

# Assessment of Transient Changes in Corneal Hydration Using Confocal Raman Spectroscopy

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**Purpose.** The degree of corneal hydration has been linked to excimer laser corneal ablation rates. Enhanced precision with excimer laser refractive surgery may result from a better understanding of the transient changes in corneal hydration. To better understand the dynamic nature of corneal hydration, bovine corneas were investigated under different surface treatments. **Methods.** Confocal micro-Raman spectroscopy was used to quantify corneal hydration. Water and acetone solutions were used to establish a quantitative response of the relative OH/CH Raman bands, which are consistent with the water and collagen protein bands in cornea, respectively. Intact bovine corneas were manually debrided (designated MD group) or lamellar flaps were created to expose stromal tissue (designated lamellar keratectomy or LK group). Raman spectra were recorded every 30 seconds for 6 minutes while the prepared cornea surfaces were exposed to quiescent air or to a forced nitrogen gas flow across the surface. **Results.** The OH and CH Raman bands yielded a linear response while the percentage of acetone was varied from 0% to 100%. For the bovine cornea under forced flow drying, the OH/CH Raman band ratio was found to decrease by 41% from the initial value for both the MD and LK treatment groups. These decreases were significantly more ( $p = 0.0051$  and  $0.054$ , respectively) than the 26% decrease in the OH/CH band ratio measured for the control corneas. In quiescent air, the control and MD groups exhibited a 7% and 6% decrease in the OH/CH ratio, respectively, while the LK treatment group revealed a 19% decrease in the OH/CH ratio. **Conclusions.** The bovine eye experiments demonstrate that significant changes in corneal hydration are realized under different drying conditions and treatment methodologies. This study elucidates the nature of transient changes in corneal hydration in a bovine model and suggests the need for further study of the role of such variations in surgical outcome for excimer laser corneal refractive procedures. **Key Words:** Cornea—Hydration—Ablation—Raman spectroscopy.

Refractive surgery continues to grow in popularity. In 2001, 1.3 million refractive procedures were performed in the United States, with laser in situ keratomileusis (LASIK) accounting for 92.3% of these procedures.<sup>1,2</sup> Along with the increasing popularity of refractive procedures have come increased patient expectations.

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Many recent advances have been made in laser and microkeratome technologies in an effort to improve the procedure by providing more precise and accurate outcomes. More work, however, needs to be directed to provide a better understanding of the interaction and subsequent response of the cornea with the laser, both at the time of surgery and during the postoperative period when the cornea is healing.

An important factor that remains not well understood is the intraoperative role of corneal hydration in the laser's ability to remove corneal tissue. Variations in the state of corneal hydration may help to explain unexpected outcomes after correctly performed refractive surgery. The corneal ablation rate is a measure of the amount of corneal tissue removed per pulse of the laser. Average corneal ablation rates are 0.2 to 0.8  $\mu\text{m}$  per laser shot for 193-nm ArF excimer lasers under clinically relevant conditions. However, current clinical excimer laser systems use an average ablation rate based on large patient populations. A significant source of error may be introduced in refractive procedures if an individual patient's cornea has an ablation rate that varies from the average clinical rate.

The corneal ablation rate has been linked to the state of corneal hydration. In an effort to determine why this variability occurs, Dougherty et al.<sup>3</sup> investigated various ablation rates, including what they referred to as the dry component ablation rate (defined as the mass of collagen and ground substance removed per surface area per laser shot) and the hydrated tissue (i.e., wet) ablation rate. They reported that the dry component ablation rate increased with decreasing hydration and that the wet tissue ablation rate decreased with decreasing hydration. The latter observation is consistent with the earlier published work.<sup>4,5</sup> Overall, researchers are in broad agreement that the degree of corneal hydration can directly affect the ablation rate of corneal tissue with 193-nm excimer laser radiation.

To elucidate the dynamic nature of corneal hydration as well as to quantify factors potentially affecting corneal hydration during clinical procedures, a quantitative measurement of corneal hydration is necessary. Pallikaris et al.<sup>6</sup> investigated the use of laser-induced breakdown spectroscopy (LIBS) to monitor corneal hydration. They reported that the atomic emission intensity of hydrogen remained constant while the atomic emission of calcium varied with corneal hydration. The LIBS technique uses a moderate laser pulse energy (tens of millijoules) to vaporize a small sample of corneal tissue, resulting in a strong shock wave and visible radiative emission. The destructive nature of LIBS is a significant limitation for in vivo analysis and therefore prevents integration into clinical studies supporting refractive procedures.

Raman spectroscopy is well suited as a technique for measuring and monitoring corneal hydration and has been investigated in several recent studies. Raman spectroscopy uses a single-wavelength excitation source (typically a low-power continuous laser beam) to probe the vibrational bond energies of constituent molecules through an inelastic light scattering process known as the Raman effect. It is minimally invasive, making it possible for integration into clinical investigations and possibly clinical systems.

The cornea is readily amenable to analysis by Raman spectroscopy due to its composition. Collagen and other proteins that are prominent in corneal tissue have characteristic vibrational frequencies, known as Raman bands or Raman shifts. Most notably, the band at  $2,940\text{ cm}^{-1}$  corresponds to the C-H stretching vibration. Water, which constitutes about 75% of normal corneal tissue,<sup>7</sup> displays a broad Raman band corresponding to the O-H stretching vibration in the region from  $3,000$  to  $3,700\text{ cm}^{-1}$  (which is commonly considered centered at approximately  $3,400\text{ cm}^{-1}$ ).

Jongsma et al.<sup>8</sup> describe the use of a confocal Raman system for *in vitro* study of rabbit eyes. This system involves Raman excitation and collection of Raman scattered light through a confocal microscope objective. The confocal implementation has the advantage of enhanced spatial surface and depth resolution, thereby reducing the size of the Raman probe volume. This enhanced resolution in turn reduces the effects of potential background fluorescence and spectral noise, and therefore improves the signal-to-noise ratio.

