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Low cerebral exposure cannot hinder the neuroprotective effects of panax notoginsenosides

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Running Title Page

Panax notoginsenosides exert neuroprotective effects

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Abbreviations: ANOVA, One-Way Analysis of Variance; BDNF, brain derived neurotrophic factor; B.L, *Bifidobacterium longum*; CCA, common carotid artery; CNS, central nervous system; CMCs, combined molar concentrations; ECA, external carotid artery; GABA, gamma aminobutyric acid; GI, gastrointestinal; ICA, internal carotid artery; IL-6, interleukin-6; IL-1β, interleukin-1β; L.B, *Lactobacillus brevis*; L.H, *Lactobacillus helveticus*; L.R, *Lactobacillus rhamnosus*; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NC, nitrocellulose; OA, occipital artery; OTUs, operational taxonomic units; PNE, *Panax Notoginsenoside* extract; R_1, notoginsenoside R_1; Rb_1 ginsenoside Rb_1; RCM, reinforced clostridium medium; Rd, ginsenoside Rd; Re, ginsenoside Re; Rg_1, ginsenoside Rg_1; SDS, sodium dodecyl sulfate; TCMs, traditional Chinese medicines; TNF-α, tumor necrosis factor-alpha; TTC, 2, 3, 5-triphenyltetrazolium chloride.

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Abstract

A bidirectional route of communication between the gastrointestinal tract and the central nervous system, termed the “gut-brain axis”, is becoming increasingly relevant to treatment of cerebral damage. *Panax Notoginsenoside* extract (PNE) is popular for prevention and treatment of cardio-cerebrovascular ischemic diseases although their plasma and cerebral exposure levels were extremely low. To date, the mechanisms underlying the neuroprotective effects of PNE remain largely unknown. In the present study, the neuroprotective effects of PNE are systematically studied via investigating the regulation of PNE on gastrointestinal microbial community and gamma aminobutyric acid (GABA) receptors. The results demonstrated that pretreatment with PNE exerted a remarkable neuroprotective effect on focal cerebral ischemia / reperfusion (I/R) injury in rats, and the efficiency was attenuated in germ-free rats. Pretreatment with PNE could significantly prevent down-regulation of *Bifidobacterium longum* (B.L) caused by I/R surgery, and colonization of B.L could also exert neuroprotective effects. More importantly, both PNE and B.L could up-regulate the expression of GABA receptors in the hippocampus of I/R rats, and co-administration of a GABA-B receptor antagonist could significantly attenuate the neuroprotective effects of PNE and B.L. The study above suggests that the neuroprotective effects of PNE may be largely attributable to its regulation on intestinal flora, and oral treatment of B.L was also useful in therapy of I/R by up-regulating GABA-B receptors.

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Introduction

Worldwide, ischemic brain injury is a major cause of dementia, disability, and death, and most of the survivors suffer from long-term sequelae including seizures, mental retardation, and neurological deficits (*Donnan et al., 2008*). Reperfusion of ischemic areas can exacerbate brain damage through release of free radicals and cytokines, platelet activation, and apoptosis (*Doyle et al., 2008*). In the past decade years, the influence of the gastrointestinal (GI) tract on the occurrence, development and treatment of brain diseases had been noted in humans, and a bidirectional communication route between the GI tract and the central nervous system (CNS) was termed the “gut-brain axis” (*Rhee et al., 2009; Maqsood and Stone, 2016*). The main bidirectional communication of the gut-brain axis mainly occurs via the vagus nerve, gut hormones (e.g., gamma aminobutyric acid (GABA), neuropeptide Y, dopamine), and gut microbiota molecules (e.g., short chain fatty acids, tryptophan) (*Braniste et al., 2014; Clark and Mach, 2016; Eisenstein, 2016*). Recent interest in gut microbiota has revealed that in addition to reflecting the body state and energy metabolism, colonic bacteria can influence curative efficacy of drugs (*Vyas and Ranganathan, 2012*). To date, more than 100 trillion microorganisms have been found in the human gut (*Yang et al., 2009*). Gut microbiota affect numerous biological functions and are linked to pathogenesis of various diseases such as obesity, cancer, and liver cirrhosis *(Lee and Hase, 2014)*. The interactions among the intestinal microbiota, GI tract, and CNS constitute the microbiome-gut-brain axis, and the research on how the microbiome-gut-brain axis influences neurological conditions may provide insight into disease etiology and treatment strategy ((*Sampson and Mazmanian, 2015; Foster et al., 2016; Sampson et al., 2016*). For example, consumption of probiotics can dramatically affect sustained attention performance as well as brain activity during social cognition (*Tillisch et al., 2013*). Recent preclinical and clinical studies have identified *Bifidobacterium longum* (B.L) and *Lactobacillus helveticus* (L.H) as putative psychobiotics with great impact on stress-related brain-gut axis disorders, behaviors, physiology, and cognitive performance (*Ohsawa et al., 2015*).

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In the past few years, researchers have focused on neural, immune, endocrine and metabolic pathways as likely mediators in this bidirectional communication (*El Aidy et al., 2015; Mayer et al., 2015*). GABA, which is a well-known neurotransmitter in development and modulation of neuropathy, can be produced in large quantities by several types of bacteria in the GI tract (*Zheng et al., 2007*). It has been reported that *Lactobacillus brevis* (L.B) FPA 3709 (prodigious producer of GABA) employs GABA-enriched soybean milk and can significantly reduce rat depressive-like behavior as effectively as the classical antidepressant fluoxetine (*Ko et al., 2013*). In another study, the ability of orally administered *Lactobacillus rhamnosus* (L.R) to reduce anxiety- and depressive-like behaviors in mice was shown to be mediated by GABA receptor expression in the brain, with changes in levels of GABA-Aα2 mRNA in brain regions associated with the specific behavior (*Bravo et al., 2011*). Effects of GABA are typically mediated through 2 major classes of receptors, GABA-A and GABA-B (*Helm et al., 2005*). Interestingly, regulation of GABA-A receptor subunits can significantly affect fear-related behaviors (*Bravo et al., 2011*), and activation of metabotropic GABA-B receptors can trigger secretion of endogenous brain derived neurotrophic factor (BDNF) (*Fiorentino et al., 2009*), which exerts neuroprotective effects against glutamate toxicity in hippocampal neurons.

Herbal traditional Chinese medicines (TCMs) are known to exert wide pharmaceutical effects in a cooperative way with multiple components and targets, yet its efficiency is poorly documented and always described in general terms without detailed characterization of herbs and diseases (*Ma et al., 2014*). The overall growing popularity of herbal TCMs has led to substantial interest in laboratory and clinical studies to evaluate their efficiency and pharmacological mechanisms (*Teschke et al., 2015*). PNE, which is an extract from the traditional Chinese herb *Panax Notoginseng*, has been commonly used to treat cardio-cerebrovascular diseases for thousands of years (*Huang et al., 2015*). In addition, PNE has a curative effect in treatment of common aural vertigo by promoting blood circulation to dissipate blood stasis. The main components of PNE, including notoginsenoside R_1 (R_1), ginsenoside Rb_1 (Rb_1), ginsenoside Rd (Rd), ginsenoside Re (Re), and ginsenoside Rg_1 (Rg_1), have been proven bioactive for prevention and treatment

of cardiovascular and cerebrovascular diseases (*Meng et al., 2012*). However, previous studies on the pharmacokinetics of *Panax Notoginsenosides* showed that bioavailability of the main notoginsenosides (R1, Rd, Re, Rg1, Rb1, Rb2) was extremely poor (< 2%), and notoginsenoside exposure in the systemic circulation was quite low (*Xu et al., 2014; Kim et al., 2015)*. For instance, notoginsenosides do not readily cross the blood-brain barrier due to poor membrane permeability, and concentrations of notoginsenosides in the rat cortex, striatum, hypothalamus, medulla oblongata, and hippocampus were much lower than 100 ng/g (*Guo et al., 2014*). Thus, the pharmacodynamic and pharmacokinetic profiles of notoginsenosides are contradictory, and their curative effect on cerebral ischemic injury remains largely unexplored.

In the present study, we aimed to study the neuroprotective effects and mechanism of PNE on I/R-induced focal cerebral ischemia via investigating the PNE regulation of I/R-related brain-gut axis disorders. The results showed that pretreatment with PNE could significantly up-regulate the relative abundance of B.L in I/R rats, and colonization of B.L could also play a protective role in focal cerebral ischemia. Finally, both PNE and B.L could up-regulate expression of GABA receptors, and their neuroprotective effects could be greatly attenuated by a GABA-B receptor antagonist.

Materials and methods

Chemicals and Standards

Panax Notoginsenoside extraction (PNE) and authentic standards of notoginsenoside R1, Rd, Rb2, Rg1, Rb1, Re were purchased from Department of Nature Medical Chemistry in Jilin University (Changchun, Jilin, China). Digoxin (internal standard, IS) was purchased from Jiangsu Institute for Food and Drug Control (Nanjing, Jiangsu, China). Saclofen, streptomycin, neomycin sulfate and 2, 3, 5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich Corporation (Sigma Aldrich, St. Louis, MO, USA). Bicuculine was purchased from Aladdin Chemical Reagent Co., Ltd (Shanghai, China). Bacteria Genomic DNA Extraction kit was purchased from Takara Bio Inc. (Nojihigashi,

Kusatsu, Shiga, Japan). Both anti-GABA B receptor 1 (ab166604) and anti-GABA B receptor 2 (ab52248) were purchased from Abcam company (Abcam, Cambridge Science Park, London, UK). SYBR Green Supermix RT-PCR Kit was purchased from Bio-RAD Laboratories Inc. (Bio-RAD Lab, Alfred Nobel Drive, Hercules, CA). Deionized water was purified using a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Billerica, MA, USA). Other chemicals and solvents were all of analytical grade.

