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The anti-inflammatory effect of picroside II and the optimizing of therapeutic dose and time window in cerebral ischemic injury in rats

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ABSTRACT

The aim is to optimize the anti-inflammatory effect and the therapeutic dose and time window of picrosede II by orthogonal test in cerebral ischemic injury in rats. The forebrain ischemia models were established by bilateral common carotid artery occlusion (BCCAO) methods in 30 *Wistar* rats. The successful models were randomly divided into sixteen groups according to orthogonal experimental design and treated by injecting picroside II intraperitoneally at different ischemic time with different dose. The concentrations of aquaporins 4 (AQP4), matrix metalloproteinases9 (MMP9) and cyclooxygenase 2 (COX2) in serum and brain tissue were determined by enzyme linked immunosorbent assay to evaluate the therapeutic effect of picroside II in cerebral ischemic injury. The best therapeutic time window and dose of picroside II in cerebral ischemic injury were 1) ischemia 2.0 h with 20 mg/kg and 1.5 h with 20 mg/kg body weight according to the concentration of AQP4 in serum and brain tissue; 2) ischemia 1.5 h with 20 mg/kg and ischemia 2.0 h with 20 mg/kg according to the concentrations of MMP9 in serum and brain tissue; and 3) ischemia 1.5 h with 10 mg/kg and ischemia 1.5 h with 20 mg/kg according to the concentrations of COX2 in serum and brain tissue respectively. According to the principle of the lowest therapeutic dose with the longest time window, the optimized therapeutic dose and time window were injecting picroside II intraperitoneally with 10 - 20 mg/kg body weight at ischemia 1.5 - 2.0 h in cerebral ischemic injury.

Keywords: Picroside II; Therapeutic Dose; Time Window; Cerebral Ischemia; AQP4; MMP9; COX-2; Rats

1. INTRODUCTION

Aquaporins 4 (AQP4) is the main channel protein which specifically allows water molecule to pass into the cell across the membrane in central nervous system (CNS) [1]. Animal experiments have shown that the expression of AQP4 protein and mRNA in cortex around the infarcts increased at ischemia 3 h, peaked at ischemia 24h and decreased after ischemia 72 h [2]. Picroside II could down-regulated the expression of AQP4 in ischemia/reperfusion injury rats and reduce the degree of brain edema [3]. Matrix metalloproteinases 9 (MMP9) belongs to the gelatin enzymes, which could injury blood-brain barrier (BBB) through degrading the basement membrane and tight junction in inflammatory reaction after brain ischemia to induce brain edema and caused neuronal death directly [4,5], while the cerebral ischemic injury reduces significantly after knocking out MMP9 gene [6,7]. Previous experiments have showed that the expression of MMP9 increased after brain ischemia, while Chinese medicines of musk [8], astragalus [9] and picroside II [10] could inhibit the expression of MMP9 after brain ischemia and reduce the degradation of extracellular matrix in BBB basement membrane and its permeability, and relieve the degree of inflammatory injury and brain edema. Cyclooxygenase 2 (COX2) mainly exists in the cerebral cortex neurons, hippocampal neurons and glial cells [11]. COX2 does not express in normal conditions [12], but express highly after cerebral ischemic injury in neurons and vascular endothelial cells, especially in the glutamate-excited neuron dendrites [13]. NS398, the selective inhibitor of COX2, has neuroprotective effect in focal cerebral ischemia mice [14]. So, AQP4, MMP9 and COX2 could be served as

indicators to evaluate edema, inflammation and BBB damage after cerebral ischemia. Our previous studies have showed that picroside II could down-regulate the expression of inflammatory factor in the ischemic penumbra area [15,16] and inhibit apoptosis caused by cerebral ischemic injury to reduce cerebral infarction volume and improve neurobehavioral function in rats [17,18]. We find that optimized therapeutic dose and time window is injecting picroside II intraperitoneally with 20 mg/kg body weight at ischemia 1.5 h in cerebral ischemic injury [19]. Given the neurobehavioral evaluation is easily influenced by subjective factors and the error of immunohistochemical staining semi-quantitative detection is bigger, the limitations of results are inevitable. In this study, through determining the concentrations AQP4, MMP9 and COX2 in the serum and brain tissue homogenate, the authors aimed to explore the optimal therapeutic dose and time window of picroside II injecting intraperitoneally in cerebral ischemic injury in rats according to the design principle of orthogonal test.

