Anterior–Posterior Strain Variation in Normally Hydrated and Swollen Rabbit Cornea

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PURPOSE. To investigate the variation in anterior and posterior straining under intraocular pressure changes for the central cornea of normally hydrated and swollen rabbit eyes.

METHODS. A new method of measuring regional corneal strains, by imaging a specific tissue location at various intraocular pressures, was developed. Sixteen freshly enucleated, New Zealand White rabbit eyes were investigated either in their normal hydration state or after swelling of the deep epithelialized cornea. The eyes were mounted on a specially designed eye fixture, and laser-scanning confocal microscopic images of a selected region in the anterior stroma or endothelium were taken at intraocular pressures of 5, 12.5, 20, 35, and 65 mm Hg. The positions of individual keratocytes or endothelial cells were used to calculate the nonhomogeneous two-dimensional strain field over the image. Corneal thickness was measured at the lowest and highest intraocular pressures (5 mm Hg and 65 mm Hg).

RESULTS. All pressure strain curves were highly nonlinear for intraocular pressures between 5 mm Hg and 65 mm Hg; the maximal posterior strains (normally hydrated, 2.1 ± 0.1%; swollen, 4.8 ± 0.8%) were larger than the maximal anterior strains (normally hydrated, 1.8 ± 0.1%; swollen, 1.5 ± 0.2%). Swelling significantly decreased the anterior strain response but increased the posterior one. The corneal thickness decreased 7.4 ± 0.4% for the normally hydrated and 6.3 ± 0.5% for the swollen corneas for an intraocular pressure step from 5 mm Hg to 65 mm Hg.

CONCLUSIONS. Bending was found to play a significant role in central corneal deformation of swollen eyes but not in the normal hydration state. Microscopic strain measurements of the cornea, using a laser-scanning confocal microscope, are a valuable tool for the assessment of regional nonhomogeneous strains in various depths and locations of the cornea. (Invest Ophthalmol Vis Sci. 1998;39:253–262)

Corneal biomechanics involves the complex interaction between its anisotropic, lamellar collagen structure and the charged, hydrated proteoglycan gel. Regional corneal strain distributions reflect these structural components and their interactions. For example, tensile loading of the cornea results in a strain response typical for anisotropic collagenous tissues,1-3 caused by the elastic properties of the lamellar collagen. However, compression and swelling tests of corneal tissue reflect predominantly the role of the viscous fluid phase and its interaction with the electronegative proteoglycan mesh.4-10 Under physiological conditions, intraocular pressure is supported through the strain response of the corneoscleral envelope, which varies with location, tissue depth, and tissue hydration.11 Most corneal strain measurements on the intact eye rely on tissue markers that are attached to the corneal surfaces. Woo et al.2 used two adhesive tape markers on the epithelium, Jue and Maurice8 used mercury droplets, and Hjortdal et al.11-13 used up to 90 mercury droplets on the epithelial and endothelial surfaces to measure the strains in one quadrant of the corneal tissue. This study describes a new method for measuring regional corneal strains at various tissue locations and depths by tracking corneal keratocytes using confocal microscopy. A local region within the corneal stroma is imaged at various intraocular pressures, and the displacements of keratocytes or endothelial cells are calculated.14 This approach has the advantage of not relying on surface markers but rather using anatomic landmarks as material points for measuring strain, which makes it noninvasive.

Numerous analytical and numerical models of corneal biomechanics have been developed with varying degrees of material complexity.15-24 But experimental verification of their assumptions and results is needed. This study addresses the strain variation between posterior and anterior locations concerning corneal hydration. It was designed with the following two questions in mind: Can corneal deformation after intraocular pressure changes be modeled as an incompressible nonlinear elastic solid or must the biphasic nature of the tissue be taken into account? Does corneal swelling lead to a measurably different deformation under intraocular pressure changes? If these questions can be answered affirmatively, then does the difference between the anterior and posterior strain response of the normally hydrated and swollen cornea support the notion that swelling relaxes the posterior layers but prestresses the anterior stroma?

