Chapter 6

UNDERSTANDING THE MICROSTRUCTURAL CHANGES THAT CAUSE CORNEAL STIFFENING

6.1 Introduction

Having established that glycation has significant therapeutic potential for keratoconus, we proceed by investigating the associated microstructural changes. Starting at the anterior surface and moving back, the cornea is composed of the following five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium[1]. The major structural element of the cornea (~ 90% of its thickness) is the stroma, which is composed of approximately 200 lamellae that consist mainly of oriented collagen fibrils embedded in a hydrated matrix of proteoglycans (PGs) and glycosaminoglycans (GAGs) (Figure 1).[2] Lamellae run parallel to the surfaces of the cornea and span its full area. There is even indirect evidence that the individual collagen fibrils in a lamella span the entire cornea.[2] Fibrils are composed of collagen type I triple helices that are enzymatically cross-linked during development (Figure 1, A and B).

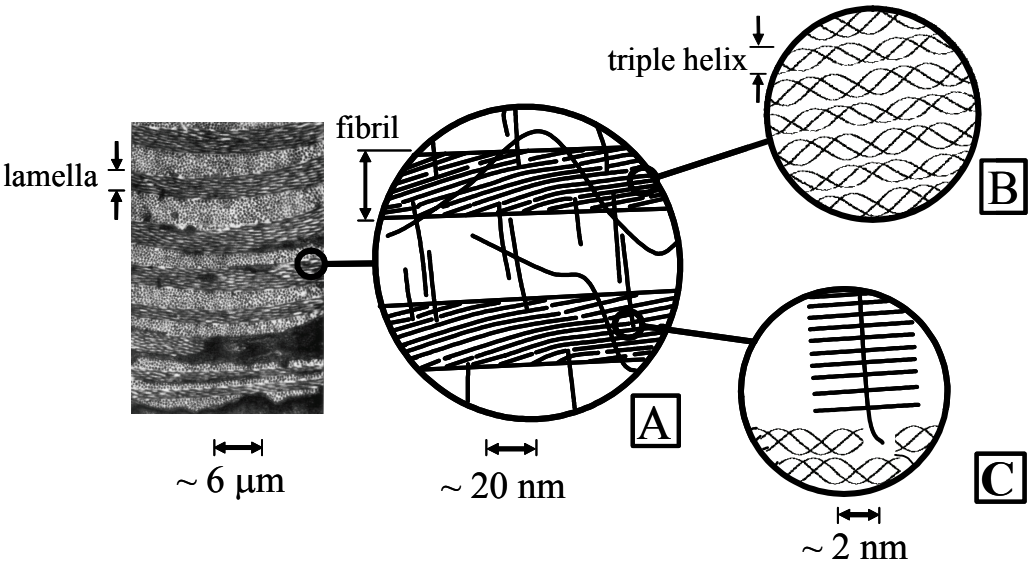

Figure 1. Stroma microstructure. [A] Represents the rigid collagen type I fibrils (D ~ 30 nm) and smaller strands of proteoglycan that compose the lamellae (2 - 3 nm thick) of the corneal stroma. [B] Shows an enlargement of part of one of the fibrils, displaying the collagen triple helices (D ~ 1.5 nm) aligned within a fibril. [C] Depicts the protein core of a proteoglycan non-covalently associated with the surface of a collagen fibril and decorated with polysaccharide chains. Micrograph was used by permission from Prof. K. Kadler, U. Manchester.

The collagen fibrils in adjacent lamellae within the stroma differ in orientation only,[2] but the result is a "stacked sheet" morphology; fibrils within a single layer run parallel to each other and nearly orthogonal to fibrils in adjacent layers. The high tensile-strength fibrils resist axial strains, while the hydrated PG/GAG matrix between layers allows the collagen fibrils to bend and translate relative to each other under off-axis stress. It has been proposed that the regular orientation and spatial distribution of the collagen fibrils is maintained by specific associations with the PGs in the matrix (Figure 1, C).[1, 3]

It is well established, and demonstrated again here, that glycation-induced crosslinks add connectivity to this native morphology within the collagen fibrils and between fibrils and matrix (see Chapter 5). Coordinated biochemical and rheological analyses have allowed us

to correlate tissue stiffening with advanced glycation endproduct (AGE) accumulation. However, it is likely that only a small fraction of the AGEs formed contribute to the mechanical properties of the tissue. Pendant adducts such as argpyrimidine cannot contribute to mechanical strength because they do not add connectivity to the system; only crosslinks contribute to the modulus. The structures of the various AGEs make it obvious which are crosslinks, but it remains unclear *where* (i.e., between which of the macromolecular components) crosslinks have the greatest impact on mechanical properties. The relative importance of contributions from crosslinks 1) within collagen fibrils vs. 2) between fibrils vs. 3) between fibrils and proteoglycans vs. 4) within or between proteoglycans, remains an open question. This is due, in part, to a lack of consensus on the importance of proteoglycans in the mechanical properties of soft collagenous tissues.

The traditional view is that proteoglycans are not mechanically significant. The mechanical strength of ocular tissues and collagenous tissues in general has been attributed solely to the orientation, density, and size of the collagen fibrils.[2, 4-6] In this model, proteoglycans are thought to be a randomly oriented "ground substance" that is distributed throughout the tissue. The primary functions of this amorphous gel are proposed to be the maintenance of tissue hydration and resistance to sudden deformations due to high viscosity.

In the modern view, proteoglycans make significant contributions to mechanical properties through specific interactions with binding sites on the surface of the collagen fibrils.[3, 7-9] In addition to hydrating the tissue, it is proposed that proteoglycans also maintain fibril spacing and orientation and contribute to tissue stiffness by bridging the span between fibrils.