2. METHODS

2.1. Establishment of Animal Models

This experiment was approved by the Ethics Committee of Qingdao University Medical College (QUMC 2011-09). 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. Total of 30 adult healthy male *Wistar* rats (weighted 230 - 250 g, SPF grade) were supplied by the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20100100). All animals were acclimatized for 7 days and allowed to take food and water freely at room temperature (23˚C ± 2˚C) and humidity-controlled housing with natural illumination and absolute diet at preoperative 12 h before operation. Five rats were randomly selected for sham group, and the rest 25 rats were established for cerebral ischemic models. The rats were anesthetized by injecting intraperitoneally 10% chloral hydrate (300 mg/kg) and fixed in supine position to conduct aseptic operation. The ischemic models of forebrain were established by bilateral common carotid artery occlusion (BCCAO) [20]. Core body temperature was keeping with a rectal probe and maintained at 36˚C - 37˚C using a homeothermic blanket control unit (Qingdao Apparatus, China) during and after the surgery operation. Four rats which had still not revived or died 2 h after surgery were rejected out, while the 21 successful models were brought into the experiment. The 5 rats of sham group were subject to the same experimental procedures except of BCCAO.

2.2. Orthogonal Experimental Design

Sixteen successful BCCAO rat models were internalized into the experiment and randomly grouped according to the principle of orthogonal experimental design of [$L_{16}(4^5)$] consisting of two impact factors with four impact levels (**Table 1**). The impact factor A is the therapeutic time widow designed four levels as 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 following 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg body weight (**Table 1**).

2.3. Intervention

Picroside II (molecular formula: $C_{23}H_{28}O_{13}$, molecular weight: 512.48) supplied by Tianjin Kuiqing Medical Technology Co., Ltd. CAS o: 39012-20-9, purity > 98%) was diluted into 1% solution with 0.1 mol/L PBS and injected intraperitoneally according to the corresponding designed doses at designed time in the orthogonal layout [$L_{16}(4^5)$]. Rats in the sham group and model group were intraperitoneally injected same amount of normal saline after 2 h of cerebral ischemia.

2.4. Sample Collection

The rats were anesthetized by injecting intraperitoneally 10% chloral hydrate (300 g/kg) after 24 h treatment with picroside II Total of 4 ml blood was collected via cardiac by thoracotomy and centrifuged (4000 rpm for 10 min) to separated serum and stored at −20˚C. Then the rats were instilled normal saline 200 ml via cardiac immediately, taken whole brain after craniotomy, removed the olfactory bulb and prefrontal brain tissue, and cut 500 mg ischemic brain tissue from optic chiasma (Bregma 0.00 mm) backwards. Add cell lysis solution (500 μl cell lysis solution +5 μl PMSF, No. P0013, Biyuntian Biotechnology Co. Ltd.) according to 1:3 proportion after grinding to powder in the pre-cooling mortar. The ultrasonic slurry was centrifuged with 12,000 r/m for 10 min at 4˚C (Eppendorf 5801, German). Abandoned the precipitation organization and then collected the supernatant to determine the protein concentration by BCA assay (Wuhan Boster Biological Engineering Co. Ltd.) and save at −20˚C for later use.

2.5. Enzyme Linked Immunosorbent Assay

We determined the levels of AQP4（E02A0467), MM-

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

P9 (E02M0329) and COX2 (E02C0080) in serum and brain tissue homogenate by the way of enzyme-linked immunosorbent kit (Blue Gene Biotech. Co. Ltd.). Before ELISA, we remelted the serum and brain tissue homogenate at room temperature, centrifuged again and then took Supernatant 100 μl for later use. The procedures: 1) Secure the desired numbers of coated wells in the holder then add 100 μl of standards or samples to the appropriate well in the antibody pre-coated microtiter plate. Add 100 μl of PBS (pH 7.0 - 7.2) in the blank control well. 2) Dispense 10 μl of balance solution into 100 μl specimens, mix well. 3) Add 50 μl of conjugate to each well (Not blank control well). Mix well. Mixing well in this step is important. Cover and incubate the plate for 1 hour at 37˚C. 4) Wash the microtiter plate using one of the specified methods indicated below: a) Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Fill in each well completely with 1× wash solution, and then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure five times for a total of FIVE washes. After washing, invert plate, and blot dry by hitting the plate onto absorbent paper or paper towels until no moisture appears. b) Automated Washing: Wash plate FIVE times with diluted wash solution (350 - 400 μl/well/wash) using an auto washer. After washing, dry the plate as above. It is recommended that the washer be set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash. 5) Add 50 μl substrate A and 50 μl substrate B to each well including blank control well, subsequently. Cover and incubate for 10 - 15 minutes at 20˚C - 25˚C (Avoid sunlight). 6) Add 50 μl of stop solution to each well including blank control well. Mix well. 7) Determine the optical density (OD) at 450 nm using a microplate reader immediately. 8) Calculating results: a) The standard curve is used to determine the amount of samples. b) First, average the duplicate readings for each standard and sample. All OD values are subtracted by the mean value of blank control before result interpretation. c) Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and draw a best fit curve e using graph paper or statistical software to generate a four paramater logistic (4-PL) curve-fit or logit-log linear regression curve. An x-axis for the optical density and a y-axis for the concentration is also a choice. The data may be linearized by plotting the log of the concentrations versus the log of the OD and the best fit line can be determined by regression analysis. d) Calculate the concentration of samples corresponding to the mean absorbance from the standard curve. Standard curve for demonstration only. e) The sensitivity in this assay is 0.1 ng/ml.

