Structural Factors That Mediate Scleral Stiffness

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PURPOSE. The intent of this study was to correlate measures of structurally relevant biochemical constituents with tensile mechanical behavior in porcine and human posterior sclera.

METHODS. Posterior scleral strips 6 × 25 mm were harvested from 13 young porcine and 10 aged human eyes and stored frozen at −20°C. Mechanical hysteresis from 10 consecutive load cycles to a peak stress of 1.0 MPa was recorded via a custom-built soft tissue tester. In a parallel study, tissue adjacent to the mechanical test specimens was apportioned for each of five assays measuring: total collagen content, nonenzymatic cross-link density, elastin content, glycosaminoglycan content, and water content.

RESULTS. The average porcine scleral modulus at 1% strain was 75% less than that measured for human tissue (0.65 ± 0.53 MPa versus 2.60 ± 2.13 MPa, respectively; P < 0.05). However, the average strain energy absorbed per loading cycle was similar (6.09 ± 2.54 kJ/m3 vs. 5.96 ± 2.69 kJ/m3) for porcine and human sclera respectively; (P > 0.05). Aged human sclera had relatively high fluorescence due to nonenzymatic cross-link density (2200 ± 368 vs. 842 ± 342; P < 0.05) and low hydroxyproline content (0.79 ± 0.17 μL/mL/g versus 1.21 ± 0.09 μL/mL/g; P < 0.05) while other measured biochemical factors were statistically similar (P > 0.05).

CONCLUSIONS. Aged human tissue had superior mechanical stiffness despite reduced collagen content, partially because of the accumulation of nonenzymatic cross-links. Differences in collagen content and cross-link density either had no effect or offsetting effects on the ability of the tissues to absorb strain energy. (Invest Ophthalmol Vis Sci. 2008;49:4232–4236) DOI: 10.1167/iovs.08-1970

A pproximately one third of the world’s population has a suboptimal eye shape that impairs visual acuity and can spur the growth of defects in the choroid and retina. Since eye shape is defined by scleral mechanical properties, their changes have been linked to the pathogenesis of several disease states, the most prevalent of which is progressive myopia. During myopization, a retinal–scleral photomechanotransduc-
weighed for each of five assays to be performed later, measuring water content, elastin content, glycosaminoglycan content, hydroxyproline content, and cross-link density. Strips for mechanical testing were stored at −20°C, along with adjacent globe tissue for biochemical assays.

Mechanical Testing

A quasistatic (\(\frac{\text{d}e}{\text{d}t} < 0.001/\text{s}\)) uniaxial tensile test was used to measure mechanical response to loading. Before the test, tissue stored at −20°C was removed from the freezer, kept sealed in a plastic bag and allowed 1 hour to defrost. Evidence suggests that a moderate storage time of 3 days does not significantly affect results from stress relaxation experiments conducted on rabbit sclera.\(^{12}\) To further bolster our confidence that a moderate frozen storage time in a sealed bag would not significantly affect results, we conducted a pilot study in which three porcine sclera specimens and one human specimen were mechanically tested within 2 days of death and then tested again after a week of frozen storage (at −20°C). We found that differences in mechanical results for each tissue specimen were statistically nonsignificant. From this evidence we made the assumption that our storage regimen that allowed up to 1 week of frozen storage would have a minimal effect on relative mechanical properties.

Average specimen thickness in the central portion of the specimen to be mechanically tested was determined by taking the mean of five readings in different locations near the center of the specimen with an ultrasonic pachymeter (Carl Zeiss Meditec, Inc., Dublin, CA). This number was then used to approximate the specimen’s cross-sectional area. A \(3 \times 3\) mm grid of nine uniformly spaced strain targets was made to adhere to the tissue by blotting it dry and using a minimal amount of cyanoacrylate. We determined average tissue strain in the direction of loading by the method described by Bass et al.\(^{12}\) Specimens kept moist at room temperature (23°C ± 1°C) with physiologic saline were cyclically loaded to a peak engineering stress of 1.0 MPa for 10 cycles. Although this peak stress is well above physiologic stress levels, pilot testing showed that results at this stress level were repeatable and therefore assumed to be nondamaging. Choosing a relatively high level of peak stress allowed observation of an expanded range of the stress-strain curve.

