Original Article
Panaxatriol saponins attenuate blood-brain barrier disruption in rats following transient middle cerebral artery occlusion

Chaosheng Li1,3*, Hui Yang1*, Zhen Hui1,5, Linjie Yu2, Sulei Wang1,5, Yijian Chen1, Yun Xu1,2,4

1Department of Neurology, Nanjing Drum Tower Hospital Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, PR China; 2Department of Neurology, Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, PR China; 3Department of Neurology, The Third People’s Hospital of Wuxi, The Clinical Medical College of Chinese and Western Medicine Affiliated to Nanjing University of Chinese Medicine, Wuxi, PR China; 4The State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210008, Jiangsu, PR China; 5Department of Neurology, Nanjing Hospital of Traditional Chinese Medicine, Nanjing 210001, Jiangsu, PR China. *Equal contributors.

Received December 5, 2016; Accepted March 2, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: Blood-brain barrier (BBB) disruption after ischemia/reperfusion (I/R) is an important factor that contributes to neuron death and infarct expansion. Finding a drug that provides BBB protection is of significant clinical value. The current study team has previously shown that panaxatriol saponins (PTS) protects against focal cerebral ischemia by reducing cerebral edema. This study used a transient middle cerebral artery occlusion (tMCAO) model to explore whether PTS can be neuroprotective. PTS treatment after reperfusion significantly decreased infarction volume and Neuroscore compared to a control group. PTS noticeably prevented BBB disruption after ischemia and reperfusion, as indicated by a reduction of EB in the brain cortex as compared with a control group. This finding was confirmed using immunofluorescence staining and immunoblotting of the tight junction-related proteins claudin-5, occludin and zonula occludens-1 (ZO-1). PTS-treated rats showed a reduction in mRNA expression and release of cytokines TNF-α, IL-1β and IL-6 in the brain. Moreover, PTS obviously inhibited elevated MMP-9 and NF-κB activity after stroke. In conclusion PTS attenuates blood-brain barrier disruption in rats after I/R, suggesting that PTS may be a promising therapeutic agent in stroke therapy.

Keywords: Ischemia/reperfusion, panaxatriol saponins, neuroinflammation, blood-brain barrier

Introduction

Stroke is a leading cause of serious long-term disability and the fifth most common cause of death, exceeded by diseases of the heart, chronic lower respiratory disease, cancer, and unintentional injury [1, 2].

There are approximately 2.5 million new stroke cases each year in China and 7.5 million stroke survivors, leading to a mortality rate of approximately 1.6 million per year, which has a large impact on the Chinese economy [3]. Currently, recombinant tissue plasminogen activator (rt-PA) is the only therapeutic drug approved by the FDA for acute ischemic stroke treatment [4]. However, intravenous rt-PA after stroke may result in ischemia and reperfusion (I/R) injury-induced blood-brain barrier (BBB) disruption leading to brain edema and hemorrhagic transformation, one of the life threatening complications after acute ischemic stroke. Consequently, an effective and safe treatment for ischemic stroke is still a challenge, especially at early stages.

The blood-brain barrier is a multicellular vascular structure that separates the central nervous system from peripheral blood circulation [5]. Acting as a diffusion barrier, the BBB is composed primarily of brain endothelial cells, astrocyte end-feet, pericytes, perivascular macrophages and a basal membrane. Its integrity is maintained by an endothelial cell monolayer through tight junctions (TJs) and the basal lamina, limiting paracellular flux of xenobiotics with
BBB protection effect of panaxatriol saponins

The exception of small, lipid-soluble molecules [6]. Previous studies have demonstrated that altered organization and expression of TJ proteins, including occludins, claudins and ZO-1, are associated with disrupted BBB function in various pathologies associated with ischemic stroke [7, 8]. TJ can therefore be targeted pharmacologically for the purpose of reducing the permeability of BBB during ischemic stroke.