METHODS

Tissue Preparation

Sixteen eyes of 12 New Zealand White rabbits weighing 2.5 to 3.1 kg were obtained through a tissue transfer protocol ap-
proved by the UCSD Animal Subjects Committee. All animals were treated according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For 8 of the 12 rabbits, only one eye was used in the study, because the confocal microscope was not available for the whole day. Ideally both eyes of each animal would have been used in a paired experiment, with measurements performed on one eye in the normal hydration state while the other was allowed to swell. Because of scheduling conflicts, no pairing was included in the statistical analysis.

Preparation for Measurements on Normally Hydrated Corneas

Eight eyes were studied in their normal hydration state. Either the left or the right eye was prepared for strain measurements under the confocal microscope within 40 minutes of enucleation. They were mounted on a specially designed eye fixture as described below and bathed in a slightly hyperosmotic buffer solution (4 g dextran 70 and 0.01 g glutathione in 100 ml phosphate-buffered saline, with the pH adjusted to 7.4) at room temperature.

Preparation for Measurements on Swollen Corneas

To study the deformation of swollen corneas, the epithelium was mechanically debrided for eight eyes, and they were allowed to swell in a solution of glutathione-enriched phosphate-buffered saline without dextran for 4 hours at room temperature. Either the left or the right eye was used. After the 4-hour swelling time, the eyes were mounted on the eye fixture, and corneal thickness was measured with the confocal microscope twice in a 20-minute period before the actual strain measurements, to guarantee that the hydration state was stable.

Mounting and Imaging of the Eye under the Confocal Microscope

The eye fixture was designed to allow intraocular pressure to be varied while the cornea was imaged under a laser-scanning confocal microscope system (MRC-1024; Bio-Rad, Richmond, CA). The mounting plate was a 3-mm thick aluminum plate with a tapered hole in which the limbal ridge of the rabbit eye rested. This allowed the whole globe of the eye to deform freely. Fixation at the limbus could introduce a nonphysiological boundary condition, which could affect central corneal strains. Mounting plates with holes ranging from 15 mm to 19 mm were available to assure a good fit for the various eye sizes. A 20-gauge needle was inserted carefully in the anterior chamber from behind the iris and was clamped to a support structure on the mounting plate to prevent distortions of the globe (Fig. 1). The needle was connected to a reservoir that could be raised and lowered to adjust the intraocular pressure. Insertion of the needle into the anterior chamber was crucial for obtaining accurate pressure loadings, as verified through measurements with a pressure transducer.

The mounting plate rested in the bath (Fig. 1) on three micrometer screws that could be adjusted individually to place the cornea as close as possible to the coverslip without actually touching it. Close positioning was important to minimize spherical aberrations and to allow imaging of the full corneal thickness within the working distance of the microscope objective. Avoiding contact between the cornea and the coverslip was important to ensure that the measured strains would only reflect the deformation response of the globe to intraocular pressure changes and not the flattening of the central cornea along a plane surface. The bath was dimensioned to fit securely on the microscope stage. The cornea was viewed through a circular window, which held the coverslip and was sealed with nail polish.

All imaging was performed with the laser-scanning confocal microscope system (MRC-1024; Bio-Rad) coupled to an inverted microscope (Diaphot; Nikon, Garden City, NY). The tissue was illuminated with the blue (488 nm) wavelength of an argon ion laser, and the back-scattered light was collected with a photomultiplier after it passed through a blue reflection filter. An objective (Fluor 20X; Nikon) with a numerical aperture of 0.75 and a working distance of 650 μm was used in all experiments. Within the 660-μm working distance of the objective, more than 900 μm of corneal tissue can be imaged because of the higher refractive index of the cornea (1.375) compared with air. All images were Kalman filtered to improve
the signal-to-noise ratio. The pixel value $P_n$ of the filtered image was calculated by:

$$P_n = \frac{I}{n} + P_{n-1}(1 - \frac{1}{n}), \quad (1)$$

where $P_{n-1}$ is the previous pixel value, $I$ is the input value, and $n$ is the frame number. In our case, two to three scanned frames were averaged.

