Video Article

An Acute Retinal Model for Evaluating Blood Retinal Barrier Breach and Potential Drugs for Treatment

Hao Wu¹, Ana R. Rodriguez², Bernd W. Spur², Venkat Venkataraman^{1,2}

¹Graduate School of Biomedical Sciences, Rowan University

²Department of Cell Biology, Rowan University School of Osteopathic Medicine

Correspondence to: Venkat Venkataraman at vvenkat2007@gmail.com

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Abstract

A low-cost, easy-to-use and powerful model system is established to evaluate potential treatments that could ameliorate blood retinal barrier breach. An inflammatory factor, histamine, is demonstrated to compromise vessel integrity in the cultured retina through positive staining of IgG outside of the blood vessels. The effects of histamine itself and those of candidate drugs for potential treatments, such as lipoxin A4, are assessed using three parameters: blood vessel leakage via IgG immunostaining, activation of Müller cells via GFAP staining and change in neuronal dendrites through staining for MAP2. Furthermore, the layered organization of the retina allows a detailed analysis of the processes of Müller and ganglion cells, such as changes in width and continuity. While the data presented is with swine retinal culture, the system is applicable to multiple species. Thus, the model provides a reliable tool to investigate the early effects of compromised retinal vessel integrity on different cell types and also to evaluate potential drug candidates for treatment.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54619/>

Introduction

A growing body of evidence supports the existence of blood retinal barrier (BRB)¹⁻⁵ and its similarity to the blood brain barrier (BBB)^{6,7}. Compromise of the BBB has been tightly linked causally or as a diagnostic marker to chronic neurodegenerative diseases such as Alzheimer's disease (AD)^{8,9} and acute conditions such as delirium¹⁰. Mechanistic insights into these pathologies and discoveries for potential drug targets are generally hampered by the limited accessibility and network intricacy of the brain. Alternatives such as *in vivo* imaging¹¹, brain organotypic culture¹², primary cell cultures^{13,14} and co-culture systems¹⁵ have been generated. However, most of these models require special instruments, long experimental periods or multiple markers to identify cells. Functional and structural similarities between BBB and BRB as well as a correlation between the dysfunctions of the two have been argued¹⁶⁻¹⁹. In addition, easier access, well-defined cell types and a layered structure have allowed the well-characterized retina as a window to the brain. The structural and functional identities of the BBB and BRB remain to be compared in detail. However, retinal pathologies, especially the BRB breach, have also been tightly associated with the progression of various diseases, including diabetes¹⁸⁻¹⁹ and AD^{21,22}. Thus, it is of interest to establish a BRB dysfunction system not only to delineate the mechanism but also to screen potential drugs. In this report, a protocol enabling BRB dysfunction using a simple acute retinal culture is developed and presented.

Increased BBB permeability and AD-like pathological changes have been established in a brain organotypic culture incubated with histamine, a pro-inflammatory mediator¹². Therefore, in the presented system, histamine was applied to the *ex vivo* retinal culture to induce BRB dysfunction. Retinas from several species, such as *Mus musculus* and *Bos Taurus*, have been tested. Due to their commercial availability and resemblance to human tissue, fresh swine eyeballs were utilized to provide the data reported here. After incubating with histamine and/or other drugs, the retinas were processed for evaluation by immunostaining for several proteins¹², such as Immunoglobulin G (IgG), one of the major components of the blood; glial fibrillary acidic protein (GFAP), a well-known marker for glial activation; and microtubule-associated protein 2 (MAP2), a neuron-specific cytoskeletal protein essential for microtubule assembly. Furthermore, the layered structure of the retina allows a detailed analysis of the processes of Müller cells and ganglion cells, such as changes in their width and continuity. Thus several additional parameters are available to assess the consequences of the BRB breach at an early stage and to evaluate the reversal effects of potential treatments as well.

In this protocol, potential reversal effects of screened drugs are evaluated from three perspectives: the leakage of blood vessels (BVs), the activation of glial cells and the damage-response of neuronal cells. Several quantification methods are utilized, for instance, the expression level shown by intensity of the immunostaining, width measurement of a process and continuity of neuronal processes shown by an enhancement filter. To better illustrate the method and to help interpret results, lipoxin A4 (LXA4), a compound endogenously synthesized in response to inflammatory injury and attenuating endothelial dysfunction²³, has been chosen for demonstration purposes.

