INTRACELLULAR CYTOSKELETON AND JUNCTION PROTEINS OF ENDOTHELIAL CELLS IN THE PORCINE IRIS MICROVASCULATURE

Authors: Hongfang Yang^{1,2,3}
Paula K Yu^{1,2}
Stephen J Cringle^{1,2}
Xinghuai Sun^{3,4,5*}
Dao-Yi Yu^{1,2}

¹Centre for Ophthalmology and Visual Science, The University of Western Australia, Perth, Australia

²Lions Eye Institute, The University of Western Australia, Perth, Australia

³Department of Ophthalmology & Visual Science, Eye & ENT Hospital, State Key Laboratory of Medical Neurobiology, Institutes of Brain Science, Shanghai Medical College, Fudan University, Shanghai 200032, China

⁴Key Laboratory of Myopia, Ministry of Health (Fudan University)

⁵Shanghai Key Laboratory of Visual Impairment and Restoration (Fudan University)

Corresponding authors:

Professor Dao-Yi Yu

Centre for Ophthalmology and Visual Science

The University of Western Australia, Nedlands, WA 6009

Tel. (618) 9381 0716

Fax. (618) 9381 0700

Email dyyu@cyllene.uwa.edu.au

*Professor Xinghuai Sun is a co-corresponding author

Department of Ophthalmology & Visual Science, Eye & ENT Hospital, State Key Laboratory of Medical Neurobiology, Institutes of Brain Science, Shanghai Medical College, Fudan University, Shanghai 200032, China

Tel. (+86) 21 6437 7134-818

Fax. (+86) 21 6437 7151

Email xhsun@shmu.edu.cn

Abstract

Recently we reported studies of the iris microvasculature and its endothelial cells using intra-luminal micro-perfusion, fixation, and silver staining suggesting that the iris vascular endothelium may be crucial for maintaining homeostasis in the ocular anterior segment. Here we present information regarding the intracellular structure and cell junctions of the iris endothelium. Thirty-seven porcine eyes were used for this study. The temporal long posterior ciliary artery was cannulated to assess the iris microvascular network and its endothelium using intra-luminal micro-perfusion, fixation, and staining with phalloidin for intracellular cytoskeleton f-actin, and with antibodies against claudin-5 and VE-cadherin for junction proteins. Nuclei were counterstained with Hoechst. The iris was flat-mounted for confocal imaging. The iris microvasculature was studied for its distribution, branch orders and endothelial morphometrics with endothelial cell length measured for each vessel order. Our results showed that morphometrics of iris microvasculature was comparable with our previous silver staining. Abundant stress fibres and peripheral border staining were seen within endothelial cells in larger arteries. An obvious decrease in cytoplasmic stress fibres was evident further downstream in the smaller arterioles, and tended to be absent from capillaries and veins. Endothelial intercellular junctions throughout the iris vasculature were VE-cadherin and claudin-5 immuno-positive, indicating the presence of both adherent junctions and tight junctions between vascular endothelial cells throughout the iris microvasculature. Unevenness of claudin-5 staining was noted along the endothelial cell borders almost in every order of vessels, especially in veins and small arterioles. Our results suggest that significant heterogeneity of intracellular structure and junction proteins is present in different orders of the iris vasculature in addition to vascular diameter and shape of the endothelia. Detailed information of topography and intracellular structure and junction proteins of the endothelium of the iris microvasculature combined with unique

structural features of the iris may help us to further understand the physiological and pathogenic roles of the iris in relevant ocular diseases.

Key words: Iris microvasculature, endothelium, micro-perfusion, cytoskeleton, junction proteins, claudin-5, VE-cadherin.

1. Introduction

The iris is the most anterior portion of the uvea lining between the anterior and posterior chambers (Hogan et al., 1971). The anterior segment consists of avascular tissues such as the cornea, lens, and aqueous humor (Tasman and Jaeger, 2005). The role of iris vasculature in maintaining intraocular homeostasis has increasingly been recognized as the major source of oxygen and nutrients supplying the anterior segment. The disruption of this homeostasis could be an important pathogenic factor in glaucoma and cataract formation (Barbazetto et al., 2004; Holekamp et al., 2005; Shui et al., 2006).

Recently we have reported the distribution of porcine iris microvasculature and its endothelial cells using intra-luminal micro-perfusion, fixation, and silver staining (Yang et al., 2015).

Our results showed that the iris has an unusual vascular distribution which consisted of abundant large vessels in the middle of the iris stroma, branching over a relatively short distance to relatively large capillaries. In addition, multiple orders of iris vessels with large input arteries and the spindle shape of the endothelia suggest a high blood inflow provides sufficient supply for material exchange, predominantly the delivery of oxygen, glucose, and other nutrients, between blood stream and iris stroma. This is assumed to be crucial for maintaining homeostasis in the ocular anterior segment, and in particular oxygen gradient homeostasis. It is also important to note that the anterior surface of iris does not form a continuous, impermeable sheet that overlies the anterior iris surface but allows aqueous humor to pass freely into the iris stroma (Freddo, 1996; Oyster, 2000). A modification of the stroma composed of a relatively dense meshwork of melanocytes and fibroblasts with associated collagen forms the anterior surface of the iris (Freddo, 1996; Hogan et al., 1971; Tousimis and Fine, 1959; Vrabec, 1952). Therefore, vascular endothelium in the iris could be a critical barrier for controlling homeostasis of the iris stroma and aqueous humor.

We hypothesized that endothelial cells were exposed to significant shear stress in the different locations and orders of iris microvasculature, the cytoskeleton of the endothelium is central to meeting all these hemodynamic challenges. The actin cytoskeleton provides a strong and dynamic intracellular scaffold that organizes integral membrane proteins with the cell's interior, and responds to environmental cues to orchestrate appropriate cell shape (Prasain and Stevens, 2009). The roles and regulation of cytoskeletal components in endothelium are critical for our understanding of endothelial function in both health and disease (Aird, 2007; Yu et al., 2014).

