Neuroprotective effects of ginsenosides on neural progenitor cells against oxidative injury

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Abstract. Ginsenosides exhibit various neuroprotective effects against oxidative stress. However, which ginsenoside provides optimal effects for the treatment of neurological disorders as a potent antioxidant remains to be elucidated. Therefore, the present study investigated and compared the neuroprotective effects of the Rb1, Rd, Rg1 and Re ginsenosides on neural progenitor cells (NPCs) following *tert*-Butylhydroperoxide (*t*-BHP)-induced oxidative injury. Primary rat embryonic cortical NPCs were prepared from E14.5 embryos of Sprague-Dawley rats. The oxidative injury model was established with *t*-BHP. A lactate dehydrogenase assay and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining were used to measure the viability of the NPCs pre-treated with ginsenosides under oxidative stress. Reverse transcription-quantitative polymerase chain reaction analysis was used to determine the activation of intracellular signaling pathways triggered by the pretreatment of ginsenosides. Among the four ginsenosides, only Rb1 attenuated *t*-BHP toxicity in the NPCs, and the nuclear factor (erythroizd-derived 2)-like 2/heme oxygenase-1 pathway was found to be key in the intracellular defense against oxidative stress. The present study demonstrated the anti-oxidative effects of ginsenoside Rb1 on NPCs, and suggested that Rb1 may offer potential as a potent antioxidant for the treatment of neurological disorders.

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Abbreviations: Keap1, kelch-like ECH-associated protein1; ARE, antioxidant response element; CAT, catalase; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H dehydrogenase (quinone 1); SOD2, superoxide dismutase2; PLL, Poly-l-lysine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; *t*-BHP, *tert*-Butylhydroperoxide; CNS, central nervous system; NPCs, neural progenitor cells; BrdU, 5-Bromo-2-deoxyuridine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; GFAP, glial fibrillary acidic protein

Key words: ginsenosides, neural progenitor cells, oxidative injury

Introduction

Neurological disorders affect ~30,000,000 individuals in China, leading to disability and contributing to mortality rates (1). These disorders are characterized by pathological changes in disease-specific areas of the brain, and the degeneration of distinct neural subsets (2). It has been well reported that neurological disorders are linked to elevated levels of oxidative stress, which is involved in modulating the biochemical changes resulting in neurological disorders (3). The supplementation of natural antioxidants is regarded as a prophylactic strategy against diseases caused by oxidative stress (4).

Ginseng, the root of *Panax* species (5,6), is one of the most frequently used herbs in China due to its potential as a general tonic or chemopreventive agent (7,8). The antioxidant action of ginseng is an area of interest in scientific investigations, which provides information for dietary supplementation and the pharmacological usage of ginseng products (9). Ginseng has been shown to have several beneficial effects in a wide range of pathological conditions, including cardiovascular disease, cancer, immunodeficiency and hepatotoxicity *in vivo* and *in vitro*. Of note, ginsenosides are the most biologically active substances found in ginseng (10). There are >30 different types of ginsenosides, which have been isolated from ginseng and classified into three major types: Panaxadiol, including Rb1,

Rb2, Rg3, Rd, Rc, Rg3, Rh2 and Rs1); panaxatriol, including Rg1, Rg2, Re, Rf and Rh1; and oleanolic acid type ginsenosides, including Ro (9). Among these, the most commonly investigated ginsenosides are Rb1, Rd, Rg1 and Re, as these four compounds are relatively more abundant in ginseng and have a wide range of actions in the central nervous system (CNS), including promoting neural survival, extending neurite growth and rescuing neurons from pathological conditions (11).

Several studies have provided evidence that ginsenoside Rb1 possesses potent neuroprotective effects on cortical neurons and dopaminergic neurons against glutamate toxicity, protects against cerebral ischemia by promoting neurogenesis, prevents MPP^+-induced apoptosis in PC12 cells, improves spatial learning, and increases levels of hippocampal synaptophysin in mice (12-16). In the CNS, Rd has been shown to be effective in decreasing the formation of reactive oxygen species (ROS) in cultured astrocytes, protecting PC12 cells from hydrogen peroxide-induced oxidative damage, mitigating neuroinflammation and nitric oxide overproduction, and attenuating neuronal oxidative damage induced by oxygen-glucose deprivation (17). Rg1 has been shown to possess neurotrophic and neuroprotective effects on dopaminergic cells against glutamate injury and MPP^+ toxicity, inhibit the mitochondrial apoptotic pathway and increase the survival of primary cultured nigral neurons against rotenone toxicity (18). It has also been demonstrated that Rg1 exerts neuroprotective effects through ameliorating amyloid pathology, modulating the production of APP and activating the protein kinase A/ cAMP response element binding protein signaling pathways (19). Re has been reported to protect mouse nigral neurons from mitochondrial permeability transition pore-induced apoptosis in a Parkinson's disease model, and this effect was considered to be attributable to upregulation in the protein expression of B cell lymphoma (Bcl)-2, downregulation in the expression levels of Bcl2-associated X protein and inducible nitric oxide synthase, and subsequent inhibition of the activation of caspase-3 (20). These previous reports suggest that the Rb, Rd, Rg1 and Re ginsenosides offer therapeutic potential in the treatment of neurological disorders.

