Minimally invasive procedures for intracerebral hematoma evacuation in early stages decrease perihematomal glutamate level and improve neurological function in a rabbit model of ICH

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1. Introduction

Spontaneous intracerebral hemorrhage (ICH) is defined as bleeding within the brain parenchyma (Aguilar and Freeman, 2010). As a major public-health concern worldwide, there is a lack of currently proven treatments available for this condition, which carries a very high morbidity and mortality rate (Adeoaye and Broderick, 2010; Rymer, 2011). Survivors often display severe neurological deficits and only 20% of the individuals who survive ICH function independently at 6 months (Adeoaye and Broderick, 2010).

The initial hemorrhage is always followed by a secondary brain injury, which induces a worse outcome in patients. The hallmark of the secondary brain injury is the BBB disruption induced by a range of factors, including inflammatory mediators, matrix metalloproteases (Keep et al., 2008) and...
perihematomal glutamate levels (Hazell, 2007). The extent of the secondary brain injury is closely related to the hematoma volume and the brain’s exposure time to the hematoma, thus, the hematoma should be removed as early as possible. However, traditional craniotomy surgeries for clearance of intracerebral hematomas do not modify the outcome or show advantages over medical treatment. Neuroendoscopy is a promising therapeutic option for spontaneous intracerebral hemorrhage with good results for hematoma removal (Nagasaka et al., 2011; Proust et al., 2007). Endoscopic hematoma evacuation provided the quick, adequate decompression of ICH (Nishihara et al., 2007). Early and complete evacuation of ICH may lead to improved outcomes in selected patients (Kuo et al., 2011). Endoscopic surgery for ICH evacuation is safe and feasible, and may promote earlier recovery (Nagasaka et al., 2011; Orakcioglu et al., 2011). The minimally invasive aspiration and thrombolysis have emerged as a promising strategy management for ICH patients (Xu et al., 2010).

Although experimental and clinical studies have demonstrated that the minimally invasive surgery, including stereotactic and endoscopic procedures, might successfully evacuate the intracerebral hematoma with limited brain tissue damage and improve the outcome of patients (Barrett et al., 2005; Miller et al., 2008, 2007; Sun et al., 2010; Wu et al., 2011a, 2011b, 2010; Zhou et al., 2011), the optimal time window for minimally invasive surgery remains to be elucidated based on pathological aspects. Clinically, the optimal time window for surgical intervention of spontaneous ICH is within 7–24 h (Wang et al., 2008b). However, the selected indices could not reflect the pathophysiological courses of perihematomal brain tissues, and it is difficult to observe the time window using a pathophysiological index in patients with spontaneous ICH.

Many studies have observed the relationship between glutamate levels of perihematomal brain tissues and secondary brain injury in models of ICH or the impact of glutamate on the outcome of patients with ICH (Chiang et al., 2006; Hartings et al., 2008; Miller et al., 2007; Qureshi et al., 2003). A few studies have observed perihematomal glutamate level changes in patients after surgery, demonstrating that elevated glutamate levels are found in the perihematomal region after ICH and are decreased during hematoma drainage (Miller et al., 2007), but little is known about the effects of minimally invasive procedures for hematoma evacuation at different time periods post-ICH on changes of perihematomal glutamate content and BBB permeability.

The present study was designed to observe the perihematomal glutamate level changes and its correlation with BBB permeability in a rabbit model of ICH treated at different times post-ICH to provide pathophysiological evidence for the optimal time window for minimally invasive surgery.

2. Results

2.1. ICH model preparation

Forty-eight rabbits were successfully prepared for ICH in the model control (MC) and minimally invasive (MI) groups. The ICHs were confirmed by a brain CT and the neurological deficit score. No rabbits were excluded from this study. There were no differences in the hematoma volume and neurological functions between the MC and MI groups before the surgical treatment, nor among the subgroups.

