

## Original Article

# Neuroprotective effect of picroside II in brain injury in mice

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**Abstract:** Various types of brain injury which led to the damage of brain tissue structure and neurological dysfunction continues to be the major causes of disability and mortality. Picroside II (PII) possesses a wide range of pharmacological effects and has been proved to ameliorate ischemia and reperfusion injury of kidney and brain. However, critical questions remain about other brain injuries. We investigated the protective effect of PII in four well-characterized murine models of brain injury. Models showed a subsequent regional inflammatory response and oxidative stress in common, which might be improved by the administration of PII (20 mg/kg). Meanwhile, a series of morphological and histological analyses for reinforcement was performed. In traumatic, ischemic and infectious induced injuries, it was observed that the survival rate, apoptosis related proteins, Caspase-3, and the expression of acute inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) were significantly alleviated after PII injection, but PII treatment alone showed no effect on them as well. The western blot results indicated that TLR4 and NF- $\kappa$ B were clearly downregulated with PII administration. In conclusion, our results suggested that PII with a recommended concentration of 20 mg/kg could provide neuroprotective effects against multi-cerebral injuries in mice by suppressing the over-reactive inflammatory responses and oxidative stress and attenuating the damage of brain tissue for further neurological recovery.

**Keywords:** Picroside II, brain injury, inflammatory cytokines, oxidative stress

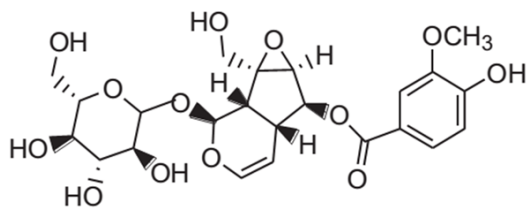
### Introduction

Brain injury constitutes to be a major health and a serious socioeconomic problem throughout the world. Each year about 52000 deaths and 80000 permanent severe neurological disabilities result from different kinds of brain injury [1]. Despite several intense studies, an effective treatment that improves the neuronal function after brain injury is yet to be identified. Picrosidell (PII) extracted from *Picrorhiza scrophulariiflora* (**Figure 1**) is a kind of traditional Chinese medicine with a wide range of pharmacological effects [2]. Previous researches have attributed their protective effects and mechanisms related to alleviate the inflammatory reaction, oxidative stress or calcium ion

overloading, etc [3]. Some animal experiments showed that Picrosidell possessed the organ protection function against kidney and brain ischemia and reperfusion [4]. This might be due to inhibition of the expression of toll-like receptor-4 (TLR4), nuclear transcription factor kappa B (NF- $\kappa$ B) and tumor necrotic factor alpha (TNF- $\alpha$ ) to make poly ADP-ribose polymerase (PARP) utilize the remnant energy to repair the nerve cells [5].

The regional inflammation that is activated in response to an extensive spectrum of brain injuries was known to cause severe neurological disabilities, including traumatic stroke, cerebral infections and radiolesions [6]. The persistency and abnormal regional inflammatory envi-

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**Figure 1.** The chemical structure of Picoside II.

ronment drives a series of pathological responses which are tightly regulated to pattern recognition receptors (PRRs), such as TLRs and Nod-like receptor proteins [7]. These stimulus in turn lead to an intracellular cascade releasing of I $\kappa$ B (Inhibitor of NF- $\kappa$ B) from nuclear factor  $\kappa$ B (NF- $\kappa$ B) translocation where it causes overexpression of inflammatory cytokines [8]. These mechanisms of Picoside II indicate its potential effects which may benefit from different kinds of brain injuries.

Therefore, we postulated that Picoside II is required for alleviating the regional inflammation that is caused by various brain injuries. Hence in the present study, we investigated the neuroprotective effects of PII on the four well-established mice models of brain injuries which included traumatic brain injury (TBI), hypoxic-ischemic reperfusion injury (I/R), radiation-induced brain injury and cecal ligation puncture (CLP) induced cerebral injury.

### Methods and materials

#### Animals

Male wide type (C57BL/6) mice, aged 6-8 weeks and weighing 30-32 g were used in all the experiments. Mice were raised with free access to food and water and in a reversed 12 h light/dark cycle in a controlled environment. The study was approved by the Ethics Committee of Dalian Medical University and all procedures were carried out in compliance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. For both Sham and experimental groups, N=6.

#### Drug treatment method

The Picoside II (Nanjing Senbeijia Co.Ltd, CAS No: 39012-20-9, HPLC>98%) was diluted into 1% solution with 0.1 mol/L PBS and were

injected intraperitoneally with several concentrations (5 mg/kg, 10 mg/kg, 20 mg/kg) [9]. After preparing the animal models, PII was injected intraperitoneally every 24H with specific concentrations of PII or with normal saline (NS).

#### TBI model

Our TBI model was induced by a weight-drop model as described previously [10, 11]. There are two sets of the trial included, neurological severity score (NSS), and brain water content and inflammation measurements. Briefly, followed by the pentobarbital sodium (10 mg/kg) anesthesia, a midline longitudinal incision was made to expose the skull. With the exposed skull, a blunt tip cone was dropped along a stainless-steel rod freely from a height (2.5 cm) onto it, a focal injury to the hemisphere was completed. After injury, the incision was closed in layers with several stitches. Sham group received anesthesia and skin incision only. Mice were randomly divided into seven groups: sham controls with or without PII, TBI-only group and TBI with three concentrations of PII (5 mg/kg, 10 mg/kg, 20 mg/kg) or normal saline group. Drugs or normal saline (NS) were injected one day before and were continuously given after TBI until the mice were sacrificed.

