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Trabecular Meshwork Stiffness in Glaucoma

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Abstract

Alterations in stiffness of the trabecular meshwork (TM) may play an important role in primary open-angle glaucoma (POAG), the second leading cause of blindness. Specifically, certain data suggest an association between elevated intraocular pressure (IOP) and increased TM stiffness; however, the underlying link between TM stiffness and IOP remains unclear and requires further study. We here first review the literature on TM stiffness measurements, encompassing various species and based on a number of measurement techniques, including direct approaches such as atomic force microscopy (AFM) and uniaxial tension tests, and indirect methods based on a beam deflection model. We also briefly review the effects of several factors that affect TM stiffness, including lysophospholipids, rho-kinase inhibitors, cytoskeletal disrupting agents, dexamethasone (DEX), transforming growth factor- β_2 (TGF- β_2), nitric oxide (NO) and cellular senescence. We then describe a method we have developed for determining TM stiffness measurement in mice using a cryosection/AFM-based approach, and present preliminary data on TM stiffness in C57BL/6J and CBA/J mouse strains. Finally, we investigate the relationship between TM stiffness and outflow facility between these two strains. The method we have developed shows promise for further direct measurements of mouse TM stiffness, which may be of value in understanding mechanistic relations between outflow facility and TM biomechanical properties.

Keywords

Trabecular meshwork; stiffness; glaucoma; outflow facility; biomechanics

1. Introduction

Elevated intraocular pressure (IOP) due to increased resistance to aqueous humor outflow within the conventional outflow pathway is an important risk factor for glaucoma (Gordon et al., 2002; Grant, 1951). The principal site of outflow resistance in this pathway is the trabecular meshwork (TM), including the inner wall of Schlemm's canal (SC) (Maepea et al., 1992; Overby et al., 2009; Stamer et al., 2012). However, the exact mechanism of how aqueous outflow resistance increases in glaucoma has remained elusive. A better

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understanding of the pathophysiology of aqueous humor drainage through the TM would be of great benefit to the development of IOP-lowering therapies for glaucoma patients.

In this context, there are several observations which suggest that TM stiffness may be important in ocular hypertension associated with glaucoma. For example, pharmacologic modulation of TM cell actomyosin tone has a significant effect on outflow facility (see below). Further, using direct measurements of TM biomechanical properties, Last et al. (2011) reported that the compressive stiffness of TM was 20 times greater in post mortem glaucomatous human eyes compared to ostensibly healthy eyes. These findings and others reviewed below have motivated studies on the relationship between TM stiffness and ocular hypertension.

Our goal in this paper was to first review the literature on trabecular meshwork stiffness, and to then present a method and preliminary data for directly measuring TM compressive stiffness in mouse eyes. The ability to make such measurements in mice is attractive because it will hopefully allow a more mechanistic understanding of how TM stiffness and fluid flow resistance are inter-related. We begin by introducing some background material on how stiffness is defined and on factors that influence tissue stiffness.

1.1. Definition of Stiffness

Stiffness is a measure of the tendency of a material to resist deformation when it is loaded, i.e. when a force is applied to it. The extent of tissue deformation can be quantified through the strain, ϵ in the simplest case, strain is defined by

$$\epsilon = \frac{\text{tissue deformation}}{\text{tissue original length}}$$

Similarly, the load is quantified through the stress, σ

$$\sigma = \frac{\text{force}}{\text{tissue cross-sectional area}}$$

A measure of tissue stiffness is then Young's modulus, E , defined as:

$$E = \frac{\sigma(\epsilon)}{\epsilon}$$

Here we explicitly note that the stress depends on the strain; in fact, for soft tissues, this dependence is usually non-linear so that Young's modulus is not a constant value, but one that varies with the strain. In this case, we can describe tissue stiffness by an effective Young's modulus, or more simply, by "the modulus". The implication is that comparison of stiffness values from different studies is strictly only valid when the extent of tissue deformation was comparable between studies.

In addition to the magnitude of strain, the effective Young's modulus of soft tissue typically depends on a number of other factors, including how the external force is applied (i.e. direction, rate of application) and whether the tissue is in tension or compression.

Importantly for any discussion of TM stiffness, it should be noted that soft tissues are much softer when they are loaded in compression vs. when they are loaded in tension.

Taking all of the above into consideration, the actual value of the effective Young's modulus should be interpreted as a general indication of tissue stiffness that may not be relevant in all situations. More realistic descriptions of tissue biomechanical behavior require more complex formulations that are beyond the scope of this article (Fung,1993; Humphrey et al., 1987). Nonetheless, measured modulus values are still useful inasmuch as they can be used for relative comparisons of tissue stiffness between samples (e.g. normal vs. glaucomatous) if the testing conditions are identical between samples.

