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Resolving Severe/Corrosive Irritant Ocular Classifications using an Alternative Dual *Ex Vivo* Assay System

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ABSTRACT

The classification of severe ocular irritation as distinct from corrosion is traditionally determined by assessing reversibility of damage to the corneal epithelium over time in live rabbits (The Draize Test). Currently, there are no regulatory accepted alternative (non-rabbit) ocular irritation assays that are capable of assessing both corneal tissue damage and reversibility (healing). The Bovine Corneal Opacity and Permeability (BCOP) assay predicts a severe/corrosive classification if the *In Vitro* Irritation Score (IVIS) is > 55; however, materials in this category that may cause reversible (non-corrosive) damage in the Draize Rabbit Eye Test are over-classified. This over-classification can lead to inaccurate hazardous shipping labels and increased manufacturing and distribution costs. Here, we demonstrate the feasibility of a dual assay system for distinguishing corrosive materials from those merely moderately to severely irritating. Twenty-one chemicals with known EPA ocular toxicity classifications (based on rabbit data) were evaluated using the BCOP assay. Test substances with an IVIS ≥ 26 were to be considered moderately irritating to corrosive and were further assayed in the Porcine Cornea Opacity Reversibility Assay (PorCORA). The PorCORA uses excised porcine corneas that are maintained in culture for up to 21 days. Test substances are topically dosed on the corneal epithelium and assessed for damage by fluorescein stain retention, similarly to the Draize method. Test substances causing stain retention persisting until Day 21 were deemed corrosive (EPA Cat. I). If the stain retention cleared before Day 21, the test substance was classified a severe irritant (EPA Cat. II). Of the 21 chemicals tested, 6/6 Category I chemicals induced irreversible damage in the PorCORA, while ocular damage caused by 15/15 Category II and Category III **(IVIS < 20)** chemicals completely reversed by Day 21. This dual *ex vivo* assay system fulfills an unmet need under current ocular hazard regulatory test guidelines by providing a non-live-animal (*ex vivo*) approach to resolving the differences between corrosive (EPA Cat. I) and severely irritating (EPA Cat. II) chemicals.

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 a rabbit test (Draize *et al*.,1944), which evaluates the effects of a single exposure of the test substance on eye tissues for a period of up to three weeks. This extended period allows the evaluation of reversibility of damage. Due to ethical issues in animal testing, there is an effort to modify current practices in toxicology to reduce, refine and replace the number of animals used in product safety testing. Although several alternative methods exist to characterize aspects of eye irritation and damage, no established method can model recovery after injury as in a Draize test. Because 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, paired with other assays (such as the BCOP, EpiOcular™, and chorioallantoic membrane-based assays) that will quantify the severity of damage, is an excellent method to replace animal-based ocular assays. Histopathology can also be used to evaluate the depth of injury and amount of recovery.

EXPERIMENTAL METHODS

BCOP Method: Excised bovine corneas were mounted in specially designed chambers which provide separate anterior and posterior chambers. After an equilibration period, a pre-exposure determination of opacity using an OP-Kit® Opacitometer (ElectroDesigns, RIOM, France), was made for each cornea by measuring against two control corneas. Each treated cornea was scored in comparison to the blanks provided with the OP-Kit® Opacitometer. Following pre-exposure opacity determination, the test substances were applied to the epithelium of each cornea 0.75 ml of liquids or 0.75 ml of a 20% dilution of solids in MEM. Liquids were flushed from the cornea after 10 minutes and solids after 4 hours. Difference in light transmission between control and treated corneas were then determined using the OP-Kit®. Following this determination, a 0.4% sodium fluorescein solution (NaFl) (liquids) or a 0.5% NaFl solution (solids) was kept in contact with the corneal epithelium. After 90 minutes, the fluid from the posterior chamber was removed and the amount of NaFl which passed through the cornea was measured as the Optical Density, at 490 nm by spectrophotometric analysis. The *In Vitro* Irritation Score (IVIS) was calculated.

