

# Neuroprotective Effects of Panax Notoginseng Saponins against Cerebral Ischemia via Improving Glutamate Metabolism Pathway: In Vivo and In Vitro

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## Research

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# Abstract

**Background:** Ischemic stroke patients suffer from relatively limited treatment options. Studies have shown that glutamate is the most important excitatory neurotransmitter in the central nervous system. However, excessive glutamate in the extracellular cause cell apoptosis, and neurodegenerative processed after cerebral ischemia stroke. Glutamate metabolism pathway is necessary for glutamate clearance after ischemic stroke. Here we investigated the *in vivo* and *in vitro* effects of *Panax Notoginseng* Saponins (PNS) on glutamate metabolism pathway.

**Methods:** we used mice impaired by middle cerebral artery occlusion (MCAO) and astrocytes exposed to oxygen-glucose deprivation/ reoxygenation (OGD/R) to test the potential mechanism of PNS. *In vivo*, we determined the cerebral infarction volume and measured brain water content. *In vitro*, we measured the astrocytes viability and evaluated the morphology of astrocyte. In addition, glutamate uptake,  $\text{Na}^+\text{-K}^+$ -ATPase activity, the expression levels of glutamate transporter GLT-1 and glutamine synthetase (GS) were determined *in vivo* and *in vitro*.

**Results:** *In vivo*, we demonstrated that PNS could significantly decrease cerebral ischemia injury and improve neurological function in mice impaired by MCAO. *In vitro*, we found that PNS increased astrocytes viability, inhibited LDH leakage, and improved morphology of astrocytes under OGD/R. Additionally, we reported that both *in vivo* and *in vitro*, PNS enhanced the glutamate uptake and  $\text{Na}^+\text{-K}^+$ -ATPase activity, and up-regulated the expression levels of glutamate transporter GLT-1 and GS.

**Conclusion:** this study suggested that PNS protects against cerebral ischemia induced brain damage. The possible mechanism is related with inhibiting glutamate accumulation by improving glutamate metabolism pathway.

## Introduction

Cerebral ischemic stroke is one of the main diseases endangering human health after malignant tumor and heart disease, which has the characteristics of high morbidity and mortality, and the high rate of relapse[1,2,3]. The mechanism of that cerebral ischemic stroke mainly involves excitability amino acid toxicity, oxidative stress, calcium overload, inflammatory reactions and cell death [4,5]. It is well known that glutamate is the most abundant amino acid in the nervous system, and plays an essential neurotransmitter between neurons [6]. However, hypoxia and ischemia may cause excessive glutamate release to the synaptic gap during ischemic stimulation, and glutamate excitotoxicity would further result in mitochondrial impairment, loss of membrane potential and  $\text{Ca}^+$  [7], changing the neurons' permeability and inducing neuronal apoptosis [8].

Astrocytes play important roles in the development and maintenance of normal brain function, particularly in preventing excitotoxicity by eliminating excessive excitatory extracellular neurotransmitters. The main mediators of glutamate clearance are glutamate transporters (GluTs) in astrocytes [9]. Of all types of glutamate transporters, glutamate transporter-1 (GLT-1/EAAT-2) plays a key

role in inhibiting glutamate-induced neurotransmission, sustaining the normal concentration of extracellular glutamate in the central nervous system, which is one of the Na<sup>+</sup>-K<sup>+</sup>-dependent transporters [10]. GLT-1 undertakes almost all the glutamate transport in the cortex and hippocampus, and predominantly exists in the astrocytes [11]. Following synaptic release, glutamate is mainly taken up by the astrocytic glutamate transporter GLT-1, and then rapidly transformed into glutamine by glutamine synthetase (GS), an astrocyte-specific enzyme [12,13]. Subsequently, the glutamine in astrocytes is transported back to the presynaptic terminals and converted to glutamate for reuse. This process is named glutamate-glutamine cycle. Interfering the glutamate-glutamine cycle or its key components such as GLT-1 and GS have been reported to influence the glutamate concentration and glutamate-induced excitotoxicity. Therefore, the important components of glutamate metabolism pathway may be potential therapeutic targets for excitotoxicity in cerebral ischemic stroke.

*Panax notoginseng* is the dried roots and rhizomes of *Panax notoginseng* (Burk.) F. H. Chen, It has been used for the treatment of hemoptysis, hemostatic, and hematoma in China and other Asian countries. *Panax Notoginseng* Saponins (PNS) is the active ingredient of total saponins purified from *Panax notoginseng*, including various saponins such as notoginsenoside R1, ginsenoside Rg1, Rb1, Rd, Re, Rf, etc. Pharmacological studies suggested that PNS can improve microcirculation, increase cerebral blood flow, suppression of platelet aggregation, reduce blood viscosity, and inhibition of inflammation reaction, which is widely used to treat cerebral ischemic stroke and cardiovascular disease [14,15]. Moreover, previous studies have shown the PNS play a key role in improving energy metabolism and glutamate uptake, protecting brain tissue *in vivo* and *in vitro* [16,17,18]. However, the effect of PNS on extracellular glutamate metabolism remains unclear.

In the present study, we assessed the potential roles of PNS on brain tissue *in vivo* and astrocytes *in vitro* following cerebral ischemia injury. Furthermore, we investigated whether PNS elicited glutamate clearance effects by mediating the crucial role of glutamate metabolism pathway.

## Methods

### Animal

Adult ICR mice (28~30 g) and neonatal (P0-1) ICR mice were obtained from Vital River Laboratory Animal Technology Co., Ltd, Beijing, China. The certificate number was SCXK (Jing) 2012-0011. Adult ICR mice were fed with a standard pellet diet and tap water *ad libitum*, and they are housed and maintained in 12 h light/darkness and standard humidity and temperature in the laboratory. The animals were acclimatized for three days before experiments. The principles of laboratory animal care guidelines, which were approved by the Animal Ethics Committee at the Beijing University of Chinese Medicine, were strictly followed (NO. BUCM-4-2017112701-5021, 27 November 2017). All efforts were made to minimize animal suffering and to reduce the animals used for experiments.

# Drug preparations

PNS (purity > 98.0%) were obtained from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China; batch no. MUST-16060601). The quality standard of PNS complied with Chinese Pharmacopoeia (2015 edition), and the contents of major effective constituents in PNS were notoginsenoside R1 (5.0%), ginsenoside Rg1 (25.0%), ginsenoside Re (2.5%), ginsenoside Rb1 (30.0%) and ginsenoside Rd (5%) (Fig. 1). PNS were kept in -20°C, and diluted with normal saline to different concentrations.

## Middle Cerebral Artery Occlusion Model

Cerebral infarction was produced following the suture middle cerebral artery occlusion (MCAO) method and modified as previously described [19]. Briefly, mouse was anesthetized with 7% chloral hydrate (60 µL/10 g, i.p.). The right common carotid artery (CCA) and external carotid artery (ECA) were exposed and carefully separated from the vagus nerve. MCAO was produced by advancing a silicone-coated nylon monofilament through the ECA into the internal carotid artery and then into the circle of Willis until a mild increase in resistance was felt. After securing the filament in place, the surgical site was closed with 6-0 Vicryl suture. Each mouse was then placed in individual cages, and the rectal temperatures of the mice were maintained at 36.0±0.5°C throughout the MCAO using warming pads. The occluding filament was removed 60 min after occlusion. In the control group, this same operation was performed but no suture was not advanced into the MCA. PNS with the concentration of 60mg/kg or 30 mg/kg was applied intragastrically 1 h after MCAO.

Mice were randomly divided into five groups (24 animals each): (1) sham group (sham), administered double distilled water (1 mL/100 g, i.g.). (2) MCAO group (MCAO), administered double distilled water (1 mL/100 g, i.g.). (3) Nimodipine group (Nim), administered nimodipine (13.5 g/kg, i.g.) 2 h after surgery, and then once a day. (4) PNS group, administered PNS (60 or 120 g/kg, i.g.) 2 h after surgery, and then once a day.

## Evaluation of Neurological deficits

The neurological deficits of MCAO mice after 72 h reperfusion were evaluated by an operator blinded to the experimental method according to Longa's method [20]: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; 4, no spontaneous locomotor activity or barrel rolling.

Following evaluations, mice were anesthetized with 7% chloral hydrate and decapitated. The ischemic cerebral cortex was rapidly removed and stored at -70°C until use for Western blot, RT-PCR, and analysis of glutamate uptake and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity.

## Measurement of infarct volume

Infarct volume was measured 72 h after reperfusion. In brief, mice were anesthetized by 7% chloral hydrate (60  $\mu$ L/10 g, i.p.), and the brains were immediately removed and frozen at  $-20^{\circ}\text{C}$  for 15 min. Brains were sectioned into 5 coronal slices (2-mm thick) that were immediately immersed in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, USA) saline solution at  $37^{\circ}\text{C}$  for 30 min in the dark, and then fixed in 4% paraformaldehyde solution. Brain slices were photographed and measured using Image-Pro Plus software. Infarct volumes are expressed as percentages of the whole brain volumes.

## Evaluation of Brain water content

The mice were anesthetized and brain tissues were harvested at 72 h after reperfusion. The brains were quickly weighed on the electronic analytical balance and recorded the wet weight. Then whole brains were dried at  $110^{\circ}\text{C}$  for 24 h to obtain the dry weight. The percent brain water content was calculated as follows:  $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$ .

## Preparation of Mouse Cortical Astrocytes Cultures

The culture of astrocytes were obtained from the cortex of five neonatal (P0-1) ICR mice in each preparation, according to the procedures described previously [21]. Briefly, the neonatal ICR mice cortex was isolated aseptically, and then minced and digested in 0.125% trypsin solution (Invitrogen Life Technologies Inc., Grand Island, NY, USA) at  $37^{\circ}\text{C}$  for 15 min. subsequently, the cortex fragment suspensions were filtered through a 150  $\mu\text{m}$  cell strainer, and centrifuged at 1000 r/min for 5 min. The cells were plated to 0.01% poly-L-lysine-coated T-25  $\text{cm}^2$  flask, and then supplemented in astrocyte medium (AM, ScienCell Research Laboratories, Carlsbad, CA, USA) supplemented with 1% astrocyte growth supplement (AGS), 2% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S), and cultured in the humidified incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  (Thermo Fisher Scientific, Waltham, MA, USA). The culture medium was renewed every 2 days. The culture cells were stained with primary antibody recognizing rabbit polyclonal glial fibrillary acidic protein (GFAP, Abcam, Cambridge, MA, USA, ab7260) (1:100), the images were captured using an ECLIPSE Ti laser confocal microscope (Nikon Corporation, Tokyo, Japan).

## OGD/R Injury to Astrocytes

To simulate the conditions of cerebral ischemia in vitro, we cultured the experimental cells in oxygen-glucose deprivation followed by reperfusion as mentioned earlier [22]. Briefly, the culture medium was replaced with free-glucose and free-serum medium, and then transferred into 95%  $\text{N}_2$  and 5%  $\text{CO}_2$  conditions for 6 h at  $37^{\circ}\text{C}$ . After 6 h, the cells were moved into the humidified atmosphere of 5%  $\text{CO}_2$  at

37°C and replaced the normal culture medium for 24 h. At the OGD/R same time, added different concentrations of PNS into cells. The astrocytes were divided six groups, namely control group, OGD/R group and PNS (10 µg/mL, 20 µg/mL, 50 µg/mL, and 100 µg/mL) group. The best effect was observed with a concentration of 20 µg/mL PNS, and thus 20 µg/mL PNS was used for further study.

## CCK-8 Assay to Measure Cell viability

Cell viability was assessed using a CCK-8 colorimetric assay. Astrocytes were seeded in 0.01% poly-l-lysine-coated 96-well plates for 24 h, and then subjected to the various treatments described previously. Then each culture well added 10 µL CCK-8 solution (Dojindo Laboratories, Kyushu, Japan) and cells were incubated for 2 h with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C, Absorbance was measured at 450 nm on enzyme-linked immunosorbent assay reader (Thermo Labsystems, Helsinki, Finland). Each experiment was repeated for three times. The cell viability from the CCK-8 assay was expressed as a percentage using the following equation:

$$\text{Viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Where *A* is the absorbance value.

## LDH Release Assay to Measure Cell Cytotoxicity

Cell cytotoxicity was measurement via the LDH release assay. Astrocytes were seeded in 96-well plate coated 0.01% poly-l-lysine, allowed to adhere overnight and incubated to experimental conditions. After incubation, a mouse LDH cytotoxicity assay was used to measure the LDH release. The assay was carried out according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

## Morphology Observation of Astrocytes

Astrocytes were seeded a density of 5 × 10<sup>4</sup> cells/mL in poly-l-lysine-coated 6-well plates with a density of 5 × 10<sup>4</sup> cells/mL and cultured for 24 h. Then, the cells were treated with various experimental conditions. After treatment, the morphology of astrocytes were visualized on an inverted microscope (Nikon Corporation, Tokyo, Japan).

