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A Study on the Extent of Brain Tissue Damage at Different Reperfusion Time Points Using a Rat Model of Cerebral Ischemia-Reperfusion Injury

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Abstract: *This study explores the development of a rat model for cerebral ischemia-reperfusion injury and evaluates the effects of varying reperfusion durations on the severity and progression of the injury. The findings aim to provide a scientific model for the advancement of more effective prevention and treatment strategies. Male Sprague-Dawley rats, each weighing approximately 260 grams, were utilized as experimental subjects. Following a one-week acclimatization period, surgical procedures were conducted. The middle cerebral artery was occluded using a suture for 90 minutes, followed by reperfusion. Brain tissue samples were collected at 1, 7, and 14 days post-reperfusion.We monitored the body weight of rats and employed the Neurological Deficit Score to assess behavioral changes across different groups. Hematoxylin and eosin (HE) staining was utilized to examine pathological damage in brain tissue at various time points, providing clear visualization of brain cell morphology. The 2,3,5-triphenyltetrazolium chloride (TTC) staining method was used to evaluate cerebral infarction volumes at different time intervals. The rats exhibited different body weight changes and neurological deficits at different time points. The volume of cerebral infarction and the extent of pathological damage in rat brain tissue exhibited significant variation across different time points. HE staining demonstrated nuclear condensation, fragmentation, and dissolution in rat brain tissue at one day post-reperfusion. By seven days post-reperfusion, irregular cellular arrangement persisted, accompanied by extensive vacuolar edema and degeneration. At fourteen days post-reperfusion, vacuolar edema decreased, although nuclear condensation, fragmentation, and dissolution continued. TTC staining revealed both red-stained normal tissue and white-stained infarct tissue at one day post-reperfusion. At seven days post-reperfusion, the volume of white infarct tissue increased further, accompanied by swelling of brain tissue. By fourteen days post-reperfusion, the volume of white cerebral infarction tissue decreased, although brain tissue remained swollen. These findings provide crucial insights into the pathogenesis of ischemic-reperfusion injury in rats, significantly enhancing our understanding of the mechanisms underlying reperfusion injury during cerebral ischemia.These findings contribute novel insights and theoretical underpinnings for the prevention and management of reperfusion injury in cerebral ischemia.*

Keywords: *MCAO/R, Ischemic Stroke, Cerebral Cortex, SD Rat*

1. Introduction

The cerebral ischemia-reperfusion injury model is of indispensable importance in the investigation of the pathological mechanisms underlying neurological diseases[1]. A substantial body of research has demonstrated that this model not only replicates the pathophysiological processes associated with cerebral ischemia-reperfusion injury following clinical ischemic stroke but also serves as an optimal animal experimental model for investigating the pathogenesis of central nervous system-related disorders, such as post-stroke depression[2-4]. In the context of research on diseases associated with cerebral ischemia-reperfusion injury, the use of Sprague-Dawley (SD) rats to establish a cerebral ischemia-reperfusion model offers significant advantages. The cerebrovascular anatomical structure of SD rats closely resembles that of humans, and the model thus established exhibits high stability and reliability, coupled with strong surgical feasibility. Furthermore, by standardizing surgical procedures, the mortality

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rate of the rats can be effectively minimized[5, 6].We recorded the body weights and neurological deficit scores of rats at different time points. After establishing the cerebral ischemia-reperfusion injury model, HE staining and TTC staining experiments were conducted at different time points. HE staining allows for the observation of pathological damage in brain tissue, while TTC staining enables the measurement of cerebral infarction volume. Together, these two methods provide morphological evidence for the success of model construction[7-9].Furthermore, establishing this model aids in deeply unveiling the key molecular regulatory networks underlying neuro-related diseases. Relevant literature indicates that the cerebral ischemia-reperfusion injury model can also be applied to investigate the pathogenesis of post-stroke depression, providing crucial theoretical and experimental support for elucidating the pathogenesis of post-stroke depression and developing targeted intervention strategies[10, 11].Currently, the construction of cerebral ischemia-reperfusion injury models still faces numerous technical challenges. Based on the current research status both domestically and internationally, there are significant methodological differences in core operational procedures, control of reperfusion time points, and standardized evaluation systems for model success. Simultaneously, this brings both challenges and opportunities to research in this field.

