Cornea

Collagenase-Mediated Tissue Modeling of Corneal Ectasia and Collagen Cross-Linking Treatments

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PURPOSE. Corneal collagen cross-linking (CXL) is a method for modifying the natural history of keratoconus and other corneal ectatic diseases. The authors evaluated the use of collagenase for generating an experimental model of ectasia to evaluate the topographic effects of CXL interventions.

METHODS. Nine human corneoscleral specimens unsuitable for transplantation were used. After epithelial debridement, mounting, and pressurization on an artificial anterior chamber, a solution of 10 mg/mL collagenase type II with 15% dextran was applied to five corneas for three hours. Three of these corneas subsequently underwent riboflavin/UV-A CXL. Scheimpflug-based tomography was performed before collagenase exposure, after collagenase exposure, and after CXL to evaluate changes in maximum axial curvature of the anterior surface (K_{max}) at three IOP levels. Results were compared to four control eyes exposed to dextran alone.

RESULTS. A statistically significant increase in K_{max} was seen across all IOP levels in the collagenase group compared to the control group (+6.6 ± 1.1 diopters [D] and +0.3 ± 0.8 D, respectively, at physiological IOP). After CXL, K_{max} decreased (−7.6 ± 2.0 D at physiological IOP). Anterior corneal aberrations increased after collagenase exposure and decreased after CXL. Light microscopy showed loss of normal stromal collagen architecture and localized edema after collagenase exposure.

CONCLUSIONS. A method for generating topographic features of corneal ectasia in human tissue is demonstrated. No significant sensitivity of K_{max} to IOP was observed. CXL caused regression of steepening and induced aberrations in this model, consistent with clinical trends. The model may be useful for testing modifications to standard CXL techniques. (*Invest Ophthalmol Vis Sci.* 2012;53:2321–2327) DOI:10.1167/iovs.11-9327

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Keratoconus, characterized by corneal ectasia and loss of visual acuity, affects approximately 1 in 2000 in the general population.[1] It was the leading indication for penetrating keratoplasty (21.2%) and anterior lamellar keratoplasty (40.2%) in the United States in 2010.[2] Ectasia progression can be modified by riboflavin/UV-A–mediated collagen cross-linking (CXL),[3,4] a more conservative method that appears to be safe[4,5] and effective in stopping keratoconus progression.[3,4,6] In 2008, a large retrospective study of CXL showed corneal topographic flattening in more than half of the eyes and statistically significant improvements in astigmatism, best-corrected visual acuity, and maximum simulated keratometry (K_{max}) values after a year. Vinciguerra et al.[7] have shown significantly less keratoconus progression compared to the untreated contralateral eye. CXL is currently being investigated in clinical trials for US Food and Drug Administration approval in the United States for treatment of keratoconus and postrefractive surgery ectasia.

However, a challenge faced by researchers is that there is currently no tissue model for study of either of these conditions. Proteinases, defective enzymatic activity, and oxidative damage have been implicated in keratoconus,[8,9] and it has been shown that levels of degradative enzymes such as acid esterase, acid phosphatase, and cathepsins B and G are elevated, and degradative enzymes such as inhibitors of α1-proteinase inhibitor and α2-macroglobulin, are reduced in keratoconus corneas.[10-13] It was thus hypothesized that keratoconus can be simulated through topical collagenase application. After promising results in a pilot study (Hong CW. *IOVS* 2011;52:ARVO E-Abstract 4386), the authors set out to evaluate the suitability of collagenase treatment for generating a model of corneal ectasia in unaffected donor corneas and to evaluate the effectiveness of a standard CXL protocol.[3]

