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# The Protective Effects of *Panax notoginseng* Saponin on the Blood-Brain Barrier via the Nrf2/ARE Pathway in bEnd3 Cells

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## Summary

**Cerebral microvascular endothelial cells (CMECs) are the main component of the blood-brain barrier (BBB), a critical determinant in the selective diffusion of substances into the brain. The injury of CMECs by oxidative stress contributes to BBB disruption, which is related to neurological diseases. Activation of the Nrf2/ARE pathway is one of the most critical endogenous defense mechanisms against cellular damage caused by oxidative stress. Substances that activate the Nrf2/ARE pathway are therefore potential neuroprotectants. We investigated the potential protective effects of the total saponins in the leaves of *Panax notoginseng* (LPNS) on oxidative-stress-induced damage in bEnd3 cells (a mouse cerebral microvascular endothelial cell line). Lactate dehydrogenase (LDH) assays indicated that LPNS protects against $H_2O_2$-induced cell death in both a concentration- and time-dependent manner. Pretreatment of bEnd3 cells with 10 µg/mL LPNS for 24 h significantly increased expression of the tight junction proteins claudin-1 and claudin-5, activated Nrf2 (shown by nuclear translocation), and up-regulated Nrf2's downstream antioxidant genes including heme oxygenase1 (*HO-1*, *HMOX1* gene) and NAD(P)H:quinone oxidoreductase 1 (*NQO-1*). This study demonstrates for the first time that the protective effects of LPNS on CMECs involve the activation of the Nrf2/ARE pathway and indicate that LPNS may be a potential therapeutic candidate for treating neurological diseases.**

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## Introduction

The blood-brain barrier (BBB) (**Figure 1**) is mainly composed of non-fenestrated cerebral microvascular endothelial cells (CMECs), astrocytes, and pericytes (1). Endothelial cells in the brain are distinct from those in the rest of the body, as they have tight junctions (TJs), which consist of proteins such as claudin-1, claudin-5, and occludin. The BBB maintains the homeostasis of the brain and acts as a selective transportation barrier at the level of CMECs, which shield the brain from harmful substances in the blood (2,3). Thus, the BBB is a critical safeguard that contributes to proper neuronal function and protects the brain from injury. Neurological diseases such as stroke, traumatic brain injuries, and Alzheimer's disease, are often accompanied by BBB dysfunction characterized by reduced CMECs viability and increased BBB permeability in response to oxidative stress (4,5).

Oxidative stress is an imbalance between cellular pro-oxidants and anti-oxidants. $H_2O_2$, a byproduct of oxidation, causes cellular damage at high concentrations when it crosses cell membranes and reaches subcellular compartments (6,7). Inside the cells, $H_2O_2$ can cause mitochondrial calcium overload and mitochondrial membrane depolarization, resulting in increased formation of mitochondrial reactive oxygen species (ROS) (8,9). Thus, an excess of $H_2O_2$ induces imbalance in ROS generation and impairs cellular antioxidant defenses, leading to cell death (10,11). CMECs are particularly susceptible to oxidative stress (12). During oxidative stress, the aggregation of ROS triggers many downstream pathways that directly mediate BBB injury, including cell damage and loss of TJs (13,14).

Under oxidative stress, cells activate endogenous antioxidant systems to neutralize ROS and maintain cellular redox homeostasis. NF-erythroid 2-related

