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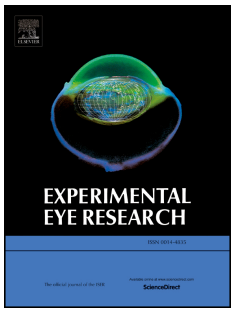

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UVA/Riboflavin Collagen Crosslinking Stiffening Effects on Anterior and Posterior Corneal Flaps

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Abstract

The UVA/riboflavin collagen crosslinking (CXL) is one of the treatment procedure for stopping the progression of keratoconus. The inclusion criterion for this procedure is a minimum corneal thickness of 400 um, which is not often met in patients with advanced keratoconus. Preoperatively swelling of thin corneas was shown to stabilize the keratectasia without any postoperative endothelial damage. Recently, we have shown that swelling porcine corneas prior to the CXL treatment had no significant effect on the resulting improvement in their tensile properties. In the present study, we extended this previous study and characterized the stiffening effects of CXL on anterior and posterior flaps as a function of their hydration. A DSAEK system was used to excise 10 mm corneal flaps from 80 porcine corneas. Individual flaps were crosslinked at different initial hydration levels by using riboflavin solutions composed of different dextran concentrations; the thickness was taken as a measure of flap hydration. A DMA machine was used to measure the tensile properties either immediately after the CXL treatment or after the thickness (hydration) of the crosslinked samples was brought down to a specific value. The average thickness of anterior groups was 670 μm, 540 μm, and 410 μm, and the average thickness of posterior groups was 845 μm, 650 μm, and 440 μm. It was found that although CXL significantly increased the tensile properties of all anterior groups, it had an insignificant effect on the stiffness of posterior flaps. Furthermore, except for the posterior flaps in 845 μm and 650 μm thickness groups, decreasing the hydration significantly increased the tensile modulus ($p < 0.05$). Finally, the anterior flaps that were crosslinked at higher hydration, i.e. swollen before CXL, showed significantly less amount of stiffening in comparison with those crosslinked at lower hydration when the tensile property measurement was done at similar hydration ($p < 0.05$).

Keywords: Corneal collagen crosslinking, Uniaxial tensile tests, Hydration, Keratoconus, Preswelling

1. Introduction:

Cornea is a transparent ocular tissue that forms the outermost layer of the eye. Besides allowing the light to pass through, it acts as a protective shield to the intraocular components. The eye mainly gets its refractive power from the combined ability of the cornea and the lens in focusing the incoming light rays on to the retina. Consequently, any damage to the cornea can cause serious vision disorders. Keratoconus is an ocular disease that results in the progressive outward bulging of the corneal tissue. The UVA corneal collagen crosslinking (CXL) is among the new treatment options to halt the progression of the disease.

The commonly used collagen crosslinking therapy, i.e. Dresden protocol, is one hour long and is divided into two stages: the soaking phase and the UVA exposure phase (Wollensak et al., 2003a). In the soaking phase, the cornea is immersed in the photosensitizer solution for thirty minutes in order to allow adequate penetration of riboflavin into the corneal stroma. In the UVA exposure phase, UVA radiation (370 nm) with a total intensity of 3 mW/cm^2 is administered for thirty minutes while drops of the photosensitizer solution are added every five minutes on the corneal surface. Because of corneal inherent tendency to swell, the photosensitizer solution contains dextran, a deturgescent agent, in order to prevent excessive swelling.

The CXL technique will damage endothelial cells if corneal thickness is less than 400 μm (Wollensak et al., 2003b; Wollensak et al., 2003c). Thus, the Dresden crosslinking procedure is not recommended for patients with thin corneas. In order to avoid this limitation, Hafezi et al. swelled thin corneas to a thickness of more than 400 μm prior to the treatment (Hafezi et al., 2009). Although they established the effectiveness of this modified crosslinking procedure in treating progressive keratoconus, they did not characterize its possible effects on the amount of

CXL stiffening. In a recent study, we showed that the extent of stiffening was the same in pre-swollen and non-swollen in vitro collagen crosslinked porcine corneas (Hatami-Marbini and Jayaram, 2018). We explained this interesting experimental observation in terms of the inhomogeneous through-thickness collagen lamella microstructure, inhomogeneous corneal swelling, and the depth dependency of the collagen crosslinking treatment. The primary objective of the present study was to provide a more comprehensive understanding of the interrelation of hydration (corneal pre-swelling) and the crosslinking treatment. Specifically, we characterized the amount of the CXL stiffening of the flaps excised from the anterior and posterior regions of the porcine corneas as a function of their hydration (thickness) at the time of crosslinking.

2. Materials and Methods:

2.1 Specimen preparation

Porcine eye globes were brought in a cooler chest from a local slaughter house within six hours of postmortem. Samples were stored inside a refrigerator at 4 ^{o}C, and were used within 24 hours of the procurement. After removing fat tissues attached to the outer surface of the sclera, epithelium debridement was performed using the blunt edge of a scalpel. A corneoscleral ring with about 2 mm of sclera was cut by a curved scissor. The corneal thickness was measured using a digital pachymeter (DGH Technology Inc., Pennsylvania) and a DSAEK system was used to excise corneal flaps. In order to create flaps from samples with similar thickness, corneal buttons were air dried until their thickness reached about 800 μm just before the flap creation. From each cornea, a 10 mm circular anterior flap was excised and the remaining of the tissue

was called the posterior flap. A custom built double-bladed punch was used to punch 5 mm wide strips from the anterior and posterior flaps. The strips were only prepared from the nasal-temporal direction in order to avoid possible variations in mechanical properties due to anisotropic properties (Elsheikh and Alhasso, 2009; Hatami-Marbini and Rahimi, 2014a).

2.2 Preparation of photosensitizer solution

An isoosmolar crosslinking solution composed of 0.1% riboflavin and 20% dextran is used in the Dresden protocol (Wollensak et al., 2003a). Dextran is a deturgescent agent that inhibits unwanted swelling of the corneal tissue. In this study, the dextran concentration in the photosensitizer solution was varied to be able to crosslink the strips at different hydration levels. Similar to our previous study (Hatami-Marbini and Jayaram, 2018), we used sample thickness as a measure of their hydration. We first conducted a preliminary swelling study to determine the required dextran concentration for attaining samples with three distinct hydration levels prior to crosslinking. Specifically, we soaked specimens in solutions with different dextran concentrations and measured their thickness on regular intervals. Based on this swelling study, we used photosensitizer solution with 2.5%, 5%, and 10% dextran concentration in order to obtain three distinct thickness groups for anterior flaps and three thickness groups for posterior flaps.

