Effect of Hydration State and Storage Media on Corneal Biomechanical Response From In Vitro Inflation Tests

Sabine Kling, MSc; Susana Marcos, PhD

ABSTRACT

PURPOSE: To evaluate corneal deformation with varying intraocular pressure and the dependency of the biomechanical response on the corneal hydration state, modulated by the storage solutions or postmortem period.

METHODS: Thirty fresh enucleated porcine eyes were used for in vitro whole eye globe inflation experiments. The eyes were separated into five groups and treated with different solutions: 20% dextran, 8% dextran, 0.125% riboflavin–20% dextran, Optisol-GS (Bausch & Lomb, Rochester, NY), and one control group of virgin (untreated) eyes. Intraocular pressure was increased (from 15 to 55 mm Hg) and decreased (to 15 mm Hg) in 5-mm Hg steps and Scheimpflug images were taken at each step. Measurements were repeated after 24 hours. Thickness and curvature changes were analyzed as a function of intraocular pressure.

RESULTS: Corneal deformation differed across conditions and hydration states. Dehydration by any dextran solution increased the hysteresis after the inflation/deflation cycle (14.29 vs 22.07 to 41.75 μm), whereas overnight hydration did not lead to a significant difference. Compared to control corneas, corneas treated with Optisol-GS showed the most similar behavior. Corneas treated with 0.125% riboflavin–20% dextran deformed most ($\Delta thickness_{max} = 38.27\ \mu m$), indicating a softening of the corneal tissue compared to control corneas (23.18 μm) and corneas treated with 8% dextran (21.01 μm) and 20% dextran (29.07 μm). Dextran instillation decreased corneal thickness on average to 56.5% at 0 hours and 72.7% at 24 hours.

CONCLUSIONS: Corneal hydration and tissue preservation changed corneal biomechanics, in particular its relaxation over a period of 24 hours.

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Quantifying corneal biomechanical properties is important for understanding different corneal pathologies and treatments. Most experimental data of corneal biomechanics have been obtained through in vitro extensiometry measurements, where stress-strain functions are obtained from corneal strips.[1-3] Alternatively, corneal inflation experiments in vitro[4-6] have allowed the analysis of the cornea in a condition closer to that of the living eye.[7] However, the experimental conditions (including time postmortem, temperature, and preservation media) affect the hydration state and microstructure of the cornea and therefore likely play a role on the biomechanical properties estimates. The pH and the osmotic tolerance of the corneal epithelium are considered to affect the corneal swelling susceptibility,[8] and swelling itself has been found to decrease the mechanical strength of the corneal tissue.[9]

Medical solutions, typically applied for preservation or treatments, are also known to modify the corneal hydration state, both in vivo and in vitro.[10] This is the case for riboflavin–dextran, used as a photosensitizer in the ultraviolet collagen cross-linking (CXL) procedure. Ultraviolet-A CXL is a technique aimed at increasing corneal rigidity,[1,11] and is generally performed with a concentration of 0.125% riboflavin in 20% dextran[1,12] (other riboflavin concentrations[11] and hypotonic solutions have also been tested).[13,14] In previous inflation experiments,[15] we found a significant stiffening of the cornea, with the CXL corneas showing an increase of 1.58 in the estimated Young modulus. It is interesting to investigate to what extent the riboflavin–dextran solution alone induces a change in the biomechanical properties of the cornea, with respect to

those of virgin (untreated) eyes. The standard photosensitizer solution for CXL contains dextran, a neutral polysaccharide well-known for its dehydrating effect. Dextran is often used to dehydrate the corneal tissue after storage in eye banks or to prevent swelling in in vitro experiments. Another medical solution is Optisol-GS (Bausch & Lomb, Rochester, NY), one of the most widespread storage media for human corneas in eye banks for preservation before transplantation.[16]

Although the ultimate goal in many applications addressing corneal biomechanics is to obtain corneal biomechanical properties in vivo, in vitro measurements are an important step forward, particularly if they are tested in conditions close to those in vivo. Porcine eye models are often used for this purpose because human and pig eyes show a similar corneal stress-strain response[16] under applied loading. Although there are some differences, such as a higher thickness, a lower viscoelastic creep,[17] and higher stiffening after CXL,[1] porcine corneas are considered a good model to study biomechanical properties and their change in response to treatments. The water content of porcine corneas[18] is slightly lower compared to human corneas (71.79% vs 78%) and therefore porcine corneas might be more susceptible to edema.

