Differential Regional Stiffening of Sclera by Collagen Cross-linking

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Abstract

Purpose: Corneal collagen cross-linking by ultraviolet light activation of riboflavin has been used clinically to enhance corneal stiffness. We sought to determine if cross-linking differentially affects scleral regions.

Methods: Adjacent, parallel strips of sclera were cut from superolateral, superomedial, inferolateral, and inferomedial quadrants of posterior and equatorial sclera of 12 human cadaver eyes. One of each pair served as control while the other was cross-linked by immersion in 0.1% riboflavin and 365 nm exposure at 6mW/cm^2 irradiance for 30 minutes. Behavior of strips was characterized using a microtensile load cell. Preloaded strips were imaged using orthogonally mounted cameras and optical coherence tomography to determine specimen dimensions including cross-sectional area. Tension was measured during 0.1 mm/s constant rate elongation.

Results: Young's modulus (YM), the slope of the relationship relating tensile stress to strain, was calculated at 8% strain, and increased significantly after cross-linking (P<0.001). In posterior sclera, mean (± standard error of mean, SEM) YM increased in the superolateral, superomedial, inferolateral, and inferomedial quadrants by 46±15%, 32±11%, 67±20%, and 53±11%, respectively. In equatorial sclera, YM increased by 139± 43%, 68±27%, 143±92%, and 68±14%, respectively. The YM of pooled equatorial quadrants increased significantly more than that of the pooled posterior quadrants.

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Conclusions: Scleral collagen cross-linking by ultraviolet activation of riboflavin differentially increases scleral YM more in the equatorial than posterior sclera, and most in the lateral, equatorial sclera. Cross-linking might be used to arrest progressive myopia or to prevent staphyloma formation.

Keywords

biomechanics; collagen cross-linking; myopia; sclera; Young's Modulus

Introduction

Myopia affects around 30% of the population in the United States and Europe, but up to 60% in Asian countries,[1, 2] with myopic progression occurring in up to 50% of such cases.[3] Severe myopia, a leading cause of blindness worldwide, is associated with sight-threatening consequences such as retinal detachment and macular choroidal degeneration.[4] Pathological scleral thinning and localized ectasia often occur in high myopia.[5] Because biomechanical properties of the sclera influence globe shape and size,[6] scleral thinning may permit further globe elongation and progressive refractive error. Pathological scleral thinning involves structural scleral abnormalities such as decreased collagen fiber diameter,[7] deficiencies in collagen fibrillogenesis,[8] and impairment of collagen cross-linking (CXL).[9] Reversing these abnormalities would beneficially change scleral biomechanical properties and might serve as a treatment for myopic progression.

Cross-linking induced by riboflavin and ultraviolet-A light (UVA) can increase corneal rigidity to treat keratoconus[10] and has emerged as the favored initial treatment.[11–14] Riboflavin serves as a photosensitizer in CXL, forming of intra- and interfibrillar covalent bonds between collagen fibers when activated by UVA.[15, 16] The riboflavin also shields underlying tissues, such as the corneal endothelium and iris, from UV irradiation.[13] Cross-linking increases corneal Young's modulus (YM), a measure of stiffness, by about 4.5 fold and typically prevents progression of keratoconus.[17,18]

Since human sclera contains approximately 50% collagen by weight, primarily type I collagen[19] much like cornea, CXL can also be used to increase scleral stiffness. Wollensak conducted CXL using riboflavin and UVA and noted a significant increase in the YM of porcine (145%) and human (31%) sclera in vitro,[20] as well as in rabbit sclera (465%) in vivo.[21] This study, however, did not consider possible regional variation as a result of CXL. In a study by Wang, CXL of anteriorly to posteriorly oriented scleral strips in the equatorial and posterior regions produced 185% and 201% increases in YM, respectively.[22] However, since scleral thickness varies markedly between the equatorial and posterior regions,[23] scleral strips in this orientation necessarily have nonuniform thickness, and so cannot reflect likely regional variations in mechanical effects of CXL. In the present study, we investigated the effect of CXL on biomechanical strength and volume of circumferentially cut scleral strips in 8 scleral regions.

Methods

Specimen Preparation.

Institutional review board review is not required for the cadaveric material studied here that was obtained in conformity with applicable local laws. Two eyes were harvested by eye banks within 48 hours of death and were obtained unfrozen but stored at just above 0° C. Ten eyes were obtained from cadavers donated to medical research and had been previously frozen. In all eyes, circumferentially oriented, parallel adjacent scleral strips measuring 2×8mm were trimmed by scalpel from the superolateral, superomedial, inferolateral, and inferomedial quadrants of the equatorial as well as posterior sclera. Equatorial strips were cut between the rectus muscle insertions, while posterior strips were cut 1 mm from edge of the optic nerve. The eye was initially hemisected sagitally, separating medial and lateral portions. Scleral strips were then excised from 4 different regions in each of the halves. (Fig. 1). One strip of each identically-prepared parallel pair was used as a control while the other underwent CXL. The retina, choroid, and episcleral tissue were removed before other procedures.

