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Optimal therapeutic dose and time window of picroside II in cerebral ischemic injury

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Abstract

A preliminary study from our research group showed that picroside II inhibited neuronal apoptosis in ischemic penumbra, reduced ischemic volume, and improved neurobehavioral function in rats with cerebral ischemia. The aim of the present study was to validate the neuroprotective effects of picroside II and optimize its therapeutic time window and dose in a rat model of cerebral ischemia. We found that picroside II inhibited cell apoptosis and reduced the expression of neuron-specific enolase, a marker of neuronal damage, in rats after cerebral ischemic injury. The optimal treatment time after ischemic injury and dose were determined, respectively, as follows: (1) 2.0 hours and 10 mg/kg according to the results of toluidine blue staining; (2) 1.5 hours and 10 mg/kg according to early apoptotic ratio by flow cytometry; (3) 2.0 hours and 10 mg/kg according to immunohistochemical and western blot analysis; and (4) 1.5 hours and 10 mg/kg according to reverse transcription polymerase chain reaction. The present findings suggest that an intraperitoneal injection of 10 mg/kg picroside II 1.5–2.0 hours after cerebral ischemic injury in rats is the optimal dose and time for therapeutic benefit.

Key Words: *nerve regeneration; picroside II; therapeutic dose; time window; brain ischemia; neuron-specific enolase; toluidine blue staining; flow cytometry; immunohistochemical assay; western blot; RT-PCR; rats; NSFC grant; neural regeneration*

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Introduction

Neuron-specific enolase (NSE), an acidic protease involved in the glycolytic pathway, is a marker enzyme of neurons and exists specifically in neurons and neuroendocrine cells (Vos et al., 2004). Normally, there is little NSE in body fluids; the highest concentration is in brain tissue, accounting for about 1.5–3.0% of the total soluble protein (Hein Née Maier et al., 2008). A growing body of evidence indicates that the content of NSE varies in different parts of the nervous system (Marquardt et al., 2009; Oksanen et al., 2009), and that NSE plays a neuroprotective role. NSE is essential in maintaining the excitability of neuronal membranes, and it is also involved in the formation of the membrane structure during the development of the central nervous system (Selakovic et al., 2005). It has been suggested that NSE is related to the regulation of the stress response, and is involved in the repair of brain cells (Selakovic et al., 2005). When neuronal injury or necrosis occurs after ischemia or hypoxia, NSE is quickly released by the neurons into the cell gap and then into the cerebrospinal fluid, or through the blood-brain barrier into peripheral blood, increasing the level of NSE in cerebrospinal fluid and serum (van Munster et al., 2009). Therefore, NSE is a specific and objective indicator by which to observe neuronal injury and necrosis in the brain (Lima et al., 2004). Recent studies have found that NSE correlates highly with the diagnosis and prognosis of ischemic brain injury, as well as the degree of injury and infarct volume (Anand et al., 2005; Jauch et al., 2006; Wunderlich et al., 2006; Brea et al., 2009; González-García Sienkiewicz-Jarosz et al., 2009; Whiteley et al., 2009; Saenger et al., 2010; Bharosay et al., 2012; Singh et al., 2013).

Increasing evidence indicates that picroside II has antioxidant, anti-inflammatory and anti-apoptotic effects (Guo et al., 2011; Meng et al., 2012). In preliminary studies, we explored the therapeutic dose and time window of picroside II in the treatment of cerebral ischemia/reperfusion injury from tests of neurobehavioral function, infarct volume and immunohistochemical staining in rats. The results suggested that picroside II has its strongest protective effect against cerebral ischemia at a dose of 20 mg/kg, 1.5 hours after ischemic injury (Pei et al., 2012). In the present experiment, we employ the orthogonal design principle to identify the optimal therapeutic dose and time window of picroside II using a variety of biological methods, in a broader attempt to qualitatively and quantitatively measure levels of NSE and neuronal apoptosis in brain tissue after cerebral ischemia.

Materials and Methods

Animals

A total of 255 adult healthy male Wistar rats of specific pathogen-free grade, weighing 230–250 g, were supplied by the Experiment Animal Center of Qingdao Drug Inspection Institute, Qingdao, Shandong Province, China (license No. SCXK (Lu) 20100100). All animals were acclimatized for 7 days to temperature (23 ± 2°C) and humidity-controlled housing with natural illumination and free access to food and water. The experiment was approved by the Ethics Committee of Qingdao University Medical College in China (approval No. QUMC 2011-09). The local legislation for ethics of animal experimentation and guidelines for the care and use of laboratory animals were followed for all animal procedures.

Experimental grouping

Animals were fasted for 12 hours before surgery. Twenty (5 × 4) rats were randomly selected as the control group, and the remaining 235 rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg) and fixed in the supine position to establish forebrain ischemic models by bilateral common carotid artery occlusion (Márquez-Martín et al., 2012). Core body temperature was maintained at 36–37°C using a rectal probe and homeothermic blanket control unit during and after surgery. Twenty-three rats that had died or not awoken within 2 hours of surgery were excluded, while the 212 successful models in which the cerebral blood flow curve (PeriFlux 5000, Swedish Perimed Medical Co. Ltd) dropped to 30% were included in the experiment. Control rats underwent identical surgical and experimental procedures except the common carotid artery was not occluded.

