

Effect of UVA/Riboflavin Collagen Crosslinking on Biomechanics of Artificially Swollen Corneas

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PURPOSE. The purpose of this study was to characterize the relation between corneal hydration and stiffening effects of the UVA/riboflavin collagen crosslinking treatment and to investigate how artificially swelling the cornea prior to this treatment procedure affects tensile property improvement.

METHODS. Porcine corneas were collagen crosslinked in vitro at different hydration levels using a number of hypoosmolar and isoosmolar riboflavin solutions. Thickness of the specimens prior to crosslinking was taken as a proxy for their hydration and was used to divide them into different thickness groups. A Dynamic Mechanical Analysis (DMA) machine was used to perform mechanical tensile tests. The hydration of specimens during the mechanical tests was kept similar to the hydration at which they were crosslinked. The recorded force was used to calculate the maximum tensile stress and tangent modulus as a function of thickness (hydration) prior to collagen crosslinking treatment.

RESULTS. Collagen crosslinking with either a hypoosmolar or isoosmolar solution significantly increased corneal tensile modulus ($P < 0.05$). Corneas that were swollen prior to crosslinking showed significantly softer tensile properties compared with those that were crosslinked at lower hydration ($P < 0.05$). Although the degree of tensile property improvement was hydration dependent, the stiffness of samples crosslinked at higher hydration was not significantly different than the stiffness of those crosslinked at lower hydration when the hydration was kept similar in the mechanical experiments.

CONCLUSIONS. Swelling porcine corneas to the different extents prior to collagen crosslinking treatment does not significantly change the amount of biomechanical improvement if tensile properties are measured at similar hydration.

Keywords: corneal collagen crosslinking, hydration, hypoosmolar solution, mechanical testing, keratoconus

The cornea is a clear front window of the eye and is responsible for a significant amount of refracting and focusing power of the eye. The human cornea is a soft transparent tissue composed of five different layers: epithelium, Bowman's layer, stroma, Descemet's layer, and endothelium. It has been shown that the mechanical behavior of the cornea is largely governed by the stroma, which is mainly composed of collagen fibers and proteoglycans.^{1,2}

Keratoconus is a vision disorder in which the cornea progressively thins and develops a conical shape in response to the intraocular pressure. The onset of this ectatic disorder is generally during puberty and may remain progressive until the age of 30 to 40 years.³ In keratoconus, the corneal tissue loses its tensile strength because of sever disruption of its microstructure.⁴ Corneal collagen crosslinking is a new treatment procedure that can stop the progression of keratoconus.⁵ In this procedure, the application of riboflavin (a photosensitizer) and UV light induces covalent bonds, otherwise known as crosslinks, between collagen fibrils. These crosslinks are believed to improve the overall strength of the tissue, halt the outward bulging, and eventually help patients restore visual acuity.

The commonly used crosslinking protocol is the Dresden protocol, which uses an isoosmolar solution composed of 0.1% riboflavin 5'-phosphate sodium and 20% dextran T-500.⁵ Previous studies have shown that the riboflavin penetration into corneal stroma takes approximately 30 minutes.^{6,7} The corneal stroma has a strong tendency to swell in contact with aqueous solution. Dextran is a deturgescents agent that limits corneal swelling during the 30-minute-long application of riboflavin solution. In other words, the presence of dextran in riboflavin solution ensures a relatively constant (and may even cause a slight decrease in) corneal thickness during operation.

In advanced keratoconus, progressive corneal thinning leads to a significant reduction in thickness. Previous studies recommended a minimum stromal thickness of approximately 400 μ m to prevent cytotoxic effects of UVA irradiation.^{8,9} Failure to satisfy this requirement causes a significant decrease in endothelial cell counts.^{9,10} One of the promising ways to treat thin corneas is to swell them preoperatively with a hypoosmolar riboflavin solution.¹¹ The proposed modification has recently been tested on patients and proved to be successful in thin (but not extremely thin) corneas.¹¹⁻¹³ Nevertheless, the amount of stiffening effect of the collagen



crosslinking procedure on biomechanical properties of these artificially swollen corneas is still unknown.

Cornea is a hydrated tissue, and its water content has an important effect on its mechanical properties.^{14–17} For instance, increasing corneal hydration decreases its tensile and compressive stiffness.^{14,17} Thus, one may hypothesize that the increase in hydration of thin corneal samples caused by swelling them before crosslinking affects the degree of biomechanical improvement. The primary objective of the current study was to test this hypothesis by characterizing the relation between the corneal hydration before treatment and stiffening effects of the collagen crosslinking. To this end, we crosslinked porcine corneas that were artificially swollen to different extents and measured their tensile properties by conducting uniaxial tensile experiments.

