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ORIGINAL ARTICLE

Corneal Light Transmission and Roughness After Refractive Surgery

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ABSTRACT

Purpose. To determine the relation between the corneal light transmission measurements and the epithelial surface properties in hen corneas after different refractive surgery techniques photorefractive keratectomy, laser in situ keratomileusis, and laser-assisted subepithelial keratomileusis, and a group with only epithelial corneal removal (deepithelialization).

Methods. Five groups of hen corneas with different treatments and a control group were analyzed at 30 days. Direct transmittance and corneal light scattering were measured by a scatterometer developed by our group. Quantitative and systematic measurements of external and internal roughness and epithelium thickness were assessed using standard techniques developed for quantitative analysis of microphotographs of the corneal epithelium.

Results. Data analysis revealed that the roughness in the epithelial surface was associated with the corneal light transmission. The direct transmittance of light showed a significant correlation with the epithelial roughness in the control ($r = -0.99$, $p < 0.05$) and photorefractive keratectomy ($r = -0.99$, $p < 0.05$) groups. However, there was no relation between the epithelial thickness and the corneal light transmission measurements.

Conclusions. The experimental results suggested that the roughness of the epithelial surfaces is related to the light transmission in the cornea.
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Key Words: corneal epithelium, direct transmittance, light scattering, refractive surgery

Refractive surgery involves a range of techniques, which have become progressively important in recent years. Ongoing developments in surgical methods coupled with the average young age of operated patients may restrict knowledge of the biological and physical changes involved. Thus, studying these areas offers an attractive line of research, because transparency and quality of vision depends on the wound healing response. During corneal wound healing after refractive surgery, the natural conformation of the corneal layers is altered, resulting in decreased transparency.[1–7] Corneal transparency has been long explored, and several theories have been suggested pertaining to the causes of light scattering.[5] Based on work by Maurice,[8] several models explain high corneal transparency as a result of its limited thickness and short-range ordering of collagen fibrils, which reduces global scattering because of interference between waves scattered by different fibrils.[9,10] However, existing models have also developed the connection between corneal crystallins and corneal cellular scattering.[11,12]

Maintaining epithelial integrity is essential for corneal optical properties. Indeed, one property of the epithelium for normal vision is to form a smooth refractive surface.[13] The epithelial surface plays an important role in the modulation of wound healing response, as epithelial signals trigger keratocyte apoptosis and the appearance of myofibroblasts in the stroma through the release of multiple cytokines and growth factors such as interleukin 1, transforming growth factor β, and tumor necrosis factor α.[14–16] After refractive surgery, the basement membrane is interrupted, and the organization of the epithelial cells is altered, with surface roughness and hyperplasia appearing. This reaction varies depending on the type of corneal epithelial injury.[4,17] When the basement membrane is removed, the epithelium must regenerate it and redevelop tight junction integrity. Remodeling can take a long time, with a

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prolonged response possibly resulting in instability of the optical outcome of refractive surgery.[4,18]

Epithelium surface, subepithelial structures, basement membrane defects and epithelium-stromal interface irregularity have been explored by several studies, which have assessed their link to corneal transparency.[4–6,19–24] Evidence of the importance of epithelial signals in the appearance of myofibroblast[16] and increased subepithelial light scattering has also been obtained from prior experiments.[6,19,20] Furthermore, greater changes in the corneal refractive index occur in the epithelial surface.[25] Alterations in the regularity of the epithelial surface during wound healing response might, thus, lead to a decrease in corneal transparency.

The aim of this study is to determine the relationship among quantitative measurements of corneal light transmission, direct transmittance (DT), corneal light scattering, and epithelial surface properties, such as roughness and thickness, during the wound healing process after various refractive surgery techniques: photorefractive keratectomy (PRK), laser in situ keratomileusis (LASIK), and laser-assisted subepithelial keratomileusis (LASEK), as well as a group with only epithelial corneal removal (deepithelialization), because each group has different intensity and location of wound healing response.