The function of the endothelium as a semi-permeable barrier can be broadly understood in terms of a balance between cell–cell and cell–matrix adhesion forces to maintain the integrity of the barrier (Curry and Adamson, 2010). Adherens, tight and gap junctions connect adjoining endothelial cells lining the vessel wall, playing pivotal roles in not only tissue integrity but also in vascular permeability, leukocyte extravasation and angiogenesis. There are complex interactions between signaling pathways that modify cell–cell adhesion. It has been reported that the adhesion protein VE-cadherin and the tight junction protein occludin, are both continuous around the periphery of venular endothelial cells in rat mesentery (Wallez and Huber, 2008). However, based on metabolic requirements, the presence and organization of inter-endothelial junctions vary in different organs, different tissues, and even in different vascular segments within the same tissue.

In this study, we examined the intracellular cytoskeleton and the distribution of key components of the adhesion mechanisms in junctions such as VE-cadherin and claudin-5 in different orders of iris microvessels.

2. Materials and Methods

Pig eyes were obtained from a local abattoir. Following enucleation, the eyes were placed in a sealed bottle of oxygenated Krebs solution and kept on ice during transfer to the laboratory (~60 minutes). Thirty seven eyes with sufficiently long temporal long posterior ciliary arteries (LPCA) and lightly pigmented irises were selected for this study (11 eyes for VE-cadherin staining, 10 eyes each for Phalloidin and claudin-5 staining, and another 6 eyes in total for double labelling). All procedures conformed to the EU Directive 2010/63/EU for animal experiments.

The dissection, cannulation and arterial perfusion in isolated eyes are fully described in our previous publications (Townsend et al., 2006; Yang et al., 2015; Yu et al., 2003). Similar techniques were used in the present study and will be briefly described. Before perfusion, the nasal LPCA and all other temporal small arteries were tied off with 9-0 nylon sutures. Details of method of perfusion staining of ocular microvasculature in our lab have been published previously (Yu et al., 2010). Briefly, the pig eye was placed temporal side facing up in an eye holder, the temporal LPCA was cannulated using glass micropipettes with tip sizes of 270 to 300 μm , and secured in place by a 9-0 nylon suture.

Syringe pumps (model 22; Harvard Apparatus, South Natick, MA) were used to deliver an adjustable flow of perfusate and the perfusion pressure was continuously monitored through conventional transducers (Cobe, Arvada, CO), each connected to a bridge amplifier (model 5B38-02; Analog Devices, Norwood, MA) and recorded on a chart recorder (LR8100; Yokogawa, Tokyo, Japan). Additionally, a 25G needle connected to a pressure transducer was inserted into the anterior chamber to record the intraocular pressure (IOP). The flow rate was chosen to ensure that the iris vascular network could be perfused completely without IOP exceeding physiological level or inducing tissue swelling. Based on pilot experiments, we chose a baseline flow rate of 150 $\mu\text{L}/\text{min}$, and then adjusted this to 100 or 75 $\mu\text{L}/\text{min}$

according to the IOP. The IOP was maintained close to the initial pressure at perfusion beginning point (around 7-15 mmHg), and under 30 mmHg after the permeabilization step using Triton X-100. Oxygenated Ringer's solution with 1% bovine serum albumin was perfused at least 30 minutes to flush out any residual blood. Then, solutions were perfused through in order with adjustments in protocols for phalloidin and antibody staining.

2.1 Perfusion labelling for actin microfilament

4% paraformaldehyde in 0.1 M phosphate buffer solution (PB) (30 minutes), 0.1% Triton-X 100 in 0.1 M PB (5 minutes), 0.1 M PB (45 minutes), the 850 μ L mixture of Alexa Fluor 546 or 635 phalloidin (30U; A22283 or A34054, Invitrogen, Carlsbad, CA) and Hoechst (1.2 μ g/ml bisBenzimide H 33258, Sigma-Aldrich, St. Louis, MO) in 0.1 M PB (90 minutes), and 0.1 M PB (40 minutes).

2.2 Intravascular VE-cadherin or claudin-5 Immunohistochemistry

A protocol similar to that of phalloidin labeling was used, but 0.1% Triton-X 100 was prolonged to 8 minutes. This was followed by 0.1M phosphate buffer wash (45mins), 10% donkey serum (1 hour), and a goat anti-VE-cadherin primary antibody (1:50, 1 hour; sc-6458, Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit anti-claudin-5 primary antibody (1:50, 1hour; SAB4200538, Sigma-Aldrich, St. Louis, MO). This was followed by a 0.1M PB wash (45 minutes), and then secondary antibodies with Hoechst (H 33258, 1:1000, Sigma-Aldrich, St. Louis, MO) in 0.1M PB was perfused (1 hour) before the final wash with 0.1 M PB (45 minutes). Secondary antibodies used in this study were donkey anti-goat IgG (1:200; Alexa Fluor 488 or 555 A11055, Invitrogen, Carlsbad, CA or ab150130, Abcam, Cambridge, UK), and donkey anti-rabbit IgG (1:200; Alexa Fluor 488 A21206, Invitrogen, Carlsbad, CA).

2.3 Intravascular immunohistochemistry double staining for microfilament, anti-VE-cadherin and anti-claudin-5

Similar protocol was used as described above. Where VE-cadherin and claudin-5 were double labelled, primary antibodies were administered at the same time in the same perfusate. Whereas for double staining using a combination of antibody and phalloidin, phalloidin conjugated with Alexa Fluor 635(A34054, Invitrogen, Carlsbad, CA) and Hoechst were included in the secondary antibody solution and perfused at the same time.