#### Radiation-induced model

Firstly, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (10 mg/kg). Then, the mice were administered with 20-Gy cranial radiation therapy (CRT), with the head centered in the exposure field [12, 13]. During the irradiation period, the rest of the body was protected with customized lead shielding. Considering the resistance of radiation to the mouse brain, we used a dose of 20 Gy in mouse which was approximately similar to the clinically relevant dose in humans [14]. The dose was delivered to the mid-plane of the brain in one session through a posterior field and the mice were fixed with a plastic clip. The irradiation was carried out with beam energy of 6 MV photons (SIEMENS, Germany), a dose rate of 2.0 Gy/min, a source surface distance (SSD) of 100 cm and an irradiation field size of 3 cm  $\times$  30 cm. The mice was divided into four groups: (1) Sham group, in which the animals did not have any radiation and treatment; (2) RT group, in which the animals were

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**Table 1.** Neurological severity scoring

Items	Detail description	Points (success/failure)
Exit circle	Ability of enter or exit a circle of 30 cm (3 min)	0/1
Mono/hemiparesis	Paresis of upper or lower limb of contralateral side	0/1
Startle reflex	Innate reflex (flinching in response to a loud hand clap)	0/1
Seeking behavior	Physiological behavior as a sign of "interest" in the environment	0/1
Straight walk	Alertness/initiative/straight walking when placed on the floor	0/1
Round stick balance	Ability to balance on a round stick 5 mm in diameter for 10 s	0/1
Beam balance	Ability to balance on a beam 7 mm in width for 10 s	0/1
Beam walk (3 cm)	Ability to cross a beam (length × width, 30 × 3 cm)	0/1
Beam walk (2 cm)	Ability to cross a beam (length × width, 30 × 2 cm)	0/1
Beam walk (1 cm)	Ability to cross a beam (length × width, 30 × 1 cm)	0/1
Maximum score		10

N=6 per group.

radiated with a dose of 20 Gy CRT; (3) PII group, in which the animals were just given PII (20 mg/kg) for two weeks without any radiation; (4) RT+PII (20 mg/kg) group, in which PII was administrated for one week before and another week after 20 Gy CRT.

### *I/R model*

Mice were anesthetized with pentobarbital sodium (10 mg/kg), and then operation was performed as described in previous reports with slight modifications [15]. Bilateral common carotid arteries were exposed via a small ventral neck incision and were occluded 10 min for twice with microvascular clips, meanwhile, between the two periods of occlusion, 15-min reperfusion was performed. 1 ml normal saline was supplied intraperitoneally after the surgery. Five groups were as follows: (1) Sham group, where the mice received the same surgical operation without occlusions, saline supplement and any injection; (2) Sham+PII (20 mg/kg) group, which was given the injection of PII (20 mg/kg) before surgery and then was administrated every 24H; (3) I/R group, which the animals had the I/R surgery treatment only; (4) I/R+NS group, in which the animals were given normal saline before I/R surgery and then was administrated every 24H; (5) I/R+PII (20 mg/kg) group, in which the mice was administered with PII (20 mg/kg) injection before I/R surgery and then administrated every 24H.

### *Infectious-induced model*

Middle-grade (50%) CLP was operated for this assay [16]. Briefly, exposing the cecum after anesthesia with pentobarbital sodium (10 mg/

kg), then 50% cecum was ligated and was punctured by a 21-gauge needle. A small amount of intestinal content was extruded to ensure the patency of the puncture. Next, the cecum was repositioned and the abdominal cavity was closed in layers with simple stitches. Sham group underwent the same procedure without ligation and puncture. Mice were divided into seven groups: sham controls with or without PII (20 mg/kg), CLP-only group and CLP with three concentrations of PII (5 mg/kg, 10 mg/kg, 20 mg/kg) or normal saline group. Drugs or NS were administrated one day before and then were given after every 24H and after CLP until mice were sacrificed.

### *Neurobehavioral score*

The neurological scoring was evaluated at 24 h and 72 h after TBI by using the NSS system. The investigators evaluated the ability of each mouse (N=6) with 10 different tasks, which demonstrated their general status. One or no points were given when the mice failed to perform the tasks (**Table 1**) [17]. All the tests were performed by two investigators who were blinded to the experiment.

### *Wet-dry weight ratios*

We used the wet-dry method to describe the severity of brain edema which was previously described [18]. Briefly, the contusion cortex and the injured brain tissues around were rapidly harvested and were quickly weighed to get the wet weight. Then, the tissues were dried in oven at 110°C for 24H, and weighed again to gain the dry weight. Finally, the brain water

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edema was calculated by the formula, the ratios=[(wet weight-dry weight)/wet weight] × 100%.

### *Morris water maze test*

To assess the cognitive changes after radiation, mice received either sham irradiation or a single dose of 20 Gy. The Morris water maze test was conducted 6 weeks after CRT. The test was performed in a circular black pool (width: 127 cm, height: 60 cm) with a black platform. The pool was filled with milk-diluted water at room temperature ( $21.0 \pm 1.0^\circ\text{C}$ ). The platform was set at approximately 1.5 cm below the water surface [19].

Facing the wall of the pool, the mice were placed in the water at 4 possible locations simultaneously, and a camera was activated. Each mouse was given up to 60 s to find the platform. The trial was terminated when the mouse located the platform within 60 s, or if the mouse failed, it was guided to find the platform by a researcher and was allowed to stay for 2-3 s. To permit them to adapt to the pool environment, each mouse was performed 3 times per day for 2 days, then tested for 3 times per day for 5 days to locate the hidden platform. The latency (the time taken to locate the platform), distance, and other data were recorded by the automated video tracking software package (EthoVision 2.3.19, Noldus, Wageningen, The Netherlands). Basically, the pool was divided into four quadrants for further analysis. The first part of the platform was moved to a different quadrant for each session for visible training, while the other part was located in the center of the target quadrant, which was identical for the mice in the hidden platform training. The behavior of the mice was recorded by using EthoVision 3.0 and analyzed for escape latency.