1.2. Tissue constituents contributing to TM stiffness

In general, tissue stiffness depends on both cells and extracellular matrix (ECM), and these two components interact in multiple ways in all tissues. Notably, in addition to matricellular signaling pathways and modification of the matrix by the resident cells, it is well known that cells directly sense and respond to the stiffness and topography of their underlying substrate (Discher et al.,2005; Engler et al.,2004; Georges et al.,2005; Russell et al.,2008). For example, fibroblasts change their internal stiffness to try to match that of a stiffer substrate by enhancing actin polymerization and cross-linking (Solon et al.,2007). Similarly, airway smooth muscle cells increase their baseline contractile tone in response to increased substrate stiffness by upregulating their contractile protein expression (West et al.,2011).

Accordingly, we expect the stiffness of human TM to depend in a complex fashion on the resident TM cells, the ECM and the interactions between the two (Fuchshofer et al.,2012; Hoare et al.,2009; Schlunck et al.,2008; Thomasy et al.,2013; Tovar-Vidales et al.,2008). It is known that TM cells are contractile (Lepple-Wienhues et al.,1991) and that elevated outflow resistance can be partly due to an increase in TM tone (Wiederholt et al.,2000). Further, TM cell contraction can direct ECM reorganization, and thus it has been hypothesized that the increased contraction state of TM cells in POAG might be associated with a stiffer TM (Fuchshofer et al.,2012). Additionally, it is suggested that stiffness changes in the ECM of TM may in turn induce alterations in the mechanical properties of the TM cell itself (Last et al.,2011). The biomechanical interaction between cells and ECM, and the likely modulation of this interaction in disease state, make it difficult to “cleanly” determine the relative contributions of cells and ECM to overall TM stiffness and stiffness changes.

2. Measurements of TM stiffness in different species

2.1. Human

The stiffness of human TM has been determined directly and indirectly in several studies. There are currently two approaches for direct measurement: compression (indentation) testing and tensile testing. In view of the Discussion in Section 1.1, modulus values obtained from these two techniques are not expected to be of comparable magnitudes. Atomic force microscopy (AFM) is an example of a compressive testing modality, with the details of the cellular deformation field depending strongly on factors such as cantilever tip geometry and indentation depth (Vargas-Pinto et al.,2013).

In an important early study, the compressive modulus of human TM was measured using AFM by Last et al. (2011). They found that TM stiffness was 20 times greater in glaucomatous eyes compared to normal eyes (glaucomatous eyes: mean, 80.8 kPa, range, 0.5 – 565.3 kPa; normal eyes: mean, 4.0 kPa, range, 0.5 – 10 kPa). However, in this study, measurements were performed on excised TM, which may affect stiffness due for example to the loss of ciliary muscle attachments and tension. Furthermore, it was possible that the cyanoacrylate glue, used to secure the tissue for AFM, may have affected the measurements (Boudou et al., 2007; Daneshvar et al., 2014; Kim et al., 2012). Most importantly, and relevant to the interpretation of all measurements on human glaucomatous TM samples, is the possible confounding effects of glaucoma medications. Post mortem glaucomatous donor eyes have almost inevitably been treated with outflow drugs, and the long-term effects of these drugs on TM stiffness is unknown but potentially significant due to e.g. sustained under-perfusion of the TM caused by beta-blockers or prostaglandins. Thus, we must bear in mind the possibility that any reported stiffness alterations in glaucomatous TM could be epiphenomenon unrelated to the pathogenesis of glaucoma per se, underscoring the importance of making similar measurements in animal models so as to potentially uncover mechanistic links between TM stiffness and outflow resistance.

Camras et al. (2012, 2014) subsequently measured the tensile stiffness of both normal and glaucomatous human TM via uniaxial tension testing, and found that Young's modulus of glaucomatous TM was approximately one-fifth that of normal TM, contradicting Last et al.'s (2011) results. A partial explanation for this difference may be related to the different modes of testing used (tensile vs compressive). Additionally, different tissue structures were involved in those two studies. In Last's study (2011), the stiffness of the JCT and inner wall of SC was primarily measured, while in Camras' study, TM stiffness was likely determined primarily by the corneoscleral portion of the TM, since it makes up the majority of TM volume. It is also notable that the stiffness values reported by Camras et al. were extremely large (many times stiffer than sclera), which is hard to understand and to reconcile with experience gained from direct manipulation of the very soft TM.

