QUANTITATIVE STUDY OF AGE-RELATED ENDOTHELIAL PHENOTYPE CHANGE IN THE HUMAN VORTEX VEIN SYSTEM

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Abbreviations: ChV = choroidal veins, PA = pre-ampulla, AM = ampulla, PtA = post-ampulla, SE = scleral entrance, IC = intra-scleral channel, ScEx = scleral exit, VX = extra-scleral vortex vein, DSP = Downstream position; ECL = Endothelial Cell Length; ECW = Endothelial Cell Width; ECA = Endothelial Cell Area; AR= Aspect Ratio; ECNL = Endothelial Cell Nuclei Length; ECNW = Endothelial Cell Nuclei Width; EC Perim. = Endothelial Cell Perimeter

Abstract

Purpose: We have previously reported significant phenotype heterogeneity in the vortex vein system. This study is to quantify the age-related change of such endothelial phenotype heterogeneity.

Method: The inferior temporal vortex vein system from 10 eyes from 7 young donors (30 ± 4.1 years) and 9 eyes from 6 aged (72 ± 4.7 years) donors were dissected after perfusion fixation and labeled for f-actin and nucleic acid. Confocal images of endothelial cells were obtained from nine anatomic regions and measurements made of the cell and nuclei sizes. The results were compared between the two age groups.

Results: Similar regional endothelial heterogeneity was observed in both age groups through the different regions of the vortex vein system. Age-related increase in endothelial cell area was observed in all the study regions. Age-associated regional differences were also observed in the endothelial length, width, and nuclei parameters. Endothelial nuclei were also found to be located further downstream within the cell in aged donor eyes.

Conclusion: Age related enlarged endothelial cells have been identified in this venous system, a likely indicator of senescence. The relationship between the endothelial senescence, regional endothelial phenotype change and endothelial dysfunction in possible pathological changes needs to be further defined.

1 Introduction

2 Vascular endothelial cells are the specialized cells at the interface between the blood and
3 smooth muscle cells or underlying tissues. It is well recognized that endothelial cells are
4 actively involved in many aspects of vascular functions like the control of vessel activity,
5 blood clotting, inflammation and the formation of new blood vessels as well as functioning as
6 gatekeepers, controlling the passage of substances and cells into and out of the bloodstream.
7 Therefore, understanding the function of vascular endothelial cells is of significance in health
8 and disease (Abraham and Dashwood 2008; Aird 2012; Chang et al. 2005; Cines et al. 1998).

9 The endothelial cells have a very slow turnover rate and limited ability to proliferate. The
10 cells eventually enter a state of irreversible growth arrest known as senescence (Brandes et al.
11 2005; Hayflick and Moorhead 1961). Recently, the molecular mechanisms involved in
12 cellular senescence have been studied and altered gene transcription has been identified
13 (Brandes et al. 2005; Erusalimsky and Kurz 2006). Senescent cells, including endothelial
14 cells, possess some characteristic morphological and functional changes such as enlarged cell
15 body and impaired endothelium-dependent vasodilator response (Burrig 1991; Thorin 2011;
16 Thorin and Thorin-Trescases 2009). The majority of studies on the effect of aging on
17 vascular endothelial cells have been conducted on endothelial cell cultures, with very few
18 studies conducted in vivo (Cavallaro et al. 2000; Erusalimsky and Kurz 2006).

19 In man, the vortex veins are the only drainage pathway for the choroidal circulation. We have
20 recently reported phenotypically heterogeneous distribution of the vein endothelial cells
21 through the different regions of the young porcine and aged human vortex vein system (Tan
22 et al. 2013; Yu et al. 2013).

Aging is considered to be one of the major risk factors for many eye diseases such as glaucoma, age-related macular degeneration and vascular occlusive diseases including central and branch retinal vein occlusion (Cheung et al. 2008; Ehrlich et al. 2008; Ehrlich et al. 2009; Ehrlich et al. 2010; Kang et al. 2011; Kang et al. 2013; Voleti and Hubschman 2013; Yu et al. 2012). The effects of aging on ocular blood flow have also been reported (Dallinger et al. 1998; Grunwald et al. 1993; Straubhaar et al. 2000). However, we have only limited knowledge of ocular senescent endothelium and its role in vascular function and pathology. The current study compares the morphologic parameters of the vortex vein system endothelium obtained from young and aged human donor eyes to look for age-related effects on the morphology of the endothelium.