Animals and Treatments

Animals Healthy Sprague-Dawley rats (220 ±10 g) were purchased from Shanghai Super -- B+K Laboratory Animal Corp. Ltd. (B+K, Shanghai, China) and acclimated for 5 days before use. All animals were kept in an environmentally controlled breeding room under controlled temperature (20 ~ 24°C) and relative humidity (40 ~ 70%) with a 12-h light/dark cycle. Standard diet and water were provided to the rats ad libitum. All procedures in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the Committee on the Ethics of Animal Experiments of China Pharmaceutical University (Nanjing, Jiangsu, China). Ethical procedures were conducted under Reduction, Replacement and Refinement (the 3 Rs rule).

Preparation of I/R model rats Rats were first anesthetized using 2 mL of enflurane in an ether jar, and maintained with 10% chloral hydrate (400 mg kg^{-1}, i.p.). Anesthetized rats underwent surgery for permanent occlusion of the middle cerebral artery (MCA) as reported previously (*Gerriets et al., 2003*). In brief, the right proximal common carotid artery (CCA), the external carotid artery (ECA), and the occipital artery (OA) were ligated by silk sutures, and the internal carotid artery (ICA) was occluded by a microvascular clip. Then a 4-0 nylon monofilament suture with a thin silicon coat was inserted into the CCA. After removal of the microvascular clip, the monofilament suture was gently slipped into the ICA 18 mm beyond the bifurcation of the CCA which resulted in permanent occlusion of the middle cerebral artery occlusion (MCAO). Reperfusion was induced at 2 h after MCAO via withdrawing the monofilament. Sham-operated animals were subjected to the

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same surgical procedures except that the CCA, ECA and OA were not ligated.

Preparation and evaluation of pseudo-germ-free (PGF) rat model For the preparation of PGF rats, antibiotic cocktail was prepared by dissolving neomycin sulfate and streptomycin in water, and Sprague-Dawley rats were orally administered the antibiotic cocktail (100 mg/kg per each antibiotic) twice a day for continuous 6 days. The experiments were performed 2 days later after the final administration.

Drug administration PNE was dissolved in normal saline and intragastrically administrated to rats at a dose of 100mg/kg for consecutive 7 days before I/R surgery. The rats in the vehicle group were administrated saline in the same way.

Neurological evaluation for the I/R model rats Neurological evaluation was performed at 26h after induction of ischemia and scored on a five-point scale according to previous reports (*Longa et al., 1989*): a score of 0 means no neurologic deficit, a score of 1 (failure to extend left forepaw fully) indicates a mild focal neurologic deficit, a score of 2 (circling to the left) is a moderate focal neurologic deficit, and a score of 3 (falling to the left) demonstrates a severe focal deficit; Besides, rats with a score of 4 cannot walk spontaneously and have a depressed level of consciousness.

TTC assessment of infarct size Cerebral infarct size was assessed with 2, 3, 5-triphenyltetrazolium chloride (TTC) staining method (*Joshi et al., 2004*). After 26 h of I/R (2-h ischemia and 24-h reperfusion), the rat brains were quickly isolated and sectioned into consecutive 2-mm-thick coronal slices with a cryomicrotome (Leica CM1950, Nussloch, GER). The brain slices were immediately immersed in 0.1% TTC medium (TTC, 0.125% w:v in 62.5 mM Tris–HCl, 13 mM $MgCl_2$, 1.5% dimethylformamide) at 37 ℃ for 15 min and incubated for another 15 min after overturning the slices. Then the stained slices were washed in phosphate buffer saline (PBS, 0.01 M) for 5 min and immobilized in a buffered formaldehyde solution for 24 h. After immobilization, color images were captured using a video camera. Further analysis was conducted to calculate the infarct volume using the Image-J analysis software according to the following equation: $[(V_C-V_L)/V_C]*100$.

In the formula, V_C is the volume of total sphere, and V_L is the volume of non-infarcted sphere.

Determination of Pro-inflammatory cytokines, BDNF and GABA in rat hippocampus

The tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels in brain tissues were quantified using the ExCell ELISA kits (ExCell Biology, Shanghai, China) following the manufacturer's instructions. The BDNF and GABA were measured using the Uscn ELISA kits (Uscn, Wuhan, Hubei, China) according to the manufacturer's instructions.

Gut Microbe Gene Sequencing

The procedures of gut microbe gene sequencing were same as our previous report (*Gerriets et al., 2003*), and 3 steps were involved in this process.

DNA extraction and PCR amplification Total genome DNA of intestinal microbiota was extracted using hexadecyl trimethyl ammonium bromide, and the DNA concentration was diluted to 1 ng μLl^{-1} using sterile water. The distinct regions (16SV4/ 16SV3/ 16SV3-V4/ 16SV4-V5) of 16S rRNA genes were amplified using specific primer (341F: CCTAYGGGRBGCASCAG, 806R: GGACTACNNGGGTATCTAAT). The reaction system (30 μL) was consisted by each primer (10 μM, 0.5 μL), Es Taq (15 μL), and DNA template (1 μL). The PCR reactions were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs), and thermal cycling consisted of initial denaturation at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 30 s. The PCR products were mixed with an equal volume of 2× loading buffer and operated electrophoresis on 2% agarose gel (contained SYB green) for detection. Samples with bright main strip between 400 to 450 bp were chosen and mixed in equi-density ratios. Then the mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Valencia, CA).

MiSeq sequencing of 16S rRNA gene amplicons The 16S rRNA gene amplicons were performed using Illumina MiSeq sequencing at Novogene Bioinformatics Technology Co.,

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Ltd (Novogene, Beijing, China). Sequencing libraries were generated using TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations. The library quality was evaluated on the Qubit @ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). Finally, the library was sequenced based on an IlluminaHiSeq2500 platform (Illumina, CA, USA).

Data analysis Paired-end reads were merged using FLASH v1.2.7 (http://ccb.jhu.edu/software/FLASH/) based on overlapping regions within paired-end reads. Quality filtering on the raw tags was performed to obtain the high-quality clean tags according to the QIIME (V1.7.0, http://qiime.org/index.html) quality control process. Tags were compared with the reference database (Gold database) using UCHIME lgorithm to detect chimera sequences. Sequence analysis was performed using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/). Sequences with ≥97% similarity were assigned to the same Operational Taxonomic Units (OTUs). Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Green Gene Database was used to annotate taxonomic information based on RDP classifier (Version 2.2, https://sourceforge.net/projects/rdp-classifier/).

B.L Cultivation and Colonization to Rats

B.L (ATCC 15707) was purchased from China Center of Industrial Culture Collection and grown under anaerobic conditions in Reinforced Clostridium Medium (RCM) which containing 0.002% Polymyxin B. Bacterial cells were harvested at 48 hour and pelleted by centrifuging for 15 min at 5000 g (4 ℃), further re-suspended at a concentration of $1*10^{10}$ mL^{-1} in RCM, and kept in frozen aliquots until use. To observe the neuroprotective effects of B.L, rats were gavaged with $1*10^{9}$ CFU bacteria (suspended in the RCM) once per day for 15 consecutive days before I/R surgery.

PCR Analysis of GABA Receptors

Total RNA of rat Hippocampus was extracted using Exiqon kit (Takara, Kyoto, Japan) and

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reverse transcribed to cDNA using PrimeScript RT reagent Kit (Takara, Kyoto, Japan). Expression levels of the GABA receptors ($A_{\alpha 2}$, $A_{\beta 2}$, $A_{\gamma 2}$, B_{1b} and B_2) in hippocampus were measured by Real Time PCR, and the primer sequences are list in **Supplemental Table 1**. Each experiment was performed in triplicate with at least 3 independent samples. Data sets were evaluated using t-test or One-Way Analysis of Variance (ANOVA) followed by Tukey's post hoc test, and expressed as mean ± SD. Statistically significant difference was set at $P < 0.05$.

Western Blotting Analysis of GABA Receptors

For Western blot analysis, rat Hippocampus was isolated and homogenized in ice-cold lysis buffer containing 1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 50 mM Tris (Ph 8.3), and a cocktail of protease and phosphatase inhibitors for 30 min and then ultrasonicated for 60-s intervals in an ice bath. Homogenates were centrifuged at 13,000 g for 15 min at 4° C. Protein concentrations were measured using a BCA protein assay kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The samples were diluted using Laemmli buffer (2% SDS, 20% glycerol, 62.5 mM Tris HCl, 5% 2-mercaptoethanol, 0.002 % bromophenol blue, 300 mM dithiothreitol, pH 6.8), and then denatured by heating to 95°C for 5 minutes. The wells of a 10% acrylamide-bisacrylamide gel were loaded with 50 μg of protein. Turn on the power of the electrophoresis instrument, and the voltage is set to 60 V. When the protein sample enters the separation gel, the voltage increased to 90V. Proteins were subsequently transferred to a nitrocellulose (NC) membrane (orb90491, Biobyt, USA), blocked in 5 % nonfat milk with 0.1 % Tween-20 in Tris-buffer for 1 hour at room temperature, and incubated overnight in the primary antibodies at 4° C.