Turning now to indirect methods, Johnson et al. (2015) used an analytical beam-bending model to obtain an estimate of TM stiffness. They visualized in vivo changes in TM and SC thickness by optical coherence tomography (OCT) as IOP was increased, and then used this information to estimate the elastic modulus of the TM. They calculated an average elastic modulus for the human TM of 128 kPa, a value between that measured by Last et al. and Camras et al.

Because the inner wall of SC is potentially important in determining outflow resistance, the stiffness of SC endothelial (SCE) cells has also been specifically investigated. Overby et al. (2014) measured the stiffness of both the cortical and subcortical components of the cytoskeleton by AFM in cultured SCE cells. Importantly, the stiffness of SCE cells from glaucomatous human eyes was found to be higher than those from healthy eyes using a 10- μ m AFM tip (glaucomatous eyes: mean $\pm$ SEM, 1.24 ± 0.11 kPa; normal eyes: 0.79 ± 0.10 kPa). One possible explanation for this finding may be that the ECM underlying SCE cells is stiffer in the glaucomatous TM vs normal eyes, since as noted above, the mechanical properties of a cell's microenvironment affect cell stiffness (Byfield et al., 2009; Solon et al.,

2007). In fact, Overby et al. (2014) investigated the influence of substrate stiffness on SC cells using AFM and optical magnetic twisting cytometry, and discovered that both normal and glaucomatous SC cells stiffen in response to increasingly stiffer substrates. More interestingly, glaucomatous SC cells exhibited higher degree of stiffening than normal SC cells. They also examined the expression of genes related to ECM remodeling and found that DCN and BMP4 had lower expression in glaucomatous SC cells compared to normal SC cells, but that DCN and CTGF were strongly up-regulated by increased substrate stiffness in glaucomatous SC cells. This confirms that substrate stiffness affects SCE cell behavior, which may in turn lead to changes in SCE cell stiffness.

2.2. Pigs

Porcine TM stiffness has been investigated by both compressive and tensile testing. The average compressive modulus was found to be 1.38 kPa, as measured by AFM (Yuan, et al., IOVS 2011: ARVO E-abstract 6693). However, the tensile stiffness reported by Camras et al. (2012; 2014) is 2490 kPa, which was three orders of magnitude higher than the compressive modulus. The huge discrepancy may again be partly due to different measurement techniques used; however, as was the case for human tissue, the TM stiffnesses measured by tensile testing were surprisingly large.

2.3. Rabbits

The mean ($\pm$ SD) stiffness of rabbit TM was measured by AFM as 1.03 ± 0.55 kPa (Raghunathan et al., 2015). Unfortunately, no detailed description of AFM measurement was provided in this study, such as tissue preparation and measurement locations (e.g. which part of the TM was indented). The same study reported that after topical administration of 0.1 % dexamethasone (DEX) in vivo for 3 weeks, the elastic modulus of TMs in DEX-treated eyes was 3.89 ± 2.55 kPa, which was significantly larger than that in control eyes. This is particularly interesting in view of the known tendency of DEX to increase intraocular pressure (Weinreb et al., 1985; Whitlock et al., 2010).

2.4. Rats

TM stiffness was measured in rats using AFM by Huang et al. (2015). In their study, Evans blue dye was perfused into the eye before measurement to help locate the TM. The anterior segment was then dissected and flat-mounted with the TM facing upwards. They found the geometric mean TM stiffness to be 162 Pa; however, there were some limitations to this study. Evans blue is not specific to the TM, making it difficult to definitively identify the TM under the AFM microscope. Also, the integrity of TM was not examined at locations where measurements were made. Finally it is conceivable that Evans blue dye itself may affect stiffness as it binds to the tissue.

2.5. Mice

In a glaucoma mouse model (overexpressing BMP-2 in conventional outflow tissue), Young's modulus of the TM was estimated by means of spectral domain optical coherence tomography (G. Li et al., 2014). In this study, the relationship between IOP and SC lumen area was used to determine TM stiffness, using the same beam deflection model described

by Johnson et al.(2015). TM stiffness was estimated to be 2.16 kPa in control eyes; in BMP-2 treated eyes this value increased by approximately 20% on day 7 and more than doubled on day 10 after treatment. One limitation of the theoretical model used this study is that all parameters were assumed to be identical for all SCs, which is likely not valid since in reality variations in parameters such as undeformed height of the SC (SC height when IOP equals collector channel pressure) will occur from one eye to another.

3. Factors and agents affecting TM stiffness

In addition to disease state, there are a number of factors and agents that are known to alter TM stiffness; here, we selectively review several important studies in this area.