## Glutamate Uptake Measurement

The ischemic hemisphere cortex tissues and cell supernatants were prepared after treating with varying experimental conditions. For data from *in vivo*, the cortex was isolated from the ischemic hemisphere of mouse. After homogenizing the cortex, glutamate uptake was determined by Glutamic Acid assay kit (Nanjing Jiancheng, China) according to the manufacturer's protocols. Protein concentration was estimated by Bradford assay using the BCA (Bicinchoninic acid) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

For data from *in vitro*, well plates, and the glutamate uptake was measured using the Amplex Red Glutamic Acid assay kit (Invitrogen Life Technologies Inc., Grand Island, NY, USA). After 6 h of OGD astrocyte culture medium was replaced by HEPES buffer containing 25 mM glucose and 500  $\mu$ M glutamate. After 24 h incubation, 50  $\mu$ L of supernatants was transferred into 96-well plates, and then mixed with 50  $\mu$ L substrate mixture and incubated at 37°C for 30 min. Fluorescence was measured using an automated microplate reader at a wavelength of 530 nm (vs. reference wavelength of 590 nm). Glutamate concentrations were calculated from the standard curve with known glutamate amounts.

## Na<sup>+</sup>-K<sup>+</sup>-ATPase Activity Measurement

The ischemic hemisphere cortex tissues or astrocytes were prepared after treating with varying experimental conditions. After treatment, the ischemic hemisphere cortex tissues or astrocytes were performed by using lysis buffer. The Na<sup>+</sup>-K<sup>+</sup>-ATPase activity was determined using an ATPase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. ATPase activity was expressed as  $\mu$ mol of released inorganic Pi by 1 mg of protein per hour. Protein concentration was estimated by Bradford assay using the BCA (Bicinchoninic acid) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

## Immunofluorescent staining

For images from *in vitro*, the astrocytes were fixed in cold 4% paraformaldehyde for 30 min, permeabilized by 0.1% Triton X-100 for 10 min, blocked with normal goat serum for 30 min. Immunostaining was performed using rabbit polyclonal anti-GLT-1 (Abcam, Cambridge, MA, USA, ab41621, 1:200) and mouse monoclonal anti-GS (Abcam, Cambridge, MA, USA, ab64613, 1:200) overnight at 4°C. After washed by PBS, goat anti-rabbit IgG/FTIC (Zhong Shan Jin Qiao, Beijing, China, 1:100) and goat anti-rabbit IgG/TRITC (Zhong Shan Jin Qiao, Beijing, China, 1:100,) for 1 h at room temperature. Then, the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The images were visualized on an ECLIPSE Ti laser confocal microscope (Nikon Corporation, Tokyo, Japan) and calculated with Image-Pro Plus 6.0 software (Media Cybernetics, Silver spring, MD, USA).

## Real time-PCR analysis

Total RNA was extracted from cortex of ischemic hemispheres or cultured astrocytes using TRIzol reagent (Invitrogen Life Technological, Carlsbad, CA, USA) according to the manufacturer's protocol and total RNA purity were detected by spectrometric analysis. 8  $\mu$ l of RNA was transcribed using TIANScript RT kit (Thermo, USA). The PCR amplification was performed as follows: pre-incubation at 95°C for 10 min, amplification at 95°C for 10 s and at 59°C for 60 s. The  $\beta$ -actin was used as a normalized control. The relative expression levels of the genes were determined using the  $2^{-\Delta\Delta C_t}$  method. Forward/reverse primers were as follows: GLT-1 (forward, 5'-CGATGAGCCAAAGCACCGAA-3', and reverse, 5'-GCATCCAGGCTAGACA CCTC-3'), GS (forward, 5'-TATTACTGCGGTGTGGGAGC-3', and reverse, 5'-CCCATTCCGGATCCCCTCACA-3'), and  $\beta$ -actin (forward, 5'-GAAGCTGTGCTATG TTGCTCTA-3', and reverse, 5'-CAATAGTGATGACCTGGC CGT-3').

## Western Blot Analysis

The protein from cortex of ischemic hemispheres or cultured astrocytes was prepared and extracted in lysis buffer. The protein concentrations were measured using a BCA protein assay kit. Briefly, protein samples (15  $\mu$ g) were loaded on a 10% SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (PVDF, Millipore™, Billerica, MA, USA). The membrane was probed with primary antibody (rabbit polyclonal anti-GLT-1, Abcam, Cambridge, MA, USA, ab41621, 1:1000; mouse monoclonal anti-GS, Abcam, Cambridge, MA, USA, ab64613, 1:1000,) overnight at 4°C. After washing with PBS for three times, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1: 10000) at room temperature for 40 min. Finally, specific signals of proteins were visualized using an enhanced chemiluminescence kit. The relative densities of the bands were quantified with Image-Pro Plus 6.0 software (Media Cybernetics, Silver spring, MD, USA).

## Statistical Analysis

All data are presented as the mean  $\pm$  standard error mean (SEM). Our results were analyzed by One-way ANOVA, followed by a Student-Newman-Keuls (SNK) test. SPSS 20.0 software was used for statistical analyses (SPSS Inc., Chicago, IL, USA).  $P < 0.05$  indicated statistical significance.

## Results

### Protective effect of PNS on middle cerebral artery occlusion (MCAO) injury in mice

To confirm the potential preventative effects of PNS on mice subjected to MCAO, mice were treated with PNS (60 or 120 mg/kg/day) for three consecutive days after the induction of cerebral ischemia. The neurological scores were evaluated 72 h after MCAO. As shown in the Fig. 2A, B, staining of brain

sections with TTC revealed that treatment with PNS (120 mg/kg) and nimodipine (13.5 mg/kg) greatly alleviated the total infarct volume compared with the sham group ( $P<0.05$ ). Moreover, the neurological scores were significantly attenuated in PNS (120 mg/kg) treated mice compared with mice in the sham group ( $P<0.05$ ), whereas no significant neurological dysfunction was observed in the MCAO group (Fig. 2C). The brain water content in PNS (60, 120 mg/kg) and nimodipine (13.5 mg/kg) were markedly reduced compared with MCAO group ( $P<0.05$ ), data as shown in Fig. 2D. These results suggested that PNS treatment improved the function outcome after MCAO injury.

## **PNS increased glutamate uptake and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in MCAO mice**

We evaluated the effect of PNS on glutamate concentration and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in ischemic cortex at 72 h after MCAO. As shown in Fig. 3, comparing with the sham group, the MCAO mice showed significantly higher levels of glutamate concentration but significant lower levels of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity ( $P<0.05$ ). Compared to MCAO group, PNS (60, 120 mg/kg) markedly decreased the glutamate concentration ( $P<0.01$ ), while obviously increased the Na<sup>+</sup>-K<sup>+</sup>-ATPase activity ( $P<0.05$ ). The above data are indicated that PNS could increase glutamate uptake and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity after cerebral ischemia.

## **PNS up-regulated the expression of GLT-1 and GS in MCAO mice**

To detect the expression level of GLT-1 and GS after PNS treatment under cerebral ischemia, western blot and real-time PCR assay were used. The study indicates that MCAO resulted in the lower expression of GLT-1 and GS protein (Fig. 4A) and mRNA (Fig. 4B) in ischemic cortex compared with sham group ( $P<0.01$ ). However, the expression of GLT-1 protein ( $P<0.01$ ) and mRNA ( $P<0.05$ ) showed a significant up-regulate after PNS (60 or 120 mg/kg) treatment (Fig. 4A), and the expression of GS protein and mRNA were up-regulated after PNS (60 or 120 mg/kg) treatment although the changes were not significant (Fig. 4B). According to the above description, we assumed that PNS alleviated the degradation of glutamate metabolism related proteins induced by cerebral ischemia, which might attenuated glutamate toxicity.

## **Protective Effect of PNS on Astrocytes Injury Induced by Oxygen-Glucose Deprivation/ reoxygenation (OGD/R)**

To determine whether the effect of PNS on cytotoxicity induced in astrocytes under OGD/R, we analyzed cell viability and toxicity *in vitro* by CCK-8 assay and LDH release assay. As shown in Fig. 5, the data showed a decrease in cell viability and an increase in LDH release during OGD/R. Compared to OGD/R group, PNS (10 µg/mL - 50 µg/mL) were obviously increased cell viability (Fig. 5A), and significantly

reduced LDH released (Fig. 5B) ( $P<0.01$ ). Based on these findings, 20  $\mu\text{g}/\text{mL}$  PNS was chosen as the final treatment concentration. Next, we detected morphology observation of astrocytes. Images showed astrocytes appeared unregulated shapes and had good refraction in the control group. After OGD/R injury, the cellular refraction was faded, the cellular protrusions became shorter or even disappeared. The incubation of 20  $\mu\text{g}/\text{mL}$  PNS markedly improved cellular state, and enhanced relationship between cellular protrusions (Fig. 5C). The above data shown that PNS may attenuate OGD/R-induced cytotoxicity in astrocytes.

## **PNS increased glutamate uptake and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in astrocytes treated with OGD/R**

To investigate the effects of PNS, astrocytes were incubated with PNS (20  $\mu\text{g}/\text{mL}$ ) treatment in OGD/R conditions. Next, the glutamate uptake and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in astrocytes were measured. As shown in Fig. 6, following OGD/R injury, the glutamate uptake and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity were obviously decreased. However, 20  $\mu\text{g}/\text{mL}$  PNS treatment markedly increased the glutamate uptake (Fig. 6A) and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity (Fig. 6B) in astrocyte cultures compared to OGD/R group ( $P<0.01$ ). The results show that PNS can improve OGD/R-induced disorder of glutamate uptake and energy metabolism in astrocytes.

## **PNS up-regulated the expression of GLT-1 and GS in astrocytes treated with OGD/R**

Since GLT-1 and GS are important parts for clearing the extra-cellular glutamate accumulation, we detected the effect of PNS in OGD/R astrocyte cultures. The effects of PNS on regulating expression of GLT-1 and GS were demonstrated with RT-PCR and western blot. OGD/R injury obviously down-regulated the expression of GLT-1 and GS mRNA and protein ( $P<0.01$ ) compared with the control group. PNS (20  $\mu\text{g}/\text{mL}$ ) treatment could up-regulate the decreased expression of GLT-1 (Fig. 7A, C) and GS (Fig. 7B, D) mRNA and protein ( $P<0.01$ ) after OGD/R. To future confirm the expression of GLT-1 and GS, we observed GLT-1 and GS levels in the astrocytes by immunofluorescence staining. Images showed GLT-1 and GS fluorescence intensity were significantly reduced by OGD/R. PNS (20  $\mu\text{g}/\text{mL}$ ) were obviously enhanced the GLT-1 and GS fluorescence intensity (Fig. 7E, F). These results are consistent with those derived from western blotting and RT-PCR. These results founded that PNS may reduce the OGD/R-induced glutamate toxicity via up-regulating the expression of GLT-1 and GS.

## **Discussion**

In the current study, we found the glutamate clearance effect of PNS in cerebral ischemia injury with evidence from both *in vivo* and *in vitro* models, and the crucial role of glutamate metabolism pathway in mediating, which was represented with improving  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, up-regulated the expression of

GLT-1 and GS mRNA and protein. These results suggest that the neuroprotective effects of PNS in a stroke model both *in vivo* and *in vitro*, and provided a novel mechanism by which PNS ameliorates cerebral ischemia injury.

Stroke is a major cause of death and disability worldwide, and glutamate-induced excitotoxicity is an important factor responsible for cell death in many nervous system disorders [23]. Excessive extracellular glutamate activates ionotropic GluRs, such as NMDA, and then appearing a sequence of downstream injury mechanisms, including dysfunction of mitochondrial, inflammation, and  $\text{Ca}^{+}$  overload [24]. Thus, inhibition of the massive release of glutamate is in favor of protecting nervous system from cerebral ischemia injury. In this study, PNS decreased levels of glutamate concentration, which meant PNS alleviated excitatory toxicity in cerebral ischemia injury. In addition, PNS reduced infarct volume and improved the function outcome in MCAO mice. As known, astrocytes are a subset of glial cells, and play a key role in providing support and trophic for neurons. Astrocytes glutamate uptake is one of the most important aspects of glutamatergic neurotransmission. In the present study, we demonstrated the inhibition of glutamate uptake in astrocytes exposed to OGD/R conditions, as the same to previously studies [25,26]. PNS treatment could enhance the uptake of glutamate under OGD/R conditions. Therefore, this study indicated that PNS can cure the ischemia by alleviating excitatory toxicity.

$\text{Na}^{+}$  and  $\text{K}^{+}$  concentration gradients were maintained by  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$  in astrocytes. However, GluTs are  $\text{Na}/\text{K}$ -dependent membrane proteins, and glutamate uptake depends on cellular  $\text{Na}^{+}$  and  $\text{K}^{+}$  current [27]. Therefore,  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$  activity might relate to the GLT-1 function. A great of research found that glutamate uptake was limited on high  $\text{K}^{+}$ /low  $\text{Na}^{+}$  conditions [28,29]. In our study,  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$  activity was inhibited in the ischemic cortex in MCAO mice and astrocytes exposed to OGD/R. This could indicate GLT-1 dysfunction in cerebral ischemia. After PNS treatment,  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$  activity was markedly increased *in vivo* and *in vitro*. These data suggest that PNS might alleviate excitotoxicity during cerebral ischemia by improving  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$  activity.