3. RESULTS

3.1. The Concentrations of AQP4, MMP9 and COX2

In sham group, the expression of AQP4, MMP9 and COX2 in serum were significantly lower than those in brain tissue homogenate (t = 13.34 − 21.63, P < 0.01). No matter in serum or brain tissue homogenate, the concentrations of AQP4, MMP9 and COX2 in model group were significantly higher than those in sham group (t = 31.38 − 43.54, P < 0.01), while in treatment group were significantly lower than those in model group in every index (t = 4.58 − 9.60, P < 0.01) (**Tables 2** and **3**).

3.2. ANOVA of AQP4 (Table 4)

AQP4 in serum: The different levels of impact factor A (therapeutic time window) and impact factor B (therapeutic drug dose) had significant probability to influencing the concentration of AQP4 (P < 0.01), while no significant difference existed in impact factor C (time-dose interaction) (P > 0.05). These suggested that both therapeutic time and the drug dose impacted significantly the concentration of AQP4 in serum after cerebral ischemia, but the interactions of time and dose had no significance. All data were compared in pairs by the way of least significant difference (LSD) and the results indicated that no significant difference between 1.0 h (A1) and 2.0 h (A3) (P > 0.05), but there was significant differences between the rest ischemia time levels (P < 0.05). No significant differences between 5 mg/kg (B1) and 10 mg/kg (B2), 10 mg/kg (B2) and 20 mg/kg (B3) (P > 0.05), but there was a significant differences among the rest dose levels (P < 0.05). According to the principle of minimum drug dose and maximization therapeutic time window, the best combination (A3B3) is the best therapeutic dose and time window injecting picroside II intraperito neally with 20 mg/kg body weight at cerebral ischemia 2.0 h.

AQP4 in brain tissue: There was a significant probability among the different levels of impact factor A (therapeutic time window), B (therapeutic drug dose) and C (time-dose interaction) (P < 0.01). These indicated that all the therapeutic time window or ischemia time, drug dose and time-dose interaction influenced the concentration of AQP4 in brain tissue significantly after cerebral ischemia injury. All data were compared in pairs by the way of least significant difference (LSD) and the results indicated showed that no significant deviation between 1.0 h (A1) and 2.0 h (A3) (P > 0.05), but there was significant deviations between the rest ischemia time levels (P < 0.05). There was no significant differences between 5 mg/kg (B1) and 40 mg/kg (B4), 10 mg/kg (B2) and 20 mg/kg (B3) (P > 0.05), but significant differences existed among the rest dose levels (P < 0.05). Considering the principle of the minimum drug dose and maximization

Table 2. The results of AQP4, MMP9 and COX2 (mean ± SD).

groups	n	$AQP4_{serum}$	$AQP4_{brain}$	$MMP9_{serum}$	$MMP9_{brain}$	$COX2_{serum}$	$COX2_{brain}$
sham	5	0.331 ± 0.056	0.613 ± 0.062[a]	0.215 ± 0.053	0.428 ± 0.082[a]	0.135 ± 0.041	0.244 ± 0.043[a]
model	5	1.176 ± 0.118[b]	1.612 ± 0.203[b]	0.823 ± 0.114[b]	1.212 ± 0.116[b]	0.492 ± 0.068[b]	0.768 ± 0.065[b]
treatment	16	0.868 ± 0.178[c]	1.105 ± 0.191[c]	0.672 ± 0.111[c]	0.803 ± 0.163[c]	0.373 ± 0.086[c]	0.581 ± 0.094[c]

[a]compare with serum concentration, $P < 0.01$; [b]compare with sham group, $P < 0.01$; [c]compare with model group, $P < 0.01$.

