Long-Term Neural Regeneration in the Rabbit Following 180° Limbal Incision

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Penetrating 180° superior limbal incisions were made on the right eye of four adult New Zealand albino rabbits. The contralateral eye served as control. Corneal touch thresholds (CTT) for the central, superior and inferior cornea (2–3 mm from limbus) were determined 3, 9, 15, 24 and 30 months after surgery. In all animals, the CTT was significantly elevated in the superior region of the cornea throughout the measurement period. CTT was elevated in the central and inferior cornea 3 months following surgery and was not affected in the inferior cornea on all other occasions. The animals were then sacrificed and the corneas subjected to histochemical demonstration of acetylcholinesterase corneal nerves. All rabbits showed a reduction in the number of histochemically detectable stromal nerve trunks in the operated region. These stromal nerve trunks showed regenerative changes including abnormally curved course and a subnormal number of axons within a nerve trunk. Epithelial nerve fiber defects included absence or distorted architecture of the basal epithelial plexus and intra-epithelial terminals. These results indicate that although extensive stromal reinnervation had occurred, the extent and quality of stromal nerves was inadequate to restore a normal epithelial plexus and corneal sensitivity.

Materials and Methods. Limbal incision: Four adult New Zealand albino rabbits were subjected to a 180° penetrating perilimbal incision in the superior cornea of the right eye; the left eye acted as a control. An aspirin suppository was given 8 hr before surgery. Anaesthesia was induced by administering xylazine (6 mg/kg) and ketamine hydrochloride (33 mg/kg) intramuscularly. The eye was opened using a Beaver blade at 12 o'clock and the limbal incision was enlarged to 180° with curved corneal scissors. Porcine heparin (1000 units/ml in balanced salt solution) was instilled into the anterior chamber to prevent fibrin formation. The wound was closed with eight to ten interrupted 9-0 nylon sutures and air was injected into the anterior chamber to restore the normal chamber depth. Gentamycin sulphate was injected subconjunctivally and chloramphenicol was applied topically. Post-surgical recovery was uneventful and healing of the wound proceeded without infection or vascularization. These animal studies conformed to the ARVO Resolution on the Use of Animals in Research.

Corneal touch thresholds: Corneal touch threshold (CTT) was determined using a hand-held Cochet-Bonnet aesthesiometer (thread diameter = 0.13 mm) (Luneau Ophthalmologie, Paris, France). The aesthesiometer was calibrated at regular intervals over the duration of the study using an Ohaus precision reloading scale (Ohaus Scale Corporation, Florham Park, NJ). To obtain a measurement, the experimenter viewed the cornea and filament at close range from the side while the aesthesiometer was advanced at a steady speed with perpendicular corneal contact until flexure of the filament occurred. A blink reflex in over 50% of trials was recorded as a positive response. Corneal sensitivity was monitored by the same operator at 3, 9, 15, 24 and 30 months after surgery. On each occasion, superior, central and inferior corneas were assessed (2–3 mm from the limbus). The CTT of each region in the control and operated eyes was converted to corneal sensitivity by taking the inverse of CTT and multiplying by 1000.
Table 1. Corneal sensitivity of rabbits 3, 9, 15, 24 and 30 months following a 180° perilimbal incision

<table>
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<th>3</th>
<th>9</th>
<th>15</th>
<th>24</th>
<th>30</th>
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<tbody>
<tr>
<td>R Sup</td>
<td>0.26 ± 0.52*</td>
<td>0.00 ± 0.00</td>
<td>0.80 ± 0.53</td>
<td>0.21 ± 0.43</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>L Sup</td>
<td>3.13 ± 0.45</td>
<td>3.69 ± 1.05</td>
<td>3.01 ± 1.09</td>
<td>6.48 ± 4.81</td>
<td>1.97 ± 0.15</td>
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<tr>
<td>R Cent</td>
<td>2.64 ± 1.95</td>
<td>4.81 ± 5.75</td>
<td>2.06 ± 2.25</td>
<td>4.30 ± 3.22</td>
<td>4.77 ± 5.84</td>
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<tr>
<td>L Cent</td>
<td>5.46 ± 2.05</td>
<td>5.42 ± 2.83</td>
<td>5.33 ± 1.98</td>
<td>8.90 ± 5.28</td>
<td>5.33 ± 5.56</td>
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<tr>
<td>R Inf</td>
<td>1.31 ± 1.06</td>
<td>2.48 ± 0.66</td>
<td>2.01 ± 0.75</td>
<td>6.29 ± 5.57</td>
<td>1.65 ± 0.50</td>
</tr>
<tr>
<td>L Inf</td>
<td>3.81 ± 0.52</td>
<td>5.18 ± 3.11</td>
<td>4.07 ± 2.01</td>
<td>5.04 ± 2.75</td>
<td>2.41 ± 1.75</td>
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* Mean and standard error of corneal sensitivity (mg⁻¹ × 10⁵) within the group.

