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Challenging the creation of middle cerebral artery occlusion in rats and factors causing its failure

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Abstract

Stroke is a cause of death and disability in the world. Animal research needs animal models that are as similar as possible to the pathophysiological processes that occur in humans. The established ischemic stroke model is the middle cerebral artery occlusion (MCAO) model. The creation of this model requires sophisticated equipment and precision. Our study aims to describe the MCAO modeling procedure using simple tools and to identify causes of death. Creation of ischemic stroke animal models with MCAO procedure with simple equipment and using monofilament nylon suture 4.0 to clog the middle cerebral artery and reperfusion for 90 minutes. Neurological deficits are clinically tested with Bederson parameters after one hour of MCAO. 2.3.5 triphenyl tetrazolium chloride staining was performed to determine the area of infarction after 24 hours of MCAO. The histological picture is examined with hematoxylin-eosin staining. Two rats succeeded in modeling ischemic stroke, two rats failed in modeling ischemic stroke, two rats died during preparation, four rats died during surgery, and 10 rats died several hours after occlusion. The most common cause of death was bleeding from 4.0 nylon sutures, which are too long to pass through the middle cerebral artery, thereby puncturing the blood vessels. When creating a model of ischemic stroke, it is essential to consider the elements that contribute to rat failure and death.

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Introduction

Stroke is the primary global contributor to mortality and the second most common cause of disability (1). An ischemic stroke makes up approximately 85% of all strokes, with hemorrhagic stroke representing the remaining 15% (2). Stroke may lead to long-term disability, ultimately impacting the individual's ability to work efficiently and increasing the overall expenses associated with their care (3). The degree of functional ability restoration significantly affects the financial burden of stroke therapy (4). Thus, in vivo research needs to be developed to understand the mechanism and treatment of stroke. Most in vivo studies use ischemic stroke models. Ischemic stroke is a disease with a complex pathophysiology, so it is not easy to replicate all clinical aspects in humans as depicted in animal models (5,6). Each model has unique strengths and weaknesses (6). The middle cerebral artery occlusion (MCAO) model is widely regarded as the most representative human ischemic stroke model and is used in more than 40% of clinical trials, i.e., using intraluminal sutures to induce occlusion of the middle cerebral artery (MCA) in rodents is a well-known and highly standardized animal model (7). In existing research, the manufacture of MCAO requires a Doppler laser device to measure brain blood flow. It requires a monofilament-type silicone-coated 4.0 monofilament nylon suture (Doccol Co.)

(8-10), a rectal probe for body temperature monitoring, and a homeothermic blanket control unit to guard it (11). MCA can be blocked in various ways, the most common approach being to implant monofilaments, which carry a high risk of death in their manufacture (12). Currently, a technique is being developed by inserting monofilaments through the internal carotid artery (ICA), and it has been shown to reduce mortality and minimize variability (13). The MCAO method that utilizes the silicon monofilament technique is a minimally invasive intervention (10). In developing countries, such as Indonesia, research challenges in making MCAO models are the unavailability of advanced equipment, the difficulty of obtaining silicon-coated nylon suture 4.0 monofilament, and the high mortality risk during the creation of ischemic stroke models. Studies on strokes using model animals still need to be developed in Indonesia. The studies are on ischemic stroke models with permanent flame-blunted monofilaments (14), a stroke model study with a simple method that allows it to be applied in Indonesia (15), and a survey of green tea on the MCAO model (16,17). In our previous research on the effects of high-intensity interval training in model stroke (18), we found the enormous challenge of creating an ischemic stroke model with 90-minute reperfusion. It is this model of ischemic reperfusion stroke that simulates patients with vascular occlusion and timely recanalization as it does in humans (17).

The challenges we found in previous studies, namely the unavailability of advanced equipment, the unavailability of silicon-coated monofilaments, and the high mortality risk, therefore our study aims to describe the MCAO modeling procedure using simple tools and to identify causes of death monofilament was inserted directly through the ICA instead of the external or common carotid arteries (CCA). One of the CCA's terminal branches is the ICA (19). Second, the length of the silicone coating on the monofilament was extended to enable the embolization of the initial segments of the MCA, the anterior cerebral artery, and a portion of the posterior communicating artery (12).

Materials and methods

Ethical approve

The Faculty of Medicine Ethics Committee, University of Indonesia-Cipto Mangunkusumo Hospital, approved all veterinary procedures (KET-762/UN2.F1/ETIK/PPM.00.02/2022). Every effort was made to minimize the number of animals used and to ensure their overall well-being.