2.3 Collagen crosslinking Procedure

First, strips from each thickness group were soaked in their respective photosensitizer solutions until their thickness reached equilibrium. Then, the specimens were exposed to UVA radiations of 3 mW/cm^2 intensity at a distance of about 2 cm for 30 minutes. Few drops of the same photosensitizer solution were added to the strips during the UVA exposure. The strips in the

control groups were subjected to the same exact treatment procedure except that the UVA light was turned off during the exposure period.

2.4 Uniaxial tensile experiments

The tensile behavior of crosslinked and control flaps was measured by a DMA machine (TA instruments, Maryland). The uniaxial tensile tests were performed either right after the preparation of the strips (no change to their hydration) or after their thickness (hydration) was brought to a specific value (see the following).

Prior to mounting the specimens, their thickness and width were measured by a digital pachymeter and calliper, respectively. A tare load of 20 mN was applied in order to remove any possible slack and determine the initial length, L_0. The initial length was used to calculate the required displacement δL for subjecting the strips to axial strain $\varepsilon = 10\%$, i.e. $\delta L = \varepsilon L_0$, at a displacement rate of 2 mm/min. No bathing solution was used because the tensile experiments were fast and no significant dehydration occurred. The tensile stress was calculated by dividing the experimentally measured force by the cross-sectional area of the strips.

A total of 80 strips were tested; 40 posterior strips and 40 anterior strips. The thickness (hydration) of strips during the crosslinking procedure and during the mechanical tests was used to divide them into the following groups: A11, A22, A33, A13, A23, P11, P22, P33, P13, and P23. The letters ‘A’ and ‘P’ refer to anterior and posterior flaps, respectively. The first numeric value after the letters ‘A’ and ‘P’ gives the concentration of dextran that was used to crosslink the samples. The numbers 1, 2, and 3 denote photosensitizer solutions with 2.5%, 5%, and 10% dextran concentration, respectively. Note that there is a one-to-one relationship between dextran concentration and thickness of the strips (this relation was obtained from the preliminary

swelling study), Table 1. The second numeric value after the letters 'A' and 'P' refers to the thickness of the samples at which the mechanical tests were conducted. For instance, A11 refers to samples, which were crosslinked using the riboflavin solution with 2.5% dextran and were tested immediately after the crosslinking treatment, i.e. the thickness of samples when they were crosslinked and when they were mechanically tested was the same. On the other hand, A13 denotes samples that were crosslinked using the riboflavin solution with 2.5% dextran and were mechanically tested after dehydrating them to the equilibrium thickness that was obtained when the strips were soaked in 10% dextran solution. Five samples were crosslinked and five samples were used as control for each of the groups A11, A22, A33, P11, P22, and P33. Only five crosslinked samples were used for groups A13, A23, P13, and P23. The symbols CXL and CTR were used to distinguish between crosslinked and control samples. The experimental data were analyzed statistically by one-way ANOVA with a significance level of 0.05.

Table 1

3. Results:

The mean thickness of anterior and posterior flaps right after dissection by the DSAEK system was 360 ± 40 µm and 480 ± 50 µm, respectively. The thickness of strips at the time of crosslinking was 670 ± 40 µm (Group A11), 540 ± 80 µm (Group A22), 410 ± 45 µm (group A33), 845 ± 150 µm (Group P11), 650 ± 80 µm (Group P22), and 440 ± 50 µm (Group P33).

The thickness of samples in groups A13 and A23 at the time of crosslinking was similar to that of samples in group A11 and A22, and the thickness of samples in groups P13 and P23 was similar to that of samples in group P11 and P22, respectively. Figures 1-2 show the effect of hydration on the stress-strain curves of anterior and posterior groups. An inverse relation between the hydration and the average tensile properties of crosslinked and control strips from both anterior and posterior flaps was observed. The peak tensile stress is shown in Figure 3. The peak stress of both control and crosslinked anterior flaps increased significantly with decreasing the thickness (hydration). The difference between peak stress of control posterior groups P22 and P11 was insignificant ($p = 0.6$); nevertheless, the peak stress of posterior flaps from group P33 was significantly higher than the peak stress of groups P22 and P11 ($p < 0.05$). Furthermore, the crosslinking treatment increased significantly the peak stress of anterior strips in all groups ($p<0.05$). Nevertheless, the crosslinking procedure has insignificant effect on the peak stress of all posterior groups and did not cause any significant increase.

Figures 1-3

Figure 4 shows the stress-strain behaviour of anterior and posterior flaps from groups A13, A23, P13, and P23; these strips were crosslinked at different initial thickness (hydration) but were tested at similar thickness. It is seen that unlike strips from anterior region, the stress-strain curves for crosslinked posterior groups were similar to each other.

Figure 4

4. Discussion:

Keratoconus is a form of corneal ectasia that is known to cause vision distortion in about one in every 2000 people (Rabinowitz, 1998). UVA corneal collagen crosslinking procedure is a clinical treatment that halts the progressive thinning of the corneal tissue by the induction of crosslinks. In order to protect the endothelial cells from the toxic damage of the UVA radiations, a minimum corneal thickness of 400 microns is recommended (Wollensak et al., 2003b; Wollensak et al., 2003c).

According to the commonly used collagen crosslinking procedure, the samples should be soaked in the riboflavin and 20% dextran T-500 solution for 30 minutes in order to ensure complete penetration of riboflavin into the corneal stroma. Furthermore, the photosensitizer riboflavin solution is applied every 3-5 minutes during the following 30 minutes of the UVA exposure in order to ensure continuous supply of the riboflavin and to prevent unwanted drying of the tissue. The cornea has strong swelling properties due to the presence of the negatively charged glycosaminoglycans (Hatami-Marbini et al., 2013; Maurice, 1984; Meek, 2008). The presence of dextran, a deturgescent agent, in the riboflavin solution is necessary to prevent excessive swelling of the cornea. The thickness of the corneas of the patients with advanced keratoconus is often less than the required 400 um (Hafezi et al., 2009). Thus, the crosslinking treatment using the above protocol is not recommended for these patients due to safety concerns. The standard collagen crosslinking protocol causes a significant decrease in the endothelial cell density when corneal thickness is less than 400 μm (Kymionis et al., 2012). A modified collagen crosslinking procedure was purposed to circumvent this problem.