MATERIALS AND METHODS

Specimen Preparation

Fresh porcine cadaver eyes of approximately 6-month-old pigs were brought from a local slaughter house within 6 hours postmortem. The eyes were inspected for any visible physical damage to the cornea, and if found, were excluded from the study. All samples were tested within 24 hours of procurement. First, the epithelial layer was carefully removed using the blunt edge of a scalpel. Then, a corneal scleral ring with approximately 2 mm of sclera was extracted.

Unlike the classical Dresden protocol that uses an isoosmolar crosslinking solution composed of 0.1% riboflavin and 20% dextran, we prepared riboflavin solutions with different osmolality by varying the dextran concentration. This was done to be able to crosslink specimens at different hydration levels (note that cornea swells to different amounts when soaked in crosslinking solutions that have different percentage of dextran). To determine the required dextran concentrations, we conducted a pilot hydration study in which we soaked porcine samples in solutions made of different dextran concentrations until their thickness reached equilibrium. The equilibrium thickness was measured using a digital pachymeter (DGH Technology, Inc., Exton, PA, USA) and was used to estimate the hydration of samples using previous hydration-thickness relation $H_w = 3\ln(5t)$.¹⁸ Based on this preliminary study, we made four 0.1% riboflavin solutions (Sigma-Aldrich Corp., St. Louis, MO, USA) with 2.5% to 20% dextran and divided the specimens into four distinct hydration groups (characterized here by their thickness): group A, $t = 590 \pm 50 \mu\text{m}$ ($H_w \sim 3.3$); group B, $t = 760 \pm 20 \mu\text{m}$ ($H_w \sim 4.1$); group C, $t = 1090 \pm 20 \mu\text{m}$ ($H_w \sim 5.1$); group D, $t = 1300 \pm 70 \mu\text{m}$ ($H_w \sim 5.7$). The concentration of dextran in riboflavin solutions was 20%, 10%, 5%, and 2.5% for groups A, B, C, and D, respectively. Within each group, five samples were crosslinked, and another five were subjected to a pseudo crosslinking treatment as described in the following; the samples in treatment and control groups were not from paired eyes.

All 10 samples from each thickness group were initially soaked for at least 30 minutes in the photosensitizer solution to ensure equilibration of their thickness and complete penetration of riboflavin into the corneal stroma.^{6,7} After measuring corneal thickness, a 5-mm-wide strip was punched in the nasal-temporal direction using a custom-built double-bladed punch. Previous studies have shown that strips with 5 mm width show the best repeatability in the stress-strain measurements.¹⁶ Furthermore, only strips from the nasal-temporal direction was prepared to prevent variation due to possible anisotropic properties. We crosslinked the specimens in the crosslinked

group by placing them on a hemispherical stage and exposing them to UVA rays of intensity of 3 mW/cm² for 30 minutes using a custom-made device.^{19,20} To minimize the exposure of the specimens to white light, we performed the crosslinking inside a dark room. Furthermore, drops of photosensitizer solution were added every 5 minutes during the treatment to ensure a sufficient supply of riboflavin. The same solution, which was used during the 30-minute soaking, was used during 30-minute UV irradiance to prevent any unwanted changes in thickness (hydration) due to variation in osmolality of the photosensitizer solution. To prepare control groups, referred to here as pseudo crosslinked, we soaked them in one of the four riboflavin solutions, depending on their group assignment, for 30 minutes, put them on the hemispherical stage, and applied drops of riboflavin solution every 5 minutes without the presence of UVA rays.

Biomechanical Measurements

A DMA machine (RSA-G2; TA Instruments, New Castle, DE, USA) was used to perform uniaxial tensile tests. All mechanical tests were done immediately after the preparation of cross-linked and pseudo crosslinked samples so that their properties could be characterized without significant changes to their hydration (Fig. 1a). Prior to mounting the specimens, we measured their thickness and width using a digital pachymeter (DGH Technology, Inc.) and a digital caliper (Mitutoyo Corp., Kawasaki, Japan), respectively. Sand papers were used at the grips to prevent any slippage. The strips were mounted at a loading gap of approximately 7 mm and were secured by applying a torque on the grips. A tare load of approximately 20 mN was applied to stretch the strips and define their initial length for strain calculations. A ramp tensile strain of 10% at a rate of 2 mm/min was applied to all strips and the reaction force was recorded. The measured force and strip cross-sectional area were used to calculate the tensile stress in specimens. The above tensile experiments were very fast (less than a minute); thus, they were done in air and without immersing the samples in a bathing solution as we did in our previous work.^{15,21} We measured the thickness of random samples after the mechanical tests and confirmed that no significant dehydration occurred during the experiments.