### *Histological examination*

The mice were euthanized with deep anesthesia. After perfusion with 4% paraformaldehyde in phosphate-buffered saline, brain tissues were fixed in 10% phosphate-buffered formalin, it was embedded with paraffin and was sectioned at 4  $\mu\text{m}$  thickness. The sections were deparaffinized and were hydrated gradually, and then stained with hematoxylin and eosin (H&E). For HE staining, the sections were visualized by microscope (Olympus, Japan). Morphological assessments were conducted by an

experienced senior pathologist who was blinded to the experiment.

### *Malondialdehyde (MDA) and superoxide dismutase (SOD) assay*

The samples were homogenized and were centrifuged at  $3,000 \times g$  for 10 min. Subsequently, the supernatants were collected for analysis of the MDA level and SOD activity. The level of MDA and SOD were measured using commercially available kits according to the manufacturer's instructions. The MDA level was represented in nmol/mg protein and the SOD activity was expressed as U/mg protein.

### *Enzyme linked immunosorbent assay (ELISA)*

Total protein was determined using a bicinchoninic acid assay kit (Pierce Biochemicals). The levels of inflammatory cytokines of the brain tissue were quantified using ELISA kits that was specific for mice according to the manufacturer's instructions (TNF- $\alpha$ , from Diaclone Research, France; IL-1 $\beta$ , from Biosource Europe SA, Belgium; IL-6, from R&D System, America). The inflammatory cytokine contents in the brain tissues were expressed as pictogram per milligram protein.

### *Western blotting analysis*

Equal quantities (40  $\mu\text{g}$ ) of total protein or nuclear protein were separated in 10% SDS-PAGE gels and were transferred on to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% defatted milk for 2 h at room temperature, then was incubated for overnight at  $4^\circ\text{C}$  with primary antibodies which was as follows: rabbit anti-Cleaved Caspase-3 (1:500; Anbo Biotechnology, San Francisco, CA, USA), mouse monoclonal NF- $\kappa\text{B}$  (p65; 1:500 dilution; sc-8008, Santa Cruz Biotechnology) and rabbit polyclonal TLR4 (1:500 dilution; sc-10741, Santa Cruz Biotechnology) and rabbit anti- $\beta$ -actin (1:5000; Bioworld Technology, Minneapolis, MN, USA). After being washed with TBST for 15 mins each for three times, bands were visualized using the enhanced chemiluminescence reagent kit (Biyuntian) and then quantification was performed by optical density method using ImageJ software.

### *Statistical analysis*

Comparisons between the two groups were determined by Student's *t*-test. Data are described using mean  $\pm$  SD. *P* values less than