Table 3. $L_{16}(4^5)$ orthogonal table and test results.

Test No.	Rank No.					Serum	Brain	Serum	Brain	Serum	Brain
	A	B	C	D	E	AQP4AQP4	AQP4	MMP9	MMP9	COX2	COX2
1	1	1	1	1	1	0.712	1.081	0.656	0.738	0.343	0.530
2	1	2	2	2	2	0.715	1.103	0.627	0.757	0.363	0.492
3	1	3	3	3	3	0.731	1.105	0.608	0.729	0.372	0.550
4	1	4	4	4	4	0.786	1.109	0.698	0.739	0.405	0.608
5	2	1	2	3	4	0.947	1.071	0.707	0.877	0.397	0.584
6	2	2	1	4	3	0.807	0.832	0.567	0.616	0.269	0.451
7	2	3	4	1	2	0.763	0.773	0.457	0.616	0.226	0.413
8	2	4	3	2	1	1.057	1.039	0.571	0.785	0.310	0.550
9	3	1	3	4	2	0.812	1.263	0.756	0.766	0.386	0.562
10	3	2	4	3	1	0.695	0.909	0.654	0.732	0.265	0.532
11	3	3	1	2	4	0.599	0.862	0.513	0.636	0.284	0.544
12	3	4	2	1	3	0.979	1.205	0.716	0.722	0.434	0.684
13	4	1	4	2	3	1.008	1.356	0.819	1.133	0.496	0.706
14	4	2	3	1	4	1.047	1.291	0.822	0.912	0.437	0.710
15	4	3	2	4	1	0.941	1.246	0.736	0.927	0.475	0.658
16	4	4	1	3	2	1.283	1.434	0.838	1.165	0.513	0.726
Ⅰ	2.944	3.479	3.401	3.501	3.405	13.882	17.679	10.745	12.850	5.975	9.300
Ⅱ	3.574	3.264	3.582	3.379	3.573						
Ⅲ	3.085	3.034	3,647	3.656	3.525						
Ⅳ	4.279	4.105	3.252	3.346	3.379						
SS	0.273	0.159	0.024	0.015	0.007						

treatment time window, the best combination is A2B3, *i.e.* the best therapeutic time window and dose is injecting picroside II intraperitoneally with 20 mg/kg body weight at cerebral ischemia 1.5 h.

3.3. ANOVA of MMP9 (Table 5)

MMP9 in serum: The different levels of therapeutic time window (A) and drug dose (B) influenced significantly the concentration of AQP4 in serum ($P < 0.05$), while no significant probability in time dose interaction or impact factor C ($P > 0.05$). LSD results showed that no significant deviation between 1.0 h (A1) and 2.0 h (A3) ($P > 0.05$), but there was significant deviations between the rest ischemia time levels ($P < 0.05$). There was no significant differences between 5 mg/kg (B1) and 10 mg/kg (B2), 5 mg/kg (B1) and 40 mg/kg (B4), 10 mg/kg (B2) and 40 mg/kg (B4) ($P > 0.05$), while significant differences ($P < 0.05$) existed among the rest drug dose levels. Considering the principle of minimum dose and maximize treatment time window, the A2B3 combinations is the best treatment time window and dose injecting picroside II intraperitoneally with 20 mg/kg at cerebral ischemia 1.5 h.

MMP9 in brain tissue: Different levels of therapeutic time window (A) impacted significantly the concentration of MMP9 in brain tissue ($P < 0.05$), but drug dose

Table 4. ANOVA of AQP4.

Source of variation	SS_{Serum}	df	MS	*F*	*P*	SS_{Brain}	df	MS	*F*	*P*
Time window	0.273	3	0.091	25.55	0.01	0.338	3	0.113	35.01	0.01
Drug dose	0.159	3	0.053	14.90	0.01	0.132	3	0.044	13.65	0.01
Time × Dose	0.024	3	0.008	2.25	0.18	0.060	3	0.020	6.17	0.03
Error	0.021	6	0.004			0.019	6	0.003		

Table 5. ANOVA of MMP9.

