Evaluation of a bioartificial corneal equivalent for the prediction of the eye-irritation potential of chemicals

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Introduction

So far no single animal-free test system or appropriate testing battery is able to predict the eye-irritating potential of chemicals correctly for all 3 GHS categories. Based on a previously developed 3-dimensional corneal equivalent consisting of both epithelial and stromal cells [1, 2] we aimed at establishing a test method to reliably distinguish between eye-irritating and non-irritating substances in the first instance. In an interlaboratory trial test chemicals were applied topically on the surface of the corneal tissue and the viability of the tissues were monitored by an MTT assay. Three different prediction models were tested, and the threshold values which resulted in the best separation of the different irritation classes were determined by Receiver Operation Characteristics (ROC) analysis for each prediction model (PM).

Material and Methods

Model production:
Bioartificial corneal equivalents were produced in each laboratory independently according to an SOP. Briefly, SV40-immortalized keratocytes were embedded into a collagen gel. SV40-immortalized human corneal epithelial cells were seeded on top of the gel, and the construct was cultured for some days under submersed conditions. After the epithelial cells having reached confluency the model was lifted to the air-liquid interface. The epithelial cells gave rise to a multilayered epithelium (**figure 1**).

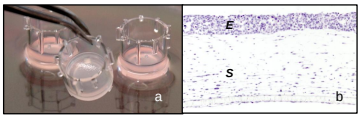

Figure 1a: Hemicornea model in a co-culture insert during the air-liquid interface culture phase. The collagen gel appears opaque. **1b:** Histological section through a fully developed corneal equivalent (H&E staining). E: epithelium; S: stroma

Test chemicals:
20 chemicals were tested under double-blinded conditions. The test chemicals comprised different chemical classes with different eye-irritating potentials according to the Draize eye irritation test (**table 1**).

No.	substance	CAS #	GHS class	state	chemical type	conc. tested
1	Ethylene glycol methyl ether acrylate	3121-61-7	1	liquid	acrylate	100%
2	para-fluoroaniline	371-40-40	1	liquid	aromatic	100%
3	Benzalkonium chloride	8001-54-5	1	liquid	cat. surfactant	1%
4	Imidazole	288-32-4	1	solid	heterocyclic	100%
5	Cyclohexanol	108-93-0	1	liquid	alcohol	100%
6	2-Methyl-1-pentanol	105-30-6	2	liquid	alcohol	100%
7	2,6 dichlorobenzoyl chloride	4659-45-4	2	liquid	acyl halides	100%
8	1-Octanol	111-87-5	2	liquid	alcohol	100%
9	Methyl cyanoacetate	105-34-0	2	liquid	acetate	100%
10	3-Chloropropionitrile	542-76-7	2	liquid	nitrile	100%
11	Dibenzyl phosphate	1623-08-1	2	solid	organo phosp.	100%
12	Ammonium nitrate	6484-52-2	2	solid	inorganic	100%
13	Cetyl pyridinium bromide	140-72-7	2	liquid	cat. surfactant	1%
14	3,3-Dimethylpentane	562-49-2	NI	solid	alkane	100%
15	3-Methoxy-1,2-propanediol	623-39-2	NI	liquid	alcohol	100%
16	1-Bromohexane	111-25-1	NI	liquid	brominated derivative	100%
17	Toluene	108-88-3	NI	liquid	aromatic	100%
18	Methyl amyl ketone	110-43-0	NI	liquid	ketone	100%
19	Trichloroacetic acid	76-03-9	NI	liquid	acid	3%
20	Ethylenglycol diethylether	629-14-1	NI	liquid	ether	100%

Table 1: List of the 20 test substances used to evaluate the hemicornea model. Indicated are the CAS No., the actual GHS class, the state, the chemical class and the concentration tested. Most of the reference substances were adopted from a selection recommended by the ECVAM.

