Panax notoginseng Saponins Promotes Stroke Recovery by Influencing Expression of Nogo-A, NgR and p75NGF, in Vitro and in Vivo

Lixing Liu,a Lingqun Zhu,*a Yihuai Zou,b Wei Liu,a Xiaqian Zhang,a Xiaqin Wei,a Bo Hu,a and Jiaxu Chenc

a Key Laboratory of Chinese Internal Medicine of Educational Ministry and Beijing, Dongzhimen Hospital, Beijing University of Chinese Medicine; b Department of Neurology, Dongzhimen Hospital, Beijing University of Chinese Medicine; Beijing 100700, China; and c Department of Chinese Medicine Diagnosis, School of Preclinical Medicine, Beijing University of Chinese Medicine; Beijing 100029, China.

Received October 2, 2013; accepted January 14, 2014; advance publication released online January 25, 2014

The spontaneous recovery of function after injury in the adult central nervous system is limited due to the several proteins, such as Nogo-A that have repulsive or inhibitory effects on growing neuritis. The Chinese herbal medicine extraction Panax notoginseng saponins (PNS) injection has been widely used and effective in repairing the function of impaired nerves, but the mechanism of this herbal medicine is still poorly understood. This project evaluated the effect of Panax notoginseng saponins on neurological functional recovery and on the expression of Nogo-A, NgR and p75 at 7, 14 and 28 d after middle cerebral artery occlusion (MCAO) in rats and also oxygen-glucose deprivation/reperfusion (OGD/R) model on SH-SY5Y cells. We found that the expression of Nogo-A, NgR and p75 of rats receiving MCAO surgery increased on the 7th day, reached a peak on the 14th or 28th day and maintained high levels and Panax notoginseng saponins significantly decreased these expressions. This may be the mechanism of Panax notoginseng saponins that contributes to the recovery of nerve function, which plays an important role in brain protection after cerebral infarction.

Key words Panax notoginseng saponin; cerebral infarction; Nogo-A; NgR; p75

Stroke is a common disease among elderly people, which severely impact their health, and can be fatal. Approximately 795000 people experience either a new or recurrent stroke each year and every minute someone has a stroke in the U.S.A., which brings a high burden to both society and the families.1,2) Although spontaneous recovery of function after injury can occur in the adult Peripheral Nervous System, the same recovery in the Central Nervous System is more limited due to several proteins with repulsive or inhibitory effects on growing neuritis, including the myelin-associated inhibitors3,4), Nogo-A,5,6) myelin-associated glycoprotein (MAG),7,8) and oligodendrocyte-myelin glycoprotein (OMgp).9,10) Among these, Nogo-A has obtained considerable interest in spinal cord trauma and ischemic stroke research as it is a neurite growth inhibitory protein that prevents axonal regeneration and plasticity both in vitro and in vivo.3,11,12) The functional component of Nogo-A that inhibits neurite outgrowth via its receptor NgR in a complex with p75.13,14) And studies showed that anti-Nogo-A treatment resulted in recovery of function and corticorubral plasticity in rat stroke model.15,16) Recently, many studies have showed that Nogo-A restricts neuron axon regeneration.17,18) Kim et al. in 200319) analyzed spinal cord injury in mice with a Nogo mutation that eliminated Nogo-A/B expression (Nogo-A/B−/− mice) and showed that numerous fibers regenerate into the distal cord segments of mice. Liebscher et al. in 200520) studied axonal regeneration after normal mice having spinal cord injury and using Nogo-A antibody and demonstrated that enhanced regeneration and reorganization of the injured central nervous system (CNS), resulting in improved recovery of function. Studies21) that suppressed NgR activity showed enhanced axon regeneration. NgR consists of a receptor complex with transmembrane collaborative receptor p75 and Lingo-1 that activates downstream signal transduction pathways.22,23) p75 is a low affinity receptor for neurotrophic factor (NGF)24) and its physiological activities generally include cooperating with Trk receptor to promote cell survival and differentiation and participating in cell apoptosis. Recently, it has been demonstrated that p75 increased its expression after cerebral ischemic injury, and blocking its signal delayed the disease and promote cell survival.25) p75 transfers inhibiting growth signals to the intracellular space, and interacts with the downstream molecular signal—RhoA to block the axon growth.26,27)

Panax notoginseng is the root of Panax notoginseng (BURK.) F. H. CHEN, and comes from the family Araliaceae. Panax notoginseng saponins (PNS) are the main biologically active constituents and Panax notoginseng saponins injection has been clinically used for the treatment of cardiovascular diseases, atherosclerosis, and cerebral ischemia.28,29) However, like most of Chinese herbal medicine, its mechanism is still not clear. Therefore, the goal of this project is to evaluate the effectiveness and try to find the mechanism of Panax notoginseng saponins in the rat middle cerebral artery occlusion (MCAO) model and cell oxygen–glucose deprivation (OGD) model.

