Human relevant approaches to assess eye irritation: Overview and comparative analysis of test methods

Webinar Series on the Use of New Approach Methodologies (NAMs) in Risk Assessment

Acceptance and Use of In Vitro and Ex Vivo Eye Irritation Test Methods

3 November 2021

Hans Raabe, VP, COO
Overview

- Overview of the Draize eye irritation test
- Corneal physiology and overview of eye irritation events of concern
- Depth of Injury Concept
- Mechanistic relevance of available non-animal test methods for eye irritation assessment
How have we traditionally conducted testing?

Rabbit Draize Test

EPA I  EPA II  EPA III  EPA IV
GHS 1  GHS 2  Non-classified

Extreme  Severe  Moderate  Mild  Very Mild

Agricultural Ingredients and Products
Consumer Products
Industrial Chemicals
Cosmetics
Draize Rabbit Eye Test Method

- Primary *in vivo* method (developed in 1944)
- Accepted by numerous agencies globally
- Test substance placed in lower conjunctival sac
- Cornea, Iris, Conjunctiva evaluated
- Animal observed over 21 days for apical events
- Conservative/hazard assessment – given differences between human and rabbit eyes
- Subjectivity and Variability
Reproducibility of the Draize Eye Test

<table>
<thead>
<tr>
<th>Prior type</th>
<th>1</th>
<th>2A</th>
<th>2B</th>
<th>NC</th>
<th>Total</th>
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<tbody>
<tr>
<td>1</td>
<td>73%</td>
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• ECHA database evaluation (UN GHS categories)
• 491 substances with at least 2 Draize eye studies
• Conditional probabilities of Draize evaluations based on a previous test result
• Ex: 46 substances had multiple Draize test results that included at least one Category 1 response
Reproducibility of the Draize Eye Test

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Most reproducible results were at the extremes

- 94% likelihood to confirm a NC prediction
- 73% likelihood to confirm a severe (GHS 1) prediction
- 10.4% of Category 1 materials predicted as NC in a subsequent test

Luechtelfeld et al., ALTEX 33(2), 2016.
### Reproducibility of the Draize Eye Test

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- Category 2A and 2B more likely to be NC than Category 2 in a subsequent test
- Minimal discrimination between Category 2B and NC
  
  (77 of 86 substances with at least one GHS 2B result also have at least one NC prediction)
- NICEATM is now curating available rabbit eye test data to repeat this analysis (for GHS categories) and to also evaluate EPA categories

Luechtelfeld et al., ALTEX 33(2), 2016.
## Sources of Test Method Variability

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Draize Eye Test</th>
<th>Non animal methods</th>
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<tbody>
<tr>
<td><strong>Dosing</strong></td>
<td>Dose volume may overfill cul-de-sac</td>
<td>Precise control of dose applied (±2%)</td>
</tr>
<tr>
<td></td>
<td>Spill-out commonly reported</td>
<td>No loss of dose during exposure</td>
</tr>
<tr>
<td><strong>Exposure time</strong></td>
<td>Actual exposure times variable due to spill and animal blinking/pawing</td>
<td>Precise control of exposure period, and dose rinse-out timing</td>
</tr>
<tr>
<td><strong>Test system</strong></td>
<td>Animal behaviors (pawing, blinking, rubbing) may affect dosing and endpoint expression; Variability among replicates</td>
<td>Test system conditions tightly controlled between replicates</td>
</tr>
<tr>
<td><strong>Endpoints</strong></td>
<td>Subjective apical observations</td>
<td>Consistency among replicates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Objective machine-read data</td>
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Using mechanistic information and human relevance

Consider strengths and limitations of all available methods with respect to:

- their relevance to human ocular anatomy
- the mechanisms of eye irritation/corrosion in humans

To link to this article: [https://doi.org/10.1080/15569527.2021.1910291](https://doi.org/10.1080/15569527.2021.1910291)
Corneal Physiology and Tissue Functions

- Squamous Epithelium
- Upper Wing Layer
- Lower Wing Layer
- Basal Cell Layer
- Bowman’s Layer
- Anterior Stroma

- Epithelium
- Bowman’s Layer
- Stroma
- Descemet’s Membrane
- Endothelium
Epithelium