The custom soft tissue tester and experimental protocol were adapted from previous tensile experiments conducted on the annulus fibrosus.\(^{13}\) Briefly, the custom testing apparatus consisted of the following: acrylic and aluminum grips designed to hold strips of sclera without significant out-of-plane compression, a precision force transducer (25 N Load Cell Model LCCA; Omega Engineering, Stamford, CT) to record forces, and a computer-controlled imaging system to calculate the strains in real time. Algorithms were adapted from software (Labview and IMAQ Vision; National Instruments, Austin, TX) that captured images of the specimen and strain targets; generated a contrast-thresholded image and located the targets within the view frame; calculated the average strain in the direction of the applied force; and calculated engineering stress as the load reading normalized by the estimated cross-sectional area (average thickness from pachymeter readings \(\times\) initial width before test).

The potentially confounding effect of residual bending stresses due to differences in thickness and curvature of each eye was mitigated by establishing a reference state at the beginning of each test and by placing strain targets only on the external outermost surface (as opposed to inserting strain marking pins through the full thickness of the sclera). The reference state was established for each specimen by loading the tissue in the grips under slight compression initially and then adjusting the grip location until a slight tensile load of 0.01 MPa was measured. Once this reference state was established, the initial distances between strain targets were recorded, and the preconditioning routine was allowed to proceed.

Data Analysis

The first nine load cycles preconditioned the tissue such that a stable response to loading could be achieved. Samples were considered properly preconditioned if the peak strain of the final preconditioning cycle was within 2% of the previous cycle. According to the method described by Fung,\(^{14}\) a continuous exponential curve of the form \(\sigma(\varepsilon) = A/B(e^{B\varepsilon} - 1)\) was fit to the discrete stress \((\sigma)\) versus strain \((\varepsilon)\) data (Fig. 2).\(^{14}\) For each sample, distinct loading, and unloading coefficients were optimized using a Levenberg-Marquardt algorithm such that a minimum of 90% of the variation in the data could be explained by the curve fit. In addition, a simplistic parameterization of modulus \((E)\) was reported as the derivative of the fitted loading curve at 1% strain, the upper end of what was identified by Downs et al.\(^{15}\) to be a physiologic range of strains. Finally, the strain energy absorbed on a load cycle was reported. The area contained within the hysteresis loop delimited by the loading and unloading curve fits gives a measure of strain energy absorbed \((W)\) on a loading cycle in SI units of Joules per

![Figure 2](https://iovs.arvojournals.org/2008/49/10/F2.png)
cubic meter. Practically, this parameter was determined by calculating the difference between integrals of the loading curve fit, \(\sigma_u(e)\), and the unloading curve fit, \(\sigma_l(e)\), up to the peak strain, \(e_{\text{max}}\).

\[ W = \int_{0}^{e_{\text{max}}} \sigma_u(e)\,de - \int_{0}^{e_{\text{max}}} \sigma_l(e)\,de. \tag{1} \]

Biochemical Assays

Five biochemical assays were run to determine water content, elastin content, sulfonated glycosaminoglycan content, hydroxyproline content, and nonenzymatic cross-link density.

Water Content. Scleral samples adjacent to the mechanical test specimen were removed for assays. Each biochemical sample was weighed after extraction to establish a wet weight. The sample was then minced to increase exposed surface area. The set of samples from each tissue was deposited into a test tube. The tubes were laid horizontally and uncapped in a 60°C oven. After 48 hours, the tissue samples were weighed, establishing a dry weight. Before-heating and after-heating weight measurements were used to determine the wet-to-dry ratio.

Elastin Content. An elastin assay and procedure (Fastin; Bio-color Ltd., Newtownabbey, Northern Ireland, UK) were used to measure elastin content via a dye-binding method. Insoluble elastin was extracted from sclera in a soluble elastin form using a 0.25-M oxalic acid digest. Bound dye was quantified using absorbance readings from a plate reader (Molecular Devices Corp., Sunnyvale, CA). The measured amount of elastin in tissue samples was determined as a percentage of tissue dry weight according to the known tissue wet weight and water content.