Orthogonal experimental design

A total of 212 successful rat models were divided randomly into a model group (5 × 4) and a treatment group (16 × 4 × 3). The treatment group was then subdivided according to the principle of orthogonal experimental design of [$L_{16}(4^5)$] consisting of two impact factors with four impact levels (**Table 1**). Impact factor A is the therapeutic time window, with four levels: 1.0, 1.5, 2.0, 2.5 hours after ischemia. Impact factor B is the therapeutic drug dose, also with four levels: 5, 10, 20 and 40 mg/kg body weight (**Table 1**). The orthogonal experimental test was repeated three times.

Drug administration

Picroside II (molecular formula: $C_{23}H_{28}O_{13}$, molecular weight: 512.48, CAS No: 39012-20-9, purity > 98%) was supplied by Tianjin Kuiqing Medical Technology Co., Ltd., Tianjin, China. Each rat was weighed and the corresponding amount of picroside II powder was diluted in 1 mL isotonic saline solution to obtain the assigned dose, and injected intraperitoneally at the time determined by the orthogonal design [$L_{16}(4^5)$] (**Table 1**). Rats in the control and model groups were intraperitoneally injected with the same volume of saline after 2 hours of cerebral ischemia.

Sample preparation

Paraffin sectioning

Twenty-four hours after injection, rats from the control group (n = 5), model group (n = 5) and treatment groups (three subgroups; n = 16 per subgroup) were randomly chosen and anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg). The rats were perfused with 200 mL normal saline and 200 mL 4% paraformaldehyde solution successively *via* the heart, and whole brains were removed and post-fixed for 2 hours in 4% formaldehyde solution before soaking in distilled water for 4 hours. Brains were embedded in paraffin, and coronal sections (5 μm thickness) were cut continually from the posterior of the optic chiasm using a microtome (Leica CM2027, Germany). The sections were dehydrated through a conventional ethanol gradient, rendered transparent using xylene, and then adhered onto poly-L-lysine-coated slides and stored at 4°C.

Flow cytometry suspension

Rats were randomly selected and anesthetized as described above, then perfused through the heart with 200 mL normal saline. Whole brains were removed and 200 mg brain tissue from the ischemic area was quickly collected into a 1.5 mL Eppendorf tube with 0.5 mL precooled PBS (0.01 mol/L). These tissue was shredded and moved into a glass tube with 2 mL EDTA-free trypsin (2.5%) to incubate for 15 minutes at 37°C, and then pipetted gently and filtered into a 1.5 mL tube (on ice) through a 200 mesh filter. The filtrate was centrifuged at 800 r/min for 5 minutes at 4°C. With the supernatant discarded, cell concentration was adjusted to 1×10^6/100 μL with 1 × Annexin binding buffer and stored at 4°C.

Obvervational indexes

Toluidine blue staining

Paraffin sections were dewaxed in dimethyl benzene and washed for 30 seconds, three times, in PBS, dyed for 1 hour in 1% toluidine blue at 56°C, washed in distilled water, placed in 70% alcohol for 1 minute, and separated in 95% alcohol. Sections were dehydrated with anhydrous alcohol, placed in xylene, and mounted with neutral gum. Five randomly-chosen, non-overlapping visual fields in the parietal cortex were observed at 400 × magnification under a light microscope (Olympus IX141, Tokyo, Japan) and the number of denatured cells in each visual field was counted; neuronal Nissl bodies were stained dark blue, karyoplasm pale blue, and the background appeared pale under the microscope. The degree of pathological damage was expressed as the denatured cell index (the number of denatured cells/total cells).

Immunohistochemical analysis

All antibodies and kits were provided by Wuhan Boster Biotechnology Co., Ltd. (Wuhan, Hubei Province, China). Paraffin sections were dewaxed in dimethylbenzene and washed in PBS for 30 seconds, three times, then incubated with rabbit anti-rat NSE primary polyclonal antibody (1:100) for 2 hours at 37°C and with horseradish peroxidase goat anti-rabbit antibody (1:200) for 30 minutes at 37°C using a streptavidin-biotin complex kit, according to the manufacturer's instructions. Staining was visualized using DAB for

