Video Article

An *In Vivo* Assessment of Blood-Brain Barrier Disruption in a Rat Model of Ischemic Stroke

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URL: https://www.jove.com/video/57156
DOI: doi:10.3791/57156

Keywords: Neuroscience, Issue 133, MCAO, Blood-brain barrier, Ischemic stroke, Rat model

Date Published: 3/11/2018

Citation: Panahpour, H., Farhoudi, M., Omidi, Y., Mahmoudi, J. An *In Vivo* Assessment of Blood-Brain Barrier Disruption in a Rat Model of Ischemic Stroke. *J. Vis. Exp.* (133), e57156, doi:10.3791/57156 (2018).

Abstract

Ischemic stroke leads to vasogenic cerebral edema and subsequent primary brain injury, which is mediated through destruction of the blood-brain barrier (BBB). Rats with induced ischemic stroke were established and used as *in vivo* models to investigate the functional integrity of the BBB. Spectrophotometric detection of Evans blue (EB) in the brain samples with ischemic injury could provide reliable justification for the research and development of novel therapeutic modalities. This method generates reproducible results, and is applicable in any laboratory without a need for special equipment. Here, we present a visualized and technical guideline on the detection of the extravasation of EB following induction of ischemic stroke in rats.

Video Link

The video component of this article can be found at https://www.jove.com/video/57156/

Introduction

Vasogenic brain edema due to blood-brain barrier (BBB) disruption remains an important complication of the ischemic stroke and a major determinant of the survival rate in the stroke patients[1,2]. The blood-brain barrier (BBB), which is formed by brain capillary endothelial cells (BCECs) and composed of distinct neurovascular components (*e.g.,* tight junctions among BCECs, pericytes, astroglial, and neuronal cells[3]), provides a specialized and dynamic interface between the central nervous system (CNS) and peripheral blood-circulation[4,5]. Insults such as ischemia-reperfusion injuries could disrupt the functional integrity of the BBB and lead to subsequent penetration of circulating leukocytes into the brain parenchyma that ultimately trigger cerebral inflammation and primary brain injuries[6,7]. Animal models are needed for the exact detection of the dysfunction of BBB following occurrence of a stroke. Such models are of great importance for studying underlying pathophysiological mechanisms and introducing new neuroprotective strategies. *In vitro* cell culture-based models of the BBB have been highly developed and used for molecular study of the BBB physiopathology[8,9,10]. Nevertheless, *in vivo* animal models, which produce ischemic damage of the BBB similar to human clinical conditions, are also very worthwhile in this regard. Quantitative detection of the extravasation of Evans blue (EB) is a well-accepted and sensitive technique that has been used for assessment of the BBB integrity and function in neurodegenerative diseases, including ischemic stroke[11,12,13,14]. This method is cost-effective, feasible, reproducible, and completely applicable in any experimental laboratory. Its implementation does not require advanced equipment, such as radioactive tracers[15] or magnetic resonance imaging (MRI)[16], that are prerequisites for other methods. In this article, we comprehensively demonstrate basic technical processes of BBB assessment using EB extravasation in rat models of ischemic stroke.

Protocol

All procedures were performed in accordance with the guidelines of Ardabil University of Medical Sciences Research Council for conducting animal studies (Ethical ID number: IR.ARUMS.REC.1394.08). In this visualized study, we used adult male Sprague-Dawley rats (300-350g) obtained from Pasture Institute (Tehran, Iran).

1. Anesthesia and Flowmetry

1. Induce anesthesia using 4% isoflurane and maintain it with isoflurane (1-1.5%) in a mixture of nitrous oxide (70% v/v) and oxygen (30% v/v) for the duration of the surgery.
2. Place an anaesthetized animal in the prone posture on a feed-back controlled heating blanket and maintain the body temperature at 37±0.5 °C by means of a rectal probe connected to this heating unit.
3. Apply a small amount of ophthalmic ointment on both eyes to prevent dryness.

