



Mewes et al.:

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

Supplementary Data

Materials and methods

Hemicornea culture

Depth of Injury (DOI) method

The hemicornea tissues were prepared as described in detail in Zorn-Kruppa et al., 2014. Briefly, immortalized human corneal keratocytes (HCK, Zorn-Kruppa, 2005) were embedded into a collagen gel which was cast into co-culture inserts of 10 mm diameter. After gelation, immortalized human corneal epithelial cells (HCE, Araki-Sasaki, 1995) were added on top of the gel. The models were cultured under submersed conditions for 5 days and then lifted to the air-liquid interface for another 7 days.

CCC method

The production of the hemicornea tissues for the CCC method was described in detail in Bartok et al. (2015). Briefly, immortalized human corneal keratocytes (HCK, Zorn-Kruppa, 2005) were embedded into a collagen gel which was cast into co-culture inserts of 10 mm diameter. In order to separate epithelium and stroma after chemical treatment, a collagenous membrane (Collagen Cell Carrier, Viscofan, Weinheim, Germany) was placed onto the surface of the just polymerized collagen gel. Then the HCE cells (HCE, Araki-Sasaki, 1995) were seeded onto the CCC membrane. The models were cultured under submersed conditions for 5 days and then lifted to the air-liquid interface for another 7 days.

Test protocol for the DOI method

For the DOI approach according to Zorn-Kruppa et al. (2014), 50 μ l or 50 mg, respectively, of the test items were topically applied onto the hemicornea tissues. After a 60-min exposure time at room temperature, the remaining chemicals were thoroughly rinsed off the surface with phosphate-buffered saline (PBS). Then the tissues were transferred into 1.5 ml MTT solution (1 mg/ml) and incubated for 2 h at 37°C and 5% CO₂. Sucrose solution was added to a final concentration of 20% and incubation

continued for another 60 min. After this 3 h incubation period the tissues were removed from the inserts, transferred into cryomolds and embedded in cryomatrix embedding resin (e.g., Thermo Scientific, UK). The tissues were then placed at 4°C for 30 min and afterwards frozen in the gaseous phase above liquid nitrogen. The frozen tissues were stored at -20°C prior to cryosectioning.

The cornea models were cut into sections of 30 μ m thickness with a cryomicrotome at a chamber temperature of -23 to -25°C and object temperature of -19°C to -21°C. From each sample 3 sections were cut from the center of the models and transferred to glass slides, where they were preserved in Fluoromount G medium (e.g., Southern Biotech, USA).

For the determination of the DOI the light microscopical images of the sections were processed with ImageJ freeware (Schneider, 2012). The total cross-sectional lengths and the formazan-stained tissue lengths were measured 5 times per section. The relative DOI (rMTT-DOI) was calculated as the ratio of the non-viable tissue thickness, where no formazan staining was present, to the total tissue thickness in percent.

The prediction model is based on two rDOI cut-off values: test chemicals that induce rMTT-DOI of $\leq 5\%$ were classified as UN GHS No Category; test chemicals that induce rMTT-DOI of $\geq 90\%$ were categorized as UN GHS Category 1; all test chemicals with an intermediate rMTT-DOI were classified as UN GHS Category 2.

Test protocol for the CCC method

For the CCC method according to Bartok et al. (2015), 50 μ l or 50 mg, respectively, of the test items were topically applied onto the epithelium in an O-ring. After exposure, the tissues were washed intensively with PBS and the O-rings were removed from the tissues. For the MTT viability assay the CCC membrane with the epithelial layer was separated from the stroma with forceps. The epithelial and stromal parts were transferred into individual wells of a 24-well plate in order to assess stroma and epithelium individually.



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To determine tissue viability, 1.5 ml MTT solution (1 mg/ml) was added to each tissue. After 3 h incubation at 37°C, the MTT solution was removed and the tissues were transferred into 1.5 ml 2-propanol for formazan extraction. The formazan content of the extract was then determined via its optical density (OD) in a spectrophotometer. Relative tissue viability was calculated from the OD as a percentage of the negative control (equals 100% viability) for each sample.

The prediction model is based on the relative viabilities (%) from both the epithelium and the stroma after chemical treatment. The differentiation of all GHS categories of tested chemicals was based on appropriate viability cut-off values set for both epithelium and stroma. According to Bartok et al. (2015), a chemical with the epithelial and stromal viability of

> 15% and > 35%, respectively, was classified as UN GHS No Category, a chemical with < 15% epithelial viability and > 35% stromal viability was categorized as UN GHS Category 2 and a chemical with < 35% stromal viability was classified as UN GHS Category 1.

Chemicals selection

The test chemicals were selected according to the following criteria: i) they must cover all 3 GHS categories, ii) they must belong to different chemical classes with different reactive groups, III) their *in vivo* classification is based on different effects on the ocular tissues, iv) they were not used in the training set, and v) they must be commercially available. The selection is listed in Table S1:

Tab. S1: Chemicals selected for this study

Chemical name	CAS-No.	Phys. state	Purity	Classification	Data source
Methyl pentynol	77-75-8	liquid	98%	Cat. 1	Spielmann et al., 1996
1,2,4-Triazole Na salt	41253-21-8	solid	90%	Cat. 1	ECETOC
Lactic acid (100%)	50-21-5	liquid	90%	Cat. 1	ICCVAM
4-Carboxybenzaldehyde	619-66-9	solid	97%	Cat. 2A	ECETOC
n-Hexanol	111-27-3	liquid	≥ 99.5%	Cat. 2A	ECETOC
Ethyl-2-methyl acetoacetate	609-14-3	liquid	95%	Cat. 2B	ECETOC
n-Butyl acetate	123-86-4	liquid	> 99%	No Cat.	ECETOC
iso-Propyl bromide	75-26-3	liquid	99%	No Cat.	ECETOC
Dodecane	112-40-3	liquid	> 90%	No Cat.	ECETOC

No Cat. – not classified (no irritation to the eye); Cat. 2 – moderately irritating to the eye; Cat. 1 – seriously irritating to the eye.

References

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