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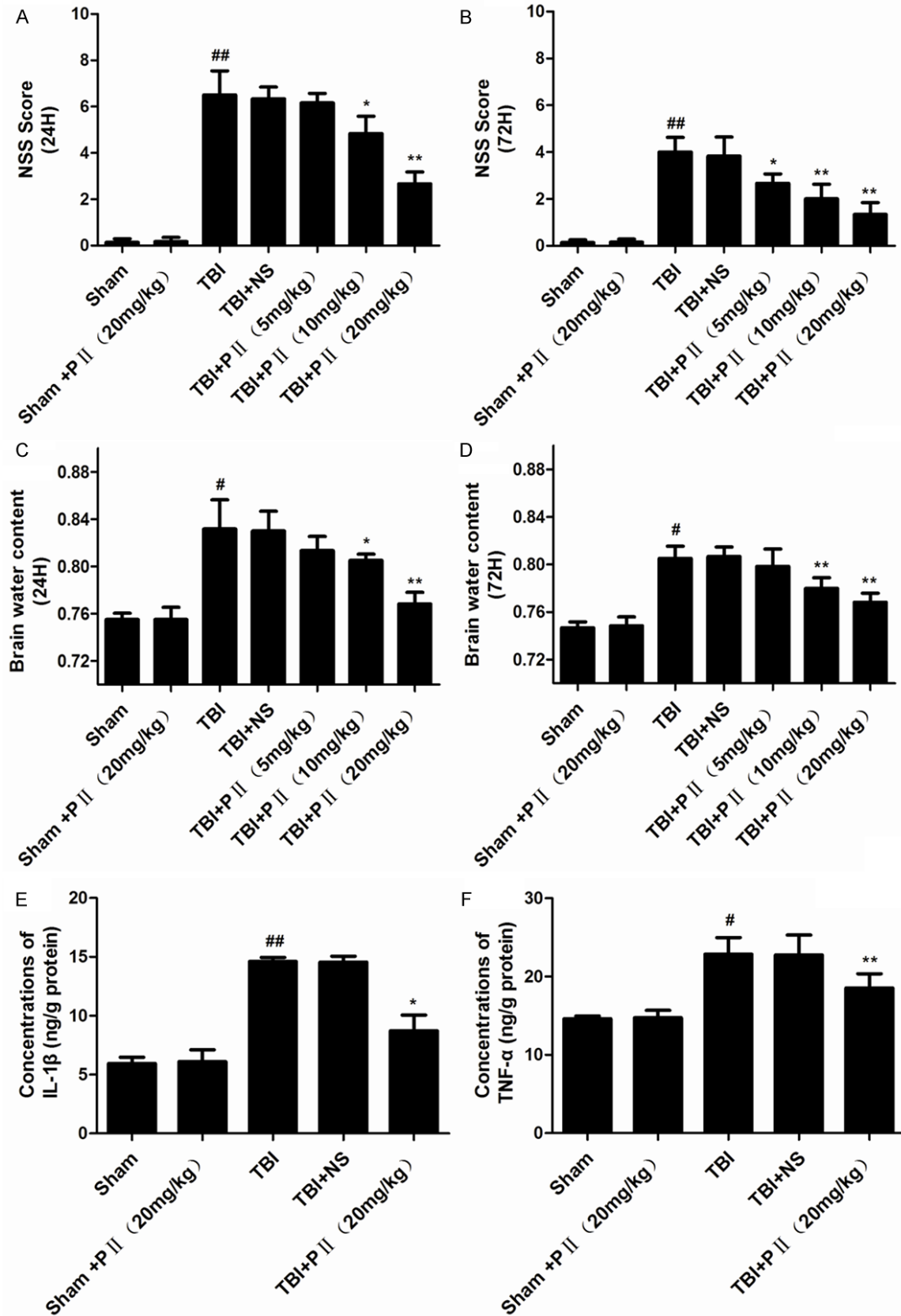
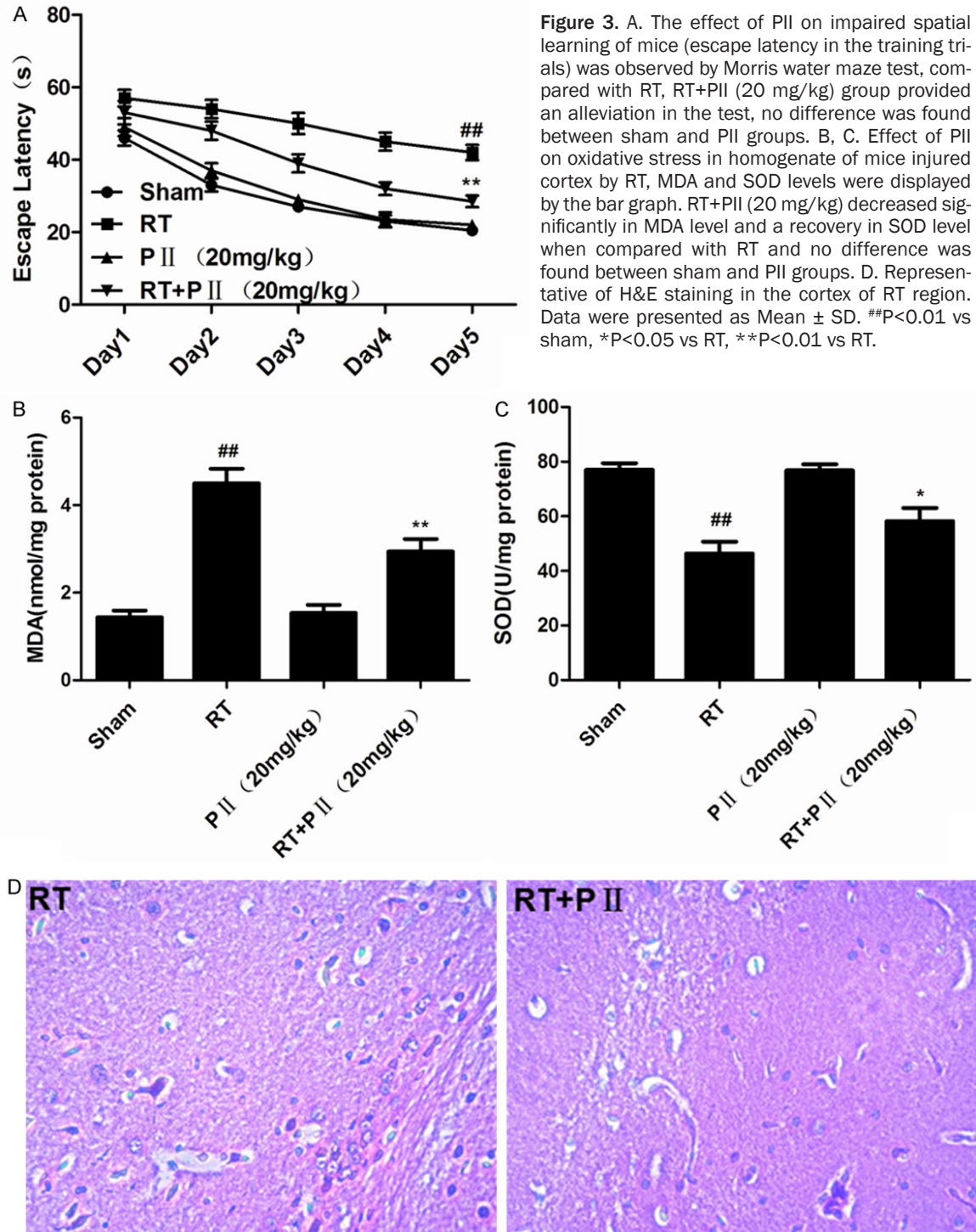


Figure 2. A-D. Effect of several concentrations of PII on NSS scoring and brain water content of mice at 24H and 72H after TBI. No difference was found between sham and sham+PII groups, and TBI+PII (10 or 20 mg/kg) group



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decreased significantly compared with TBI+NS at both time points; E, F. Inflammatory cytokines were measured by ELISA for IL-1 $\beta$  and TNF- $\alpha$  protein levels at 24H after TBI. TBI+PII (20 mg/kg) group attenuated compared with TBI+NS, and no difference was found between sham and sham+PII groups. Data were presented as Mean  $\pm$  SD. ##P<0.01 vs sham, \*P<0.05 vs TBI+NS, \*\*P<0.01 vs TBI+NS.