Material and methods

This study was approved by the human research ethics committee at The University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

Human Donor Eyes

A total of 19 eyes from 13 donors were studied with 10 eyes from 7 young donors and 9 eyes from 6 aged donors. All the eyes were perfusion fixed prior to dissection and staining. Data from 6 of the perfusion fixed eyes used previously (Yu et al. 2013) have been pooled with data from another 3 eyes in the aged group for this comparative study. All eyes were obtained from the Lions Eye Bank of Western Australia or Donate West, the West Australian agency for organ donation. We received the eye bank eyes after removal of corneal buttons for transplantation. None of the eyes used in the present study had a known history of eye disease. Details of the age, sex, and cause of death of each donor are listed in Table 1, along with the post-mortem time to enucleation and post-mortem time to fixation.

Tissue Fixation, Dissection and Labelling

All donor eyes were perfusion-fixed through the temporal long posterior ciliary artery as previously described (Yu et al. 2010a; Yu et al. 2010b; Yu et al. 2013) and the posterior globe further post-fixed by immersion. The inferior temporal vortex vein system was identified and dissected out for float labeling as in previous study of aged donor eyes (Yu et al. 2013).

Regions of Study

The vortex vein system from the inferior temporal region of human donor eyes was examined in detail. The vortex vein system may be roughly subdivided into three sections – intra-orbital, scleral and extra-orbital. The intra-orbital encompasses the draining choroidal veins which merge to form the pre-ampullas. A few pre-ampullas merge to form a single short (approximately 1mm) and slightly enlarged vestibule called the ampulla. The posterior part of the ampulla then enters the relatively rigid sclera (start of the scleral section of the system) through a foramen, and the vessel continues its travel between sheets of collagen fiber in the intra-scleral channel until it emerges extra-orbitally as one of the vortex veins. The different regions studied included the choroidal veins (ChV), pre-ampulla (PA), ampulla (AM), post-ampulla (PtA), scleral entrance (SE), intra-scleral channel (IC), scleral exit (ScEx) and extra-scleral vortex vein (VX). As far as possible, the VX, ScEx and IC regions were bisected opened to allow easy access of fluorescent probes to the endothelial cells. The endothelial cells in the vessel wall were studied for f-actin and nucleic acid distribution, and the morphology of the cells.

Confocal Imaging

Confocal imaging was performed on a Nikon C1 systems equipped with 4 (408 nm, 488 nm, 546 nm and 635 nm) laser board, coupled with Nikon i90 microscope. Images were collected

from the different regions of the outflow pathway using x20 dry or x40 oil Plan Apo Objective lenses.

Image Analysis

Confocal image sequences from the various regions were examined closely for the morphology of the endothelium. Endothelial cells and their respective nuclei were outlined using the polygon tool in Image J (1.42q, National Institute of Health, USA) and the perimeter, major and minor axis of a fit ellipse measured. The major and minor axis were inferred to be the length and width of the structure measured. The aspect ratio of each cell and their respective nuclei were computed according to the ratio between the major and minor axis of the same structure. The parameters measured included endothelial cell length (ECL), endothelial cell width (ECW), endothelial cell area (ECA), endothelial cell perimeter (EC Perim.), length of endothelial nucleus (ECNL), width of the endothelial nucleus (ECNW), endothelial nuclei area (ECNA) and the aspect ratios (AR) of cell and nuclei. The distance of the centre of the endothelial nucleus from the upstream pole of the endothelium was also measured and expressed as percentage of the total cell length as measured from the upstream pole (DSP = downstream position).