Animals

Wistar male rats aged 16–18 weeks weighing 280–400 g was obtained from Biofarma Bandung. Twenty animals were used in the optimization phase of this MCAO model creation. The rats were placed individually in day and night/12-h cycles (20) in a temperature-controlled environment at the Animal Facilities Research of Indonesia Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia.

Middle cerebral artery occlusion

The MCAO procedure in our study followed previous research (15). After an acclimatization period (21,22), the animals were transferred from the maintenance room to the operation preparation room. They were weighed first and then given anesthesia with ketamine (40 mg/kg) and xylazine (4 mg/kg) (23). After the animals slept, epilation of the neck area was carried out. The animals were then transferred to the operating room. The animals were in a prone position and fixed to the surgical table with adhesive tape. The procedure shown in figure 1 started with a 2–3 cm incision on the midline of the neck. Subsequently, the subcutaneous tissue and platysma muscles were dissected.

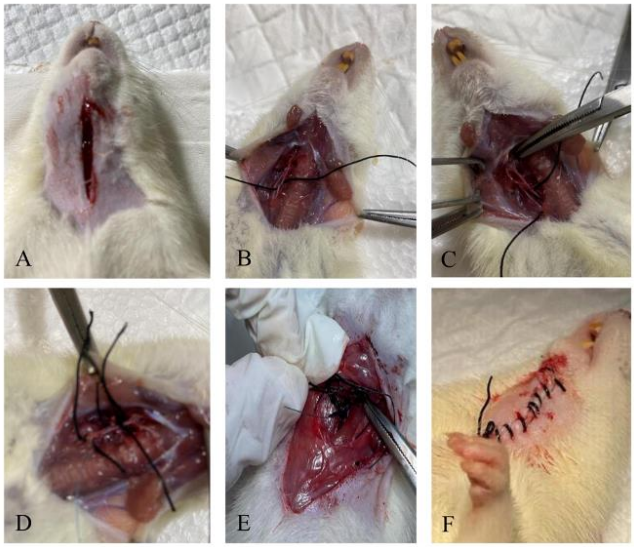

Figure 1: Stroke model-making procedure with MCAO technique, incision on the front neck A), connective tissue and muscle dissection for left CCA ligase B), ligase ECA C), ligase ICA and PPA D), Insects nylon suture 4.0 E), incision sutured back F).

The common carotid artery (CCA) was sought through gland cutting and dissection of the digastric and sternohyoid muscles. The CCA was isolated from the vagus nerve and connective tissue. CCA was ligated with a 4.0 silk suture. Afterward, we located the external carotid artery (24) and ICA. The ECA and CCA were ligated with a 4.0 silk suture. Moreover, we traced the pterygopalatine artery (25) and ligated it with a 4.0 silk suture. A small hole was made in the ICA with the tip of a 1-cc syringe. When creating an aperture, it is essential to exercise caution to prevent the occurrence of hemorrhaging. Then, a 4.0 nylon suture was

inserted into the blood vessels of the MCA through a small hole in the ICA. The 4.0 nylon suture was inserted carefully into the ICA to prevent bleeding.

The 4.0 nylon suture was maintained in the blood vessel for 90 min. Observations were made during occlusion. After 90 minutes, the 4.0 nylon suture was unplugged. If bleeding occurs while removing the 4.0 nylon suture, bleeding should be stopped immediately. Once the bleeding stops, the incision should be stitched back. Lidocaine gel was applied to the wound to relieve pain and discomfort after surgery, and 0.5 ml of saline was given intraperitoneally. After surgery, the animals were housed individually for 24 hours with free access to water and food and then returned to their home cages.

Behavior deficit neurology

Clinically, the effect of MCAO was confirmed with neurological deficits (26). A rating scale of 0–5 was used to evaluate neurological outcomes, with slight modifications made by Bederson (27). Normal rats were given a score of 0, while dead or unresponsive rats were scored 5. A score of 1 was shown in the case of forelimb defects, 2 in the case of a deformity of the front legs and body swing, 3 in the case of a defect in the forelimbs, body swing, and rotation, 4 in the case of forelimb defects and decreased level of consciousness and 5 forelimb defects and unresponsiveness to stimuli or death. The evaluation was performed one hour after the MCAO procedures.

Infarct area assessment

Twenty-four hours after MCAO, euthanasia was performed by giving the rats lethal dosages of ketamine and xylazine. A surgical procedure involving the skull bone was conducted as the rats slept. The brain was removed from the animals, put in a brain blocker, and then cut into 2-mm-thick coronal sections at bregma −2.00. The infarct area assessment procedures followed earlier research (10). The segment was stained for 20 min at 37°C in a 0.5% 2.3.5 triphenyl tetrazolium chloride (TTC) saline solution. TTC staining was utilized to estimate the size of a brain infarction (28). The white color showed the infarction area, which was analyzed with ImageJ software.