Source of variation	SS_{Serum}	df	MS	*F*	*P*	SS_{Brain}	df	MS	*F*	*P*
Time window	0.110	3	0.037	16.39	0.01	0.286	3	0.095	12.49	0.01
Drug dose	0.055	3	0.018	8.25	0.02	0.065	3	0.022	2.85	0.13
Time × Dose	0.008	3	0.003	1.16	0.40	0.002	3	0.001	0.10	0.96
Error	0.013	6	0.002			0.046	6	0.008		

(B) and time dose interaction (C) had no significant probability ($P > 0.05$). LSD results showed that no significant deviations between 1.0h(A1) and 1.5 h (A2), 1.0 h (A1) and 2.0 h (A3), 1.5 h (A2) and 2.0 h (A3) ($P > 0.05$), but there was significant deviations between the rest ischemia time levels ($P < 0.05$). There was a significant difference between 5 mg/kg (B1) and 20 mg/kg (B3) ($P < 0.05$), while no significant differences among the rest dose levels ($P > 0.05$). On the base of minimum dose and maximization treatment time window, the best combination 1s A3B3, that is the best treatment time window and dose is respectively ischemia 2.0 h and 20 mg/kg of picroside II in cerebral ischemic injury.

3.4. ANOVA of COX2 (Table 6)

COX2 in serum: The influences of therapeutic time (A) and drug dose (B) had significant probabilities on the concentration of COX2 in serum ($P < 0.05$), and time-dose interaction (C) had no significant probability ($P > 0.05$). LSD results showed that no significant deviations between 1.0 h (A1) and 2.0 h (A3), 1.5 h (A2) and 2.0 h (A3) ($P > 0.05$), but there was significant deviations between the rest ischemia time levels ($P < 0.05$). There was no significant differences between 5 mg/kg (B1) and 40 mg/kg (B4), 10 mg/kg (B2) and 20 mg/kg (B3) ($P > 0.05$), while significant differences among the rest dose levels ($P < 0.05$). Given the minimum dose and maximize treatment time window, the best combination is A2B2, i.e. the best treatment time window and dose of picroside II is respectively ischemia is 1.5 h and 10 mg/kg body weight.

COX2 in brain tissue: The different therapeutic time window (A) had a significant probability on the concentration of COS2 in brain tissue ($P < 0.05$), but no significant differences ($P > 0.05$) among the different levels of drug dose (B) and time-dose (C). LSD results indicated that no significant deviations between 1.0 h (A1) and 1.5 h (A2), 1.0 h (A1) and 2.0 h (A3) ($P > 0.05$), while there were significant deviations between the rest ischemia time levels ($P < 0.05$). There was significant differences between 10mg/kg (B2) and 40 mg/kg (B4), 20 mg/kg (B3) and 40 mg/kg (B4) ($P < 0.05$), and no significant differences among the rest dose levels ($P > 0.05$). According to the principle of minimum dose and maximizing treatment time window, the best combination (A2B3) is the best treatment time window and dose is injecting picroside II intraperitoneally with 20 mg/kg body weight at cerebral ischemia 1.5 h.

4. DISCUSSION

AQPs, the four polymers structure, is membrane channel proteins family associated with the water transport function through membrane [1]. It consisted of four monomer (relative molecular mass: 30000 kD) and each monomer contain six transmembrane regions and five rings. The rings B and D located internally while the rings A, C and E externally cell membrane. Rings B and E have the specific and hydrophobic structure of AQPs family and participate in regulating the activity of aquaporin [21]. In the central nervous system, AQP4 mainly distribute on cell membrane of astrocytes and ependymal cells in the brain and spinal cord tissue [22], and form water-potassium ion channel complex, which has the function of water transfer, in the site of important water transport with internal flow-potassium ion channels [23]. During the early stages of cerebral ischemia, the energy depletion and the disorder of intracellular regulation lead to the accumulation of waters in the cells to cause cellular edema. Meanwhile, astrocytes is the main edema cell, especially its foot processes around the capillaries is the main site of AQP4 expression in the brain area [24]. Previous experiment showed that AQP4 leaded to the formation of brain edema in the early or acute cerebral ischemia [25]. AQP4 gene knockout could

Table 6. ANOVA of COX2.

