

## **The Porcine Corneal Opacity and Reversibility Assay is an alternative assay that provides evidence of reversible eye irritancy**

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### **ABSTRACT**

MB Research has developed an *ex vivo* alternative assay that can assess both potential eye irritation and reversibility using an SOT Grant for Alternatives Research from Colgate-Palmolive. In the Porcine Corneal Opacity and Reversibility Assay (PorCORA), excised porcine corneas (byproducts from the meat industry) are cultured for up to 28 days. Corneas are treated topically with test substances and the resulting damage to the corneal surface is measured by visualizing sodium fluorescein (NF) stain retention with UV light. The reversibility of the corneal damage is demonstrated by measuring the changes in retention of fluorescein stain in the same cornea over time in culture. As corneal re-epithelialization occurs, the area of fluorescein stain retention decreases.

There has been favorable correlation between recovery in the *in vivo* Draize rabbit eye irritation results (ECETOC, 1998 Eye Irritation: Reference Chemicals Data Bank) and the PorCORA for the chemicals tested (100% ethanol, 1% benzalkonium chloride, 3% sodium dodecylsulphate, 10% sodium hydroxide, phosphate buffered saline). *In vivo* data for this project will come from this data bank to avoid the use of more animals. Verification of the re-epithelialization process was done histologically as well as by confocal microscopy (see presentation by Piehl, Gilotti, Ball & Cerven). When present, damage to the corneas was visualized by fluorescein staining of the corneas prior to fixation. Re-epithelialization was demonstrated within 14 days for 100% ethanol, 21 days for 1% benzalkonium chloride, and 10 days for 3% sodium dodecyl sulfate. Corneas treated with 8% sodium hydroxide retained up to 21 days. On the rare occasion that corneas treated with phosphate buffered saline retained fluorescein, re-epithelialization was demonstrated within 3 days. Time course of re-epithelialization was demonstrated by histopathology of treated corneas from 1, 2, 3, 7, 14 and 21 days post treatment.

## **INTRODUCTION**

Regulatory agencies require manufacturers to characterize the risk of eye irritation/damage, and mandate the use of animals. Eye irritation is still characterized using Draize methodology developed in 1944. The Draize rabbit eye test evaluates the effect of a test substance on eye tissues for a period of up to three weeks. This extended time period allows the evaluation of reversibility of any damage. Due to the ethical issues involved in the testing of animals, there is an effort to modify current practices in toxicology to Reduce, Refine and Replace the number of animals currently involved in product safety testing. Although there are several alternative methods to characterize aspects of eye irritation and damage, no established method can model recovery after injury as in a Draize test. Some regulatory classification methods of ocular irritancy depend upon the time for an ocular injury to completely heal (OECD, 1967; WHMIS, 1988; HMIS, 1996; EPA, 1997). We have developed an alternative assay that measures corneal damage and recovery for extended periods in excised porcine corneas. PorCORA, in combination with other assays to quantify the severity of the irritation (such as the BCOP (Bovine Corneal Opacity and Permeability test), MatTek Epi-Ocular and CAMVA (Chorioallantoic Membrane Vascular Assay), is an excellent candidate to replace the animal based ocular assays. Histopathology can also be used to further evaluate the depth of injury and amount of recovery.

## **METHODS AND MATERIALS**

### Long-term Culture of Excised Corneas.

Porcine eyes were excised at the abattoir and delivered in cooled Hank's Balanced Salt Solution (HBSS) with antibiotics. Corneas were transferred to a sterile field and visually inspected for defects. Corneas with defects were discarded. The eyes were disinfected by immersion in 1% povidone-iodine solution for 2 minutes, and rinsed immediately by immersion in sterile phosphate-buffered saline (PBS), then immersed for 15 minutes in 0.1% gentamicin in PBS. Corneas were excised from the eyes leaving a 2-5 mm rim of sclera for easier handling. The corneas were rinsed extensively in a series of pools of sterile HBSS. The corneas were re-examined and those with defects were discarded. Each excised cornea was suspended epithelial-side down over a 24-well plate. The wells of the plate were filled with enough HBSS to support the suspended corneas. Molten agar/gelatin/medium mixture was added to the endothelial corneal cavities a few drops at a time to ensure that the agar gelled directly to the endothelial cells. The cavities were filled with the mixture and allowed to cool at room temperature.

