OPTIMIZATION OF AN IN VITRO LONG TERM CORNEAL CULTURE ASSAY

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Abstract

The long-term culture of corneas has been proposed as an in vitro model to evaluate potential eye irritation and post-treatment recovery following chemical exposures. Porcine eyes were obtained within 24 hours of sacrifice and disinfected prior to excising corneas. Corneas were filled with an agar/gelatin gel in M199 medium to support the corneas, and were cultured at 37ºC, 5% CO2, 90% RH in M199 medium to the limbus. The corneas were moistened by brief immersion in medium every 2.7 hours using a modified plate rocker. Corneas were treated with either SLS, EtOH, or H2O (controls). The corneas were rinsed with PBS, cultured for a pre-determined post exposure time, and fixed in buffered formalin. H&E-stained control corneas showed normal morphology after 4 days, similar to excised/immediately fixed corneas. Controls were characterized by an intact epithelium with viable squamous, wing, and basal cells. The stroma showed minimal swelling with frequent viable keratocytes. The endothelium was typically intact. Some stromal swelling near the sclera was noted after 5 to 7 days. Corneas treated with 3% SLS or EtOH showed complete epithelial cell damage or loss 24 hours after treatment, as well as loss of viable keratocytes in the upper stroma. After 48 hours, epithelial cell sheet migration was observed into the damaged zone. After 120 hours, the regeneration of a stratified epithelium was observed. These results confirm the ability to culture porcine corneas for at least 120 hours, as well as demonstrate the potential for further optimization of evaluating recovery of damaged corneas.

Introduction

In vitro eye irritation tests of commercial consumer products and chemicals typically model the potential for ocular irritation in the test system, however, they do not directly model tissue recovery. In product development or pre-clinical testing settings, materials that are found to be non-irritating in an *in vitro* assay, may proceed in a specific corporate testing strategy. However, for materials that show some level of ocular irritation *in vitro*, there may not be sufficient information in some cases to estimate the potential for recovery. In those cases where the depth of injury is limited to the epithelium and perhaps the upper stroma (a depth of injury which may readily be reversible) it would be desirable to demonstrate recovery *in vitro*. Therefore, the long-term culture of porcine corneas has been proposed as an *in vitro* model to evaluate potential eye irritation and post-treatment recovery following chemical exposures.

As part of their corporate program directed towards developing and utilizing *in vitro* methods for product development and safety testing, The Gillette Company has sponsored the optimization of a long-term corneal culture assay system using porcine corneas. The techniques employed are modifications of those of Foreman (1996), Xu (2004) and Boulton (2003). Porcine eyes were obtained as a byproduct of abattoir operations and cultured in vitro within 24 hours after sacrifice. There were several technical goals in establishing and optimizing the assay system for routine application. These goals included preparing reproducible agar gels to support the normal corneal architecture, controlling microbial contaminants in the slaughter-house derived tissues, automating the periodic moistening of the air-exposed epithelium, and developing a positive control chemical which could induce recoverable damage in the corneal epithelium. The achievement of these goals was determined by visual and histological evaluation of the cultured corneas.

Materials & Methods

Porcine Eyes
Porcine eyes were obtained from Sioux-Preme Pork Products (Sioux City, IA) within 24 hours after sacrifice. The eyes were excised and shipped to the laboratory in cooled saline solution. Preparation of the corneas was performed upon receipt.

Preparation of Corneas
Porcine eyes were transferred to a sterile field during corneal excision. The corneas were visually inspected for defects, such as neovascularization, pigmentation, opacity, and scratches; those with defects were discarded.

Disinfection Process
- Immersion in 1% povidone-iodine solution for 2 minutes
- Rinsed in sterile PBS
- Soaked in 0.1% gentamicin-PBS solution for 15 minutes

After the disinfection process, the corneas were excised from the whole globe eyes.

Excision of the Cornea
- Corneas were excised from the whole globe eyes leaving a 3 to 5 mm circular rim of sclera intact
- Corneas were serially rinsed in multiple wells each containing 5 to 7 mL of pre-warmed, sterile HBSS

After excision, an agar/gelatin/M199 mixture was added to the endothelial cavity to support the rounded corneal architecture.