Furthermore, to characterize the tensile properties of the preswollen crosslinked cornea at their natural hydration, we crosslinked 15 more samples at average thickness (hydration) of group A, group C, and group D. Unlike what we did above (Fig. 1a), we ran mechanical tests on these specimens after air drying (or swelling) them until their average thickness (hydration) reached that of group B (i.e., $t_B \sim 760 \mu\text{m}$; Fig. 1b). Five samples were tested in each group, and the thickness of samples prior to crosslinking was 600 ± 20 (group E1), 1080 ± 40 (group E2), and $1280 \pm 20 \mu\text{m}$ (group E3).

Data Analysis

An exponential function $\sigma = \alpha(e^{\beta\varepsilon} - 1) + \sigma_0$ was used to numerically represent the tensile stress-strain curves. Here, σ_0 is the initial tare stress, and α and β are the fitting parameters. The Levenberg-Marquardt curve-fitting algorithm was used to find the best fit constants for the experimental measurements. The goodness of the exponential fits was determined by calculating fit coefficients of determination R^2 . All experimental data were reported as mean \pm SD, and the behavior of specimens in different groups was assessed using the 1-way ANOVA with significance level of 0.05.

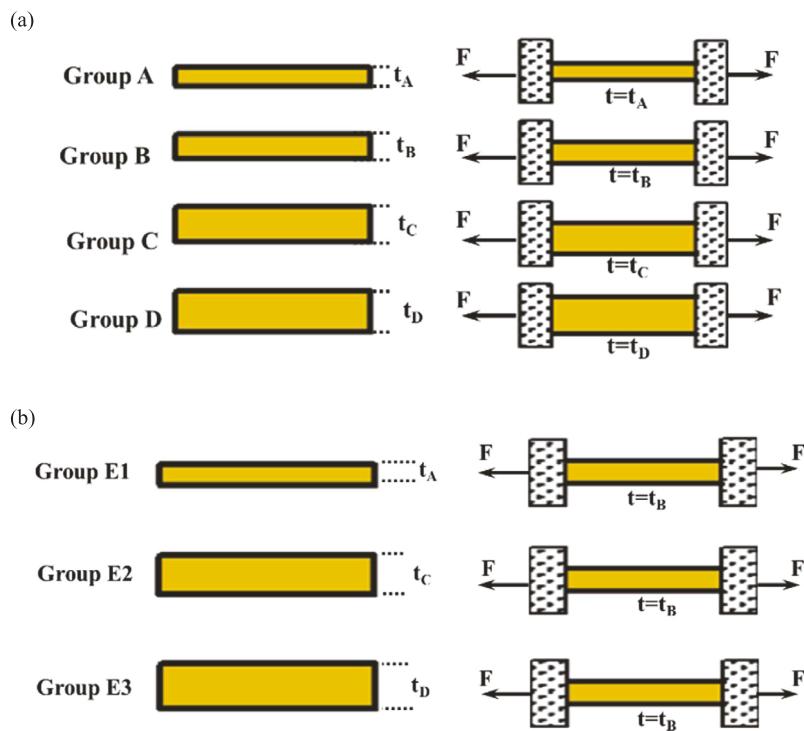


FIGURE 1. Schematic plot of the experimental study. (a) Samples were treated at different thickness (hydration) levels, and the mechanical measurements were done immediately after the treatment procedure. $t_A = 590 \pm 50 \mu\text{m}$, $t_B = 760 \pm 20 \mu\text{m}$, $t_C = 1090 \pm 20 \mu\text{m}$, and $t_D = 1300 \pm 70 \mu\text{m}$. (b) Samples were treated at different thickness (hydration) levels, but the mechanical measurements were done after the thickness (hydration) was brought to a predetermined amount.

RESULTS

Figure 2 compares the stress-strain behavior of crosslinked and pseudo-crosslinked samples. Tensile properties increased with decreasing the thickness (hydration) of samples in both groups. Furthermore, the average stress-strain curves for each group were accurately represented by an exponential relation ($R^2 > 0.99$). The Table gives the average and standard deviation (SD) of fit constants. The hydration of the samples in different groups is also given in this Table. Using the stress-strain curves given in Figure 2, we calculated the maximum tensile stress σ_{\max} and tangent modulus E_t of different groups at 10% strain. Figure 3 shows that both maximum stress and tangent modulus increased with decreasing sample thickness (hydration). Furthermore, this figure confirms that there was a significant difference between the behavior of crosslinked and pseudo-crosslinked specimens in each thickness group ($P < 0.05$). Figure 4 compares the behavior of samples that were crosslinked at different thickness (hydration) but tested at a similar hydration level. No significant difference between the tensile behavior of these samples was observed.

