STATE OF THE ART

Stroke is one of the most prevalent diseases in the world and is a leading cause of death and disability among adults. Although since 1970 scientists have been trying to develop and improve cerebral ischemia models, it became obvious that the ideal model must be in concordance not only with the purpose of the study but also in line with the anatomical features of the species. Nowadays there is an increasing interest in developing ideal animal (CC BY-NC-SA 4.0) License (http://creativecommons.org/licenses/by-nc-sa/4.0/), allowing third parties to copy and redistribute the material in any medium or format and to remix, transform, and build upon the material, provided the original work is properly cited and states its license.

Address for correspondence / Adres korespondencyjny: Adina Huțanu, University of Medicine and Pharmacy of Târgu-Mureș, Gheorghe Marinescu 38; 540139 Târgu-Mureș, Romania, e-mail: adina.huutanu@umftrm.ro; phone: +4 744 300 508
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Various rodent models have been developed for examining either focal or global cerebral ischemia so far, but the most used model is the rat middle cerebral artery occlusion by intraluminal filaments, followed by reperfusion, and 2,3,5-triphenyltetrazoliumchloride or Cresyl Violet/Nissl staining for evaluation of the region of interest. Like other surgical procedures, this one is prone to various complications (excessive bleeding, vascular or nerve lesions) and failure related to the surgical technique. We focused on detailed surgical techniques, along with data from literature, in order to reduce the complications and increase the chance of experiment success. Magnetic Resonance Imaging evaluation with 3D reconstruction of the ischemic area opens new perspectives on investigations into the ischemic brain, having the advantage of being noninvasive as an alternative to gold histological standard for measuring the infarct size, being the only one accesible in surviving subjects.

Keywords: stroke, middle cerebral artery occlusion, cerebral ischemia

Introduction

Stroke is one of the most prevalent diseases in the world and is a leading cause of death and disability among adults. Although since 1970 scientists have been trying to develop and improve cerebral ischemia models, it became obvious that the ideal model must be in concordance not only with the purpose of the study but also in line with the anatomical features of the species. Nowadays there is an increasing interest in developing ideal animal models for stroke research, models for risk factors and prevention, for pathophysiology, models for therapeutic approaches (reperfusion, neuroprotection, neuroregeneration and anti-inflammatory drugs) [1].

Orădan A, Huțanu A, Horváth E, Chiriac L, Dobreanu M. Improved rat stroke model by intraluminal middle cerebral artery occlusion: a special emphasis on surgical technique. Health Problems of Civilization. 2017; 11(3): 202-210. doi: 10.5114/hpc.2017.70008.
Pathophysiology of ischemic stroke

Focal cerebral ischemia characterized by blood flow reduction in a particular brain region, or global ischemia which affects the entire cortex have been used in this regard. Ischemic stroke has a complex pathophysiology involving oxygen and energy failure, cell ion homeostasis disturbance and increased intracellular calcium levels, free radicals production and acidosis, increased glutamate excitotoxicity, overproduction of proinflammatory cytokines, complement or resident immune cells activation, disruption of the blood-brain barrier (BBB) with consecutive leukocyte infiltration. The consequences of the above processes are necrosis in the core region, where the blood cessation is complete, and apoptosis in the peri-infarct area or penumbra [2].

There are several models of cerebral ischemia in rodents to evaluate the pathophysiology and various therapeutic approaches. Permanent or temporal focal ischemia induced by middle cerebral artery occlusion (MCAO) are methods that greatly mimic stroke in humans. Rodents are preferred above all other species, due to their similarity with humans; the rodent model met almost all the conditions required to manipulate, induce, evaluate and treat stroke [3]. The first model for ischemic stroke was developed in rats by Koizumi et al. in 1986 by intraluminal MCAO [4]. Several optimised models for MCAO in rats were described [5], [6]; in order to minimize intra- and perioperative complications. Thus, we validated this experimental model in our laboratory by establishing the concordance between morphological modifications and ischemic tissue volume measured by MRI. The evaluation of the magnitude of the ischemic damage is a sensitive subject. Usually, 2,3,5-triphenyltetrazolium chloride (TTC) is used for tissue staining in order to quantify the size of infarction and to distinguish between core, penumbra and normal tissue [7]. Kramer et al demonstrated that TTC stained cerebral tissue can be used for further protein and gene analyses [8], but this could be a problem if the tissue must undergo lipidomic investigations. TTC staining may be replaced by Cresyl Violet/ Nissl (CV/Nissl) staining [9], an efficient routinely used method to determine cerebral infarct volume and area, which offers an opportunity to test many critical markers by immunohistochemistry [10]. Magnetic Resonance Imaging (MRI) opens new perspectives on investigations into the ischemic brain, having the advantage of being noninvasive as an alternative to gold histological standard for measuring the infarct size [11] and enabling dynamic evaluation of BBB breakdown and vasogenic edema formation [12], being the only one accessible in surviving subjects.