The membrane was washed (4 ×10 min),and then incubated in appropriate secondary antibodies: antirabbit HRP (KGAA35, KeyGEN BioTECH, Nanjing, China) diluted 1:10,000 for 1 hour at 4°C; and bound immunoglobulins were visualized with the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) on G:BOX chemiXR5. Gray scale analysis was carried out using Gel-Pro32 software.

The Influence of GABA Receptor Antagonists on PNE Efficacy

The rats in vehicle group were treated with iso metric medium. To investigate the blunting effects of GABA-A receptor and GABA-B receptor antagonists on the neuroprotective effect of PNE and B.L, bicuculline (GABA-A receptor antagonist, 0.2 mg kg^{-1}) was administrated to rats via intraperitoneal injection, and saclofen (GABA-B receptor antagonist, 0.1 mg kg^{-1}) was intravenously administrated to rats at 30 min before I/R surgery.

Determination of Notoginsenoside Concentrations in Normal and I/R Rat Brain

The method for simultaneous quantitative analysis of multiple notoginsenosides (notoginsenoside R1, Rg3, Rd, Rg2, Rb2,Rf, Rg1, Rb1 and Re) was developed based on UFLC–MS/MS in our previous study (*Zhou et al., 2015*). The method was fully validated in accordance with US-FDA Bio-analytical Method Validation Guidance with respect to selectivity, linearity, sensitivity, accuracy, precision, matrix effect, recovery, stability, *etc*. The results demonstrated that the method could meet the requirement of determination of ginsenosides in biological matrices, and the specific steps were as follows.

Sample preparation Both I/R model and normal rats were administrated with PNE intragastricly at a dose of 100 mg kg^{-1}. The rat plasma and brain specimens were quickly collected at 30 min, 2 h and 10 h post dose and frozen at -80 ℃. Before analysis, brain tissues were homogenized and extracted using *n*-butanol as follows: (i) To each tube containing 100 μL of homogenate, 10 Ml of internal standard (digoxin, 0.1 μg mL^{-1}) and 1.0 mL of *n*-butanol was added. (ii) The mixture was vortex-extracted for 2 min, and centrifuged for 5 min at 10,000 × g. (iii) The n-butanol extract was evaporated to dryness in a rotary evaporator and the residue was reconstituted in 200 μL of acetonitrile.

Instrument, parameters and conditions The ultra-fast liquid chromatography–triple quadrupole mass spectrometer system (UFLC–MS/MS-8050 system, Shimadzu Corporation, Kyoto, Japan), composed of a Shimadzu 30 AD liquid chromatography system and a 8050 triple quadrupole mass spectrometer, was used to quantitatively

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analyze notoginsenosides in biological matrices. Data acquisition was performed using the LabSolutions LCMS Ver. 5.6 software (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved on C18 reversed phase LC column (Thermo ODS 5 m 50 mm × 2.1 mm I.D.). The electrospray ionization source was operated in negative mode. The optimized MRM parameters are list in **Supplemental Table 2**, and the other operating parameters were as follows: 250 ◦C of desolvation temperature; 400 ◦C of heat block temperature; 3 l min^{-1} of nebulizer gas flow rate; and 5 l min^{-1} of drying gas flow rate.

Results

Intracephalic and Plasma Concentrations of Notoginsenosides in Normal and I/R Rats

To elucidate deposition and protective mechanisms of PNE in the brain, intracephalic concentrations of 6 main PNE components (R1, Rd, Re, Rg1, Rb1, Rb2) were determined using a UFLC-MS/MS-8050 system. As illustrated in **Figure 1 A to 1C**, detectable levels of R1, Rd, Re, Rg1, Rb1, and Rb2 were observed in rat brains collected at 2 h, and intracephalic concentrations of the rats in the I/R group were higher than those in the normal group. For the brain tissues collected at 30 min, only R1, Rg1, and Rb1 could be quantified, and the concentrations of Rd, Re, and Rb2 were lower than 1 ng g^{-1}.At 10 h, only trace levels of R1 and Rb1 were detectable (~1 ng g^{-1}) in the normal and I/R model rat brains. Besides, the combined molar concentration of R1, Rd, Re, Rg1, Rb1 and Rb2 were calculated to express the exposure of PNE in rat brains. At 30 min, the combined molar concentrations (CMCs) of notoginsenosides in normal and I/R model rat brains were 0.0867 and 0.1311 nMol/g, respectively. The CMCs were 0.0867 and 0.1311 nMol/g in normal and I/R model rat brains collected at 2h, which were significantly higher than those collected at 30 min. At 10 h, the CMCs decreased to 0.003 nMol/g in normal and I/R rat brains.

The plasma concentrations of notoginsenosides were also measured in the present study (**Figure 1D ~1F**). Obviously, the concentrations of R1, Rd, Re, Rg1, Rb1, and Rb in rat

plasma were significantly higher than those in rat brains. To demonstrate the distribution characteristics more clearly, the concentration rations of brain to plasma were calculated for the notoginsenosides. All the rations were found below 1.0 (**Supplemental Table 3**). In sum, the exposure of notoginsenosides was quite low in the rat brain.

Neuroprotective Effects of PNE in Rats with Focal Cerebral Ischemia

To investigate neuroprotective effects of PNE, infarct volume in the brain after reperfusion for 24 h was estimated using TTC staining. As shown in **Figure 2A**, cerebral infarcts were pronounced after establishing the I/R model (2-h ischemia and 24-h reperfusion); the cerebral infarct volume was 37.74 ± 3.94% in the I/R group (**Figure 2B**). Compared with the I/R group, infarct size in the PNE treatment group was significantly decreased with a cerebral infarct volume of 29.60 ± 5.16%. In addition, neurological deficits were assessed by scoring specific behaviors. As shown in **Figure 2C**, the neurological deficit score of the I/R group (2.87 ± 0.41) was significantly higher than the score of the control group. Following pretreatment with PNE (100 mg kg^{-1} d^{-1}), significant decreases in neurological deficit scores (1.90 ± 0.22) were observed compared with the I/R group ($P < 0.001$), which indicates that PNE improved neurological deficits caused by cerebral I/R. These results robustly demonstrate that PNE exerts a remarkable neuroprotective effect from cerebral I/R injury in rats.

The Role of Gut Microbiota in Neuroprotective Effects of PNE

PGF model rats were used to investigate the role of gut microbiota in the neuroprotective effects of PNE. As shown in **Figure 2D**, cerebral infarcts were prominent after establishing the I/R model in PGF rats. Cerebral infarct volume was 36.81 ± 4.61% in the PGF+I/R group (**Figure 2E**). In the PNE treatment group, infarct size was reduced to 29.60 ± 5.16%. In addition, the neurological deficit score of the PGF + I/R group was slightly higher than that of the PNE treatment group. The neurological deficit scores of the PGF + I/R and PNE treatment groups were 2.79 ± 0.53 and 2.25 ± 0.25, respectively (**Figure 2F**). However, comparisons of cerebral infarct volumes and neurological deficit scores between PGF +

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I/R and I/R rats indicated that the neuroprotective effects of PNE in the PGF + I/R rats were much lower than in normal I/R rats. These results suggest that gut microbiota play an important role in neuroprotective effects of PNE.

I/R-induced brain injury is closely associated with enhanced inflammatory responses and lower BDNF levels. Therefore, pro-inflammatory cytokine and BDNF levels in control, I/R, I/R + PNE, PGF, PGF + I/R, and PGF + I/R + PNE groups were determined to verify further the role of gut microbiota in neuroprotective effects of PNE. As shown in **Figure 2G** and **2H**, levels of IL-1β and IL-6 in the hippocampus of the I/R group were significantly increased compared with the control group (one-way ANOVA, $P < 0.01$). After pretreatment with PNE, levels of IL-1β and IL-6 were significantly decreased (one-way ANOVA, $P < 0.01$). IL-1β and IL-6 levels in the PGF group were similar to those in the control group, and no significant difference was found between the I/R and PGF + I/R groups. In the PGF + I/R + PNE group, IL-1β and IL-6 levels were lower than those in the PGF + I/R group. However, IL-1β levels in the PGF + I/R + PNE group were markedly higher than in the I/R + PNE group (one-way ANOVA, $P < 0.05$), and IL-6 levels in the PGF + I/R + PNE group were also higher than in the I/R + PNE group to some extent. These results demonstrate that gut microbiota greatly influence regulation of pro-inflammatory cytokines by PNE in I/R rats.

BDNF is a member of the nerve growth factor family and is involved in a plethora of functions within the CNS including neuronal survival and differentiation (Bjorkholm and Monteggia, 2016). Thus, regulation of BDNF expression was assessed to investigate its role in the neuroprotective effects of PNE. As shown in **Figure 2I**, levels of BDNF were significantly decreased in I/R rats compared with the control group, while PNE treatment could significantly attenuate this tendency (one-way ANOVA, $P < 0.05$). However, PNE could not up-regulate expression of BDNF in PGF + I/R rats, and expression of BDNF in the I/R + PNE group was significantly higher than in the PGF + I/R + PNE group. Thus, these results suggest that gut microbiota influence regulation of BDNF expression during PNE-induced protective effects on the brain after cerebral I/R.

