



# Endothelial glycocalyx layer in the aqueous outflow pathway of bovine and human eyes



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## ABSTRACT

The glycocalyx layer on the vascular endothelium is known to have an important role as a transport barrier and in the mechanotransduction of fluid shear stress. The detailed structure and distribution of the glycocalyx in the bovine and human aqueous humor outflow pathways has not yet been reported. The purpose of this study was to determine whether this layer exists in the bovine and human aqueous outflow pathways and to compare the distribution and thickness therein. Enucleated bovine ( $N = 4$ ) and human ( $N = 4$ ) eyes were fixed using Alcian Blue to preserve the glycocalyx. The glycocalyx distribution and thickness (in regions where it was seen) were measured on the trabecular beams (TM), Schlemm's canal (SC)/aqueous plexus (AP), and collector channels (CC). The glycocalyx, which appears as a layer of hair-like brushes, coats the surface of the endothelium non-uniformly in the bovine and human aqueous outflow pathways with a thickness in bovine eyes of 68–122 nm and in human eyes of 52–166 nm (25th to 75th percentiles). The distribution of the glycocalyx in different regions of the outflow pathway is not the same between bovine and human eyes. In both species, the glycocalyx was most uniform in the CCs. Less coverage of glycocalyx was found in the AP than the TM in bovine eyes, while more coverage was found in SC than the TM in human eyes. Most interestingly, glycocalyx was also found filling most pores of the endothelium of AP/SC in both bovine and human eyes. Glycocalyx was usually not found coating the inner membranes of the giant vacuoles (GVs); however, in GV's with a visible pore, glycocalyx was frequently observed on the inner membranes of the GV's. Based on our findings and those from the vascular endothelium, it is likely that the glycocalyx in SC plays a role in transduction of shear stress and perhaps regulation of outflow resistance.

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## 1. Introduction

It is well-known that flow-induced shear stress acting on vascular endothelial cells leads to cell elongation and cell alignment in the direction of flow (Dewey, 1983; 1984; Remuzzi et al., 1984). Surprisingly, even the relatively low shear stress in Schlemm's canal (SC) leads their endothelial cells to align with the flow (Ethier et al., 2004). This alignment is mediated by the glycocalyx and “the presence of the glycocalyx is necessary for the endothelial cells to respond to fluid shear,” (Yao et al., 2007).

The glycocalyx also plays an important role in the mechanotransduction of fluid shear stress (Tarbell and Ebong, 2008;

Tarbell, 2010; Weinbaum et al., 2007; Pries et al., 2000), particularly as related to activation of endothelial nitric oxide synthase (eNOS) and subsequent nitric oxide (NO) release. The latter mediates flow-induced vasodilation in vascular endothelium. Modifications to the glycocalyx including removal of heparan sulfate, hyaluronic acid, or sialic acid abolishes the release of nitric oxide and consequent flow induced vasodilation (Tarbell and Ebong, 2008). In addition, transgenic mice overexpressing eNOS have lower IOP and aqueous outflow resistance (Stamer et al., 2011).

It is thus of interest to characterize the morphological structure of the glycocalyx in the aqueous humor outflow pathway. In this study, we preserved this labile layer using Alcian Blue 8GX, and then examined its thickness and distribution in the aqueous outflow pathway of both bovine and human eyes. We found this layer to be irregular in both the bovine and human outflow pathways with significant differences between the two species.

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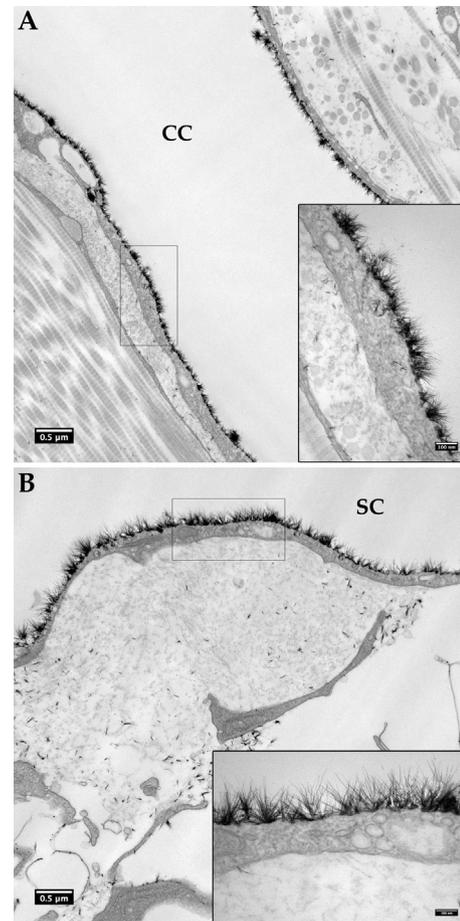
## 2. Materials and methods

