Panax notoginseng saponins attenuate oxygen-glucose deprivation/reoxygenation-induced injury in human SH-SY5Y cells by regulating the expression of inflammatory factors through miR-155

Lanqing Meng#, Jun Lin#, Qing Huang, Ping Liang, Jianmin Huang, Chongdong Jian, Chong Lin, Xuebin Li*

Department of Neurology, Affiliated Hospital of Youjiang Medical College for Nationalities

*Corresponding author: Dr. Xuebin Li, 18 Zhongshan Second Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. Email: gxbsmlq@126.com; Tel: +86-13507766338.

#Contributed equally
Summary

Panax notoginseng saponins (PNS) have been widely used in China to treat stroke. Accumulating evidence has found that miR-155 plays critical roles in the pathology of ischemic stroke. Here we investigated whether PNS plays a protective effect against oxygen-glucose deprivation/reoxygenation (OGD/R)-induced focal inflammation and injury in SH-SY5Y cells by regulating miR-155 expression. Treatment with PNS at a concentration less than 160 µg/mL had no effect on the proliferation of SH-SY5Y cell. In OGD/R-induced SH-SY5Y cells, 160 µg/mL PNS treatment promoted cell proliferation and cell cycle progression, as well as decreased inhibited apoptosis and miR-155 expression. However, overexpression of miR-155 attenuated the promotion effects of PNS on cell proliferation and cell cycle, apoptosis inhibition in OGD/R-induced SH-SY5Y cells. Moreover, 160 µg/mL PNS treatment decreased the levels of IL-1β, IL-6, and TNF-α in OGD/R-induced SH-SY5Y cells, whereas overexpression of miR-155 reversed PNS-induced decreases in the levels of IL-1β, IL-6, and TNF-α in OGD/R-treated SH-SY5Y cells. In conclusion, PNS attenuated OGD/R-induced injury in human undifferentiated SH-SY5Y cells by regulating the expression of inflammatory factors through miR-155.

Keywords: Panax notoginseng saponins; SH-SY5Y; miR-155; oxygen-glucose deprivation; reoxygenation; inflammatory factors
Stroke is the second leading cause of death or disability worldwide.\textsuperscript{1} Ischemic stroke, which accounts for 80\% of all strokes, is usually triggered by insufficient blood supply, and blood reperfusion in the brain that causes cerebral ischemia and reperfusion injury.\textsuperscript{2} Transient cerebral ischemia can cause severe neuronal damage, and secondary neuronal death. Cerebral ischemia and reperfusion injury are complex pathological processes, including inflammation, apoptosis, and neuronal stress in the brain tissue.\textsuperscript{3} At present, it remains lack of effective clinical drugs for cerebral ischemia and reperfusion injury.\textsuperscript{4} Medicinal plants, such as the popular traditional Chinese herb \textit{Panax notoginseng}, known as Sanqi, has been widely used to treat cerebrovascular and cardiovascular disorder in traditional Chinese medicine.\textsuperscript{5} Panax Notoginseng Saponin (PNS; total saponin extraction), one of the most abundant bioactive compounds in \textit{P. notoginseng}, has been shown to have a neuroprotective and anti-inflammatory effect and promote stroke recovery \textit{in vivo} and \textit{in vitro}.\textsuperscript{6-8} However, molecular mechanisms underlying the pharmacological activity of PNS against ischemic injury remain to be investigated.