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PNE Regulation of Gut Microbiota Populations

Intestinal bacterial community structures were characterized based on 16S rRNA amplicon Illumina sequencing to investigate intestinal flora associated with protective effects of PNE in the brain. The taxon abundance of each sample was divided into phylum, class, order, family, and genera levels. First, 58414, 61311, 60633, 62915, 66084, and 65872 16S rRNA valid sequence reads were obtained from samples in the control, I/R, I/R + PNE, PGF, PGF + I/R, and PGF + I/R + PNE groups, respectively (**Supplemental Figure 1**). This indicated that the 16S rRNA gene sequence database of each group was abundant enough to capture most microbial diversity information. To clearly highlight differences in bacterial community structures among the groups, stacked column charts of the dominant bacterial genera were constructed based on the top 10 most abundant gut microbes in each group using the QIIME toolkit (**Supplemental Figure 2**). As shown in **Figure S2A**, Bacteroidetes, Firmicutes, and Proteobacteria were identified as the major phyla of the rat GI bacterial community. The relative abundance of Proteobacteria and Firmicutes in the I/R group was much lower than in the control group, and pretreatment with PNE could significantly reduce changes in intestinal bacterial community structure caused by I/R surgery. Similarly, large differences in bacterial class and order were observed between the control and I/R groups. Furthermore, PNE could shift the gut microbiota populations of the I/R group to be more like those of the control group (**Supplemental Figure 2B** and **2C**). For PGF rats, the gut microbiota populations were dominated by *Blautia*, *Erysipelatoclostridium*, *Alistipes* and *Paraprevotella*, which belong to the *Firmicutes* and *Bacteroidetes* phyla. After comparing gut microbiota populations in the PGF, PGF + I/R, and PGF + I/R + PNE groups, both I/R surgery and pretreatment with PNE could change intestinal bacterial community structures of PGF rats, although the magnitude of the changes were weakened compared with that in normal rats.

According to previous reports, L.R, L.B, L.H, and B.L could play a crucial role in the bidirectional gut-brain axis via modulating stress-induced behavioral deficits, immune changes and gut dysbiosis (*Bravo et al., 2011; Barrett et al., 2012; Borrelli et al., 2016;*

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Bharwani et al., 2017). To explore further the types of intestinal bacteria that mediate brain protection, the relative abundances of L.R, L.B, L.H, and B.L in control, I/R, and I/R + PNE groups were measured using qPCR. As shown in **Figure 3A**, the relative abundances of these 4 species of bacteria were down-regulated after I/R surgery, and pretreatment with PNE could significantly up-regulate the relative abundance of L.B (one-way ANOVA, $P < 0.05$) and B.L (one-way ANOVA, $P < 0.01$) in I/R rats. Given that PNE regulation of B.L was much higher than that of other bacteria, we compared the population of B.L in control, I/R, I/R + PNE, PGF, PGF + I/R and PGF + I/R + PNE groups. As shown in **Figure 3B**, the population of B.L in PGF, PGF + I/R and PGF + I/R + PNE groups was much lower than that in control, I/R and I/R + PNE groups. PNE could significantly prevent the decrease in B.L caused by I/R surgery, but it did not have a significant effect on B.L in PGF and PGF + I/R rats.

Neuroprotective Effects of B.L for I/R Model Rats

According to the results above, the decrease in relative abundance of B.L in I/R rats could be prevented by pretreating with PNE for 7 consecutive days. Thus, we investigated whether B.L exerted neuroprotective effects. Rats were pretreated with 1×10^9 CFU of B.L per day for 15 consecutive days before I/R surgery. TTC staining was used to examine cerebral infarcts 24 h after I/R surgery. **Figure 4A** shows representative images of coronal sections from the sham-operated (control), I/R, and B.L treated groups. Cerebral infarcts were obvious after establishing the I/R model. Infarct size in the B.L treatment group was significantly decreased compared with that in the I/R group; cerebral infarct volume decreased from 36.0 ± 4.3% to 25.0 ± 9.2% (**Figure 4B**). In addition, after pretreating with B.L, significant decreases in neurological deficit scores (1.96 ± 0.27) were observed compared with the I/R group (2.83 ± 0.47), which indicated that B.L improved neurological deficits caused by cerebral I/R (**Figure 4C**). Furthermore, B.L could significantly down-regulate levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in I/R rats (**Figure 4D** to **Figure 4F**). Similarly, I/R surgery significantly decreased levels of BDNF in the rat hippocampus, and B.L treatment could efficiently attenuate the down-regulation of

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BDNF caused by I/R surgery. These results suggest that colonization by B.L can exert neuroprotective effects in I/R rats.

Effects of PNE and B.L on the Expression of GABA Receptor in the Rat Hippocampus

Recent studies have suggested that in addition to playing an important role in maintaining functional homeostasis in the brain, GABA receptors are vulnerable to pathological factors, especially during ischemia *(Fiorentino et al., 2009; Cheng et al., 2010; Fu et al., 2015; Nasrallah et al., 2017*). To elucidate further the mechanism underlying therapeutic effects of PNE during I/R, effects of PNE and B.L on expression of GABA receptors were investigated. Expression levels of GABA-A receptors (α2, β2, γ2) and GABA-B receptors (R1b, R2) in the hippocampus of normal and PGF rats were assessed using RT-PCR. For normal rats, expression level ratios of GABA-A receptors (α2, β2, γ2) and GABA-B receptors (R1b, R2) in the hippocampus from I/R and control groups were less than 1, which indicated that I/R surgery could decrease expression levels of GABA receptors (**Figure 5A**, one-way ANOVA, $P < 0.05$). PNE pretreatment could significantly attenuate the decrease in expression levels of GABA receptors in the I/R group. For PGF rats, I/R surgery could also distinctly decrease expression of GABA receptors in the hippocampus. However, the ability of PNE to regulate expression of GABA receptors in PGF rats was much lower than in normal rats (**Figure 5B**, one-way ANOVA, $P < 0.05$). These results suggest that up-regulation of GABA-receptors by PNE was attenuated by antibiotic treatment due to a shift in gut microbiota populations. We also examined whether B.L could regulate GABA receptors in the hippocampus. Expression level ratios of GABA-A receptors (α2, β2, γ2) and GABA-B receptors (R1b, R2) in the B.L colonized group were much higher than in the I/R group (**Figure 5C**, one-way ANOVA, $P < 0.05$). Thus, these results suggested that B.L could prevent decreased expression levels of GABA receptors in the I/R group.

Given that both PNE and B.L could enhance expression of GABA receptors in I/R rats, GABA concentrations in the hippocampus were determined to investigate further PNE

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regulation of the GABAergic system. As shown in **Figure 5D**, I/R surgery led to an increase in GABA levels, and pretreating with PNE could significantly decrease GABA concentrations in the I/R group. Similarly, colonization with B.L could prevent an increase in GABA levels caused by I/R surgery. For PGF rats, I/R surgery could also enhance GABA levels. However, the effect of PNE on GABA levels in PGF + I/R rats was not as significant as in normal I/R rats.

Effects of GABA-A and GABA-B Receptor Antagonists on Neuroprotective Effects of B.L

Given that both PNE and B.L could up-regulate expression of GABA receptors in the hippocampus of I/R rats, we investigated whether GABA-A or GABA-B receptors mediate the neuroprotective effects. A GABA-A receptor antagonist (bicuculline, Bic) or GABA-B receptor antagonist (saclofen, Sac) was used to inhibit GABA-A or GABA-B receptor activities, respectively. As shown in **Figure 6A**, neuroprotective effects of B.L were significantly attenuated when the bacteria were co-administered the GABA-B receptor antagonist Sac. In contrast, the GABA-A receptor antagonist Bic had no significant effect. The cerebral infarct volumes of rats in the I/R, I/R + B.L, I/R + B.L + Bic and I/R + B.L + Sac groups were 36.0 ± 4.3%, 25.0 ± 9.2%, 26.2 ± 3.1% and 34.9 ± 5.1%, respectively (**Figure 6B**). The neurological deficit scores of rats in the I/R, I/R + B.L, I/R + B.L + Bic, and I/R + B.L + Sac groups were 2.83 ± 0.46, 1.96 ± 0.28, 2.10 ± 0.77 and 2.80 ± 0.25, respectively (**Figure 6C**). No obvious differences in expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were found between the I/R + B.L and I/R + B.L + Bic groups. When saclofen was co-administered with B.L, the neuroprotective effects of B.L were dramatically attenuated, and expression levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the I/R + B.L + Sac group were much higher than in the I/R + B.L and I/R + B.L + Bic groups (**Figure 6D** to **6F**). These results strongly suggested that up-regulation of GABA-B receptors might be a key factor in mediating neuroprotective effects of B.L and PNE.

The influence of PNE and B.L on the GABA-B receptors (R1, R2) was further verified

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based on Western blotting analysis. As shown **Figure 7**, the I/R surgery led to a decrease in the expression of GABA-B R1 receptor, and the expression of GABA-B R2 receptor in I/R group was significantly lower than that of the control group. Importantly, pretreating with PNE could up-regulate the expression of GABA-B R1 and GABA-B R2 receptors in the hippocampus of I/R model rats. Similarly, colonization with B.L could prevent a decrease in the expression of GABA-B R1 and GABA-B R2 receptors caused by I/R surgery.