Histopathology evaluation

Twenty-four hours after MCAO, the animals were sacrificed, and the brain slices were post-fixed with 4% paraformaldehyde in Phosphate Buffer Saline (pH 7.4). The paraffin-embedded brains were cut into 5µm sections (29,30). The staining was done using hematoxylin and eosin (HE) (31). This staining is to determine cellular morphology after MCAO. The HE procedure followed that from the histology laboratory of the Faculty of Medicine, Universitas Indonesia. Finally, a light microscope examined every slide (32,33).

Statistical analysis

The narrative analysis was presented using GraphPad Prism 9.0. Research data were collected and assessed before statistical analysis was conducted (34). The data from the clinical examination of neurological deficits and the area of infarction are presented as graphs.

Results

Twenty rats underwent MCAO surgery. Two rats successfully modelled ischemic stroke, two rats failed to model ischemic stroke, two rats died during preparation, four rats died during the operation, and ten rats died a few hours after being blocked. We conducted a neurologic deficit assessment utilizing the Bederson criteria. Clinically examined neurological deficits were given to rats one hour after the MCAO. In addition, following a twenty-four-hour period after MCAO, the animals were euthanized, and brain tissue was procured. To ascertain the extent of infarction within the brain, TTC staining was executed. The results of the Bederson examination are shown in figure 2, and TTC staining is shown in figure 3. When the nylon suture was inserted into a blood vessel through the ICA to the MCA, the 4-0 nylon suture would sometimes penetrate the blood vessel, causing bleeding in the brain area, as figure 4 shows bleeding and a pale brain color. We performed HE staining to determine the histopathological features of rat brains with MCAO models and hemorrhage rat brains. Figure 5 shows the results of HE staining in rat brains 24 h after MCAO. Hemorrhage showed neuron cell damage characterized by an irregular shape and a shrinking size in the cortical brain areas.

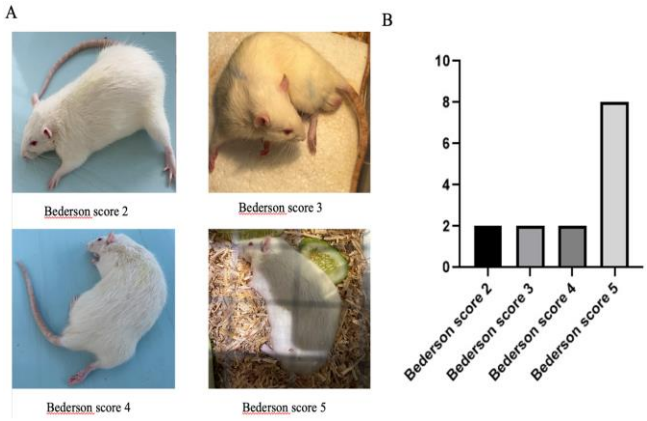

Figure 2: Result of deficit neurologist examination, Bederson score 2,3,4 dan 5 A), Sum of each of Bederson's scores B).

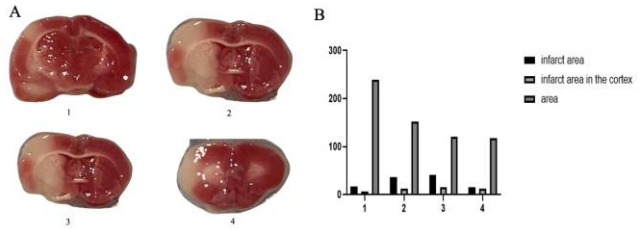

Figure 3: TTC staining results in cerebral artery occlusion stroke model after 24 hours of occlusion, white color indicates infarction area; 2-mm-thick coronal sections at bregma −2.00 A), comparison of infarction area value, infarction in the cortex and area B).

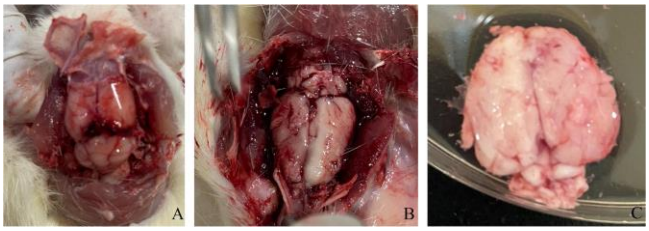

Figure 4: Intracranial hemorrhage occurs when nylon sutures penetrate blood vessels; cranial bones are opened, showing a blood clot in the brain A); bleeding indicating the area of infarction, still in the bones of the skull B); brain tissue that has been transferred to a dish C).

