QUANTITATIVE STUDY OF THE MICROVASCULATURE AND ITS ENDOTHELIAL CELLS IN THE PORCINE IRIS

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Abstract

The roles of the iris microvasculature have been increasingly recognised in the pathogenesis of glaucoma and cataract; however limited information exists regarding the iris microvasculature and its endothelium. This study quantitatively assessed the iris microvascular network and its endothelium using intra-luminal micro-perfusion, fixation, and staining of the porcine iris. The temporal long posterior ciliary artery of 11 isolated porcine eyes was cannulated, perfusion-fixed and labelled using silver nitrate. The iris microvasculature was studied for its distribution, orders and endothelial morphometrics. The density of three layers of microvasculature was measured. Endothelial cell length and width were measured for each vessel order. 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 the microvasculature located in the superficial and deep stroma as well as the pupil edge. The average vascular density of the middle, superficial, and deep layers were $38.9 \pm 1.93 \%$, $10.9 \pm 1.61 \%$ and $8.0 \pm 0.79 \%$ respectively. Multiple orders of iris vessels (capillary, 6 orders of arteries, and 4 orders of veins) with relatively large capillary and input arteries ($319.5 \pm 25.6 \mu\text{m}$) were found. Significant heterogeneity of vascular diameter and shape of the endothelia was revealed in different orders of the iris vasculature. Detailed information of topography and 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, vascular biology, microvascular networks.

1. Introduction

The iris vasculature is the major source of oxygen and nutrients supplying the anterior segment. The anterior segment is a highly specialized structure consisting of avascular tissues such as the cornea, lens, and aqueous humor (Tasman and Jaeger, 2005). It is thought that the iris vasculature not only has a role in maintaining intraocular homeostasis, it is also likely to have a role in the sequential survival as well as function of the cells in the anterior segment when the iris vasculature undergoes pathological changes.

The impact of the iris vasculature on the anterior segment is not well defined. Recent non-invasive imaging techniques such as ultrasound biomicroscopy and optical coherence tomography have provided some insights suggesting a possible role in the pathogenesis of angle closure glaucoma (Wang et al., 2010). Significant changes in iris volume have been observed using these imaging devices and postulated to be a major pathogenic factor for primary angle closure glaucoma (Aptel et al., 2012; Baskaran et al., 2013; Mak et al., 2013; Oyster, 2000; Quigley, 2009; Seager et al., 2014). The dynamic processes leading to changes in iris and choroid volume could have a more significant and mechanistic effect to its surrounding structures than a mere anatomical observation (Quigley, 2009). Hence the mechanisms involved in iris volume change as well as the consequence from the iridal volume change could well be an important pathogenic factor in glaucoma.

Secondly, both clinical and experimental studies demonstrated significant oxygen gradients are present normally in the anterior chamber. We now know that changes in oxygen gradients and oxidative stress 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). Exposure of the lens to elevated intraocular oxygen and the

subsequent increase in reactive oxygen species (ROS) (Barbazetto et al., 2004; Truscott, 2005) is a risk factor for nuclear cataracts (Barbazetto et al., 2004; Holekamp et al., 2005; Siegfried et al., 2010). It has been said that increased oxidation may be the hallmark of age-related nuclear cataract (Truscott, 2005). Recent reports that vitrectomy and cataract surgery can increase oxygen delivery to the outflow pathway and directly increases 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). The iris microvasculature being the predominant source of oxygen in the region most likely plays a critical role in maintaining oxygen homeostasis.

