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A method for predicting the success of Pulsinell's four-vessel occlusion rat model by LDF monitoring of cerebral blood flow decline

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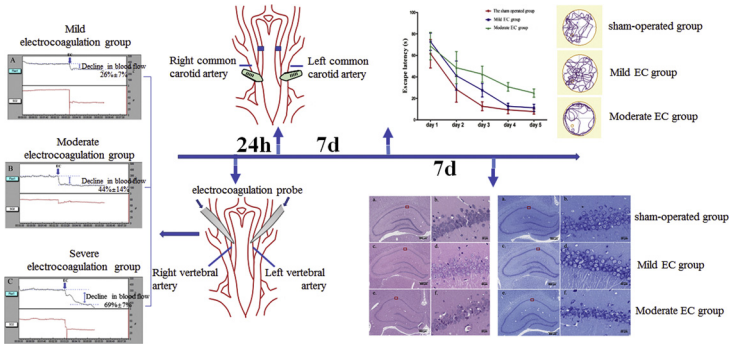
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Since the electrocoagulation of the bilateral vertebral artery in the Pulsinelli's four-vessel occlusion method must rely on the experimental experience of the researchers, it has no objective quantitative guidance. The high mortality or insufficient brain injury in the animals occur early in the experiment, requiring more animals to account for those not suitable.

New method: In this study, Laser Doppler flowmetry (LDF) was used to monitor the decline in blood flow during electrocoagulation in rats to control the degree of brain injury.

Results: Rats were divided into the sham-operated, mild electrocoagulation, moderate electrocoagulation and severe electrocoagulation groups. In this three electrocoagulation groups, the decline in cerebral blood flow of rats was 26% ± 7%, 44% ± 14% and 69% ± 7% and the corresponding mortality rates were 0%, 33% and 100%, respectively. Rats in the moderate electrocoagulation group, which indicated that the model was successful, had a low mortality rate, showed a high degree of brain injury.

Comparison with existing methods: The position of the vertebral artery cannot be directly visualized and the degree of cerebral ischemia can only be adjudicated by the experimental experience of the researcher, with no

Abbreviations: EC, electrocoagulation; ANOVA, analysis of variance; H&E, hematoxylin and eosin; LDF, Laser Doppler flowmetry; LSD, least Significant Difference; min, minutes; mNSS, modified Neurological Severity Score; MCAO, middle cerebral artery occlusion; s, seconds; SD, Sprague-Dawley

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objective guideline by observation. We used LDF to monitor the decline in blood flow during and after electrocoagulation, allowing us to effectively control the degree of cerebral ischemia and thus the mortality of animals.

Conclusions: With the addition of LDF monitoring, Pulsinelli's four-vessel occlusion can be used as a stable and reliable model for global cerebral ischemiareperfusion injury.

1. Introduction

Global cerebral ischemia-reperfusion injury in animals is a common model used to study the mechanisms and treatments of ischemic encephalopathy, vascular dementia, cardiac arrest, severe shock and other diseases. It is of great significance to establish a stable and reliable animal model for the study of clinical diseases. The most common models of global cerebral ischemia are the two-vessel occlusion (Eklof and Siesjo, 1972), the three-vessel occlusion (Kameyama et al., 1985), and the four-vessel occlusion. Among them, the two-vessel occlusion is a model of chronic and progressive brain hypoperfusion. A disadvantage of this model is that it may not represent global cerebral ischemia. The advantage is that the surgical procedure is simple and convenient. Scholars in China have regularly applied this method to study chronic cerebrovascular diseases. The three-vessel occlusion model may often result in severe injury to the surrounding tissue and may easily result in the death of the animals. The four-vessel occlusion model is a better model for simulating the process of global cerebral ischemic injury in clinical practice and is widely used internationally (Hwang et al., 2017; Sadelli et al., 2017). However, based on our previous research experience (Wang et al., 2017, 2018), we have found that the surgical procedure of the four-vessel occlusion is complicated. In particular, successful electrocoagulation of the vertebral artery relies on the experimental experience of the researcher, such as observing the surrounding alar foramen becoming white to obtain vertebral artery occlusion. There is no objectively consistent guidance for this observation. Early in the experiment, researcher error can create a high mortality of animals with excessive electrocoagulation. Insufficient electrocoagulation can only be detected later, after the rats complete the modified Neurological Severity Score (mNSS) and the Morris water maze test. These insufficiencies indicate the need for an objective standard in producing global cerebral ischemia-reperfusion injury in rats.

Laser Doppler flowmetry (LDF) is a real-time dynamic and sensitive detection technique that reflects the microcirculatory blood flow (Frerichs and Feuerstein, 1990; Wardell et al., 2016). Ster first reported detecting the blood flow of the skin by a laser Doppler blood flowmeter. Laser Doppler blood flow detection technology is now widely used in various fields. Sommer et al. used non-invasive LDF to detect local cerebral microcirculation during cerebral aneurysm surgery and found that this monitoring method greatly improved the early detection of microcirculatory disorders during surgery (Sommer et al., 2017). LDF is frequently used to monitor and confirm the reduction of local cerebral blood flow in a rat model of the middle cerebral artery occlusion (MCAO) of stroke (Morris et al., 2016; Wen et al., 2017). Studies have suggested that LDF can also be used to predict the prognosis of MCAO of stroke in rats (Hedna et al., 2015). However, this technique has not been applied to the four-vessel occlusion method of global cerebral ischemia-reperfusion injury model.