The corneas and supporting gel were inverted and transferred to large deep-well dishes and incubated at 37°C, 5% CO<sub>2</sub> and 90% relative humidity (RH) for approximately 24 hours prior to dosing. M199 media was supplemented with FBS, NaHCO<sub>3</sub>, glutamine, gentamicin, amphotericin B and penicillin/streptomycin. The media was added so as to cover the limbal-conjunctivae, but leaving the rounded corneal epithelium exposed to air. The cultures were placed on a modified rocker that briefly and periodically pivot the culture dishes from a horizontal position to approximately a 45° angle, causing a blink effect that moistened the epithelium with medium.

## ***METHODS AND MATERIALS (cont'd)***

### Dosing Procedures.

24 hours after the initiation of cultures, the culture media was removed from the dishes. Corneal surfaces were treated directly with 10 ul of 100% ethanol (EtOH), 1.0% benzalkonium chloride (BAK), 3% sodium dodecylsulphate (SDS), 10% hydrogen peroxide (NaOH) or phosphate buffered saline (PBS) for 5 minutes. Immediately following the five-minute exposure, the corneas were rinsed drop-wise with 2 ml of sterile PBS. Once the rinsing medium appeared free of test substance, the corneas were transferred to a new sterile dish and 40 ml of media was added to the plate. This volume was sufficient to cover the limbal-conjunctiva (leaving the epithelium exposed to air) and completely submerged the corneas when periodically tilted by the rocker. Culture media was replaced daily.

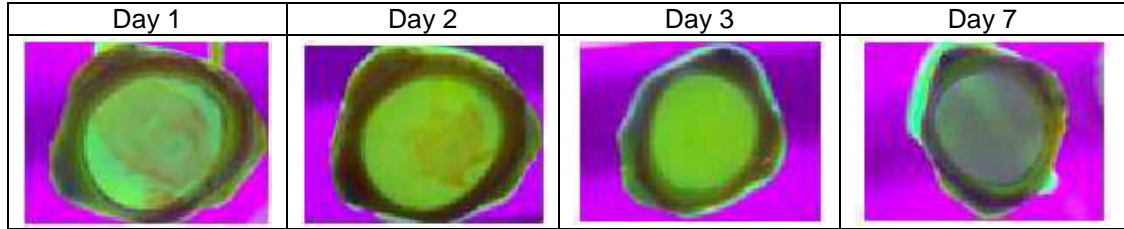
### Fluorescein Staining.

Corneal cultures were treated with ocular irritants as described above. One hour after treatment and once daily thereafter, the media was removed from the plate and 2% fluorescein in PBS was added drop-wise to the apical surface of the cornea under sterile conditions. Excess fluorescein was rinsed immediately with sterile PBS and media was replaced in the cultures. Fluorescein retention was visualized under UV illumination and scored. Corneas were restained and monitored repeatedly until there was no visible stain retention. Corneas were fixed in 10% neutral buffered formalin for at least twenty-four hours. Tissues were embedded, sectioned, and mounted onto slides for further analysis by Dr. Ray Brown, DVM. Normal histological evaluation of the slides included hematoxylin and eosin stain.

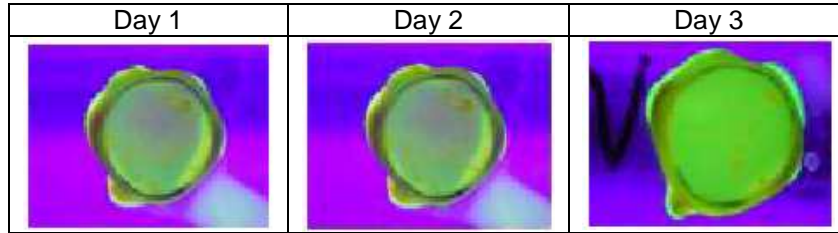
**The Porcine Corneal Opacity and Reversibility Assay**