The current study investigated the differences in corneal deformation patterns on changes in intraocular pressure (IOP) across different hydration conditions of the corneal tissue. This helped us to identify the best preservation media or photosensitizer solution and optimal baseline conditions for the investigation of the corneal tissue's biomechanical properties.

MATERIALS AND METHODS

EXPERIMENTAL METHODS

Eyes. Thirty fresh, enucleated porcine eyes (left and right eyes packed together) were obtained from a local slaughterhouse (prior to scalding) and used within 4 hours postmortem. The eyes were separated equally into five groups and treated with different medical solutions (**Table 1**). In four groups, measurements were performed immediately and at 24 hours. In the Optisol-GS group, measurements were performed at 24 hours only. Eyes were allowed to hydrate (swell) over night. These times and storage conditions were chosen to study differences due to storage time. Human cadaver eyes often are stored in eye banks before being used for biomechanical measurements and typically undergo such a hydration and dehydration process.

Dextran/Riboflavin–Dextran Treatment. Dextran was applied for a short period of time (30 minutes), because it is typically done to dehydrate corneas of eye bank eyes[16] or with riboflavin–dextran solutions

TABLE 1				
Summary of the Different Conditions Studied				
	No. of Eyes		**De-epithelialization**	
Group	**0 Hours**	**24 Hours**	**0 Hours**	**24 Hours**
Virgin (untreated)	6	6	No	Yes
8% dextran	6	6	Yes	Yes
20% dextran	6	6	Yes	Yes
0.125% riboflavin–20% dextran	6	6	Yes	Yes
Optisol-GS	–	6	–	Yes
Optisol-GS is manufactured by Bausch & Lomb, Rochester, NY.				

in CXL treatment.[12] De-epithelialization to allow the solutions to better diffuse into the cornea was achieved with a hockey epithelium removal knife. Different concentrations of dextran solutions were tested: 8% dextran, 20% dextran, and 0.125% riboflavin–20% dextran (which is the photosensitizer solution typically used in CXL treatments) in different sets of eyes. All solutions were diluted in a 0.9% NaCl solution. Solutions were instilled for 30 minutes (one drop every 3 minutes) before measurements were started. Measurements were repeated after 24 hours. The eyes were wrapped hermetically with aluminum foil for storage at 4°C in a refrigerator. Hypothermia typically allows the preservation of the corneal endothelium for 7 to 14 days,[19] but corneas swell at low temperatures and hence the 24-hour results may not be representative of what might happen in vivo.

Optisol-GS Treatment. Optisol-GS is used specifically for long-term storage of corneas. Therefore, eyes treated with Optisol-GS were only measured 24 hours after storage. The eyes were immersed in Optisol-GS solution overnight and stored at 4°C. It was necessary to de-epithelialize the corneas before starting the measurements to allow proper corneal imaging.

Control Eyes. Virgin (untreated) eyes were used as control eyes, without undergoing any treatment. Epithelial transparency loss prevented measurements of the intact eyes after 24 hours. For this reason, the eyes were de-epithelialized immediately after the first session. The eyes were then wrapped hermetically with aluminum foil and stored for 24 hours before repeating the measurements.

Scheimpflug Imaging. Corneal geometry measurements were obtained using an imaging system (Pentacam 2.73R18; Oculus Optikgeräte GmbH, Wetzlar, Germany) based on the Scheimpflug principle. We have presented validations of the accuracy of the anterior and posterior corneal shape measured with this system[20] using a hybrid porcine-plastic model eye, which
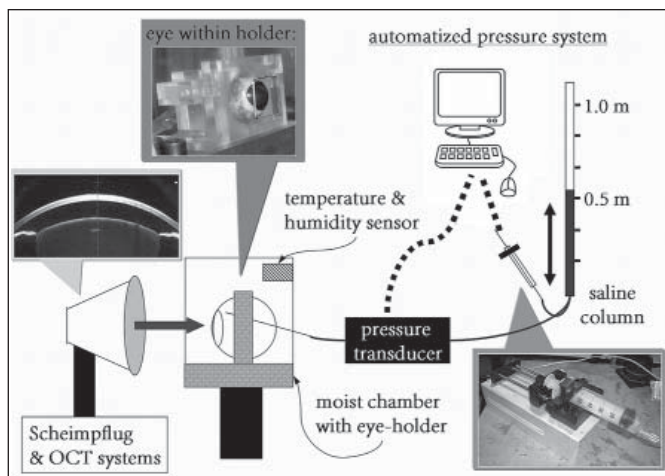
Figure 1. Set-up for the in vitro whole eye globe inflation experiments. OCT = optical coherence tomography