IVIS = Mean Opacity Score + [15 X (Mean Permeability Score)]

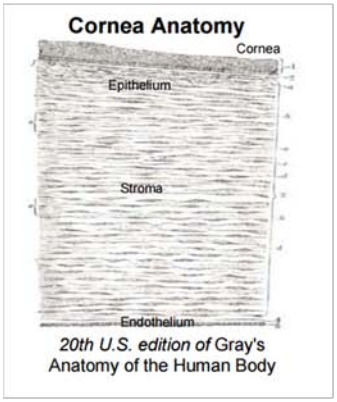
PorCORA Method: Long-term Culture of Excised Corneas. Porcine eyes were evaluated at the abattoir and shipped to the laboratory in cooled Hank's Balanced Salt Solution (HBSS) with antibiotics. Corneas were visually inspected for defects and those with defects were discarded. The eyes were disinfected by immersion in 1% povidone-iodine solution, and rinsed in sterile phosphate-buffered saline (PBS). Then the eyes were immersed for 15 minutes in PBS containing 0.1% gentamicin. Corneas were excised from the eyes leaving a 2-5 mm rim of sclera for handling. The corneas were rinsed extensively in a series of pools of sterile HBSS, re-examined, and those with defects were discarded. Each excised cornea was suspended epithelial-side down over a 24-well plate. Plate wells were filled with enough HBSS to support the suspended corneas. Molten agar/gelatin/medium mixture was added to the endothelial corneal cavities drop-wise 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_2, 90% relative humidity for approximately 24 hours prior to dosing. M199 media was supplemented with FBS, $NaHCO_3$, amphotericin B, and penicillin/streptomycin. The media was added to cover the limbal-conjunctiva, while leaving the rounded corneal epithelium exposed to air. The cultures were placed on a rocker modified to briefly and periodically pivot the culture dishes from horizontal position to an angle of approximately 45°, to moisten the epithelium.

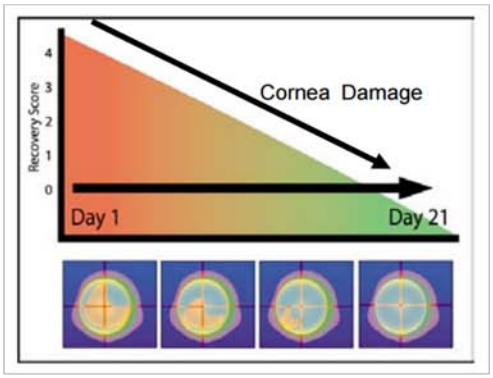
EXPERIMENTAL METHODS (continued)

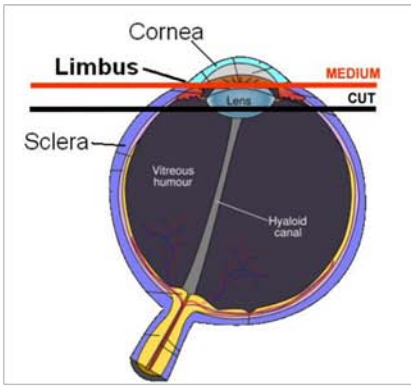
Dosing Procedures. Twenty-four hours after the initiation of cultures, the culture media was removed from the dishes. Corneal surfaces were treated directly with 10 µl of dPBS, 10% NaOH, 100% EtOH, or with 20 mg of solid test substances. The corneas were rinsed drop-wise with 2 ml of sterile PBS after 5 minutes of exposure. Once the corneal surface 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 covered 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. On Days 1, 2, 3, 7, 10, 14, and 21 after dosing, media was removed from the plate and 2% fluorescein in PBS was added to the apical surface of the cornea under sterile conditions. Excess fluorescein was rinsed immediately with sterile PBS. Fluorescein retention was visualized via a white light transilluminator.