DISCUSSION

The primary objective of the present study was to determine the strengthening effects of corneal collagen crosslinking when this procedure is performed on samples at different hydration levels. In other words, this study was done to determine how swelling the cornea before the crosslinking treatment affects the collagen crosslinking biomechanical improvement. In corneal collagen crosslinking procedure, crosslinks are introduced in the stroma by subjecting the tissue to riboflavin solution and UVA irradiation. Upon exposure of the riboflavin to UVA rays, singlet oxygen

molecules, necessary for induction of crosslinks, are produced.²² By absorbing UVA radiations, the riboflavin also acts as a protective shield for the internal ocular components such as the endothelial cells, lens, and retina against possible UVA damages.²³ In the common crosslinking protocol, aka the Dresden protocol, the riboflavin photosensitizer solution contains 20% dextran T-500, a neutral polysaccharide with significant deturgescence effect. Prior to being used in corneal crosslinking, dextran was known among ophthalmologists as one of the main components of storage media (e.g., Optisol), which are widely used by eye banks for preservation of human corneas before transplantation. The cornea has an inherent tendency to imbibe water and significantly swell due to the presence of negatively charged proteoglycans.^{1,2} Dextran is present in crosslinking solution and corneal storage media to prevent unwanted corneal swelling and to keep thickness variation to a minimum.

The cytotoxic effect of UVA irradiation on endothelial cells becomes significant if the corneal thickness is less than 400 μm .^{8,9} It is known that keratoconus leads to corneal thinning. For example, a central corneal thickness of 448 ± 58 was found in 51 patients in a recent study.²⁴ The cornea continues to get thinner as the disease progresses and can reach a thickness of less than 300 μm in patients with advanced keratoconus. There have been previous studies showing that the application of isoosmolar riboflavin and 20% dextran alone, as suggested by the Dresden protocol, induces an additional decrease in the thickness of these corneas.²⁵ Thus, the standard crosslinking protocol is expected to impose an increased risk of damaging endothelium in patients with advanced keratoconus. Indeed, Kymionis et al.¹⁰ observed a significant decrease in endothelial cell density in corneas of patients with minimum corneal thickness of less than 400 μm .

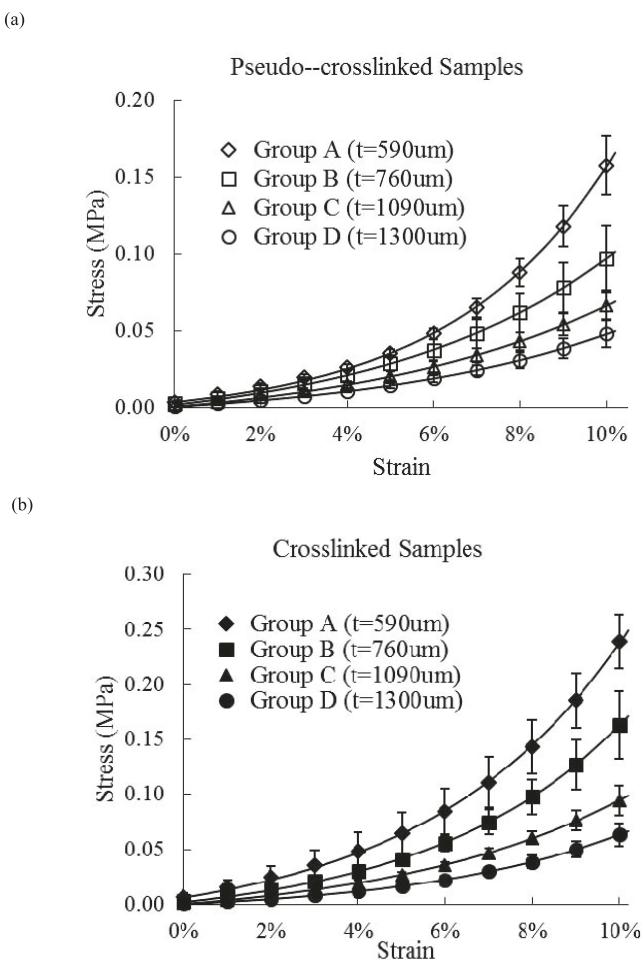


FIGURE 2. (a) Effect of thickness (artificially swelling/deswelling) on tensile stress-strain response of porcine corneal samples undergone pseudo collagen crosslinking treatment. (b) Effect of thickness (i.e., artificially swelling/deswelling samples) prior to the collagen crosslinking treatment on tensile stress-strain behavior of porcine cornea. The solid lines represent the exponential fits of the average data.

after they were treated by the standard collagen crosslinking protocol.