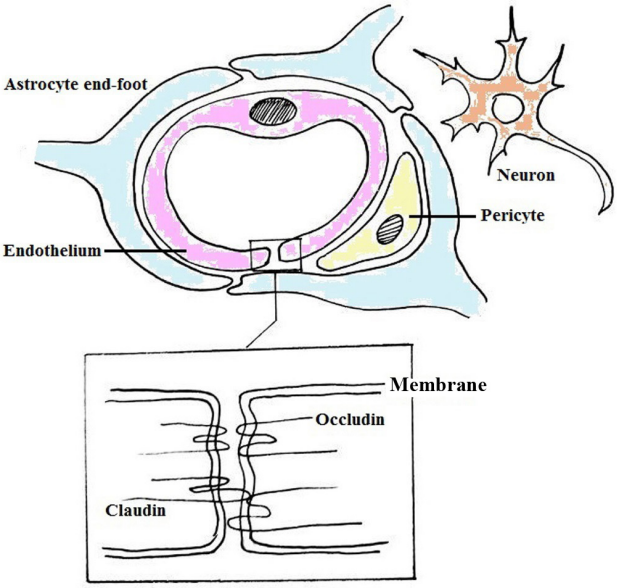


**Figure 1. Structure of the BBB.** The blood-brain barrier (BBB) is mainly composed of cerebral microvascular endothelial cells (CMECs), astrocytes, and pericytes. The magnified section shows the presence of tight junctions between endothelial cells.

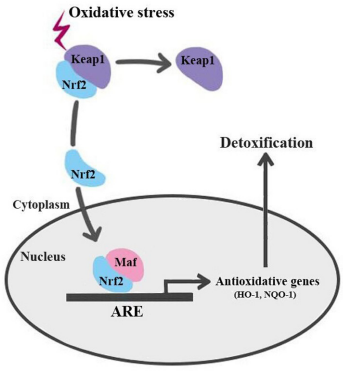


**Figure 2. Nrf2/ARE pathway.** Under oxidative stress, Nrf2 disassociates from Keap1 and translocates to the nucleus, where it combines with small Maf and activates the antioxidative response element (ARE) to induce the transcription of antioxidative genes such as HO-1 and NQO-1.

factor 2 (Nrf2) is one of the most critical proteins of the endogenous antioxidant mechanisms (**Figure 2**). Under normal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) (15). However, during oxidative stress, the cysteine residues of Keap1 become oxidized. Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to antioxidant response elements (ARE). Once activated, AREs induce antioxidant and phase II detoxification enzymes such as heme oxygenase1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1), which have antioxidation, detoxification, and radical scavenging functions (16,17). These observations suggest that oxidative stress is a critical factor of BBB breakdown and implicate substances that activate the Nrf2/ARE pathway as potential neuroprotectants in conditions like stroke and Alzheimer's disease (18).

In recent years, numerous herbal drugs have been reported to have antioxidative effects on brain injury. Unfortunately, most clinical trials did not find these drugs to be effective (19). Nevertheless, CMECs are an important potential site for new therapies (20). *Panax notoginseng* (Burk.) F.H. Chen (21), a traditional Chinese medicine also named Sanqi, has been widely used in clinics for thousands of years as a haemostatic. Recently, the *Panax notoginseng* saponins (PNS), the main active compounds in the root, have been shown to have potential for inhibiting platelet aggregation and plasma coagulation (22); reducing total cholesterol, triglycerides, and low-density lipoprotein-cholesterol in serum(23); protecting myocardium cells (24); and dilating cerebrovascular and anti-cerebral infarction tissue (25). To date, some preparations of PNS, such as Sanqi Panax Notoginseng injection and the Xuesaitong soft capsule, have been approved by State Food and Drug Administration of China. They are currently used in western medicine to treat acute ischemic stroke (26), acute intracerebral hemorrhage (27), and angina pectoris (28).

Even though *P. notoginseng* can protect the brain from neurological diseases both in experiments and

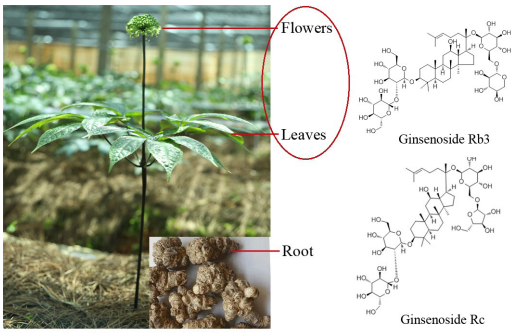


**Figure 3. *Panax notogensing* used in experiment.** Photograph of the *Panax notogensing* plant and roots. The saponins (mainly ginsenoside Rb3 and Rc) used in these experiments were extracted from the aerial parts (primarily the leaves).