The physiological structure of the middle cerebral artery and the blood supply of the basal ganglia of rats are similar to those of humans. Rats are the most frequently used model animals because of the characteristics of good vitality, strong anti-infective ability and excellent homozygosity within the species. Therefore, we used the Pulsinelli's four-vessel occlusion method to establish a rat model of global cerebral ischemia-reperfusion injury in this study. With the purpose of establishing a more stable and scientific model of global cerebral ischemia-reperfusion injury, the cerebral blood flow of Sprague-Dawley (SD) rats during the surgical process was monitored by LDF and the decline in cerebral blood flow was used to determine the hypoxic state of the rat brain during surgery. The mNSS score, Morris water maze test, hematoxylin and eosin (H&E) and Nissl staining were used to assess the degree of brain injury, to determine the stability and reliability of this rat model.

2. Material and methods

2.1. Experimental animals

Thirty healthy adult male SD rats, weighing 250–300 g, 7–8 weeks old, specific-pathogen-free grade, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., license number SCXK 2012-0001(Beijing, China) and raised in an animal barrier laboratory of the Institute of Rehabilitation Medicine of China. All animal procedures used in the present study were approved by the Ethics Committee of Capital Medical University (approval number AEEI-2018-096). The experimental animals were randomly assigned by random number tables into the sham-operated group (n = 10) and the global cerebral ischemia-reperfusion injury model group (n = 20). Rats were conditioned for at least 3 days before the experiment and were given free access to drinking water. The feeding environment was kept at an indoor temperature at 24–26 °C, with air humidity at 60–70%, ambient noise at < 60 dB and in a 12:12-h light–dark cycle.

2.2. Pulsinelli's model preparation

The global cerebral ischemia-reperfusion injury rat model was established by using the modified four-vessel occlusion method introduced by Pulsinelli et al (Meade et al., 2000; Pulsinelli and Brierley, 1979). After 24 hr fasting before surgery, rats were anesthetized by inhalation of isoflurane (4% gas concentration) with an isoflurane vaporizer (Matrx; Midmark Corp., Dayton, OH, USA). During surgery period, all rats were on continuous airway anesthesia using isoflurane (Lunan better Pharmaceutical Co.Ltd., Shandong, China, 2% gas concentration). A homeothermic blanket (Temperature Control Unit HB 101/2, Spain) was used to maintain the body temperature of the animal at 37 °C ± 0.5 °C. Each rat was placed in the prone position and fixed on the operating table. Gauze was placed beneath the neck so that the head could be tilted downward by about 30° along the vertical axis to facilitate exposure of the atlas. The animals were depilated routinely, and the skin was disinfected with Anerdian (iodine 0.2 ± 0.02%, chlorhexidine acetate 0.45 ± 0.045%, ethanol 65 ± 5%; Disinfection Technologies Ltd., Shanghai, China). A 3–4 cm incision on the neck skin centered over the atlas was made along the posterior midline. The latissimus dorsi and trapezius muscles were dissected bluntly to expose the atlas. The bilateral transverse foramens then could be seen and the vertebral arteries were passed inferior to these foramens. An HV-300E diathermy electrocoagulation probe (Beijing Jinhengwei Technology Development Co., Ltd., Beijing, China) was inserted 1–2 mm into the transverse foramen toward the caudal end and the bilateral vertebral arteries were coagulated until the declined value of cerebral blood flow was observed by LDF. Then, the incision was sprinkled with penicillin powder, disinfected again and a layered closure was carried out.

Twenty-four hours later, the rat was anesthetized again by isoflurane gas. The rat was placed in the supine position and fixed on the operating table. The hair on the neck was removed and the skin

disinfected with Anerdian. A 3 cm incision was made along the midline of the neck and the bulla mastoidea and fascia were dissected bluntly using ophthalmic forceps to expose the bilateral carotid triangles. The fascia was continuous dissected bluntly by using the vascular clamp to expose the pulsating carotid sheath farther down. The fascia on the surface of the carotid artery was dissected. The common carotid artery and the vagus nerve were separated. The common carotid artery was raised using an ophthalmic curved forceps and a silk thread was passed under the artery for later application. The bilateral common carotid arteries were clipped with non-invasive vascular clamps for 20 min (minutes) (the ischemic phase); perfusion was restored by removing the vascular clamps 20 min later. Finally, the incision was sprinkled with penicillin powder and layered closure was carried out.

In the Pulsinelli's model group, the modified Pulsinelli's four-vessel occlusion method mentioned above was used to establish the global cerebral ischemia-reperfusion injury rat model. In the sham-operated group, the bilateral transverse foramens at the atlas were segregated, but the bilateral vertebral artery was not electrocoagulated and the bilateral carotid artery was separated but not clamped.

2.3. Laser Doppler flowmetry (LDF) monitored changes in cerebral blood flow during surgery in rats

In this study, a high-power LDF (Moor VMS-LDF1-HP; Moor Instruments Inc., Wilmington, DE) was used to detect the decline in blood flow of the bilateral vertebral arteries during electrocoagulation. The rat was placed in the prone position on the operating table and the hair on the head was removed. A 2 cm longitudinal incision was made between the tip of the nose and the posterior edge of the occipital bone. The periosteum was then separated to expose the cranium between the right sutura coronalis and the sutura lambda. Exudate was wiped with a cotton swab soaked with hydrogen peroxide. The LDF probe was fixed to the cranium with glue on the right parietal lobe (4 mm behind the anterior fontanelle and 2 mm to the right, lateral to the hippocampus). After 2 min, saline was injected into the middle of the base and a laser Doppler probe was inserted. Before electrocoagulation of the vertebral artery, the blood flow in the head of the rat was monitored by LDF; monitoring continued for 3 min and then was stopped. The cerebral blood flow was closely monitored during electrocoagulationl. After each electrocoagulation, the cerebral blood flow of the rat was continuously monitored for 2–3 min and the process was repeated until the cerebral blood flow declined to/reached the expected value.