Hafezi and coworkers modified the classical crosslinking procedure and did not use dextran in the riboflavin solution in order to preoperatively swell the corneas of patients with an average stromal thickness of about 320 um (Hafezi et al., 2009). They confirmed that keratectasia was stabilized without any sign of endothelial damage. Despite the success of this new crosslinking procedure in halting the progression of the disease, the extent of corneal stiffening could be less than what it would be if the standard crosslinking procedure had been used. In a recent study, we crosslinked porcine corneas at different initial hydration and characterized changes in their mechanical properties (Hatami-Marbini and Jayaram, 2018). We observed that corneas crosslinked at different thickness (hydration levels) but mechanically tested at the same thickness (hydration) showed the same amount of increase in tensile strength. Consequently, we concluded that the extent of stiffening produced in the cornea is independent of the thickness at which it is crosslinked. We speculated that this observation could be because crosslinks mainly occur in the anterior regions whose microstructure does not significantly alter by artificially swelling corneal strips. The primary objective of the present study was to better understand this counterintuitive observation by crosslinking corneal flaps excised from the anterior and posterior regions.

The collagen fibrils of the cornea are organized into bundles, known as lamellae, which run parallel to the surface of the tissue. It is well-known that corneal tissue shows depth dependent mechanical properties due to the clear microstructural differences in the anterior and posterior regions. The microstructure of the cornea is inhomogeneous through-the-thickness, i.e. anterior lamellae interweave while the posterior ones lie parallel to each other (Jester et al., 2010; Maurice, 1984; Meek, 2008). It is also known that the presence of negatively charged proteoglycans creates a strong swelling tendency in the cornea when immersed in an ionic solution (Hodson, 1971). Corneal swelling (increasing the hydration) causes a significant

decrease in the tensile and compressive properties of the tissue (Hatami-Marbini, 2014; Hatami-Marbini and Etebu, 2013; Hatami-Marbini and Rahimi, 2014b, 2015; Hjortdal and Jensen, 1995; Søndergaard et al., 2013). The results shown in Figure 3 shows that control anterior flaps had stronger tensile properties compared to the flaps excised from the posterior region. This observation is in agreement with previous studies in the literature (Dias and Ziebarth, 2013; Kohlhaas et al., 2006; Randleman et al., 2008; Scarcelli et al., 2012). Furthermore, the tensile properties of both anterior and posterior strips had an inverse relation with the hydration (thickness). This observation is also in agreement with previous studies on hydration dependent mechanical properties of the corneal tissue (Hatami-Marbini, 2014; Hatami-Marbini and Rahimi, 2014b, 2015). However, Figure 3 shows that the peak stress of posterior groups P11 ($t = 850\ \mu m$) and P22 ($t = 660\ \mu m$) was almost similar.

The collagen fibrils and proteoglycans (PGs) are primary structural ingredients of the corneal extracellular matrix. The proteoglycans are attached to collagen fibrils via their core proteins and form interfibrillar bridges between neighboring collagen fibrils by their glycosaminoglycan (GAG) side chains (Cheng et al., 2013; Lewis et al., 2010; Scott, 1992). Although the precise function of interfibrillar GAG duplexes in corneal mechanics is not fully known, recent studies suggest that their intrinsic elasticity as well as the interaction of their fixed charges with themselves and the free ions inside the interstitial fluid are important in defining the corneal biomechanics (Cheng et al., 2013; Hatami-Marbini and Pinsky, 2011). It has also been proposed that if GAG chains are held close to each other, despite the presence of strong electrostatic repulsion, relatively strong supramolecular organizations will be formed by intramolecular hydrogen bondings (Han et al., 2008; Lewis et al., 2010; Scott, 1992, 2001). In order to explain similar tensile properties of posterior strips in P11 and P22 groups as shown in Figure 3, let us

represent the corneal stroma as a composite material composed of collagen fibril reinforcements embedded in a hydrated proteoglycan matrix (Hatami-Marbini and Rahimi, 2014a). As the hydration of posterior strips increases, the relative distance between neighboring GAGs increases and weaker supramolecular organizations are formed, which reduces the effective elastic properties of the proteoglycan matrix. This reduction in tensile properties of posterior flaps reaches a plateau probably because the relative distance between GAG chains (due to excessive swelling of the posterior strips) becomes so large that they do not form strong GAG duplexes and the mechanical contribution of proteoglycan matrix, subsequently, reaches its limit. This conceptual model also explains the reduction in tensile properties of anterior strips with increasing their hydration. Nevertheless, it is noted that the collagen lamellae are interwoven with each other in the anterior strips. The collagen interweaving limits the amount of swelling of anterior strips and also contribute to the overall tensile properties of anterior flaps because of entanglements (Doi, 1996).

Figure 3 shows that although the CXL treatment significantly increased the tensile properties of anterior flaps (independent of their initial hydration), it had insignificant effect on the mechanical properties of the posterior flaps. This is an important observation that requires an explanation. First note that, in most of previous studies, full thickness corneas have been crosslinked and it has been shown via different methods that stiffening effect of the CXL procedure is limited to the anterior region (Kohlhaas et al., 2006; Scarcelli et al., 2012; Schumacher et al., 2012; Seiler and Hafezi, 2006; Wollensak et al., 2004). Here, anterior flaps and posterior flaps were crosslinked separately and no stiffening effect was observed in posterior flaps.

Hayes et al. claimed that cross-links due to the CXL treatment should occur at the surface of collagen fibrils and within the proteoglycan rich coating surrounding them (Hayes et al., 2013).

Considering the conceptual structural composite model presented in the previous paragraphs, the results shown in Figure 3 for the crosslinked anterior flaps agree with this hypothesis, i.e. CXL therapy increases tensile properties of these flaps because it stiffens both the proteoglycan matrix and collagen fibrils. For posterior flaps, we can assume that crosslinks still occur in the proteoglycan matrix and the surface of collagen fibrils. However, due to the lack of collagen interweaving and not formation of strong supramolecular organizations, they only cause a slight (insignificant) increase in tensile properties. In spite of this explanation, future studies using imaging techniques or mechanical measurements at micro/nano scales are required to provide a better explanation for this observation, i.e. why no stiffening is observed in the crosslinked posterior flaps.