4. Shave the surgical region on the left side of the skull with electric clippers. Use betadine solution with a gauze pad to disinfect the skin. Rinse this region with a sterile pad containing 70% ethanol and repeat both steps for three cycles[17].
5. For analgesia, inject 0.2 mL of 0.5% bupivacaine subcutaneously into the surgery region[17].
6. Make a 1 cm long skin incision on the skull (extending from the lateral canthus of the left eye to the base of left ear) and dissect the left temporalis muscle to expose the skull. Then, create a small burr hole 5 mm lateral to and 1 mm posterior to the bregma[18] to ease placement of the tip of the Doppler flowmeter pencil probe.
7. Turn the rat from the prone to supine position, and then put the laser pencil probe into the previously drilled skull blind-hole to monitor the regional cerebral blood flow (rCBF).
8. Record each animal's baseline rCBF and consider this as 100%. Notably, middle cerebral artery occlusion (MCAO) is started whenever a decrease in rCBF of more than 80% is detected[19,20].

2. Induction of the Focal Cerebra Ischemia

1. Shave the neck region and disinfect the skin with betadine solution and 70% ethanol. Inject 0.2 mL of 0.5% bupivacaine subcutaneously in surgery site for analgesia[17].
2. Make a 2 cm long surgical incision in the ventral surface of the neck to access the left common carotid artery (LCCA). Isolate the LCCA from the neighboring fascia and the vagus nerve to access bifurcation of the external carotid artery (ECA) and the internal carotid artery (ICA).
3. Ligate permanently either the LCCA or ECA employing a 5-0 silk suture and dissect ICA free to the level of pterygopalatine artery.
4. Loosely place a tie of 5-0 silk suture around LCCA, and then temporarily ICA clamp with a vascular micro-clip.
5. Make a small incision on the LCCA prior to the previously placed loose tie, and then insert a 4-0 silicon-coated nylon suture into the luminal space of ICA and tighten the suture around the LCCA to secure the nylon suture and prevent blood leaking.
6. Remove the vascular micro-clip from the ICA. Then, advance a silicon coated intraluminal filament until observing a marked decline in rCBF that indicates occlusion of the MCA origin. At the end of ischemic period (90 min), start reperfusion by withdrawing the intraluminal suture[21].

3. Jugular Vein Cannulation and Evans Blue (EB) Injection

1. Make a 1 cm longitudinal incision in the neck to the left side of the midline and then bluntly dissect away superficial fascia to access the external branch of the left jugular vein (LGV). Then, permanently ligate the cranial end of the LGV with 5-0 silk suture. Loosely place two ties around the vein, and then temporarily clamp the cardiac end of the LGV with a vascular micro-clamp.
2. Make a small incision on the LGV between two sutures, and then insert heparinized serum filled catheter into the luminal space of the LGV and advance it approximately 10 mm. Afterward, tighten the ligatures around the vein to secure the catheter and prevent bleeding. Inject small amounts of serum to avoid collapsing the vein.
3. At the beginning of the reperfusion period, slowly inject EB dye (1 mL/kg of 2% EB solution in saline) over a 5 min period. Subsequently, wash the cannula by injection of 0.5 mL normal saline and withdraw the injection cannula from the vein[12,22].
4. Finally, suture the neck and head incisions and inject 0.05 mg/kg buprenorphine intraperitoneally (IP) and repeat the injection every 6-8 h during reperfusion period for post-operative analgesia[17].
5. Inject 5 mL of pre-warmed saline(IP) to provide hydration in the recovery cage[17].
6. Place the animal in a special recovery cage equipped with temperature and air conditioning control and monitor the recovery of the animal from anesthesia.

4. Assessment of the Blood Brain Barrier Permeability

1. After 24 h of reperfusion, deeply anesthetize the animal with sodium thiopental. Then, open the thoracic cavity by making a small hole beneath the sternum.
2. Make a small opening in the right atrium and inject 250 mL of pre-warmed 0.9% saline (37 ˚C) at a pressure of 110 mmHg through the left ventricle for 15 min to wash EB away from the circulation until the normal saline exits from the atrium becomes colorless[23].
3. Immediately remove the brain from the skull and place it in the brain matrix. Dissect the olfactory bulb and cerebellum, and then using a clean razor blade, separate the right hemisphere of the brain from the left (Lesioned) along the midline.
4. Weigh each hemisphere and homogenize them separately in 2.5 mL of phosphate buffered saline, and mix this with 2.5 mL of trichloroacetic acid (60%) for 2 min using a vortex machine.
5. Centrifuge brain samples for 30 min at 1,322 x g and allow the samples to cool down in a 4 °C refrigerator for 10 min.
6. Use the supernatants to estimate the EB absorbance by a spectrophotometer at 610 nm[23].
7. Calculate EB concentrations against a standard curve and express results as µg/g of the brain tissue.

