Effects of Eyelid Closure and Disposable and Silicone Hydrogel Extended Contact Lens Wear on Rabbit Corneal Epithelial Proliferation

Patrick M. Ladage, David H. Ren, W. Matthew Petroll, James V. Jester, Jan P. G. Bergmanson, and H. Dwight Cavanagh

PURPOSE. To examine the rabbit corneal epithelial cell proliferation rate after extended wear of disposable or silicone hydrogel contact lenses or prolonged eyelid closure.

METHODS. One randomly chosen eye of 40 New Zealand White rabbits was assigned to silicone hydrogel contact lens wear \((n = 15, \text{SH})\), disposable hydrogel contact lens wear \((n = 6, \text{DH})\), eyelid suturing \((n = 15, \text{SUT})\), or no intervention \((n = 4)\). Contralateral eyes served as the control. After 24 hours or 1 week of lens wear, 5-bromo-2-deoxyuridine (BrdU) was injected intravenously to label dividing corneal epithelial cells, and animals were killed 24 hours after injection. Corneas were stained with monoclonal anti-BrdU antibody and FITC-conjugated secondary antibody. A series of continuous digital images of the whole-mounted epithelium were collected from the superior to inferior limbus, and the number of BrdU-labeled cell pairs was counted.

RESULTS. SH, DH, and SUT caused a significant decrease in BrdU-labeled pairs of cells over the entire corneal epithelium at day 2 compared with the number in contralateral control eyes \((P < 0.001)\). One week of SUT or SH caused a significant increase centrally in BrdU-labeled cells \((P < 0.01)\). BrdU labeling at the limbus in all groups was not significantly different from the control. Unexpectedly, the proliferation rate of the control corneas was also significantly affected by contralateral lens wear and suturing.

CONCLUSIONS. Short-term overnight SH, DH, and SUT all significantly suppressed the cell proliferation rate in the rabbit corneal epithelium. However, adaptation, with central hyperproliferation of cells, appeared to occur at 8 days. The effects of lens wear and eyelid suturing on the cell proliferation rate in contralateral control eyes suggests a central mechanism that regulates corneal epithelial proliferation.

One of the main functions of the corneal epithelium is to protect the cornea and inner ocular components against mechanical damage and the invasion of virulent microorganisms. As a self-sustained renewable tissue, the corneal epithelium balances epithelial cell division (proliferation), movement (centripetal and vertical migration), and surface cell death (exfoliation) to maintain constant thickness and overall integrity. In the normal cornea, proliferation exclusively occurs in the basal cell layer of the corneal epithelium and limbus. Each basal epithelial cell may undergo a few rounds of cell division before becoming postmitotic. One cell is terminally differentiated, it moves up toward the corneal surface, undergoes apoptosis, and exfoliates into the tear film. All self-renewing tissues such as the corneal epithelium harbor stem cells, which are vital for long-term maintenance of homeostasis. Stem cells characteristically are slow-cycling cells that retain an unconstrained capacity to produce daughter cells with a life span often as long as the expected life of the host. There is accumulating evidence that the stem cells of the corneal epithelium are solely located in the basal epithelial cell layer of the limbus.

We hypothesize that a disturbance of the delicate epithelial homeostatic balance during contact lens wear may lead to a diminished effectiveness of the corneal epithelial barrier against infection. Contact lens wear is known to alter both surface cell exfoliation and cell proliferation in the corneal and limbal epithelia. Several clinical human studies have reported a decrease in the number of exfoliating surface epithelial cells during both daily and overnight lens wear, even though conventional disposable lens wear appears to increase the size of exfoliated epithelial cells and silicone hydrogel lens wear may not. Furthermore, using ethidium homodimer staining, Yamamoto et al. showed that both soft and rigid gas-permeable (RGP) contact lens wear significantly reduces the total number of dead and dying cells on the epithelial surface of the central cornea compared with non–lens-wearing control corneas, but a notable finding was that there is no significant difference in the number of exfoliating cells between low- and high-oxygen-transmissible lens materials. Similar results were encountered with apoptotic surface markers such as TUNEL labeling and annexin V staining. Wear of all types of lenses reduces the number of central apoptotic surface epithelial cells significantly. In addition to decreasing normal corneal epithelial surface cell exfoliation, contact lens wear also decreases basal epithelial cell proliferation. Hamano and Hori were the first to show decreases in mitotic figures of up to 90% in the central and midperipheral epithelium of the rabbit cornea after 2 days of low-O2 soft overnight lens wear. Moreover, demonstrated a 81% and 22% decrease in the cell proliferation rate in the central corneal epithelium after 2 days of low- and hyper-O2 rigid gas-permeable (RGP) lens wear, respectively, whereas at the limbus a concomitant unexpected but significant increase in the proliferation rate was recorded with both types of RGP lenses. A follow-up study confirmed these results and demonstrated signs of adaptation.