3.1. Lysophospholipids

Two lysophospholipids, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), are known to increase outflow resistance and thus have the potential to be involved in the regulation of aqueous humor outflow. In TM cells, it has been shown that LPA increased the expression of proteins such as α -smooth muscle actin (α -SMA), fibroblast specific protein-1 (Pattabiraman et al.,2014), and connective tissue growth factor (CTGF) (Chudgar et al., 2006). Although the influence of LPA and S1P on TM stiffness has not been directly measured, it has been suggested that both may activate a wide variety of intracellular signaling pathways (such as Rho/Rho kinase and protein kinase) that would affect contraction and actin cytoskeletal organization, which would in turn affect TM cell stiffness (P. V. Rao,2014). Consistent with this understanding, S1P and LPA caused an increase in cell stiffness of up to 200 per cent in primary cultures of human SCE (Zhou et al.,2012).

3.2. ROCK inhibitors

The TM is known to express many components of the Rho signaling pathway such as ROCK1 and ROCK2 (Rao,2001). In recent years, Rho-associated protein kinase (ROCK) inhibitors have emerged as a potential treatment option for glaucoma due to their IOP lowering effect (Daneshvar et al.,2014; Honjo et al.,2002). Specifically, the ROCK inhibitors Y-27632, H-1152 and AR-12286 have been shown to induce IOP lowering (Honjo et al., 2001; Tamura et al.,2005; Williams et al.,2011). The exact mechanism by which ROCK inhibitors work on the TM to increase outflow facility is not completely understood. Nevertheless, it has been shown that ROCK inhibitors could increase matrix metalloproteinase expression in TM cells which may reorganize ECM and widen intracellular spaces in the TM. Most relevant to this review, evidence suggests that ROCK inhibitors work by relaxing the TM through reduction of actomyosin contractile tone (S. K. Wang et al.,2014).

3.3. Cytoskeletal disrupting agents

Latrunculin B and the serine-threonine kinase inhibitor H-7 are cytoskeletal disrupting agents, that dramatically increase outflow facility in human and monkey eyes (Ethier et al., 2006; Tian et al.,1999). Latrunculin-B substantially decreased human TM cell stiffness after 30 min of exposure in vitro (McKee et al.,2011), while H-7 has been shown to inhibit cell

contractility, and expand the intercellular spaces in the JCT, accompanied by removal of extracellular material, which likely affected TM stiffness (Sabanay et al.,2000).

3.4. DEX

Treatment with ocular glucocorticoids such as DEX causes increased IOP in approximately one third of the normotensive population. Studies have shown that TM cell stiffness in human patients increased approximately two-fold after just three days of treatment with DEX, and this was correlated with activation of extracellular signal-related kinase $\frac{1}{2}$ and overexpression of α -SMA (Raghunathan et al.,2015). Furthermore, the matrix deposited by DEX-treated human TM cells was approximately four-fold stiffer than that deposited by control cells, and there was increased expression of matrix proteins such as fibrillin, myocilin and decorin (Raghunathan et al.,2015), demonstrating that chronic treatment with DEX can alter TM cell and matrix stiffness. This is consistent with whole tissue measurements in rabbit eyes described above.

3.5. TGF- β_2

Several investigators have found that the concentration of TGF- β_2 is significantly increased in the aqueous humor of POAG patients (Inatani et al.,2001; Tripathi et al.,1994; Trivedi et al.,2011). Early studies demonstrated that TGF- β_2 stimulates a very significant increase in the expression of ECM molecules by TM cells (Flugel-Koch et al.,2004; Han et al.,2011; J. Li et al.,2000; Welge-Lussen et al.,2000; Zhao et al.,2004; Zhao et al.,2005), and importantly, is also involved in the induction of irreversible cross-linking of TM fibronectin (Welge-Lussen et al.,2000). Moreover, TGF- β_2 is known to induce the expression of cross-linking enzymes for elastin and collagen (Sethi et al.,2011). It is possible that this increase in cross-linking and deposition may have contributed to the increased TM stiffness observed by Last et al. (2011) in glaucomatous eyes (Sethi et al.,2011).

3.6. NO

Several studies have indicated that nitric oxide (NO) has an important role in IOP homeostasis. A study using isolated strips of bovine TM also indicated that TM contracts in response to L- nitroarginine, an inhibitor of NO formation (Wiederholt et al.,1994). A more recent study using a gel contraction assay demonstrated that NO donors could relax human TM cells (Dismuke et al.,2014). Thus, one possible mechanism proposed for the increase in outflow facility induced by NO is via TM relaxation (Cavet et al.,2014).

