

The effect of picoside II on the expressions of AQP4, MMP9 and COX2 in cerebral ischemic injury in rats*

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ABSTRACT

The aim is to study the neuroprotective effect and optimize the therapeutic dose and time window of picoside II by orthogonal test in cerebral ischemic injury in rats. The forebrain ischemia models of rats 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 picoside II intraperitoneally with different therapeutic dose at different ischemic time. The contents of aquaporins 4 (AQP4), matrix metalloproteinases 9 (MMP 9) and cyclooxygenase 2 (COX2) in brain tissue were determined by enzyme linked immunosorbent assay to evaluate the therapeutic effect of picoside II on cerebral ischemic injury. The results indicated that the best therapeutic time window and dose of picoside II in cerebral ischemic injury should be 1) ischemia 1.5 h with 20 mg/kg body weight according to the content of AQP4; 2) ischemia 2.0 h with 20 mg/kg according to the content of MMP9; and 3) ischemia 1.5 h with 20 mg/kg according to the content of COX2 in brain tissue. It is concluded that the optimized therapeutic dose and time window is injecting picoside II peritoneally with 10 - 20 mg/kg body weight at ischemia 1.5 - 2.0 h in cerebral ischemic injury according to the principle of lowest therapeutic dose with longest time window.

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

1. INTRODUCTION

In central nervous system (CNS), aquaporins 4(AQP4)

mediates transportation of water across cell membranes [1]. Previous animal experiments proved that AQP4 expression increased in the cortex around the infarct area at cerebral ischemia 3 h in rats [2], and could be down-regulated to attenuate cerebral edema by treating with picoside II [3]. Matrix metalloproteinases 9 (MMP 9) could damage the blood-brain barrier (BBB) by degrading the basement membrane and tight intercellular junction to induce cerebral edema in inflammatory reaction process, and also cause neuronal death and cerebral injury directly [4,5], while knocking out MMP9 gene could reduce cerebral injury obviously [6]. Some experiments proved that MMP9 expression increased after cerebral ischemia and be inhibited by picoside II [7] to attenuate inflammatory injury in cerebral ischemia. After cerebral ischemic injury, cyclooxygenase 2 (COX2) expression increased significantly in cortical and hippocampal neurons and glial cells [8], while COX2 gene knocking out reduced obviously the infarction volume in rats [9]. The author's research results shown that picoside II could inhibit the expressions of relative inflammatory factors in the ischemic penumbra [10,11] and reduce infarction volume after cerebral ischemia in rats [12,13], and also explored primarily the therapeutic doses and time window of picoside II in treating cerebral ischemia [14,15]. This experiment aims to further explore the therapeutic doses and time window of picoside II in treating cerebral ischemia from three aspects of inflammatory reaction, BBB permeability and brain edema by measuring the contents of AQP4, MMP9 and COX2 in brain homogenates.

2. MATERIALS AND METHODS

2.1. Animal Models

This experiment was approved by the Ethics Committee of Qingdao University Medical College (QUMC 201109). The local legislation for ethics of experiment on animals

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and guidelines for the care and use of laboratory animals were followed in all animal procedures. 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). All animals were acclimatized for one week in the laboratory and allowed free access to food and water in a room temperature ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and absolute diet 12 h before operation. Firstly, five rats were randomly assigned to sham-surgery group, and the rest 25 rats were anesthetized by injecting intraperitoneally 10% choral hydrate (0.3 ml/kg) and fixed in supine position to conduct aseptic operation to establish forebrain ischemia model by separating and ligating bilateral common carotid artery (BCCAO)[16]. The rectal temperature was maintained at $36^{\circ}\text{C} - 37^{\circ}\text{C}$ during and after the operation. Four animals which still unawaked 2 h after operation or died were excluded and the 21 successful models were internalized into the experiment. Rats in the shamoperation group underwent the same surgical procedures except of occlusion of bilateral common carotid artery.