Addition of Agar/gelatin/M199
Agar/gelatin/M199 mixture: 1% low gelling temperature agar, 1% porcine skin gelatin, M199 medium, supplemented with gentamicin, penicillin/streptomycin, and amphotericin B maintained at 37ºC.

- Corneas were suspended epithelial-side down in 24-well plate wells partially-filled with HBSS to support the cornea's natural shape
- The agar/gelatin/M199 mixture was added drop-wise (2 to 3 drops at a time) into the endothelial corneal cavity and allowed to gel at room temperature
- This process was repeated until the endothelial cavity was completely filled, and the agar/gelatin had completely solidified
- Corneas were inverted and transferred into large deep-well dishes

Fig. 1a. Addition of Agar/gelatin/M199

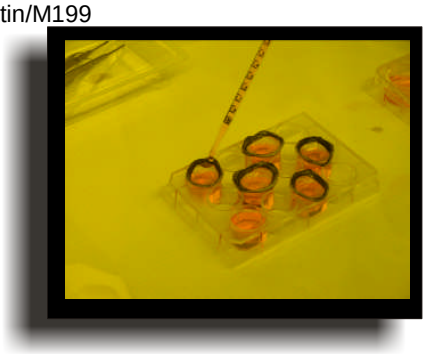
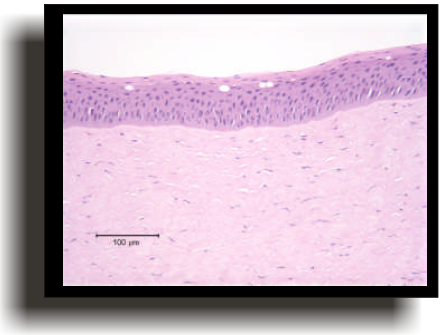


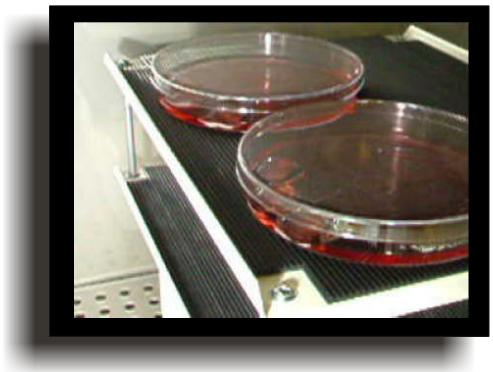
Fig. 1b. Freshly-excised porcine corneas

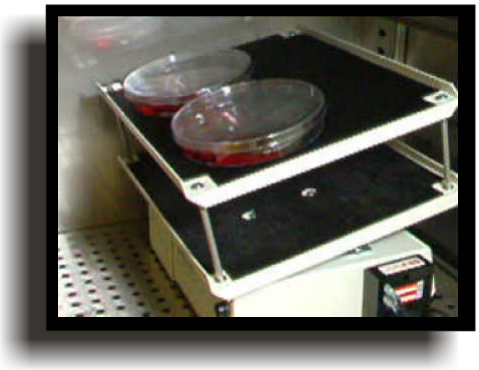


Corneal Culture

M199 culture medium: M199 medium (with Earle's salts) with 10% FBS, and supplemented with gentamicin, penicillin/streptomycin, and amphotericin B.

- M-199 culture medium was added to each dish until it covered the limbal-conjuctivae (40 mL), leaving the rounded corneal epithelium exposed to air
- The cultures were placed onto a modified plate rocker and incubated at 37ºC, 5% CO_2, and 90% RH for approximately 24 hours prior to dosing
- The modified plate rocker was programmed to briefly pivot the culture dishes from a horizontal position to approximately a 45º angle every 2.75 hours. This allowed the corneas to be briefly submerged in medium to moisten the epithelium.





Treatment of Test Chemicals

Porcine corneas were treated approximately 24 hours after initiating the cultures.