at the clinic, its effect on BBB impairment and related mechanisms is not fully understood yet. Our previous work has shown that saponin extracted from the leaves of *P. notoginseng* (LPNS), which contain mainly ginsenosides Rb3 and Rc (**Figure 3**), has antioxidative effects on primary astrocytes, another important component of the BBB (29). It is important to explore the effect and mechanism of LPNS as a potential curative for BBB disruption related to oxidative stress. This study examines the antioxidative effects of LPNS in bEnd3 cells, a mouse cerebral microvascular endothelial cell line. The cell viability, TJ protein expression, and the Nrf2 pathway were tested to determine the protective effects of LPNS on CMECs.

## Results

### Reduction in $H_2O_2$-induced cell death by LPNS

Because BBB disruption in neurological diseases is characterized in part by reduced CMECs viability in response to oxidative stress, we examined whether LPNS protects against $H_2O_2$-induced cell death. We determined the viability of bEnd3 cells following $H_2O_2$-induced cell damage by using a lactate dehydrogenase (LDH) assay. The leakage of LDH from damaged cells can be measured spectrophotometrically in culture media using an enzymatic reaction that results in a red formazan product, thus measuring cellular toxicity and death.

We first treated the cells with LPNS at concentrations of 2.5, 5, or 10 μg/mL or vehicle (DMSO) for 24 h prior to exposing the cells to $H_2O_2$. Cells cultured in normal conditions without $H_2O_2$ exposure were used as a background measurement that was subtracted from the $H_2O_2$-insult groups. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons between groups was used to identify which concentration of LPNS could induce the greatest protective effect. Compared to vehicle control (expressed as one hundred percent), pretreatment with 5 and 10 μg/mL LPNS significantly reduced LDH release to 75 ± 9% ($p < 0.01$) and 68 ± 4% ($p < 0.01$), respectively, in $H_2O_2$-induced bEnd3 cell injury (**Figure 4A**). Compared to the