GluTs are membrane functional proteins that regulate extracellular glutamate concentration. In astrocytes, GLAST and GLT-1 are crucial GluTs. GLAST is mainly identified in the cerebellum and requested to maintain extracellular glutamate at a normal level [30]. GLT-1 is found in different regions, which are cerebral cortex, hippocampus, and the striatum. More than 90% of glutamate uptake is depended on GLT-1 in these areas [31]. The studies found that dysfunction or knockout of GLT-1 gene leads to elevation of extracellular glutamate and exacerbation of acute cortical injury [32]. In contrast, targeted over-expression of GLT-1 decreased the glutamate overflow and reduced the cellular and behavioral deficits induced by ischemic insult [33]. In the present study, the expression of GLT-1 mRNA and protein levels were decreased obviously *in vivo* and *in vitro*. PNS administration significantly up-regulated the expression of GLT-1 mRNA and protein levels in the ischemic cortex in MCAO mice and astrocytes exposed to OGD/R, as previously reported by others [34,35,36]. Additionally, metabolic amination of glutamate to glutamine by GS could facilitate the extracellular glutamate clearance by decreasing the intercellular glutamate concentration, thus it has a protective role after cerebral ischemia

[37], and GS is only expressed in astrocytes. We have demonstrated a decrease in the expression of GS mRNA and protein in ipsilateral brain tissues of MCAO mice and cortical astrocytes exposed to OGD/R. The expression of GS mRNA and protein were up-regulated in the PNS treatment in vivo and vitro. These results suggest that PNS alleviates excitatory toxicity by restoring the expression of GLT-1 and GS.

## Conclusion

In conclusion, we showed that PNS could promote extracellular glutamate clearance via up-regulating the expression of GLT-1 and GS in MCAO mice and astrocytes exposed to OGD/R. This study revealed that administration with PNS play a potential therapeutic strategy for cerebral ischemic stroke.

## Abbreviations

PNS: panax notoginseng saponins; MCAO: middle cerebral artery occlusion; OGD/R: oxygen-glucose deprivation/reoxygenation; GLT-1: glutamate transporter-1; GS: glutamate synthetase; TTC: 2,3,5-triphenyltetrazolium chloride; DAPI: 4,6-diamidino-2-phenylindole.

## Declarations

## Acknowledgements

Not applicable

## Author Contribution

YQ, QM, YL, and CLF performed the experiments and analyzed the data; MKT and YQ were the major contributor in designing the research; YQ and QM were the major contributor in whiting the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets used and/or analyzed in this study will be available with reasonable request.

# Ethics approval and consent to participate

All experimental protocols were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (China). The principles of laboratory animal care guidelines, which were approved by the Animal Ethics Committee at the Beijing University of Chinese Medicine, were strictly followed.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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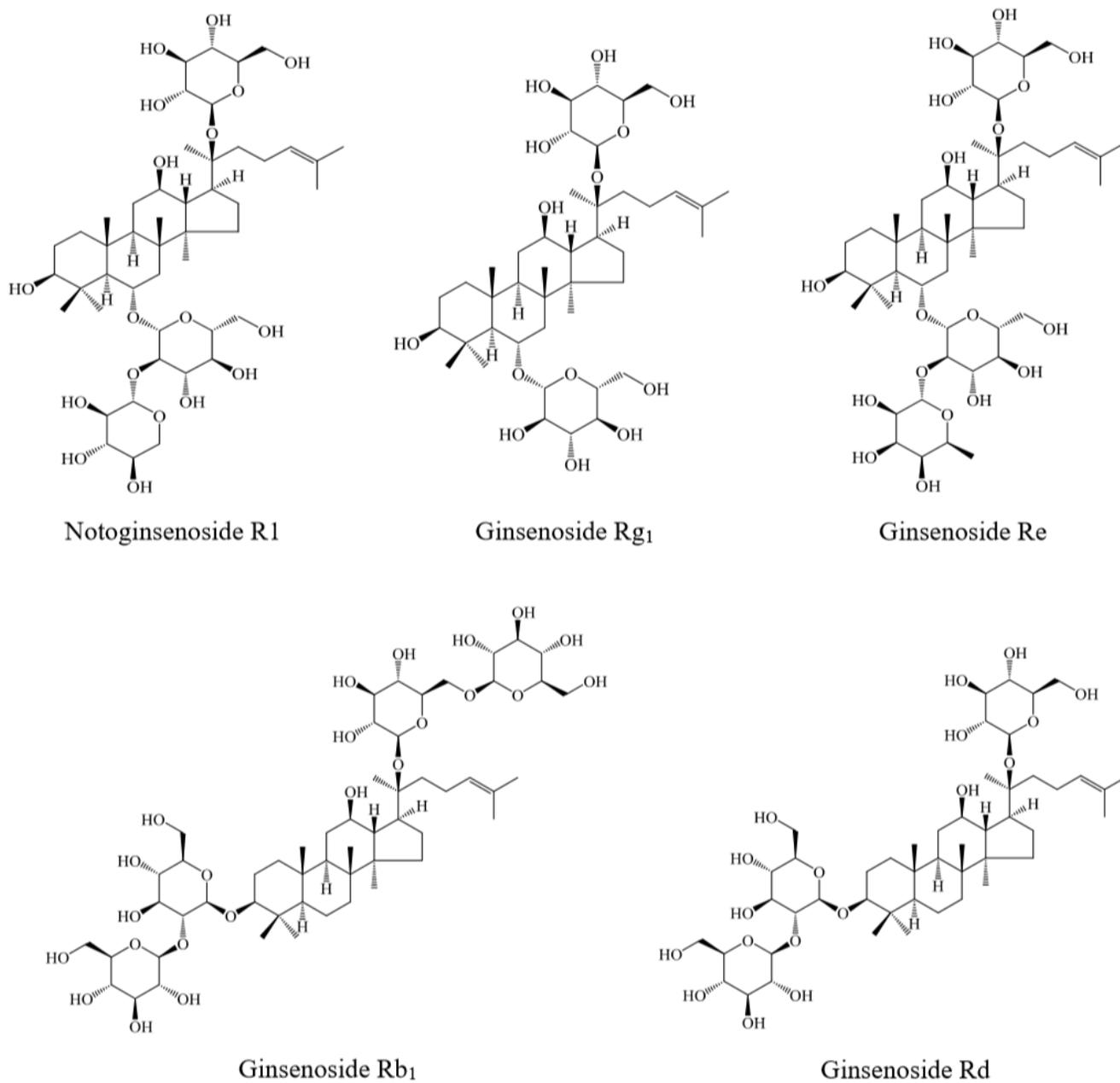
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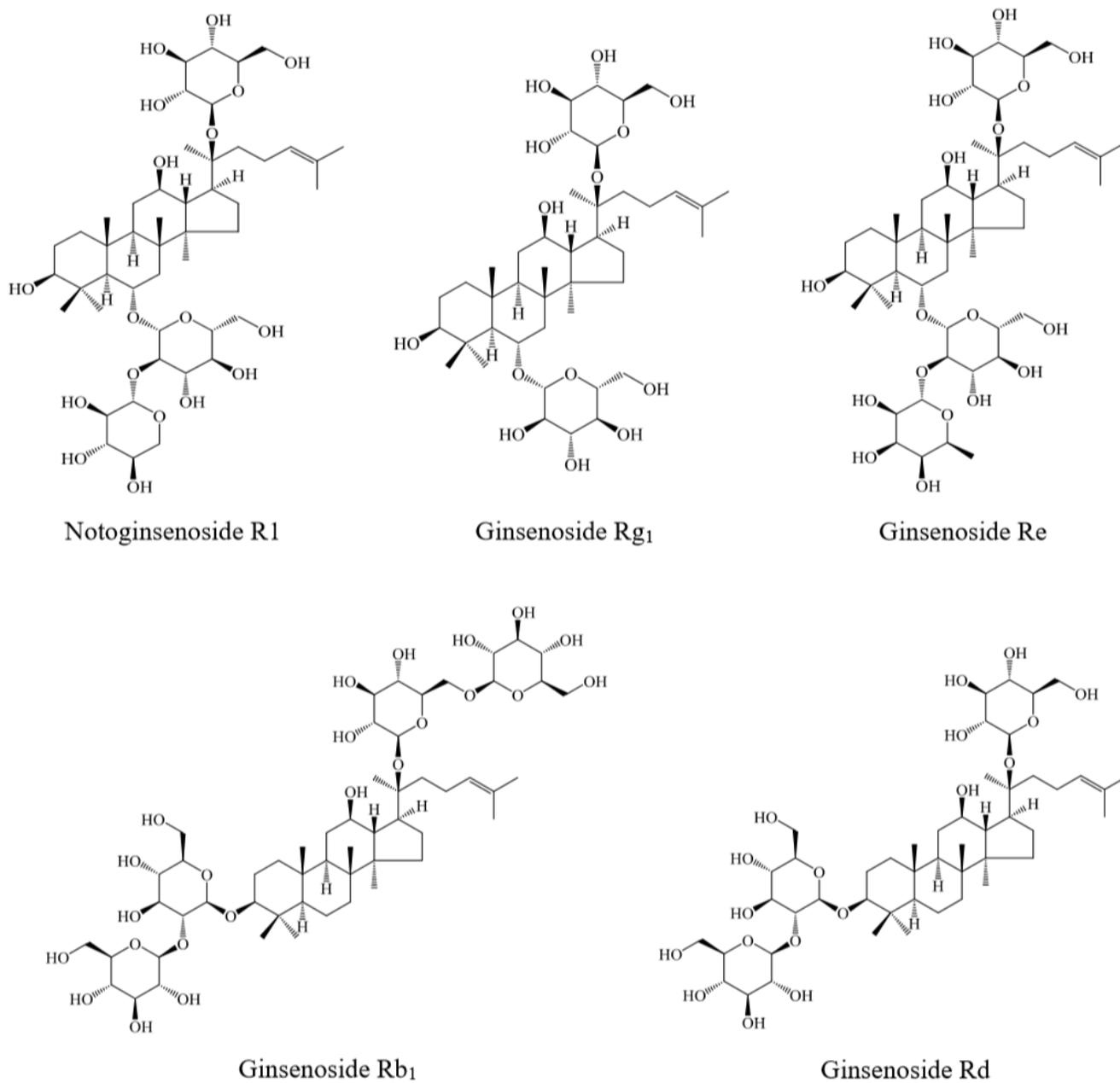
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## Figures



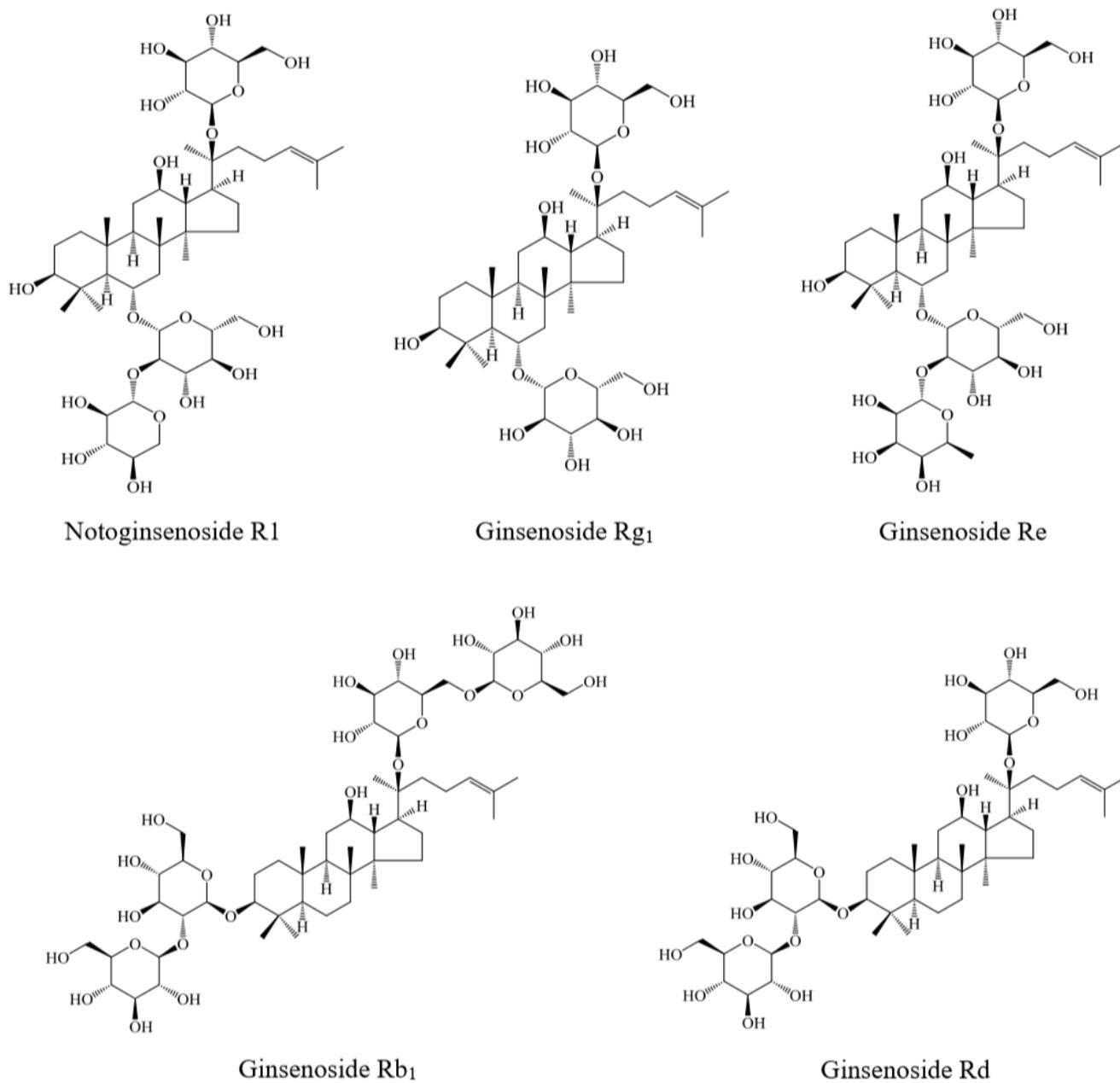
**Figure 1**

Chemical structures of the main compounds in *Panax Notoginseng* Saponins.