The confocal Raman system, because of the very small probe volume and high depth resolution, enables investigation of the hydration gradient throughout the cornea thickness. Using the same confocal Raman system discussed above, Bauer et al.<sup>9</sup> measured corneal hydration in both *in vitro* and *in vivo* rabbit corneas as well as in collagen-based phantom media. They reported, using the ratio of the water O-H stretch Raman intensity to the C-H stretch intensity as a metric, that hydration was constant throughout a sample of albumin with a uniform water distribution. These findings suggest that the measured hydration is a true function of physical hydration and is independent of the probe depth, even in samples with nonuniform water distribution, such as corneal tissue. In a more recent study, Bauer et al.<sup>10</sup> found that the relative hydration of the anterior surface of the human cornea *in vivo* decreased with the application of the dehydrating drug Muro 128. This important study demonstrated the utility of confocal Raman spectroscopy for measuring corneal hydration and was the first time that *in vivo* Raman spectra were successfully obtained from the corneal tissue of humans.

This paper reports on the use of a confocal Raman system for the assessment of bovine corneal hydration as a function of time corresponding to different surface treatment methodologies.

## MATERIALS AND METHODS

Whole bovine eye globes were collected within 30 minutes of animal sacrifice and stored in phosphate-buffered saline at ambient temperature ( $\sim 22^\circ\text{C}$ ) prior to experiments. All Raman measurements were made between 2 and 4 hours postmortem. Eyes were collected and stored in pairs for each bovine and subsequently used in pairs for the Raman measurements as discussed below. After performing Raman spectroscopy, select bovine cornea were har-

vested and measured at the cornea center using a precision linear caliper ( $12.5\text{ }\mu\text{m}$  accuracy). The bovine corneas were found to have an average full thickness of  $750 \pm 50\text{ }\mu\text{m}$  (compared with average human central corneal thickness of  $520\text{ }\mu\text{m}$ ).

A confocal micro-Raman spectrometer system (LabRam Infinity, Jobin Yvon) was used for all measurements. Figure 1 shows a schematic diagram of the Raman system, including the main excitation and collection components. The excitation source was a 632.8-nm helium-neon laser operating with nominal output energy of 15 mW. The laser beam was focused on the sample using a 10 $\times$  objective resulting in a focused beam spot of  $35\text{ }\mu\text{m}$  on the corneal sample surface. The laser beam was attenuated with a neutral density filter prior to entering the microscope objective to yield a laser beam power of 2.8 mW on the sample surface. It was determined through experimental repeatability and examination using visible microscopy that no effects on the corneal tissue were induced by the laser beam intensity under these conditions. Raman scattered light was collected in the backscatter mode through the microscope objective and dispersed onto a 1024-pixel CCD detector array using a 600 grooves/mm grating. The grating was centered to provide a spectral window between 735 and 862 nm over the CCD array, corresponding to a Raman shift ranging from  $2,215$  to  $4,205\text{ cm}^{-1}$ . The effective spectral dispersion was  $0.12\text{ nm/pixel}$ , equal to about  $1.6$  to  $2.3\text{ cm}^{-1}/\text{pixel}$  over the specified wavelength range. The Raman microscope was configured for confocal microscopy. The confocal aperture was set to a diameter of  $500\text{ }\mu\text{m}$ , which resulted in an effective integration depth of  $300\text{ }\mu\text{m}$ , thereby ensuring a volume-integrated measurement of corneal tissue rather than a surface-weighted measurement. All Raman spectra were recorded using a single acquisition time (no spectral averaging) of 10 seconds for the corneas and for the water/acetone solutions.

To assess the signal linearity of the Raman technique as a relative measure of hydration, known solutions of acetone and deionized water were prepared and analyzed. All solutions were pre-

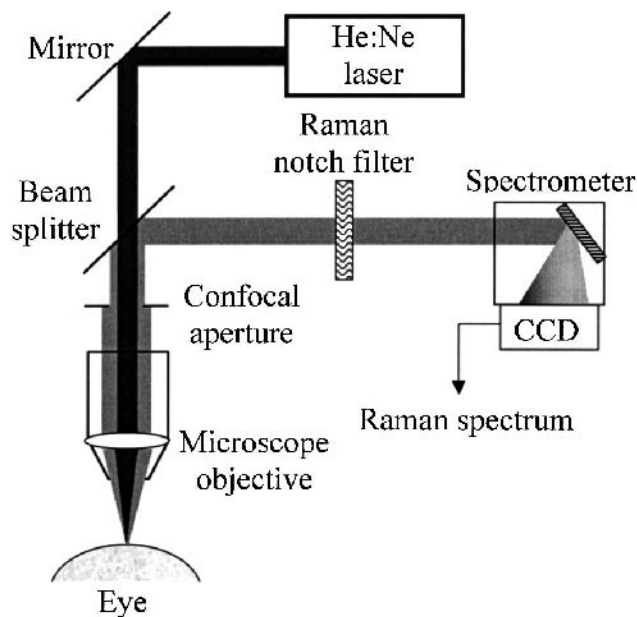


FIG. 1. Schematic of experimental confocal Raman spectrometer system.

pared using ultrapurified, deionized water (Fisher Scientific) and electronic grade acetone (Fisher Scientific). To eliminate changes in solution concentration due to preferential acetone evaporation, all solutions were prepared and used immediately.

Next, 30 attached bovine corneas (whole globes) were equally divided into three experimental groups. The first group (10 corneas) was designated the control group, and the intact corneas were used as collected and stored. The second group (10 corneas) was designated the MD group, as the central corneas were manually debrided using a scalpel edge to create a 10- to 12-mm optical zone (OZ). The third group (10 corneas) was designated the lamellar keratectomy (LK) group, as corneal lamellar flaps were manually cut using a scalpel and removed from the surface. The total flap thickness of three different flaps ranged from 100 to 250  $\mu\text{m}$ , as directly measured with the precision calipers. Each of the three experimental groups (Control, MD, and LK) was comprised of 10 corneas from five bovines.