Cross-linking.

Each treated strip was immersed in 0.1% dextran-free riboflavin for 30 min, and then exposed to 365nm UVA light at 6mW/cm^2 irradiance for 30 min using the LightLink CXL Corneal Cross-Link System (LightMed, San Clemente, CA). Riboflavin was applied drop wise onto the specimens every 30 s during CXL. To avoid dehydration, treated and control strips were kept in Ringer's solution in petri dishes placed on ice until tensile testing.

Tensile Testing.

Tensile testing was performed in a horizontally mounted load cell incorporating a precise train gauge (LSB200, FUTEK, Irvine, CA) with 5mN force resolution attached to a linear motor (Ibex Engineering, Newbury Park, CA) having 20nm distance resolution. Scleral strips were tested in an environmental chamber simulating physiological conditions at 36°C and 100% humidity for 5 minutes. Excess moisture was wiped away. Scleral strips were anchored in serrated clamps separated by 5 mm to set initial specimen length, and preloaded to 0.05 N tension to eliminate slack. Preloaded strips were photographed with two orthogonally mounted digital cameras (Canon 70D and Canon 5D) to determine specimen dimensions, and also imaged using optical coherence tomography (OCT, Thorlabs Inc., Newton NJ) to measure cross-sectional area for stress calculation.

Young's Modulus Calculation.

Tensile force was divided by mean specimen cross-sectional area in four OCT images obtained at 0.5 mm intervals along the specimen length to calculate stress. The YM, the ratio of tensile force to stress, was analyzed at 8% strain. In each of the 8 scleral regions, 10 control and 10 cross-linked samples were tested. Of the 12 specimens prepared for each region, two were typically damaged by ruptures due to preparation or clamping, so data from only the 10 specimens free of artifacts is included in the reported results. The sample size in the current study is similar to sample sizes in relevant literature[20, 21, 24].

Cross-linking Shrinkage Analysis.

Twenty specimens, measuring approximately 3.5×3.5 mm, were excised from the posterior (10 specimens) and equatorial (10 specimens) regions. Excess moisture was removed, and the specimen equilibrated in the environmental chamber for 10 minutes prior to OCT imaging. The specimen was then immersed in 0.1% riboflavin for 30 minutes. Next, each specimen was placed on an inclined platform to prevent riboflavin pooling, and exposed to 365nm UVA at 6mW/cm^2 irradiance for 30 minutes to produce CXL, during which specimens were dropwise irrigated with riboflavin every 30 seconds. After CXL, specimens were placed in the environmental chamber for 10 minutes, and were re-imaged by OCT.

Specimen thickness and volume were determined using OCT before and after CXL. The entire length of each specimen was cross-sectionally imaged at 125 μm intervals, requiring approximately 28 image planes in which specimen boundaries were then manually traced to determine cross-sectional areas. Summed areas were multiplied by 125 μm to calculate specimen volume. This current study only investigated CXL shrinkage in the posterior and equatorial sclera because we lacked sufficient scleral tissue to study CXL shrinkage in all 8 scleral regions individually.

Statistical Analysis.

In each region, paired t-tests were conducted for YM to compare control and cross-linked samples. An unpaired t-test was used to determine regional variation in YM. A secondary analysis was conducted to assess the effect of outlier values, with removal of all values more than 2 standard deviations (SD) from the mean. Since outlier removal did not change the overall conclusions, in the interest of rigor we report results of all data acquisitions that were free of obvious experimental artifacts.

Results

Stress-Strain Analysis.

Significant increases in YM were evident after CXL in all 8 scleral regions. Figure 2 shows example stress-strain curves of individual samples (dotted curves) and their overall average (solid curve) in the posterior superolateral region.

Average stress-strain curves for posterior sclera are shown in Fig. 3. In posterior superolateral sclera, mean YM (± SEM) increased 46±15% from 23.2±2.5 MPa to 32.4±2.9 MPa. In posterior superomedial sclera, YM increased 32±11% from 26.2±3.7 MPa to 34.7±5.8 MPa. In posterior inferolateral sclera, YM increased 67±20% from 22.1±2.0 MPa to 34.6±3.0 MPa, similar to the 53±11% increase in posterior inferomedial sclera from 26±4 MPa to 37±5 MPa. Data are summarized in Table 1 and Fig. 1.