The differences between these two models become particularly relevant in the context of glycating corneal tissue. The predominant view in the literature is that glycation-related tissue stiffening is due to fibril stiffening.[10] We test this hypothesis and attempt to differentiate between the contributions of fibril stiffening and matrix-related changes by comparing the effects of glycation on isolated collagen fibers (from mouse tails) to the effects of glycation on the cornea. Hereafter, "fiber" will be used to denote a coherent, crosslinked subunit of a mouse tendon (typically ~ 100 μm diameter) and "fibril" will generally refer bundles of laterally-associated collagen triple helices (~30 nm diameter) found in the stroma (Figure 1, A and B). Collagen fibers (not found in cornea tissue) are bundles of fibrils.

Previous mechanical analyses of native and engineered cornea focused on the tensile strength of strips of tissue, which does not probe the tissue as a whole. Tensile measurements on cornea strips are dominated by the tensile strength of the collagen fibrils whose axes are coincident with the axis of elongation. We have performed oscillatory shear measurements, which deform the cornea along the axis perpendicular to the planes of the lamellae so that the collagen fibrils are not loaded along their axes. This method of analysis allows us to probe the tissue as a whole and not simply the collagen fibrils.

To determine the relative contribution of changes in the mechanical properties of the collagen fibrils, we have examined glycated mouse tail tendon fibers. Mouse tail tendons are composed almost entirely of bundles of collagen type I fibers and provide a well-studied model system for measuring the mechanical properties of these fibers.[4] The "biomechanics" approach is to measure the stress-strain relation of the fibers directly,

under approximately physiological conditions, by applying a uniaxial load to fibers submersed in a physiological buffer.[4] In these experiments, the stresses that develop as a result of straining fibers at a constant rate are measured. The resulting stress-strain relation reflects the response of native fibers (hydrogen bonds intact) to mechanical loading. Small strains and loads reflect normal physiological conditions, while the failure behavior is relevant to injuries.

Unfortunately, the methods used in the biomechanics literature to characterize fresh tendon[4] are inconsistent with the methods used in the glycation literature to measure the mechanical impact of crosslink accumulation.[11] The metric most commonly used to quantify the mechanical properties of glycated collagen fibers is the tendon breaking time (TBT) test.[11-13] In the TBT procedure, ~ 2 g weights are suspended from mouse tendon fibers submersed in 7 M urea at 40° C. The tendon breaking time begins with hanging the weight on the fibril and submersing it and ends at the time the weight falls to the bottom of the container. While breaking time is well correlated with animal age and the accumulation of covalent crosslinks,[11, 12] it provides little insight into the impact of those crosslinks on the mechanical properties of the fibers *in vivo*. We note that this is a point of confusion in the literature (discussed further in Section 6.5).

Applying this "biomechanics" approach to glycated tissues, we have succeeded in correlating AGE accumulation in glycated mouse-tail collagen fibers with mechanical property changes under nondenaturing conditions. We will show that the mechanical properties of intact collagen fibers are less sensitive to AGE accumulation than those of denatured fibrils. However, we will show that the biomechanics approach, but not the TBT

test, is directly related to the physiologically-relevant impact of glycation on collagen fibrils. With a known, quantitative correlation between AGE formation and the mechanical strength of intact collagen fibrils, we proceed to show that treatment-induced cornea stiffening cannot be explained by changes in the properties of its collagen fibrils. Instead, glycation must also change the way the collagen interacts with the PG/GAG matrix.

6.2 Materials and Methods

Mouse-Tail Collagen Fiber Glycation – Fresh tendons were removed from the tails of 6-week and 6-month old mice and incubated for 24 hours at 37° C in 100 mM phosphate-buffered saline (PBS controls), 0.1 mM methylglyoxal (in PBS), or 0.5 mM methylglyoxal (in PBS). After incubation, tendons were rinsed and placed in PBS at 5° C and either analyzed immediately by GC/MS in Professor Vincent Monnier's lab at Case Western Reserve University (CWRU, Department of Pathology) or shipped overnight to Caltech for mechanical analysis. Mice were bred, sacrificed, and dissected at treated at CWRU (strain C57BL6). Phosphate-buffered saline and methylglyoxal (MGO) were purchased from Sigma Chemical Co. (St. Louis, MO). MGO was distilled prior to use.

HPLC Analysis – A minimum of 3 tendons from each treatment were individually hydrolyzed in 6 N HCl for 18 hours at 110° C. Total lysine and caboxyethyl-lysine (CEL), a surrogate marker for modification of collagen by MGO, were quantified by method of internal standard using GC/MS. These experiments were performed by Christopher Strauch under the supervision of Professor V. Monnier.

Mechanical Analysis – Tendons readily separate into strands ("fibers") when sectioned. If placed in air, fibers dry and shrink rapidly; therefore, fibers were kept in buffer at all times. Precise fiber diameters were measured using optical microscopy with the tendons submersed in physiological saline. Diameters ranged from 75 μm – 200 μm, and lengths were cut to ~ 1.25 cm. It is significant that only fibers in this range were used: fibers less than 75 μm in diameter are easily damaged and above 200 μm results no longer reflect intrinsic properties (e. g., there is a systematic decrease in apparent failure strain and stress with increasing diameter > 200 μm).

Fibers were then loaded on an Instron 5542 Universal Materials Testing Machine (Instron Corp., MA) by gently clamping the ends of the fiber between opposing steel plates and immersing the assembly in a PBS bath at 37° C. Clamping must be done with great care and consistency: excessive clamping force results in the fiber failing prematurely at the edge of the clamp, and insufficient force allows the fiber to slip.[14] Failure strains never exceeded 12%, indicating that fibrils did not slip.[14] Clamps were then gently drawn apart to straighten the fiber. Fibers were considered fully extended when further separation of the clamps induced a measurable normal force. Sample length was then measured as the distance between clamps.