0.05 were considered to be statistically significant. All analyses were performed using Gra-

phPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA).

**Table 2.** Picoside II effect on spatial learning and memory in spatial probe test of mice irradiated by 20-Gy

Groups	Percent of time in target quadrant (%)	Time of crossing over the platform
Sham	68.34 ± 4.58	6.41 ± 1.53
RT	33.61 ± 3.05 <sup>##</sup>	2.14 ± 0.98 <sup>##</sup>
Pil (20 mg/kg)	67.88 ± 3.88	6.82 ± 1.17
RT+Pil (20 mg/kg)	50.61 ± 3.47 <sup>**</sup>	5.37 ± 1.26 <sup>**</sup>

N=6 per group; <sup>##</sup>P<0.01 vs Sham group; <sup>\*\*</sup>P<0.01 vs RT group; RT: radiation therapy.

**Results**

*Picoside II suppresses inflammation and improve neurological function in TBI*

Neurological function evaluation [20] based on the NSS scores system and brain water content was operated at 24H and 72H after TBI, as shown in **Figure 2A-D**. Both NSS and brain water content evaluation tests indicated the same tendency, which substantially increased in the injury model compared with sham group at each time point. The results revealed that Pil at a concentration of 20 mg/kg significantly alleviated the neurological deficits and brain edema (<sup>##</sup>P<0.01 vs sham and <sup>\*\*</sup>P<0.01 vs TBI+NS), and at concentrations 5 mg/kg and 10 mg/kg did not provide any such protective effects. Pil had no effects on the sham group. It was implied that a concentration of 20 mg/kg might had protective effect in mice and hence was used in the subsequent trials.

For the production of inflammatory cytokines, we measured TNF-α and IL-1β at 24H after TBI by ELISA shown in **Figure 2E, 2F**. Both TBI and sham groups showed a dramatic increase after TBI (<sup>##</sup>P<0.01 vs sham), but after treatment with drugs, it was significantly decreased in Pil after TBI (<sup>\*</sup>P<0.05 vs TBI+NS).

*Picoside II alleviates irradiation-induced impairments*

Morris water maze test were performed for evaluating the spatial learning skill. The mice with radiation therapy (RT) reached the visible platform as rapidly as sham group in all the trials, and showed no occurrence of any basic neurological dysfunction [21]. In the hidden platform of water maze test, it was observed that latency was shorter in RT+Pil group than

RT. Compared with the sham, RT group latency was significantly extended (<sup>##</sup>P<0.01 vs sham) (**Figure 3A**). Meanwhile the percent of time in the target quadrant in RT group was shorter than RT+Pil group (<sup>\*\*</sup>P<0.01), time of crossing over the platform in RT group decreased when compared with RT+Pil as well (<sup>\*\*</sup>P<0.01) (**Table 2**).

The oxidative stress in the mice brain which was subjected to radiation injury are shown in **Figure 3B, 3C**. Compared to the sham group, MDA level had tripled in the RT group and had a decline in the SOD level in RT group at 24H after RT (<sup>##</sup>P<0.01 vs sham), while treatment with Pil at 20 mg/kg attenuated the irradiation-induced MDA level and showed an increase in the SOD level (<sup>\*\*</sup>P<0.01 or <sup>\*</sup>P<0.05 vs RT). The Pil group demonstrated no effects over sham group. H&E staining also showed that microglial-positive cells presumably undergoing apoptosis was decreased in RT+Pil [22], and the tissues in RT+Pil had a better order and original structure (**Figure 3D**). All these results indicated that Pil alleviated the radiation-induced impairments.

