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Histopathology in the isolated chicken eye test and comparison of different stainings of the cornea

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ABSTRACT

The isolated chicken eye (ICE) test, developed at our Institute, is accepted by the OECD for identification of severe eye irritants. The OECD ICE Guideline (No. 438) encourages preservation of the treated eyes for possible histopathology of the cornea, which is believed to strengthen evidence of absence or presence of irritation and to help clarify borderline effects by assessment of the corneal Depth-of-Injury. Histopathology of the cornea in addition to the normal slit-lamp microscope assessment of corneal effects has already been performed routinely in ICE tests at our Institute, using two standard stainings (H&E and PAS). In this study, three other stainings (AZAN, EVG and Trichrome), more specific for collagen-rich membranes such as basement- and Bowman's membranes were examined with corneas exposed to four model compounds ranging from non- to severely irritating (corrosive). PAS appeared to be the superior staining method. Surprisingly, the well-known eye corrosive sodium hydroxide (NaOH, solid) did not visibly compromise the integrity of Bowman's or the basement membrane. Based on our experience, histopathology of the treated cornea is confirmative in relation to the standard assessment of eye irritation by slit-lamp observation in the ICE and in certain cases can help to evaluate borderline effects. Besides establishing the depth of injury, additional investigation of corneal limbal stem cell damage after chemical exposure might be appropriate to determine reversibility or irreversibility of eye effects.

1. Introduction

The isolated chicken eye (ICE) test, developed at our Institute, is one of two validated alternative methods accepted by the OECD for identification of severe irritants (OECD, 2009; Prinsen and Koëter, 1993; Prinsen, 1996). The OECD ICE Test Guideline No. 438 encourages preservation of the treated eyes in order to perform histopathology. Histopathology of the cornea is believed to strengthen evidence (absence or presence) of eye irritation and to help clarify borderline effects, especially those that are expected to be reversible or irreversible. The corneal Depth-of-Injury approach was introduced as an additional tool to more precisely determine the extent of initial corneal injury (Maurer et al., 2002; Jester, 2006; Jester et al., 2010; Scott et al., 2010). This approach is based on histopathology performed by light microscopy and *in vivo* confocal microscopy of rabbit corneas and by using biomarkers of cell death and viability. In general, it is believed that with non- or (mild) irritants the effects are limited to the epithelium of the cornea, while moderate to severe irritants also affect the deeper layers of the cornea such as stroma and endothelium. In their well thought-out publication, Maurer et al. recommended that any *ex vivo* or *in vitro* replacement of the rabbit eye irritation test should meet the following criteria: (1) assessment of injury should be three-dimensional, as injury is a three-dimensional process, (2) extent of injury may be assessed by extent of cytotoxicity within the cornea, (3) assessment of injury to epithelium, stroma and endothelium, (4) differentiate injury that is diffuse from injury that is focally extensive, and (5) assessment of injury at different time points. The ICE test including histopathology meets by far these pre-requisites and, moreover, histopathology of the cornea in addition to the normal slit-lamp microscope assessment of corneal effects is already performed in ICE tests at our Institute for more than ten years. Most of these tests are performed for sponsors and the results are confidential. However, an article concerning the performance of the ICE test with household cleaning products and including histopathology has been published (Schutte et al., 2009).

From the perspective of reversibility/irreversibility, damage not only to the epithelium, stroma and endothelium of the cornea, but also to the other structures present in the cornea, such as basement membrane or Bowman's- and to a lesser extent Descemet's

Abbreviations: AZAN, Azocarmine & Aniline; EU-CLP, European Union-Classification, Labelling and Packaging; EVG, Elastic Van Gieson; H&E, haematoxylin & eosin; ICE, isolated chicken eye; OECD, Organisation for Economic Co-operation and Development; PAS, periodic acid-schiff; TNO, Toegepast Natuurwetenschappelijk Onderzoek (Organization for Applied Scientific Research); Trichrome, Masson's trichrome; UN-GHS, United Nations-Globally Harmonised System.

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membranes, are important to evaluate. After all, extracellular matrices, such as the basement membrane, are the essential framework for wound healing (Wagoner, 1997).

Initially, the traditional haematoxylin & eosin (H&E) staining was used for the histopathological evaluation of the cornea. In an attempt to further improve the evaluation of the cornea, the Periodic Acid-Schiff (PAS) staining was applied. This turned out to provide a colour spectrum with more contrast. The various specific structures of the cornea became better discernable, which was helpful during microscopic evaluation of the corneal lesions. Therefore, it was decided to use PAS as the standard staining method in the ICE test from then on. Three other stainings, known to be more specific for collagen-rich membranes, were examined and compared to the H&E and PAS stainings. Chicken corneas were obtained from standard ICE tests with substances classified according to the usual classification systems, i.e. UN-GHS (2007) and EU-CLP (2008); (formerly EC criteria for labelling of EC, 1993). Three substances represented the categories non-classified (NC), irritating (Cat2 sub-divided into 2A and 2B in the UN-GHS classification system) and severely irritating (Cat1), and one represented a borderline case between Cat2 and Cat1. The focus of this investigation was on the quality and applicability of the different staining techniques in relation to the morphology of the various cell structures and the pathology. Publications on the *ex vivo* histopathology of the cornea in the open literature are scarce or the work is confidential. Statements with respect to the corneal Depth-of-Injury theory and histopathological evaluation of the cornea in the ICE test are based on the many years of experience in this field at our Institute.