2.2. Effect of the minimally invasive procedures on functional neuroscore

In the MI group, the neurological deficit score on the day when the rabbits were sacrificed decreased significantly at different time points (6–24 h) compared with the MC group (t = 21, 21, 24.5, 25.5, respectively). A significant difference was observed (p < 0.05, Fig. 2), suggesting that the minimally invasive surgery for evacuating the intracerebral hematoma improved the neurofunctional outcome. The Kruskal–Wallis test detected a significant difference of the neurological deficit score among the MI subgroups (χ² = 17.298); however, there were no significant differences among the MC subgroups (χ² = 1.358). The Bonferroni method was used to determine comparisons between any two MI subgroups.

Fig. 1 – Brain CT of the rabbits showing the hematoma changes before and after the minimally invasive surgery. (A) The arrow pointed to the hematoma (hyperdensity) in the basal ganglion of the rabbit’s brain. (B) The hematoma was evacuated successfully 48 h after the minimally invasive surgical procedures.
We found that the 6 h and 12 h groups showed the most favorable outcome compared with the other subgroups.

2.3. Effects of minimally invasive procedures for ICH

A brain CT showed that there were no significant differences in the hematomal volume between the MI (0.287 ± 0.020 ml) and MC groups (0.285 ± 0.018 ml) after the ICH model was prepared successfully. Postoperative brain CT and histological sections showed that the hematoma was evacuated successfully in all of the subgroups. A significant difference in the hematoma volume was observed between the MC and MI subgroups when the rabbits were sacrificed ($t=45.71$, $p<0.05$, Fig. 3), suggesting that performing minimally invasive procedures within 24 h after ICH could effectively evacuate the hematoma.

2.4. Changes of perihematomal glutamate levels

Glutamate levels decreased remarkably after the hematoma was evacuated by the minimally invasive procedures. There was a significant difference between the MI and MC groups ($t=6.20$, $p<0.05$, Fig. 4), suggesting that the minimally invasive procedures reduced the perihematomal glutamate content. The perihematomal glutamate contents were different among the minimally invasive subgroups ($F=9.06$, $p<0.01$). They were the lowest in the 6 h group, and increased gradually as the time-window increased, peaking in the 24 h group. The decrease in the glutamate levels in the 6 h and 12 h subgroups was more significant compared to the other subgroups ($p<0.05$), suggesting that minimally invasive procedures for evacuating ICH in early stages could significantly reduce perihematomal glutamate content (Fig. 4).

A significant difference was also observed in the perihematomal glutamate content between any two subgroups in the MC group ($F=13.89$, $p<0.05$). A more significant increase of glutamate content was observed in the 18 h and 24 h subgroups of the MC group compared with the 6 h and 12 h subgroups, suggesting that in the late stages after ICH, the perihematomal glutamate content showed a remarkable increase.

2.5. Changes in BBB permeability

After the rabbits underwent the minimally invasive procedures for evacuating the ICH, the perihematomal Evans blue content remarkably decreased. Although the perihematomal Evans blue content varied in different MI subgroups ($F=14.30$, $p<0.05$), they had significantly decreased content compared with the MC group ($t=3.40$, $p<0.05$, Fig. 5). Among the MI

Fig. 2 – The neurological deficit score between the MC and MI groups on the day when the animals were sacrificed. The neurological deficit score in different MI subgroups decreased significantly compared with the same MC subgroup. A significant difference of the neurological deficit score in the MI group was also observed among the subgroups. Notably, the 6 h group showed the most favorable outcome compared with the other three subgroups.

Fig. 3 – Comparison of the intracerebral hematomal volume between the MC and MI groups within 48 h after performing the minimally invasive procedures. A significant difference in the hematoma volume was observed between the MC and MI groups on the day when the rabbits were sacrificed, suggesting that the minimally invasive surgery could effectively remove the intracerebral hematoma.