2.4. The modified neurological severity score (mNSS)

The mNSS score includes exercise, sensory, reflex and balance assessment, with a minimum of 0 points and a maximum of 18 points. Six rats each were selected from the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group. The mNSS of each rat was measured at 3, 7 and 14 days after global cerebral ischemia reperfusion. Scores range 0–18: 0 points indicates normal, 1–6 points is a mild injury, 7–12 points is a moderate injury, and 13–18 points is a severe injury. A double-blind method was used for scoring and the average value was recorded.

2.5. Morris water maze test

The Morris water maze (Model XR-XM101, Hong Kong Biotechnology Co., Ltd., Hong Kong, China) was used to measure the learning and memory ability in rats after the global cerebral ischemia-reperfusion injury. Six rats each were selected from the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group. The Morris water maze test was started on the 7th day after reperfusion in rats, and conducted for a total of 6 days. The Morris water maze test consists of two parts: the place navigation test and the spatial probe trial (Wang et al., 2018).

2.5.1. The place navigation test

After completing the experimental protocol, the rats were trained 4 times a day in the morning for 5 consecutive days. During training, the rats were randomly released into the pool at water entry points of the four different quadrants, facing the wall of the pool. All rats had the same water entry point in the training. The trajectory, duration and swimming speed of each rat in climbing onto the platform (escape latency) were observed and recorded. The maximum duration of the latency was set to 120 s (s). If the rat found the hidden platform in the water and stayed on the platform for more than 3 s, it was considered to have found the platform. The swimming time for exploring the platform of the rat, which was defined as the latency period, was recorded by the software system. If the platform was not found by the rat within 120 s and it then had to be artificially navigated to the platform, the latency was set as 120 s. If the rat found the platform within 120 s, it was allowed to stay on the platform for 10 s. The average of the four latency periods per day was taken as the escape latency for the day for that animal.

2.5.2. The spatial probe trial

The spatial probe trial was started on the 6th day of training. The platform hidden under the water was removed. The fourth quadrant was selected as the water entry point and the rat was placed in the pool facing the wall of the pool for 120 s. The time of the first arrival on the platform (latency), the percentage of time spent in the quadrant, the percentage of distance spent in the quadrant, the average speed and the swimming trajectory of the rat were recorded by the software.

2.6. Hematoxylin and eosin (H&E) and Nissl staining

On the 14th day after reperfusion, 6 rats each from the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group were sacrificed, then perfused transcardially with paraformaldehyde. The brain tissue was isolated and fixed in 4% paraformaldehyde in a 4 °C refrigerator for 24 h. Paraffin embedded tissue sections were made according to the conventional method.

Paraffin sections were deparaffinized in conventional xylene and washed in distilled water. They were stained in the haematoxylin solution for 1 min, rinsed in water for 10 min, differentiated in 0.6% HCl in 70% ethanol for 30 s, rinsed in water for 20 min, counterstained with the eosin solution for 3 min, dehydrated in gradations of ethanol, cleared in xylene and mounted in neutral balsam.

The sections were stained with toluidine blue for 5–10 min, washed in distilled water, cleared in xylene for 5 min and mounted in neutral balsam.

2.7. Image analysis

The scanning and analysis of H&E and Nissl stained tissue images were performed using the TissueGnostics Multispectral Imaging Quantitative Analysis System (HistoFAXS; TissueGnostics, Vienna, Austria) at high magnification (x20) using the built-in image acquisition applications. The histomorphology changes of neurons in the hippocampal CA1 area were observed by microscopy and counts of neurons in the CA1 area of the hippocampus on Nissl-stained sections were made and statistically analyzed.

3. Statistical analysis

Statistical analysis was performed using the Statistical Product and Service Solutions (SPSS) version 22.0 software (IBM Corp., Armonk, NY) and GraphPad Prism7 software (GraphPad Software, Inc., San Diego, CA) for graphing. Data were first tested for normality and homogeneity of variances and expressed as mean ± standard deviation. If the normality and homogeneity of variances were satisfied, one-way analysis of variance (ANOVA) was employed to compare

differences among three groups. The Least Significant Difference (LSD) test was used for pairwise comparisons. If the normality was not satisfied, the rank sum test was performed. The significance level α was set as 0.05.

4. Results

4.1. Laser Doppler flowmeter monitored the blood flow of the bilateral vertebral artery before and after electrocoagulation

The percentage of decline in blood flow of the bilateral vertebral artery after electrocoagulation in the mild, moderate and severe electrocoagulation groups were compared by ANOVA. The results showed that the variances were homogeneous ($P > 0.05$) and there was a statistical difference ($F = 20.733$, $P < 0.01$) among groups. Post hoc analysis was performed using the LSD test. The decline in blood flow after electrocoagulation in the mild, moderate and severe electrocoagulation groups were 26% ± 7%, 44% ± 14% and 69% ± 7%, respectively (Fig. 1).

4.2. Mortality rate

All 6 rats in the mild electrocoagulation group survived, with a mortality rate of 0. Of the 9 rats in the moderate electrocoagulation group, three died, two after electrocoagulation and one after clamping the carotid artery, for a mortality rate of 33%. All 5 rats in the severe electrocoagulation group died.