Enucleated bovine eyes ( $N = 4$ ) were obtained from a local abattoir and delivered on ice within 6 h post-mortem. Enucleated human eyes ( $N = 4$ ) from anonymous donors without any known history of ocular diseases were obtained from National Disease Research Interchange (Philadelphia, PA) within 24 h post-mortem. The bovine eyes were either perfusion-fixed ( $N = 2$ ) or immersion-fixed ( $N = 2$ ), while the human eyes were perfusion-fixed. Perfusion-fixed eyes were perfused at 15 mmHg with Dulbecco's phosphate-buffered saline (DPBS) with 5.5 mM of  $D$ -glucose for at least 30 min at 34 °C, the anterior chambers exchanged with 1% glutaraldehyde and 4% paraformaldehyde in DPBS containing 30 mmol/L  $MgCl_2$  and 0.05% (w/v) Alcian Blue 8GX, and then perfused with the same solution for an additional hour. The eye was then hemisected and immersion-fixed in the same solution overnight. Immersion-fixed eyes were dissected to remove the lens and vitreous humor before immersing in the same fixation solution as perfusion-fixed eyes for at least 12 h. All eyes were dissected into small tissue wedges and post-fixed in 1% aqueous osmium tetroxide and 1% lanthanum nitrate hexahydrate for 2 h at room temperature, followed by en-bloc staining with uranyl acetate, dehydration in ascending series of ethanol, then replaced with propylene oxide and finally embedded in 100% Epon-Araldite. Ultra-thin sections (85 nm) of the trabecular outflow pathway were cut and examined with a transmission electron microscope (JOEL JEM-1011, Tokyo, JAPAN).

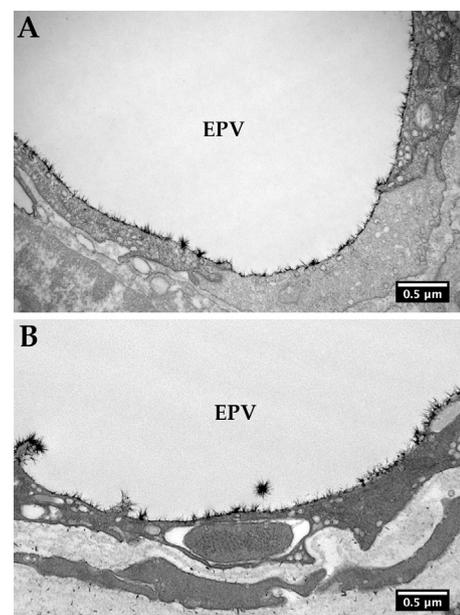
Electron micrographs [bovine:  $\geq 12$  images/eye; human:  $\geq 18$  images/eye] were taken randomly at different regions (trabecular meshwork: TM; Schlemm's canal: SC; aqueous plexus: AP; collector channels: CC) of the aqueous outflow pathway and pooled for each region from all eyes to calculate the percent distribution and thickness of the glycocalyx. Percent distribution of glycocalyx on the endothelial surface (length of glycocalyx covering endothelial surface/total length of endothelial surface) was determined by the intensity plot profile of glycocalyx close to and parallel to the luminal membrane to include all the observable glycocalyx (including collapsed glycocalyx) using ImageJ (NIH) (bovine:  $\geq 16$  measurements/region; human:  $\geq 48$  measurements/region). A preset intensity was applied across all images to separate the glycocalyx from the background. In those regions where the glycocalyx was seen, its thickness was measured at randomly chosen regions of the TM, in the AP/SC, and CCs. Episcleral veins were used as positive controls. The results given in box-plots represent the 25th and 75th percentile, while the center line represents the median, with whiskers extending to a maximum of 1.5 interquartile range. The statistical analysis was done using R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria.). Because the datasets were not normally distributed as determined using the Shapiro–Wilk test, the Mann–Whitney U nonparametric test was used to assess the statistical significance of difference seen in the distribution and thickness of glycocalyx in different regions.

## 3. Results

The glycocalyx, which appears as a layer of hair-like brushes similar to the endothelial glycocalyx reported previously (van den Berg et al., 2003) (Fig. 1), validated our modified Alcian Blue staining method adapted for the aqueous outflow pathway. Fig. 2 showed our positive controls, the glycocalyx in the episcleral veins of the bovine and human aqueous outflow pathways. We did not find any significant differences in the glycocalyx distribution in different regions of the outflow pathway when comparing immersion- and perfusion-fixed bovine eyes (glycocalyx distribution between immersion vs. perfusion: AP: 3–20% vs. 3–65%  $p = 0.49$ ;



**Fig. 1.** Glycocalyx in the bovine and human aqueous outflow pathways. Glycocalyx appears as a layer of hair-like structures in bovine collector channel (A) and human Schlemm's canal (B) of the aqueous outflow pathway. Insets show the glycocalyx at a higher magnification.



