Assessment of Blood-Brain Barrier Permeability in a Cerebral Ischemia-Reperfusion Model in Rats; A Pilot Study

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Summary

Animal models are an important tool for studying ischemic mechanisms of stroke. Among them, the middle cerebral artery occlusion (MCAO) model via the intraluminal suture method in rodents is closest to human ischemic stroke. It is a model of transient occlusion followed by reperfusion, thus representing cerebral ischemia–reperfusion model that simulates patients with vascular occlusion and timely recanalization. Although reperfusion is very beneficial for the possibility of preserving brain functions after ischemia, it also brings a great risk in the form of brain edema, which can cause the development of intracranial hypertension, and increasing morbidity and mortality. In this paper, we present the results of our own transient reperfusion model of MCAO in which we tested the permeability of the blood-brain barrier (BBB) using Evans blue (EB), an intravital dye with a high molecular weight (68,500 Da) that prevents its penetration through the intact BBB. A total of 15 animals were used in the experiment and underwent the following procedures: insertion of the MCA occluder; assessment of ischemia by 2,3,5 - Triphenyltetrazolium chloride (TTC) staining; assessment of the BBB permeability using brain EB distribution. The results are presented and discussed. The test of BBB permeability using EB showed that 120 minutes after induction of ischemia, the BBB is open for the entry of large molecules into the brain. We intend to use this finding to time the application of neuroprotective agents via ICA injection in our next stroke model.

Keywords

Cerebral ischemia–reperfusion model • Middle cerebral artery occlusion • Blood-brain barrier • 2,3,5 -Triphenyltetrazolium chloride • Evans blue

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Introduction

According to the World Health Organization, in the past 10 years, stroke has remained the world's main threat to human health, because of its high morbidity, high mortality, and high disability [1]. This is why cerebral ischemia research is constantly in the spotlight, and the development of effective drugs to prevent and treat stroke has remained the goal of researchers in this field.

Animal models are an important tool for studying ischemic mechanisms of stroke. Among them, the middle cerebral artery occlusion (MCAO) model via the intraluminal suture method in rodents is closest to human ischemic stroke, widely accepted and well-standardized because of its simpler procedure and stable infarct volume. It is a model of transient occlusion followed by reperfusion, thus representing cerebral ischemia–reperfusion model that simulates patients with vascular occlusion and timely recanalization. The timely recanalization of blood vessels after cerebral ischemia is the most direct and effective therapeutic method. Although reperfusion is very beneficial for the possibility of preserving brain functions after ischemia, it also brings a great risk in the form of brain edema, which can cause an increase in the intracranial space and the development of intracranial hypertension, threatening vital functions and increasing morbidity and mortality [1].

Many studies have used the reperfusion model of MCAO to develop neuroprotective agents [1-10]; on the other hand, only a small number of studies deal with the state of the blood-brain barrier (BBB) permeability, which is a determinant for the development of brain edema [11-15].

In this paper, we present the results of our own

transient MCAO reperfusion model in which we tested BBB permeability using Evans blue (EB). It is an intravital dye whose molecular weight after binding to albumin is high (68,500 Da) and thus its penetration into the brain signals an open BBB for large substances. The main goal of this experiment is to find an adequate timing of the application of neuroprotective substances with a high molecular weight for our next experimental stroke model being prepared.

Methods

All experiments were approved by the Ethical Committee of the First Faculty of Medicine (Charles University in Prague) and were in agreement with the Guidelines of the Animal Protection Law of the Czech Republic and Guidelines for the treatment of laboratory animals EU Guidelines 86/609 / EEC. For experiments, male rats of the Wistar strain weighing 400 g of our own breed were used.

Animals

A total of 15 animals were used in the experiment and underwent the following procedures: insertion of the middle cerebral artery (MCA) occluder; assessment of ischemia by 2,3,5 -Triphenyltetrazolium chloride (TTC) staining; assessment of the blood-brain barrier (BBB) permeability using brain Evans Blue (EB) distribution.

Insertion of the MCA occluder

Spontaneously breathing rat under the inhalation anesthetic isoflurane (Forane ®, AbbVie Ltd.) in concentration of 2 volume % underwent in the supine position the skin incision along the midline between the upper end of the sternum and the mandible. The left common carotid artery (CCA) and its branches (the internal carotid artery, ICA and the external carotid artery, ECA) were exposed with a standard microsurgical technique and ECA was ligated beyond the bifurcation (Fig. 1A). An intraluminal occluder was introduced into the CCA trunk from the arteriotomy and advanced until the origin of the middle cerebral artery (MCA) was blocked (distance from CCA bifurcation was 20-22 mm for a 400 g rat) and fixed with suture (Fig 1A). The occluder was removed after 90 minutes, the distal and proximal ends of the exposed CCA were ligated, and the skin incision was closed with a continuous suture. After awakening, the animal was placed for 24 hours in a home environment, at room temperature, with unlimited access to food and drink.