Each group was further divided into two subgroups of five corneas. The corneas were paired for this division; therefore, each subgroup received a single eye from each of the five bovines. The first subgroup was exposed to quiescent ambient air for a period of 6 minutes, with a Raman spectrum recorded every 30 seconds. The second subgroup was exposed to a forced flow of compressed nitrogen tangential to the cornea surface, with a Raman spectrum recorded every 30 seconds for 6 minutes. The forced nitrogen flow was achieved using a flow rate of 9 L/min through a 3.5-mm inner diameter stainless steel tube. The tube was positioned about 3 cm from the cornea with the nitrogen flow directed tangentially across the cornea surface. The mean flow velocity at a distance of 12 mm from the exit of the tube was 20 m/s, and the mean velocity directly above the corneal surface was about 7 to 11 m/s, as measured using a hot-wire velocity probe. The Reynolds number (gas density  $\times$  velocity  $\times$  tube diameter/gas viscosity) of the nitrogen flow was approximately 1,000, which corresponded to a laminar flow of nitrogen.

For the corneas exposed to the nitrogen flow, an initial Raman spectrum was recorded prior to initiation of the flow. With this experimental matrix, changes in corneal hydration due to drying under either ambient or forced flow conditions were assessed using paired groups ( $n = 5$ ) for the control, MD, and LK treatments.

### Clinical Relevance

While this study is designed to assess transient changes in bovine cornea hydration, it is useful to relate the current experimental conditions to those corresponding to clinical excimer laser refractive procedures. A clinical excimer laser system uses vacuum suction to draw air across the surface of the cornea, which causes aspiration of the ablation plume. To provide a comparison with clinical systems, velocity measurements were recorded within the flow field induced above a human subject with a clinical LASIK system (VISX Star S3) under representative clinical conditions. The aspiration tube (22-mm diameter) was positioned about 3 to 4 cm from the human cornea and pointed toward the corneal surface such that the axial centerline of the tube was positioned about 3 cm above the corneal surface. The velocity of air induced by the aspirator near the tube inlet was 20 m/s, and the velocity of the induced flow field above the corneal surface was about 1 to 1.3 m/s. While the clinical aspirator entrance velocity and the current nitrogen jet exit velocity were identical, the flow velocity above the corneal surface was markedly greater for the nitrogen jet case.

This increased velocity reflects the relative confinement of the nitrogen jet momentum (i.e., flow field) to the centerline of the exit nozzle. In contrast, the clinical aspirator draws in air equally from all directions resulting in a more diffuse and lessened flow field several centimeters from the entrance.

It is noted that the micro-Raman measurements were recorded in a sealed (light tight) sample chamber, which precludes the direct emulation of the open-air flow patterns created by clinical system aspirators. Furthermore, no active control of ambient air humidity was available in the current study. It is noted that several research groups have reported the effects of humidity or the need to control humidity during LASIK procedures.<sup>11-13</sup>

## RESULTS

### Acetone-Water Study

Acetone ( $\text{CH}_3\text{COCH}_3$ ) contains two  $\text{CH}_3$  groups but lacks an O-H bond, while water contains only two O-H bonds. Accordingly, pure acetone will display an intense Raman peak near 2,900  $\text{cm}^{-1}$  corresponding to the C-H stretching mode, and pure water will display a broad, prominent Raman band at 3,400  $\text{cm}^{-1}$  (3,100–3,700  $\text{cm}^{-1}$ ) corresponding to the O-H stretching mode. These two bands are analogous to the collagen protein bands and water bands observed in the Raman spectra of corneal tissue, respectively. In pure solutions of either acetone or water, these two bands are mutually exclusive, while binary mixtures of acetone and water will exhibit both the C-H and the O-H bands, with the relative intensities corresponding to their respective molar fractions. To confirm the quantitative use of the C-H to O-H Raman band intensities as a relative measure of hydration, experiments were performed in a series of water/acetone solutions ranging from pure water to pure acetone.

A representative Raman spectrum corresponding to 80% water and 20% acetone solution is shown in Fig. 2. The C-H band at

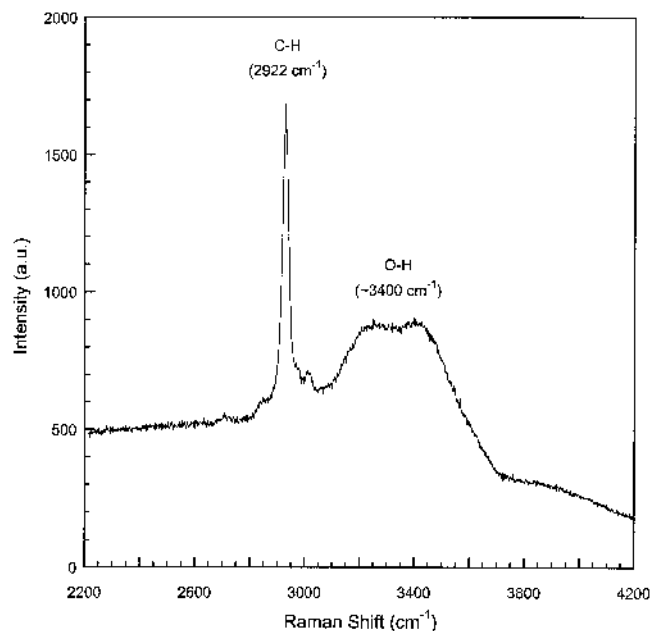
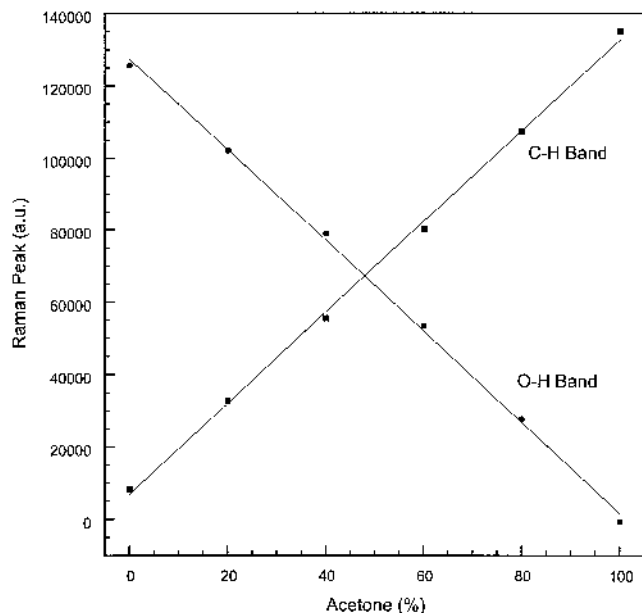


FIG. 2. Representative Raman spectrum for a solution of 80% water (by volume) and 20% acetone shows the C-H vibrational stretch of acetone and the O-H stretch of water.