**Fig. 2.** Glycocalyx in the bovine and human episcleral veins. Epithelial veins (EPV) served as a control. The glycocalyx was relatively uniform in both bovine (A) and human (B) episcleral veins.

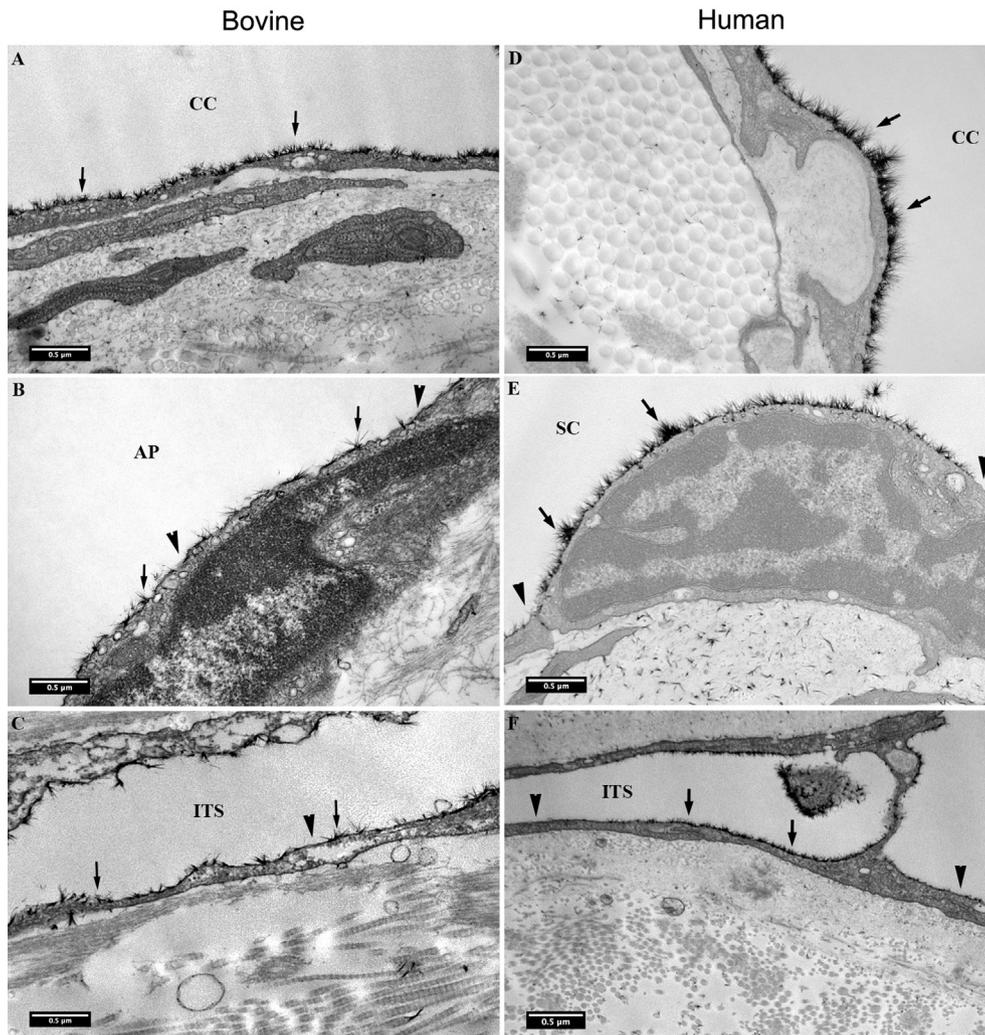
TM: 21–43% vs. 28–43%,  $p = 0.52$ , respectively), and thus, for bovine eyes, immersion- and perfusion-fixed data were pooled for the analysis.

The distribution of the glycocalyx was non-uniform in both the bovine (Fig. 3A–C) and human (Fig. 3D–F) aqueous outflow pathways. In both species, the glycocalyx was most uniform in the CCs (Fig. 3A, D). The distribution of glycocalyx was more variable in the proximal aspects of the outflow pathway, but differed somewhat between the two species. In the bovine eyes, the glycocalyx distribution was most variable on the wall of the AP, with some regions showing little or no coverage (Fig. 3B); the TM showed somewhat more uniform coverage than the AP but was less uniform than that seen in the CCs (Fig. 3C). The human eyes showed a different pattern, with the most variable coverage in the TM, and a somewhat more uniform coverage of SC (Fig. 3E, F).