Fig. 4 – Perihematomal glutamate level changes in the different groups after the hematoma was evacuated by the minimally invasive procedures. The perihematomal glutamate levels in the different MI subgroups significantly decreased after the hematoma in the rabbit's brain was evacuated by the minimally invasive procedures. There was a significant difference between the MC and MI groups. A significant difference was also found among the different MI subgroups, and the glutamate levels were the lowest in the 6–12 h groups.
subgroups, the perihematomal Evans blue content were the lowest in the 6 h MI subgroup, and increased as the time window was prolonged, reaching a high peak in the 24 h MI subgroup. The increase of perihematomal Evans blue content in the 24 h MI subgroup was of statistical significance compared to the other subgroups (p<0.05), suggesting that evacuation of the intracerebral hematoma by minimally invasive procedures within 24 h after onset of hemorrhage could also reduce the perihematomal BBB permeability, however, the secondary brain injury was still more serious. Thus, minimally invasive procedures performed in early stages could reduce the perihematomal Evans blue content and subsequently mitigate brain edema, where such effects are more apparently specific for the clearance of intracerebral hematoma by minimally invasive procedures in 6 h after the ICH (Fig. 5). In the MC group, the Evans blue content was also different among the subgroups (F=29.16, p<0.05) and was the lowest in the 6 h and 12 h groups but the highest in 24 h group, suggesting that the secondary brain damages deteriorated gradually as the time after ICH increased.

3. Discussions

Spontaneous intracerebral hemorrhage remains a devastating disease. Although minimally invasive surgery has showed promising results in recent years, the pathophysiological time window of minimally surgery remains to be elucidated. It is difficult to observe the perihematomal changes in patients with ICH treated by minimally invasive procedures. In the present study, using a previously established model of ICH (Andaluz et al., 2002; Frantzias et al., 2011), we successfully generated a rabbit ICH model by stereotactic injection of non-anticoagulant autologous arterial blood into the basal ganglion of the rabbit’s brain. We observed the effect of performing a minimally invasive procedure in different time window on the neurofunction by such large animal models. The results showed that the minimally invasive procedures performed within 24 h could effectively remove the hematoma. The neurological functions were improved remarkably in the MI group compared with the MC group, and performing the minimally invasive surgery during the early stages displayed a more significant outcome. These results are consistent with our previously published studies (Wu and Zhong, 2010).

After the ICH was evacuated by the minimally invasive procedures, perihematomal glutamate levels decreased significantly compared with the MC group, as the time window of the minimally invasive procedures prolonged, the decrease in the glutamate levels gradually became less obvious. Although the glutamate levels varied among the different surgical subgroups, a significant decrease was observed in each MI subgroup compared to the MC group. The same phenomenon was observed in perihematomal Evans blue. These findings suggest that the minimally invasive procedures for hematoma evacuation could effectively reduce the perihematomal glutamate levels, as well as the BBB permeability, both of which contribute to the secondary brain damages following ICH.