METHODS

Human corneoscleral tissue not suitable for transplantation was obtained from the Cleveland Eye Bank. The study was performed in compliance with the tenants of the Declaration of Helsinki. Corneas were mounted and pressurized on an artificial anterior chamber (ALTK System; Moria USA, Doylestown, PA) by using saline infusion with an in-line pressure transducer and continuous pressure monitor. For each eye, the epithelium was removed with a surgical blade. Intraocular pressure was decreased by reducing the height of the saline reservoir. A solution of 10 mg/mL collagenase type II (Worthington, Lakewood, NJ) in balanced salt solution and 15% dextran was applied to the surface of the mounted corneas within the concavity resulting from the pressure reduction for three hours. The intraocular pressure was then restored to physiologic pressure and the solution was removed by using cotton swabs. Control eyes were subject to the same protocol but collagenase was absent from the applied solution. Nine corneal–scleral specimens were obtained: five of them were subject to collagenase application and four of them were used as controls.
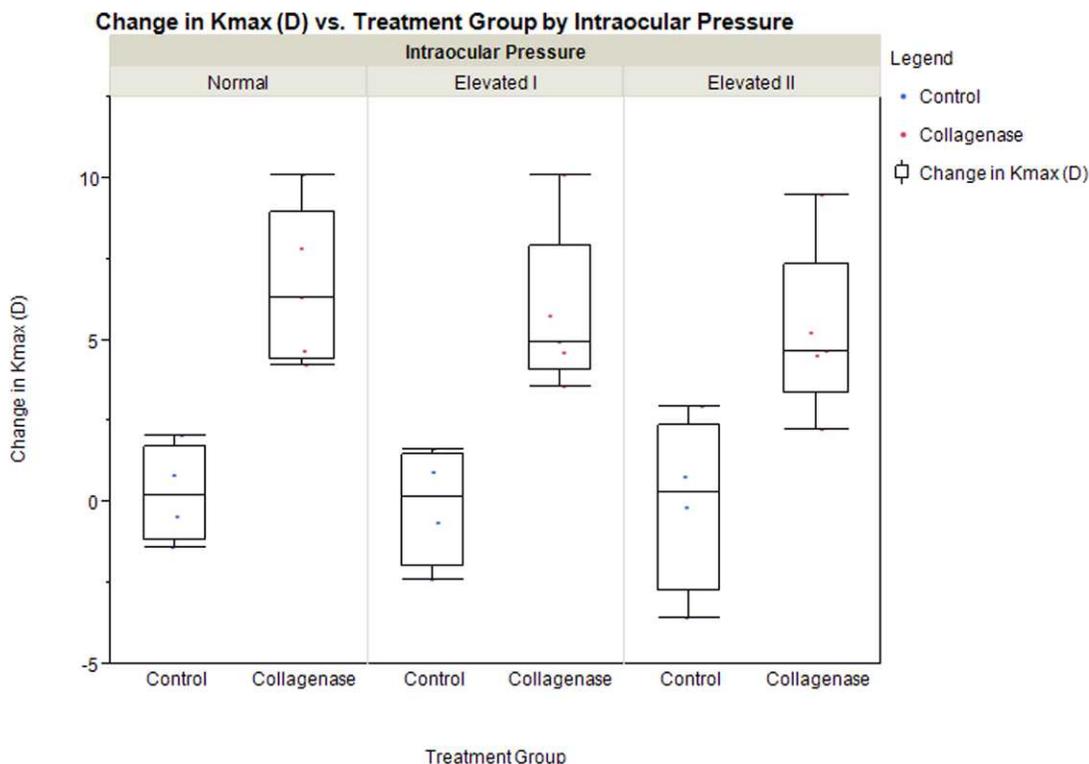
Figure 1. Change in K_{max} in control and collagenase corneas across all IOP levels.

Three of the corneas that were exposed to collagenase were subsequently subjected to cross-linking while on the mount to preserve corneal orientation for serial topographic comparisons. These corneas underwent cross-linking according to the technique described by Wollensak et al.,[3] wherein a drop of 0.1% riboflavin was applied to the surface of the cornea every five minutes for 30 minutes, followed by UV-A irradiation for 25 minutes starting from the second addition of riboflavin at an average beam intensity of 3 mW/cm^2 and diameter of 9 mm. Tomographic maps before and after collagenase, as well as after cross-linking, were taken with a Pentacam (software v. 1.17r60, Oculus Inc., Wetzlar, Germany) for each cornea at intraocular pressures of 15, 30, and 45 mm Hg (denoted by normal, elevated I, and elevated II, respectively). Three measurements were taken at each pressure step, and the mean value of each variable was reported. Maximal axial curvature of the corneal surface and the anterior surface wave front aberrations were obtained and recorded. Wave front aberrations were fit up to 10th-order Zernike polynomials, with a pupil diameter of 6 mm and a refractive index of 1.376. Statistical significance was assessed with the two-tailed Mann-Whitney U test with a 5% significance level. Analyses were performed with JMP (software v. 9.0.0, SAS Institute, Cary, NC).