2.4 Flat-mounting of Iris and Confocal Scanning

The eyeballs were cut in half at the ora serrata and the anterior segments were immersion fixed in 4% paraformaldehyde in 0.1M PB for 30 minutes at room temperature, followed by buffer immersion wash for another 30 minutes. Then the perfused half of iris was carefully dissected out from the root connected to the ciliary body, and cut into 4 to 5 sectors. The posterior pigment epithelial layer of iris was carefully removed with the aid of dissecting microscope. The iris sectors were immersed in RapiClear® 1.47 (Sunjin Lab, Taiwan) for 2 hours for optical clearing before it is flat-mounted with anterior face up for confocal imaging. Confocal imaging was performed on Nikon i90 microscope and Nikon C1 system with EZ-C1 software (v. 3.20, Nikon), equipped with 4 (408 nm, 488 nm, 546 nm and 635 nm) laser board. Confocal images of all iris sectors were acquired at low magnification first (x2 and x4, Nikon Plan Apo lenses), to help orientate and identify the areas of interest. Specific regions were zoomed in with high-power objective lens (x40, Nikon plan apochromatic oil lens, NA1.0) for detailed imaging of labelled structures at cellular level. Z-stacks were captured at 0.3 μ m optical section increments along the z-plane for each order of arteries and veins, according to our classification with the aid of silver staining published previously.

2.5 *Image analysis*

Confocal image sequences from all the vascular orders were examined carefully. Quantitative measurements were performed only when both cell and nuclear borders were clearly defined on confocal images. Endothelial cells and their corresponding nuclei were outlined and sketches of them were made using Adobe Illustrator CS4 (version 14.0, Adobe Systems Inc.). Morphometric measurements of vessel diameter, endothelial cell length, and nuclei position were done using Image J (1.45s, National Institute of Health, USA). Endothelial cell length (ECL) measurements were taken as the longitudinal distance from the upstream pole to the downstream pole. And nuclei position was defined as distance from the nuclear edge to the cell apex in the downstream direction of blood flow (hereafter denoted as variable e). (Kang et al., 2011) The ratio of e to ECL was considered as normalized nuclei position regardless of endothelial cellular size. Numbers of vessels and endothelial cells measured were dependent on the order of vessels according to our previous paper (Yang et al., 2015). Briefly, for vessel diameter measuring, 2 (major arterial circle (MAC)) to 15 (pre-capillary arteries) arteries, 20 capillaries, and 5 (the biggest veins) to 15 (post-capillary veins) veins of each order were measured in each eye. While for endothelial cell dimensions, 7cells/vessel $\times$ 2 vessels (MAC) to 3cells/vessel $\times$ 5 vessels (pre-capillary arteries), 1cell/vessel $\times$ 10 vessels for capillaries, and 2cells/vessel $\times$ 10 vessels (post-capillary veins) to 6 cells/vessel $\times$ 3 vessels (the biggest veins) of each eye were measured.

All images for this manuscript were processed using Image Pro Plus (Media Cybernetics, Version 7.0) including the projection of confocal Z-stacks as 2-D image and pseudo colouring. All figures were prepared using Adobe Illustrator CS4.

2.6 *Statistical analysis*

All statistical testing was performed using Sigmaplot (v.12.0). We tested differences in measurements between the orders of vessels using one-way ANOVA with post hoc factor comparison performed using Student's t-test with Bonferroni correction or Tukey's test where data passed normality test, or one-way ANOVA on ranks with Dunn's test where data set was not normally distributed. All results were expressed as mean $\pm$ standard deviation unless otherwise stated.

3. Results

3.1 *General*

37 adult porcine eyes in total were perfused. Among them, 10 eyes were perfuse-stained with phalloidin, 10 eyes for claudin-5, 11 eyes were used for VE-cadherin, and the other 6 eyes for double labelling. The temporal half of iris in all the perfused eyes showed complete staining of vasculature. Phalloidin labelled the f-actin in vascular smooth muscle cells and endothelial cells, whilst endothelial cell borders were clearly labelled for VE-cadherin and claudin-5. Nuclei were counterstained with Hoechst. Hence information of both the distribution of porcine iris vascular network and individual endothelial cells could be collected in this study. As what has been revealed in our previous paper using silver nitrate perfusion staining (Yang et al., 2015), there are three layers of vasculature in porcine iris: superficial concentrically aligned capillary meshwork, deep microvascular plexus running radially, and relatively big and tortuous vessels sandwiched in the middle. However, information of intracellular structure (f-actin) and cellular junctions of endothelial cells were of greater interest in this study.

3.2 *F-actin of iris vasculature*

Morphologic characteristics and intracellular structure of endothelial cells of various orders of iris vessels are shown in Figures 1 and 2. The orders of microvascular network are according to the Horton-Strahler nomenclature (Tuma et al., 2008; Yu et al., 2014). F-actin fibres within vascular endothelial cytoplasm and smooth muscle cells wrapping around vessels were clearly stained via intra-luminal perfuse-staining with phalloidin. Stress fibres and peripheral border staining were seen within endothelial cells with remarkable variations in different orders. Stress fibres distributed parallel to the cellular longitudinal axis and the direction of blood flow, whilst peripheral bundles lay along cell borders, allowing individual endothelial cells available to be recognized. Strong staining of stress fibres was found in endothelial cells on large arteries, predominantly on A6, A5 and A4 (Figure 1). An obvious decrease in cytoplasmic stress fibres is evident further downstream in the smaller arterioles, and tended to be absent from A3 onwards (Figure 1). Stress fibres were absent in endothelial cells of A3 in 6 out of the 10 eyes labelled with phalloidin. However, disorganized dotted or fragmented appearance of f-actin tagged by phalloidin was seen in the endothelial cytoplasm of the capillaries, veins, and lower orders of arterioles (Figure 1 and 2). As to peripheral bundles, they appeared as more than one layer in high orders of arteries, usually in A4-A6, which sometimes made the endothelial cell borders hard to be determined accurately. The width of peripheral bundle became thinner in endothelial cells of small arterioles in the iris arterial system, but thickened again from lower order to higher ones in the venous system. The configuration of vascular smooth muscle cells around the iris microvasculature was able to be demonstrated due to the f-actin content highlighted by phalloidin binding. They were packed closely together much like a band wrapping circumferentially around arterioles and appeared the clearest and the most intense as several layers in the major arteriole half circle, but becoming looser and thinner in the lower orders of arterioles. There are no smooth muscle

cells around the iris capillaries. However, venules had irregularly and loosely arranged smooth muscle cells that were much less regular in shape and f-actin fluorescence intensity than those in arteries (Figure 2). In the wall of lower venous order, smooth muscle appeared as scattered patches at different angles, but the arrangement of smooth muscle cells around higher order veins became more ordered with an increased presence of smooth muscle cell-bundles perpendicular to the direction of flow.