Thirdly, differential protein concentration is known to exist in the aqueous humor and ciliary process stroma. The protein concentration in the aqueous humor (50mg/dl) is less than 1% of that in plasma (8g/dl) (Smith et al., 1985) whereas that in ciliary process stroma is 74% of that in plasma (Bill, 1968). This is likely a consequence of differential permeability to protein in the iridal and ciliary vasculature. Evidence has been found where intravascular horse radish peroxidase appeared in the iridal stroma having leaked from ciliary body but not from the iridal vasculature (Freddo et al., 1990a). Similarly, transport in the reverse direction back into the blood stream found only anionic molecules are transported back via plasmalemmal vesicles of endothelial cells in both the retinal and iridal vasculature with cationic molecules not returned by this pathway (Raviola and Butler, 1985). This further suggests heterogenic property of the endothelium in the iridal and ciliary vasculatures. If we are to assume that open communications exist freely between the interstitial spaces of the iris and the anterior chamber, then it may be further speculated that the iris microvasculature and its endothelium holds the control mechanism to maintain homeostasis in the anterior

segments. In recent decades, the vascular endothelium has emerged not merely as an inert barrier but also an active signal transducer for circulating influences as well as the key regulator of material exchange (Deanfield et al., 2007). Previous studies using corrosion casts had provided some qualitative information on the iris vasculature (Funk and Rohen, 1987; Funk and Rohen, 1990; May, 2011; Ninomiya et al., 2008; Olver and McCartney, 1989a; Olver and McCartney, 1989b; Risco and Nopanitaya, 1980; Rohen and Funk, 1994). Whilst limited information is available on the iridal endothelium both in the structural and functional aspects, this study provided some detail information on iridal endothelium morphometrics and vessel density. This information could help us to further understand and investigate the structural difference in iridal endothelium as well as in considering the role of the iris microvasculature in normal and disease conditions.

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). Eleven eyes with sufficiently long temporal long posterior ciliary arteries (LPCA) and lightly pigmented irises were selected for this study. 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; 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., 2010b) and were modified for silver nitrate labelling of the iris microvasculature. 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 290 to 320 μm , and secured in place by a 9-0 nylon suture. A flow rate of 200 $\mu\text{l}/\text{min}$ was delivered using a syringe pump (model 22; Harvard Apparatus, South Natick, MA) throughout the whole perfusion sequence except for the silver staining part. Firstly, residual blood was washed out with filtered oxygenated Ringer's solution containing 1% bovine serum albumin for 30 minutes. This was followed by filtered 4% paraformaldehyde in 0.1 M phosphate buffer perfused for 5 minutes. Silver nitrate labelling of cell border was based on a modified protocol of P Baluk's method (Baluk et al., 1997). Five solutions were perfused in rapid succession: 0.9% NaCl 2 min; 5% glucose (2ml hand pushed in 2 minutes); 1% AgNO_3 (1 ml hand pushed over 1minute); 5% glucose (1-1.5 ml hand pushed for 1 minute); and 4% paraformaldehyde pump perfused for 3 minutes. The anterior segments were subsequently immersion fixed in 4% paraformaldehyde overnight. The rationale of using silver nitrate staining in this study was to obtain clear morphology of endothelial cells from the iris stroma (Baluk et al., 1997).

The iris was carefully dissected out from the root connected to the ciliary body. To bleach the pigments covering the surfaces of the iris, irises were kept in 10% H_2O_2 in 0.05 M Tris HCl buffer, pH 7.6, for up to 48 hours at room temperature. After bleaching, the iris was exposed to bright light to visualise the silver deposit at cell borders. The iris was then immersed in RapiClear® 1.47 (Sunjin Lab, Taiwan) for 2 hours for clearing.

The intact cleared iris was flat mounted for light microscopy imaging using the Nikon i90 and NIS basic software (v.3.8, Nikon). Low magnification Z-stack images were taken to a depth of 210 μm using a x4 objective lens and up to 250 μm using a x10 objective lens. This enabled three-dimensional data collection on the distribution of iris microvasculature.

Specific vascular segments were examined using higher power objective lenses (x40 plan apochromatic oil lenses) with each z-stack consisting of a depth of optical sections collected at 1 μm increments along the z-plane, for detailed structures in the vascular endothelium and

smooth muscle cells. Z-projection images were made using Image Pro Plus (v.7.0, Media Cybernetics, Inc.) and sketches of endothelium were made to outline the cell shape using Adobe Illustrator CS4.