Average stress-strain graphs for equatorial sclera are shown in Fig. 4. The YM of equatorial superolateral and equatorial inferolateral sclera was similarly increased by CXL by 139±43% and 143±92%, respectively. The YM of equatorial superolateral sclera was increased by CXL from 17.1±3.8 MPa to 30.8±3.5, while that of equatorial inferolateral sclera increased from 23.3±4.3 MPa to 44.3±4.8 MPa. Likewise, CXL increased the YM of

superomedial and equatorial inferomedial sclera similarly by 68±27% and 68±14%, respectively. The YM of equatorial superomedial sclera was increased by CXL from 23.3±3.0 MPa to 34.7±3.8 MPa, while that of equatorial inferomedial sclera increased from 21.4±3.2 MPa to 32.8±3.0 MPa. Data are summarized in Table 1 and Fig. 5.

Un-paired t-testing showed that the increase in YM produced by CXL was significantly greater in the pooled equatorial than in the pooled posterior regions (P<0.01), and the increase in the pooled lateral regions was significantly greater than in the pooled medial regions (P<0.05, Fig. 5). However, CXL affected YM similarly in the pooled superior (71±14% increase) and inferior regions (118±29% increase) with P=0.14.

Scleral Shrinkage.

Posterior sclera was on average significantly thicker at 1.00±0.03mm than equatorial sclera at 0.77±0.03mm prior to CXL (P<0.001). Cross-linking generally reduced scleral thickness, as illustrated by the OCT cross section in Fig. 6. Pooling all quadrants, CXL reduced scleral volume 17±1% in the equatorial and 13±1% in posterior sclera (P<0.001).

Figure 7 illustrates relative reduction of both thickness and volume in posterior and equatorial sclera. Correlation analysis indicated that 90% of the volume reduction was due to thickness reduction, with little change in length and width.

Outliers.

After removal of YM values lying more than ±2SD from the mean of all sample results before and after CXL, average SEM for the remaining values decreased by 16% in posterior and 21% in equatorial sclera. Nevertheless, after outlier removal, the increase in YM due to CXL remained significantly greater in equatorial than posterior sclera (P< 0.01), and greater in lateral than medial sclera (P<0.05). These relative changes are the same as illustrated in Fig. 5 when considering the entire date set, an approach that avoids possible bias and which is therefore the basis for the principal data reported here.

Discussion

Cross-linking using riboflavin and UVA increased YM significantly in all regions of human sclera. The effect was varied topographically, being relatively greater in the equatorial than posterior region (Fig. 5). This difference is in part attributable to regional variation in scleral thickness. Consistent with previous studies,[23] we found that the average posterior sclera thickness at 1.00mm to be significantly greater than the equatorial sclera thickness averaging at 0.77mm. Limited to a fixed tissue depth, UVA illumination therefore cross-linked a greater percentage of the total thickness of the equatorial than posterior sclera, and thus produced a greater increase in relative stiffness. In addition, we found that CXL increases YM significantly more in the lateral than in medial sclera. It should be noted that the irradiance and time of UV exposure were chosen to provide a stronger scleral crosslinking effect than the standard Dresden protocol for corneal cross-linking. Since other dosages of UV exposure were not investigated, specific protocols for *in vivo* scleral CXL would likely require optimization.

Another factor in differential regional effect of CXL may be a ceiling effect suggested by Fig. 5. After CXL, mean pooled YM similarly averaged about 33 MPa in posterior, equatorial, lateral, and medial regions. The differential effect of CXL might also be understood as increasing scleral YM to a maximum value regardless of reginal variation in untreated YM. Thus, the lower the untreated YM, the greater the relative increase in YM due to CXL.

Previous studies used calipers to measure volume changes post CXL[21, 22, 24]. In this study, we measured volume more accurately by OCT, showing that CXL significantly reduces equatorial and posterior sclera volume (Figs. 6 and 7), almost entirely due to thinning similar to that of corneal CXL[25] In vivo, corneal thickness returned to baseline after CXL treatment for keratoconus[26]. It is unknown if similar reversal of scleral thinning might occur after CXL in vivo.

The current results differ substantially from those of Wang *et al.*[22], the only other study to evaluate regional variations in human sclera tissue due to CXL. The Wang *et al.* study reported human YM in the quantitatively implausible range of 200–470 MPa,[22] roughly an order of magnitude greater than typically observed in sclera. Such unrealistic scleral values are more typical of muscle at approximately 480 MPa,[27, 28] or tendon, approximately 560 MPa.[28–31] Our study of sclera yielded a mean YM of 23 ± 1 (SEM) MPa, similar to the report of Wollensak, who did not investigate, however, possible regional variations.[20] Prior studies of scleral YM[20, 22] applied loading to scleral strips that had been cut sagitally from anterior to posterior, a direction in which systematic variation in specimen thickness compromises accurate computation of YM. Consistent with the anatomical literature[23], we found equatorial to be significantly thinner than posterior sclera. Assuming that the thinnest cross-section is applicable to the entire specimen would exaggerate apparent YM by exaggerating computed stress, which is calculated as force divided by cross sectional area. Finally, in previous studies where CXL was performed in whole globes prior to specimen excision[6, 22], control specimens were obtained from different eyes, introducing intersubject variability. The present approach mitigated interindividual variability by using adjacent scleral strips in the same eye as controls.

