



Picroside II Could Protect The Cerebral Ischemic Injury by Reducing The Content of Free Radical and Enhancing The Activity of Antioxidase in Rats

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ABSTRACT

The aim is to optimize the anti-oxidation and the drug dose and medication time of picroside II by orthogonal test in cerebral ischemia 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 injected picroside II intraperitoneally with different dose at different ischemic time for treatment. The contents of malondialdehyde (MDA), nitric oxide (NO) and hydrogenperoxide (H₂O₂) in brain tissue were respectively determined by thiobarbituric acid assay, nitratase reduase assay and chemiluminescence immunoassay. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) in brain tissue were respectively determined by xanthinoxidase assay, chemical colorimetry and chemiluminescence immunoassay. The results indicated that the best therapeutic time window and dose of picroside II in cerebral ischemic reperfusion injury was (1) ischemia 1.5h with 10mg/kg, 1.5h with 20mg/kg and 1.5h with 20mg/kg body weight according to the contents of MDA, NO and H₂O₂ in brain tissue; (2) ischemia 1.5h with 20mg/kg, 2.0h with 20mg/kg and 1.5h with 10mg/kg body weight according to the activities of SOD, GSHPx and CAT in brain tissue. From the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window is injecting picroside II intraperitoneally with 10-20mg/kg at 1.5-2.0h after cerebral ischemic injury.

Keywords

Cerebral ischemia; Free radicals; Anti-oxidase; Picroside II; Therapeutic dose; Time window; Rats

Academic Discipline And Sub-Disciplines

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INTRODUCTION

Nitric oxide (NO), a kind of free radicals, can react with O₂- to synthesize more toxic free radicals of OH⁻ and NO₂⁻ and induce DNA damage and apoptosis[1]. After cerebral ischemia reperfusion, the regional brain tissue released more excited amino acids (EAAs) to activated inducible nitric oxide synthase (iNOS) [2], then oxygen free radicals attack polyunsaturated fatty acids of bio-membrane and cause lipid superoxide reaction [3-4] to induce apoptosis or necrosis[5-6]. Glutathione peroxidase (GSHPx) and catalase (CAT) could transform hydrogen peroxide (H₂O₂) into O₂ and H₂O to clear the toxic effects of OFR[7-8] and reduce the cerebral ischemic injury [9-13]. The concentration of malondialdehyde (MDA) could reflect the free radicals and lipid superoxide degree in tissue[14], and the activity of superoxide dismutase (SOD) reflect the capacity of clearing OFR in organism[15]. It has been confirmed that picoside II could decrease the PC12 cell damage induced by H₂O₂ and increase the survival rate of PC12 cells[16-19]. Recent animal studies suggested that picoside II could protect the neurological function of rats by inhibiting the expressions of inflammatory cytokines and reducing neuronal apoptosis following cerebral ischemia[20-21]. The authors administered picoside II with different drug dose at different ischemia time in MCAO rat models, and the results showed that the best medication time and drug dose of picoside II should be ischemia 1.5h with 20 mg/kg body weight[22]. This experiment aims to further explore the anti-oxidant effect and the best medication dose and medication time of picoside II in treating cerebral ischemia through detecting the OFR contents and anti-oxidase activity in brain tissue.

MATERIALS AND METHODS

1. Animal models

The local legislation for ethics of experiment on animals and guidelines for the care and use of laboratory animals were followed in all animal procedures. This experiment was approved by the Ethics Committee of Qingdao University Medical College (QUMC 2011-09). Total of 65 healthy adult male Wistar rats (specific-pathogen free grade, 230-250g body weight) were provided by Qingdao Laboratory Animal Center (SCXK (LU) 20100100) and were housed under controlled conditions with natural light, a temperature at 23±2°C and humidity-controlled housing for one week before surgery. The animals were allowed free access to food and water and absolute diet at 12h before surgery. Five rats (n=5) were randomly selected for sham group, and the rest 60 rats were subjected to establish cerebral ischemic models. The animals were anesthetized by intraperitoneally injection of 10% chloral hydrate at the dosage of 300mg/kg body weight. The ischemic models were established by bilateral common carotid artery occlusion (BCCAO) [23]. Rectal temperature was monitored and maintained between 36 and 37°C using a homeothermic blanket control unit (Qingdao Apparatus, China) during and after the surgery operation. Seven rats which had still not revived or died 2h after surgery were rejected out, while the 53 successful models which were randomly divided into model group (n=5) and treatment group (n=16×3) were brought into the experiment. The 5 rats of sham group were subject to the same experimental procedures except of BCCAO.