The tensile testing procedure, based on prior literature, consisted of a preconditioning period followed by a linear extension at fixed rate until the fiber failed.[4] Preconditioning was achieved by three successive cycles of extending the fiber to up to 3% strain and back down to 1% at a constant rate of 3%/min (at or below this rate, properties are essentially rate-independent). Precondition was performed as described in the literature to reduce

variability in the high-strain behavior.[4] Apparent tensile stress and strain were recorded throughout the preconditioning period (Figure 2). The linear elastic modulus was calculated from the results before or during preconditioning. After the third return to 1% strain, the sample was extended until it failed, while the apparent tensile stress and strain were recorded. If the breaking point of the fiber was at the edge of the clamp, the result was discarded to eliminate errors due to edge effects.

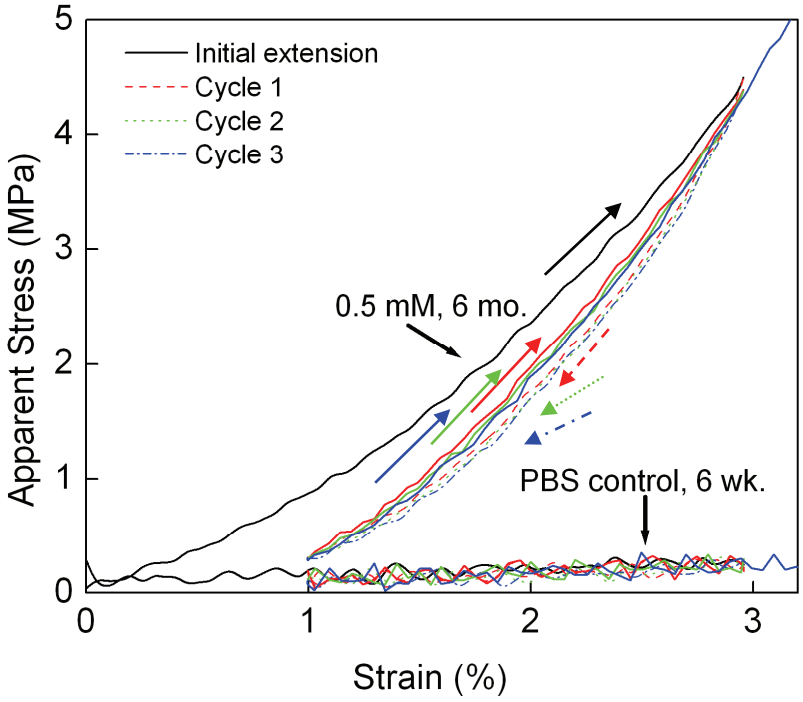

Figure 2. Preconditioning collagen fibrils by cycling from 1 - 3% strain three times provides a common flow history for all samples. Unglycated controls show essentially no hysteresis, whereas tendons glycated with methylglyoxal appear to relax significantly after the first cycle.

6.3 Advanced Glycation Endproducts in Mouse Tail Collagen

Mouse tails were glycated using MGO rather than GA because it glycates tissues more rapidly than GA, while yielding similar AGEs (see Chapter 1).[15, 16] MGO is also physiologically significant; it is a known degradation product of glyceraldehyde-3-phosphate *in vivo*.[17] MGO is formed from GA via a simple dehydration reaction and from GA-3-phosphate via loss of phosphate.[15] Formation of MGO is thought to be a significant mechanism of glycation in the presence of glyceraldehyde, although direct reaction of glyceraldehyde with proteins is prevalent. MGO is useful for examining glycation *in vitro*, however it is unsuitable for tissue engineering due to its high toxicity.

Carboxyelthyl-lysine (CEL) was used as a surrogate marker for glycation levels of mouse tail tendon.[15, 17] CEL is a good surrogate for argpyrimidine because it represents a comparable fraction of the total quantity of AGEs formed with GA and MGO (approximately 1 – 2%).[16, 17]

Collagen fibers taken from 6-week and 6-month old mouse tail tendons incubated with 0.1 mM MGO and 0.5 mM show a dose-dependent rise in AGEs as indicated by levels of CEL (Figure 3). The tendons of 6-month old mice are less susceptible to glycation (μmol CEL / mol lysine = 291 ± 8 for 0.1 mM MGO and 544 ± 100 for 0.5 mM MGO) than 6-week old mice (μmol CEL / mol lysine = 550 ± 9 for 0.1 mM MGO and 1265 ± 68 for 0.5 mM MGO). The maturation of lysyl oxidase-derived crosslinks into trifunctional crosslinks, etc. and the formation of glucosepane crosslinks (the primary AGE thus far identified *in vivo*) appear to block potential glycation sites.[18]

