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Panax notoginseng saponin attenuates hypoxia/reoxygenation-induced oxidative stress in cortical neurons★

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Abstract
The present study monitored the effect of 2, 10, and 50 mg/L of *Panax notoginseng* saponin exposure following hypoxia-reoxygenation injury in fetal rat cortical neurons. Results showed that varying doses of *Panax notoginseng* saponin significantly enhanced the cell viability of neurons, reduced malondialdehyde content, increased superoxide dismutase activity, inhibited mRNA and protein expression of inducible and neuronal nitric oxide synthase, and decreased the release of nitric oxide in hypoxia/reoxygenation injured cells. In particular, 50 mg/L of *Panax notoginseng* saponin was the most effective dose. These findings suggest that *Panax notoginseng* saponin can attenuate neuronal oxidative stress injury caused by hypoxia/reoxygenation in a dose-dependent manner.

Key Words
Panax notoginseng saponin; neuron; oxidative stress; superoxide dismutase; malondialdehyde; nitric oxide; neuroprotection; nerve injury; neural regeneration

Research Highlights
(1) *Panax notoginseng* saponin can enhance neuron viability following hypoxia/reoxygenation-induced injury.
(2) *Panax notoginseng* saponin can reduce malondialdehyde and nitric oxide production in neurons following hypoxia/reoxygenation-induced injury.
(3) *Panax notoginseng* saponin can increase superoxide dismutase activity in neurons following hypoxia/reoxygenation-induced injury.
(4) *Panax notoginseng* saponin can decrease inducible and neuronal nitric oxide synthase expression in neurons following hypoxia/reoxygenation-induced injury.
(5) *Panax notoginseng* saponin is neuroprotective against hypoxia/reoxygenation-induced injury in cortical neurons.

Abbreviations
SOD, superoxide dismutase; MDA, malondialdehyde; NO, nitric oxide; H/R, hypoxia/reoxygenation

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INTRODUCTION

During the process of aerobic metabolism, oxygen radical overproduction or failure of clearance may cause oxidative stress, resulting in lipid peroxidation and damage to the cell membrane. This event leads to mitochondrial dysfunction, lysosome rupture, cytolysis, and tissue edema, all of which initiates a free radical chain reaction[1-2]. Although the relationship between oxidative stress and brain injury is not completely understood, several studies suggest that oxidative damage may be involved in cerebral disease, diabetes, and nephrosis through aggravation of inflammatory reactions, induction of genetic modifications and participation in signal transduction pathways[3-5]. In addition, the activities of antioxidants such as superoxide dismutase (SOD), glutathione and glutathione peroxidase are significantly reduced, and malondialdehyde (MDA) production has been shown to be markedly increased in the brain following hypoxia-reoxygenation[6-7]. Nitric oxide synthase regulates nitric oxide (NO) production in cells. However, when excessive NO is produced, NO reacts with the superoxide anion (O^{2-}•) and generates the detrimental peroxynitrite anion ($OONO^-$). As a kind of stabile oxide, $OONO^-$ can cause membrane lipid peroxidation, protein injury, and damage DNA chain structures[8], and thus contribute to brain injury. Therefore, it is of no surprise that oxidative stress has been considered a key factor of brain ischemia/reperfusion injury.

Panax notoginseng, known as *Sanqi*, a popular traditional Chinese herb, has been widely used worldwide for the treatment of cerebrovascular and cardiovascular disorders[9]. *Panax notoginseng* saponin, one of the most abundant and bioactive compounds extracted from *Panax notoginseng*, has been shown to have a neuroprotective and anti-inflammatory effect *in vivo* as well as *in vitro* [10-13]. In addition, studies have demonstrated that *Panax notoginseng* saponin has protective properties against ischemia/reperfusion-induced injury in the rat brain[14-15]. Further investigations have revealed that the inhibitory effect of *Panax notoginseng* saponin on cerebral ischemia may involve blood vessel relaxation and inhibition of platelet aggregation[16-19]. Previous reports also indicate that pretreatment with *Panax notoginseng* saponin up-regulates the protein expression of brain-derived neurotrophic factor, nerve growth factor and basic fibroblast growth factor in rat ischemic brain tissue, which protects cortical neurons from cerebral ischemia/reperfusion injury[20-22]. More recent studies suggest that inhibition of the JNK signal transduction pathway, which is involved in mitochondrial-mediated apoptosis, may contribute to the protective effect of *Panax notoginseng* saponin against anti-cerebral ischemia/reperfusion injury[23].

