Short Communication

In Vitro Eye Irritation Testing Using the Open Source Reconstructed Hemicornea – a Ring Trial

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Summary

The aim of the present ring trial was to test whether two new methodological approaches for the *in vitro* classification of eye irritating chemicals can be reliably transferred from the developers' laboratories to other sites.

Both test methods are based on the well-established open source reconstructed 3D hemicornea models. In the first approach, the initial depth of injury after chemical treatment in the hemicornea model is derived from the quantitative analysis of histological sections. In the second approach, tissue viability, as a measure for corneal damage after chemical treatment, is analyzed separately for epithelium and stroma of the hemicornea model. The three independent laboratories that participated in the ring trial produced their own hemicornea models according to the test producer's instructions, thus supporting the open source concept. A total of 9 chemicals with different physicochemical and eye-irritating properties were tested to assess the between-laboratory reproducibility (BLR), the predictive performance, as well as possible limitations of the test systems.

The BLR was 62.5% for the first and 100% for the second method. Both methods enabled to discriminate Cat. 1 chemicals from all non-Cat. 1 substances, which qualifies them to be used in a top-down approach. However, the selectivity between No Cat. and Cat. 2 chemicals still needs optimization.

Keywords: *in vitro* eye irritation testing, open source 3D hemicornea equivalent, depth of injury, ring trial, test performance

1 Introduction

In order to replace the Draize Eye Irritation Test (OECD TG 405) different approaches to develop animal-free alternative test methods have been pursued. Currently, five *in vitro* methods are available that have undergone formal validation and eventually gained regulatory acceptance (OECD TG 437, 438, 460, 491, 492). Four of them, the Isolated Chicken Eye test (ICE), the Bovine Corneal Opacity and Permeability assay (BCOP), the Short Time Exposure test (STE) and the Reconstructed human Cornea-like Epithelium test (RhCE) can be used to identify chemicals that do not require GHS classification (No Cat.) or that induce serious eye irritation (GHS Cat. 1). In contrast, the fluorescein leakage assay is only accepted for the classification of serious eye damage. Thus, classification of a chemical as a GHS Cat. 2 substance is based on the exclusion principle: if a chemical is not identified as a Cat. 1 substance or as a non-irritant for the eye, it is assigned to GHS Cat. 2.

In order to overcome this limitation in predictivity, two test methods, both based on a bioartificially produced 3D human corneal equivalent (hemicornea), were developed (Zorn-Kruppa et al., 2014; Bartok et al., 2015). Both methods predict the eye-irritation potential of chemicals for all 3 GHS categories within one test. Assay performance, reliability and predictivity of both methods have been demonstrated in the developers' laboratories with sets of reference chemicals representing all GHS categories and different physicochemical properties (Zorn-Kruppa et al., 2014; Bartok et al., 2015; Tandon et al., 2015).

The hemicornea model consists of a differentiated epithelium on top of a collagen gel populated with stromal keratocytes (Zorn-Kruppa et al., 2004, 2005; Engelke et al., 2013). With this complex tissue architecture mimicking epithelium and stroma

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of the human cornea, the hemicornea comprises the essential properties that had been recognized by an expert group to be a prerequisite for any test method to predict all GHS categories (Scott et al., 2010).

For the first method, the "depth of injury" (DOI) concept, based on extensive analyses performed by Jester and colleagues on isolated rabbit eyes (Jester et al., 2010), was adapted. The depth of tissue damage after chemical exposure is determined on histological sections of the treated hemicornea models and is a measure for the eye-irritating potential of the test item.

In the second method (collagen cell carrier – "CCC" approach), epithelium and stroma of the hemicornea are physically separated by an artificial collagen membrane inserted at the interface between both tissues before seeding of the epithelial cells. After chemical exposure of these modified models, the epithelium is stripped off the stroma, and cell viability is analyzed separately for each tissue. This procedure takes into account the observation that certain chemicals are known to damage epithelium and stroma differently (Jester et al., 1998a,b, 2001, 2006, 2010; Maurer et al., 2001, 2002).

The aim of the present ring trial is to test whether the protocols for both tests can be reliably transferred from the developers' laboratories to other sites to measure both the between-laboratory reproducibility (BLR) and the predictive performance.

2 Materials and methods

The protocols for the production of the hemicornea models and the performance of the eye irritation test have been previously published by Zorn-Kruppa et al. (2014) and Bartok et al. (2015). More details, including the list of test items used in this ring trial, can be found in the supplementary file (doi: 10.14573/altex.1610311s).