Panaxnotoginseng root (Burk., F.H. Chen,) has been widely used in China for thousands of years. Traditional Chinese medicine considers it effective for the prevention and treatment of cardiac and cerebral vascular diseases and diabetes and for treating pain and bleeding [9]. Panaxatriol saponins (PTS), the main active ingredient of Panaxnotoginseng, has been clinically used in China for the treatment of cerebral infarction. It has been reported that PTS can attenuate oxygen-glucose deprivation injury in PC12 Cells [10] and protect against focal cerebral ischemia by reducing cerebral edema, upregulating heat shock protein HSP70 expression and downregulating the transfer of BBB [11]. However, the effect of PTS on BBB injury after focal cerebral I/R has not been explored. Using a tMCAO rat model, this study explores whether PTS can maintain BBB integrity and improve behavioral score after ischemia/reperfusion.

Material and methods

tMCAO rat model

All animal surgical procedures and experimental protocols were approved by the Committee of Experimental Animal Administration of Nanjing University. Male Sprague-Dawley rats (body weight 250-300 g) were supplied by the Animal Center of Nanjing Drum Tower Hospital and were housed in a 12/12 h dark/light cycle at a temperature of 21°C ± 2°C and humidity of 60%-70% for more than 1 week before the experiments. All rats were allowed free access to standard rodent food and tap water before the experiment. MCAO was performed using the intraluminal suture method as previously described [12, 13]. Rats were randomly assigned to a MCAO group and a PTS-treated MCAO model group. Briefly, the middle cerebral artery of each rat was occluded using a 4-0 monofilament nylon suture with heat-rounded tip. The vessel was occluded for 2 hours and the suture was then removed to allow reperfusion. During surgery and reperfusion, body temperature was maintained in the range of 36.5°C to 37.5°C by means of a heating blanket and regional cerebral blood flow (rCBF) of the middle cerebral artery territory was monitored using a laser Doppler computerized main unit (Perimed AB, Sweden). Successful occlusion was characterized as a decrease of rCBF to 20% of baseline; otherwise, animals were excluded. The same procedure without suture insertion was conducted on the sham group.

Drug treatments

PTS extracted from Panaxnotoginseng was provided by Huasun Group Co., Ltd. (Sichuan, China). As determined by HPLC in Figure 1. The ginsenoside Rg1, notoginsenoside R1, and ginsenoside Re are the main components of PTS with the concentrations of 50%, 11% and 6% respectively. PTS was dissolved in PBS at a dose of 8 mg/mL. According to previous literature, rats were randomly treated with 25, 50, and 100 mg/kg doses of PTS. As a result, 50 mg/kg of PTS was chosen as optimum. Rats in the sham-operated and the vehicle-treated ischemic model groups were each further divided to create four groups: sham-veh, sham-pts, MCAO-veh and MCAO-pts. The rats were treat-
ed intraperitoneally with PTS or PBS once per day for 3 days.

**Infarct volume measurement**

Infarct volume in MCAO-treated rats was measured using 5-triphenyltetrazolium (TTC) staining. After 24 and 72 hours of reperfusion, ten rats from each group were sacrificed and the brains were cut into a series of 2 mm thick coronal sections. The brain sections were stained with a 2% TTC (Sigma, St Louis, MO, USA) solution at 37°C for 30 min and then fixed in 4% (w/v) paraformaldehyde 24 hours later. Stained sections were scanned with a computer-controlled digital camera (Olympus, Japan). Infarct volume was calculated using Image-Pro Plus 6.0 (IPP) software (Media Cybernetics, USA). The value of the infarct volume was presented as the percent of the contralateral hemisphere after a correction for edema in the ipsilateral cortex.

**Neurological function assessment**

After 6 h, 1 d, 3 d and 7 d of reperfusion, neurological function was evaluated according to Longa Score [14], as follows: score 0, no neurological deficit; score 1, failed to fully extend the contralateral forepaw; score 2, circled around while walking; score 3, tumbled to its side while walking because of hemiplegia; and score 4, lost consciousness or died. Assessments were by an observer who was blinded to drug treatment.