Finally, Figure 4 shows the stress-strain response of strips that were crosslinked at different thickness (using riboflavin solution containing 10%, 5%, and 2.5% dextran) but were mechanically tested at the same hydration level. The corresponding stress-strain curves for crosslinked posterior strips were all similar to the stress-strain curve of the control group. This further supports the conclusion that CXL treatment has an insignificant effect on tensile properties of posterior flaps. Figure 4 shows that the hydration of anterior flaps prior to the CXL treatment significantly influenced the amount of CXL stiffening. The largest stiffening was for A33 samples, which were crosslinked at lower hydration (average thickness ~ 410 μm), and the smallest amount of stiffening was observed in A13 strips, which were significantly swollen prior to the CXL treatment (average thickness ~ 670 μm). Note that the average thickness of all anterior strips, independent of their thickness during the CXL treatment, was about 410 μm during the mechanical measurements, i.e. the difference in the behavior of various groups is not due to hydration (Hatami-Marbini, 2014; Hatami-Marbini and Etebu, 2013; Hatami-Marbini and

Rahimi, 2014b). This observation can be explained as follows. The effective depth of collagen crosslinking is about 300-400 um (Kohlhaas et al., 2006; Scarcelli et al., 2012; Schumacher et al., 2012; Seiler and Hafezi, 2006; Wollensak et al., 2004). Specifically, it is believed that about 70% of the UVA radiations are absorbed in the top 200 μm and 20% in the subsequent 200 μm (Kohlhaas et al., 2006). With increasing the thickness (hydration) of the anterior samples; their effective crosslinked thickness reduces and they subsequently stiffen to a lesser amount. Thus, artificially swelling the cornea prior to CXL treatment changes the amount of stiffening only if it causes significant swelling in the anterior lamellae.

A limitation of the present study is that corneal samples were cut in half through their thickness in order to obtain anterior and posterior flaps. There exists a transition region through the corneal thickness such that the highly interwoven top lamellae become almost parallel to each in the posterior part (Jester et al., 2010; Maurice, 1984; Meek, 2008). Thus, the microstructure of posterior and anterior strips in the present study is expected to be inhomogeneous. Future studies could avoid this limitation by investigating the properties of very thin strips from posterior and anterior region. Furthermore, the present study used uniaxial testing method to characterize the mechanical behavior of cornea. This testing technique is unable to represent the in-vivo condition and is primarily suitable for comparative studies (Hatami-Marbini and Rahimi, 2014b). For example, errors may be introduced into measurements because of damages that occur to the internal microstructure of collagens in the posterior and anterior flaps during strip preparation. Also it is noted that posterior collagen lamellae which are not aligned with the direction of strip testing may not be placed under tension. Nevertheless, non-aligned fibrils contribute to tensile properties of anterior stroma because of the presence of interweaving. Thus, the observed difference between mechanical properties of anterior and posterior stroma may be partially due

to this artifact of strip testing. Another limitation is that we used porcine corneas instead of human corneas. Also, keratoconus affects the organization of collagen fibrils; thus, similar studies using diseased human tissues are required to provide a better characterization of CXL stiffening when the tissue is artificially swollen. Furthermore, we did not characterize riboflavin penetration into samples at different dextran concentration. However, a clear yellow discoloration of all treated specimens (a sign of riboflavin penetration) was observed upon application of crosslinking solution. Despite these limitations, the present work provided interesting and novel data regarding the CXL treatment. First, anterior flaps are significantly stiffer than the posterior flaps. Moreover, similar to what was observed previously on full thickness corneas, increasing the hydration of both posterior and anterior corneal flaps significantly reduces their tensile properties. Furthermore, although the collagen crosslinking increased the stiffness of the anterior flaps, it had insignificant effect on the stiffness of posterior flaps. Thus, the interweaving of anterior collagen lamellae is expected to be one of the key factors in effectiveness of the collagen crosslinking treatment.

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Figure Captions:

Figure 1. The tensile stress-strain response of a) anterior and b) posterior flaps from the control groups. The mean thickness was 670 µm (group A11), 540 µm (group A22), 410 µm (group A33), 845 µm (group P11), 650 µm (group P22), and 440 µm (group P33).

Figure 2. The tensile stress-strain response of collagen crosslinked a) anterior and b) posterior flaps. The specimens were crosslinked at the thickness (hydration) of their respective group and their tensile behavior was immediately measured.The mean thickness was 670 µm (group A11), 540 µm (group A22), 410 µm (group A33), 845 µm (group P11), 650 µm (group P22), and 440 µm (group P33).

Figure 3. Maximum tensile stress of a) posterior and b) anterior corneal samples. With increasing the thickness (hydration), both control and crosslinked anterior samples showed weaker tensile properties. Although collagen crosslinking caused a significant increase in the maximum stress of anterior specimens ($P<0.05$), it had an insignificant influence on mechanical properties of posterior flaps.

Figure 4. The tensile stress-strain response of the collagen crosslinked a) anterior and b) posterior flaps. Samples were subjected to collagen crosslinking treatment at different thickness (hydration) levels but the mechanical measurements were done at the same thickness of 410 µm and 440 µm for anterior and posterior samples, respectively. The mean thickness of different groups at time of crosslinking was 670 µm (group A13), 540 µm (group A23), 410 µm (group A33), 845 µm (group P13), 650 µm (group P23), and 440 µm (group P33).

References:

Cheng, X., Hatami-Marbini, H., Pinsky, P.M., 2013. Modeling Collagen-Proteoglycan Structural Interactions in the Human Cornea. Springer Netherlands, Dordrecht, pp. 11-24.

Dias, J.M., Ziebarth, N.M., 2013. Anterior and posterior corneal stroma elasticity assessed using nanoindentation. Exp Eye Res 115, 41-46.

Doi, M., 1996. Introduction to polymer physics. Oxford university press.

Elsheikh, A., Alhasso, D., 2009. Mechanical anisotropy of porcine cornea and correlation with stromal microstructure. Exp Eye Res 88, 1084-1091.

Hafezi, F., Mrochen, M., Iseli, H.P., Seiler, T., 2009. Collagen crosslinking with ultraviolet-A and hypoosmolar riboflavin solution in thin corneas. Journal of Cataract & Refractive Surgery 35, 621-624.

Han, L., Dean, D., Daher, L.A., Grodzinsky, A.J., Ortiz, C., 2008. Cartilage Aggrecan Can Undergo Self-Adhesion. Biophysical Journal 95, 4862-4870.

Hatami-Marbini, H., 2014. Hydration dependent viscoelastic tensile behavior of cornea. Annals of biomedical engineering 42, 1740-1748.

Hatami-Marbini, H., Etebu, E., 2013. Hydration dependent biomechanical properties of the corneal stroma. Experimental eye research 116, 47-54.

Hatami-Marbini, H., Etebu, E., Rahimi, A., 2013. Swelling pressure and hydration behavior of porcine corneal stroma. Current eye research 38, 1124-1132.

Hatami-Marbini, H., Jayaram, S.M., 2018. Effect of UVA/Riboflavin Collagen Crosslinking on Biomechanics of Artificially Swollen Corneas. Investigative Ophthalmology & Visual Science 59, 764-770.

Hatami-Marbini, H., Pinsky, P., 2009. On Mechanics of Connective Tissue: Assessing the Electrostatic Contribution to Corneal Stroma Elasticity. Mater. Res. Soc. Symp. Proc. 1239-VV03-07.