MATERIALS AND METHODS

Focal Ischemia. Animals Adult male Sprague-Dawley rats (250–300 g) were housed in standard cages, with free access to food and water under a 12:12-h light cycle with controlled temperature and humidity for at least one week prior to drug treatment or surgery. The study conforms to the “Guide for the Care and Use of Laboratory Animals” published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/
All procedures were approved by the Institutional Animal Care and Use Committee of Dongzhimen Hospital (Beijing, China). All efforts were made to minimize animal suffering and reduce the number of animals used.

Model of Focal Ischemia Animals were randomly selected into the surgery group and the control group. To make the focal and permanent MCAO model, we applied the improved Longa method. Briefly, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and then isolated. The proximal parts of the ECA and CCA were ligated with nylon suture and a microvascular clip was placed on the ICA origin. And then introduced a nylon intraluminal suture into the ICA and the microvascular clip was removed and advanced intracranially to block blood flow. Finally fixed the intraluminal suture and sutured skin. EZ-Longa evaluation (30) was used to assess the neurological deficit 2 h after emergence from anesthesia. Only the rats that fit the EZ-Longa evaluation (1–3 grade) and did not have any surgical complications were enrolled into the study.

Drug Treatment PNS injection was purchased from Kunming Pharmaceutical Corporation (Batch No.: 12J1B09), and the content analyses were Notoginsenoside R1: 9.8%, Ginsenoside Rb1: 32.3%, Ginsengoside Rg1: 35.3%, Ginsengoside Re: 4.0%, Ginsenoside Rd: 4.9%, R1+Rg1+Rb1+Re+Rd: 86.3%. The surgery rats were randomly assigned into 1) model group, 2) PNS low-dose group (3.6 mg/100 g/d, intraperitoneal injection), 3) PNS high-dose group (7.2 mg/100 g/d) and 4) positive control group-nimodipine group (1.44 mg/100 g/d, oral administration, Bayer). Drug treatment was administrated 5 h after surgery, and EZ-Longa evaluation and weight were measured at each time point. 6–8 animals were sacrificed to get the brain tissue from each group at each time point (7, 14, 28 d) and their brain tissue was measured for changes in No -

Immunohistochemistry We used 7 rats in each group at different time point and the brain tissue was fixed in 10% formaldehyde for 48 h. Then the brain tissue was embedded in paraffin, and sectioned at 4 µm thickness. The sections were incubated at different primary antibody: Nogo-A antibody (1:25, sc-2566, Santa Cruz, U.S.A.); NgR (1:400, ab26291, Abcam, U.S.A.) and p75 antibody (1:400, ab8874, Abcam, U.S.A.). And then the anti-rabbit secondary biotinylated antibody was applied. DAB kit (Boster Bio, China) was used to develop sections.

Western Blot Analysis We got 4 rat brain tissue in each group at different time point. Protein was extracted using the RIPA lysate kit (C1053, Applygen Technologies Inc., China). We loaded 40µg protein in each lane of a polyacrylamide-dodecyl sulfate (SDS) gel. And then incubated with the appropriate primary antibodies overnight at 4°C Nogo-A antibody (1:1000, sc-2566, Santa Cruz, U.S.A.); NgR (1:1000, ab26291, Abcam, U.S.A.) and p75 antibody (1:1000, ab8874, Abcam, U.S.A.); β-actin antibody (1:4000, ZSGB-Bio., China). The secondary antibodies were goat anti mouse immunoglobulin G (IgG) and goat anti rabbit (1:3000, C1308 and C1309, Applygen Technologies Inc., China) (1:3000 dilution, Santa Cruz, sc-2786, sc-2004). We used the chemiluminescent (ECL) reagent and optical density of each band was analyzed by the quantity one software.

