Toxicology in Vitro 29 (2015) 72–80

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Development of an *in vitro* ocular test system for the prediction of all three GHS categories

M. Bartok [a,*], D. Gabel [a], M. Zorn-Kruppa [b], M. Engelke [a]

[a] School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany
[b] Department of Dermatology and Venerology, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany

ARTICLE INFO

Article history:
Received 3 July 2014
Accepted 5 September 2014
Available online 27 September 2014

Keywords:
Eye irritation
Human hemi-cornea model
Prediction model
Predictive capacity
GHS classification

ABSTRACT

In the present study we considered a new approach that allows the individual quantification of damages induced in the epithelium and stroma of an *in vitro* hemi-cornea model after chemical treatment. We aimed at a stand-alone test system for classification according to the classification of the globally harmonized system of classification and labelling of chemicals (GHS).

We have modified a previously developed 3D hemi-cornea model by the insertion of a collagen membrane between epithelium and stroma. This membrane allows the separation and independent assessment of these compartments after topical exposure to potential eye irritants. The cell viability quantified by MTT assay was used as the toxicological endpoint. The prediction model based on the results obtained from 30 test chemicals uses a single exposure period and the combination of cut-off values in tissue viability from both epithelium and stroma.

The *in vitro–in vivo* concordance of the test system is 77%. All of the GHS category 1, 80% of the GHS category 2 and 50% of the GHS not categorized chemicals are predicted correctly. In conclusion, the test system predicts and discriminates GHS category 1 and GHS category 2 chemicals, but is over-predictive for GHS not categorized materials.

1. Introduction

In order to ensure consumer and environmental protection, chemicals and raw materials are subjected to extensive toxicological assessment according to the specific legislation regarding different industries and countries. The assessment of the eye-irritating potential of substances is an essential part of the toxicological examinations. To date, the rabbit Draize eye irritation test (Draize et al., 1944) is still the only OECD-approved test for the determination of the whole range of eye irritating effects from mild to severe (OECD, 2002). However, the Draize eye test has been increasingly criticized due to its lack of reproducibility, overestimation of human responses, and cruelty to animals (Weil and Scala, 1971).

In the past, a number of new *in vitro* methods have been developed in order to replace the Draize test. One group of test systems is based on isolated animal eyes, e.g. the Bovine Corneal Opacity and Permeation (BCOP) test and the Isolated Chicken Eye (ICE) test (Gautheron et al., 1992; Prinsen and Koeter, 1993; Barile, 2010; Verstraelen et al., 2013). A second group takes advantage of the reactions evoked by chemicals in incubated hen eggs (Eskes et al., 2005) or invertebrates (Adriaens et al., 2005, 2008). One test system is based on the perturbation and denaturation of corneal proteins; these processes are supposed to mimic the disruptive effect to ocular irritants (Eskes et al., 2014). Another group uses cultured cells of different origin to assess the eye-irritating potential of chemicals (Eskes et al., 2005; McNamee et al., 2009; Hartung et al., 2010; Takahashi et al., 2010, 2011; Sakaguchi et al., 2011).

As a further approach, 3D cornea epithelial models have been reconstructed (Doucet et al., 2006; Van Goethem et al., 2006; Huhtala et al., 2008; Seaman et al., 2010; Kaluzhny et al., 2011; Kolle et al., 2011; Katoh et al., 2013). Approaches using 3D epithelial models only discriminate between irritants (I) and non-irritants (NI) based on a classification (PM) with one viability cut-off value (Van Goethem et al., 2006; Kaluzhny et al., 2011). Due to the absence of stroma, further discrimination between chemicals classified as GHS2 (moderate irritants) and GHS1 (severe irritants) is not foreseen.

Currently, only one cell-based test system (Fluorescence leakage, FL) and two *ex vivo* animal test systems (BCOP and ICE) have reached official regulatory acceptance (OECD, 2012, 2013a,b). However, these test systems allow only the prediction of serious eye damage (GHS1, i.e., BCOP, ICE and FL), and of

* Corresponding author. Tel.: +49 421 2003253; fax: +49 421 2003249.
E-mail address: m.bartok@jacobs-university.de (M. Bartok).

http://dx.doi.org/10.1016/j.tiv.2014.09.005
0887-2333/

chemicals not requiring classification (GHSnc, i.e., BCOP and ICE), but not of GHS2. No single *in vitro* assay has been developed and validated as a full regulatory replacement for the Draize Eye Irritation test. Instead, the tests developed so far are intended to be used within the framework of an integrated testing strategy, either in a top–down or in a bottom–up approach (McNamee et al., 2009; Scott et al., 2010; Hayashi et al., 2012). These approaches, as *in vitro* alternatives to animal testing for the safety assessment, reveal their strengths preferably in the discrimination between severe and non-severe chemicals. Test systems predicting GHS2 substances directly are not yet available.

According to Scott et al. (2010), only those reconstructed corneal models that include both the epithelium and the stroma allow discrimination between all three GHS classes: severe/corrosive (GHS1), mild/moderate (GHS2) and not classified (GHSnc). This perception is based on studies by Jester and Maurer (Jester et al., 1998a,b, 2001, 2006, 2010; Maurer et al., 2002), who showed that the surface area and depth of initial corneal injury (DoI) in the epithelium and stroma, caused by chemicals of various classes in the rabbit eye, strongly correlate with the macroscopically observable severity and duration of ocular injury.

Consequently, we previously generated a 3D cornea model based on human SV40-immortalized corneal cell lines (Engelke et al., 2004; Zorn-Kruppa et al., 2004, 2005; Seeber et al., 2008; Manzer et al., 2009) which comprises both epithelium and stroma to provide an animal-free tool for eye irritation testing (Engelke et al., 2013) as well as for *in vitro* drug permeation studies (Hahne and Reichl, 2011; Hahne et al., 2012).