MicroRNAs (miRNAs or miRs) are a group of small (19-23 nucleotides in length) endogenous non-coding RNA molecules.\textsuperscript{9} miRNAs play a vital role in the modulation of various biological events, including signal transduction, differentiation, proliferation, and apoptosis.\textsuperscript{10} Emerging studies have suggested that miRNAs are participated in the regulation of cell survival and inflammation of cerebral ischemia.\textsuperscript{11, 12} miR-155 is located on chromosome 21.\textsuperscript{13} Increasing reports have suggested that miR-155 is closely involved in the regulation of cell apoptosis, immunity, inflammation, and blood-brain barrier.\textsuperscript{14-16}

In this study, we suspected that miR-155 might play a vital role in the effect of PNS on the ischemic injury-related inflammation. We first investigated the effect of PNS on the proliferation of SH-SY5Y cells. Next, we used oxygen-glucose deprivation/reperfusion...
(OGD/R)-induced SH-SY5Y cells, an *in vitro* model of ischemic neurocyte injury,[17, 18] to study the effects of PNS on expression of inflammatory cytokines, cell proliferation, cell cycle, and apoptosis. Additionally, we studied whether PNS attenuates OGD/R-induced injury in SH-SY5Y cells by regulating miR-155 expression.

MATERIALS AND METHODS

**Drug**  PNS was purchased from Guangxi Wutong Pharmaceutical (Group) CO., LTD (China Food and Drug Administration (CFDA) approval number: Z20025652; Guangxi, China), which mainly include ginsenoside Rb1, ginsenoside Rg1, notoginsenoside R1 and ginsenoside Re.

**Cell culture and transfection** Human undifferentiated SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Thermo Scientific, Waltham, MA, USA) at 37°C in a humidified incubator with 5% CO₂. The medium was replaced twice each week. Cells in exponential phase of growth were used for the subsequent experiments. miR-155 mimic (5′-UUAAUGCUAAUCGUAGUGGU-3′) and miRNA negative control (NC: 5′-UUUGUACUACAAAAGUACUG-3′) were purchased from Ribo. Bio (Guangzhou, China). Cell transfection was performed using Lipofectamine 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. After 24 h of transfection, cells were used for the following experiments.
Cell proliferation assay  SH-SY5Y cells containing 1 × 10⁴ cells were inoculated into 96-well plates and treated with 0, 20, 40, 80, 160, 320, or 640 μg/mL PNS (Guoyaozhunzi: Z20025652; Guangxi Wutong Pharmaceutical (Group) CO., LTD, Guangxi, China;) for 0, 24, 48, or 72 h. Cell proliferation was detected by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) according to the manufacturer’s protocol. Next, the absorbance was detected at 490 nm using a microplate absorbance reader.

Oxygen-glucose deprivation/reoxygenation (OGD/R) injury  To mimic ischemic injury in vitro, SH-SY5Y cells were pretreated with PNS at 160 μg/mL in a DMEM medium for 24 h. Cells in the normal group were cultured in normal culture condition. Cells in the OGD/R group were washed twice with PBS, seeded in glucose-free DMEM (Hyclone) and kept at 37°C for 5 h in a hypoxic chamber using an AnaeroPack Campylo culture system (Mitsubishi Gas chemical, Inc., Tokyo, Japan) following the manufacturer’s protocol. Next, the cells were transferred to normal culture medium and kept in an incubator with 5% CO₂ at 37°C for 24, 48, or 72 h for the following experiments.

Quantitative real-time PCR (qRT-PCR)  Total RNA was extracted from cells using Trizol (Invitrogen). Reverse transcription was performed using a TaqMan™ microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and Prime Script™ RT Reagent Kit (Takara, Dalian, China. qRT-PCR was performed using a standard SYBR Green PCR Kit (Takara) and Mir-X miRNA qRT-PCR SYBR Kit (Clontech, Inc, Palo Alto, CA, USA) in an ABI PRISM® 7500 real-time PCR system (Applied Biosystems). The levels of miRNA and mRNA were normalized against those of U6 and GAPDH. Relative fold changes in the transcripts were calculated using the 2⁻ΔΔCT method. Each sample was measured in triplicate. The primer sequences were as follows. miR-155: forward, 5ʹ-TGAACCTCTCTGCTG