Glutamate-related excitotoxicity is associated with secondary brain damages and the level of glutamate was closely associated with the outcome of patients with ICH. Patients with a spontaneous ICH who present with more serious brain injuries have a higher concentration of glutamate in the brain and, consequently, a worse prognosis (Wang et al., 2008a). BBB disruption is accompanied by the development of vasogenic brain edema in the surrounding areas, a very life-threatening event after ICH. In addition to brain edema formation, BBB disruption following ICH also contributes to the influx of leukocytes, and the entry of potentially neuroactive agents into the perihematomal brain, all of which may result in brain injury (Keep et al., 2008). The degree of BBB breakdown was directly correlated with late functional outcome (Lampl et al., 2005). Increased levels of glutamate have been associated with increased BBB permeability and brain edema. Studies have demonstrated that the glutamate level in perihematomal brain tissue increases and results in disruption of the BBB and increased BBB permeability, thereby increasing brain edema. Evans blue is a type of dark blue powdered dye that is almost incapable of penetrating the BBB under normal conditions. The Evans blue assay is a popular method for the quantification of BBB disruption. A small amount of Evans blue can be observed in the normal brain of animals after intravenous injection (Wu et al., 2011a). In the present study, the Evans blue content in the periphery of the hematoma were decreased in each of the MI groups, compared with the MC group, suggesting that the BBB permeability decreased after removing the intracerebral hematoma by minimally invasive procedures. The Evans blue contents were significantly decreased in the MI subgroups, especially in the 6 h and 12 h subgroups, compared to the MC groups. Further statistical analysis demonstrated that the perihematomal glutamate levels were positively correlated with the BBB permeability, as well as neurological function. In the 6 h MI subgroup, the perihematomal glutamate content, Evans blue content and neurological functions were minimal. As the time window of minimally invasive surgery
increased, the decrease of perihematomal glutamate became less obvious; however, the Evans blue content and neurological functions were the same. Thus, reducing the effects of glutamate or decreasing its levels in the perifocal brain tissues might be helpful in the protection of the BBB and of neurological function. Blocking the effect of glutamate with its receptor antagonists MK-801 and felbamate have been shown to reduce the formation of brain edema and help restore BBB permeability in experimental subarachnoid hemorrhages and diffuse brain injuries (Germano et al., 2007; Imer et al., 2009); memantine, an NMDA receptor antagonist, has been found to reduce inflammatory infiltration and apoptosis and to induce functional recovery after ICH (Lee et al., 2006). Increased local cerebral glucose utilization in pig models of ICH has been shown to be blocked by NMDA and AMPA glutamate receptor antagonists (Sharp et al., 2008). However, these promising achievements in basic research have not been transferable into clinical treatments. Reducing the glutamate level in perifocal brain tissues by minimally invasive procedures for hematoma evacuation might be an alternative choice because standard open craniotomy for clearance of intracerebral hematoma commonly causes damage to the uninjured brain tissue overlying the hematoma. Minimally invasive surgery combines the benefits of surgical clot removal with limited tissue damage and shorter surgery duration (Thiex, 2011). These ideas have been demonstrated in our previously published studies (Wu et al., 2007; Wu and Zhong, 2010). In the present study, perihematomal pathophysiological changes were used as indices to observe the time window of minimally invasive procedures. We demonstrated that the optimal time window was within 6–12 h after the onset of hemorrhage, without considering the aspects of re-hemorrhage.

Earlier therapeutic time window (3–6 h) would be helpful in reducing the perihematomal secondary brain damages and improving neurological functions. The effects of performing the minimally invasive surgery in super-early stage on the outcome of treatment were not observed in the current study. This would be a limitation of the present study. A further experimental study would be required for observing the effect of performing the minimally invasive surgery in 3–6 h after ICH to evacuate the hematoma on the outcome of neurofunction, and the perihematomal pathophysiological changes. If the hematoma could be removed at a hyperacute stage (within 6 h after ICH) after onset, the brain damages would be reduced more significantly and a more favorable outcome would be expected. In clinical practice, the 6–24 h window is usually the earliest surgical procedures can be performed. The interval between the onset of ICH and the ICH evacuation is prolonged by patient presentation to the hospital, diagnostic work-up, procedures for randomization, arrangements for stereotactic navigation, and surgical placement of a catheter into the clot with subsequent radiographic confirmation. So we selected 6 h as one of the time points to observe the outcome of neurofunction and the perihematomal changes.

As the animals were sacrificed within 54–72 h after the ICH was induced to observe the perihematomal pathophysiological changes, we were unable to observe the long-term outcome of neurological function. Further clinical observations and more research are needed to observe and understand the long-term outcomes of patients with ICH treated with minimally invasive procedures.