**FIG. 3.** Integrated peak area of the C-H and O-H Raman band intensities as a function of acetone volume percentage. The solid lines are linear least-square fits.

2,922  $\text{cm}^{-1}$  due to the acetone fraction and the O-H band at  $\sim 3400 \text{ cm}^{-1}$  due to the water fraction are both well defined in the spectrum. The C-H and O-H Raman bands were analyzed to provide a quantitative measure of solution hydration. A baseline was fit for each spectrum and the absolute peak areas were calculated for each Raman band. The O-H and C-H Raman peak intensities are plotted as a function of acetone content in Fig. 3. As the acetone volume fraction increased from zero to 100%, the C-H peak area increased linearly and the O-H peak area decreased linearly. All measurements were recorded in triplicate, and each data point in Fig. 2 is the average value. The data are characterized by a high degree of precision, with the average relative standard deviation equal to 0.4% and 0.8% for the O-H and C-H peaks, respectively. The data were fit with linear least-square lines (as shown in the plot) with resulting regression coefficients ( $R$ ) greater than 0.999. It is noted that the two curve fits have nearly identical but inverted slopes, namely, 1,260 for the C-H peak data and negative 1,257 for the O-H peak data.

### Bovine Eye Study

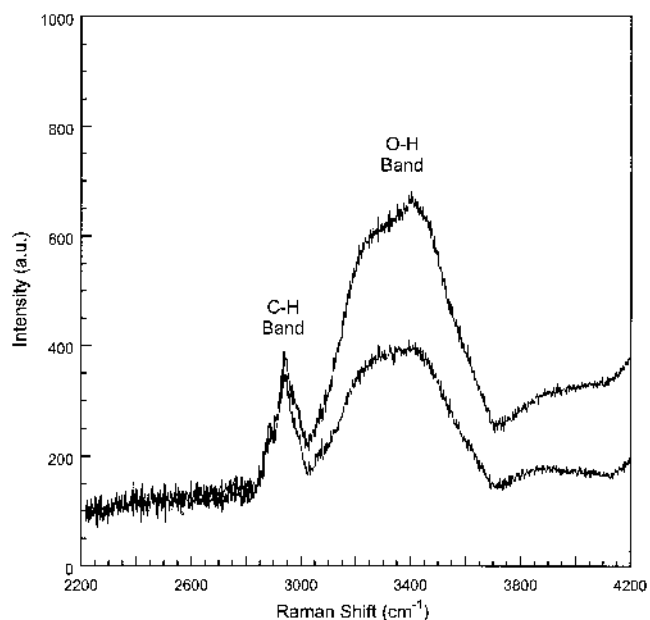
A series of experiments was performed to assess the transient state of corneal hydration corresponding to different surface treatments, as described above. Raman spectroscopy was used to assess the relative hydration as a function of drying time for exposure to quiescent surrounding air and for exposure to a forced flow of nitrogen gas. For the current study, relative hydration is defined as the ratio of the integrated O-H Raman band to the integrated C-H Raman band. The response curves of the water-acetone study demonstrate the quantitative response of these Raman peak intensities (O-H and C-H), making their ratio an ideal measure of relative hydration as pertaining to the concentrations of corneal water and corneal protein. It is noted that earlier studies support the use of O-H to C-H Raman bands as a quantitative measure of hydration.<sup>9,10</sup> Quantitative data are presented directly as the O-H to C-H ratios; however, for brevity, additional discussion may refer sim-

ply to corneal hydration, which is considered analogous to the reported O-H to C-H ratios.

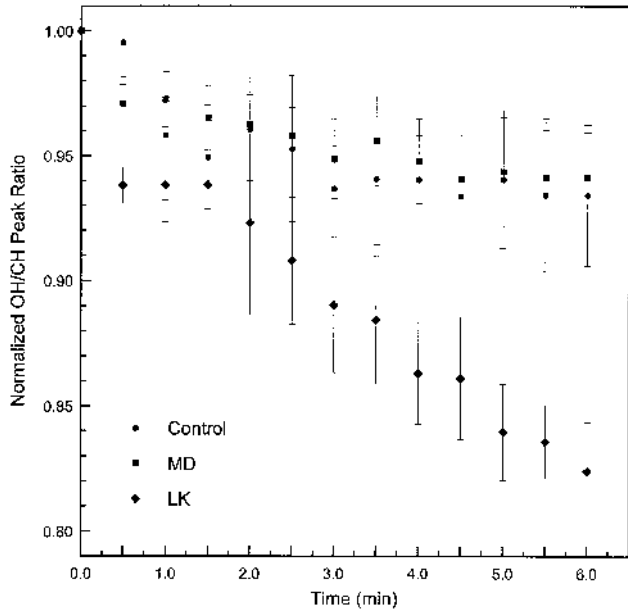
All bovine corneas were measured for a continuous period of 6 minutes by recording Raman spectra (10-second average) every 30 seconds. Figure 4 shows two representative Raman spectra, one recorded from a manually debrided cornea at the outset of the forced nitrogen flow experiment and one recorded from the same cornea after 5 minutes of exposure to nitrogen flow. As demonstrated in the spectra, the peak area of the C-H band ( $2,900 \text{ cm}^{-1}$ ) remained relatively constant while the O-H band ( $3,100\text{--}3,700 \text{ cm}^{-1}$ ) intensity decreased markedly after 5 minutes. The O-H to C-H Raman peak area ratio decreased from 8.7 to 5.0, which corresponds to a decrease of 43% in relative hydration after 5 minutes of drying.