Glycosaminoglycan Content. Glycosaminoglycan content, hydroxyproline content, and cross-link density were assessed for each tissue sample using a papain digest and distinct assay protocols. Tissue samples were cut from the posterior globe to obtain a wet weight of 10 to 25 mg. The wet weight was recorded, and each tissue was minced to 25 mg. The sample was then minced to increase exposed surface area. The set of samples from each tissue was deposited into a test tube. The tubes were laid horizontally and uncapped in a 60°C oven. After 48 hours, the tissue samples were weighed, establishing a dry weight. Before-heating and after-heating weight measurements were used to determine the wet-to-dry ratio.

Hydroxyproline Content. After the papain digest, the total hydroxyproline (HPr) content of each tissue sample was determined with the Woessner assay.16 HPr content was normalized by sample dry weight.

Nonenzymatic Cross-link Density. Fluorescence of the papain digest supernatant is an indicator of nonenzymatic cross-link content.17–19 Nonenzymatic, glycation-type cross-links form as the result of the attachment of the carbonyl group of glucose, followed by a ketoamine rearrangement. The cross-links formed due to the Maillard-type reactions that follow then yield fluorophores characteristic of a reaction of a sugar with a protein. To measure fluorescence, a 100-μL aliquot of each papain supernatant was removed to a black ELISA plate, and fluorescence was measured in the aforementioned plate reader at an excitation wavelength of 370 nm with emission sensitivity set at 440 nm.

RESULTS

The porcine and human samples were not different in water content, s-GAGs, and elastin (Table 1; \(P > 0.13\)). The mean hydroxyproline content in aged human tissue was 53% less than that of porcine tissue (0.79 ± 0.17 vs. 1.21 ± 0.09; Table 1; \(P < 0.05\)). Mean fluorescence due to nonenzymatic cross-links for human sclera was 1.6 times that of porcine sclera (2200 ± 368 vs. 842 ± 342; Table 1; \(P < 0.05\)).

Human sclera had a modulus \(E\) roughly three times that of porcine tissue (2.60 ± 2.13 vs. 0.65 ± 0.53; Table 2; \(e = 1.0\%\), \(P = 0.03\)). Despite this difference in the stress-versus-strain profiles, the average strain energy absorbed on a load cycle \(W\) was very similar for each group; 5.96 ± 2.13 kJ/m3 for human sclera and 6.09 ± 2.54 kJ/m3 for porcine sclera. Human tissue tended to have a higher modulus while retaining its ability to absorb strain energy (Fig. 3).

DISCUSSION

We hypothesized that collagen architecture and associated cross-links define sclera biomechanical behavior. Our data indicate that the principal determinant of scleral stiffness is collagen cross-linking: strikingly, while aged human sclera contained significantly less collagen than the porcine sclera, it achieved superior stiffness via increased collagen cross-link density. It has been established that, in mammals, nonenzy-

### Table 1. Biochemical Results Summary

<table>
<thead>
<tr>
<th></th>
<th>H2O</th>
<th>s-GAG</th>
<th>Elastin</th>
<th>HPr*</th>
<th>NE x-Links*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine ((n = 13))</td>
<td>3.29 (0.23)</td>
<td>4.65 (0.53)</td>
<td>8.20 (1.90)</td>
<td>1.21 (0.09)</td>
<td>842 (342)</td>
</tr>
<tr>
<td>Human ((n = 10))</td>
<td>3.56 (0.75)</td>
<td>6.09 (1.29)</td>
<td>7.20 (1.80)</td>
<td>0.79 (0.17)</td>
<td>2200 (368)</td>
</tr>
</tbody>
</table>

H2O given as (wet wt./dry wt.); s-GAG & HPr in μL/mL digest/dry wt.; Elastin in % dry wt.; N.E. x-links in fluor/HPr.

* Statistically significant difference between groups \((P < 0.05)\).

### Table 2. Mechanical Results Summary

<table>
<thead>
<tr>
<th></th>
<th>(E^*)</th>
<th>(W)</th>
<th>(A(\text{Load})^*)</th>
<th>(B(\text{Load})^*)</th>
<th>(A(\text{UnLoad})^*)</th>
<th>(B(\text{UnLoad})^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine ((n = 13))</td>
<td>0.65 (0.53)</td>
<td>6.09 (2.54)</td>
<td>1.01 (1.63)</td>
<td>42.54 (17.49)</td>
<td>0.35 (0.53)</td>
<td>57.53 (23.15)</td>
</tr>
<tr>
<td>Human ((n = 10))</td>
<td>2.60 (2.13)</td>
<td>5.96 (2.69)</td>
<td>2.23 (1.71)</td>
<td>34.24 (22.20)</td>
<td>1.16 (1.04)</td>
<td>45.00 (25.64)</td>
</tr>
</tbody>
</table>

\(E\) given in MPa; \(W\) in kJ/m3; \(A\) and \(B\) are unitless mean coefficients from \(\sigma = \frac{A}{B} (e^p - 1)\) curve fit.