**Western blotting**

Protein was extracted from the ischemic region of cerebral cortices according to previously described procedure and was then quantified using a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA). Equal portions of total protein samples were separated by sodium dodecyl sulfate-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, membranes were incubated overnight at 4°C with primary antibodies at the following dilutions: claudin-5 (1:1000, Millipore, USA), MMP-9 (1:1000, Cell Signaling Technology, USA), GAPDH (1:2000, Bioworld, USA), I-κB-α (1:1000, Cell Signaling Technology, USA), NF-κB p65 (1:1,000, Cell Signaling Technology, USA), occluding (1:500, Life Technologies, USA), zona occluden-1 (ZO-1) (1:500, Life Technologies, USA), β-actin (1:5000, Santa Cruz Technology, USA). Membranes were incubated with the respective secondary antibodies for 2 h and then reacted with enhanced chemiluminescence substrate (Bioworld, Rockford, IL, USA). The protein bands were imaged using Quantity One image software (Bio-Rad, Hercules, CA, USA) and relative intensity was calculated using Image J software (National Institutes of Health, USA).

**Enzyme linked immunosorbent assay (ELISA)**

Amounts of IL-1β, IL-6, and TNF-α were quantified using an ELISA kit (R&D systems, Minneapolis, MN, USA) according to manufacturer’s instruction. OD at 450 nm was recorded using a fluorescence spectrometer (Bio-Rad, USA) and the concentration of target protein was calculated according to the standard curve. Results were expressed as pg/mg protein.

**Real-time PCR**

Total RNA was extracted from ipsilateral hemispheres using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as previously described, and was reverse-transcribed to cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative RT-PCR was performed in the presence of a fluorescent dye (Takara, Dalian, China) using an ABI 7500 machine (USA). The cycle threshold (CT) mean of triplicate wells of each sample was obtained; if a CT value differed by more than 0.5 from the other two, it was excluded. The CT value was normalized to GAPDH expression. The expression levels of mRNAs were reported as fold changes vs. sham-veh group.

Primers (Invitrogen, USA) were as follows: IL-1β: F: CACCTCTCAAGCAGACACAG, R: GGTTCCATGGTGAAGTCAAC; IL-6: F: GAGACATGGGAAGTTGGGG, R: CTTCCAGCAGTTGCCTTCT; TNF-α: F: CTCTTCAAGGGACAAGGCTG, R: TCACAGAGCAATGACTCCAAAG; GAPDH: F: GGCTCTCTGCCTCCCTGTTCTA, R: CGGCCAAATCCGATTCAACAGAAG.

**Immunofluorescence staining**

The rats were perfused with 0.9% saline and 4% paraformaldehyde (PFA) under anesthetic. Brain cryosections (20 μm in thickness) were incubated with anti-Rat CD31 (Abcam, UK) pri-
BBB protection effect of panaxatriol saponins

Figure 2. Effects of PTS on infarct volumes and neurological function. Rats were subjected to 2 h MCAO followed by treatment of vehicle (PBS) or PTS. A. Dose-dependent effects of PTS on infarct volumes evaluated by TTC at 72 h (n = 10 per group). B. Neurological performance of rats in each group assessed with longa score at 6 h, 1 d, 3 d, and 7 d after reperfusion (n = 10 per group), *P < 0.05 vs. MCAO-veh. C. Statistical bar graph of brain infarct size in vehicle or PTS-treated rats at 1 d and 3 d after MCAO. *P < 0.05 versus MCAO-veh group, (n = 10 per group), *P < 0.05 vs. MCAO-veh. D. A sample of brain slices TTC staining at 72 h after MCAO.

Primary antibody, rabbit anti-occludin (Abcam, UK) and rabbit anti-ZO-1 (Cell Signaling Technology, USA) overnight at 4°C. The brain sections were then incubated with Alexa Fluor secondary antibody (1:500, Invitrogen, USA) for 2 h at room temperature. Cell nuclei were stained with DAPI. Images of sections were acquired using an Olympus microscope with a BX51 digital camera and then processed with IPP6.0 software.