To protect the endothelium in thin corneas, Hafezi et al.¹¹ used a modified version of the Dresden protocol. They preoperatively swelled thin corneas of 20 patients with average stromal thickness of ~ 320 μm to thickness of at least 400 μm .¹¹

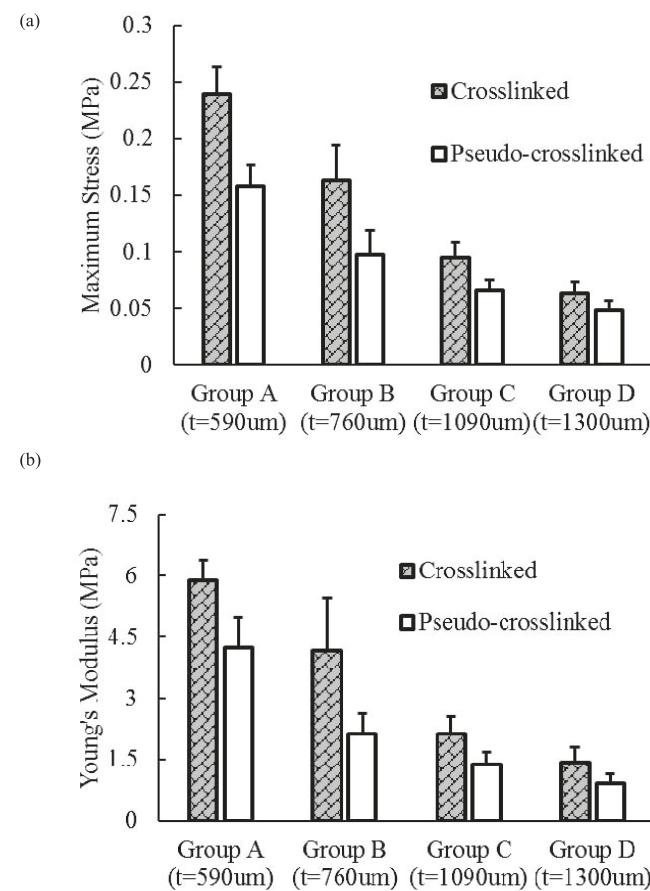


FIGURE 3. Effect of thickness (artificially swelling/deswelling) of collagen crosslinked and pseudo collagen crosslinked porcine corneal samples on (a) maximum tensile stress and (b) Young's modulus at 10% strain. A significant increase in maximum stress and Young's modulus ($P < 0.05$) was observed in all of the thickness groups.

The corneas were swollen by administrating hypoosmolar dextran-free riboflavin solution during the whole treatment period. Keratctasia was stabilized and no clinical sign of endothelial damage was observed in these patients. Later, the proposed approach, although with no signs of endothelial damage, failed to arrest the progression of the disease in a patient with a stromal thickness of 268 μm .¹³ Despite the above studies and similar reports in the field,^{11–13} the important question that remains to be answered is whether crosslinking using hypoosmolar riboflavin solution will affect

TABLE. Fit Constants and Average Coefficients of Determination When the Experimental Measurements Were Fitted With an Exponential Relation, $\sigma = \alpha(e^{\beta c} - 1) + \sigma_0$

Groups	Type	t (μm)	H_w	α (MPa)	β	σ_0 (MPa)
A	CXL	590 ± 50	3.29 ± 0.19	0.028 ± 0.008	23.3 ± 2.0	0.007 ± 0.002
	Pseudo-CXL			0.015 ± 0.001	24.2 ± 1.6	0.007 ± 0.001
B	CXL	760 ± 20	4.10 ± 0.10	0.019 ± 0.002	22.3 ± 1.6	0.006 ± 0.001
	Pseudo-CXL			0.014 ± 0.001	22.7 ± 1.7	0.005 ± 0.002
C	CXL	1090 ± 20	5.12 ± 0.07	0.012 ± 0.002	21.8 ± 2.0	0.004 ± 0.002
	Pseudo-CXL			0.007 ± 0.001	22.8 ± 1.4	0.004 ± 0.002
D	CXL	1300 ± 70	5.68 ± 0.14	0.009 ± 0.003	20.5 ± 2.9	0.003 ± 0.001
	Pseudo-CXL			0.006 ± 0.001	21.5 ± 1.9	0.003 ± 0.001
E1	CXL	600 ± 20	3.31 ± 0.11	0.018 ± 0.001	23.7 ± 1.2	0.003 ± 0.002
E2	CXL	1080 ± 40	5.11 ± 0.10	0.019 ± 0.003	23.0 ± 2.4	0.006 ± 0.001
E3	CXL	1280 ± 20	5.63 ± 0.05	0.019 ± 0.003	23.9 ± 2.4	0.003 ± 0.003