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 picoside II at the dose of 5mg/kg, 10mg/kg, 20mg/kg and 40mg/kg (Table 1).

3. Treatment of picoside II

The molecular formula of picoside II is C₂₃H₂₈O₁₃, and molecular weight is 512.48. Picoside II (CAS No:39012-20-9) was purchased from Tianjin Kuiqing Medical Technology Co.Ltd. and its purity was more than 98 %. Picoside II was diluted with physiological saline to the content of 10g/L and injected intraperitoneally based on the corresponding designed doses at designed time in the orthogonal experiment [L16(45)]. Rats in the sham group and model group were intraperitoneally injected same amount of normal saline after 2 h of cerebral ischemia. The orthogonal experiment [L16(45)] was repeated 3 times.

Table 1. Orthogonal experimental design of [L16(45)]

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 24h, the animals were reanesthetized by intraperitoneally injection of 10% chloral hydrate at the dosage of 300mg/ml body weight and then immediately perfused with 200ml normal saline via cardiac. Taking the whole brain and removing the olfactory bulb and prefrontal brain tissue, cutting 500 mg ischemic brain tissue from optic chiasma backwards to grind into powder in the pre-cooling mortar, and then adding cell lysis solution according to 1:3 proportion (500 μ l cell lysis solution + 5 μ l PMSF, No. P0013, Biyuntian Biotech. Co. Ltd.). After ultrasonic slurry, the brain sample mixture was centrifuged with 12000r/min for 10min at 4°C condition (Eppendorf 5801, Germany), then the supernatant was collected to determine the protein concentration by BCA assay (Wuhan Boster Bioltech. Co. Ltd) and stored at -20 .

5. Evaluating indexes

5.1. Anti-oxidase: The activities of superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) in brain tissue were respectively determined by xanthinoxidase assay, chemical colorimetry and chemiluminescence immunoassay. According to the instructions of kits (Nanjing Jiancheng Biological Science Co. Ltd.), the brain tissue sample supernatant 100 μ l is collected to determine the optical density at 550 nm (SOD), 412nm (GSHPx) and 751 (CAT) immediately using a microplate reader. The activity of sample is corresponding to the mean absorbance from the standard curve and the sensitivity is U/L.

5.2. Oxygen free radicals (OFR): The concentrations of malondialdehyde (MDA), nitric oxide (NO) and hydrogenperoxide (H₂O₂) in brain tissue were respectively determined by thiobarbituric acid assay, nitratase reduase assay and chemiluminescence immunoassay. The experiment procedure was operated as the instructions of kits (as above). The brain tissue samples were re-melted at room temperature and centrifuged again to collect supernatant 100 μ l for determining the optical density (OD) at 550 nm (MDA), 550nm (NO) and 405 (H₂O₂) immediately using a microplate reader. The standard curve is used to determine the amount of samples and the concentration of samples is corresponding to the mean absorbance from the standard curve and the sensitivity is mmol/L (MDA), μ mol/L (NO) and mmol/L (H₂O₂) respectively.

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. The results

The orthogonal experiment [$L_{16}(4^5)$] was repeated 3 times and the average values of the index test results were used in the statistical analysis (Table 2-3). In the rats of model group, the contents of MDA, NOS and H₂O₂ in brain tissue increased, while the activities of SOD, GSHPx and CAT in brain tissue decreased significantly than those in sham operation group ($t=12.32-43.86$, $P<0.01$). After treatment with picoside II, the contents of MDA, NOS and H₂O₂ in brain tissue reduced, while the activities of SOD, GSHPx and CAT in brain tissue arise significantly than those in the rats of model group ($t=3.88-7.64$, $P<0.01$).