Based on the protective effects of *Panax notoginseng* saponin against cerebral ischemic injury and the key factors involved in cerebral ischemia/reperfusion injury, we hypothesize that *Panax notoginseng* saponin may exert a similar antioxidant effect following hypoxia/reoxygenation (H/R) injury. Therefore, the present study was designed to investigate the effect and mechanism of action of *Panax notoginseng* saponin on H/R injury in rat cortical neurons.

RESULTS

Panax notoginseng saponin enhanced cell viability in H/R-injured cortical neurons

The effect of *Panax Notoginseng* saponin on cell viability of cultured cortical neurons was first evaluated. Rat primary cortical neuron cultures were injured by H/R in the presence or absence of *Panax notoginseng* saponin for a 24-hour period. Results showed that the absorbance value, which represented the viability of cultured cortical neurons, dramatically increased in the presence of *Panax notoginseng* saponin compared with those exposed to H/R alone ($P < 0.01$). Interestingly, the protective effect of *Panax notoginseng* saponin (2, 10, 50 mg/L) was dose-dependent under our experimental conditions as measured by MTT test (Figure 1).

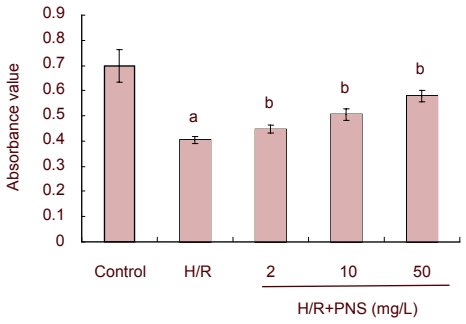

Figure 1 Effect of *Panax notoginseng* saponin (PNS) on cell viability in hypoxia/reoxygenation (H/R) injured cortical neurons.

Cortical neurons were pretreated with PNS (2, 10, 50 mg/L) and stimulated with H/R for 24 hours. Cell viability was measured using the MTT assay.

Data are expressed as mean ± SD, $n = 6$ per condition. $^aP < 0.01$, *vs*. control group (normal neurons); $^bP < 0.01$, *vs*. H/R-treated cortical neurons (one-way analysis of variance).

Effect of *Panax notoginseng* saponin on SOD, MDA, and NO levels following H/R-injured cortical neurons

The potential antioxidant activity of *Panax Notoginseng* saponin was tested by evaluating the production of oxidation mediators (SOD, MDA, NO) from cultured cortical neurons. Cultures of rat primary cortical neurons were stimulated with H/R in the presence or absence of *Panax Notoginseng* saponin for 24 hours. H/R-stimulated cortical neurons showed a remarkable increase in NO and MDA levels, and a reduction in SOD levels in the cell-conditioned media ($P < 0.01$). Pretreatment of cortical neurons with *Panax Notoginseng* saponin (2, 10, 50 mg/L) significantly reduced H/R-induced NO and MDA production, and increased SOD production in a dose-dependent manner (Figure 2).