• Protection from xenobiotic and foreign material insults
• Provides an optical interface
• Maintains ideal stromal hydration state
• Bowman’s Layer and basal membrane provide structure and matrix for basal cell layer
• Basal cells – proliferative cells maintain basal layer matrix; are source for upward epithelial development and stratification; corneal wound healing through sheet migration and rapid proliferation
• Wing cells – intermediate cells expressing precursors of tight junctions; provide significant structural support
• Squamous cells – protective barrier / zona occludens
Stroma and Endothelium

- Stroma: makes up 80% of the corneal cross-section
- Optical clarity and light transmission functions
- Keratocytes – sparse but networked cells involved in maintenance of organized collagen fiber bundles
- Disorganized collagen fibers result in opacities
- Disruption of keratocytes induces inflammatory response to stimulate keratocyte proliferation, migration and reestablishment of collagen fibers
- Descemet’s Membrane provides structure and anchoring matrix for endothelial cell layer
- Endothelium – non-proliferative single cell layer maintains ideal stromal hydration
Depth of injury is predictive of the degree and duration of injury

“Regardless of the process leading to tissue damage, extent of initial injury is the principal, mechanistic factor determining the outcome of the ocular irritation”

Maurer et al, 2002
Damage Limited to the Superficial Conjunctival or Corneal Epithelium

CELLULAR RESPONSE
Upon exposure to the squamous epithelium, chemicals may induce
- cell stress responses
- release of chemokines and cytokines
- changes in relevant biomarkers
- breakdown of the tight junctions
- loss of cell to cell adhesion molecules
- changes in cell metabolism/respiration
- necrotic or apoptotic damage
- epithelial cell death

ORGAN RESPONSE
- increased corneal or conjunctival permeability/loss of barrier function
- susceptibility to xenobiotics
- conjunctival hyperemia and discharge
- swelling of the conjunctival tissues
- transient and mild corneal swelling
- sloughing of superficial epithelial cells
- induction of wound healing response and basal cell regeneration/turnover
- limited inflammatory response and neutrophil migration

Rapid recovery of the corneal and conjunctival tissues typical
CELLULAR RESPONSE
Upon penetration into the squamous epithelium and upper wing cells, or the conjunctival layers, chemicals may induce
- cell stress responses
- release of chemokines and cytokines
- changes in relevant biomarkers
- breakdown of the tight junctions
- damage to the desmosomes
- loss of cell to cell adhesion molecules
- changes in cell metabolism/respiration
- necrotic or apoptotic damage
- cell death

ORGAN RESPONSE
- increased corneal permeability/loss of barrier function
- Increased susceptibility to xenobiotics
- corneal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- sloughing of mid to lower epithelial tissues
- increased induction of wound healing response and basal cell regeneration/turnover
- increased potential for inflammatory response and neutrophil migration

Damage Limited to the Wing Cell Layer of the Epithelium

Recovery of the corneal and conjunctival tissues likely
Damage Into The Lower Wing Cell and Basal Cell Layers

**CELLULAR RESPONSE**

Upon penetration into the lower wing cells, and/or into the basal cell layers, chemicals may induce
- cell stress responses
- release of chemokines and cytokines
- loss of cell to cell adhesion and cell to basement membrane adhesion
- changes in cell metabolism/respiration
- necrotic or apoptotic damage
- cell death
- changes in basement membrane? *

**ORGAN RESPONSE**

- increased corneal permeability/loss of barrier function
- susceptibility to xenobiotics
- corneal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- sloughing of lower epithelial tissues
- increased induction of wound healing response and basal cell regeneration/tturnover increased
- inflammatory response and neutrophil migration

Recovery of the corneal tissues expected but prolonged.
* Basement membrane integrity is essential
Damage Into the Corneal Stroma

**CELLULAR RESPONSE**

Upon penetration through the epithelium into the corneal stroma, chemicals may induce
- cell stress responses
- retraction of keratocyte cell to cell network
- release of chemokines and cytokines, primarily IL-1α and TNFα
- induction of extracellular matrix / collagen synthesis
- activation of matrix metalloproteases result in loss of cell to cell adhesion and local tissue restructuring
- changes in cell metabolism/respiration
- necrotic or apoptotic damage

