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To Optimize the Therapeutic Dose and Time Window of Picroside II in Cerebral Ischemic Injury in Rats by Orthogonal Test

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ABSTRACT

The paper aims 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 divided into sixteen groups according to orthogonal experimental design and treated by injecting picroside II intraperitonenally at different ischemic time with different dose. The concentrations of neuron-specific enolase (NSE), neuroglial marker protein S100B and myelin basic protein (MBP) in serum were determined by enzyme linked immunosorbent assay to evaluate the therapeutic effect of picroside II in cerebral ischemic injury. The results indicated that best therapeutic time window and dose of picroside II in cerebral ischemic injury were ischemia 1.5 h with 20 mg/kg body weight according to the concentrations of NSE, S100B and MBP in serum. It is concluded that according to the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window are injecting picroside II intraperitonenally with 20 mg/kg body weight at ischemia 1.5 h in cerebral ischemic injury in rats.

Keywords: Picroside II; Cerebral Ischemia; Therapeutic Dose; Time Window; Rats

1. Introduction

S100B, a neuroglial marker protein, participates in cell multiplication, cytoskeleton regulation and other biological activities. When there are plentiful S100B between cells, it may promote the expression of inflammatory reaction factors, and induce neuron apoptosis [1]. Clinical study [2] has found that S100B, a neuroglial marker protein, elevates significantly in the serum of patients with ischemic stroke, and S100B concentration is closely related to ischemic stroke type, severity, infarction volume and mortality [3]. Neuron-specific enolase (NSE), which exists specifically in the neurons and neuroendocrine cells [4], involves in the formation of membrane structures and the repair of brain cells [5]. The level of NSE in CSF reaches its peak on the third day after cerebral ischemia in rats, and is positively correlated with the final infarction volume [6]. The up-regulation of NSE level in serum lags behind that in brain tissue for 2 hours in permanent cerebral ischemia model [7,8]. Myelin basic protein (MBP) is an important myelin structural protein. It is beneficial to stabilize the structure and function of the central nerve system (CNS) [9], and its level change can reflect the severity of the injury in CNS and myelin damage [10]. The mRNA expression of MBP is a small amount in normal adult rat brain, and the expression decreases after cerebral ischemic injury, however, as ischemic time goes on, the expression of MBP mRNA increases slowly [11]. Our previous studies have shown that in rats with cerebral ischemia for 1.5 h, intraperitoneal injection of picroside II (20 mg/kg) can suppress the expression of inflammatory cytokines and neuronal apoptosis [12-15]. This study attempts to detect the changes of NSE, S100B and MBP levels in serum to further explore the optimal therapeutic dose and the time window of picroside II for treatment of cerebral ischemic injury.

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2. Material and Methods

2.1. Animal Model Establishment

Thirty healthy, male, specific-pathogen free, *Wistar* rats, weighting 230 - 250 g, were provided by the Laboratory Animal Center of Qingdao Drug Inspection Institute (certificate No. SCXK (Lu) 20100010). Five rats were selected for the sham control group randomly, and the remaining 25 were used to establish the model of cerebral ischemia. The rats were deprived of food for 12 hours before surgery, and anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/kg), and the bilateral carotid arteries were isolated and occluded to establish forebrain ischemia model [16]. Body temperature was monitored using a rectal probe, and was maintained at 36˚C - 37˚C using a homeothermic blanket control unit during surgery. 4 rats were excluded for unconsciousness in two hours after the surgery or death, and the remaining 21 successful models were included in the study. The sham control group also had the operation, but no occlusion of the bilateral carotid arteries were performed.

2.2. Group Design

The 21 successful animal models were randomly divided into 5 for model group and 16 for treatment group. Animal models of the treatment group were grouped by four levels and two factors [$L_{16}(4^5)$] orthogonal experimental design. Therapeutic time window was factor A, and four levels were set including cerebral ischemia for 1.0 h, 1.5 h, 2.0 h, 2.5 h. Therapeutic dose was factor B, and 5, 10, 20, 40 mg/kg body weight were set as four levels.

2.3. Interventions

Picroside Ⅱ (CAS No.: 39012-20-9, purity> 98%) was provided by the Tianjin Kuiqing pharmaceutical company, and was diluted to 1% solution with saline solution. According to [$L_{16}(4^5)$] orthogonal experimental design, appropriate dose of picroside Ⅱ was injected intraperitoneally on corresponding ischemic time. Equal amount of saline were injected at cerebral ischemia 2 h for sham control group and model group (**Table 1**).