Hatami-Marbini, H., Rahimi, A., 2014a. Effects of bathing solution on tensile properties of the cornea. Exp Eye Res 120, 103-108.

Hatami-Marbini, H., Rahimi, A., 2014b. The relation between hydration and mechanical behavior of bovine cornea in tension. Journal of the mechanical behavior of biomedical materials 36, 90-97.

Hatami-Marbini, H., Rahimi, A., 2015. Evaluation of hydration effects on tensile properties of bovine corneas. Journal of Cataract & Refractive Surgery 41, 644-651.

Hayes, S., Kamma-Lorger, C.S., Boote, C., Young, R.D., Quantock, A.J., Rost, A., Khatib, Y., Harris, J., Yagi, N., Terrill, N., 2013. The effect of riboflavin/UVA collagen cross-linking therapy on the structure and hydrodynamic behaviour of the ungulate and rabbit corneal stroma. PLoS One 8, e52860.

Hjortdal, J.O., Jensen, P.K., 1995. In vitro measurement of corneal strain, thickness, and curvature using digital image processing. Acta ophthalmologica Scandinavica 73, 5-11.

Hodson, S., 1971. Why the cornea swells. Journal of theoretical biology 33, 419-427.

Jester, J.V., Winkler, M., Jester, B.E., Nien, C., Chai, D., Brown, D.J., 2010. Evaluating Corneal Collagen Organization Using High Resolution Non Linear Optical (NLO) Macroscopy. Eye & contact lens 36, 260-264.

Kohlhaas, M., Spoerl, E., Schilde, T., Unger, G., Wittig, C., Pillunat, L.E., 2006. Biomechanical evidence of the distribution of cross-links in corneastreated with riboflavin and ultraviolet A light. Journal of Cataract & Refractive Surgery 32, 279-283.

Kymionis, G.D., Portaliou, D.M., Diakonis, V.F., Kounis, G.A., Panagopoulou, S.I., Grentzelos, M.A., 2012. Corneal collagen cross-linking with riboflavin and ultraviolet-A irradiation in patients with thin corneas. American journal of ophthalmology 153, 24-28.

Lewis, P.N., Pinali, C., Young, R.D., Meek, K.M., Quantock, A.J., Knupp, C., 2010. Structural interactions between collagen and proteoglycans are elucidated by three-dimensional electron tomography of bovine cornea. Structure (London, England : 1993) 18, 239-245.

Maurice, D.M., 1984. The cornea and sclera, in: Davason, H. (Ed.), The eye. Academic Press, pp. 1-184.

Meek, K., 2008. The cornea and sclera, in: Fratzl, P. (Ed.), Collagen: structure and mechanics. Springer Science, pp. 359-396.

Rabinowitz, Y.S., 1998. Keratoconus. Survey of ophthalmology 42, 297-319.

Randleman, J.B., Dawson, D.G., Grossniklaus, H.E., McCarey, B.E., Edelhauser, H.F., 2008. Depth-dependent cohesive tensile strength in human donor corneas: implications for refractive surgery. Journal of refractive surgery (Thorofare, N.J. : 1995) 24, S85-89.

Scarcelli, G., Pineda, R., Yun, S.H., 2012. Brillouin Optical Microscopy for Corneal Biomechanics. Investigative Ophthalmology & Visual Science 53, 185-190.

Schumacher, S., Mrochen, M., Wernli, J., Bueeler, M., Seiler, T., 2012. Optimization model for UV-riboflavin corneal cross-linking. Invest Ophthalmol Vis Sci 53, 762-769.

Scott, J.E., 1992. Morphometry of cupromeronic blue-stained proteoglycan molecules in animal corneas, versus that of purified proteoglycans stained in vitro, implies that tertiary structures contribute to corneal ultrastructure. Journal of anatomy 180 (Pt 1), 155-164.

Scott, J.E., 2001. Structure and function in extracellular matrices depend on interactions between anionic glycosaminoglycans. Pathol Biol. 49, 284-289.

Seiler, T., Hafezi, F., 2006. Corneal cross-linking-induced stromal demarcation line. Cornea 25, 1057-1059.

Søndergaard, A.P., Ivarsen, A., Hjortdal, J., 2013. Reduction of Stromal Swelling Pressure after UVA-Riboflavin Cross-Linking. Investigative Ophthalmology & Visual Science 54, 1625-1634.

Wollensak, G., Spoerl, E., Seiler, T., 2003a. Stress-strain measurements of human and porcine corneas after riboflavin–ultraviolet-A-induced cross-linking. Journal of Cataract & Refractive Surgery 29, 1780-1785.

Wollensak, G., Spoerl, E., Wilsch, M., Seiler, T., 2003b. Endothelial cell damage after riboflavin-ultraviolet-A treatment in the rabbit. Journal of cataract and refractive surgery 29, 1786-1790.

Wollensak, G., Spoerl, E., Wilsch, M., Seiler, T., 2004. Keratocyte apoptosis after corneal collagen cross-linking using riboflavin/UVA treatment. Cornea 23, 43-49.

Wollensak, G., Spörl, E., Reber, F., Pillunat, L., Funk, R., 2003c. Corneal endothelial cytotoxicity of riboflavin/UVA treatment in vitro. Ophthalmic research 35, 324-328.

Groups		Dextran (%)	Thickness (μm)
Anterior	A11	2.5	670 ± 40
	A22	5	540 ± 80
	A33	10	410 ± 45
Posterior	P11	2.5	845 ± 150
	P22	5	650 ± 80
	P33	10	440 ± 50

Table 1. Dextran concentration and thickness of samples in different groups.

Fig. 1a)