Three laboratories participated in the ring trial: Henkel AG & Co. KGaA (Lab 1), University Medical Center Hamburg-Eppendorf (Lab 2, developer lab for DOI approach), and Jacobs University Bremen (Lab 3, developer lab for CCC approach). The participating laboratories produced their own hemicornea models for both methodological approaches. The test chemicals were taken from the same batch and distributed blinded to all participants. Unless stated otherwise, all chemicals were tested in 3 independent runs of each method in each laboratory.

3 Results and discussion

3.1 DOI method

Nine chemicals were tested initially. However, lactic acid could not be classified due to tissue disintegration after substance application, concordantly observed in all three laboratories. Of the remaining 8 chemicals, 5 were tested with concordant results in all laboratories, while 3 were classified discordantly, leading to a between-laboratory reproducibility of 62.5%. The within-laboratory reproducibility was 62.5%, 87.5% and 62.5% for laboratories 1, 2, and 3, respectively (data not shown).

Two out of 3 chemicals classified as GHS Cat. 1 could be analyzed in this approach. As mentioned, the collagen gel dissolved after topical application of lactic acid (100%), which led to complete hemicorneal disintegration. Thus, the DOI could not be determined. The 2 other chemicals both also induced massive damage of the hemicorneal tissue. 1,2,4-triazole Na salt was concordantly predicted correctly as being corrosive to the eye (GHS Cat. 1) (Tab. 1). The test of methyl pentynol led to discordant results, because the DOI mean was below the 90% cut-off value in laboratory 3. Only two of 3 independent runs resulted in values > 90% DOI.

The results for the moderately eye-irritating chemicals (GHS Cat. 2) were quite heterogeneous with mean DOI values between 1 and 91%. Ethyl-2-methyl acetoacetate was concordantly predicted correctly in all laboratories. 4-carboxy benzaldehyde was clearly misclassified as a non-irritant in two laboratories and in the third laboratory, the mean DOI of 5.16% was only slightly above the cut-off value of 5%, which separates non-classified from Cat. 2 chemicals. As 4-carboxy benzaldehyde is a solid, the misclassification (false negative) probably resulted from its low solubility on the tissue surface. In contrast to liquid substances, most of the applied solid matter was not in cell contact and did not penetrate the tissue. The results for n-hexanol were discordant due to its prediction as a Cat. 1 chemical in laboratory 3. However, the respective mean DOI value is only marginally above the cut-off value of 90% which separates Cat. 1 and 2. Analysis of the data generated in 3 independent runs in Lab 3 revealed that 2 out of 3 runs resulted in DOI values above and one value below the 90% cut-off value. Thus, the individual results are also discordant, with the median characterizing n-hexanol as a Cat. 1 chemical.

Of the 3 chemicals that were classified as non-irritants in the Draize test, only dodecane was classified correctly in all 3 test laboratories. Topical exposure to n-butyl acetate and iso-propyl bromide resulted in 23-41% mean damage to the corneal tissue; hence, they were classified as GHS Cat. 2 chemicals (false positives). All 3 chemicals were classified concordantly in the laboratories.

The results of this part of the ring trial confirmed a tendency that was already observed during the developmental phase and protocol transfer of this method (Zorn-Kruppa et al., 2014). The DOI data for the Cat. 1 chemicals all fell into a narrow range of values above the 90% cut-off threshold, which identified them as corrosive to the eye. In contrast, the DOI values for the Cat. 2 chemicals varied over a broad range, a fact that is also reflected in the prediction model.

This study reveals limits with regard to the applicability of chemicals that disintegrate the tissue structure, i.e., pH-extreme chemicals like acid and alkaline solutions are apparently not compatible with the hemicornea model, which is based on a collagen gel. The collagen gel liquefies upon exposure to an acid and hence cannot be fixed and stained for further analysis. A similar effect was observed with aqueous SDS solutions, which also disintegrate the tissue structure (data not shown). Thus, it must be determined whether test substances fall into the applicability domain of the method. A clear advantage of

Tab. 1: Depth of Injury (DOI) in hemicornea models after topical exposure to chemicals of different eye-irritation potentials