**RESULTS**

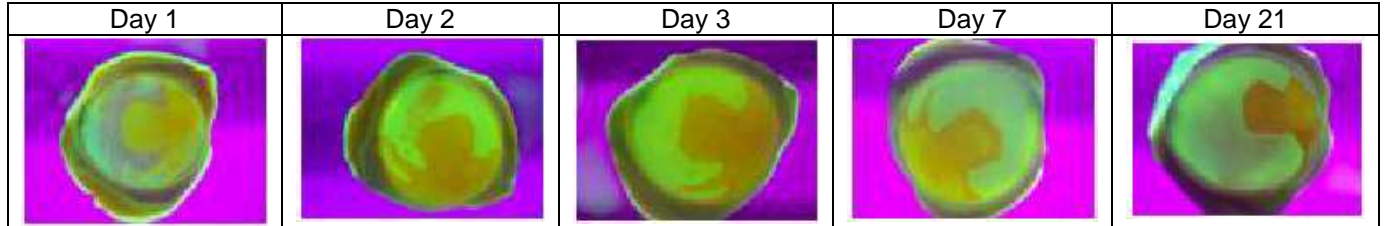
**PBS**



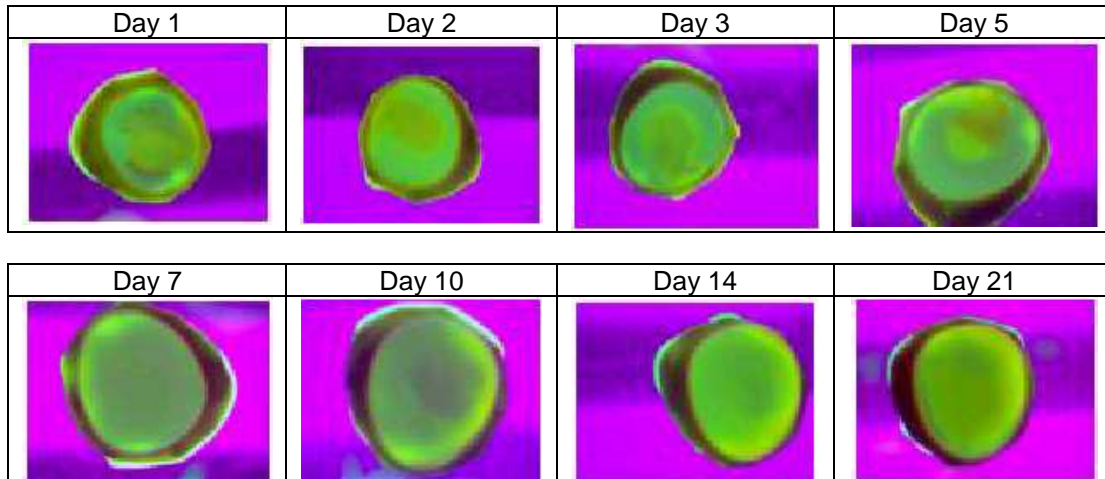
**3% SDS**



**10% NaOH**

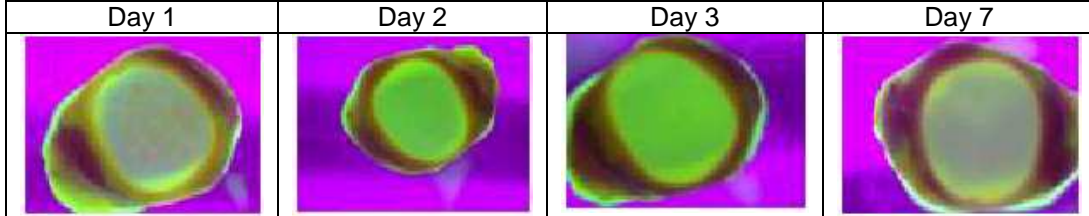


**1.0 % BAK**



**RESULTS (cont'd)**

**100% EtOH**



**PBS, Day 1**



**Untreated, Day 1**



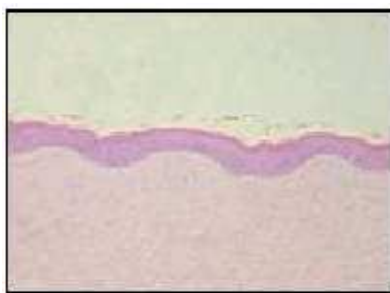
**3% SDS, Day 8**



**1% BAK, Day 21**



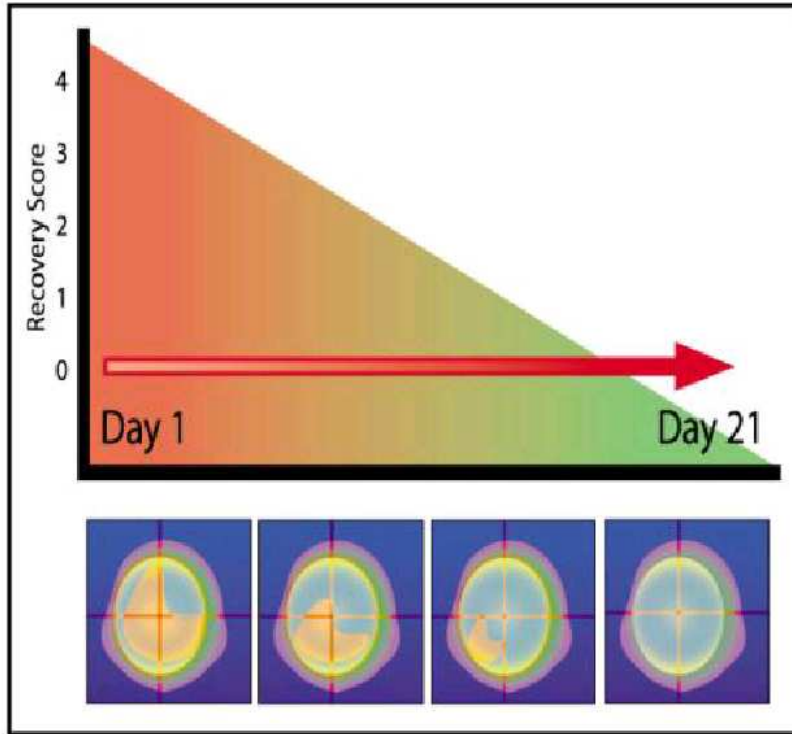
**100% EtOH, Day 11**



**10% NaOH, Day 21**



**RESULTS (cont'd)**



Score	Corneal Grading Criteria <sup>1</sup>
0	No Stain Retention.
1	One Quarter (or less) but not zero.
2	Greater than one quarter, but less than one half.
3	Greater than one half, but less than three quarters.
4	Greater than three quarters up to the whole area.

<sup>1</sup> Draize, J.H., et al., J. Pharm. Exp. Ther., 82:377-390, 1944.

## The Porcine Corneal Opacity and Reversibility Assay

### DISCUSSION

Corneas were cultured for up to 21 days with minimal morphological changes. All corneas were dosed with 10 µl of the test substance.

The chart below indicates recovery for each chemical tested. The results for PorCORA and ECETOC are the number of days until there was no more macroscopic opacity. The histology column indicates the last day that damage was observed microscopically.

#### Days to Clear

	PorCORA	ECETOC	Histology
EtOH (100%)	14	14	14
BAK (1.0%)	21	21	21
SLS (3.0%)	10	3-7	3
NaOH (10%)	21	21	21
PBS	3	NA	10-14

According to ECETOC data, 3.0% SLS should clear up between 3 – 7 days. Our corneas were cultured for 3 days. On Day 3, most corneas had minimal damage that was also indicated in the histology report. 10% NaOH was cultured for 21 days and retained the NaFL staining for all 21 days. The histology report indicated tissue damage deep into the stroma as well as the epithelial layer.

For corneas treated with 100% EtOH, the area of fluorescein staining decreased over time, thus indicating reversibility. In 10% NaOH treated corneas, the area of stain retention did not significantly decrease over time, indicating irreversible damage, however, corneas treated with 1% BAK showed some recovery, but significant damage was still observed at Day 19. Corneas treated with 3% SDS had no more fluorescein stain retention by Day 6, indicating recovery. No significant retention was visualized in corneas treated with PBS by Day 3. These results show PorCORA's potential to discriminate eye irritants that cause reversible damage from those that irreversibly damage corneal epithelia.

