Video Article

Quantification of Neurovascular Protection Following Repetitive Hypoxic Preconditioning and Transient Middle Cerebral Artery Occlusion in Mice

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Abstract

Experimental animal models of stroke are invaluable tools for understanding stroke pathology and developing more effective treatment strategies. A 2 week protocol for repetitive hypoxic preconditioning (RHP) induces long-term protection against central nervous system (CNS) injury in a mouse model of focal ischemic stroke. RHP consists of 9 stochastic exposures to hypoxia that vary in both duration (2 or 4 hr) and intensity (8% and 11% O_2). RHP reduces infarct volumes, blood-brain barrier (BBB) disruption, and the post-stroke inflammatory response for weeks following the last exposure to hypoxia, suggesting a long-term induction of an endogenous CNS-protective phenotype. The methodology for the dual quantification of infarct volume and BBB disruption is effective in assessing neurovascular protection in mice with RHP or other putative neuroprotectants. Adult male Swiss Webster mice were preconditioned by RHP or duration-equivalent exposures to 21% O_2 (*i.e.* room air). A 60 min transient middle cerebral artery occlusion (tMCAo) was induced 2 weeks following the last hypoxic exposure. Both the occlusion and reperfusion were confirmed by transcranial laser Doppler flowmetry. Twenty-two hr after reperfusion, Evans Blue (EB) was intravenously administered through a tail vein injection. 2 hr later, animals were sacrificed by isoflurane overdose and brain sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC). Infarcts volumes were then quantified. Next, EB was extracted from the tissue over 48 hr to determine BBB disruption after tMCAo. In summary, RHP is a simple protocol that can be replicated, with minimal cost, to induce long-term endogenous neurovascular protection from stroke injury in mice, with the translational potential for other CNS-based and systemic pro-inflammatory disease states.

Video Link

The video component of this article can be found at https://www.jove.com/video/52675/

Introduction

As the leading cause of adult disability and the fourth leading cause of death, stroke is one of the most debilitating disease states facing the adult population of the United States.[1] Animal models of stroke allow for experimental investigation of new methods of reducing ischemic injury and improving post-stroke recovery. One novel avenue for such translational research is preconditioning. Preconditioning is the intentional use of a non-damaging stimulus to reduce damage from a subsequent, and more severe, injury.[2] Hypoxic preconditioning has been shown to produce pleiotropic changes in the brain that provide protection against stroke in both *in vivo* and *in vitro* studies.[3] However, a single exposure to hypoxia only offers short-term neuroprotection, inducing less than 72 hr of tolerance against ischemia in adult mice.[4] Even after four weeks of 14 hr daily exposures to hypobaric hypoxia, Lin et al. found that neuroprotection was only sustained for one week.[5] Repetitive hypoxic preconditioning (RHP) is characterized by stochastic variations in frequency, duration, and intensity of hypoxic exposures. In contrast to a single preconditioning challenge, RHP induces a cerebroprotective phenotype that lasts up to eight weeks in mice.[6] RHP reduced infarct volumes, blood-brain barrier (BBB) disruption, vascular inflammation, and leukocyte diapedesis for weeks after the final hypoxic exposure. RHP specifically reduced inflammation in the ischemic brain by reducing T cell, monocyte, and macrophage populations, while maintaining B cell populations in the ischemic hemisphere.[7] In fact, RHP induced an immunosuppressive phenotype in mice prior to any CNS injury, including stroke. RHP-treated B cells isolated from RHP-treated healthy mice exhibited a unique anti-inflammatory phenotype, with a downregulation of both antigen presentation and antibody production. The overall reduction in pro-inflammatory adaptive immune mechanisms makes RHP an excellent methodology to induce endogenous immunosuppression for not only CNS-specific inflammatory diseases, but also systemic injury or disease models that include a pro-inflammatory pathology.

RHP reduces both infarct volume and BBB disruption following a transient middle cerebral artery occlusion (tMCAo). Animal models of stroke, such as the commonly used tMCAo, dramatically improve the understanding of the pathophysiology of stroke, as well as the design of more effective neurotherapeutics. First developed by Koizumi *et al.*, in 1986,[8] the tMCAo procedure is a widely used method of inducing stroke in rodents and one of the preferred methods for investigating inflammation following reperfusion. As the methods for tMCAo evolve, the more recent use of silicone-coated filaments further reduce the risk of subarachnoid hemorrhage compared to other models[9,10] and improve reliability,

though unfortunately tMCAo often produces a wide variation in infarct volumes.[11-13] Most of these studies delineate infarction regions in coronal brain sections by staining with 2,3,5- triphenyltetrazolium chloride (TTC), considered a gold standard for infarct quantification because it is a simple and inexpensive way to produce vivid, replicable results. TTC serves as a substrate of dehydrogenases present in mitochondria. When brain slices are exposed to the TTC solution, TTC is selectively taken into living cells where its non-soluble reduction product, formazan, precipitates to a deep red color in viable mitochondria. Because of mitochondrial dysfunction in the ischemic tissue, this tissue remains white, allowing for differentiation of damaged and healthy tissue.[14]