In the present study, the anti-oxidative effects of four ginsenosides (Rb1, Rd, Rg1 and Re) on NPCs were investigated and compared. NPCs can be utilized for functional tissue engineering as a potential treatment for neurologic diseases (21). They are defined by their ability to self-renew through mitotic cell division and differentiate into neurons, astrocytes and oligodendrocytes (22,23). The results of the present study may provide evidence on the optimal ginsenoside for use as a potent antioxidant in the treatment of neurological disorders.

Materials and methods

Chemicals and reagents. Ginsenosides Rb1, Rg1, Rd and Re were provided in powder form (>98% purity) by Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). The powder was dissolved in saline. Dulbecco's modified Eagle's medium (DMEM) nutrient mix F12, goat serum, fetal bovine serum (FBS), 0.05% (w/v) trypsin/EDTA, phosphate-buffered saline (PBS) powder and N2 and B27 supplements were supplied by Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Poly-l-lysine (PLL), laminin, 4',6-diamidino-2-phenylindole (DAPI),bovineserumalbumin(BSA),5-Bromo-2-deoxyuridine (BrdU), *tert*-Butylhydroperoxide (*t*-BHP), paraformaldehyde, mouse anti-BrdU (B8434), rabbit anti-glial fibrillary acidic protein (GFAP; SAB4501162) and mouse anti-β-tubulin III (Tuj-1; T8578) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Lactate Dehydrogenase (LDH) Cytotoxicity Assay kit (cat. no. 11644793001) and In Situ Cell Death Detection kit (cat. no. 11684817910) were obtained from Roche (Basel, Switzerland). The goat anti-mouse 488 antibody, goat anti-rabbit 568 antibody, Click-iT EdU Alexa Fluor® 594 Imaging kit (cat. no. C10339) and the Qubit® RNA BR Assay kit (cat. no. Q10210) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The RNeasy® Mini kit (cat. no. 74134) was purchased from Qiagen (Hilden, Germany), and the primers, PrimeScript™ RT Master Mix (Perfect Real Time) kit (cat. no. RR036A) and SYBR® Premix Ex Taq™ II (Tli RNase H Plus; cat. no. RR820A) were supplied by Takara Biotechnology, Co., Ltd. (Dalian, China). The MicroAmp® Optical 96-well reaction plates with barcode were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). Mouse anti-Nestin (MAB353) was purchased from EMD Milipore (Billerica, MA, USA); epidermal growth factor (EGF) and basic fibroblast factor (bFGF) were purchased from Peprotech (Rocky Hill, NJ, USA); and mouse anti-receptor interacting protein (Rip) was provided by Dr Xiaoming Xu of the University of Louisville (Louisville, USA). All other chemicals and reagents were of analytical grade.

Primary culture of cortical NPCs. A total of eight pregnant female Sprague-Dawley (SD) rats (weight, 300-350 g; age, 3-4 months) were obtained from the Animal Unit at the University of Macau (Macau, China). The rats were maintained in a temperature-controlled room under a 12-h light/dark cycle, with ad libitum access to food and water. The present study was approved by the Committee on the Care and Use of Laboratory Animals at the University of Macau (Macau, China). The primary rat embryonic cortical NPCs were prepared from E14.5 embryos derived from the SD rats using a modified protocol (24,25). Briefly, the cortex was separated from the surrounding tissue following removal of the meninges. The cortex was transferred into a 15 ml centrifuge tube containing culture medium (10 μl/ml N2, 20 μl/ml B27, 20 ng/ml EGF and 20 ng/ml bFGF in DMEM/F12) and dissociated into a single-cell suspension ($5x10^6$ cells/ml) by gentle mechanical trituration through a fire-polished Pasteur pipette. The dissociated cells were filtered through a cell strainer and then cultured in a T25 flask in suspension. The cells were incubated in a humidified incubator at 37˚C in 5% CO_2. Half of the culture medium was replaced every 2-3 days. After 5-6 days, the cells had grown in neurospheres with the diameter of ~150 μm. The cells in the neurospheres were passaged at the ratio of 1:6. These sub-cultured cells were designated as 'first passage' (P1) cells. The third passage (P3) cells were used for all subsequent experiments.

Establishment of the oxidative injury model. *t*-BHP is commonly used as a model substance for evaluating the mechanisms of cellular alterations resulting from oxidative stress in cells and tissues (26). In the present study, the P3 NPCs

were dissociated into single cells, and then seeded into 96-well plates coated with PLL (25 μg/ml) and laminin (13.3 μg/ml) at a density of $1x10^4$ cells per well. The cultures were grown at 37˚C humidified CO_2 incubator for 36 h and then treated with 50, 100, 200 and 300 μM *t*-BHP for 2.5 h at 37˚C. The cytotoxicity of *t*-BHP in the whole cells culture was determined using an LDH cytotoxicity assay. A toxicity rate of ~35-45% induced by *t*-BHP at specific concentrations was considered to be an optimal *t*-BHP-induced oxidative injury model.