Herein, we present in detail the surgical procedure for transient MCAO with modification for minimal complications of vasculature and nerve lesions, followed by MRI evaluation and 3D reconstruction of the ischemic area, and morphological study of damaged tissue using histological Hematoxylin Eosin (H&E) and CV staining.

Animal selection

All procedures were conducted according to the guidelines for care and use of animals in research (Directive 2010/63/EU of the European Parliament and the Council on the protection of animals). The experimental studies were approved by the Ethical Committee of the University of Medicine and Pharmacy of TârguMureș, Romania. The experiment was carried out on adult male rats (280-320 g). Before and during the experiment all animals were maintained in a climate-controlled environment (temperature 21-25 C°, humidity 60%, and natural circadian day-night cycle) and had will have “ad libitum” access to water and food. A MCAO followed by reperfusion after 90 minutes was performed; 24 hours after reperfusion the ischemic region was evaluated by MRI with 3 D reconstruction. Behavioral tests for neurological assessment were performed at 90 minutes (the reperfusion time) and 24 hours after reperfusion, by a person blinded to the experiment. A five point scale was used for neurological deficit evaluation, from 0–no deficit– to 4– unable to walk [2], [13]. Only animals with a neurological deficit greater than 2 points were used for further investigation. Animals were sacrificed after 24 hours to assess morphological and molecular changes.

Additionally, brains were collected in 10% Neutral Buffered Formalin for histological examination. Experimental model consisted in skin incision and neck dissection, carotid complex dissection, insertion followed by monofilament occluder withdraw 90 minutes thereafter.

Middle Cerebral Artery Occlusion (MCAO)

The most used models for focal cerebral ischemia is MCAO method using a specially manufactured monofilament thread with a silicone tip which is passed through the internal carotid artery (ICA) until the silicone tip reaches the origin of the middle cerebral artery (MCA) thus occluding it [6]. For our research, we used a reusable silicon rubber-coated monofilament, size 4-0, diameter 0.185 mm with a coating diameter of 0.39 +/- 0.02 mm (Doccol Corporation, USA).
Rat cerebral vasculature anatomy

The rat carotid system is similar to the one described in humans, although a few differences are noticeable. The common carotid artery (CCA) bifurcates to form the external (ECA) and ICA. When carrying out the dissection for an MCAO procedure one should carefully observe, dissect and ligate the branches of the ECA, the occipital artery (OA) and the superior thyroid artery (STA) in order to facilitate the suture insertion. An important branch that needs to be mentioned is the pterygopalatine artery (PPA) – the only extracranial branch of the ICA. After entering the skull, the ICA gives off the hypothalamic artery (HTA), the anterior choroidal artery (AChA), and then it bifurcates into the middle cerebral artery (MCA) and anterior cerebral artery (ACA) [14].

Rat positioning and field preparation

After isoflurane-induced anesthesia (3.5%), the rats were positioned supine on the heating pad (Doccol Corporation, USA) and fixed into the breathing mask firmly. During the surgical procedure, anesthesia was maintained with 1.5% isoflurane [15]. To obtain a better exposure of the left carotid, the head was elevated using cotton pads placed underneath and rotated slightly to the right (Figure 1a).

Figure 1a. The positioning and anesthetic method of the rats. The rat fixed in the anesthesia mask with a cotton swab underneath its head in order to better expose the carotid artery

No other restraint methods were used in order to minimize trauma or induce breathing difficulties. The neck was shaved between the manubrium and chin using clippers. Using an iodine based solution (Betadine) the shaved region was draped three times, and sterile drapes were applied over the rat, leaving only the cervical region exposed. Bupivacaine (0.2 ml) was injected subcutaneously along the marked incision in order to minimize postoperative pain [16].