Evaluation of Evans blue dye (EBD) extravasation

Rats were injected with 2% EBD (sigma, St Louis, MO, USA) (w/v) in phosphate-buffered saline (PBS, pH 7.5) sterilized by pass through a Millipore® GP 0.22 µm filter (Millipore, Bedford, MA, USA) and stored at 4°C. Intravenous (i.v.) injections were made to either of the dorsal veins of the tail. After injection, animals were returned to their cage and allowed food and water ad libitum. The brain tissues were collected, then they were weighted and suspended with a 1:3 volume of a 50% trichloroacetic acid solution (dissolved in saline). The trichloroacetic acid-suspended tissue was homogenized and the resulting lysate was centrifuged at 10000 rpm for 20 min. The final supernatant was diluted with a three-fold volume of 95% ethanol and subjected to a photospectrometric detection of EBD absorbance at 620 nm.

Statistical analysis

Data were presented as mean ± SEM and analyzed using SPSS 16.0 statistical software.
BBB protection effect of panaxatriol saponins

(SPSS, Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) method was used to analyze differences among multiple groups. Differences between two groups were statistically evaluated using Student’s t-test. A value of $P < 0.05$ was considered statistically significant.

**Results**

*PTS exerts a neuroprotective effect against ischemia/reperfusion injury in tMCAO rats*

TTC staining was used to explore the effect of PTS on reducing infarct volume in experimental ischemia/reperfusion injured rats at different doses (Figure 2A). A dose of 50 mg/kg/day PTS was then administered for subsequent experiments. Results suggest that treatment of PTS dramatically decreased infarct size ($P < 0.05$) (Figure 2C).

To characterize the neurological effects of PTS treatment, NSS were used to assess behavior at different times after I/R. The resulting differences in neurological function were significant between the MCAO-veh and MCAO-pts treated groups ($P < 0.05$) (Figure 2C).

PTS reduced ischemia/reperfusion-induced BBB disruption

BBB disruption is a type of brain damage that occurs after cerebral ischemia/reperfusion. To explore whether PTS has a positive effect on BBB disruption in rat brains 72 h after reperfusion, BBB permeability was tested using Evans blue extravasation assay. Compared with the contralateral hemisphere, a significant increase in Evans blue content was observed in the ipsilateral side. Compared with the sham-group, the EB content of the MCAO-group significantly increased ($P < 0.05$) (Figure 3A), suggesting that PTS may attenuate BBB disruption.

To further corroborate the effects of PTS on BBB integrity, TJ proteins ZO-1, occludin, claudin-5 and CD31 were measured using immunofluorescence microscopy. Meanwhile, protein expression levels were measured using western blotting. In the sham-veh and sham-pts groups, some claudin-5 and CD31 signals in rat cortices were found to perfectly overlap (Figure 4A, top panel). However, alignment of claudin-5 signals was mostly destroyed in the ipsilateral of the MCAO-veh group, indicating that there is a disruption of BBB (Figure 4A, middle panel). Disruption of claudin-5 signal alignment was largely reduced in the MCAO-pts group compared with MCAO-veh group, suggesting that BBB disruption after ischemia/reperfusion was attenuated (Figure 4A, bottom panel). Consistently, it was demonstrated using western blot analysis that reduction of claudin-5 levels was significantly reduced after PTS treatment (Figure 4B, 4C).

Figure 3. PTS lessened Evans blue extravasation in rats. A. Bar graph shows a quantification analysis of Evans blue contents in brain tissue (n = 6 per group). B and C. Images show Evans blue extravasation in sham-veh, sham-pts, MCAO-veh and MCAO-pts at 3 days after tMCAO. Data are mean ± SD, *P < 0.05, MCAO-pts versus MCAO-veh group.
Figure 4. PTS protects the decrease in TJ protein expression. (A, D, G) Representative immunofluorescence confocal images in different groups of claudin-5 (green), occludin (green) and ZO-1 (green) localized at the periphery of endothelial cells with marker CD31 (red). (B, E, H) Representative western blots of claudin-5, occludin and ZO-1 (C, F, I). Quantitative analysis of claudin-5, occludin and ZO-1. Values are mean ± SEM, n = 3 per group. **P < 0.01 vs. Sham-veh group, #P < 0.05, ###P < 0.01 vs. MCAO-veh group.
Similarly, the occludin and ZO-1 proteins were also examined with immunofluorescence and western blot (Figure 4D-I). The results of TJ proteins demonstrated that PTS could alleviate ischemia/reperfusion-induced blood-brain barrier disruption.