The Raman-based hydration data for all bovine cornea experiments are summarized in Figs. 5 and 6, which correspond to the quiescent air drying and forced flow drying conditions, respectively. For all data analysis, the O-H/C-H peak ratios were normalized with respect to their initial values for each cornea. For each experimental group, the normalized values were then averaged corresponding to each drying time; thus, each data point represents an average of five corneas from five different bovines. For all experimental conditions, the average relative standard error was 6.3%. For the forced flow data, the normalizing initial O-H/C-H ratio was recorded 30 seconds prior to the nitrogen flow initiation. Therefore, in Fig. 6 the O-H/C-H ratios corresponding to zero flow time correspond to the actual first 10 seconds of nitrogen flow, and the starting ratio corresponds to a time of negative 30 seconds.

Several key features characterize the corneal hydration data presented in Figs. 5 and 6. In the case of quiescent air drying, the O-H/C-H peak ratio of both the control corneas and the MD corneas remained relatively constant, decreasing to 93.4% [5.6% rela-

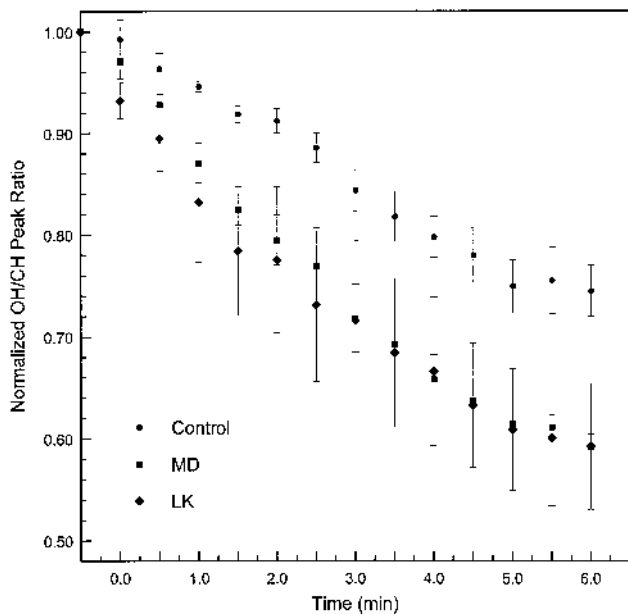


**FIG. 4.** Raman spectra from a manually debrided bovine cornea corresponding to the outset (upper spectrum) of forced flow drying and following 5 minutes of exposure to forced flow drying (lower spectrum). The C-H protein band and O-H water band are labeled, and the spectra have been baseline corrected.



**FIG. 5.** Normalized ratio of integrated O-H band intensity to integrated C-H band intensity of bovine corneal tissue as a function of time for exposure to quiescent air drying for the three treatment groups (Control, MD, and LK). The O-H/C-H ratio is normalized by the initial (time equals zero) value of the ratio.

tive standard deviation (RSD)] and 94.1% (3.8% RSD) of the initial values, respectively. In contrast, the LK corneas revealed a more significant decrease in hydration over 6 minutes, ending with an average O-H/C-H peak ratio equal to 81.1% (3.5% RSD) of the initial hydration values. For the case of forced nitrogen gas drying, all three corneal treatments revealed a marked decrease in the O-H/C-H peak ratio over time, with the MD and LK eyes experiencing much more pronounced decreases than the control eyes.



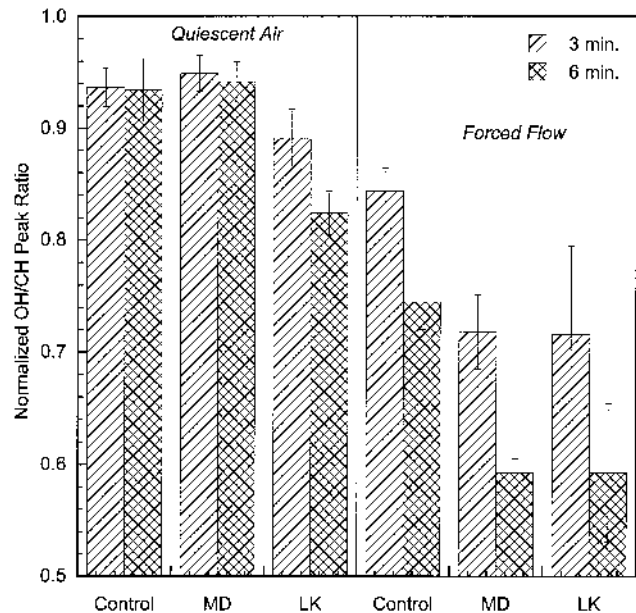
**FIG. 6.** Normalized O-H/C-H ratio of bovine corneal tissue as a function of time for exposure to forced flow drying for the three treatment groups (Control, MD, and LK). The O-H/C-H ratio is normalized by the initial, preflow (time equals negative 0.5 min) value of the ratio.

The statistical significance of the data is discussed below. In concert, the Raman data demonstrate a marked difference in the extent of corneal dehydration realized with the two different drying scenarios, namely, quiescent air and forced flow, as well as the different corneal treatment methods.

With all data shown in Figs. 5 and 6 normalized to an initial value of 1.0, the final hydration level (O-H/C-H ratio) indicates the degree of reduction in corneal tissue hydration over the course of the 6-minute drying period. For comparison of all experimental conditions, the normalized O-H/C-H ratios are summarized in Fig. 7 after 3 and 6 minutes of drying. The average final decreases in corneal hydration for the different treatment methodologies, as measured by the percentage of decrease in O-H/C-H ratio after 6 minutes, are summarized in Table 1. Using the two-sided Student *t* test with a 99% confidence level ( $\alpha = 0.01$ ), there is no statistically significant difference between the control and the MD groups exposed to 6 minutes of quiescent air drying ( $p = 0.816$ ). In contrast, the LK group revealed a statistically significant reduction in the O-H/C-H ratio in comparison to the control corneas ( $p = 0.00486$ ) and the MD corneas ( $p = 0.000219$ ) after drying for 6 minutes in quiescent air.

