



Research Article

Picroside II Could Reduce the Concentration of NO and Enhance the Activity of GSHPx in Cerebral Ischemic Injury in Rats

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Abstract

Picroside II could reduce the oxidative damage induced by cerebral ischemia-reperfusion and improve nerve behavior function of rats. The aim of this study was to 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 intraperitoneally at different ischemic time with different dose. The concentration nitric oxide (NO) in serum and brain tissue were determined by nitrate reductase assay. The activity of glutathione peroxidase (GSHPx) in serum and tissue were determined by chemical colorimetry. The optimized composition of the therapeutic dose and time window of picroside II in cerebral ischemic injury was (1) ischemia 1.5h with 20mg/kg body weight according to the concentrations of NO in serum and brain tissue; (2) ischemia 1.5h with 10mg/kg and ischemia 2.0h with 20mg/kg body weight according to the activities of GSHPx in serum and brain tissue. From the principle of lowest therapeutic dose with longest time window, the optimized composition of the therapeutic dose and time window of picroside II in cerebral ischemic injury is ischemia 1.5-2.0h with 10-20mg/kg body weight.

Keywords: Picroside II, Cerebral ischemia, NO, GSHPx.

Introduction

As a free radical, nitric oxide (NO) reacts with O₂⁻ to produce OH⁻ and NO₂⁻, which are more toxic than NO and contribute to disturbing the function of the respiratory chain, induction of DNA damage and cell apoptosis and aggravation of ischemia-

reperfusion injury (Ito et al.,2010). Under physiological condition, there is no expression of inducible nitric oxide synthase (iNOS). The release of local excitatory amino acid (EAA) was increased after cerebral ischemia-reperfusion, which activates iNOS and induces NO synthesis (Choi et al., 2010; Wang et al., 2011). This

effect is associated with lipid peroxide (Caso et al., 2008; Bi et al., 2009) and induction of cell apoptosis or death (Brown et al., 2010, Perluigi et al., 2010; Rey et al., 2011). After cerebral ischemia, increased production of free radical promoted degeneration and necrosis of vascular endothelial cell, aggravated infarction edema and destroyed reconstruction of blood supply in infarct area (Bailey et al., 2004). Under normal circumstances, the presence of effective antioxidant system is required to maintain the physiological levels of free radicals. Glutathione peroxidase (GSHPx) can convert H₂O₂ into H₂O and eliminate the toxic action of oxygen free radical (Yan et al., 2008; Wang et al., 2010). Cell culture assay shows that picoside II can reduce damage of H₂O₂-induced PC12 cells and improve cell survival rate (Li et al., 2002, Tao et al., 2003; Guo et al., 2007). Picoside II, which is an iridoid glucoside, is an active constituent extracted from the traditional Chinese medicine (TCM) hu-huang-lian. Animal experiments indicate that picoside II might regulate the expressions of iNOS, SOD and Caspase-9 (Li et al., 2010; Sun et al., 2011) to inhibit the neuronal apoptosis induced by cerebral ischemia reperfusion injury and improve the neurological function of animals (Li et al., 2010; Li et al., 2010). Author's former research indicated that the optimized therapeutic dose and time window is injecting picoside II intraperitoneally with 10-20mg/kg body weight at ischemia 1.5h in cerebral ischemic injury in rats (Pei et al., 2012). The authors aimed to further explore the optimum therapeutic dose and time window according to the quantitative measurement of NO and GSHPx level in blood and brain homogenates.

Materials and Methods

Animal Models

A total of 78 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). All animals were given time to adapt to the laboratory environment, allowed free access to food and water in a housing with natural illumination for a week. The room temperature was controlled at 23 ± 2°C. All rats were randomly divided into sham-operation group with 10 rats, model group with 10 rats and treatment group with 48 rats. All animals were fasted 12h 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 (Márquez-Martín et al., 2012). The successful rat models of global forebrain ischemia were internalized into the experiment group (ten 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. The Local Ethical Committee of Experimental Animals of Medical College of Qingdao University approved that all the steps of this study were compliant with ethical rules.

Orthogonal Experimental Design

Total 48 successful global forebrain ischemia rat models of treatment group were internalized into the experiment and divided randomly according to the principle of orthogonal experimental design of [L16(4⁵)] consisting of two impact factors with four impact levels (Table 1). The impact factor A is the therapeutic time widow designed four levels: 1.0 h, 1.5 h, 2.0 h, 2.5 h after ischemia. The impact factor B is the therapeutic drug dose which has four levels as follows: 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg body weight. The orthogonal experiment 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

Treatment Methods

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 (Pei et al., 2012) in the orthogonal layout [L16(4⁵)]. Rats in sham group were simultaneously suffered the same doses saline solution.

Sample Collection

After treatment with picroside II for 24h, total of rats were anesthetized with 10% chloral hydrate (300 ml/kg) and collected 4ml blood through heart to centrifuge at 4000 rpm for 10 min to separate serum, and stored at -20°C. And then the rats 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°C. The supernatant liquid was collected and stored at -20°C. The protein concentration in the supernatant was determined by the BCA method (Wuhan Boster Biological Engineering Co., Ltd, China).