Biological and Pharmaceutical Bulletin Advance Publication
Cell cycle and apoptosis analysis  
Cell cycle and apoptosis assay was performed using a cell cycle detection kit and Annexin V-FITC Apoptosis detection kit (Keygentec, Nanjing, China) in flow cytometry (BD Biosciences, San Jose, CA, USA). After OGD/R treatment for 24, 48, and 72 h, SH-SY5Y cells (1 × 10⁶) were harvested and fixed in 500 μL 70% ice-cold ethanol for 2 h at 25 °C room temperature. SH-SY5Y cells were washed three times with cold PBS and incubated in propidium iodide (PI, 400 μL) and RNase (100 μL) for 30 min at 37°C in the dark for detecting cell cycle. SH-SY5Y cells were incubated in 5 μL of Annexin-V fluorescein isothiocyanate and 5 μL of PI in the dark for 15 min at 25 °C for detecting cell apoptosis. Each experiment was repeated three times.

Statistical analysis  
All data were analyzed using SPSS version 19.0 statistical analysis package (SPSS Inc., Chicago, IL, USA). All data are expressed as mean ± standard deviation (SD) and analyzed by t-test or ANOVA if appropriate. A value of P < 0.05 was considered statistically significant.

RESULTS

Effects of PNS on SH-SY5Y cell proliferation  
To determine the optimum PNS concentration that has no effect on SH-SY5Y cell proliferation, SH-SY5Y cells were treated with various concentrations of PNS, and cell proliferation was examined using MTS assay.
Compared with 0 µg/mL PNS treatment, 320 and 640 µg/mL PNS treatment markedly decreased SH-SY5Y proliferation at 24 h, 48 h, and 72 h, while PNS at concentrations less than 160 µg/mL showed no marked difference in cell proliferation. Therefore, 160 µg/mL PNS treatment was selected for the subsequent experiments.

**PNS treatment decreased miR-155 expression in OGD/R-induced SH-SY5Y cells**

To investigate whether miR-155 is a potential target for PNS in treating OGD/R-induced injury, miR-155 expression were measured using qRT-PCR in OGD/R-induced SH-SY5Y cells. Result showed that OGD/R treatment significantly upregulated miR-155 expression in SH-SY5Y cells at 24, 48, and 72 h, which was significantly attenuated by PNS treatment (Fig.2). Additionally, miR-155 mimic was transfected into OGD/R-induced SH-SY5Y cells. Results showed that miR-155 expression in the PNS + miR-155 group were significantly increased compared with that in the PNS + NC group at 24 h, 48 h, and 72 h after transfection (Fig.2).

**Overexpression miR-155 attenuated the proliferation-promoting effects of PNS in OGD/R-treated SH-SY5Y cells**

The proliferation of OGD/R-treated SH-SY5Y cells was examined by the MTS assay (Fig.3). The results showed that SH-SY5Y cell proliferation was significantly inhibited after OGD/R treatment at 24, 48, and 72 h, while PNS treatment significantly improved the proliferation of OGD/R-induced SH-SY5Y cell. Additionally, the proliferation of OGD/R-induced SH-SY5Y cells in PNS + miR-155 mimic group was significantly decreased compared with that in PNS + NC group.

**Overexpression of miR-155 attenuated the promoted effects of PNS on cell cycle in OGD/R-treated SH-SY5Y cells**

Cell cycle distribution in all groups was measured by flow cytometry (Supplementary Fig. S1 and Fig.4). The proportions of cells in the G1 and S phase at 24, 48, and 72 h significantly increased and decreased, respectively, in OGD/R-induced
SH-SY5Y cells compared with those in SH-SY5Y cells. PNS treatment significantly decreased and increased the proportions of cells in the G1 and S phase, respectively, at 24, 48, and 72 h compared with those in OGD/R-treated SH-SY5Y cells. Additionally, SH-SY5Y cells treated with OGD/R + PNS + miR-155 mimic showed significantly increased and decreased proportions of cells in the G1 and S phase, respectively, at 24, 48, and 72 h compared with cells treated with PNS + NC under OGD/R.