Topologic description of vessel trees by the Horton-Strahler nomenclature was performed in this study. The Horton-Strahler scheme starts at the capillary level and proceeds centripetally, and the convergence of two equal order branches gives one increase in the order of vessels (i.e. where two capillaries joined together to form a first-order arteriole (A-1) and two A-1 arterioles joined together to form a second-order arteriole (A-2)).

Morphometric measurements of vessel diameter, endothelial cell length and width were made NIS basic software (v.3.8, Nikon). Arteries are recognized as vessels with spindle endothelial cells and thick smooth muscle cells wrapping around; whereas veins have polygonal shaped endothelial cells and connected to arteries by capillaries. As iris microvessels are not uniform in their diameters along their lengths, every vessel of each order was measured at five positions and averaged for diameter measurement. Endothelial cell length measurements were taken as the longitudinal distance from the upstream pole to the downstream pole. Endothelial cell widths measurements were taken as the greatest perpendicular width to the endothelial cell length. Considering the iridal vascular structure branching like a tree, the lower order of vessels (i.e. the further off from the major arterial circle (MAC)) were observed, the more and the slimmer vessels there were. Hence for vessel diameter measuring, 2 (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 $\times$ 2vessels (MAC) to 3cells/vessel $\times$ 5vessels (pre-capillary arteries), 1cells/vessel $\times$ 10vessels for capillaries, and 6cells/vessel $\times$ 3vessels (the biggest veins) to 2cells/vessel $\times$ 10vessels (post-capillary veins) of each eye were measured.

A quarter of iris from the perfused half of each porcine eye was randomly chosen for vascular density measurements. Vessel density was measured as percentage of vessels per defined area of interest. The three iris vascular layers (superficial, middle and deep) were measured separately. Vessels were traced manually using Adobe Photoshop CS3 and then density analysed using Image-Pro Plus 7.0.

Statistical analysis was carried out using SigmaStat (v.3.5). Kolmogorov-Smirnov testing was performed first to determine whether the data were normally distributed. Normally distributed data were analysed with ANOVA with post hoc factor comparison performed using Student's t-test with Bonferroni correction or Tukey's test. Non-normally distributed data were analysed using ANOVA on ranks with the Dunn's test used for post hoc paired analysis. Measurements were first compared according to vessel orders among all porcine eyes using one-way ANOVA, mean values of each eye were then pooled for further analysis. Comparisons between capillary and arteries or veins was analysed using one-way ANOVA with multiple comparisons versus control group. All results were expressed as mean $\pm$ standard deviation unless otherwise stated.

3. Results

3.1 General

Perfusion of the 13 adult porcine eyes did not show any anastomosis between the temporal and nasal half of the iris. Only the temporal half of the iris vasculature was completely stained via cannulation and perfusion labelling through the temporal LPCA. The cell border of endothelial cells as well as arteriole smooth muscle cells were clearly outlined by the silver deposits. The thorough and clear labelling allowed us to reliably acquire detailed information of the topographic distribution and the endothelial morphology of the iris vasculature.

3.2 Distribution of iris vasculature and vascular density in three vascular networks

Consistent with iris histological section (Figure 1), morphologically different distribution of vasculature was identified through the whole thickness of the iris (Figure 2). Three iris vascular networks were identified as a superficial microvascular network, a middle vascular network with relatively large vessels, and a deep microvascular network.

Close to the anterior surface of iris, the superficial microvascular network comprised of capillary meshwork that are more concentrically aligned and inter-connected by smaller arterioles or venules (Figure 2 B and B'). Beneath this superficial microvascular network, a dense vascular network containing abundant and substantially larger iris vessels (Figure 2C and C') were seen in the middle stroma occupying the main bulk of the iris stroma. These large iris vessels ran radially to and from the pupil margin and are interconnected by capillaries close to the pupil margin. The pupil margin capillaries occupied the innermost ring of the iris and accounted for approximately 8.3 ± 0.03 % ($n=13$) of the radial area extending from the pupil margin. The large vessels in mid-stroma displayed a three-dimensional vascular configuration with arteries located generally more superficially than

veins after branching in from the major iris artery half ring at the iris root. Arteries were generally tortuous, whereas veins were fairly straight. A deep microvascular layer consisted of a fine network of capillaries, small arterioles and venules were found beyond the mid-stromal vascular layer. This deep microvascular network was close to the iris epithelium, running radially and tortuously (Figure 2C and C').