Attenuating Effects of GABA-B Receptor Antagonists on Efficacy of PNE

Given that neuroprotective effects of B.L could be greatly attenuated by a GABA-B receptor antagonist, we examined whether a GABA-B receptor antagonist also attenuated neuroprotective effects of PNE. The GABA-B receptor antagonist, saclofen, was co-administered with PNE to confirm that neuroprotective effects of PNE were mediated by B.L and the GABAergic system. As shown in **Figure 8A** to **8C**, neuroprotective effects of PNE were drastically attenuated by the GABA-B receptor antagonist. The cerebral infarct volumes of rats in the I/R + PNE and I/R + PNE + Sac groups were 25.0 ± 9.2% and 33.4 ± 3.0%, respectively, and neurology deficit scores of rats in the I/R + PNE and I/R + PNE + Sac groups were 1.68 ± 0.41 and 2.54 ± 0.29, respectively. Similarly, levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the I/R + PNE + Sac group were significantly higher than in the I/R + PNE group.

Discussion

This study demonstrates a specific, previously unreported neuroprotective mechanism of PNE based on the microbiome-gut-brain axis. In our previous studies, the optimal dosage of PNE was screened via comparing the neuroprotective effects of PNE under different doses (20, 50, 100 and 250 mg/kg). The results revealed that the neuroprotective effects of PNE were dose-dependent within the 20 to 100 mg/kg dosage range, and the efficiency apparently increased with the increasing of the dose. At 100 mg/kg and 250 mg/kg doses, there was no significant difference in the neuroprotective effects of PNE. Hence, 100

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mg/kg of PNE was used to investigate the neuroprotective effect of PNE in the present study. We found that pretreatment with PNE (100 mg/kg) for 7 consecutive days before cerebral I/R could significantly decrease levels of pro-inflammatory cytokines, reduce the volume of brain infarcts, attenuate neurological deficits, and maintain BDNF levels in the hippocampus even though exposure to each component of PNE was extremely low in the brains of normal and I/R rats. For herbal saponins, oral bioavailability is often limited due to low membrane permeability, high molecular weight, fast clearance in the GI tract, and efflux transporters (*Hao et al., 2016*). Clinically, some commercial PNE formulations cure cerebral diseases mainly after intravenous administration, as exemplified by *XUE SAI TONG* injection. However, inconvenient medication and adverse reactions caused by injection have limited application of PNE to a large extent (*Guo et al., 2014*). In the present study, we found that cerebral concentrations of notoginsenoside monomers in the I/R group were higher than in the control group. This phenomenon could be explained by increased permeability of a compromised blood-brain barrier after ischemic brain insult. However, most of the notoginsenoside monomers were undetectable at 10 h after oral administration, which indicated that neuroprotective effects of PNE may not be mediated directly by cerebral notoginsenoside monomers. The contradiction between pharmacokinetic and pharmacodynamic profiles of PNE led us to hypothesize that PNE may exert neuroprotective effects indirectly by acting on peripheral targets.

Recent studies have revealed that intestinal microflora play a role in development of cells and tissues, affect physiological, nutritional, and immunological processes, as well as directly and indirectly alter pharmacokinetics and pharmacological activities of drugs, especially those that are orally administered (*Wallace et al., 2010; Haiser and Turnbaugh, 2012*). Given that the residence time of notoginsenosides in the intestine is significantly longer than in other sites (*Liu et al., 2009*), interactions between notoginsenosides and intestinal microflora are worth exploring. In our previous study, we found that intestinal microflora greatly affect metabolism of notoginsenosides. For example, Proteobacteria can affect deglycosylation metabolism of notoginsenosides by regulating activities of glycosidases, and up-regulation of Bacteroidetes can promote redox metabolism of

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notoginsenosides in intestinal microflora (*Xiao et al., 2016*). In the present study, a PGF rat model was established to investigate the role of gut microbiota in neuroprotective effects of PNE. We found that neuroprotective effects of PNE in PGF + I/R rats were much lower than in normal I/R rats. Thus, gut microbiota play an important role in mediating neuroprotective effects of PNE. The influence of PNE on the gut microbiota community was investigated to clarify the bidirectional regulation of intestinal flora and notoginsenosides. The results revealed that I/R surgery could dramatically alter the structure of the gut microbiota community, and cerebral I/R-induced changes in gut microbiota could be partially prevented by pretreating with PNE for 7 consecutive days. In recent years, there has been a growing interest in targeting the gut microbiome for a beneficial impact on behaviors related to psychiatric disorders including anxiety, depression, autism spectrum disorder, obsessive-compulsive disorder, and memory impairment (*Wang et al., 2016*). To date, multiple probiotic bacteria with psychotropic potential, including strains of the genera *Lactobacillus* (*Messaoudi et al., 2011a*) *Bifidiobacterium* (*Messaoudi et al., 2011b*), and *Enterococcus* (*Divyashri et al., 2015*), have been identified as drug action targets or potential therapeutic psychobiotics. For example, *L.R is a* very promising potential psychobiotic with demonstrated pharmacological activity in mice and humans (*Borrelli et al., 2016*). Oral administration of *L.B* can significantly inhibit lipopolysaccharide production and P16 expression in Lipopolysaccharides-stimulated macrophages (*Jeong et al., 2016*). In addition, a single strain of *B.L* or *L.H* can improve anxiety-like behaviors in immune-deficient mice and chronically restrained rats (*Ohland et al., 2013; Savignac et al., 2014*). In the present study, the relative abundances of CNS disease therapy-related strains (*L.R, L.B, L.H,* and *B.L)* were measured to investigate the therapeutic target and mechanism of PNE for cerebral I/R. The results demonstrated that the relative abundances of all 4 species of bacteria were down-regulated after I/R surgery, and pretreating with PNE could significantly up-regulate the relative abundance of *B.L.* We chose *B.L* as a potential therapeutic bacterial species for cerebral I/R since it had been reported to improve anxiety-like behavior and normalize levels of hippocampal BDNF in mice (*Bercik et al.,*

2011). After colonizing *B.L* (1×10^9 CFU) by gavage 15 d before I/R surgery in rats, the I/R surgery-induced effects on infarct size, neurological deficit score, pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), and BDNF could be prevented.

GABA is the primary inhibitory neurotransmitter in the mammalian CNS and acts on GABA-A and GABA-B receptors (*Vollenweider et al., 2006*). Numerous studies have demonstrated that GABAergic neurotransmission in the hippocampus is closely related to modulation of behavior and memory, and GABA receptors play a pivotal role in treatment of transient focal cerebral ischemia (*Vollenweider et al., 2006; Wang et al., 2007; Han et al., 2008; Cheng et al., 2010*). To bridge the gap between PNE and GABA receptors, the influence of B.L on expression of GABA receptors was investigated. The results revealed that pretreatment with B.L could up-regulate expression of GABA-A and GABA-B receptors in the rat hippocampus. In addition, PNE pretreatment could significantly up-regulate expression levels of GABA receptors in the hippocampus of rats in the I/R group. To confirm the role of B.L in PNE regulation of GABA receptors, we compared expression of GABA receptors in the hippocampus of normal and PGF rats. The results suggest that I/R surgery can decrease expression of GABA receptors in the hippocampus of PGF rats and that the ability of PNE to regulate expression of GABA receptors is much lower in PGF rats compared with normal rats.

Numerous GABAergic neurons are located in the hippocampal dentate gyrus, and GABA-A and GABA-B receptors in this region are the 2 major classes of receptors that regulate synaptic plasticity and learning and memory processes (*Shahidi et al., 2008*). The GABA-A receptor, which is a ligand-gated ion channel, is a pentameric structure assembled from 5 of the 19 known protein subunits, including α1–6, β1–3, γ1–3, δ, ε, π, ρ1–3, and θ (*McGinnity et al., 2017*). Experiments in animals suggest that alteration of GABA-A receptor-mediated neurotransmission is closely related to a wide variety of neurological and psychiatric disorders including anxiety, epilepsy, and schizophrenia (*Macdonald et al., 2004; Charych et al., 2009*). Recently, Li *et al*. reported that GABA-B receptors in the hippocampal dentate gyrus, which is involved in impairment of spatial learning and memory due to vascular dementia, have been suggested as a potential new

target for alternative treatment of cognitive dysfunction in vascular dementia (*Li et al., 2016*). To date, the potential influence of GABA receptors on the treatment of focal cerebral I/R has not been studied. To investigate whether GABA-A or GABA-B receptors mediate neuroprotective effects of PNE, bicuculline (GABA-A receptor antagonist) and saclofen (GABA-B receptor antagonist) were used to inhibit GABA-A and GABA-B receptor activities, respectively. The results suggest that neuroprotective effects of B.L and PNE are significantly attenuated when co-administered with the GABA-B receptor antagonist saclofen, whereas the effect of GABA-A receptor antagonist bicuculline was not as obvious as saclofen.