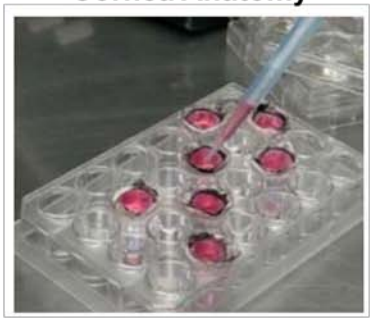
PROCEDURE







Cornea Anatomy

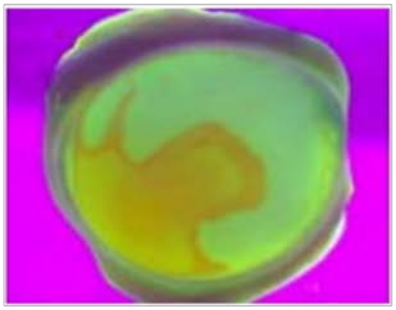


Corneas Filled with Agar / Gelatin Plug

21-Day Study: Cornea Damage Reversibility

Porcine Corneas - Fluorescein Staining

Cornea Excision



Fluorescein stain (orange) delineates damaged tissue

RESULTS

TABLE 1

Assay	Alternative Ocular Dual *ex vivo*			PorCORA		BCOP	
EPA Category	Cat I	Cat II	Cat III	Cat I	Cat II	Cat I & II	Cat III
Conditions	PDC > 21 OR IVIS > 65	IVIS > 6 AND PDC ≤ 21	IVIS ≤ 6 AND PDC ≤ 10	PDC > 21	PDC ≤ 21	Severe/ Corrosive (IVIS >55)	IVIS <20
Accuracy	100%	80%	80%	100%	100%	55%	75%
Sensitivity	100%	67%	67%	100%	100%	100%	55%
Specificity	100%	71%	86%	100%	100%	40%	100%