Vascular density was measured from superficial, middle and deep layers of the iris vasculature. The averaged density of the middle layer, which mainly consisted of relatively large arteries and veins, was $38.9 \pm 4.9 \%$ ($n = 11$). The averaged density of the superficial and deep layers was $10.9 \pm 4.1 \%$ ($n = 11$) and $8.0 \pm 2.0 \%$ ($n = 11$) respectively. Vascular density of middle layer was significantly greater than that of either superficial or deep layer (both $P \leq 0.001$), but no significant difference was found between superficial and deep layers ($P = 0.185$).

3.3 Vascular orders and vessel diameters

Using the Horton-Strahler's nomenclature, the iris vasculature was found to have 5.6 ± 0.08 ($n=44$) orders of artery and arterioles and 3.6 ± 0.09 ($n=30$) orders of veins and venules. It was noted that instead of the convergence of 2 lower order vessels to form a larger higher order, there were often 1-3 tributaries joining each of the 2nd and 3rd order of arteries. Similarly, 1 to 4 tributaries joined the 1st order venules, and 1 to 2 tributaries joined the 2nd and 3rd order veins and venules. These twigs of tributaries gave rise to or drain blood from superficial and deep capillaries.

The diameter of the arteries and veins were often not uniform along their lengths within the same order. Particularly for the venules, the point of convergence were commonly observed as a constricted part that becomes broader as the higher order vein continues on. However, generally, arteries became narrower along the way running from major arterial circle to

capillaries around pupil margin, whilst the veins were wider when coming back from the pupil and collecting blood from the lower orders. Table 1 showed iris vessel diameter in different vessel orders. It was notable that the diameter of the major artery (order A6) was relatively large ($319.48 \pm 25.6 \mu\text{m}$, $n=11$) indicating the blood supply was relatively rich and the diameter of the iris arteries was steeply reduced with each order. The diameter of small arterioles was only $28.05 \pm 2.3 \mu\text{m}$ ($n=11$). Statistics showed significant differences between diameter of each order of both arteries and veins (One way ANOVA $p \leq 0.001$, Dunn's test all $p < 0.05$). But no significant differences existed among all the 11 experimented eyes (One way ANOVA both $p \leq 0.05$).

3.4 Endothelial Cell Morphometrics (Table 2)

Microstructural heterogeneity of the iris microvasculature was studied. Arterial endothelial cells were generally more spindle-shaped and longer than those seen in veins. Figures 3, 4 and 6 show high magnification images from different orders of iris arterioles (A1 to A6) and venules (V1 to V4). The shape of the endothelial cells and most of the smooth muscle cells were outlined by peripheral border silver deposits. Outlining and sketches were made for a few of the endothelial cells from each vessel order. With increasing order of vessels in both arteries (i.e. upstream) and veins (i.e. downstream), the endothelial cells became more slender. In the 1st and 2nd order venules, endothelial cells assumed a polygonal shape, but were more elongated in the 3rd and 4th order veins.

Endothelial cell lengths and widths are shown in Table 2 as a function of vessel order in the iris arterial and venous system. The arterial endothelial cells averaged 87.4 to 113.5 μm in length and 10.0 to 8.5 μm in width from A1 to A6, whereas the venous endothelial cells averaged 49.1 to 58.7 μm and 12.0 to 9.8 μm from V1 to V4. In the arteriole system, one way ANOVA showed endothelial lengths from A1 to be significantly shorter than those from

A3~A6 ($p \leq 0.001$, and all the Tukey test between A1 and any of A3~A6 showed $p < 0.05$). However, no significant difference was found between A1 and A2, or any two orders among A3 to A6 (p values of all the pairwise multiple comparison using Tukey test were not less than 0.05).). Similarly, endothelial cell width was not all the same among all arterial orders (one way ANOVA $p \leq 0.001$), but there was no significant difference among the lower three orders of arterioles (Tukey test all $p \geq 0.05$), and neither among A4~A6 (Tukey test all $p \geq 0.05$).

