Review Article

Alternative Methods for the Replacement of Eye Irritation Testing

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Summary

In the last decades significant regulatory attempts were made to replace, refine and reduce animal testing to assess the risk of consumer products for the human eye. As the original *in vivo* Draize eye test is criticized for limited predictivity, costs and ethical issues, several animal-free test methods have been developed to categorize substances according to the global harmonized system (GHS) for eye irritation. This review summarizes the progress of alternative test methods for the assessment of eye irritation. Based on the corneal anatomy and current knowledge of the mechanisms causing eye irritation, different *ex vivo* and *in vitro* methods will be presented and discussed with regard to possible limitations and status of regulatory acceptance. In addition to established *in vitro* models, this review will also highlight emerging, full thickness cornea models that might be suited to predict all GHS categories.

Keywords: eye irritation, Draize eye test, OECD guideline, corneal equivalent

1 Introduction

The human eye is responsible for the optical perception of the surrounding world. To allow light to reach sensory cells on the retina the eye is protected only by a relatively thin transparent tissue called the cornea. In contrast to human skin, the cornea epithelium is not cornified and is thus more prone to mechanical or chemical injury. Due to the eyes' vulnerability and their outstanding importance for the individual, standardized test protocols have been developed to assess the potential hazard exerted by chemical substances and consumer products on the human eye.

The current standard assays, such as the Draize eye test, predict the effect on the human eye by the reactions observed in different animal models. However, due to ethical concerns, scientific reasons and a change in international legislation there is an increasing demand to replace the current *in vivo* methods by alternative approaches. These alternative approaches involve the use of different *in silico*, *ex vivo* and *in vitro* models. Although *in silico* methods also are promising tools to classify test chemicals, this review will focus on biological *ex vivo* and *in vitro* assays that can be employed for the assessment of eye irritation. Important for that is to understand the ocular anatomy and how substances interact with the eye. Moreover, the progress and challenges of complex corneal models will be evaluated as a potential solution for current limitations in ocular toxicology.

Anatomy of the eye

Vertebrates can rely on a sensitive and highly developed optical sensory organ for orientation and communication. Even though its function requires an exposed position, evolution has developed many protective measures to preserve its functionality. Being embedded in muscle, fat and connective tissues within the orbits, the human eye is generally considered to be well-

Abbreviations

BCOP; bovine corneal opacity and permeability; CM; cytosensor microphysiometer; EIT; EpiOcular™ eye irritation test; EURL ECVAM; The European Union Reference Laboratory for alternatives to animal testing; FL; fluorescein leakage; GHS; global harmonized system; HCE; human corneal epithelium; Het-CAM; hen's egg test chorio-allantoic-membrane; ICE; isolated chicken eye; IRE; isolated rabbit eye; OECD; Organisation for Economic Co-operation and Development; PorCORA; porcine corneal ocular reversibility assay; RCE; reconstructed human corneal epithelium

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protected from mechanical injury. Further protection is given by the eye's physiology itself. It consists of three distinct coats – fibrous tunic, uvea and retina – surrounding the inner aqueous humour. The fibrous tunic, the outermost region, maintains the eyeball's form and consists of the cornea and the sclera (Drenckhahn, 2008). The sclera is a thick opaque tissue covering 95% of the eye forming a connective tissue coat, which protects the eye from both internal and external forces. Moreover, it is the anchor tissue for the extraocular muscles. The cornea as the most anterior part of the eye is transparent and transmits the light into the lens and the retina. It is approximately 11 mm in diameter and 600 μm in thickness and contains no blood vessels. Hence, the cornea is chosen for most eye irritation tests because it represents the first and vital barrier of the eye.