Drug treatment. To determine the neuroprotective effects of the four ginsenosides, the cultured NPCs were pre-treated with 0, 0.1, 1, 10 and 100 ìM Rb1, Rg1, Rd or Re, respectively, for 24 h at 37˚C, followed by drug washout with 0.01 M PBS. The cells were then treated with 300 ìM *t*-BHP for another 2.5 h. The cell viability was measured using the LDH assay and further confirmed using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

TUNEL assay. The free 3-OH DNA ends were detected *in situ* using an In Situ Cell Death Detection kit, according to the manufacturer's protocol. Briefly, after washing the cells three times with ice-cold PBS, the cells were fixed by incubation with fixation solution (Sigma-Aldrich) for 1 h, followed by incubation with permeabilization solution (Sigma-Aldrich) for 2 min on ice. The fixed cell samples were incubated in the TUNEL reaction medium for 1 h at 37˚C in the dark. Following completion of the reaction, the cells were washed using PBS, transferred into 2 μg/ml DAPI solution, and mounted on slides. The number of apoptotic nuclei and the total number of nuclei were determined under a fluorescence microscope (Axio Imager A2; Carl Zeiss AG, Oberkochen, Germany).

Lactate dehydrogenase (LDH) release assay. Cell death in the NPCs was quantified by measuring the release of LDH into the medium. As the enzyme is released from cells with damaged membranes, the efflux of LDH is closely associated with the extent of damage or destruction of the NPCs (27). To confirm cortical NPC injury, the activity of LDH in the medium following oxidative injury was determined using the Cytotoxicity Detection kit, according to the manufacturer's protocol. Briefly, the treated cells were lysed for 45 min at 37˚C in PBS supplemented with 1X Triton X-100 (0.1%; Invitrogen; Thermo Fisher Scientific, Inc.), followed by centrifugation at 1,000 x g for 10 min at 37˚C. The sample supernatants were transferred to a 96-well enzymatic assay plate and reacted with the substrate mix from the Cytotoxicity Detection kit in the dark for 30 min at room temperature. The absorbance of the samples was measured at 490 nm, according to the filter of the SpectraMax^R M5 Multi-Mode microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). Each experiment was repeated three times independently.

Immunocytochemical analysis. The P3 NPCs in a single cell suspension were cultured on cover slips, which were coated with PLL/laminin (1:1 ratio), at a density of $1x10^4$ cells/cm^2 in a 24-well plate. For differentiation experiments, growth factors were removed from the culture medium and 1% FBS was added. The cultures were allowed to differentiate for up to 5 days.

The cells on the cover slips were then fixed with freshly prepared 4% paraformaldehyde solution in PBS at room temperature for 20 min. Following several washes with 0.01 M PBS, the cells were processed for immunocytochemistry. The following primary antibodies were used to stain the cells: Monoclonal anti-Nestin antibody (1:500) for NPCs; monoclonal anti-Tuj-1 antibody (1:500) for neurons; polyclonal anti-GFAP antibody (1:1,000) for astrocytes; and monoclonal anti-Rip antibody (1:50) for oliogodendrocytes. The cultures were incubated with the primary antibodies in PBS with 1% BSA, 10% normal goat serum and 0.3% Triton X-100 overnight at 4˚C. The samples were then washed twice with PBS and incubated in secondary antibody conjugated to fluorescent Alexa 568 or 488 (1:500; Thermo Fisher Scientific, Inc.) for 45 min at room temperature. To visualize the nuclei, the cells were mounted in anti-fade solution containing DAPI for 10 min. The fluorescence images were captured using a fluorescence microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR was used to evaluate the mRNA expression levels of the antioxidant gene in response to oxidative injury. The cells were pretreated with 10 μM Rb1 for 24 h, after which total RNA was extracted using the RNeasy® Mini kit. The RNA concentrations were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.) with a Qubit® RNA BR Assay kit. Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT Master Mix kit, according to the manufacturer's protocol. Amplifications were performed in duplicate in 20 μl reaction volumes containing 1X SYBR® Premix Ex Taq™ II (Tli RNase H Plus), 0.2 μM of each primer and 2 μl target DNA, to quantitatively detect the gene expression levels of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heme oxygenase-1 (HO-1), superoxide dismutase 2 (SOD2), NAD(P)H: quinone oxidoreductase 1 (NQO1) and catalase (CAT). The relative expression level of each target gene was normalized to the housekeeping gene, β-actin. All primer sequences used are listed in Table I. Subsequently, qPCR was performed using the 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec, followed by melting curve analysis. Each sample was assessed in triplicate and the $2^{-\Delta\Delta Cq}$ method was used to analyze the relative transcription data (28).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The two-tailed Student's t-test was used to make comparisons between two groups and one-way analysis of variance followed by Tukey's post-hoc test was used to analyze differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of NPCs. In the presence of the EGF and bFGF mitogens, the majority of cells showed bipolar or multipolar morphology with small cell bodies, and were immunoreactive