For the venous side, no significant difference was found among V1, V2 and V3 either in endothelial cell length ($p > 0.05$) or width ($p > 0.05$) by one-way ANOVA with Tukey rank test. However, significantly longer and narrower endothelium were found in V4 (all $p < 0.05$).

Compared with capillaries, endothelial cell length was significantly longer in A2~A6 (one-way ANOVA $p \leq 0.001$, Dunn's method all $p < 0.05$), while only endothelial cells from A5 and A6 showed significantly narrower than those from capillaries (one-way ANOVA $p \leq 0.001$, Dunn's method all $p < 0.05$). Endothelial cells from V1~V3 were shorter than from capillaries (one-way ANOVA $p \leq 0.001$, Dunn's method all $p < 0.05$), while significantly wider endothelial cells were found from V1 and V2 (one-way ANOVA $p \leq 0.001$, Dunn's method all $p < 0.05$).

Outline of smooth muscle cells (SMC) may be seen from the cell border silver chloride deposits oriented circumferentially around arterioles. The SMC border labelling is the clearest in the major arteriole circle (MAC), and became less intensely stained in the lower orders of arteries. The SMC border labelling was absent in capillaries and venous iris vessels (Figures 5 and 6). Nuclei may be identified in some endothelium when silver chloride is deposited inside the cytoplasm but not at the location of the nuclei (Figure 6A).

4. Discussion

The major findings in this study are: (1) the iris has high density of microvasculature and unusual pattern of the vascular distribution, (2) the iris vasculature consisted of abundant relatively large vessels in the middle of the iris stroma supplying the microvasculature located in both superficial sides of stroma and pupil edge within a short distance, (3) multiple orders of iris vessels (capillary, 6 orders of the arteries and 4 orders of the veins) with relatively large arteries in A6, (4) large diameter of capillary and relatively long spindle shaped endothelial cells in different orders of iris vasculature. All these findings suggest that iris has high blood flow rate and capability for sufficient nutrient and waste exchange between the blood stream and the iris stroma.

The iris has previously been studied with regard to specific features relevant to fluid exchange with the aqueous humor. It was found that the anterior surface of the iris is covered with endothelium during embryologic development. At or soon after birth, the endothelial covering disappears and 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). This meshwork of cells 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). However, the process of fluid and molecules exchange between blood vessels, stroma and aqueous humor 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). There does not appear to be any apparent diffusion barrier between the interstitial spaces of the iris and the anterior chamber. Therefore, aqueous flow, iris vasculature and ciliary body vasculature may offer different roles to keep a certain concentration gradient of each molecule in aqueous

humor, 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). Also some molecules can selectively be transported through iris endothelium (Raviola and Butler, 1985). Therefore, the roles of iris microvasculature particularly its endothelium need to be further explored.

Intra-luminal microperfusion and staining technique has been established in our laboratory and used for study on retinal and choroidal vasculature (Adam et al., 1997; Chan et al., 2013; Kang et al., 2011; Kang et al., 2013; Tan et al., 2012; Tan et al., 2013; Yu et al., 2014; Yu et al., 2010a; Yu et al., 2010b; Yu et al., 2013; Yu et al., 2012). Advantages of this technique include full perfusion fixation of the tissues and intact vasculature and endothelium in situ. As far as we know, detailed information of each order of the iris vasculature and endothelium has not been reported before. This technique of perfusion staining allowed us to precisely investigate vascular endothelial cells in each order of iris vasculature gaining quantitative information of the size and shape of endothelial cells. In addition we were able to study layer distributions of iris vasculature within the iris tissue.