In general, five layers can be distinguished in the human cornea: (l) the epithelium, (II) Bowman's membrane, (III) the lamellar stroma, (IV) Descemet's membrane and (V) the endothelium (Fig. 1) (Drenckhahn, 2008). The epithelium consists of 5-7 layers of non-cornified epithelial cells with high mitotic activity, resulting in a turnover time of 7-10 days (Daniels et al., 2001). It serves as a barrier to pathogens and helps to maintain the stroma at an appropriate level of hydration via a high number of cell junctions. Bowman's membrane is a thin (10-15 μm) cell-free layer composed of collagen type I and type V forming a fine-meshed network of tenuous fibrils. It separates the lamellar stroma from the epithelium. With a thickness of 500 μm the corneal lamellar stroma is the thickest part of the cornea and provides structural integrity. Produced by stromal keratocytes, the collagen is structured in a highly organized manner, which is crucial for the cornea's transparency. Within the stroma 200-250 collagen lamellae house the collagen fibrils that are densely packed with a strict parallel and orthogonal orientation to allow high transparency (Meek and Boote, 2004). Descemet's membrane, like Bowman's membrane, is a cell-free layer of collagen type VIII. It has a thickness of approx. 10 μm and separates the stroma from the endothelium. It thickens with age and shows a banded anterior and a more amorphous posterior morphology (Johnson et al., 1982). It is secreted by a single layer of endothelial cells that have a predominantly hexagonal shape and show almost no self-renewing potential. The endothelial layer is in direct contact with the aqueous humour, the fluid filling the anterior chamber of the eye, and allows an exchange of nutrients and fluids with the rest of the eye (Drenckhahn, 2008).

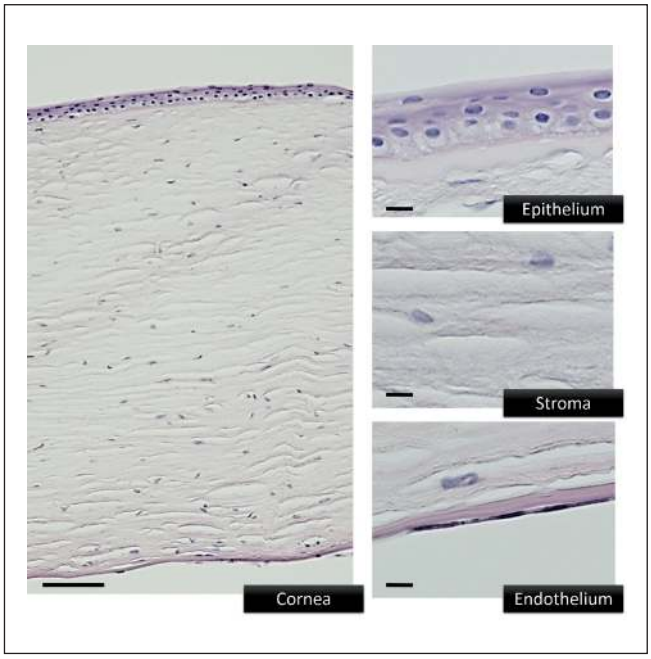

Fig. 1: Histology of the human cornea
Scale bars indicate 100 μm in the left figure and 10 μm in the right panel. The keratinocytes form a stratified epithelium on top of Bowman's layer. The stroma, mainly consisting of collagen type I, harbors the keratocytes and the endothelium on top of Descement's membrane completes the cornea.

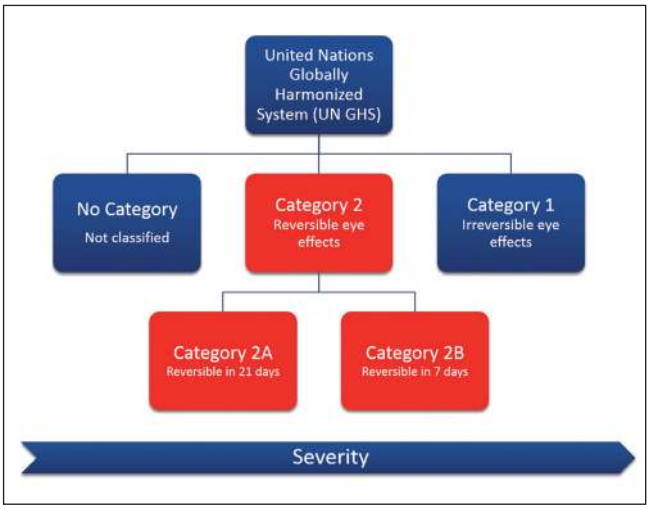

Fig. 2: The United Nations Globally Harmonized System (UN GHS) of eye irritation

Modes of action in eye irritation

Thousands of substances able to irritate or harm human eyesight can be found not only in pharmaceuticals and cosmetics but also in many consumer products. Therefore, risk management is an important aspect of public health management (Wilhelmus, 2001).

