A comparison of healing of corneal epithelial wounds stained with fluorescein or Richardson's stain. JOHN L. UBELS, HENRY F. EDELHAUSER, AND KRISTINA H. AUSTIN.

The effects of fluorescein and Richardson's stain on corneal epithelial wound healing were compared in eyes of rabbits whose corneas had the epithelium removed by scraping or by n-heptanol. One eye of each rabbit was stained with fluorescein and the other eye was stained with Richardson's stain at intervals throughout the healing process, and the wounds were photographed for planimetry and determination of re-epithelialization rate. Corneal thickness was also measured throughout the re-epithelialization. These studies showed that Richardson's stain, as compared with fluorescein, decreases re-epithelialization rate, delays wound closure, and slows the return of the edematous cornea to normal thickness. Therefore fluorescein rather than Richardson's stain should be used to stain epithelial defects in corneal wounds. Healing was also monitored by measurement of the area of the wound over time. Corneal thickness was also measured as an index of the functional integrity of the regenerating epithelium.

Materials and methods

Experimental procedures. Albino rabbits (2 to 2.5 kg) were sedated with ketamine HCl (30 mg/kg) and the corneas of the animals were anesthetized with a drop of proparacaine HCl (0.5%). A central epithelial wound was made on both corneas of each animal by means of either the n-heptanol method of Citron et al. or the scraping method described by Ho et al. A filter paper disc 6.5 mm in diameter was used for heptanol wounds, and scraped corneas were debrided within a 6.5 mm trephine mark. The right eye of each animal was stained by applying a moistened fluorescein sodium ophthalmic strip to the bulbar conjunctiva and rinsing the eye with an ophthalmic irrigating solution.

The clinical problems of corneal epithelial erosion and persistent epithelial defects have led to several studies of the mechanisms and biochemistry of corneal wound healing. Evaluation of a corneal wound or infection requires the use of a topically staining technique. The most common method used clinically has been fluorescein staining. Fluorescein has also had wide usage in ophthalmic research and has no known toxic effects on the corneas of humans or rabbits.

A second stain, known as Richardson's stain, has recently been used in laboratory studies of corneal epithelial wound healing. This is a histologic stain containing methylene blue, azure II, and borax; when applied to the cornea, this stain yields a dark blue, well-delineated stain at the location of an epithelial defect. It is visible under white light and can be conveniently photographed without special filters.

Several characteristics of Richardson's stain, however, raise questions about its suitability as a stain for corneal wounds. The pH is basic, 8.6, the osmolality is 15 mOsm and, most significantly, methylene blue is reported to be toxic to human and rabbit corneas. Comparison of two reports in the literature indicates that healing of chemically wounded corneas may occur more rapidly in fluorescein-stained corneas than in corneas stained with Richardson's stain. A controlled study was therefore undertaken to compare corneal healing rates with the use of fluorescein and Richardson's stain. Rates of wound closure were determined by measurement of the area of the wound over time. Corneal thickness was also measured as an index of the functional integrity of the regenerating epithelium.
Table I. Corneal epithelial wound healing rates of eyes stained with fluorescein or Richardson’s stain

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Fluorescein OD Rate*</th>
<th>r</th>
<th>Richardson’s OS Rate*</th>
<th>r</th>
<th>n-Heptanol</th>
<th>Fluorescein OD Rate*</th>
<th>r</th>
<th>Richardson’s OS Rate*</th>
<th>r</th>
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<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.94</td>
<td>0.44</td>
<td>0.88</td>
<td>6</td>
<td>0.92</td>
<td>0.94</td>
<td>0.58</td>
<td>0.95</td>
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<td>2</td>
<td>0.75</td>
<td>0.91</td>
<td>0.41</td>
<td>0.88</td>
<td>7</td>
<td>0.92</td>
<td>0.94</td>
<td>0.68</td>
<td>0.94</td>
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<td>3</td>
<td>1.00</td>
<td>0.99</td>
<td>0.80</td>
<td>0.80</td>
<td>8</td>
<td>1.00</td>
<td>0.94</td>
<td>0.58</td>
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<tr>
<td>4</td>
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<td>0.97</td>
<td>0.86</td>
<td>0.92</td>
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<td>0.92</td>
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<tr>
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<td>0.94</td>
<td>0.56</td>
<td>0.94</td>
<td>10</td>
<td>0.91</td>
<td>0.92</td>
<td>0.61</td>
<td>0.92</td>
</tr>
<tr>
<td>6</td>
<td>0.92</td>
<td>0.94</td>
<td>0.80</td>
<td>0.80</td>
<td>11</td>
<td>0.89</td>
<td>0.95</td>
<td>0.58</td>
<td>0.95</td>
</tr>
<tr>
<td>Mean</td>
<td>0.99†</td>
<td>0.61</td>
<td></td>
<td>0.61</td>
<td></td>
<td>0.94†</td>
<td>0.63</td>
<td></td>
<td>0.63</td>
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<tr>
<td>SE</td>
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<td>0.092</td>
<td></td>
<td>0.092</td>
<td></td>
<td>0.019</td>
<td>0.002</td>
<td></td>
<td>0.002</td>
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</table>

r = correlation coefficient.
*Expressed as mm²/hr.
†Significant difference between fluorescein and Richardson’s (p ≤ 0.05).