**Experimental Protocol**

Figure 2 shows the time course for the strain experiments in swollen and normally hydrated corneas. The intraocular pressure was varied stepwise from 5 mm Hg to 65 mm Hg, and the images were taken at 5, 12.5, 20, 35, and 65 mm Hg. The pressure was always increased slowly to allow the region of strain if the selected area was imaged at a different depth. The pixel value was calculated by:

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No images were taken at pressures below 5 mm Hg because of the slow convergence to a new steady state at these pressures.5 Each eye was preconditioned for three cycles to ensure reproducibility of the corneal strain and thickness measurements. During the preconditioning cycles, great care was taken to align the symmetry-axis of corneal deformation with the optical axis of the microscope. Good alignment minimized the rotation of the imaged keratocytes out of the focal plane and made tracking keratocytes easier. An intraocular pressure increase of only 5 mm Hg could result in a radial movement of the whole cornea to 300 μm. This movement had to be compensated for by adjusting the micrometer screws on the mounting plate until the desired area was imaged at a z-position similar to the one used before the pressure step. The adjustment using the micrometer screws was important not only for the limited working distance of the objective but because spherical aberrations could introduce an artificial strain if the selected area was imaged at a different depth.

After the preconditioning cycles, corneal thickness was measured by determining the difference in the z-positions of the microscope objective that brought the basal lamina and the endothelium into focus. The basal lamina was chosen as the anterior reference point because the epithelium was mechanically debrided for the swollen corneas. A location on the endothelium was then selected and imaged at intraocular pressures of 5, 12.5, 20, 35, and 65 mm Hg. Corneal thickness was again measured at an intraocular pressure of 65 mm Hg. The pressure was lowered, and the loading cycle with thickness measurements was repeated at a location in the anterior stroma (<50 μm below the basal lamina). One full measurement cycle was conducted in less than 20 minutes, with any single measurement at each load step never exceeding 7 minutes. Figure 3 shows confocal images of a particular endothelial location at intraocular pressures of 5 mm Hg and 65 mm Hg as used for the strain analysis. One experiment on the normally hydrated corneas had to be terminated because of the leakage of the coverslip seal, thus reducing the group size to seven.

For the tests on the swollen eyes, great care was taken to conclude the measurements with the same time restrictions. This was most difficult for the endothelial location because the image quality was impaired by corneal haze in the anterior stroma (Fig. 4). This resulted generally in a higher error in the posterior strain measurements on the swollen corneas and in one case made it impossible to identify positively the endothelial location. This reduced the group size to seven eyes for the posterior strain measurement of the swollen corneas at an intraocular pressure of 65 mm Hg.

**Image and Data Analysis**

Images were analyzed using the public domain National Institutes of Health Image 1.58 program (written by Wayne Rasband at NIH). Images of the same location at different intraocular pressures were stacked together, and image coordinates of 15 to 40 anatomic landmarks (keratocyte nuclei, endothelial cells) that could be identified in all images were saved in pixels to a spreadsheet. The identification of a set of landmarks was time consuming, especially for the images of keratocyte nuclei. The observer had to confirm that the chosen keratocyte nuclei were visible in all frames of a loading cycle and whether a characteristic feature along the edge of the chosen keratocytes could be identified in all cases. For images of the anterior stroma of swollen corneas, in which the shape of each keratocyte was visible instead of its nucleus, it was easier to identify characteristic features along the edges of the keratocyte network. For endothelial cell images, the cells corners were used. Because of eye movement under intraocular pressure changes, the individual images and, hence, the digitized image coordinates of the landmarks could be shifted from one frame to another. Using Excel 5.0 (Microsoft, Redmond, WA) these translations in the image coordinates of the landmarks were removed by aligning the centroids of the data sets. The alignment was not necessary for subsequent finite element analysis but was helpful in assuring that the individual data points were processed in the correct order.