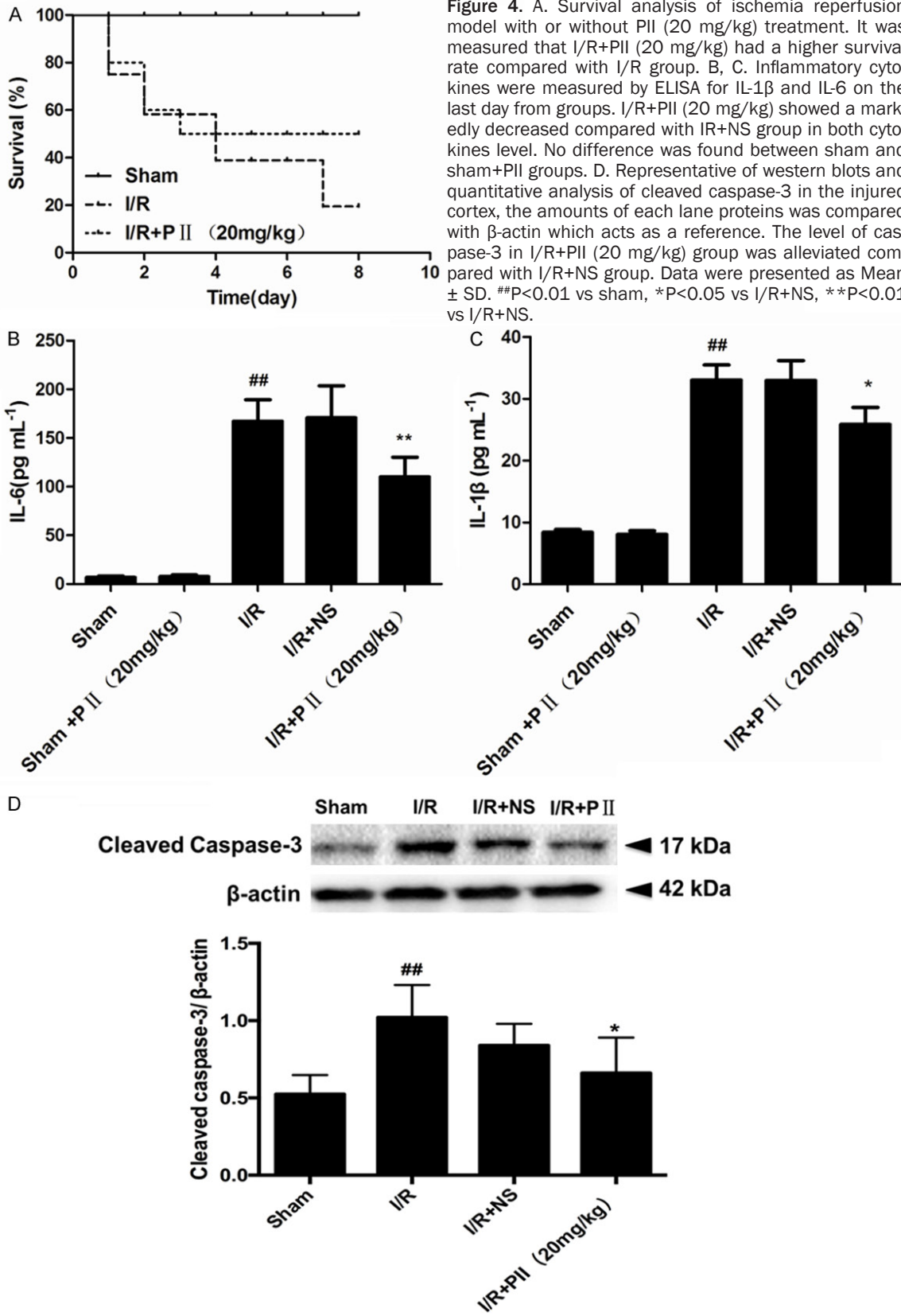
*Neuroprotective effective of picoside II on transient ischemia and reperfusion (I/R)*

30 mice (10 mice per group) were used for our survival rate analysis [24]. It was revealed that mice in I/R+Pil group were more active during the recovery days, both feeding and drinking. Overall, I/R dramatically reduced the life span of mice, while survival rate of I/R+Pil group was prolonged compared with I/R group (**Figure 4A**).

The effects of Pil on down-regulation of inflammation were further confirmed by ELISA (**Figure 4B, 4C**). Recovery after surgery on day 8, the protein levels of IL-6 and IL-1β in the cerebral cortex of ischemic mice were significantly enhanced in I/R group and I/R+NS group when compared with sham group (<sup>##</sup>P<0.01 vs sham). However, I/R+Pil at 20 mg/kg produced a pronounced decrease on both inflammatory cytokines (<sup>\*\*</sup>P<0.01 or <sup>\*</sup>P<0.05 vs I/R+NS).

The potential neuroprotective effect of Pil on neuron apoptosis, caspase-3, and the apoptosis-related protein was assessed [25]. Expression of cleaved caspase-3 was remarkably up-

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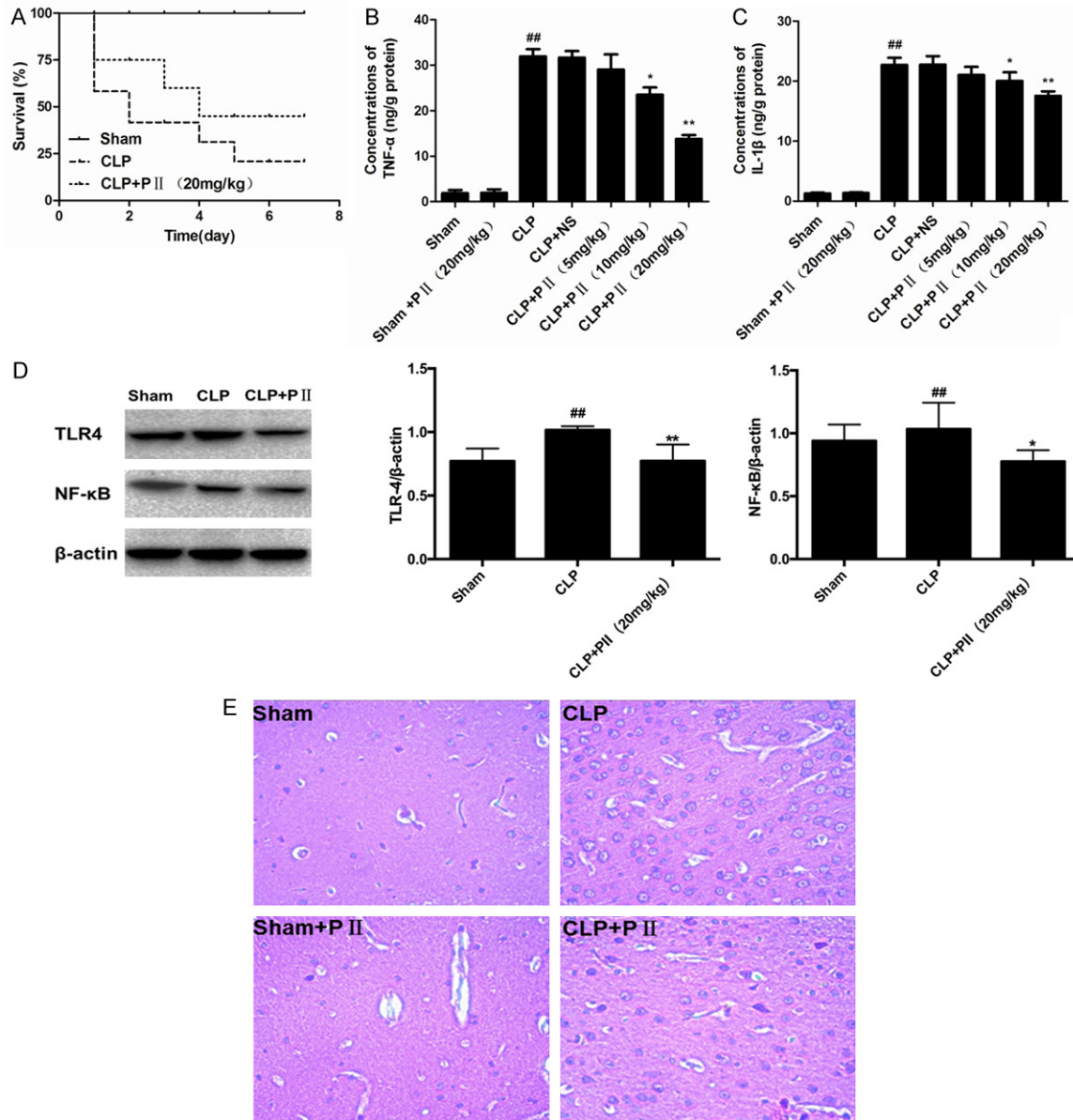
**Figure 4.** A. Survival analysis of ischemia reperfusion model with or without PII (20 mg/kg) treatment. It was measured that I/R+PII (20 mg/kg) had a higher survival rate compared with I/R group. B, C. Inflammatory cytokines were measured by ELISA for IL-1 $\beta$  and IL-6 on the last day from groups. I/R+PII (20 mg/kg) showed a markedly decreased compared with I/R+NS group in both cytokines level. No difference was found between sham and sham+PII groups. D. Representative of western blots and quantitative analysis of cleaved caspase-3 in the injured cortex, the amounts of each lane proteins was compared with  $\beta$ -actin which acts as a reference. The level of caspase-3 in I/R+PII (20 mg/kg) group was alleviated compared with I/R+NS group. Data were presented as Mean  $\pm$  SD. <sup>##</sup> $P < 0.01$  vs sham, <sup>\*</sup> $P < 0.05$  vs I/R+NS, <sup>\*\*</sup> $P < 0.01$  vs I/R+NS.