4. Experimental procedures

4.1. Materials

4.1.1. Main reagents

Formamide (molecular formula: HCONH₂, Chongqing Chuanjiang Chemical Reagent Factory), urethane (molecular formula: C₃H₇NO₂, Wuxi Yangshan Biochemical), Evans blue (Beijing Hengye Zhongyuan Chemical), 4% paraformaldehyde (Wuhan Boster Biological Technology), urokinase (Guangdong Livzon Pharmaceutical), glutamate (Sigma), derivatization reagent borate buffer (Agilent Technologies, USA), FMOC reagent Agilent PN5061-3337 (Agilent Technologies, USA), OPA reagent Agilent PN5061-3335 (Agilent Technologies, USA), 2,4-DNFB (Japan) and HPLC-grade acetonitrile and methanol (Germany) were used in this study.

4.1.2. Main instruments

For this study, we used the following instruments: a ZH-Lanxing B-Type rabbit stereotaxic apparatus (Huabei Zhenghua Biological Instrument & Equipment), electronic scales (Satourious, Germany), a Rainbow Type-722 grating spectrophotometer (Shandong Gaomi Rainbow Analytical Instrument), a 5415R high-speed centrifuge (Frozen, Heraeus Company), micropipettors (Eppendorf), a 200-2 constant temperature oven (Shanghai Luda Laboratory Apparatus), a digital display thermostat water bath HH-2 (Guohua Electric Appliance), a desktop general centrifuge (TGL-16B; Shanghai Anting Scientific Instrument Factory), a –80 °C freezer (Forman Scientific Company), a refrigerator (Qingdao), a pH meter (410 A, ORION, USA), an Agilent 1313A automatic sampler (Agilent Technologies, USA), a column oven (Agilent Technologies, USA) and scales (Beijing Gangdong Hengye Instrument).

4.2. Experimental groups

The present study was approved by the Animal Care and Use Committee of Guiyang Medical College.

Forty-eight rabbits (3.2–3.5 kg, male or female) were provided by the Animal Center of Guiyang Medical College. These rabbits were randomly divided into a model control group (MC group, including 24 rabbits) and a minimally invasive group (MI group, 24 rabbits). The animals in each group were equally divided into 4 subgroups, defined as the 6 h group, 12 h group, 18 h group and 24 h group, with each subgroup including 6 rabbits. An ICH model was induced in all of the animals in the MC and MI groups.
4.3. Animal preparations

4.3.1. ICH model preparation

The rabbits were anesthetized by injecting 20% urethane (5 ml/kg) into the ear vein. The anesthetized rabbits were fastened to the stereotaxic apparatus. A 3 cm incision was made along the midline. The skulls of the rabbits were drilled, and using a #12 needle and a 1 ml syringe, 0.5 ml autologous arterial blood was taken from the central ear artery. Air was completely removed from the syringe, leaving 0.3 ml of blood. The needle was then inserted vertically and quickly into the skull (12 mm deep), and the blood was slowly injected into the basal ganglia. A CT scan was performed 3 h later. A high-density shadow in the basal ganglia region with no shadow in the lateral ventricle was considered a successful ICH induction.

The rabbits were sent back to the animal room and housed as usual after successful ICH induction. All of the animals recovered from anesthesia within 5 h after the intravenous injection of 20% urethane. The total anesthesia time was 3–5 h.

Exclusion criteria included visualization of back flow along the needle track, blood in the ventricle and death of the rabbits.

4.3.2. Minimally invasive surgery for evacuation of the ICH

In the MI group, the minimally invasive surgery for evacuating the intracerebral hematoma was performed 6 h, 12 h, 18 h or 24 h after successful ICH induction.

The rabbits were anesthetized again by injecting 20% urethane (5 ml/kg) into the ear vein. They were then placed in the stereotaxic apparatus. Using the former drill hole, a #7 needle was inserted into the hematoma, and the liquid part of the hematoma was aspirated. We then injected 5000 U of urokinase (dissolved in 0.5 ml of 0.9% sodium chloride solution) into the hematoma. The needle was kept in place for 15 min, followed by slow aspiration while withdrawing the needle. The rabbits were placed back into the breeding room and housed as usual until they were sacrificed.