Table 2. The detecting results of MDA, NO, H₂O₂, SOD, GSHPx and CAT

groups	n	MDA (mmol/L)	NO (μ mol/L)	H ₂ O ₂ (mmol/L)	SOD (U/L)	GSHPx (U/L)	CAT (U/L)
Sham	5	6.360 \pm 0.68	1.12 \pm 0.25	12.13 \pm 1.25	40.86 \pm 4.76	635.65 \pm 25.16	1.15 \pm 0.16
Model	5	9.60 \pm 0.82 ^a	2.67 \pm 0.34 ^a	17.36 \pm 1.56 ^a	25.44 \pm 3.29 ^a	426.17 \pm 20.65 _a	0.78 \pm 0.10 ^a
Treatment	48	7.61 \pm 1.00 ^b	1.74 \pm 0.65 ^b	15.17 \pm 1.58 ^b	32.13 \pm 6.11 ^b	552.90 \pm 92.17 _b	0.95 \pm 0.24 ^b

^a Compared with sham group, $t=12.32-43.86$, $P<0.01$; ^b Compared with model group, $t=3.88-7.64$, $P<0.01$

Table 3. [L₁₆ (4⁵)] orthogonal table and the test results

Test No.	Rank No.					MDA	NO	H2O2	SOD	GSHP	CAT
	A	B	C	D	E	mmol/	μmol/	mmol/	U/L	U/L	U/L
1	1	1	1	1	1	7.62	1.88	15.06	30.76	497.11	1.04
2	1	2	2	2	2	7.44	2.13	14.80	31.45	513.20	1.07
3	1	3	3	3	3	7.30	1.93	14.61	32.91	505.64	1.18
4	1	4	4	4	4	7.54	2.05	15.08	34.62	508.64	0.82
5	2	1	2	3	4	7.19	2.11	14.29	32.00	519.46	0.86
6	2	2	1	4	3	6.81	1.05	13.61	38.26	639.57	1.50
7	2	3	4	1	2	6.06	0.65	12.14	39.94	723.05	1.36
8	2	4	3	2	1	6.22	0.96	14.23	33.41	633.36	0.92
9	3	1	3	4	2	7.16	1.08	17.31	37.69	603.71	0.75
10	3	2	4	3	1	6.39	1.04	13.70	38.83	612.87	1.11
11	3	3	1	2	4	8.20	1.28	14.04	38.57	689.53	0.94
12	3	4	2	1	3	8.70	2.78	16.38	31.58	612.68	0.79
13	4	1	4	2	3	8.49	2.34	16.71	22.39	459.08	0.71
14	4	2	3	1	4	8.56	1.80	16.04	27.23	437.65	0.77
15	4	3	2	4	1	8.30	1.93	16.56	25.36	466.37	0.73
16	4	4	1	3	2	9.70	2.77	18.17	19.15	424.40	0.67
I	29.90	30.46	32.33	30.94	28.53	121.68	27.78	242.73	514.15	8846.3	15.22
II	26.28	29.20	31.63	30.35	30.36						
III	30.45	29.86	29.24	30.58	31.30						
IV	35.05	32.16	28.48	29.81	31.49						
SS	9.71	1.21	2.57	0.69	0.84						

2. ANOVA of MDA contents (Table 4)

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 picoside 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.

3. ANOVA of SOD activity (Table 4)

The different levels of influential factor A had significant probability to influence the activity of SOD in brain tissue ($P < 0.05$), while no significant probability among the different levels of influential factor B and the time-dose interaction ($P > 0.05$). The results of LSD under the significance level of 0.05 revealed that statistical differences were found between 1.0h(A1) and 1.5h(A2), 1.5h(A2) and 2.0h(A3), 1.5h(A2) and 2.5h(A4), 2.0h(A3) and 2.5h(A4), but no significant deviations between the other ischemia time levels. There were significant differences between 10mg(B1) and 40mg(B2), 20mg(B3) and 40mg(B4) ($P < 0.05$), but no statistical differences between the other drug dose levels ($P > 0.05$). Given the minimum injection dose and maximized medication time, the best combinations is A2B3, that is to say the minimum injection dose and maximized medication time of picoside II is injecting intraperitoneally with 20 mg/kg at cerebral ischemia 1.5h.