RHP also reduces BBB disruption in the ischemic hemisphere.[6] Therefore, the dual quantification of BBB integrity within the same brains as TTC-based infarct volume determinations[15] would provide useful information about the full efficacy of endogenous protection, and potential causal relations between BBB disruption and infarction in untreated and treated animals. The influx of peripheral blood through a disrupted BBB, secondary to stroke, increases leukocyte populations, pro-inflammatory cytokines, oxidative stress, vasogenic edema, and hemorrhagic transformation in the ischemic hemisphere, ultimately increasing the rates of infection and mortality in patients with ischemic stroke.[16,17] A common method of measuring BBB disruption in animal models is through quantification of Evans blue (EB) dye leak into the brain.[15,18-21] EB selectively binds to serum albumin, a globular protein (MW=65 kDa) that does not cross the BBB in uninjured animals.[22] Following ischemic stroke, EB infiltrates the brain, and fluoresces at 620 nm, allowing for measurement of optical density within the perfused injured parenchyma.[22] The optical density is directly proportional to the permeability of the BBB when EB has been washed out of the post-mortem cortical vasculature by transcardiac perfusion. With the immediate processing of TTC-stained brains in animals with EB administration, both the infarct volume and BBB disruption can be effectively quantified. It should be noted, however, that neuronal injury and BBB disruption are not concomitant processes in the post-stroke brain,[23,24] so the selection of time of sacrifice is an important consideration.

The protocol that follows details the RHP method, the tMCAo method for inducing a temporary arterial occlusion that models middle cerebral artery occlusions in human patients, and the dual histological methods for determining neural and vascular stroke injury endpoints. TTC measures cell death and cumulative tissue damage, allowing for the quantification of an overall infarct volume, while EB provides for the hemispheric quantification of BBB damage.

Protocol

NOTE: This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at UT Southwestern Medical Center which abides by the National Institutes for Health (NIH) policy for experimental animal usage.

1. Repetitive Hypoxic Preconditioning

1. Custom design four flowmeters on gas regulators and attach to standard 15 L induction chambers with PVC tubes to allow compressed gas from the oxygen (O_2) tanks to flow into the chambers via an inlet port. See Equipment and Materials for more details about custom design.
2. Divide mice into 2 groups: Repetitive Hypoxic Preconditioning (RHP) Group, which receive exposures to 8% and 11% O_2, and the Control Group, which receive exposures to 21% O_2 (room air) concurrently. See **Table 1** for the variations in frequency, intensity (8 and 11% O_2 or 21% O_2), and duration (2 or 4 hr) of RHP exposures.
3. Remove the top filter lid of each cage and place the cage, with food and water bottles intact, in the chambers connected to their respective O_2 tanks. Close and secure the lid of the chamber.
    1. Open the main gas valve for the tanks and set the initial flow for each induction chamber to 2 L per min (LPM) for the first 5 mins of exposure. Reduce the flow rate to 1 LPM for the remainder of the exposure.
    2. At the end of the exposure, reduce the flow to to 0 LPM and close the gas valve for the tanks.
    3. Open the chamber lids and replace the filter top lid on each cage. Place the cages in standard housing until the next hypoxic exposure.
4. Spray down each induction chamber with NPD (Steris) or an equivalent disinfectant/deodorizer after each use.
5. Expose both 21% and RHP mice at the same time of day over the course of two weeks as described in **Table 1**.

2. Transient Middle Cerebral Artery Occlusion (tMCAo)

1. See Discussion for more details on the timing of stroke following final RHP exposure.
2. Prepare an aseptic surgery workplace. Clean surrounding workplace with 70% Ethanol or an equivalent disinfectant and autoclave all surgical tools.
    1. Set up the Laser Doppler Flowmetry (LDF) instrument to measure relative changes in cerebral blood flow (CBF). Turn on the heating pad to 37 °C. Turn on the incubator to 34 °C.
3. Anesthetize mice with a brief exposure to a mix of 4% isoflurane/70% NO_2/30% O_2 in a small induction chamber. Confirm proper anesthesia by lightly pinching the paw. If the mouse withdraws its paw, return the mouse to the induction chamber and continue isoflurane exposure.
4. Remove mice from the anesthesia induction chamber and quickly insert the mouse's nose in the nose cone. Open the gas flow to the nose cone, closing off flow to the anesthesia induction chamber.
    1. Without changing the 70% NO_2/30% O_2 gas mixture, fix isoflurane at 1.8% as the maintenance dose for the remainder of the procedure. Breathing should remain slow and regular throughout the procedure but if breathing becomes rapid and shallow, increase the isoflurane dose. Maintenance dose may vary between equipment manufacture and animal used in the experiment.
5. Using a microshaver, shave the hair on the temporal region between the corner of the eye and the ear as well as ventral midline of the neck. Remove excess fur and apply ocular lubricant with a sterile cotton swab to keep the corneas from drying out during the procedure. Wipe down incision area with alcohol pads and swab providone-iodine with a sterile cotton swab to maintain aseptic conditions.
6. Administer analgesics according to rodent surgical guidelines.