In a previous study (Engelke et al., 2013) we proved the reproducible construction and the transferability of the production of the complex 3D models into other laboratories. With this model, we developed a prediction model based on 20 test chemicals that reliably predicted GHS category 1 chemicals. The model did, however, not sufficiently discriminate between all 3 GHS categories

Since we assume that the individual assessment of the viability in the epithelium and stroma is essential for the GHS classification, we have expanded our previous model to include a membrane between these two compartments. This membrane allows us to separate the epithelium and stroma after exposure and to determine the cell viability individually. We have tested the model using thirty chemicals of balanced GHS classification. Based on suitable viability cut-off values for both the epithelium and stroma we developed a prediction model which allows the discrimination of all three GHS categories.

As a reproducible tissue reconstruction is crucial for the reliability of an *in vitro* test system we have assessed the intra-laboratory reproducibility. Acceptance criteria were defined based on the viabilities of the negative control (NC) and batch control (BC) in the epithelium (NC_{epi}, BC_{epi}) and stroma (NC_{stroma}, BC_{stroma}) from all batches produced over a 6-month period.

2. Materials and methods

2.1. Materials and reagents

Tissue culture flasks, flat bottomed multi-well plates (6-, 12-, 24- and 96-well plates), penicillin/streptomycin, PBS without Ca^{2+}, Mg^{2+} (PBS−−) and PBS with Ca^{2+}, Mg^{2+} (PBS++) were purchased from Biochrom (Berlin, Germany). $CaCl_2$, PBS−− tenfold concentrated powder, isopropanol, Nunc cell culture inserts (0.5 cm^2 surface area, 3 μm pore size, polycarbonate) were obtained from OmniLab (Bremen, Germany) and Bola Teflon O-rings (1 cm outer and 0.7 cm inner diameter) were from Bohlinger GmbH, Grünsfeld, Germany). The silicon O-rings were from Dichtungstechnik (Bensheim, Germany) and the electrospun membranes were from The Electrospinning Company (Harwell Oxford, UK). The rat tail collagen solution was obtained from CellSystems (Troisdorf, Germany). The media 199, Ham's F12 and TrypLE Express were from Invitrogen (Darmstadt, Germany). Triton X-100, thiazolyl blue tetrazolium bromide (MTT reagent), NaOH, $NaHCO_3$, Hepes, Bouin's solution was purchased from Sigma–Aldrich (Schnelldorf, Germany). The Collagen Cell Carrier (CCC) was from Viscofan Bioengineering (Weinheim, Germany). Keratinocyte Basal Medium (KBM) plus Bullet-kit as well as chemically defined Keratinocyte Growth Medium (KGM-CD) were obtained from Lonza (Basel, Switzerland).

2.2. Test chemicals and control materials

The test chemicals were chosen on the basis of eye irritation classifications derived from individual *in vivo* rabbits. We selected 30 test chemicals from the database of the Technical Report of the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC, 1998). Of these chemicals, 24 are liquids and 6 are solids. We have only selected 6 solid test substances, because similar to the *in vivo* test, testing of solids *in vitro* is more challenging, especially if particles remain on the tissue surface after the washing procedure. Solids may result in false classification as GHSnc only because they are not soluble under applied conditions. Details of the 30 selected chemicals, together with their *in vivo* eye irritation classification, are shown in Table 1. The test materials covered the whole range of eye irritation potencies and represented different chemical classes. PBS++ and 0.3% Triton X-100 served as the negative control (NC) and as the batch control (BC), respectively.

2.3. Solutions for the cell cultures and hemi-cornea reconstruction

The reconstruction buffer for neutralizing the acidic solution of the collagen was prepared by mixing together 2.2 g $NaHCO_3$ and 4.77 g Hepes in 100 ml of 0.5 N NaOH solution. The tenfold concentrated F99 medium was prepared according to Engelke et al. (2013). The O-ring attachment solution was made by mixing together 100 μl F99 medium, 250 μl reconstruction buffer, 100 μl KGM medium and 600 μl collagen solution. The MTT solution was prepared by first dissolving the MTT reagent in double-distilled water to a concentration of 5 mg/ml. This stock solution was stored at −20 °C for a maximum of 6 months in the dark. It was diluted with KGM medium to a final concentration of 1 mg/ml before use.

2.4. Cell culture and hemi-cornea reconstruction

The human corneal epithelial (HCE) cell line used in this study was immortalized by Araki-Sasaki et al. (1995). This cell line was kindly provided by Stephan Reichl, Technische Universität Braunschweig, Germany, who received it from the RIKEN cell bank (Tsukuba, Japan). The HCE cells were used between the 87th and 112th passage number. The HCE cells form a multilayered epithelium when cultivated at the air–liquid interface under serum-free conditions (Seeber et al., 2008). The human corneal keratocytes (HCK) were immortalized and established by Zorn-Kruppa et al. (2005) and Manzer et al. (2009). Also, the HCK cell line was only used for a limited number of passages (passage 13–26 in this study). Both HCE and HCK were cultured as described by Engelke et al. (2013).

The hemi-cornea models were prepared in three steps: preparation of the stroma equivalent, attachment and fixation of the CCC-membrane, and addition of the epithelial cells. For the stroma equivalent, per model 30 μl of F99 medium, 75 μl of reconstruction buffer, 30 μl of KGM medium containing 50,000 HCK cells and 180 μl of the collagen solution was mixed; 250 μl of this solution was placed into the cell culture insert and left for about 25 min