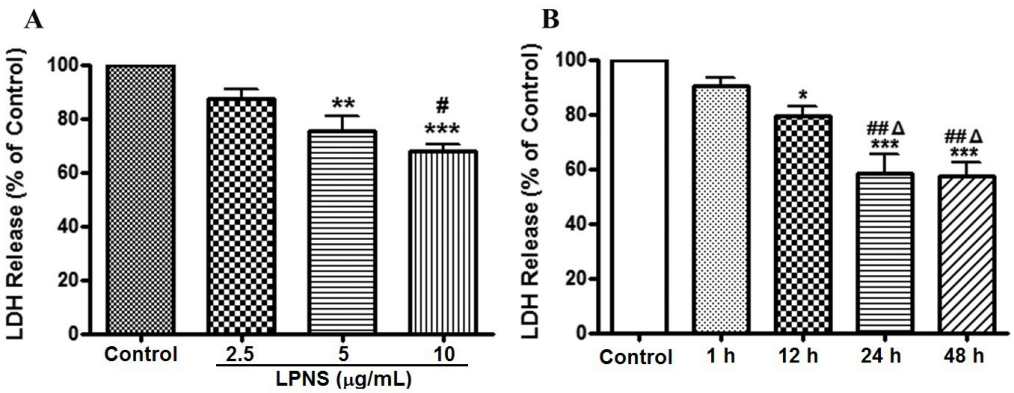


**Figure 4. Effect of LPNS on $H_2O_2$-induced release of lactate dehydrogenase (LDH) from cultured bEnd3 cells.** A: Cells were pre-incubated for 24 h in the presence of different LPNS concentrations (2.5, 5, 10 μg/mL) or vehicle (DMSO) and exposed for 4 h to 0.5 mM $H_2O_2$ in FBS-free medium. The results are expressed as a percent of LDH release from vehicle-treated cells exposed to $H_2O_2$ (control). Values are means ± SEM, n = 4 independent experiments. ANOVA followed by the Tukey's *post-hoc* test. **, *** indicate significant differences relative to controls at the $p < 0.01$ and $p < 0.001$ levels, respectively; # significant differences from 10 μg/mL LPNS compared to 5 μg/mL at the $p < 0.05$ level. B: LDH release from bEnd3 cells pretreated with 10 μg/mL LPNS for different durations (up to 48 h) before exposure to 0.5 mM $H_2O_2$. *, *** indicate significant differences relative to controls at the $p < 0.05$ and $p < 0.001$ levels, respectively; ##, significant differences at $p < 0.01$ for 24 h and 48 h compared to 1 h; Δ, significant differences at $p < 0.05$ for 24 h and 48 h compared to 12 h.

2.5- or 5-μg/mL treatment groups, 10 μg/mL LPNS had the largest protective effect on cells.

We then treated the cells with 10 μg/mL LPNS for 1, 12, 24 and 48 h prior to exposing cells to $H_2O_2$ to identify a time course for protection. Compared to vehicle, the pretreatment of cells with 10 μg/mL LPNS for 12, 24, and 48 h reduced LDH to 79 ± 6%($p < 0.05$), 58 ± 12% ($p < 0.01$), and 57 ± 8%($p < 0.01$), respectively, of the control value (**Figure 4B**). The time course indicated that pretreatment of cells with LPNS for 24 h resulted in the largest protective effect on cells.

CMECs death caused by oxidative stress may contribute to disruption of the BBB. Our results suggest that LPNS can increase endothelial cell viability in response to oxidative stress, which may in turn protect the BBB.

## LPNS upregulates the expression of tight junction proteins

TJs are the primary structure regulating the selective permeability of the BBB. The decreased expression of TJ components, such as claudin and occludin in CMECs, is another hallmark of BBB disruption. To further elucidate the protective effects of LPNS on the BBB, we determined the TJ proteins' expression by immunofluorescence after LPNS treatment. A Student's *t*-test was used to identify the differential expression of TJ proteins between cells receiving LPNS treatment and the vehicle control, and hence indicate whether LPNS contributes to BBB integrity. We treated the cells with 10 μg/mL LPNS for 24 h and detected the expression of TJ proteins with immunofluorescence. Fluorescence intensity was quantified with a fluorometric plate reader. As illustrated in **Figure 5**, bEnd3 cells pretreated with 10 μg/mL LPNS for 24 h significantly increased the expression of the tight junction proteins claudin-1 (2.00 ± 0.17 fold) and claudin-5 (1.86 ± 0.16 fold) ($p < 0.01$), compared to the vehicle control. However, no effects were observed on occludin expression (1.18 ± 0.11 fold change). These results indicate that LPNS can increase TJ protein expression, which may decrease the permeability of endothelial cells and protect the BBB.

## Upregulation of Nrf2 and its downstream antioxidant systems by LPNS

The most important endogenous antioxidative stress system in cells is mediated by the Nrf2/ARE pathway. Under oxidative stress, Nrf2 disassociates from Keap1 and translocates to the nucleus, where it activates downstream response proteins and induces the phase II detoxification enzymes. The stress-responsive HO-1 enzyme prevents cells from dying by catabolizing free heme and reducing programmed cell death. NQO-1 is capable of reducing quinone compounds and preventing the formation of reactive oxygen species, and thus, directly scavenges superoxide anion radicals to protect cells from death. In this study, Nrf2, NQO1, and HO-1 expression were measured by western blot to determine whether LPNS can exhibit antioxidant activity by upregulating the intracellular phase II enzyme. The nuclear Nrf2 levels in bEnd3 cells were 2.14 ± 0.27 fold higher than controls ($p < 0.01$, **Figure 6**) after 1 h treatment with 10 μg/mL LPNS. After pretreatment with 10 μg/mL LPNS for 24 h, the expression of the Nrf2 downstream response proteins NQO-1 and HO-1 increased 2.47 ± 0.40 fold ($p < 0.01$, **Figure 7B**) and 2.39 ± 0.43 fold ($p < 0.01$, **Figure 7C**), respectively, compared to controls. Representative western blots further support the hypothesis that LPNS can activate the Nrf2/ARE pathway to enhance the endogenous antioxidative stress system, hence protecting endothelial cells.