7. Make an incision through the temporal skin between the eye and the ear. Expose the temporalis muscle. Using surgical micro-scissors, cut the temporal muscle ligament at the temporal ridge within the area of white muscle striation.
    1. Gently push the muscle bulk laterally with forceps to visualize the middle cerebral artery (MCA) through the skull. After incision of the temporal muscle, the area may fill with blood. Gently use a cotton swab to staunch any potential bleeding.
    2. Target the the LDF probe tip to the MCA area. Record the vessel chosen as this area varies between mice.
    3. Hold the LDF in place and flush with the skull until a stable red blood cell flux is read and record this value as the baseline CBF. Ideal baseline CBF on a Laser Doppler Flowmetry are >600 flux, but this will vary with manufacturer. If baseline CBF is <400 flux, the flow recording is most likely from a nearby vein, or an incomplete placement of the probe over the target vessel.

8. After baseline CBF is established, reposition the mouse so that the neck is exposed. Support its head and keep the mouse under steady anesthesia from the nosecone.
9. Make a ventral midline incision from just below the mandible to the clavicle.
    1. Using forceps, blunt dissect all superficial fascia to expose the left common carotid artery (CCA). Separate the CCA from connective tissue and the vagus nerve.
    2. Permanently ligate the CCA with a 6.0 silk suture. Place the ligating suture as proximal as possible to allow enough room for occluding filament placement.
    3. Loop and loosely tie a second silk 6.0 suture around the CCA distal to the occluding suture. Be careful not to occlude the artery as the occluding filament will subsequently be threaded through the carotid.
    4. Use an 8 x 2 mm light micro serrafine areterial clamp to clamp the CCA distal to the loosely-tied silk suture. Gently lift the CCA with forceps and make a small longitudinal incision, as proximal to the ligating suture as possible with 3 mm vannas.
    5. Thread a 12 mm silicon-coated 6.0 gauge nylon occluding filament through the incision to enter the artery lumen, and then advance it a few mm. Loosely tighten the second loose silk suture around the tip of the occluding filament to ensure blood flow does not push the filament out of the CCA and remove the arterial clamp.
    6. At the first bifurcation of the CCA into the internal carotid artery (ICA) and the external carotid artery (ECA), thread the occluding filament into the right branch of the first bifurcation to enter the ICA.Advance the occluding filament 9 to 10.5 mm past the second silk suture into the left internal carotid artery (ICA).
    7. Shortly after entering the ICA, advance the occluding filament into the left branch at the second bifurcation between the ICA and the pterogopalantine artery (PPA). Visualization of the PPA is unlikely so proceed until you feel a mild resistance with full placement of the occluding filament. Lifting the ICA with forceps may help the filament to thread more easily into the left branch of the second birfurcation. Tighten the second silk suture.
    8. Turn the mouse so the incision over the MCA is visible. With the LDF equipment, confirm that the CBF is blocked through LDF readings. A successful occlusion is a >80% reduction from baseline CBF.
    9. Completely tighten and double-knot the second silk suture around the occluding filament when the proper position is achieved. If necessary, slightly push in or pull out the occluding filament to achieve CBF criteria for a successful occlusion (*e.g.* >80% reduction from baseline CBF).
    10. Close the neck and head opening with 6.0 nylon sutures.

10. Place mice in the 34 °C incubator for the duration of the occlusion. Recommended length of occlusion is 60 min, but this varies for age, strain-dependent differences in cerebrovascular anatomy,[25] and the extent of injury desired (mild, moderate, severe). Make sure that animals regain consciousness within mins of coming off anesthesia.
11. Re-anesthetize the animals with isoflurane, as described in step 2.3, 5 min before the end of the pre-defined occlusion period, open the scalp incision and confirm that the MCA perfusion is still reduced using transcranial LDF readings. If CBF is not sufficiently reduced (*e.g.* <20% baseline CBF), the MCA has reperfused at some point during the occlusion and the mouse should be excluded from further experimentation.
12. Open the midline neck incision and loosely tie a third silk suture around the CCA, distal to the second silk suture but proximal to the CCA bifurcation to ensure that the external carotid artery (ECA) will remain viable after the filament is removed.
    1. Cut or untie the knot that holds the occluding filament (*i.e.,* second silk suture) and withdraw the occluding filament slowly. Once removed, quickly close the third silk suture around the CCA to minimize backflow of blood from the ICA. Double-knot this suture and close the incision with 6.0 nylon sutures.
    2. Reposition the mouse to quantify the level of CBF after 5 min of reperfusion. Successful reperfusion is generally defined as a CBF of >50% of baseline CBF, but, as with occlusion flow, investigators can establish their own criterion. If animals exhibit a CBF below 50% of their baseline, it is likely the MCA is 'permanently' occluded, and thus represents another study exclusion criterion.
    3. Close both incisions with 6.0 nylon sutures. Provide saline, anesthetic (Lidocaine), and antibiotics according to rodent guidelines. However some small doses of antibiotics (3 mg/kg of minocycline) have been found to be neuroprotective following stroke.[26]

13. After regaining consciousness in the heated incubator following surgery, place mice in a clean, sterile cage. Provide moistened food or nutritional hydration dietary gel supplements and a petri dish of water as the animals will have restricted mobility following stroke. Monitor animals closely during recovery for excessive post-surgical pain and death.