Hydration of the specimens are calculated from the following equation: $H_w = 3\ln(5t)$.¹⁸

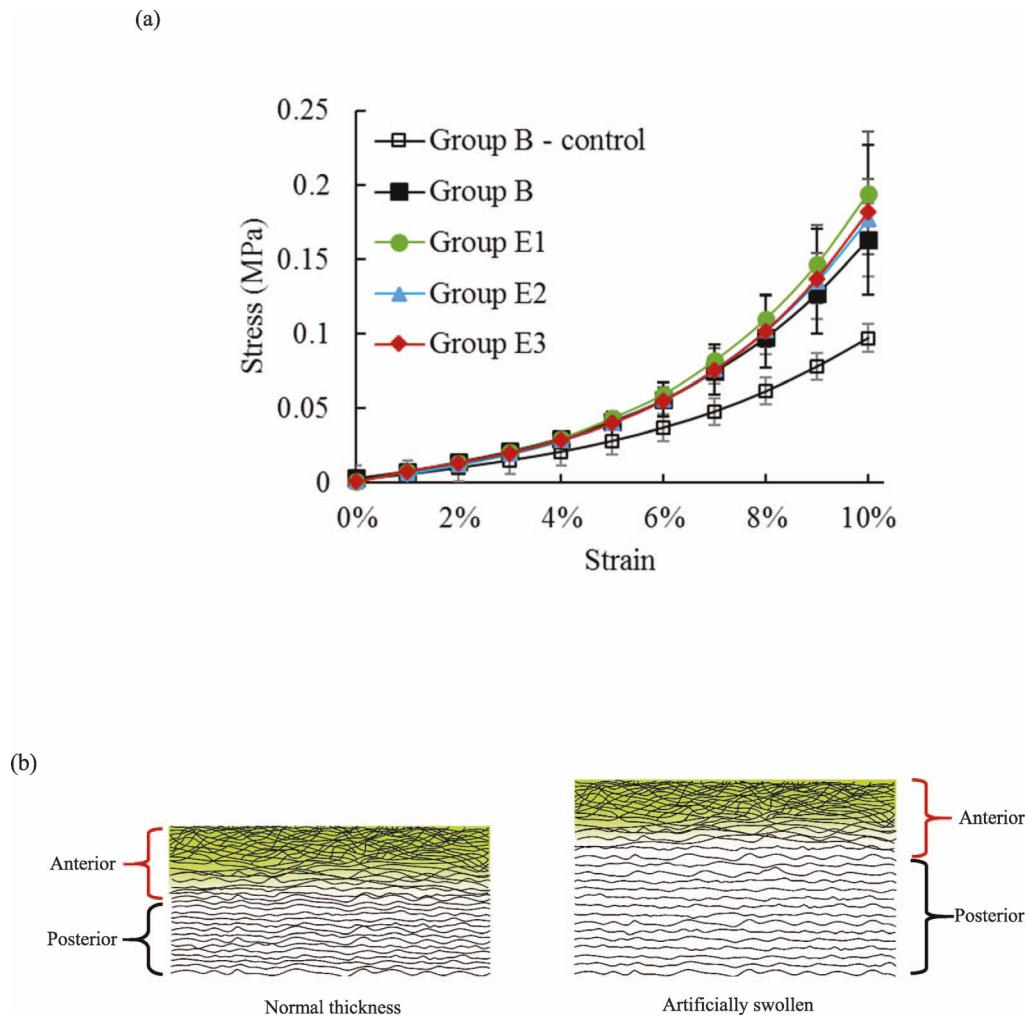


FIGURE 4. (a) Stress-strain behavior of porcine corneas that were crosslinked at different thickness (hydration) but tested at the same thickness (hydration). Samples in groups E1, E2, and E3 were crosslinked at thickness 600 ± 20 , 1080 ± 40 , and $1280 \pm 20 \mu\text{m}$, respectively, but tested at the thickness of group B ($t \sim 760 \mu\text{m}$). The behavior of pseudo crosslinked samples in group B is also plotted to show the strengthening effect of the collagen crosslinking treatment procedure. The solid lines represent the exponential fit of the average data. (b) Schematic plot showing possible microstructural level properties responsible for the observed properties. The anterior collagen lamellae do not significantly swell in artificially swollen cornea, and their relative distance remains almost the same. Thus, almost the same portion of corneal thickness is expected to be crosslinked when it is swollen prior to the treatment procedure. The green color represents the depth of crosslinking treatment.

the effectiveness of corneal collagen crosslinking procedure. In other words, will the increase in intermolecular and interfibrillar spacings of collagen fibrils in artificially swollen corneas reduce the biomechanical effects of riboflavin/UV collagen crosslinking?