Chemical	*In vivo*	Lab 1		Lab 2		Lab 3		BLR
	GHS cat.	DOI (%)	*In vitro* class	DOI (%)	*In vitro* class	DOI (%)	*In vitro* class	
Methyl pentynol	Cat. 1	94.02 ± 5.87	Cat. 1	95.97 ± 2.84	Cat. 1	89.88 ± 3.19	Cat. 2	dis
1,2,4-Triazole Na salt	Cat. 1	99.20 ± 0.70	Cat. 1	99.98 ± 0.02	Cat. 1	99.69 ± 0.12	Cat. 1	con
Lactic acid (100%)	Cat. 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	con
4-Carboxybenzaldehyde	Cat. 2A	3.48 ± 2.28	No Cat.	5.16 ± 2.23	Cat. 2	1.98 ± 0.67	No Cat.	dis
n-Hexanol	Cat. 2A	78.02 ± 1.72	Cat. 2	75.83 ± 11.11	Cat. 2	90.30 ± 5.26	Cat. 1	dis
Ethyl-2-methyl acetoacetate	Cat. 2B	8.94 ± 4.41	Cat. 2	26.13 ± 5.88	Cat. 2	9.13 ± 11.03	Cat. 2	con
Dodecane	No Cat.	1.40 ± 0.28	No Cat.	0.14 ± 0.09	No Cat.	1.06 ± 0.08	No Cat.	con
n-Butyl acetate	No Cat.	23.72 ± 9.48	Cat. 2	36.74 ± 1.54	Cat. 2	25.90 ± 2.52	Cat. 2	con
iso-Propyl bromide	No Cat.	22.58 ± 6.81	Cat. 2	30.29 ± 1.77	Cat. 2	40.61 ± 2.96	Cat. 2	con

Mean DOI values ± SD for 3 independent test runs of 3 hemicornea tissues each are shown for all laboratories. For every laboratory, the resulting GHS classification according to the prediction model is indicated. No Cat. – not classified (no irritation to the eye); Cat. 2 – moderately irritating to the eye; Cat. 1 – seriously irritating to the eye; n.d. – not determined. The values highlighted in grey indicate the false predictions. The BLR of test results for every chemical is mentioned (dis – discordant results; con - concordant results) as well as the *in vitro* classification based on the majority of results achieved in the 3 laboratories. Dodecane was tested only twice in Lab 1.

Tab. 2: Relative viability data generated with the CCC method (epithelium and stroma separated after topical exposure)

Chemical	*In vivo* GHS category	Viability (%) ± SD						*In vitro* classification (identical in all labs)
		Epithelium			Stroma			
		Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
Methyl pentynol	Cat. 1	1.09 ± 0.26	1.94 ± 0.27	1.92 ± 0.68	17.78 ± 10.19	18.17 ± 2.22	16.36 ± 4.51	Cat. 1
1,2,4-Triazole Na salt	Cat. 1	2.67 ± 0.17	3.68 ± 0.73	3.82 ± 0.24	8.23 ± 1.24	19.01 ± 2.16	26.12 ± 12.76	Cat. 1
Lactic acid (100%)	Cat. 1	1.81 ± 0.53	2.65 ± 0.30	3.04 ± 1.54	1.26 ± 0.86	0.50 ± 0.15	2.23 ± 0.76	Cat. 1
4-Carboxybenzaldehyde	Cat. 2A	55.07 ± 26.22	59.52 ± 6.26	69.51 ± 14.61	76.41 ± 26.22	99.76 ± 9.34	105.31 ± 26.52	No Cat.
n-Hexanol	Cat. 2A	1.38 ± 0.71	2.28 ± 0.60	2.84 ± 0.27	38.94 ± 13.76	35.72 ± 11.82	67.83 ± 3.57	Cat. 2
Ethyl-2-methyl acetoacetate	Cat. 2B	6.48 ± 6.76	2.24 ± 0.58	5.20 ± 3.22	68.42 ± 29.25	78.14 ± 9.47	83.35 ± 8.57	Cat. 2
Dodecane	No Cat.	79.59 ± 31.66	75.13 ± 6.03	107.10 ± 26.63	83.72 ± 25.66	97.49 ± 5.20	96.60 ± 1576	No Cat.
n-Butyl acetate	No Cat.	1.76 ± 1.53	2.08 ± 0.84	2.05 ± 0.29	50.02 ± 20.07	68.05 ± 21.67	81.10 ± 17.47	Cat. 2
iso-Propyl bromide	No Cat.	4.11 ± 3.21	2.09 ± 0.40	2.83 ± 0.56	35.90 ± 2.44	36.71 ± 3.43	36.81 ± 5.81	Cat. 2

The columns show the mean values of relative tissue viability for three independent experiments ± SD. Each test run was performed with 3 hemicornea models. In the right column the *in vitro* classification, based on the majority of classifications from the 3 labs, is indicated: No Cat. – not classified (no irritation to the eye); Cat. 2 – moderately irritating to the eye; Cat. 1 – seriously irritating to the eye. Relative tissue viability is calculated related to the respective negative control (100% tissue viability). The fields highlighted in grey indicate false negative or false positive results as compared to the *in vivo* classification, which were common to all labs. Ethyl-2-methyl acetoacetate was tested only twice at JU Bremen.

the DOI method is that it provides information on the mode of action of a given chemical in the corneal tissue, since it is based on histological methods.

