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*Full Length Research Paper*

# The protection and optimizing for the therapeutic dose and time window of picroside II in cerebral ischemic injury in rats

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Abstract

**The aim of the study is to optimize the therapeutic dose and time window of picrosede II by orthogonal test in cerebral ischemic injury in rats. Methods, 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 intervened by injecting picroside II intraperitonenally at different ischemic time with different dose. The concentrations of aquaporins 4 (AQP4), matrix metalloproteinases9 (MMP9) and cyclooxygenase 2 (COX2) in serum were determined by enzyme linked immunosorbent assay to evaluate the therapeutic effect of picroside II on cerebral ischemic injury. Results, the best therapeutic time window and dose of picroside II in cerebral ischemic injury were (1) ischemia 2.0h with 20mg/kg body weight according to the concentration of AQP4; (2) ischemia 1.5h with 20mg/kg according to the concentrations of MMP9; and (3) ischemia 1.5h with 10 mg/kg according to the concentrations of COX2 in serum. Conclusion, from the principle of lowest therapeutic dose with longest time window, the optimized therapeutic dose and time window is injecting picroside II intraperitonenally with 10-20mg/kg body weight at ischemia 1.5-2.0h in cerebral ischemic injury.**

**Key words:** Picroside II, therapeutic dose, time window, cerebral ischemia, AQP4, MMP9, COX2, rats.

## INTRODUCTION

Aquaporins (AQPs) belong to a kind of membrane channel protein family which are of tetramer structure, and it's action relevant water across membrane, for example, AQP4 participate mediating water molecule's across membrane in central nervous system (Zelenina, 2010). Animal experiment had showed that the expression of AQP4 had enhanced in cerebral ischemic injury in rats (Sun et al., 2009), while it has been reduced after treated by picsroide 2, accompany that the scene of edema in central nervous system had get be improved (Li et al., 2010). Matrix metalloproteinase's 9 (MMP9) belongs to the gelatin enzymes. When the body get cerebral ischemia, the expression of MMP9 would increase in brain tissue. Some study had showed that picroside Ⅱ( Li et al., 2010) and huangji injection (Zhu et al., 2011) could improve the scene of inflammatory injury and brain edema in cerebral ischemia by inhibiting the expression of MMP9 in brain tissue to reduce the degradation and protect the permeability of blood-brain barrier (BBB) basement membrane's extracellular matrix. In the normal condition, an enzyme called cyclooxygenase 2 (COX2) mainly

expressed in glutamate excited neurons and may be associated with the high density of excitatory amino acids receptor (Li et al., 2008; Kim et al., 2004;Breder et al., 1995). The expression of COX2 significantly enhanced on neurons in cerebral ischemia. Someone had found that a selective inhibitors (NS398 ) of cox-2 had showed the nerve protective effect (Candelario-Jalil and Fiebich, 2008) in focal cerebral ischemia in mice. Our previous study had showed that picroside Ⅱ cauld reduce cerebral infarction volume due to cutting the expression of inflammatory factors associated that related to ischemic penumbra area in rat (Li et al., 2010; Guo et al., 2011), to inhibit apoptosis of cell (Li et al., 2010; Li et al., 2010), and had discussed the dose and time window (Li et al., 2012; Pei et al., 2012 ) of picroside Ⅱ in cerebral ischemia injury. The aim of this experiment is that further discuss the best dose and time windows of picroside Ⅱ in cerebral ischemia injury from inflammation and BBB permeability and brain edema and so on, regarding the blood level of AQP4 and MMP9 and COX2 as indicators.

## MATERIALS AND METHODS

### Animal Model

Total of 70 Healthy adult male SPF *Wistar* rats with weighting 230-230g were purchased from the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (Lu) 20100010). Before experiment, in order to adapt the environment, animal lived in laboratory for 7 days under natural sunshine and normal temperature (23±2□) with freely eating and drinking. Then, five rats were randomly divided into control group, the rest 65 rats were subjected to established the model of brain ischemia. By fasting twelve hours preoperative, every animal were anesthetized by injecting 10% chloral hydrate anesthesia intraperitoneally with 0.3 ml/kg, fixed with lying on its back. Every animal was subjected into forebrain ischemia model by ligation bilateral common carotid arteries (Márquez-Martín et al., 2012). Two hours after the operation, twelve rats which unawake or died were getting rid of experiment. Meanwhile, animals of control group were subjected the same operation without ligation of bilateral common carotid arteries.