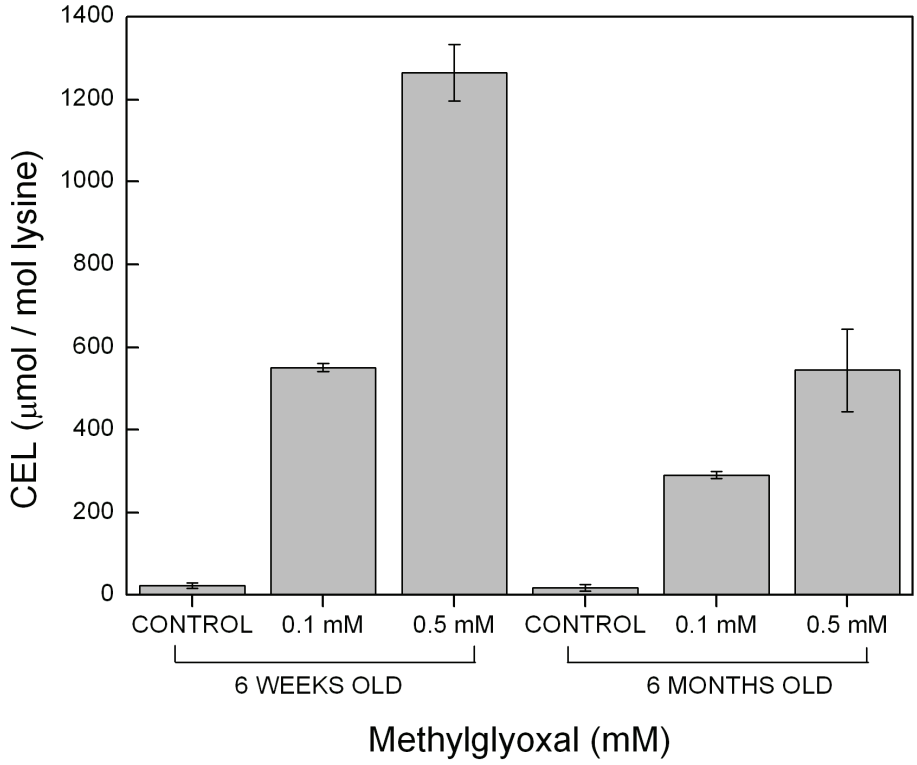

Figure 3. CEL increases with increased exposure to MGO. The effect on young tendon is stronger than the effect on old tendon.

The highest level of glycation achieved (AGEs / mol lysine) in 6-week old tendons is an order of magnitude lower than the glycation achieved in corneas treated with 2% GA. The reason we examine the collagen fibers at a lower level of AGE accumulation is that collagen fibers become stiff and fragile when glycated to the level of the corneas in Chapter 5 (V. Monnier, personal communication). We will return to this observation in the context of quantitative measures of glycation-induced changes in the mechanical properties of collagen fibrils.

6.4 Mechanical Impact of Glycation on Mouse Tail Tendons

The complex stress-strain behavior of collagen fibers under uniaxial extension is well documented, precluding application of a simple viscoelastic model or characterization by a single parameter.[4] Nevertheless, a comparison of the entire stress-strain curve of glycated vs. unglycated fibers, from the strain-free state through failure, does reveal significant mechanical changes that accompany AGE formation (Figure 4). With increasing strain, four regimes may be identified: an initial, linear regime in which the apparent stress increases gradually and proportional to the strain; a transition to nonlinear behavior in which the fibers exhibit strain-hardening; a pseudo-linear regime with an effective modulus much greater than the linear one; and lastly, yield and failure.