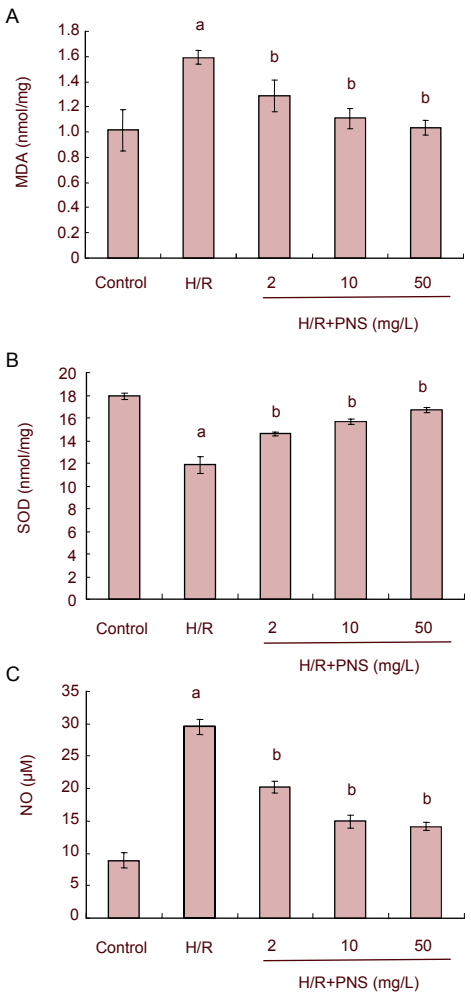

Figure 2 Effect of *Panax notoginseng* saponin (PNS) on malondialdehyde (MDA; A) and superoxide dismutase (SOD; B) release and nitric oxide (NO) production (C) in hypoxia/reoxygenation (H/R)-induced cortical neurons.

Neurons were pretreated with PNS (2, 10, 50 mg/L) and stimulated with H/R for 24 hours. Data are expressed as mean ± SD, $n = 6$ per condition. $^aP < 0.01$, *vs*. control group (normal neurons); $^bP < 0.01$, *vs*. H/R-treated cortical neurons (one-way analysis of variance).

Panax notoginseng saponin remarkably inhibited inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) mRNA expression in H/R-injured cortical neurons

iNOS and nNOS expression at the transcriptional level was detected by real-time reverse transcription-PCR analysis. As expected, H/R markedly increased mRNA expression of iNOS and nNOS in rat primary cortical neurons ($P < 0.01$). *Panax notoginseng* saponin remarkably inhibited iNOS and nNOS mRNA expression in H/R-injured cortical neurons ($P < 0.05$ or $P < 0.01$), with 50 mg/L *Panax notoginseng* saponin having the most significant effect ($P < 0.01$; Figure 3).

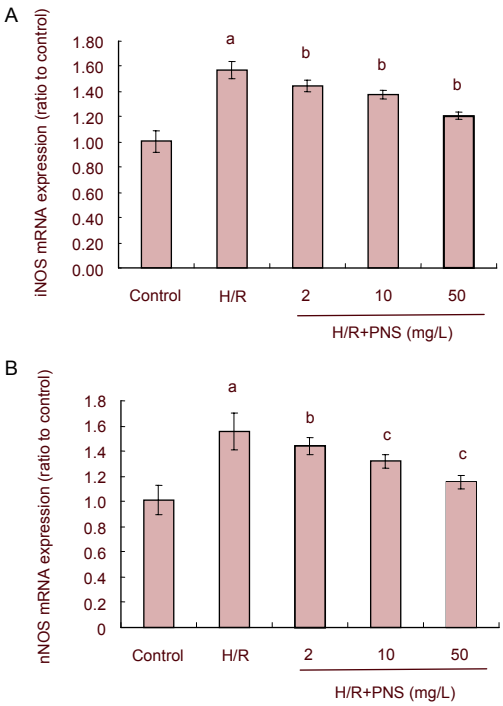

Figure 3 *Panax notoginseng* saponin (PNS) suppresses hypoxia/reoxygenation (H/R)-induced inducible nitric oxide synthase (iNOS; A) and neuronal nitric oxide synthase (nNOS; B) mRNA expression in primary cultures of cortical neurons.

Primary cultured cortical neurons were pretreated with PNS (2, 10, 50 mg/L) and stimulated with H/R for 24 hours. iNOS and nNOS mRNA levels were measured by real-time reverse transcription-PCR.

Data are expressed as mean ± SD, $n = 6$ per condition. $^aP < 0.01$, *vs*. control group (normal neurons); $^bP < 0.05$, $^cP < 0.01$, *vs*. H/R-treated cortical neurons (one-way analysis of variance).