2.2. Design and Group

Of 21 successful modes, five cases were randomly assigned to model group and the rest 16 cases to the treatment group, which were randomly subgrouped according to the principle of orthogonal experimental design of [$L_{16}(4^5)$] consisting of two impact factors with four impact levels. The impact factor A is the therapeutic time widow designed four levels of 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 5mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg body weight (**Table 1**).

2.3. Intervention Methods

Picoside II (molecular formular: $\text{C}_{23}\text{H}_{28}\text{O}_{13}$, molecular weight: 512) was provided by Tianjing Kuiqing Medical Technology (CAS No: 39012-20-9, purity > 98%). Picoside II was diluted into 1% solution with 0.9 g/L sodium chloride and injected intraperitoneally according to the corresponding designed dose at designed time in the orthogonal layout [$L_{16}(4^5)$]. Rats in the sham operation group and model group were intraperitoneally

injected same dose of 9 g/L sodium chloride after operation 2 h (**Table 1**).

2.4. Sample Collection

After 12 h following the administration, rats were anesthetized by injecting intraperitoneally 10% choral hydrate (0.3 ml/kg) and perfused with 200 ml of normal sodium solution from the heart into the aorta. The brains were completely taken out, and olfactory bulb and prefrontal brain were removed. 500 mg forebrain ischemic tissue were cut backward from the optic chiasm (Bregma 0.00 mm) put into pre-cooling mortar to grind into powder. Added cell lysis solution (500 μl cell lysis solution + 5 μl PMSF, No. P0013, Biyuntian Biotechnology Co. Ltd.) according to 1:3 proportion after grinding into powder in the pre-cooling mortar. The ultrasonic slurry was centrifugated with 12,000 r/min for 10 min at 4°C by freezing centrifuge (Eppendorf 5081, Germany). Then collected the supernatant to determine the protein concentration by BCA assay (Wuhan Boster Biological Engineering Co. Ltd.) and stored at -20°C for later use.

2.5. ELISA Assay

The contents of AQP4, MMP9 and COX-2 in brain tissue were determined by enzyme linked immunosorbent assay kits of AQP4 (E02A0467), MMP9 (E02M0329) and COX-2 (E02C0080) provided by Blue Gene Co. Ltd. According to the instructions of kits, remelte the brain tissue homogenate at room temperature and centrifuge again to 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 automatically 5 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 minutes at 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

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 \times 5	1.5 \times 5	2.0 \times 5	2.5 \times 5
10 mg/kg (B2)	1.0 \times 10	1.5 \times 10	2.0 \times 10	2.5 \times 10
20 mg/kg (B3)	1.0 \times 20	1.5 \times 20	2.0 \times 20	2.5 \times 20
40 mg/kg (B4)	1.0 \times 40	1.5 \times 40	2.0 \times 40	2.5 \times 40

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 using graph paper or statistical software to generate a four parameter 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. e) The sensitivity in this assay is 0.1 ng/ml.

2.6. 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 and therapeutic dose had significant deviation or not, and whether their interaction on each detected index had significant deviation or not, meanwhile to explore the best therapeutic drug dose and the therapeutic time window.

3. RESULTS

In sham group, the contents of AQP4, MMP9 and COX2 in brain tissue were significantly lower than those in model group ($t = 31.38 - 43.54$, $P < 0.01$). In treatment group, the contents of AQP4, MMP9 and COX2 in brain tissue were significantly lower than those in model group ($t = 4.58 - 9.60$, $P < 0.01$). Shown as **Tables 2** and **3**.

3.1. AQP4 (Table 4)

There were significant influences of different levels of administration time (factor A), drug doses (factor B) and the interaction of time-dose (factor C) on the concentration of AQP4 in brain tissue after ischemia injury ($P = 0.01 - 0.03$). All data were compared in pairs by the way of least significant difference (LSD) and the results indicated that no significant difference ($P > 0.05$) between the concentration of AQP4 at administration 1 h (A1) and 2 h (A3), while there were significant differences be-

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

groups	n	AQP4	MMP9	COX2
Sham group	5	0.613 ± 0.062	0.428 ± 0.082	0.244 ± 0.043
Model group	5	1.612 ± 0.203 ^a	1.212 ± 0.116 ^a	0.768 ± 0.065 ^a
Treatment group	16	1.105 ± 0.191 ^b	0.803 ± 0.163 ^b	0.581 ± 0.094 ^b

^aCompare with sham group, $P < 0.01$; ^bcompare with model group, $P < 0.01$.