Topical treatment:
A volume of 50 µl of each test substance (liquids and solids) was applied topically onto the surface of the corneal equivalents. For each chemical three independent runs with triplicate tissues were performed. Tissues were incubated for 10, 20 and 60 minutes, respectively, thoroughly washed with PBS and transferred to the MTT solution (1.5 ml; 1 mg/ml). After 3 hours incubation the formazan was extracted with 2 ml 2-propanol and the optical density (OD) determined in a spectrophotometer at λ = 550-600 nm.

Quality control:
PBS-treated tissues after 60 minutes incubation time served as a negative control (NC). A 0.3% TRITON X-100 solution was used as a batch control (BC). Both, NC and BC were determined for every singly tissue lot. Only batches with 1.2 >OD_{NC}> 0.5 and 0.5 >OD_{BC}>0.1 qualified for further analysis. Mean values with an SD >18% were excluded from the analysis. Prior to the testing all chemicals have been assessed for their intrinsic property to reduce the MTT reagent.

Calculations and prediction models:
For each sample the relative viability was calculated from the OD as percentage of the negative control (= 100% viability). From triplicate tissues the mean +/- standard deviation (SD) has been determined. The mean values for each substance and each time point were plotted against the rel. viability. The time-dependent course of viability could be described best with a 3-parametrical exponential function of the following type (see also **figure 2**):

$$Y = Y_0 + a * \exp^{(-bx)}$$

Y_0 (%): asymptote; a (%): amplitude; a + Y_0 = 100 %; b (1/min): decay constant

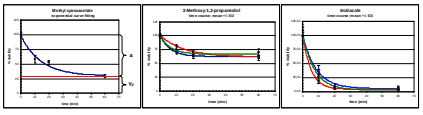

Figure 2 depicts representative examples of curve fittings for 3 chemicals with different eye-irritation potentials for 3 independent runs each. In the 1st diagram the main variables of the exponential function are explained.

Two different prediction models (PM) are based on the exponential regression:
1.) Y_0/b (%* min); 2.) ET50 (min)
A third PM assesses the relative viability after 60 minutes incubation time, compared to the NC.

Literature:
[1] Engelke et al. (2004) Altern. Lab. Anim. 32, 345-53
[2] Zorn-Kruppa et al. (2005) Altern Lab Anim, 33, 37-45

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Results and Discussion

All independently produced hemicornea models clearly matched the previously defined quality criteria. The mean optical densities after the MTT viability assay of the PBS-treated negative controls were situated in a range between 0.7 and 0.8 (**figure 3**). The TX-100-treated batch controls revealed values between 0.25 and 0.35. The mean OD's were similar in both labs.

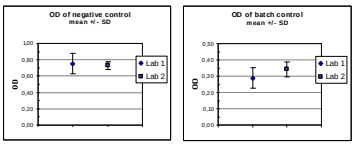

Figure 3: Intra- and interlaboratory variability of the optical densities of negative and positive controls after MTT determination of tissue viability. Indicated are the mean +/- SD values for at least 20 separate tissue batches per lab.

By applying a Receiver Operation Characteristics analysis on our data sets we identified those threshold values which resulted in the best distinction between non-classified substances and irritants for each of the 3 prediction models in each lab. On the basis of these thresholds a 2-way contingency table analysis was performed and the predictive capacity of the PMs evaluated in both labs (**table 2**).

prediction model	threshold	sensitivity %	specificity %	ppv %	npv %	accuracy %	wlc%	
Y0/b [%*min]	300	69	71	82	56	70	75	Lab 1
ET50 [min]	25	62	57	70	40	70	65	
cut-off 60' [% viability]	40	77	57	77	57	70	75	
prediction model	**threshold**	**sensitivity %**	**specificity %**	**ppv %**	**npv %**	**accuracy %**	**wlc%**	
Y0/b [%*min]	350	69	86	90	60	75	65	Lab 2
ET50 [min]	20	77	86	91	67	80	75	
cut-off 60' [% viability]	40	77	86	91	67	80	70	