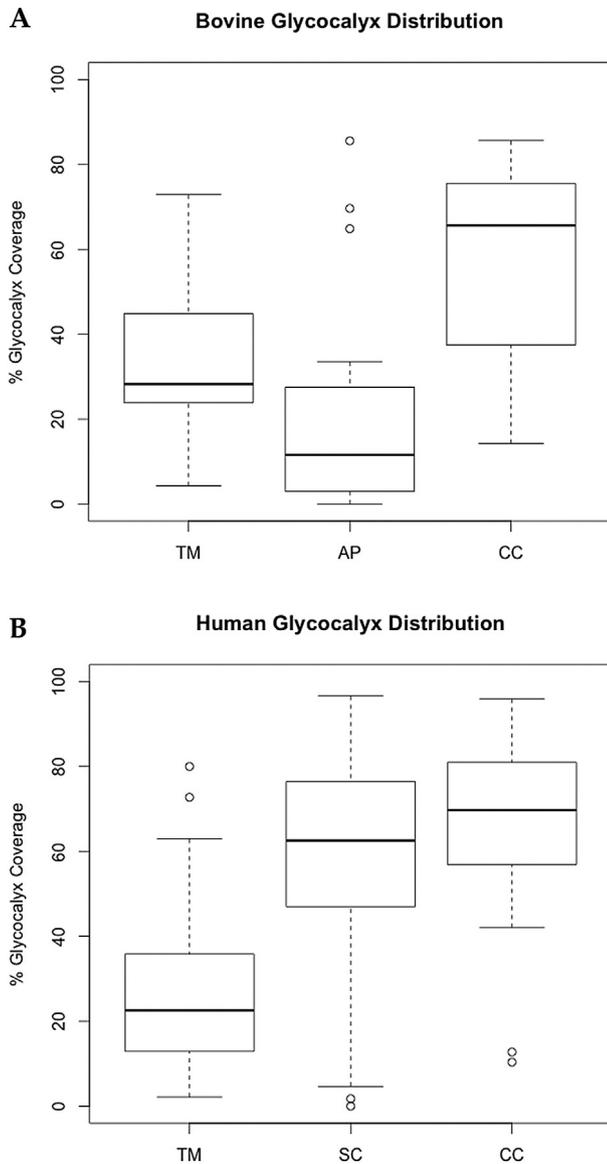
Quantitative studies confirmed these differences in glycocalyx distribution between the two species. In both bovine and human eyes, the surface coverage by glycocalyx was most complete in the CC (bovine: 37–76%,  $n = 23$  images; human: 57–81%,  $n = 52$  images) (Fig. 4); this was followed in the bovine by the TM at 27–43%

( $n = 16$ ), and then AP at 3–27% ( $n = 26$ ) ( $p < 0.01$ ) (Fig. 4A); in the human, this order was reversed with the coverage in SC at 47–76% ( $n = 110$ ) and the TM at 13–36% ( $n = 49$ ) ( $p < 0.01$ ) (Fig. 4B). In bovine eyes, the thickness of the glycocalyx was very uniform in those regions where it was found: AP (70–122 nm,  $n = 158$ : 25th to 75th percentiles), TM (68–124 nm,  $n = 199$ ), and CC (80–114 nm,  $n = 404$ ) ( $p > 0.05$ ) (Fig. 5A). In human eyes, the glycocalyx was thickest in the CC (109–166 nm,  $n = 521$ ) followed by SC (88–154 nm,  $n = 1043$ ), and then the TM (52–88 nm,  $n = 288$ ) ( $p < 0.001$ ) (Fig. 5B).

Ultrastructural observations revealed that the glycocalyx covered the plasma membranes of the TM, SC/AP, and CC endothelial cells (Fig. 3). Alcian Blue stained the glycocalyx coated on the luminal surfaces of the SC/AP, while very sparse coating was seen on the basal surfaces of the endothelial cells of SC/AP. Additionally, the plasma membranes of some juxtacanalicular tissue (JCT) cells also showed sparse distribution of glycocalyx in both human and bovine eyes (Fig. 6). Also very noteworthy was the Alcian Blue staining pattern associated with the giant vacuoles (GVs) of the endothelium of SC. In both bovine and human eyes, glycocalyx was



**Fig. 3.** Glycocalyx in the bovine (A–C) and human (D–F) aqueous outflow pathways. A, D: In the bovine and human collector channels (CC), the glycocalyx (black arrows) was relatively uniform. B, E: In the bovine aqueous plexus (AP), the glycocalyx (black arrows) was variable and less uniform than that in the CC, with some regions showing little or no glycocalyx (arrowheads); in human Schlemm's canal (SC), the glycocalyx was relatively uniform but less so than in the CC with some regions showing little or no glycocalyx (arrowheads). C, F: On the bovine trabecular beams, the glycocalyx (black arrows) was also less uniform than that in the CC with some regions showing little or no glycocalyx (arrowheads); on the human trabecular beams, the glycocalyx was less uniform than that in the CC and SC with some regions showing little or no glycocalyx (arrowheads). ITS: Intertrabecular space.