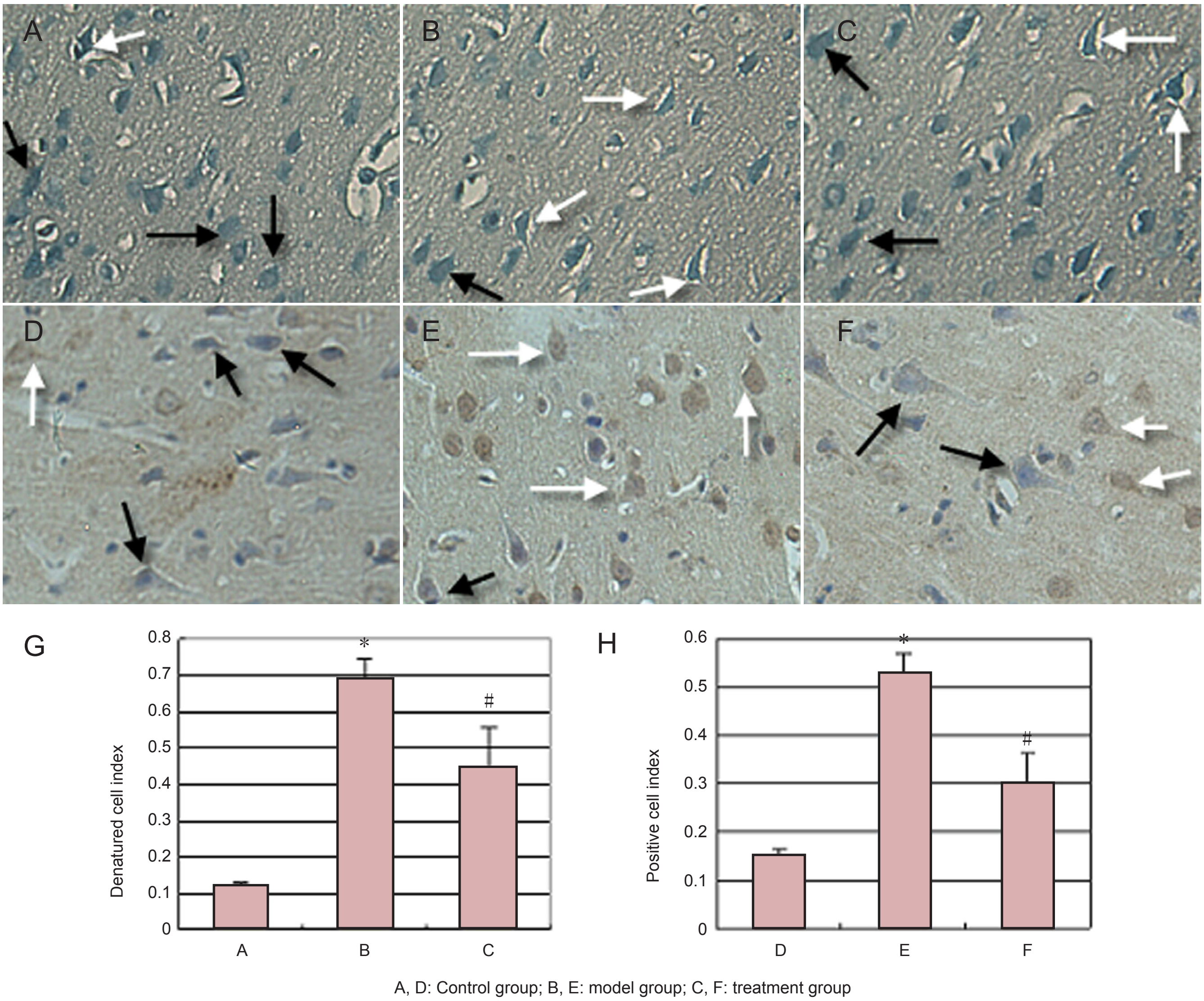

Figure 1 Neuronal morphology and expression of neuron-specific enolase (NSE) in the parietal cortex of rats with cerebral ischemia after picroside II treatment.

(A–C) Toluidine blue staining of neurons in the parietal cortex of control (A), model (B) and picroside II treated (C) rats. Black arrows, normal neurons showing dark blue Nissl bodies, pale blue karyoplasm and pale background. White arrows, neurons with pyknotic or absent karyoplasms (× 400).

(D–F) Immunohistochemistry of NSE expression in parietal cortical neurons of control (D), model (E) and picroside II treated (F) rats. White arrowheads, NSE-positive cytoplasm appears brown or tan. Black arrowheads, NSE-negative cells appear blue after hematoxylin counterstain (× 400).

(G) The denatured cell index was significantly greater in the model group than in the control group (*$P < 0.05$), and significantly lower in the picroside II treatment group than in the model group (#$P < 0.05$).

(H) The NSE-positive cell index was significantly greater in the model group than in the control group (*$P < 0.05$), whereas it was significantly lower in the picroside II group than in the model group (#$P < 0.05$).

(G, H) One-way analysis of variance was used for the comparison of multiple sets of data, and differences were identified by the least significant difference *post-hoc* test.

Table 3 Comparison of denatured cell index (DCI), positive cell index (PCI), early apoptosis ratio (EAR), relative gray value ratio of protein (RVP), and relative gray value ratio of mRNA (RVM)

Group	*n*	DCI	PCI	EAR	RVP	RVM
Control	5	0.110±0.022	0.145±0.021	1.37±0.146	0.931±0.064	0.667±0.037
Model	5	0.691±0.052^a	0.530±0.040^a	5.22±0.420^a	1.484±0.086^a	0.920±0.073^a
Treatment	16×3	0.444±0.112^b	0.294±0.071^b	2.467±1.073^b	0.706±0.431^b	0.614±0.166^b

$^a P < 0.05$, *vs*. control group; $^b P < 0.05$, *vs*. model group (one-way analysis of variance and least significant difference test). All data are represented as mean ± SD.

Table 1 Orthogonal experimental design of [$L_{16}(4^5)$]

Therapeutic dose	Ischemia 1.0 h (A1)	Ischemia 1.5 h (A2)	Ischemia 2.0 h (A3)	Ischemia 2.5 h (A4)
5 mg/kg (B1)	1.0×5	1.5×5	2.0×5	2.5×5
10 mg/kg (B2)	1.0×10	1.5×10	2.0×10	2.5×10
20 mg/kg (B3)	1.0×20	1.5×20	2.0×20	2.5×20
40 mg/kg (B4)	1.0×40	1.5×40	2.0×40	2.5×40

h: Hours.

Table 2 Primers of target gene neuron-specific enolase (NSE) and GAPDH

Primer	Primer sequence	Product size (bp)
NSE	S: 5′-GGG CAC TCT ACC AGG ACT TTG-3′ A: 5′-CCG ACA TTG GCT GTG AAC TT-3′	106
GAPDH	S: 5′-CGT TGA CAT CCG TAA AGA CCT C-3′ A: 5′-TAG GAG CCA GGG CAG TAA TCT-3′	110

S: Sense; A: antisense.