### Design groups

Successful animal models were randomly divided into models group (n=5) and treatment group (n=16×3). Then, the animal models of treatment group animals were grouped according to the principles of orthogonal test which involve two factors, four levels [$L_{16}(4^5)$]. The therapeutic time window is factor A which is set four levels, such as 1.0h, 1.5h, 2.0h and 2.5h; The therapeutic doses is factor B, which also is set four levels, such as 5mg/kg, 10mg/kg, 20mg/kg, 40mg/kg. The orthogonal test was repeated three times Table 1.

### Treating methods

Picroside II (CAS No: 39012-20-9, purity > 98%, molecular formula: $C_{23}H_{28}O_{13}$, molecular weight: 512.48) was provided by Tianjin KuiQing pharmaceutical company, and diluted into 1% solution with application of saline solution. According to the designing of the orthogonal table [$L_{16}(4^5)$], every dosage of picroside II which associated with a corresponding ischemia time was injected intraperitoneally. Meanwhile, animals of control group and model group were treated with same dose normal saline after been operated two hours.

### ELISA

After treatment 24h, every animal were anesthetized by injecting 10% chloral hydrate anesthesia intraperitoneally with 0.3 ml/kg. Then, 4ml blood of rat was taken from the heart, and the serum was separated by centrifugations at 4000r/min for 10 min. The levels of AQP4 (E02A0467), MMP9 (E02M0329) and COX2 (E02C0080) in serum were determined by enzyme linked immune-sorbent assay (Blue Gene Co, Ltd.). Testing steps: 1) take ELISA plate coating anti-NSE, anti-S100B and anti-MBP, 2) put 100μL standard solution into blank micro holes separately according to the order of the specimens, and then join 150 μl solution enzyme markers to every hole, 3) add 100μL distilled water to blank controller, 4)seal elisa plate, incubate 1h in 37□,wash 5 times and dry, 5)add color developing agent A and B 50 μl to every hole(Do not contain blank control group), incubate 10 min in the dark 20-25 □, 6)add stop buffer 50 μl to every hole, 7)determine the value of OD at 450 nm using ELIASA (Bio Rad-680, the United States) within 30 min, 8) detect its concentration on standard curve according to the OD value, expressed in ng/ml.

**Table 1.** Orthogonal test design group of [$L_{16}(4^5)$]

| Treatment 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 |

### Statistical Analysis

SPSS17.0 software was used for statistical analysis. According to the different levels of ischemia (delivery) time and dose and time-dose interaction of experimental measurements have significant effect, analysis of optimal dose and time window.

## RESULTS

### Detection results

In model group, the results of AQP4 (1.18±0.12), MMP9 (0.82±0.11) and COX2 (0.49±0.07) were significantly higher than those in control group of AQP4 (0.33±0.06), MMP9 (0.22±0.05) and COX2 (0.14±0.04) ($t$=31.38-43.54, $P$<0.01). After treatment, the results of AQP4 (0.87±0.18), MMP9 (0.67±0.11) and COX2 (0.37±0.09) in treated group were significantly lower than those in model group ($t$=4.58-9.60, $P$<0.01) Table 2.

### Variance analysis of the content of AQP4

Factor A (delivery time) and factor B (dosage) after cerebral ischemia different levels of serum content of AQP4 were significant differences (P <0.05), but the time - dose interaction (C) no significant difference (P> 0.05). Number of applications least significant difference (LSD) method for each set of data pairwise comparison shows: AQP4 content delivery time 1.0h (A1) and 2.0h (A3) There was no significant difference between (P> 0.05), the rest to time between drug levels were significantly different (P <0.05); AQP4 content dose 5 mg / kg (B1) and 10 mg / kg (B2), 10 mg / kg (B2) and 20 mg / kg (B3) no significant difference between (P> 0.05), the remaining dose levels showed significant difference between (P <0.05). Therefore, administration time and dose combinations with A1B1 or A1B2 or A1B3 or A3B3 A3B1 or A3B2 or better. According to the dose and treatment time window minimization principle of maximizing comprehensive analysis of A3B3 combination best, that is the best treatment time window and the dose of ischemic 2.0h intraperitoneal injection 20 mg / kg body weight.