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Target gene	Direction	Sequence	Association no.
β-actin	Forward	5'-GTCGTACCACTGGCATTCTG-3'	NM_031144
	Reverse	5'-CTCTCAGCTGTGGTGGTGAA-3'	
NRF-2	Forward	5'-GCAACTCCAGAAGGAACAGG-3'	NM-031789.1
	Reverse	5'-CAGTGAGGGGATCGATGAGT-3'	
HO-1	Forward	5'-TGCTCGCATGAACACTCTG-3'	NM_012580.2
	Reverse	5'-TCCTCTGTCAGCAGTGCCT	
SOD2	Forward	5'-GGCCAAGGGAGATGTTACAA-3'	NM_001274771
	Reverse	5'-GCTTGATAGCCTCCAGCAAC-3'	
NQO1	Forward	5'-GCCCGGATATTGTAGCTgAA-3'	NM_017000.3
	Reverse	5'-GTGGTGATGGAAAGCAAGGT-3'	
CAT	Forward	5'-TTATGGCCTCCGAGATCTTTTC-3'	NM_012520
	Reverse	5'-ACCTTGGTCAGGTCAAATGGAT-3'	

NRF-2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1; SOD2, superoxide dismutase 2; NQO1, NAD(P)H: quinone oxidoreductase 1; CAT, catalase.

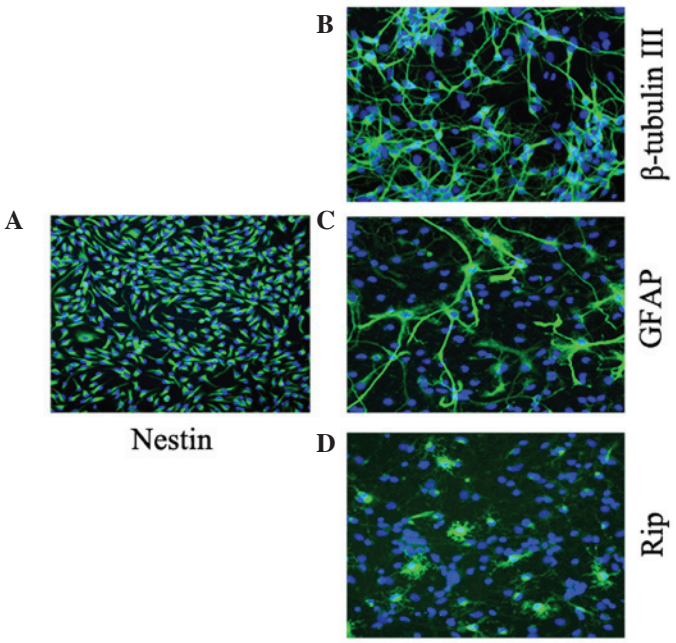

Figure 1. Characterization on NPCs. (A) Image shows P3 NPCs cultured in basic fibroblast factor- and epidermal growth factor-supplemented culture medium. Cells were either bipolar or multipolar with immunoreactivity for Nestin. When cultured in differentiating medium, the NPCs differentiated into (B) β-tubulin III-positive neurons, (C) GFAP-positive astrocytes and (D) Rip-positive oligodendrocytes. Scale bar=150 μm in A; 50 μm in B-D. NPCs, neural progenitor cells; GFAP, glial fibrillary acidic protein; Rip, receptor interacting protein.

for Nestin, an effective marker for NPCs (Fig. 1A), confirming that the cells remained in an immature stage. Following replacement of the mitogens with 1% FBS, the NPCs began to differentiate. At day 5 of culture in the differentiating medium, the NPCs had successfully differentiated into Tuj-1-positive neurons (Fig. 1B), GFAP-positive astrocytes (Fig. 1C) and Rip-positive oligodendrocytes (Fig. 1D).

Neuroprotective effects of the four ginsenosides on t-BHP-induced cytotoxicity in NPCs. The present study used *t*-BHP to establish a model of oxidative injury. As shown in Fig. 2A, *t*-BHP treatment induced cell toxicity in a concentration-dependent manner. The NPCs treated with 50, 100, 200 and 300 μM for 2.5 h exhibited a cytotoxicity rate of 5.43±1.40, 9.07±2.20, 13.13±1.80 and 37.67±2.52%, respectively. As a toxicity rate of 35-45% induced by *t*-BHP was considered to be an optimal oxidative stress model, the oxidative injury induced by 300 μM *t*-BHP for 2.5 h was selected for the subsequent experiments to investigating the anti-oxidative effect of the four ginsenosides.