3.3 *Junction Proteins*

Endothelial intercellular junctions throughout the iris microvasculature were positively labelled for VE-cadherin and claudin-5, indicating the presence of both adherent junction and tight junction. Figure 3 shows representative confocal images for endothelial cells from selected orders of iris microvasculature (A6, A2, capillaries, V3 and V4). The projected confocal images were collected using x40 objective lens showing endothelial f-actin, VE-cadherin and claudin-5 labelling. There are some remarkable differences between VE-cadherin and claudin-5 labelling. VE-cadherin appeared as a continuous, smooth and even labelling at the endothelial cell borders and clearly outlined the iris microvascular endothelial cells. For claudin-5, however, although the labelling was continuous, it was not of uniform width. Some clusters or dots were seen along the endothelial border, and diffuse fluorescent dusts were noted in some endothelial cytoplasm (Figure 3e). The unevenness of claudin-5 staining was noted along the endothelial cell borders almost in every order of vessels, especially in veins and small arterioles. With decreasing fluorescent intensity in small arterioles and big veins, claudin-5 labelling was absent in V4 in 6 out of the 10 experimented porcine eyes. Double staining via the intraluminal perfusion technique is another way to allow us to compare or correlate any of the three markers to one another. Paired images in Figure 4 shows double labelling for F-actin and VE-cadherin; F-actin and claudin-5; and VE-cadherin and claudin-5. VE-cadherin and claudin-5 seem well colocalized at this level, but f-

actin picked up smooth muscle around the pupil margin (sphincter) in addition to the vessels. Figure 5 shows an example of high magnification confocal images of VE-cadherin and claudin-5 double staining, and counterstained by Hoechst. Generally, VE-cadherin and claudin-5 roughly co-localized with each other at cell borders. However, uneven distribution of claudin-5 is clearly seen along the interendothelial junction, with some areas with thickened lines or dots overlying the VE-cadherin.

3.4 Endothelial Morphometrics

Endothelial cell border were clearly labelled using the endothelial specific antibody for VE-cadherin. The nuclei were clearly labelled using Hoechst. Eyes labelled for f-actin also showed cell border clearly. However, in vessels with mural smooth muscle cells, the endothelial border labelling was masked by the rich f-actin microfilament content of the overlying smooth muscle cells. Therefore, only the 11 eyes labelled for VE-cadherin were used to conduct the quantitative measurements. Vessel diameter, endothelial cell length and nuclei position results were shown in Table 1. One-way ANOVA didn't show any significant differences among the 11 eyes measured. Then one-way ANOVA tests were performed on the pooled data from all 11 eyes to increase statistical power.

The diameter of vessels varied significantly between consecutive vessel orders, in agreement with our previous report (Yang et al., 2015). Overall, the arteriole diameters were significantly smaller than that obtained from the previous study where silver nitrate method was used. However, the diameters in the venous vessels were fairly comparable across the two studies.

In this study, the average diameter of the major artery (order A6), the biggest artery in iris, was $279.40 \pm 18.0 \mu\text{m}$ (n=11) and that of the largest veins was $159.40 \pm 9.4 \mu\text{m}$ (n=11), while average the diameter of capillaries was only $17.21 \pm 1.6 \mu\text{m}$ (n=11).

The endothelial cell shape was comparable with previous report (Yang et al., 2015), with more spindle-shaped cells in the arteries and polygonal cells in the veins. Endothelial cell length averaged 92 to 116 μm in the artery system, 50 to 57 μm in veins and approximately 86 μm in capillaries. There was a gradual shortening of cell length through various arterial orders since A6 and elongating along venous orders downward from V1. One-way ANOVA showed endothelial lengths from A1 to be significantly shorter than those from A2~A6 (all $p \leq 0.001$), and those from A6 and A5 were much longer than the other lower orders of arterioles (all $p \leq 0.001$ or 0.05). For the venous side, V1 and V2 were notably shorter than V3 and V4 in endothelial cell length (all $p < 0.05$). Endothelial cell lengths of capillaries were significantly shorter than those on any order of arterioles (all $p \leq 0.001$), but longer than any order of venules (all $p \leq 0.001$).

With the counterstaining of Hoechst, both the shape and position of nuclei could be analysed. Sharing the same tendency of endothelial cellular shape along various vessel orders, the shape of endothelial nuclei differed from order to order of iris vessels. The endothelial cells of the largest arteries contained the longest elliptical nuclei. And the long axis of nuclei lay parallel to the longitudinal axis of the endothelial cells. Nuclei of endothelial cells in lower orders of arterioles and capillaries appeared shorter and wider, close to round in morphology. Through low orders of venules after capillaries, the nuclei of endothelial cells transited back from roundish shape to an elongated contour in relative big veins.

The proximity of endothelial nucleus position to the downstream pole is expressed as a ratio to the total cell length. For A6 endothelium, *e*-to-endothelial-cell-length ratio was around 0.4. This means the nuclei were positioned close to the centre of endothelial cells. Comparing the positions of endothelial nuclei in vessels more downstream than A6, all were significantly closer to the downstream pole of the endothelia when compared with those in A6 (all

$P < 0.05$). In particular, nuclei were displaced downstream notably to the direction of blood flow in capillaries, A1 and A2 compared to A6 (all $P < 0.01$) (Figure 6).