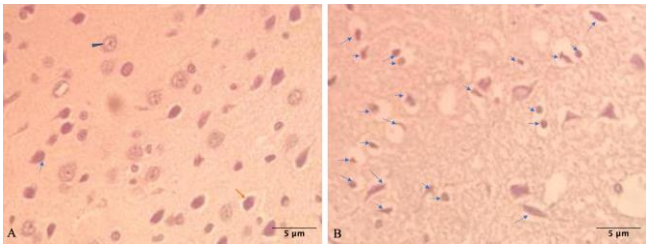

Figure 5: Haematoxylin and Eosin staining at 24 hours after MCAO, without bleeding: pyramidal neuron (arrow); nonpyramidal neurons, round nucleus, and nucleoli (arrowhead); shrunken nucleus of the neurons (red arrows) A); after MCAO with bleeding: pyknosis and shrunken nucleus of the neurons (arrow) B) x400. MCAO: middle cerebral artery occlusion.

Discussion

Our study aims to describe the MCAO modelling procedure using simple tools and to identify causes of death. Twenty rats had MCAO surgery; of these, ten rats died a few hours after the operation, four rats died in the operating room, two rats died in the preoperative period, two rats were unable to mimic an ischemic stroke, and two were successful in DOIng so. The factor that led to the death of 10 rats after a few hours of surgery and four rats during surgery was bleeding due to nylon-suture monofilament that pierced the blood vessels. We created the MCAO model using the anesthetic ketamine–xylazine. Administering ketamine–xylazine through subcutaneous injection is an efficient anesthetic method for MCAO rat models, offering numerous benefits, including minimal mortality rates, cost-effectiveness, safety, convenience of administration, and the absence of any specialized equipment requirements (35).

The filament used in this study was a 4.0 nylon suture instead of a silicone-coated 4.0 monofilament nylon suture because obtaining it in Indonesia is challenging. The most commonly used monofilaments are Doccol® monofilaments (36-38). A 4-0 monofilament nylon suture with a silicone-coated tip is inserted 18–20 mm into the lumen of the ICA, with the body weight of the rats being 280–320 g (39) and 200±20 g (40). At the beginning of our research, we used a 4-0 nylon suture with a length of 2,5–3,0 mm, with the body weight of the rats being 280–400 g. We did this to facilitate the entry of monofilaments into the ICA, considering that the rats we operated on weighed up to 400g. Next, we use nylon suture monofilament with a 1.8-2.2 mm length. The choice of monofilament size should be based on the weight of the rats utilized in the investigation (38).

The stroke model was created with the MCAO technique using simple equipment and a 4-0 nylon suture, which resulted in the induction of clinical manifestations characterized by a Bederson score of 3. The Bederson score for rats that experienced bleeding ranged from 4-5 and 2 for rats that failed to demonstrate an ischemic stroke model. TTC results showed an infarct area consistent with MCA vascularization. In modeling the MCAO, inserting a 4-0 nylon suture into the blood vessels through the ICA to the MCA is the most challenging technique. This technique requires a high level of skill and a sense of taste. When inserting nylon, you must be careful; if the nylon has entered about 18 mm and there is a feeling of obstruction, then the monofilament insertion is stopped. If there are no obstacles, the stabbing is continued until the length of the incoming nylon is around 22 mm. If the size of the 4-0 nylon suture that enters a blood vessel exceeds 22 mm in length, it can puncture the blood vessel and cause intracerebral bleeding. In addition, to prevent the tip of the 4-0 nylon suture from penetrating blood vessels, the nylon tip was blunted by heating it in an incandescent lamp.

In establishing the MCAO model, we identified the cause of ischemic stroke model failure and rat death. Among the 20 rats available, two rats experienced ischemic stroke with a Bederson score of 3, two rats did not experience a stroke with a Bederson value of 2, and 16 rats died. Among the rats that died, two died during handling, four died from bleeding during surgical procedures, and ten died after several hours (4, 6, and 8 hours) of occlusion, with a Bederson score of 4–5. In two ischemic stroke model rats, one rat was sacrificed 24 hours after occlusion, and one rat was maintained for up

to two weeks (14 days). Two rats did not experience ischemic stroke when the nylon was removed; the length of the nylon entering the ICA was less than 18 mm. During the surgical procedure, four rats died when a small hole was made in the ICA. Ten rats were killed after several hours. The next day, euthanasia was carried out to remove brain tissue using the skull bone. Blood clots in the brain indicate bleeding, while white brain tissue indicates cerebral infarction. Analysis showed that the length of nylon entering the ICA in the ten rats that died after several hours was 25–30 mm.