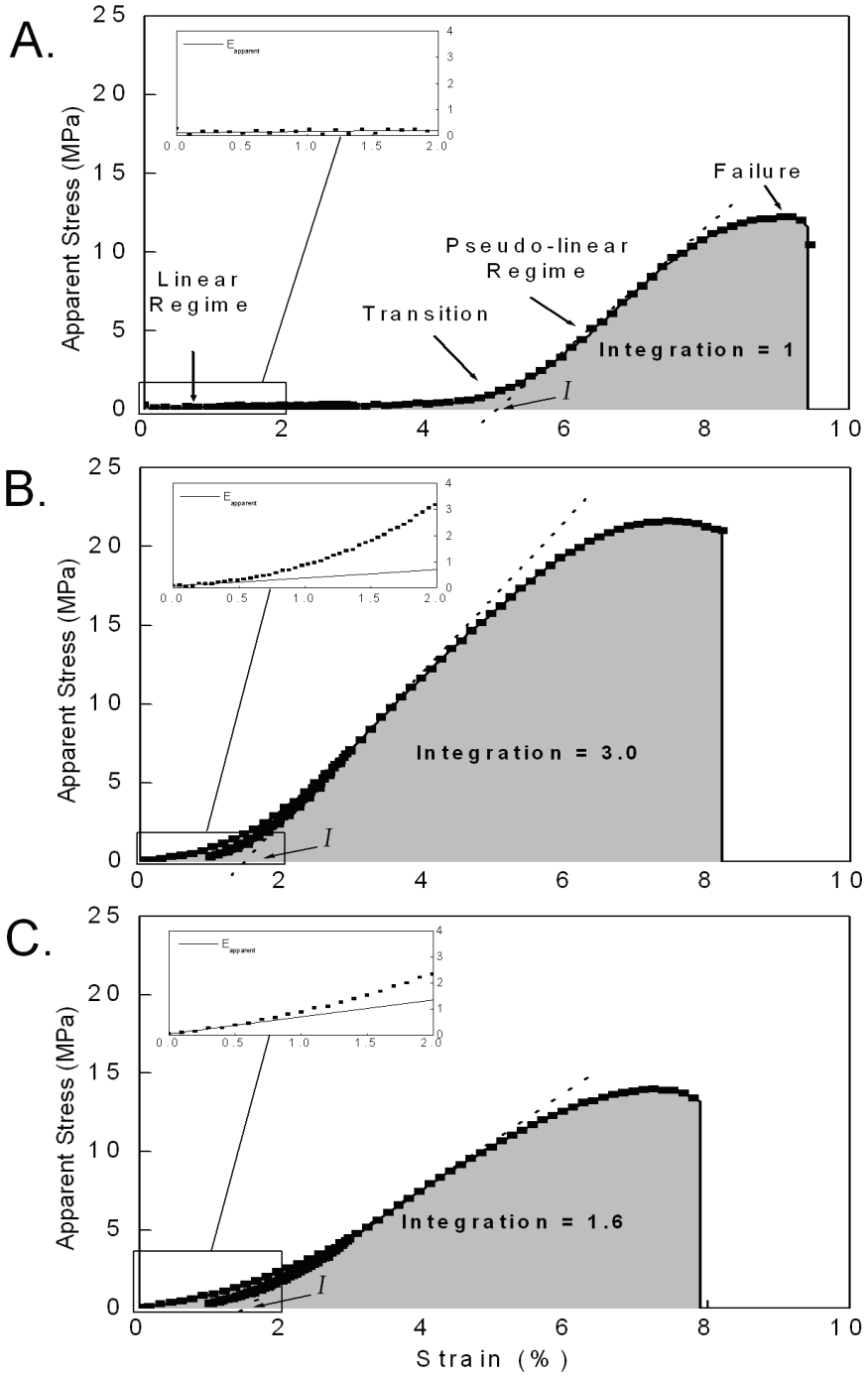

Figure 4. Uniaxial extension stress-strain plots representative of 6 mo. collagen fibrils treated with PBS (A.), 0.1 mM MGO (B.) and 0.5 mM MGO (C.). The four characteristic regimes are: linear, transition, pseudo-linear, and failure. Glycation shortens the linear regime (shifting the x-intercept, "I") and increases the "integration," of the stress-strain relation (toughness). 6 week and 6 month results were similar except where embrittlement became apparent on 6 week tendons treated with 0.5 mM MGO.

The initial linear regime of the stress-strain curve is often lumped together with the transition regime and referred to as the "toe region."[4, 19] Though it is accepted that this toe region is indicative of normal physiological strains, it is not normally analyzed because variability in tissue handling can lead to inconsistent Young's modulus measurements.[20] Instead, most investigations precondition fibers at low strains and focus on the properties of the pseudo-linear regime. Preconditioning is designed to give the fibers a well-defined, reproducible flow history.[4, 19]

We are interested not only in the behavior of the fibers under high loads, but also under physiologically-relevant loads; therefore, we focus on both the linear and the failure regimes. While true moduli cannot be obtained from the linear regime, *relative* differences in this regime can be used to assess the mechanical impact of different levels of AGE accumulation. At the small strains encountered *in vivo*, the treated fibers already show nonlinear, strain-hardening behavior and substantial hysteresis that do not set in until much higher strains for unglycated samples (Figure 2). The most significant glycation-induced changes in mechanical properties appear to be an increase in apparent modulus at low strain amplitude, a loss of extensibility, and an increase in toughness. In the present work we show that all of these parameters are significantly affected by glycation, but that the dependence is nonlinear at high levels of AGE accumulation.

Low-Strain (Young's) Modulus

The apparent modulus, $E_{apparent}$, in the low-strain regime increases significantly with glycation (Figure 5, A). The apparent modulus of fibers increased by ~ 500% for both 6-

week old mice, $E_{apparent}$ = 0.11 ± 0.1 (PBS control) to 0.61 ± 0.04 MPa, and 6-month old mice, $E_{apparent}$ = 0.08 ± 0.02 (PBS control) to 0.58 ± 0.17 MPa, when glycated with 0.5 mM MGO. As mentioned above, this low-strain behavior is particularly significant because it reflects physiological conditions. It has been argued that strain-hardening occurs at the upper limit of the normal physiological range of motion for collagen fibers.[4]