30 seconds and the sections were counterstained with hematoxylin for 5 seconds at room temperature. Immunopositive cells appeared as brown particles under a light microscope (Olympus IX141). Negative control sections were incubated with 0.01 mol/L PBS instead of NSE primary antibody and no positive reaction was found. Five randomly-chosen, non-overlapping visual fields in the parietal cortex were observed in each section at 400 × magnification under a light microscope to calculate the positive cells. Positive cell index (number of positive cells/total cells in the visual field) was used to determine NSE expression.

Flow cytometry

Apoptosis was determined in the samples by flow cytometry, using an Annexin V-FITC apoptosis detection kit (Nanjing Keygen Biotechnology Co., Ltd., Nanjing, Jiangsu Province, China) and FACSCalibur system (Becton Dickinson Co., Ltd., New Jersey, USA) with an excitation wavelength of 488 nm and emission at 535 nm and 575 nm. Early apoptotic ratio was analyzed using FlowJo 7.6 software (TreeStar, San Carlos, CA, USA).

Western blot analysis

Rats were randomly selected and anesthetized as described above. Ischemic brain tissue (200 mg) was harvested from the parietal cortex and placed into a 1.5 mL Eppendorf tube, and mixed with cell lysis buffer (No. P0013, Biyuntian Biotech Co., Ltd., Beijing, China) at a ratio of 1:4. The mixture was ground and homogenized ultrasonically at −4°C in an ice bath, and centrifuged (Eppendorf 5801, Hamburg, Germany) at 10,949 × *g* for 10 minutes at 4°C. The protein concentration in the supernatant was determined using the BCA-100 protein quantitation kit (Shenneng Biotech. Co., Ltd., China), and samples were stored at −20°C until protein separation. NSE proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (5% stacking gel at 120 V followed by 12% gel at 75 V) and transferred onto a polyvinylidene difluoride membrane (40 minutes at 360 mA). The membrane was rinsed with PBS and Tween-10, three times for 5 minutes each time, then incubated with rabbit anti-rat NSE primary monoclonal antibody (1:500; Ab53025, Abcam, Hong Kong, China) and horseradish peroxidase goat anti-rabbit antibody (1:10,000; ZB-2301, Beijing Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 2 hours at 37°C. Proteins were visualized using enhanced chemiluminescence reagents (GE Healthcare Japan, Tokyo, Japan) and scanned in a Bio-Rad 2000 gel imaging system (Bio-Rad, Hercules, CA, USA). Gray values of the protein bands were analyzed using Quantity One software (Bio-Rad). Gray value (pixel intensity) was used to quantify protein content, and the value for each sample was normalized against that of β-actin (42 kDa) as an internal control. The relative value of target protein was calculated as follows: gray value of NSE/gray value of β-actin. The experiment was repeated three times.

RT-PCR

Rats were randomly selected and anesthetized as described above, 24 hours after injection. Ischemic brain tissue (200 mg) was harvested from the parietal cortex and placed into a 1.5 mL Eppendorf tube with 1 mL RNA-Solv Reagent (Omega Bio-Tek. Inc, Norcross, GA, USA), and the sample was minced and ground. The mixture was oscillated ultrasonically for 30 seconds, incubated for 5 minutes at room temperature, and centrifuged for 15 minutes (4°C, 12,000 × *g*). The supernatant was transferred to a new Eppendorf tube containing 0.2 mL chloroform, shaken for 15 seconds, and placed on ice. After 10 minutes, the supernatant was collected into a fresh EP tube and centrifuged for 15 minutes (4°C, 12,000 × *g*), and 0.5 mL isopropyl alcohol was added and gently mixed. The supernatant was placed on ice for 10 minutes, centrifuged (4°C, 12,000 × *g*) for 15 minutes and discarded. The precipitate was washed using 1 mL of 75% alcohol, mixed and centrifuged (4°C, 7,500 × *g*) for 5 minutes, and then the supernatant was carefully discarded. The precipitate was dried for 30 minutes under a fume hood, 30 μL of 0.1% diethylpyrocarbonate-treated water was added, and the sample was placed in a water bath at 57°C for 10 minutes. The purity and quantity of RNA were determined with an ultraviolet spectrophotometer (Bekaman DU640, Pasadena, CA, USA) and stored at −20°C.

Primer sequences for the target gene (NSE) and internal control (GAPDH) are listed in **Table 2**. PCR was performed under the following conditions: 95°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, and finally an extension step at 72°C for 3 minutes. The quantity of mRNA was expressed as the following ratio: gray value of NSE mRNA/gray value of GAPDH mRNA. The experiment was repeated three times.

Statistical analysis

Data are expressed as mean ± SD. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance was used for the comparison of multiple sets of data, and differences were identified by the least significant difference *post-hoc* test. Data was considered to be significant when $P < 0.05$.

Results

Overall results

In the control group, neuronal Nissl bodies appeared as dark blue plaques, the karyoplasm was pale blue and the background was pale; in the model group, pyknosis was visible, with nuclei appearing fragmented or even absent; in the treatment group, neuronal morphology was improved compared with the model group (**Figure 1**). The denatured cell index and positive cell index (**Figure 1; Table 3**), early apoptotic ratio (**Figure 2; Table 3**), relative value of protein (**Figure 3; Table 3**) and relative value of mRNA (**Figure 4; Table 3**) were significantly higher in the model group than in the control group ($P < 0.05$). After treatment with picroside II, all indices were significantly lower than those of the model group ($P < 0.05$; **Figures 1–4; Table 3**).

