Experimental Eye Research 172 (2018) 36–44

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

# Regional differences in endothelial cell cytoskeleton, junctional proteins and phosphorylated tyrosine labeling in the porcine vortex vein system

Priscilla Ern Zhi Tan[a], Paula K. Yu[a,b], Hongfang Yang[a], Stephen J. Cringle[a,b], Dao-Yi Yu[a,b,*]

[a] Centre for Ophthalmology and Visual Science, The University of Western Australia, Perth, Australia
[b] Lions Eye Institute, The University of Western Australia, Perth, Australia

ARTICLE INFO

*Keywords:*
Endothelial cells
Endothelial phenotype heterogeneity
Vortex vein system
Choroid
Cytoskeleton
Porcine eye
Junctional proteins
Intramural cells

ABSTRACT

We previously demonstrated endothelial phenotype heterogeneity in the vortex vein system. This study is to further determine whether regional differences are present in the cytoskeleton, junctional proteins and phosphorylated tyrosine labeling within the system.

The vortex vein system of twenty porcine eyes was perfused with labels for f-actin, claudin-5, VE-Cadherin, phosphorylated tyrosine and nucleic acid. The endothelial cells of eight different regions (choroidal veins, pre-ampulla, anterior ampulla, mid-ampulla, posterior ampulla, post-ampulla, intra-scleral canal and the extra-ocular vortex vein) were studied using confocal microscopy.

There were regional differences in the endothelial cell structures. Cytoskeleton labeling was relatively even in intensity throughout Regions 1 to 6. Overall VE-Cadherin had a non-uniform distribution and thicker width endothelial cell border staining than claudin-5. Progressing downstream there was an increased variation in thickness of VE-cadherin labeling. There was an overlap in phosphorylated tyrosine and VE-Cadherin labeling in the post-ampulla, intra-scleral canal and extra-ocular vortex vein. Intramural cells were observed that were immune-positive for VE-Cadherin and phosphorylated tyrosine. There were significant differences in the number of intramural cells in different regions.

Significant regional differences with endothelial cell labeling of cytoskeleton, junction proteins, and phosphorylated tyrosine were found within the vortex vein system. These findings support existing data on endothelial cell phenotype heterogeneity, and may aid in the knowledge of venous pathologies by understanding regions of vulnerability to endothelial damage within the vortex vein system. It could be valuable to further investigate and characterize the VE-cadherin and phosphotyrosine immune-positive intramural cells.

## 1. Introduction

The vortex vein system of the eye is the primary drainage conduit for the choroidal circulation (Kutoglu et al., 2005), but there has been minimal research conducted on its role in both retinal and choroidal diseases. The venous system has been noted to be more complex than the arterial system (Meissner et al., 2007), with venous diseases having a ten-fold greater incidence as compared with arterial diseases (Monos et al., 1995). The vortex vein system physically occupies a substantial region of the choroid, and has been previously described (Hogan et al., 1971; Kaufman and Alm, 2003), and recently characterized into eight specific regions(Tan et al., 2013). Whilst research has been conducted on the pathogenesis of venous diseases (Bergan et al., 2008; Cines et al., 1998; Esmon, 2009; Lopez and Chen, 2009), research into the mechanism of pathophysiology of the vortex vein system of the eye has been scarce.

Many similarities have been observed between the porcine and the human vortex veins. Whilst the human eye is typically drained by 4–6 vortex veins (Yu et al., 2013), the porcine eye are generally drained by four vortex veins (Ninomiya and Inomata, 2006) located obliquely in the four quadrants near the equator. Significant endothelial cell phenotype heterogeneity has been found in the eight different regions of the human and porcine vortex vein system, and indicates likely a reflection of the hemodynamic variations within the vortex vein system (Tan et al., 2013; Yu et al., 2013). Previous demonstrations of phenotypic heterogeneity were based on endothelial cell border f-actin labelling which appeared intact (Tan et al., 2013; Yu et al., 2013). However, with such noticeable and measureable phenotypic heterogeneity, another important aspect to be investigated is whether junctional proteins heterogeneity is also present within the vortex vein

* Corresponding author. Lions Eye Institute, 2 Verdun Street, Nedlands, 6009, Western Australia.
*E-mail addresses:* priscilla.ez.tan@gmail.com (P.E.Z. Tan), paula.yu@uwa.edu.au (P.K. Yu), 511278186@qq.com (H. Yang), Steve@lei.org.au (S.J. Cringle), dyyu@lei.org.au (D.-Y. Yu).

https://doi.org/10.1016/j.exer.2018.03.028
Received 5 March 2018; Accepted 29 March 2018
Available online 30 March 2018

system which may point to variation in permeability and vulnerability in this drainage pathway. Previous studies have supported endothelial adherens and tight junctions being modulated by hemodynamic forces and having a role in the regulation of vascular permeability and inflammation (Curry and Adamson, 2010; Orsenigo et al., 2012). Additionally, it has been reported that the presence of tyrosine phosphorylated VE-Cadherin in veins *in vivo* may indicate a vulnerability for increased permeability (Orsenigo et al., 2012). It is currently unknown if a similar pattern of tyrosine phosphorylated VE-Cadherin would also be present in the venous part of the choroid. We still know very little overall about the structural barrier of the vascular endothelium in the choroidal and vortex vein system. Knowledge of junctional proteins and their functional phenotype regional differences are important for the further understanding of the normal vortex vein system and its venous pathologies.

The purpose of this study is to determine whether there are qualitative regional differences in the distribution of junctional proteins, f-actin and in relation to phosphorylated tyrosine within the porcine vortex vein system. We propose that there would be regional differences reflective of endothelial cell phenotype heterogeneity within different regions of the vortex vein system. We used porcine eyes as our baseline animal model due to the similarity of the anatomy and physiology to the young normal human eye, and will enable close comparisons in this field with future studies of human eyes.