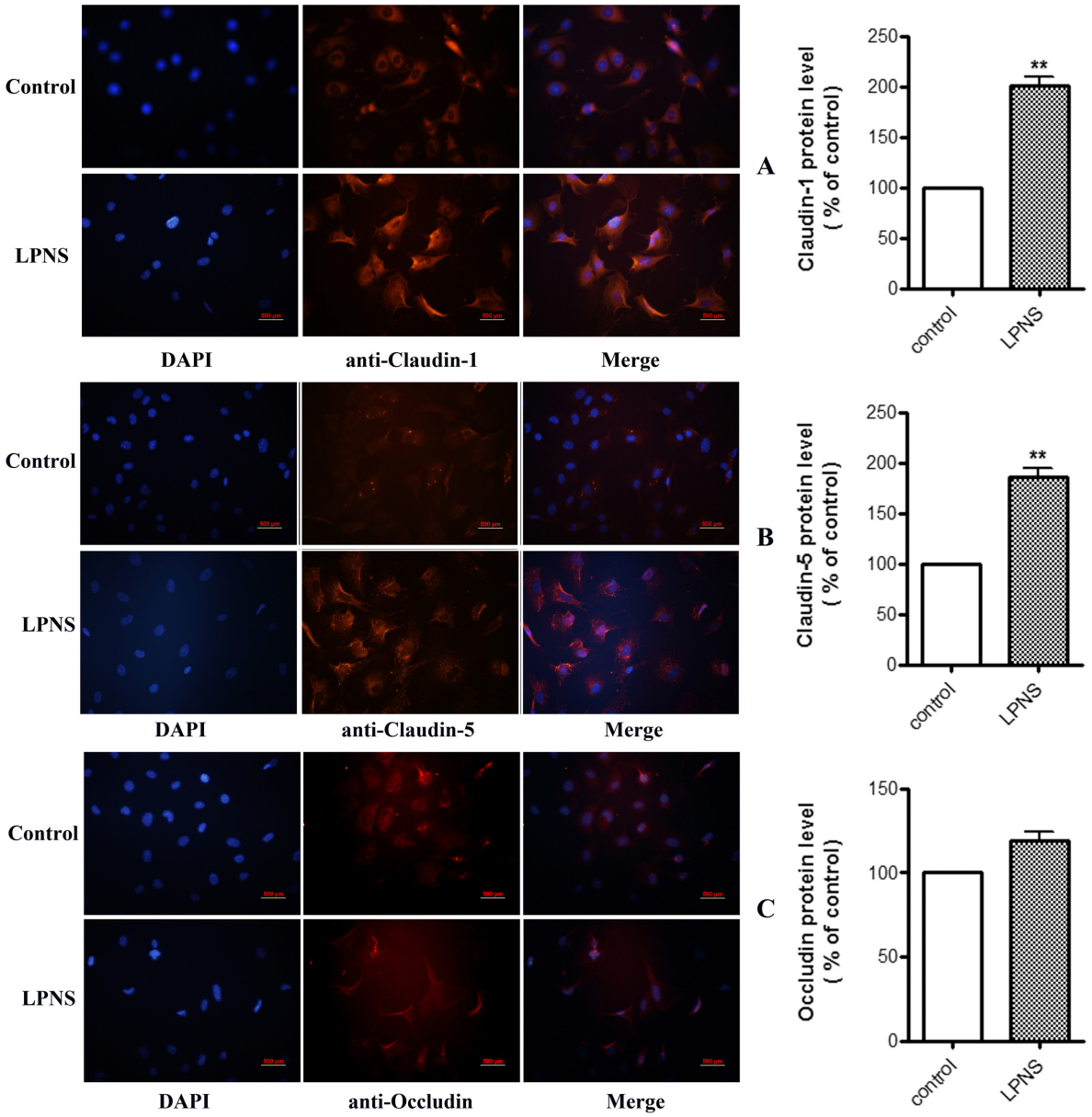


**Figure 5. Effects of LPNS on tight junction protein expression in bEnd3 cells.** Cells grown on 24-well plates were incubated for 24 h with 10 μg/mL LPNS and then were fixed and stained with an anti-claudin-1 antibody (A), anti-claudin-5 antibody (B), or anti-occludin antibody (C). The immunocytochemistry results were quantified by fluorescence density as described in the Methods. Photomicrographs represent results obtained in three experiments performed in duplicate. Scale bar = 500 μm.