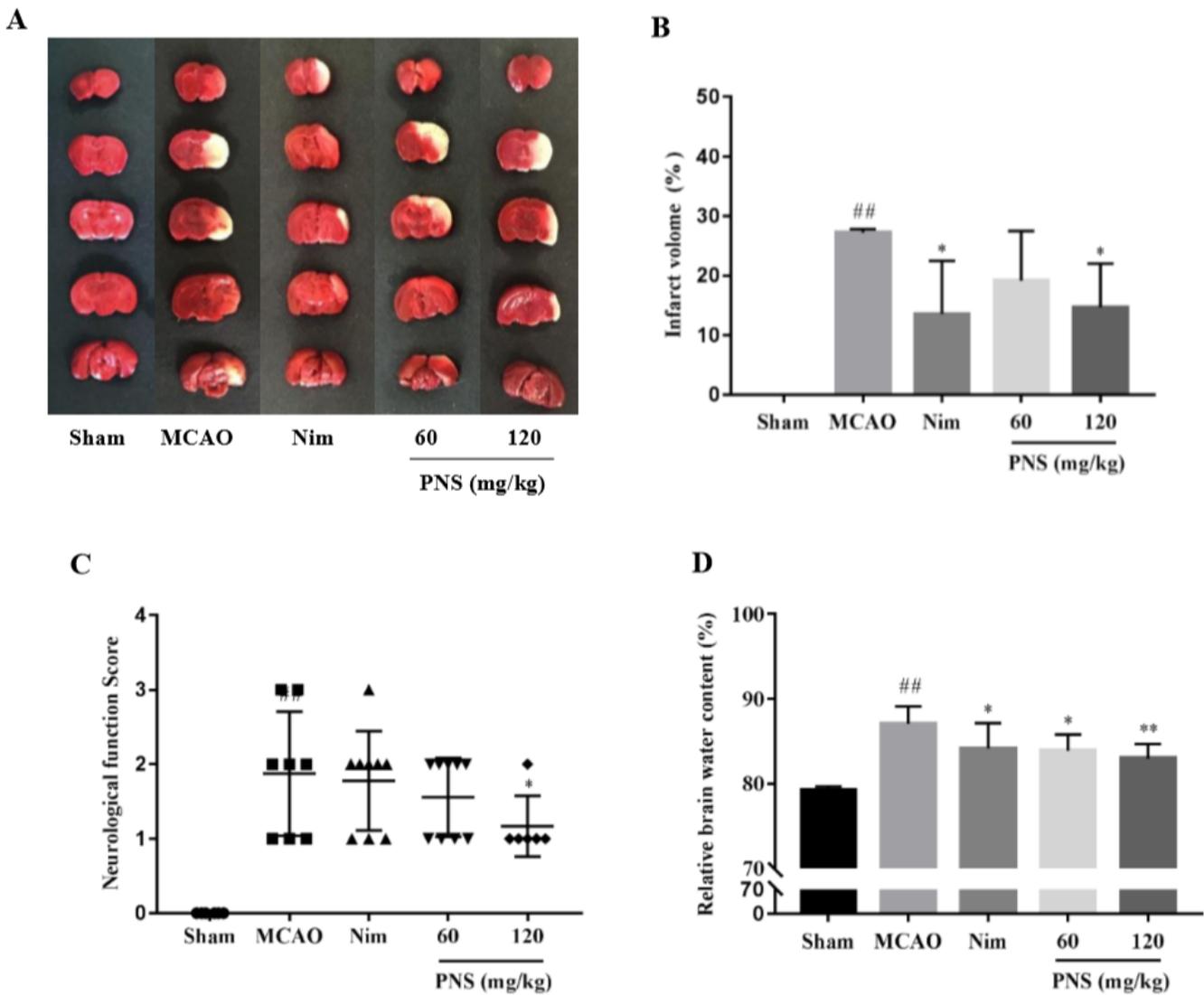
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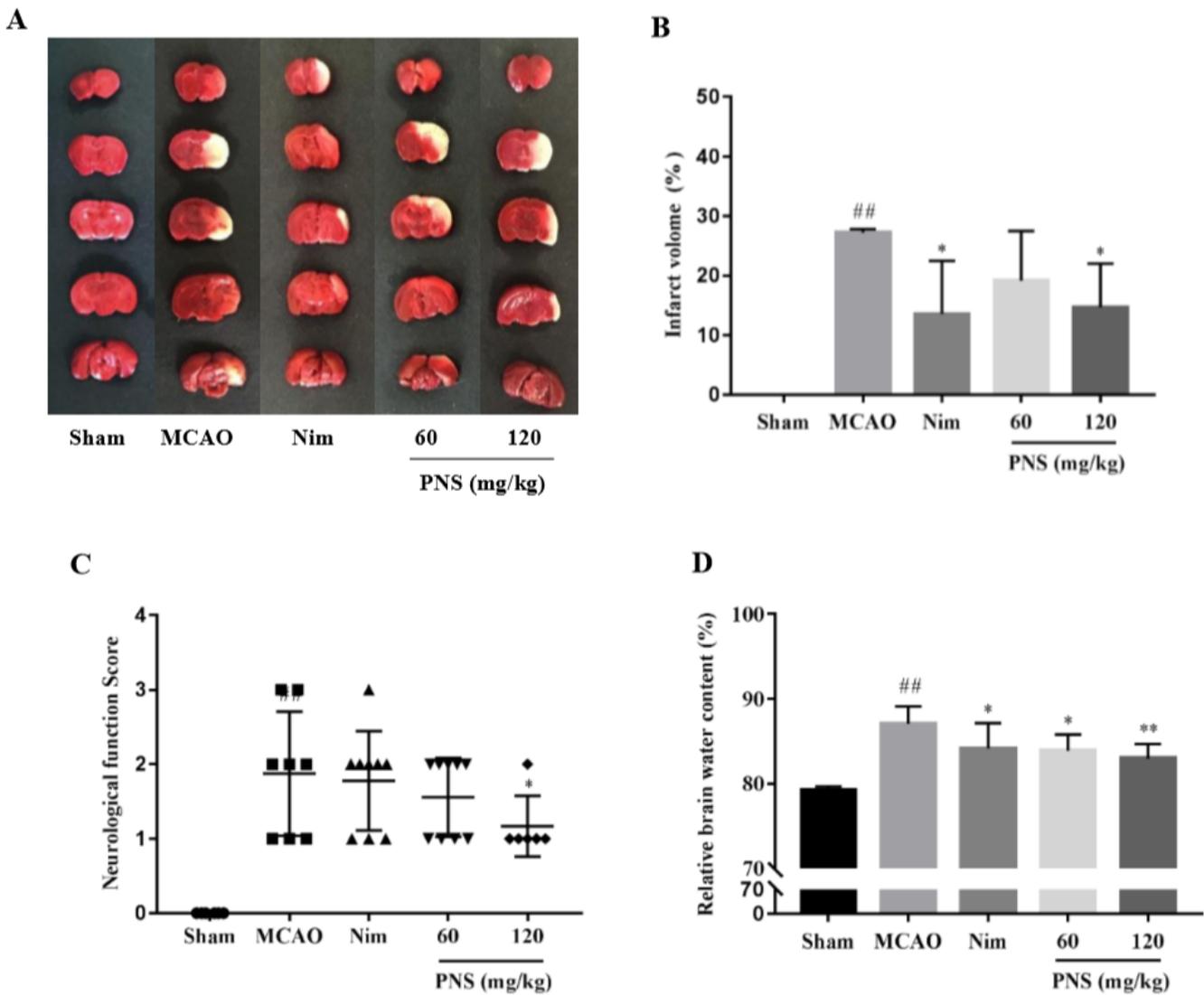
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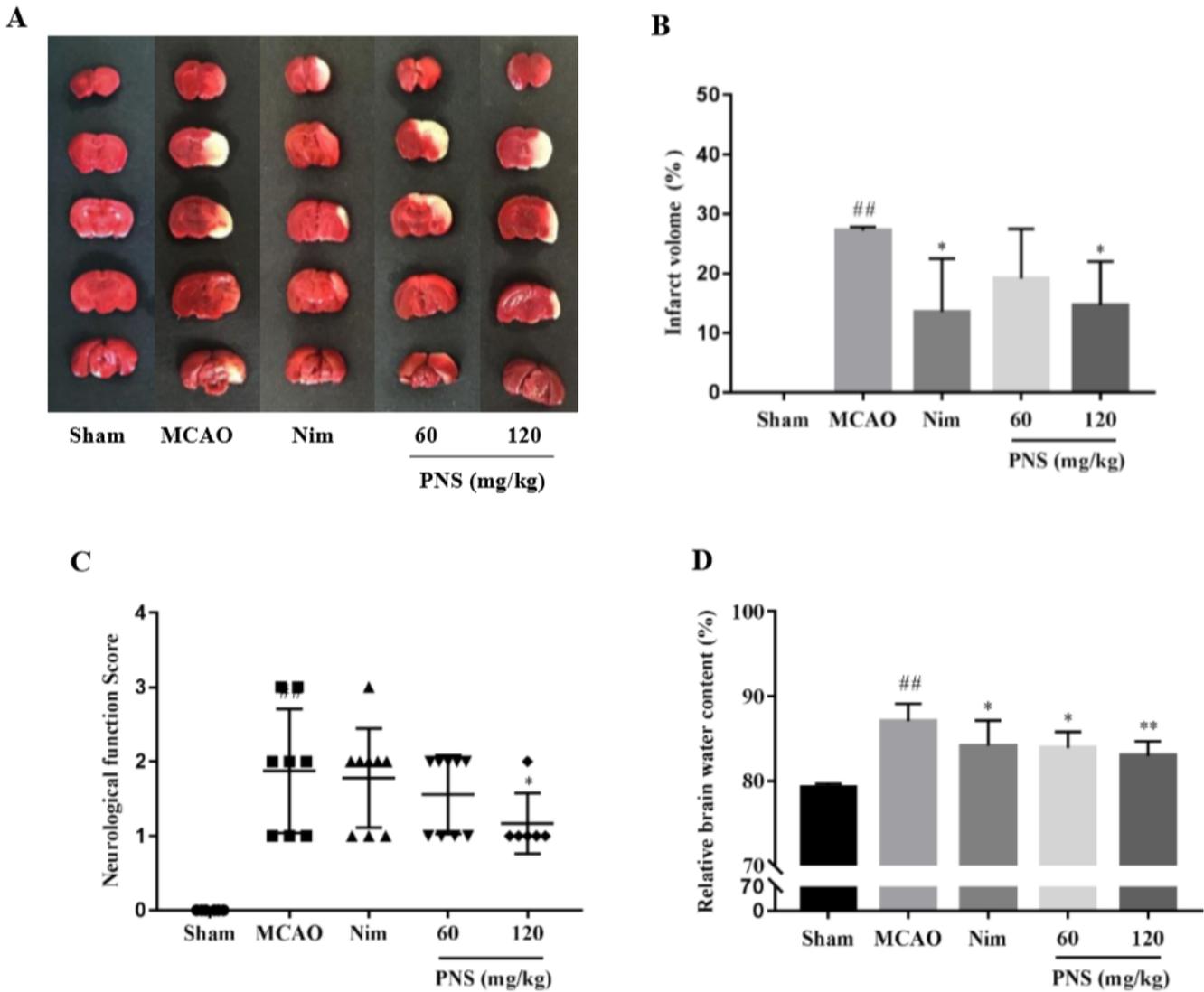
**Figure 2**

Preventive effects of PNS on brain injury after MCAO in mice. PNS (60 or 120 mg/kg) was orally administered for three consecutive days after inducing brain ischemia. (A) Images of cerebral infarction (white, infarct tissue; red, non-infarct tissue) were TTC-stained brain sections. (B) Quantitative analysis of infarct volume base on TTC staining was presented as % whole brain volume. (C) Neurological deficit by Longa scoring system. (D) Brain water content. Data was presented as mean  $\pm$  SEM,  $n=6\sim9$  pre group,  $\#P < 0.05$ ,  $\#\#P < 0.01$  compared with the sham group,  $*P < 0.05$ ,  $**P < 0.01$  compared with the MCAO group.