Thus, the findings of the present study suggest that I/R surgery alters intestinal flora and down-regulates the population of B.L. The decrease in B.L levels then leads to down-regulation of GABA receptor expression. After pretreating with PNE, I/R-related shifts in intestinal flora can be prevented to some extent, and the relative abundance of B.L can be significantly enhanced. Enhanced B.L levels can then up-regulate GABA-A and GABA-B receptor expression in the rat hippocampus, and the up-regulated GABA-B receptors play a protective role in ischemic brain damage. To the best of our knowledge, this is the first report to elucidate cerebral protective effects of PNE that involve gut microbiota. It is important to note that B.L plays a key role in PNE treatment of cerebral I/R by up-regulating GABA-B receptors.

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Footnotes

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Legends for Figures

Figure 1 Intracephalic and plasma concentrations of Panax notoginosides (R1, Rd, Re, Rg1, Rb1, Rb2) at 30 min, 2 h and 10 h after intragastric administration of 100mg/kg of PNE to normal and I/R model rats (n= 6) . (A) Intracephalic concentrations at 30 min, (B) Intracephalic concentrations at 2 h, (C) Intracephalic concentrations at 10 h, (D) Plasma concentrations at 30 min, (E) Plasma concentrations at 2 h, (F) Plasma concentrations at 10 h.

Figure 2 Coronal sections of TTC-stained brains (A), infarct volume (B) and neurology deficit score (C) of normal, I/R model and I/R +PNE rats (n= 6 per group). Coronal sections of TTC-stained brains (D), infarct volume (E) and neurology deficit score (F) of PGF, PGF+I/R model and PGF+I/R+PNE rats (n= 6 per group). Levels of IL-1β (G), IL-6 (H) and BDNF (I) in rat hippocampus (n= 6 per group).

Figure 3 Relative abundance of the dominant bacterial in the rat intestinal microflora (n= 6 per group). (A) The relative abundances of L.R, L.B, L.H, and B.L in control, I/R and I/R + PNE rat intestinal microflora, (B) The relative abundance of B.L in control, I/R, I/R + PNE, PGF, PGF + I/R, and PGF+I/R+PNE rats.

Figure 4 The neuroprotective effects of B.L (n= 6 per group). (A) Coronal sections of TTC-stained brains, (B) Infarct volume. (C) Neurology deficit score, (D) IL-1β, (E) IL-6, (F) TNF-α, (G) BDNF.

Figure 5 Effects of PNE and B.L on GABA receptor expression and GABA concentration in the rat hippocampus (n= 6 per group). (A) The relative expression of GABA receptors in I/R and I/R+PNE rats, (B) The relative expression of GABA receptors in GF, GF+I/R and GF+I/R+PNE rats, (C) The relative expression of GABA receptors in I/R and I/R+B.L rats, (D) GABA concentrations in rat hippocampus.

Figure 6 Effects of GABA-A and GABA-B receptor antagonists on B.L-induced neuroprotection (n= 6 per group). (A) Coronal sections of TTC-stained brains. (B) Infarct volume of rat brains. (C) Rat neurology deficit score. (D) IL-1β levels. (E) IL-6 levels. (F) TNF-α levels. (G) BDNF levels.

Figure 7 The influence of PNE and B.L on the expressions of GABA-B receptors (R1, R2) determined by Western blotting (n= 6 per group). (A) The corresponding protein band of GABA-B R1, GABA-B R2 and GAPDH; (B) Gray scale analysis of the GABA-B R1 protein expression; (C) Gray scale analysis of the GABA-B R2 protein expression.

Figure 8 Effects of GABA-B receptor antagonists on efficacy of PNE (n= 6 per group). (A) Coronal sections of TTC-stained brains, (B) Infarct volume of rat brains, (C) Rat neurology deficit score, (D) IL-1β levels, (E) IL-6 levels, (F) TNF-α levels.

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Figures

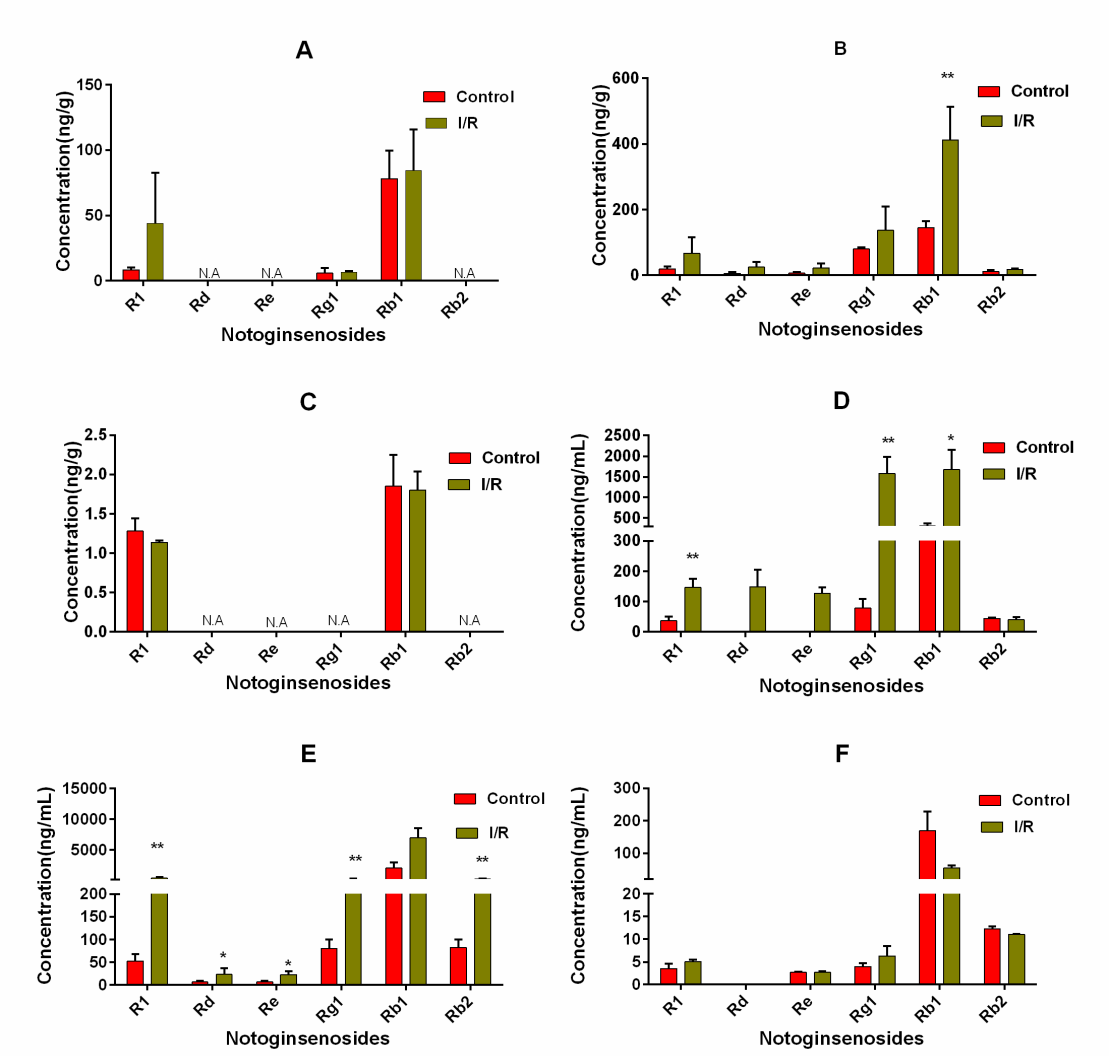

Figure 1

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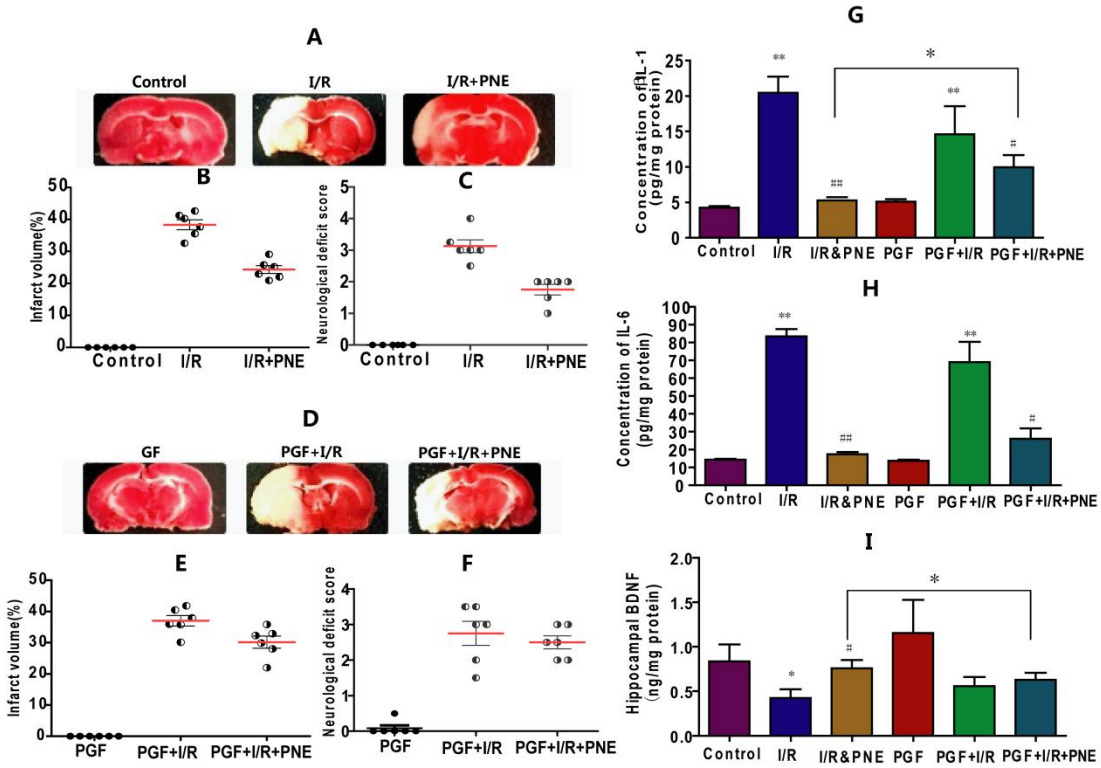