2.4. ELISA

The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/kg) 24 hours after drug administration. 4 ml blood was taken through the heart and centrifuged at 4000 r/min for 10 minutes, and the serum were separated. And then the levels of NSE (E02N0025), S100B (E02S0042), MBP (E02M0034) were measured in serum by the enzyme-linked immunosorbent assay kit (Blue Gene biotech company). The ELISA plate coated with anti NSE, S100 and MBP specific antibody was used, 100 μl standard solution was added in the blank micropores according to the order of the samples, 100 μl samples were added in the blank micropores, and 100 μl distilled water was added in the blank control, and 50 μl enzyme marker solution was added in each hole (the blank control holes were not included); the enzyme label plate was sealed with sealing compound, and incubated for 1 hour at 37˚C. The microtiter plate was washed five times using distilled or de-ionized water, and dried thoroughly with absorbent paper; 50 μl substrate A & B was added to each well (the blank control holes were not included) and incubated for 10 minutes in darkness at 20˚C - 25˚C, followed by addition of 50 μl stop solution to each well; The OD value was calculated by an enzyme monitor (Bio-Rad 680, USA) at 450 nm for each set of reference standards and samples; the concentration can be found in the standard curve according to the OD value of the samples, expressed in ng/ml.

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

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

2.5. Statistical Analysis

SPSS 17.0 statistical software was applied to process statistical analysis, to analyze the contribution of different levels of ischemia (drug administration) time and dose to the test indicators, as well as the interaction of ischemia time and dose to the test indicators, and comes to the best dose and the combination of the time window according to the results.

3. Results

3.1. Test Results

The model animal group had significantly elevated serum NSE (6.773 ± 0.812), S100B (0.762 ± 0.110) and MBP (0.675 ± 0.083) levels compared with the sham control group, in which the serum NSE, S100B and MBP levels were 2.368 ± 0.532, 0.234 ± 0.051 and 0.227 ± 0.042 ($t = 2.79 - 5.97$, $P < 0.05$). And the model animal group after treatment had more decreased serum NSE (5.613 ± 1.362), S100B（0.634 ± 0.153）and MBP (0.305 ± 0.099) levels than before ($t = 2.33 - 3.91$, $P < 0.05$).

Table 2 is an orthogonal experiment of [L16(45)].

Table 2. [$L_{16}(4^5)$] orthogonal design and test results.

Test No.	Rank No.					Serum	Serum	Serum
	A	B	C	D	E	NSE	S100	MBP
1	1	1	1	1	1	4.784	0.631	0.565
2	1	2	2	2	2	4.976	0.651	0.655
3	1	3	3	3	3	4.494	0.640	0.517
4	1	4	4	4	4	5.074	0.710	0.573
5	2	1	2	3	4	5.268	0.696	0.598
6	2	2	1	4	3	4.122	0.537	0.387
7	2	3	4	1	2	4.076	0.415	0.341
8	2	4	3	2	1	5.385	0.461	0.404
9	3	1	3	4	2	7.086	0.753	0.756
10	3	2	4	3	1	6.396	0.409	0.623
11	3	3	1	2	4	3.290	0.375	0.526
12	3	4	2	1	3	7.478	0.664	0.603
13	4	1	4	2	3	7.319	0.780	0.759
14	4	2	3	1	4	7.523	0.783	0.722
15	4	3	2	4	1	5.433	0.740	0.726
16	4	4	1	3	2	7.098	0.891	0.823
Ⅰ	19.328	24.457	19.294	23.861	21.998	89.802	10.136	9.578
Ⅱ	18.851	23.017	23.155	20.970	23.236			
Ⅲ	24.250	17.293	24.488	23.256	23.413			
Ⅳ	27.373	25.035	22.865	21.715	21.155			
SS	12.544	9.407	3.696	1.343	0.857			

Test No. are rat models (No. 1 - 16) and Rank No. are impact factor's levels. Data listed in the table of serum NSE, S100 and MBP are the experimental results of corresponding rat models. Data listed in line I, II, III, IV and SS are the ANOVA result of serum NSE, the ANOVA results of Serum S100 and MBP are omitted.

3.2. NSE Analysis

Significant difference was showed in the content of NSE in serum (**Table 3**) on different levels of administration time (factor A) and dosage (factor B) ($P < 0.05$), but the interaction of administration time and dosage (factor C) showed no significant difference ($P > 0.05$). With the method of least significant difference (LSD) in each two groups, only in the levels of administration time of 1.0 h (A1) and 1.5 h (A2), 2.0 h (A3) and 2.5 h (A4), the content of NSE were not different significantly ($P > 0.05$), and other levels of administration time all showed significant difference ($P < 0.05$); no significance was found in administration dose of 5 mg/kg (B1) and 10 mg/kg (B2), 10 mg/kg (B2) and 40 mg/kg (B4), 5 mg/kg (B1) and 40 mg/kg (B4) ($P > 0.05$), and other levels of administration dose all showed significant difference ($P < 0.05$). From the point of view of the maximum therapeutic time window and minimum administration dose, A2B3 is the optimal combination, in other words, the best treatment time window and dose were ischemia for

Table 3. Variance analysis of the content of NSE in serum.

source of variation	SS	df	MS	*F*	*P*
ischemia time	12.544	3	4.181	11.41	0.01
drug dose	9.407	3	3.136	8.55	0.01
time × dose	3.696	3	1.232	3.36	0.10
deviation	2.199	6	0.367		

1.5 h, 20 mg/kg, respectively.