- Keratocyte cell death

**ORGAN RESPONSE**

- susceptibility to xenobiotics
- progressive ulceration and tissue necrosis
- notable stromal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- induction of wound healing response and basal cell regeneration/turnover
- recruitment of neutrophils / inflammatory response in stroma
- fibrosis resulting in disorganized collagens
- pannus and neovascularization
- loss of endothelium

Recovery becomes less likely with progression of the depth and degree of injuries
**Damage involving the Corneal Endothelium**

**CELLULAR RESPONSE**  
Upon penetration through the corneal epithelium and stroma, chemicals may induce  
- cell stress responses, leading to changes in cell adhesion  
- release of chemokines and cytokines  
- changes in relevant biomarkers  
- activation of matrix metalloproteases  
  result in loss of cell to cell adhesion and cell to Descemet's membrane adhesion  
- changes in cell metabolism/respiration  
- necrotic or apoptotic damage  
- **Endothelial cell death**

**ORGAN RESPONSE**  
- notable lower corneal swelling and swelling-related corneal opacity  
- loss of endothelium  
- loss of keratocytes in lower stroma

No meaningful recovery of cornea
Full thickness Cornea epithelium, stroma and endothelium

Available non-animal test methods model different portions of the cornea.

Its important to understand the relationship of those test methods to the various corneal layers to appreciate the mechanistic relevance in eye irritation assessments.
Isolated Chicken Eye Test

Bovine Corneal Opacity and Permeability Assay

Fluorescein Leakage Assay

Squamous epithelium

Short Time Exposure Assay

Full thickness corneal models

Bovine Corneal Opacity and Permeability Assay

Isolated Chicken Eye Test

Reconstructed Human Cornea-like Epithelium Test

Epithelium models
Squamous epithelium models

- Model the upper-most squamous layer
  - Relevant to tight junction and barrier disruption
  - Validated methods do not use human cells
- Cell viability / cell death can be determined
- Concentration-based prediction models correlate to severe and/or non-irritants
- Depth of injury not modeled
  - Mechanistically limited to discriminating non-irritants from irritants
Reconstructed human corneal epithelium models

- Model the stratified human corneal epithelium
- Cell viability / cell death are determined
- Cytokine release / expression can be measured
- Depth of injury into epithelium modeled
  - Discriminate among non, mild and moderate irritants
Full corneal thickness models

- Model all layers of the cornea
  - non-human species used; human eyes are rare
- Opacity, swelling, loss of barrier measured
- Histopathology can be very helpful for DOI
- Other endpoints possible (viability, cytokine)
- Model penetration and injury in all corneal layers
  - Discriminate among all categories
EPA OPP Non-animal Testing Strategy for Cleaning Products with Anti-Microbial Claims

Evaluate components

Oxidizing chemistry?
- Yes
  - BCOP
    - In vitro score
      - < 25: Category III
      - 25 < 75: Category II
      - ≥ 75: Category I

- No
  - Expected severe or moderate?
    - No
      - Water soluble?
        - No
          - No
            - No
              - No

    - Yes
      - EpiOcular

- Yes
  - Cytosensor
    - In vitro score
      - ≥ 75: Category I
      - 25 < 75: Category II
      - < 25: Category III

To distinguish Category I from II, conduct BCOP

Category IV
- ≥ 80 mg/ml
- ≥ 70 min

Category III
- ≥ 2 but < 80 mg/ml
- ≥ 4 but < 70 min

Category II
- < 2 mg/ml
- < 4 min
RhCE Test Method Overview
Measuring chemical-induced cell death

Tissue Treatment
Chemicals or formulations are applied without dilution to model real life exposures

Prepare aliquots for spectrophotometry

Tissue Rinsing
After exposure, tissues are rinsed, immersed in medium for 12 minutes, and then incubated for a post-treatment incubation

Post-treatment Expression Incubation

Isopropanol Extraction

MTT Reduction

25
MTT endpoint for cell cytotoxicity assessment

Extracted MTT is thoroughly mixed and transferred to a 96-well plate.

The 96-well plate/MTT-isopropanol samples are quantified using a microplate reader. Optical Density (OD) at 550 to 570 nm is measured.

$\text{OD}_{550}$ values are used to calculate relative viability values.