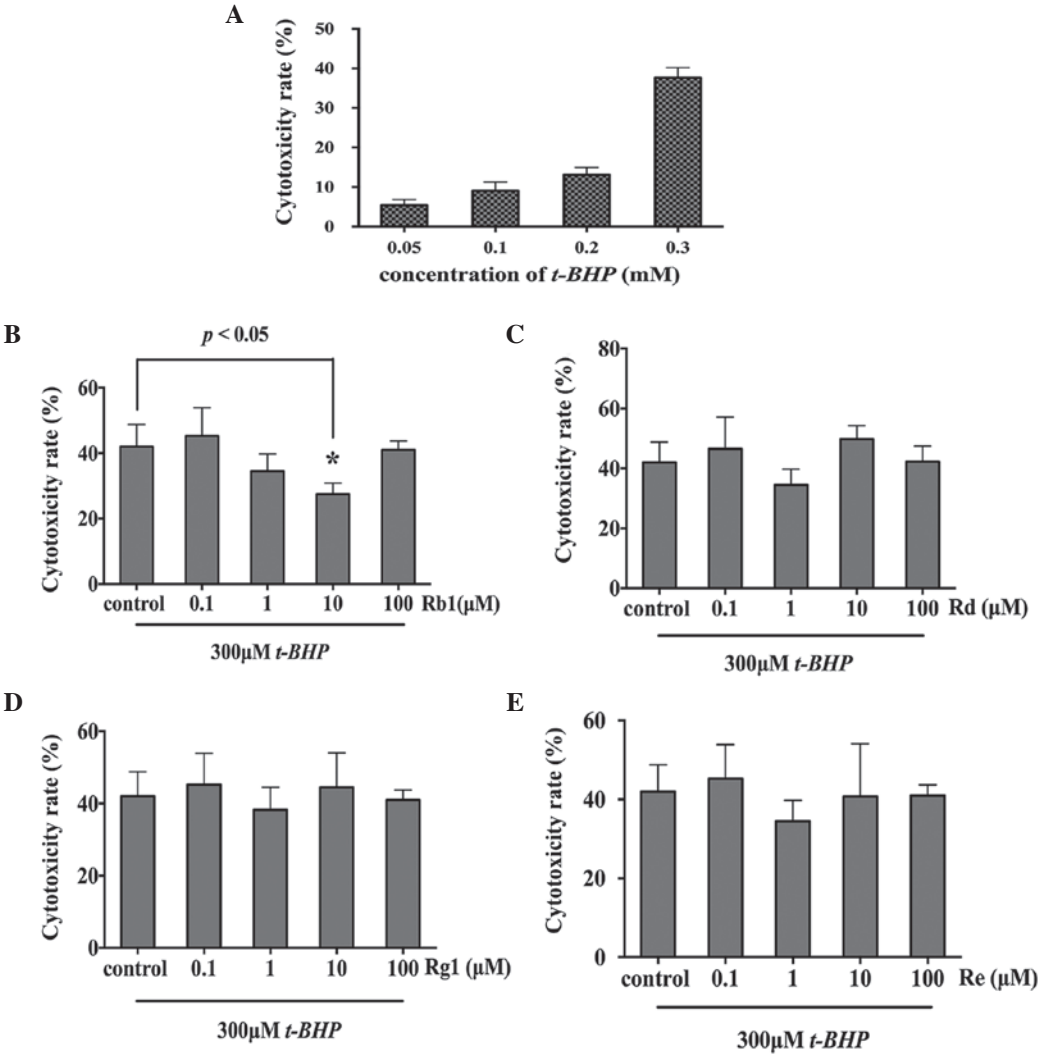

Figure 2. Evaluation of the effects of the Rb1, Rd, Rg1 and Re ginsenosides on *t*-BHP-induced cytotoxicity in NPCs using LDH assay. (A) Establishment of *t*-BHP-induced oxidative injury model. (B) LDH assays revealed that pretreatment with 10 μM Rb1 for 24 h significantly reduced oxidative stress on the NPCs. Results are expressed as the mean ± standard deviation (n=4), *P<0.05, vs. control group. LDH assays revealed that pretreatment with (C) Rd, (D) Rg1 and (E) Re at different concentrations showed no anti-oxidative effects on the NPCs. NPCs, neural progenitor cells; *t*-BHP; *tert*-Butylhydroperoxide; LDH, lactate dehydrogenase.

The NPCs were pretreated with different concentrations of Rb1, Rd, Rg1, Re (0.1, 1, 10 and 100 μM) for 24 h, followed by treatment with 300 μM *t*-BHP for 2.5 h, respectively. Cell viability was measured using an LDH assay and TUNEL staining. The results of the LDH assay suggested that only 10 μM Rb1 showed a protective effect against oxidative stress, with a cytotoxicity rate of 27.5±2.87% in the 10 μM Rb1-pretreated group, compared with 42±5.87% in the control group (P=0.0208; Fig. 2B). Rd, Rg1 and Re had no neurooprotective effects against oxidative injury (Fig. 2C-E). The neuroprotective effects of Rb1 were confirmed by the TUNEL assay, with an apoptotic index of 12.5±2.20% in the 10 μM Rb1-pretreated group, compared with 23±3.02% in the control group (P=0.0003; Fig. 3A-C). The TUNEL staining demonstrated that the remaining three ginsenosides, Rd, Rg1 and Re, exhibited no neuroprotective effects on the NPCs against oxidative injury (Fig. 3D-F).

