
PREVALIDATION OF A NEW *IN VITRO* RECONSTITUTED HUMAN CORNEA MODEL TO ASSESS THE EYE IRRITATING POTENTIAL OF CHEMICALS.

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INTRODUCTION

Adequate risk assessment in the context of ocular safety, is mainly related to the generation of quality toxicological data on the eye irritation potential of chemical, pharmaceutical and cosmetic products. The determination of acute eye irritation potential is therefore included in international regulatory requirements for the testing of chemicals. Eye irritation testing using laboratory animals have largely remained unchanged for many years. The Draize eye test (Draize *et al.*, 1944) became a governmentally endorsed method to evaluate the safety of materials meant for use in or around the eyes. The test procedure, which is described in OECD testing guideline 405 (OECD, 2002), involves a standardized protocol for instilling materials onto the cornea and conjunctiva of rabbits. However, advances in ocular toxicology are challenging the validity, precision, relevance, and need of the Draize eye test (Wilhelmus, 2001). Since in the Draize test the visual scoring procedure is based on a rather subjective evaluations, significant levels of variability were observed (Weil and Scala, 1971). Further, it is recognized that the response in the rabbit is not always predictive of that found in humans (Curren and Harbell, 2002). Taken together, both ethical and scientific reasons stimulated the development and validation of several alternative *in vitro* methods (Curren and Harbell, 1998; Worth and Balls 2002).

One of the available alternatives is the Human Corneal Epithelial (HCE) model of SkinEthic Laboratories (Nice, France). Besides the possibility of multiple endpoint analysis, this 3-dimensional *in vitro* model has the advantage of assessing both water-soluble and water-insoluble chemicals, pharmaceuticals and cosmetic ingredients and formulations.

OBJECTIVES

The main objective of this study was to evaluate the relevance (predictivity) and reliability (reproducibility) of the HCE model for ocular irritation of chemicals. This small-scale interlaboratory study (protocol optimization, transfer and performance), was based on the established ECVAM prevalidation scheme (Curren *et al.*, 1995; Worth and Balls, 2002). Both intra- and interlaboratory reproducibility was assessed, followed by the evaluation of the model's predictive ability to discriminate between irritant and non-irritant chemicals.

THE RECONSTITUTED HUMAN CORNEAL EPITHELIUM (HCE)

The reconstituted human corneal epithelial (HCE) model from SkinEthic (Nice, France) consists of immortalized human corneal epithelial cells cultured in chemically defined medium on an inert permeable polycarbonate insert at the air liquid interface. Histologically, the construct appeared as a multi-layered, stratified epithelium with an overall thickness of 60 microns, similar to that of the normal human corneal epithelium. Ultra-structurally, numerous intermediate filaments, desmosomal and hemidesmosomal junctions were seen and Western blot analysis showed the presence of the cornea-specific differentiation marker cytokeratin-3 (Nguyen *et al.*, 2003). Reconstituted human epithelial constructs to assess eye irritation, have been reported to allow routine screening of cosmetic test formulations (Doucet *et al.*, 1998; Doucet *et al.*, 1999).

EXPERIMENTAL APPROACH

Twenty reference test chemicals were chosen primarily on the basis of eye irritation classifications derived from the rabbit data described in the ECETOC database (ECETOC, 1998). The experimental set-up was based on the method described by Doucet *et al.* (1998) with modifications. In brief, chemicals were applied directly onto the surface of the epithelial culture for accurately timed exposure periods (10, 20, 30 and 60 minutes) and the induced cytotoxicity was monitored as a decreased MTT metabolism relative to the negative (vehicle) control cultures. The percentage viability was then calculated for each culture and linear regression analysis performed to identify the most relevant exposure time. Since the *in vivo* MMAS value is not considered to be the most excellent endpoint for evaluating *in vitro* scores (Prinsen, 1999), a classification prediction model was defined by using a specific cut-off value and compared to the calculated Globally Harmonized Classification System (GHS) for Chemicals (United Nations, 2003).

