ISSN 2347-6893

The influence of picroside II on the expressions of NSE, S100B and MBP in cerebral ischemia of rats

Hongwei Li [1], Yue Wang [2], Xiaodan Li [3], Li Zhao [3]

Department of Bascic Medicine, Taishan Vocational College of Nursing, Taian 271000, China

E-Mail: lihongwei3691@126.com

Department of Neurology, Qingdao Municipal Hospital, Qingdao 266011, China

E-Mail: wangyuehh@126.com

Institute of Intgrative Medicine, Qingdao University, Qingdao 266021, China

E-Mail: lixiaodanqd@163.com

ABSTRACT

The aim is to study the neuroprotection and optimize the therapeutic dose and time window of picroside II by orthogonal test in cerebral ischemic injury in rats**.** The forebrain ischemia models were established by bilateral common carotid artery occlusion (BCCAO) methods. The successful models were randomly grouped according to orthogonal experimental design and treated by injecting picroside II intraperitonenally with different dose at different ischemic time. The concents of neuron-specific enolase (NSE), neuroglial marker protein S100B and myelin basic protein (MBP) in brain tissue were determined by enzyme linked immunosorbent assay to evaluated the therapeutic effect of picroside II in cerebral ischemic injury**.** The Results proved that the best therapeutic time window and dose of picroside II in cerebral ischemic injury were (1) ischemia 1.5h with 10mg/kg body weight according to the concentration of NSE in brain tissue; (2) ischemia 1.5h with 20mg/kg according to the concentration of S100B; and (3) ischemia 1.5h with 10mg/kg according to the concentration of MBP. It is Concluded that the optimized therapeutic dose and time window is injecting picroside II intraperitonenally with 10-20mg/kg body weight at ischemia 1.5h in cerebral ischemic injury in rats according to the principle of lowest therapeutic dose with longest time window.

Keywords

Picroside II; Therapeutic dose; Time window; Cerebral ischemia; NSE; S-100; MBP; Rats

Academic Discipline And Sub-Disciplines

Neurobiology, Neurology and Integrative Medicine

Subject Classification

Biology and Medicine

Type (Method/Approach)

Quasi-experimental

ISSN 2347-6893

INTRODUCTION

Neuron-specific enolase (NSE) is an acidic protease and located specifically in neurons and neuroendocrine cells[1], which takes part in forming and repair process of membrane structures of brain tissue [2]. Hatfield et al.[3] demonstrated that NSE level in cerebrospinal fluid of rats with cerebral ischemic injury was positive correlated with infarct size and it was a marker of cerebral ischemic injury[4]. A traditional Chinese medicine, Shuxuetong injection has the remarkable neuronal protective effect and can obviously decrease the serum NSE level [5]. Neuroglial marker protein S100B is an acidic Ca2+-binding protein that has several regulatory roles in many functional events and processes such as cell proliferation, cytoskeletal structure regulation. When S100B exists between cells, it could stimulate the expression of inflammatory reaction factor and induce neuronal apoptosis [6]. Animal experiments indicated that S100B expression increased significantly one hour of ischemia and related to cerebral ischemia degree level[7]. Myelin basic protein (MBP) is localized in Schwann cell cytoplasm and myelin sheaths of the grigeminal ganglion[8].Without enough MBP can induce dysmyelination and MBP level can be indicative of the extent of myelin injury[9]. Animal experiments [10] showed that there was little expression of MBP mRNA in a normal adult rat brain. The relative levels of MBP mRNA decreased after ischemia, and recovered gradually at the time went by. Author's former research indicated that injecting picroside II intraperitonenally with 10-20mg/kg body weight at ischemia 1.5h in rats might reduce the expressions of NSE to inhibit the neuronal apoptosis induced by cerebral ischemia reperfusion injury and improve the neurological function of rats[11-14]. Because of the fact that neurological scores and immunohistochemical staining are easily influenced by subjective factors, the authors aimed to further explore the optimal therapeutic dose and time window of picroside II injecting intraperitoneally in cerebral ischemic injury in rats according to the quantitative measurement of NSE, S-100 and MBP level in brain tissue by enzyme linked immunosorbent assay.