of Mishima and Hedbys. These readings were taken before wounding and immediately prior to photography. The central fixation lights were easily visualized on the surface of wounded corneas. Although it is known that proparacaine can inhibit wound healing, it was necessary to anesthetize wounded corneas so that the animal would open its eye to allow corneal thickness measurements to be made.

The animals were euthanized by an overdose of sodium pentobarbital at the conclusion of the experiments.
Fluorescein—Richardson's Stain

Fig. 2. Corneal thickness during healing of an epithelial wound. A, Scraped wound. Thicknesses were compared by paired t test at 12, 24, 48, and 75 hr. Dagger, No significant difference; asterisk, significant difference (p < 0.05) mean ± S.E., n = 5. B, Heptanol wound. Thicknesses were compared at 24, 30, 54, and 72 hr. Dagger, No significant difference; asterisk, significant difference (p < 0.05) mean ± S.E., n = 6.

Data analysis. Corneal re-epithelialization rates for each eye of each animal were determined by linear regression. These healing rates were then compared for each experimental group by the paired t test (p < 0.05). This is the most accurate method of comparing healing rates, since it has been reported that healing characteristics are more similar within animals than among animals.* Corneal thicknesses were also compared by t test (p < 0.05) at selected times during the healing process.

Results. Initial wound areas varied from 40 to 60 mm². In the case of heptanol wounds, this is due to diffusion of the heptanol away from the filter paper applicator. In scraping, the knife blade occasionally crossed the trephine mark, resulting in some variation of wound size. There was, however, no correlation between initial wound size and healing rate. This has also been shown by Ho et al.²

The average healing rate of corneas wounded by scraping and stained with fluorescein was 0.99 mm²/hr. Staining with Richardson’s stain decreased the average healing rate to 0.61 mm²/hr (Table I). Staining of a heptanol wound with fluorescein or Richardson’s stain resulted in healing rates of 0.94 and 0.63 mm²/hr, respectively.

Graphs of pooled data expressed as percent initial wound area (Fig. 1) show that fluorescein-stained corneas wounded by scraping or by heptanol healed at a constant rate and that these rates are similar to those listed in Table I. The use of Richardson’s stain resulted in a decrease in healing rate after 40 hr, yielding a sigmoid healing curve. As illustrated in Fig. 1, A, a line can be fitted only to the early part of this curve and results in the calculation of a healing rate of 0.87 mm²/hr.

By comparison with fluorescein, Richardson’s stain caused a delay in corneal wound closure. In four out of five animals with scraped corneal wounds, the fluorescein-stained wound closed earlier than the wound stained with Richardson’s stain. In five out of six animals with heptanol wounds, the fluorescein-stained wound closed earlier than the wound stained with Richardson’s stain. The epithelium of the cornea stained with Richardson’s stain sloughed at 78 hr in three of the animals with heptanol wounds.

Immediately after epithelial wounding the cornea swells rapidly (Fig. 2). Scraped corneas stained with fluorescein continued to swell for 12 hr and then returned to control thickness by 72 hr after wounding. Corneas wounded with heptanol continued to swell for 24 hr and reached a significantly greater thickness than scraped corneas (0.78 vs. 0.68 mm). When stained with fluorescein, the heptanol-wounded corneas did not reach control...
thicknesst by 72 hr; however, subsequent experiments indicated that control thickness was reached by 90 hr after wounding.

Whether corneas are scraped or wounded with heptanol, there is no significant difference in the maximum thickness reached by corneas stained with Richardson's stain as compared with those stained with fluorescein. The thickness of corneas stained with Richardson's stain, however, reached a plateau and the deswelling phase of corneal healing was significantly delayed (Fig. 2). Measurements on several animals indicated that the thickness of corneas stained with Richardson's stain may not return to normal for as long as 1 week after wounding.

**Discussion.** The results of this study show that, as compared with fluorescein, the use of Richardson's stain results in a decrease in wound healing rate, a delay of wound closure, and slowing of the return of the edematous cornea to normal thickness. Although the cellular mechanisms by which the components of Richardson's stain inhibit the normal corneal healing process are not precisely known at this time, the delay in wound closure indicates possible interference with the sliding of epithelial cells. The thickness data indicate that Richardson's stain may also delay the establishment of a functional epithelial barrier.