**Fig. 4.** Glycocalyx distribution. In bovine eyes (A), the ordering of decreasing percentage of the endothelial surface covered by glycocalyx was CC > TM > AP ( $p < 0.01$ ). In humans, more uniform glycocalyx was seen in SC than in AP of bovines. This ordering was different: CC > SC > TM ( $p < 0.01$ ). TM: Trabecular Meshwork; AP: Aqueous Plexus; SC: Schlemm's Canal; CC: Collector Channel.

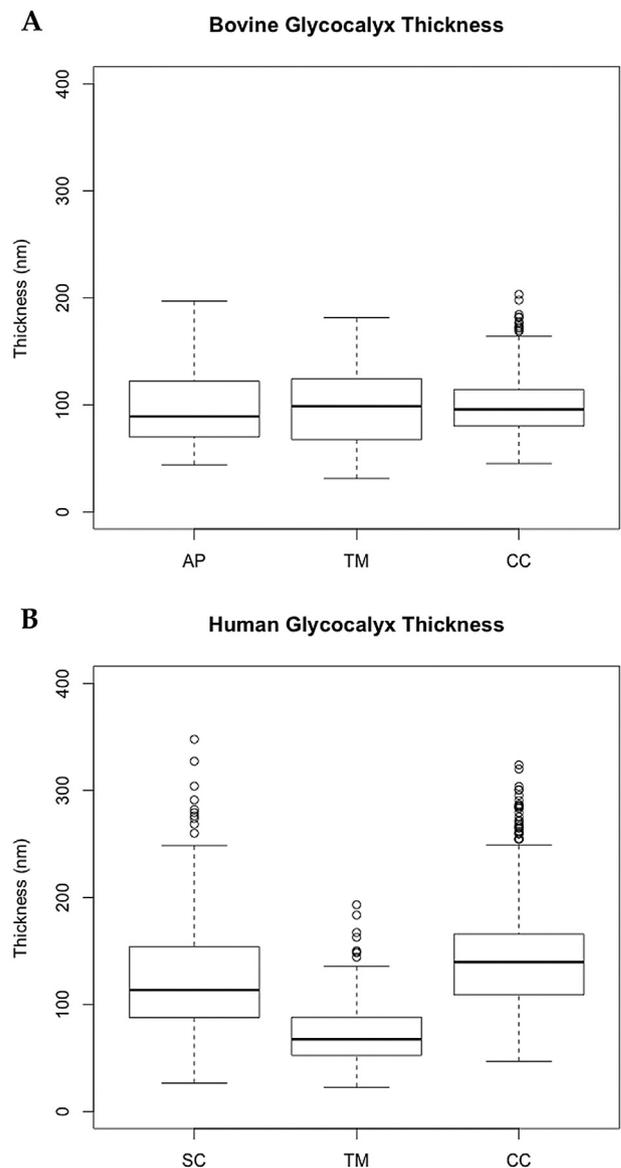
usually seen only coating the outer membrane and not inner membrane of the GV of the AP (Fig. 7A, B) or SC (Fig. 7C, D) whether the sections were passing through a basal opening or not. No Alcian Blue staining was found filling the lumens of either GVs or SC/AP, consistent with their being empty spaces. Lastly, glycocalyx distributions appeared similar on the outer surfaces of GVs in SC/AP as compared to the surrounding endothelium.

A most interesting finding in our study was that most transcellular pores passing through the endothelium of SC/AP were filled with glycocalyx, including both GV associated and non-GV associated pores (Fig. 8). This was true for 10 of the 12 pores seen in human eyes and 8 of 9 pores seen in bovine eyes. Interestingly, in those GVs with a visible pore, particularly those in which a basal opening to the GV was also apparent, glycocalyx was found not only coating the outer membrane of the GVs but also sparsely coating the inner membrane of the GVs closest to the basal opening and closest to the pore (Fig. 8B,C). Glycocalyx was also seen in the