## Discussion

This study examined the protective effects and related mechanisms of total saponin found in the leaves of *P. notoginseng* (LPNS) on oxidative stress-induced CMECs damage *in vitro*.

There are many factors inherent to the brain and cerebral endothelial cells that increase the likelihood of oxidative stress causing cell damage. The death of CMECs caused by excessive oxidative products, such as hydrogen peroxide ($H_2O_2$) and oxygen free radicals, has been extensively studied (30,31). Substances that serve as antioxidants can protect CMECs from oxidative stress-induced injury (32,33). In our study, an LDH assay revealed that pretreatment with LPNS significantly increases cell viability in $H_2O_2$-insulted bEnd3 cells, showing that LPNS can protect endothelial cells from the cytotoxicity created by oxidative stress.

Oxidative stress not only leads to CMECs death, but can also increase the permeability of endothelial cells by breaking down TJs (34). TJ proteins, such as claudin and occludin, exist between the cerebral endothelial cells and act as a complex cellular gate that maintains

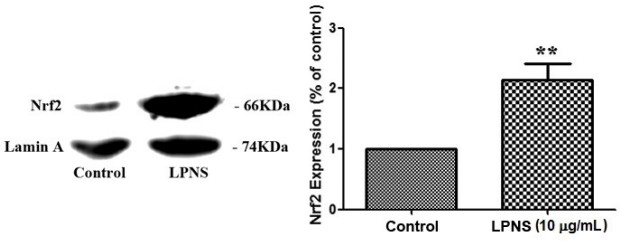


**Figure 6. Effects of LPNS on nuclear Nrf2 levels in bEnd3 cells.** A: Representative Western blot of nuclear Nrf2 in bEnd3 cells after 1 h treatment with vehicle or 10 μg/mL LPNS. B: Quantification (fold changes) of the Nrf2 Western blots. Values are means ± SEM, n = 3. **, indicate significant difference from vehicle-treated control at the $p < 0.01$ level.

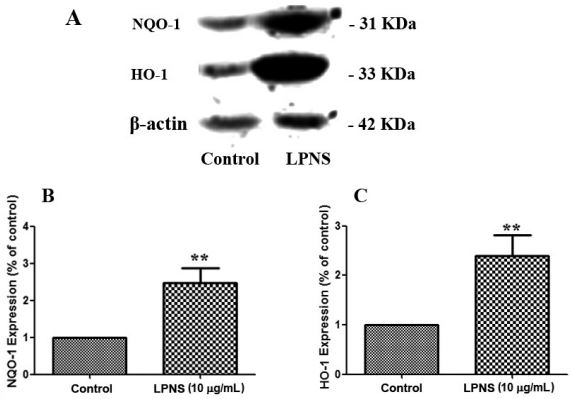


**Figure 7. Effects of LPNS on HO-1 and NQO-1 protein levels in cultured bEnd3 cells.** A: Representative NQO-1 and HO-1 western blots in bEnd3 cells after 24 h treatment with vehicle or 10 μg/mL LPNS. B: Quantification (fold changes) of the NQO-1 western blots. C: Quantification (fold changes) of the HO-1 western blots. Values are means ± SEM, n = 3. **, indicate significant difference from vehicle-treated control at the $p < 0.01$ level.