There are significant changes of the content of AQP4 in serum（$P$<0.05）among different levels of groups with factor A (treatment time) and groups with factor B (treatment dose) while it had no significant change （$P$＞0.05）under interaction of time-dose(C). The comparing result, which is between every two groups data by using the least significant difference method, showed that the content of AQP4 in serum had no significant change (P>0.05) between groups of time 1.0 (A1) and time 2.0 (A3), however, significant change appeared (P<0.05) between the rest groups of time window; By the same way, The comparing result, which is between every two groups data by using the least significant difference method, showed that no significant changes (P>0.05) of the content of AQP4 in serum had be listed, dose 5 mg/kg (B1)and dose 10 mg/kg (B2)、dose 10 mg/kg (B2)and dose 20 mg/kg (B3), but, significant change appeared (P<0.05) between the rest groups of treating dose; Considering the result above, the better choice of combination of time and dose in this experiment could be listed：A1B1、A1B2、A1B3、A3B1、A3B2、A3B3. According the principle of the minimum dose and the maximize treatment time window, the best choice is A3B3, In a word, the best time window is the point round two hours after ischemia happened, and the best dose of injection intraperitoneal is of 20 mg/kg body weight Table 3.

### Variance analysis of the content of MMP9

Different levels of administration time (A) and dose (B) on cerebral ischemia serum MMP9 levels were significantly affected (P <0.05), but the timing of

**Table 2.** Orthogonal table of $L_{16}(4^5)$ and the results

| Test | Rank No. | | | | | AQP4 | MMP9 | COX2 |
|---|---|---|---|---|---|---|---|---|
| No. | A | B | C | D | E | ng/ml | ng/ml | ng/ml |
| 1 | 1 | 1 | 1 | 1 | 1 | 0.712 | 0.656 | 0.343 |
| 2 | 1 | 2 | 2 | 2 | 2 | 0.715 | 0.627 | 0.363 |
| 3 | 1 | 3 | 3 | 3 | 3 | 0.731 | 0.608 | 0.372 |
| 4 | 1 | 4 | 4 | 4 | 4 | 0.786 | 0.698 | 0.405 |
| 5 | 2 | 1 | 2 | 3 | 4 | 0.947 | 0.707 | 0.397 |
| 6 | 2 | 2 | 1 | 4 | 3 | 0.807 | 0.567 | 0.269 |
| 7 | 2 | 3 | 4 | 1 | 2 | 0.763 | 0.457 | 0.226 |
| 8 | 2 | 4 | 3 | 2 | 1 | 1.057 | 0.571 | 0.310 |
| 9 | 3 | 1 | 3 | 4 | 2 | 0.812 | 0.756 | 0.386 |
| 10 | 3 | 2 | 4 | 3 | 1 | 0.695 | 0.654 | 0.265 |
| 11 | 3 | 3 | 1 | 2 | 4 | 0.599 | 0.513 | 0.284 |
| 12 | 3 | 4 | 2 | 1 | 3 | 0.979 | 0.716 | 0.434 |
| 13 | 4 | 1 | 4 | 2 | 3 | 1.008 | 0.819 | 0.496 |
| 14 | 4 | 2 | 3 | 1 | 4 | 1.047 | 0.822 | 0.437 |
| 15 | 4 | 3 | 2 | 4 | 1 | 0.941 | 0.736 | 0.475 |
| 16 | 4 | 4 | 1 | 3 | 2 | 1.283 | 0.838 | 0.513 |
| Ⅰ | 2.944 | 3.479 | 3.401 | 3.501 | 3.405 | 13.882 | 10.745 | 5.975 |
| 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 | | | |