Test Chemicals

- SLS 1, 2, or 3%
- EtOH (ethanol) 100%
- H_2O (sterile deionized water) (negative controls)
- Untreated controls

Exposure Methods Evaluated

- Teflon dosing rings (1.0 cm diameter) - Rings were placed onto the corneas; 40 µL of test chemicals were dosed into the rings
- Filter paper discs (1.3 cm diameter) - Test chemicals were soaked into the filter paper prior to application onto the corneas
- Nylon meshes (0.8 cm diameter) - Meshes were placed onto the corneas; 25 µL of test chemicals were applied to the meshes

Exposure Times

Initial time course trials ranging from 2 to 10 minutes

Treatments were terminated by gently rinsing the corneas with approximately 2 mL of PBS, until the corneas appeared free of test chemical.

Corneas were transferred to new dishes and cultured in fresh M199 medium as described above.

Post-Exposure Incubation and Tissue Harvest

Corneas were maintained in culture for a variety of post-exposure periods [6, 24, 48, 96, and 120 hours] to allow for expression of toxic effects and potential tissue recovery

- Visual and microscopic observations were made daily
- Cultures were refed with 40 mL of fresh M199 medium daily
- After the post-exposure incubation period, the corneas were fixed in 10% neutral buffered formalin for at least 24 hours
- Tissues were embedded, sectioned, mounted onto slides and stained with hematoxylin and eosin [Pathology Associates, Inc. (Frederick, MD) or Histo Techniques, Ltd. (Powell, OH)]
- Tissues were evaluated for histological changes at the Institute for In Vitro Sciences, Inc.

RESULTS

We have been able to maintain porcine corneas in culture for at least 7 days, and have demonstrated recovery of a fully stratified corneal epithelium 120 hours after a 5-minute exposure to 3% sodium lauryl sulfate (SLS). Within 24 to 48 hours after induction of chemical wounds in porcine epithelia by SLS or ethanol exposure, we observed sheets of viable cells presumably migrating along the basal membrane into the damaged zone. In several fields, these sheets of viable cells were observed to displace non-viable damaged epithelium. The morphology of the cells within the migrating epithelial sheets was typically rounded or flattened. Zhao (2003) demonstrated that the majority of re-epithelialization in mechanically-wounded bovine corneas occurred by the sliding of entire epithelial cell sheets from the limbal region, rather than by individual cell movements. Zhao reported that two morphologies were noted during the migration phase; oval shaped cells predominated in the first several rows behind the leading edge, and behind these, were cells with a more flattened appearance.

Control Corneas

H&E-stained preparations of H_2O-treated control corneas showed that normal morphology could be maintained in culture for up to 96 hours (Fig. 2). Control corneas were characterized by an intact epithelium with viable squamous, wing, and basal cells. The stroma showed frequent viable keratocytes, and minimal swelling indicative of a functional endothelium. The endothelium was typically intact.

Control corneas that were cultured from 5 to 7 days showed normal epithelial morphology. However, some loss of the endothelium and notable stromal swelling was typically observed at the periphery near the sclera (Fig. 3).

Fig. 2a. Control corneas (H_2O for 5 minutes) 6 hours post-exposure

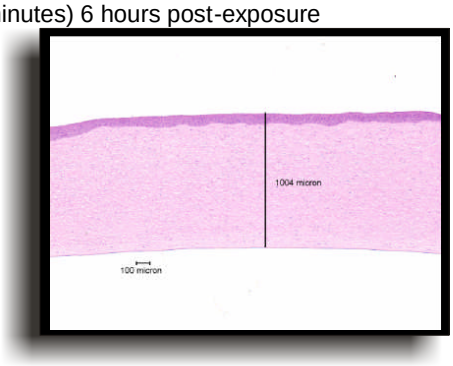


Fig. 2b. Control corneas (H_2O for 10 minutes) 48 hours post-exposure - epithelium and upper stroma

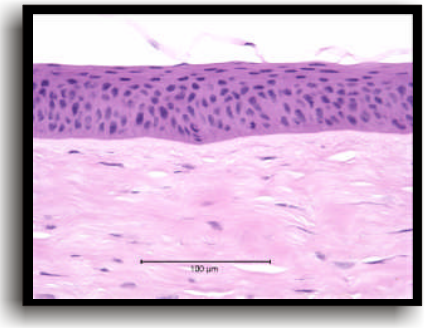


Fig. 2c. Control corneas (H_2O for 10 minutes) 48 hours post-exposure - endothelium and lower stroma