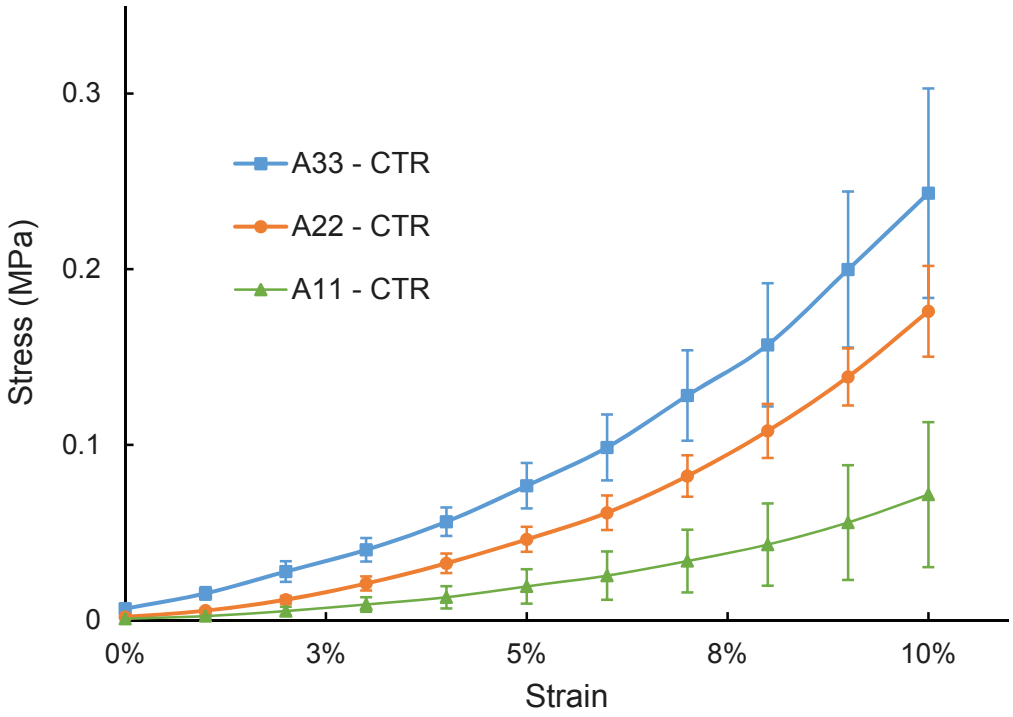

0.3
0.2
0.1
0
Stress (MPa)
A33 - CTR
A22 - CTR
A11 - CTR
0%
3%
5%
8%
10%
Strain

Fig. 1b)

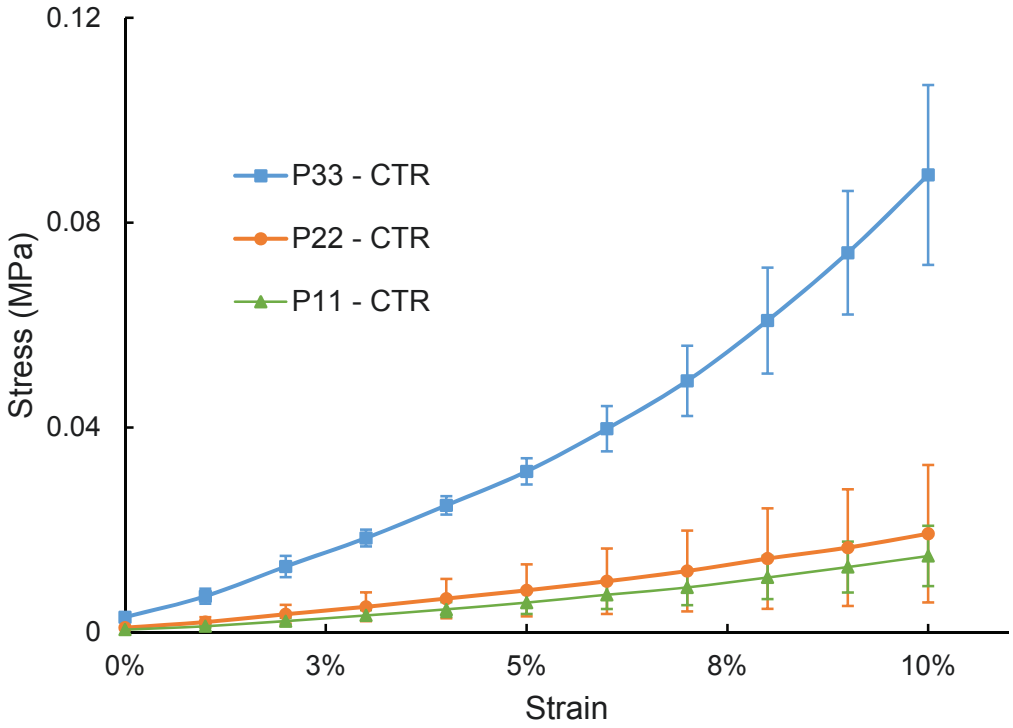

0.12
0.08
0.04
0
Stress (MPa)
P33 - CTR
P22 - CTR
P11 - CTR
0%
3%
5%
8%
10%
Strain

Fig. 2a)

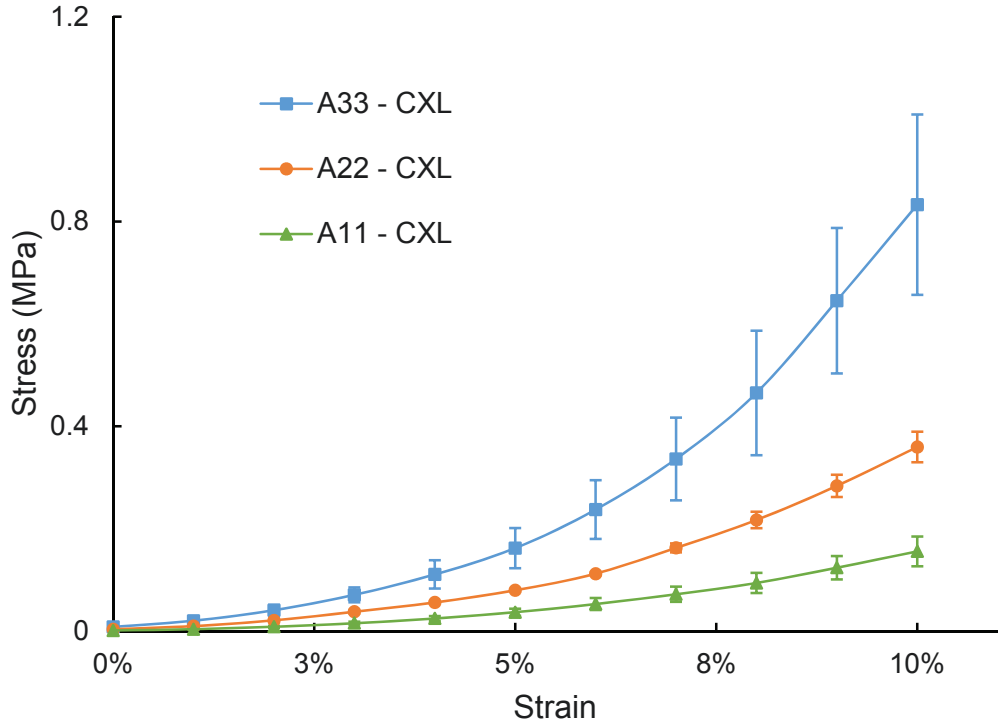

1.2
0.8
0.4
0
Stress (MPa)
A33 - CXL
A22 - CXL
A11 - CXL
0%
3%
5%
8%
10%
Strain

Fig. 2b)