Panax notoginseng saponin significantly suppressed iNOS and nNOS protein expression in H/R-injured cortical neurons

To determine protein levels of iNOS and nNOS in supernatants, the enzyme linked immunosorbent assay

was performed. As expected, H/R markedly increased protein expression of iNOS and nNOS in rat primary cortical neurons ($P < 0.01$; Figure 4). *Panax notoginseng* saponin significantly suppressed iNOS and nNOS protein expression in H/R-injured cortical neurons ($P < 0.01$), particularly the 50 mg/L dose (Figure 4).

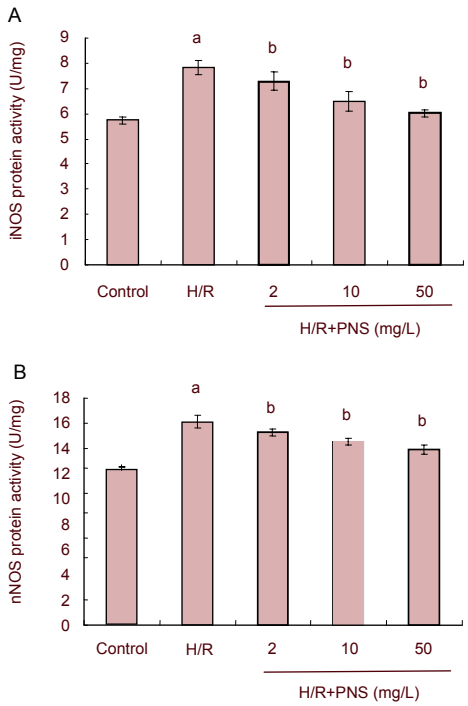

Figure 4 *Panax notoginseng* saponin (PNS) suppresses hypoxia/reoxygenation (H/R)-induced inducible nitric oxide synthase (iNOS; A) and neuronal nitric oxide synthase (nNOS; B) protein expression in primary cultures of cortical neurons.

Primary cultured cortical neurons were pretreated with PNS (2, 10, 50 mg/L) and stimulated with H/R for 24 hours. iNOS and nNOS protein expression was measured using the enzyme linked immunosorbent assay.

Data are expressed as mean ± SD, $n = 6$ per condition. $^{a}P < 0.01$, *vs*. control group (normal neurons); $^{b}P < 0.01$, *vs*. H/R-treated cortical neurons (one-way analysis of variance).

DISCUSSION

During oxidative stress, NO increases and produces peroxynitrite ($ONOO^-$) when combined with the superoxide anion, which leads to aggravated oxidative damage[24]. SOD activity and MDA content directly reflects the ability of agents to scavenge free oxygen radicals in cells[25]. Results from the present study showed that SOD levels were significantly decreased, but that both NO and MDA levels significantly increased in H/R injured cortical neurons, indicating that oxidative damage was induced in cortical neurons.

In the present study, *Panax notoginseng* saponin reduced the production of NO and MDA, but increased the production of SOD following H/R treatment in primary cultured cortical neurons in a dose-dependent manner. Pretreatment with *Panax notoginseng* saponin also suppressed H/R-induced iNOS and nNOS mRNA expression. It was also observed that *Panax notoginseng* saponin could afford neuroprotection through inhibition of iNOS and nNOS protein expression.

Panax notoginseng saponin has been regarded as a common bioactive constituent[26-28]. Previous reports demonstrated that *Panax notoginseng* saponin has a protective effect against cerebral ischemia/reperfusion injury. Oxidative stress is a key contributor to cerebral ischemia/reperfusion injury. Over several decades the question of whether *Panax notoginseng* saponin acts as an antioxidant during cerebral ischemia/reperfusion injury has been widely debated. Results from the present study confirmed the pharmacological activity of *Panax notoginseng* saponin in H/R-stimulated cortical neurons. In addition, we found that *Panax notoginseng* saponin inhibited mRNA and protein expression of iNOS and nNOS, which reduced NO content. Activation of nuclear factor-κB has been reported to be important for iNOS gene expression[29]. Nicotinamide adenine dinucleotide phosphate oxidase, a main source of oxidative stress, can generate O^{2-} and $ONOO^-$, which turns into NO when combined together. However, it remains unclear whether *Panax notoginseng* saponin can inhibit nicotinamide adenine dinucleotide phosphate oxidase and decrease the production of NO and superoxide anions through activating nuclear factor-κB. Therefore, the mechanism of action of *Panax notoginseng* saponin requires further investigation.