Statistical Analysis

All statistical testing was performed using SigmaStat (ver. 3.1; SPSS, Chicago, IL). We tested for regional significant differences in the parameters studied between the age groups using simple t-tests. One-way ANOVA was used to test the effect of age on various endothelial parameters measured. One-way ANOVA was also used to test the effect of region on various endothelial cell parameters. Two-way ANOVA was used to test the combined effect of region and age on the parameters measured.

Results

Donor Demographics

The average age of donors in the two groups were 30 ± 4.1 ($n = 7$) and 72 ± 4.7 ($n = 6$) years, respectively. The average time to perfusion was 20 ± 2.4 hours for the younger donors, and 15 ± 2.7 hours for the aged donors; and the average time to enucleation was 13 ± 2.8 hours for the younger donors and 9 ± 1.7 hours for the aged donors. Details of the donors are shown in Table 1.

Study Regions

Figure 1 displays the study regions of the vortex vein system as previously described.(Yu et al. 2013)

Figures 2 to 4 display the typical appearance of endothelial cells in the study regions in the young and aged human vortex vein system. Overall, the cell borders were clearly visible, as were the nuclei. There was a gradual thinning and elongated appearance of the endothelial cells in the choroidal regions of ChV to AM (Figure 2). In the transit regions of PtA to IC1 (Figure 3), the endothelial cells not only became slimmer, but also shorter. The endothelial cells in the last three regions (Figure 4) of the system were significantly shorter than the IC1 region (Figure 5).

Endothelia in Young Vortex Vein System

The different endothelial cell shapes in the study regions of the young vortex vein system follows a similar pattern as previously reported in the aged vortex vein system.(Yu et al. 2013) There was a significant (t-test, $p < 0.001$) but gradual increase in endothelial cell length (mean values of 52.9, 60.1 and 64.0 μm) accompanied by a corresponding reduction in cell

width (mean values of 18.2, 16.6 and 14.3 μm respectively) from the choroidal vein (ChV) through the pre-ampulla (Pre-AM) into the ampulla (AM) regions. As previously reported in the aged vortex vein system, the young vortex vein system also saw a sharp increase in cell length ($82.0 \pm 1.80 \mu\text{m}$, t-test ChV $p < 0.001$) and reduction in cell width ($10.9 \pm 0.26 \mu\text{m}$, $p < 0.001$) in the post-ampulla (Post-AM or PtA) region. There was a significant reduction in cell length ($66.8 \pm 1.66 \mu\text{m}$, $p < 0.001$) at the scleral entrance (SE) accompanied by a significant ($p < 0.001$ compared with ChV) reduction in cell area (ECA). In the first half of the intrascleral canal (IC1), endothelial cells were significantly elongated ($71.6 \pm 1.22 \mu\text{m}$, $p = 0.036$) than at SE. However, there is a significant drop in endothelial cell length ($46.2 \pm 1.94 \mu\text{m}$, $p < 0.001$) in the second half of the intrascleral canal (IC2) accompanied by a significant reduction ($p < 0.001$) in ECA. The shorter endothelial length persisted through the scleral exit (ScEx) and into the extra-orbital vortex vein regions (VX) where cells measured an average length of 44.0 μm and 37.3 μm respectively.

Endothelial nuclei followed a similar pattern of elongation in the cell with increase in nuclei length from the ChV downstream to the PtA regions. This is clearly reflected in the graphs showing significant changes in nuclei length (Figure 6), as well as aspect ratios of the endothelial cell and nuclei (Figure 7) through the study regions. The nuclei were the most elongated in the posterior ampulla (PtA) region and then became significantly shorter ($p < 0.001$) further downstream. A corresponding and significant ($p < 0.02$) drop in nuclei area was observed in step with the drop in cell area (Figures 5 & 6) from the scleral exit to the extra-orbital vortex vein regions.

Age-related Endothelial Cell and Nuclei Shape Difference

Significant differences have been noted between the age groups. Notably, the aged endothelia were significantly larger in cell area in all regions studied (Figure 5). Regional and aged-related differences were also observed.