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over time during hyper-O₂ RGP lens wear, but not with low-O₂ RGP lens wear. 23

The introduction of silicone hydrogel lenses, with oxygen transmission levels far exceeding currently available hydrogel lenses, offers great clinical promise with significantly less hypoxia-mediated changes, such as corneal swelling, limbal hyperemia, and neovascularization. 24–26 Furthermore, it has been suggested that this new generation of contact lens materials may lead to a decrease in the incidence of lens-associated microbial keratitis. 27–29 However, it has not been established thus far what effect silicone hydrogel lens wear has on the homeostasis of the corneal epithelium.

Prolonged eyelid closure mimics the physiological hypoxic effects of low-oxygen-transmissible contact lens wear. It causes an acid shift in pH, 26 swelling of the corneal stroma, 29 a decrease in glycogen reserves, and a decrease in corneal sensitivity 30 and eventually may lead to serious complications in the corneal epithelium. 31 With normal eyelid closure during sleep, the corneal epithelium principally receives its oxygen from the blood vessels of the palpebral conjunctiva and the aqueous of the anterior chamber. As a consequence, available oxygen levels drop from an atmospheric oxygen percentage of 21% (open eye) at sea level to approximately 7.7% (closed eye). 32 The effects of chronic hypoxia during prolonged eyelid closure on the corneal epithelium, however, have yet to be studied at the cellular level. Eyelid closure provides an excellent model to study the effects of hypoxia with no associated eyelid blinking forces on the corneal epithelium or the mechanical presence of a contact lens.

The purpose of this study was to assess the proliferation rate of the rabbit corneal epithelium after 2 and 8 days of overnight extended wear of silicone hydrogel or disposable hydrogel lenses, with eyelid suturing without lenses serving as a non-lens-wearing hypoxic control.

**METHODS**

The 40 New Zealand White rabbits used in these experiments were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were screened for ocular disease with a handheld biomicroscope before experimental procedures. The rabbits were housed in individual cages at a room temperature of 19°C to 23°C, under relative humidity of 30% to 50% and maintained in a constant 12-hour light–dark cycle. Food and water were provided ad libitum. Forty rabbits were assigned to six groups: (1) 2 days of silicone hydrogel lens wear (n = 8), (2) 8 days of silicone hydrogel lens wear (n = 7), (3) 2 days of disposable hydrogel lens wear (n = 6), (4) 2 days with eyelid sutured (n = 8), (5) 8 days with eyelid sutured (n = 7), and (6) no experimental treatment in either eye (unaltered control group; n = 4). One eye of each rabbit was randomly chosen to be the experimental eye, with the other eye serving as the control. Group 6 was added as an additional control for possible sympathetic reactions in the contralateral control eyes after the manipulation of the experimental eyes (contact lens wear or suturing). It was not mechanically feasible to maintain the disposable hydrogel lens in the cornea continuously for periods longer than 2 to 5 days.