Diminished CXL is an important factor in the weakening process of myopic sclera[9] as in myopic human eyes that have significantly lower scleral YM.[32] There is a natural increase in CXL with advancing age[9] and in diabetics.[33] In older people, the natural increase in CXL is believed to slow myopic progression.[34] Similarly, the increase glycation-induced CXL in diabetic patients has been proposed to explain their reduced axial myopia.[33] It is therefore hypothesized that if natural CXL is associated with retardation of myopic progression, then artificially inducing CXL using UVA/riboflavin might be used therapeutically to retard axial elongation and reduce the risk of blindness.[35] Other treatment options exist, such as scleral reinforcement surgery,[36] in which a strip of cadaveric sclera is wrapped around the globe to prevent further elongation.[37, 38] In one study, scleral reinforcement was usually successful in arresting the progression of myopia.[39] However, such procedure is both highly invasive and relies on the availability of healthy scleral donor tissue, which is currently becoming scarcer with the increasing prevalence of myopia.

Complications of high myopia, such staphylomata, may be sight-threatening. Staphylomata form as localized scleral ectasia having a radius of curvature less than the surrounding scleral curvature.[40] Localized posterior scleral ectasia is an important component of several vision-threatening myopic maculopathies.[41–43] It is theorized that the prevention of posterior staphyloma could prevent further visual impairment related to maculopathies.[41] Because the formation of staphylomata involves localized scleral weakening, CXL might be a prophylactic measure to reinforce scleral tissue. The topographically differential effect of CXL might be useful as a treatment for staphylomata formation in various regions of the globe.