Furthermore, it is noteworthy that this method achieved the best results of the study in the developer's lab (Lab 2), indicating that the method requires good technical skills.

3.2 CCC method

All 9 chemicals were concordantly classified by the CCC method in all participating laboratories, which corresponds with a between-laboratory reproducibility of 100%.

All chemicals classified as GHS Cat. 1 by the Draize test were predicted correctly in all 3 laboratories (Tab. 2). Relative viabilities were clearly below the cut-off thresholds of 15% and 35% for the epithelium and the stromal compartment, respectively. In contrast to the DOI method, the eye-irritating potential of lactic acid could be determined using the CCC approach, because the epithelial part could be removed from the stroma and transferred to another well while the stroma remained in the insert. Cell viability could be determined in both compartments.

Two out of 3 GHS Cat. 2 chemicals were predicted correctly, with mean epithelial viability values below 7%. Ethyl-2-methyl acetoacetate was classified as Cat. 2 with relatively high stromal tissue viabilities. This chemical is classified as GHS Cat. 2B and the *in vitro* result reflects its lower *in vivo* eye irritating potential. 4-carboxy benzaldehyde was misclassified as a non-irritant in all test laboratories, based on both high epithelial and stromal viability. Poor solubility and hence poor bioavailability are considered to be responsible for this result in the CCC test, as described in the DOI test.

Of the 3 non-irritating chemicals only dodecane was predicted correctly in all laboratories. In contrast, both n-butyl acetate and iso-propyl bromide were classified as GHS Cat. 2 chemicals. Both chemicals resulted in very low epithelial viabilities below 5%, and for iso-propyl bromide even the mean stromal viabilities of about 36-37% were very close to the 35% cut-off value, which distinguishes Cat. 1 and 2.

The results of the eye irritation test conducted with the CCC method correspond with those generated with the DOI approach. GHS Cat. 1 chemicals were all predicted correctly, characterized by low epithelial and stromal relative tissue viability. The selectivity between Cat. 1 and Cat. 2 chemicals was good, and no Cat. 2 or non-irritant chemical was overpredicted as Cat.1. These observations confirm the outcome from previous studies conducted on hemicornea tissues. In contrast, the discriminatory power between Cat. 2 and No Cat. chemicals is still too low.

4 Conclusions

Irrespective of whether the relative viability after topical treatment with chemicals was determined in an MTT assay of the whole tissue (Engelke et al., 2013), or whether sets of chemicals different from those used in the current study were assessed with the CCC and DOI methods, respectively (Zorn-Kruppa et al., 2014; Tandon et al., 2015; Bartok et al., 2015), the GHS Cat. 1 chemicals were always clearly separated from the other chemicals. Thus, both hemicornea-based test methods presented in this paper are suited to be used in a top-down approach to single out Cat. 1 chemicals with high reliability, a condition already previously requested by an EURL-ECVAM expert team in 2005 (Scott et al., 2010). The 2 non-irritating chemicals that had been classified as false positives and one Cat. 2 chemical that was misclassified as a non-irritant generated identical results in both methods.

In conclusion, the ring trial presented here shows that both hemicornea-based *in vitro* methods to assess the eye irritation potential of chemicals can be successfully transferred to other laboratories. However, a lower between-laboratory reproducibility was observed for the DOI method. This difference could be attributed to the high complexity of the DOI method, requiring good technical skills, and to some borderline values in the close vicinity of the respective cut-off values of the prediction model (e.g., for the Cat. 1 chemical methyl pentynol). The predictive capacities were comparable for both assays and thus confirmed results from previous studies conducted with the hemicornea (Engelke et al., 2013; Bartok et al, 2015; Tandon et al., 2015; Zorn-Kruppa et al., 2014), whereas the selectivity of both assays must be optimized.

Both methods presented in this paper repeatedly revealed their strength to clearly distinguish Cat. 1 chemicals from all non-Cat.1 substances. Thus, they could be used in a top-down approach to identify those chemicals leading to severe eye damage (Scott et al., 2010). In addition, these methods are both open source, meaning that all protocols underlying tissue production and assay performance have been made publicly available for the indicated purpose without any legal and intellectual property restrictions, given that the predefined quality criteria are met.

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Conflict of interest

The authors declare that they have no conflict of interests.

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