In accordance to Adriaens et al. (2014) the drivers of eye irritation can be grouped depending on the specific molecular mechanisms underlying the modes of action. Membrane lysis is caused by disruption of the lipid bilayer through exposure to materials such as surfactants. Moreover, denaturation and coagulation of macromolecules, especially proteins, by acids, alkali or organic solvents can lead to ocular damage. Also, saponification, the hydrolysis of lipids by alkaline actions causing membrane lysis and coagulation with a progressive tendency is a major cause of eye irritation. Chemical reactivity comprises all chemicals that interact with cellular components (e.g., alkylation, oxidative actions on macromolecules) able to lead to cell stress, coagulation and lysis. A delayed onset has to be con-

sidered with this group since the initial reaction can be subtle (e.g., peroxides, mustards and bleaches) (Maurer et al., 2002; Jester, 2006; Scott et al., 2010).

To ensure consumer safety, products have to be labeled according to their potential to harm the human eye. The globally harmonized system of classification and labeling of chemicals (GHS) defines three categories for eye irritation. Substances that do not cause adverse effects do not require labeling (no category), whereas substances that lead to reversible effects in the eye are classified as category 2. Depending on the period that is needed to restore these effects, category 2 substances are subdivided into category 2a, if effects are reversed after 21 days and category 2b, if effects only persist for 7 days. In case effects are irreversible, substances are classified as category 1 (Fig. 2).

To assess these categories different *in vivo*, *ex vivo* and *in vitro* test methods have been developed of which the Draize eye test has been the reference method since its development over 50 years ago (OECD, 2012a; UN, 2013).

2 The *in vivo* Draize eye test to assess eye irritation

Traditionally eye irritation has been examined using various animal models such as rabbits. However, the methodology was not consistent until in 1944 the Food and Drug Administration toxicologist J. H. Draize published a standardized method that was subsequently excessively used and became known as the Draize eye test (Draize et al., 1944).

Briefly, the test uses albino rabbits, which are evaluated before exposure to ensure eye integrity (Wilhelmus, 2001). Three to six animals are exposed to a dosage volume of 0.01 to 0.1 ml. In case of solid test substances, the compounds are ground to a fine powder and then applied. The instillation is made into the lower conjunctival cul-de-sac whilst the animal is restrained but conscious; blinking is allowed, although the eyelids can be held together for several seconds after instillation. Evaluations are made 1, 24, 48 and 72 hours after exposure and can be extended up to a 21 day period. Any signs of corneal opacity, iritis and conjunctival redness are recorded and graded using a defined scoring system. After the tests are completed test animals are sacrificed (Hartung et al., 2010; OECD, 2012a). In accordance with the GHS classification the derived score values can be used to classify substances into the 3 categories of eye irritation (UN, 2003): category 1 is defined by Draize scores of corneal opacity of ≥ 3 and/or iritis > 1.5. Category 2 includes Draize scores for corneal opacity of ≥ 1 and/or iritis > 1 and/or conjunctival redness ≥ 2 and/or chemosis ≥ 2.

The Draize eye test was adopted in 1981 by the Organisation for Economic Co-operation and Development (OECD) as test guideline (TG) 405. The guideline was improved and updated three times in 1987, 2002 and 2012 (OECD, 2002, 2012a). The improvements include a prior weight-of-evidence analysis to ensure the necessity of the test, the use of analgesics and anesthetics to reduce animal suffering and a sequential tiered testing strategy starting with only one animal to reduce the overall number of animals used in the test (OECD, 2012a). Furthermore, a variant of the Draize eye test was developed, the "low volume eye test". In comparison to the original test the test substance volume is reduced to 10 μl and is directly applied to the corneal surface in order to underpredict the Draize eye test and thereby be less overpredictive for the human eye (Griffith et al., 1980).

The Draize eye test has drawn scientific, economic and ethical criticism (Doucet et al., 2006). The main points of criticism are the methodology itself (Wilhelmus, 2001), species-specific differences regarding the eye's physiology (Sharpe, 1985), use and interpretation of test scores (Curren and Harbell, 1998), reproducibility and relevance of the findings (York and Steiling, 1998; Adriaens et al., 2014), economic costs and ethical issues (Sharpe, 1985; Wilhelmus, 2001). In addition, recent legislative changes, i.e., the REACH Regulation (Regulation (EC) of No 1907/2006), intensified the demand for alternative test systems as animal testing is to be reduced while at the same time toxicity assessments of chemicals remain required (EC, 2006).