**PTS reduced ischemia/reperfusion-induced pro-inflammatory cytokine production**

Pro-inflammatory cytokines play an important role in BBB disruption after stroke. Since PTS could protect BBB, further evaluation was conducted to determine if PTS could reduce the level of pro-inflammatory cytokine production in tMCAO models. Results showed that the mRNA levels of TNF-α, IL1-β and IL-6 were greatly upregulated in MCAO-veh and MCAO-pts groups. However, PTS largely reduced transcription levels of TNF-α, IL1-β and IL-6 (Figure 5D-F). At the same time, the same effect was found in protein expression levels of TNF-α, IL1-β and IL-6 in the MCAO-pts group (Figure 5A-C). Therefore, results show that PTS may be a potent suppressor of ischemia/reperfusion-induced neuroinflammation.

**PTS inhibited ischemia/reperfusion-induced MMP-9 expression**

To further investigate whether PTS affects the upstream molecular pathway of TJs, MMP-9 expression was analyzed using western blotting at 72 h after reperfusion (Figure 6A, 6B). The expression of MMP-9 was significantly increased in the MCAO-veh and MCAO-pts groups compared with the sham-veh group (P < 0.01). PTS significantly inhibited the expression of MMP-9 compared with the MCAO-veh group (P < 0.05).

**PTS inhibited NF-κB pathway in tMCAO rats**

Once it was determined that PTS may reduce ischemia/reperfusion-induced pro-inflammatory cytokine production, this study explored whether the effect was mediated by the NF-κB pathway. Protein levels of I-κB-α and NF-κB-P65 were tested using western blot at 72 h after reperfusion (Figure 6C, 6E and 6G). The expression of I-κB-α was significantly reduced in the MCAO-veh and MCAO-pts groups compared with the sham group (P < 0.01). PTS significantly increased the expression of I-κB-α (P < 0.05).
BBB protection effect of panaxatriol saponins

Figure 6. PTS inhibited MMP-9 expression and NF-κB pathway in cerebral ischemia of rats. (A, C, E and G) Representative western blots of MMP-9, IκB-α, nuclear NF-κB P65 and cytoplasm NF-κB P65 in different groups (B, D, F and H). Quantitative analysis of MMP-9, IκB-α, nuclear NF-κB P65 and cytoplasm NF-κB P65. Data are presented as mean ± SEM, n = 6 per group. #P < 0.05, **P < 0.01.
but significantly inhibited the nuclear translocation of NF-κB-P65 compared with the MCAO-veh group (P < 0.05).

Discussion

In this study, an ischemia/reperfusion-induced cerebral injury rat model was used to demonstrate a novel neuroprotective effect of Panaxnotoginseng extraction. Experiments were performed to show that PTS significantly reduced infarct volumes following TTC staining and alleviated the neurological deficit of the rats after tMCAO. In addition, study results indicated that the neuroprotective effects of PTS were largely associated with anti-inflammatory effects, along with improved BBB integrity and reduced MMP-9 expression levels.

BBB disruption is a key event in the pathogenesis of stroke [15], as it can worsen the prognosis of patients by the formation of intracerebral vasogenic edema and hemorrhagic transformation. Therefore, maintenance of BBB integrity is a potential strategy to protect the brain from ischemia/reperfusion-induced injury [15-17]. BBB disruption after stroke has two phases. The first phase, biphasic permeability, is closely associated with hemorrhagic transformation and is present at 3-8 h post-reperfusion [18]. The second phase, vasogenic edema, is present 18-96 h after reperfusion and is one of leading causes of clinical deterioration and death subsequent to ischemia [18, 19].