MATERIALS AND METHODS

1. Animal models

A total of 30 adult healthy male Wistar rats, SPF grade, weight 230–250 g, were supplied by the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20100010). Five rats were randomly selected as a sham-operation group, and the remaining 25 rats were used to establish cerebral ischemic models. All animals were given time to adapt to the laboratory environment, allowed free access to food and water in a temperature and humidity-controlled housing with natural illumination for a week, and fasted 12 h before operation. The rats were anesthetized by injecting intraperitoneally 10% chloral hydrate (300 ml/kg) and fixed in supine position to conduct aseptic operation. Global forebrain ischemia models were established by bilateral common carotid artery occlusion (BCCAO) of rats[15]. Core body temperature was checked by a rectal probe and maintained at 36–37 °C. Twenty-one successful global forebrain ischemia model rat models were internalized into the experiment group (four rats that unconsciousness or dead 2 hours after operation were excluded). The rats in the sham operation group underwent the same surgical procedures except BCCAO operation.

2. Orthogonal Experimental Design

Total of (16×3) successful BCCAO models in treatment group were randomly grouped based on the orthogonal experimental design of [L16(45)] which contained two influential factors with four influential levels (Table 1). The medication time window, which was designed four levels at ischemia 1.0h, 1.5h, 2.0h, 2.5h, is the influential factor A. The influential factor B is the drug dosage, which was designed to inject picroside II at the dose of 5mg/kg, 10mg/kg, 20mg/kg and 40mg/kg (Table 1).

3. Treatment of picroside II

Picroside II (Tianjin Kuiqing Medical Technology Co., Ltd., CAS No: 39012-20-9, purity >98%, Molecular formula: C23H28O13, Molecular weight: 512.48) was diluted into 1% solution with saline solution and injected intraperitoneally according to the corresponding designed doses and ischemia time in the orthogonal layout [L16(45)]. Rats in sham group were simultaneously suffered the same doses saline solution.

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

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

4. Specimen collection

After treatment with picroside II for 24h, total of rats were anesthetized with 10% chloral hydrate (300 ml/kg), and were perfused with 200ml saline solution through heart. The brain was taken out completely through fast craniotomy and the olfactory bulb and prefrontal brain tissue were excised. Five hundreds milligram brain tissue were prepared backward from optic chiasma (Bregma 0.00mm), grinded to power in pre-cooling mortar and then homogenized by ultrasonic wave to extract cellular proteins with lysis buffer (500ul lysis buffer + 5ul PMSF, No.P0013, Beyotime Institute of Biotechnology. Shanghai, China) according to the proportion of 1:3, centrifuged with refrigerated centrifuge (Model Eppendorf 5801, Germany) at 12,000 r/min for 10 min at 4℃. The supernatant liquid was collected and stored at −20℃. The protein concentration in the supernatant was determined by the BCA method (Wuhan boster Biological Engineering Co., Ltd., China).

5. Enzyme Linked Immunosorbent Assay (ELISA)

The NSE (E02N0025), S100B(E02S0042)and MBP(E02M0034) levels of brain tissue homogenate were measured using an enzyme-linked immunosorbent assay (ELISA) Kit (BlueGene Biotech. Co. Ltd.). Before the measurement, samples were resolved at room temperature and centrifuged again. The 100ul supernatant liquid was collected. The experimental procedures were as follow: The microtiter plate coated with NSE, S100B and MBP specific antibody were prepared before starting assay procedures. Firstly, we secured the desired number of coated wells in the holder and added 100ul of standards or samples to the appropriate well of the antibody pre-coated microtiter plate. A hundred microlitre samples (100 ul) were added to blank well and 100 ul distilled water was added to blank control well. Fifty microlitre (50 ul) of enzyme marking solution was added to each well (not include blank control well), mixed thoroughly, sealed and incubated for 1 h at 37 °C. The microtiter plate was washed five times with wash buffer and bloted on absorbent paper to remove any residual buffer. Fifty microlitre (50 ul) of substrate A and B was added to each well, sealed and incubated for 10 minutes at 25 °C. Finally, 50 ul of stop solution was added to each well, thoroughly mixed. Read optical density (O.D.) with microtiter plate reader (Model Bio-Rad 680, USA) at 450 nm within 30 minutes of adding stop solution. The standard curve is generated by plotting O.D. on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis. The concentration of the samples can be determined from the standard curve. The sensitivity by this assay is 1.0 ng/ml.