Neuroprotective effect of picroside II in the treatment of cerebral ischemic injury and analysis of the optimal therapeutic dose and time window

Toluidine blue staining

With toluidine blue staining (**Figure 1**), a significant effect ($P < 0.05$) on the denatured cell index (**Table 4**) was observed with impact factor A (time after ischemic injury) but not B (drug dose) or C (time × dose interaction) ($P > 0.05$). This evidence indicates that the therapeutic time window (or cerebral ischemic time) has a significant influence on the pathological changes in neurons after cerebral ischemic injury, whereas drug dose does not influence neuronal pathology and there are no interactions between therapeutic time window and drug dose. Significant differences ($P < 0.05$) were found between the following groups: 1.0 h (A1) and 1.5 h (A2); 1.0 h (A1) and 2.0 h (A3); 1.0 h (A1) and 2.5 h (A4); 1.5 h (A2) and 2.5 h (A4); 2.0 h (A3) and 2.5 h (A4). No significant difference was found between 1.5 h (A2) and 2.0 h (A3) ($P > 0.05$). There was a significant difference ($P < 0.05$) between the 10 mg/kg (B2) and 40 mg/kg (B4) dose groups, while no significant differences were found between the other therapeutic doses ($P > 0.05$). According to the principle of minimizing medication dose and maximizing therapeutic time window, the best combination was revealed as A3B2 (2.0 h/10 mg), *i.e.*, for optimal response, 10 mg/kg picroside II should be injected intraperitoneally at 2.0 hours of cerebral ischemia.

NSE immunohistochemistry

Analysis of NSE expression by immunohistochemistry (**Figure 1**) showed that different levels of impact factor A (time) had a statistically significant effect on the NSE-immunopositive cell index ($P < 0.05$; **Table 4**), whereas factors B (dose) and C (interaction) had no statistically significant effect ($P > 0.05$), indicating that the time to treatment had a significant effect on NSE expression following cerebral ischemia, whereas the injected dose and the time × dose interaction have no notable effect. Significant differences were found between the following times post-ischemic injury: 1.0 h (A1) and 2.5 h (A4); 1.5 h (A2) and 2.5 h (A4); 2.0 h (A3) and 2.5 h (A4) ($P < 0.05$). In addition, significant differences ($P < 0.05$) were found between the following dose groups: 10 mg/kg (B2) and 40 mg/kg (B4); 20 mg/kg (B3) and 40 mg/kg (B4). According to our comprehensive evaluation, the optimal combination is A3B2 (2.0 h/10 mg), *i.e.*, intraperitoneal injection of 10 mg/kg picroside II at 2.0 hours of cerebral ischemia.

Flow cytometry

Significant differences in early apoptotic ratio were observed between various levels of impact factors A (time) and B (dose) ($P < 0.05$), but not in impact factor C (time-dose interaction; $P > 0.05$) in the flow cytometry study (**Figure 2**). This indicates that the therapeutic time window and the drug dose, but not their interaction, influence the early apoptotic ratio after cerebral ischemia injury (**Table 4**). Significant differences ($P < 0.05$) in treatment time were observed between the following groups: 1.0 h (A1) and 2.0 h (A3); 1.0 h (A1) and 2.5 h (A4); 1.5 h (A2) and 2.0 h (A3); 1.5 h (A2) and 2.5 h (A4); and 2.0 h (A3) and 2.5 h (A4). Furthermore, there were significant differences ($P < 0.05$) in drug dose in the following groups: 5 mg/kg (B1) and 10 mg/kg (B2); 5 mg/kg (B1) and 40 mg/kg (B4); 10 mg/kg (B2) and 20 mg/kg (B3); 10 mg/kg (B2) and 40 mg/kg (B4); 20 mg/kg (B3) and 40 mg/kg (B4). Therefore, the best combination is A2B2 (1.5 h/10 mg), *i.e.*, for optimal therapeutic benefit, picroside II should be injected intraperitoneally at a dose of 10 mg/kg and at 1.5 hours of cerebral ischemia.

Western blot analysis

According to quantitative analysis, NSE expression differed across all groups (**Figure 3**). Expression of NSE in the treatment group was significantly lower than that in the model group. One-way analysis of variance showed that impact factor A (time), but not factor B (dose) or C (interaction), had a significant effect on the expression of NSE ($P < 0.05$), highlighting the important effect of treatment time on NSE expression, while drug dose and time × dose interaction had no notable effect (**Table 4**). Least significant difference analysis revealed significant differences ($P < 0.05$) between the following treatment times: 1.0 h (A1) and 1.5 h (A2); 1.0 h (A1) and 2.0 h (A3); 1.5 h (A2) and 2.5 h (A4); 2.0 h (A3) and 2.5 h (A4). No notable differences were found between any of the dose groups ($P > 0.05$). These results indicate that the optimal combination is A3B2 (2.0 h/10 mg), *i.e.*, an intraperitoneal injection of 10 mg/kg picroside II at 2.0 hours of cerebral ischemia.

RT-PCR

NSE mRNA transcription levels in rat brain tissue were different in each group, and the expression of NSE mRNA was lower after treatment than in the model group (**Figure 4**). One-way analysis of variance showed that factors A (time), B (dose) and C (time × dose interaction) had no statistically significant effect on the expression of NSE mRNA ($P > 0.05$) (**Table 4**). Least significant difference results revealed significant differences ($P < 0.05$) between the following treatment time groups:

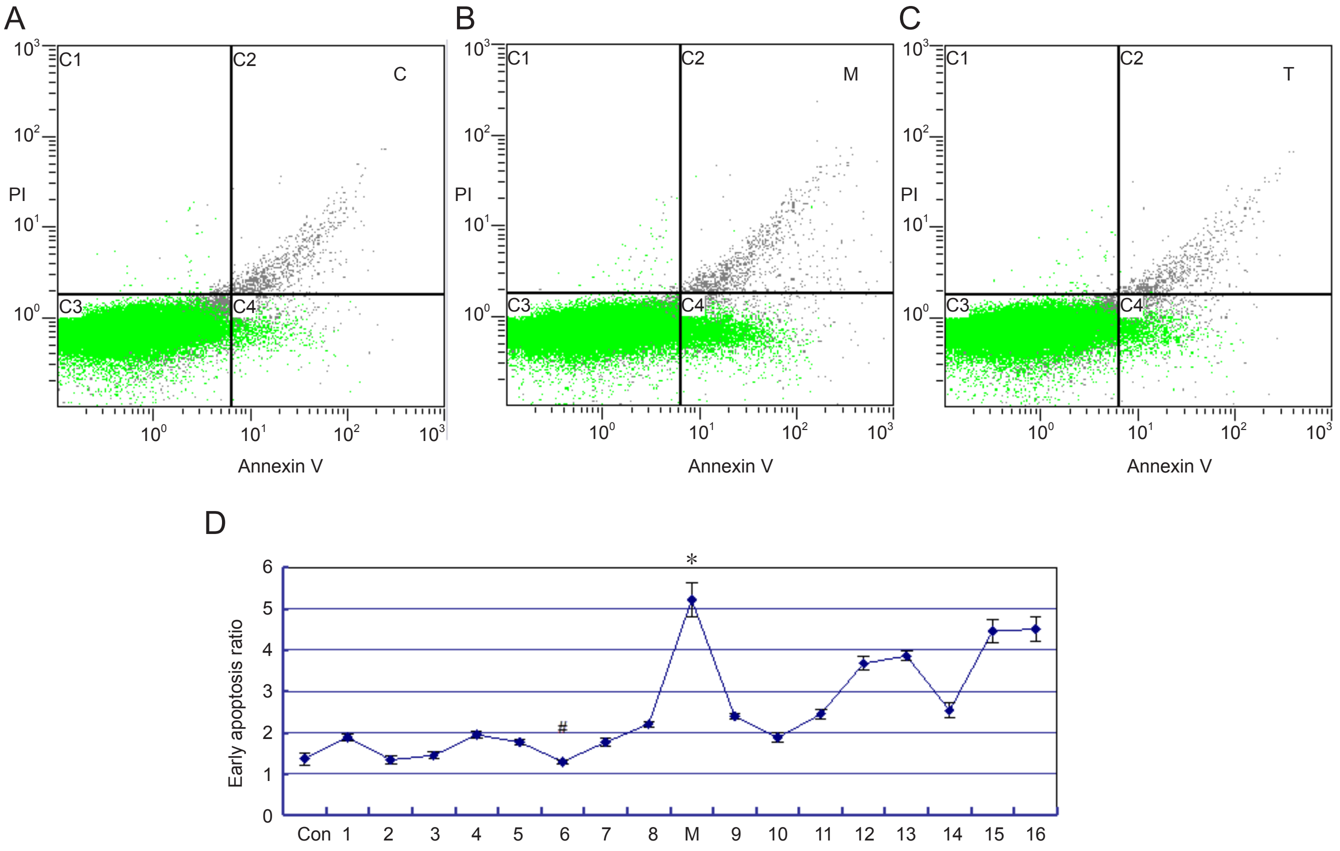

Figure 2 Neuronal apoptosis in the parietal cortex of rats with cerebral ischemia, with or without picroside II treatment (flow cytometry).
Annexin V-FITC staining and FACScan Calibur (excitation wavelength, 488 nm; emission wavelength, 535 and 575 nm) were used to detect neuronal apoptosis (A–C), and early apoptosis ratio (D) was analyzed by FlowJo 7.6 software. (1–16) Treatment groups in which rats received various doses of picroside II at various time points. Data are presented as mean ± SD. Early apoptosis ratios were 1.37 ± 0.146 in the control group (A), 5.22 ± 0.420 in the model group (B), and 2.467 ± 1.073 in the treatment group (C) with picroside II 10 mg/kg. After modeling, the early apoptosis ratio was significantly greater than that in the control group, and significantly lower after treatment with picroside than in the model group. **P* < 0.05, *vs.* control group (Con); #*P* < 0.05, *vs.* model group (M) (one-way analysis of variance and the least significant difference test). FITC: Fuorescein isothiocyanate.