2. Materials and methods

2.1. Test substances

The four materials and their regulatory classifications selected were:

- physiological saline; non-classified/negative control (Eurovet, Bladel, The Netherlands)
- liquid surfactant containing cleaning product; Category 2/2A/R36 (source: confidential)
- liquid surfactant containing cleaning product; Category 2/2A/R36 borderline Category 1/R41 (source: confidential)
- NaOH, solid (purity > 97%); Category 1/R41 (Sigma–Aldrich, Germany).

Chicken corneas treated with these materials were obtained from routinely performed ICE tests, which constituted assessment of corneal swelling, opacity, fluorescein retention by damaged epithelial cells using the Haag–Streit slit-lamp microscope over a 4 h period and after a 10 s treatment with the test substance (OECD TG 438, 2009; Prinsen and Koëter, 1993; Prinsen, 1996). On the basis of the severity (maximum mean score of three eyes) of the observed findings for corneal swelling, corneal opacity and fluorescein retention, the effects were divided into four classes, viz. I = no effect; II = slight effect; III = moderate effect; IV = severe effect. The final irritation classification is determined by the combination of the three classes obtained for the three endpoints (corneal swelling, corneal opacity and fluorescein retention) into predefined classification schemes (Prinsen and Koëter, 1993; Prinsen, 1996). In addition, to allow for numerical ranking and comparison an Irritation Index was calculated. This index is based on the addition of the maximum mean scores obtained for the parameters according to the following formula: Irritation Index = maximum mean corneal swelling + maximum mean opacity (×20) + mean fluorescein score (×20). The factor of 20 is included to give equal weight to the scores obtained for opacity and fluorescein retention in the index compared to the maximum swelling possible (ca. 60%).

Table 2
PAS staining of the cornea.

Step	Treatment of the slides
1	Deparaffinization
2	Periodic acid 0.5% for 10 min
3	Rinsing in water for 5 min
4	Rinsing in aqua dest. two times for 1 min
5	Schiff reagent for 30 min
6	Rinsing in water for 30 min
7	Haematoxyline for 30 s
8	Short rinse in water
9	Dehydration, xylol, malinol
10	Pertex mounting medium

2.2. Preservation of the cornea

Our experience with histopathology of the chicken cornea showed that specific fixatives, e.g. Davidsons, often suggested for fixation of eyes appeared not to be necessary. The treated corneas (eyes) were collected in a neutral aqueous phosphate-buffered 4% solution of formaldehyde at termination of the ICE test, i.e. 4 h after treatment. For that purpose, the eyes were first cut in half with a scalpel just behind the level of the lens and through the vitreous body. The half with the cornea and lens was placed in a glass container with approximately 20 ml of formalin. After fixation for at least 24 h, the tissue was trimmed with scissors in such a way that a thin piece containing the entire cornea and the adjacent sclera were embedded in paraffin wax. Longitudinal serial slides (sec-

Table 1
Slit-lamp examination: maximum mean scores for corneal swelling, opacity and fluorescein retention, irritation categories assigned, Irritation Index, and regulatory classifications.

Test material	Maximum mean score for			Irritation class[1]	Irritation Index[2]	Classifications (UN-GHS[3]/EU-CLP[4]/EC-standards[5])
	Swelling %	Opacity	Fluorescein retention			
Saline	0	0.0	0.0	I; I; I	0	NC/NC/NC
Cleaning product 1	11	2.2	2.0	II; III; III	94	Category 2A/Category 2/R36
Cleaning product 2	18[6]	3.0	2.0	II; IV; III	118	Category 2A[7]/Category 2[7]/R36[7]
NaOH, solid	44	4.0	3.0	IV; IV; IV	184	Category 1/Category 1/R41

[1] I = no effect; II = slight effect; III = moderate effect; IV = severe effect.
[2] Irritation Index = maximum mean corneal swelling + maximum mean opacity (×20) + mean fluorescein score (×20).
[3] NC = not classified; Category 2B = mild irritant; Category 2A = irritant; Category 1 = irreversible effects on the eye/serious damage to the eye.
[4] NC = not classified; Category 2 = Irritating to eyes; Category 1 = irreversible effects on the eye/serious damage to the eye.
[5] NC = not classified; R36 = Irritating to eyes; R41 = risk of serious damage to eyes. EC-standards as published in the Official Journal of the European Communities, L 110 A, Volume 36, 4 May 1993.
[6] Wrinkling of the epithelium.
[7] Considered borderline with Category 1 and R41 because of the severe opacity and wrinkling of the epithelium.