Two additional corneas from the same donor were used to characterize the histopathologic effects of collagenase. One cornea was exposed to collagenase and the contralateral cornea was exposed to dextran as a control. Each cornea was mounted and pressurized separately on a Barron artificial anterior chamber (Precision Instruments, LLC, Grand Blanc, MI). This artificial anterior chamber was not used in experiments requiring Scheimpflug imaging owing to the difficulty of successful scan acquisition, a problem that may be related to the geometry of the chamber or its optical properties. However, the chamber performed similarly to the Moria system with regard to pressure control, which was the sole reason for mounting the corneas in this portion of the study. The epithelium was removed with a surgical blade and the pressure lowered to form a concavity into which the appropriate solution was applied for three hours. Each solution was removed with cotton swabs and the corneas were cut into halves. Hemicorneal specimens were fixed in formalin, paraffin embedded, and processed routinely for light microscopy with hematoxylin-eosin (H&E) and PAS staining.

RESULTS

An increase in K_{max} was observed in corneas that were exposed to collagenase compared to those exposed to dextran (Fig. 1). The difference between the groups was statistically significant. The mean $K_{max} \pm$ SE (standard error) before and after treatment, as well as the differences, are reported for each

TABLE 1. Comparison of K_{max} (Absolute Value and Change) in Control and Collagenase Corneas

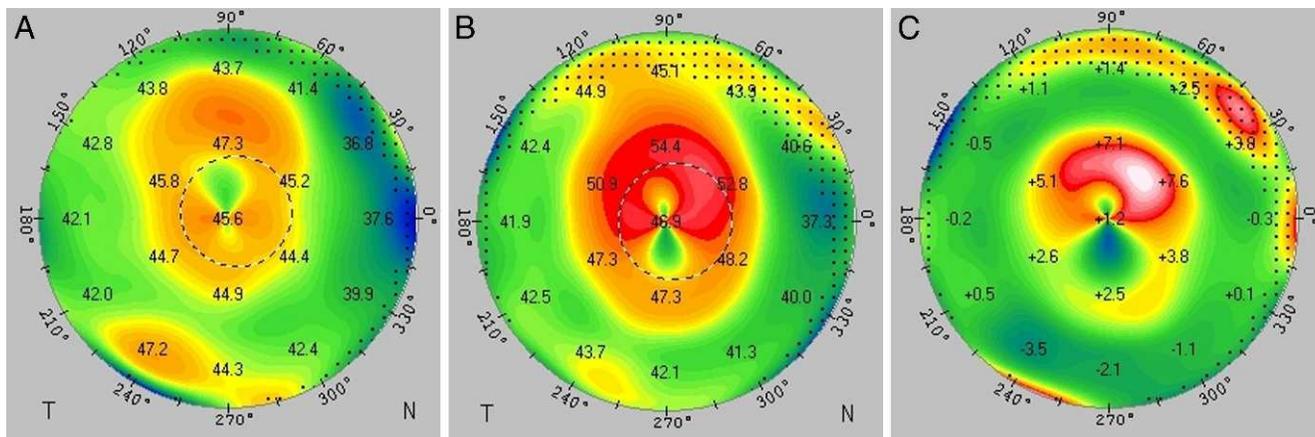| IOP	Group	Pre K_{max} (D)	Post K_{max} (D)	ΔK_{max} (D)	P Value
Normal	Collagenase	47.5 ± 2.1	54.1 ± 1.2	$+6.6 \pm 1.1$	0.0143
	Control	50.4 ± 1.5	50.7 ± 1.1	$+0.3 \pm 0.8$	
Elevated I	Collagenase	47.9 ± 0.9	53.7 ± 1.0	$+5.8 \pm 1.1$	0.0143
	Control	50.2 ± 1.4	50.1 ± 1.1	-0.1 ± 0.9	
Elevated II	Collagenase	48.1 ± 0.8	53.3 ± 1.1	$+5.2 \pm 1.2$	0.0275
	Control	50.8 ± 2.0	50.8 ± 0.8	$+0.0 \pm 1.4$	

