



Biphasic Blood-Brain Barrier Openings after Stroke

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Abstract

Blood Brain Barrier (BBB) dysfunction results in post-stroke brain edema through influx of solutes, immune cells, and blood; however, the time-course of BBB permeability post-stroke remains controversial. Currently, treatments after stroke are time-specific, with little opportunity for effective treatments if the short time-window passes. To investigate BBB permeability after stroke, C57/BL6 male mice was undergone 1 hour transient Middle Cerebral Artery Occlusion (tMCAO); Evans blue and fluorescein extravasation were used as indicators of BBB permeability in this study. Brains were stained with 2,3,5-triphenyltetrazolium chloride and Cresyl violet to determine infarction. We observed that the BBB exhibits biphasic openings-once at 6 hours and once at 72 hours, and brain damage was observed from 6 hours to 72 hours. This novel study demonstrates that post-stroke brain damage is progressive, yet BBB exhibits biphasic openings. Through evaluation of post-stroke mechanisms, the BBB permeability is important to reveal a potential therapeutic window for stroke victims.

Keywords: Blood-Brain Barrier (BBB); Stroke; Transient Middle Cerebral Artery Occlusion (tMCAO); Evans blue; Fluorescein

Introduction

Stroke is the third cause of death and the leading cause of incapacity globally [1]. After acute stroke, treatments include thrombolytic therapy through administration of a tissue Plasminogen Activator (tPA) and surgical removal of clot. It's important to note, however, that there is only a 4.5 hours window to administer tPA [2] and 24 hours time window for use of clot retrieval devices [3]. These time windows have been clinically determined, yet it is important to further investigate post-stroke mechanisms to extend the treatment windows for other neuroprotective medications.

The Blood Brain Barrier (BBB) is composed of Tight Junctions (TJs) and an interface primarily of cerebrovascular endothelial cells, basement membrane, astrocytes (end feet) and pericytes. The BBB functions to wall-off the central nervous system from peripheral circulation. Dysfunction of the BBB is linked to post-stroke brain edema in clinic patients and stroke animal models [4,5]. As the BBB is disrupted, solutes, ions, blood, and immune cells (such as neutrophils) are able to infiltrate into the brain to take part in the evolution of delayed brain injury post-ischemia [4-6].

While BBB dysfunction has been implicated in stroke, the time-course of post-stroke BBB permeability remains debated. Specifically, it is questionable whether a progressively or biophysically opening of the BBB follows brain ischemia. For example, in clinic patients who received tPA treatments, BBB disruption has been visualized through Magnetic Resonance Imaging (MRI) 2 hours post tPA treatment and has been associated with brain edema evolution [7]. However, Giraud et al. [8] reported that BBB disruption is exceptional during the first 3 hours after stroke onset and argued that delayed BBB alteration is associated with stroke severity and vasogenic edema [8]. In rat MCAO studies, Kastrup et al. [9] reported BBB changes at 4.5-6 hours following reperfusion by means of MRI and Gd-DTPA extravasation. Conversely, Kidd et al. [10] demonstrated BBB disruption at 24 hours post-MCAO reperfusion using an Evans blue extravasation assay, and in murine MCAO model, Kuntz et al. [11] detected a peak of BBB opening at 4 hours following 1 hour tMCAO and post-stroke reperfusion. However, another group reported that the BBB demonstrated one-time opening at 24 hours post-stroke using tMCAO model in mice [12]. These controversial

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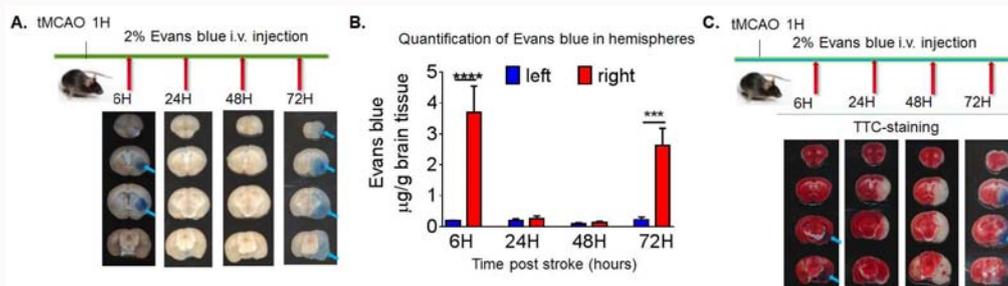


Figure 1: The BBB exhibits biphasic opening evaluated by Evan Blue extravasation in post-stroke mice.

studies suggest further need to clarify the time-course for BBB opening and reveal the pathological mechanisms in stroke.

In this study, using a transient Middle Cerebral Artery Occlusion (tMCAO) model, time windows of BBB opening after stroke were investigated. Interestingly, the BBB demonstrated a two-phase opening detected by Evan's blue and fluorescein following 1 hour tMCAO.