METHODS

Animals, Surgery, and Deepithelialization

Twenty-one eyes of Iber Braun adult hens, *Gallus gallus domesticus* (weight, 2 kg) were used in the study. The animals were cared for following the guidelines of the Association for Research and Vision in Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Refractive surgery procedures (PRK, LASIK, and LASEK) were performed using a Summit Apex Plus excimer laser (Summit Technology, Waltham, MA). All the hens were operated on with the same parameters, each eye being ablated using a 6.00-mm diameter optical zone with no transition zone and receiving −6.00 diopters (D) of ablation with a programmed depth of 68 μm. In previous experiments, the hen was used by our group as an animal model to study the wound healing response after PRK[3,26] and LASIK.[27] Topical administration of 50 μl of recombinant nerve growth factor (NGF) was applied three times a day for a week (stock solution of 200 μg in 1 ml of balanced saline solution, personal gift of A. Lambiase) to a group of three hens operated on using PRK (PRK-NGF). In LASEK surgery, the epithelium was treated with alcohol (20% ethanol in balanced saline solution) for 40 s, using a 7.5-mm trephine as reservoir, to detach it from the underlying tissue. The epithelium was detached using the "in-the-bag" technique, and the exposed tissue was treated with the excimer laser, the epithelium finally being placed back over the cornea. Epithelial corneal removal (deepithelialization) was performed with a small scalpel. A group of untouched corneas was used as control.

For surgery and deepithelialization, the hens were anesthetized with an intramuscular injection of ketamine hydrochloride (37.5 mg/kg; Ketolar, Parke-Davis S.A., Barcelona, Spain) and xylazine hydrochloride (5 mg/kg; Rompun, Bayer AG, Leverkusen, Germany) followed by topical application of 0.5% tetracaine chlorohydrate and 1 mg of oxybuprocaine (Colircusí Anestésico Doble, Alcon cusí, S.A., Barcelona, Spain). At 30 days, the animals were killed by an overdose of pentobarbital (Dolethal; Vetoquinol, Madrid, Spain) when the animal was under general anesthesia.

Hens were divided into six groups [control (n = 3), deepithelialization (n = 4), PRK (n = 3), PRK-NGF (n = 3), LASIK (n = 4), and LASEK (n = 4)] to study DT, light scattering, and epithelial surface properties.

Biophysical Measurements: Corneal Light Transmission

Measurements of light transmittance and light scattering were taken immediately after killing as corneas needed to be excised for scattering and transmittance measurements. The excised cornea was placed in the cornea holder filled with a liquid maintenance medium, ringer lactate (Frenesius Kabi España, S.A., Barcelona, Spain), at a constant temperature and continuous flow. Data processing of the scatterometer provided the values of light transmission across the cornea, that is, transmittance in the forward direction (DT) and the half-width of the angular distribution of the scattered light for intensities corresponding to 1/100 and 1/1000 of the maximum intensity [half-width of the angular distribution for intensity corresponding to 1/100 (SA 1/100) and half-width of the angular distribution for intensity corresponding to 1/1000 (SA 1/1000)]. Further information concerning the experimental scatterometter, its calibration, and the ex vivo scattering measurements was presented in a previous study.[28] Immediately after the corneal light transmission measurements, the corneas were fixed with buffered formalin 10% to perform the histological analysis and the epithelial measurements.

Tissue Processing and Light Microscopy

All the corneas were fixed with buffered 10% formalin for 24 h, then washed in buffer phosphate 0.1 M, and embedded in paraffin wax. In an effort to avoid differences in shrinkage and dehydration for a better comparison among samples, all the corneas were processed with the same times.

Sections were stained with hematoxylin-eosin stain. The sections were examined under an Axiophot light microscope (Zeiss, Oberkochen, Germany), and microphotographs were obtained with a SPOT Digital Camera (Diagnostic Instruments, Sterling Heights, MI) taken at 200× magnification.

Measurements of the Epithelial Surface

Quantitative and systematic measurements of external and internal roughness and thickness of the epithelium were assessed using standard routines developed by our group for quantitative analysis of microphotographs of the corneal epithelium written in Matlab (Mathworks, Nattick, MA). Analysis routines are available on request.

The program works on color images of the cornea (Fig. 1A, center). We studied various parameters that may define the color and intensity of each pixel (e.g., red, green, blue content used for the red-green-blue representation of color or hue, saturation, or value of the hsv color representation). We found that saturation best discriminates the epithelium from the stroma for photographs

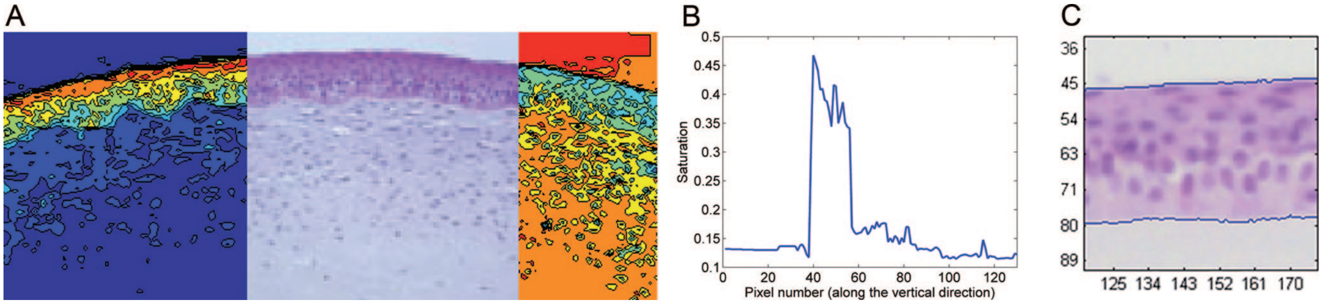


FIGURE 1.