3. Evans Blue (EB) Injection

1. Inject EB 22 hr after reperfusion and should circulate in the bloodstream for 2 hr prior to sacrifice and TTC staining.
2. Make an EB solution for injection (2% EB in saline) and gently mix at room temperature. Filter solution through filter paper or push through a 0.2 µm filter attached to a small syringe to remove undissolved EB and store at room temperature.
3. Prepare the amount of EB needed for injection (4 ml/kg of body weight) Draw the desired amount of dye into a 0.3 cc insulin syringe with a 29 gauge needle and ensure that the solution is at room temperature
    1. Restrain mouse using flat bottom restrainer. Hold the tail so that the lateral vein is uppermost. Lateral veins are located on either side of the centerline of the tail. Hold the tip of the tail to keep the mouse steady for injection.
    2. Insert the needle into the vein approximately 3.5 mm, being careful not to perforate the vein. Confirm that the needle is in the vein by drawing back on the syringe and looking for traces of blood.

3. Inject all of the dye over the course of 1 min. If the solution is going into the vein there should be minimal resistance as pressure is applied to the syringe. Confirm successful systemic venous administration of EB by an immediate color change in the tail, limbs, and eyes of the mouse.
4. Remove the needle from the tail and gently apply pressure using clean gauze in order to stop bleeding.

4. Begin timing when the mouse's skin turns blue. Allow the EB to circulate for 2 hr to penetrate the weakened BBB.

4. 2,3,5- Triphenyltetrazolium Chloride (TTC) Staining

1. Perfusion and TTC staining should occur 24 hr after reperfusion.
2. Prepare a 2% TTC solution prior to the designated time of sacrifice. Add 10 g of TTC powder to 500 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4. Heat solution to 37 °C in water bath to facilitate solubilizing of the TTC. CAUTION: TTC powder and solution are a skin, lung, and eye irritant. Wear appropriate personal protective equipment when handling these materials.
    1. Once the powder has completely dissolved, immediately transfer to a bottle, cover in foil, and store at 4 °C. TTC and tissue stained with TTC are light sensitive.

3. At 24 hr after tMCAo and 2 hr after EB administration, sacrifice the animal with an isoflurane overdose in a small induction chamber. Perfusion should begin immediately following sacricice in order to minimize autolysis which begins in the absence of oxygen following death.
4. Quickly secure the animal on a Styrofoam platform with forearms pinned through the paws. Cut a lateral incision through the abdominal wall from the midline just below the ribcage. Carefully cut through the diaphragm to expose the heart.
    1. Start the perfusion pump at 5 ml/min flow rate with a 60 cc syringe filled with ice cold 0.01 M PBS and connected to a 27 guage winged infusion needle. Place the tip of the needle about 0.5 cm into the left ventricle of the heart and cut the right atrium. Progressively diluted venous blood should flow out of the atrium during the perfusion until venous blood appears colorless. Transcardially perfuse 30 ml 0.01 M phosphate buffered saline (PBS) through the heart.

5. Add 5 ml of TTC solution into transparent 20 scintillation bottles.
6. Immediately following perfusion, decapitate the animals and dissect out the brains using small scissors and a spatula if necessary. Examine the brains to exclude animals which underwent subarachnoid hemorrhage at the Circle of Willis, secondary to suture placement. Check that the hemisphere contralateral to the occluded MCA appears pale, without noticeable EB leakage or edema.
7. Pour PBS into an acrylic brain matrix designed to make 1.0 mm thick coronal sections of a mouse brain. Place the brain, dorsal side up, into the matrix and immediately pour PBS over the brain. Keep the brain moist.
    1. Remove the olfactory bulbs by inserting a stainless steel 0.21 mm thick blade into the second slot from the rostral side of the matrix.
    2. Remove the cerebellum by inserting a blade in the fourth slot from the caudal side of the matrix.
    3. Insert a blade into the middle slot of the remaining slots in the matrix. Insert the remaining blades, evenly bisecting the remaining tissue to ensure the most even distribution of tissue during the slicing.
    4. Once all blades have been inserted, add 1 to 2 drops of PBS to the brain to moisten it.
    5. Remove the blades one at a time from the matrix beginning with the rostral region. Keep the first 7 slices for TTC analysis after tMCAo. Use a small spatula to carefully transfer the slices from the blade to the TTC-filled vial.

8. After all sections are in the vial, cap it and place in a warm water bath until the sections turn pink. Gently turn the bottle in the bath if necessary to avoid section overlap, which could lead to uneven staining. Then dispose of the TTC and pour a 4% paraformaldehyde solution into the vial to cover brain sections to terminate the TTC chemical reaction.
9. Immediately arrange the sections on a clean 1" x 3" glass slide and orient the sections from rostral to caudal.
    1. When the sections are arranged on the slide, scan the slide using a standard scanner. Set the resolution at a minimum of 600 dpi for image analysis. Be sure to include the name of the animal and a metric ruler in the scanned image.
    2. Flip over the slide and scan the back side to ensure all data is collected.