The importance of iris and iris vasculature are increasingly being recognised. It had been postulated that significant iris volume changes could be a major pathogenic factor for angle-closure glaucoma (Aptel et al., 2012; Baskaran et al., 2013; Mak et al., 2013; Oyster, 2000; Quigley, 2009; Seager et al., 2014). Our results of rich vasculature and their unique arrangements may provide some initial information of the possible role of iris vasculature in iris volume changes. It is postulated that the rich vascular network in mid-stromal layer may be the effector in mediating the iris volume change, and that the superficial and deep capillary meshworks is possibly the site of dynamic fluid and molecule exchange. Exact mechanisms may also involve unique structure of iris surface, sponge-like iris stroma, fluid exchange mechanisms between iris microvasculature and stroma as well as vascular activities.

Iris vasculature is also known to be a major source of oxygen gradients in aqueous humor and may keep appropriate oxygen levels in key structures such as the cornea, lens, and trabecular meshwork. No doubt that iris blood flow and vascular endothelium play critical roles in maintaining selective permeability of different size molecules, including oxygen (Abraham and Dashwood, 2008; Aird, 2012). Our results have shown quantitative information of vascular endothelial cells in each order of iris vasculature. These phenotype differences in the different orders may provide hemodynamic information of iris circulation and suggest high shear stress in A6 to A1 orders (Abraham and Dashwood, 2008; Aird, 2012; Yu et al., 2014). It is shown in this study that vascular endothelial cellular length decreased significantly from higher orders (A3~A6) to lower ones (A1 and A2). Additionally, the 4th order of iris arteries in this study shared similar diameter and endothelial cells dimensions with the highest order of porcine retinal arteries shown in our previous study of porcine retinal vasculature (Yu et al., 1997). Comparing smaller arterioles with similar diameters in the two ocular sites, the iris endothelium appeared shorter and wider. By contrast, endothelial cells of the highest order iris veins are more slender than those in retina even with comparable venous diameter. Based on the relationship between shear stress and endothelial cell shape, it could be speculated that blood supply is abundant at the iris root, and blood flow decreased more steeply along vascular circulation from relatively big arteries to small arterioles. This is reasonable because most ramifications were found occurring at A4 and downstream, especially at A3 and A2, causing a dividing effect for blood flow and consequently lower shear stress. While on the venous side, convergence happened more frequently at V2 and V3 and gave rise to V4, resulting in relative higher shear stress and more slender endothelial cells on V4.

The iris area is much smaller than the retina, but the diameter of supplying arteries of the iris were approximately double that of the large retinal artery in the porcine retina (Su et al., 2005). That suggested a massive blood inflow provides not only high blood flow rate, but

also sufficient supply for material exchange, predominantly the delivery of oxygen, glucose, and other nutrients, between blood stream and iris stroma which may be crucial for metabolic demand for maintaining homeostasis in the ocular anterior segment, and in particular oxygen gradient homeostasis. Studies also supported this hypothesis by finding that anterior chamber oxygen is mostly derived from the iris and ciliary body vasculature and by diffusion across the cornea (Shui et al., 2005). Considering recent evidence from both clinical and experimental studies demonstrating the increased development of nuclear cataract as a consequence of disturbed ocular oxygen gradients caused by vitrectomy (Barbazetto et al., 2004; Holekamp et al., 2005), any iris vascular abnormality would probably affect the oxygen gradients in anterior chamber with pathological consequences. Moreover, there is added potential risk of corneal de-compensation after traumatic procedures such as laser iridotomy (Szumny and Szelag, 2014; Wang et al., 2014; Youm et al., 2014) .

In our study, extravascular smooth muscle cells were outlined throughout iris arteries by silver chloride deposits, although the staining intensity decreased downstream of the vascular bed. However, such smooth muscle related silver deposit was totally absent in venules and veins. This could be due to differing permeability and/or adhesion properties, or related to mechanical factors such as shear stress (Yu et al., 1997).