2. Experimental Materials

2.1 Experimental Animals

Eighteen SPF-grade adult male SD rats (provided by Guangdong Weitong Lihua Experimental Animal Co., Ltd.) were housed separately. After a 7-day acclimation period, surgical procedures were carried out. All experimental procedures were conducted in strict adherence to the “3R principles” (Replacement, Reduction, and Refinement) and the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.2 Reagents and Instruments

HE staining solution (Solarbio Science & Technology Co., Ltd.); 2,3,5-triphenyltetrazolium chloride (TTC) staining solution (Regen Bio-Technology Co., Ltd.); isoflurane (RuiPu Bio-Technology Co., Ltd.); upright fluorescence microscope (Model: ECLIPSE CI-L); cryostat microtome (Model: Crystar NX50OPD).

3. Methods

3.1 Establishment of cerebral ischemia-reperfusion injury model in rats

The rats underwent a 7-day acclimatization period and were subjected to a 24-hour fasting regimen prior to the induction of the model, while maintaining normal water intake. The Zea-Longa[12] method was employed to establish a rat model of right middle cerebral artery embolism. The procedure was conducted as follows: Following anesthesia, the rats were positioned supine on the surgical table. A midline incision was made on the neck, and the muscles and mucosa were carefully dissected. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were identified and isolated, as depicted by green, blue, and orange arrows in Figure 1A. The ECA was ligated near the bifurcation of the CCA (Figure 1B, indicated by the orange arrow), followed by ligation of the CCA distal to the bifurcation (Figure 1C, indicated by the orange arrow). The ICA was temporarily occluded using a microvascular clamp, and a small incision was made above the CCA ligation site to facilitate the insertion of the thread embolus. A slip knot was tied above the entry point of the thread embolus into the CCA. The vascular clamp was subsequently released, and the thread embolus was carefully advanced along the internal carotid artery until the designated marking point reached the bifurcation of the common carotid artery, The thread embolus was then secured using a ligature (refer to Figure 1 D, indicated by the orange arrow), the excess suture material was trimmed, and the surgical wound was closed. Following 1.5 hours of occlusion of the middle cerebral artery, the thread embolus was meticulously withdrawn to initiate reperfusion. Post-surgery, the rats were placed in a temperature-controlled incubator. Upon regaining consciousness, their neurological deficits were evaluated using the Longa scoring system[13].

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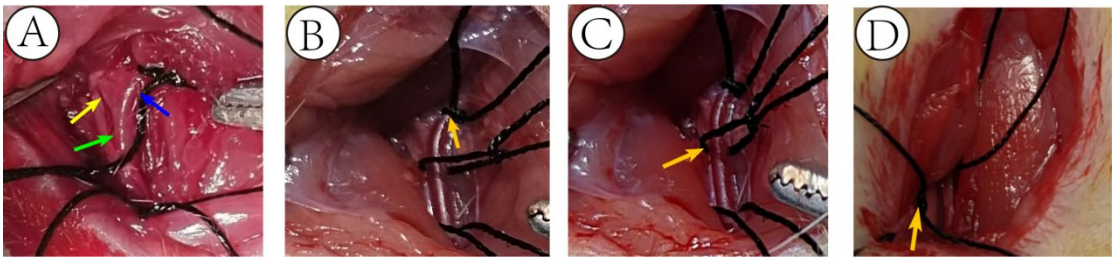

Figure 1: Establishment of a rat cerebral ischemia-reperfusion injury model. A: Intraoperative exposure and identification of cervical vasculature. B: Ligation of the ECA at the level of the CCA bifurcation.C: CCA ligation at a site 2 cm from the carotid bifurcation.D: Insertion and fixation of the intraluminal filament

3.2 Neurological function assessment and body weight measurement

Measure the body weight of rats at the corresponding time points and assess their neurological deficit using the Zea-Longa 5-point scale. This scoring system is defined as follows: a score of '0' denotes normal limb motor function; '1' indicates partial impairment in the affected forelimb, characterized by an inability to fully extend; '2' signifies spontaneous circling towards the paretic side during locomotion; '3' represents a loss of balance, resulting in lateral falls to the affected side during ambulation; and '4' indicates a complete inability to ambulate independently, accompanied by disturbances in consciousness. According to this scoring system, rats with scores ranging from 1 to 3 were considered to have successfully developed a model, making them suitable for subsequent experimental procedures.