RT-PCR We used 5 rats brain tissue in each group at different time point. The total RNA of each rat cerebral cortex was isolated using SV Total RNA Isolation System (Z3100, Promega, U.S.A.). Firststrand cDNA was synthesized using Reverse Transcription System (A3500, Promega, U.S.A.). The primers were designed and synthetized by Augctbio (China): Nogo A Forward Primer 5'-AGG GAT GTG CGT GCT GCT AG-3' and Reverse Primer 5'-GTT GCT TTC CGT GTG CTA G-3', NgR Forward Primer 5'-GGGCAA CCT CAC GCA TCT CT-3' and Reverse Primer 5'-TCATGAG TGC GGC CAA

Fig. 1. The Evaluation of Body Weight Mass and Neurologic Function of Rats after MCAO

PNS improve the recovery of body weight and neurological function of rats at 7, 14, and 28 d after MCAO (6–7 rats in each group). (A) Body weight of rats with MCAO surgery significantly decreased after surgery (p<0.01). There were no significant differences (p>0.05) at the 7th and 14th day between treatment groups and MCAO model group. On the 28th day, PNS high dose group and Nimodipine group showed a significant difference compared with MCAO model group (p<0.05 or p<0.01) with no significant difference between these two groups. (B) Neurological function of rats with MCAO surgery significantly decreased after surgery and treatment groups showed significant differences with the MCAO model group (p<0.05 or p<0.01). On the 28th day, PNS high-dose group and Nimodipine group were almost at the normal levels (p<0.01). *p<0.05, **p<0.01 versus control group; *p<0.05, ***p<0.01 versus model group.
Fig. 2. Expression of Nogo-A, p75 and NgR at Different Recovery Time Points (Immunohistochemistry)

Expression of Nogo-A, p75 and NgR in the peri-infarct area after MCAO surgery (7 rats in each group). Nogo-A, NgR and p75 visualized a brown color in the peri-infarction area (40×10) at each time point. (A) Nogo-A expression at different time point. (B) NgR expression at different time points. (C) p75 expression at different time points (scale bar=30μm).
GGTCC-3'; p75 Forward Primer 5'-GGCTCGGAAGCTGTCTCCT-3' and Reverse Primer 5'-CTCTGACATTAGCAGCATCAGG-3'; β-actin Forward Primer 5'-AGATTCGTAAGCGAGTCG-3' and Reverse Primer 5'-CCA GGAGGAAAGG-3'. RT-qPCR was performed using Power SYBR Green PCR Master Mix (4367659, ABI, U.S.A.).

**Cell Culture** Human neuroblastoma SH-SY5Y cells were routinely cultured in Dulbecco's modified Eagle's medium.

---

**Fig. 3. Expression of Nogo-A, p75 and NgR at Different Recovery Time Points (Western Blot)**

Expression of Nogo-A, p75 and NgR at different recovery time points (Western blot, 4 rats in each group). (A, B) At each time point, the expression of Nogo-A in MCAO model group was higher than the control group ($p < 0.01$) and peaked on the 14th day then decreased by the 28th day. On the 7th day, PNS and Nimodipine groups had lower expression than the MCAO model group but this difference was not significant ($p > 0.05$). On day 14, the PNS high-dose and the Nimodipine groups were significantly different from the MCAO model group ($p < 0.01$). On day 28, all treatment groups were significantly different from the MCAO model group ($p < 0.01$) and there was a significant difference between the high and low-dose group ($p < 0.01$). In figure B, D and F, a) control group, b) MCAO model group, c) PNS low-dose group, d) PNS high-dose group, e) Nimodipine group (**$p < 0.01$ versus control group; $p < 0.05$, **$p < 0.01$ versus model group; $p < 0.05$, **$p < 0.05$ between high and low-dose group).
Bromide (MTT) Assay

1×10^4 SHSY5Y cells were seeded into 96 well culture plates and allowed to attach. After 24 h, 20 μL of 5 mg/mL MTT was added to each well and allowed to incubate for 2–4 h followed by 150 μL dimethyl sulfoxide (DMSO) to each well. Finally, the optical density (OD) value was measured by the microplate reader (Bio-Tek, U.S.A.).

RESULTS

Body Mass Index and Neurologic Function Evaluation

Weight of each group was recorded pre-surgery and on the 7th, 14th and 28th day after MCAO surgery. Body mass index is the ratio between the weight at pre-surgery and different time points after the surgery. The body mass index of each group decreased 7 d after MCAO but recovered by the 14th day. On the 28th day, the weight increased more in the groups of high-dose PNS and Nimodipine than the model group. The expression of Nogo-A, NgR and p75 protein expressions were tested using the methods of MTT and immunohistochemistry.

Statistical Analysis

Statistical analysis was performed using the SPSS 16.0 software. Results are reported as means±S.D. and analyzed with one-way ANOVA (α=0.05).