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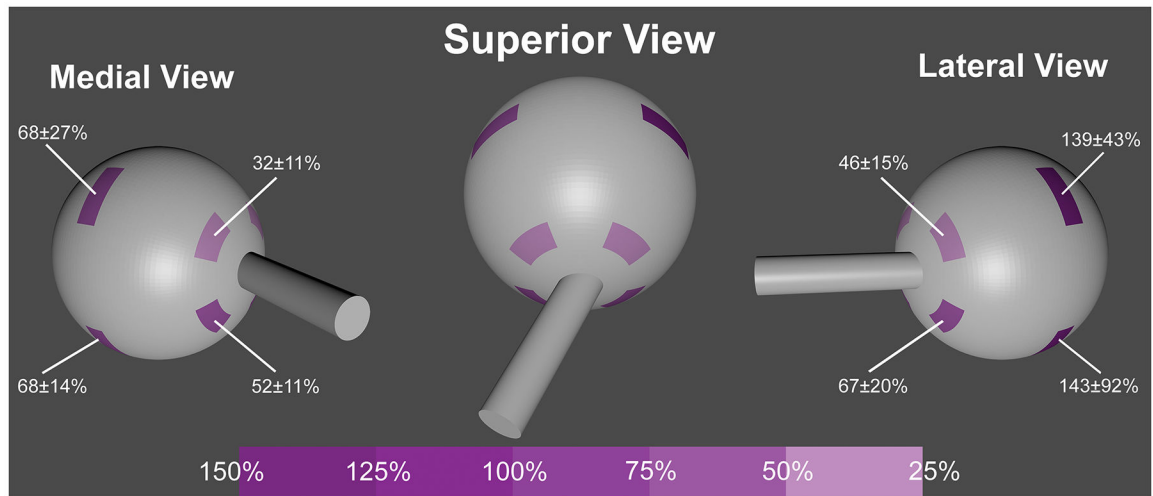
Fig. 1.
Scleral regions tested and mean percent increase in Young's modulus (± standard error of mean) following cross-linking.
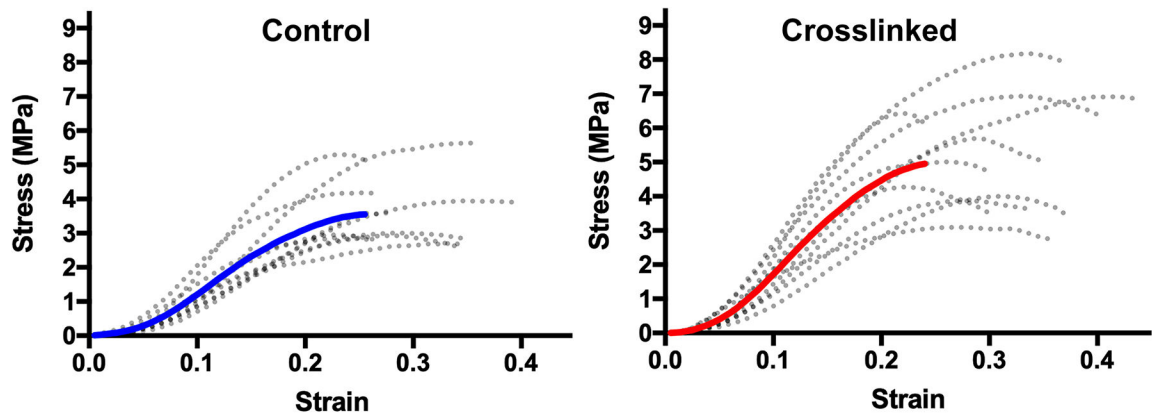
Fig. 2.
Individual stress-strain curves of 10 samples from the posterior superolateral sclera (dotted