**Contact Lens Fitting**

Table 1 shows the test contact lenses used in this study. Soft contact lenses easily dehydrate on the rabbit cornea because of the rabbit’s low blinking rate 24 and are more frequently lost than rigid contact lenses. Without intervention, the test lens (etafitcon A) often became so dry that the shape of the lens altered on the corneal surface, causing it to be dislodged easily by blinking. Preservative-free rewetting drops (Refresh Plus; Allergan, Irvine, CA) were therefore routinely administered every 2 hours during the day to prevent lens dehydration. Initially, pilot experiments in this study were performed before and after nictitating membranectomy. The success rate of soft lens retention was higher when the nictitating membrane retained intact. The nictitating membrane was therefore preserved. In a previously published study, 25 the presence or absence of the nictitating membrane has been shown not to affect corneal epithelial cell proliferation.

**Eyelid Suturing**

Before suturing, the rabbits were anesthetized with 30 to 50 mg/kg ketamine (Ketaset; Fort Dodge, Fort Dodge, IA) and 3 to 5 mg/kg xylazine (Rompun; Bayer, Shawnee Mission, KS). A 4.0 black braided silk thread (Ethicon Inc., Somerville, NJ) was used to suture the upper and lower eyelids with two square patterns. The needle penetrated the eyelid of the inferior eyelid approximately 4 to 6 mm under the cilia, passed through the orbicularis muscle, and emerged at the mucocutaneous junction (tarsal glands). Thereafter, the needle and thread penetrated the mucocutaneous junction of the superior eyelid all the way through to the surface skin 4 to 6 mm above. Approximately 2 mm toward the middle, the same steps were repeated to complete the square, but from superior to inferior eyelid. Two squares were made in each eye, to ensure full eyelid closure.

**Experimental Design**

One day (groups 1, 3, and 4) or 7 days (groups 2 and 5) after lens fitting and eyelid suturing at 9 AM, anesthetized rabbits were injected intravenously with 5-bromo-2-deoxyuridine (BrdU; 200 mg/kg) in phosphate-buffered saline (PBS; 50.5 mg/mL), to detect proliferating cells. Animals were humanely killed at 9 AM the next day with pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) to ensure enough time for cells to complete the mitotic phase after taking up BrdU, in the 8 phase of the cell cycle. The cell proliferation rate in the corneal epithelium is affected by a circadian rhythm, with the lowest rate in the late afternoon and the highest in the early morning in the rabbit. Therefore, all steps in this study (lens fitting and eyelid suturing, BrdU injection, and death) were performed at 9 AM to control for the effects of circadian rhythm. 3,35 The time points of BrdU injection (days 1 and 7) were chosen to reflect the most common clinical wearing

<table>
<thead>
<tr>
<th>Lens Type*</th>
<th>Material Name</th>
<th>Dk/t†</th>
<th>EOP‡</th>
<th>Thickness§ (mm)</th>
<th>Water Content</th>
<th>Base Curve</th>
<th>Diameter (mm)</th>
</tr>
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<tbody>
<tr>
<td>High Dk/t soft</td>
<td>Etafitcon A</td>
<td>27</td>
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<td>58%</td>
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<tr>
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<td>19.9</td>
<td>0.09</td>
<td>36%</td>
<td>8.6</td>
<td>14.00</td>
</tr>
<tr>
<td>Eyelid suturing</td>
<td>NA</td>
<td>7.8</td>
<td>7.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Classification according to Benjamin WJ. 33
† Dk/t is oxygen transmissibility measured in saline at 35°C by the polarographic method/edge effect correction (International Standard Organization 9913). 10 10 (cm/sec)(mL O₂/mL mm Hg).
‡ EOP is equivalent oxygen percentage (21% is normal at sea level).
§ Thickness at ~3.00 D for the soft contact lenses.
¶ Surevue lens, Vistakon Inc., Jacksonville, FL.
Epithelial Proliferation after Contact Lens Wear or Eyelid Closure

**RESULTS**

**Normal Eyes**

Figure 1 shows the cumulative data from the non-lens-wearing corneas (n = 62, including pooled data from a previous RGP study).23 The superior and inferior limbal regions contained the least number of BrdU-labeled cells. The immediately adjacent peripheral superior corneal epithelium showed the highest degree of Brdu labeling. A montage of BrdU-labeled sections illustrates the BrdU-labeling profile in a normal cornea from superior to inferior limbus (Fig. 2).