Effect of PNS on the Expression of Nogo-A, p75 and NgR at Different Recovery Time Points

In the MCAO model group, Nogo-A had significantly higher expression compared to the control group on the 7th day (p<0.05), and peaked on the 14th day (p<0.01), then decreased slightly but still maintained a high level on the 28th day (p<0.01). Rats in the Nimodipine and the high-dose PNS groups showed lower expression in the MCAO model group (p<0.01) and maintained a high level on the 28th day (p<0.05). The Nimodipine and PNS high dose groups showed significant differences with the MCAO model group at every time point (p<0.05 or p<0.01). The expression in PNS low dose group decreased but this decrease was not significantly different with the model group until on the 28th day (p<0.05). The expression of NgR in the MCAO model group increased gradually (p<0.01) and maintained a high level on the 14th and 28th day. All treated groups showed a significant difference with the MCAO model group (p<0.01) and there was significant difference between high and low dose group (p<0.01) on the 28th day. The expression of p75 mRNA in the MCAO model group increased gradually (p<0.01) and maintained a high level on the 14th and 28th day. All treated groups showed a significant difference with the MCAO model group (p<0.01) and there was significant difference between high and low dose group (p<0.01) on the 28th day. (C) Expression of p75 mRNA. The expression of NgR in the MCAO model group increased gradually (p<0.01) and maintained a high level on the 14th and 28th day. All treated groups showed a significant difference with the MCAO model group (p<0.01) and there was significant difference between high and low dose group (p<0.01) on the 28th day. (C) Expression of p75 mRNA.

Fig. 4. Expression of Nogo-A, p75 and NgR at Different Recovery Time Points (qPCR)

Nogo-A, NgR and p75 mRNA expressions at different recovery time points (5 rats in each group). (A) Expression of Nogo-A mRNA. There was no difference in the control group at each time point, and in the surgery groups, the mRNA expressions were higher than the control group (p<0.01). PNS high dose and the Nimodipine groups showed significant differences with the MCAO model group at every time point (p<0.05 or p<0.01). The expression of Nogo-A mRNA was significantly higher than the control group; # p<0.05, ## p<0.01 versus control group; * p<0.05, ** p<0.01 versus model group; $ p<0.05$ between high and low-dose group.)
expression than MCAO model group \((p<0.01)\). There was significant differences between high and low dose groups on the 14th and 28th day \((p<0.05\) or \(p<0.01)\). The NgR expression in the MCAO model group was higher than the control group \((p<0.01)\). Seven days after MCAO, the PNS high-dose group and the Nimodipine group showed a significant difference with the control group \((p<0.01)\). PNS high-dose group had lower expression after 14 d \((p<0.01)\) and 28 d than the model group \((p<0.01)\). On the 14th and 28th day, there was a significant difference between high-dose and low-dose group \((p<0.01)\). p75 expression showed a significant increase on the 7th day in the MCAO model group \((p<0.01)\), that peaked on the 14th day and showed a decrease by the 28th day. p75 expression in the Nimodipine and PNS high-dose groups significantly decreased from 14th day \((p<0.01)\). All treatment groups had lower expression than the MCAO model group on the 28th day \((p<0.05\) or \(p<0.01)\). The contralateral area in each treated group had lower Nogo-A, NgR and p75 expression than the model group, but there were no significant differences between groups (shown in the supplement data).

**Effect of PNS on the mRNA Expressions of Nogo-A, p75 and NgR at Different Recovery Time Points** In the control group, the mRNA expressions of Nogo-A, p75 and NgR did not change significantly. In the surgery groups, Nogo-A increased during the first 7 d and peaked at the 14th day followed by a decrease on the 28th day. All treated groups showed a decrease and were significantly different from the MCAO model group on the 28th day \((p<0.05\) or \(p<0.01)\). The PNS high dose and Nimodipine groups were significantly different from the MCAO model group \((p>0.05)\). The expression of NgR mRNA in the MCAO model group increased gradually \((p<0.01)\) reaching a high level on the 14th day and peaking on the 28th day. All treated groups were significantly different from the MCAO model group and there was significantly different between the high and low dose group. The expression in the Nimodipine group was significantly lower than the MCAO model group \((p<0.05)\) on the 7th day and expressions in the PNS high dose group was significantly different from the MCAO model group on the 14th day. The expression of p75 in the MCAO model group increased gradually \((p<0.01)\) and peaked on the 14th day then decreased by the 28th day. The expression of p75 in treated groups decreased but not significantly different from the MCAO model group. The Nimodipine and PNS high dose groups were significantly different from the control group, \((p<0.05\) or \(p<0.01)\).

---

![Fig. 5. Effect of PNS on Cell Viability and the Expression of Nogo-A, NgR and p75 in Different Groups](image-url)
different from the MCAO model group ($p<0.05$) at the 14th day. On the 28th day, all the treated groups were significantly different from the MCAO model group ($p<0.05$ or $p<0.01$) (Fig. 4).