**Overexpression of miR-155 attenuated the inhibitory effects of PNS on cell apoptosis in OGD/R-treated SH-SY5Y cells**  
Apoptotic cells were measured by a flow cytometry (Supplementary Fig. S2 and Fig.5). Apoptosis was significantly increased after OGD/R treatment at 24, 48, and 72 h, while PNS treatment significantly attenuated the apoptosis of OGD/R-induced SH-SY5Y cell. Additionally, treatment with PNS + miR-155 mimic significantly increased cell apoptotic rates at 24, 48, and 72 h in OGD/R-treated SH-SY5Y cells compared with those in cells treated with PNS + NC.

**Overexpression of miR-155 reversed PNS-induced decreases in the levels of IL-1β, IL-6, and TNF-α in OGD/R-treated SH-SY5Y cells**

Fig.6 revealed that OGD/R treatment at 24 h, 48 h, and 72 h significantly upregulated the levels of IL-1β, IL-6, and TNF-α in SH-SY5Y cells, these were significantly attenuated by PNS treatment. Additionally, PNS + miR-155 mimic treatment significantly increased the levels of IL-1β, IL-6, and TNF-α at 24, 48, and 72 h in OGD/R-treated SH-SY5Y cells compared with those treated with PNS + NC.

**DISCUSSION**

PNS have several biological effects, such as antioxidant, anti-inflammatory, antiaging, neuroprotective, and anticancer activities. However, the underlying mechanisms of the
neuroprotective properties of PNS remain poorly understood. In this study, our results showed that miR-155 expression was significantly upregulated in OGD/R-treated SH-SY5Y cells, whereas 160 µg/mL PNS decreased miR-155 expression in OGD/R-treated SH-SY5Y cells. In SH-SY5Y cells, OGD/R treatment inhibited proliferation and transition from the G1 to S phases, promoted apoptosis, and increased the levels of IL-1β, IL-6, and TNF-α. However, treatment with 160 µg/mL PNS attenuated OGD/R-induced inflammation and injury in SH-SY5Y cells. Additionally, overexpression of miR-155 attenuated the neuroprotective effects of PNS on OGD/R-induced injury in SH-SY5Y cells. These results indicated that PNS exerted protective effects against OGD/R-induced injury in SH-SY5Y cells by regulating the expression of IL-1β, IL-6, and TNF-α through miR-155.

Inflammatory is closely related to the severity and clinical prognosis of cerebral injury following acute ischemic stroke. After cerebral ischemia, IL-1β, IL-6, and TNF-α were released and brain tissues were injured. PNS has been used in clinical treatment of cerebrovascular diseases due to its neuroprotective effects against central nervous system ischemia/reperfusion injury by decreasing inflammation and apoptosis. In our study, we observed that 160 µg/mL PNS promoted cell proliferation and cell cycle progression from the G1 to S phase, as well as inhibited apoptosis and the levels of IL-1β, IL-6 and TNF-α in OGD/R-treated SH-SY5Y cells. These results suggested that treatment with 160 µg/mL PNS attenuated OGD/R-induced injury in SH-SY5Y cells by regulating the secretion of inflammatory factors.

miR-155 can regulate the pathological processes of ischemic cerebrovascular diseases. For example, Garrido et al. found that miR-155 silenced can decrease brain tissue damage, protect brain microvasculature, and promote the recovery of animal functions. Moreover, highly expressed miR-155 can regulate the development of inflammatory T cells, promote
inflammation cytokines secretion, while downregulation of miR-155 can reduce the inflammation secretion.28) Wen et al.29) showed that miR-155 expression was significantly enhanced in ischemic cerebral tissues and OGD-treated BV2 cells compared with that in control mice and BV2 cells. Consistent with this result, our research showed that OGD/R treatment increased miR-155 expression in SH-SY5Y cells, similar with the result of Wen et al.29), whereas 160 µg/mL PNS treatment decreased miR-155 expression in OGD/R-treated SH-SY5Y cells. Moreover, overexpression of miR-155 reversed the protective effects of PNS on OGD/R-induced injury by inhibiting cell proliferation and promoting cell apoptosis and the expression of IL-1β, IL-6, and TNF-α in OGD/R-treated SH-SY5Y cells. These results suggested that miR-155 promoted the secretion of inflammatory cytokines and enhanced OGD/R-induced injury in SH-SY5Y cells. These results also suggested that PNS attenuated OGD/R-induced injury in SH-SY5Y cells by inhibiting miR-155 expression.