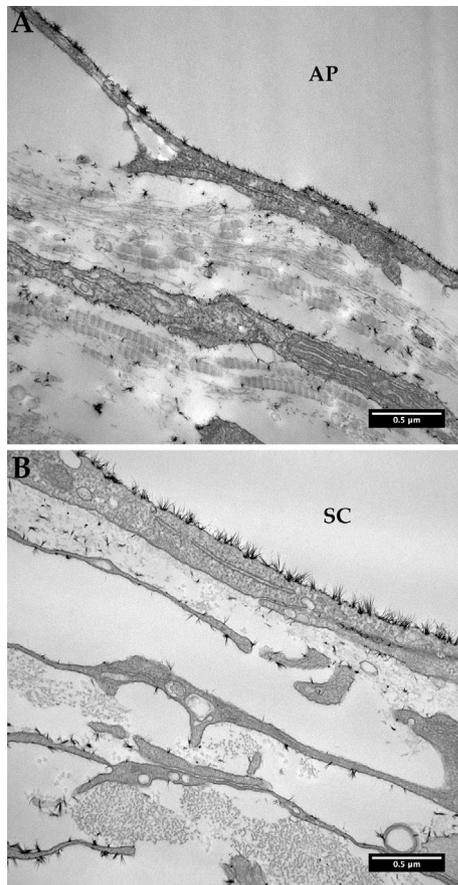
extracellular matrix nearest the basal openings of the pore-associated GVs (Fig. 8B,C).

#### 4. Discussion

The glycocalyx is comprised of glycoproteins bearing acidic oligosaccharides and terminal sialic acids, proteoglycans, and glycosaminoglycans (GAGs) (Reitsma et al., 2007). Previous studies have used electron microscopy to examine the structure of glycocalyx in blood vessels, including rat myocardial capillaries (van den Berg et al., 2003), rat aorta (Devaraj et al., 2009), and human umbilical vein (Chappell et al., 2009). Richardson (Richardson, 1982) previously examined the presence of GAGs on the surface of the aqueous outflow pathway in feline using ruthenium red, but that label is known to collapse the glycocalyx (Tarbell and Ebong, 2010), and thus the existence, detailed structure, and distribution of glycocalyx in the outflow pathway have remained unclear. To better



**Fig. 5.** Glycocalyx thickness. In bovine eyes, the glycocalyx thickness was not significantly different on the surfaces of the AP, TM, or CC ( $p > 0.05$ ), whereas in human eyes, the thickness in these three areas was different (CC > SC > TM,  $p < 0.001$ ). TM: Trabecular Meshwork; AP: Aqueous Plexus; SC: Schlemm's Canal; CC: Collector Channel.



**Fig. 6.** Glycoalkyx in the juxtacanalicular tissue (JCT) Region. Glycoalkyx was found sparsely distributed on the plasma membranes of some JCT cells in both bovine (A) and human (B) eyes, which was much less than the respective endothelial cells of aqueous plexus (AP)/Schlemm's canal (SC) in the same image.

preserve the labile glycoalkyx, we applied the modified Alcian Blue protocol published by van den Berg and colleagues in this study (van den Berg et al., 2003). Alcian Blue, a copper-containing phthalocyanine, is a polyvalent basic dye that, when used in the fixative at physiological pH, can bind to glycoalkyx (extracellular glycoproteins and glycosaminoglycans) forming insoluble precipitates to preserve the labile glycoalkyx during dehydration and embedding processes (Hayat, 1993). While it is possible that the Alcian Blue did not assess all aspects of the outflow pathway (and thus absence of staining does not necessarily imply absence of glycoalkyx), our study has attempted to minimize that possibility by cutting tissue into small segments prior to Alcian Blue-fixative immersion overnight.

We found that the glycoalkyx in the aqueous outflow pathway was less thick than that reported for vascular endothelium and also less uniform. The thickness of the glycoalkyx, in regions where it was found, ranged from 68–122 nm in the bovine outflow pathway and 52–166 nm in the human (25th to 75th percentiles). This is less than the range reported for vascular glycoalkyx (200–500 nm), measured using comparable methods (Tarbell and Ebong, 2010).

Glycoalkyx is reported to be uniformly distributed in rat and mouse aortas but not in the microvessels, with perhaps half either not covered, or covered with a much thinner glycoalkyx (Yen et al., 2012). Some arterioles have been reported to be without glycoalkyx. These findings are, in general, consistent with our findings in the episcleral veins, CCs and SC; however, in the TM of human eyes and in the AP of bovine eyes, glycoalkyx was found to be even more