Before the animals were sacrificed, a CT scan was performed to demonstrate the efficacy of the surgical procedures (Fig. 1). Histological sections were prepared in all of the animals for residual hematoma evaluation. The hematoma volume was calculated on a CT using software provided by the manufacturer.

4.3.3. MC group treatment

The minimally invasive surgical procedures for evacuating the ICH performed in the MI group were also performed in the MC subgroups, but without aspirating the hematoma and injecting urokinase into the hematoma cavity.

4.3.4. Medical treatment of the animals

Animals in each group received only an intramuscular injection of penicillin (400,000 U) to prevent infection, and they were housed as usual until they were sacrificed. No other medical treatment was administered.

4.4. Neurological deficit score measurement

A neurological deficit scale was used to determine neurological function (Purdy et al., 1989). The scale included tests for motor function (1–4), consciousness (1–4), head turning (0–1), circling (0–1) and hemianopsia (0–1). A total score of 11 indicated maximum impairment (comatose or dead rabbit), whereas 2 denoted complete normality. The neurological deficit scores were measured by an observer blinded to the conditions of the animals.

4.5. Brain tissues preparation

All of the animals were sacrificed 48 h after the ICH was evacuated by the minimally invasive surgery. Thus, the animals were sacrificed within 54 h of ICH induction in the 6 h subgroup, 60 h in the 12 h subgroup, 66 h in the 18 h subgroup and 72 h in the 24 h subgroup. A 2% Evans blue (2 ml/kg) solution was injected into the ear vein 2 h before the animals were euthanized. The animals were then anesthetized using 20% urethane. The animal’s chest was quickly opened to expose the heart. A tube was inserted from left ventricle into the aortic root, with a small hole cut into the right auricle to allow the tube to exit. The rabbits were then perfused transcardially with 400 ml of 0.9% sodium chloride solution, followed by 100 ml of 4% paraformaldehyde after flow-through became clear. The brain was then extracted and placed on ice. Using the needle track as the center to prepare coronal and sagittal sections, the brain on the hematoma side was cut and divided into four parts: front-inner, front-outer, back-inner and back-outer. A total of 5 mm of brain tissue surrounding the hematoma was collected from each area mentioned above. The front-inner part and the back-outer part were used for amino acid testing, and the front-outer part and the back-inner part were used for Evan’s blue testing.

4.6. Determination of perihematomal glutamate levels

The perihematomal glutamate content was determined by high performance liquid chromatography (HPLC).

4.6.1. Chromatographic conditions

The chromatographic column used was the ZORBAX Eclipse-AAA (4.6 × 150 mm, 5 μm). The mobile phase A was 40 mM Na2HPO4, pH 7.8 (5.5 g NaH2PO4·H2O + 1 l water, and NaOH was used if needed to make the pH 7.8) and the mobile phase B was 45:45:10 (v/v/v) ACN:MeOH:water. The column was run with a flow rate of 2 ml/min. Phase B increased from 0% to 57% between 0 and 18 min and from 57% to 100% between 18.1 and 18.6 min. It remained at 100% between 18.6 and 22.3 min and decreased from 100% to 0% between 22.3 min and 23.2 min. Between 23.2 and 26 min Phase B remained at 0%. The column temperature was 40°C, and the sampling volume was 10 μl. The wavelength of the diode array was 262 nm, and the reference wavelength was 324 nm.

4.6.2. Derivative solution preparation

A total of 25 mg OPA was dissolved in 1 ml methanol. Sodium borohydride buffer (4 mol/l) was then added (pH 10.4) and the solution was stirred. The final solution was stored at 4°C.
4.6.3. Standard solution preparation
Glutamate and 0.2 mol/l NaHCO₃ (pH 9.8) was used to make a standard stock solution at a concentration of 1 g/l.