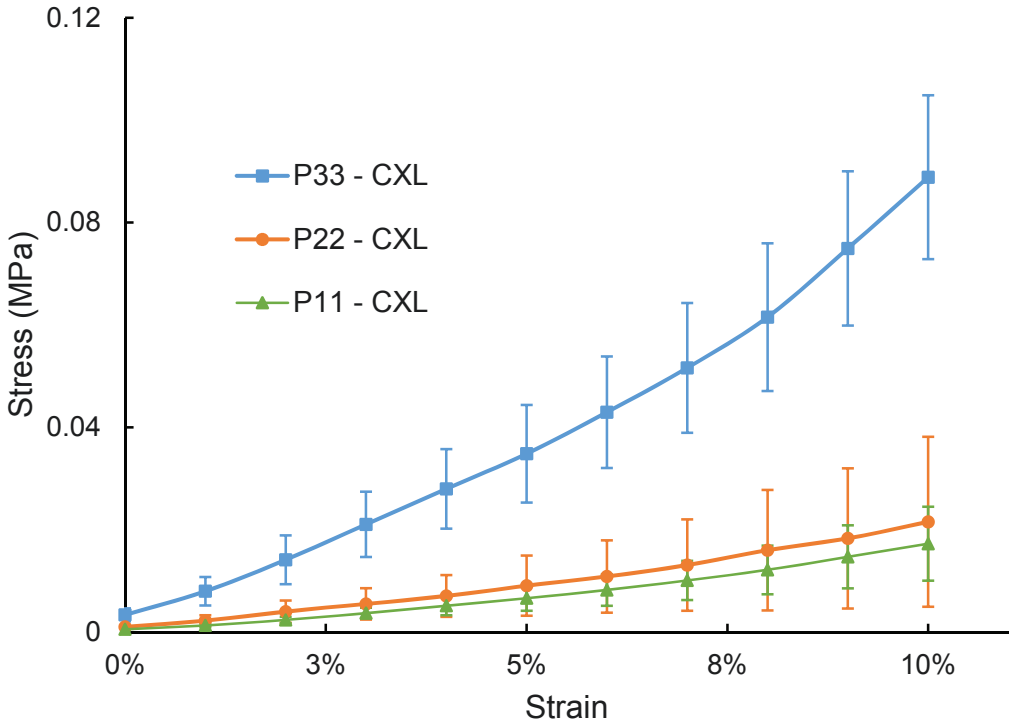

0.12
0.08
0.04
0
Stress (MPa)
P33 - CXL
P22 - CXL
P11 - CXL
0%
3%
5%
8%
10%
Strain

Fig. 3a)

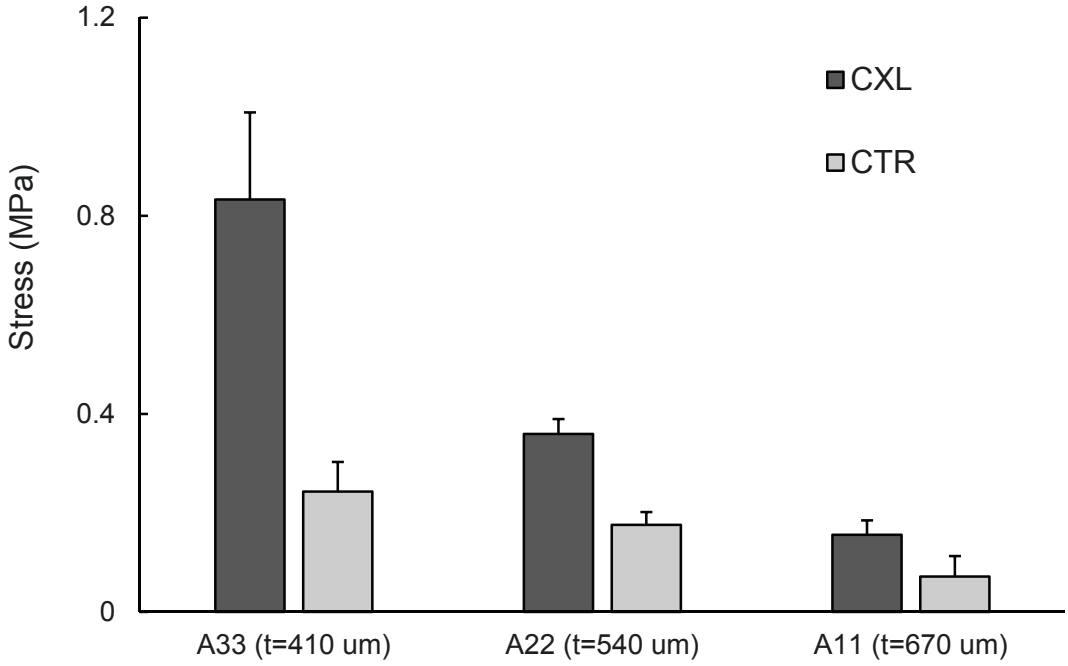

1.2
0.8
0.4
0
Stress (MPa)
CXL
CTR
A33 (t=410 um)
A22 (t=540 um)
A11 (t=670 um)

Fig. 3b)