(A) Photograph of corneal epithelium, showing different parameters of the image. Left: False color image showing the saturation value of each pixel. Center: True color image. Right: False color image showing the red level of each pixel. (B) Saturation value throughout a vertical line in the center of a corneal image. The region of higher saturation is the epithelium. The algorithm uses both this high value and the high gradients in the borders to determine epithelium edges. (C) Example of a control cornea measurement, showing the detection of epithelial borders (dark lines). A color version of this figure is available online at www.optvissci.com.

(Fig. 1A, right). Thus, the epithelium has a higher saturation than the stroma and the background, with a sharp change in transition (Fig. 1B). Therefore, high values of saturation characterize the epithelium well, whereas high gradients of saturation (high contrast) characterize the epithelium edge. We used these two criteria to build an algorithm to detect the epithelial edge. In all cases, we visually checked the result and corrected wherever necessary until the reconstructed edge matched that identified by visual inspection of the color image to within one pixel.

Once the edges were correctly detected, three fits were performed: anterior edge, posterior edge, and average. A conic curve was fitted to each edge, and roughness was estimated from the deviations between the real edge and the fitted curve. Average epithelium thickness and standard deviation were also computed. Thickness was measured perpendicular to the conic curve that best fits both epithelial edges.

Statistics

Principal component analysis (PCA) is a way of reducing the dimensionality of the data space by retaining most of the information in terms of variance. PCA was applied to summarize the information in epithelial roughness and corneal light transmission (DT and corneal light scattering). The variables related to epithelial roughness and corneal light transmission were included after a logarithmic transformation. We will use these "PCA releases" of the measured variables. This will allow us to analyze the relation among these characteristics and study their differences along the treatment groups, thus avoiding redundant information.

Differences between treatments were examined using t-test with Bonferroni multiple comparisons correction. In this analysis, we used three groups: control, LASIK, and surface epithelial procedures (deepithelialization, PRK-NGF, PRK, and LASEK). Pearson's correlation coefficients were calculated to examine the relationship between measures.

RESULTS

Principal Component Analysis

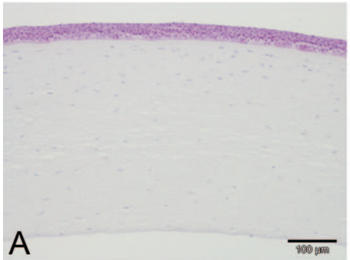
PCA was applied to summarize the information in each group of variables, epithelial roughness [external and internal roughness (ER and IR)], DT (DT for red and green wavelength), and corneal light scattering (SA 1/100 and SA 1/1000 red and green). The high correlation between the variables included in each PCA is reflected by the high percentage of variability accounted for by the first principal component: 90, 97, and 93%, respectively. The newly obtained variables in each PCA basically correspond to "size variables," as shown by their loadings: 0.65 for IR and 0.76 for external roughness; 0.62 for DT red and 0.76 for DT green; 0.44 for SA 1/100 red, 0.55 for SA 1/100 green, 0.48 for SA 1/1000 red, and 0.51 for SA 1/1000 green. These new variables, thus, capture a large percentage of all the information and can be interpreted as an average of the original variables. We use these "first PCA" variables in the following analysis as a measurement of epithelial roughness, DT, and corneal light scattering in the subjects rather than the original ones.

Corneal Light Transmission: DT and Light Scattering

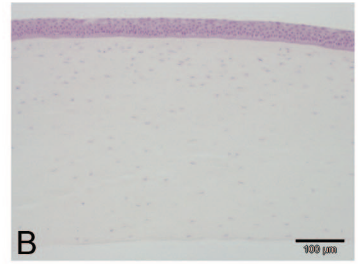
Quantitative data from the corneal light transmission measurements in the analyzed groups are showed in Fig. 2 (DT, SA 1/100, and SA 1/1000). The DT and corneal light scattering showed similar magnitude in the control and in the LASIK groups ($p = 0.42$ and $p = 0.64$, respectively). The DT was higher in the control and LASIK groups than in the surface epithelial procedures ($p < 0.05$).