Detection Index

The NO levels and GSHPx activities of serum and brain homogenate were measured by method of nitrate reductase and chemical colorimetry. According to the kit (Nanjing Jiancheng biology technology company) instructions, the serum or brain tissue homogenate were resolved at room temperature and centrifuged. The 100μl supernatant liquid was collected. We measured absorbance value with UV spectrophotometer (Beckmann DU640, USA) at a wavelength of 550nm (NO) and 412nm (GSHPx) and then calculated the content of NO (μmol/L) content and the activity of GSHPx (U/ml).

Statistical Analysis

SPSS 17.0 software was used for data statistical analysis. According to the result, multi-group comparison was made by analysis of orthogonal test whether different level of administrating time dose and the therapeutic time window.

Results**Measurement Results**

In model group, the contents of NO in serum and brain tissue homogenate were increased significantly than those in sham-operated group and the activities of GSHPx were obviously decreased than those in sham-operated group (t=12.32~43.86, P<0.01). In treatment group, the contents of NO in serum and brain tissue homogenate were obviously decreased than those in model group and the activities of GSHPx were increased significantly than those in model group (t=3.88~7.64, P<0.01. See tables 2 and 3.

Table 2: The Measurement Results Results of NO and GSHPx

groups	n	Serum NO (μmol/L)	Brain NO (μmol/L)	Serum GSHPx (U/L)	Brain GSHPx (U/L)
Sham group	5	45.56±6.72	1.12±0.25	328.15±9.85	635.65±25.16
Model group	5	80.33±9.26a	2.67±0.34a	267.14±8.32a	426.17±20.65a
Treatment group	48	65.95±8.90b	1.74±0.65b	305.81±25.05b	552.90±92.17b

^a Compare with sham-operated group, $t=12.32\sim43.86$, $P<0.01$; ^b compare with model group, $t=3.88\sim7.64$, $P<0.01$

Table 3: L16 (45) Orthogonal Layout and the Results of Measurement

Test No.	Rank No.					Serum	Brain	Serum	Brain
	A	B	C	D	E	NO	NO	GSHPx	GSHPx
1	1	1	1	1	1	67.50	15.06	293.67	497.11
2	1	2	2	2	2	66.67	14.80	303.26	513.20
3	1	3	3	3	3	62.22	14.61	310.61	505.64
4	1	4	4	4	4	71.11	15.08	293.99	508.64
5	2	1	2	3	4	71.67	14.29	291.84	519.46
6	2	2	1	4	3	62.11	13.61	345.92	639.57
7	2	3	4	1	2	51.39	12.14	361.64	723.05
8	2	4	3	2	1	56.94	14.23	313.47	633.36
9	3	1	3	4	2	66.67	17.31	304.29	603.71
10	3	2	4	3	1	50.56	13.70	325.71	612.87
11	3	3	1	2	4	54.17	14.04	322.92	689.53
12	3	4	2	1	3	71.33	16.38	307.40	612.68
13	4	1	4	2	3	78.22	16.71	273.55	459.08
14	4	2	3	1	4	72.62	16.04	301.76	437.65
15	4	3	2	4	1	76.25	16.56	272.65	466.37
16	4	4	1	3	2	75.83	18.17	270.20	424.40
I	267.50	284.06	259.61	262.84	251.25	1055.26	242.73	4892.88	8846.32
II	242.11	251.96	285.92	256.00	260.56				
III	242.73	244.03	258.45	260.28	273.88				
IV	302.92	275.21	251.28	276.14	269.57				
SS	614.62	267.92	173.06	66.34	69.98				

ANOVA Analysis of NO Content

There was significant difference ($P<0.01$) of the content of NO in serum among various ischemia time (factor A) but no significant difference ($P>0.05$) found among 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 there was significant difference ($P<0.05$) of NO content in serum between medication time (ischemia time) 1.0h (A1) and 2.5 h (A4), 1.5 h (A2) and 2.5 h (A4), 2 h (A3) and 2.5 h (A4), but no significant difference existed between each other for the rest groups ($P>0.05$). There was significant difference of NO content between therapeutic dose 5mg/kg (B1) and 20mg/kg (B3) ($P < 0.05$), 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 time window and therapeutic dose is ischemia 1.5h with 20mg/kg body weight.

There was no significant difference ($P > 0.05$) of NO content in 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 there was significant difference ($P < 0.05$) of NO content between medication time 1.0h (A1) and 1.5 h (A2), 1.5 h (A2) and 2.5 h (A4), while there was no significant difference between each other for the rest groups ($P > 0.05$). There was no significant difference ($P > 0.05$) among various therapeutic dose. Therefore, according to the principle of lowest therapeutic dose with longest time window, A2B3 is the best combination group, i.e., the optimized time window and therapeutic dose is ischemia 1.5h with 20mg/kg body weight.