* Statistically significant difference between groups \((P < 0.05)\).
mature cross-links accumulate with age and cause stiffening, and this may be the predominant factor mediating scleral stiffness in aged humans. The human sclera tended to have a higher modulus than the porcine sclera while having a comparable ability to absorb strain energy at a low strain rate. This suggests that sclera can have a relatively high cross-link density and retain its ability to absorb energy.

This study bolsters a shortage of published information on human scleral mechanics. Curtin reported on the importance of a wide-angle collagen weave and swelling as factors that rendered the human posterior sclera more extensible than anterior sclera. The histologic analysis of microstructure in this study, although interesting, provided no quantifiable biochemical data. Avetisov et al. advanced the field of sclera research by reporting fundamental trends in soluble collagen content and tensile strength with age. They noted a strongly pronounced inverse correlation between the soluble collagen fraction and the modulus of elasticity. Our data support trends identified by Avetisov et al. and provides a more rigorous quantification of how biochemical constituents may influence stiffness.

In addition to expanding data available from human sclera, our study showed how human data may compare with data from an animal model. Many animal models have been used in recent scleral research, including chick, monkey, rabbit, pig, and tree shrew. Clinically, these models are valuable because of the assumption that similar constituents and similar biological activity in an animal model may have results that are translatable to humans. To date, this assumption has not been adequately evaluated, as there are no previous parallel studies that offer data from both animal and human sclera. Our study indicates that juvenile animal models likely have lower nonenzymatic cross-link density that ought to be taken into account for appropriate comparisons with elderly human eyes. The human sclera data presented herein may help contextualize data from other animal models.

Future research calls for a more rigorous biochemical characterization of both human and animal sclera. In this study, we assumed that potential postmortem enzymatic activity and the aforementioned storage regimen would not have a significant effect on the relative outcome of assays, although we saw no trends in our data indicating that differences in storage times had a pronounced effect on results. Nevertheless, our human tissue biochemical and mechanical data are limited by the fact that the human eyes were stored at 4°C for an average of 30 days before dissection. Further biochemistry determining the effect of postmortem tissue processing on structurally relevant biochemical constituents should be performed to strengthen our initial assumption.

Further work is also needed to quantify all types of cross-links present in various models. In this study, we quantified relative levels of nonenzymatic cross-links via fluorescence reading. This accounts only for the cross-links due to collagen’s inherent glycosylated lysine and hydroxylysine residues. As in most connective tissues, collagen in the mature sclera also undergoes cross-linking due to nonreducible hydroxyprolylinium residues. Our conclusions rely on the assumption that both enzymatic and nonenzymatic cross-links accumulate with age in a similar fashion and that neither plays a disproportionate role in the mechanics. This assumption should be verified, such that all the covalent bonds that serve to stabilize the collagen network can be evaluated. Further study focusing on the measurement of cross-links should be conducted with a pentosidine assay or high-pressure liquid chromatography techniques to quantify exhaustively the cross-links present in the tissue.

A question prompted by the data in this study is whether age-related cross-links accumulate in a manner that might serve to stabilize the myopic eye. Given that cross-linking seems to be a principal factor that determines scleral modulus, exogenous cross-linking strategies may be promising treatments for stabilizing the sclera. Indeed, several studies on this topic have been published, and others are under way. Conversely, it has been suggested in a vascular model of age-related macular degeneration that the stiffening of the sclera with age may spur macular degeneration. These studies highlight the growing need to understand how structural constituents, particularly collagen cross-links, influence mechanical properties.

In summary, accumulated collagen cross-links probably account for a significant increase in modulus in human tissue and suggest an effective means of modulating sclera properties. Animal model research viewed in the context of this and other human data may yield a more valuable clinical surrogate. Finally, further biomechanics research accounting for both enzymatic and nonenzymatic cross-links is needed.