5. Infarct Volume Quantification

1. Quantify the infarct volume by using a standard image analysis software (*e.g.,* ImageJ).
2. Scan images at a high resolution (*e.g.* 600 dpi) for adequate analysis. Crop images. Standardize the scale for all images using the metric ruler included in the scanned image.
3. Calculate the total volume of the contralateral hemisphere using the following formula. Repeat this formula to calculate the total volume of the ipsilateral hemisphere.
   Sum of total contralateral hemisphere of each slice x slice thickness
4. Calculate the indirect infarct volume. Control for ipsilateral edema from stroke by using the healthy, contralateral hemisphere as a control.[27]
   Use the following formula to calculate the indirect infarct volume:
   Total volume of contralateral hemisphere -
   (Total volume of ipsilateral hemisphere –Average volume of 3 measurements of infarct)

6. Blood-brain Barrier (BBB) Integrity Quantification

1. To prepare for EB quantification, first weigh 2.5 inch weigh boats. Record the weight and label two weigh boats for each animal: one for the ipsilateral hemisphere and one for the contralateral hemisphere.
2. After scanning the TTC-stained sections, bisect each section with a disposable razor blade into ipsilateral and contralateral hemispheres. Place the ipsilateral hemispheres from all 7 sections in a weigh boat and record the weight. Repeat for contralateral hemisphere.
3. Immediately transfer the weight boats to an oven set for 56 °C for 48 hr.
4. Weigh the dried sections. Transfer both hemispheres into separate 1.5 ml microcentrifuge tubes.

1. Calculate the amount of formamide needed for each hemisphere (8 ml/g of dry tissue), and add to their respective microcentrifuge tubes. Formamide is light sensitive and cover all formamide-treated samples in foil from this point onward.
2. Transfer the microcentrifuge tubes to an incubator set to 56 °C for another 48 hr.

5. After the 48 hr, pipette out the blue supernatant into another set of labeled microcentrifuge tubes. Push tissue to the bottom of the tube to maximize the volume of extracted supernatant. Keep the tubes of supernatant and dispose of all tissue.
6. Prepare exponential serial dilutions of EB in formamide for the standard curve. Include 1 blank (formamide only) and then 10 exponential solutions from 0.125 ug/ml through 64 ug/ml of EB in formamide.
    1. Pipette 300 µl of the dilutions made for the standard curve into a 96-well plate. Pipette 300 µl of the supernatant into corresponding wells.
    2. Measure the absorbance on a spectrophotometer at 620 nm.
    3. Compare the standard curve of the EB dilutions with the absorbance of the supernatant samples. The optical density is directly proportional to the integrity of the BBB.
    4. Assume the optical density of the contralateral hemisphere as background and use the formula (ipsilateral-contralateral)/contralateral to determine fold change. For further details about statistical analysis see Martin *et al.*, 2010.[18]

Representative Results

This study included 25 male Swiss Webster mice that were 10 weeks of age at the start of randomization into RHP (n = 10) or 21% O_2 (n = 15) groups. Two weeks after the final RHP exposure, surgical procedures were performed, with groups blinded and counterbalanced between days. Following tMCAo, 1 mouse died during post-operative recovery and 1 mouse was excluded from the study because it did not meet the reperfusion CBF criterion. Both excluded mice were from the 21% O_2 group. In accordance with ARRIVE guidelines,[28] Table 2 shows surgical parameters. Indirect infarct volumes, hemispheric swelling (*i.e.*, edema), and Evans blue (EB) extravasations are shown in **Figure 2**. All data were analyzed with unpaired t-tests (mean, standard deviation shown), and outliers detected with a False Discovery Rate less than 1% (Graphpad Prism), which removed 4 outliers (2 from 21%, 2 from RHP) from the EB data set.

In this cohort, RHP reduced infarct volumes by almost half (33.9 ± 23.0 mm^3) compared to 21% O_2-treated mice (62.6 ± 42.6 mm^3), but this difference was non-significant (p = 0.10). However, an *a priori* Power analysis predicted a required n = 10 per group, which we did not achieve with the RHP-treated mice after outliers were removed. This should be considered in designing future experiments using RHP. There was no effect of RHP on hemispheric swelling, a metric derived from the TTC volume analysis that could be equated with edema.[6] RHP-treated mice exhibited a trend (p = 0.05) in the reduction of BBB disruption within the ischemic hemisphere normalized to the contralateral hemisphere. **Figure 2D** shows a range of values for EB leak for both treatment groups.