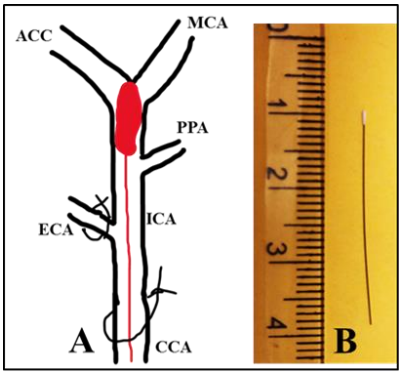

Fig. 1. A) schematic representation of occluder (in red) insertion; CCA-common carotid artery; ICA-internal carotid artery, MCA-middle cerebral artery, ACC-anterior cerebral artery PPA-pterygopalatine artery; ECA-external carotid artery. **B**) silicone rubber coated monofilament obtained commercially (www.doccol.com), diameter of the tip (A in red, B in white) 0,45 mm, length of the coating 2 mm for a 400 g rat.

Assessment of ischemia by TTC staining

24 hours after removal of the occluder, the animal was euthanized by inhalation of a lethal dose of isoflurane, the brain was removed and stored in a -20 °C freezer for 30 minutes. The sections were coronal plane cut into a thickness of 2 mm, immersed in 2 % TTC (2,3,5-Triphenyltetrazolium chloride) solution, and placed in an incubator at 37 °C for 30 min. Brain sections were rotated frequently to achieve uniform staining, followed by washing with PBS (Phosphate-buffered saline) and photography. After staining, the infarct area was stained white, while other areas were stained red.

2,3,5 -Triphenyltetrazolium chloride is a marker of metabolic function and represents a reliable indicator of ischemic areas in experimental stroke models. TTC is a colorless water-soluble dye that is reduced by the mitochondrial enzyme succinate dehydrogenase of living cells into a water-insoluble, light sensitive compound (formazan) that turns healthy/normal tissue deep red. In contrast, damaged/dead tissue remains white showing the absence of living cells, and thereby indicating the infarct region [2].

TTC staining was used and evaluated in 3 animals with intraluminal MCA occlusion for 90 min and further in control animals – healthy animals (3x), and animals that underwent left CCA ligation (3x).

Assessment of BBB permeability using brain EB distribution

Spontaneously breathing rat under the inhalation anesthetic isoflurane (Forane ®, AbbVie Ltd.) in concentration of 2 volume % underwent in the supine

position the skin incision along the midline between the upper end of the sternum and the mandible. The left common carotid artery (CCA) and its branches (the internal carotid artery, ICA and the external carotid artery, ECA) were exposed with a standard microsurgical technique and ECA was ligated beyond the bifurcation. A polyethylene catheter was introduced into the bifurcation through a small arteriotomy of the CCA, and 2 % EB at a dose of 2 ml/kg was applied into ICA at a rate of 0.45 ml/min. The catheter was removed after EB application, the distal and proximal ends of the exposed CCA were ligated, the skin incision was closed with a continuous suture, and the rat was left under inhalation anesthesia. Within 30 min after the end of EB injection, the animal was euthanized and the brain was fixed by transcardial perfusion with a 4 % paraformaldehyde solution in pH 7.4 phosphate buffer for a period of 15 min. The brain was removed, and then fixed in the same solution for another 24 h. Each brain was then sliced on a vibratome into coronary sections 30 μm thick and then, without further staining, placed on slides for microscopic examination. The sections were studied under a fluorescence microscope for intracellular and extracellular EB distribution in cortex and hippocampus of ipsilateral, left hemisphere.

This procedure as described was performed in 3 animals and in additional 3 animals EB was administered by use of a catheter inserted into the ICA through CCA arteriotomy after removal of the MCA occluder. That is, 3 animals represent the control group and other 3 animals are the experimental group with intraluminal occlusion of the MCA for 90 min followed by EB application to the ICA.

Results

Assessment of ischemia by TTC staining

Brain sections from healthy animals and those that underwent left CCA ligation were stained red, while the infarcted area in animals with intraluminal MCA occlusion for 90 min was stained white (Fig. 2).