6. Statistical Analysis

Apply SPSS 17.0 software to analyze data. In order to compare whether there were significant differences among different medication time and drug dose, whether there were significant differences between different interactions on detected indexes, as well as to explore the optimal drug dose and treatment time, multiple comparison was made.

RESULTS

1. Measurement Results

The contents of NSE and S100B in brain tissue of experimental group were increased significantly than those in sham-operated group (t=22.38-37.04, P<0.01). The contents of MBP in experimental group were obviously decreased than that in sham-operated group (t=2.33, P<0.05). After treatment, the contents of NSE and S100B were obviously decreased than those in experiment group (t=2.79-5.97, P<0.05). The content of MBP in brain tissue were increased than that in experimental group (t=3.91, P<0.05).

Table 2 The results of AQP4, MMP9 and COX2 (ng/ml)

groups	n	NSE	S100B	MBP
Sham group	5	5.415±0.546	0.443±0.064	0.434±0.060
Model group	5	9.410±0.620 [a]	0.821±0.086 [a]	0.212±0.030 [a]
Treatment group	16	7.404±1.328 [b]	0.623±0.151 [b]	0.305±0.099 [b]

[a] Compare with sham-operated group, *P*<0.01; [b] compare with model group, *P*<0.01

2. Result Analysis of NSE

There was significant difference (P<0.05) of NSE content of ischemia brain tissue among various medication time (factor A), various therapeutic dose (factor B) and various interaction between medication time and therapeutic dose (factor C). Pairwise comparisons among data of all independent groups by least-significant-difference (LSD) method showed that no significant difference (P > 0.05) of NSE content between medication time 1.0h (A1) and 1.5 h (A2), 1.0 h (A1) and 2.0 h (A3), while there was significant difference between each other for the rest groups (P < 0.05). There was no significant difference of NSE content between therapeutic dose 5mg/kg (B1) and 40mg/kg (B4), 10mg/kg (B2) and 20mg/kg (B3) (P > 0.05), but a significant difference existed between each other for the rest groups (P < 0.05). Therefore, according to the principle of lowest therapeutic dose with longest time window, A2B2 maybe the best combination group, i.e., the optimized therapeutic dose and time window should be injecting picroside II intraperitonenally with 10mg/kg body weight at ischemia 1.5h.

ISSN 2347-6893

Table 3 ANOVA of NSE Content

Variation source	SS	df	MS	*F*	*P*
Ischemia time	18.082	3	6.027	42.73	0.01
Therapeutic dose	5.357	3	1.786	12.66	0.01
Time×Dose	2.158	3	0.719	5.10	0.04
Error	0.846	6	0.141		

3. Result Analysis of S100B

There was significant difference (P<0.05) of S100B content of ischemia brain tissue among various medication time (factor A) or among various therapeutic dose (factor B), but no significant difference (P>0.05) found among various interaction between medication time and therapeutic dose (factor C). Pairwise comparisons among data of all independent groups by LSD method indicated that no significant difference (P>0.05) of S100B content between medication time 1.0h (A1) and 2 h (A3), while there was significant difference between each other for the rest groups (P<0.05). There was significant difference (P<0.05)of S100B content between therapeutic dose 5mg/kg (B1) and 10mg/kg (B2), 5mg/kg (B1) and 20mg/kg (B3), but no significant difference existed between each other for the rest groups (P>0.05). Therefore, according to the principle of lowest therapeutic dose with longest time window, A2B3 is the best combination group, i.e., the optimized therapeutic dose and time window is injecting picroside II intraperitonenally with 20mg/kg body weight at ischemia 1.5h.