Figure 2

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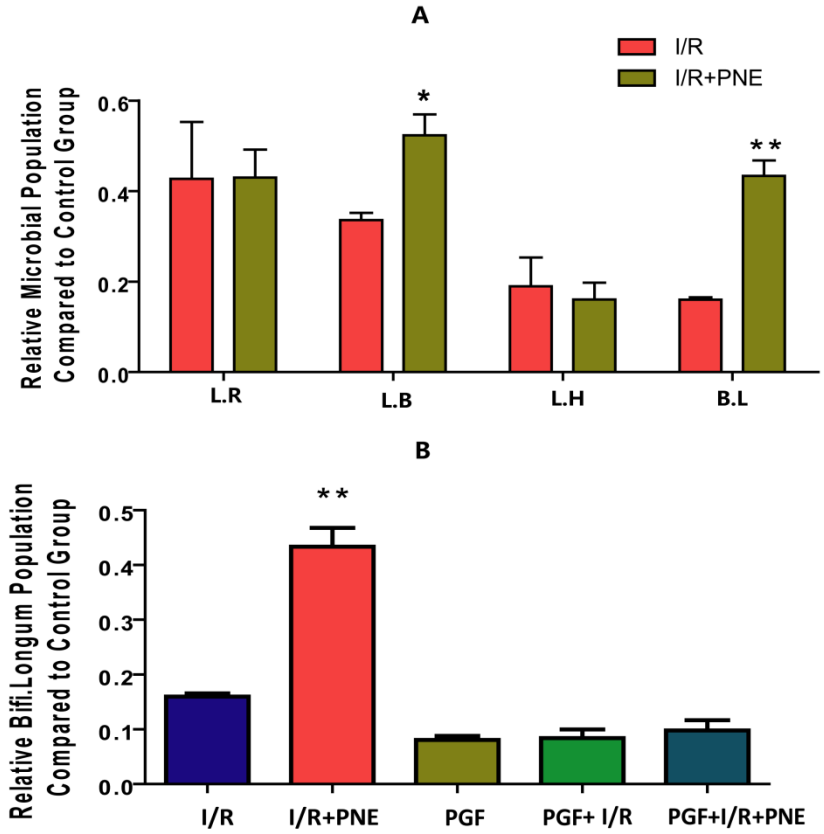

Figure 3

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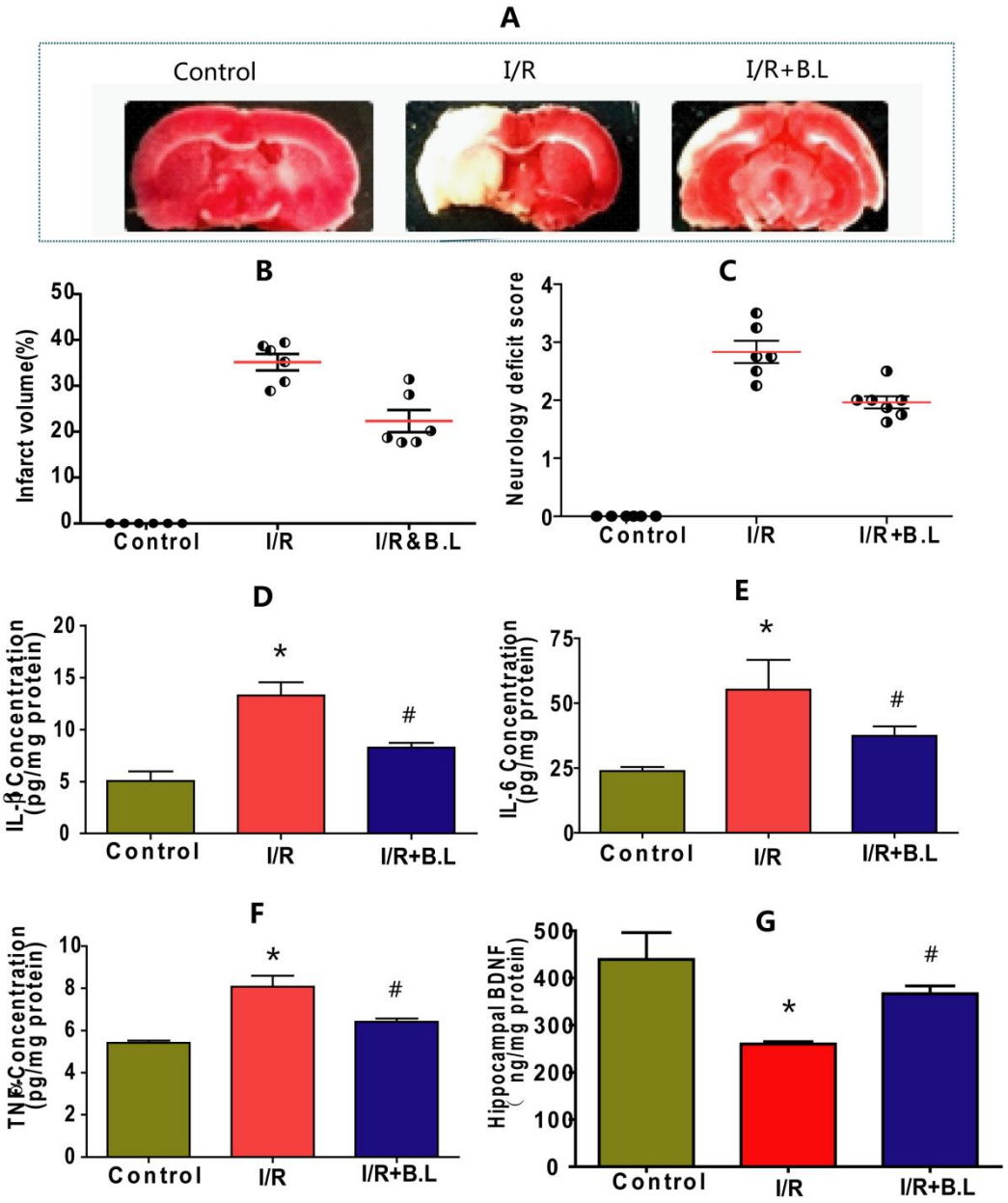

Figure 4

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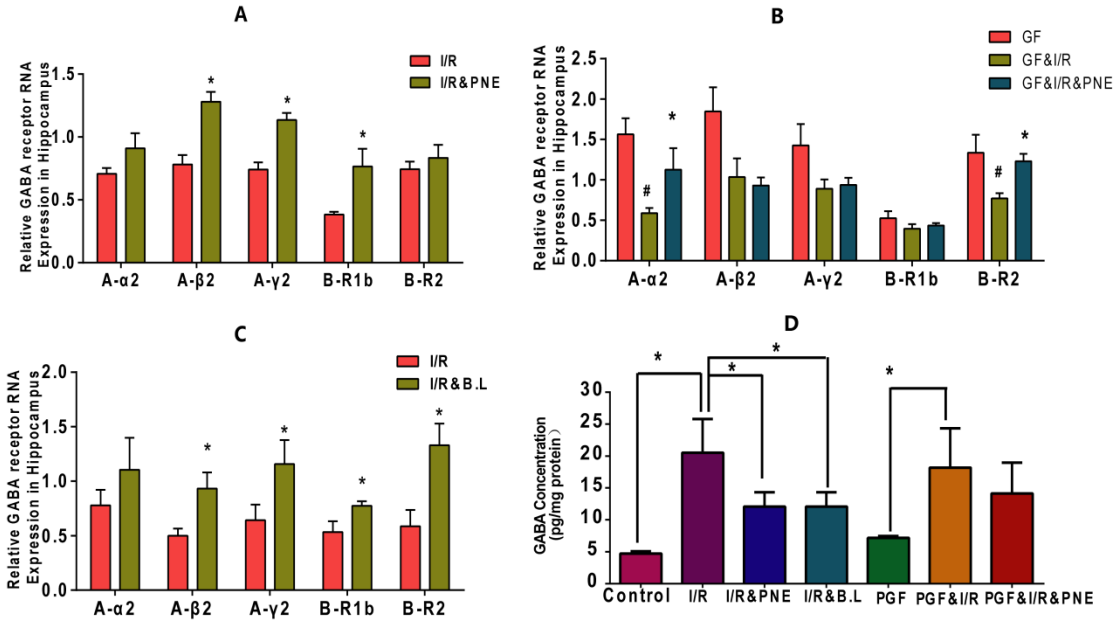