4.6.4. Biosample preparation
The brain tissue was defrosted, weighed and placed into a dry glass homogenizer. Diluted hydrochloric acid (1.5 w/v; 0.1 mmol/l) was added, and the homogenized brains were placed into an ultrasonicator (Temperature: 4 °C; pulse for 2 s, rest for 2 s; intensity: 20%; 15 times in total). The samples were then centrifuged at 1200 rpm for 20 min at 4 °C. Borate saline buffer (2.5 ml) was then added to the supernatant solution and mixed for 20 min, followed by the addition of 0.5 μl OPA. The sample was then mixed for 30 s, FMOC (0.5 μl) was then added and the solution was mixed for another 30 s. Finally, water (32 μl) was added, and the final sample was mixed for an additional 30 s, and 10 μl of the sample was used.

4.6.5. Calculation of glutamate concentration
The peak area of glutamate from the HPLC was integrated and used as an external standard for the samples. The glutamate concentration for 1 g of brain tissue was then calculated according to the sample quality.

4.7. Measurement of BBB permeability

4.7.1. Experimental methods
Evans blue was used as a tracer to measure BBB permeability. 2 h before each experiment, 2% Evans blue (2 ml/kg) was injected into the rabbit's ear vein. After 2 h, the brain tissue was quickly removed. The tissue surrounding the hematoma was weighed (with an accuracy of 0.1 mg) and then placed into a test tube with 4 ml of formamide. The tube was then capped and placed in a 54 °C water bath for 24 h to allow the Evans blue solution to spread throughout the brain tissue. The samples were then centrifuged at 2400 rpm for 5 min. A spectrophotometer was used (λ = 632 nm) to measure the absorbance of the supernatant, which was removed using a straw and placed into a quartz cuvette. The absorbency was measured using formamide alone as a blank control.

4.7.2. Setting up the standard curve
Evans blue (4 mg) was placed into a volumetric flask and weighed (within an accuracy of 0.1 mg). A total of 100 ml of NS was added, and the solution was stirred. From this solution, 0.3 ml was removed and placed in 5.7 ml of formamide to make the standard buffer solution. A total of 3 ml of this solution was serially diluted into seven tubes, each containing 3 ml of formamide. The amount of Evans blue in each of the seven tubes was 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/ml. The tubes were capped and placed into a 54 °C water bath for 24 h. The absorbance was then measured as previously described. Linear regressions were then calculated for the absorbencies and Evans blue content. The final equation was y = 0.0055x + 0.0608 (R² = 0.9833).

4.7.3. Computational method of the Evans blue content
We used the formamide method to measure the Evans blue content in the brain tissue to gauge the severity of the BBB damage. The formula used was as follows: Evans blue content in brain tissue (μg/g wet brain) = B × formamide (ml)/wet weight (g), where B refers to the Evans blue content of the sample (μg/ml) given by the linear regression equation according to the standard curve.

4.8. Statistical analysis
All of the data were analyzed using SPSS 11.5. The basic data are expressed as the mean ± standard deviation (X ± SD). A t-test was performed to make comparisons between the MI and MC groups. A repeated measures ANOVA was used to make comparisons of the glutamate content and Evans blue content across the entire time series. When a difference was detected by ANOVA, a LSD-t was used to make comparisons between every two subgroups. For the neurological deficit score, a Wilcoxon test was used to detect the differences between the MC and MI groups, and a Kruskal–Wallis test was used for comparisons among the subgroups. When a difference among the subgroups was detected by the Kruskal–Wallis test, a Bonferroni post hoc test was used to make comparisons between every two subgroups (including the MI and MC subgroups). A p value of less than 0.05 was considered statistically significant. The statistical analysis was performed in consultation with the Department of Biostatistics, Guiyang Medical College.

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