Title: Progesterone treatment in two rat models of ocular ischemia

Authors:
Rachael S Allen, BS\textsuperscript{1A,B}, Timothy W Olsen, MD\textsuperscript{1B}, Iqbal Sayeed, PhD\textsuperscript{1A}, Heather A Cale, BS\textsuperscript{1A}, Katherine C Morrison\textsuperscript{1A}, Y Oumarbaeva\textsuperscript{1A}, I Lucaci\textsuperscript{1A}, Jeffrey H Boatright, PhD\textsuperscript{1B}, Machelle T Pardue, PhD\textsuperscript{1B, 2}, Donald G Stein, PhD\textsuperscript{1A}

\textsuperscript{1}Emergency Medicine, \textsuperscript{2}Department of Ophthalmology, \textsuperscript{1}Emory University, Atlanta, GA 30322; \textsuperscript{2}Rehab R&D Center of Excellence, Atlanta VA Medical Center, Decatur, GA 30033

Supported in part by an Unrestricted grant from Research to Prevent Blindness (RPB)

Corresponding Author:
Donald G. Stein, Ph.D.
Emory University Department of Emergency Medicine
1365B Clifton Road NE, Suite 5100
Atlanta, GA 30322
Telephone: (404) 712 - 2540
Fax: (404) 727 - 2388
E-mail: donald.stein@emory.edu
Supplemental Methods

Methods utilized in rAION experiments

Fundus Photographs

Animals were anesthetized via 5% isoflurane inhalation (in a N₂/O₂ 70%/30% mixture). The cornea was anesthetized with topical proparacaine (1%), and pupils were dilated with topical tropicamide (1%) and phenylephrine hydrochloride (2.5%). The camera objective lens was placed in contact with a layer of ultrasound gel on the corneal surface, and images were captured (RetCam II, Clarity Medical Systems, Pleasanton, CA, USA).

Visual Evoked Potential (VEP)

After rats were anesthetized (ketamine 80 mg/kg and xylazine 16 mg/kg), the cornea was anesthetized with topical proparacaine (1%), and pupils were dilated with topical tropicamide (1%) and phenylephrine hydrochloride (2.5%). Cortical responses were recorded using a 1 cm platinum needle electrodes placed subcutaneously over the left and right visual cortex. Ground and reference electrodes were placed in the tail and forehead skin, respectively. Using a commercial amplifier and acquisition system (UTAS-E3000, LKC, Gaithersburg, MD, USA), light-adapted VEPs were recorded three days after rAION for one eye at a time, while the other eye was covered with a patch. Both ipsilateral and contralateral VEPs were recorded, with contralateral VEPs being quantified. 200 responses to Ganzfeld strobe flashes with an intensity of 137 cd s/m² were averaged.
Immunohistochemistry for Brn3a

Eyes were enucleated from euthanized rats and fixed with 10% buffered formalin. Corneas and lenses were removed and the resulting eye cups digested in hyaluronidase (1:500 in 1xPBS, Sigma, 1mg/mL) at room temperature for 30 minutes. Retinas were rinsed, dissected, and permeabilized in PBS/0.5% Triton (PBST) at -80°C for 15 minutes. Retinas were thawed, rinsed in PBS/0.5% Triton, and blocked in 2% donkey serum in PBS/0.25% Triton at room temperature for 30 minutes. Retinas were then incubated with Brn3a primary antibody (Goat polyclonal, 1:500 in PBST, Santa Cruz, sc-31984) over two nights at 4°C. Next, retinas were rinsed in PBST and incubated with Alexa Fluor 488 secondary antibody (donkey anti-goat, 1:500 in PBST, Invitrogen, A11055) for 1 hour at room temperature. Retinas were rinsed in PBST, dissected into flat mounts, and mounted with aqueous mounting media. Retinal ganglion cells were counted for the whole flat mount using the automatic cell counting tool of commercial imaging software (Image Pro©, Media Cybernetics; Rockville, MD, USA). Retinal ganglion cell counts were corrected for retinal area.

Methods utilized in MCAO experiment

MCAO surgery

Animals were anesthetized via 5% isoflurane inhalation (in a N₂/O₂ 70%/30% mixture) and remained sedated with 2% isoflurane inhalation. A pulse oximeter (SurgiVet, model V3304; Waukesha, WI, USA) was used to measure and sustain blood oxygen saturation (SpO₂) at 90%. Body temperature was monitored with a rectal probe and sustained between 36.5°C and 37.5°C using a heating lamp.