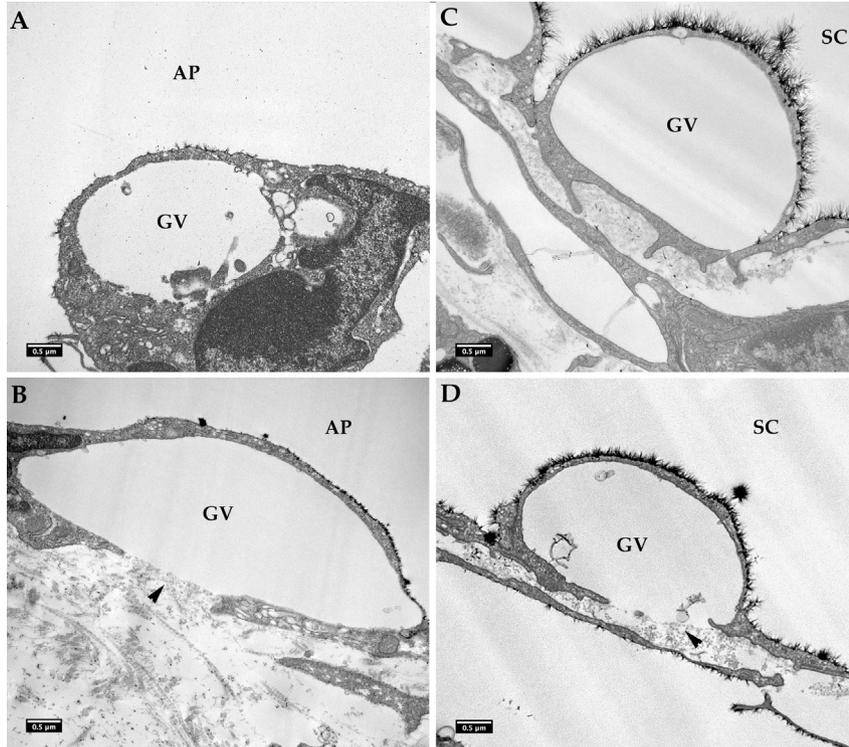
sparse and variable than in most other microvessels. The variability in the distribution of the glycoalkyx in the outflow pathway that we found may relate to non-uniform flow of aqueous humor through these tissues (Overby et al., 2009; Keller et al., 2011; Battista et al., 2008; Yang et al., 2013). This non-uniform flow in the aqueous outflow pathways results in heterogeneously distributed shear forces similar to variable glycoalkyx distribution in the vascular system resulting from heterogeneous blood flow distribution (van den Berg et al., 2003; van den Berg et al., 2009).

The finding of a glycoalkyx in SC/AP allows for speculation as to its role as a possible regulator of aqueous outflow resistance, and thereby, IOP. Changes in shear stress levels in SC/AP would be expected to be transduced by the glycoalkyx and modulate NO release (Tarbell and Ebong, 2010). In SC/AP, this autocrine response would then be expected to relax these cells, a response that has been associated with decreased outflow resistance (Zhou et al., 2012). Thus, for example, if IOP were to increase, this would cause narrowing of SC/AP (Johnstone and Grant, 1973; Van Buskirk, 1982; Battista et al., 2008; Zhang et al., 2009), thus increasing the shear stress level in SC/AP (shear stress is inversely proportional to the size of SC/AP), and thereby initiating this regulatory response to lower the IOP.

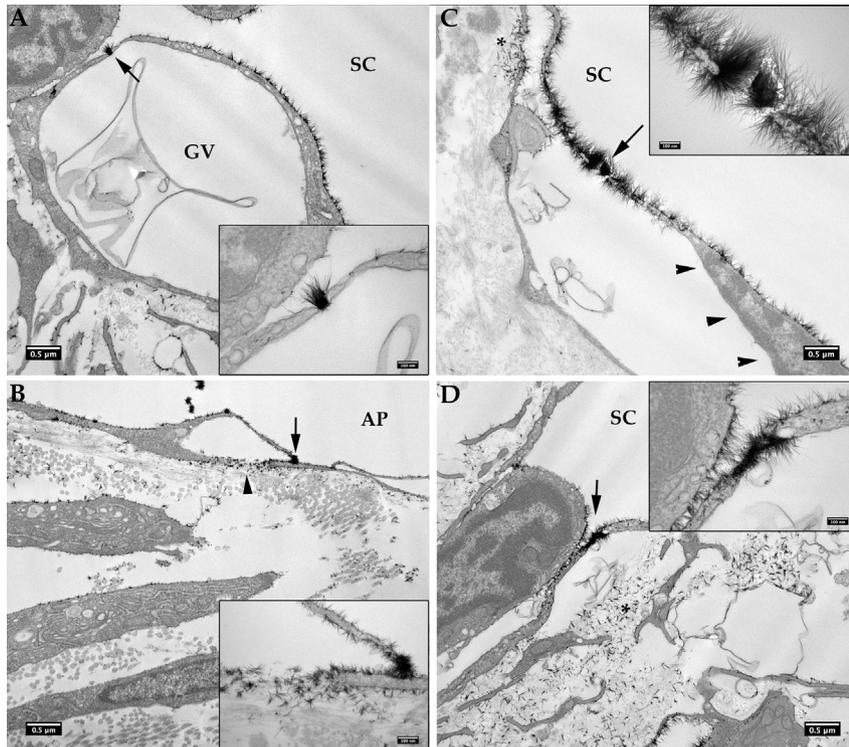
Overall, our observation of glycoalkyx staining pattern in the aqueous outflow pathway delineated by Alcian Blue appeared similar to previous observation using cationic ferritin (de Kater et al., 1989; Ethier and Chan, 2001; Tripathi et al., 1987), ruthenium red (Richardson, 1982; Grierson et al., 1977), and colloidal iron (Grierson and Lee, 1975; Armaly and Wang, 1975; Tripathi et al., 1987) but with much better ultrastructure. The outer membrane of giant vacuoles had significantly more continuous glycoalkyx coverage compared to that of the inner membrane, which is likely due to the differences in the sialic acid residues between the luminal and basal lining of giant vacuoles (Tripathi et al., 1987). Furthermore, we found that most inner wall pores were filled with glycoalkyx. Although a previous study had observed the accumulation of cationic ferritin at possible pore locations, that study used the scanning electron microscope with which the authors were only able to identify the surface but not inside of the pores (Ethier and Chan, 2001). Since the inner wall pores are thought to be passages for aqueous humor to enter SC (Johnson, 2006), glycoalkyx filling the pores may play a role in regulating aqueous outflow resistance.