demonstrated that the optical distortions are properly corrected and the system reliably measured anterior and posterior corneal curvature. Three-dimensional image acquisition with this system (25 meridional sections per image) was performed automatically and in synchronization with an inflation system, as previously described.[15]

Experimental Set-up. The measurement set-up for corneal imaging on variable IOP is illustrated in **Figure 1**. A detailed description of the set-up has been previously published.[15] Briefly, a water column was filled with 0.9% saline solution and connected to the eye globes to change the IOP. A pressure transducer converted the IOP into an appropriate input signal for a customized MATLAB (MATLAB R2009a; Math-Works, Natick, MA) routine and allowed to automatize the inflation process. The MATLAB program also controlled the pumping system (NE-500; New Era Pump Systems, Inc., Wantagh, NY), which changed the IOP by varying the height of the water column. Temperature and humidity within the eye chamber were continuously monitored and recorded.

Experimental Protocols. Each enucleated porcine eye was imaged separately. Motion during the experiment was prevented by fixing the eyes in a customized eye holder, which could accommodate the individual differences in diameter across eyes. Within the holder, we oriented the eye similarly as it was naturally oriented in the pig (ie, the longer side horizontally and the optical nerve head upwards), preserving the natural superior-inferior orientation. The experiments were performed with the porcine eye within a moist chamber to guarantee a constant humidity. To allow undistorted Scheimpflug imaging, a small window was cut in the box. Control measurements (moist chamber without eye) showed that relative humidity

was kept constant at 48% (23°C room temperature). A more detailed description of the measurement set-up can be found in Kling et al.[15]

One full inflation procedure took approximately 20 minutes. First, 15 mm Hg of IOP (assumed as the physiological IOP of pigs) were adjusted for reference. Then, IOP was increased from 5 to 55 mm Hg steps, and then decreased at 5-mm Hg intervals. Each pressure step was held constant for 1 minute before a Scheimpflug image of the anterior segment of the eye was collected. This ensured that the cornea had adapted a constant shape before the measurement. Measurements were performed without prestressing[21,22] to avoid the drastic changes in the mechanical relaxation behavior (particularly the viscoelastic behavior) normally produced when preconditoning the tissue,[23] and to allow evaluation of the corneal viscoelastic properties of the original first-stretch behavior in natural conditions. Also, the fact that in our inflation set-up the corneal tissue was loaded in the same direction as in vivo conditions and the globes were constantly exposed to the IOP made it unnecessary to precondition the tissue, because it was never allowed to relax before the measurements and allowed the fibers toward the load direction.[24,25] These favorable conditions resulted in highly repeatable curvature and thickness deformation on IOP variation, even without tissue prestressing.

Data Analysis. Corneal thickness and mean corneal curvature were obtained for all conditions and IOP levels (in a central zone of 6 mm). The mean corneal thickness was directly taken from the Scheimpflug imaging software. The apical mean radii of curvature were obtained from conic fittings of the anterior and posterior corneal elevation data. Mean corneal curvature represents the radius of the most similar sphere and was used in previous studies to estimate corneal stress. However, because changes in corneal thickness are more directly related to the corneal biomechanical properties (the curvature changes also depend largely on the overall geometry and scleral properties), we set our main focus on corneal thickness changes happening under three-dimensional stress-strain testing.

The corneal hydration state was estimated by the normalized thickness:

$$H = \frac{th}{th_0} \qquad (1)$$

where th is corneal thickness in treated eyes at different times and th_0 is corneal thickness in control eyes at 0 hours. H = 1 refers to normally hydrated corneas.