Where no blink response could be elicited by a thread length of 0.5 cm, zero corneal sensitivity was recorded.

Histochemical demonstration of acetylcholinesterase (AChE) activity: At 30 months following surgery, each animal was killed by intravenous injection of sodium pentobarbital. Corneas were excised with a small scleral rim using curved corneal scissors, and immersed in cold (4°C) 4% phosphate buffered (0.1M) formaldehyde solution for 2 hr. Following fixation, the samples were rinsed one to three times in the same buffer at 4°C. The tissue was stored in cold (4°C) phosphate buffered sucrose solution (15%). AChE activity was demonstrated with the Lewis-Shute modification of Koelle’s copper thiocholine technique.²¹⁰

The numbers of thick stromal nerve bundles in the superior and inferior halves of each cornea were determined by two observers (KT and TT) independently. Where the results differed, the estimation was repeated and averaged. Figure 1A shows the nerves which were counted as a stromal nerve trunk in the control eye of rabbit 1.

Results. Table 1 shows the mean corneal sensitivity 3, 9, 15, 24 and 30 months after surgery. Corneal sensitivity was significantly reduced in the superior cornea 2–3 mm from the operated limbus on all occasions (one-tailed student t-test). Thread lengths of 0.50 to 0.75 cm elicited a blink response occasionally, but not at the 50% level. Mean sensitivity in the center of the cornea was reduced slightly on every measurement occasion; however, this was not statistically significant. Sensitivity in the inferior region of the cornea when compared to the control eye was reduced at 3 months but not affected for the rest of the measurement period (two-tailed student t-test). Thirty months after 180° limbal incision, corneal sensitivity in the superior region of the cornea was still significantly depressed, although there appeared to be normal corneal sensitivity in the central and inferior regions of the cornea.

The number of stromal nerve trunks in the superior and inferior halves of each cornea were determined. Nine to thirteen stromal nerve bundles were observed in the control corneas of albino rabbits. No statistical difference was observed in the number of stromal nerve trunks in the inferior half of the cornea between control and operated eyes [5.75 ± 0.96 (mean ± SD) compared with 5.25 ± 0.50]. However, all operated corneas showed a reduction in the number of histochemically detectable stromal nerve bundles in the superior half of the cornea, 3.25 ± 1.71 compared with 5.50 ± 1.29 respectively; (P < 0.05, one-tailed student t-test).

It was evident from the whole-mount preparations that there was a considerable amount of variation in the extent of stromal regeneration between rabbits. Rabbit 2 displayed the least amount of neural regeneration within the group (Fig. 1B). Only one stromal nerve trunk was observed in the superior half of the operated eye compared with five in the superior region of the control eye. This rabbit displayed the greatest reduction in corneal sensitivity and developed persistent epithelial lesions a few months after surgery. Rabbit 4 (Fig. 1C) showed an average amount of stromal regeneration. Four stromal nerve trunks were observed in the operated sector compared with seven in the superior sector of the control eye. Rabbit 1 (Fig. 1D) displayed the greatest amount of regeneration within the group with five in the operated eye compared to six in the control eye.

Although numerous stromal nerve trunks had regenerated, they showed structural differences when compared to normal nerve trunks. These regenerated trunks, in the superior cornea, had an abnormally curved course when compared with the radial pattern taken by the normal stromal trunks entering the inferior cornea (Fig. 1C). In addition, the regenerated fibers (Fig. 2A) had more spaces between axon profiles, indicating lowered axon numbers when compared to a normal stromal nerve trunk of a similar diameter. Morphological changes include spaces between axon profiles and irregularity of caliber.