Roughness and Thickness of the Epithelial Surface

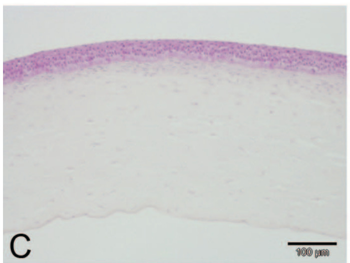
Fig. 2 presents examples of the microphotographs from the six corneal groups, and it shows quantitative analysis of the corneal microphotographs [epithelial thickness (ET), ER, and IR]. The epithelial surface is apparently not uniform in the PRK-NGF, PRK, and LASEK groups. This analysis generally evidenced a similar level of epithelial roughness and ET to the control and LASIK groups. By contrast, an increase in epithelial roughness was apparent in those procedures with epithelial surface alteration in comparison with the control and LASIK groups ($p < 0.05$, respectively). When analyzing ET, a larger increase was observed in the epithelial surface procedures in comparison with the control and LASIK groups ($p < 0.05$).



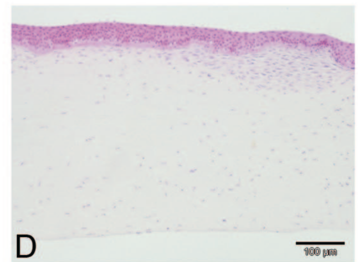
CONTROL		
ET	33.78 ± 2.24	
ER	1.50 ± 0.52	
IR	1.63 ± 0.78	
DT	R	0.789 ± 0.057
	G	0.703 ± 0.099
SA1/100	R	1.142 ± 0.094
	G	1.254 ± 0.160
SA 1/1000	R	3.018 ± 1.005
	G	3.749 ± 1.351



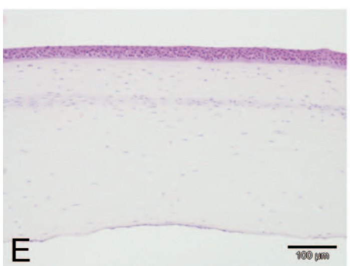
DEEPITHELIALIZATION		
ET	40.04 ± 10.39	
ER	2.89 ± 2.90	
IR	2.88 ± 3.23	
DT	R	0.543 ± 0.187
	G	0.495 ± 0.189
SA1/100	R	2.193 ± 1.075
	G	2.466 ± 1.202
SA 1/1000	R	5.695 ± 3.124
	G	6.898 ± 2.874



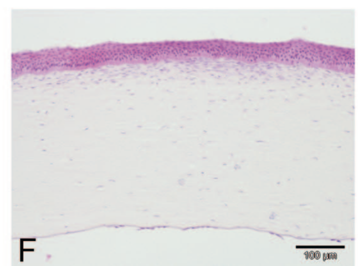
PRK-NGF		
ET	47.77 ± 7.30	
ER	2.35 ± 1.04	
IR	4.67 ± 2.06	
DT	R	0.582 ± 0.131
	G	0.618 ± 0.216
SA1/100	R	1.582 ± 0.282
	G	1.649 ± 0.286
SA 1/1000	R	4.753 ± 0.927
	G	5.233 ± 0.601



PRK		
ET	40.04 ± 9.18	
ER	3.83 ± 2.17	
IR	7.21 ± 2.05	
DT	R	0.599 ± 0.078
	G	0.492 ± 0.067
SA1/100	R	1.572 ± 0.061
	G	1.713 ± 0.057
SA 1/1000	R	4.514 ± 0.233
	G	4.969 ± 0.209



LASIK		
ET	32.29 ± 3.66	
ER	1.31 ± 0.67	
IR	2.29 ± 1.24	
DT	R	0.795 ± 0.061
	G	0.750 ± 0.072
SA1/100	R	1.157 ± 0.092
	G	1.401 ± 0.226
SA 1/1000	R	3.732 ± 1.287
	G	4.696 ± 1.311



LASEK		
ET	40.41 ± 3.70	
ER	3.67 ± 2.30	
IR	5.57 ± 2.21	
DT	R	0.452 ± 0.088
	G	0.274 ± 0.060
SA1/100	R	1.497 ± 0.389
	G	1.702 ± 0.651
SA 1/1000	R	4.424 ± 1.928
	G	5.324 ± 2.732

FIGURE 2.

Cross sections of healing hen corneas stained with hematoxylin-eosin stain after various refractive surgery procedures and deepithelialization at 30 days. (A) Control; (B) Deepithelialization; (C) PRK-NGF; (D) PRK; (E) LASIK; (F) LASEK. Magnification ×200. The figure shows quantitative data from epithelial measurements and corneal light transmission. Epithelial measurements: ET, ER, and IR. Corneal light transmission: DT, SA 1/100, and SA 1/1000. R, red wavelength, 632.8 nm; G, green wavelength, 543.5 nm. A color version of this figure is available online at www.optvissci.com.