Table 4. ANOVA of MDA content and SOD activity

Source of variation	SS _{MDA}	df	MS	F	P	SS _{SOD}	df	MS	F	P
Time window	9.71	3	3.24	12.60	0.01	435.32	3	145.11	28.64	0.01
Drug dose	1.21	3	0.40	1.57	0.29	62.08	3	20.69	4.08	0.07
Time x Dose	2.57	3	0.86	3.33	0.10	32.34	3	10.78	2.13	0.20
Error	1.54	6	0.26			30.40	6	5.07		

4. ANOVA of NO content (Table 5)

A significant difference ($P < 0.05$) existed among each level of influential factor A and B, but no significant probability found in influential factor C ($P > 0.05$). The results of LSD under the significance level of 0.05 revealed that although the differences on the content of NO in brain tissue between 1.0h (A1) and 1.5h(A2), 1.5h(A2) and 2.5h(A4) were statistically significant, no significant deviations were found between the other ischemia time levels. There was no significant differences among the other injection dose levels ($P > 0.05$). On the basis of the minimum injection dose and maximized time window, the A2B3 is the best combinations, so the minimum injection dose and maximized medication time of picoside II is injecting intraperitoneally with 20 mg/kg at cerebral ischemia 1.5h.

5. ANOVA of GSHPx activity (Table 5)

The different levels of therapeutic time window (A) influenced significantly the activity of GSHPx ($P = 0.05$), while no significant probability between different drug doses (B) and time-dose interaction (C) ($P > 0.05$). The results of LSD under the significant level of 0.05 revealed that no significant deviations between 1.0h(A1) and 2.5h(A3), 1.5h(A2) and 2.0h(A3), but there were significant deviations between the other ischemia time levels. There was a statistical significance between 5 mg/kg (B1) and 20 mg/kg (B3) ($P < 0.05$), while no significant differences existed among the other drug dose levels ($P > 0.05$). Considering the principle of minimum dose and maximize treatment time window, the A3B3 combinations is the best treatment time window and dose injecting picoside II intraperitoneally with 20mg/kg at cerebral ischemia 2.0h.

Table 5. ANOVA of NO content and GSHPx activity

Source of variation	SS _{NO}	df	MS	F	P	SS _{GSHPx}	df	MS	F	P
Time window	2.50	3	0.83	4.54	0.05	100379.50	3	33459.83	20.72	0.01
Drug dose	1.25	3	0.42	2.28	0.18	12134.89	3	4044.96	2.51	0.16
Time x Dose	1.54	3	0.51	2.79	0.13	5236.77	3	1745.59	1.08	0.43
Error	1.10	6	0.18			9688.57	6	1614.76		

6. ANOVA of H₂O₂ content (Table 6)

The influences of therapeutic time (A) and drug dose (B) had significant probabilities on the content of H₂O₂ ($P < 0.05$), and time-dose interaction (C) had no significant probability ($P > 0.05$). The results of LSD under the significant level of 0.05 revealed that no significant deviation existed between 1.0h(A1) and 2.0h(A3), but statistically significant deviations between the other ischemia time levels. The differences on H₂O₂ content in brain tissue between 5mg/kg(B1) and 40mg/kg(B4), 10mg/kg(B2) and 20mg/kg (B3) were not found, while significant differences existed among the other dose levels ($P < 0.05$). Given the minimum dose and maximize treatment time window, the best combination is A2B2, that is to say the optimal treatment time and the optimal injection dose of picoside II is respectively ischemia 1.5h and 10 mg/kg body weight.

7. ANOVA of CAT activity (Table 6)

The different therapeutic time window (A) and drug dose (B) had a significant probability on the activity of CAT in brain tissue ($P_A = 0.01$, $P_B = 0.03$), but no significant differences among the time-dose interaction (C) ($P_C = 0.26$). The results of LSD under the significant level of 0.05 indicated that there were significant deviations between 1.0h(A1) and 2.5h(A3), 1.5h(A2) and 2.0h(A3), 1.5h(A2) and 2.5h(A4), while no significant deviations between the rest ischemia time levels. There was significant differences between 5mg(B1) and 40mg(B3), 10mg(B2) and 20mg(B4) ($P < 0.05$), and no significant differences among the other dose levels ($P > 0.05$). According to the principle of minimum dose and maximizing treatment time window, the best combination (A2B2) is the best treatment time and the optimal injection dose is respectively cerebral ischemia 1.5 h and 10mg/kg.