This study analyzed the effect of PTS on BBB permeability and brain edema at 72 h after reperfusion. Results demonstrated that PTS treatment significantly attenuated BBB disruption after ischemia/reperfusion.

Tight junctions (TJs) are important elements of BBB, which is located in the tightly sealed monolayer of brain endothelial cells [20]. TJs are formed by a combination of transmembrane occludin and claudin-5, which play important roles in regulating the integrity and proper functions of the BBB [5, 21]. In experimental models of ischemic stroke, reduced expression of claudin-5 [22, 23] and disruption of interaction between claudin-5 and occluding [24] have been reported. ZO-1, a 220 kDa protein that links transmembrane proteins of the TJ (i.e., occludins, claudins) to the actin cytoskeleton [25], is decreased after ischemic stroke [26]. In this study, disruption of tight junction proteins (claudin-3, claudin-5 and zon-1) was observed in the brain after transient MCAO, while PTS treatment normalized expression levels of these proteins.

Many proteinases, in particular matrix metalloproteinases (MMPs), play a vital role in maintaining the integrity of BBB after stroke [27, 28]. Among MMPs, MMP-9 has been extensively studied for its involvement in BBB disruption after stroke [29]. It has been reported that MMP-9 knockout mice are resistant to BBB disruption after transient focal-cerebral ischemia [29]. Disruption of tight junctions has been positively associated with MMP-9 activity and TJ proteins such as claudin-5 and occludin have been shown to be degraded by MMP-9 through cleavage of portions of their extracellular domains [30-32]. Accordingly, decreasing the activity of MMP-9 is beneficial for maintaining TJ proteins. This study observed robust upregulation of MMP-9 in the brain after transient MCAO, which was significantly decreased after PTS treatment. These results suggest that the protective effect of PTS on BBB disruption may be associated with the inhibition of MMP-9 and the regulation of TJ proteins.

There is substantial evidence showing that post-ischemic inflammation plays an important role in the pathogenesis of ischemic stroke [33, 34]. The expression of critical TJ proteins, occludin and ZO-1, were also influenced by pro-inflammatory stimuli [35], accompanied by BBB disruption. Many anti-inflammatory materials have therapeutic effect in tMCAO animal models [36, 37]. Real-time PCR and ELISA data of the current study show that increased expression of cytokines TNF-α, IL-1β and IL-6, after tMCAO, was significantly attenuated by PTS treatment, suggesting that PTS has anti-neuro-inflammatory properties.

NF-κB, a pivotal transcription factor, is essential for immune and stress responses in the brain [38]. It is composed of subunits p65 and p50. When cells are stimulated, NF-κB is activated and translocated into the nucleus, inducing transcriptional activation of potentially deleterious pro-inflammatory genes [39]. In rodents, activation of NF-κB occurs after experimental stroke; inhibiting the NF-κB signaling pathway by pharmacological and genetic approaches has been reported to be neuroprotec-
BBB protection effect of panaxatriol saponins

tive in experimental stroke models [39, 40]. This study found that PTS treatment could suppress NF-κB nuclear translocation and, therefore, assumes that NF-κB plays an important role in the pathway in which PTS exerts its neuroprotective effect.

In summary, PTS protected tMCAO rats from BBB disruption via maintenance of TJ proteins (claudin-5, occludin and ZO-1). The underlying mechanism of the neuroprotective effect of PTS was also associated with down-regulation of the NF-κB pathway which leads to a decreased level of neuroinflammation after tMCAO.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81230026, 81630028) and Jiangsu Science and Technology Department (BE2016610). The authors would like to thank Teri Brad for assistance of English editing of this paper.

Disclosure of conflict of interest

None.

Address correspondence to: Yun Xu, Department of Neurology, Nanjing Drum Tower Hospital Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, 321 Zhongshan Road, Nanjing 210008, Jiangsu, PR China. Tel: +86 25 83105208; Fax: +86 25 83317016; E-mail: xuyun20042001@aliyun.com

References


BBB protection effect of panaxatriol saponins