3.3. S100B Analysis

Significant difference was showed in the content of S100B (**Table 4**) on different levels of factor A (P = 0.05), but the factor B, as well as time-dose interaction (C), had not taken effect on serum S100B levels after ischemia significantly ($P > 0.05$). With the method of least significant difference (LSD) in each two groups, only in the levels of administration time of 1.5 h (A2) and 2.5 h (A4), 2.0 h (A3) and 2.5 h (A4), the content of NSE were different significant ($P < 0.05$) , and none of the other levels of administration time showed any difference ($P > 0.05$); significance ($P < 0.05$) was found in administration dose of 5 mg/kg (B1) and 20 mg/kg (B3), and other levels of administration dose showed no difference ($P > 0.05$). From the point of view of the maximum therapeutic time window and minimum administration dose, A2B3 is the optimal combination, namely, the best treatment time window and dose were ischemia for 1.5 h, 20 mg/kg, respectively.

3.4. BMP Analysis

Significant difference was showed in the content of BMP (**Table 5**) on different levels of factor A ($P < 0.05$), but no significance was found between BMP levels after ischemia, and there were no significant difference in time-dose interaction (C) ($P > 0.05$). With the method of least significant difference (LSD) in each two groups, only in the levels of administration time of 1.0 h (A1) and 2.0 h (A3), the content of NSE were not different significantly ($p > 0.05$), and other levels of administration time all showed significant difference ($p < 0.05$); significance was found in administration dose of 5 mg/kg (B1) and 20 mg/kg (B3) ($p < 0.05$), and other levels of administration dose showed no difference ($p > 0.05$). From the point of view of the maximum therapeutic time window and minimum administration dose, A2B3 is the optimal combination, that is, the best treatment time window and dose were ischemia for 1.5 h, 20 mg/kg, respectively.

Table 4. Variance analysis of the content of S100B in serum.

source of variation	SS	df	MS	*F*	*P*
ischemia time	0.184	3	0.061	5.87	0.03
drug dose	0.075	3	0.025	2.38	0.17
time × dose	0.029	3	0.010	0.93	0.48
deviation	0.063	6	0.010		

Table 5. Variance analysis of the content of BMP in serum.

source of variation	SS	df	MS	F	P
ischemia time	0.216	3	0.072	15.08	0.01
drug dose	0.040	3	0.013	2.81	0.13
time × dose	0.013	3	0.004	0.93	0.48
deviation	0.029	6	0.005		

4. Discussion

S100B mainly exists in the astrocytes and Schwann cells of nervous system. Tracing S100B may advance the extension of neuron axons and enhance the survival rate of neurons, while a great deal of it may generate toxic and side-effect [1]. A large number of activated S100B protein which is produced by glial cells is released into the extracellular after brain tissue injury [17], and then leaks into CSF and blood through damaged blood brain barrier, and S100B level in serum is positively correlated with the severity of the injury of cerebral ischemia [18]. Brouns and his group have also found that the concentration of S100B, which is in the cerebral spinal fluid (CSF) of the patients with acute ischemic stroke, is highly correlated to the severity of the injury and the prognosis of cerebral ischemia [19]. Under normal circumstances, NSE content is not high in blood, however, along with the necrosis of neurons and the disintegration of nerve myelin, NSE is released into CSF from the cells after cerebral ischemia injury, and then leaks into blood through the blood brain barrier. The more severe the brain tissue is injured, the more nerve cells are damaged, and the more NSE is released into the blood, therefore, the detection of changes of NSE levels in CSF or in serum becomes diagnosis markers of neuronal damage [20]. Normal MBP content in CSF is less than 6.95 mg/L, however, when the ischemic cerebral injury happens, cerebral ischemia and hypoxia may be due to oligodendrocyte death and demyelination. At the same time, MBP will flow into the CSF and further into blood with the damage of the blood brain barrier, which is caused by brain injuries. Otherwise, the increase of MBP synthesis may be stimulated by stress conditions themselves, including ischemia, hypoxia and others [21]. So to a certain degree, the detection of the serum MBP can show whether there are brain injuries, and MBP becomes a specific protein marker of demyelination [22]. Our study confirmed that the content of MBP in serum of the model group was significantly higher than that in the sham control group, and reduced in different degrees after treatment. Our results showed that the contents of NSE, S100B and MBP in serum of the model group were significantly higher compared with that of the sham control group, and decreased significantly after treatment, sug-

gesting a protective role of picroside II on both ischemic brain tissue and blood brain barrier.

This experiment was a [L16(45)] orthogonal experiment design, in which four time points of 1 h, 1.5 h, 2 h and 2.5 h after brain ischemia were set and four levels of picroside II dose were given (5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg). The expressions of NSE, S100B and MBP in serum were measured, and the optimal therapeutic dosage and time window of picroside II were analysed. These results showed that there was a significant difference for the therapeutic effects of picroside II in administration time and the dose. From the principle of the lowest therapeutic dose with the longest time window, A2B3 was the optimal combination and the best treatment time window and dose were ischemia for 1.5 h and intraperitoneal injection of 20 mg/kg. For the mechanism of cerebral ischemic injury was very complicated, only four indexes, as mentioned here, were observed in this study, which must bring some variances. Therefore, the exact mechanism and optimal therapeutic time window and dosage of picroside II should be evaluated combined with other detection indexes.

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