**Table 3.** serum AQP4 variance analysis

| Source of variation | SS | df | MS | *F* | *P* |
|---|---|---|---|---|---|
| time | 0.273 | 3 | 0.091 | 25.55 | 0.01 |
| dose | 0.159 | 3 | 0.053 | 14.90 | 0.01 |
| Time×dose | 0.024 | 3 | 0.008 | 2.25 | 0.18 |
| error | 0.021 | 6 | 0.004 | | |

administration and dose interaction (C) no significant effects (P> 0.05). LSD for pair wise comparison of each set of data shows: MMP9 content delivery time 1.0h (A1) and 2.0h (A3) There was no significant difference between (P> 0.05), the remaining time of administration, there were significant differences in the level (P <0.05); the dose of 5mg/kg (B1) and 10mg/kg (B2), 5 mg / kg (B1) and 40 mg / kg (B4), 10mg/kg (B2) and 40 mg / kg (B4) There was no significant difference between

**Table 4.** serum MMP94 variance analysis

| Source of variation | SS | df | MS | F | P |
|---|---|---|---|---|---|
| time | 0.110 | 3 | 0.037 | 16.39 | 0.01 |
| dose | 0.055 | 3 | 0.018 | 8.25 | 0.02 |
| Time×dose | 0.008 | 3 | 0.003 | 1.16 | 0.40 |
| error | 0.013 | 6 | 0.002 | | |

(P> 0.05), the remaining dose levels were significantly different between (P <0.05). According to the dose and treatment time window minimization principle of maximizing comprehensive analysis of A2B3 combination best, ie the best treatment time window and the dose of ischemia 1.5h, 20 mg / kg.

There are significant changes of the content of MMP9 in serum（$P$<0.05）among different levels of groups with factor A (treatment time) and groups with factor B (treatment dose) while it had no significant change （$P$＞0.05）under interaction of time-dose(C). The comparing result, which is between every two groups data by using the least significant difference method, showed that the content of MMP9 in serum had no significant change (P>0.05) between groups of time 1.0 (A1) and time 2.0 (A3), however, significant change appeared (P<0.05) between the rest groups of time window; By the same way, The comparing result, which is between every two groups data by using the least significant difference method, showed that no significant changes (P>0.05) of the content of MMP9 in serum had be listed, dose 5 mg/kg (B1) and dose 10 mg/kg (B2)、dose 5 mg/kg (B1)and dose 40 mg/kg (B4), 10mg/kg (B1) and dose 40 mg/kg (B2). but, significant change appeared (P<0.05) between the rest groups of treating dose; Considering the result above, the better choice of combination of time and dose in this experiment could be listed：A1B1、A1B2、A1B3、A3B1、A3B2、A3B3. According the principle of the minimum dose and the maximize treatment time window, the best effect of combination is A2B3. In a word, the best time window is the point round 1.5 hours after ischemia happened, and the best dose of injection intraperitoneal is of 20 mg/kg body weight **Table 4.**

## Variance analysis of the content of COX2

Different levels of administration time (A) and dose (B) on cerebral ischemia COX2 serum levels were significantly affected (P <0.05), time - dose interaction (C) had no significant effect (P> 0.05) . LSD for pairwise comparison of each set of data shows: COX2 content delivery time 1.0h (A1) and 2.0h (A3), 1.5h (A2) and 2.0h (A3) There was no significant difference between (P> 0.05) the remaining time of administration levels were significantly different between (P <0.05); COX2 content dose 5 mg / kg (B1) and 40 mg / kg (B4), 10 mg / kg (B2) and 20 mg / kg (B3) no significant difference between (P> 0.05), the remaining dose levels showed significant difference between (P <0.05). From the dose and treatment time window minimized to maximize the angle of analysis to A2B2 combination of the best that the best treatment time window and the dose of ischemia 1.5h, 10 mg / kg.