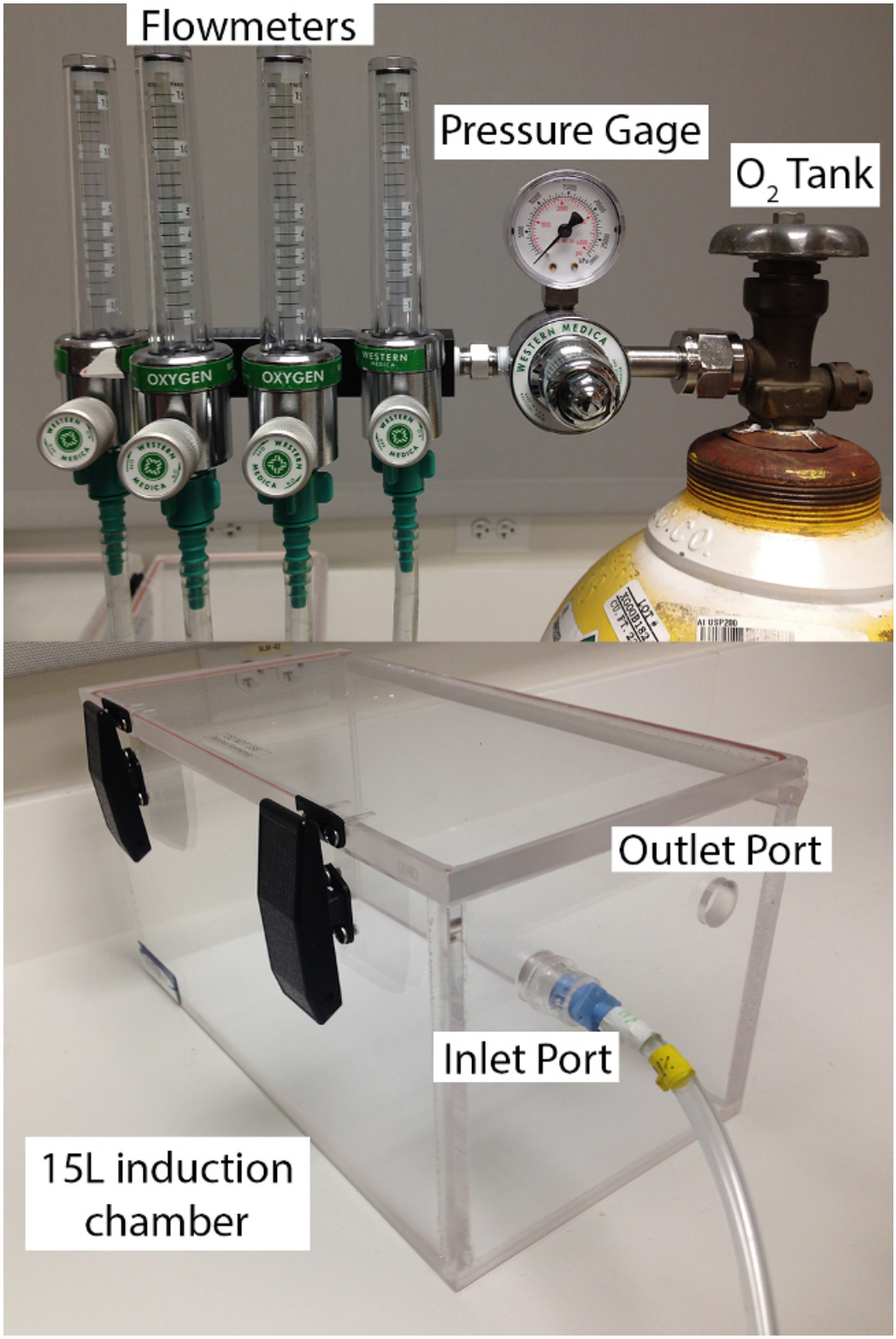

Figure 1: Custom-designed RHP chambers. Upper panel depicts the custom-built flowmeters for individual monitoring of air flow in up to four chambers. Air impermeable tubing is shown leaving the flowmeter and attaching to the inlet port of the 15 L induction chamber in the lower panel. Outlet port remains open during RHP to allow for air circulation.