Table 4 ANOVA of S-100B Content

Variation source	SS	df	MS	*F*	*P*
Ischemia time	0.294	3	0.098	70.73	0.01
Therapeutic dose	0.022	3	0.007	5.39	0.04
Time×Dose	0.016	3	0.005	3.76	0.08
Error	0.008	6	0.001		

4. Result Analysis of MBP

There was significant difference (P<0.05) of MBP content of ischemia brain tissue among various medication time (factor A), but no significant difference (P>0.05) among various therapeutic dose (factor B) or among various interaction between medication time and therapeutic dose (factor C). Pairwise comparisons among data of all independent groups by LSD method showed that no significant difference (P>0.05) of MBP content between medication time 1.0h (A1) and 2 h (A3), 1.5 h (A2) and 2.0 h (A3), while there was significant difference between each other for the rest groups (P < 0.05). There was significant difference of MBP content between therapeutic dose 5mg/kg (B1) and 10mg/kg (B2) (P < 0.05), but no significant difference between each other for the rest groups (P>0.05). Therefore, according to the principle of lowest therapeutic dose with longest time window, A2B2 is the best combination group, i.e., the optimized therapeutic dose and time window is injecting picroside II intraperitonenally with 10mg/kg body weight at ischemia 1.5h.

Table 5 ANOVA of MBP Content

Variation source	SS	df	MS	*F*	*P*
Ischemia time	0.090	3	0.030	11.83	0.01
Therapeutic dose	0.018	3	0.006	2.43	0.16
Time×Dose	0.022	3	0.007	2.90	0.12
Error	0.015	6	0.003		

There was significant probability between the different levels of influential factors A (therapeutic time window) on the content of MDA in brain tissue (P < 0.01), but no significant difference found in the influential factor B (drug dose) and influential factor C (time-dose interaction) (P>0.05). This indicated that the different therapeutic time window (or cerebral ischemia time) influenced significantly the content of MDA in brain tissue after cerebral ischemia injury, while no significant influences existed in the picroside II drug dose and the interactions of therapeutic time window and drug dose. All data were compared in pairs by the way of LSD-t test and the further analysis under the significance level of 0.05 revealed that the differences on the content of MDA in brain tissue between 1.0 (A1) and 2.5h(A4), 1.5h (A2) and 2.5h (A4) were

statistically significant, but no note deviations was found between any other medication time levels. No significant difference was found among the different levels of drug dose (P>0.05). According to the principle of lowest drug dose with longest time window, the best combination is A2B2 (1.5h/10mg), or the best time window is ischemia 1.5h and the best drug dose is 10mg/kg.