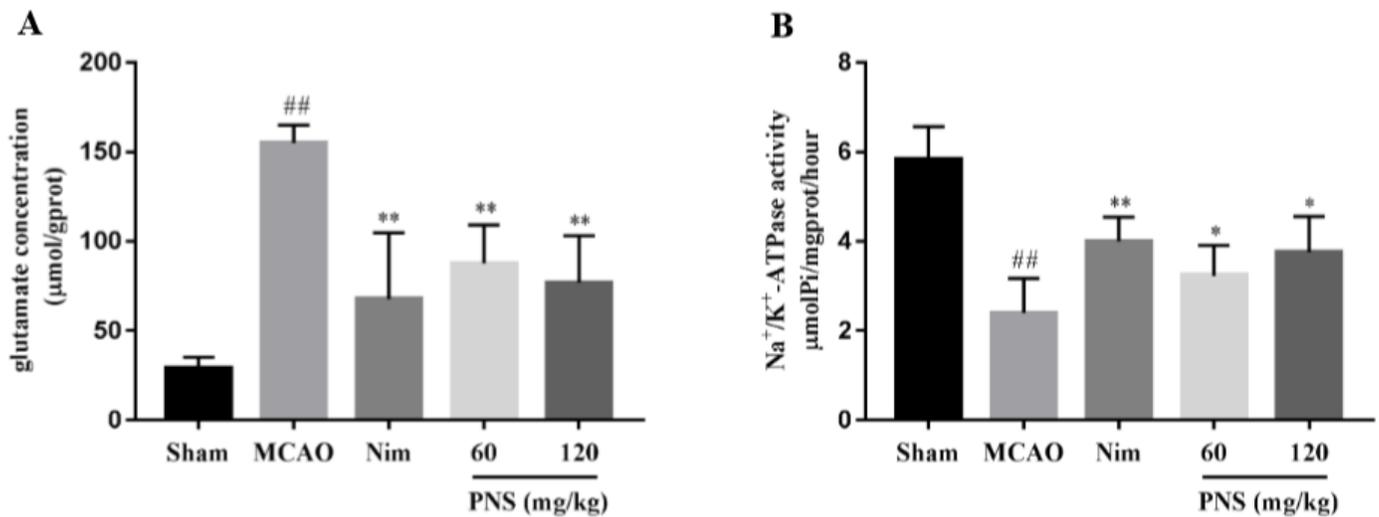
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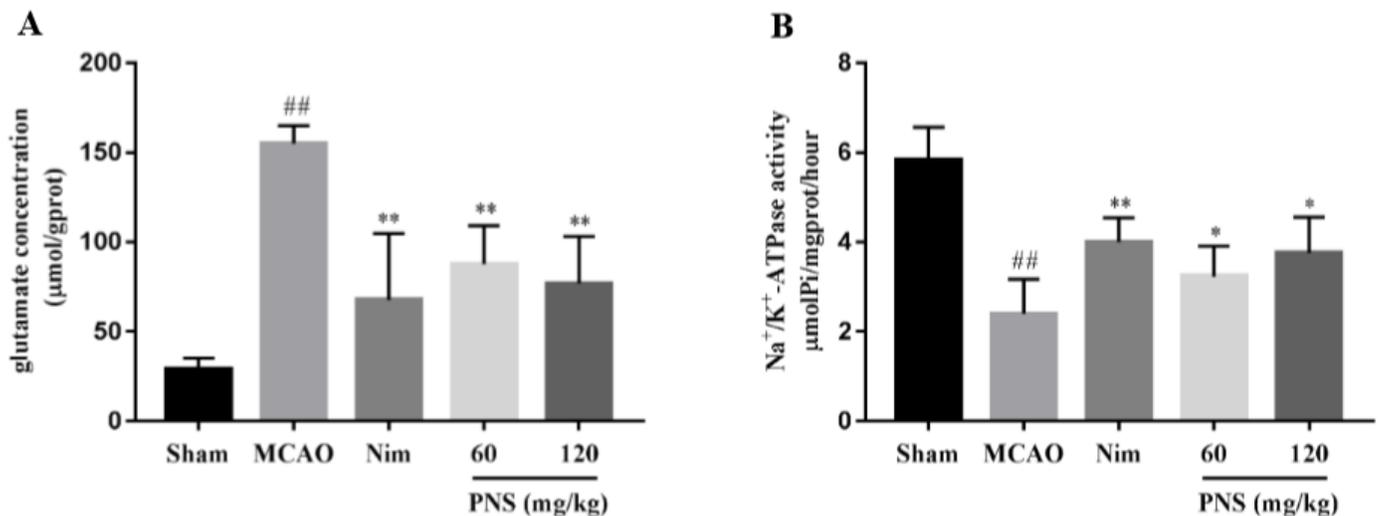
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**Figure 3**

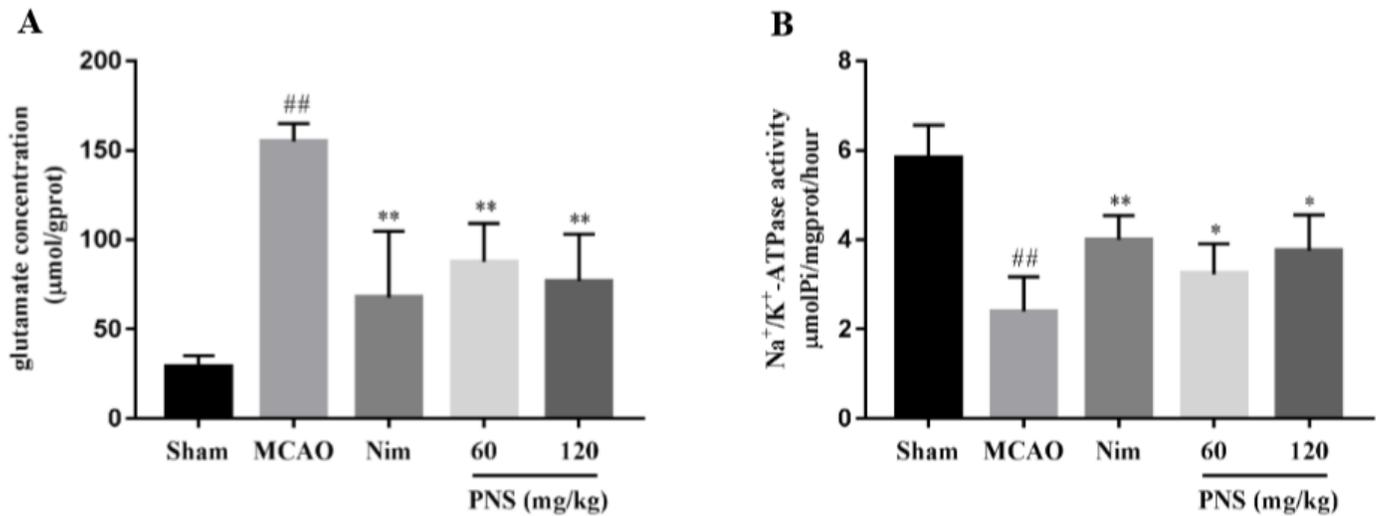
PNS increased glutamate uptake and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity after MCAO in mice. PNS (60 or 120 mg/kg) were orally administered for three consecutive days after inducing brain ischemia. (A) Glutamate concentration, (B) Na<sup>+</sup>-K<sup>+</sup>-ATPase activity were determined by colorimetric method. Data was presented as mean ± SEM, n=6~9 pre group, #P < 0.05, ##P < 0.01 compared with the sham group, \*P < 0.05, \*\*P < 0.01 compared with the MCAO group.



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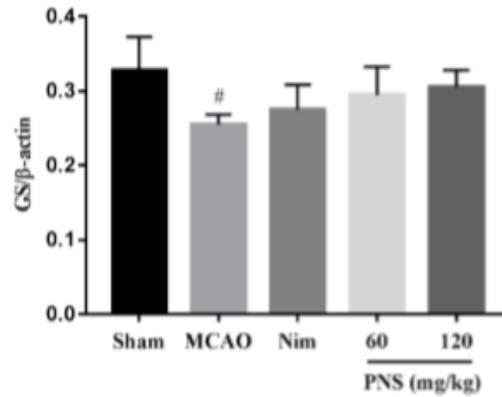
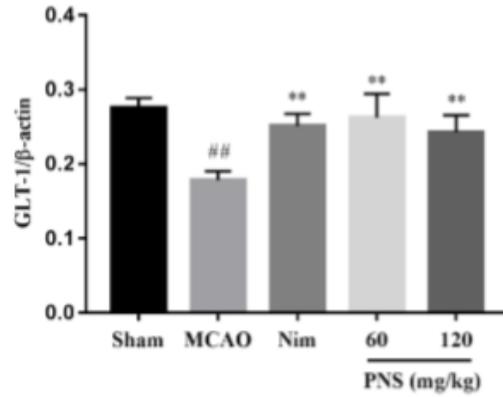
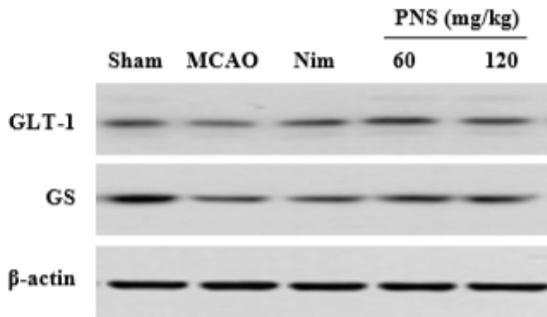
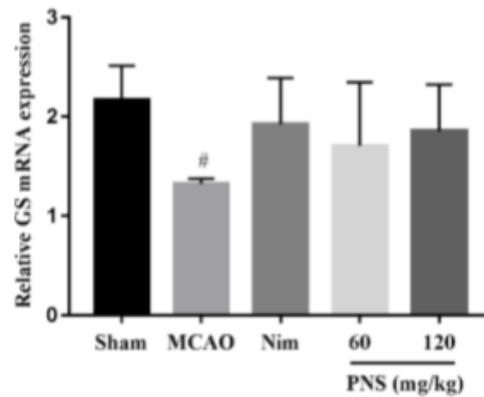
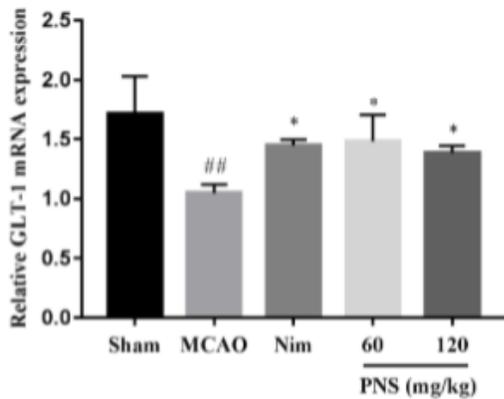
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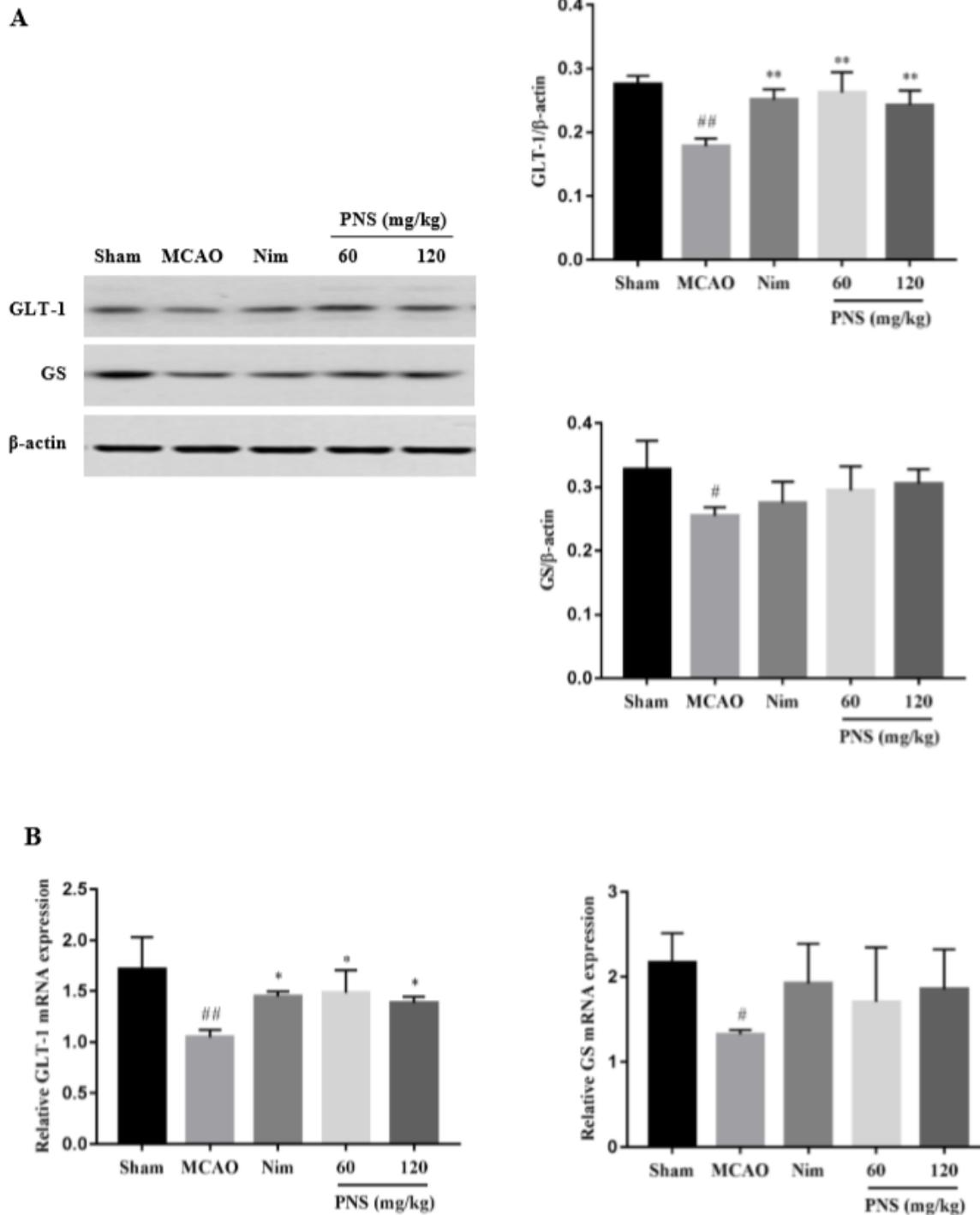
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**A****B****Figure 4**