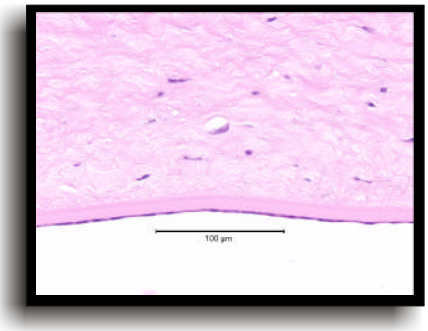


Fig. 3a. Control corneas (H_2O for 5 minutes) 120 hours post-exposure

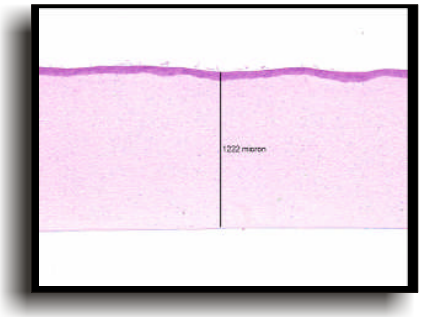


Fig. 3b. Control corneas (H_2O for 5 minutes) 120 hours post-exposure - epithelium and upper stroma

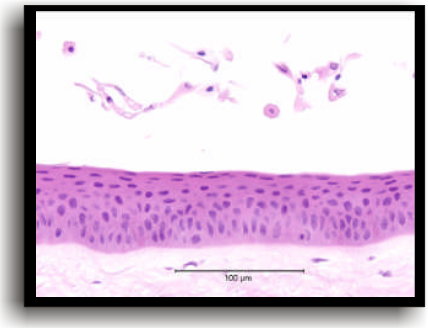
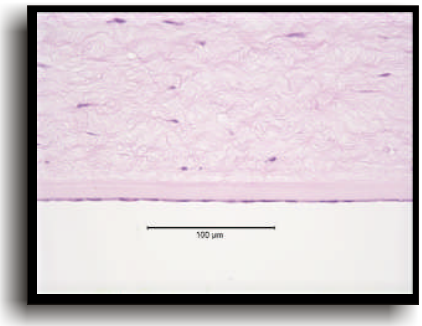


Fig. 3c. Control corneas (H_2O for 5 minutes) 120 hours post-exposure - endothelium and lower stroma



Visual observations of the corneas show that slight increases in opacity occur temporally during the culture period, and may be attributable to slight increases in stromal hydration or changes in the agar/gelatin. However, the level of opacity is minimal such that microscopic evaluation of cells throughout the tissue can be achieved (Fig 4).

Fig. 4a. Bovine corneas 1 day in culture

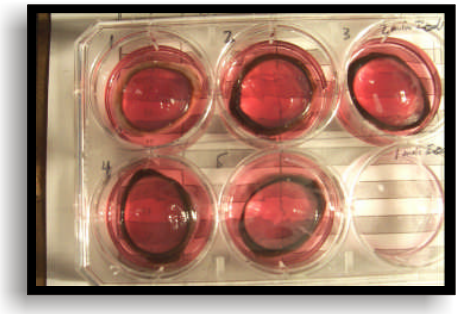
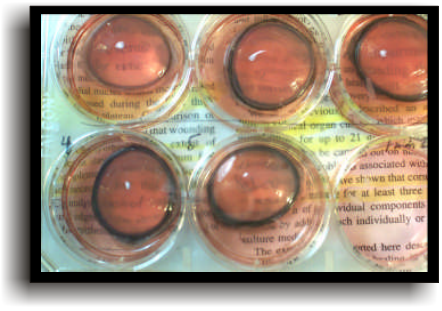


Fig. 4b. Bovine corneas 4 days in culture show minimal increases in opacity



Test Chemical Treated Corneas

Corneas treated with 1% SLS for 2 minutes showed no pathological changes at 6 and 24 hours after exposure.