4. Discussion

In this study, we have provided some major and interesting findings in addition to our previous study using silver staining which mostly outlined the shape of the endothelium. These major findings in this study include: (1) High blood flow and shear stress exists in the iris vasculature particularly in larger arteries evidenced by spindle-shaped endothelial cells containing distinct stress fibres in the arteries, (2) Iris endothelium has stable barrier function evidenced by defined peripheral border staining of f-actin, VE-cadherin and claudin-5 of individual endothelial cell in each order of vessels, and (3) significant heterogeneity of intracellular structure and junction proteins was revealed in different orders of the iris vasculature in addition to vascular diameter and shape of the endothelia evidenced by different staining of stress fibres and junction proteins. All these findings further suggest that endothelium of iris microvasculature has certain intracellular structure and junction properties to adapt high blood flow rate within different orders and capability for sufficient nutrient and waste exchange between the blood stream and the iris stroma.

Recently, the roles of iris and its vasculature are increasingly being recognised in both physiological and pathological conditions. Iris blood flow and vascular endothelium play critical roles in maintaining selective permeability of different size molecules (Abraham and Dashwood, 2008; Aird, 2012). Anterior chamber oxygen is mostly derived from the iris and ciliary body vasculature and by diffusion across the cornea. The iris microvasculature being the predominant source of oxygen in this region most likely plays a critical role in maintaining oxygen homeostasis and may keep appropriate oxygen levels in key structures such as the cornea, lens, and trabecular meshwork. Changes in oxygen gradients could

induce oxidative stress and subsequently increase reactive oxygen species (ROS) in trabecular meshwork and lens tissue which are major pathogenic factors for cataract and glaucoma (Barbazetto et al., 2004; Holekamp et al., 2005; Shui et al., 2006). The lens is a structure which normally has a relatively low oxygen level (Barbazetto et al., 2004; Holekamp et al., 2005; Shui et al., 2006). Association of oxygen stress and ROS to nuclear cataracts formation has been well emphasized (Barbazetto et al., 2004; Holekamp et al., 2005; Siegfried et al., 2010; Truscott, 2005). Recent reports that intraocular surgery can disturb ocular oxygen gradients, increase oxygen delivery to the outflow pathway and directly induce oxidative stress (Chang, 2006; Luk et al., 2009; Siegfried et al., 2010). Oxidative stress may also present an important pathogenic step in primary open-angle glaucoma by inducing human trabecular meshwork degeneration, favoring an intraocular pressure (IOP) increase, thus priming the glaucoma pathogenic cascade (Izzotti et al., 2009; Sacca et al., 2005). Hence, any iris vascular abnormality would probably affect the oxygen gradients in anterior chamber with pathological consequences.

Of interest, the cell borders between neighbouring endothelial cells stained by VE-cadherin were observed to be a bit wider and denser than those highlighted by claudin-5. It suggests bigger cleft between lateral membranes of adjoining endothelial cells at the VE-cadherin anchoring places than at the claudin-5 anchoring sites, which supports the different tightness of adherent junction and tight junction. Given that adherent junction is organised along the entire area of interendothelial cell contact and a complex network of anastomosing tight junction strands is embedded within the adherent junction, it could be speculated claudin-5 and VE-cadherin together function as a two-pronged fence blocking paracellular pathway. Our data also suggested that the alignment of tight junction molecules between neighbouring endothelial cells was not uniform, probably less aggregation of this molecule at the thinned or

less strongly stained points. So the veins and small arterioles are possibly more prone to get involved in the processes inducing vascular leakage.

The process of fluid and molecules exchange between blood vessels, stroma and aqueous humour has not been delineated, although it is known that the stroma of the iris is a sponge-like layer composed of an interwoven, collagenous framework in a matrix of hyaluronidase-sensitive substance (Tasman and Jaeger, 2005). The root of iris is connected with ciliary body, and no apparent diffusion barrier between the interstitial spaces of the iris and the anterior chamber (Freddo, 1996; Oyster, 2000). Previous studies have supported the pathway that plasma constituents might diffuse from the ciliary body stroma into the iridial stroma and, finally into the anterior chamber (Freddo et al., 1990; Raviola and Butler, 1985). Therefore, aqueous flow, iris vasculature and ciliary body vasculature may offer different roles to keep a certain concentration gradient of each molecule in aqueous humour. Also some molecules can selectively be transported through iris endothelium (Raviola and Butler, 1985). Hence, the roles of iris microvasculature particularly its endothelium need to be further explored.

Our previous results of rich vasculature and their unique arrangements may provide some initial information of the possible role of iris vasculature in iris volume changes (Yang et al., 2015). However, limitation of our previous study is using silver staining which can only show the border information of endothelium. In this study, we have focused on intracellular cytoskeleton and junction proteins of the endothelial cells in the different orders of iris microvasculature to further understand the roles of iris vascular endothelial cells in physiological and pathological conditions.

Our results in this study show that marked dissimilarities exist in different orders in terms of pattern of stress fibres and peripheral band of actin within endothelial cells. Strong staining of stress fibres found in endothelial cells on relatively large arteries, predominantly on A6, A5

and A4 indicated the presence of shear stress. Instead of obvious and organized stress fibres, irregular pattern of f-actin tagged by phalloidin showed in vessels downstream from A3. Meanwhile, peripheral borders staining of f-actin was found thinner in small arterioles and venules. It is understandable that endothelial cells are constantly exposed to mechanical stimuli as mechano-sensors and subsequently convert mechanical stimuli into intracellular signals that elicit a morphological or genetic response. The endothelium must be capable of delicately balancing and dynamically regulating both barrier function and selective permeability to solutes and immune cells. Endothelial cells must resist significant, and in some settings extreme, mechanical forces including fluid shear, hydrostatic pressure, and cyclical stretch. The variations of cytoskeleton of the endothelium in the different orders are critical to maintain endothelial functions and meeting all these challenges. The patterns and regulation of cytoskeletal components in endothelium may help us to understand the endothelial function in both health and disease.