PNS up-regulate expression level of GLUT-1 and GS protein and mRNA in the mouse cerebral cortex after MCAO. PNS (60 or 120 mg/kg) were orally administered for three consecutive days after inducing brain ischemia. Western blot (A) and real-time PCR (B) assay were performed to detect the effect of PNS on GLUT-1 and GS protein and mRNA expression levels in ischemic cortex after cerebral ischemia. Data was

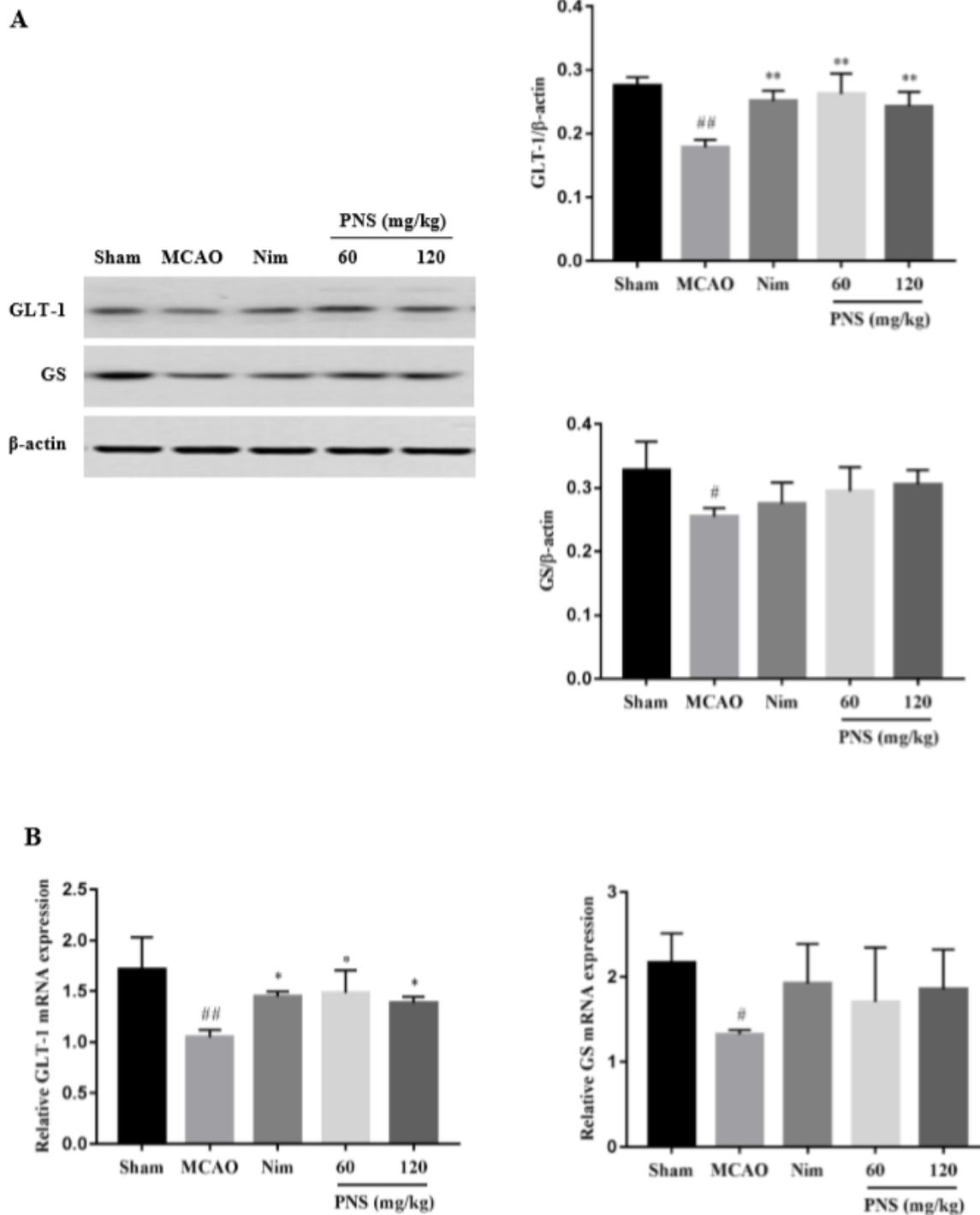
presented as mean  $\pm$  SEM, n=4 pre group, #P < 0.05, ##P < 0.01 compared with the sham group, \*P < 0.05,\*\*P < 0.01 compared with the MCAO group.



**Figure 4**

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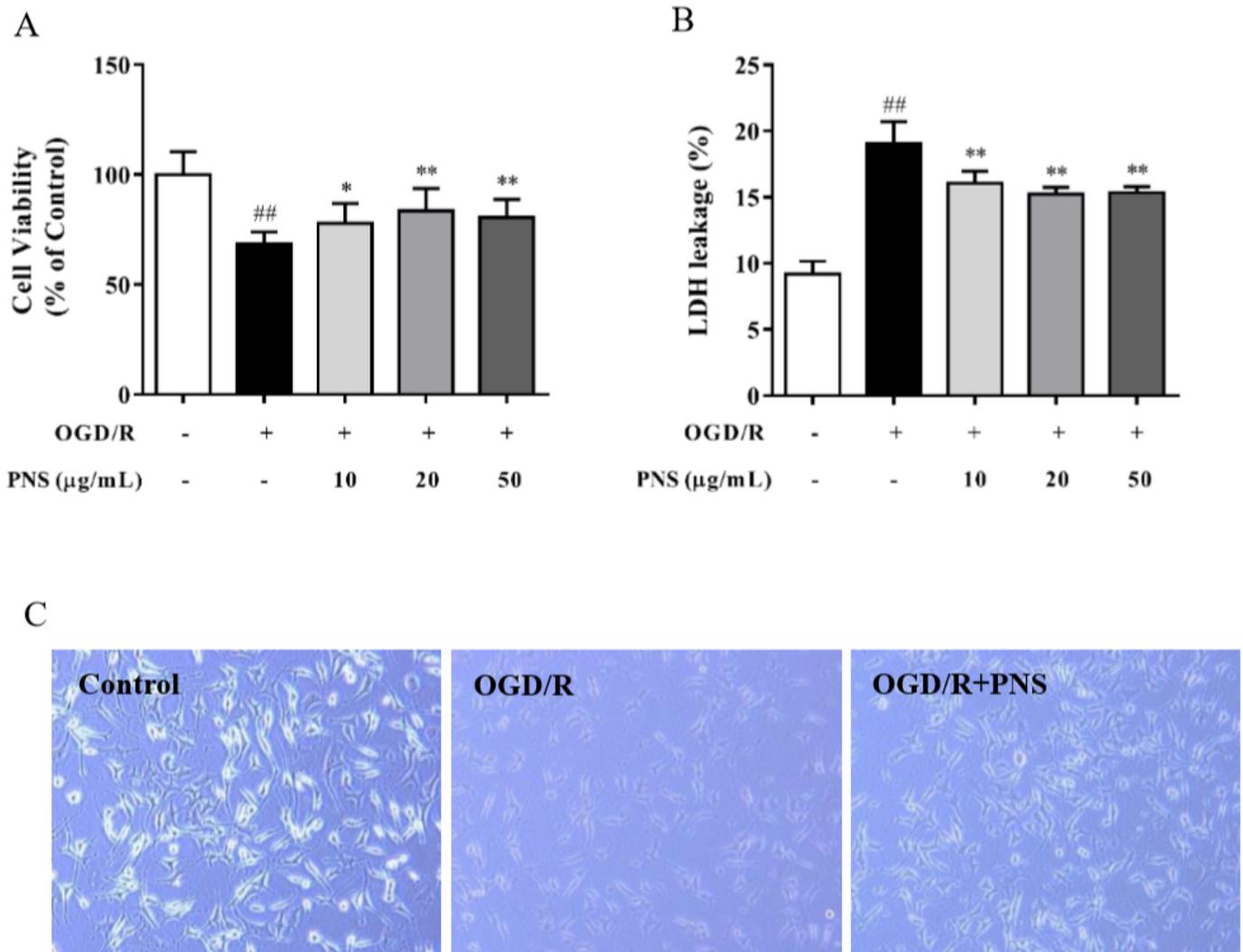
GLT-1 and GS protein and mRNA expression levels in ischemic cortex after cerebral ischemia. Data was presented as mean  $\pm$  SEM, n=4 pre group, #P < 0.05, ##P < 0.01 compared with the sham group, \*P < 0.05,\*\*P < 0.01 compared with the MCAO group.