rising in I/R and I/R+NS groups as compared with sham group (<sup>##</sup> $P < 0.01$  vs sham), and decr-

eased by PII (20 mg/kg) administration (<sup>\*</sup> $P < 0.05$  vs I/R+NS) (**Figure 4D**). Overall, PII display-



## Picoside II protects brain injury in mice



**Figure 5.** A. Survival analysis of CLP-induced infectious brain injury model with or without PII (20 mg/kg) administration. It was observed that the survival rate of CLP+PII (20 mg/kg) group was significant higher than CLP group. B, C. Inflammatory response was assessed by ELISA for levels of IL-1 $\beta$  and TNF- $\alpha$  on the day of sacrificing. The level of both cytokines had a sharp decrease in CLP+PII (20 mg/kg) group when compared with CLP+NS. D. Representative western blots showed the effects of PII on TLR4 and NF- $\kappa$ B (p65) expression after observation,  $\beta$ -actin was used to show equal amounts of protein loading in the lanes. The levels of them had an attenuation in CLP+PII (20 mg/kg) group compared with CLP group. E. Histological examination was performed to compare the amount of positive-microglial cells and the severity of inflammatory infiltrations in mice. Data were presented as Mean  $\pm$  SD. <sup>##</sup>P<0.01 vs sham, <sup>\*</sup>P<0.05 vs CLP+NS, <sup>\*\*</sup>P<0.01 vs CLP+NS.

ed neuroprotective effects against I/R model in mice.

*Picoside II treatment improve survival rate and inflammation response in CLP-induced brain injury*

A total of 30 mice (10 mice for each group) were used for survival analysis and a recovery rate

was observed in them for seven days [26]. CLP group displayed a sharp increase in mortality in two days as compared with other groups. Mice in CLP+PII (20 mg/kg) group suffered from body weight loss, however, the mortality rate was ameliorated by PII injection. Sham group showed a 100% survival rate and observed no paralysis and convulsions (**Figure 5A**).

## Picoside II protects brain injury in mice

The expression levels of TNF- $\alpha$  and IL-1 $\beta$  were evaluated by ELISA (**Figure 5B, 5C**). The levels of TNF- $\alpha$  and IL-1 $\beta$  were significantly greater in CLP-induced model than the sham group ( $^{##}P<0.01$  vs sham). Nonetheless, treatment with PII (20 mg/kg) dramatically reduced the levels of these inflammatory cytokines followed by CLP, and PII showed no effects on sham group ( $^{**}P<0.01$  vs CLP+NS). Also, the levels of TLR4 and NF- $\kappa$ B protein expressions were measured by western blotting (**Figure 5D**). It was obvious from the results that the levels of both were up-regulated in CLP model when compared with those in sham group ( $^{##}P<0.01$  vs sham). On the other hand, PII injection attenuated the CLP-induced protein expressions ( $^{**}P<0.01$  or  $^{*}P<0.05$  vs CLP).

The histological features were evaluated by H&E staining (**Figure 5E**). It was clear that microglial-positive cells were increased with neuron deformation in CLP model when compared with sham group. Mice with PII administration showed an improvement in neurons and the quantity of microglial-positive cells.