Corneas treated with 3% SLS for 2 minutes induced slight hyper-eosinophilia in the squamous epithelium 24 hours after exposure, suggestive of minimal irritation limited to the epithelium. Considering the limited depth of the injury, the corneas would be expected to fully recover (Jester, 2001).

Corneas treated with 3% SLS for 5 or 10 minutes exhibited complete epithelial cell damage or loss at 6 and 24 hours after treatment, as well as loss of viable keratocytes in the upper stroma (Fig. 5). By 48 hours after treatment, evidence of epithelial cell sheet migration along the basal membrane into the damaged zone was observed (Fig. 6). Corneas treated with 3% SLS for 5 minutes exhibited a complete fully-stratified epithelium 120 hours after treatment (Fig. 7).

Fig. 5a. 3% SLS (5 minutes) 6 hours post-exposure

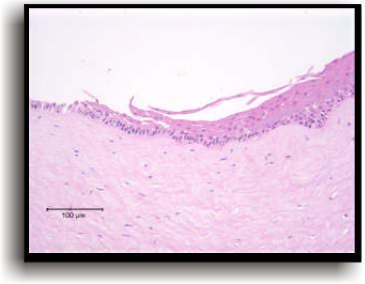


Fig. 5b. 3% SLS (5 minutes) 6 hours post-exposure

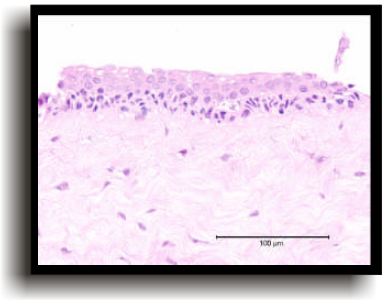


Fig. 5c. 3% SLS (5 minutes) 24 hours post-exposure

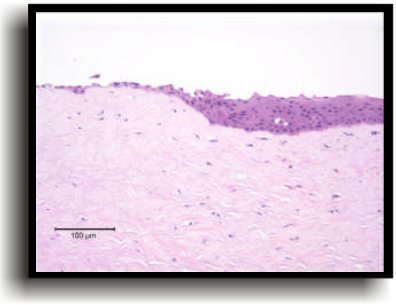


Fig. 6a. 3% SLS (5 minutes) 48 hours post-exposure

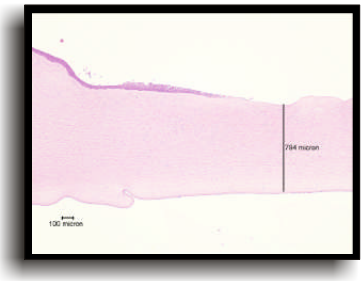


Fig. 6b. 3% SLS (5 minutes) 48 hours post-exposure

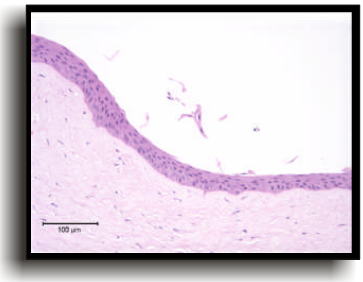


Fig. 6c. 3% SLS (5 minutes) 48 hours post-exposure

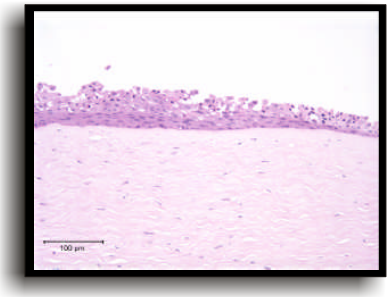


Fig. 7a. 3% SLS (5 minutes) 120 hours post-exposure - epithelium and upper stroma

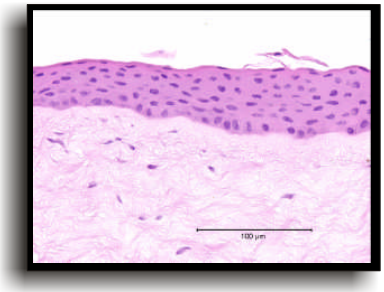
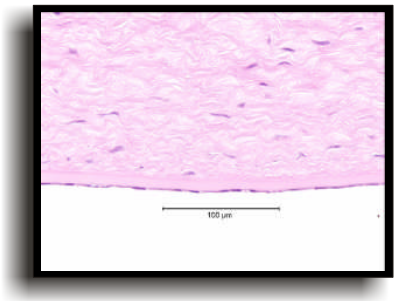