Regional and age-related increase in cell area, length and width

Images and measurements obtained from the young vortex vein system were compared against those from the aged vortex vein system. Whilst the pattern of phenotypic differences was similar in the different study regions of the two groups, significant differences (two-way ANOVA p value < 0.001) in cell and nuclei parameters are also found between the age groups in specific regions; as can be seen from the confocal images (Figures 2 to 4) of the endothelium, as well as the measurements displayed in graph (Figures 5, 6 and 7) and table forms (Tables 2.1, 2.2). The venous endothelial cells from the aged donor eyes were significantly larger in cell area (Figures 2, 3, 4, 5-ECA) either as a result of a significant increase in cell width (Figure 5-ECW, ChV to SE and ScEx regions) or cell length (Figure 5-ECL, from SE to VX regions) compared to the endothelia from younger donor eyes. Significant increase in the cell perimeter as reflected by f-actin labeling was also observed at the ChV, and from the SE to VX regions.

The choroidal regions of ChV and PA (Figure 2, 5) saw a significant increase in cell width and area without accompanying increase in cell length. This is reflected in the significant reduction in endothelial cell aspect ratio at these two regions (Figure 7). The nuclei area also did not differ significantly for ChV and PA between the two age groups, although significant reduction in nuclei length and increase in nuclei width were noted (Figure 6, 7). The ampulla (AM) region endothelia showed the least differences between the two age groups except for the significant age-related increase in cell width and area (Figures 5, 6 & 7).

The PtA, SE and IC1 formed the transition regions at the choroidal and scleral parts of the vortex system. The PtA is similar to the AM, showing significant increase only in cell area and width. The SE region found larger cells with smaller nuclei in the aged eyes. The endothelial cells were significantly longer and wider, as reflected by the significantly larger cells and increased cell perimeter (Figure 5). However, the aspect ratio of the cells did not change in the SE region (Figure 7). The SE region also showed a reduction in nuclei length, width and area accompanied by a more downstream nuclei location within the cell (Figure 6).

The first part of the intra-scleral channel (IC1) noted a significant age-related increase in cell length, perimeter and area (Figures 3 & 5). However, no significant increase in cell width was observed in the aged IC1. Nor was significant age-related differences noted for the IC1 nuclei (Figure 6). However, the nuclei were located more downstream in the aged-IC1 than young-IC1 (Figure 6-DSP). The second half of IC2 found significantly elongated cells (Figure 5-ECL) in the aged eyes. This is accompanied with a significant increase in nuclei length (Figure 6-ECNL) and a significant reduction in nuclei width (Figure 6-ECNW). Both the cell and nuclei area were significantly greater in this region of the aged eyes. The DSP of nuclei remained significantly greater in the aged eyes in the IC2 region (Figure 6-DSP).

At the exit from the sclera (ScEx), the aged endothelia continued to be significantly longer, wider, larger and with a larger perimeter than the younger endothelia (Figure 5). The nuclei remained more elongated but significantly broader than those in the younger eyes.

At the extra-orbital vortex vein region, the aged endothelia remained significantly more elongated and larger (Figure 5) than those from the young eyes. The aged-VX endothelial nuclei are also significantly more elongated and larger, and assumed a more downstream position when compared to the younger eyes (Figure 6).

Statistical Analysis of Morphometric measurements

The averaged morphometric data of endothelial cells from the nine study regions of the two age groups are presented in Tables 2.1, 2.2 as well as in Figures 5 and 6. One-way ANOVA ($p < 0.001$) confirmed the study region to be a significant factor in affecting the measurements obtained. It also confirmed age group to be a significant ($p < 0.001$) factor in affecting the measurements obtained; the only exception being ECNW where p value equals 0.482. Combining the two factors involved, two way ANOVA found a significant ($p < 0.001$) difference as a result of age as well as region on all parameters (ECL, ECW, ECNL, ECNW, ECA, ECNA, EC Perim., and DSP) measured. The differences as the result of study regions is significant ($p < 0.001$) for all parameters measured.

Discussions:

The present study identified an age-related and region-specific morphological change in the venous endothelia of the human vortex vein system.