MATERIALS AND METHODS

Design

An *in vitro* cell culture experiment.

Time and setting

The experiment was performed in the Key Laboratory for Prescription of National Educational Ministry of China and Research Center of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, China from March 2008 to March 2009.

Materials

Animals

Pregnant (16 days) female Wistar rats were provided by

Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China (Animal permit No. SCXK (Jing) 2006-0009). Use of animals was approved by the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China[30].

Drugs

Panax notoginseng was purchased from Hangzhou Chinese Herbal Pieces Factory, China. Its purity was 82.50%. All herbal materials were identified by Professor Liurong Chen, College of Pharmaceutical Sciences Zhejiang University, China, and were consistent with the regulations of the Chinese Pharmacopoeia.

To obtain *Panax notoginseng* saponin extracts, 500 g crude material of *Panax notoginseng* (*Sanqi*) were extracted and purified according to the following procedures. Crushed powder was added into 4 L of 70% (v/v) ethanol, and extracted twice for 2 hours with refluxing. Extracts were evaporated by a rotary evaporator. Next, *Panax notoginseng* saponin was purified on D101 resins and washed with 0.1% (v/v) ammonia, water, and 70% (v/v) ethanol. The fraction washed by ethanol was collected and evaporated. The residue was dried in a freezing dryer.

Methods

Isolation and primary culture of cortical neurons

Primary rat cortical neurons were isolated from the embryonic cortex of female Wistar rats pregnant for 16 days. The tissue was trypsinized (0.25% (w/v)) and the reaction was then stopped by addition of Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, Utah, USA). Cell suspensions were prepared with DMEM/F12 containing 10% (v/v) FBS after being centrifuged for 10 minutes at 800 r/min. Neuron density was adjusted to 1×10^5/mL and the cell suspension was plated into 96-well or 6-well plates coated with 0.01% (w/v) polylysine (Sigma, St. Louis, MO, USA). Cells were maintained in DMEM/F12 medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 U/L streptomycin, 2% (v/v) B-27 and 5% CO_2 at 37°C. After 4 hours, the medium was replaced with serum free DMEM/F12 (100 U/mL penicillin, 100 U/L streptomycin and 2% (v/v) B-27), and half of the medium was replaced every two days. Cells grown for 4 days were used in the experiment.

Drug intervention

Control group: neurons were cultured in FBS-free medium for 48 hours.

H/R group: neurons were cultured in FBS-free medium, and perfused with 94% N_2, 5% CO_2 and 1% O_2 for 24 hours at 37°C using a modulator chamber. At the end of hypoxic exposure, neurons were removed from the chamber and maintained in a regular incubator (containing 95% air and 5% CO_2 at 37°C) for 24 hours for reoxygenation[31-33].

Panax notoginseng saponin groups: neurons were cultured in FBS-free medium supplemented with different doses (2, 10, 50 mg/L) of *Panax notoginseng* saponin [34]. The following procedures were the same as in the H/R group.

MTT assay for cortical neuron viability

After H/R and *Panax notoginseng* saponin treatment, cortical neurons in the logarithmic phase of growth were harvested and their density was adjusted to 1×10^4/mL. The neurons were cultured in 5% CO_2 at 37°C with MTT (0.5 mg/mL; 100 μL per well; Sigma) for 4 hours. The supernatant was discarded when ianthinus crystals formed. Each well was then added with dimethyl sulphoxide (100 μL per well) and shaken for 1 minute to dissolve the crystals. The absorbance was measured at 570 nm using a microplate reader (Multiskan Ascent, THERMO LABSYSTEMS, Barrington, IL, USA). Afterwards, the mean absorbance of six wells was calculated. The procedure was repeated three times.