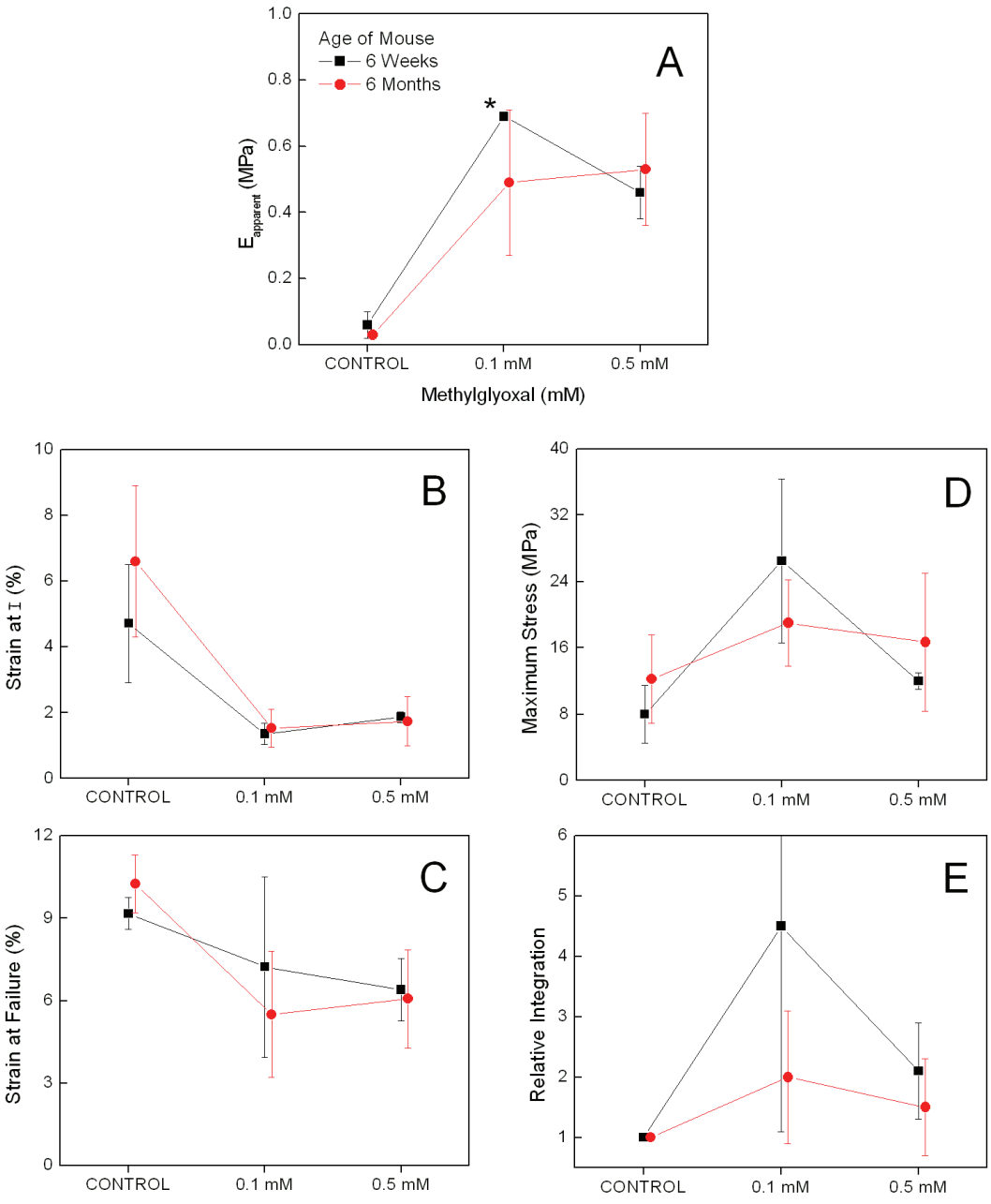

Figure 5. Key changes in the mechanical properties of mouse tail tendon fibers as a function of glycation: (A) Young's modulus increases with moderate glycation (0.1 mM MGO) but does not increase with additional glycation (0.5 mM MGO). (B) The linear regime shrinks, shifting the transition regime to lower strains. (C) Failure strain decreases monotonically with glycation in the 6 week fibers, while the effect saturates at 0.1 mM MGO in 6 month fibers. (D) "Ultimate tensile strength" (UTS), or stress at failure, of 6 mo. fibers increases with glycation but saturates at 0.1 mM MGO, 6 week fibers are strengthened with 0.1 mM MGO but become brittle with additional glycation. (E) Toughness (area under the stress-strain curve) increases with moderate glycation but falls with excessive crosslinking.

Loss of Extensibility

Glycation also correlates with a reduction in extensibility (Figure 5, B and C). The loss of extensibility is accompanied by a narrowing of the low-strain regime as the transition from linear to pseudo-linear behavior broadens and shifts to lower strains (Figure 4). To quantify this shift to lower strain, we extrapolate the line tangent to the high-strain regime down to its x-intercept "*I*" (Figure 4). On average, the maximum slope of the tangent line changes little with treatment condition or mouse age; thus, *I* is a good indicator of extensibility loss. Glycation reduces *I*, however, the effect appears to saturate (Figure 5, B).

Failure Strain and Stress

Loss of extensibility is also manifest in the reduction in the maximum strain attainable before failure (Figure 5, C). When pushed to the point of failure, untreated tendons generally stretch to ~ 10% strain before breaking (consistent with the literature[4, 19]), whereas glycated tendons predominantly break at 6 – 7% strain. Fibers from 6-week old mice showed a monotonic, dose-dependent reduction in failure strain, whereas the effect was saturated in mature fibers with 0.1 mM MGO. It is likely that enzymatic crosslinks and preexisting glycation-related modifications reduce the number of AGEs required to decrease extensibility. Enzymatic crosslinks alone, however, are insufficient to reduce extensibility and may even increase it: in the absence of further glycation, the average extensibility of mature fibers is larger than immature fibers. The increase in failure stress (ultimate tensile strength, or UTS) that accompanies enzymatic crosslinking is also seen for moderate glycation (Figure 5, D).

Collagen fibers from 6 month old mice are capable of bearing significantly higher loads than fibers from 6 week old mice; however, moderate levels of glycation reverse their relative failure stresses. The average failure stress of untreated 6 month fibers is ~ 50% higher than 6 week fibers. Moderate glycation (0.1 mM MGO) causes a much smaller increase in failure stress (60% rather that 230%) in the mature fibers, apparently due to the presence of enzymatic crosslinks. Enzymatic crosslinks (formed primarily on Lys residues[21]) appear to prevent the accumulation of excessive crosslinks, which appear to cause fibers to become brittle. Failure stresses of 0.5 mM MGO-treated mature fibers were not significantly different from 0.1 mM specimens; however, the failure stress of immature fibers decreased by over 50% relative to 0.1 mM treatment. An increase in failure stress with moderate crosslinking followed by a decrease at high levels of crosslinking is also consistent with prior literature.[19]

The combination of changes in failure stress and strain results in changes in the toughness of the fibers. Toughness is characterized by the integration of the area beneath the stress-strain curve and is an indicator of the total strain energy stored in the sample (Figure 4). Variability in the shape of the stress-strain curves from one fiber to another made the failure point difficult to define (some fibers failed with a sharp reduction in stress, while others failed more gracefully). Therefore, the integration was performed up to the strain at the peak stress. The toughness of 6 month fibers increased by an average factor of x2 when treated with 0.1 mM MGO, but only x1.4 when treated with 0.5 mM MGO (Figure 5, E). The effect on 6 week fibers was even more dramatic, increasing the toughness by an average factor of x4.4 with 0.1 mM and x2 with 0.5 mM treatment. Tendons treated with

0.5 mM MGO severed at the edge of the clamps more frequently upon loading and during tensile-strength experiments, further implicating brittleness as the reason for reduced toughness compared with 0.1 mM-treated fibers (Figure 5, D). Graphically, the increase in integration appears to reflect the shortening of the low-strain regime combined with the lengthening of the long, nonlinear high-strain regime. These increases in the magnitude of the stress are sufficient to counter balance the reduction in area that results from the reduced failure strains, even in 0.5 mM specimens. From a physical perspective, this indicates that glycation causes the collagen fibers to resist small deformations with greater force and that the fibers can withstand high stresses over a wider range of strains.