The iris is the anterior portion of the uvea. Iris blood flow has been measured by radioactive labelled microspheres (Alm and Bill, 1972; Alm and Bill, 1973; Duijm et al., 1996) and laser Doppler flowmetry (Chamot et al., 2000). Iris blood flow can be modulated by ocular perfusion pressure and drugs (Cole and Rumble, 1970; Kogure, 1982; Nagasubramanian, 1975). Alm and Bill (Alm and Bill, 1972) reported a study where flow was determined in iris, other intraocular tissues and brain of the cat using radioactively labelled microspheres . Their data demonstrated a much higher blood flow in the iris (97 g/min per 100 g tissue) than in the retina (19 g/min per 100 g tissue), the optic nerve (14 g/min per 100 g tissue) and the

brain (34 g/min per 100 g tissue). Our data strongly support a high blood flow rate in the iris with the high density of vasculature in the iris that has a prescribed pattern of the vascular distribution. The six orders of arterioles extend from a cluster of large vessels in the middle of the iris stroma. The long spindle shaped endothelium in these numerous larger vessels are supportive evidence of a high flow volume and flow rate. These large vessels in mid-stroma are able to sustain 2 to 3 orders of smaller branches within short distances to support the microvascular beds at the pupil edge as well as at the superficial and deep stroma. The presence of large and less tortuous venous vessels is also supportive as the receiving end of a high flow volume.

The limitation of this study is our data from porcine eyes. Ideally, data should be obtained using human donor eyes. However, human iris blood is supplied by not only LPCAs but seven anterior ciliary arteries which are always left as stubs embedded in extraocular muscles after enucleation, so that perfusate often cannot effectively be directed through the iris vasculature because of leakage from the anterior ciliary arteries. 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. The rationale of using porcine eye as a model was based on the structural similarities between porcine and human eye (Pond and Houpt, 1978; Prince et al., 1961). The structure of the porcine retina demonstrates a typical primate-like architecture (Beauchemin, 1974; Riva et al., 1986). The overall arrangement of iris vascular layers with the larger arteries and veins sandwiched between a superficial and a deep compact capillary plexus also concurs with previous finding using corrosion cast technique on porcine and primate eyes (Funk and Rohen, 1990; Rohen and Funk, 1994; Simoens, 1985). Similarly as reported in primate eyes (Rohen and Funk, 1994), the large porcine iris arteries were more tortuous whereas the veins tend to be straighter. However, previous studies were not able to provide the detailed morphological information at cell level

as presented in this study due to technical limitations. We have the added advantage in using the porcine eyes with the availability to choose eyes with lightly pigmented iris to enable detail study at a cellular level.

In summary, the distribution and endothelium of the iris microvasculature have been quantitatively assessed in this study. Combining information gained from this and future studies with unique structural features of the iris, such as the absence of endothelium in the surface and sponge-like stroma, may help us to further understand the physiological and pathogenic roles of the iris in relevant ocular diseases.

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Legends

Figure 1: Haematoxylin and eosin stained histology section of a porcine iris.

The iris as a diaphragm is bathed by aqueous humor separating the anterior and posterior chambers of the eye. The two magnified inserts (A and B) correspond with outlined regions shown in the upper picture. “A” showed the most anterior border composed of a relatively dense meshwork of melanocytes and fibroblasts with associated collagen. Note that this meshwork of cells does not form a continuous sheet that overlies the anterior iris surface (arrowheads). The small arrows indicate small vessels and larger vessels are indicated by larger arrows. In figure B, small blood vessels and capillaries were located anterior to a continuous pigment epithelium (arrowheads). Smaller vessels and capillaries (smaller arrows) were close to the pigment epithelium while larger blood vessels (larger arrows) were located in middle stroma. Scale bar = 500 μm in the upper image, and 333 μm in A and B.

Figure 2: Light microscope images of iris vascular network.

Low magnification images were taken from temporal side of iris vasculature illustrating the distribution of the iris vascular network. “A” shows iris vasculature of the middle iris region and “B to D” was taken from the outlined region in “A”. Three layers of iris vasculature were identified. B, C and D showed the light microscopic image of the superficial capillary, middle vascular and deep capillary layers in this iris respectively, with the corresponding layer in focus and outlined in colour. B’, C’ and D’ show the schematic drawing of the corresponding layers in solid colour. In “B’”, red indicates arterioles and blue indicates venules. Scale bar equals 800 μm in A and 400 μm in B-D.