FIGURE 2. Axial curvature maps of anterior cornea surface before (**A**) and after collagenase exposure (**B**). (**C**) Subtraction map.
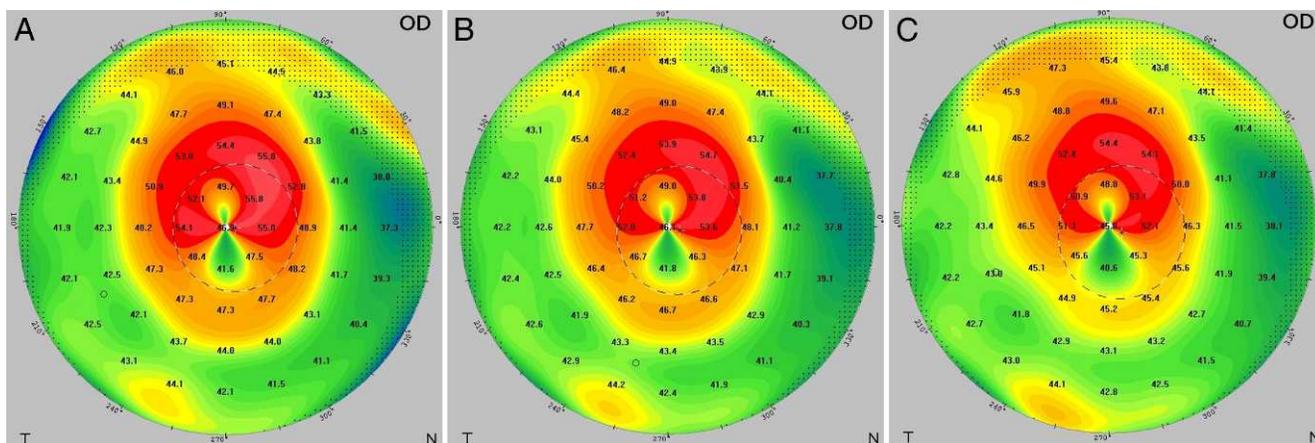

FIGURE 3. Axial curvature maps of anterior cornea surface after collagenase exposure at 15 mm Hg (**A**), 30 mm Hg (**B**), and 45 mm Hg (**C**).

group of eyes stratified by IOP (Table 1). The two-tailed Mann-Whitney U test at the 5% significance level was used for statistical comparisons since normality could not be assessed with the current sample size. A difference map of a representative cornea at physiological IOP showed increased curvature over most of the cornea (Fig. 2). No notable pressure dependence was observed (Fig. 3).

In the three corneas that underwent CXL after collagenase application, K_{max} decreased substantially after CXL. Stratified by IOP category, the mean $K_{max} \pm$ SE before and after cross-linking, as well as the differences, are reported for each group of eyes (Table 2). The absolute and difference maps of one cornea are shown, and the marked decrease in curvature after CXL is illustrated (Fig. 4). No pressure dependence was observed in the post-CXL group (Fig. 5).

Changes in mean root mean square (RMS) total, low-order term RMS, and high-order term RMS after collagenase

TABLE 3. Mean Changes in Anterior Corneal Aberrations in Control and Collagenase Corneas

	IOP	Control (μm)	Collagenase (μm)
Total RMS	Normal	−0.091 ± 0.247	+0.889 ± 0.237
	Elevated I	+0.020 ± 0.192	+0.844 ± 0.266
	Elevated II	+0.025 ± 0.206	+0.814 ± 0.271
RMS LOA	Normal	−0.086 ± 0.240	+0.891 ± 0.238
	Elevated I	+0.027 ± 0.189	+0.846 ± 0.267
	Elevated II	+0.040 ± 0.197	+0.821 ± 0.274
RMS HOA	Normal	−0.017 ± 0.035	+0.103 ± 0.030
	Elevated I	−0.029 ± 0.031	+0.091 ± 0.033
	Elevated II	−0.066 ± 0.067	+0.081 ± 0.031

HOA, higher-order aberrations; LOA, lower-order aberrations.

TABLE 2. K_{max} after Cross-Linking of Collagenase-Exposed Corneas

IOP	Pre K_{max}(D)	Post K_{max}(D)	ΔK_{max}(D)
Normal	52.6 ± 1.0	46.5 ± 0.9	−7.6 ± 2.0
Elevated I	52.1 ± 0.9	47.2 ± 1.0	−6.1 ± 1.3
Elevated II	52.2 ± 0.8	47.0 ± 1.2	−5.6 ± 1.0