**Effect of PNS on the SH-SY5Y Cells OGD/R Model**

When SH-SY5Y cells were treated with different concentrations of PNS for 24 h, 48 h, 72 h, PNS had a proliferation effect when using the concentration of 320 µg/mL ($p<0.01$), 500 µg/mL ($p<0.05$) and 640 µg/mL after 24 h exposure. After 48 h and 72 h, concentrations of 320 µg/mL, 500 µg/mL, 640 µg/mL and 1000 µg/mL had a proliferation effect ($p<0.01$). After 72 h exposure, the concentration of 640 µg/mL group had a better effect than the 320 µg/mL group ($p<0.01$). After 3 h of OGD and then 24 h OGD/R, the cell viability decreased significantly ($p<0.01$), but when treated with PNS low dose (320 µg/mL) and high dose (640 µg/mL), a protective function was observed with the high dose showing a greater effect ($p<0.01$) (Fig. 5). After OGD/R, Nogo A protein and its receptor NgR and p75 expressions increased significantly ($p<0.01$), when treated PNS, the Nogo A, NgR and p75 expressions decreased in both low and high dose groups ($p<0.01$) (Fig. 6).

**DISCUSSION**

Peripheral region to the cerebral infarction ischemic is the main focus of treatment. Comprehensive protective treatment in the early and acute phase of ischemic injury with cell protective agent can restore the blood supply to the ischemic area, and improve microcirculation to block the pathological process of cerebral infarction. The results shown here demonstrate that the expression of Nogo-A, NgR and p75 in the MCAO model group was higher than that of the control group. Expressions increased on the 7th day, peaked on the 14th or 28th day and were maintained at high levels. The expressions of all treatment groups were less than those seen during the same time period in the MCAO model groups and this may relate to the fact that during the early phase of cerebral infarction, nerve cells experience ischemia, degeneration and necrosis with cerebral edema, rupture and dissolution of neurons along with decrease in protein and neurotransmitter synthesis when neurons are in this damaging stress phase and nerve repair after stress has not been fully initiated. Thus, Nogo A expression level is not high on the 7th day, but after one week and the body fully initiates repair mechanisms, such as accelerated nerve tissue and connective tissue repair, proliferative changes in the tissue around infarction accelerated regeneration, or the nerve near the lesion site and oligodendrocyte cell proliferation. Concurrently, Nogo A increases after receiving a signal of repair, and then inhibites growth of neurons. We hypothesize that the slow recovery of neurological function after cerebral infarction may be due to the hyper-expression of Nogo A. Nogo-A, NgR, p75 mRNA and protein expression significantly increased after early brain damage to a peak expression, and then Nogo-A and p75 decreased during the early stage of recovery phase. NgR mRNA and protein expression had no significant decrease after the peak and maintained a high expression level at 28 d after MCAO. This lasting high expression of NgR mRNA and protein may play a more important role in the signaling pathways of Nogo protein to inhibit the growth of neurons. In Fig. 5, it showed that the cell viability of 160 µg/mL PNS treatment was lower than the control group, and at the concentration of the 80 µg/mL, the cell viability also showed a decrease trend and lower than the control group at 72 h time point. This phenomenon may due to the two-way adjustment function of the Chinese medicine, and the further research will be needed to test the pharmacodynamics

![Fig. 6. Nogo-A, NgR and p75 Expression after OGD/R](image)
in vivo about this phenomenon.

The micro-environment after cerebral infarction of the mammal is quite complex as it includes neurotrophic factors that promote synaptic regeneration, and inhibitors that inhibit axon regeneration. This study showed that PNS can effectively lower the expression level of the inhibitor Nogo A, thereby contributing to the recovery of neural function, which plays an important role in brain protection. In addition, PNS have a dose-dependent effect which can provide a foundation for further clinical usage of PNS for the treatment of ischemic cerebrovascular disease.

Promoting blood circulation and removing blood stasis is the most common and fundamental method for the treatment of ischemic stroke using Chinese medicine. PNS injection is made of the extraction of the traditional Chinese medicine Panax notoginseng which is from the Araliaceae Panax genus and can promote blood circulation and remove blood stasis. In conclusion, PNS can decrease the expressions of Nogo-A and its receptor NGR, p75 protein and mRNA after cerebral infarction, which could be part of its mechanism of action to promote recovery of brain function after cerebral infarction.

Acknowledgment  This work was supported by the Natural Science Foundation of China (30772803).

REFERENCES