Skin incision and neck dissection

Using a number 15 blade, a skin incision was carried out between the manubrium and chin, exposing the superficial cervical fascia. We used a long incision in order to facilitate access to the entire carotid complex (CCA, ICA, ECA), although after experience has been achieved, a smaller incision centered at the carotid bifurcation can be performed without the need of extension. In order to minimize bleeding and maintain a clean operating field, electrocautery was used at this stage. The next step was to incise the cervical fascia using microsurgical dissecting scissors (S&T SDC-15) without damaging important vascular structures, such as the external jugular vein and its branches. The dissection was carried out on the midline through the glandular tissue until the sternohyoid muscle was reached. A self-retaining retractor was placed on the skin and glandular tissue exposing the sternohyoid, sternomastoid and digastric muscles. At this point, the deep fascia was sharply dissected separating the three muscles and exposing the carotid artery (Figure 1b).
For a better exposure of the artery, the omohyoid muscle, which runs between the sternomastoid muscle and carotid, was cut at its most cranial point possible and tucked underneath the sternomastoid. Care must be taken when cutting the muscle in order not to damage the carotid artery and the adjacent veins. After dissecting the muscles, the retractor was placed on the sternohyoid medially and sternomastoid laterally exposing the carotid complex.

**Carotid complex dissection**

After the complete exposure of the carotid artery, the following steps represent the dissection of the artery from the surrounding structures in order to facilitate the insertion of the monofilament. The CCA runs parallel to the trachea and after it bifurcates, the ECA runs medially whereas the ICA runs laterally towards the skull base underneath the digastric muscle. Together with the artery we also encountered a very rich venous plexus surrounding it together with the vagus nerve, lateral to the CCA and ICA. The veins were carefully dissected, ligated, coagulated and cut if necessary in order to minimize bleeding. Care was taken at this stage not to damage the artery. The vagus nerve was also kept safe in order to minimize life-threatening complications such as a severe tachycardia.

By dissecting a small portion of the CCA and ICA, allowed the placement of microvascular clamps which were used at another stage of the procedure, but also allowed applying the clamps in the event of a carotid injury which may require repair. The next step was dissection of the ECA as cranially as possible and ligation of it. By ligating the ECA as soon as possible in the procedure we also lowered the risk of bleeding in the event of carotid injury. If an injury occured by applying clamps of the CCA and ICA we did not have any backflow into the carotid complex, therefore a proper repair of the artery could be carried out. One important branch of the ECA was the STA which was ligated and transected at this stage. After the ligation of the ECA and transection of the STA, we continued the dissection of the ECA towards the bifurcation of the CCA where we encountered another branch, the OA, which was ligated and transected. The OA emerges from the ECA immediately after the bifurcation of the CCA and it runs parallel with the ICA. Care was taken when dissecting this portion because the tissue in the carotid bifurcation is a fibrous tissue and bleeding can occur easily, sometimes from a small arterial branch emerging from the bifurcation (Figure 1c).
Next, the ICA was dissected from the vagus nerve and surrounding tissue until the emergence of the PPA. As described in the anatomy section, the only branch of the ICA is the PPA which emerges laterally from the ICA, as the ICA runs slight medial towards the skull base. Due to this anatomical disposition, when inserting the occluder, it tends to enter the PPA rather than continuing through the ICA. Some authors recommend ligation of the PPA, or applying a temporary clip in order to occlude the PPA [5], and force the occluder in the ICA, however we did not consider this necessary. After the dissection of the ICA was completed, one makes sure that the entire carotid complex is freely dissected from all the surrounding tissue in order to move on to the next step, the insertion of the monofilament occluder.

**Insertion of the monofilament occluder**

Microvascular clamps were applied on the CCA and ICA, and a small arteriotomy was made in the ECA below the ligature point. A heparinized solution was used to cleanse the field of any residual blood. Before the arteriotomy was made, a ligature was placed below the future arteriotomy. The occluder size 4-0, diameter 0.185 mm, with a coating diameter of 0.39 +/- 0.02 mm (Doccol Corporation, USA) was inserted into the ECA up until the CCA bifurcation, then the ligature was tied around the occluder, and the ECA was cut completely at the point of the arteriotomy. The clamp on the ICA was removed and the occluder advanced in the ICA until resistance is felt. Care was taken not to insert it in the PPA, as described above. If the occluder insertion is not deeply enough, it might not obstruct the MCA, and in case of too deep insertion, it might rupture the ACA and cause a subarachnoid hemorrhage.

The skin incision was closed with 5-0 silk suture, and the rats were allowed to recover from anesthesia. After 90 minutes the animals were anesthetized again, the incision was opened, and the endoluminal suture removed. The incision was closed, and Tramadol and saline were administered subcutaneously to help reduce postoperative pain and maintain optimal hydration.