curves), with average curves solid. Note greater stress for cross-linked specimens.
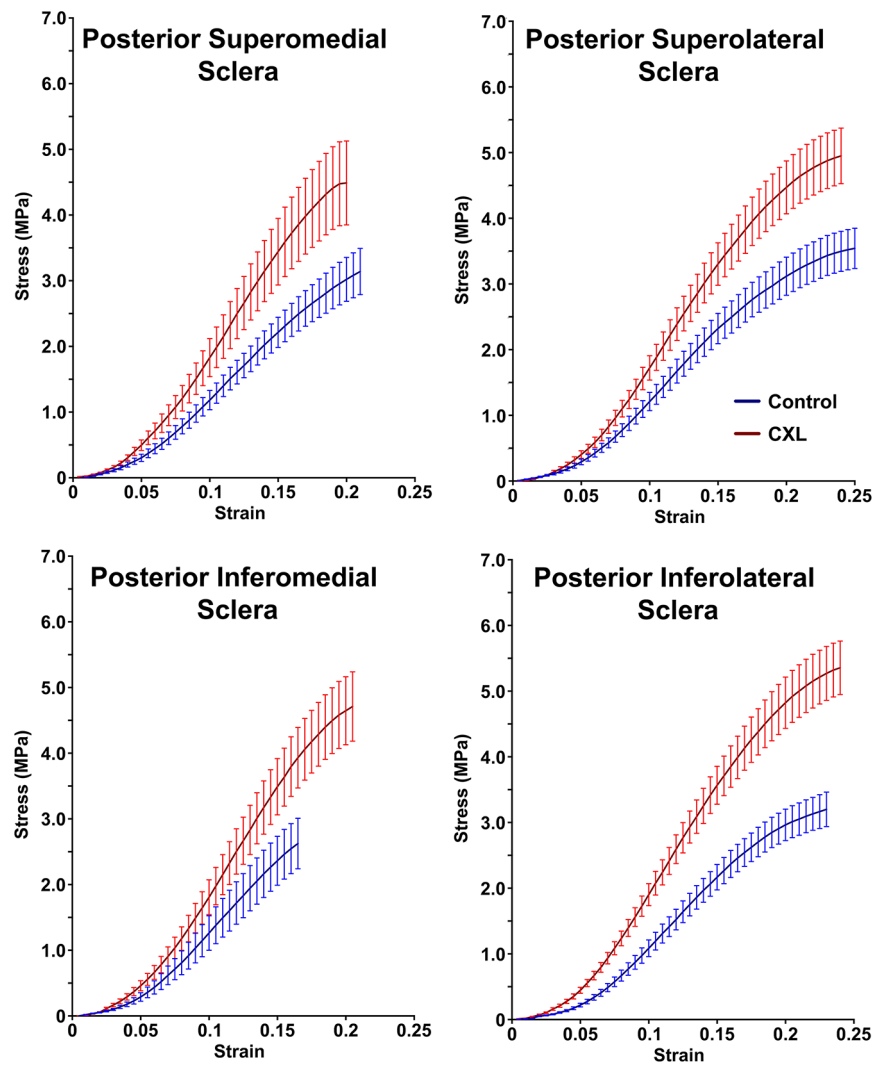
Fig. 3.
Stress-strain curves in posterior superomedial, superolateral, inferomedial and inferolateral sclera for control and cross-linked (CXL) specimens. Error bands ± standard error of mean.
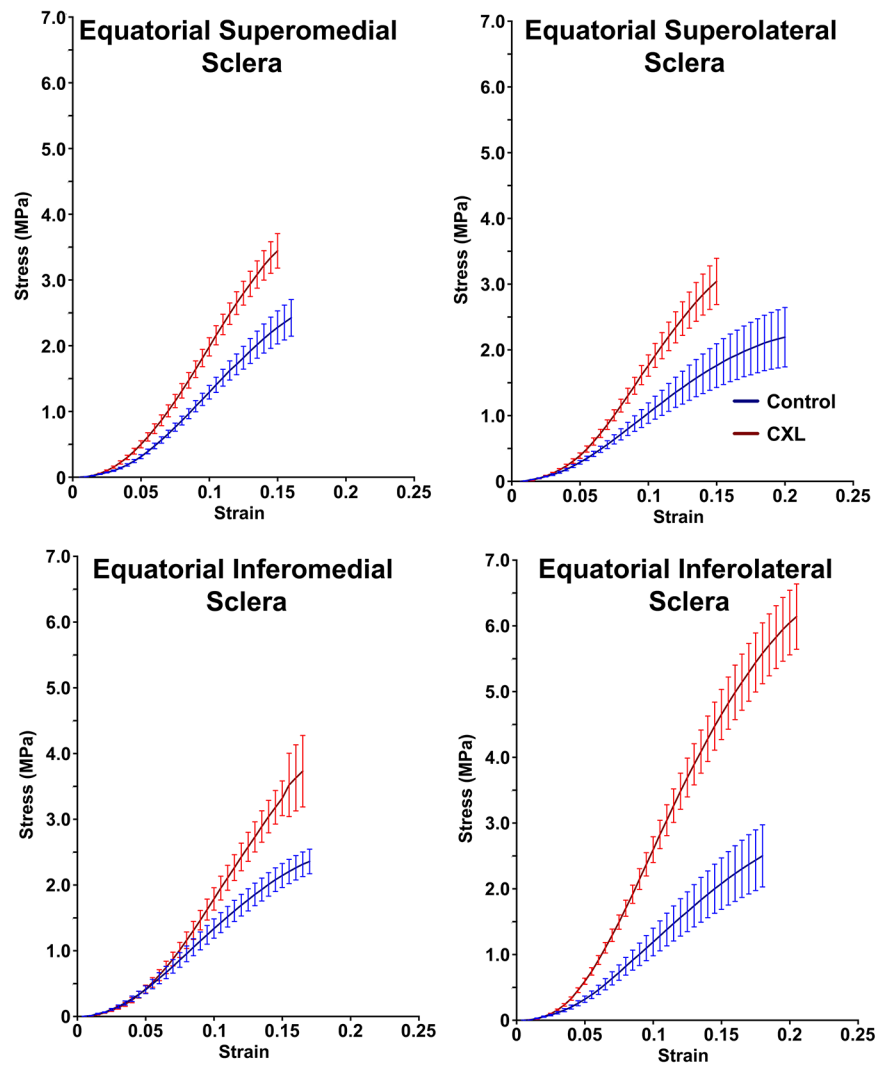
Fig. 4.