TABLE 4. Mean Changes in Anterior Corneal Aberrations after Cross-Linking

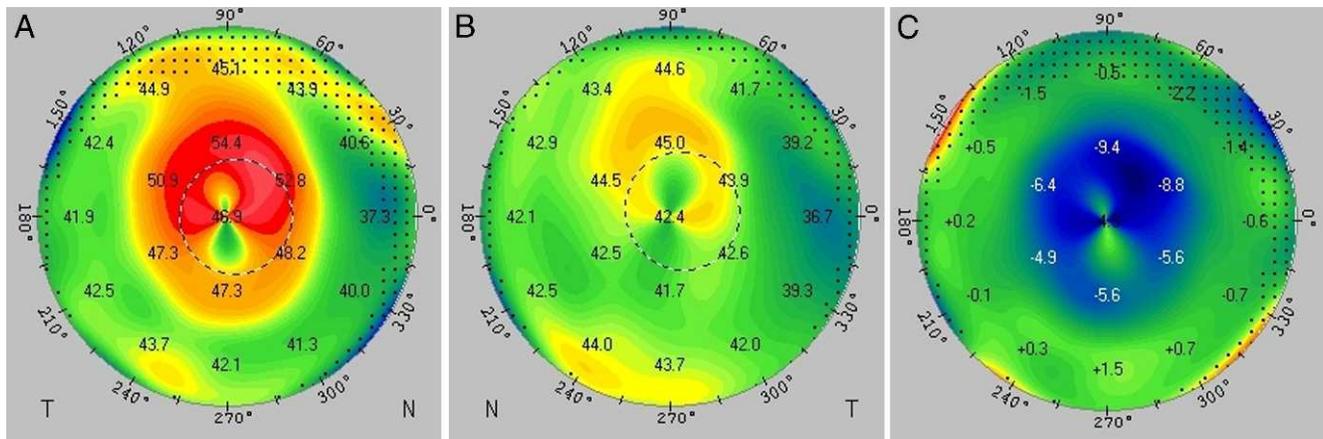| IOP	ΔRMS Total (μm)	ΔRMS LOA (μm)	ΔRMS HOA (μm)
Normal	−0.474 ± 0.243	−0.463 ± 0.243	−0.100 ± 0.024
Elevated I	−0.357 ± 0.256	−0.349 ± 0.256	−0.080 ± 0.0237
Elevated II	−0.465 ± 0.275	−0.460 ± 0.276	−0.072 ± 0.022

FIGURE 4. Axial curvature maps of anterior cornea surface after collagenase exposure before cross-linking (**A**) and after cross-linking (**B**). (**C**) Difference map.

application are reported in Tables 3 and 4. Aberrations increased significantly after collagenase application (Table 3) and decreased substantially after CXL treatment (Table 4). Inspection of posterior corneal surface tracings from the Scheimpflug tomography system revealed edge detection errors that prevented meaningful quantitative analyses of corneal thickness after collagenase and cross-linking treatment.

Light microscopy in an eye exposed to collagenase demonstrated changes in collagen organization within the central and superficial regions of the cornea most directly exposed to collagenase. Compared to the contralateral control, loss of lamellar structure and of interlamellar clefts was observed with edema and overall corneal thickening (Fig. 6).

DISCUSSION

Keratoconus is an important cause of vision loss and a leading indication for corneal transplantation that lacks a readily available tissue model for preclinical study of potential treatments. Keratoconus has been associated with abnormal enzymatic activity[14-17] and increased levels of degradative enzymes.[18,19] Aberrant maintenance of corneal stromal collagen or matrix related to enzymatic dysregulation may be related to ultrastructural collagen abnormalities like those described in histologic,[20] x-ray diffraction,[21] and second-

harmonic imaging studies[22] of keratoconic tissue. Abnormalities of collagen structure presumably contribute to a decrease in corneal material strength,[23,24] which has been shown to be a major factor in the pathomechanics of keratoconus and its characteristic topographic features.[25-27]