Rb1 pretreatment activates anti-oxidative genes in cultured NPCs. The present study subsequently investigated the potential mechanism underlying the anti-oxidative effect induced by Rb1. Firstly, the changes in the mRNA expression levels of Nrf2 were measured using RT-qPCR analysis. Nrf2 belongs to the basic-leucine zipper family and coordinately upregulates the constitutive and inducible transcription of a wide array of genes involved in drug metabolism, detoxification and antioxidant defenses (29). The mRNA expression level of Nrf2 was increased 2-fold following pretreatment of the cultured NPCs with 10 μM Rb1 for 24 h. However, pretreatment of the NPCs with the other three ginsenosides did not elevate the expression of Nrf2 (Fig. 4A). The mRNA expression levels of the Nrf2-responsive genes, HO-1, SOD2, NQO1 and CAT were then examined. A 1.5-fold increase in the expression of HO-1 was observed in the 10 μM Rb1-pretreated cells, whereas the other three ginsenosides had no effects on the activation of the downstream HO-1, SOD2, NQO1 or CAT genes (Fig. 4B).

Discussion

Ginseng is reported to have a wide range of therapeutic and pharmacological applications, and has been widely used to

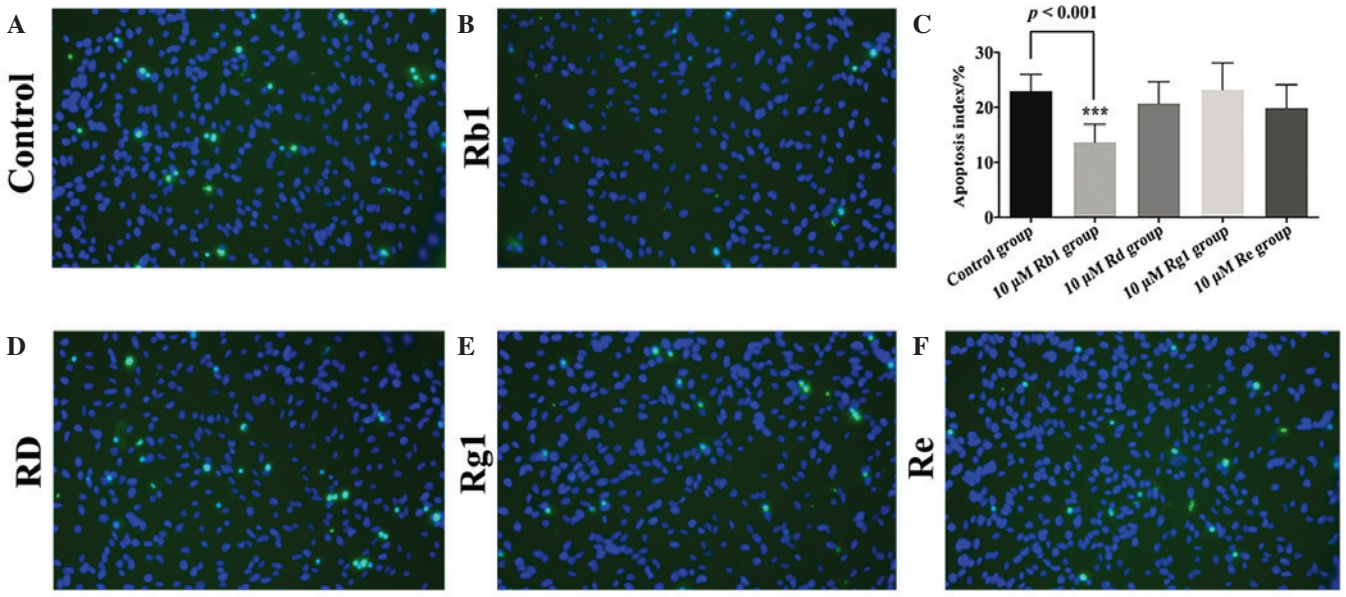

Figure 3. Pretreatment with 10 μM Rb1 significantly reduces the apoptosis of NPCs induced by *t*-BHP-cytotoxicity. Representative photomicrographs of TUNEL staining in the (A) control group, (B) 10 μM Rb1-treated group, (C) 10 μM Rd-treated group, (D) 10 μM Rg1-treated group and (E) 10 μM Re-treated group. (F) Quantitative analyses of the TUNEL staining confirmed that the percentage of TUNEL-positive cells was significantly reduced in the 10 μM Rb1-treated group, compared with the control. Cell apoptosis index values are presented as the mean ± standard deviation from six independent experiments. ***P<0.001. Scale bar=200 μm. NPCs, neural progenitor cells; *t*-BHP; *tert*-Butylhydroperoxide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