3.7. Senescence

A recent study (Morgan et al.,2015a) demonstrated that senescence may be a causal factor in human TM stiffening. They found that the stiffness of senescent TM cells increased approximately two-fold, indicating that such cells are intrinsically stiffer as measured by AFM. Further, it has been shown that secreted frizzled related protein-1 (SFRP1), a potent inhibitor of a key pathway involved in proliferation regulation, can induce human TM cell stiffening (Morgan et al.,2015b). Increased expression of SFRP1 has also been observed in TM cells grown on substrates that mimic the stiffness of glaucomatous TM (McKee et al., 2011; Raghunathan et al.,2013).

4. Novel measurements of TM stiffness in mice

We here describe preliminary work to develop a new technique for measuring TM stiffness in mice. Eventually we anticipate that this technique will be one part of a strategy designed to mechanistically relate TM stiffness and outflow facility.

4.1. Background and Rationale

Mice have proven to be an excellent model for studying outflow pathway physiology for several reasons. For example, the conventional outflow pathway in mice is similar to that in human eyes, both anatomically and functionally (Overby, Bertrand, et al.,2014) with mice possessing both a laminated TM and a continuous SC. Moreover, outflow facility in mice is responsive to compounds that similarly affect outflow facility in human eyes (Boussommier-Calleja et al.,2012). Finally, genetically distinct mouse strains have significantly different IOPs (Savinova et al.,2001), providing a “natural experiment” to identify factors affecting outflow facility. Notably, it has been reported that there is a strong correlation between IOP and outflow facility across three mouse strains (CBA/J, C57BL/6J and BALB/CJ), with 70% of the variation in IOP being attributable to variations in outflow facility (Boussommier-Calleja et al.,2013). This provides an opportunity to investigate the relationship between TM stiffness and outflow facility in the absence of drug effects or pathology.

In view of the above, we were motivated to use mice to investigate whether there is a correlation between TM stiffness and outflow resistance. Since facility has been measured in CBA/J, C57BL/6J and BALB/CJ strains, we originally planned to use all three strains for this study. However, we discovered that it was nearly impossible to distinguish the TM from adjacent tissue in BALB/CJ albino mice (see below), and therefore have investigated only CBA/J and C57BL/6J strains in this preliminary study.

To date, no direct measurements of mouse TM stiffness have been published, likely due to the significant challenge of obtaining measurements on the small, delicate TM of this species. Fortunately, AFM presents an attractive means for determining the mechanical properties (stiffness) of mouse TM, since it has high force sensitivity and spatial resolution (Last et al.,2010). We first tried an ‘*en face*’ approach, in which AFM measurements were performed on the inner surface of an anterior segment wedge in which the iris had been reflected posteriorly to expose the angle and the uveal surface of the TM. Stiffness data were acquired along a line starting in the cornea and ending in the sclera, nominally including the uveal surface of the TM (K. Wang et al.,2015). However, identification of the TM anterior and posterior boundaries was extremely challenging with this approach. Additionally, post-measurement histologic analysis indicated that the dissection process damaged the TM in some samples, while in others the TM was likely not even exposed to the cantilever during AFM measurements. Therefore, we abandoned the *en face* approach and considered alternative techniques.

Cryosectioning, combined with AFM, has previously been used to measure stiffness in ocular tissues, namely human lamina cribrosa and porcine cornea (Braunsmann et al.,2012; Seifert et al.,2014). We reasoned that a similar protocol could be used to measure TM stiffness in mice. This goal of this preliminary study was therefore to evaluate the feasibility

of using AFM to measure TM stiffness in fresh mouse eyes which had been frozen and cryosectioned.

4.2. Methods

Measurements were performed in eyes of 4 C57BL/6J (age: 12–22 weeks) and 8 CBA/J (age: 11–15 weeks) female mice from Jackson Laboratory. Mice were sacrificed using CO₂, and the eyes were enucleated within 5 minutes of death and stored in Dulbecco's phosphate buffered saline (DPBS, Mediatech inc) at 4°C until use. Each eye was perfused to measure outflow facility, using the iPerfusion system as described elsewhere (Sherwood et al., 2016). Eyes were then perfused with DPBS plus 5.5mM glucose at 8 mmHg for a further 30 minutes, coated with optimal cutting temperature compound (O.C.T.; Tissue-Tek), frozen (see below) and stored at –80°C. During the freezing process, the perfusion needle remained in the anterior chamber to maintain IOP.