As far as we know, age-related increase in venous endothelial cell area from in situ human donor tissues has not been reported before. Whilst site specific endothelial phenotypic heterogeneity have been described mainly in artery with relatively large caliber (Aird 2012) and correlated with changes in endothelium-dependent vasodilatation function, expression of oxidative stress marker, nitric oxide synthase level, pro- and anti-atherogenic markers (Simmons et al. 2012) and arteriosclerotic vulnerability; age-related or senescence induced change in vascular endothelium has been more controversial due to the perceived quiescent state of the endothelial cells. However, observations consistent with signs of senescence, such as increase in the frequency of tetrasomy correlated with telomere shortening have been demonstrated in the aortic endothelial nuclei (Aviv et al. 2001) obtained from human donor tissues and studied in situ. This is supported by previous reports of age-related increase in size of endothelial cell (Chang et al. 2005; Erusalimsky and Kurz 2006) from large vessels

1 such as the human umbilical vein and bovine aorta ~~from~~ in cell culture studies (Johnson and
2 Longenecker 1982). Increase in cell size was also observed after multiple passages of
3 microvascular endothelial cells. In vitro evidence points to age-dependent cell size increment
4 to be present both in vascular and microvascular endothelium. The significant difference in
5 cell area, length, width and nuclei parameters demonstrated in the current report supports the
6 presence of senescence in the microvascular endothelium of the vortex vein system. Our
7 previous report on the increase in endothelial length at aged retinal artery-vein crossing (Yu
8 et al. 2012) concur with the current observation of increase in cell parameters with aging.
9 The authors recognize the importance of associating functional change with the reported
10 morphological change and have attempted to label immunohistochemically for endothelial
11 junctional proteins such as Claudin-5 and VE-cadherin in relation to permeability change and
12 pro-inflammatory marker such as Interleukin-1 α . However, the data obtained was quite
13 variable and not conclusive. It is unclear whether the variable results are a consequence of the
14 vortex vein endothelium itself, individual variations, post-mortem time, aging or disease
15 process or other variables that exists in post-mortem human donor tissues.

16 Whereas the increase in cell area was found across the whole vortex vein system, significant
17 increase in length was found only from scleral entrance to extra-orbital vortex veins, regions
18 that are closely associated with the sclera. The presence of more elongated cells in these
19 regions of the aged donor eyes is suggestive of a higher shear stress based on cell culture
20 studies where cells tend to elongate after prolonged exposure to increased shear stress (Sato
21 and Ohashi 2005). It may be speculated that the demonstrated stiffening of sclera with aging
22 (Geraghty et al. 2012) may have increased the resistivity in the flow of the intrascleral
23 channel by being less compromising to the venous pressure. It is known that the pulsatile
24 ocular blood flow is also reduced with age (Lam et al. 2003), but no reduction to the
25 episcleral venous pressure has been detected (Toris et al. 1999), supporting the hypothesized

1 increase in resistivity of the intrascleral channel. The absence of significant elongation of
2 endothelia in the choroidal (ChV, PA, AM and PtA) parts of the vortex vein system is also
3 suggestive of a role for the aging sclera which the scleral part of endothelia are closely
4 associated with and their basement membrane also anchored onto. Perhaps the interplay of
5 all these factors contributed to the elongated appearance of the vascular endothelium within
6 the sclera.

7 Flattening of cell has been reported in various cell culture studies (Chang et al. 2005;
8 Erusalimsky and Kurz 2006; Johnson and Longenecker 1982; Pospelova et al. 2013) and is
9 consistent with our observations of increased cell width in the majority of regions.