Using the same statistical test, it is concluded that there is no difference between the relatively large decrease in the O-H/C-H ratio realized with the MD and LK treatment groups ( $p = 0.999$ ) after 6 minutes of forced nitrogen flow. There is, however, a statistically significant difference in the decrease in the O-H/C-H ratio between the control corneas and the MD corneas ( $p = 0.0051$ ) under forced drying conditions. Under the same forced flow conditions, a similar difference is reported between the control corneas and the LK corneas ( $p = 0.0538$ ) using a confidence level of 94% ( $\alpha = 0.06$ ). The relaxed confidence level reflects the somewhat larger standard deviations realized with the LK treatment protocol.

In comparing the effects of quiescent and forced flow drying for a given corneal treatment, the final O-H/C-H ratios are statistically



**FIG. 7.** Normalized O-H/C-H ratio of the bovine corneas after 3 and 6 minutes of exposure to quiescent air or forced flow drying for the three treatment groups (Control, MD, and LK).

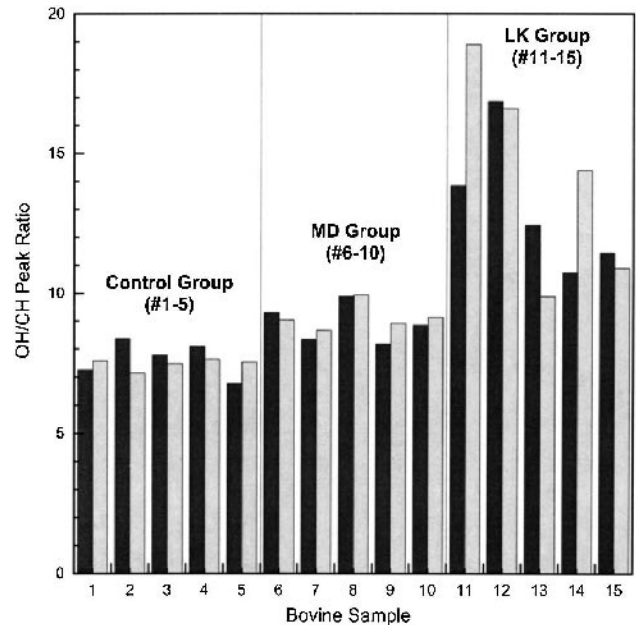
**TABLE 1.** Final percent decrease in corneal hydration ( $\pm$  standard deviation,  $n = 5$ ) as measured using the O-H/C-H peak area ratio from the Raman spectra of bovine corneas after six minutes under quiescent air or forced flow drying. The percent decrease is with respect to the initial hydration state

Cornea treatment	Quiescent air	Forced flow
Control	6.6% $\pm$ 0.4%	25.5% $\pm$ 1.7%
MD	5.9% $\pm$ 0.2%	40.7% $\pm$ 1.7%
LK	18.8% $\pm$ 0.7%	40.7% $\pm$ 8.5%

different for the control eyes ( $p = 0.00109$ ) and the MD eyes ( $p = 4.16 \times 10^{-7}$ ) with a confidence level of 99% ( $\alpha = 0.01$ ). In comparing quiescent and forced flow for the LK treatment, the two drying scenarios were also statistically different ( $p = 0.0181$ ) for a confidence level of 98% ( $\alpha = 0.02$ ).

While the above analysis details the degree to which the O-H/C-H Raman ratio decreases after 6 minutes for the various experimental conditions, it is also useful to assess the change observed in a shorter time interval. In the current study, rather significant decreases in corneal hydration, as measured by the O-H/C-H ratio, were evident in as little as 1 minute, as observed in Figs. 5 and 6, for the different experimental conditions. After 1.5 minutes of forced gas flow, the average O-H/C-H ratio for the control, MD, and LK groups were reduced to 91.9%, 82.4%, and 78.4%, respectively, of their initial values. Using a two-sided Student  $t$  test, the decrease in the O-H/C-H ratio was statistically significant for the MD corneas ( $p = 0.00079$ ) at a confidence level of 99% ( $\alpha = 0.01$ ) and for the LK corneas ( $p = 0.0761$ ) at a confidence level of 92% ( $\alpha = 0.08$ ) after only 1.5 minutes with respect to the control value. As a final item concerning Figs. 5 and 6, it is worth noting that the rate of decrease in the O-H/C-H ratio for all six experimental conditions was nearly linear albeit with a few aberrant data points. There was, however, a slight upturn in the O-H/C-H ratio near the end of the drying sequence, perhaps indicating a "leveling off" of the dehydration effects beyond approximately 5 minutes.

As all the data presented above were normalized with respect to the initial O-H/C-H Raman peak ratios, it is useful to examine the absolute values of the ratios associated with the various treatments. All the bovine eyes (control, MD, and LK) were obtained in pairs from freshly killed cows. For a given bovine pair, each eye was treated identically and subsequently subjected to either quiescent or forced flow drying. The initial Raman spectrum of each cornea was used to calculate the initial O-H/C-H ratio used for the normalization of all subsequent data. The absolute O-H/C-H ratio for each of the corneas at the initial, physiologically hydrated condition is presented in Fig. 8. Each bovine sample number contains a pair of shaded and unshaded bars corresponding to a pair of eyes from a single cow. No attempt was made to identify the left and right eyes. As observed in the figure, the initial O-H/C-H peak ratio was similar for both eyes in a given pair and for all eyes in a given treatment group, notably so with the control and MD groups. The greatest variability in initial hydration existed for the LK group. This is attributed to the variability from eye to eye in the depth of the corneal flap as well as to the somewhat rough surface texture of the resulting corneal stroma, realized with the manual cutting of corneal tissue. The resulting surface microstructures can be characterized by high surface-to-volume ratios, which can lead to local dehydration in very short time scales in view of the earlier results. With this in mind, a relatively large variation in absolute



**FIG. 8.** The initial value of the O-H/C-H ratio of bovine corneal tissue at the outset of each experiment for the various treatment groups (Control, MD, and LK). Each pair of shaded bars represents the two eyes from a single bovine.

hydration, as assessed using the micro-Raman system, is expected for the flap treatment group.