4. DISCUSSION

Enolases are divided into five kinds of isozymes as follows: type αα, ββ, γγ, αβ and αγ, of which γγ enolases, frequently designated as neuron-specific enolases (NSE), are located mainly in neurons and neuroendocrine cells. NSE has a molecular weight of 78 kDa and its active site is mainly located in the three dimensional structure of γ subunit[16]. Normally, the content of NSE in brain tissue or cerebrospinal fluid is low. Cerebral ischemia or cerebral anoxia increases the expression of NSE mRNA. The more serious brain tissue or neuron injury, the more NSE released to blood circulation. Therefore, NSE level in cerebrospinal fluid or serum is a marker enzyme of cerebral ischemic injury diagnosis and severity of brain tissue injury [17]. S100B is mainly localized in neural astrocytes and Schwann cells[18]. During brain damage, amount of active S100B protein is released from astrocytes and the serum levels of S100B are positively correlated with multiple forms of CNS damage[19]. Results in vitro culture of spongiocyte suggested that high concentrations of S100B in culture solution can modulate microglia/astrocyte activity by decreasing their neuroprotective action and modifying microglia/astrocyte interaction through inhibiting glial-derive growth factors [20]. Over-expression of human S100B aggravated brain damage after permanent middle cerebral artery occlusion in transgenic mice[21]. MBP is a major myelin protein component, and is responsible for the compaction of the myelin sheath. It is induced in response to collateral axonal sprouting in CNS of human adult. Normally, the MBP content in cerebrospinal fluid is less than 6.95mg/L. During cerebral ischemic injury, cerebral hypoxia -ischemia serve as a stimulus for the increased MBP expression [22]. Our results suggested that the contents of NSE and S100B in brain tissue of experiment group were increased significantly than those in sham-operated group, while the content of MBP in experiment group were significantly decreased than that in sham-operated group. This phenomenon indicates that the expression of NSE and S100B is increased after brain ischemic injury and is released into the blood circulation because of cerebral ischemia injuring the blood-brain barrier. After treatment with picroside II, the content of NSE and S100B were obviously decreased than those in experimental group, and the content of MBP in brain tissue were increased than those in experimental group, which reflects the neuroprotective effects of picroside II on cerebral ischemia and blood-brain barrier.

In this experiment, the authors designed four time points at 1 h, 1.5 h, 2 h and 2.5 h after brain ischemic injury in rats, and inject intraperitoneally picroside II with four therapeutic doses of 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg. The experiment was carried out according to orthogonal table of [L16(45)] to explore the best therapeutic dose and best time window of picroside II in treating cerebral ischemic injury by determining the expressions of NSE, S100B and MBP. The results indicate that there is significant difference in therapeutic effect among various medication times and various therapeutic doses of picroside II. But the results are not consistent with the best combination of different indexes. According to the principle of lowest therapeutic dose with longest time window, A2B3 maybe the best combination group, i.e., the optimized therapeutic dose and time window should be injecting picroside II intraperitonenally with 10-20mg/kg body weight at ischemia 1.5h. Because the mechanism of cerebral ischemic injury is very complicated and only above indexes was observed in this experiment, it is impossible to be no deviation in results. Therefore, in combination with other indexes the exact action mechanism of picroside II and the best medication time window and therapeutic dose need to be further studied in further experiments.

CONCLUSIONS

From the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window is injecting picroside II peritonenally with 10-20mg·kg-1 at 1.5h after cerebral ischemia.

ACKNOWLEDGEMENTS

This work is supported by the National Natural Science Foundation of China (Grant No. 8104109, 81274116).

REFERENCES

[1]Joseph J, Cruz-Sánchez FF, Carreras J. Enolase activity and isoenzyme distribution in human brain regions and tumors. J Neuroehem,1996,66(6):2484-2490.

[2]Selakovic V, Raicevic R, Radenovic L. The increase of neuron-specific enolase in cerebrospinal fluid and plasma as a marker of neuronal damage in patients with acute brain infarction. J Clin Neurosci, 2005, 12(5): 542-547.

[3]Hatfield RH, McKernan RM. CSF neuron-specific enolase as a quantitative marker of neuronal damage in a rat stroke model. Brain Res, 1992,577(2):249-252.

[4]Sienkiewicz-Jarosz H, Gałecka-Wolska M, Bidziński A, Turzyńska D, Sobolewska A, Lipska B, Płaźnik A, Ryglewicz D. Predictive value of selected biochemical markers of brain damage for functional outcome in ischemic stroke patients. Neurol Neurochir Pol, 2009,43(2):126-133.

[5] Zhen J, Chen T, Kong M, Li ZD, Kou L, Liu HW, Zhang L. Effect of Shuxuetong injection on neuron-specific enolase of serum and recovery of function in patients with acute cerebral infarction. Chin J Chinese Materia Medica, 2011,36(18):2584-2587.

[6] Yardan T, Erenler AK, Baydin A, Aydin K, Cokluk C. Usefulness of S100B protein in neurological disorders. J Pak Med Assoc, 2011, 61(3):276-281.