Originally developed to investigate the ophthalmological effects of pharmaceuticals and cosmetics, the Draize eye test naturally fails to address the full toxicological potential of any given substance tested, as it doesn't cover all forms of possible exposure scenarios. Also, exposure time and concentrations strongly differ from commonly assumed human accidental exposures. Furthermore, the scoring itself relies on preselected and weighted points, the signs recorded are those easily observed, whereas non-macroscopic aspects are not taken into consideration for the scoring (Curren and Harbell, 1998).

Although results from category 1 or no category suffer less from variations due to biological and subjective factors, middlerate or borderline substances are more likely to present ambiguous outcomes (Prinsen, 2006; Wilhelmus, 2001).The general use of animal models to model humans is controversial owing to different physiological and anatomical properties compared to the human eye, such as constituents of the tear film, relatively larger corneal surface and a thinner cornea without Bowman's membrane (Sharpe, 1985). Moreover, the site of application, the components rate of release or the animal's reflex tearing during substance application can have a significant influence (Prinsen, 2006). Also the examiners' subjective grading can lead to wrong conclusions (Curren and Harbell, 1998). Another point of criticism is the variations between the test animals causing high variations in regards of reproducibility of application and evaluation. Even though this drawback can be addressed by increasing the sample sizes, this is not a solution worth striving for, both from an economical and an ethical point of view.

3 Organotypic methods

Although it was acknowledged that it is unlikely that the Draize eye test can be replaced by a single *in vitro* test, as early as the 1980s and 1990s, multilaboratory studies were undertaken to find alternatives for the Draize eye test. Among these methods, organotypic models were introduced to evaluate the potential of chemicals to cause eye irritation. These tests do not require the use of live animals, but employ corneas of animal origin that

have been removed from animals post-mortem. Thus, organotypic methods are not considered animal experiments by legal authorities. Most frequently bovine, rabbit and chicken eyes are used to investigate eye irritation (Tab. 1).

Organotypic models were first introduced by Burton et al. in 1981 using isolated rabbit eyes (IRE) (Zerger et al., 2014; Burton et al., 1981). A modified test protocol was established in 1993 using isolated chicken eyes (ICE) (Prinsen and Koëter, 1993; Prinsen, 1996). Both methods use eyes as a waste from the food industry. Enucleated eyes are assessed for corneal integrity by applying sodium fluorescein to the corneal surface. If intact, the cornea is positioned vertically in a stainless steel clamp. The clamp is placed in a temperature-controlled superfusion apparatus to maintain the tissue in a healthy state. Before applying test substances, corneal integrity is again examined macroscopically. After treatment, irritative effects are measured by assessment of corneal opacity, swelling, morphological damage and fluorescein retention. Test substances are exposed for 10 seconds and monitored over a period of 4 h. The ICE test method is suitable to classify substances as severe irritants (GHS category 1) and non-irritants (GHS no category). An OECD guideline for the ICE test was formulated in 2009 and updated in 2013 (OECD, 2009b, 2013a). Although the IRE test has not been officially validated, it can be used as an in-house screen for severely irritating materials within a battery of *in vitro* assays (ICCVAM, 2010).

Tab. 1: List of available alternatives for the assessment of eye irritation
* Negative results require further testing, ** positive results require further testing.

Test model	System	Readout	Limitations	Status of validation	Reference
Bovine Corneal Opacity Test (BCOP)	Bovine cornea in cornea holder	Cat. 1*	Overprediction of ketones and alcohols Underprediction of solids	OECD TG 437 (2009)	Gautheron et al., 1992; Invittox protocol 124, 1997
		No cat.**	Overpredictive	Guideline for Bottom-Up Approach	Gautheron et al., 1992; Invittox protocol 124, 1997
Isolated Chicken Eye Test (ECVAM)	Chicken eye in holder	Cat. 1*	Underprediction of surfactants and solids	OECD TG 438 (2009)	Prinsen and Koëter, 1993; Invittox protocol 80, 1994
		No cat.**	Over prediction of alcohols	Guideline for Bottom-Up Approach	Adopted guideline in 2013
Cytosensor Microphysiometer (CM)	Influence on metabolic rate of L929 fibroblast cells	Cat. 1*	Applicable to water soluble substances and mixtures. High false negative rate.	Validated (2009)	McConnell et al., 1992
		No cat.**	Applicable to water soluble surfactants and water soluble surfactant containing mixtures. High false positive rate.	Validated (2009)	McConnell et al., 1992
Fluorescein Leakage (FL)	Influence on tight junctions of monolayer cultured cells	Cat. 1*	Applicable to water soluble substances and mixtures. High false negative rate.	Validated (2009)	Tchao, 1998
EpiOcular™ Eye irritation test (EIT)	Epithelial model	Cat. 1/ cat. 2 against No cat.	Applicable to both hydrophilic and hydrophobic test substances in either liquid or solid state	Validated (2014); OECD TG 492 (2015)	Pfannenbecker, 2013
SkinEthic™ HCE test method	Epithelial model	Cat 1/ cat. 2 against No cat.		Under pre-validation	Alepee, 2013
Vitrigel®-Eye Irritation Test	Epithelial model			MAFF-sponsored validation study is on-going	Yamaguchi, 2013