Table 3. [$L_{16}(4^5)$] orthogonal layout and the results.

Test NO.	Rank NO.					AQP4	MMP9	COX2
	A	B	C	D	E	ng/ml	ng/ml	ng/ml
1	1	1	1	1	1	1.081	0.738	0.530
2	1	2	2	2	2	1.103	0.757	0.492
3	1	3	3	3	3	1.105	0.729	0.550
4	1	4	4	4	4	1.109	0.739	0.608
5	2	1	2	3	4	1.071	0.877	0.584
6	2	2	1	4	3	0.832	0.616	0.451
7	2	3	4	1	2	0.773	0.616	0.413
8	2	4	3	2	1	1.039	0.785	0.550
9	3	1	3	4	2	1.263	0.766	0.562
10	3	2	4	3	1	0.909	0.732	0.532
11	3	3	1	2	4	0.862	0.636	0.544
12	3	4	2	1	3	1.205	0.722	0.684
13	4	1	4	2	3	1.356	1.133	0.706
14	4	2	3	1	4	1.291	0.912	0.710
15	4	3	2	4	1	1.246	0.927	0.658
16	4	4	1	3	2	1.434	1.165	0.726
I	4.398	4.771	4.209	4.350	4.275	17.679	12.850	9.300
II	3.715	4.135	4.625	4.360	4.573			
III	4.239	3.986	4.698	4.519	4.498			
IV	5327	4.787	4.147	4.450	4.333			
SS	0.338	0.132	0.060	0.140	0.060			

Table 4. ANOVA of AQP4 in brain tissue.

Sources of variation	SS	df	MS	F	P
Ischemic time	0.338	3	0.113	35.01	0.01
Therapeutic dose	0.132	3	0.044	13.65	0.01
Time × dose	0.060	3	0.020	6.17	0.03
Error	0.019	6	0.003		

tween the rest time levels of AQP4 concentrations ($P < 0.05$). There was no significant difference between the concentration of AQP 4 between drug dose of 5 mg/kg (B1) and 40 mg/kg (B4), 10 mg/kg (B2) and 20 mg/kg (B3) ($P > 0.05$), but the significant differences existed among the other levels of doses ($P < 0.05$). According to the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window maybe injecting picoside II peritonally with 20

mg/kg body weight at ischemia 1.5 h in cerebral ischemic injury, which is the combination of A2B3.

3.2. MMP9 (Table 5)

There were significant differences between different therapeutic time (A) on the concentration of MMP9 in brain tissue following cerebral ischemia ($P < 0.05$), but no significant difference existed between drug doses (B) and the interaction of time-dose on the concentration of MMP9 in brain tissue ($P > 0.05$). The results of LSD showed that no significance ($P > 0.05$) between the concentration of MMP9 at administration time 1.0 h (A1) and 1.5 h (A2), 1.0 h (A1) and 2.0 h (A3), 1.5 h (A2) and 2.0 h (A3), while there were significant differences between the other administration time levels ($P < 0.05$). There were significant differences ($P < 0.05$) between the concentration of MMP9 in a drug dose of 5 mg/kg (B1) and 20 mg/kg (B3), and no significant difference found between that of the other doses ($P > 0.05$). From the principle of lowest therapeutic dose with longest time window, the combination of A3B3 is the best, that is injecting picoside II peritonally with 20 mg/kg body weight at ischemia 2 h in cerebral ischemic injury.