A midline incision was made at the ventral surface of the neck, and a 6-0 silk suture was
used to separate and ligate the right common carotid arteries. A microvascular clip was used to occlude the internal carotid and pterygopalatine arteries. Next, a 4-0 silicon-coated monofilament (0.35–0.40 mm long) (Doccol Co., Albuquerque, NM, USA) was inserted through the external carotid artery and into the internal carotid artery and pushed an estimated 20 mm distal to the carotid bifurcation to block the opening of the middle cerebral artery and the adjacent ophthalmic artery. The filament remained in place for 120 minutes, followed by reperfusion. Upon removal of the filament, the wound was sutured, and rats were transferred to a heating blanket to recover from anesthesia. Laser-Doppler flowmetry (LDF), an established and reliable system for monitoring cerebral blood flow changes due to induction of focal cerebral ischemia [1], was used to monitor cerebral blood flow. Animals with mean ischemic cerebral blood flow greater than 35% of baseline LDF were excluded to reduce variability and ensure relative uniformity of the ischemic insult [2]. The LDF probe (Moor Instruments, Wilmington, Delaware, USA) was placed over the ipsilateral parietal cortex from 5 minutes prior to occlusion to 5 minutes after reperfusion.

Electroretinogram (ERG)

The cornea was anesthetized with topical proparacaine (1%), and pupil were dilated with topical tropicamide (1%) and phenylephrine hydrochloride (2.5%). A DTL fiber was placed in contact with the corneal surface of each eye through a layer of methylcellulose to record retinal responses. Platinum needle ground and reference electrodes were placed in the tail and in the cheek below the eye, respectively. Using a commercial amplifier and acquisition system (UTAS-E3000, LKC, Gaithersburg, MD, USA), dark-adapted ERG responses were recorded to a series
of Ganzfeld strobe flashes (with intensities increasing from 0.00039 to 137 cd s/m$^2$).

Interstimulus time increased from 10 to 60 s as light intensity increased, and 3 to 10 responses were averaged per flash stimulus.

TTC staining

Brains were placed in ice-cold saline upon removal. Beginning 1 mm posterior to the anterior pole and using a rat brain matrix (Harvard Apparatus), brains were sliced into 7 serial coronal sections (2 mm thick). Slices were stained using 2% TTC in saline for 15 minutes at 37°C in the dark and then fixed using 10% buffered formalin. TTC is reduced by metabolically active tissue to form a red product, while stroke tissue remains white due to its compromised metabolic enzymes. Stained slices were scanned using a high-resolution scanner (Epson Perfection 2400 Photo). A subset of TTC tissue was analyzed due to defective TTC.

Retinal morphology and immunohistochemistry

Eyes were fixed in 10% buffered formalin, processed through a series of graded ethanols, and embedded in paraffin. Sections (5 mm) were cut on a rotary microtome. Sections containing optic nerve were stained with 0.1% cresyl violet. Images were captured, and retinal ganglion cells were counted in three sections and averaged per eye, with six fields being counted per section (three on either side of the optic nerve, spaced approximately 100 microns apart).

For labeling of GFAP and glutamine synthetase (GS) in Muller cells, sections were blocked in 0.1 M Tris-buffered saline (TBS; pH 7.4) containing 3% normal serum for 30 minutes, then incubated overnight at 4°C in primary antibodies diluted in the blocking serum.
Primary antibodies used were rabbit anti-GFAP (1:500; Millipore; Billerica, MA, USA; AB5804) and mouse anti-GS (1:1000; Millipore; MAB302). Primary and secondary antibodies from different species were used simultaneously for double labeling. After primary antibody incubation, sections were rinsed 3 times with 0.1 M TBS. Sections were then incubated in the corresponding fluorophore-conjugated secondary antibody solution (goat anti-rabbit; 1:500; Alexa Fluor 546; A11071 and goat anti-mouse; 1:500; Alexa Fluor 488; A11001) for 1 hour at room temperature. Following secondary antibody incubation, sections were rinsed 3 times with 0.1 M TBS, mounted with a DAPI mounting medium, and coverslipped. One image per retina was acquired superior to the optic nerve. A fluorescent microscope (Olympus BX41, Olympus America Inc.) was used to acquire all images and a histogram tool (Photoshop, Adobe, San Jose, CA, USA) was used to quantify GS staining intensity in a field of retina 300 pixels wide spanning the length of the retinal Muller cells, which was normalized to background brightness.

Supplemental References