Endothelial cells lining the vessel wall are connected by adherens, tight and gap junctions. These junction proteins play important roles in tissue integrity but also in vascular permeability, leukocyte extravasation and angiogenesis (Wallez and Huber, 2008). VE-cadherin and claudin-5 are specific to endothelial cells (Breier et al., 1996; Lampugnani et al., 1992; Morita et al., 1999; Wallez and Huber, 2008). Endothelial cell junction resistance to flow stress is dependent upon VE-cadherin, and more specifically upon its binding to plakoglobin (Schnittler et al., 1997). Claudin family members are the major tight junction transmembrane constituents, exhibiting homophilic and heterophilic (with other claudin subtypes) adhesive activities through their extracellular domains and forming the tight junction strands (Furuse and Tsukita, 2006).

Our results showed that VE-cadherin and claudin-5 were highly expressed in endothelial intercellular junctions throughout the iris vasculature, indicating the presence of both

adherent junction and tight junction. However, there are some remarkable differences between VE-cadherin and claudin-5 labelling. Even though both continuously highlighted endothelial cell borders in the iris microvasculature, VE-cadherin appeared as a smooth and even labelling at the endothelial cell borders, while the labelling of claudin-5 was not of uniform width. Sometimes the signal of claudin-5 was not restrained at the cell borders, diffuse fluorescent dusts could be noted in some endothelial cytoplasm. Although the unevenness of claudin-5 staining was noted along the endothelial cell borders almost in every order of vessels, however, it was more obviously found in veins and small arterioles. For single layered vascular endothelial cells to function as barriers there must be some seal to prevent solutes diffusion from blood stream through the paracellular route. The tight junctions have been thought responsible for this intercellular sealing. However, tight junctions are not simple barriers; they show ion and size selectivity and their barrier function varies significantly in tightness depending on the tissue and cell type, and physiological requirements. Such regulated and diversified permeability of tight junctions is required for dynamically maintaining the each environment. In this study we have characterised significant phenotype heterogeneity of endothelial cells and gradual changes in cytoskeleton pattern in the different orders of iris microvasculature which suggests that endothelial cells in each vessel order have specific hemodynamic environment. Therefore, such environment requires endothelial cells to perform specific functions. Our findings of intracellular cytoskeleton, intercellular junction proteins in different orders represent the coupling between endothelial cells and its cellular compositions and their physiological environment. Under pathological conditions, vascular remodelling usually occurs in response to locally generated mediators, injury, inflammatory cell infiltration, or changes in hemodynamic conditions. It could involve structural alterations in the vascular walls including the changes in size and cellular compositions. Currently, most of the vascular remodelling studies focus on large

arteries and arterioles but only a few have investigated remodelling in micro vessels, a site that allows macromolecules to escape, resulting in tissue oedema and organ dysfunction (Yuan and He, 2012). We have previously reported vascular modelling in the specific sites of the retinal vein, arteriovenous crossing and lamina cribrosa region where the risk sites for retinal vein occlusion are (Kang et al., 2011; Kang et al., 2013; Yu et al., 2014; Yu et al., 2012). Identifying the structural and functional changes in iris microvascular endothelial cells and their impact on microvessel permeability will be crucial for a better understanding of the pathogenesis of related vascular diseases and thereby benefit the development of targeted therapies.

Some technical difficulties limit us to use human donor eyes for this study. Mainly perfusate often cannot effectively be directed through the iris vasculature because of leakage from the anterior ciliary arterial stubs. In addition, the choroidal and uveal connections to the iris are often severed during the removal of the corneal button, rendering incomplete perfusion of the human iris (Yang et al., 2015). Given the structural similarities between porcine and human eye (Pond and Houpt, 1978; Prince et al., 1961) and the advantage of being able to choose porcine eyes with lightly pigmented iris, we conducted this study using porcine eyes.

In summary, the distribution and endothelium of the iris microvasculature have been quantitatively assessed along with intracellular cytoskeleton and intercellular junction proteins of endothelial cells in the different orders of iris microvasculature in this study.

Combining information gained from this and future studies with specific location and unique structural features of the iris, such as the absence of endothelium in the anterior surface and sponge-like stroma, may help us to further understand the physiological and pathogenic roles of the iris in relevant ocular diseases.

Acknowledgements

The authors thank the staff at the Linley Valley Abattoir for supplying the pig eyes, and Mr Dean Darcey for manufacture and maintenance of the perfusion systems. Grant support was provided by the National Health and Medical Research Council of Australia. The first author is supported by the Chinese Scholarship Council.

References

- Abraham, D., Dashwood, M., 2008. Endothelin--role in vascular disease. *Rheumatology*. (Oxford) 47 Suppl 5, v23-v24.
- Aird, W.C., 2007. *Endothelial Biomedicine*, Cambridge University Press, New York.
- Aird, W.C., 2012. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med* 2, 1-13.
- Barbazetto, I.A. et al. 2004. Oxygen tension in the rabbit lens and vitreous before and after vitrectomy. *Exp. Eye Res* 78, 917-924.
- Breier, G. et al. 1996. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* 87, 630-641.
- Chang, S., 2006. LXII Edward Jackson lecture: open angle glaucoma after vitrectomy. *Am J Ophthalmol* 141, 1033-1043.
- Curry, F.R., Adamson, R.H., 2010. Vascular permeability modulation at the cell, microvessel, or whole organ level: towards closing gaps in our knowledge. *Cardiovasc. Res.* 87, 218-229.
- Freddo, T.F., 1996. Ultrastructure of the iris. *Microsc. Res Tech.* 33, 369-389.
- Freddo, T.F. et al. 1990. The source of proteins in the aqueous humor of the normal rabbit. *Invest Ophthalmol. Vis. Sci.* 31, 125-137.
- Furuse, M., Tsukita, S., 2006. Claudins in occluding junctions of humans and flies. *Trends Cell Biol.* 16, 181-188.
- Hogan, M.J., Alvarado, J.A., Weddell, J.E., 1971. *Histology of the Human Eye: An Atlas and Textbook*, W.B. Saunders Company, Philadelphia.
- Holekamp, N.M. et al. 2005. Vitrectomy surgery increases oxygen exposure to the lens: a possible mechanism for nuclear cataract formation. *Am J Ophthalmol* 139, 302-310.
- Izzotti, A. et al. 2009. Sensitivity of ocular anterior chamber tissues to oxidative damage and its relevance to the pathogenesis of glaucoma. *Invest Ophthalmol Vis Sci* 50, 5251-5258.
- Kang, M.H. et al. 2011. Morphometric characteristics of central retinal artery and vein endothelium in the normal human optic nerve head. *Invest Ophthalmol Vis Sci* 52, 1359-1367.
- Kang, M.H. et al. 2013. Alterations to vascular endothelium in the optic nerve head in patients with vascular comorbidities. *Exp Eye Res* 111, 50-60.