5. Production of the EB Standard Curve

1. Prepare 10 sample solutions of EB with concentrations of 1-10 µg of EB in 5 mL of phosphate buffered saline.
2. Measure the absorbance values of each sample at 610 nm using a spectrophotometer.
3. Make a scatter chart using a spreadsheet and present EB concentrations on the X axis and the absorbance values on the Y axis (**Figure 1**).
4. Define a linear trend-line for the curve with its equation. Then, use it to obtain the EB concentration of samples.
5. Report the final results of EB extravasation as µg/g of the brain tissue weight.

6. Sham Operation

1. Perform the same surgical process with rats in sham-operated group (including making an incision in the neck region and EB injection) but exclude the MCAO.

Representative Results

There was no significant difference in EB levels in the right hemisphere versus the left hemisphere of the sham-operated rats (1.06 ± 0.1 µg/g and 1.1 ± 0.09 µg/g, respectively). As shown in **Figures 2A-2B**, induction of transient ischemia (90 min ischemia/ 24 h reperfusion) caused a significant difference in EB levels (10.41 ± 0.84 µg/g, p <0.001) in the left hemisphere of ischemic rats, as compared to the respective hemisphere in the sham-operated rats. Collectively, these findings indicate that under normal conditions, EB cannot readily cross the BBB into cerebral parenchyma and cerebral ischemic insults induce the extravasation of EB through an enhanced permeability ofthe BBB (**Figures 2A and 2B**).

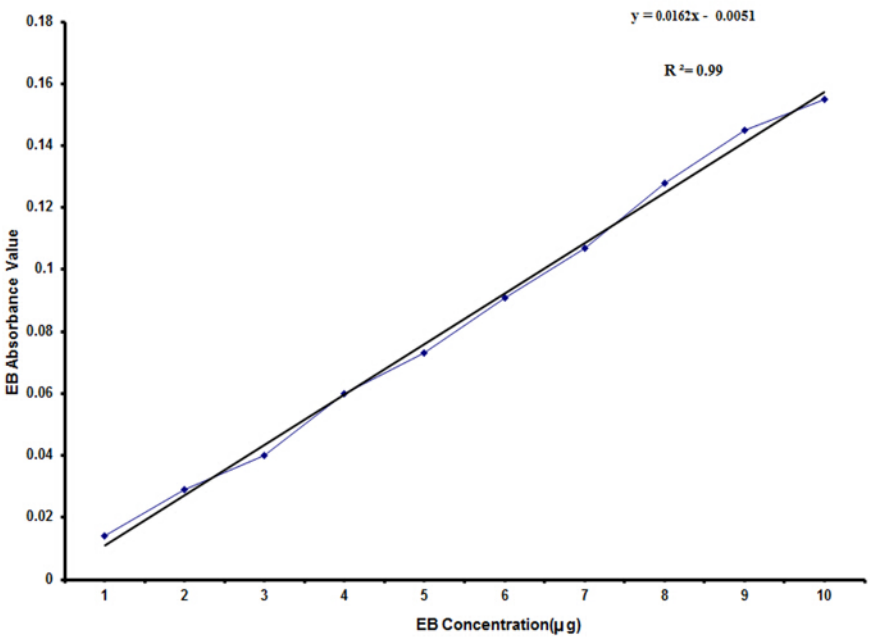

Figure 1: The standard curve is used to determine the EB concentration from the absorbance values. Please click here to view a larger version of this figure.