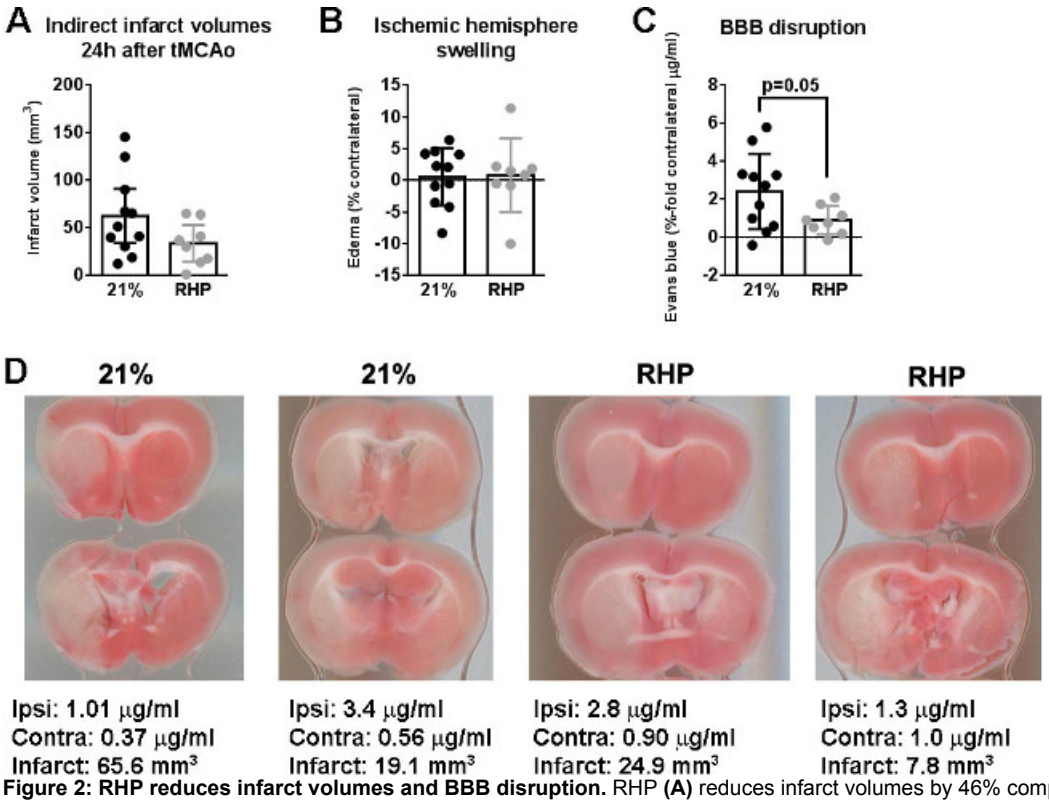

Figure 2: RHP reduces infarct volumes and BBB disruption. RHP **(A)** reduces infarct volumes by 46% compared to control mice, but has **(B)** no affect on hemispheric swelling derived from the TTC data. **(C)** In the same animals, RHP reduces BBB disruption (p = 0.05) as defined by Evans Blue leak normalized to the contralateral hemisphere. **(D)** Representative TTC stains from both RHP-treated and 21% O_2-exposed control mice with corresponding infarct volumes and Evans Blue leak shown below each sample. Values shown are mean +/- standard deviation.

Discussion

A single exposure to systemic hypoxia (*i.e.,* 2 hr of 11% O_2) in mice "transiently" protects the brain from tMCAo,[29] meaning the epigenetic response to the hypoxic preconditioning challenge is short-lasting, and the baseline phenotype is restored within days. Repetitive presentations of the hypoxic preconditioning stimulus dramatically extend the duration of the neuroprotective phenotype.[6] Many studies have shown that the frequency, magnitude, and duration of the repetitive stimulus train are critical determinants of this response. For example, simply repeating the same intensity and duration of hypoxia (2 hr of 11% oxygen) 3 times per week (M-W-F) over 2 weeks did not extend the therapeutic window for neurovascular protection from tMCAo,[6] but was sufficient to induce long-term ischemic tolerance in the retina.[30] Interestingly, although 5 exposures to systemic hypoxia (5 hr of 8% O_2) spaced 6 days apart protected against tMCAo induced 3 days after the last hypoxic exposure, neuroprotection was lost if the 5 hypoxic exposures were placed 3 days apart.[31] The reason for this difference remains unclear, but may have been due to the duration of the severe hypoxia relative to this protocol and/or insufficient recovery time between hypoxic challenges of this severity.

In short, titration of the repetitive hypoxic challenge across the domains of frequency, magnitude, and duration, seems critical to the development of tolerance while not inducing injury, not to mention tissue-specific and perhaps species-specific effects. For example, 3 of 9 of the RHP exposures involve exposure to 8% O_2 for 4 hr, though 6 hr of 8% O_2 induces hippocampal neuronal death.[32] Therefore, there must be a strict adherence to the RHP protocol with regard to duration and severity of hypoxic exposures to induce endogenous protection. Although the potential effect of circadian rhythm on preconditioning-induced protection needs further exploration,[33] performing RHP exposures at the same time of day for a particular cohort reduces the risk of any potential confounding effect. In terms of dose-response and efficacy, the most robust RHP-mediated protection from focal stroke occurred when the tMCAo was induced 2 weeks following the final hypoxic exposure,[3] at a time when RHP-treated healthy mice exhibit an immunosuppressive phenotype (in the absence of ischemic injury).[7] This 2 week time point may not coincide with the period of maximal protection against other acute CNS injuries, or in other organ systems. Both the RHP protocol, and the temporal features of the epigenetic response, will undoubtedly need optimization for each new translational use.