- Lampugnani, M.G. et al. 1992. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J. Cell Biol.* 118, 1511-1522.
- Luk, F.O. et al. 2009. Presence of crystalline lens as a protective factor for the late development of open angle glaucoma after vitrectomy. *Retina* 29, 218-224.
- Morita, K. et al. 1999. Endothelial Claudin: Claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *The Journal of Cell Biology* 147, 185-194.
- Oyster, C.W., 2000. The Human Eye, Structure and Function. *Ophthal. Physiol. Opt.* 20, 349-350.
- Pond, W.G., Houpt, K.A., 1978. The Biology of the Pig, Cornell University Press, Ithaca, NY.
- Prasain, N., Stevens, T., 2009. The actin cytoskeleton in endothelial cell phenotypes. *Microvasc Res* 77, 53-63.
- Prince, J.H., Diesem, C.D., Eglitis, I., Ruskell, G.L., 1961. Anatomy and histology of the eye and orbit in domestic animals, Charles C Thomas, Springfield, Illinois.
- Raviola, G., Butler, J.M., 1985. Morphological evidence for the transfer of anionic macromolecules from the interior of the eye to the blood stream. *Curr Eye Res* 4, 503-516.
- Sacca, S.C. et al. 2005. Oxidative DNA damage in the human trabecular meshwork: clinical correlation in patients with primary open-angle glaucoma. *Arch Ophthalmol* 123, 458-463.
- Schnittler, H.J. et al. 1997. Role of cadherins and plakoglobin in interendothelial adhesion under resting conditions and shear stress. *Am. J. Physiol* 273, H2396-H2405.
- Shui, Y.B. et al. 2006. Oxygen distribution in the rabbit eye and oxygen consumption by the lens. *Invest Ophthalmol Vis Sci* 47, 1571-1580.
- Siegfried, C.J. et al. 2010. Oxygen distribution in the human eye: relevance to the etiology of open-angle glaucoma after vitrectomy. *Invest Ophthalmol. Vis. Sci.* 51, 5731-5738.
- Tasman, W., Jaeger, E.A., 2005. Duane's Clinical Ophthalmology on CD-ROM.
- Tousimis, A.J., Fine, B.S., 1959. Ultrastructure of the iris: the intercellular stromal components. *Arch Ophthalmol* 62, 974-976.
- Townsend, R. et al. 2006. Confocal laser doppler flowmeter measurements in a controlled flow environment in an isolated perfused eye. *Exp Eye Res* 82, 65-73.
- Truscott, R.J., 2005. Age-related nuclear cataract-oxidation is the key. *Exp Eye Res* 80, 709-725.
- Tuma, R.F., Duran, W.N., Ley, K., 2008. Handbook of Physiology: Microcirculation, 2nd ed. Academic Press (Elsevier), San Diego.
- Vrabec, F., 1952. The anterior superficial endothelium of the human iris. *Ophthalmologica* 123, 20-30.
- Wallez, Y., Huber, P., 2008. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochim. Biophys. Acta* 1778, 794-809.
- Yang, H. et al. 2015. Quantitative study of the microvasculature and its endothelial cells in the porcine iris. *Exp Eye Res* 132, 249-258.
- Yu, D.Y. et al. 2003. Isolated preparations of ocular vasculature and their applications in ophthalmic research. *Prog Retin Eye Res* 22, 135-169.

- Yu, D.Y. et al. 2014. Functional and morphological characteristics of the retinal and choroidal vasculature. *Prog Retin Eye Res* 40, 53-93.
- Yu, P.K. et al. 2010. The structural relationship between the microvasculature, neurons, and glia in the human retina. *Invest Ophthalmol Vis Sci* 51, 447-458.
- Yu, P.K. et al. 2012. Age-related changes in venous endothelial phenotype at human retinal artery-vein crossing points. *Invest Ophthalmol Vis Sci* 53, 1108-1116.
- Yuan, D., He, P., 2012. Vascular remodeling alters adhesion protein and cytoskeleton reactions to inflammatory stimuli resulting in enhanced permeability increases in rat venules. *J. Appl. Physiol* 113, 1110-1120.

Legends

Figure 1. F-actin labelling in endothelium of iris arteries

Confocal microscope images and schematic outlines show endothelial morphology in each order of the porcine iris arteries (A1-A6). F-actin within both endothelial cells and smooth muscle cells are tagged by phalloidin (red colour). Nuclei were counterstained with Hoechst (blue colour). Cell border located f-actin (peripheral border staining) outlines the shape of endothelium (yellow dotted lines). A more structured filamentous structure (stress fibres) could be seen inside the endothelial cells in relatively big arteries (A4-A6). Strong staining of smooth muscle cells made relatively weak appearance of peripheral border staining and stress fibres. Endothelial cells in the iris arteries are spindle shaped, particularly in A6, A5 and A4. All the endothelial cells oriented in the direction of blood flow (red arrow denotes blood flow direction). Red lines in the schematics represent cytosolic f-actin stress fibers. The small white arrows point to smooth muscle band wrapping around arteries and big veins. Scale bar measures 25µm in each column.

Figure 2. F-actin labelling in endothelium of iris capillary and veins.