Figure 5

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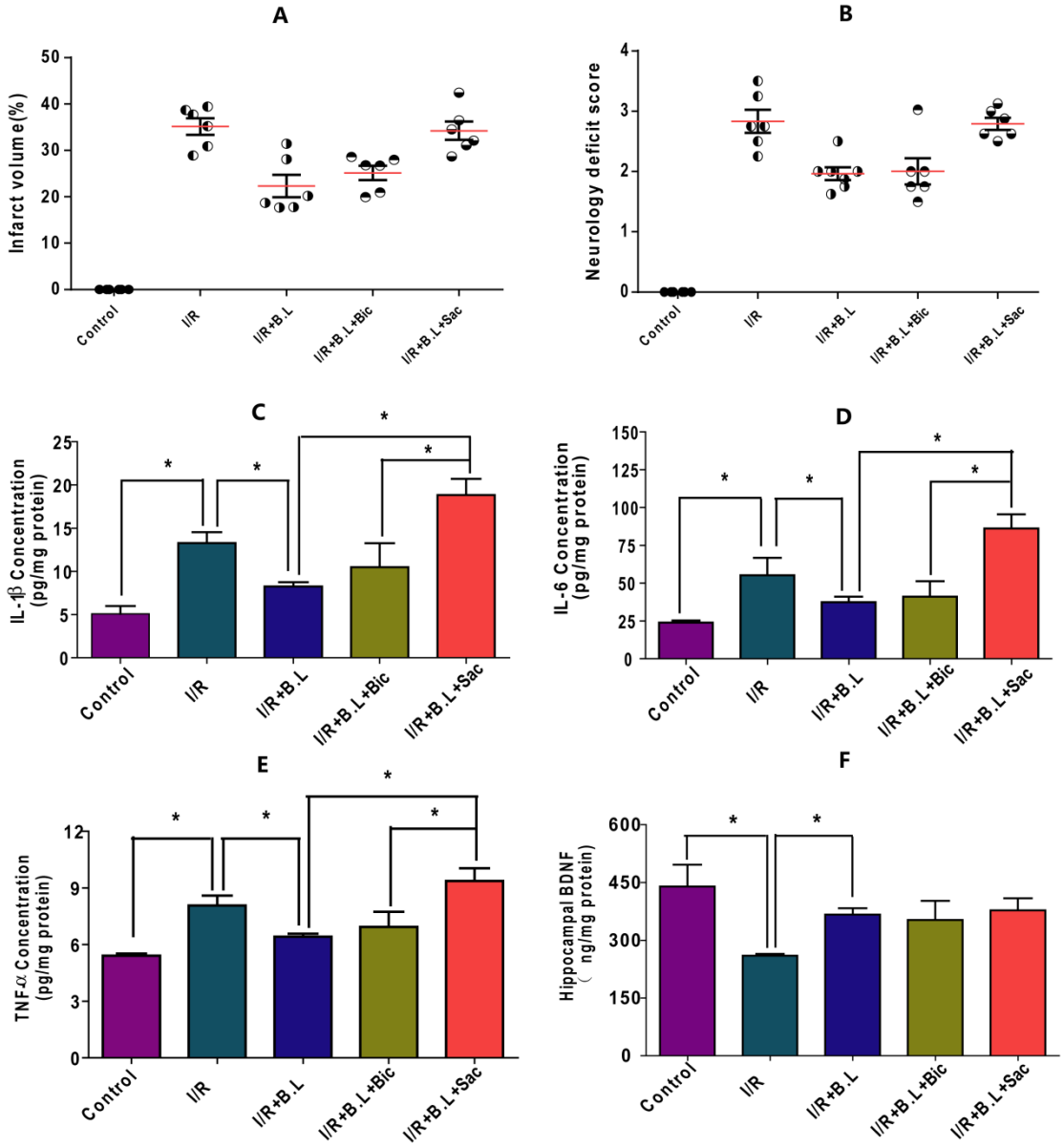

Figure 6

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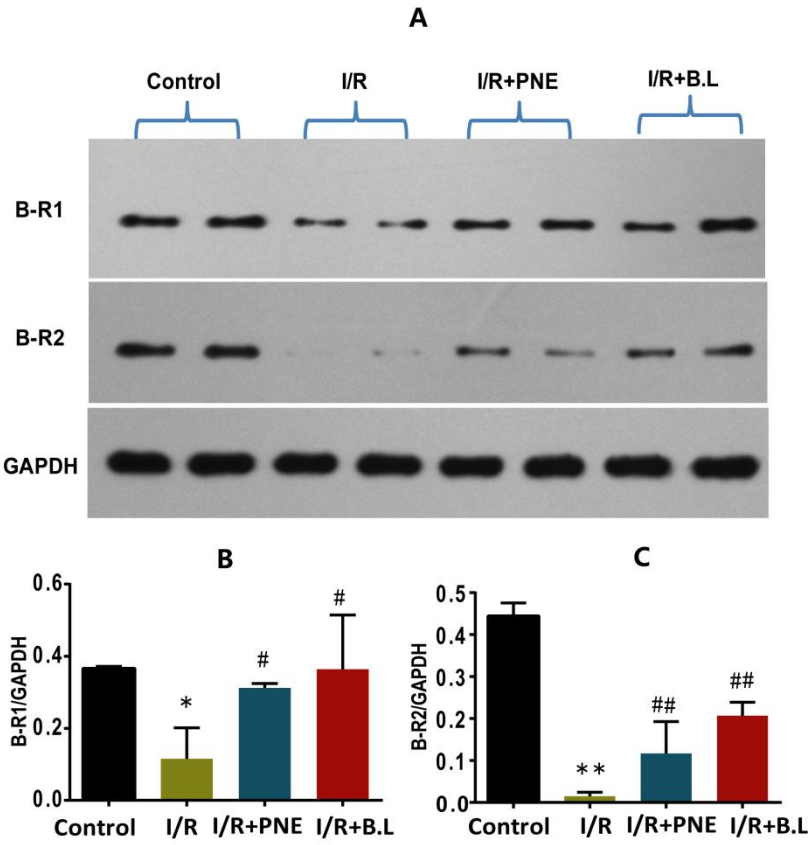

Figure 7

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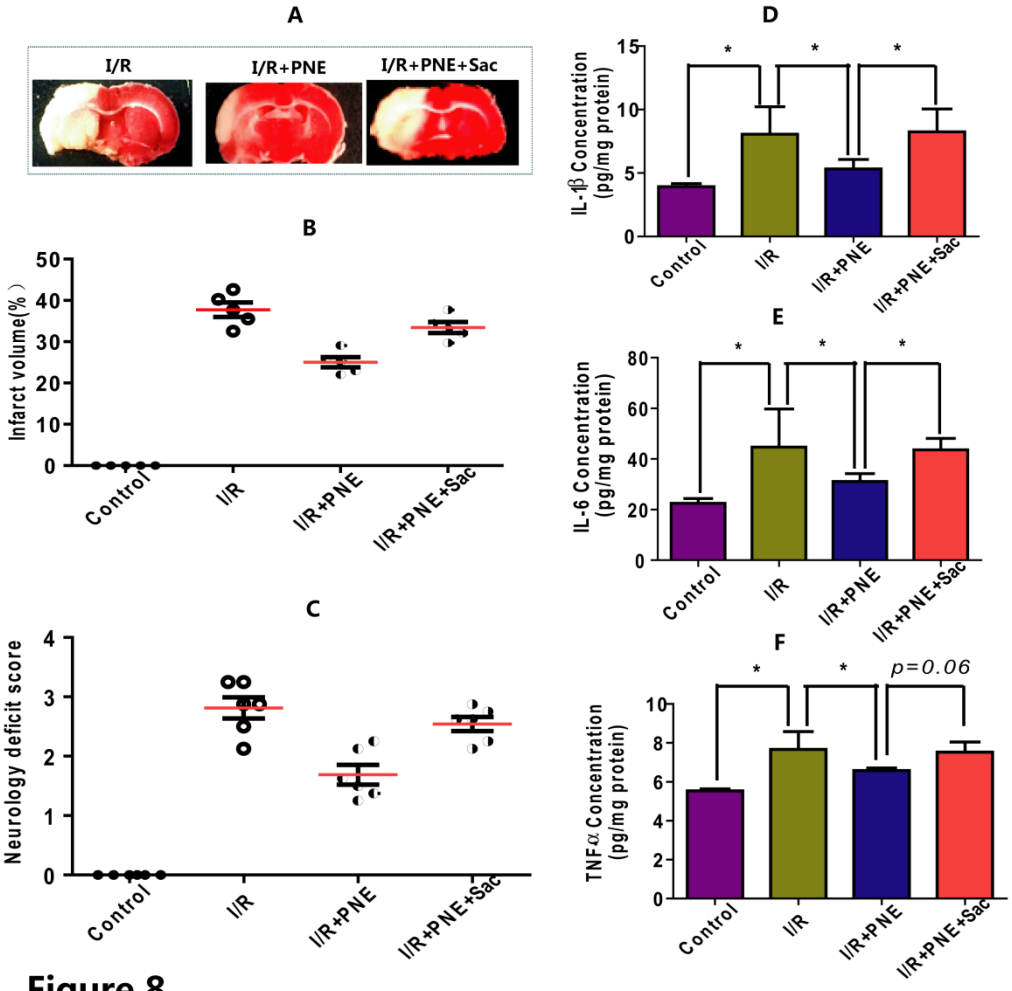

Figure 8