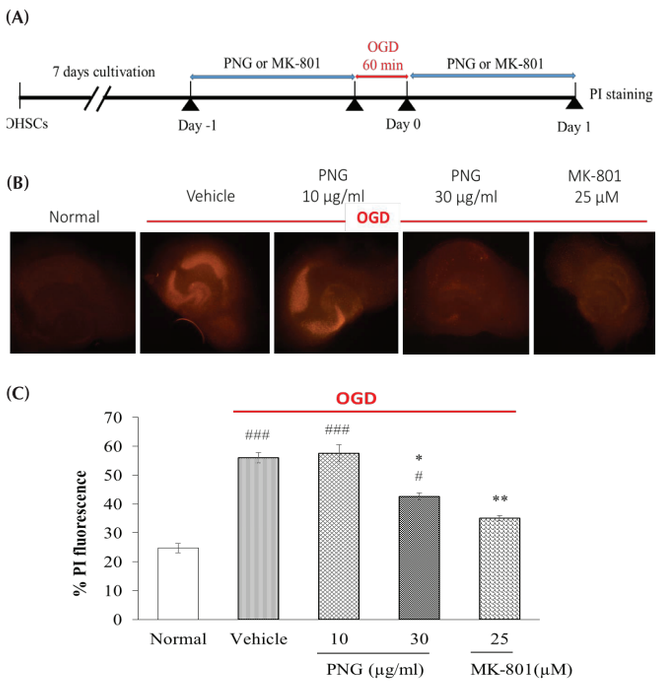

Fig. 3. Effects of PNG extract on OGD-induced neuronal cell damage in OHSCs. (A) Experimental protocol. OHSCs were treated with PNG extract for 24 h before OGD and 24 h after the 60-min period of OGD. Neuronal cell damage was determined by measuring the PI uptake signal at 24 h after OGD treated. **(B)** Images of hippocampal neuronal damage of OHSCs treated with 60-min OGD in the vehicle and presence of PNG extract (10, 30 µg/ml) and MK-801 (25 µM). **(C)** % of total PI fluorescence caused by 60-min OGD. Data represents Mean±S.E.M. (n=4). ###p<0.001 compared to normal OHSCs; *p<0.05, **p<0.01 vs. OHSCs treated with vehicle.

Preventive effects of ginsenosides Rg1 and Rb1 against OGD-induced neuronal cell damage in OHSCs

Ginsenosides Rb1 and Rg1 are the most abundant ginsenosides presenting in PNG root [5]. In this study, the PNG extract containing 15.86% ginsenoside Rg1 and 12.04% ginsenoside Rb1 raised the possibility that ginsenosides Rg1 and Rb1 may play a role on the neuroprotective effect of the PNG extract against OGD-induced ischemic neuronal cell damage. To test this possibility, we next employed OGD as an *in vitro* model of ischemia to evaluate the anti-ischemia effects of ginsenosides Rg1 and Rb1. The percentage of PI fluorescence of the vehicle-treated OGD group significantly increased compared to the signal of the normal control slices (shown in Fig. 4). Treatment with ginsenoside Rg1 (5 µM) insignificantly reduced the PI uptake signal in comparison with the vehicle-treated OGD group. However, the exposure of ginsenosides Rg1 (25 µM) and Rb1 (5, 25 µM) in OGD-treated OHSCs significantly decreased the percentage of total PI fluorescence signal. Treatment of MK-801 (25 µM) significantly attenuated OGD-induced neuronal cell damage. The protective activity observed with the ginsenoside Rb1 treatment was similar to that caused by MK-801.

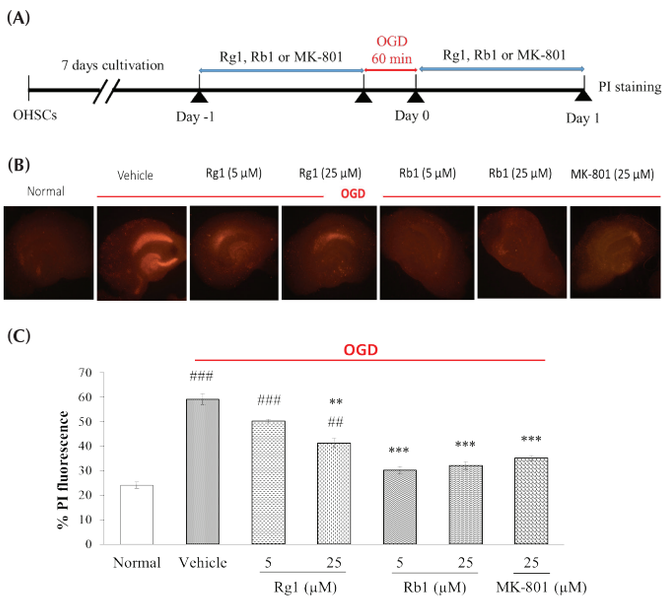

Fig. 4. Effects of ginsenosides Rg1 and Rb1 on OGD-induced neuronal cell damage in OHSCs. (A) Experimental protocol. OHSCs were treated with ginsenosides Rg1 and Rb1 for 24 h before OGD and 24 h after the 60-min period of OGD. Neuronal cell damage was determined by measuring the PI uptake signal at 24 h after OGD treated. **(B)** Images of hippocampal neuronal damage of OHSCs treated with 60-min OGD in the vehicle and presence of ginsenosides Rg1, Rb1 (5, 25 µM) and MK-801 (25 µM). **(C)** % of total PI fluorescence caused by 60-min OGD. Data represents Mean±S.E.M. (n=4). ##p<0.01, ###p<0.001 compared to normal OHSCs; **p<0.01, and ***p<0.001 vs. OHSCs treated with vehicle.