Figure 3: Endothelial cell morphology in large iris arteries.

Light micrographs showing borders of silver-stained endothelial cells in large iris arteries.

The left and middle panels show images of 4th (C, C'), 5th (B, B') and 6th (A, A') order arteries. Red arrow heads in the left panels point to smooth muscle cell border labelled with silver nitrate in each order of artery. Pink dash lines in the middle panel outlined a few of the endothelial cells in each vessel order. The schematic drawings on the right panel are a replica of the outlined endothelial cells shown in solid black lines. Scale bar = 50 μm

Figure 4: Endothelial cell morphology in small iris arteries.

The left and middle panels show images of 3rd (A, A'), 2nd (B, B'), and 1st (C, C') order arteries. Red arrow heads in the left panel point to smooth muscle cell border labelled with silver nitrate in each order of artery. Pink dash lines in the middle panel outlined a few of the endothelial cells in each vessel order. The schematic drawings on the right panel are a replica of the outlined endothelial cells shown in solid black lines. Scale bar = 50 μm in the 3rd and 2nd artery order images (A, A', B and B'). Scale bar = 25 μm in the 1st order images (C and C').

Figure 5: Endothelial cell morphology in an iris capillary.

The left panel show images of iris capillary. Pink dash lines in the middle panel outlined a few of the endothelial cells in iris capillaries. The schematic drawings on the right panel are replica of the outlined endothelial cells shown in solid black lines. Scale bar = 50 μm .

Figure 6: Endothelial cells in iris veins.

“A” to “D” in the left panels presented images of 1st to 4th order of iris veins respectively.

Individual endothelial cells have been outlined in the middle panel in pink dashed lines (A' to

D'). Red arrow heads indicated the nuclei which were spared by the silver chloride labelling.

Right panel shows schematics of the border of the endothelial cells. Scale bar = 50 μm .

Table 1: Iris vessel diameter according to vessel order

Vessel Order	Vessel Diameter (μm)
C	15.58 $\pm$ 1.47(11)
A1	28.05 $\pm$ 2.32(11)
A2	49.31 $\pm$ 4.54(11)
A3	83.72 $\pm$ 5.63(11)
A4	132.64 $\pm$ 12.94(11)
A5	225.40 $\pm$ 15.26(11)
A6	319.48 $\pm$ 25.65(11)
V1	36.95 $\pm$ 2.92(11)
V2	74.47 $\pm$ 6.58(11)
V3	114.69 $\pm$ 5.36(11)
V4	160.85 $\pm$ 14.00(11)

*Values are mean $\pm$ standard deviation (number of eyes measured)

Table 2: Endothelial cell dimensions according to vessel order

Vessel Order	Endothelial Length (μm)	Endothelial Width (μm)
C	81.35 $\pm$ 6.88(11)	10.04 $\pm$ 0.87(11)
A1	87.36 $\pm$ 6.17(11)	9.69 $\pm$ 0.93(11)
A2	100.59 $\pm$ 8.82(11)	9.91 $\pm$ 0.91(11)
A3	104.72 $\pm$ 7.76(11)	10.14 $\pm$ 0.95(11)
A4	109.54 $\pm$ 7.53(11)	9.38 $\pm$ 0.52(11)
A5	110.41 $\pm$ 8.28(11)	8.10 $\pm$ 0.69(11)
A6	113.54 $\pm$ 10.04(11)	8.45 $\pm$ 0.81(11)
V1	49.13 $\pm$ 4.82(11)	12.01 $\pm$ 0.76(11)
V2	49.91 $\pm$ 4.52(11)	12.53 $\pm$ 1.20(11)
V3	52.74 $\pm$ 3.33(11)	11.00 $\pm$ 1.07(11)
V4	58.70 $\pm$ 3.13(11)	9.82 $\pm$ 0.90(11)

*Values are mean $\pm$ standard deviation (number of eyes measured)

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6