Protocol

All protocols were carried out in compliance with the policies of the Institutional Animal Care and Use Committee where applicable.

1. Preparation

1. Prepare the stabilization media with 75% Dulbecco's Modified Eagle Medium (DMEM), 25% Hanks' Balanced Salt Solution (HBSS). Mix well, aliquot and store at -20 °C until use.
2. Prepare the Phosphate Buffered Saline (PBS) and sterilize by autoclaving. Store the solution at room temperature. Warm the PBS to 37 °C before the experiment (5 ml per well).
3. Prepare 10%, 20% and 30% sucrose in PBS. Filter all the solutions individually over separate 0.22 µm filters for sterilization. Save the solutions at 4 °C.
4. Prepare the histamine stock solution in PBS to a concentration of 90 mM. Sterilize the solution by filtering over a 0.22 µm filter. Aliquot and save the solution at -80 °C. Avoid multiple freeze-and-thaw cycles.
5. Make fresh 4% paraformaldehyde (PFA) in PBS before the experiment. Place it on ice.
Caution: Keep PFA in a fume hood and wear appropriate protective equipment.
6. Thaw the stabilization medium (20 ml per retina). Add Penicillin-Streptomycin to a final concentration of 100 U/ml. Warm part of the medium (15 ml per retina) to 37 °C in incubator before the experiment. Leave the rest of the medium on ice.
7. Place the HBSS (10 ml per retina) on ice.

2. Retinal Organotypic Culture

1. Transfer the samples to a tissue culture hood. Open the eyecup by cutting around the lens. Using a brush, gently remove the vitreous humor without pulling on the retina. Gently detach the retina from the cut edge with a brush to expose the optic disc. Sever the optic nerve with a razor and release the retina.
2. Transfer the retina into a Petri dish and carefully rinse the retina once using cold HBSS. Place the sample in HBSS on ice. Dissect as many retinas out as needed by repeating Step 2.1 and this step.
3. Cut the retina in half symmetrically with a razor. Gently transfer samples to a 6-well plate with 3 ml of stabilization media per well. Allow equilibration for 30 min at 37 °C in an incubator with the atmosphere containing 5% CO₂.
4. Prepare the following reagents during the incubation at Step 2.3: Dilute the histamine stock solution to the desired concentration (450 µM) in warm medium. Dissolve LXA4 (stored under Argon) into the medium to a final concentration of 250 ng/ml.
NOTE: When measuring the effect of LXA4, one half from a single retina is used for histamine alone, while the other half is used for histamine with LXA4.
5. Aspirate the stabilization medium carefully and add the prepared medium to each well (3 ml per well). Incubate the samples at 37 °C for 1 hr in an incubator with the atmosphere containing 5% CO₂.
6. Rinse the samples once in warm, sterile PBS.

3. Prepare the Samples for Immunostaining

1. Aspirate PBS from the plates. Add 4% PFA (3 ml per well) and incubate for 15 min at room temperature.
2. Quickly aspirate the PFA. Rinse the samples once using PBS. Transfer the samples to 10% sucrose (5 ml per well) and incubate for 2 to 4 hr at 4 °C.
3. Transfer the samples to a 30% sucrose (5 ml per well) and incubate overnight at 4 °C. Mix one part of the commercial freezing medium with two parts of 20% sucrose to make the working freezing medium. Leave it at 4 °C overnight or longer until the air bubbles disappear.
4. Transfer the samples to the working freezing medium and allow equilibration for 5 min at room temperature. Trim each retina into rectangles (roughly 3 mm by 5 mm).
5. Carefully transfer the samples into the working freezing media contained in a cylindrical container (about 1 cm radius x 2 cm height) devised from aluminum foil. Ensure that the retinal slices are vertical (oriented to provide a cross-section of the retina upon sectioning) and freeze them in liquid nitrogen. Save the blocks at -80 °C until use.
6. Section each block on a cryostat into 14 µm thick slices and mount the sections on glass slides. Store the slides at -80 °C until use.