Table 2: Predicitive capacity of the different PMs. ppv = positive prediction value; npv = negative prediction value; wlc = within laboratory concordance: concordance between the 3 independent runs in one lab each. Bold letters: best predictivity

For 2 PMs the optimal threshold values differed slightly between the 2 labs, whereas the threshold for the PM based on the viability after 60 minutes was identical. The inter-lab differences are a consequence of the incomplete selectivity of the test system, meaning that some of the data are located very close to the optimal threshold value. A test system is supposed to exhibit highest selectivity when the data points of the substances to be separated are located as far as possible away from the threshold at the opposite borders of the data range.

At lab 1 the highest sensitivity was achieved with the 60' viability prediction model and the highest specificity with the Y_0/b PM. At lab 2 specificity was equally high (86%) for all 3 PM, and the best sensitivity was achieved with the ET50 and the 60 minutes viability PMs.

In **table 3** the *in vivo- in vitro* correlation is presented for both labs and all prediction models. GHS category 1 substances were always classified correctly, whereas we faced some false negative results amidst the category 2 chemicals. Two chemicals were classified false negative by both labs: 2,6-dichlorobenzoyl chloride (7) is unsoluble in H_2O and therefore supposed not to penetrate the tissue model. 3-chloropropionitrile (10) was classified as non irritating according to the old EU system, and the effects on the rabbit eye are only temporarily.
Among the GHS category "not classified" the 3% trichloroacetic acid solution (19) was predicted false positive. With a pH value of ~ 1.5 the solution is highly acidic and thus potentially cytotoxic, although it did not affect cornea and iris in the Draize test.
Additionally some other chemicals were falsely predicted either in a single lab or with a single PM. The interlaboratory concordance varied between 95% and 70%. The highest correlation between the *in vitro* and the *in vivo* data was observed at lab 2 for the PMs based on the ET50 and the viability after 60 minutes (80% each).

No. RC	GHS	Y0/b (%min)		ET50 (min)		cut-off 60' (%)	
		Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
1	1	I	I	I	I	I	I
2	1	I	I	I	I	I	I
3	1	I	I	I	I	I	I
4	1	I	I	I	I	I	I
5	1	I	I	I	I	I	I
6	2	I	I	NI	I	I	I
7	2	NI	NI	NI	NI	NI	NI
8	2	I	I	I	I	I	I
9	2	NI	NI	NI	I	I	I
10	2	NI	NI	NI	NI	NI	NI
11	2	I	I	I	I	I	I
12	2	I	I	NI	I	NI	I
13	2	NI	NI	I	NI	I	NI
14	0	NI	NI	NI	NI	NI	NI
15	0	NI	NI	NI	NI	NI	NI
16	0	NI	NI	NI	NI	NI	NI
17	0	I	NI	I	NI	I	NI
18	0	NI	NI	NI	NI	NI	NI
19	0	I	I	I	I	I	I
20	0	NI	NI	I	NI	I	NI
in vivo- in vitro correlation		70%	75%	60%	80%	75%	80%
interlaboratory concordance		95%		70%		80%	

Table 3: *in vivo-in vitro* correlation for the 20 test substances, based on the optimal threshold values for each lab. Green: correctly classified; red: falsely classified; I = irritant; NI = non irritant
The interlaboratory concordance is a measure for the number of chemicals which are classified identically in the labs. The *in vivo-in vitro* correlation describes the percentage of correctly predicted chemicals with the Draize data as a benchmark.
No. of reference chemicals refer to **table 1.**

Conclusion

- The hemicornea can be reproducibly manufactured with high quality in independent labs, as revealed by low intra- and interlaboratory variability.
- Dependent on the prediction model the hemicornea is able to distinguish eye-irritating chemicals from non-eye-irritating substances with high sensitivity and specificity.
- Thus, the corneal equivalent is a promising tool to be included into an animal-free testing strategy for the prediction of the eye-irritating potential of chemicals.

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