**High-Dk/t Disposable Hydrogel Soft Contact Lens**

One day after Brdu labeling (2 days of disposable lens wear), an overall significant difference was recorded in BrdU-labeling between lens-wearing and control corneas (P < 0.001; two-way ANOVA; Fig. 3). The cell proliferation rate in the central cornea was suppressed by 40.8% (P = 0.007, power 0.87; paired t-test). It should be noted that one fixed limitation of this method was that the high-Dk/t soft lens tended to move downward on the rabbit cornea. Thus, the superior limbus and 1 to 2 mm of the superior corneal epithelium were not consistently covered by the lens and were intermittently exposed to atmospheric oxygen. The central cornea, however, was consistently covered by the test lens.

**Eyelid Closure with Suturing**

The response of the eye with eyelid closure (hypoxia, no eyelid blinking forces, no contact lens) was significantly different from the open-eye control (P < 0.001; two-way ANOVA; Fig. 6). There were 47% fewer BrdU-labeled cells in the central epithelium of the eyelid-sutured eyes than in control eyes (P = 0.014, power 0.68, paired t-test). Labeling at the limbus was not affected by eyelid closure. At 8 days, a dramatic and significant increase in BrdU labeling was present in the central cornea (+62%; P = 0.007; two-way ANOVA; Fig. 7).

**DISCUSSION**

Recently, the U.S. Food Drug Administration has approved two novel types of silicone hydrogel lenses for continuous use of up to 30 nights. Several clinical studies have shown a virtual elimination of hypoxia-associated contact lens effects on the ocular surface physiology, which has led to the prediction that corneal infection rates with these hyper-oxygen-transmissible hydrogel lenses will decline.16 Our understanding of the effects of silicone hydrogel lenses on corneal epithelial homeostasis is currently limited. This is the first study to investigate the corneal epithelial cell proliferation rate in the rabbit cornea after short-term silicone hydrogel or conventional disposable hydrogel lens wear or prolonged eyelid clo-

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Corneas were fixed in situ in PBS with 1% paraformaldehyde for 3 minutes, and tissue in a vertical stripe extending from the superior to inferior limbus was then cut from the cornea, with the superior muscle used as a reference point. Subsequently, the tissues were processed through a series of staining and washes: washed 3 minutes in PBS with 1% Triton X-100 and 1% dimethyl sulfoxide (DMSO; TD buffer), placed in acetone (−20°C) for 3 minutes, washed in TD buffer for 3 minutes, placed in 0.3 N HCl for 3 minutes, washed in TD buffer for 3 minutes, incubated in whole goat serum 1:10 for 30 minutes at 37°C, and stained overnight in monochromic anti-BrdU antibody (1:30) and washing buffer (Roche Molecular Biochemicals, Indianapolis, IN) at room temperature with agitation (100 turns per minute). The second day, the tissues were washed with TD buffer three times for 30 minutes and placed in FITC-conjugated goat anti-mouse secondary antibody (1:20; ICN, Costa Mesa, CA) overnight at room temperature with agitation (100 turns per minute). On the final day, the tissues were washed three times for 30 minutes each in TD buffer.

**Immunocytochemistry**

Whole corneas were mounted epithelium side up on a glass slide and assessed by epifluorescence microscopy. Sequential, digital fluorescence microscopic images (410 μm vertically by 884 μm horizontally) were collected from the superior limbus to central cornea and also from the inferior limbus to the central cornea. The limbus was clearly recognized as an area of low BrdU labeling compared with the adjacent peripheral corneal epithelium and the goblet-cell–containing conjunctival epithelium. The number of nuclei appearing as pairs was counted per image (0.36 mm²) using the Image Tool program developed at the University of Texas Health Science Center (UTHSCSA; San Antonio, TX; available at http://ddsdx.uthscsa.edu/dig/itdesc.html).