In previous studies, our group has shown that hydration plays a significant role in defining the biomechanical properties of the cornea. Corneal mechanical properties as measured by uniaxial tensile tests and unconfined compression experiments showed a significant decrease with increasing hydration.^{14–17} The hydration-dependent biomechanics has also been observed in collagen crosslinked corneas.^{19,20} In the present study, we crosslinked porcine corneas at different initial thickness (hydration) using isoosmolar solution (composed of 20% dextran) and three different hypoosmolar solutions (composed of 10%, 5%, and 2.5% dextran) to determine the relation between the hydration of the samples and the stiffening effect of the collagen crosslinking procedure.

The in vivo thickness of porcine cornea is estimated to be approximately $700 \mu\text{m}$.^{18,26} Thus, although group A and group B represent the cases in which the porcine samples were

crosslinked at approximately their in vivo thickness (hydration), groups C and D are cases when corneas were artificially swollen before being crosslinked. Figure 2 shows that, similar to previous studies on bovine corneas,^{15,16} the behavior of porcine cornea is hydration dependent. For example, the maximum tensile stress, σ_{max} , of samples in group A ($t = 590 \pm 50 \mu\text{m}$) was significantly higher than samples in group D ($t = 1300 \pm 70 \mu\text{m}$; $P < 0.05$). Although a significant increase in Young's modulus was observed in all four groups due to the collagen crosslinking ($P < 0.05$), this increase was hydration dependent. In other words, biomechanical improvement due to the collagen crosslinking in samples, which were swollen to a higher thickness (hydration) prior to the treatment procedure, was not as high as those that were swollen to a lower thickness (hydration). Note that the stiffening effect can be accurately measured if the comparison is done using control and crosslinked samples at similar thickness (hydration). Otherwise, the hydration differences will introduce errors in the experimental measurements.^{15–17}

Figure 4 compares the mechanical behavior of samples, which were swollen to different extents prior to being collagen

crosslinked, but their mechanical properties were measured while they all had almost similar hydration. The thickness (hydration) of samples during crosslinking was 600 ± 20 ($H_w \sim 3.3$), 1080 ± 40 ($H_w \sim 5.1$), and $1280 \pm 20 \mu\text{m}$ ($H_w \sim 5.6$); and their thickness (hydration) during the mechanical tests was similar to that of group B (i.e., $760 \pm 20 \mu\text{m}$; $H_w \sim 4.1$). No significant difference ($P = 0.88$) was observed in Young's modulus of these groups. This is an important observation indicating that if corneas are artificially swollen and cross-linked, the improvement in their mechanical properties will be similar to the case in which they are not swollen. In the following, we attempt to explain this observation in terms of corneal microstructure.

The corneal stroma is composed of many parallel-to-the-surface sheets of collagen fibrils, known as lamellae. In collagen lamellae, thin collagen fibrils are regularly packed in a hydrated proteoglycan matrix.^{1,2} The proteoglycans are negatively charged and create a strong tendency in the cornea to swell when immersed in an ionic solution.^{27,28} Polarized light imaging has shown that lamellae interweave at the anterior stroma while they lie parallel to each other in the posterior region. High-resolution microscopy has also shown that there exists clear depth-dependent collagen interconnectivity in central cornea.²⁹ Different anterior and posterior collagen microstructure is directly correlated to the depth-dependent mechanical properties. High-resolution Brillouin images of the cornea showed a clear depth-dependent variation of elastic modulus.³⁰ Furthermore, the anterior corneal stroma has been shown to have significantly higher cohesive tensile strength than the posterior cornea.³¹ The elastic modulus of the anterior stroma is larger than the modulus of its posterior part because of significantly interwoven organization of collagen fibers in this region.