In this human donor eye study, collagenase exposure produced a significant increase in anterior corneal curvature and generated topographic changes on the scale of those observed in clinical keratoconus. Similarly, anterior corneal aberrations increased significantly after collagenase application and not in control corneas, which were exposed only to vehicle. Higher magnitudes of corneal aberrations are associated with keratoconus severity[28,29] and have been incorporated into some keratoconus grading classification schemes.[30] Histopathology revealed a loss of organized lamellar structure in the collagenase-treated sample relative to the control sample, especially in the anterior-central region, which corresponded to the zone of exposure. Since the collagen lamellar mesostructure normally resists fluid imbibition, collagenase-induced changes appear to favor a loss of resistance to the stromal swelling pressure present as a result of a hydrophilic glycosaminoglycan matrix, which manifests as edema, corneal thickening, and a loss of characteristic stromal clefts on histology. Although human corneal collagen comprises predominantly type I constituents, the histologic and topographic effects of the type II collagenase used in this
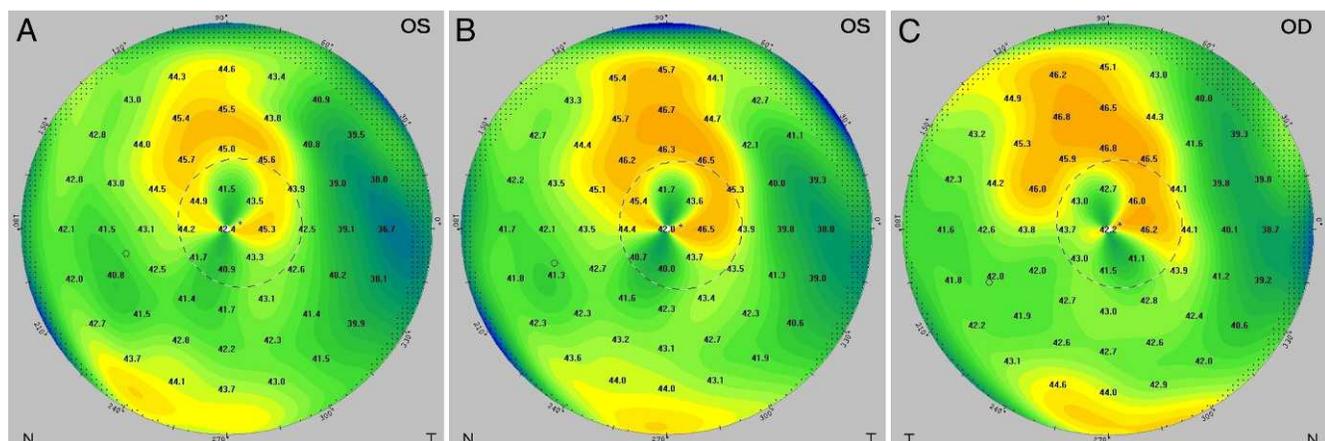

FIGURE 5. Axial curvature maps of anterior cornea surface after cross-linking at 15 mm Hg (**A**), 30 mm Hg (**B**), and 45 mm Hg (**C**).
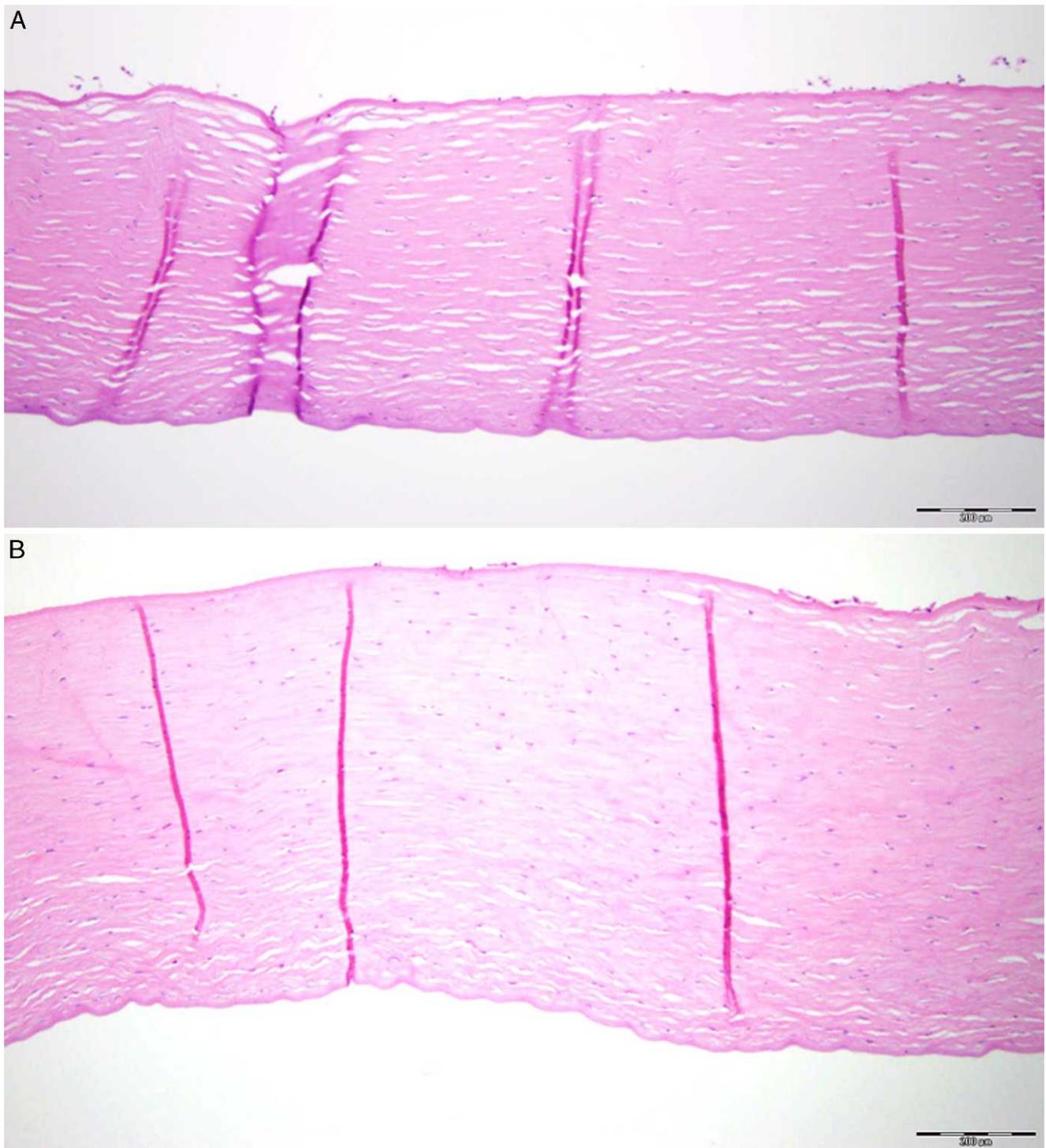
FIGURE 6. H&E stain of control cornea (**A**) and collagenase-treated cornea (**B**), ×20 magnification.