**Fluorescence Microscopy and Digitizing**

Whole corneas were mounted epithelium side up on a glass slide and assessed by epifluorescence microscopy. Sequential, digital fluorescence microscopic images (410 μm vertically by 884 μm horizontally) were collected from the superior limbus to central cornea and also from the inferior limbus to the central cornea. The limbus was clearly recognized as an area of low BrdU labeling compared with the adjacent peripheral corneal epithelium and the goblet-cell–containing conjunctival epithelium. The number of nuclei appearing as pairs was counted per image (0.36 mm²) using the Image Tool program developed at the University of Texas Health Science Center (UTHSCSA; San Antonio, TX; available at http://ddsdx.uthscsa.edu/dig/itdesc.html).

**Statistics**

Statistical analysis was performed on computer (Sigmastat 2.03; SPSS, Inc., Chicago, IL). Two-way analysis of variance with factor location and group and the paired t-test were used, with statistical significance set at P < 0.05.
sure. A significant decrease in epithelial basal cell proliferation (−33.8% centrally) was observed in the silicone hydrogel lens group after 48 hours. Both disposable lens wear and eyelid suturing caused even greater suppression in the central corneal epithelium, with respectively −40.8% and −47% decreases in BrdU labeling. Hamano and Hori21 have reported that 48 hours of continuous contact lens wear with very-low-oxygen-transmissible 2-hydroxyethyl methacrylate (HEMA) soft lenses severely decreases the total number of mitotic figures in the central corneal epithelium (by as much as −94%). More recently, we have shown with the same technique as used in this study that 48 hours of RGP lens wear in the rabbit cornea decreased the epithelial proliferation rate by −57% (Dk/t = 97) and −80% (Dk/t = 10).23 Thus clearly, all contact lens wear, regardless of lens type (rigid versus soft) or oxygen transmissibility, reduced the proliferation rate of the corneal epithelium to some degree during the first 48 hours of overnight lens wear. The extent of reduction appears to be partly dependent on the oxygen transmissibility of the lens material. Low-Dk/t lenses suppressed the proliferation rate the most. However, both hyper-oxygen-transmissible silicone hydrogel and RGP lenses have oxygen levels approaching that of the open eye at sea level; yet, they still suppressed the proliferation rate by approximately one third. Thus, it appears that in addition to oxygen availability, other factor(s) may have inhibitory effects on the proliferation rate of corneal epithelial basal cells. The decrease in proliferation could be a nonspecific tissue response to sudden changes in the environment.

The normal corneal epithelial thickness is very tightly regulated; therefore, the corneal epithelial cell proliferation rate may be driven partly by the demand for new epithelial cells. If fewer epithelial cells exfoliate from the corneal epithelial surface, a coupled decrease in basal cell proliferation, to maintain normal corneal epithelial thickness, may result. During contact lens wear, the overall normal apoptotic surface exfoliation rate of the corneal epithelium in humans and rabbits is clearly suppressed.13,15,16,18–20 It is essential to note that oxygen availability under the lens does not seem to be the predominant driving factor in surface cell exfoliation. All contact lenses suppress the exfoliation rate to a similar degree. Thus, the decreases in proliferation rate in the corneal epithelium during contact lens wear may be caused partly by a decrease in epithelial surface cell exfoliation. Hypoxia during wear of lower-oxygen-transmissible contact lenses may further amplify this suppression by interfering with cellular metabolic pathways. In further support of the hypothesis that the proliferation and exfoliation rates are coupled, we recently have observed in the rabbit cornea that an increase in focal localized epithelial cell loss on the corneal surface leads to a localized increase in the proliferation rate of basal epithelial cells immediately beneath it.

FIGURE 2. Compilation picture showing the distribution of BrdU-labeled cells from the superior conjunctival to central epithelium in a normal rabbit cornea 24 hours after BrdU injection.

FIGURE 3. Proliferation rate after 2 days of high-O2 soft lens wear. It should be noted that superiorly the contact lens did not cover the cornea because of decentration problems. Again, centrally there was a significant decrease in cell proliferation. SL, superior limbus; IL, inferior limbus.