Materials and Methods

Mice

Institutional Animal Care and Use Committees at West Virginia University (WVU) approved criteria for procedures prior to experimentation. C57/BL6J male mice (3 months to 6 months old, 25 g to 30 g; Jackson's Laboratory, ME) were used for all procedures.

tMCAO

Mice were anesthetized with 4% to 5% isoflurane and maintained to the point where animals did not respond to toe pinch. 1% to 2% isoflurane was used to sustain unconscious state *via* face-mask in oxygen-enriched air. Focal cerebral ischemia was achieved by occlusion of the right middle cerebral artery for 60 minutes with a 6.0 monofilament suture (Doccol, Sharon, MA). Rectal body temperature of $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ was maintained throughout surgery. A successful occlusion was confirmed by changes of regional cerebral blood flow using Laser Speckle Imager (Moor Instruments, United Kingdom). Local analgesia (bupivacaine 1 mg/kg) was used daily following tMCAO. Mice were euthanized at 6, 24, 48, and 72 hours of reperfusion.

BBB permeability assay

Evans blue (Sigma, CA, 2% in saline; 4 mL/kg) and fluorescein were used as measures for BBB permeability. For Evans blue detection, the dye was intravenously administered through tail vein 30 minutes prior to euthanization. Animals were transcardially perfused with saline, brains were sectioned with a 2 mm brain matrix, and images were taken by a photo scanner. Hemisphere samples were weighed, homogenized with 400 μL PBS, and precipitated with 50% trichloroacetic acid (Sigma, CA) overnight. All samples were centrifuged at 1,000 rpm for 30 minutes to separate out the brain tissue in pellet prior to measuring. Absorption was quantified at 610 nm with a plate reader (BioTek, Winooski, VT). To quantify Evans Blue penetration through the brain barrier, an Evans blue standardized curve was used. Results were quantified as microgram/gram brain tissue.

For fluorescein detection, Texas Red (6 mg/Kg, Alfa Aesar) and Rhodamine-123 (6 mg/Kg, Life Technology) were intravenously administered through tail vein and allowed 30 minutes circulation.

All brains were retrieved after transcardial perfusion and frozen immediately in -80°C isopentane. Leica CM30505 Cryotome was used to cut brain slices (20 μm) for imaging. All brain sections were inspected for fluorescence. Cresyl Violet and Hematoxylin and Eosin (H & E) staining was performed on frozen slides additionally according to published protocols. Fluorescent images were obtained from a microscope (Olympus MVS10, Japan) and bright field images were taken through a slide scanner (Olympus VS120, Japan) at Microscope Imaging Facility at WVU.

Triphenyl Tetrazolium Chloride (TTC) staining

Brains were cut in 2 mm brain matrix; sections were stained with 2% TTC (sigma, Saint Louis, MO) in Phosphate Buffered Solution (PBS) at 37°C for 30 minutes. Brain images were photographed using a photo scanner (CanonScan 9000F, Japan).

Statistical analysis

All statistical analyses were performed with PRISM 5 software (GraphPad Software, La Jolla, CA). Group analysis was conducted through one-way ANOVA followed by post-hoc Tukey's test.

Results

The BBB exhibits biphasic opening evaluated by Evan blue extravasation in post-stroke mice. To determine the magnitude of BBB dysfunction post-stroke, C57/BL6 male mice underwent transient Middle Cerebral Artery Occlusion (tMCAO) and were injected with 2% Evans blue as a marker for BBB penetration and permeability 30 minutes prior to euthanization (Figure 1A). Interestingly, Evans Blue penetrated the BBB not only at 6 hours, but also at 72 hours as indicated by the blue arrows (Figure 1A). Quantification of Evans Blue extravasation into the hemispheres revealed a 3.5-fold and 3-fold increase at 6 hours and 72 hours, respectively, post-stroke in the right hemisphere of the brain where the occlusion occurred (Figure 1B). To note, no significant Evans blue extravasation was observed at 24 hours and 48 hours post-stroke (Figure 1B). To confirm brain infarction following BBB opening, a separate cohort of mice underwent tMCAO and were injected with 2% Evans Blue 30 minutes prior to euthanization. Brains were stained with TTC to visualize infarction. Evans Blue extravasation was observed similarly at 6 hours and 72 hours. The infarction, though not visible at 6H, was detected at 24 hours, 48 hours and 72 hours by TTC-staining (Figure 1C). These data suggest that BBB opens biphasically at 6 hours and 72 hours post-stroke, while infarction increases progressively post stroke.

The biphasic BBB opening is visualized by fluorescein in post-stroke mice

As it is reported that Evans blue may bind to albumin and

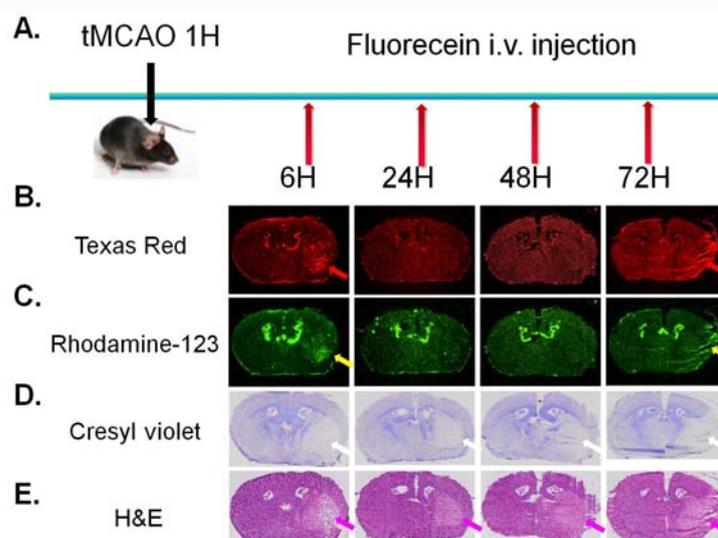


Figure 2: Biphasic BBB openings are visualized by Fluoresceins in post-stroke mice.