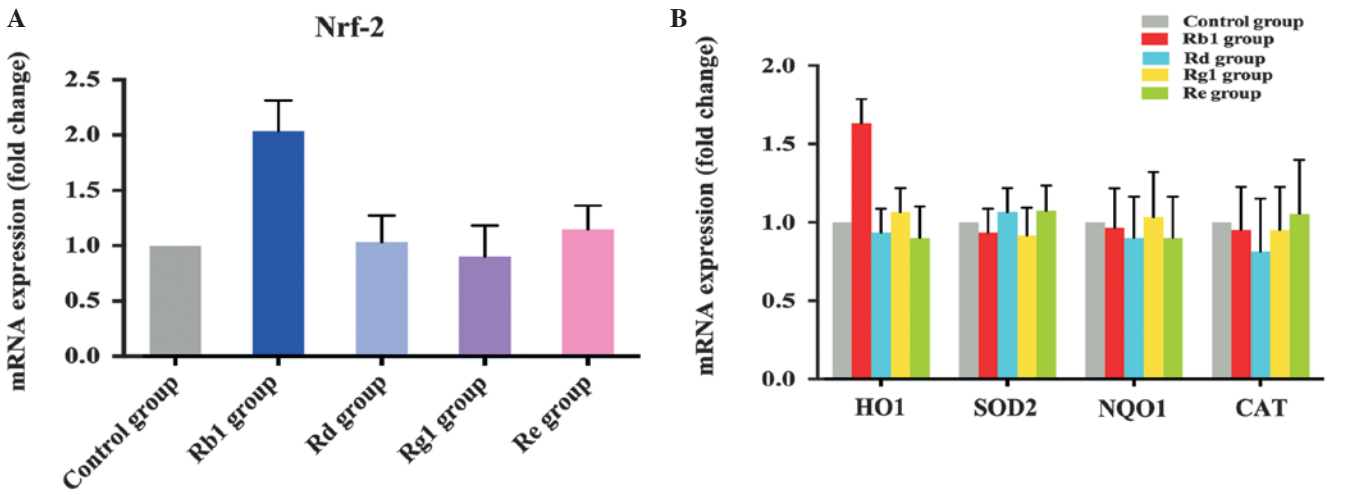

Figure 4. mRNA expression levels of Nrf2, HO-1, SOD2, NQO1 and CAT in NPCs following pretreatment with Rb1, Rd, Rg1 and Re. (A) RT-qPCR analysis demonstrated that the expression of Nrf2 was increased 2-fold in NPCs following pre-treatment with 10 μM Rb1 for 24 h, compared with the control, whereas no changes in the expression of Nrf2 were observed following pre-treatment with 10 μM Rd, 10 μM Rg1 or 10 μM Re. (B) RT-qPCR measurement of levels of HO-1, SOD2, NQO1 and CAT in NPCs following pre-treatment with 10 μM Rb1, 10 μM Rd, 10 μM Rg1 and 10 μM Re for 24 h, followed by incubation with 300 μM *t*-BHP for 2.5 h. Rb1 treatment significantly increased the expression of HO-1, compared with the control group, but did not increase the expression levels of SOD2, NQO1 or CAT. Rd, Rg1 and Re had no effect on the expression levels of HO-1, SOD2, NQO1 or CAT in the NPCs. NPCs, neural progenitor cells; *t*-BHP; *tert*-Butylhydroperoxide; Nrf-2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1; SOD2, superoxide dismutase 2; NQO1, NAD(P)H: quinone oxidoreductase 1; CAT, catalase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

treat various diseases and improve health for thousands of years in Asia (7). Accumulating evidence has indicated that ginsenosides are the principle pharmacologically active ingredients of ginseng. An increasing number of studies are being performed to investigate purified ginsenoside alone to examine the mechanism of function of ginseng, rather than using whole ginseng root (30-35). Each ginsenoside is suggested to have distinct effects in pharmacology and distinct mechanisms due to their unique structures (36). At present, ~40 ginsenoside compounds have been identified, among which Rb1, Rd, Rg1 and Re are the most commonly investigated ginsenosides due to their quantitative abundance in ginseng root (9). The present study investigated and compared the neuroprotective effects of four types of ginsenosides on NPCs against oxidative stress. The results showed that only Rb1 exhibited a protective effect on the NPCs, whereas the Rd, Rg1 and Re ginsenosides