Hjortdal11,13 showed that meridional and circumferential strains can differ significantly for a given location; therefore, the strains within an image cannot be expected to be equibiaxial. It is also possible that they are nonhomogeneous, for example, in the area adjacent to a keratorefractive cut. To extract these nonhomogeneous, nonuniform, two-dimensional strains accurately, a customized finite element package called DCMISS, developed by the Cardiac Mechanics Research Group at the University of Cali-

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Figure 3. Confocal images of one endothelial location at intraocular pressures of 5 mm Hg (top) and 65 mm Hg (bottom). Twenty-eight cell corners that could be identified in both images were used for the finite element strain analysis (see Fig. 5). Image size: 543 × 362 μm.

A set of smoothing weights governed the degree to which local variations in strain were allowed to reduce the fitting error. The significance of the choice of smoothing weights was further illustrated in the error analysis. All measurements were performed as close as possible to the corneal center, thereby resulting in no preferred orientation of the principal strains throughout the image and no difference in values beyond statistical variations. Therefore both principal strain values were averaged.

Calculation of Volume Change and Bending

Anterior and posterior strains are not independent of each other but represent the boundary deformation of the central
corneal shell. Therefore, it is important to investigate the shell deformation corresponding to the measured strains and thickness changes.

Figure 6 illustrates the possible geometric changes caused by the inflation of a rotationally symmetric shell segment. This deformation includes a flexural bending of the central arc as well as an extension of its length \((\phi r)\) and a change in corneal thickness. The measured strains reflect the extension of the posterior and anterior arc length, respectively. The measured corneal thicknesses \((D,d)\) allow the calculation of a homogeneous transverse strain value. Thus, we modeled the three-dimensional kinematics of the cornea as follows:

\[
\phi r_1 = \alpha \Phi R_1 \tag{2}
\]

\[
\phi r_2 = \beta \Phi R_2 \tag{3}
\]

\[
d = \gamma D \tag{4}
\]

where \(r_1\) and \(R_1\) are the deformed and undeformed posterior radii of curvature, \(r_2\) and \(R_2\) are the deformed and undeformed anterior radii of curvature, and \(d\) and \(D\) are the deformed and undeformed corneal thickness, respectively. \(\alpha, \beta,\) and \(\gamma\) are the respective extension ratios and corneal thickness changes. A similar relation can be formulated for the volume \(X\) ratio.
Deformation of a quadrilateral finite element to fit the undeformed 28 data points (O) of the shown endothelial location (see Fig. 3) to the deformed ones (+). The solid line shows the undeformed rectangular element; the dashed lines show the deformed one. The degree of nonhomogeneity in the resultant strain field is controlled by a set of smoothing weights.

\[ \frac{dv}{dV} = \delta dV \]

Using an initial anterior radius of 7.5 ± 0.5 mm29 and inserting the measured values for \( \alpha \), \( \beta \) and \( \gamma \), one can compute the relative bending flexure \( \frac{df}{dQ} \) and volume ratio \( \frac{dv}{dV} \).

The amount of bending flexure illustrates the deviation of the deformation from a homogeneous inflation of a spherical shell. The volume ratio illustrates the deviation from a local incompressibility.

### Error and Statistical Analysis

Identification and digitization of the corresponding image points are the major sources of error in this analysis. Not all images were taken at exactly the same corneal depth, which can result in a different relative location of the identified cell corners or keratocyte nuclei. In addition, all image measurements were made manually by positioning a cursor on the image location using the mouse. The latter error was estimated to be within 2 pixels (with no preferred orientation) by repeated measurements on test images. The first error is more difficult to assess, but the least-squares method provides an indirect way of estimating it. The choice of smoothing weights used depends on a compromise between a minimal root-mean-square error for the overall fit of all data points and a minimal standard deviation for the three strain values (\( E_{11} \), \( E_{22} \), and \( E_{12} \)) for a small image area (smoothness of the result). A choice of small smoothing weights favors a large variation in local strains to obtain a near perfect fit of all data points. Contrary high smoothing weights allow the fit to move away from the actual data points to obtain a nearly homogeneous strain distribution. Assuming that a large variation in each strain value \( E_{11} \), \( E_{22} \), and \( E_{12} \) within a small corneal area (image area, <1 mm²) would be nonphysiological, the analysis led to a systematic error of no greater than 2.2 pixels (3.5 pixels for the posterior location in the swollen eye because of the poorer image quality) for a single data point. This error corresponds well with the digitization error described above. In the actual fit, smoothing constraints in the ideal range (minimal strain variation in combination with minimal root-mean-square error of the fit [see above]) were used. This results in a root-mean-square error of the fit of less than 1.3 pixels and a standard deviation in the strain values within the center of the image in all cases of less than 0.0017.