proteins in plasma [13], the stability of Evans blue extravasation in BBB experiments is uncertain. To detect BBB permeability at different time points in post-stroke mice, two sodium fluorescein, Texas Red (325 Da) and rhodamine-123 (380 Da), were chosen (Figure 2A). Interestingly, Texas Red (Figure 2B) and rhodamine-123 (Figure 2C) clearly revealed the biphasic opening at 6 hours and 72 hours time points consistently with the Evans blue extravasation assay (Figure 1). However, neither Texas Red nor rhodamine-123 was detected at 24 hours and 48 hours post-stroke. Using the same brain samples, we evaluated Cresyl violet staining for brain infarction and H & E staining for pathological changes. The data demonstrated that infarction (Figure 2D) and neuronal death (Figure 2E) were detected at all time points following stroke. These data suggest brain damage through 6 hours post-stroke to 72 hours; however the BBB only opens at certain time points.

Discussion

Using both Evan's blue assay and fluorescein indicators, our novel data clearly demonstrate biphasic BBB openings following tMCAO in mice. We demonstrate this BBB opening accompanied by brain infarction and neuronal death at all time points post-stroke. These data suggest that post-stroke brain damage is visualized 6 hours through 72 hours, while the BBB exhibits biphasic openings at two time-points.

While our data suggests a biphasic opening at 6 hours and 72 hours post-stroke, it has been reported that BBB opens at other time points in MCAO model. Durukan et al. [14] reported a persistent permeability using 90 minutes tMCAO rats at one-week follow-up by MRI; however, Pillai et al. [15] demonstrated the biphasic course of BBB opening (at 4 hours and 48 hours) with a significant reduction in BBB permeability at 24 hours from 1 hour tMCAO rats. The biphasic openings of BBB could vary because different stroke models were used. In permanent MCAO model, the BBB opening may be progressive owing to the failure of the cerebrovascular endothelial cells to reform the BBB and the permanent degradation of TJs [9]. In transient MCAO model, we observed that MCAO mice demonstrate the early opening at 6 hours and late opening at 72 hours post-stroke reperfusion. The removal of filaments usually results in transient occlusion of MCA, but a blood thrombus might form inside of brain

artery during filament occlusion and causes a permanent occlusion of the vessel after the filament removal. In addition, other factors might account for the BBB opening using tMCAO model, including stability of stroke surgery, bleeding and temperature control during stroke surgery, anesthetic reagents, occlusion period, post-surgery temperature maintenance, circulation time for BBB indicators, animal species and strains, and animal gender and age factors. Our study fails to address all these factors individually; however, it is important to consider them for future evaluations of BBB permeability post-stroke.

The mechanisms that contribute to BBB breakdown have been investigated, some of which involve the immune system, Matrix Metalloproteins (MMPs), and mitochondrial dysfunction. For example, in previous studies, it was reported that challenge with LPS resulted in an increased infarct volume post-stroke and that post-BBB disruption, neutrophils extravasation into the brain parenchyma and contributed to brain insult [4]. MMPs that degrade TJs are reportedly upregulated following stroke, and recent studies indicate dysfunction of MMPs (ex. MMP2 and MMP9) have been associated with complications post-stroke, such as neuronal damage, excitotoxicity and BBB opening [6,16-18]. We recently demonstrate that mitochondrial impairment has been linked to dysfunction of cerebrovascular endothelial cells and breakdown of TJs [4]. The peak of hypothermia has been observed at 6 hours post-tMCAO [19], and this time point is consistent with the early BBB opening following tMCAO. As the hypothermia may be linked to mitochondrial dysfunction, body temperature could be an indicator of BBB disruption. In addition, we recently demonstrated that miR-34a alters mitochondrial function and changes BBB permeability [20]. Taken together, mitochondrial dysfunction in cerebrovascular endothelial cells could initiate the TJ breakdown and MMPs may further cause degradation of TJs. This can result in neutrophil infiltration, and ion, solute, blood, etc. extravasation into the brain parenchyma and thus, further brain edema and injury through inflammatory mechanisms.

Since the only current treatments for acute stroke are administration of tPA (within 4.5 hours of symptom onset) and usage of retrieval devices (24 hours of symptom onset), determining the BBB opening is important for controlling brain edema and preventing other adverse effects. Ideally, it is expected that BBB openings after

stroke could provide a window for the delivery of neuroprotective medications into the brain.

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