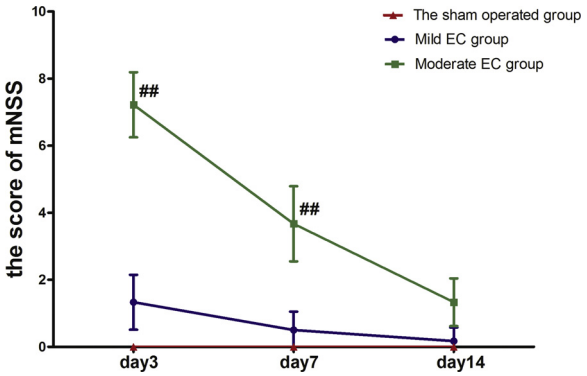

Fig. 2. The modified Neurological Severity Score (mNSS) of rats in the sham-operated group, the mild and moderate electrocoagulation groups at 3, 7 and 14 days after reperfusion. Data are presented as mean ± SD. $^{\#\#}P < 0.01$ compared with the sham-operated group.

4.3. The mNSS to detect neurological impairment after global cerebral ischemia and reperfusion

The Kolmogorov-Smirnov normality test of the mNSS scores on the 3rd and 7th and 14th day of rats in the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group showed that the assumption of normal distribution was not satisfied ($p < 0.05$). Therefore, the Kruskal–Wallis rank sum test was used to analyze the mNSS score. The mNSS scores of the three groups differed

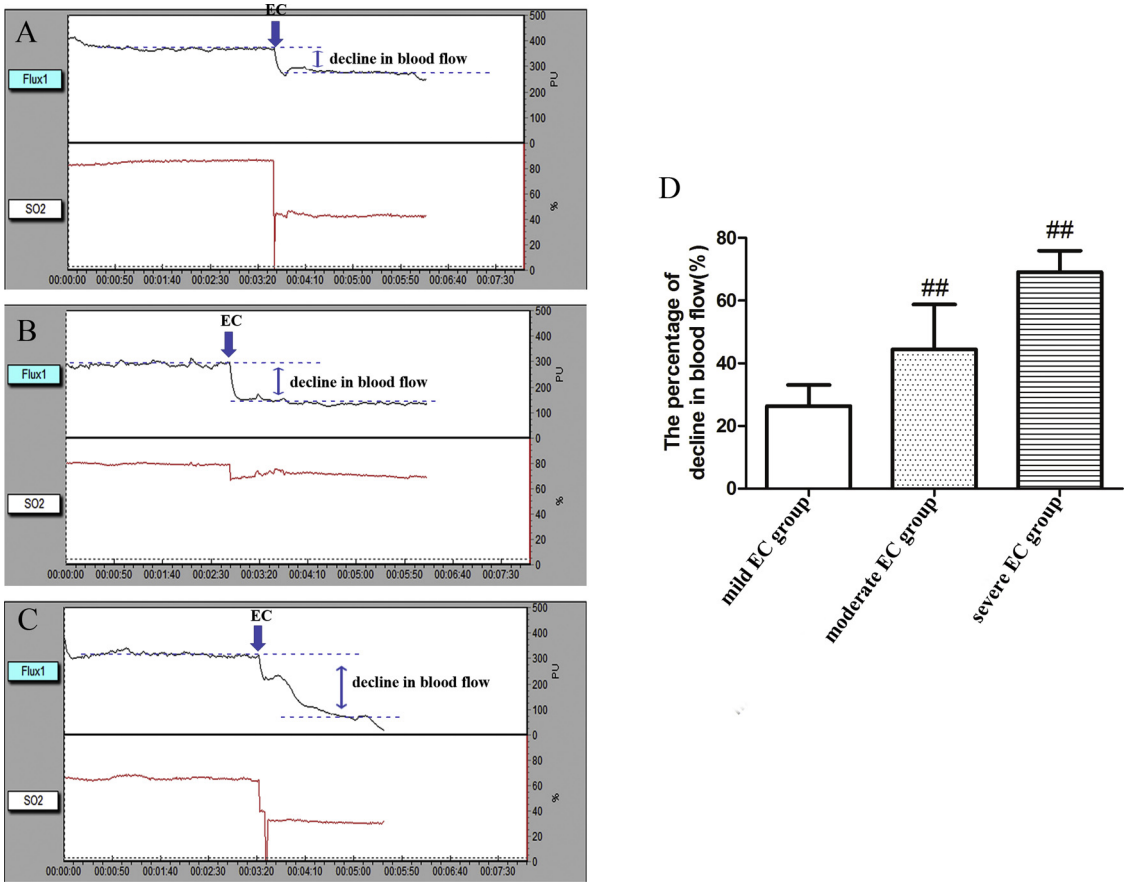

Fig. 1. The comparison of the decline in cerebral blood flow when electrocoagulation applied on vertebral artery at 3 different levels. A high-power LDF (Moor VMS-LDF1-HP; Moor Instruments Inc., Wilmington, DE) was used to detect the decline in blood flow of the bilateral vertebral arteries during electrocoagulation. A. the mild electrocoagulation group. B. the moderate electrocoagulation group. C. the severe electrocoagulation group. D. Percentage decline in blood flow of the vertebral artery after electrocoagulation. Blood flow rapidly declined after electrocoagulation (arrow). Data are presented as mean ± SD. $^{\#\#}$ $P < 0.01$ compared with the mild electrocoagulation group.

significantly on the 3rd and 7th day (H = 18.568, P < 0.01; H = 17.9, P < 0.01, respectively). The mNSS scores of rats in the sham-operated group is 0. The mNSS scores of rats in the moderate electrocoagulation group were significantly higher than those in the sham-operated group and the mild electrocoagulation group at the 3rd and 7th day (p < 0.05). There were no statistical differences in the mNSS scores of rats in the sham-operated group and the mild electrocoagulation group at the 3rd and 7th day (p > 0.05) (Fig. 2).