A monotonic increase in the O-H/C-H ratio was noted as the Raman measurements progressed from the surface of the cornea to deeper into the cornea stroma after removal of the flap. The average initial O-H/C-H ratio was  $7.56 \pm 0.46$  for the control eyes,  $9.03 \pm 0.58$  for the MD group, and  $13.61 \pm 3.13$  for the LK group ( $n = 10$  for each group). A two-sided Student  $t$  test with a confidence level of 99% ( $\alpha = 0.01$ ) revealed statistically significant differences between the control eyes and MD eyes ( $p = 8.8 \times 10^{-6}$ ), between the MD eyes and the LK eyes ( $p = 0.000874$ ), and between the control eyes and the LK eyes ( $p = 0.000158$ ). In other words, the control eyes, which were left untouched and therefore investigated on the anterior portion of corneal tissue with full epithelium, had the lowest level of relative hydration at the initial measurement. The MD eyes, which were investigated on the anterior portion of debrided corneal tissue, were noticeably more hydrated initially than the control eyes. Finally, the LK eyes, which were investigated on the surface of the exposed corneal stroma, were noticeably more hydrated initially than both the control eyes and the MD eyes. In conclusion, corneal hydration as measured by the Raman technique increases with progressively deeper locations in corneal tissue. These results are in excellent agreement with the findings reported by Bauer et al., in which it was determined that the level of corneal hydration (O-H/C-H ratio) increased as the cornea was probed from anterior to posterior.<sup>9</sup>

## DISCUSSION AND CONCLUSIONS

The primary conclusions of the current study are summarized as follows.

1. The confocal Raman technique is an effective way to measure relative corneal hydration using the C-H protein bands and the O-H water bands.

2. For all treatment groups, the relative state of corneal hydration, as measured by the ratio of the integrated O-H peak intensity to the integrated C-H peak intensity, decreased with time. The decrease in corneal hydration is statistically significant within the first 90 seconds of corneal exposure to quiescent air or forced flow drying.
3. For all treatment groups, the O-H/C-H ratio decreased to a greater extent and at a greater rate if the cornea is exposed to a forced flow of nitrogen gas as compared with quiescent air drying.
4. The O-H/C-H ratio varied depending on the surface treatment method. Removing the epithelium (i.e., MD) does not decrease the O-H to C-H ratio relative to controls for quiescent air drying, but does decrease the ratio relative to controls when corneas are exposed to forced flow drying. Creating a corneal flap (i.e., LK) decreased the ratio relative to controls for corneas exposed to quiescent air and forced flow drying.
5. The absolute degree of corneal hydration, as measured by the O-H/C-H ratio, increased, as measurements were made deeper into the cornea.

In addition to the conclusions enumerated above, specific comments are offered with respect to the current findings, including discussion of the relative physical processes associated with transient changes in corneal hydration, and the clinical relevance of the present findings.

The present water/acetone Raman study demonstrates the quantitative nature of the O-H and C-H Raman peak area ratios as a metric for relative hydration. The Raman data for these experiments are characterized by a high degree of precision and yield an ideal inverse relationship between the two response functions (i.e., Raman band peak areas) for binary mixtures of the two compounds. The water/acetone data support the current use of the O-H to C-H Raman band ratio as a quantitative measure of relative corneal hydration. This is consistent with earlier studies in which the O-H/C-H ratio was reported as a quantitative metric for water content in an albumin model<sup>9</sup> and for changes in corneal hydration following application of a dehydrating drug.<sup>10</sup> As such, the confocal Raman system is well suited for the assessment of transient changes in corneal hydration corresponding to different surface treatment methodologies. For the remainder of the discussion, it should be understood that relative corneal hydration refers to the O-H/C-H Raman peak ratio.

The bovine eye experimental data support the present conclusion that significant changes in corneal hydration are realized under different drying conditions and surface treatment methodologies. More significantly, exposure of the corneal stroma (LK group) to a forced airflow condition can induce a 10% to 20% decrease in corneal hydration in as little as 2 minutes. With these findings in mind, additional comments are offered with regard to the role of induced airflow velocity in corneal dehydration. Using the slope of the O-H to C-H Raman intensities as a function of time (Figs. 4 and 5), the rates of corneal dehydration were evaluated in the temporal region between 1 and 3.5 minutes of drying time, a region that is characterized by an approximately linear decay. For the control and MD corneas, the rate of dehydration was increased by a factor of 7.4 and 10.6, respectively, with forced flow drying as compared with the quiescent air drying. In contrast, for the LK group, the rate of corneal dehydration only doubled with forced flow as compared with quiescent air drying. Note, however, that the LK baseline quiescent drying case was significantly greater

than the quiescent drying realized with either the control or MD corneas.

In concert, the dehydration rate data offer insight into the relative importance of water diffusion within the corneal tissue and mass transfer from the corneal tissue to the surrounding gas. The data suggest that the rate of dehydration is significantly influenced by the rate of water diffusion to the corneal surface under forced flow conditions. This finding would explain the more significant increase in the rate of dehydration (forced flow to quiescent) with the MD corneas as compared with the control corneas. The epithelial cells are expected to present an additional barrier to water diffusion to the cornea surface. As a result, the forced flow condition is limited for the control case to a dehydration rate that is less than that observed with the MD corneas. When comparing the MD and LK conditions under forced drying, it is reasonable to conclude that the presence of the relatively thick bovine basement membrane in the MD corneas does not appear to offer an appreciable additional resistance to water diffusion to the surface as compared with the corneal stroma exposed by flap removal. Hence, these two treatment conditions were characterized by similar dehydration rates under forced flow drying, presumably due to the overall limitation of water diffusion through the corneal tissue to the surface. These findings suggest a "saturation" effect with forced flow dehydration. Hence, additional increases in air velocity contribute little to the rate of corneal tissue dehydration, which is controlled primarily by the rate of water diffusion through the corneal stroma.