During development of this assay, we anticipated utilizing a software-driven analysis to measure damage to corneal tissues as the corneas remained in culture over 21 days. After careful consideration of the amount of labor and time needed for individual cornea evaluation with ImageJ (NIH Software), it was apparent that a more practical approach to assessment of irritation was necessary. The assessment of corneas and the assignment of a score was not only easier to implement, but also closer to the eye irritation studies using the area of cornea involved, similar to the Draize methodology.

An additional group of approximately thirty materials from the ECETOC *in vivo* eye irritation data bank will be evaluated in the next twelve months under a continuing SOT Colgate-Palmolive grant. Solid chemicals will be included for the first time in this validation. Favorable correlation between these two models would support the use of PorCORA as part of a suite of alternative eye irritation tests that would replace the Draize rabbit eye test.

There are several alternative methods to characterize aspects of eye irritation and damage, but no established method can model recovery after injury as in a Draize test. PorCORA was developed to fill this void by measuring corneal damage and recovery for extended periods in excised porcine corneas. Combined with other alternative assays, such as the HET-CAM or CAMVA for assessment of conjunctival injury and vascular damage and the BCOP for assessment of acute ocular irritation, PorCORA is the missing piece that allows evaluation of corneal healing.

## **CONCLUSIONS**

- ◆ PorCORA is an effective alternative ocular assay to measure reversibility.
- ◆ PorCORA can be used to evaluate the same tissues for up to 21 days.
- ◆ Results of fluorescein staining compare favorably to histopathological evaluation and ECETOC data.

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