4.4. Morris water maze test of learning and memory ability in rats after global cerebral ischemia-reperfusion

In the Morris water maze test, the escape latency, time spent in the target quadrant and swimming trajectories of rats in the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group were determined to be normally distributed and then analyzed using one-way ANOVA. The result showed equal variances across data (P > 0.05), and post hoc analysis was performed using the LSD test.

The escape latencies of rats in the sham-operated group, the mild electrocoagulation group and the moderate electrocoagulation group decreased as the training days increased. The results showed no significant difference in escape latencies between groups on the first day (F = 1.637, P > 0.05). In the four days of training, rats in the moderate electrocoagulation group had significantly longer escape latencies than those in the sham-operated group (P < 0.01). The escape latency at the 3rd, 4th, and 5th day of rats in the moderate electrocoagulation group was significantly longer than in the mild electrocoagulation group (P < 0.01). There was no significant difference in the escape latencies at the 1 st, 4th, and 5th day of rats in the sham-operated group and those in the mild electrocoagulation group (P > 0.05).

During the probe trial, the time spent in the target quadrant and the swimming trajectories of the rats in the moderate electrocoagulation group were statistically significantly smaller than those of the sham-operated group and the mild electrocoagulation group (P < 0.01). There was no significant difference in the time spent in the target quadrant and swimming trajectories of the rats in the mild electrocoagulation group and the sham-operated group (P > 0.05). The swimming trajectories of rats during the probe trial in the mild electrocoagulation group and the sham-operated group were more concentrated in the target quadrant with the platform, which became more oriented. The swimming trajectories of rats in the moderate electrocoagulation group showed a typical edge-type swimming pattern, which was biased towards the surrounding area, and these rats spent less time in the target quadrant (Fig. 3).

4.5. Effects of global cerebral ischemia-reperfusion injury on neurons in the hippocampal CA1 region

4.5.1. H&E staining results

In the sham-operated group, the neurons in the hippocampal CA1 area were arranged neatly in 4–5 layers, cells showed a clear structure and normal shape, the nucleus was round or elliptical, and the structure of the nucleolus and the nuclear membrane was apparent with uniformed staining. There was no significant difference in appearance of the neurons in the CA1 area of rats in the mild electrocoagulation group and the sham-operated group. However, in the moderate electrocoagulation group, the nucleus structure of neurons in the hippocampal CA1 area had disappeared substantially, exhibiting nuclear condensation and deep blue staining; the cell arrangement was disorganized and the number of normal neurons was small (Fig. 4).

4.5.2. Nissl staining results

In the sham-operated group and the mild electrocoagulation group, the neurons in the hippocampal CA1 area were large and round, the cytoplasm was evenly colored, and the Nissl bodies around the

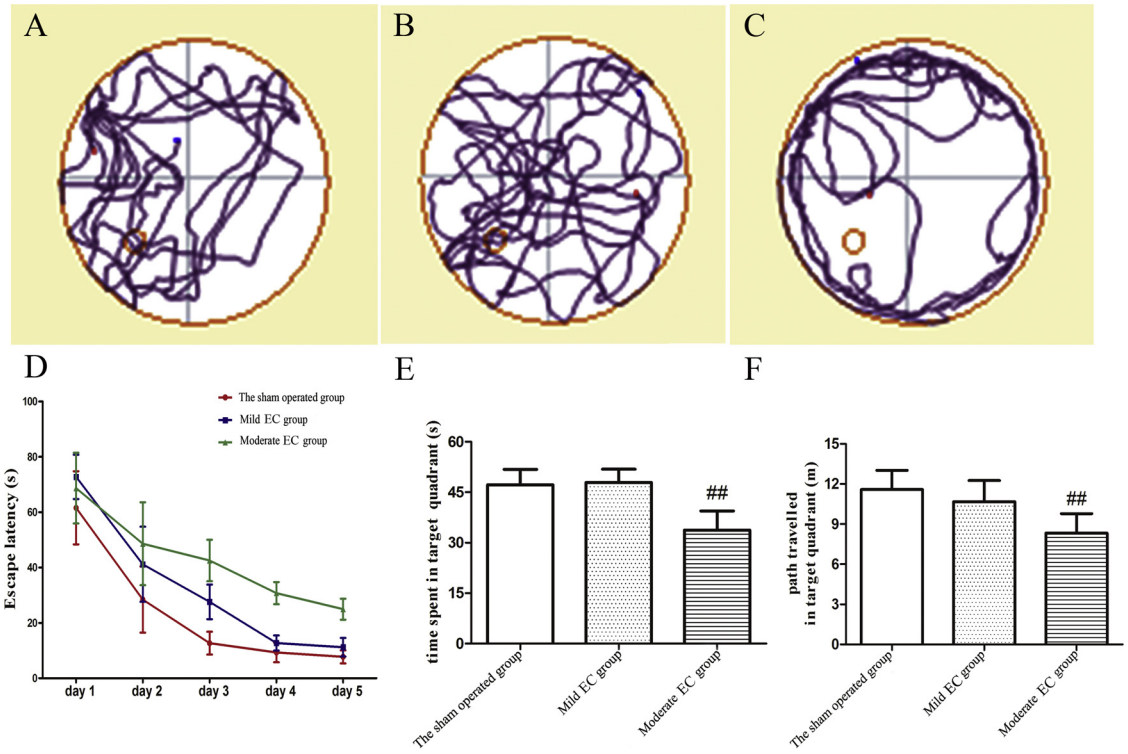

Fig. 3. The swimming trajectories of the rats during the probe trial. A. the sham-operated group. B. the mild electrocoagulation group. C. the moderate electrocoagulation group. D. the escape latencies of rats in the sham-operated group and the Pulsinelli's model groups during the five days of training. E. the time spent in the target quadrant of the rats in the sham-operated group and the Pulsinelli's model groups. D. the distances in the target quadrant of the rats in the sham-operated group and the Pulsinelli's model groups. Data are presented as mean ± SD. $^{\#\#}P < 0.01$ compared with the sham-operated group.