The white area marked with an asterisk (Fig. 2C) corresponds to the absence of viable cells and documents the area of ischemia. The fact that the brain section is red not only in healthy animals (Fig. 2A) but also in those that have undergone left ACC ligation (Fig. 2B) confirms that this procedure does not threaten the viability of brain cells. This is explained by the fully functional circle of Willis, which is completely developed in rats [3].

The distribution of EB in the control group (EB applied to the ICA of healthy animals) was strictly extracellular, while in the experimental group with intraluminal occlusion of the MCA for 90 min followed by EB application to the ICA, the tracer was detected both in the extracellular compartment and in the cells (Fig. 3).

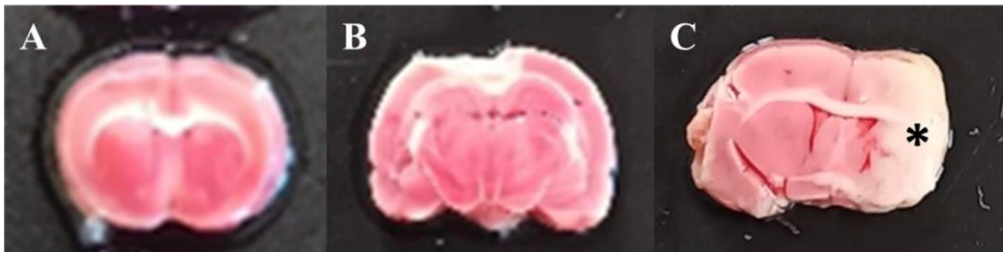

Fig. 2. A) healthy animal; **B**) animal with left CCA ligation; **C**) animal with intraluminal MCA occlusion for 90 min, * white infarcted area

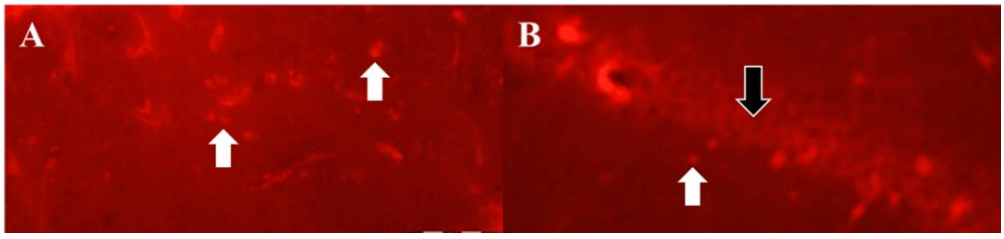

Fig. 3. Fluorescence microscopy. **A**) control group (healthy animal) with extracellular distribution of EB (white arrows); **B**) experimental group with intraluminal occlusion of the MCA for 90 min followed by EB application to the ICA with extracellular distribution of EB (white arrows) and intracellular distribution of EB in neuronal population of hippocampal area CA3 (black arrow).

Assessment of BBB permeability using brain EB distribution

Evans blue (EB, MW 961 Da) is one of the largest tracers, which are used to test the BBB permeability. Five minutes after its application into the blood stream it becomes strongly, though reversibly, bound to the albumin fraction of proteins to give rise to a high-molecular complex (EBA 68 500 Da) [16]. The pure dye and the EBA complex have a MW greater than 180 Da, preventing passage through the intact barrier. However, both forms cross the BBB after application directly to the brain via the ICA and are detectable in the brain by fluorescence microscopy. Clearly, each form enters the brain at a different state of BBB permeability.

In healthy animals (control group) the entry of the pure dye (MW 961 Da) into the brain can be attributed to a number of factors which together influence the intact barrier on its luminal side. We summarized a list of these factors in one of our earlier experimental models of BBB permeability [17]: rapid increase in intravascular and, consequently, hydrostatic pressure; sudden hypervolemia in a limited area; presence of a hypertonic solution on the luminal side of the BBB, and the direct effect of the staining agent, which resembles the side effect of an angiographic contrast medium. The mentioned factors explain the presence of a small amount of EB in the form of a pure dye (MW 961 Da) in the extracellular compartment in healthy animals (control group).

The ischemia/reperfusion injury includes cellular and BBB damage, which explains the distribution pattern of EB in the experimental group [11,18].

Discussion

The majority of rodent ischemic stroke models target the middle cerebral artery (MCA), with either transient or permanent occlusion. The main methods of MCA occlusion (MCAO) are: (1) mechanical – e.g. blocking the origin of the MCA intraluminally with a filament, using clips and/or sutures to tie off the artery or applying compression to stop the flow through the artery; (2) electrocoagulation – coagulating the blood and destroying the structure of the artery using fine diathermy forceps; (3) pharmacological – e.g. applying vasoconstrictor substances such as endothelin-1 directly onto the artery or injecting it into neighbouring tissue to induce prolonged, local ischemia; (4) thrombo/embolic – introducing a pre-formed blood clot to block the MCA, or its distal branches, inducing local thrombosis [4].