There are significant changes of the content of COX2 in serum（$P$<0.05）among different levels of groups with factor A (treatment time) and groups with factor B (treatment dose) while it had no significant change （$P$＞0.05）under interaction of time-dose(C). The comparing result, which is between every two groups data by using the least significant difference method, showed that the content of COX2 in serum had no significant change (P>0.05) between groups of time 1.0 (A1) and time 2.0 (A3), time 1.5h (A2) and time 2.0(A3). however, significant change appeared (P<0.05) between the rest groups of time window; By the same way, The comparing result, which is between every two groups data by using the least significant difference method, showed that no significant changes (P>0.05) of the content of COX2 in serum had be listed, dose 5 mg/kg (B1)and dose 40 mg/kg (B4)、dose 10 mg/kg (B2)and dose 20 mg/kg (B3), but, significant change appeared (P<0.05) between the rest groups of treating dose; According the principle of the minimum dose and the maximize treatment time window, we can draw a conclusion that the best choice is A2B2, In a word, the best time window is the point round two hours after ischemia happened,

**Table 5.** serum COX2 variance analysis

| Source of variation | SS | df | MS | *F* | *P* |
|---|---|---|---|---|---|
| time | 0.071 | 3 | 0.024 | 21.72 | 0.01 |
| dose | 0.022 | 3 | 0.007 | 6.82 | 0.02 |
| Time×dose | 0.012 | 3 | 0.004 | 3.71 | 0.08 |
| error | 0.007 | 6 | 0.001 | | |

and the best dose of injection intraperitoneal is of 20 mg/kg body weight Table 5.

## DISCUSSION

In the central nervous system, AQP4 are mainly distributed on cell membranes of astrocytes and ependymal cells in the brain and spinal cord tissue(Yang et al., 2011), participating the adjusting action of water channel. Under normal condition, AQP4 coexist with potassium ion channels which be with internal flow action, can function in the service of water's translation with the channels of potassium ion (Zhang and Verkman, 2008). Some experimental results show that, in the early period of ischemic or acute ischemic in cerebral brain, AQP4 can function in the promotion of the edema in brain (Manley et al., 2000), while the gene of AQP4 been knocked out had showed protective effect on the rats that had suffered acute cerebral ischemic injury. As usually, in normal brain tissue ,MMP9 has a extremely low expression which is only of the forms of enzymes in the original. MMP9 enzymes, however, can be activated and the activated MMP9 participated inflammatory response in cerebral ischemia injury process (Rosenberg et al., 2001). In cerebral ischemia, a variety of cytokines, inflammatory mediators was released , which combined with MMP9 binding sites with the expression of MMP9 increasing and the activity of MMP9 strengthening, At the same time, a large number of protein enzymes were released to active MMP9. Actived MMP9 damage blood-brain barrier by degradation and destruction on the basement membrane and tight connection of brain cells. This damage can lead to head edema and cerebral hemorrhage after cerebral ischemia , and contribute directly to neuron death and brain damage. (Sun et al., 2008).Under normal condition, COX2 only has a low expression on neurons. However in pathological conditions, oxygen free radicals and inflammation participate the ischemic reperfusion damage, in additional, COX2 is regarded as a especial target of treatment (Liu et al., 2010) in nerve injury. In this study, the results show that, the content of AQP4, MMP9 and COX2 in serum in model group were higher significantly than it in the control group, while after treatment, the significantly decline emergence in treat group. These data has proved that the treatment by picroside II had certain protective effect in cerebral ischemia injury.

This experiment was design to divide into groups according to the principle of orthogonal experiment[$L_{16}(4^5)$] .At first, the time window of ischemia in rats had been listed:1.5 h, 1 h, 2 h and 1.5 h , and the corresponding dose of picroside Ⅱ had been listed: 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg, Then, the indicator in serum, involving AQP, MMP9 and COX2, were detected. At last, the results showed that different time window and dose of picroside Ⅱ contribute directly to different effect. Considering the principle of minimum dosage and to maximize treatment time window, The combination of A2B2 and A3B3 are better than the other, that is say, in cerebral ischemia the best effect of treatment of picroside II is of dose from 10 to 20 mg/kg body weight, and the time window from 1.5h to 2.0h. The mechanism of cerebral ischemia injury, however, is very complex (Guo et al.,2010) and this study only observe the change of some indicator above, objectively, it is hard to avoid all deviation. As my idea, it is necessary to use more index indictor to evaluate the best way of combination of treatment time window and dose.

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