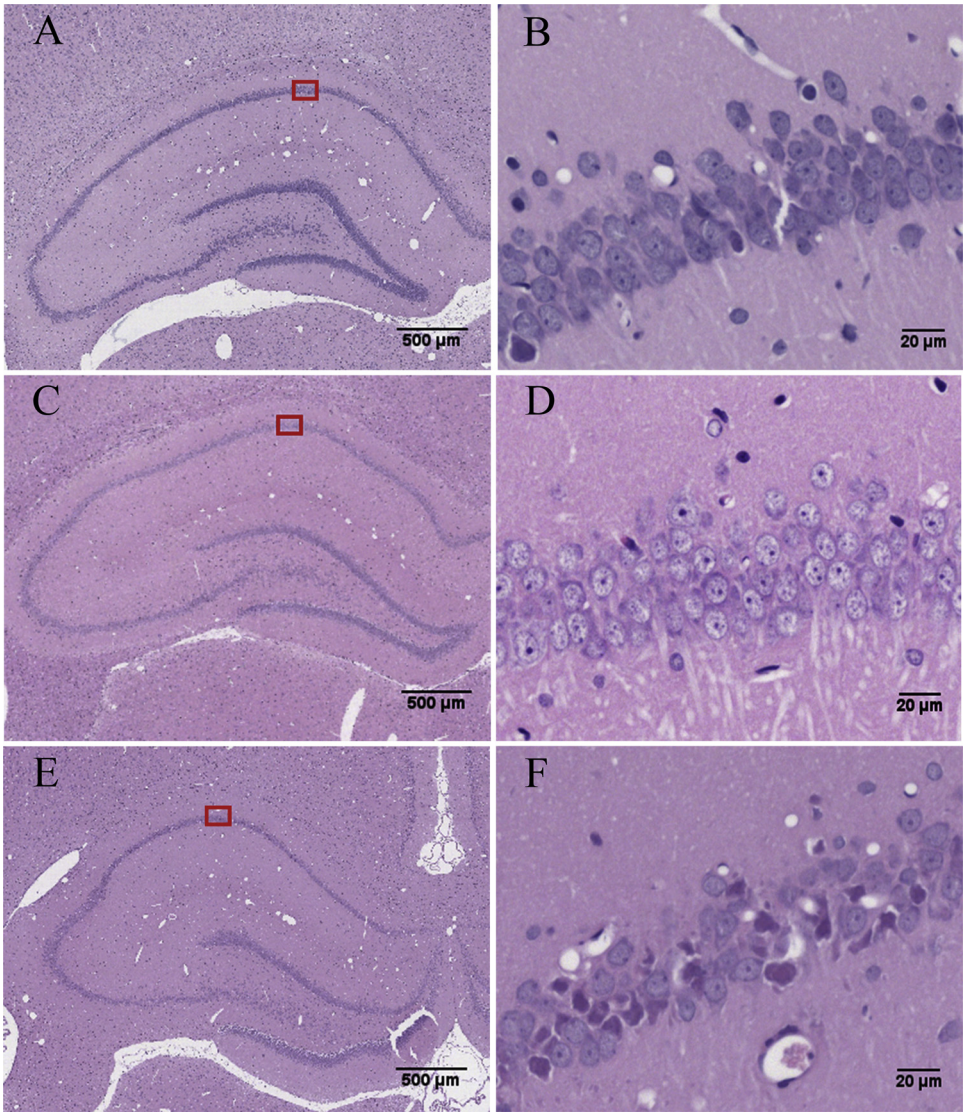

Fig. 4. Hematoxylin and eosin (H&E) staining in the hippocampal CA1 area of rat brain tissues in the sham-operated group and the Pulsinelli's model groups. A and B. the sham-operated group. C and D. the mild electrocoagulation group. E and F. the moderate electrocoagulation group.

cytoplasm were uniformly blue with intact and normal shape. In the moderate electrocoagulation group, the neurons in the hippocampal CA1 area were pyknotic, forming dense plaques; the gaps between cells became larger; the cell arrangement was loose; and the number of regular morphological neurons was small (Fig. 5). Neuron counting in the hippocampal CA1 area was performed at high magnification and analyzed by one-way ANOVA. The results showed that the moderate electrocoagulation group had significantly fewer normal neurons than either the sham-operated group or the mild electrocoagulation group ($F = 62.55$, $P < 0.01$) (Fig. 6).

5. Discussion

Pulsinelli's four-vessel occlusion is a common experimental model for global cerebral ischemia-reperfusion injury and vascular dementia. Through many years of experiments, we have found that it is difficult to control the degree of electrocoagulation and cerebral ischemia when performing electrocoagulation of the bilateral vertebral arteries. The result is often either high mortality of the animals in the early stage of the experiment or insufficient brain injury, detected only after observation of the neurological function later in the experiment.