Fig. 7b. 3% SLS (5 minutes) 120 hours post-exposure - endothelium and lower stroma



Corneas treated with EtOH for 5 or 10 minutes exhibited complete epithelial cell damage or loss 24 hours after treatment, as well as loss of viable keratocytes in the upper stroma. By 48 hours after treatment, evidence of epithelial cell sheet migration along the basal membrane into the damaged zone was observed, with displacement of the EtOH-fixed epithelium by the migrating cell sheet (Fig. 8).

Fig. 8a. EtOH (5 minutes) 6 hours post-exposure

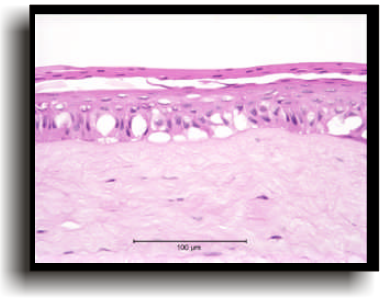


Fig. 8b. EtOH (5 minutes) 24 hours post-exposur. Note columnar cells in EtOH-fixed epithelium

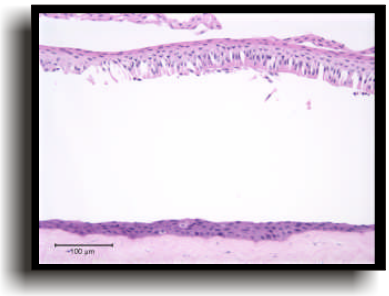
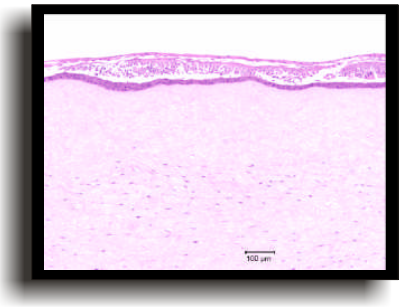


Fig. 8c. EtOH (5 minutes) 48 hours post-exposure



Comparison of Dosing Devices: dosing ring, filter paper, and nylon mesh

Dosing ring:

Pros

- more precise definition between exposed and unexposed epithelium
- maximum uniform dose rate (40 µL per 0.78 cm2)
- histological evaluation revealed definitive zone of treated and untreated tissue

Cons

- dosing site not always well contained; prone to leak, especially if cornea had irregular shape

Filter paper discs:

Pros

- dosing site contained; no excess run-off from application site

Cons

- did not provide precise definition between exposed and unexposed epithelium
- variability in actual dose available to tissue (dependant upon absorption/saturation)
- contact area smaller than diameter since the filter paper did not follow the corneal curvature
- histologies did not always reveal zone of damaged epithelium

Nylon mesh:

Pros

- uniform exposure within contact area

Cons

- did not provide precise definition between exposed and unexposed epithelium
- dosing site not well contained; excess readily flowed off of site
- contact area smaller than diameter since the mesh did not follow the corneal curvature
- histologies did not always reveal zone of damaged epithelium

CONCLUSIONS

- Results confirm the ability to culture porcine corneas for at least 96 to 120 hours with normal full thickness morphology

- Model provides a viable platform for the evaluation of recovery after induction of chemical damage

- SLS and EtOH-induced damage in porcine corneas is histologically consistent with observations from other *ex vivo* models. The *in vitro* cornea's ability to exhibit re-epithelialization after exposure to EtOH is consistent with corneal recovery exhibited *in vivo* in rabbits and in humans.

- 3% SLS exposed for 5 minutes may be used as an assay system positive control to demonstrate epithelial damage as well as subsequent re-establishment of a fully stratified epithelium

- Additional endpoints such as live / dead staining using calcein AM / ethidium homodimer-1 for laser confocal microscopy for depth of injury and recovery analyses may be included.

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