Our study showed clinical symptoms one hour after MCAO with scores of 2,3,4 and 5, as shown in Figure 2. The rats successfully examined with Bederson parameters were 14; 6 rats were not examined with Bederson because they had died during preparation and the surgical procedure. The rats with Bederson values of 4 and 5 could survive only a few hours (4, 6, and 8 hours), after which the rats died. The rats with a Bederson value of 3 showed a model of ischemic stroke that could survive up to 14 days. This is in line with previous research, which stated that the average Bederson score of 3 indicates being able to live with apparent neurological disorders (15,18). The Bederson parameter used is 0-5, according to Bieber (27).

The results of the HE histological examination also show the difference between ischemic stroke and bleeding. In ischemic stroke, shrinkage in neurons begins to appear, while in a haemorrhagic stroke, pyknosis and shrunken nucleus of the neurons. Our study is based on previous research on neuron shrinkage (41) and degeneration (42). The histological picture after the stroke is consistent with the previous study, but the measurement time is different; the last study took measurements after 48 hours of the MCAO (43) and took measurements after 24 hours of the MCAO, which is in line with previous research conducted animal experiments by assessing neurological deficits after 24 hours with TTC staining, immunoblotting, and qPCR (44). Neurological dysfunction occurs within seconds to minutes after reduced perfusion, resulting in neuronal death, and necrotic and irreversible cell death will occur within minutes or hours if blood flow is not immediately restored (45). In addition, previous studies have examined the neuroprotective effects of global ischemia after 2 and 6 hours of reperfusion (46). The limitation of our study is that we did not conduct further examination of the unsuccessful strokes and the rats that died during the preparation and surgical procedures.

Conclusion

In conclusion, with simple equipment, animal models of ischemic stroke can show clinical features, infarction volume, and histopathology, which can be used for future research. Further research can be conducted on interventions or drug administration for the recovery process. In creating models, it is necessary to pay attention to the factors leading to the failure and death of rats.

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Conflict of interest

The authors declare no conflicts of interest.

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تحدي إحداث انسداد الشريان الدماغي الأوسط في الفئران والعوامل المؤدية لفشله

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الخلاصة

السكتة الدماغية هي سبب الوفاة والعجز في العالم. تحتاج الأبحاث الحيوانية إلى نماذج حيوانية مشابهة قدر الإمكان للعمليات الفسلجية المرضية التي تحدث في الانسان. نموذج السكتة الدماغية الإقفارية المعمول به هو نموذج انسداد الشريان الدماغي الأوسط. يتطلب إنشاء هذا النموذج معدات متطورة ودقيقة. تهدف دراستنا إلى وصف إجراء انسداد الشريان الدماغي الأوسط باستخدام أدوات بسيطة وتحديد أسباب الوفاة. إنشاء نماذج حيوانية للسكتة الدماغية الإقفارية مع إجراء انسداد الشريان الدماغي الأوسط بمعدات بسيطة واستخدام خيوط نايلون أحادية الشعيرات ٤,٠ لسد الشريان الدماغي الأوسط وإعادة التروية لمدة ٩٠ دقيقة. يتم اختبار العجز العصبي سريريا مع معايير بيدرسون بعد ساعة واحدة من انسداد الشريان الدماغي الأوسط. استخدمت صبغة 2.3.5 triphenyl tetrazolium chloride لتحديد منطقة الاحتشاء بعد ٢٤ ساعة من انسداد الشريان الدماغي الأوسط. تم فحص الصورة النسيجية باستخدام صبغة الهيماتوكسيلين-ايوزين. نجح نموذج السكتة الدماغية الإقفارية في فأران وفشل في فأران، ومات فأران أثناء التحضير، وماتت أربعة فئران أثناء الجراحة، وماتت ١٠ فئران بعد عدة ساعات من الانسداد. كان السبب الأكثر شيوعا للموت هو النزيف من خيوط نايلون ٤,٠، التي كانت طويلة جدا لتمر عبر الشريان الدماغي الأوسط، وبالتالي ثقب الأوعية الدموية. من الضروري عند إنشاء نموذج للسكتة الدماغية مراعاة العناصر التي تساهم في فشل الفئران وموتها.

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