We used two somewhat different protocols to freeze the eyes. In the first, which was used for all C57BL/6J mouse eyes, the perfusate contained 15% glycerol (Sigma-Aldrich, St. Louis, MI) as a cryoprotectant (Meryman, 2007; Seshoka et al., 2016) and the eyes were fast-frozen by direct contact with dry ice. In the second, carried out on all CBA/J mouse eyes, cryoprotectant was not added to the perfusate, and eyes were snap-frozen by immersion in 2-methylbutane (Sigma-Aldrich, St. Louis, MI) cooled by liquid nitrogen (Erickson et al., 2011; Steu et al., 2008). We switched to the second approach in an effort to reduce the risk and minimize the size of ice crystals forming in the tissue, which could possibly affect TM mechanical properties, and to achieve more uniform freezing of the eye. Also, it is possible that cryoprotectants may cause stiffening of collagen, while ice crystals are unlikely to have a significant effect on TM biomechanical properties (Masic et al., 2015; Paten et al., 2016; Stemper et al., 2007). No evidence of large ice crystals were observed; however, this does not preclude the possibility of smaller crystals forming, detectable only at the ultrastructural level (Schwabe et al., 1980). For each eye, 10–20 µm thick sagittal sections were cut on a Microm Cryostar NX70 cryostat (Dreieich, Germany). Sections were collected on adhesive glass slides (Plus gold slide, Electron Microscopy Sciences, Hatfield, PA) and stored in ice-cold PBS prior to AFM analysis, which was carried out immediately after sectioning.

Samples were transferred to an MFD-3D AFM (Asylum Research, Santa Barbara, CA) and kept continuously immersed in DPBS during measurements at room temperature. Silicon nitride cantilevers with an attached borosilicate sphere (radius, 5 µm; nominal spring constant, 0.1 N/m; Novascan Technologies, Inc., Ames, IA) were calibrated by measuring the thermally induced motion of the unloaded cantilever before measurements and relating the resultant spring energy to the thermal energy via the equipartition theorem. An open SC lumen was identified on each cryosection and used as a landmark to aid in locating the TM, which itself was identified as the tissue occupying the region between the inner wall of SC and the root of the ciliary body/iris. Force measurements were then performed on this presumptive TM, with three measurements taken at each measurement site, at an indentation rate of 8 µm/s, with typical indentation depth of 0.5 – 1 µm. Force curves were fit to a Hertz model (Equation 1) for a sphere in contact with a flat surface to determine the local

compressive modulus (Igor Pro software, 6.34A). This standard Hertz model assumes that the tissue deformation during indentation is purely elastic.

$$E = \frac{3(1 - \nu^2)F}{4R^{1/2}\delta^{3/2}} \quad (1)$$

In Equation 1, E is Young's modulus, F is the force causing cantilever bending, R is the radius of the sphere, δ is the actual sample indentation, and ν is the sample's Poisson's ratio which was taken as 0.5. The contact point was determined using routines in the Igor Pro software that identify deviation from the linear region of noncontact and the onset of increased slope. Figure 1 shows typical force-indentation curves obtained from indentations made on TM and sclera as well as the fitted curves (dashed lines).

At each measurement location, the average value of the three measurements was taken as the modulus at that single location. The stiffness of the TM for each eye was defined as the average moduli of all measurement sites in the TM (average measurement sites per cryosection: 6, range: 3–10), from at least one cryosection (average cryosections per eye: 1, range: 1–2). The TM stiffness for each mouse was then taken as the average moduli of its two eyes.

During AFM indentations, we noticed that the desired measurement location and the cantilever tip may not have been in precisely the same spot when observed from the bottom camera of the AFM due to the cryosection having a finite thickness (10–20 μm) and small, inevitable misalignment between the direction in which the cantilever moved and the normal to the tissue plane. To estimate the associated uncertainty in measurement location, the cantilever tip was first moved to barely touch the surface of a glass slide and the bottom camera was focused on that surface. The cantilever tip was then raised by 20 μm and the camera was refocused on the cantilever tip. Finally, the distance between the cantilever tip before and after the refocusing was used to estimate the lateral (in-plane) uncertainty of the measurement location, which was 2.75 μm .

4.3. Results

Six eyes from 4 C57BL/6J mice and 13 eyes from 8 CBA/J mice were successfully perfused to measure outflow facility (Table 1). Outflow facilities for C57BL/6J and CBA/J mice, as defined by Sherwood et al. (Sherwood et al., 2016) at a reference pressure of 8 mmHg, were 5.3 ± 2.9 and 7.4 ± 4.5 nl/(minmmHg) respectively (mean $\pm$ SD). This difference was not statistically significant ($p > 0.05$, Wilcoxon rank sum test).

A SC lumen could be observed in cryosections, serving as a landmark for the TM. Typical stiffness measurement locations are shown in Figure 2, where Locations 1–7 represent the TM region in each panel. The TM stiffnesses (mean $\pm$ SD) for C57BL/6J and CBA/J mice were 3.22 ± 1.84 kPa (3 mice, 2 cryosections on average for each mouse) and 3.84 ± 3.37 kPa (7 mice, 2 cryosections on average for each mouse) respectively.