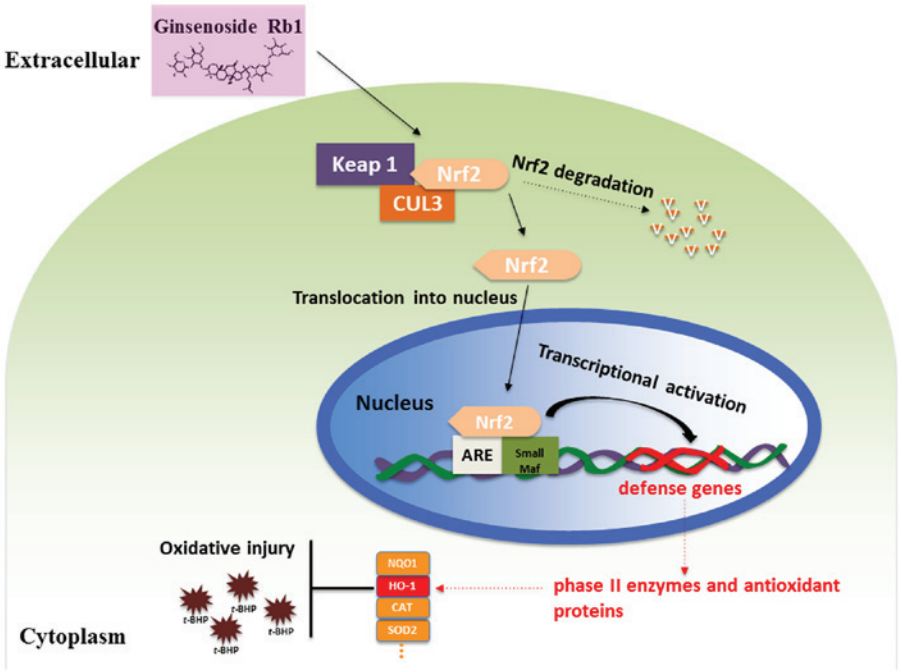

Figure 5. Suggested mechanisms underlying the cellular responses to oxidative injury and protective effects of Rb1 pre-treatment via the Nrf2/HO-1 signaling pathways. Keap1, kelch-like ECH-associated protein1; Maf, musculoaponeurotic fibrosarcoma; ARE, antioxidant response element; CAT, catalase; HO-1, heme oxygenase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H dehydrogenase (quinone 1); SOD2, superoxide dismutase 2; CUL3, cullin 3.

exhibited no protective effects towards NPCs under oxidative stress.

Oxidative stress is defined as the general principle of imbalance between the formation and detoxification of ROS. When not sufficiently scavenged, these small molecules may cause DNA damage, or mutations and lipid peroxidation, leading to membrane damage (37). Substantial evidence has indicated that oxidative stress is a major contributor to the pathophysiology of a variety of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and acute CNS injuries, including spinal cord injury and traumatic brain injury (2,38,39). Ginsenosides have been confirmed to exert protective effects, attributed to their antioxidant ability through increasing internal antioxidant enzymes and acting as a free-radical scavenger (40-42). It has been suggested that the administration of 100 or 200 mg/kg/day of ginsenosides through drinking water improves memory loss in senescence accelerated (SAMP8) mice, and increases serum antioxidant levels (43).

The association between the structure of ginsenoside and its anti-oxidative or pro-oxidative activity has been investigated in free radical-induced hemolysis of human erythrocytes (44,45). The exact mechanisms underlying the differences in protective effects of ginsenosides on NPCs against oxidative stress remain to be elucidated. A number of studies have suggested that Rb1 has beneficial effects in the treatment of oxidative stress (46-51). The present study also demonstrated that pretreatment with Rb1 significantly protected NPCs against oxidative injury, and upregulated Nrf2 and its downstream antioxidant-responsive gene, HO-1. It is generally considered that the activation of the Nrf2 may further upregulate the transcription of multiple antioxidant response element (ARE)-controlled genes, and finally initiate the expression of a variety of antioxidant enzymes and phase II drug-metabolizing enzymes (52,53). HO-1, which belongs to the heat shock protein family, is an inducible enzyme, which catalyzes the first and rate-limiting step in oxidative degradation (54). Evidence has indicated the critical role of HO-1 and its enzymatic by-products in anti-inflammation, anti-oxidation, and more diverse biological functions (55,56).

The mechanism underlying the response of the Nrf2/HO-1 signaling pathway to oxidative stress on NPCs by pretreatment with Rb1 is shown in Fig. 5. Under homeostatic conditions, Nrf2 signaling is repressed by Kelch-like ECH-associated protein 1 (Keap1), which has been identified as a Cullin3-dependent substrate adaptor protein. Nrf2 is found to bind to Keap1 and be sequestered in the cytoplasm, where it is ubiquitinated and subsequently degraded (57). When treated with ginsenoside Rb1, Nrf2 is activated and triggered to translocate into the nucleus, where it elicits a series of anti-oxidative responses. A complex, which consists of Nrf2 protein, a group of small musculoaponeurotic fibrosarcoma proteins and a *cis*-acting enhancer, ARE, is then formed, which is essential for the anti-oxidative response to cell injury induced by *t*-BHP by activating the transcription of the downstream anti-oxidative gene, HO-1 (58,59).

The present study provided an overview on the pharmacological activity of ginsenoside Rb1, Rd, Rg1 and Re, in terms of the neuroprotective effects on NPCs against oxidative injury. Only Rb1 was shown to have protective effects, by activating Nrf2/HO-1 pathway, in an experimental model of oxidative injury, whereas Rd, Rg1 and Re had no protective effects on the NPCs against oxidative injury. Future investigations are warranted to further examine the mechanisms underlying the

protective actions of ginsenoside Rb1 against oxidative injury, and to investigate the therapeutic potential of Rb1 in animal models.

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