All errors reported hereafter are the standard errors of the mean values within each group. All \( P \) values were obtained through a three-way ANOVA (two within the group factors [intraocular pressure, location] and one between group factors [hydration state]).

### Results

#### Strain Measurements

The average homogeneous in-plane strain at posterior and anterior locations for normally hydrated corneas is shown in Figure 7. The relationships between strain and intraocular pressure were highly nonlinear, and the cornea was fairly extensible for intraocular pressures below physiological values but nearly inextensible for pressures above physiological lev-
Figure 7. Average homogeneous in-plane strain for the anterior and posterior central regions of seven normally hydrated corneas.

Corneal Thickness Measurements

The average corneal thickness diminished with a rise in intraocular pressure from 5 mm Hg to 65 mm Hg for both the normally hydrated and the swollen corneas (Fig. 10). For the normally hydrated corneas, the thickness was 367 ± 4 μm at an intraocular pressure of 5 mm Hg and dropped to 340 ± 6 μm at 65 mm Hg. In the swollen state, the thicknesses were 669 ± 8 μm and 627 ± 8 μm at respective intraocular pressures of 5 mm Hg and 65 mm Hg. The decrease in corneal thickness with increasing intraocular pressure was statistically significant (P < 0.005) for both hydration states.

Table 1. Volume Change and Bending of the Corneal Segment

<table>
<thead>
<tr>
<th>Hydration State</th>
<th>Anterior Radius (mm)</th>
<th>Bending Flexure (\frac{dϕ}{dV})</th>
<th>Volume Ratio (\frac{dv}{dV})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.5</td>
<td>1.03 ± 0.04</td>
<td>0.962 ± 0.008</td>
</tr>
<tr>
<td>Swollen</td>
<td>7.5</td>
<td>0.73 ± 0.10</td>
<td>0.994 ± 0.013</td>
</tr>
</tbody>
</table>

Discussion

The advantages of laser-scanning confocal microscopy for measuring corneal strains are threefold. First, it does not rely on tissue markers but images corneal cells and measures microscopic strains. This makes it noninvasive, because it is used in vivo and clinically. Second, this technique is not restricted to strain measurement on the corneal surface but allows measurement at virtually any desired position within the tissue. This could allow detailed measurements of the biomechanical properties of corneal wound tissue in vivo and could provide a better understanding of differential swelling or the shearing properties of the cornea. Third, a finite element-based nonhomogeneous strain analysis allows the quantification of complex nonuniform deformations, which may occur in the areas of keratorefractive cuts or sutures after corneal transplants.

The present study addressed the anterior and posterior strain response of the normally hydrated and swollen stroma in the central cornea. The results for the normal hydrated cornea correspond well with the findings of Hjortdal et al. for human tissue, taking the difference in material stiffness into account. The general nonlinear strain response is in good agreement with the results of Jue and...
Maurice, who showed an approximate 2.5% strain for the intact rabbit cornea for a comparable load range. The data for the normally hydrated cornea further support the idea of a homogeneous load distribution throughout the corneal thickness under physiological pressures as suggested by McPhee and Maurice. The decrease in corneal thickness with increasing intraocular pressure—as well as larger posterior than anterior straining—is generally viewed as a result of the incompressibility of the tissue. Inflation of a shell leads to an increase in surface area. Incompressibility requires constant shell volume. Hence, the result is a decrease in shell thickness that compensates for the surface increase. Furthermore, the same reasoning requires that the relative increase in surface area is larger posteriorly, which is one reason for the observed difference in anterior and posterior straining. In the normally hydrated cornea, the increased posterior straining is also an effect of the small but significant decrease in local corneal volume. The latter provides valuable insight into the first question raised in the introduction. The data presented here suggest that, even within minutes after load changes, local fluid shifts are possible and do occur to accommodate and redistribute stress concentrations. Therefore, great care must be taken concerning the manner in which the incompressibility assumption, common in most corneal modeling, is incorporated. For a reliable model of corneal biomechanics, it is necessary to investigate further the interaction of fluid flows within the corneal stroma and corneal load.