Source of variation	SS_{Serum}	d*f*	MS	*F*	*P*	SS_{Brain}	df	MS	*F*	*P*
Time window	0.071	3	0.024	21.72	0.01	0.088	3	0.029	14.96	0.01
Drug dose	0.022	3	0.007	6.82	0.02	0.027	3	0.009	4.55	0.06
Time × Dose	0.012	3	0.004	3.71	0.08	0.005	3	0.002	0.88	0.50
Error	0.007	6	0.001			0.012	6	0.002		

protect acute cerebral ischemia in rats and delay the pathological changes occurring at ischemia 7 - 14 days [26]. But in chronic cerebral ischemia mice model, AQP4 gene knockout will aggravate the chronic brain injury and cause more serious brain damage and loss of neurons after cerebral ischemia injury 35 days [27]. Our study showed that in sham group the concentration of AQP4 in serum were significantly lower than that in brain tissue, and both in serum and brain tissue in model group were significantly higher than those in sham group, which suggesting a large amount of AQP4 leaked into the blood through the damaged BBB in model group rats. After treatment with picroside II, the concentrations of AQP4 both in serum and brain tissue decreased significantly than those in model group, which prompting picroside II play a protective effect to nerve cells and BBB.

Matrix metalloproteinases (MMPs) are a group of zinc ions dependent protease family, and mainly de-gradating and remodeling the extracellular matrix. Normally, MMP9 expression is extremely low in brain and only existed as primary enzyme form. MMP9 were activated and then joined in the process of inflammatory reaction in cerebral ischemia injury [28]. MMP9 promoter region contains many binding sites of growth factors, cytokines and protease, these signaling pathways are activated and then joined in the activation of protein-1 or nuclear factor kappa B to regulate the expression of MMP9. When brain ischemia occurred, various cytokine and inflammatory mediator released and combined with MMP9 binding sites to promote its expression and enhance its activity, at the same time, the released protein enzymes activated MMP9 to de de-gradate and destruct the basement membrane and tight connection, and lead to damage of BBB and secondary brain edema. At the same time, MMP9 can directly cause neuron death and brain injury [4,5]. In this study, the concentration of MMP9 in serum were significantly lower than that in brain tissue of sham group rats, and both in serum and brain tissue in model group rats were significantly higher those in sham group rats, which suggesting the expression of MMP9 enhanced and induced inflammatory reaction to damage BBB after ischemia. With pcroside II treatment, the concentration of MMP9 both in serum and brain tissue decreased significantly than those in model group rats, which indicated that picroside II could play an important role to repair cerebral ischemia damage by reducing the permeability of BBB.

Cyclooxygenases (COX), molecular weight 71 kD, is the limited enzyme to catalyze arachidonic acid producing prostaglandin and thromboxane. COX consisted of COX1, COX2 and COX3 types. In normal condition, COX2 did not express and expressed lowly in some neurons and other cells. After cerebral ischemic reperfusion injury, oxidative stress and inflammation could induce the high expression of COX2 and aggravate neuronal damage, so COX2 is considered as one of the important targets for treating brain injury [29,30]. In this study, the concentration of COX2 in serum were significantly lower than that in brain tissue homogenate in sham group, and both in serum and brain tissue in model group were significantly higher those sham group, which suggesting COX2 induced inflammation to brain damage after cerebral ischemia. With picroside Ⅱ treatment, the concentrations in serum and brain tissue of COX2 reduced significantly than those in model group rats, which speculating picroside II might decrease the expression of COX2 and play an anti-inflammatory action to protect cerebral ischemic injury.

Orthogonal test has the following advantages: balanced sampling, fewer test, better representativeness, so it can better accomplish the experiment. In this experiment, the authors designed four time points at ischemia 1.0 h, 1.5 h, 2.0 h and 2.5 h, and injected 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 $[L_{16}(4^5)]$ to explore the best therapeutic dose and best time window of picroside II in treating cerebral ischemic injury. Through the concentrations of AQP4, MMP9 and COX2, the treatment effects of picroside II had significant differences between the different levels of therapeutic time and drug dose, and the different detecting index had varied best combination. Considering minimization of medication dose and maximization of therapeutic time window, it is suggested the best choose of A2/A3, B2/B3 composition, i.e. the best therapeutic time window is at 1.5 - 2.0 h after ischemia and the best therapeutic dose of picroside II is intraperitoneally injecting with 10 - 20 mg/kg body weight. Because the mechanism of cerebral ischemia injury is very complex, the exact pharmacological mechanism of picroside II and the best treatment

time window and give medicine dose are still need further testing with other indexes.

5. CONCLUSION

This study suggested that the optimal composition of the therapeutic dose and time window of picroside II in treating cerebral ischemic injury should be injecting picroside II intraperitoneally with 10 - 20 mg/kg body weight at ischemia 1.5 - 2.0 h according to the principle of minimization of medication dose and maximization of therapeutic time.

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