Stress-Strain Plots and Corneal Hysteresis. Corneal thickness was plotted against IOP (increasing and decreasing) similarly to standard stress-strain graphs,
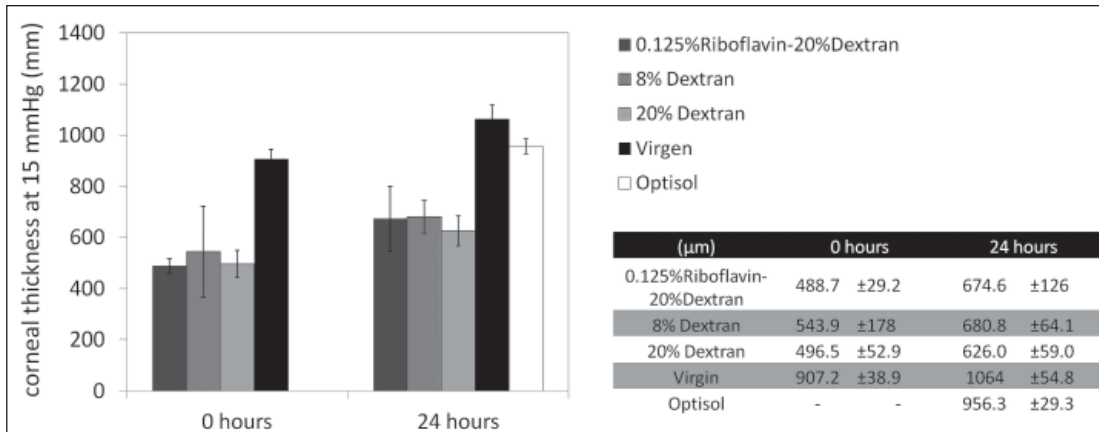
(µm)	0 hours		24 hours	
0.125%Riboflavin-20%Dextran	488.7	±29.2	674.6	±126
8% Dextran	543.9	±178	680.8	±64.1
20% Dextran	496.5	±52.9	626.0	±59.0
Virgin	907.2	±38.9	1064	±54.8
Optisol	-	-	956.3	±29.3

Figure 2. Initial corneal thickness before intraocular pressure variation for all tested conditions at 0 and 24 hours.

TABLE 2

Hysteresis (Remaining Thickness Deformation) After IOP Variation for Different Conditions at 0 and 24 Hours

	Hysteresis (µm)	
Group	0 Hours	24 Hours
Virgin (untreated)	-14.29	-13.29
8% dextran	-22.07	-21.12
20% dextran	-31.54	-33.76
0.125% riboflavin–20% dextran	-41.75	-43.97
Optisol-GS	–	2.42

IOP = intraocular pressure
Optisol-GS is manufactured by Bausch & Lomb, Rochester, NY.

TABLE 3

Normalized Corneal Thickness Before Measurements at 0 and 24 Hours

	Corneal Hydration	
Group	0 Hours	24 Hours
Virgin (untreated)	1	1.17
8% dextran	0.54	0.74
20% dextran	0.6	0.75
0.125% riboflavin–20% dextran	0.55	0.69
Optisol-GS	–	1.05

Optisol-GS is manufactured by Bausch & Lomb, Rochester, NY.

with stress (IOP) on the y axis and strain on the x axis. Stress is related to but not equivalent to the IOP, because estimation of the stress should consider geometrical deviations of the cornea from an ideal sphere, among other effects. In this context, corneal hysteresis was defined as the amount of remaining corneal deformation (in µm) after a cycle of increased/decreased IOP variation.

RESULTS

EXPERIMENTAL RESULTS

Corneal Thickness. The mean corneal thickness (measured over a central zone of 6 mm) varied strongly across the different hydration conditions (**Figure 2**). The largest differences were observed between the dehydrated conditions (8% dextran and 0.125% riboflavin–20% dextran) and the other conditions (control and Optisol-GS). Corneal hysteresis was lowest for control corneas and highest for corneas treated with 0.125% riboflavin–20% dextran (**Table 2**). No differences were observed between measurements at 0 and 24 hours. The application of any of the dextran solutions (30 minutes prior to measurements) produced