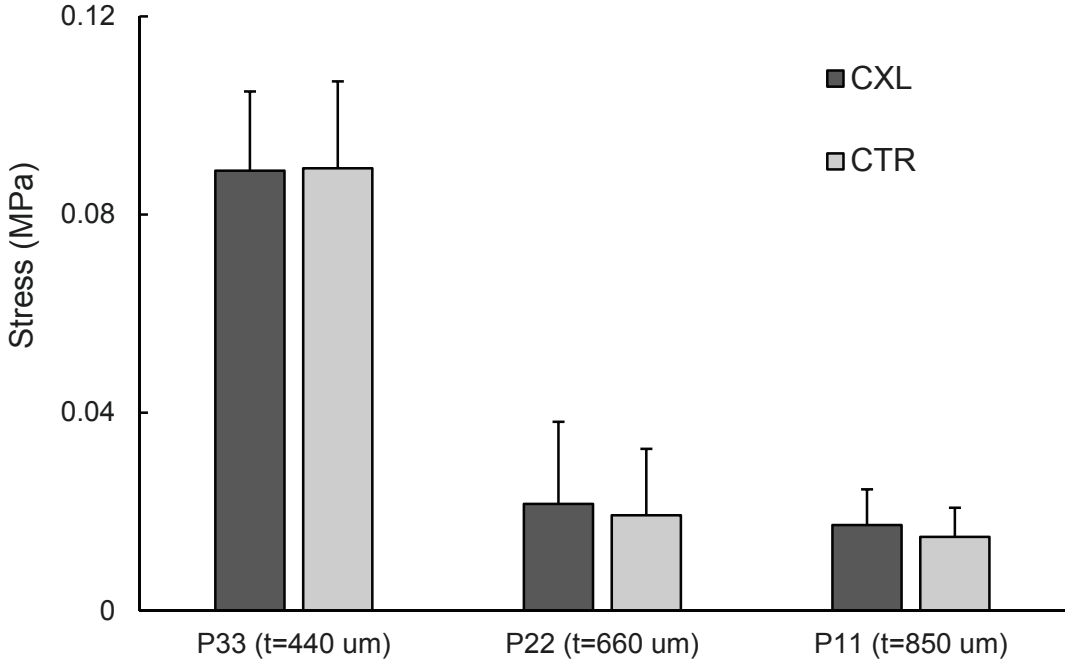

0.12
0.08
0.04
0
Stress (MPa)
CXL
CTR
P33 (t=440 um)
P22 (t=660 um)
P11 (t=850 um)

Fig. 4a)

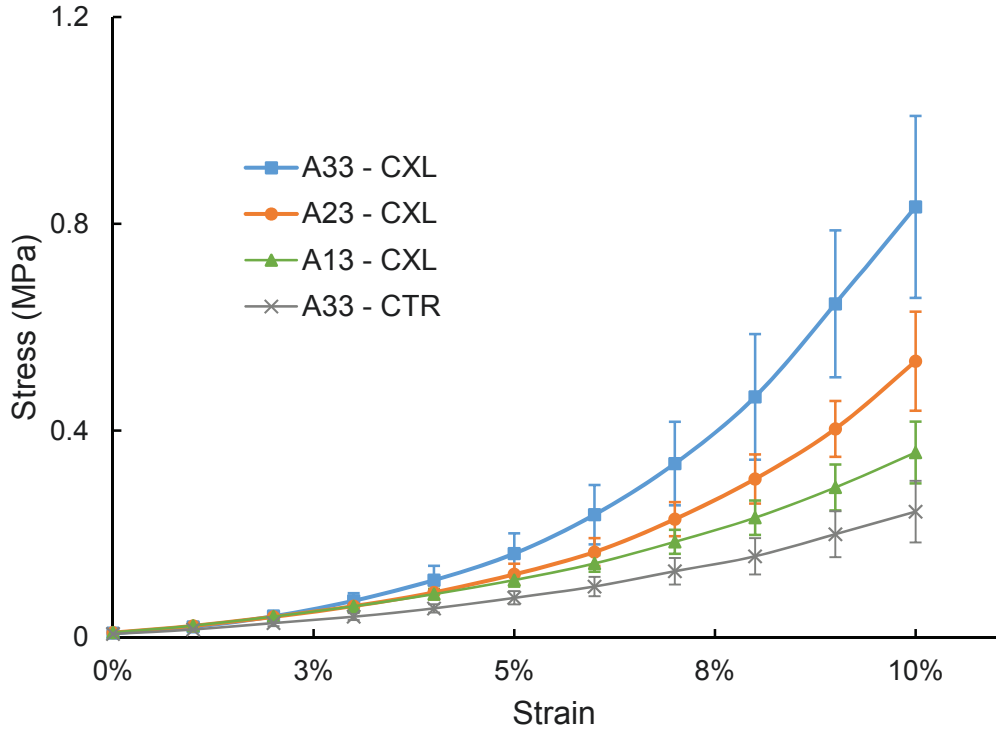
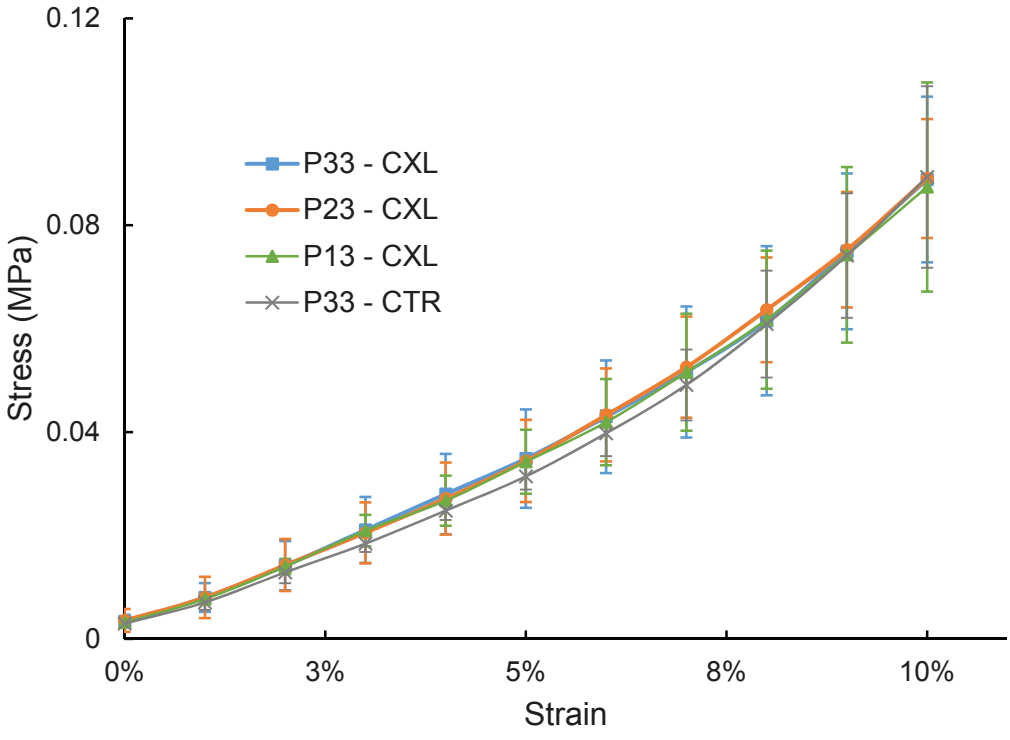

1.2
0.8
0.4
0
Stress (MPa)
A33 - CXL
A23 - CXL
A13 - CXL
A33 - CTR
0%
3%
5%
8%
10%
Strain

Fig. 4b)


0.12
0.08
0.04
0
Stress (MPa)
P33 - CXL
P23 - CXL
P13 - CXL
P33 - CTR
0%
3%
5%
8%
10%
Strain

Highlights

- Characterizing corneal inhomogeneous through-thickness tensile properties
- Measuring the stress-strain response of anterior and posterior flaps under tension
- Determining the interrelation of hydration and collagen crosslinking treatment