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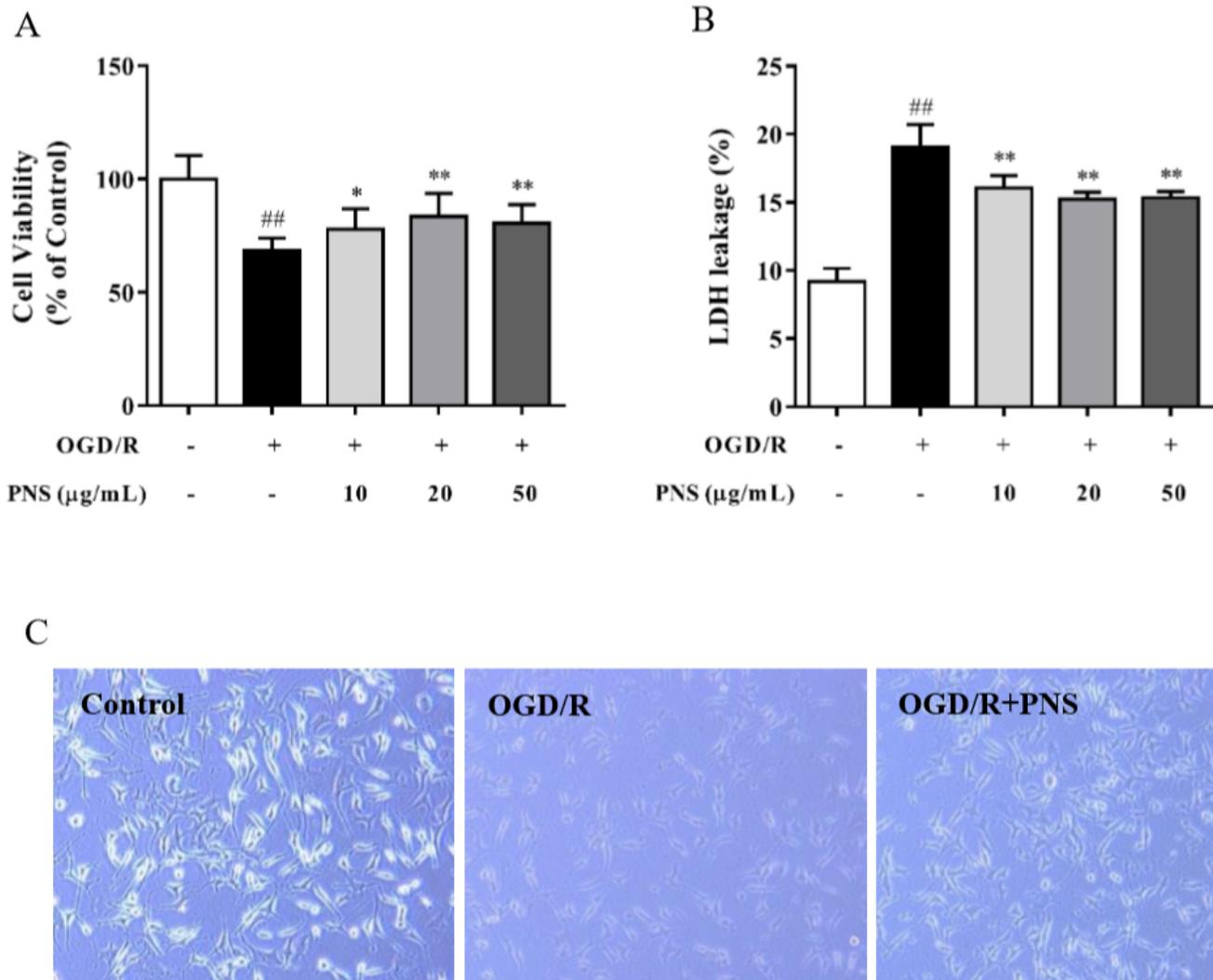
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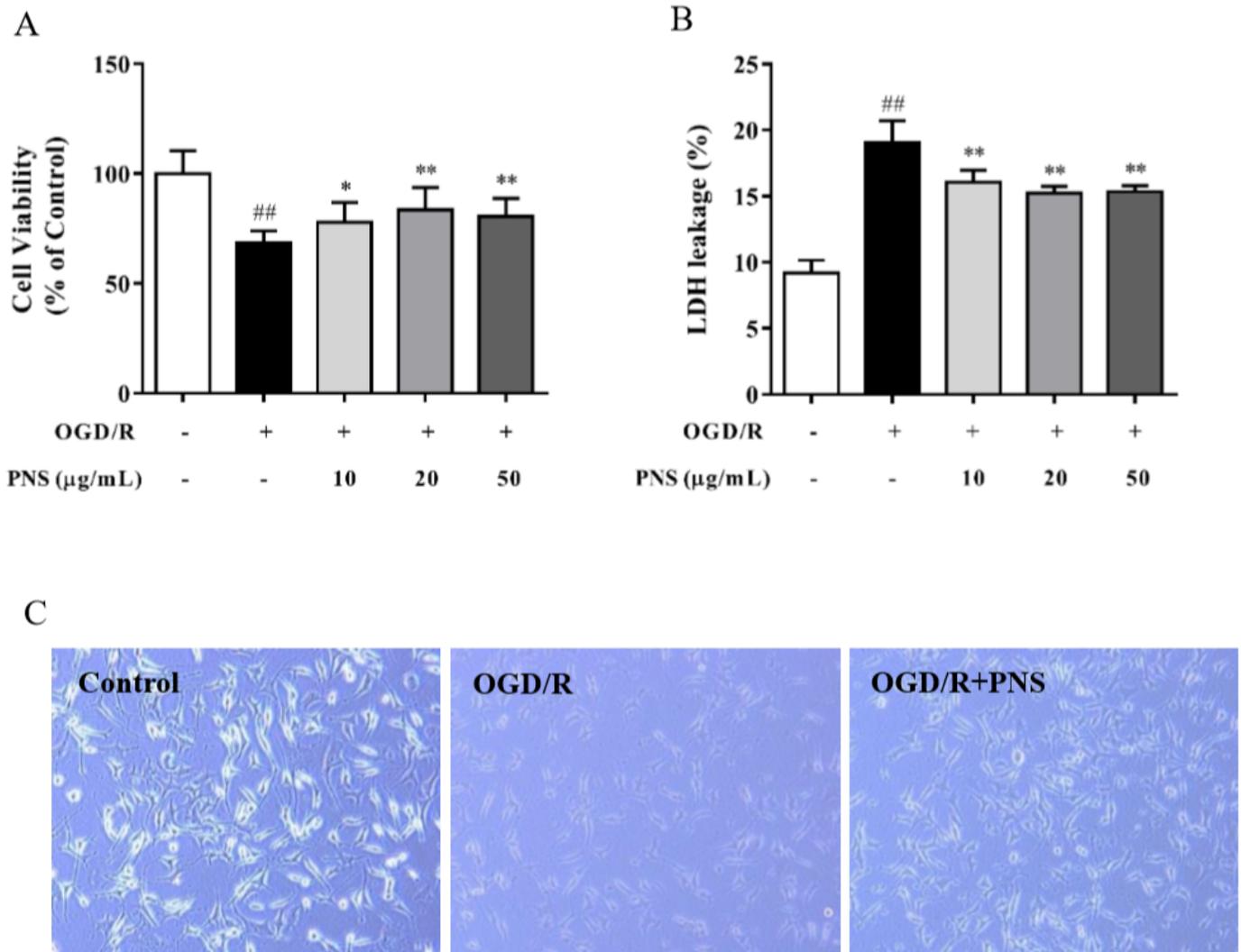
**Figure 5**

Protection of PNS from OGD/R-induced cytotoxicity in astrocytes. OGD/R-incubated astrocytes were treated with various concentrations of PNS (10, 20, 50 and 100  $\mu\text{g/mL}$ ). (A) Cell viability was examined using CCK-8 assay. PNS (10, 20 and 50  $\mu\text{g/mL}$ ) markedly increased cell viability. (B) Cell cytotoxicity was examined by LDH release assay. PNS obviously inhibited the LDH release. (C) The morphology of astrocytes was obtained by using an inverted microscope. PNS (20  $\mu\text{g/mL}$ ) significantly improved cell state, magnification:  $\times 100$ . Data are expressed as the mean  $\pm$  SEM (n=10). ##P < 0.01 vs. Control group; \*\*P < 0.01, \*P < 0.05 vs. OGD/R group.