a decrease in corneal thickness to 56.5% at 0 hours and 72.7% at 24 hours compared to the control cornea (**Table 3**). The initial corneal thickness was 510 ± 24.4 µm (0 hours)/660 ± 24.5 µm (24 hours) in eyes treated with dextran and 907 ± 36.3 µm (0 hours)/1,064 ± 53.1 µm (24 hours) in control eyes. All treated corneas were de-epithelialized. Control corneal thickness included the corneal epithelium at 0 hours, but not at 24 hours. The changes from 0 to 24 hours were similar in all groups (Δth_{0h-24h} = -154 ± 29.1 µm), indicating that overnight swelling is independent of the prior state of corneal dehydration.

Figure 3 shows the change in corneal thickness with IOP (with respect to the initial value at 15 mm Hg) in all conditions (circles and triangles). An increase in IOP produced a reduction in corneal thickness in all groups, regardless of the prior hydration state. The maximum mean thickness decrease (**Table 4**) in control corneas differed significantly from that found for corneas treated with 8% dextran ($P = .04$), 20% dextran ($P = .001$), and 0.125% riboflavin–20% dextran ($P = .03$), but not with respect to those treated with Optisol-GS ($P = .43$). Measurements at 0 and 24 hours were statistically similar (confidence interval = 95%) for all conditions, except for 0.125% riboflavin–20% dextran ($P = .02$).
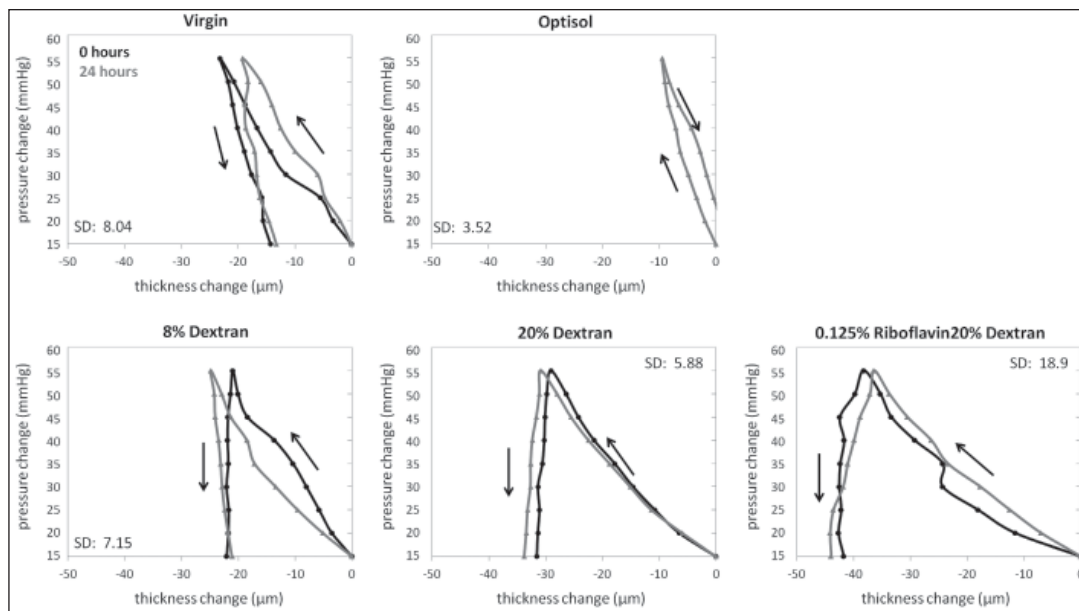
Figure 3. Corneal thickness response to intraocular pressure variation. The black lines represent 0 hours data and the grey lines 24 hours data. Intraocular pressure variation is with respect to 15 mm Hg initial intraocular pressure.