In a recent study of corneal epithelial wound healing,

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933104/)  data from the first 24 hr of healing were used to determine a re-epithelialization rate for Richardson's stained corneas. Our data (Fig. 1, A) show that because of the sigmoid shape of the healing curve when Richardson's stain is used, the use of data from the first 30 hr of healing will result in an overestimation of healing rate and an underestimation of wound closure time.

The results of the present study also show that valuable data about the toxicity of a chemical substance can be obtained by measurement of corneal thickness during the healing of a corneal epithelial wound. Thus it is recommended that pachometry be used in studies of corneal epithelial wound healing and in the evaluation of topical drugs, chemicals, and household substances. In addition to the comparison of fluorescein and Richardson's stain by measurement of corneal thickness, we have also shown by this method a possible toxic effect of heptanol on the cornea, since corneal thickness is greater after a heptanol wound than after scraping. Two other groups have also reported damage to the cornea caused by heptanol. Cintron et al.

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933104/)  showed that the anterior stromal keratocytes are damaged by heptanol treatment. Hirst et al.,

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933104/)  who compared several methods of epithelial debridement, found that although epithelial hemidesmosomes begin to regenerate by day 3 of healing in a scraped cornea, no epithelial hemidesmosomes are present at day 7 in the heptanol-wounded cornea. This lack of junctional complexes may, in part, account for the greater swelling of the heptanol-wounded cornea. The absence of hemidesmosomes may also explain the greater tendency of the epithelium of the heptanol-wounded corneas stained with Richardson's stain to slough.

In conclusion, it is clear that Richardson's stain inhibits corneal wound healing and that this stain is not acceptable for use in laboratory studies of corneal re-epithelialization. Fluorescein is known to be safe for many clinical applications, and it can be easily photographed with black and white film, using a Wratten 47B filter on an electronic flash and a Wratten 12 filter on the camera lens, or with color film, using a cobalt filter on the electronic flash. Fluorescein, rather than Richardson's stain, should therefore be used to stain epithelial defects in future studies.

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**Key words:** corneal epithelium, fluorescein, Richardson's stain, wound healing, pachometry

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Episcleral venous pressure was measured by a noninvasive method with a modified force-displacement transducer in six laboratory-quality normotensive and 12 glaucomatous beagles. The dogs were anesthetized by ketamine-xylazine, acepromazine-ketamine, and halothane. Simultaneous intraocular pressure (IOP) and blood pressure were recorded. The mean episcleral venous pressures in normotensive beagles were 11.4 to 11.6 mm Hg with the three methods of anesthesia; in the glaucomatous beagles the mean episcleral pressures were 10.6 to 12.5 mm Hg. There were no significant differences in episcleral venous pressure (p < 0.19 and greater) and blood pressure (p < 0.53 and greater) between the normotensive and glaucomatous beagles. IOP was significantly different between the normotensive and glaucomatous beagles anesthetized with acepromazine-ketamine (mean IOP, 23.4 and 34.2 mm Hg, respectively; p < 0.02) and halothane (mean IOP, 19.9 and 27.4 mm Hg, respectively; p < 0.001) but not significant with anesthesia with ketamine-xylazine (mean IOP, 26.0 and 37.8 mm Hg, respectively; p < 0.12). Episcleral venous pressure was unchanged as the disease progressed in the glaucomatous beagle. (Invest Ophthalmol Vis Sci 23:131-135, 1982.)

Noninvasive measurements of episcleral venous pressure in man and animals have used direct pressure to temporarily blanch and collapse the vessel by various devices and a stream of air.1-4 These methods have employed a torsion balance, pressure chamber with transparent membranes, and force-displacement transducer. Measurement of episcleral venous pressures in rabbits and cats by direct cannulation under general anesthesia yielded highly reproducible results and had a distinct end point but was an invasive procedure.5-8 Pressure-chamber methods using various membranes provided values similar to direct cannulation.7 The torsion-balance system, evaluated in man and rabbits, resulted in greater difficulty in obtaining an end point and provided higher values.5 With an isometric force-displacement system, episcleral venous pressures of normotensive and glaucomatous humans were not significantly different.2 However, in one family with glaucoma, episcleral venous pressure was elevated.9 The episcleral venous pressure in persons with ocular hypertension was significantly lower than that in subjects with normal levels of intraocular pressure (IOP) and glaucoma.4 Normal episcleral venous pressures in rabbits, cats, and man range from 5 to 15 mm Hg. Pressure chamber measurements of the episcleral venous pressure in the dog yielded a range of 10 to 18 mm Hg.6 The values in these species are remarkably similar in spite of the different venous pathways of the anterior segment.