Concerning the second question raised in the introduction, the results for the swollen corneas showed measurable alterations in the anterior and posterior deformation compared to...
Strains in the center of the swollen cornea

**FIGURE 9.** Average homogeneous in-plane strain for the anterior and posterior central regions of eight swollen corneas.

with the normally hydrated corneas. The decrease in anterior strain for the swollen tissue is mostly a result of the high amount of corneal bending but could reflect a certain amount of prestressing of the anterior tissue under swelling. The latter would make the tissue appear stiffer in the low pressure range and is supported by the statistically significant decrease ($P < 0.01$) in anterior strain compared with the normally hydrated tissue for the pressure increase from 5 to 12.5 mm Hg. Similarly, the increase in posterior strain after swelling is a consequence of the high amount of corneal bending, but the observed change in the shape of the curve (Fig. 9) and the decreased stiffening for high intraocular pressures support the notion that swelling relaxes the posterior stroma. The measured posterior strains are, therefore, in all probability not the consequence of high loads in the posterior stroma but a passive extension of the relaxed lamella caused by bending.

The high amount of bending requires a nonhomogeneous variation in corneal strain from the center toward the limbus. This was possible because the design of the eye fixture did allow the whole globe to deform under intraocular pressure changes. (The eyes were not fixed at the limbus.) The bending could be the consequence of differences in the amount of swelling throughout the cornea or alterations in the stress distribution. Measurements of corneal thickness at various regions of swollen corneas and measurements of changes in corneal curvature could provide further insight into these questions, but they were beyond the scope of this study. The same is true for measurements of corneal hydration and differential swelling. Castoro and Bettelheim showed that the characteristics of corneal swelling are most likely related to the proteoglycan content and composition throughout the cornea. Cristol et al. drew a similar conclusion in their study of the differences in corneal swelling induced through either the anterior or the posterior surface. To which extent variations in proteoglycan distribution and corneal hydration are responsible for the increase in corneal bending is unclear. The method presented here could further our understanding of the combined effects of biomechanical and biochemical factors on corneal deformation, because it enables measurements of relative thickness changes at various depths of the stroma in combination with measurements of the in-plane tissue strains. The lack of a volume change for the deformation of the swollen corneas does not necessarily imply that the swollen tissue is fully incompressible. It may only be the consequence of corneal bending in combination with the altered load-carrying characteristics.

Another striking observation was that the appearance of the anterior keratocyte after swelling changed (Fig. 4). Generally only the keratocyte nuclei are visible in confocal microscopy images of the unstained stroma, but changes in corneal hydration resulted in images in which the whole cell body and the cell network could be imaged. Similar images were previously shown only after staining with 5-chloromethylfluorescein diacetate. The exact mechanism behind this change in appearance is unknown, but it could be the consequence of changes in keratocyte hydration or changes in the refractive index of the surrounding tissue.

**FIGURE 10.** Average corneal thicknesses of the seven normally hydrated and the eight swollen eyes for intraocular pressures of 5 mm Hg and 65 mm Hg. The decrease in corneal thickness with increasing intraocular pressure is statistically significant ($P < 0.005$) for both hydration states.
Regional strain measurements of the form presented here are a useful addition to marker-based macroscopic strain measurements. They enable one to investigate strain variations on a microscopic scale at virtually any point and depth within the cornea. This becomes especially important if one considers measurements close to keratorefractive cuts or other local tissue variations, such as wounds or edema. It is to be hoped that the combination of microscopic and macroscopic strain measurements can provide the experimental background for establishing a set of physically significant material parameters that will allow accurate predictions of corneal deformation for a wide range of surgical procedures.

References