REPRODUCIBILITY

Twenty reference chemicals were tested (triplicate cultures) at 4 different time points. Correlation analyses showed that the 10 minutes treatment time is the most predictive and that the interlaboratory reproducibility was high (Table 1).

Table 1: Reproducibility of the viability after a 10 min treatment

	J&J PRD	Novartis	Pfizer	SkinEthic
J&J PRD	-	0.98	0.96	0.82
Novartis	0.91	-	0.97	0.87
Pfizer	0.93	0.92	-	0.84
SkinEthic	0.84	0.83	0.84	-

Pearson correlation coefficients (n=20): values above the dashes; slopes of the linear regression lines: values below the dashes. The correlation coefficients and slopes of the regression lines are significant ($p \leq 0.001$).

RELEVANCE

To evaluate the predictive capacity of the HCE model to classify test compounds as irritants or non-irritants, a **classification prediction model** (PM) was defined based on a viability cut-off value of 60%. When the viability after 10 min. exposure is $\geq 60\%$, the test chemical is considered non-irritant (NI).

Reproducibility of the PM: the κ -statistics is a measure of agreement between the classification according to the different laboratories. When 60% was selected as cut-off value there was a high level of agreement between the laboratories resulting in a perfect concordance between J&JPRD, Pfizer and Novartis. SkinEthic classified only 1 chemical different from the other laboratories.

Table 2: Agreement of the classifications between the different laboratories

	J&J PRD	Novartis	Pfizer	SkinEthic
J&J PRD	-	1.00 \pm 0.00	1.00 \pm 0.00	0.88 \pm 0.12
Novartis	-	-	1.00 \pm 0.00	0.88 \pm 0.12
Pfizer	-	-	-	0.88 \pm 0.12
SkinEthic	-	-	-	-

κ -statistics of PM60 (cut-off 60%) => significant at $p \leq 0.001$ (n = 20).

Relevance of the PM: the predictivity of the HCE model was evaluated using the GHS classification system. Although other PMs resulted in better specificities, the PM60 is more stable but showed relatively low specificity values (Table 3).

Table 3: performance of the prediction model using 60% viability as cut-off value

	SkinEthic	J&J PRD	Pfizer	Novartis
Sensitivity	100	100	100	100
Specificity	67	56	56	56
Positive prediction	79	73	73	73
Negative prediction	100	100	100	100
Accuracy	85	80	80	80

The reason for the low specificity might be related to the fact that the *in vivo* Draize test data reported in the ECETOC data bank (1998) underestimated some of the chemicals which were classified as irritants (I) by the HCE model. Indeed, when additional *in vivo* data are taken into account, the obtained *in vitro* classification could be confirmed and thus probably mimics better the real *in vivo* situation. For example, some of the compounds which were classified as irritant by the HCE model and as non-irritant in the ECETOC report were cited in the Hazard Substances Data bank (TOXNET®) as eye irritating to eyes. Consequently, higher specificity values can be calculated, without changing the sensitivity of the model. A detailed discussion of this approach and further comparisons with other *in vitro* models will be reported in Van Goethem *et al.* (in preparation).

CONCLUSIONS

Based on the obtained results and the analyses performed, following conclusions can be made:

- a standardized protocol was developed and will be made available in the coming publication;
- the *in vitro* HCE model provides a quality controlled, reproducible and rapid *in vitro* test system that can identify eye irritants and that has the potential to enter formal validation;
- the HCE model is characterized by a high level of reproducibility (reliability);
- it became clear that the selection of quality *in vivo* data is one of the major issues when evaluating the relevance of a new *in vitro* model;
- optimization of the prediction model might result in a more fine-tuned classification model; further protocol modifications (e.g. treatment time, diluted test chemicals; cytokine analysis; etc...) can increase the predicting capacity of the test system.

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