4. Immunostaining

1. Wash the sections with 5% sucrose phosphate buffer (SPB, 5% sucrose in PBS, pH 7.4) once. Block the non-specific binding sites by incubating the sections in blocking buffer (5% SPB with 10% normal goat serum and 0.5% Triton X-100) for 1 hr at room temperature.
2. Incubate the sections (on slides) with the appropriate primary antibody (GFAP or MAP2, diluted at 1:500 in blocking buffer) for 1 hr. Aspirate the solution. Wash three times in 5% SPB. For staining the endogenous IgG, skip this step.
3. Incubate the sections with the corresponding secondary antibodies (Cyanine3/FITC conjugated donkey anti-mouse/anti-rabbit IgG, diluted at 1:800 in the blocking buffer) for 1 hr.
4. Wash the sections thoroughly with 5% SPB three times. Prepare the samples for microscopy by mounting them in mounting medium containing DAPI and covering with coverslips.
5. Using a laser confocal microscope, capture images through a 20X lens under identical parameters such as laser intensity, gain value, dwell time, etc. across groups. Set the excitation/emission wavelengths for the blue channel (to detect DAPI) as 408/447 nm, for the red channel (to detect Cyanine3) as 561/785 nm and for the green channel (to detect FITC) as 488/525 nm.

5. Image Analysis

1. Quantify percentage of leaky blood vessels according to the following criteria¹²: Include only blood vessels with endothelial cell nuclei within the plane of section in the count; consider a blood vessel leaky if it shows a gradient of immunostaining surrounding the vessel.
2. To determine the expression level, select regions of interest and measure the mean grey value using the image analysis software.
3. To measure the width of the process, choose an area of the section where the processes are distinct (the outer nuclear layer, ONL, for example). Then choose an optical field where such processes are visible and continuous. Set the scale bar according to the microscopic setting, draw a line across the process and perform the measurement.
4. To analyze the continuity of the process, select regions of interest, apply the filter called variance which enhances the edges in the image by replacing each pixel with its neighborhood variance, automatically adjust the contrast and brightness, count the lines and normalize the count by the area.
5. Calculate statistical significances using a two-tailed Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Plot the result as Mean $\pm$ SEM.

Representative Results

We present a low-cost, time-efficient and easy-to-use system to evaluate potential treatments that could protect against the BRB breach induced by histamine. IgG is constrained within the vessels in control retina (**Figure 1A**), but leaks out of the blood vessels upon histamine exposure (**Figure 1B**), confirming that the model is successfully established.

LXA4 was chosen to demonstrate the screening for drugs against BRB breach in the presented system. BRB dysfunction is attenuated upon treatment with LXA4 (**Figure 1D**), which has no significant effect by itself (**Figure 1C**). The reversal is quantitated as a percentage of the leaked blood vessels and the result is significant (**Figure 1E**). Two other retinal cell types, Müller glial cells (**Figure 2**) and ganglion cells (**Figure 3**), are further assessed. LXA4 treatment reverses the decrease in the width of the Müller glial cell processes (**Figure 2E**) shown through GFAP staining (**Figure 2, A-D**) but has no significant effects on the continuity of the dendritic processes in ganglion cells (**Figure 3G**), shown by MAP2 staining (**Figure 3, A-E**). It is also noted that no significant change in the expression level of either proteins is detected.

Figure 1: The Blood Retinal Barrier is Compromised upon Histamine Exposure and Rescued by LXA4 Treatment. IgG immunostaining (red) is presented from retina which was untreated (A), damage-induced with histamine only (B), treated with LXA4 only (C), or exposed to both histamine and LXA4 (D). In control (A) and LXA4 treated (C) groups, IgG is restricted within the blood vessels (BV) while in the histamine group (B), IgG is detected out of the BVs where it forms leakage clouds indicated by dotted circles. Further addition of LXA4 rescues the vessel dysfunction (D). Scale bar, 20 μ m. (E) Quantitative measurement of the leaky BVs across the tested groups. Statistical significance is determined by a two-tailed Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Mean $\pm$ SEM ($n = 18$) is plotted in the graph. [Please click here to view a larger version of this figure.](#)