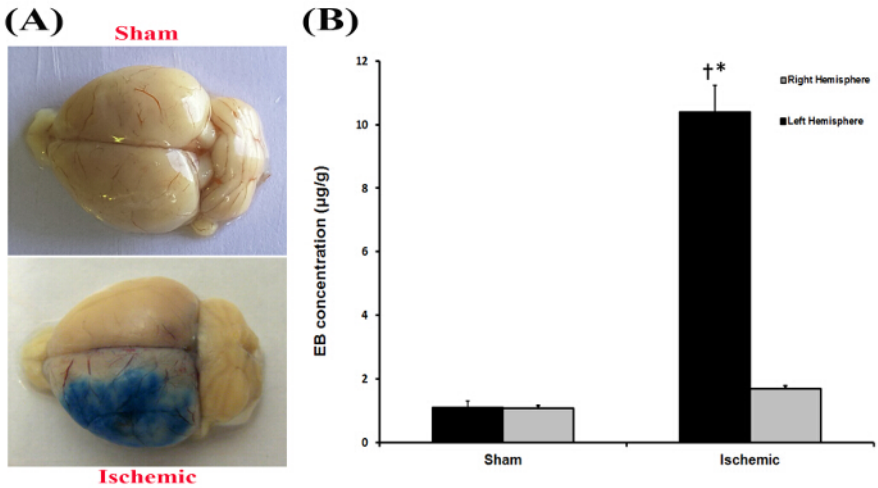

Figure 2: Assessment of BBB disruption by EB extravasation 24 hours after ischemic stroke. The photograph of the brains in the sham-operated and ischemic animals (**A**). The intensity of EB extravasation in the brain tissue (blue color) arises from extent of the BBB disruption in the lesioned hemisphere. EB concentration in samples prepared from the left (lesioned) and right hemispheres of the brain in the sham-operated and ischemic animals (**B**) (n=6, *p<0.001 compared to left hemisphere in sham group, p<0.001 compared to ipsilateral hemisphere of same group). Please click here to view a larger version of this figure.

Discussion

Thus far, various methods such as autoradiography and detection of the radioactive tracers[24,25], immunofluorescence microscopy[26,27], and EB extravasation technique[20,23] have been used to evaluate the blood-brain barrier damage. EB dye is strongly able to bind to the serum albumin and is used as a tracer for detecting vascular leakage and quantifying the BBB breakdown[11,28,29]. As a highly accepted and reliable method, the EB extravasation technique provides a direct estimation upon the integrity of the BBB that is affected by different cerebral injuries including ischemic stroke.

In vivo assessment of the BBB allows researchers to study possible pathophysiological mechanisms of ischemia induced vasogenic brain edema and to find new therapeutic interventions. This model does not require special facilities and can produce credible results with a high success rate in experiments (more than 80%)[13,20]. With direct access to the brain tissue, this model enables highly accurate assessments of the BBB integrity but is restricted to long-term studies.

Pathological changes in the BBB caused by ischemic stroke develop in three phases: acute (within hours), sub-acute (hours to days), and chronic (days to months)[30,31]. Obviously, the earliest therapeutic interventions produce valuable protective effects in the acute pathological phase. EB dosage and the time point of injection are two crucial parameters for obtaining reliable results due to the dynamic nature of the BBB following ischemic insults. Hence, injection of the EB dye slowly via a vein cannula using the appropriate dose (1 mg/kg of 2% EB solution in saline) after the beginning of the reperfusion period is an important factor and allows the study of pathophysiological changes in the early stages of stroke.

Several experimental methods have been introduced to study ischemic stroke. In this experimental model, we used MCAO with the intraluminal filament method that creates conditions similar to human stroke[21,32]. This technique is simple and reliable; however, its execution needs to take into consideration some technical points to further enhance the performance of the technique and ensure its accuracy. Body temperature should be kept within the physiological range during the surgery, while blood pressure and blood gases must be monitored[33,34,35]. Constant recording of the rCBF with a laser Doppler flowmeter and using a suitable prepared silicone-coated filament can not only increase the MCAO success rate, but also reduce the mortality rate.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors are thankful to the Vice Chancellor for Research of the Ardabil University of Medical Sciences (Ardabil, Iran) for the financial support (grant No: 9607).

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