Pulsinelli's four-vessel occlusion method lacks an objectively adjudicated guideline for determining the cerebral ischemia during electrocoagulation. This study found, using LDF to monitor blood flow, that cerebral blood flow decreased 26% ± 7% after electrocoagulation in rats in the mild electrocoagulation group, which indicated that the brain injury was insufficient and the model was unsuccessful. The cerebral blood flow decreased 44% ± 14% in rats with a low mortality rate in the moderate electrocoagulation group, which indicated that the brain injuries were sufficient and the model was successful. The cerebral blood flow decreased at 69% ± 7% in rats in the severe electrocoagulation group, and all of them died. In this study, we used LDF to monitor the decline in blood flow during and after electrocoagulation, allowing us to effectively control the degree of cerebral ischemia and thus the mortality of animals. With the addition of LDF monitoring, Pulsinelli's four-vessel occlusion can be used as a stable and reliable model for global cerebral ischemia-reperfusion injury.

At present, rodent animals such as rats are often used as experimental animals in studies simulating human cerebral ischemia domestically and internationally. The main reason is that the cerebral blood supply in rats is formed by the carotid artery and the vertebral-basilar artery joining together to form the arterial ring, which then branches to the local blood supply, a cerebrovascular anatomical structure similar to that of humans. Besides, rats are inexpensive and easy to obtain, and

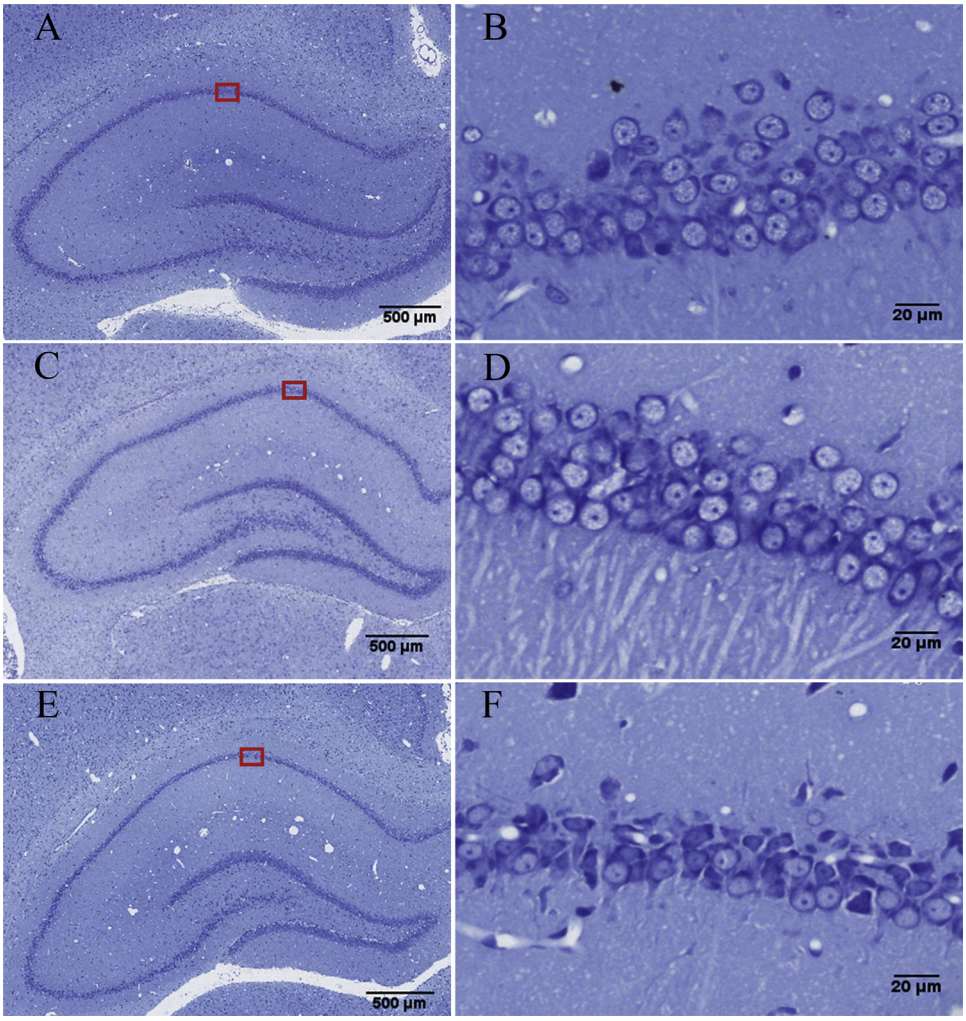

Fig. 5. Nissl staining in the hippocampal CA1 area of the sham-operated group and the Pulsinelli's model groups. A and B. the sham-operated group. C and D. the mild electrocoagulation group. E and F. the moderate electrocoagulation group.

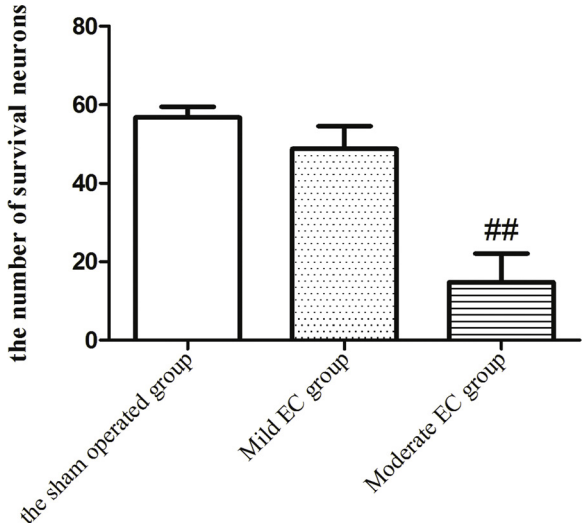

Fig. 6. Survival cell counts in the hippocampal CA1 area of the sham-operated group and the Pulsinelli's model groups. Data are presented as mean ± SD. $^{\#\#}$ $P < 0.01$ compared with the sham-operated group.