BBB integrity. Under physiologic conditions, the BBB is relatively impermeable. However, malfunction of the BBB induced by ROS contributes to the disruption of TJs and leads to leakage of the BBB (35,36). More molecules can diffuse into the extravascular space, which can harm the CNS (37). Claudin is specifically involved in the active regulation of small molecule permeability at the BBB (38). Drugs that increase claudin-5 expression can increase transendothelial resistance and decrease BBB permeability (39,40). In our study, pretreatment of bEnd3 cells with LPNS upregulated the expression of claudin-1 and claudin-5, indicating that LPNS can decrease the permeability of endothelial cells, thereby maintaining BBB integrity.

BBB disruption is closely related to oxidative stress, which has been proposed as an early event that aids in progressive neurodegeneration (41). Since LPNS has been shown to protect the brain, it is necessary to know whether LPNS has antioxidative effects. Nrf2 plays a key role in defending against oxidative stress. When oxidative stress occurs, Nrf2 is released and enters the nucleus. There, it binds to ARE, allowing the transcription of antioxidants. Substances that activate the Nrf2 pathway can protect the cerebral microvasculature from BBB disruption in neurological disease. Previous reports suggest that pharmacological activation of Nrf2 signaling significantly restores the loss of TJ proteins and prevents BBB disruption after brain injury (42). Conversely, the deletion or down-regulation of Nrf2 enhances cell susceptibility to the toxic effects of pro-oxidants and results in a loss of BBB integrity (43,44). This evidence suggests that the Nrf2 pathway is a promising therapeutic target for preventing BBB breakdown in neurological diseases (45). Our study revealed that after treating bEnd3 cells with LPNS, Nrf2 accumulates in the nucleus and its downstream antioxidative enzymes (HO-1, NQO-1) are upregulated, showing that LPNS has the potential to promote antioxidation.

In summary, LPNS could protect CMECs from oxidative stress, upregulate the expression of TJ proteins, and activate the Nrf/ARE pathway. Our results support the hypothesis that LPNS can stimulate antioxidative responses in bEnd3 cells and attenuate the effects of ROS-mediated endothelial cell death and TJ breakdown to protect BBB integrity. These protective effects involve activation of the Nrf2/ARE pathway. All of these findings indicate that LPNS could be a promising candidate to treat neurological diseases.

There are still some limitations to our studies. First, we only tested the effects of LPNS on tight junctions and the Nrf2 pathway in normal cells, but not in injured cells under oxidative stress. It is important to understand whether LPNS can exert these up-regulation effects under oxidative stress. Second, even though our results suggest that LPNS can protect CMECs by reducing cell death from oxidative stress, increasing the expression of TJ proteins, and activating Nrf2/ARE, we cannot prove a direct relationship between the upregulation of these proteins and the reduction in cell death. Additional experiments should be performed to clarify this relationship. In the future, we will block the protection from LPNS by selectively knocking down Nrf2 expression by siRNA-based gene silencing or by an Nrf2 inhibitor (*e.g.*, all-trans-retinoic acid) to determine the specific role of LPNS in the maintenance of BBB integrity.

## Materials and Methods

Fetal bovine serum (FBS) was purchased from Gibco (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Antibiotic-Antimycotic (100X), and trypsin-EDTA were obtained from Invitrogen (Shanghai, China). $H_2O_2$ was from Adamas Reagent Co., Ltd (China).

The total saponin from the leaves of *Panax notoginseng* was a gift from Yunnan Hongyun Bioengineering Technology Co., Ltd. (Kunming, China). The predominant ingredients of LPNS are ginsenoside Rc (11.72%), Rb3 (17.58%), Rb1 (2.80%), Rb (22.61%), and Rd (3.87%) (29).

### Cell cultures

bEnd3 cells (ATCC, China) were cultured as previously described (46). Briefly, cells were seeded on 48-well plates or 10-cm dishes. The culture medium was changed every 48 h in DMEM with 4.5 g/L glucose and L-glutamine containing 10% FBS, 2% glucose, and 1% antibiotic-antimycotic agents. Cells were grown in a humidified incubator with 95% air and 5% $CO_2$ at 37°C and passaged (1:12-16) twice a week.