Figure 2B shows the distorted architecture of the peri-corneal, stromal and sub-epithelial plexus in the operated region, when compared with the normal plexus (Fig. 2C) in the inferior cornea of the same eye (Rabbit 3, RE). In the normal cornea the stromal
nerve trunks were composed of numerous axons, which branched off dichotomously from the main trunk in several divisions as it travelled anteriorly toward the center of the cornea. These collateral branches form the sub-epithelial plexus. In the operated cornea, the stromal fibers displayed a variation in caliber along their length, as well as distorted architecture at sites of branching (Fig. 2B, arrowheads).
Whereas the sub-epithelial plexus in a normal cornea travels anteriorly toward the stromal-epithelial interface to form the epithelial plexus, this plexus in the operated cornea travels in a disorganized fashion, wandering in depth as well as the direction of travel. The density of this plexus appeared markedly reduced.

The basal epithelial leashes in the operated regions had lost their orderly appearance, and were replaced by abnormal nerves near the stromal epithelial interface (Fig. 2D, arrowheads). Numerous single epithelial axons were also seen penetrating the scar region (Fig. 2E). They travelled for a limited distance in a straight path at the basal epithelial level, sometimes criss-crossing or forming irregular groupings of epithelial fibers.

**Discussion.** Examination of corneal reinnervation 30 months after a 180° limbal incision showed that normal innervation had not been fully reestablished in the superior cornea. Our evidence for this conclusion comes from measurements of corneal sensitivity and histochemical demonstration of corneal nerves. Corneal sensitivity within the superior half of the cornea was significantly reduced throughout the measurement period when compared with the control eye. This was evident in all animals despite considerable variability in the CTT on the same animal between measurements, possibly due to suppression of a blink reflex in the apprehensive animal. Other observers have reported the capricious nature of corneal sensitivity measurements in rabbits.5

Early studies of corneal reinnervation in rabbits claimed that sensitivity had recovered after 2–3 months.5,6 While our results do not preclude this possibility, the criteria for establishing normal sensitivity in these studies were not well defined, and the measurement methods can no longer be considered accurate. Our measurements, taken over an extended period, clearly established that incomplete recovery of sensitivity is a common sequel to a limbal incision in rabbits. This is consistent with the findings of Draeger and Martin11 that normal corneal sensitivity was not restored even 8 years after cataract surgery in human patients.

There was a large individual variation in the extent of neural regeneration within our group. This variation could be due to the quality of the wound healing, since other experimenters have reported that scar tissue and poor wound apposition could act as a barrier to neurone growth.

In spite of a large individual variation in neural regeneration within our group of four rabbits, all showed a reduction in the number of histochemically detectable stromal nerve bundles in the superior half of the operated cornea. These regenerating nerve fibers showed structural differences when compared to normal nerve trunks. The regenerating nerve bundles were abnormally curved and appeared to contain less axons than equally thick bundles in control corneas. The epithelium in the operated sector also showed reduced density of intra-epithelial terminals as well as disturbed architecture of the basal epithelial plexus and nerve terminals. The sprouting of axons within the epithelium is characteristic of that observed in the distal region following nerve section and reanastomosis.2,8,12

Previous histological studies of the time course of reinnervation in the rabbit have yielded conflicting results. While Mohan et al7 considered regeneration to be complete after 4–7 weeks, a recent careful examination by Rozsa et al9 found that large patches (1–2 mm²) of denervated cornea were still present at the sub- and intra-epithelial levels after 2 months. Our findings are consistent with those of Rozsa et al. In addition, they suggest that the process of reinnervation may not be complete even after longer recovery periods.

The pattern of neural reinnervation found in the rabbit following a limbal incision is in marked contrast to that found in the human after corneal grafting. Tervo et al2 found that almost all stromal nerve trunks ceased at the edge of the host cornea. The epithelial nerve leashes apparently traversed the scar area and supplied some corneal sensitivity in addition to the few stromal nerves that were seen in the donor cornea. The difference between the neural regeneration in human corneal grafts and rabbit corneas after perilimbal incision may not be explained purely as a species difference. In perilimbal incisions the Schwann cell channels may remain in reasonable apposition to the proximal nerve trunks, which could assist stromal reinnervation. Where grafted tissue is introduced, no such continuity exists, making stromal nerve regeneration more difficult. Although a perilimbal incision does not grossly misalign the Schwann cell channels, scar tissue formation may cause sufficient disruption to displace a significant number of regenerating fibers from their path, thus leading to a reduced number of stromal nerve trunks with a subnormal number of axons, as observed.