FIGURE 4. Proliferation rate after 2 days of hyper-O2 soft lens wear. SL, superior limbus; IL, inferior limbus.
whereas no reaction is seen in the surrounding epithelial cell layer tissue.³⁸

After 1 week of silicone hydrogel lens wear, a significant burst of proliferating cells was detected in the central and superior peripheral epithelium. A similar pattern emerged after 1 week of prolonged eyelid closure. There was a significant increase in BrdU-labeled cells in the central epithelium, but the superior and inferior peripheral epithelium showed suppressed labeling. Clearly, the initial response of the corneal epithelium to the contact lens or eyelid suturing the first 2 days did not persist, and a noticeable adaptive reaction of "proliferative recovery" occurred. The important question remains: whether this is caused by a delay of cells entering the cell cycle or whether the corneal epithelium is physiologically adapting to the new condition. Unfortunately, we were not able to determine whether this recovery also occurred in the lower-oxygen-transmissible disposable soft contact lens group at 8 days, because it was not possible for the rabbit to retain the lens continuously for an extended period. Future studies are needed to determine the long-term effects (more than several months) of contact lens wear or eyelid suturing on the corneal epithelial cell proliferation rate. Does the proliferation rate eventually decrease and stabilize at prelens baseline levels or remain lower than normal?

The stem cells of the corneal epithelium are thought to reside exclusively in the limbal epithelium.¹¹,¹² During short-term (2 days), continuous low- and hyper-Dk/t RGP lens wear, an unexpected increase in BrdU labeling was noted in the normally low proliferating limbal epithelium.²³ Similar to events observed during central wound healing, it was hypothesized that this limbal upregulation of cell division was required to compensate for the severe suppression of proliferation in the central epithelium. Alternatively, it was postulated that the mechanical rubbing of the RGP lens edge locally stimulates cell division, as the large diameter (14.00 mm) RGP rabbit test lenses were designed to cover the corneal epithelium from limbus to limbus.²³ At day 2 in the present study, no increases in BrdU labeling at the limbus were found with either soft lens or after eyelid suturing. Therefore, the upregulation of limbal epithelial cell proliferation that occurred during RGP lens wear at day 2 was most likely due to the mechanical rubbing of the lens edge at the surface, which indicates that, at least at day 2 in this study, the limbus did not directly respond to the decrease of in cell proliferation in the central corneal epithelium.

Unexpectedly, a statistically significant difference was found between the non-lens-wearing control eyes of the experimental groups, when cumulative pooled data from this and a previous study²³ were analyzed (P = 0.027; one-way ANOVA). The control eyes of the rabbits wearing the high-oxygen-transmissible lenses in one eye, noticeably contained more BrdU-labeled cells than did the control eyes of the low-oxygen-transmissible lenses (Fig. 8). Compared with the cell proliferation in corneas of control rabbits wearing no contact lens in either eye, the high-O₂ soft and RGP lenses showed increased proliferation in the contralateral control eyes whereas, by contrast, the low- and medium-O₂ lenses, showed a reverse effect. Taken together, the data indicate that the manipulation of one cornea with a contact lens affects the proliferation rate on the non-lens-wearing, contralateral cornea. Estil et al.³⁹ have reported a similar cross talk reaction during wound healing. Inducing a wound in one cornea caused an increase in cell proliferation in the contralateral control cornea. In addition, Fonn et al.²⁴ found that the corneal swelling rate was significantly different between the control eyes of patients wearing a high-oxygen-transmissible lens unilaterally than in the control subjects wearing the low-oxygen-transmissible lens. Thus, corneal epithelial homeostasis in both eyes

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933226/)  
**Figure 5.** Proliferation rate after 8 days of hyper-O₂ soft lens wear. SL, superior limbus; IL, inferior limbus.

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933226/)  
**Figure 6.** Proliferation rate after 2 days with eyelids sutured. SL, superior limbus; IL, inferior limbus.

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933226/)  
**Figure 7.** Proliferation rate after 8 days with eyelids sutured. SL, superior limbus; IL, inferior limbus.
appears to be coupled systemically through mechanism(s) that remain to be established.

References