In addition to the significant difference in microstructure and mechanics of posterior and anterior stroma, it is well developed that the collagen crosslinking procedure creates primarily crosslinks in the anterior corneal stroma.^{30,32-35} The depth-dependent stiffening of the collagen crosslinking has been shown by different methods such as mechanical tests of anterior and posterior flaps dissected from crosslinked corneas and Brillouin microscopy of treated samples.^{30,34} Other methods have also showed that collagen crosslinking primarily affects the anterior part of stroma. For example, keratocyte apoptosis was observed to be limited to the anterior stroma and a demarcation line was observed in the anterior corneal stroma of collagen crosslinking corneas.^{32,33} Finally, the spatial distribution of the corneal stiffening due to the collagen crosslinking has been captured by a numerical model.³⁵ There are also significant differences between swelling behavior of anterior and posterior cornea. Thus, the changes in hydration at the macro scale (or changes in collagen interfibrillar spacing at the micro scale) caused by application of hypoosmolar or isoosmolar solution occur mainly in the posterior stroma, where the collagen lamellae are parallel to each other.^{36,37} For instance, Müller et al.³⁷ observed that top $100 \mu\text{m}$ of the anterior stroma showed strong resistant to swelling when immersed in water. Putting all these together, we can conclude that when samples are artificially swollen by using a hypoosmolar riboflavin solution, the microstructure of the anterior part of the cornea, where crosslinks are induced, does not alter significantly. Thus, these samples should behave similarly to those crosslinked using the common crosslinking solution. (Note that both groups are required to be tested at the same thickness (hydration) to nullify the hydration effects on measured properties.^{16,17}) The results shown in Figure 4 support this discussion. The slight difference in the curves corresponding to the mechanical response of samples cross-linked at different thickness (590, 750, 1100, and 1300 μm)

and tested at 750 μm could be either because the hydration of anterior layers varied slightly as thickness (hydration) of the whole tissue increased or because of the intrinsic differences between the mechanical properties of specimens. Although this study did not include any data on behavior of keratoconic corneas, its findings can be used to speculate why crosslinking with hypoosmolar riboflavin solution is successful for thin corneas but not for extremely thin corneas. First, it needs to be mentioned that the disease in extremely thin corneas has often progressed thus far that their inflammatory profile is expected to be too strong to be mediated by biomechanical stiffening. Hafezi reported that corneal collagen crosslinking using hypoosmolar riboflavin solution failed to arrest the disease progression in a patient with very thin cornea.¹³ The preoperative stromal thickness of this patient was 268 μm ; thus, the treatment was not effective possibly because the thickness of the anterior portion was not sufficient. In other words, the collagen crosslinking mostly affects the anterior corneal stroma and the posterior stroma does not stiffen significantly by crosslinking.^{34,38} Future studies (possibly on keratoconus corneas) are required to determine the required minimum thickness of anterior corneal stroma for a successful crosslinking treatment and to explain better implications of the present study for effects of collagen crosslinking on keratoconic corneas. Future studies are also required to determine whether a distinct anterior stromal layer is even present in extremely thin corneas and whether its presence makes a difference in effectiveness of collagen crosslinking treatment.

In the current study, we used thickness of the samples as a measure of their hydration and the thickness-hydration relation for normal porcine corneas was used to estimate the hydration of different groups. Hayes et al. showed that the crosslinking procedure had no significant effect on the hydrodynamic response of the cornea.³⁹ Similarly, our preliminary studies showed that riboflavin/UVA collagen crosslinking did not change corneal hydration-thickness relation. However, intrinsically thick or thin corneal samples may exist in each thickness group. Despite this possibility, we still expect a significant difference between the hydration of strips in different groups due to the significant (150 to 200 μm) thickness difference between them. Moreover, it is noted that ophthalmologist surgeons use the thickness as a surrogate for determining the corneal hydration prior crosslinking treatment. A limitation of this work is that we characterized the effect of collagen crosslinking on hydrated porcine cornea, which were swollen from their normal thickness, and not on keratoconus corneas. Although the findings are consistent with previous reports in literature on the effects of collagen crosslinking on thin keratoconus corneas, future studies are required to fully characterize the efficacy of collagen crosslinking using hypoosmolar solution on diseased corneas. Despite these limitations, this study determined the mechanical improvements due to riboflavin/UVA collagen crosslinking of swollen/deswollen corneas. We found that with increasing the hydration of porcine cornea, their stiffness reduced. Furthermore, we found that the crosslinking treatment significantly improved corneal biomechanics independent of their hydration at the time of treatment. Also, in comparison with hydrated crosslinked porcine corneas ($t >> 666 \mu\text{m}$), specimens crosslinked close to *in vivo* porcine cornea thickness ($t \sim 666 \mu\text{m}$) showed a significantly stiffer response. Nevertheless, almost similar improvement in mechanical properties was found when the specimens were tested at approximately their *in vivo* thickness (hydration). Thus, we can conclude that the thickness (hydration) of the cornea before crosslinking might not have a significant effect on the degree of biomechanical improvement due to riboflavin/UVA collagen crosslinking treatment procedure. In closing, we note

that although the success of corneal crosslinking is correlated with thickness (hydration), this should not be perceived as the only factor guaranteeing the success of this treatment procedure.

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