Discussion

In this study, we employed mouse OHSCs to investigate the anti-ischemia effects of *Panax notoginseng* root extract and its ginsenosides Rg1 and Rb1 on neuronal damage

caused by OGD. Our finding strongly suggested that ginsenosides Rg1 and Rb1 play an important role in the neuroprotective action of PNG against ischemic damage and that the neuroprotective effect of ginsenoside Rb1 was stronger than that of ginsenoside Rg1.

The hippocampus is composed of the CA1, CA2, and CA3 pyramidal neurons and dentate gyrus (DG) granule cells. Interestingly, organotypic hippocampal cultures retain a complex three-dimensional organization of nervous tissues that are the same as that of *in vivo* brain tissues. Because of the important *in vivo*-like organization of nervous tissues of OHSCs, it is commonly used as an *in vitro* model for studying neuroprotective agents in neurodegenerative diseases [9]. In OHSCs, OGD is widely used as an *in vitro* model of ischemia. Thus, OHSCs exposed to OGD can provide a surrogate system to evaluate neuronal cell damage following an ischemic injury. In the ischemia models, the hippocampus is one of the most susceptible regions of the brain. Functionally, the excessive activation of glutamate and increase in intracellular calcium play an important role in the acute phase of neuronal damage. Recently, many studies have strongly suggested that apoptosis is considered to have a significant role in delayed neuronal death [9]. In addition, in many cases, it is very difficult to have a large number of substances to test in animal models. Thus, this is a suitable model for screening neuroprotective effects of pharmacological agents. In our study, we applied the 60-min optimum duration of OGD to induce ischemia in OHSCs. The exposure of OHSCs to OGD for 30, 60 and 90 min significantly increased the percentage of total PI fluorescence in a manner depending on the duration of OGD exposure. However, with 30 min OGD, neuronal cell death was not sufficient to evaluate the neuroprotective effects of the tested drugs. In contrast, within 90 min, the percentage of PI fluorescence of vehicle-treated OHSCs was almost equivalent to NMDA-treated OHSCs, which could lead to inaccuracies in the evaluation of the effects of tested drugs. Therefore, 60 min was the most suitable period for OGD for screening neuroprotective effects of pharmacological agents.

To our knowledge, this is the first study to investigate the neuroprotective effects of PNG on OGD-induced neuronal cell damage in OHSCs. The results showed that the treatment with 10-µg/ml PNG extract failed to reduce OGD-induced neuronal cell damage under our experimental conditions. In contrast, 30-µg/ml PNG extract significantly decreased ischemic neuronal cell damage. In our previous study, we demonstrated that 150 mg/kg of PNG root ethanol extract exerted neuroprotective effects on brain injury using middle cerebral artery occlusion for 60 min in mice [4]. Thus, the results of this study confirm the neuroprotective effects of PNG in ischemic-induced neuronal injury. Besides, *in vivo* studies do not answer the question of whether the test drug has a direct effect on the target organ or if it is converted to an active substance. In these *in vitro* experiments using OHSCs, our data suggests that the neuroprotective effects of PNG in ischemic-induced neuronal injury are at least partially involved in the direct effect of PNG on hippocampal neuronal cell under ischemic conditions.

In the present study, we investigated whether treatment of OHSCs with ginsenosides Rg1 and Rb1 was able to prevent neurotoxicity resulting from OGD. Because of their amphipathic property, ginsenosides have the ability to intercalate into the plasma membrane. Consequently, this leads to alterations of membrane fluidity and affects the function of the membrane [11]. Ginsenosides Rb1 and Rg1 are the most abundant ginsenosides present in PNG roots. Based on high performance liquid chromatography analysis, the PNG ethanolic extract was estimated to contain 15.86% ginsenoside Rg1 and 12.04% ginsenoside Rb1. The present study revealed ginsenosides Rg1 (25 µM) and Rb1 (5, 25 µM) reduced OGD-induced neuronal cell damage in the OHSCs. These results demonstrated that ginsenosides Rg1 and Rb1 play an important role on the neuroprotective effects of PNG against ischemic damage in OHSCs. Interestingly, the effect of Rb1 was comparable to that of MK-801, a specific NMDA receptor antagonist. Several recent studies have reported the mechanism of action of Rg1 and Rb1. Rg1 inhibits calcium influx through NMDA receptors and other voltage-dependent ion channels. Additionally, Rg1 modulates the production of intracellular nitric oxide synthase, so it promotes hippocampal neurogenesis. Rb1 helps to regulate homeostasis in the brain by regulating the expression of neuropeptide Y. Moreover, by enhancing the Nrf2/Ho-1 pathway, Rb1 can protect neuronal cells against oxidative injury in ischemia [5]. Besides, the present data highlights that, compared to ginsenoside Rb1, ginsenoside Rg1 had much less protective effect on cerebral ischemic injury. These findings are supported by a previous report that ginsenosides Rg1 and Rb1 exerted neuroprotective effects against ischemic injury with ginsenoside Rb1 having the strongest effect out of all the tested ginsenosides [12]. Ginsenosides are structurally divided into protopanaxadiol-type ginsenosides and protopanaxatriol-type ginsenosides. Ginsenoside Rb1 belongs to the protopanaxadiol type while ginsenoside Rg1 belongs to the protopanaxatriol type ginsenosides. This suggests the important role of the protopanaxatriol-type structure on the neuroprotective action of PNG. The difference between the two groups is

the attached position of sugar moieties. However, to the best of our knowledge, there is no research that explains the relationship between the different structures and the neuroprotective mechanism of the ginsenosides.

Conclusions

The present study demonstrated that ginsenosides Rg1 and Rb1 play an importance role on the neuroprotective action of PNG against ischemic damage and that the neuroprotective effect of ginsenoside Rb1 was stronger than that of ginsenoside Rg1.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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