Stress-strain curves in equatorial sclera for control specimens and after cross-linking (CXL).
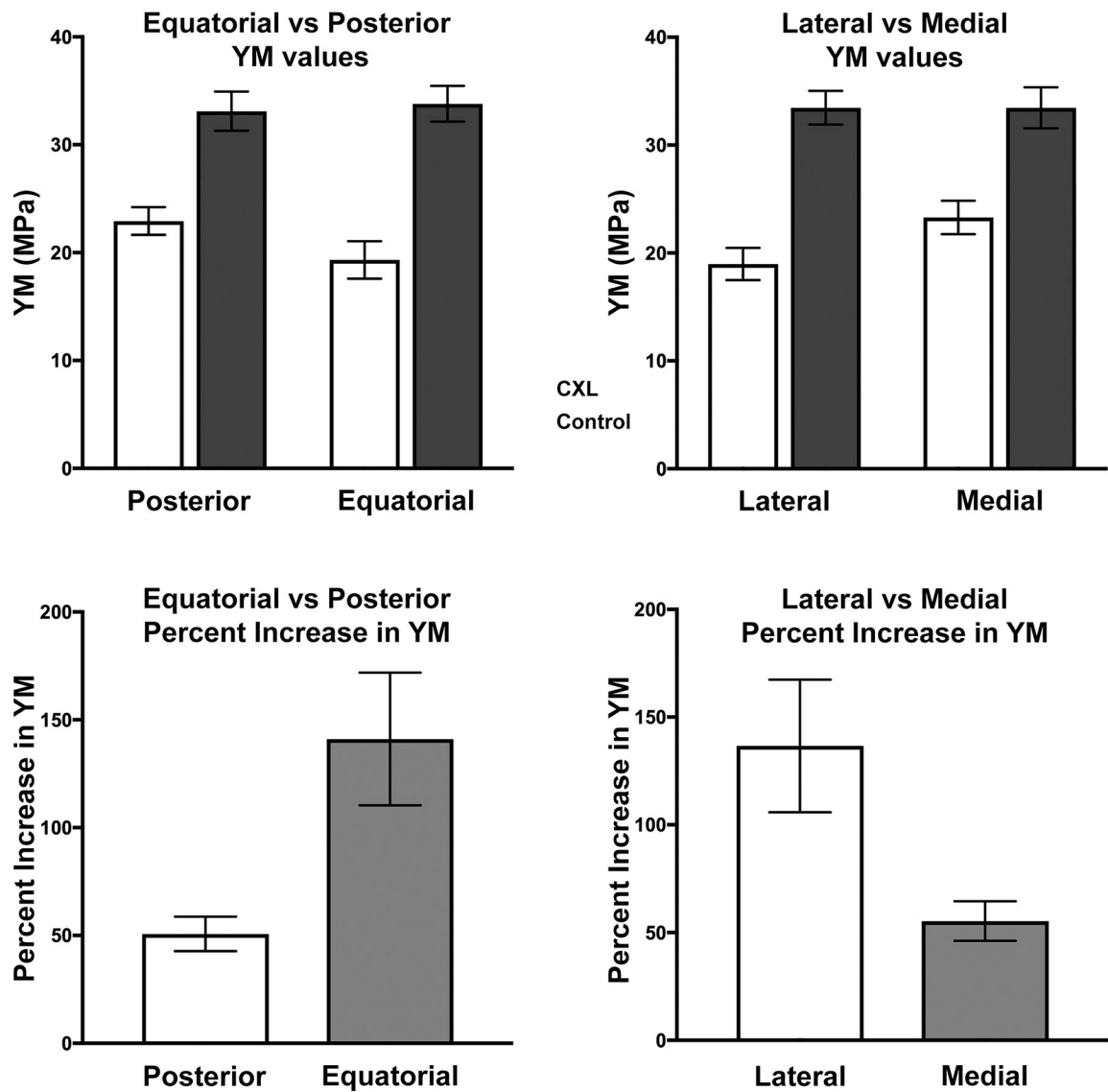Error bands ± standard error of mean.