TABLE 4

Corneal Thickness Change at Maximal IOP for Different Conditions at 0 and 24 Hours

Group	Thickness Change (µm) at IOP$_{max}$	
	0 Hours	24 Hours
Virgin (untreated)	-23.18	-19.24
8% dextran	-21.01	-24.85
20% dextran	-29.07	-30.89
0.125% riboflavin–20% dextran	-38.27	-36.45
Optisol-GS	–	-9.48

IOP = intraocular pressure
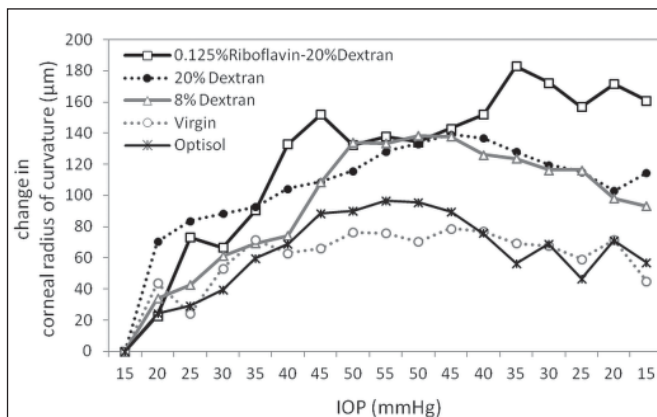Optisol-GS is manufactured by Bausch & Lomb, Rochester, NY.

Figure 4. Changes in corneal radius of curvature as a function of intraocular pressure (IOP) variation. Data are averaged anterior and posterior changes and 0 and 24 hours measurements.

Corneal Curvature. Corneal curvature data were noisier than thickness data (mean standard deviation = 89.2 vs 8.96 µm). **Figure 4** shows the changes in mean (anterior and posterior) corneal curvature as a function of IOP variation, averaged over measurements at 0 and 24 hours. The variation of corneal curvature with IOP shows similar trends to those found for the thickness variations with IOP: control corneas and corneas treated with Optisol-GS deformed less than corneas treated with dextran and showed less remaining deformation after IOP decreased to physiological levels.

Corneal Hydration. The corneal hydration states estimated for different treatments and times after enucleation are listed in **Table 3**. Corneas treated with dextran showed a similar degree of dehydration, and control corneas and corneas treated with Optisol-GS were approximately ×1.67 more hydrated. Although hydration increased after 24 hours in all conditions (~1.3), the ratio of hydration between control corneas and those treated with dextran remained constant.

Epithelium and Corneal Transparency. Control corneas could not be measured at 24 hours because when IOP increased to 40 mm Hg the epithelium lost its transparency, preventing the acquisition of Scheimpflug images of the posterior corneal surface. Also, a custom spectral-domain optical coherence tomography system[26,27] was tested and failed to obtain a feasible image. As soon as the epithelium had been removed, there was no further problem in obtaining high quality images from the anterior and posterior corneal surfaces. We also encountered epithelial transparency loss after storing eyes with epithelium for 24 hours in Optisol-GS.

DISCUSSION

We investigated the biomechanical response of the cornea in a cycle of decreased and increased IOP in a

whole eye globe inflation experiment and found that different solutions typically used in storage or treatment of the cornea result in different corneal deformation patterns on IOP variation. This suggests that changes in the biomechanical properties (elastic and viscoelastic) of the cornea occur as a result of the modulation of corneal hydration produced by these solutions and a potential impact of those solutions on the corneal microstructure. The changes in corneal thickness as a function of IOP variation (**Figure 3**) reveal interesting differences across conditions.

The slope of the curves in the IOP increasing range (15 to 55 mm Hg) is related to the stiffness of the corneal tissue. Compared to control corneas, corneas treated with dextran showed a flatter slope and corneas treated with Optisol-GS showed a steeper slope. These results suggested that corneas treated with dextran were less stiff than control corneas or those treated with Optisol-GS, although the initial corneal thickness was also likely to play a role in this variation (because corneal geometry and the expansion behavior is different).

The most obvious differences across conditions occurred in the amount of hysteresis (remaining deformation after the IOP variation cycle). A higher hysteresis was associated with a higher dextran concentration compared to control corneas. The presence of riboflavin enhanced this effect further, whereas Optisol-GS reduced it. The corneal deformation in the IOP decreasing range (55 to 15 mm Hg) depends to a high degree on its viscoelastic properties (such as the relaxation time). A faster recovery should occur in control corneas and those treated with Optisol-GS compared to those treated with dextran.

We have shown that the corneal hydration condition played an important role in the biomechanical response of the cornea because different solutions affect corneal hydration differently. However, hydration alone is not the only cause for the observed differences in the biomechanical response of the cornea, because the measurements at 0 and 24 hours (which showed significant differences in the hydration state) only showed slight differences in the deformation pattern with IOP.