10 Endothelial nuclei also showed corresponding elongation (IC2, ScEx and VX) and widening
11 (ChV, PA and ScEx) with increase in the two dimensional nuclei area measurements (IC2,
12 ScEx and VX) in the aged eyes. In particular, the nuclei position was noted to be more
13 downstream in the scleral part of the aged donor vortex vein system. From cell culture study,
14 the nuclei is said to be approximately 9 times stiffer (Maniotis et al. 1997) than the cytoplasm
15 of the endothelial cells. Another study reported the endothelial nucleus as an intracellular
16 stress-bearing organelle that remodels in response to increased shear stress (Deguchi et al.
17 2005; Flaherty et al. 1972). Perhaps it is the innate rigidity of the nuclei that has contributed
18 to the comparatively less morphological differences in the endothelial nuclei between the two
19 age groups. It is also known that the nuclei rigidity could be somewhat negated by
20 interfering with microfilaments, intermediate filaments or microtubules (Maniotis et al. 1997).
21 It may be speculated that the significant change in intracellular position of the nuclei could be
22 reflective of changes in the intracellular cytoskeleton arrangement in the aged endothelium.
23 Furthermore, the histones in the nucleosomes of cells are known to be altered by methylation
24 and demethylation as a chronic response to changes in microenvironment. More studies will
25 be required to examine the underlying cause and mechanisms involved.

1 In conclusion, whilst we have reported an increase in cell size, changes in nuclei parameters
2 and the more downstream location of the nuclei to be a possible indicator of endothelial
3 senescence, further studies are required to determine how this phenotypic change may have
4 contributed to the decrease in ocular blood flow reported in aged eyes. The mechanobiology
5 behind the phenotype heterogeneity in relation to fluid mechanics remains to be clarified
6 (Dahl et al. 2010). The genetic, molecular level and any epigenetic changes that accompany
7 the phenotypic change will need to be characterized to further our understanding of the
8 microvascular endothelia such as that of the vortex vein system in normal physiological,
9 aging and pathophysiological conditions. To identify site-specific age-related changes of
10 endothelium and their molecular mechanisms involved may potentially help to develop some
11 possible interventions for the retinal vascular diseases.

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Legends

Figure 1. Fluorescent, photographic images and schematic drawings of the study regions (ChV – Draining veins; PA – Pre-ampulla; AM – Ampulla; PtA – Post-ampulla; SE – Scleral Entrance; IC1 – Intrasccleral channel first half; IC2 – intrasccleral channel second half; ScEx – Scleral Exit; and VX – Extra-orbital vortex vein) within the human vortex vein system. Schematic drawings of regions in (B) and (E) correspond with areas labeled on respective photos (A) and (D). (A) Low magnification fluorescent image of the inferior temporal drainage area from the choroidal perspective. The ChV and PA are brightly fluorescent after phalloidin labeling. The AM is less brightly labeled due to the relative absence of smooth muscle cells. The intra-orbital ChV, PA, AM and PtA regions are lightly colored and displayed schematically in solid colors in (B). (C) Low magnification photo of the scleral and extra-orbital portion of the vortex vein system. Dashed line outline the vessel embedded within sclera. (D) Low magnification of the same vessel in (C) after dissecting free from the sclera. The SE, IC1, IC2, ScEx and VX are clearly visible. Blood cells were often seen to be retained in the VX region but would clear after micro-bisection. (E) Schematic drawing of intra-scleral and extra-orbital regions present in (D).

Figure 2. Confocal images of endothelia sampled from the choroidal draining veins (left panel), pre-ampulla (middle panel), and ampulla (right panel) regions of eyes from young (top row) and aged (bottom row) donor eyes. The images have been pseudo-colored with red representing the f-actin location, and green for nucleic acid. Orange and white dotted lines on the images outlined the endothelial cell and nuclei shape respectively. The outlines are repeated on the right hand side of each image in black for ease of visibility. Endothelia in most of the study regions of aged donor eyes were wider than those from the same regions of

1 young donor eyes. Scale bar in the right bottom image measures 20 μm and is applicable to
2 all images in this figure.

3 Figure 3. Confocal images of endothelia sampled from the post-ampulla (left panel), scleral
4 entrance (middle panel), and first half of the intrascleral canal (right side panel) regions of
5 eyes from young (top row) and aged (bottom row) donor eyes. The images have been
6 pseudo-colored with red representing the f-actin location, and green for nucleic acid. Orange
7 and white dotted lines on the images outlined the endothelial cell and nuclei shape
8 respectively. The outlines are repeated on the right hand side of each image in black for ease
9 of visibility. Endothelial from aged donor eyes appeared to be broader than those in the
10 younger donor eyes. Endothelia in the scleral entrance and first half of intrascleral canal of
11 aged donor eyes were also longer than those from the same regions of young donor eyes.
12 Scale bar in right bottom measures 20 μm and is applicable to all images in this figure.