The most common method of focal ischemic stroke is intraluminal thread occlusion of the MCA. The model was first described by Koizumi et al in 1985 [5] and modified by Longa et al in 1989 [6]. The basic technique involves introducing a filament with a round tip into the internal carotid artery (ICA) and advancing it to block the origin of the MCA [7].

From a technical point of view, this model causes reproducible infarcts in the MCA region, it does not require craniectomy and allows reperfusion by withdrawing the occlusive fiber. Its design offers several different modifications that are worth mentioning.

The following time intervals are crucial for choosing a suitable model: the duration of ischemia, the onset of reperfusion and the development of brain edema. Rats with reperfusion after occlusion lasting for 0.5–1 h have only mild infarction and can quickly recover after surgery, which does not meet the requirements of long-term testing. When the vessel is occluded for 3 or 4 hours, reperfusion can lead to an increase in infarct volume, corresponding to permanent ischemia with high mortality. Transient ischemia lasting $\geq$ 2 h induces spontaneous hyperthermia, which is associated with hypothalamic damage in rats; this is not seen in human clinical stroke. Clinical results show that optimal results are observed if thrombolytic therapy is initiated within the first 90 minutes after the onset of symptoms [1]. From the above, it follows that in terms of time, reperfusion performed 90–120 minutes after MCAO best corresponds to the experimental requirements. And this model is also suitable for the study of ischemic brain edema.

The intraluminal filament in MCAO transient model can be introduced through an opening in the common carotid artery (CCA) or the external carotid artery (ECA). There are no fundamental differences between the two approaches, although some preferential aspects are indicated. Percie du Sert et al note that approach via the ECA results in cauterization of the ECA and therefore disrupts the blood supply to the ECA territory, including the facial musculature with masseter damage, weight loss, and drinking disorders. This will not happen by inserting the filament via the CCA with permanent ligation. They point out that no pathology was reported even in sham animals that had one carotid artery ligated [4]. Our results confirm this experience. TTC staining demonstrated viable cells in healthy animals with left CCA ligation (Fig. 2B). On the other hand, Fluri et al prefer the ECA approach because it is a better choice for transient MCAO as it maintains the anatomical integrity necessary for

reperfusion [8]. Moreover, Liu et al remind that most intraluminal models that appear in the literature were induced through the ECA approach [9]. Based on a meta-analysis of rodent data, Li et al conclude that the CCA approach is easy to operate with a high modelling success rate and is suitable for studying brain edema, while the EAC approach is very challenging for novices and is suitable for studying ischemia-reperfusion brain injury caused by complete ischemia-reperfusion [10]. We use the CCA approach because, after the occluder is removed, a catheter for EB application is inserted through the same opening, and in addition, this approach is more suitable for studying ischemic edema.

Many studies have reported that the incidence of clinical left-hemisphere ischemia is higher than that of right-hemisphere ischemia, and that left-sided ischemia is associated with higher mortality and a worse prognosis. The uneven distribution of patients with clinical left- and right-hemisphere ischemic stroke suggests that preclinical studies can preferentially select the left-hemisphere ischemic animal model to promote the clinical translation of the results [1]. The side of occlusion can be altered and is often a matter of the surgeon's preference. Right-handed surgeons tend to find that occluding the left MCA is easier [4]. Both of the above views played a role in choosing the left CCA approach in our model.

The physical properties (tip diameter, tip length, tip shape, and flexibility) of the occluder tip play a critical role in infarct variation. Matching the size of the occluder to the size of the animal theoretically would improve the consistency of the model. For different rodent weights, it is recommended to obtain commercially available occluders. In general, the smallest diameter and shortest coating that produces consistent MCA territory injury should be used. It has been reported that the optimal occluder diameter for rats weighing 275-320 g is around 0.38 mm for silicon rubber coated monofilaments. The inserted distance of the occluder is critical to a model's success. For the rat model, the distance from the CCA bifurcation is 18-20 mm for a 300 g, and 20-22 mm for a 400 g rat [1,4,9]. According to above mentioned recommendations we use the commercially available occluders (http://www.doccol.com/) with diameter of the tip (Fig. 1 A in red, B in white) 0.45 mm, length of the coating 2 mm for a 400 g rat.