For the sham group, the initial steps of the procedure were carried out with the exposure of the carotid artery, but without its dissection. Pain relief medication was administered after the procedure just like in the other groups.

**MRI quantification of the infarct region**

MRI was performed on BrukerBioSpec 70/16 USR scanner, operated at 7 Tesla. The protocol used for geometry and acquisition was described elsewhere [17]. The TurboRARE 3D protocol was preferred because it allows retrospective gating, provides better contrast to noise ratio (CNR), reducing the animal preparation time. After MRI acquisition, the ischemic region was reconstructed and evaluated by 3D Slicer (https://www.slicer.org/), an open-source software platform. In figure 2a a representative image of ischemic region was visualised in T2 weighted MRI protocol acquisition, while in figure 2b a 3D reconstruction of the ischemic region can be seen.
Morphological analyses

To control the effectiveness of the experimental surgical procedure, the removed brains were fixed in 10% Neutral Buffered Formalin for 1 day, and they were processed by standard histological procedures. For histological and immunohistochemical analysis a standard paraffin block was obtained from each sample.

A series of 5-6 µm thick sections were cut from the block. The serial sections were stained using H&E complemented with CV/Nissl staining in order to evaluate the extent of the ischemic core (dead neurons) and penumbra (containing degenerated, yet viable neurons) and the major neuronal damage. Sham-operated rats brains served as controls. Representative morphological cellular changes were analysed as: focal acute eosinophilic necrosis associated with neural cell vacuolization, neural degeneration, pycnotic nucleus associated with vacuolation of neuropils.
Figure 3a. Quantifiable microscopic changes of rat brain tissue (H&E stain) imaged following MACO: focal acute eosinophilic necrosis associated with neural cell vacuolization (short arrow), neural degeneration: pycnotic nucleus associated with vacuolation of neuropil (long arrow) and area of edema (arrowhead) (20x magnification).

Figure 3b. Infiltration of inflammatory cell around the ischemic tissue Cresyl Violet staining marks ischemic zone as a pale area surrounded by intensely coloured intact nervous tissue (arrow) (CV/Nissl stain, 20x magnification).

Figure 3c. Coronal section of rat brain stained with CV/Nissl stain, after 24 h post-reperfusion. The ischemic area extends in cortex and adjacent brain areas and appears as a pale and imprecisely demarcated tissue (magnification 3.4x).
To determine the ischemic tissue percentage on the total volume of the right hemisphere, a serial CV/Nissl-stained sections captured with Mirax Digital Slide Scanner image acquisition system (Panoramic Viewer 1.15.4) were used. The representative ischemic fields (area lightly stained with CV/Nissl) were photographed, the digital images were saved in TIFF format and imported for analysis into the Image J 1.40 software [18]. The ischemic areas on serial section were summed to obtain the volume of damaged tissue and the ischemic volume was expressed as a percentage of total volume. The morphological changes occurring after MCAO confirmed the accuracy of our experimental method. H&E staining enhanced by CV staining offers a detailed overview of light microscopic features of the ischemic tissue, parallel application of these methods helps to highlight the substantial morphological changes following acute ischemia: neural cell vacuolization, neural degeneration, cell edema and the presence of inflammatory cells (Fig.3a-c).

In addition, these stains offer the possibility to determine the injury volume using an image analysis system to compare with the results obtained with MRI analysis. Evaluating the ischemic volume CV/Nissl staining in comparison with TTC offers the possibility to use the brain tissue for further histological processing and immunohistochemistry.

Conclusions

Intraluminal MCAO model most closely simulates human ischemic stroke events. The effectiveness of the method can be verified by behavioural tests, but the size of the ischemic lesions can only be highlighted by MRI, while the severity by the histological analysis of the cerebral tissue. For more advanced studies, immunohistochemistry with cell-specific markers is required to be used. We considered unnecessary ligation or applying a temporary clip on pterigopalatin artery, only visualization being enough to minimize the risk of inserting occluder into it.

Disclosure and acknowledgements

We are grateful to Dr. Berce Cristian from the animal facility of the University of Medicine and Pharmacy Iuliu Hațieganu Cluj-Napoca for technical assistance, and Dr. Flaviu Turcu from the University Babes-Bolyai Cluj-Napoca, Faculty of Physics, National Magnetic Resonance Center for his technical support regarding MRI acquisitions. This work was supported by an internal research grant from the University of Medicine and Pharmacy Târgu-Mureș (Project nr. 17803/1/22.12.2015).

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