3.3 TTC Staining of Rat Brain Tissue

Tissue On the 1st, 7th, and 14th days following cerebral ischemia-reperfusion injury in rats, the brain tissues were meticulously excised from the animals post-anesthesia. The excised rat brain tissues were promptly placed in a -20℃ freezer for 20 minutes to achieve solidification. Subsequently, the frozen brain tissues were sectioned coronally on ice to produce coronal slices approximately 2 mm in thickness. These sections were immersed in a 2% TTC staining solution, wrapped in aluminum foil to prevent light exposure, and incubated at 37℃ for 32 minutes. To ensure consistent staining, the brain slices were rotated every 8 minutes. The red-stained slices indicated normal brain tissue, whereas white-stained slices signified cerebral infarction tissue. Each stained slice was photographed individually to assess the volume of cerebral infarction.

3.4 HE Staining of Rat Brain Tissue

On the 1st, 7th, and 14th days following cerebral ischemia-reperfusion in rats, the animals were anesthetized and subjected to cardiac perfusion with paraformaldehyde to facilitate brain extraction. The harvested brain tissue was subsequently fixed in 4% paraformaldehyde for a duration of 24 hours. Thereafter, the tissue underwent gradient dehydration using sucrose solution. Coronal sections were prepared and embedded in OCT compound, followed by cryosectioning into 10 μm thick slices, which were mounted on glass slides. Hematoxylin and eosin (H&E) staining was performed to observe morphological changes in the brain tissue under an optical microscope.

3.5 Statistical Analysis

This study used SPSS 27.0 statistical software for data analysis. Measurement information was expressed as mean ± standard deviation ($\overline{x}\pm s$), and independent samples t-test was used for comparison between two groups, and analysis of variance (ANOVA) was used for comparison between multiple groups. Data are expressed at least three independent experiments.

4. Results

4.1 Neurological function assessment and body weight measurement results

Body weight measurements and neurological deficit assessments were conducted on postoperative

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days 1, 7, and 14. Rats exhibited varying degrees of neurological deficits at different time points, with deficits reaching their most severe stage on day 7. Body weights across all groups remained different, showing a gradual decrease over time, as shown in (Table 1).

Table 1. Results of Neurological function assessment and body weight measurement (n=8, $\bar{x}\pm s$)

Group	*body weight*	Longa scores
1day	248.90±4.65	1.50±0.76
7day	206.40±3.57	1.75±0.71
14day	202.40±4.22	1.63±0.92

4.2 Results of TTC Staining

Following TTC staining, the red regions indicated normal brain tissue, whereas the white regions signified areas of cerebral infarction. At 1 day post-reperfusion, both normal red tissue and white infarcted tissue were observed in the brain (Figure 2A). By 7 days post-reperfusion, an increase in the volume of white infarcted tissue was noted, along with evidence of brain swelling (Figure 2B). At 14 days of reperfusion, the volume of white cerebral infarction tissue decreases, but brain swelling persists (Figure 2C).

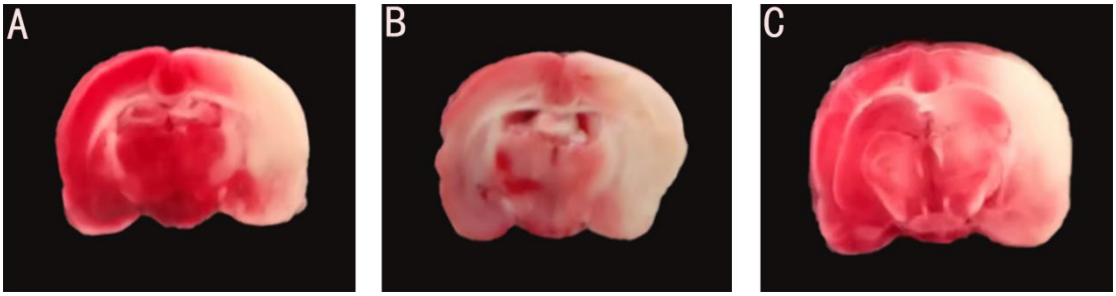

Figure 2: Rat brain tissue after TTC staining at different time points. A: TTC staining image of rat brain tissue at 1 day after reperfusion. B: TTC staining image of rat brain tissue at 7 days after reperfusion. C: TTC staining image of rat brain tissue at 14 days after reperfusion