**Table 6.** ANOVA of H₂O₂ content and CAT activity

Source of variation	SS _{H₂O₂}	df	MS	F	P	SS _{CAT}	df	MS	F	P
Time window	22.29	3	7.431	15.26	0.01	0.42	3	0.14	9.24	0.01
Drug dose	8.71	3	2.903	5.96	0.03	0.29	3	0.10	6.25	0.03
Time x Dose	3.36	3	1.120	2.30	0.18	0.08	3	0.03	1.73	0.26
Error	2.92	6	0.487			0.09	6	0.02		

4. DISCUSSION

The previous study proved that progesterone could play a neuroprotective effect by elevating the activities of SOD and GSHPx in ischemic tissue to clear OFR[24], reducing intercellular Ca²⁺ concentration to influence the transmit system of amino acid[25], and recovering the concentration of inositol triphosphate (IP3) in cerebral ischemic area[26]. Modern pharmacological researches suggested that a number of active ingredients in traditional Chinese drugs, such as salvia miltiorrhiza bge [27] and bilobalide B[28] could significantly light mitochondrial damage caused by cerebral ischemia/reperfusion in rats, reduce the concentration of MDA, elevated the activity of SOD and GSHPx, and inhibit neuronal apoptosis induced by mitochondrial signal pathway. After cerebral ischemia/reperfusion, calycosin [29] and liquiritin [30] could decrease the concentrations of MDA and protein-hydroxy compound as a dose-dependent pattern, increase the activities of SOD, CAT and GSHPx, at the same time, inhibit the expression of 4-Hydroxy-2-nonenal (4-HNE) to reduce the number of positive cells labeled by 8-Hydroxy-2'-deoxyguanosine (8-OHdG). So they played an important neuroprotective effect by antioxidant mechanism and improve the neurobehavioral function of rats.

Li et al. [16-17] reported that picroside II, a active ingredient extracted from *Picrorhiza scrophulariiflora* of traditional Chinese drugs, could enhance the PC12 cells axonal growth induced by basic fibroblast growth factor and staurosporine or dbcAMP (N6,2'-O-dibutyl adenosine 3',5'cyclic-phosphate), which related to enhance the intracellular expression of mitogen-activated protein kinase-dependent signaling pathway at backward position. Guo et al. [18-19] found that picroside II could directly scavenge OFR in the body and enhance antioxidant capacity to reduce PC12 cell damage caused by H₂O₂. Recent animal experiment indicated that picroside II might elevate the antioxidant capacity of ischemic brain tissue, reduce the oxidant damage caused by ischemia/reperfusion, and improve the neurobehavioral functions of rats[31-32]. The results of this experiment indicated that the concentrations of MDA, NOS and H₂O₂ in brain tissue increased, while the activities of SOD, GSHPx and CAT decreased significantly after cerebral ischemia in rat. After treatment by picroside II, the concentrations of MDA, NOS and H₂O₂ in brain tissue decreased and the activities of SOD, GSHPx and CAT increased significantly than those in model group rats. The results suggested that picroside II might play a neuroprotective role by enhancing the activity of endogenous antioxidant and clear away the extra OFR in the body. From the orthogonal experimental design of [L₁₆(4⁵)], there were significance differences between the different levels of therapeutic time window and drug dose on the concentrations of oxygen free radicals and the activities of anti-oxidase in brain tissue, and the best combination of time-dose was not coincident according to the different detecting indexes. According to the principle of minimum dose and maximizing treatment time window, the best combination is A2B3 or A3B2, namely the optimal treatment time and injection dose is injecting picroside II intraperitoneally with 10-20mg/kg at 1.5-2.0h following cerebral ischemic injury. Because the mechanism of cerebral ischemia injury is very complex, the exact pharmacological mechanism of picroside II and the optimal medication time and medicine dose needs further testing with other indexes.

CONCLUSIONS

From the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window is injecting picroside II peritonally with 10-20mg·kg⁻¹ at 1.5-2.0h following cerebral ischemic injury.

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