Table 4: ANOVA Analysis of NO Content

Variation source	SS Serum	df	MS	F	P	SS Brain	df	MS	F	P
Ischemia time	614.62	3	204.87	9.29	0.01	2.50	3	0.83	4.54	0.06
Therapeutic dose	267.92	3	89.31	4.05	0.07	1.25	3	0.42	2.28	0.18
Time×Dose	173.06	3	57.69	2.62	0.15	1.54	3	0.51	2.79	0.13
Error	132.33	6	22.06			1.10	6	0.18		

ANOVA Analysis of GSHPx Activity

There was significant difference ($P < 0.05$) of the activity of GSHPx in serum among various ischemia time (factor A) and various therapeutic dose (factor B), but the interaction between medication time and therapeutic dose (factor C) was negligible ($P > 0.05$). Pairwise comparisons among data of all independent groups by least-significant-difference (LSD) method showed that there was no significant difference ($P > 0.05$) of the activity of GSHPx in serum between medication time (ischemia time) 1.0h (A1) and 2 h (A3), 1.5 h (A2) and 2 h (A3), but significant difference existed between each other for the rest groups ($P < 0.05$). There was no significant difference of the activity of GSHPx in serum 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 is the best combination group, i.e., the optimized time window and

therapeutic dose is ischemia 1.5h with 10mg/kg body weight.

There was significant difference ($P < 0.05$) of the activity of GSHPx in brain tissue among various ischemia time (factor A), but the therapeutic dose (factor B) and the interaction between medication time and therapeutic dose (factor C) were negligible ($P > 0.05$). Pairwise comparisons among data of all independent groups by least-significant-difference (LSD) method showed that no significant difference ($P > 0.05$) of the activity of GSHPx in brain tissue between medication time 1.0h (A1) and 2.5 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 the activity of GSHPx in brain tissue between therapeutic dose 5mg/kg (B1) and 20mg/kg (B3) ($P < 0.05$), 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, A3B3 is the best combination group, i.e., the optimized time window and

therapeutic dose is ischemia 2.0h with 20mg/kg body weight.

Table 5: ANOVA Analysis of GSHPx Activity

Variation source	SS Serum	df	MS	F	P	SS Brain	df	MS	F	P
Ischemia time	5230.40	3	1743.47	12.30	0.01	100379.50	3	33459.83	20.72	0.01
Therapeutic dose	2471.13	3	823.71	5.81	0.03	12134.89	3	4044.96	2.51	0.16
Time×Dose	862.88	3	287.63	2.03	0.21	5236.77	3	1745.59	1.08	0.43
Error	850.23	6	141.71			9688.57	6	1614.76		

Discussion

Modern medical studies show that the active ingredients of traditional Chinese medicine have good neuroprotective effect. *Salvia miltiorrhiza* (Zhang et al., 2010) and calycosin (Guo et al., 2012) could reduce mitochondrial injury induced by cerebral ischemia-reperfusion in rats, significantly decrease contents of MDA (Malondialdehyde) and protein hydroxyl compounds, upregulate the activity of SOD (Superoxide dismutase), CAT (Catalase) and GSHPx, and at the same time, inhibit the expression of 4-Hydroxy-2-nonenal. Chrysophanol formulations (Li and Zhang et al., 2010) could significantly increase the activities of GSHPx in whole blood and SOD in plasma in rats with cerebral ischemia reperfusion, and improve the prognosis in ischemic cerebrovascular disease. Liquiritin (Sun et al., 2010) could decrease the content of MDA in brain tissue of mice and increase the activities of CAT and GSHPx. Salidroside (Yan et al., 2008) can obviously reduce the content of NO in rats with cerebral ischemia reperfusion, and improve the damage of neurons induced by ischemia reperfusion, the mechanism of which may be the enhanced expression of downstream step in pathways of intracellular mitogen-activated protein kinase (Li et al., 2002; Li et al., 2000). Cell culture assay shows that picoside II has obvious protective effect on damage of H₂O₂-induced PC12 cells, which has a relationship with effect of direct scavenging oxygen free radicals and enhancing cell itself function of antioxidant system (Tao et al., 2003). Experiments on animal models indicate that picoside II could improve the antioxidant capacity of brain tissue in cerebral ischemic injury in

rats, reduce the oxidative damage induced by cerebral ischemia-reperfusion and improve nerve behavior function of rats (Zhao et al., 2010).

As the detection index of this experiment, NO can directly reflect changes of free radical content in cerebral ischemic injury and GSHPx can reflect the change of antioxidant content in cerebral ischemic injury. 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 picoside 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 picoside II in treating cerebral ischemic injury by determining the expressions of NO and GSHPx. The results indicate that there is significant difference in therapeutic effect among various medication times and various therapeutic doses of picoside II. But the results are not consistent with the best combination of different indexes.

Conclusion

According to the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window should be injecting picoside II intraperitoneally with 10-20mg/kg body weight at ischemia 1.5-2.0h.

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