Confocal microscope images and schematic outlines show endothelial morphology in the porcine iris capillaries (C) and each order of veins (V1-V4). The red colour represents the f-actin labelling, whilst the blue colour represents the labelling of nucleic acid by Hoechst. Yellow dotted lines on the images highlight the endothelial shapes. Endothelial cells in the iris capillaries are spindle shaped, while those in the veins are polygonal, shorter and wider. All the endothelial cells oriented in the direction of blood flow (red arrow denotes blood flow direction for capillaries and veins). Lack of cytosolic f-actin stress fibers in the capillaries and veins is clearly evidenced. The small white arrows point to smooth muscle band wrapping

around arteries and big veins. Scale bar measures 25 μm , and V1-V4 share the same scale bar.

Figure 3. Distribution of VE-cadherin, claudin-5 and f-actin in endothelia of porcine iris microvasculature.

Representative confocal images of iris endothelia labelled for a single junction protein. The left column showed vessels labelled by VE-cadherin, the middle column showed claudin-5, and the right column showed f-actin. The blue colour shows the nuclei counterstained by Hoechst. Images across the same row represent endothelial cells from vessels of the same order and included the major arterial half circle (A6), the 2nd order of arteries (A2), capillaries (C), and the 3rd (V3) and 4th (V4) order veins. VE-cadherin was shown to be precisely concentrated at cell-cell borders of endothelial cells of all segments of blood vessels in the porcine iris. Endothelial cell borders highlighted by claudin-5 in the middle column were not as even and neat as those labelled by VE-cadherin corresponded in the left column. In the f-actin panel, endothelia are barely seen in some areas due to masking by dense and heavily stained smooth muscle cells wrapping around big arteries, or cluttered outside big veins. Scale bar, 50 μm .

Figure 4. Double staining of the porcine iris vasculature viewed under low magnification.

The top row is confocal images dual-stained with f-actin and VE-cadherin. The middle row is with f-actin and claudin-5, and the bottom is with combination of VE-cadherin and claudin-5. Left column: dual-channel projections showing overlap between any two markers upon double staining; middle and right columns: separate images for each marker corresponding to the left sector. White arrows indicate sphincter close to pupil detected only with phalloidin perfusion staining. Scale bar, 1 mm.

Figure 5. Distribution of VE-cadherin and claudin-5 on iris interendothelial junction under high magnification

Projected image of confocal data taken from the porcine major arterial half circle with intravascular double staining. Both VE-cadherin (red) and claudin-5 (green) were expressed and localized at endothelial cell borders. However, two junction molecules are not precisely overlapped at inter-endothelial junction. Note the smooth junction arrangement of VE-cadherin at each cell and the overlying uneven distribution of claudin-5 in the dual-channel image at the bottom. White arrows point to some remarkably thickened spots of claudin-5 at cell borders in the merged image. The blue colour represents the nuclei. Scale bar equals 50 μm .

Figure 6. Intracellular location of endothelial cell nuclei in different orders of iris vessels.

The normalized position of nuclei is expressed as a ratio of variable e to endothelial cell length. A is a plot of mean value and standard errors of the e -to-endothelial cell length ratio in each order of the porcine iris vessels. B is a group of images representing the 6th order arteries (a), capillaries (b) and the 4th veins (c) respectively, showing the difference of nuclear position in various order of iris vessels. Using A6 as the reference point for comparison, statistical significance (* denotes $p < 0.05$; † denotes $p < 0.01$, ‡ denotes $P < 0.001$.) was found. A= arteries, C=capillaries, V= veins, the number after A or V indicates the order of vessels. Red arrow on the top of B illustrates the direction of blood flow. Scale bar indicates 25 μm and is applicable to all images in B.

Tables

Table1. Morphometric dimensions according to vessel order in the porcine iris.

Vessel Order	Vessel diameter(μm)	ECL(μm)	Distance e (μm)	e -to-ECL ratio
A6	279.4 $\pm$ 18.00(11)	115.7 $\pm$ 6.06(11)	44.7 $\pm$ 4.10(11)	0.386 $\pm$ 0.0370(11)
A5	191.1 $\pm$ 16.16(11)	110.2 $\pm$ 3.6(11)	38.4 $\pm$ 3.96(11)	0.347 $\pm$ 0.0293(11)
A4	124.5 $\pm$ 8.43(11)	105.4 $\pm$ 4.60(11)	34.7 $\pm$ 4.26(11)	0.333 $\pm$ 0.0358(11)
A3	79.9 $\pm$ 7.70(11)	102.6 $\pm$ 4.26(11)	34.4 $\pm$ 3.88(11)	0.337 $\pm$ 0.0356(11)
A2	52.8 $\pm$ 4.68(11)	104.8 $\pm$ 4.26(11)	34.3 $\pm$ 3.18(11)	0.318 $\pm$ 0.0367(11)
A1	30.9 $\pm$ 2.89(11)	91.9 $\pm$ 4.79(11)	28.8 $\pm$ 4.02(11)	0.314 $\pm$ 0.0403(11)
C	17.2 $\pm$ 1.62(11)	86.2 $\pm$ 3.61(11)	27.8 $\pm$ 3.24(11)	0.325 $\pm$ 0.0331(11)
V1	27.2 $\pm$ 2.54(11)	50.3 $\pm$ 3.47(11)	17.6 $\pm$ 2.39(11)	0.355 $\pm$ 0.0457(11)
V2	59.3 $\pm$ 4.69(11)	51.1 $\pm$ 2.94(11)	18.4 $\pm$ 2.21(11)	0.351 $\pm$ 0.0476(11)
V3	117.5 $\pm$ 7.61(11)	53.5 $\pm$ 2.44(11)	18.9 $\pm$ 1.29(11)	0.354 $\pm$ 0.0366(11)
V4	159.4 $\pm$ 9.43(11)	57.3 $\pm$ 2.55(11)	20.2 $\pm$ 2.40(11)	0.350 $\pm$ 0.0324(11)

Distance e refers to nuclei position (Distance from cell apex to nucleus in the downstream direction of blood flow. A6 indicates the biggest arteries (major arterial circle), A1 is the pre-capillary arterioles, C represents capillaries, V1 is post-capillary venules, and V4 is the biggest vein. Values are mean $\pm$ standard deviation (number of eyes measured).

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A Ratio of e/ECL on each order of vessels

B

Figure 6