[7] Büyükuysal RL. Protein S100B release from rat brain slices during and after ischemia: comparison with lactate dehydrogenase leakage. Neurochem Int. 2005, 47(8):580-588.

[8]Wunderlich MT, Wallesch CW, Goertler M. Release of neurobiochemical markers of brain damage is related to the neurovascular status on admission and the site of arterial occlusion in acute ischemic stroke. J Neurol Sci, 2004, 227(1):49-53.

[9]Lamers KJ, Vos P, Verbeek MM, Rosmalen F, van Geel WJ, van Engelen BG. Protein S-100B, neuron-specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) and blood of neurological patients. Brain Res Bull, 2003,61(3):261-264.

[10]Chen YZ, Yi Q, Liu G, Shen X, Xuan LH, Tian Y. Cerebral white matter injury and damage to myelin sheath following whole-brain ischemia. Brain Res. 2013,1495(1):11-17.

[11]Li Q, Guo YL, Li Z, Xu XY. The interference of picroside ll on the expressions of Caspase-3and PARP following cerebral ischemia reperfnsion injury in rats. Chin Pharmacol Bull, 2010,26(3):342-345.

[12] Li Z, Li Q, Guo YL, Qin LH, Luan LJ. Interference effect of picroside II on cerebral ischemia reperfusion injury in rats. Acta Anat Sinica, 2010,41(1):9-12.

[13] Guo YL, Xu XY, Li Q, LI Z, Du F. Anti-inflammation effects of picroside Ⅱ in cerebral ischemic injury rats. Behavioral Brain Function, 2010, 6:43-53.

[14] Pei HT, Su X, Zhao L, Li HY, Guo YL, Zhang MZ, Xin H. Primary study for the therapeutic dose and Time window of picroside Ⅱ in treating cerebral ischemic injury in rats. Int J Mol Sci, 2012, 13(3):2551-2562.

[15] Márquez-Martín A, Jiménez-Altayó F, Dantas AP, Caracuel L, Planas AM, Vila E. Middle cerebral artery alterations in a rat chronic hypoperfusion model. J Appl Physiol, 2012, 112(3):511-518.

[16]Uinn GB, Reeves IG, Day IN. Mapping of antigenic sites in human neuron specific enolase by expression subcloning. Clin Chem, 1994,40(5):790-795.

[17]Wunderlich MT, Lins H, Skalej M, Wallesch CW, Goertler M. Neuron-specific enolase and tau protein as neurobiochemical markers of neuronal damage are related to early clinical course and long-term outcome in acute ischemic stroke. Clin Neurol Neurosurg, 2006, 108(6): 558-563.

[18] Murabayashi M, Minato M, Okuhata Y, Makimoto M, Hosono S, Masaoka N, Okada T, Yamamoto T, Mugishima H, Takahashi S, Harada K. Kinetics of serum S100B in newborns with intracranial lesions[J]. Pediatr Int, 2008, 50(1): 17-22.

[19] Gerlach R, Demel G, König HG, Gross U, Prehn JH, Raabe A, Seifert V, Kögel D.Active secretion of S100B from astrocytes during metabolic stress. Neuroscience, 2006, 141(4): 1697-1701.

[20] Reali C, Pillai R, Saba F, Cabras S, Michetti F, Sogos V. S100B modulates growth factors and costimulatory molecules expression in cultured human astrocytes. J Neuroimmunol. 2012, 243(1-2):95-99.

[21] Mori T, Tan J, Arendash GW, Koyama N, Nojima Y, Town T. Overexpression of human S100B exacerbates brain damage and periinfarct gliosis after permanent focal ischemia. Stroke. 2008, 39(7):2114-2121.

[22]Gregersen R, Christensen T, Lehrmann E, Diemer NH, Finsen B.Focal cerebral ischemia induces increased myelin basic protein and growth-associated protein-43 gene transcription in peri-infarct areas in the rat brain. Exp Brain Res, 2001, 138(3):384-392.