One of the greatest limitations of the tMCAo procedure is the heterogeneous distribution of infarct volumes, as shown by this representative data set. The collateral circulation provided by the Circle of Willis, leptomeningeal anastomoses, and dorsal collateral junctions can lead to inconsistent infarct volumes.[34] Variations between the Circle of Willis in different strains of mice, as well as the presence and patency of posterior communicating arteries,[25,35] can further reduce consistency of infarct volumes within groups. Robust sample sizes and replication are often necessary to produce conclusive results and should be included in the design of the experiment after powering accordingly. Other methods of stroke induction, particularly distal focal ischemic methods such as occlusion of the primary MCA branch via a burr hole in the lateral skull, or photothrombosis of distal branches of the MCA, often produce less variation in stroke volume. Photothrombotic strokes are limited, however, as they produce simultaneous extracellular and intracellular edema, a phenomenon not seen in ischemic strokes in humans.[36] Conversely, tMCAos are more directly applicable to the clinical setting as over 60% of all strokes in humans occur due to obstruction of the middle cerebral artery.[37] Finally, the anesthesia used during tMCAo, isoflurane, has been shown to induce neuroprotection.[38] In order to account for this neuroprotection,

isoflurane levels should be consistent between surgeries and between treated and untreated experimental groups, and maintained at <3% to reduce its potential neuroprotective effects.[39]

Time is also critical in the TTC staining process. Although there is great variation in the stroke literature about the time in which TTC staining can be performed after ischemia, varying from 4 hr to 7 days post-stroke,[18,40] TTC staining should occur at 24 to 48 hr after stroke for the most consistent and clearly delineated infarct volumes. TTC infarct volumes have been found to stabilize 24 hr after stroke but after 48 hr, an influx of macrophages into the ischemic brain makes defining the infarct volume difficult.[14,40] Furthermore, the staining of brain tissue with TTC should immediately follow sacrifice. Delaying staining will decrease the quality of the stain because of increased mitochondrial death resulting from animal death, not cerebral infarction. A similar increase in mitochondrial death will occur if the brain is not kept moist during the insertion of the blades. While this protocol clearly illustrates the benefit of infarct analysis with TTC staining, other immunohistological stains can be used to quantify the infarct volume following tMCAo. Cresyl violet or fluoro-jade staining have less rigid time requirements for staining after sacrifice, but these classic stains require very thin sections obtained by cryostat or microtome, and thus require more time for integrative summation and analysis.[14] Furthermore, these stains cannot be used in conjunction with EB quantification of BBB disruption, as developed by others,[15] eliminating the possibility of direct comparison between infarct volume and BBB integrity in a given brain. It should be noted that RHP-treated animals typically exhibited pink as opposed to pure white volumes.[6] In order to avoid washout of infarct in RHP-treated mice, we use a shorter staining period for TTC that results in more pink healthy tissue as opposed to dark red. Evans Blue processing may also darken the infarct area so pure white infarcts are unlikely with the dual quantification staining presented in this protocol.

In order to compare EB quantification within and between groups, all animals must undergo equivalent circulation times for EB. Others have injected EB immediately following reperfusion and allowed EB to circulate for up to 72 hr,[41] or at 4 hr post-tMCAo to circulate for 4 hr,[15] as alternative approaches. The amount of EB that crosses the disrupted BBB and enters brain tissue represents the integral accumulation of albumin-bound dye from time of injection to time of sacrifice. Thus, the method described in this paper reveals the status of the BBB at precisely the time between 22 and 24 hr of reperfusion, and may miss earlier or later openings or closings of the barrier. Conversely, the method employed by Wang et al. reveals the status of the BBB for 72 hr following stroke, including all changes in BBB status during that 3 day post-stroke time.[42] Neither protocol is better or worse; although there remains a risk of “missing” a transient opening of the BBB by using shorter circulation times, it allows investigators to identify specific temporal features of BBB pathophysiology and to pair it with other methods, as shown here. Others have combined TTC and EB by injecting EB intracardially 1 min after transcardial perfusion.[18] However, this method produced inconsistent results and is a far more complicated procedure than the protocol described above.

One of the most promising future directions for RHP is the translational application of this preconditioning protocol to other disease states. Intermittent hypoxic exposures have been found to be protective in pro-inflammatory CNS conditions other than ischemia. In a rat model of Alzheimer’s, 2 weeks of 4 hr daily exposures to hypobaric hypoxia reduced the impairment of memory retention and loss of cortical neurons after intracerebral injections of beta-amyloid.[43] Intermittent hypoxia (2 weeks of 15 hr daily exposures to hypobaric hypoxia) was also found to be protective in rats following L-buthionine-[S,R]-sulfoximine (L-BSO) infusion, a model of Parkinson’s which impairs striatal dopaminergic transmission.[44] However, these models of Alzheimer’s and Parkinson’s only investigated the short-term effects of hypoxic preconditioning.[43,44] Investigating the long-term neuroprotection induced by RHP may be a fruitful future direction for research. Furthermore, the neuroprotection induced by RHP may extend to improved BBB integrity in mouse models of Alzheimer’s, which could be analyzed with the EB.[45] The dual TTC and EB analysis would be even more useful in Parkinson’s model as it induces striatal lesions[46] and BBB disruption,[47] which can be quantified with TTC[48] and EB,[49] respectively. Overall RHP is a simple, powerful form of preconditioning with great translational potential as it uniquely induces long-term, anti-inflammatory, and neuroprotective effects. The dual quantification of TTC and EB could also be easily applied to other disease states that involve mitochondrial disruption, cell death, and BBB leakage.

Disclosures

The authors have nothing to disclose.

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