have been investigated in detail with abundant relevant research data (Minnerup et al., 2008). In addition, Pulsinelli's four-vessel occlusion is currently a recognized technique for the global cerebral ischemia model domestically and internationally. The surgical process begins with electrocoagulation of the vertebral artery, resulting in permanent occlusion, followed by the separation and clamping of the bilateral carotid arteries to create global cerebral ischemia. This method can simulate the process of global cerebral ischemic injury in the clinic practice and is also widely used in animal models internationally. However, the surgical procedures used in Pulsinelli's four-vessel occlusion are complex, especially in electrocoagulation of the bilateral vertebral artery. The position of the vertebral artery cannot be directly visualized and the degree of cerebral ischemia can only be adjudicated by the experimental experience of the researcher, with no objective guideline by observation.

Laser Doppler blood flowmetry is a continuous real-time blood flow monitoring technique that can reflect the total local microcirculatory blood perfusion (Toussay et al., 2019). It works by transmitting fiber optic light and monochromatic light to the brain tissue. The afferent optical fibers then receive different laser signals, including the scattered light from the moving red blood cells of the brain tissue and the reflective light of the stationary tissue structures, which serves as the reference light (Pafitanis et al., 2017). The blood flow in the brain tissue is obtained when the photodetector converts the optical signal into a flux signal (Sutherland et al., 2014). Laser Doppler flow monitoring techniques are widely used in the MCAO stroke model (Cuccione et al.,

2017; Ingberg et al., 2018) to monitor local brain tissue ischemia after tethering (Hedna et al., 2015), but has not been used in Pulsinelli's four-vessel occlusion method. Therefore, this study aimed to determine whether monitoring the decline of cerebral blood flow after electrocoagulation by LDF could predict whether the model was successful, in order to reduce the mortality of experimental animals and improve the efficiency of the experiment.

We used LDF to monitor the decline in blood flow after electrocoagulation. The experimental animals were divided into 3 groups according to the decline in blood flow. Mortality and degree of brain injury were then observed. The mNSS scores are comprehensive assessments of motor, sensory, reflexive and balance abilities (Ma et al., 2016), which can objectively reflect neurological deficits in rats (Shen et al., 2006). The Morris water maze test is the most commonly used cognitive behavioral method for detecting the learning and memory abilities of rodents, with high reliability (Ahmadi et al., 2017; Oz et al., 2017). The hippocampus is the brain area most relevant to spatial learning and memory abilities and a region vulnerable to hypoxic conditions (Deng et al., 2010; Deuker et al., 2016; Schmidt-Kastner, 2015; Schmidt-Kastner and Freund, 1991). Therefore, this study used the mNSS score, Morris water maze test and number of normal neurons in the hippocampus to assess brain injury.

Previous studies have found that a 45% reduction in cerebral blood flow affects the energy metabolism of the brain (Eklof and Siesjo, 1972); therefore, we segregated electrocoagulation according to a decline in cerebral blood flow of $< 45\%$, 45% and $> 45\%$. Our results showed that the average blood flow of rats in the three groups decreased after electrocoagulation of the bilateral vertebral arteries: $26\% \pm 7\%$ in the mild, $44\% \pm 14\%$ in the moderate and $69\% \pm 7\%$ in the severe electrocoagulation group. The rats in the severe electrocoagulation group all died. This result suggests that electrocoagulation of the bilateral vertebral artery to a blood flow decline of $69\% \pm 7\%$ may result in excessive mortality. None of the rats in the experimental group with a blood flow decline of $26\% \pm 7\%$ after electrocoagulation died, but the results of the mNSS, the water maze test and Nissl staining showed a low degree of brain injury, indicating that this occlusion model was unsuccessful. Only the rats in the experimental group with a decrease in blood flow of $44\% \pm 14\%$ after electrocoagulation had low mortality and results of mNSS, the water maze test and Nissl staining showing a high degree of brain injury, indicating that this occlusion model was successful.

LDF is not the only instrument available to detect cerebral blood flow; laser speckle contrast imaging is also commonly used to observe cerebral ischemia (Junyun et al., 2015; Wood et al., 2016). However, due to the thick skull of the rat, the skull bone must be mechanically thinned or removed when using the laser speckle instrument for observation, a laborious and time-consuming task that is prone to damage the brain tissue and blood vessels. Once a circular section of the skull is removed, the experimental rats are susceptible to infection after surgery and cannot perform the Morris water maze test. In contrast, LDF is a simpler technique for dynamic monitoring of cerebral blood flow. In this study, LDF was used at the same point to observe the changes in blood flow both before and after electrocoagulation of the vertebral artery, to obtain the relative value of the declined cerebral blood flow and to predict the brain injury of SD rats after electrocoagulation. This method can reduce the mortality of experimental animals and minimize the damage to them, so that the follow-up Morris water maze test can be carried out. It also holds the potential to enhance the experimental efficiency and improve the availability of experimental animals.

In summary, LDF monitoring revealed that a decline in cerebral blood flow of $44\% \pm 14\%$ after electrocoagulation was most suitable for the Pulsinelli's four-vessel occlusion method, to minimize the mortality of animals and ensure the success of this model. We also confirmed that this method is both feasible and easy-to-use.

Declaration of Competing Interest

None.

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