Correlation Between Epithelial Surface and Corneal Light Transmission

Taking into consideration data from all the eyes, a negative correlation between the epithelial roughness and DT was found ($r = -0.48$, $p = 0.02$). We also observed that DT was correlated to corneal light scattering ($r = -0.64$, $p < 0.01$). These correlations appear to be an "average effect" between the high correlations observed in the control and PRK groups and the moderate correlation observed in the remaining groups. In the control group, the correlation among epithelial roughness (e.g., ER), DT, and corneal light scattering (SA) was, in absolute terms, above 0.99 (ER-DT, $r = -0.99$, $p < 0.05$; ER-SA, $r = -0.99$, $p = 0.05$; DT-SA, $r = 0.99$, $p = 0.05$). In the PRK-NGF, correlation among the variables ER, DT, and SA was, in absolute terms, above 0.95. In the PRK group, a high correlation value was found between ER and DT ($r = -0.99$, $p < 0.05$). In deepithelialization, moderate correlations were observed in the LASIK and LASEK groups, with these values not proving statistically significant. There was no relation between the ET and the corneal light transmission measurements.

DISCUSSION

In this study, quantitative histologic measurements were analyzed to determine whether objective differences exist in roughness of the epithelial surfaces and ET after wound healing in diverse refractive surgery procedures and deepithelialization and whether there is any relationship between these measurements and corneal light transmission.

Previous studies have explored the link between the epithelium and corneal transparency, using rabbits as the most suitable animal model.[4,6,19,20] Nonetheless, in this study, we chose the hen as the experimental model because, histologically, its cornea resembles human cornea in size and structure, it is thinner centrally and thickens toward the periphery. In addition, the proportions of its layers are very similar.[29] Furthermore, in the central cornea, our analyzed area, the fibrillar collagen arrangement is similar to human cornea.[30,31] This experiment was performed based on measurements made 30 days after the treatments. This time point was selected because at 30 days, epithelial organization has not yet been reestablished, corneal light transmission has not yet recovered, and the haze peak tends to occur at ~30 days.[2–4]

One challenge involved in the study of complications arising during wound healing response is to obtain quantitative measurements from histologic observations, particularly measurements of the epithelium-stromal interface. However, the algorithm presented solves this problem, extracting quantitative and systematic information concerning certain histologic parameters from corneal microphotographs, in particular, ET and its uniformity over the whole area photographed, as well as roughness of both external and internal surfaces.

In this study, we propose using the first PCA variables as a measurement of epithelial roughness, DT, and corneal light scattering rather than the original ones. In each analysis, this first PCA appeared as a "size" variable, highly correlated with the mean of the original variables and able to account for most of the information in the data.

Several treatments with different wound healing response and an untreated control group were analyzed to characterize the epithelial surface and light transmission across the cornea. Various authors ascribe subepithelial light scattering to irregularities in the epithelium and to subepithelial structures.[4,5,21,22] McCally et al.[6] obtained objective corneal light scattering measurements and observed greater irregularities in the basement membrane in the high-scattering group. In addition, it has been suggested that the main cause of scattering in the postablation cornea is the subepithelial region, this area being characterized by irregularities.[19,20] However, none of these suggestions have related quantitative measurements of epithelial roughness to objective measurements of corneal light transmission. Roughness is associated with epithelial-stromal interactions. The corneal wound healing response that occurs after PRK or LASEK is usually more intense than after LASIK for the same correction.[23] Quantitative measurements of epithelial properties after PRK and LASEK showed a higher ET and a greater roughness in comparison with control and LASIK groups. The hypothesis that the roughness of the epithelial surface is related to corneal light transmission is supported by the correlation of epithelial roughness with DT in the control, PRK, and PRK-NGF groups.

It has been evidenced that corneal scattering was significantly lower after LASIK than after PRK[32] and that there was no significant increase in forward light scatter at 1 month after PRK compared with a control group.[33] Although light scattering in the epithelial surface procedures appears to be higher in comparison with LASIK or control, the differences observed in this experiment were not statistically significant. The role of ET and its link to light transmission was also explored; no significant relation being found.

To sum up, measurements performed in this study have shown that roughness in the epithelial surface is related to corneal light transmission, particularly for the high correlations observed in the control and PRK groups. However, further studies using a larger sample size are needed to measure all the correlations more accurately and, in general, to gain a deeper insight into the basis of corneal transparency during wound healing.

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