## 2. Materials and methods

### 2.1. General

Twenty post-mortem white landrace pig eyes were used, sourced from a local abattoir. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the University of Western Australia Animal Ethics Committee.

### 2.2. Preparation

Porcine eyes were freshly enucleated, placed in carbogen bubbled Ringer's solution, delivered to our laboratory on ice, and used within the hour. Porcine eyes with non-pigmented or very lightly pigmented irises and sufficiently long length of superior temporal vortex vein were preferentially chosen for experimentation. Non-pigmented or very lightly pigmented irises were preferentially chosen because it allowed the greatest clarity of view of the endothelium, as the choroid of porcine eyes with pigmented irises contained a lot of melanocytes which obscured our view of the endothelial cells. The eyes were then oriented via fundoscopy and the position of the superior oblique muscle. All musculature and orbital fat were carefully dissected away.

### 2.3. Preparation of porcine vortex vein system prior to labeling

Our laboratory has provided the methodology for targeted retinal and choroidal endothelial labeling in previous reports (Yu et al., 2010, 2012). We applied these techniques to the vortex vein system and have published our findings in both porcine and human eyes (Tan et al., 2013; Yu et al., 2013). In this study we used similar methodologies to further visualize the intracellular structures within and surrounding the vortex vein system. This study follows directly from a previous article, which investigated whether region-dependent endothelial heterogeneity was present within the porcine vortex vein system (Tan et al., 2013).

The porcine eye was dissected to locate the temporal long posterior ciliary artery, and superior and inferior temporal vortex vein. For phalloidin labeling, the superior temporal vortex vein was cannulated as per our previous paper (Tan et al., 2013). We took assiduous precautions to ensure all air bubbles were carefully eliminated prior to cannulation and securing of the vessel, and throughout the perfusion process. For labeling with claudin-5, VE-Cadherin and phosphorylated tyrosine, the temporal long posterior ciliary artery was cannulated using glass micropipettes with tip sizes of 270 μm–300 μm, and the cannula secured with 8–0 vicryl sutures and attached to a syringe pump (model 22; Harvard Apparatus, South Natick, MA). The pump supplied a modifiable flow of solution, and the perfusion pressure was monitored through conventional transducers (Cobe, Arvada, CO), which was connected to a bridge amplifier (model 5B38-02; Analog Devices, Norwood, MA) and logged on a chart recorder (LR8100; Yokogawa, Tokyo, Japan). The perfusion pressure recorded ranged from 25 mmHg to 30 mmHg at flow rate of 100–150 μL/min. Based on pilot studies, this is sufficient pressure for the choroidal vasculature network to be entirely perfusion labelled. The perfusion pressure was also kept low to avoid leakage from choroidal capillaries and damage to the endothelial cells.

Following cannulation, the following sequence of filtered solutions were perfused through the temporal vortex vein system: (1) 30-min washout of blood with 1% bovine serum albumin in oxygenated Ringer's solution at 150 μL/min; (2) 30-min fixation at 150 μL/min with 4% paraformaldehyde in 0.1 M phosphate buffer; (3) 8-min wash with 0.1% Triton X-100 in 0.1 M phosphate buffer at 150 μL/min; and (4) 30-min wash with 0.1 M phosphate buffer solution at 100 μL/min. The flow rate was reduced after perfusion of Triton X-100 to avoid fluid leaking out into the extravascular stroma, as this non-ionic surfactant detergent permeabilizes the membranes of living cells.

### 2.4. Phalloidin perfusion labeling

Eight eyes chosen for perfusion labeling for actin microfilament additionally had the inferior temporal vortex vein and other potential outflow arteries and veins tied off with 8–0 vicryl sutures. Microfilaments and cell nuclei were then labelled using a mixture of phalloidin conjugated to Alexa Fluor 546 (30U; A22283, Invitrogen, Carlsbad, CA) and an iodide dye (YO-PRO-1; 6.6 μM; Invitrogen), which was slowly perfused over a period of 3 h. This was done by manually pushing the labeling solution through the system very slowly and staining for 1 h, before removing the outflow sutures and repeating these steps for two more hours. Once staining was completed, a final 10-min washout of 0.1 M filtered phosphate buffer solution was perfused to remove excess dye.

The eyes were subsequently immersed in a fixative solution of 4% paraformaldehyde overnight prior to dissection and flat mounting of the choroid and vortex vein system for imaging.

### 2.5. Claudin-5 immunolabeling

After the phosphate buffer wash, a 1 mL filtered solution of 10% donkey serum in 0.1 M phosphate buffer was perfused over an hour. This was conducted via manual pushing of the labeling solution slowly through the system in increments of a third of the volume every 20 min for an hour. This was followed by another hour of perfusion of a rabbit anti-claudin-5 primary antibody (1:50; SAB4200538, Sigma-Aldrich, St. Louis, MO), and a further wash with 0.1 M phosphate buffer for 45 min. Donkey anti-rabbit IgG secondary antibody (1:200; Alexa Fluor 488 A21206, Invitrogen, Carlsbad, CA) with Hoechst (H33258; 1:1000, Sigma-Aldrich, St. Louis, MO) in 0.1 M phosphate buffer was manually perfused over three 20 min intervals before a final 45 min wash with phosphate buffer.

The three porcine eyes perfused with claudin-5 and Hoechst were immediately dissected and flat-mounted for imaging.

### 2.6. VE-cadherin and phosphorylated tyrosine immunolabeling

For the nine eyes labelled with VE-Cadherin and phosphorylated tyrosine, a 1 mL filtered solution of 10% donkey serum in 0.1 M