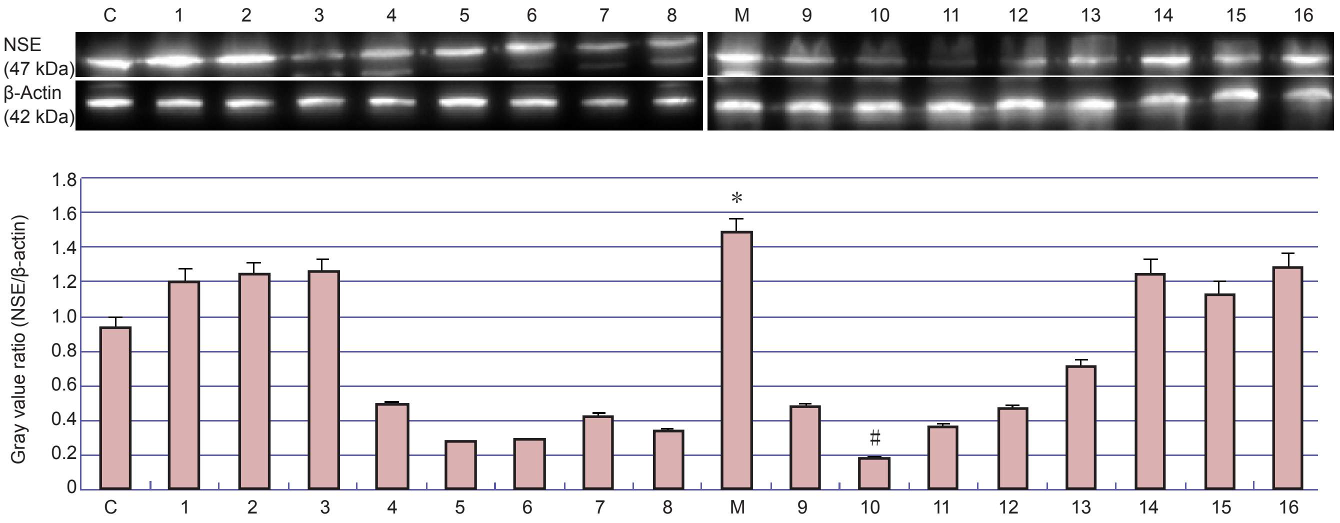

Figure 3 Western blot analysis of neuron specific enolase (NSE) protein expression in parietal cortex of rats with cerebral ischemia, with or without picroside II administration.
β-Actin was used as a loading control. (1–16) Treatment groups in which rats were treated with picroside II at various time points and doses. Results are expressed as the relative gray value ratio (mean ± SD). NSE expression in the model group (M) was significantly greater than in the control group (C), and lower in the treatment groups (1–16) than in the model group. **P* < 0.05, *vs.* control group; #*P* < 0.05, *vs.* model group (one-way analysis of variance and the least significant difference test).

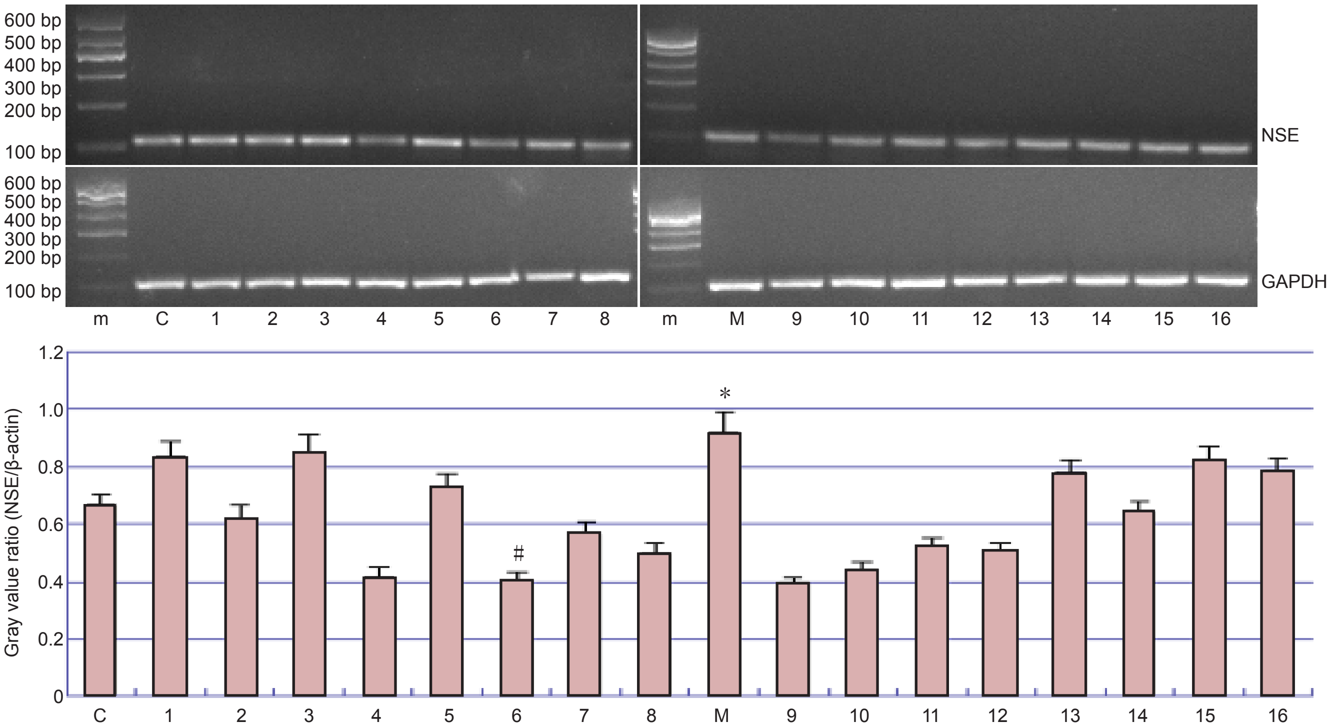

Figure 4 The mRNA expression of neuron specific enolase (NSE) in the parietal cortex of rats with cerebral ischemia, with or without picroside II administration.

GAPDH was used as a loading control. m: molecular weight marker. (1–16) Treatment groups in which rats received picroside II at various doses and time points. NSE RT-PCR results are expressed against GAPDH as relative gray value ratio (mean ± SD). Compared with the control group (C), the relative value of NSE mRNA was significantly greater in the model group (M), and lower in the treatment groups (1–16) than in the model group. $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* model group (one-way analysis of variance and the least significant difference test).