4.3 Results of HE staining

To further determine the pathological damage to brain tissue, brain tissue samples from different time points were selected for hematoxylin and eosin (HE) staining. As shown in Figure 3, the upper panel represents the brain tissue before OCT embedding, while the lower panel shows the rat brain tissue after HE staining at different time points. At 1 day of reperfusion, irregular arrangement of cells in the rat brain tissue was observed, with most cells showing phenomena such as nuclear condensation, nuclear fragmentation, and nuclear dissolution (Figure 3A).After 7 days of reperfusion, it was observed that the arrangement of cells in the rat brain tissue remained irregular, with most cells exhibiting significant vacuolar edema and degeneration (Figure 3B). After 14 days of reperfusion, it was found that the cells in the rat brain tissue still exhibited considerable vacuolar edema, along with phenomena such as karyopyknosis, karyorrhexis, and karyolysis (Figure 3C).

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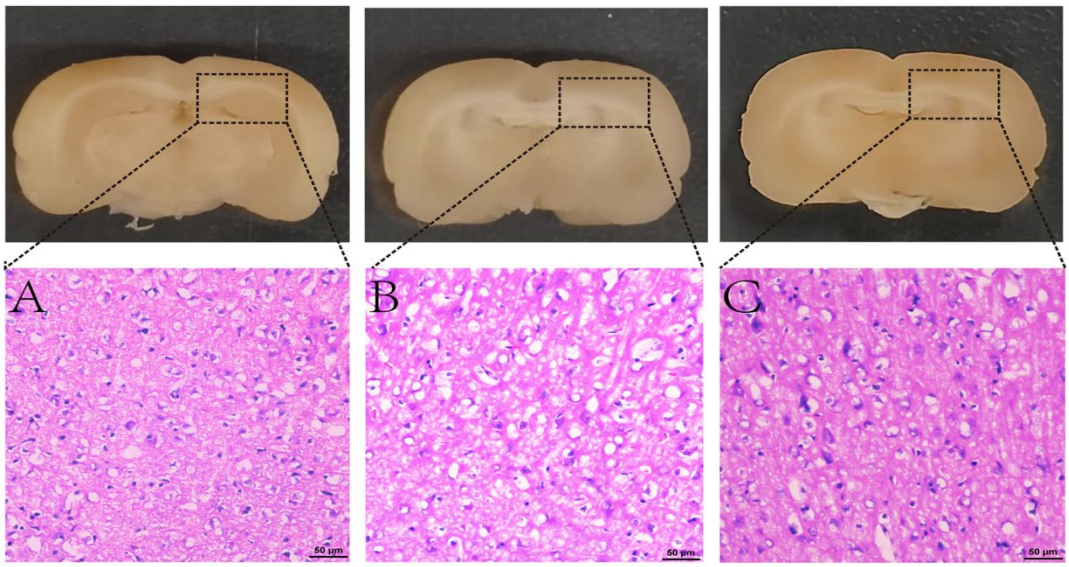

Figure 3: The top panel shows the rat brain tissue before embedding at different time points, while the bottom panel displays the rat brain tissue after HE staining at different time points(40×10). A: HE staining image of rat brain tissue at 1 day after reperfusion B: HE staining image of rat brain tissue at 7 days after reperfusion C: HE staining image of rat brain tissue at 14 days after reperfusion