Viability is presented relative to negative control tissue values

\[
\% \text{ of Control} = \frac{\text{Test Material } \text{OD}_{550}}{\text{Negative Control } \text{OD}_{550}}
\]
Time-to-toxicity Concept in RhCE Models

US EPA Antimicrobial Cleaning Products (AMCP)
- To discriminate between EPA III and IV or identify EPA Cat I (without further testing)
- Multiple exposure time protocol
- Continuum of responses across eye irritation spectrum
- Also used in product development to create progressively milder/safer formulations
- Rank-order candidate formulations
  - Can include benchmarks for data interpretation

$ET_{50}$ (estimated time to reduce viability to 50% of control), plot relative viability over exposure time
Eye Irritation Test (EIT) Data Evaluation
OECD TG 492 for Eye Irritation

Uses a single fixed exposure time (liquids are treated for 30 minutes; solids for 6 hours)
  • Viability is assessed by MTT reduction, and the following prediction model applied

For Bottom-up strategy to identify GHS “No Category”
  • Viability > 60% - test chemical does not require labeling for eye irritation/serious eye damage (GHS No Cat)
  • Viability ≤ 60% - test chemical classified as requiring classification and labelling as an irritant
  • does not distinguish between GHS category 1 or 2 – further testing indicated

Overall Accuracy 80%
Sensitivity 96%
False Negative Rate 4%
Specificity 63%
False Positive Rate 37%

Assay performance when used to identify chemicals that do not induce either moderate or severe eye irritation or damage (GHS No Category)
Bovine Corneal Opacity and Permeability (BCOP) - Overview

Measuring changes in corneal opacity and loss of barrier function

Bovine corneas are mounted in corneal chambers with glass windows. Cultured in EMEM at 32°C

Initial opacity values determined using an opacitometer

Bovine eyes are obtained as a byproduct of meat production

No live animals used
• Treat test chemical
  – 10 minutes (liquids)
  – 4 hours (solids) 20% aqueous preparation
• Rinse / incubate (2 hours for liquids)
  (expression of toxic effects)
• Read post-treatment opacity
• Induction of opacity (up to 150+ units)
• Loss of corneal barrier function

measured by determining fluorescein permeation after 90 minutes (OD$_{490}$)
BCOP Prediction Models

In Vitro Score = Opacity + (15 x OD$_{490}$)

Prediction Model Developed by Merck* (non regulatory use)

<table>
<thead>
<tr>
<th>In Vitro Score</th>
<th>Predicted Irritation Potential</th>
</tr>
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<tbody>
<tr>
<td>≤ 25</td>
<td>Mild</td>
</tr>
<tr>
<td>25.1 – 55</td>
<td>Moderate</td>
</tr>
<tr>
<td>&gt; 55.1</td>
<td>Severe</td>
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Prediction Model per OECD TG 437 (for UN GHS classification and labeling)

<table>
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<tr>
<th>In Vitro Score</th>
<th>UN GHS</th>
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<tbody>
<tr>
<td>≤ 3</td>
<td>No Category</td>
</tr>
<tr>
<td>&gt;3 ≤ 55</td>
<td>No standalone prediction can be made</td>
</tr>
<tr>
<td>&gt; 55</td>
<td>Category 1</td>
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The assay provides a continuum of responses across the eye irritation spectrum from mild to severe

Histological Evaluation

Histopathology of progressive surfactant-induced corneal epithelial erosion and stromal swelling.

**Fig a.** Negative Control cornea showing intact epithelium and organized upper stroma.

**Fig b.** Loss of squamous and upper wing layers, results in increases in FL$_{490}$. 

- **Opacity** = 1.7
- FL OD$_{490}$ = 0.302
- IVIS = 6.2

**Fig c.** Complete loss of epithelium results in high FL$_{490}$. Marked stromal edema and disorganization results in modest opacity.

- **Opacity** = 7.7
- FL OD$_{490}$ = 2.540
- IVIS = 45.8
Assays should complement each other
(integrate mechanisms and evidence)

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**Rabbit Draize Test**

- **BCOP / ICE**
- **RhCE (Time-to-toxicity)**
- **RhCE EIT**
- **2D Cells**

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Thank You for Your Participation!

For more information on additional assays to address ocular irritation, please visit:

www.iivs.org