3.3. COX2 (Table 6)

There were significant influences of administration time (A) on the concentration of COX2 in brain tissue following cerebral ischemia ($P < 0.05$), but no significant difference existed between drug doses (B) and the interaction of time-dose (C) on the concentration of COX2 in brain tissue ($P > 0.05$). The result of LSD proved that no significant difference ($P > 0.05$) between the concentration of COX2 at administration time 1.0 h (A1) and 1.5 h (A2), 1.0 h (A1) and 2.0 h (A3), but there were signifi-

Table 5. ANOVA of MMP9 in brain tissue.

Sources of variation	SS	df	MS	F	P
Ischemic time	0.286	3	0.095	12.49	0.01
Therapeutic dose	0.065	3	0.022	2.85	0.13
Time × dose	0.002	3	0.001	0.10	0.96
Error	0.046	6	0.008		

Table 6. ANOVA of COX2 in brain tissue.

Sources of variation	SS	df	MS	F	P
Ischemic time	0.088	3	0.029	14.96	0.01
Therapeutic dose	0.027	3	0.009	4.55	0.06
Time × dose	0.005	3	0.002	0.88	0.50
Error	0.012	6	0.002		

cant differences between the other time levels ($P < 0.05$). There were significant differences ($P < 0.05$) between the concentration of COX2 in drug doses of 10 mg/kg (B2) and 40 mg/kg (B4), 20 mg/kg (B3) and 40 mg/kg (B4), while no significant difference between that in the other doses ($P > 0.05$). From the principle of lowest therapeutic dose with longest time window, the combination of A2B3 is the best, that is injecting picoside II peritonally with 20 mg/kg body weight at ischemia 1.5 h in cerebral ischemic injury.

4. DISCUSSION

AQP4, mediating water transportation across cells and consisting of 4 subunits, is a member of the water channel protein family [1]. It is a major specific water channel protein in CNS, participating in regulating the activity of the water channel [17]. It's distributed within the glial and ependymal cell membrane in brain tissue and myeloid tissue [18]. In the early stage of cerebral ischemia, AQP4 expression increased in the glial cell, especially in the foot processes surrounding capillary vessels, which promoted the occurrence of cerebral edema [19]. AQP4 gene knockout plays a protective effect on acute cerebral ischemic injury [20]. Under normal conditions, MMP9 expression is very low, and various cytokines and inflammatory factors are released and combine with the binding site of MMP9 after cerebral ischemia, which contributes to increasing and activating the expression of MMP9 [21]. The activated MMP9 degrades and damages the basement membranes and tight intercellular junction, which resulted in the compromise of the BBB and secondary cerebral edema. It also could result in neuronal death and cerebral injury directly [4,5]. COX is one of nuclear signal transporting targets and expresses in some cells such as neuron in a low level under normal condition. COX expression increases following cerebral ischemia, which is considered as an important target of treating neuronal injury [22].

This experiment was carried out according to orthogonal table of $[L_{16}(4^5)]$, and was designed four time points at 1 h, 1.5 h, 2 h and 2.5 h after brain ischemic injury. The rats were injected intraperitoneally picoside II with four therapeutic doses of 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg. The concentrations of AQP4, MMP9 and COX2 in brain tissue were determined to explore the best therapeutic dose and best time window of picoside II in treating cerebral ischemic injury. The results indicated that there were significant differences between administration time and therapeutic doses of picoside II in treating cerebral ischemia, and the best combination is not accordant by different indexes. Considering minimization of medication dose and maximization of therapeutic time window, it is suggested the best choose of A2B2 and A3B3 combination, that is injecting

picroside II intraperitoneally with 10 - 20 mg/kg body weight at ischemia 1.5 - 2.0 h in cerebral ischemic injury. Because the mechanism of cerebral ischemic injury is very complicated [23] and only above indexes was observed in this experiment, the results could not possibly all be right. So the exact mechanism, the best therapeutic dose and administration time of picroside II need to be further studied in further experiments.

5. CONCLUSION

This paper suggested that the best therapeutic dose and time window of picroside II in treating cerebral ischemic injury should be injected 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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