Weinbaum et al. (2003) estimated the specific hydraulic conductivity of glycoalkyx to be  $3.2 \text{ nm}^2$ . Using Darcy's law, we can estimate the flow resistance of a glycoalkyx-filled pore of diameter  $1 \mu\text{m}$  and thickness  $0.1 \mu\text{m}$  and compare this to the resistance of empty pore of the same dimensions. We found that a glycoalkyx-filled pore has potentially a far higher flow resistance than an empty pore, confirming that the glycoalkyx filling the pores could potentially play a role in regulating aqueous outflow resistance.<sup>1</sup> If so, the bulk of aqueous flow might pass through pores that are not filled with glycoalkyx. However, Ethier and Chan showed that removal of sialic acid (a component of glycoalkyx) with neuraminidase does not alter aqueous outflow resistance (Ethier and Chan, 2001). Further studies are warranted to understand the role of glycoalkyx in the aqueous outflow pathway.

<sup>1</sup> For a glycoalkyx-filled pore, Darcy's law gives a flow resistance of  $\mu L/(KA)$  with aqueous humor viscosity  $\mu$ , pore length  $L$ , permeability of the glycoalkyx  $K$ , and  $A = \pi R^2$  with pore radius  $R$ ; for an empty pore, Sampson's law give a resistance of  $3\mu/R^3$  and thus the ratio of these two resistances is  $LR/(3\pi K)$ . With  $K = 3.2 \text{ nm}^2$  (Weinbaum et al., 2003), assuming  $L = 200 \text{ nm}$  (a typical thickness of the wall of a giant vacuole), this ratio is 3300 for a  $1 \mu\text{m}$  diameter pore.



**Fig. 7.** Glycocalyx on the giant vacuoles. In bovine eyes, glycocalyx only sparsely coated the outer membrane, not the inner membrane of a giant vacuole (GV) of aqueous plexus (AP) endothelium whether a basal opening was not present (A) or present (B, arrowhead). The same is true in human eyes; glycocalyx is usually seen only coating the outer membrane but not inner membrane of a GV of Schlemm's canal (SC) endothelium whether a basal opening is not observed (C) or is observed (arrowhead, D). More uniform glycocalyx was seen on the outer membrane of GVs in human eyes than in bovine eyes.



**Fig. 8.** Glycocalyx in the pores. A: A pore in a GV (arrow, insert) of a human eye is filled with glycocalyx, which also coats the outer membrane, but not the inner membrane of the GV; note the membranous material apparently in the passage through the GV. B: Glycocalyx is seen filling a pore (arrow, insert) in a GV, coating the outer membrane, and sparsely coating the inner membrane of the GV close to the basal opening and the pore, as well as the extracellular matrix near the basal opening (arrowhead) of the GV in a bovine eye. C: Glycocalyx was seen within a pore of a giant vacuole (arrow), and coating the outer surface and left side of the inner membrane of the giant vacuole, which is close to a basal opening (\*), but not the right side of the inner membrane of the giant vacuole (arrowheads) in a human eye. D: Glycocalyx was seen filling a pore not associated with the giant vacuole (arrow). Insets show the pore filling with glycocalyx at a higher magnification. Significant Alcian Blue staining was also seen in the basal side of extracellular matrix (\*).

In summary, this study has demonstrated the detailed structure and distribution of the glycocalyx in both bovine and human aqueous outflow pathways, which is species-dependent. The implications of a non-uniform glycocalyx in the aqueous outflow pathway remain to be clarified. More importantly, based on our findings that showed the glycocalyx lines the wall of SC and fills most of the pores entering SC and findings from vascular endothelium, it is likely that the glycocalyx in SC plays a role in transduction of shear stress and perhaps regulation of outflow resistance.

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