In contrast to forced flow drying, quiescent drying is more likely limited by mass transfer from the corneal surface to the bulk surrounding air. This conclusion is consistent with the similar dehydration rates observed with quiescent air drying for the control and MD corneas. The greater rate of corneal dehydration observed with the MD group under quiescent drying is assumed to be due to an increase in available corneal stromal surface area and to an increase in the absolute surface water content secondary to the slight hydration gradient realized within the corneal tissue (Fig. 7).

The presence of a pronounced hydration gradient, with hydration increasing with depth from the anterior surface, directly couples to the transport of water to the corneal surface. As noted above, the LK data are characterized by a larger experimental variability as compared with the control and MD data. It is expected that a thicker flap will produce a corneal surface characterized by increased hydration at the interface, due to the hydration gradient, thereby promoting mass diffusion within the cornea and an enhanced rate of dehydration. In view of these comments, the range of flap thickness (100–250  $\mu\text{m}$ ) in the current study is most likely a significant contributor to the variability in the LK results.

Final comments are offered with respect to the effects of boundary conditions on corneal dehydration. Relative humidity of the surrounding environment may affect the rate of corneal dehydration. Accounting for the entrainment of ambient air via diffusion into the forced jet flow, the relative humidity of the forced flow at the cornea interface is estimated to be 75% of the ambient air value, which was consistent at about 50%. If the relative humidity of the nitrogen gas flow were increased to 50%, the present dehydration rates would be expected to decrease by about 20% for gas-transfer limited dehydration, notwithstanding the above discussion regarding the overall limitation of water diffusion through the corneal tissue to the surface, which is unaffected by ambient humidity.

### Clinical Relevance

The current study suggests that changes in the corneal hydration state may occur with common excimer laser refractive surgery preoperative treatment methodologies, namely, epithelium removal and lamellar keratectomy. Given the generally accepted dependency of excimer laser corneal ablation rates on the absolute state of corneal hydration, such dynamic changes in corneal hydration may subsequently affect the outcome of clinical refractive procedures.

The exact mass transfer problem of corneal drying is a complex problem involving mass diffusion in both the corneal tissue and gas stream, coupled with the fluid dynamic boundary layer flow. A solution for mass transfer based on a constant surface water content, binary mass diffusion of water vapor in air, and a simplified set of boundary flow equations yields a mass transfer rate proportional to the square-root of the free stream gas velocity.<sup>14</sup> Based on this analysis, the nominal eight-fold decrease in the velocity of the forced airflow realized with a clinical aspirator (as compared with the current study) is expected to result in an approximately three-fold decrease in corneal dehydration rate with respect to the current data reported for the forced nitrogen jet. However, as discussed below, the current data suggest a saturation effect for forced flow dehydration; hence, surface mass transfer (i.e., from corneal tissue to the gas stream) may not completely control the cornea flap dehydration conditions under all forced flow conditions. Clearly, additional research is necessary to further elucidate the important mechanisms of corneal dehydration under more clinically relevant conditions. Nonetheless, the current data emphasize the importance for surgeons to standardize the time between lifting the LASIK flap and initiating excimer laser therapy, particularly if the vacuum aspiration tube has been put in place. The importance of standardization was recognized in the protocol used for an early study on LASIK.<sup>15</sup>

It is also noted that clinical studies have reported variability with respect to the mean flap thickness ranging from  $\pm 20$  to  $\pm 50$   $\mu\text{m}$ ,<sup>16,17</sup> which may contribute to variations in clinical outcome. Furthermore, human corneas are about 30% thinner than bovine corneas; hence, human corneas may have a greater corneal hydration gradient, leading to enhanced dehydration rates and greater variation in dehydration rates with changing flap thickness. The relative humidity of the surrounding environment may also affect the rate of corneal dehydration, as noted above. Humidity levels reported in the literature regarding LASIK studies range from about 40% to 50%,<sup>11,12</sup> and while various clinical systems may recommend humidity levels, overall, the important point is that the humidity should be as constant as possible from procedure to procedure.

Finally, surface hydration of the cornea may be directly affected by excimer laser ablation, which may significantly affect the boundary conditions. Oshika et al.<sup>18</sup> suggested that shock waves generated during laser ablation might cause moisture to pool centrally within the ablation zone. They further concluded that this pooling contributed to the formation of steep central islands, a condition where significantly less tissue is ablated locally. Maldonado-Codina et al.<sup>19</sup> reported temperature increases of nearly 9°C over the course of various PRK procedures ranging in correction from 2.00 to 10.00 D. Temperature changes resulting from laser ablation will certainly modify the boundary conditions for the mass transfer problem, which may affect any dynamic changes in corneal hydration.

In conclusion, this study demonstrates the potential utility of using Raman spectroscopy to analyze the cornea in vivo with the goal of better understanding excimer laser treatment methodologies. It elucidates changes in corneal hydration that occur with time. Specifically, the study shows that corneal hydration varies depending on the exposure time, type of corneal exposure (quiescent air or forced flow drying), and type of surface treatment (MD or LK). The data offer possible insight into explaining unexpected refractive surgical outcomes for successfully administered laser treatments. This study further reinforces the importance of surgeon consistency in surgical technique, particularly with respect to the initiation of excimer laser treatment after lifting the flap (or removing the epithelium) and the positioning of the vacuum tube. Ultimately, it is expected that a better knowledge and understanding of the underlying physics and corneal physiology will lead to enhanced precision and accuracy of laser refractive surgery.

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