Differences in the mechanical properties of collagen fibers treated with 0.1 mM vs. 0.5 mM MGO are either insignificant (strain at *I*, $E_{apparent}$) or somewhat deleterious (decreasing failure strain, maximum stress, and toughness), in spite of the fact that the concentration of AGEs is much higher for the 0.5 mM treatment (Figure 3). Clearly, the mechanical properties of collagen fibers are not linearly dependent upon the quantity of AGEs. There is likely a dose-dependent regime, but it appears that treatment with 0.5 mM MGO is well beyond that regime. The relative lower amount of CEL incorporated into the 6 month tendons and relative saturation of the physical endpoints at 0.1 mM MGO may not only reflect preexisting blockage of lysine residues at 6 months of age, but also kinetic differences in rates of CEL adduct formation vs. MGO-mediated crosslinks (Figures 3-5). A comprehensive assay of all modifications would be needed to precisely understand the relationship between crosslink formation and changes in modulus, etc; unfortunately, at the present time only a few of the MGO-derived crosslinks are known.

Glycation with 0.5 mM MGO appears to be so extensive that collagen fibers (particularly immature fibers) become somewhat brittle. This may be a result of the way in which stress is distributed in the fiber. Under normal conditions, stress on a fiber is distributed among all of the fibrils and collagen triple helices. When sufficient stress is applied that a triple helix breaks, in the absence of AGEs it does so independent of the other helices. The stress released by the break is then redistributed across the rest of the helices in the fibril. However, if the helix is tightly crosslinked to a neighboring helix, the stress released by the break in the first is transferred directly to its neighbor. Further, the added stress will be localized to the portion of the helix that lies between its crosslinks to the first fibril. The localized stress could then break the second helix, releasing yet more strain energy and allowing the failure to propagate like a crack.

Our findings call into question the oft-repeated assertion that the source of glycation-induced tissue stiffening is intramolecular crosslinking within or between the triple helices of a collagen fibril:[11, 22, 23] at levels of glycation in which the mechanical properties of collagen fibers saturate or decline, the properties of softer collagen-rich tissues (e. g. cornea) continue to increase. The bulk properties of highly-glycated tissues such as cornea do not reflect the brittle state of their constituent fibrils; therefore, stiffening must be more complex than previously assumed.

6.5 Proteoglycans May Play a Role in Tissue Stiffening

Correlations between AGE accumulation, tendon breaking time, and changes in bulk mechanical properties have been used to argue that glycation-induced tissue stiffening is

the direct result of crosslinks that strengthen collagen fibrils;[11, 13, 24] however, we believe this explanation of glycation-induced tissue stiffening to be incomplete. We propose that significant changes in the mechanical properties of soft collagenous tissues such as the cornea must also involve changes in the matrix surrounding the fibrils—most likely via collagen-proteoglycan and proteoglycan-proteoglycan crosslinks.

The Correlation between TBT and Tissue Stiffening is Insufficient to Establish Causality

In the TBT assay, hydrogen bonds and hydrophobic interactions are dissolved in the 7 M urea. This leaves only the covalent crosslinks (enzymatic + AGEs), which are insufficient to keep unglycated fibrils intact under the 2 g load traditionally applied (a 2 g load corresponds to ~ 2.5 MPa for a 100 um fibril).[11] Thus, under denaturing conditions, as AGE crosslinks accumulate, fibril breaking times increase. Under nondenaturing conditions, however, we found that immature fibers (age 6 weeks) with similar diameters to those used in published TBT studies were capable of withstanding loads of no less than 6 g and that mature, unglycated fibers (age 6 months) were capable of withstanding loads up to 20 g in PBS at 37˚ C. Thus, retention of their noncovalent bonds increased the load capacity of the collagen fibrils by a factor of 3 at least. Clearly, noncovalent bonds are responsible for most of the mechanical strength of collagen fibrils *in situ*.

It was assumed that TBT was a good indicator of age-related increases in collagen stiffness *in situ* because of the observed correlations between AGE accumulation and TBT[12, 15, 24] and between AGE accumulation and tissue stiffening.[13, 25, 26] However, we have shown that high levels of AGEs can accumulate (known to increase TBT) without increasing tensile

strength. The assumption that glycation was linearly correlated with increased fiber strength *in situ* resulted in the further assumption that increase in fibril strength was related to the increase in tissue stiffness.[26, 27] In light of our axial extension results, however, it becomes necessary to consider alternative explanations for glycation-induced tissue stiffening at moderate to high levels of glycation.

The Role of Proteoglycans in Glycation-Induced Corneal Stiffening

With knowledge of the relationship between glycation levels and collagen tensile strength, we can estimate the contribution of fibril changes to changes in the cornea as a whole. All mechanical measurements of treated tail tendons indicate that the effect of moderate glycation (treatment with 0.1 mM MGO) is significant; however, glycating with 0.5 mM creates 2 – 3 times more AGEs without a significant increase in mechanical strength. By comparison, corneas treated with 1% GA and 2% GA contain x15 and x30 times more AGEs than the highest levels measured in 0.5 mM MGO-treated collagen fibers, respectively. Thus, if glycation-induced stiffening of cornea were due primarily to stiffening of collagen fibrils, our mouse tail results would predict that 1% GA would already be well into the saturated regime so there would be no further increase in the modulus of corneas treated with 2% vs. 1% GA. This prediction is incorrect: the shear modulus of porcine corneas increased by ~ 300% upon treatment with 1% of GA and by 600% after treatment with 2% GA.

The natural geometry of corneal collagen guides our interpretation of the observed changes in modulus (Figure 1).[3, 28] As stated previously, the major structural element of the cornea

is the stroma, which is composed of lamella of oriented collagen fibrils. We hypothesize that glycation stiffens the cornea and other collagenous tissues primarily by crosslinking fibrils to their associated proteoglycans, which would restrict the translation of collagen fibrils rather than reinforcing/stiffening them. Because the lamellae are believed to compose distinct planes that span the entire cornea,[2] shear modulus measurements on the corneal buttons are analogous to twisting a stack of papers: the resistance to deformation will depend more upon the friction between the sheets of paper than on the moduli of the sheets within the stack. Glycation may "glue the sheets (lamellae) together" in addition to stiffening the fibrils within the sheets.