13 Figure 4. Confocal images of endothelia sampled from the second half of the intrascleral
14 canal (left panel), scleral exit (middle panel), and extra-orbital vortex vein (right panel)
15 regions of eyes from young (top row) and aged (bottom row) donor eyes. The images have
16 been pseudo-colored with red representing the f-actin location, and green for nucleic acid.
17 Orange and white dotted lines on the images outlined the endothelial cell and nuclei shape
18 respectively. The outlines are repeated on the right hand side of each image in black for ease
19 of visibility. Endothelial from aged donor eyes were longer and broader than those in the
20 younger donor eyes. Scale bar in bottom right image measures 20 μm and is applicable to all
21 images in this figure.

22 Figure 5. Morphometric data (endothelial cell length - ECL, width - ECW, area - ECA and
23 perimeter – EC Perim.) in the different regions of the vortex vein system. Solid circles
24 represent data from young donor eyes, open circles represent data from aged donor eyes.

Standard errors are displayed. Two-ways ANOVA on the effect of age and study regions showed statistical significance ($p < 0.001$) between the two age groups for all four parameters displayed. “*” and “†” indicate significant difference ($p < 0.009$ and $p < 0.05$ respectively) between the two age groups at specific regions.

Figure 6. Morphometric data (endothelial nuclei length - ECNL, width - ECNW, area - ECNA and downstream position - DSP) in the different regions of the vortex vein system. Solid circles represent data from young donor eyes, open circles represent data from aged donor eyes. Standard errors are displayed. Two-ways ANOVA on the effect of age and study regions showed statistical significance ($p < 0.001$) between the two age groups in all parameters displayed. “*” indicates significant ($p < 0.006$) regional differences between the two age groups. “†” indicates significant ($p < 0.05$) regional differences between the two age groups.

Figure 7. The length to width aspect ratios of endothelial cells and nuclei in the study regions. “*” indicates significant difference ($p < 0.006$) between the two age groups at specific regions.

1 Tables

2 Table 1. Donor eyes details. Double line separates the two age groups.

3

Donor Eye ID	Age	Sex	Cause of Death	Time to enucleation	Time to perfusion
A	15	M	Suicide	10 h	22.5 h
B	15	M	Suicide	10 h	22.5 h
C	30	M	Motor Vehicle Accident	22 h	24 h
D	40	M	Cardiac arrest	8 h	21 h
E	18	F	Cardiac arrest	16 h	20 h
F	18	F	Cardiac arrest	16 h	20 h
G	36	M	Cardiac arrest	21.5 h	23 h
H	36	M	Cardiac arrest	21.5 h	23 h
I	33	F	Suicide	4 h	7 h
J	39	M	Respiratory failure	12 h	20 h
K	71	M	Motor Vehicle Accident	11 h	15 h
L	71	M	Motor Vehicle Accident	11 h	15 h
M	82	M	Cardiac arrest	12 h	15 h
N	71	M	Acute abdominal aortic aneurysm	4 h	17 h
O	71	M	Acute abdominal aortic aneurysm	4 h	17 h
P	84	F	Cerebral Vascular Accident	4.5 h	6.5 h
Q	64	M	Motor Vehicle Accident	12.5 h	25 h
R	64	M	Motor Vehicle Accident	12.5 h	25 h
S	56	F	Sepsis	10.5 h	13 h