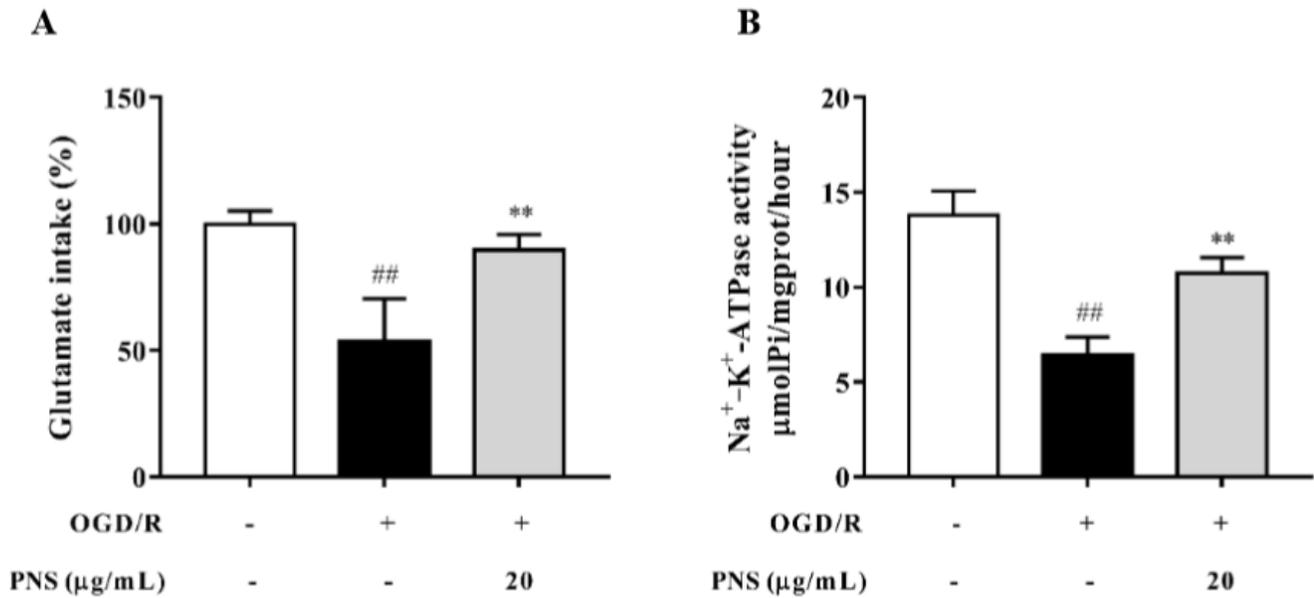
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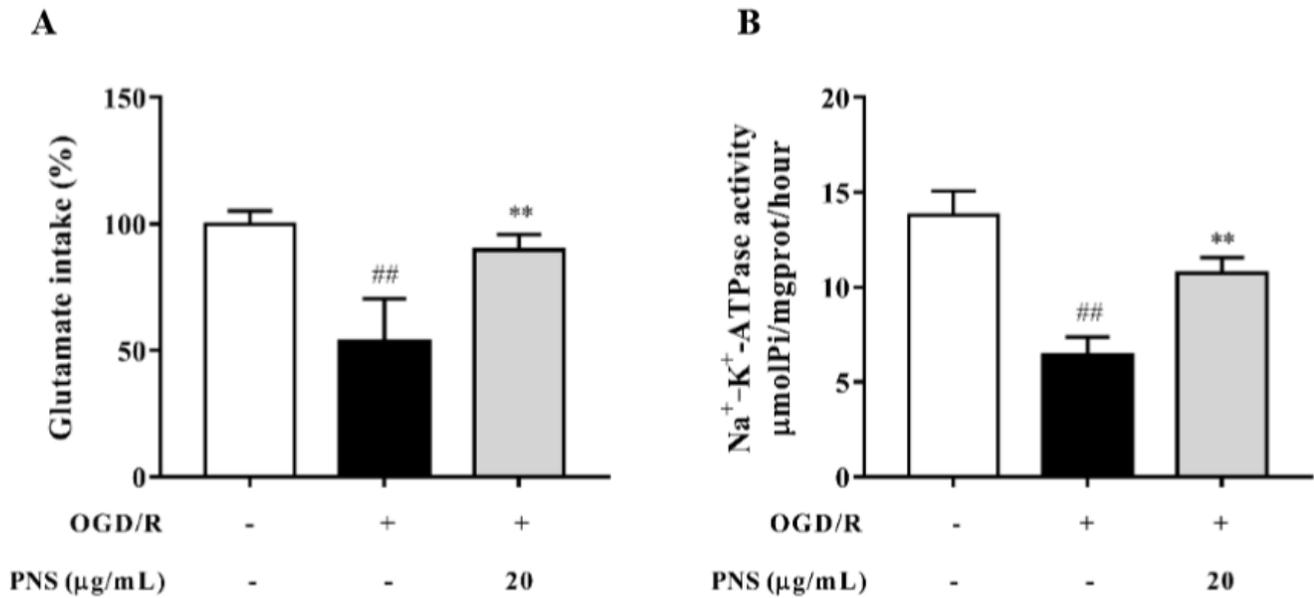
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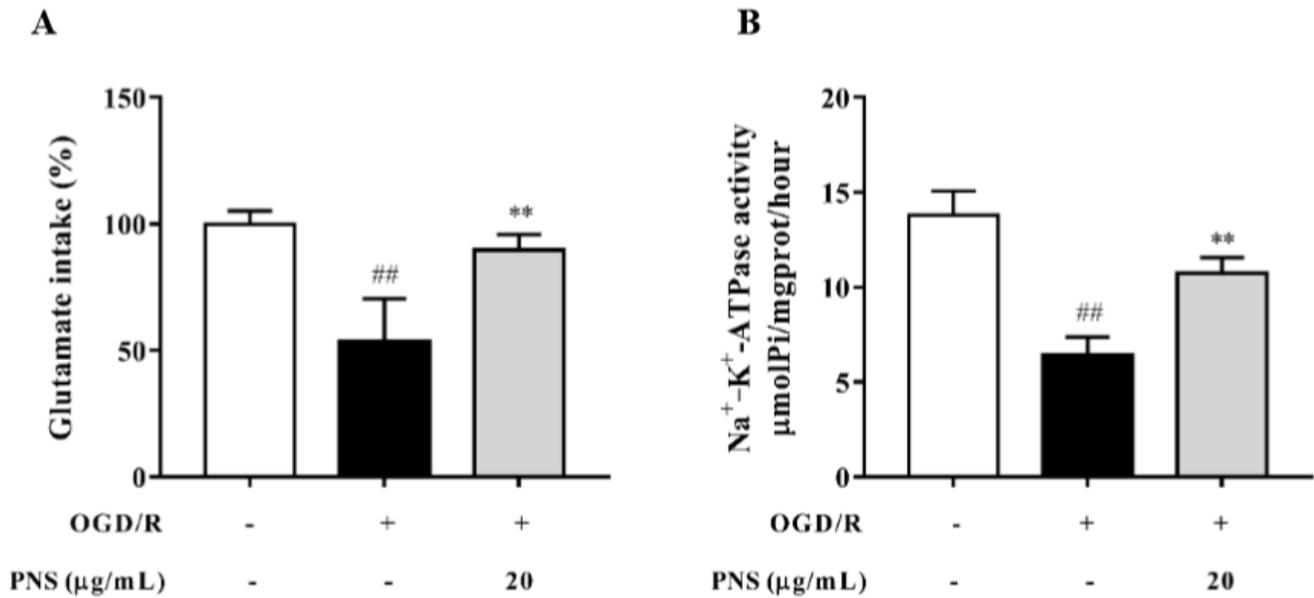
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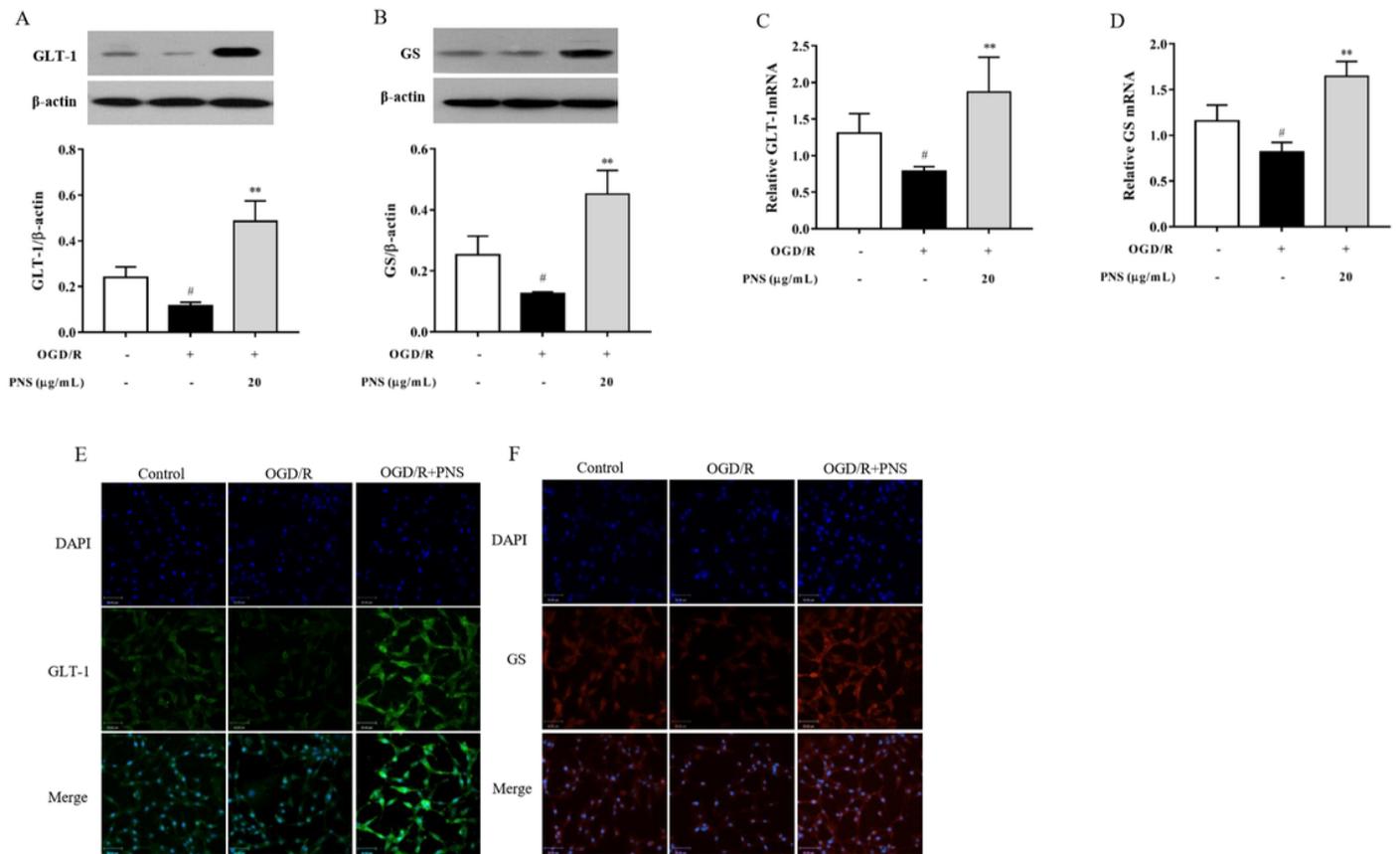
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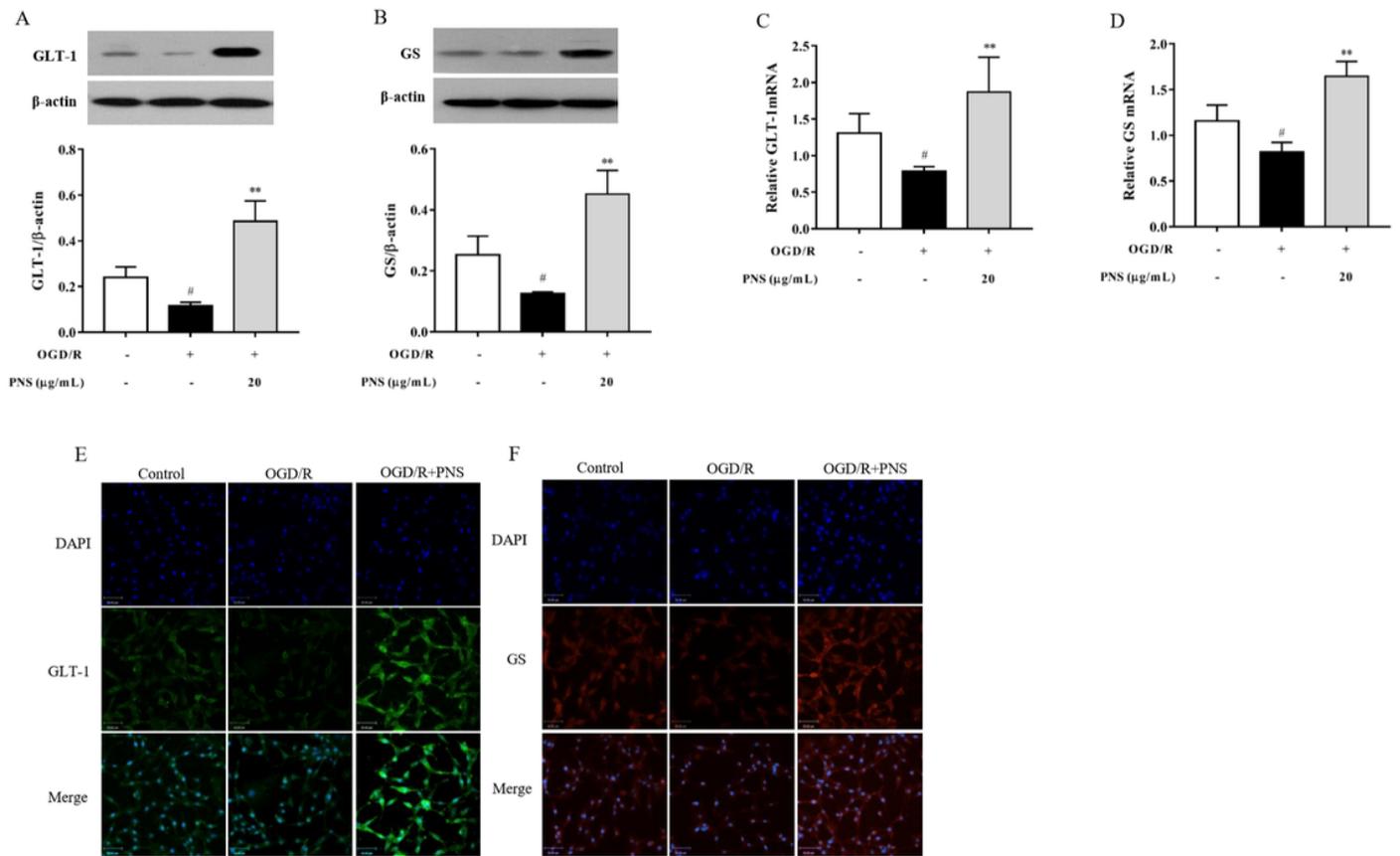
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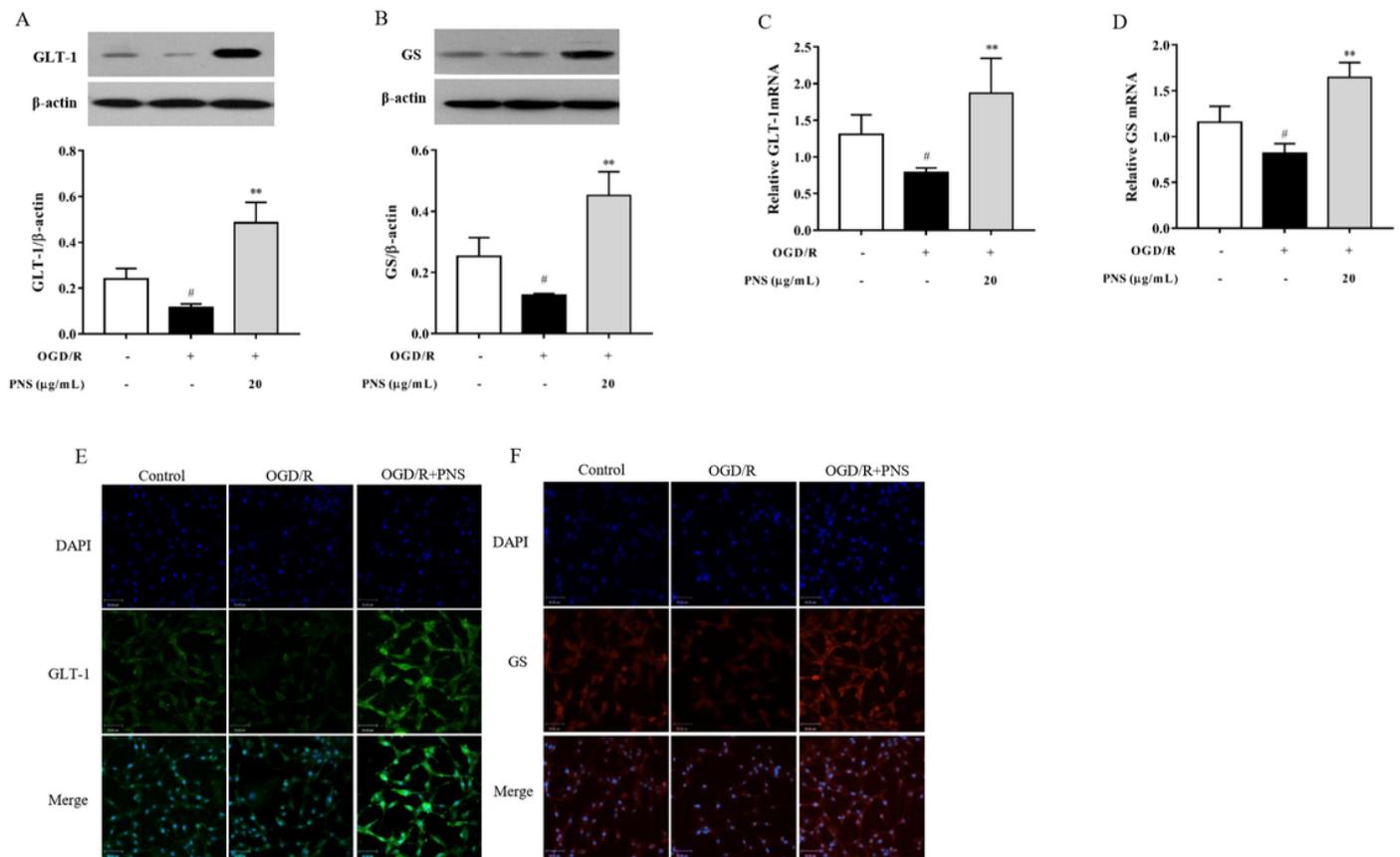
**Figure 7**

PNS up-regulated the expression of GLUT-1 and GS in OGD/R-treated astrocytes. (A) Western blotting analysis of GLUT-1, (B) Western blotting analysis of GS, (C) RT-PCR analysis of GLUT-1, (D) RT-PCR analysis of GS, (E) Immunofluorescent staining for GLUT-1 (green), the nuclei were stained blue by DAPI. (F) Immunofluorescent staining for GS (red), the nuclei were stained blue by DAPI. Data are expressed as the mean  $\pm$  SEM (n=3). ##P<0.01 vs. Control group; \*\*P<0.01 vs. OGD/R group.



**Figure 7**

PNS up-regulated the expression of GLUT-1 and GS in OGD/R-treated astrocytes. (A) Western blotting analysis of GLUT-1, (B) Western blotting analysis of GS, (C) RT-PCR analysis of GLUT-1, (D) RT-PCR analysis of GS, (E) Immunofluorescent staining for GLUT-1 (green), the nuclei were stained blue by DAPI. (F) Immunofluorescent staining for GS (red), the nuclei were stained blue by DAPI. Data are expressed as the mean  $\pm$  SEM (n=3). <sup>#</sup>P<0.01 vs. Control group; <sup>\*\*</sup>P<0.01 vs. OGD/R group.



**Figure 7**

PNS up-regulated the expression of GLT-1 and GS in OGD/R-treated astrocytes. (A) Western blotting analysis of GLT-1, (B) Western blotting analysis of GS, (C) RT-PCR analysis of GLT-1, (D) RT-PCR analysis of GS, (E) Immunofluorescent staining for GLT-1 (green), the nuclei were stained blue by DAPI. (F) Immunofluorescent staining for GS (red), the nuclei were stained blue by DAPI. Data are expressed as the mean  $\pm$  SEM (n=3). <sup>#</sup>P<0.01 vs. Control group; <sup>\*\*</sup>P<0.01 vs. OGD/R group.