Using rheometry in combination with biochemistry we have shown that the accumulation of collagen fibril AGEs and increase in TBT at high levels of glycation do not correlate with stronger collagen fibrils. Thus, the simultaneous rise of cornea modulus and accumulation of AGEs on collagen should not be taken to imply causality. Applying the tools of biomechanics to glycated tissue has allowed us to clarify this mechanistic aspect of glycation-induced tissue stiffening.

More generally, our findings suggest that proteoglycans and other apparently nonstructural tissue components may play a more significant role in the biomechanics of healthy and diseased soft collagenous tissues than previously thought. As noted in Chapter 3, the cartilage community is already aware of the importance of proteoglycans in joints; however, other research communities (such as the glycation and ocular biomechanics communities) have yet to explored the biomechanical role of PGs. We hope that this work will help bridge the gap between these fields and bring the tools and understanding of each

to the other. This may be of particular importance in developing novel therapeutics for treating glycation-induced pathologies related to skin[6, 29, 30] and cardiovascular tissues[25, 31, 32] in diabetes and aging.

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Appendix A – Internal Review Board letter of approval for use of human tissue

CALIFORNIA INSTITUTE OF TECHNOLOGY
Committee for the Protection of Human Subjects

Principal Investigator(s): Dr. Julie Kornfield with Charles Nickerson (graduate student)

Title of Project: Exploration and Engineering of the Chemical and Mechanical Properties of the Vitreous Humor

Funding Agency (if applicable)/identification number: Vitreo Retinal Technologies, Inc.

The above application:

☒ is judged to be exempt under paragraph(s) Sec.101(b)(4). Once ratified, no further interaction with the Committee is necessary unless there is a change in protocol affecting the human participants in this research and/or the project terminates.

☐ was provided with expedited review and approval on _____ with the following qualifications:

_____.

This project will be submitted to the Full Committee for ratification at its next meeting scheduled for Fall 2003. Unless exempt, it will be reviewed annually, but in any event, changes in protocol are to be submitted for further review and approval and the Committee must be advised should any complications arise that adversely affects, or has the potential to adversely affect, the human subjects. As applicable, a current copy of any partner organization's IRB approval must be obtained for this Committee's files.

We certify that this review was carried out in accordance with the requirements of Part 46, "Protection of Human Subjects" of Title 45 of the Code of *Federal Regulations* and the "U.S. Department of Health and Human Services (DHHS) Federal-Wide Assurance (FWA) for the Protection of Human Subjects for Domestic (U.S.) Institutions," California Institute of Technology Assurance #FWA00003897.

Name: Charles R. Plott

Title: Chairman, Committee for the Protection of Human Subjects

Signature: Charles R Plott Date: July 14, 2003

* * * * * * * * * * * *

Name: Richard P. Seligman

Title: Senior Director, Office of Sponsored Research

Signature: Richard P. Seligman Date: July 15, 2003

cc. HSC Files

Appendix B – Experimental Protocol for in vivo tests in Mexico

Eye Test Protocol

Proposed Initial experiments for in vivo studies in Mexico City

Summary of Experiment

Quantify the degree of liquefaction in pig eyes 1 and 15 days after injection with 100 ul of 12% carbamide solution pH~6. (8 animals required) and with 100 ul of 24% carbamide solution pH ~9 (3 animals required) compared to untreated animal eyes (2 animals required). Total = 13 animals required.

Experimental Protocol

Animals #1, #2, #3, and #4 – On day 1 (September 7) treat L. eye with 12% carbamide, pH ~6, R. eye with 0.9% saline, slaughter 15 days later (day 16-Sept. 23): Animal #H1 and #H2- On day 1 treat L. eye with 24% carbamide, pH ~9, R. eye with 0.9% saline, slaughter 15 days later (day 16-Sept. 23).

Animals #5, #6, #7, and #8 – On day 14 (September 21) treat L. eye with 12% carbamide, pH ~6, R. eye with 0.9% saline, slaughter 1 day later (day 15-Sept. 22): Animal #H3- On day 14 treat L. eye with 24% carbamide, pH ~9, R. eye with 0.9% saline, slaughter 1 day later (day 15-Sept. 22).

Animal #9 and #10 – On day 15 (September 22) slaughter animals (to give the opportunity for final instrument check and test runs)

Tests to run

Day 15 (Sept. 22) – I will test the rheology of the 1-day treatment eyes (animals #5-#8 and #H3) and the untreated eyes (animal #9&#10). Please have a clinician test the IOP of the animals prior to slaughter and try to gauge liquefaction using their methods.

Day 16 (Sept. 23) – I will test the rheology of the 15-day treatment eyes (animals #1- #4, #H1, and #H2). Again, please have a clinician test the IOP and liquefaction of the animals prior to slaughter.

Daily Schedule:

Day	1 (Sept. 7)	14 (Sept. 21)	15 (Sept. 22)	16 (Sept. 23)
Treatment	Animal #1-#4- L. eye 12% urea, pH ~6 R. eye saline Animal #H1&#H2 L. eye 24% urea, pH ~9 R. eye Saline	Animal #5- #8- L. eye 12% urea, pH ~6 R. eye saline Animal #H3 L. eye 24% urea pH ~9 R. eye Saline		
Collection			Animals #5- #10 & #H3 - Please check IOP & liquefaction then sacrifice the animals	Animals #1- #4, #H1 & #H2 - Please check IOP & liquefaction then sacrifice the animals

* Note: All injections should be 100 µl. I will arrive on day 13 and leave on day 17

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