Fig. 5.
Top. Young's Modulus (YM, top row) and its percentage increase (bottom row) in control specimens, and after cross-linking (CXL), in posterior, equatorial, lateral, and medial sclera. Error bars – standard error of mean.
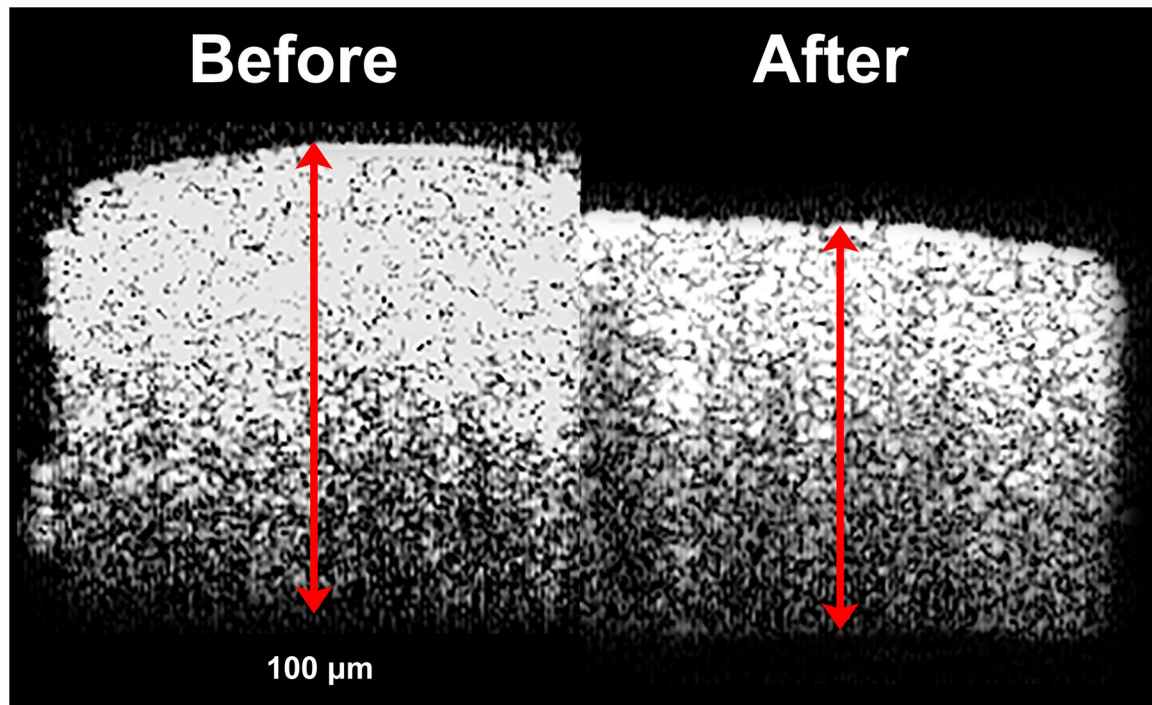
Fig. 6.
Cross sectional optical coherence tomography of the same scleral specimen before and after cross-linking.
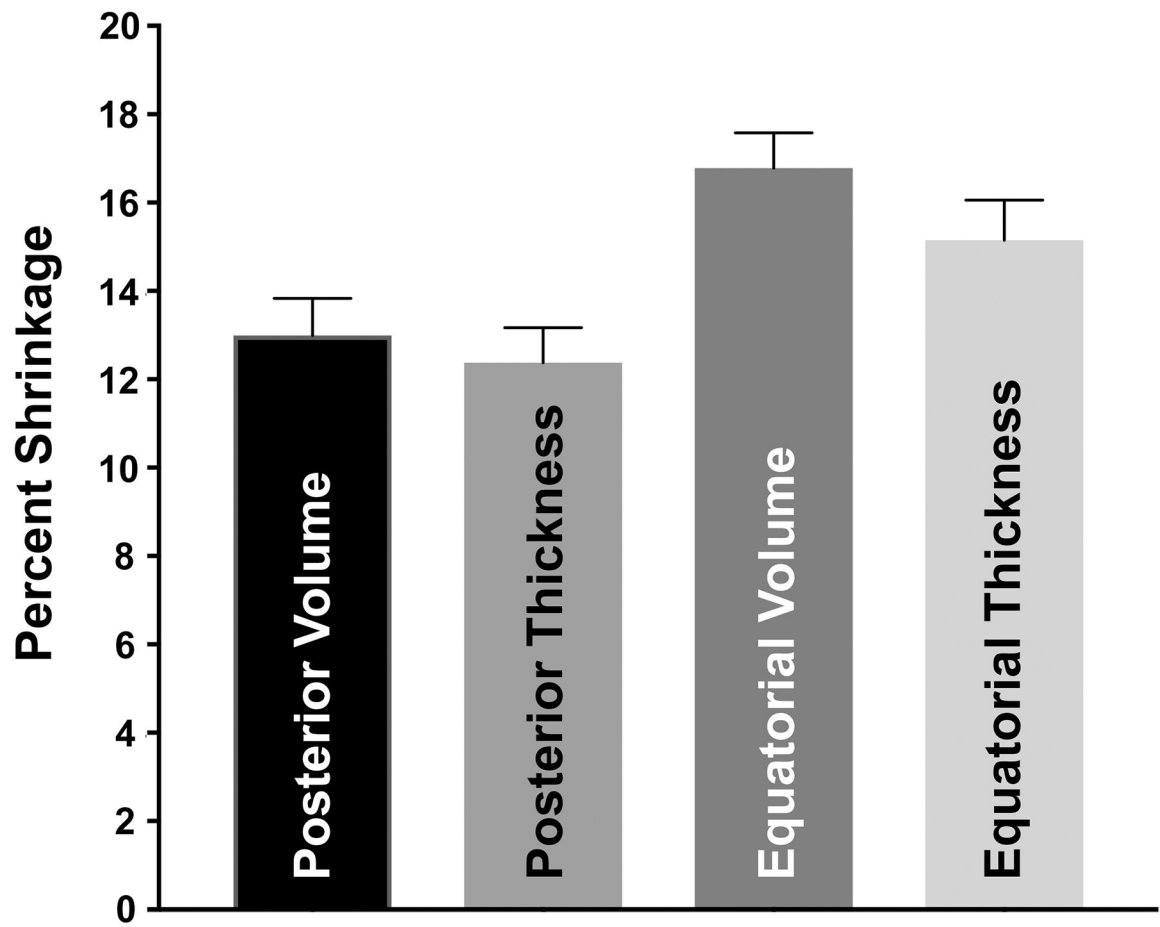
Fig. 7.
Shrinkage due to cross-linking in posterior and equatorial sclera.

Table 1.

Effect of Cross-linking on Scleral Young's Modulus

Regions	Control YM (MPa, SEM)	Cross-linked YM (MPa, SEM)	Percent Increase (SEM)	Significance
Posterior Superolateral	23.2±2.5	32.4±2.9	46±15%	0.01
Posterior Superomedial	26.2±3.7	34.7±5.8	32±11%	0.05
Posterior Inferolateral	22.1±2.0	34.6±3.0	67±20%	0.01
Posterior Inferomedial	25.7±4.5	36.6±5.1	53±11%	0.001
Equatorial Superolateral	17.1±3.8	30.8±3.5	139±43%	0.001
Equatorial Superomedial	23.3±3.0	34.7±3.8	68±27%	0.005
Equatorial Inferolateral	23.3±4.3	44.3±4.8	143±92%	0.001
Equatorial Inferomedial	21.4±3.2	32.8±3.0	68±14%	0.001

SEM – standard error of mean. Significance by 2-tail t-testing.