Table 4 One-way analysis of variance of denatured cell index (DCI), positive cell index (PCI), early apoptotic ratio (EAR), relative value of protein (RVP) and relative value of mRNA (RVM)

Source of variation		*SS*	*df*	*MS*	*F*	*P*
DCI	Time window	0.170	3	0.057	71.70	0.01
	Drug dose	0.008	3	0.003	3.23	0.10
	Time × Dose	0.005	3	0.002	1.91	0.23
	Error	0.005	6	0.001	–	–
PCI	Time window	0.043	3	0.014	9.10	0.01
	Drug dose	0.018	3	0.006	3.72	0.08
	Time × Dose	0.004	3	0.001	0.90	0.51
	Error	0.010	6	0.002	–	–
EAR	Time window	12.275	3	4.092	48.83	0.01
	Drug dose	3.530	3	1.177	14.04	0.01
	Time × Dose	0.948	3	0.316	3.77	0.08
	Error	0.503	6	0.084	–	–
RVP	Time window	0.203	3	0.068	4.51	0.06
	Drug dose	0.088	3	0.029	1.97	0.22
	Time × Dose	0.031	3	0.010	0.69	0.59
	Error	0.090	6	0.015	–	–

1.0 h (A1) and 2.0 h (A3); 2.0 h (A3) and 2.5 h (A4). However, there were no significant differences between dose groups ($P > 0.05$). Thus, the optimal combination is A2B2, *i.e.* 10 mg/kg picroside at 1.5 hours of cerebral ischemia.

Discussion

The orthogonal design balances sampling across different factors, increasing the statistical representation of each group while reducing the number of necessary tests (Liu et al., 2010). In the present study, we applied the orthogonal layout to the entire experiment to ensure comprehensive comparisons using a smaller number of experiments.

As a key enzyme in the glycolytic pathway and widely distributed in various tissues, enolase catalyzes 2-phosphoglycerate into 2-phosphoenolpyruvate during glucose metabolism. Enolase has five isozymes (αα, ββ, γγ, αβ and αγ). NSE is the γγ isozyme and exists in neurons and neuroendocrine cells (Wu et al., 2004). It is a biological macromolecule with molecular weight of 78 kDa and has stable physicochemical properties (Wu et al., 2004). Normally, the concentration of NSE in brain tissue, cerebrospinal fluid or blood is low. When hypoxic-ischemic brain damage occurs, however, its expression in brain increases (Hou, 2003). It is different from lactate dehydrogenase, aldolase and creatine kinase, which cannot combine with actin in cells, and can be released readily from ischemic or necrotic cells with the increase of apoptotic neurons and disintegration of the myelin sheath. There are a large number of studies addressing the biological markers of brain injury (for example, Jickling et al., 2011; Whiteley et al., 2011), and many experiments have revealed that the content of NSE increases significantly after cerebral ischemia injury (Brouns et al., 2010; Kaca-Oryńska et al., 2010; Ji et al., 2012). In a recent clinical trial, it was found that the concentration of NSE in serum within the first 72 hours of acute stroke was significantly higher than that in a control group (Singh et al., 2013), showing that the concentration of NSE in serum is correlated with the severity of neurological injury after acute cerebral ischemia and has high predictive value in the prognosis of neurological function. It was also confirmed in patients with ischemic cerebrovascular disease that the rise of NSE in serum is associated with the degree of neuronal necrosis and reflects the extent of neuronal ischemic injury; high expression of NSE in serum can prompt ultra-early cerebral infarction (Fan et al., 2011; Huang et al., 2012). Furthermore, the elevation of NSE shows two peaks after cerebral ischemia, the first appearing 7–18 hours after onset, the second in 2–4 days (Al-Rawi et al., 2009). Here, we found that the number of NSE-immunopositive cells, expression of NSE protein, and NSE mRNA transcription level all increased after modeling. NSE can therefore be used as a marker of ischemic brain injury and be valuable in clinical diagnosis.

Picroside II is one of the active ingredients of the traditional Chinese medicine Picrorhizae, the functions of which are to reduce heat, humidity, fever, dampness and steam, cool the blood, and purge bile (Jiangsu New Medical College, Dictionary of Chinese Traditional Drugs, 1996). Cell culture experiments confirmed that picroside II could reduce H_2O_2-induced injury in PC12 cells and improve cell survival (Li et al., 2002a), and its antioxidant effect has also been demonstrated (Li et al., 2007d). Our early animal experiments confirmed that picroside II could inhibit the expression of inflammatory cytokines, Toll-like receptor 4, nuclear factor κB, caspase-3, and tumor necrosis factor α, as well as the expression of inflammatory factors in the cerebral ischemic penumbra after middle cerebral artery occlusion and reperfusion, thus inhibiting neuronal apoptosis induced by ischemia (Guo et al., 2010; Li et al., 2010b, c, e, f). The present study indicates that both the reverse transcription level of NSE mRNA and its protein expression in the ischemia model group were significantly higher than in the control group, showing that NSE can be used as a marker to judge the extent of brain injury. Compared with the model group, Nissl body damage and apoptosis were lower after picroside II treatment, and the expression of NSE protein and reverse transcription of mRNA were also lower to varying degrees. Together, the above evidence shows that picroside II can protect the brain against different levels of ischemic injury. Intervention of picroside II at 1.5–2.0 hours of ischemia had a greater effect on the protection against brain injury than administration at other times, while there was no significant difference between different doses of picroside II. Therefore, although the optimal dose of picroside II needs further study, our results suggest that the effective therapeutic time window might be more important than the dose used.

Given the principle of obtaining the lowest therapeutic dose with the longest time window, the optimal therapeutic response in a rat model of cerebral ischemia is after an intraperitoneal injection of picroside II with 10 mg/kg body weight, 1.5–2.0 hours after injury.

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