Since AChE nerves are derived from the ophthalmic branch of the trigeminal nerve and the majority if not all are sensory in function,10 our study allows a comparison between a functional response to a physical stimulus and histochemical evidence of neural regeneration. We have provided sensitivity data and histochemical evidence that neural regeneration is incomplete 30 months after a 180° penetrating limbal incision in the rabbit. Although some neural reorganization has occurred in the stroma, the quality...
Fig. 2. (A) Higher magnification photo of a regenerating nerve showing a subnormal number and irregularity of caliber of axons (Rabbit 2 RE). Magnification \( \times 70 \). (B) Regenerated peri-corneal stromal nerve plexus showing distorted architecture (arrowheads) and reduced nerve density (Rabbit 3 RE). Magnification \( \times 31 \). (C) Normal peri-corneal stromal nerve plexus taken from the inferior region of Rabbit 3, RE. Magnification \( \times 26 \). (D) Abnormal nerves just distal to the limbal scar. Microscope is focused at the stromal-epithelial interface (Rabbit 4 RE). Magnification \( \times 41 \). (E) Numerous single epithelial axons seen penetrating the scar region. They travel for a limited distance at the basal epithelial level. Taken from the superior region of Rabbit 4, RE. Magnification \( \times 41 \).

and extent of stromal and epithelial reinnervation was insufficient to restore normal sensitivity. Corneal sensitivity was reduced to a much greater extent than would be suggested by the post-surgical stromal nerve density. This study supports the observations of Beuerman and Kupke, that a normal epithelial plexus, not merely the presence of stromal nerve trunks, is necessary for the return of normal sensitivity. As neural regeneration was not complete even in the long term after a penetrating incision, it is possible that epithelial permeability and wound repair, which has been shown to be affected by sensory denervation, may be permanently impaired. 

Key words: nerve regeneration, sensitivity, acetylcholinesterase, cornea, rabbit
Acknowledgments. The authors wish to thank Dr Daniel O’Leary for his valuable suggestions throughout this study and Ms. Annic Ansselin for helpful comments on the manuscript.

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References


Corneal Storage in MK Medium and K-Sol®

Effect on Ionic and Non-Ionic Fluxes

David S. Hull, Rosa Berdecia, and Keith Green

Rabbit corneas were stored at 4°C for 3, 7 or 14 days in either modified MK medium or K-Sol®. Corneal endothelial permeability to inulin following storage in modified MK was significantly less at each time examined than that found in corneas stored for either 3, 7 or 14 days in K-Sol. Inulin permeability after storage in K-Sol was increased at all times relative to unstored control corneal tissue, but only at 7 and 14 days in MK medium. Dextran permeability was similar following 3 days of storage in either solution, but dextran permeability following storage in modified MK was significantly less than the values found in corneas stored for 7 and 14 days in K-Sol. Dextran permeability was not significantly increased relative to control, at any storage time in MK medium but was increased at 7 and 14 days in K-Sol. Inulin and dextran permeabilities after storage in MK medium were maintained more closely to values found in fresh tissue than corneas stored in K-Sol. Net endothelial sodium fluxes following storage in modified MK medium were markedly less than those found in corneas stored for 3, 7 and 14 days in K-Sol. Net sodium fluxes are maintained better in K-Sol than in MK medium relative to control values. Net bicarbonate fluxes following storage in modified MK medium were significantly less than the 3-day values in K-Sol, but similar to the values after 7 and 14 days of K-Sol storage. All net ion fluxes, except for sodium at 7 days storage in K-Sol, were significantly lower than control values. MK medium appears to preserve endothelial barrier function better than K-Sol, but K-Sol preserves net ionic movement better than MK medium. Invest Ophthalmol Vis Sci 28:2088–2091, 1987

MK medium has been used to store corneas for periods of 72–96 hr prior to usage for penetrating keratoplasty.1,2 K-Sol® (Cilco, Inc., Huntington, WV) is a new corneal storage medium in which 2.5% chondroitin sulfate replaces the dextran in tissue culture medium.3,4 It is thought that the chondroitin sulfate in this solution enhances the storage characteristics of tissue culture medium, thereby allowing for longer storage periods. It was the purpose of this investigation to evaluate and compare corneal endothelial sodium ion, bicarbonate ion, inulin, and dextran fluxes following storage for 3, 7, and 14 days in...