CONCLUSION

The present study showed that PNS attenuated OGD/R-induced injury in human undifferentiated SH-SY5Y cells by regulating the expression of inflammatory factors through miR-155. miR-155 is represent a potential target for PNS to prevent and treat inflammatory brain damage after cerebral ischemia. We will conduct further experiments to verify whether PNS can regulate stroke development through miR-155 in vivo.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
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Figure legends:

Fig. 1: Effects of different concentration of PNS on the proliferation of SH-SY5Y cells at different times. *P <0.05, **P <0.01, ***P <0.001 vs 0 µg/mL PNS.

Fig. 2: Effects of PNS on miR-155 expression in OGD/R-treated SH-SY5Y cells. (**P <0.001, vs OGD/R-treated SH-SY5Y group. ###P <0.001 vs PNS + NC group under OGD/R)

Fig. 3: Effects of PNS and overexpression of miR-155 on the proliferation of oxygen-glucose deprivation/reoxygenation (OGD/R)-treated SH-SY5Y cells. (**P <0.001, vs OGD/R-treated SH-SY5Y group. ###P <0.001 vs PNS + NC group under OGD/R)

Fig. 4: Effects of PNS and overexpression miR-155 on cell cycle in OGD/R-treated SH-SY5Y cells. A-C: Cell cycle distribution in OGD/R-treated SH-SY5Y cells at 24, 48, and 72 h in all groups. (**P <0.001, vs OGD/R-treated SH-SY5Y group. ###P <0.001 vs PNS + NC group under OGD/R)

Fig. 5: Effects of PNS and overexpression of miR-155 on cell apoptosis in OGD/R-treated SH-SY5Y cells. A-C: Apoptosis rate after OGD/R treatment for 24, 48, 72 h in all groups. (**P <0.001, vs OGD/R-treated SH-SY5Y group. ###P <0.001 vs PNS + NC group under OGD/R).

Fig. 6: Effects of PNS and miR-155 on the levels of IL-1β, IL-6, and TNF-α in OGD/R-treated SH-SY5Y cells. The levels of IL-1β (A), IL-6 (B), and TNF-α (C) in OGD/R-treated SH-SY5Y cells at 24, 48, and 72 h were measured by qRT-PCR. (**P <0.01, ***P <0.001, vs OGD/R-treated SH-SY5Y group. ###P <0.01, ###P <0.001 vs PNS + NC group under OGD/R)
Figure 1
Figure 2
Figure 3

[Graph showing OD₄₉₀ over time (0h, 24h, 48h, 72h) for different treatments labeled as SH-SY5Y, SH-SY5Y, PNS, PNS+NC, and PNS+miR-155. The graph includes statistical significance indicators (***, ###).]
Figure 4

[Bar charts showing cell cycle distribution for different conditions labeled as G1, S, and G2.]
Figure 5

A

B

C

Apoptotic rate (%)

OGD/R

Apoptotic rate (%)

OGD/R

Apoptotic rate (%)

OGD/R

SH-SYSY

SH-SYSY

PNS

PNS+NC

PNS+miR-155

SH-SYSY

SH-SYSY

PNS

PNS+NC

PNS+miR-155

SH-SYSY

SH-SYSY

PNS

PNS+NC

PNS+miR-155

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Figure 6

A

B

C

IL-1β

IL-6

TNF-α

Relative expression

24h 48h 72h

SH-SYSY

SH-SYSY

PNS

PNS+NC

PNS+miR-155

ODG/R

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