Detection of MDA and NO content, and SOD activity

The media of cells treated with H/R and *Panax notoginseng* saponin was collected to measure MDA and NO content, and SOD activity according to the manufacturer's specifications (MDA, SOD, NO biochemical test kit; batch No.20080728, 20080728, 20090318, Nanjing Jiancheng Institute of Biological Engineering).

Real-time reverse transcription-PCR assay of iNOS and nNOS mRNA expression

Neurons were harvested by centrifugation and washed twice with PBS. Total RNA was extracted from the cells using Trizol reagent according to the manufacturer's instructions (batch No. 0901; Shanghai Shenggong Technology Co., Ltd.). An ultraviolet-visible spectrophotometer was used to detect total RNA content and purity. Quantitative real-time PCR analysis was used to assess the mRNA expression of iNOS and nNOS. Total RNA (2 μg) was used as the template for reverse transcription, and single-stranded cDNA was synthesized according to the instructions printed on the

PrimeScript™ RT-PCR kit (batch No BK7903; Shenzhen Lvtai Treasure Biological Engineering Co., Ltd.). The PCR reaction was performed in a 25-μL mixture including 12.5 μL of 2 × SYBR® Green RT-PCR master mix (Invitrogen, Carlsbad, CA, USA) and 0.2 μM cDNA template (1 μL) . Each gene-specific primer was designed according to information gathered from published literature (Table 1; Shanghai Shenggong Biological Technology Co., Ltd.). Amplification was performed using the following steps: pre-denaturalization at 95°C for 10 seconds, 40 cycles of 95°C for 5 seconds and 60°C for 31 seconds. The level of mRNA expression were normalized to that of the housekeeping gene β-actin. Data were analyzed using ABI®7300 RT-PCR software (Foster City, CA, USA).

Table 1 Primer sequences of iNOS, nNOS and β-actin

Gene	Primer sequence (5' to 3')	Size (bp)
iNOS	Forward: CCT TGT TCA GCT ACG CCT TC Reverse: GGT ATG CCC GAG TTC TTT CA	227
nNOS	Forward: TGG CAG CCC TAA GAC CTA TG Reverse: AGT CCG AAA ATG TCC TCG TG	293
β-actin	Forward: TCA TGA AGT GTG ACG TTG ACA TCC GT Reverse: CCT AGA AGC ATT TGC GGT GCA CGA TG	110

iNOS: Induced inducible nitric oxide synthase; nNOS: neuronal nitric oxide synthase.

Enzyme linked immunosorbent assay for iNOS and nNOS protein expression

The supernatant of each group was centrifuged at 1 000 r/min for 10 minutes and iNOS and nNOS protein expression was detected according to the iNOS, nNOS enzyme linked immunosorbent assay test kit (R&D Co., Ltd., Shanghai; batch No. 20090312). Standard substance (50 μL) and 50 μL of sample from the supernatant of each group was added into the corresponding reaction wells and mixed for 30 seconds. The mixture was incubated in sealed plates at 37°C for 30 minutes, and then the plates were washed five times. Afterwards, 100 μL of concentrated enzyme-linked material was added into each well. The above procedures were repeated before addition of 100 μL of chromogenic solution into each well. The plates were then incubated in the dark for 15 minutes. After adding 50 μL of stop buffer into each well the absorbance at 450 nm was measured using a microplate reader. Protein concentration was calculated using the standard curve equation.

Statistical analysis

Enumeration data were expressed as mean ± SD. One-way analysis of variance was performed using SPSS 11.5 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

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Author contributions: Chen Yan conceived and designed this study, and wrote the draft of the manuscript. Jinqiang Zhu, Xiaoxu Jia, Chao Wang and Shaoxia Wang collected the data and provided technical support. Liyuan Kang supervised the study and guided the work. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: The experiment was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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