On the contrary, significant differences were observed across different treatment conditions. Although the water content of porcine corneas[18] was slightly lower compared to those of humans, the rate of dehydration produced by dextran occurred similarly in human[28,29] and porcine corneas. Interestingly, although the corneal swelling seemed to occur similarly in all conditions after 24 hours (except to a lesser extent with Optisol-GS), the response of corneal thickness on varying IOP differed significantly across conditions. These results suggested that, apart from the modula-

tion of corneal hydration, the specific composition of the solutions has additional impact on the biomechanical properties of the cornea.

According to Fratzl and Daxer, the corneal tissue dries in two stages.[30] Only the inter-fibril substance dehydrates initially, but then the fibrils reduce in diameter after a certain level of dehydration is exceeded. This model is consistent with our findings with dextran. As dehydration of the extracellular matrix dominates overall corneal thickness, this probably happens up to a 510-µm thickness (porcine corneas). This corneal thickness was similar in corneas treated with dextran at 0 hours, although it would be expected to be higher for higher dextran concentrations. Beyond this first dehydration level, the level of subsequent fibril dehydration (following the model referred by Fratzl and Daxer[30]) will be reached.

Riboflavin is a smaller molecule (376.36 g/mol) than dextran (103 to 2×106 Daltons), and therefore may alter the structural properties of the collagen lamellae more easily. This could explain the different biomechanical behavior of corneas treated with 0.125% riboflavin–20% dextran, in comparison with corneas treated with 20% dextran without riboflavin. Wollensak et al. observed an increase in collagen fiber diameter after riboflavin–dextran ultraviolet-A CXL[31] and a decrease in the corneal swelling,[32] although other studies on chemical CXL (ie, without riboflavin as photosensitizer) reported reduced hydration of the collagen fibrils after CXL.[33,34] We suggest that this increase in fibril diameter might come from the riboflavin.

An important implication of this study pertains to the design and evaluation of CXL treatments on corneal stiffening. Previous inflation experiments[15] showed a significant stiffening of the cornea, with the CXL corneas showing an increase of 1.58 in the estimated Young modulus. Similar to other studies, the control condition involved de-epithelialization of the cornea and riboflavin–dextran solution instillation. However, the results from this study suggest an overall weakening of the corneal tissue after riboflavin–dextran instillation (flatter slope in **Figure 3**). Although this weakening effect is likely temporary, taking this effect into account becomes important when conducting experimental tests of the efficacy of CXL that include measurements immediately after the treatment or in studies where corneas soaked with riboflavin–dextran are used as a control condition.[1,11,12,15,35] On the other hand, the decrease in corneal thickness may increase CXL efficiency, because the compression of the collagen fibers would bring more of the stromal lamellae within the 250-µm range of the CXL effect. The use of alternative photosensitizer solutions to riboflavin

is under debate. Hypotonic solutions (eg, 0.9% NaCl) are already being studied as an alternative to dextran and to prevent the corneal dehydration and thinning during the treatment.[13] Our study suggests that both dextran and riboflavin may play a role in the modulation of the biomechanical properties of the cornea in the short term following treatment.

The current study has important implications for the selection of appropriate experimental conditions to estimate biomechanical properties of the cornea. In many cases, particularly with eye bank specimens, immediate access to tissue is not possible. We showed that none of the medical solutions containing dextran preserved its biomechanical response. This should be taken into account when performing biomechanical experiments on tissue that has been stored. The availability of experimental corneal deformation will allow building of more accurate analytical and numerical models and increased predictability of surgical results. In a first step, corneal deformation data can be used for inverse modeling to obtain the corresponding biomechanical parameters, for each condition. Corneal shape and thickness can serve as inputs for Finite Element Models. Both changes in corneal curvature and thickness showed similar same trends on the relative amounts of deformation and hysteresis. However, considering that the curvature deformation is strongly influenced by the overall ocular geometry and by scleral properties, models can rely on corneal thickness changes as input data for future models or, more comprehensively, include experimental data on the scleral geometrical and mechanical properties to provide a complete model of eye inflation.

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