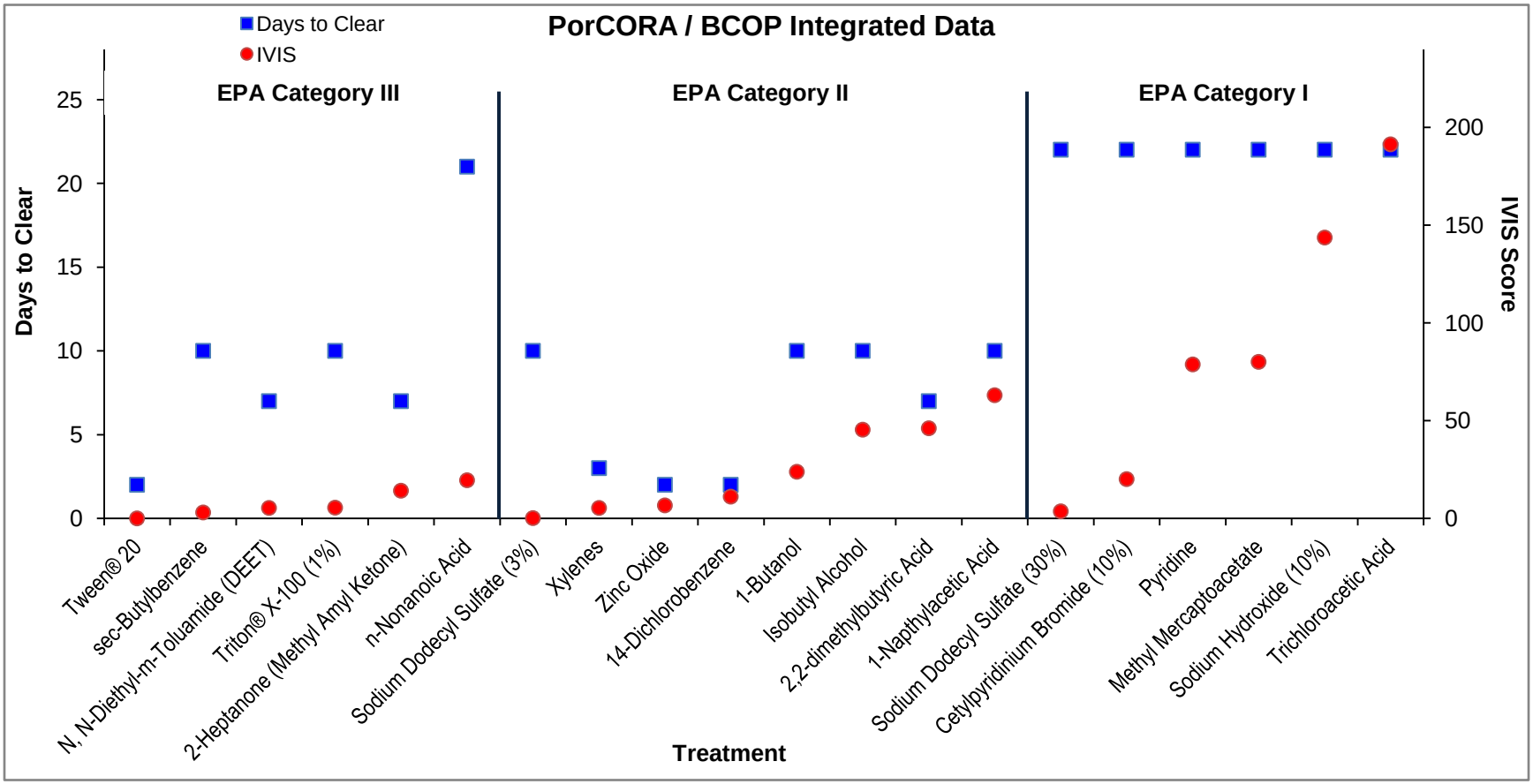
TABLE 2

Test Substance	EPA	BCOP IVIS	PorCORA Day Clear	PorCORA Result
Sodium Dodecyl Sulfate (30%)	I	3.54	>21	Irreversible
Cetylpyridinium Bromide (10%)	I	20.01	>21	Irreversible
Pyridine	I	78.77	>21	Irreversible
Methyl Mercaptoacetate	I	79.99	>21	Irreversible
Sodium Hydroxide (10%)	I	143.73	>21	Irreversible
Trichloroacetic Acid (30%)	I	191.38	>21	Irreversible
Sodium Dodecyl Sulfate (3%)	II	0.17	10	Reversible
Xylenes	II	5.24	3	Reversible
Zinc Oxide	II	6.56	2	Reversible
1,4-Dichlorobenzene	II	11.04	2	Reversible
1-Butanol	II	23.82	10	Reversible
Isobutyl Alcohol	II	45.37	10	Reversible
2,2-Dimethylbutyric Acid	II	46.11	7	Reversible
1-Napthylacetic Acid	II	63.00	10	Reversible
Tween® 20	III	-1.69	2	Reversible
sec-Butylbenzene	III	3.06	10	Reversible
N,N-Diethyl-m-Toluamide (DEET)	III	5.30	7	Reversible
Triton® X-100 (1%)	III	5.48	10	Reversible
2-Heptanone (Methyl Amyl Ketone)	III	14.17	7	Reversible
n-Nonanoic Acid	III	19.45	21	Reversible

An additional chemical (#21) was tested; it was EPA Cat. IV and correctly predicted; data not shown

RESULTS (continued)

Figure 1



CONCLUSIONS

- For the chemicals tested, both the dual assay and PorCORA resolve corrosive (EPA Category I) from severely irritating chemicals (EPA Category II) with 100% Accuracy and 100% Sensitivity.
- This Alternative Dual *ex vivo* Assay consisting of a combination of PorCORA and BCOP assays correctly discriminates EPA Categories II from III with an Accuracy of 80% of the tested chemicals.

REFERENCES

J. H. Draize, G. Woodard, and H. O. Calvery. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 82:377-390, 1944.

ACKNOWLEDGEMENT

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