The MCAO model used, is the closest to a human ischemic stroke and results in interruption of cerebral blood flow (CBF). This sudden and profound reduction in CBF restricts the supply of vital oxygen and nutrients to the brain tissue, leading to ischemia, cell injury and ultimately cerebral infarction. The infarct that results from the cerebral ischemia is comprised of two distinct regions: the core and the penumbra. The infarct core represents an area of tissue that has succumbed to the ischemia and undergone rapid neuronal cell death. Surrounding the core is the penumbral tissue, neurons in this area are compromised by the reduction in CBF but are still viable. If ischemia continues then neurons within the penumbra may progress to become irreversibly damaged and undergo cell death. However, with adequate and timely reperfusion neuronal loss may be reduced, but this process is associated with the risk of developing ischemia/reperfusion injury. It is caused by a series of pathological cascade reactions triggered by the recovery of oxygenated blood flow into the ischemic brain tissue with development of brain edema due to increased BBB permeability [12,18].

One of the most significant contributors to BBB breakdown in stroke is activation of matrix metalloproteinases (MMPs). This includes MMPs that are activated by hypoxia-inducible factor-1α (HIF-1α)-dependent mechanisms (i.e., MMP-2) and MMPs whose activation is triggered by cytokines (i.e., TNF-α, IL-1β) such as MMP-3 and MMP-9. MMP-2 and MMP-9, directly compromise the BBB by degrading tight junction constituent proteins - claudin-5, occludin, ZO-1, and VE-cadherin. Next to MMPs, integrins, transmembrane glycoprotein receptors for the extracellular matrix, also play a significant role in BBB breakdown. Physiologically, integrins interact with constituents of the basement membrane (i.e., collagen IV, fibronectin, laminin, heparin sulfate proteoglycans such as perlecan) to regulate BBB permeability and transport. In ischemic stroke, integrins are rapidly degraded, which leads to BBB breakdown and subsequent edema. Reorganization of tight junction proteins following focal cerebral ischemia is also mediated by vascular endothelial growth factor and nitric oxide [11,13].

Ischemia/perfusion injury also includes cellular damage that follows primary ischemic injury, is modified by reperfusion, and precedes BBB opening. Ischemia leads to energy loss due to failure of primary active transport (ATP-dependent Na+/K+ ATPase), ionic imbalance, and loss of cellular homeostasis. Extracellular water flows into the cell interior, resulting in an increase in intracellular fluid volume. Morphologically, this process results in alterations in membrane surface architecture with prominent bleb formation. This intracellular edema begins

within 30 minutes of MCA occlusion and persists for up to 24 hours after reperfusion [19]. The subsequent increased cell damage is related to the oxidative/nitrosative stress reaction after reperfusion, in which free radicals play an important role [18]. The final stage of ischemia/reperfusion damage at the cellular level is oncosis, which results in a significant increase in cell volume and is manifested by damage to the plasmalemma membrane and other organelle membranes along with the loss of membrane phospholipids [19]. Overall, disruption of cellular homeostasis and damage to cytoplasmic cell membranes are the main features of ischemia/perfusion injury at the cellular level.

In a transient MCAO model, increased BBB permeability was demonstrated by accumulation of Evan's blue (EB) in the brain as early as 2 h after induction of ischemia and persisted for 48–72 h [11,14,15]. In our MCAO model, EB was administered via a catheter inserted into the ICA through the CCA arteriotomy after removal of the MCA occluder, i.e., after intraluminal occlusion of the MCA for 90 min. After another 30 min, the rat brain was perfused with paraformaldehyde. The coexistence of cellular and BBB damage during ischemia/reperfusion injury and at an appropriate time interval (120 min after induction of ischemia and 30 min after initiation of reperfusion) explains our finding of EB distribution in both extracellular and intracellular compartments (Fig. 3 B). The mentioned time intervals deserve a more detailed explanation. The goal of our work was the technical evaluation of possibilities of our laboratory to induce focal ischemia and to establish a time window for the application of a high MW tracer that does not penetrate the intact BBB. We used the preferred MCAO time of 90 minutes according to literature data and the timing of EB application 30 minutes after occluder removal, i.e. after 30 minutes of reperfusion. Based on our previous experiments [17, 20, 21, 22], we considered a time interval of 30 min to be optimal for studying BBB opening by different methods. A time of 120 minutes (90 minutes of occlusion + 30 minutes of reperfusion) is also the shortest possible time to demonstrate increased BBB permeability according to literature data [14, 15].

We can conclude that our transient MCAO model has met expectations. From a technical point of view, our results are comparable to literature data. The test of BBB permeability using EB showed that 120 minutes after induction of ischemia, the BBB is open for the entry of large molecules into the brain. We intend to use this finding to time the application of neuroprotective agents via ICA injection in our next stroke model.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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