### Discussion

The regional inflammation of brain, a process which aimed at restoring the microenvironment and homeostasis after an injury, might be more severe if prolonged or increased [27, 28]. Variability in the brain injury systems, which included traumatic, ischemic reperfusion, radiation-induced and infection-induced brain injuries could start with an acute, uncontrolled regional inflammation and end up with neurological dysfunction, disability and even death [29, 30]. Picoside II, a traditional Chinese medicine with a wide range of pharmacological effects demonstrates neuroprotective effects on the organs [4, 31]. Current studies showed that the effects of Picoside II in the brain and kidney were associated with reduction in oxidative stress and inflammation [4]. Nonetheless, critical questions still remain as to whether it works for other types of brain injuries. Using four well-established models of brain injury, we show that Picoside II could attenuate different acute injuries and neuron damages, which was dependent on anti-inflammatory and improvement of immune modulatory activities by blocking the TLR4/NF- $\kappa$ B signal pathway.

A growing body of evidence indicated that the persistence of inflammatory infiltration respon-

se is highly implicated in the pathophysiology of TBI, such as tissue edema, blood-brain barrier disruption as well [32]. IL-1 $\beta$  and TNF- $\alpha$ , the key pro-inflammatory cytokines after acute injuries were flourished in the early stage which started a continuous regional inflammatory response. Our study revealed that PII markedly alleviated the brain tissue edema, damage to the brain-blood barrier and an improvement of the NSS score. It is indispensable to control the inflammatory responses flourished during the tissue injuries with a milder response. Moreover, it was strongly supported that the inflammatory cytokines showed a shrinkage with the concentration of 10 or 20 mg/kg of PII, and we had no clue that whether 5 mg/kg had the protective effects or not. PII reduced brain edema and neuronal apoptosis and improved neurological function after TBI.

It was widely accepted that most of the patients who had CRT for more than half year were tended to develop irradiation-induced brain injury, which included cognitive changes, learning or memory deficits [33]. Previous researches suggested that the over-activated microglial cells and oxidative stress played a key role in this kind of brain injury [34, 35]. Our study demonstrated that 20 mg/kg injection of PII after radiation exposure ameliorated the learning and memory deficits, suppressed the over-oxidative stress and microglial cells activation in mice. In the results of escape latency, we concluded that there were no significant differences on 1-3 recovery days, then we observed for the latency which was longer in RT group than RT+PII group on day 5. On the other hand, reactive oxygen species (ROS) can lead to a direct strike to the membranes and proteins causing an indirect damage by activating the pro-apoptotic pathways [36, 37]. With the up-regulation of SOD and down-regulation of MDA, the anti-oxidative stress in our model also implied that 20 mg/kg PII could exert significant therapeutic effects on the brain injury, which in turn benefitted the brain-blood barrier as well [38]. Our histological examination results showed that the activated microglial cells in RT group were more than RT+PII group and the morphology of neuron appeared more natural after PII treatment. A similar case reported that lancemaside A could reverse the scopolamine-induced memory and learning deficits by expression of brain-derived neurotrophic factor and phosphorylated cAMP response element binding

protein [39]. PII treatment with 20 mg/kg provided a structural and biochemical protection in the mouse model of radiation-induced brain injury.

Organ dysfunction caused by ischemic reperfusion was alleviated by PII or other traditional Chinese medicines which have been studied from few years, especially in kidney, lung and brain. Within hours of ischemia, pro-inflammatory mediators may reach a sharp level that contributes to the severe regional neuro-inflammatory responses and tissue damage. IL-1 $\beta$  and IL-6 are the key cytokines that participate in the pathological process. Previous studies indicated that IL-1 $\beta$  and IL-6 could promote the secretion when suffering from ischemia by autocrine-like function [40]. In the present study, both of these cytokines showed a sharp increase in the I/R group when compared with sham group. Caspase 3, the apoptosis-related protein was dramatically increased after I/R surgery and was decreased by PII (20 mg/kg) administration. No significant differences have been found in sham+PII and I/R+NS groups. These data showed that PII provided neuroprotection possibly via suppressing acute inflammatory cytokines release and anti-apoptosis response.

Infection-induced brain injury is one of the most happened but easily ignored disease could bring an irreversible damage to the brain-blood barrier and neurons, which led to the central hyperthermia and neurological function deficits [41]. As a classic model of sepsis, CLP assay displayed about a two-fold increase in the survival rate in PII group as compared with no drug treatment. To study whether global infectious brain injury could be attenuated by PII as a regional brain injury, we used several concentrations as previously described. A stronger protection effects were observed when administered by PII 20 mg/kg. NF- $\kappa$ B regulates these inflammatory and apoptosis related genes as a nuclear transcription factor. TLR4 signals, a typical pathogenic pattern recognition receptor, can cause the subsequent activation of NF- $\kappa$ B (p65) and mitogen-activated protein kinase signal pathways. The protein expression of TLR4, NF- $\kappa$ B and inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  were significantly decreased in the CLP+PII group, which strongly suggested that PII could alleviate the infectious brain injury by blocking the TLR4/NF- $\kappa$ B pathway.

In conclusion, our study demonstrates a comprehensive and novel evaluation for PII effects on different brain injuries with four well-established models. It also revealed a common view that PII could provide a neuroprotective effect by blunting the inflammatory responses and neuron apoptosis during the early stages of injury. This involves blockage of TLR4/NF- $\kappa$ B pathway that attenuates the over-oxidative stress which in turn shows an improvement in the neurological deficits and neuronal damage. We further recommended that PII 20 mg/kg concentration show more benefits. Overall, our research provides a deeper insight into the PII therapy for brain injury, however, further clinical studies that require confirmation in humans is highly required.

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### Disclosure of conflict of interest

None.

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