study were marked and presumably depend on some degree of nonspecific enzymatic activity. Clinical[31] and computational modeling[25,27] studies have demonstrated increases in focal curvature values in keratoconic corneas in response to increased IOP, but the current study was insufficiently powered for a similar analysis and did not suggest a similar trend in the collagenase model.

The increases in corneal thickness noted on histology after collagenase exposure could be interpreted as paradoxical given that reduced corneal thickness is one of the major clinical features of keratoconus. As described above, the authors suspect that corneal edema is a secondary phenomenon related to a loss of normal lamellar resistance to swelling after collagenase exposure that should not be confused with a natively thicker cornea that has a larger-than-normal number of tension-bearing collagen elements. Recent computational modeling studies suggest that reductions in corneal elastic modulus and reductions in corneal thickness both contribute

to corneal steepening in keratoconus.[25,27] Roy and Dupps[27] have assessed the impact of each factor independently in a patient-specific finite element model and found that topographic changes of early keratoconus could be produced without any requirement for corneal thinning when a localized reduction in corneal hyperelastic properties was imposed. The ability of the collagenase tissue model to produce significant increases in corneal curvature despite a lack of simultaneous thinning is consistent with these findings and suggests that a focal material weakness is the principal driver of topographic change in the present tissue model. The study design only allowed for observation of the acute posttreatment changes in corneal thickness. Further study in a tissue-culture setting that ensures preservation of endothelial cell function could provide information on thickness changes beyond the acute postcollagenase period and would allow one to determine whether or not an active endothelium would lead to eventual stromal compaction. Hydration conditions were similar between collagenase and control corneas, and changes in anterior corneal curvature—the primary outcome measure in this study—are unlikely to be explained by hydration changes alone given evidence that anterior curvature is insensitive to corneal hydration.[32,33]

In conclusion, human donor corneas exposed to collagenase showed a significant increase in curvature and anterior corneal aberrations and reductions in the same metrics after a standard cross-linking treatment. The response to cross-linking mirrored that of clinical cross-linking.[3,4,7] Although this model may be limited in reproducing the specific pathophysiological processes that occur in keratoconus, these findings suggest that collagenase-treated donor corneas may be a useful topographic model for the study of keratoconus and modifications to standard cross-linking treatments.[27]

Acknowledgments

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