When outflow facility was plotted against TM stiffness for individual mice (Figure 3), facility and TM stiffness were not significantly correlated, although the number of samples was very small. We note that cross-plotting of facility and TM stiffness was carried out on a mouse-by-mouse (rather than an eye-by-eye) basis so that statistical confounding effects of non-independence of paired eyes were avoided.

5. Discussion

This preliminary study presents the first method to directly measure the local compressive modulus of mouse TM. We also investigated the correlation between TM stiffness and outflow facility between two mouse strains.

5.1. Outflow facility

The outflow facility (mean $\pm$ SD) we measured for C57BL/6J mice (5.3 ± 2.9 nl/minmmHg) was close to that reported by Sherwood et al. (2016) in the same mouse strain, namely 5.9 ± 1.1 nl/minmmHg, validating the facility measurement approach. An additional comment about facility data is warranted: the mean facility we measured for the two strains appeared to be 2–3 times higher than values reported in Boussoimmier-Calleja's (Boussoimmier-Calleja et al., 2013) study. This may be attributed to differences in the fitting method for the flow-IOP traces (Sherwood et al., 2016). Specifically, in Boussoimmier-Calleja's study, facility was assumed to be constant with respect to IOP, and flow-pressure data were fitted with a straight line for 4 pressure levels. In our study, a more sophisticated and accurate nonlinear equation was used for fitting and eyes were perfused at 9 pressure levels, with the facility obtained from this fitted curve at a reference IOP of 8 mmHg.

5.2. TM stiffness

It is useful to compare our mouse data with previously reported TM stiffnesses (Table 2). Focusing on compressive measurements of ostensibly normal, untreated TMs (bold entries), our TM stiffness values for both mouse strains are generally consistent with other measurements, lying slightly (10%) below those of normal human TM measured by Last et al. One exception is the rat TM, which appears to be extremely soft (about 20 times softer than mouse TM); it is unclear if this difference is real or reflects experimental (technique) differences. Generally, the technique we propose here seems to yield TM stiffness values that are reasonable, as judged by overall consistency with previously reported values in other species.

5.3. Relationship between outflow facility and TM stiffness

Surprisingly, we found no significant correlation between outflow facility and TM stiffness from individual eyes across the two strains of mice. However, it is important to understand that this data is preliminary and is thus based on a small number of samples; studies are now ongoing to expand the sample size to better understand this issue. Of course, this lack of correlation is likely due, in part, to factors other than TM stiffness influencing outflow facility in mice

5.4. Limitations

There are a number of limitations in this study. For example, in the AFM measurement of tissue stiffness it is important to ensure that the cantilever tip is indenting the TM. However, there is a slight uncertainty in measurement location (see Section 4.2). Although the deviation between the desired and actual measurement locations was less than the radius of the sphere on the cantilever tip, measurements towards the “edges” of the TM could lead to the local contact region lying slightly outside of the TM. Therefore, it was necessary to keep the cantilever sphere away from SC and CB as much as possible so that there was a higher probability of measuring the TM when contacting the tip.

The freeze-thaw procedure for tissue preparation in stiffness measurements may also have influenced the mechanical properties of the tissue. Studies have shown that freeze-thaw processing can effectively lyse cells within lung tissue and does produce minor disruptions of the ECM ultrastructure in porcine skin (Cortiella et al., 2010; Prasertsung et al., 2008). Thus, further studies should investigate how freeze-thaw treatment of TM and freezing rate may affect tissue stiffness. This issue was confounded by the fact that we changed freezing protocols when we started measurements on the second mouse strain, so that the contribution of the protocol switch to TM stiffness differences between the two strains is unknown.

Another issue of note is that the aqueous outflow is segmental in mouse eyes (Swaminathan et al., 2013), suggesting that TM stiffness might also be location-dependent. Thus, TM stiffness measured from one or two cryosections may not necessarily represent the average TM stiffness in that eye. Future studies will acquire measurements on more cryosections/eye to investigate this effect. Additionally, we will mark the orientation of the eye in vivo so we can determine which quadrant the cryosections are cut from.

6. Conclusions and future directions

There is growing evidence suggesting that mechanical properties of TM may be involved in ocular hypertension associated with glaucoma. We know that TM stiffness may be affected by pharmacological agents as well as intrinsic alterations in TM and SC cells. In addition, the ECM may have important effects on TM cellular behavior and, notably, TM cells and their ECM are inextricably linked, with alterations in one affecting the other. By combining the techniques of cryosectioning and AFM, we were able to develop a technique to measure TM stiffness in the mouse eye. Although this study is preliminary, the results are sufficiently promising to merit further development. We expect that this approach will be used in future work to study issues such as identification of molecular factors and associated genes involved in TM stiffness regulation. Progress in this area may lead to further understanding of the role of TM stiffness in ocular hypertension, which may help guide the design of future glaucoma therapies.