### $H_2O_2$ treatment

A 30% solution of $H_2O_2$ was diluted in FBS-free DMEM and added to the cultures to a final concentration of 0.5 mM. In the first set of experiments, bEnd3 cells were pretreated with vehicle (DMSO) or LPNS at 2.5, 5, or 10 μg/mL. The medium was then replaced and cells incubated with FBS-free DMEM with or without freshly made 0.5 mM $H_2O_2$ for 4 h. In each experiment, cultures exposed to $H_2O_2$ were compared with normal controls (FBS-free DMEM not containing $H_2O_2$) and vehicle-treated cells exposed to $H_2O_2$. In a second set of experiments, bEnd3 cells were pretreated with 10 μg/mL LPNS for 1 to 48 h before exposure to 0.5 mM $H_2O_2$ to examine the time course of protection.

### Lactate dehydrogenase release assay

Cell injury was assessed by measuring extracellular lactate dehydrogenase (LDH) release. After cells were exposed to 0.5 mM $H_2O_2$ for the designated time, LDH release into the medium was measured using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, China) at 492 nm with a microplate reader (Infinite M200 PRO, Tecan, Switzerland). The amount of LDH released from each test sample was expressed as a percentage of the control and vehicle-treated cells exposed to $H_2O_2$.

### Cytoimmunofluorescence

bEnd3 cells were seeded with 30000 cells/well in a 24-well plate. After 24 h culture, the cells were incubated with 10 μg/mL LPNS for another 24 h and then fixed with 4% paraformaldehyde in PBS for 20 mins, followed by a 30-min incubation with 10% normal goat serum in PBS to block nonspecific binding. Primary antibodies against claudin-1 (rabbit polyclonal, clone ab15098, dilution 1:200, Abcam, China), claudin-5 (rabbit polyclonal, clone ab15106, dilution 1:100, Abcam, China), and occluding (rabbit polyclonal, clone ab31721, dilution 1:100, Abcam, China) were added for 60 mins, then washed in PBS (4×6 min) at room temperature and exposed to goat anti-rabbit IgG H&L (clone ab150083, dilution 1:1000, Abcam, China) for 45 mins in the dark. After washing again in PBS (4×6 min), cells were covered with DAPI and read with the fluorometric plate reader (Infinite M200 PRO, Tecan, Switzerland) at 652 nm/668 nm or pictured by fluorescence microscope with appropriate filters.

### Western blotting studies

bEnd3 cells treated with 10 μg/mL LPNS and harvested at 1h were used to examine the nuclear translocation of NF-erythroid 2-related factor 2 (Nrf2). Nuclear fractions were isolated using a Nuclear and Cytoplasmic Protein Extraction Kit following the manufacturer's instructions (Beyotime, China). Cells pretreated with 10 μg/mL LPNS for 24 h were collected and lysed with RIPA buffer (Beyotime, China) for heme oxygenase1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1) detection. Supernatants were collected, and the protein concentration was determined by BCA assay kit (Beyotime, China).

Sixty micrograms of each protein sample was run through a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Millipore, China). Membranes were blocked in 5% non-fat dry milk for 1 h and then incubated with polyclonal antibodies (rabbit anti-rat Nrf2, 1:400, Santa Cruz, China; rabbit anti-rat Lamin A, 1:200, Boster, China; rabbit anti-rat HO-1, 1:500, Proteintech, China; rabbit anti-rat NQO-1, 1:200, Santa Cruz, China) for 1 h. Immunoreactive proteins were visualized with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (EZ-ECL Kit, Biological Industries, China) and were then pictured with a gel imaging system (BIO-RAD Gel Doc 2000). The relative densities of the Nrf2, HO-1, and NQO-1 bands were measured and quantified. Nrf2 was then normalized against Lamin A while HO-1 and NQO-1 were normalized against β-actin. The protein levels were expressed as a percentage of the control value.

### Statistical analysis

Data from at least three independent experiments are presented as mean ± SEM and were analyzed statistically by *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons between groups. A $p$ value less than 0.05 was considered statistically significant. All analyses were performed with SPSS.

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