Figure 2: Müller Cell Processes are Compromised by Histamine and Rescued by LXA4 Treatment. Immunostaining for GFAP is presented from retina which was untreated (A), damage-induced with histamine only (B) treated with LXA4 only (C) or exposed to both histamine and LXA4 (D). Positive staining is obtained in the processes of Müller cells across the retina and around the BVs. Scale bar, 20 μ m. (E) The width of the Müller cell processes from all groups are presented. About 30 individual processes were measured per group. Statistical significance is determined by a two-tailed Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Mean $\pm$ SEM ($n = 30$) is plotted in the graph. [Please click here to view a larger version of this figure.](#)

Figure 3: The Continuity of Ganglion Cell Processes is not Affected by LXA4. (A) Immunostaining against MAP2 is presented from retina which was untreated (A), damage-induced with histamine only (B) treated with LXA4 only (C) or exposed to both histamine and LXA4 (D). Positive staining is obtained in the processes and cell bodies of ganglion cells. A sample image of MAP2 staining (E) together with its corresponding filter-enhanced image (F) is presented. The continuous processes are indicated by arrowheads. Scale bar, 20 μ m. (G) The density of the continuous, MAP2-positive ganglion cell processes from all groups are presented. Statistical significance is determined by a two-tailed Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Mean $\pm$ SEM ($n = 18$) is plotted in the graph. [Please click here to view a larger version of this figure.](#)

Discussion

In this report, we present a powerful *ex vivo* acute retinal model of BRB dysfunction using the swine retina. This model system does not require special instruments and can be easily adapted under most laboratory settings. However, to obtain a successful result, several steps require close attention. After obtaining the eyeballs from the source, they must be kept at 4 °C or on ice and processed as soon as possible. When the effect of a treatment is being analyzed, two halves of the same retina must be used - one half to detect changes solely due to experimental procedures (baseline) and the other to detect the effect of the treatment. This approach also allows corrections for animal variations such as age, sex and health.

This protocol can be successfully applied to retina from various species, such as *M. musculus* and *B. Taurus* (data not shown). Using commercial swine eyeballs is a significant cost-saving factor compared to maintaining the animals. Furthermore, in this study, the incubation time with histamine is only 60 minutes. This short incubation time provides the advantage of teasing out the early responses. It is noted that the study was carried out over different durations (20 min to 120 min) and different histamine concentrations (up to 700 μ M). Effects are observable at 30 min and at 225 μ M histamine. For illustrative purposes, the result obtained at 60 min with 450 μ M histamine is presented. In its present form, the following are the limitations of the model: (1) use of commercially obtained eyeballs increases the variability (but significantly cuts down on the cost); (2) the short incubation time may not allow complete manifestation of the response to the treatment - for example, those that require *de novo* protein synthesis. Modifications may help including longer incubation periods that could enable monitoring delayed responses.

LXA4 is chosen as the drug to investigate possible protection against the histamine-induced BRB damage. LXA4 is an endogenous compound that plays a key role in inflammatory events. It is produced in response to stress *in vivo* and has a short half life²³. To mimic these conditions, a single addition of LXA4 was carried out along with histamine. This model system could also be used to further investigate if a prior treatment with LXA4 (or any other drug) would yield protection as well as to evaluate the duration of the protective treatment.

To the best of our knowledge, this is the first report of a retinal culture system with an experimentally-induced BRB breach. Evidently, exogenous perturbations other than histamine, such as lipopolysaccharides (LPS)²⁴ and vascular endothelial growth factor (VEGF)²⁵, can also be utilized to induce BRB breach. The effects of those perturbations and their potential treatments can be investigated on different cell types such as endothelial, Müller glial and neuronal cells in the presented model. Analyses may be performed on the entire retina or on one or more specific layers. Available cell-specific markers for retinal cells and synaptic layers allow an efficient detection of the responses via a large combination of parameters. Thus, we have established a retinal culture system for the rapid screening of products that protect against BRB dysfunction.

Disclosures

The authors have nothing to disclose.

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