5. Discussion

The rat model of cerebral ischemia-reperfusion injury is of considerable research significance in the investigation of neurological diseases. This model has consistently served as a valuable tool for elucidating the pathogenesis of neurological disorders, identifying potential therapeutic targets, and facilitating drug development[14, 15]. Moreover, comprehensive research on this model is anticipated to advance our understanding of ischemic stroke pathogenesis and expedite the development of effective therapeutic interventions. Despite its utility, the current body of research concerning the modeling methodology, core operational procedures, control of reperfusion timing, and the establishment of a standardized evaluation system for model success remains relatively underdeveloped. Inadequate control of the reperfusion time point has resulted in increased mortality rates among rats, and some studies have opted to euthanize rats shortly after successful modeling to mitigate concerns about high mortality, leading to unnecessary animal use[16-18]. Consequently, it is imperative to investigate the pathological damage occurring at various time points to optimize the model's efficacy and ethical application.The selection of animals for the rat cerebral ischemia-reperfusion injury model is of paramount importance. Rats are frequently employed in replicating experimental models of cerebral ischemia due to their anatomical and functional cerebrovascular similarities to humans, robust vitality, and high resistance to infection[5]. The most commonly used experimental rat strains include SD rats and Wistar rats. In this study, SD rats were preferred over Wistar rats due to several practical advantages: reduced intraoperative hemorrhage, enhanced surgical accessibility owing to clearly exposed cervical vasculature, and decreased variability in the middle cerebral artery[6, 19]. These anatomical features facilitate the consistent formation of stable parietotemporal cortical infarcts with larger ischemic necrotic areas following middle cerebral artery occlusion. Additionally, to mitigate the confounding neuroprotective effects of estrogen, only male rats were utilized, thereby ensuring experimental consistency and objectivity[20].In relation to the body weight of surgical rats, a low body weight results in excessively thin intracranial vessels, complicating the insertion of the thread embolus and thereby increasing the complexity of the surgical procedure. Conversely, an excessively high body weight leads to thickened and deformed vessel diameters, rendering it unsuitable to completely occlude the middle cerebral artery blood flow with the thread embolus, which may result in model failure[21, 22]. Research has indicated that maintaining the body weight of rats within the range of 250 to 300 grams is optimal[23]. Consequently, this study utilized male Sprague-Dawley rats weighing approximately 260 grams for experimentation.

Following the selection of the rats, model preparation commenced.The cerebral ischemia-reperfusion injury model was established using the middle cerebral artery embolization technique. The procedure commenced with the preparation of the rat's neck skin, followed by the systematic separation of the skin

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and subcutaneous tissue in layers. Subsequently, the common carotid artery, external carotid artery, and internal carotid artery were carefully isolated in proximity to the trachea (Figure 1a). The external carotid artery was then ligated (Figure 1b), succeeded by the ligation of the common carotid artery (Figure 1c). A vascular clamp was applied to the internal carotid artery, and an incision was executed above the ligation site of the common carotid artery. Through this incision,a polyester fiber thread with a rounded tip was introduced into the common carotid artery, and a slipknot was fashioned above the thread's entry point. Upon releasing the vascular clamp, the thread was gradually advanced along the internal carotid artery. Once the thread reached a depth of approximately 18-20 mm, it was secured in place (Figure 1d). The middle cerebral artery was occluded for a duration of 1.5 hours, after which the thread was carefully withdrawn.Subsequent evaluations of body weight and neurological deficits indicated that rats experienced significant changes in body weight and neurological impairments at various time points(Table 1). Histological examinations usingTTC (Figure 2) and HE (Figure 3) staining were subsequently conducted to validate the successful establishment of the model, revealing differential degrees of pathological damage in brain tissue at each time point.

In summary, this study presents a straightforward and practical model with high success rates, illustrating varying degrees of brain tissue damage at different reperfusion intervals. The model's success is corroborated byTTC and HE staining, which demonstrate distinct pathological damage at each time point. This model exhibits excellent stability, fulfilling the requirements for subsequent experimental investigations. These findings are of significant importance for advancing our understanding of the pathogenesis in cerebral ischemia-reperfusion injury models, providing new insights and theoretical foundations for the prevention and treatment of this condition.

Author information

Mengxue Zang and Xichao Zang contributed equally to this work and share first authorship.The authors declare no conflict of interest

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