4

1 Table 2.1. Morphometric data from Young donor eyes. Mean $\pm$ S.E. has been displayed.

2

Region	ECL (μm)	ECW (μm)	ECNL (μm)	ECNW (μm)
ChV	52.9 $\pm$ 1.47, 120	18.2 $\pm$ 0.46, 120	15.9 $\pm$ 0.25, 120	8.4 $\pm$ 0.11, 120
Pre-AM	60.1 $\pm$ 2.18, 94	16.6 $\pm$ 0.57, 94	16.9 $\pm$ 0.27, 94	8.5 $\pm$ 0.14, 94
AM	64.0 $\pm$ 1.83, 136	14.3 $\pm$ 0.39, 136	17.7 $\pm$ 0.18, 136	8.2 $\pm$ 0.11, 136
Post-AM	82.0 $\pm$ 1.80, 88	10.9 $\pm$ 0.26, 88	18.3 $\pm$ 0.28, 88	7.7 $\pm$ 0.14, 88
SE	66.8 $\pm$ 1.66, 211	10.7 $\pm$ 0.19, 211	17.7 $\pm$ 0.23, 211	7.5 $\pm$ 0.10, 211
IC1	71.6 $\pm$ 1.22, 187	11.7 $\pm$ 0.32, 187	17.3 $\pm$ 0.21, 187	7.9 $\pm$ 0.13, 187
IC2	46.2 $\pm$ 1.94, 93	15.5 $\pm$ 0.53, 93	14.2 $\pm$ 0.34, 93	8.8 $\pm$ 0.14, 93
ScEx	44.0 $\pm$ 1.96, 62	14.0 $\pm$ 0.63, 62	14.8 $\pm$ 0.55, 62	7.9 $\pm$ 0.23, 62
VX	37.3 $\pm$ 0.70, 138	17.4 $\pm$ 0.33, 138	12.5 $\pm$ 0.14, 138	8.9 $\pm$ 0.10, 138

3 Table 2.2. Morphometric data from Aged donor eyes. Mean $\pm$ S.E. has been displayed.

Region	ECL (μm)	ECW (μm)	ECNL (μm)	ECNW (μm)
ChV	53.6 $\pm$ 1.13, 173	24.0 $\pm$ 0.39, 173	14.8 $\pm$ 0.17, 173	9.1 $\pm$ 0.11, 173
Pre-AM	58.8 $\pm$ 1.52, 137	20.3 $\pm$ 0.41, 137	15.6 $\pm$ 0.21, 137	8.9 $\pm$ 0.14, 137
AM	62.7 $\pm$ 1.10, 170	16.2 $\pm$ 0.43, 170	17.4 $\pm$ 0.22, 172	8.2 $\pm$ 0.14, 172
Post-AM	81.4 $\pm$ 2.12, 73	12.8 $\pm$ 0.39, 73	18.2 $\pm$ 0.33, 73	7.6 $\pm$ 0.15, 73
SE	73.9 $\pm$ 1.51, 196	12.2 $\pm$ 0.34, 196	17.3 $\pm$ 0.20, 196	7.3 $\pm$ 0.13, 196
IC1	81.0 $\pm$ 2.43, 72	11.8 $\pm$ 0.44, 72	17.3 $\pm$ 0.29, 72	7.7 $\pm$ 0.22, 72
IC2	66.5 $\pm$ 1.62, 100	16.8 $\pm$ 1.10, 100	17.5 $\pm$ 0.23, 100	7.8 $\pm$ 0.15, 100
ScEx	55.6 $\pm$ 1.79, 78	16.4 $\pm$ 0.62, 78	15.9 $\pm$ 0.34, 78	8.6 $\pm$ 0.16, 78
VX	40.5 $\pm$ 1.07, 43	18.3 $\pm$ 0.62, 43	14.4 $\pm$ 0.39, 43	9.0 $\pm$ 0.26, 43

4 ECL = Endothelial Cell Length; ECW = Endothelial Cell Width; ECNL = Endothelial Cell
5 Nuclei Length; ECNW = Endothelial Cell Nuclei Width; ChV = Choroidal Vein; Pre-AM =
6 Pre-ampulla; AM = Ampulla; Post-AM = Posterior Ampulla; SE = Scleral Entry; IC =
7 Intrasceral Channel; ScEx = Scleral Exit; VX = Extra-ocular Vortex Vein

8

1 Figures

Figure 1

A. Young

B. Aged

1

2

Figure 2

A. Young

B. Aged

Figure 3

A. Young

B. Aged

1

2 Figure 4

Figure 5

1

2

3 Figure 6

1
2 Figure 7
3