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Figure 1:

Typical advancing force-indentation data (points) and fits (dashed lines) to raw data for TM (red) and sclera (blue) locations. The force experienced by the cantilever tip is zero when the cantilever is far away from the sample surface (negative indentation), and increases as the cantilever tip touches and then indents the sample surface.

Figure 2:

Anterior angle in representative cryosections observed from the AFM for C57BL/6J (left) and CBA/J (right) mice. SC, TM and CB are labeled. Numbers indicate where AFM measurements were made in the TM. Section thickness: 20 µm (left) and 10 µm (right). Average TM stiffnesses were 1.21 ± 0.78 kPa (left panel) and 1.47 ± 0.71 kPa (right panel).

Figure 3:

Cross-plot between TM stiffness and outflow facility from individual mice for C57BL/6J (n=3) and CBA/J (n=6) strains. For each mouse, the outflow facility was taken as the average of the facilities from both eyes, while the TM stiffness was taken as the average stiffness measured from one or two cryosections from each eye. Only mice where both facility and TM stiffness were measured in the same eyes were included. The solid line and equation represent the linear regression of the pooled data.

Table 1: Outflow facility and TM stiffness (mean $\pm$ SD) for C57BL/6J and CBA/J mice *

Mouse Eye	Strain	Freezing Approach	Age (weeks)	Facility nl/(min·mmHg)	TM Stiffness (kPa)
C1M1OS	C57BL/6J	First approach (see text)	12	2.3	ND
C1M2OD			12	3.6	3.68 ± 0.93
C1M2OS			12	3.4	1.21 ± 0.65
C1M3OD			22	8.7	5.32 ± 3.8
C3M1OD			13	4.4	1.36 ± 0.81
C3M1OS			13	8.7	2.41 ± 1.41
				Mean ± SD: 5.3 ± 2.9	3.22 ± 1.84
C4M3OD	CBA/J	Second approach (see text)	14	ND	2.91 ± 2.59
C4M3OS			14	4.9	ND
C4M4OD			15	3.9	ND
C4M4OS			15	3.0	1.47 ± 0.71
C4M5OD			15	11.8	0.96 ± 1.01
C4M5OS			15	3.4	6.52 ± 2.87
C5M1OD			11	6.3	1.07 ± 0.36
C5M1OS			11	6.0	0.55 ± 0.22
C5M2OD			11	11.4	12.51± 5.31
C5M2OS			11	3.6	4.12 ± 0.79
C5M3OD			11	7.2	ND
C5M3OS			11	8.9	0.93 ± 0.17
C5M4OD			11	ND	5.2 ± 3.43
C5M4OS			11	3.9	12.25 ± 3.92
C5M5OD			11	17.7	ND
				Mean ± SD: 7.4 ± 4.5	3.84 ± 3.37

*C#M# = Corhort#Mouse#; OD = oculus dextrus; OS = oculus sinister; SD = standard deviation; ND = not determined.

Table 2:

TM stiffnesses reported to date, Bold values are most methodologically comparable.

Method		Tissue	Modulus (kPa)	Reference
Direct	Compressive (AFM)	Human TM (JCT, normal)	4.0 ± 2.2^a	Last et al.
		Human TM (JCT, glaucomatous)	80.8 ± 32.5^a	Last et al.
		Porcine TM	1.38^n	Yuan et al.
		Rabbit TM	1.03 ± 0.55^a	Raghunathan et al.
		Rabbit TM (DEX treated)	3.89 ± 2.55^a	Raghunathan et al.
		Rat TM	0.162 ± 0.0012^b	Huang et al.
		mouse TM (C57BL/6J)	3.22 ± 1.84^a	This study
		mouse TM (CBA/J)	3.84 ± 3.37^a	This study
	Tensile (uniaxial tension test)	Human TM (normal)	51500 ± 13600^b	Camras et al.
		Human TM (glaucomatous)	12500 ± 1400^b	Camras et al.
		Porcine TM	2490 ± 1.5^b	Camras et al.
Indirect	Beam-bending model	Human TM (in-vivo)	128^n	Johnson et al.
		Mouse TM	2.16^n	Li et al.

^a: mean $\pm$ SD;

^b: geometric mean $\pm$ geometric SE;

ⁿ: not specified.