The bovine corneal opacity and permeability (BCOP) assay uses bovine eyes from abattoir animals, which are enucleated, brought to the laboratory, inspected for defects, isolated from the rest of the eye and placed in a cornea holder. Corneas are exposed to surfactants and liquids for 10 min or for 4 h to solid test substances. Subsequently, they are rinsed and incubated for 2 h. Toxic effects of the test substance to the cornea are measured as the induction of opacity and increased permeability. Corneal opacity is measured quantitatively as the amount of light transmission through the cornea. Sodium fluorescein dye that passes all layers of the cornea and reaches the lower chamber provides information about the permeability of the tissue. An OECD guideline was adopted in 2009 and updated in 2013 (OECD, 2009a, 2013c). The BCOP assay is suitable to screen for severe irritants (GHS category 1) and non-irritants (GHS no category). Instead of bovine corneas, Piehl et al. (2010) developed the porcine corneal ocular reversibility assay (PorCORA). Porcine corneas more accurately resemble the human cornea with regard to thickness and structure. Since this assay is able to detect reversible effects, it has great potential for risk assessment, as it might be used to classify substances of all three GHS. The assay has yet not been adopted by regulatory authorities.

The hen's egg test – chorioallantoic membrane (Het-CAM) assay does not use corneal tissue but the chorioallantoic membrane of fertilized and incubated chicken eggs. The eggs can be used up to the tenth day after fertilization as the development of the embryonic nervous system is not completed before that day. It is assumed that the test material has similar effects on the membrane as on the eye. The test substance is classified according to its potential to cause hemorrhage, lysis and coagulation of the blood vessels in the membrane. Thus, the Het-CAM assay is the only organotypic method, which directly addresses conjunctival effects. Initially developed by Luepke et al. (Luepke, 1985; Luepke and Kemper, 1986) the test shows a high predictivity for mild and non-irritating test materials. The Het-CAM assay is in ongoing validation by the Brazilian Centre for the Validation of Alternative Methods (BraCVAM) and validated but not yet recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Alcohols, sticky materials, colored chemicals and solids that cause physical abrasion are prone to misclassification (ECVAM, 2015).

Between 2003 and 2006, a retrospective validation study of four organotypic assays (BCOP, ICE, IRE and Het-CAM) was conducted in which the ability of these assays to detect severe eye irritants was investigated. Two test methods, BCOP and the ICE test, were approved as scientifically valid within a top-down approach to identify "ocular corrosives and severe irritants" (ICCVAM, 2007; ESAC, 2007), which lead to two OECD guidelines in 2009 (OECD, 2009a,b). The BCOP assay appears to be the only suitable assay for the identification of non-classified materials (ICCVAM, 2010). In April 2013, updated test guidelines expanded the use of BCOP and ICE test methods in a bottom-up approach to identify chemicals as "not classified for eye irritation". For the ICE assay, high false positive results occur for alcohols and high false negative results for solids and surfactants. For the BCOP assay, high false positive rates were found for ketones, solids, alcohols and non-irritating substances (OECD, 2013c).

Organotypic methods offer a three-dimensional tissue architecture, in which some of the structural and functional characteristics of the *in vivo* animal eye are retained. Nevertheless, these test procedures are still dependent on animal tissues and thus face the same limitations as *in vivo* tests in regard of interspecies differences. Therefore, several *in vitro* tests have been developed that replace test animals or tissues of animal origin with either two-dimensional cell cultures or three-dimensional reconstructed tissues.

4 *In vitro* tests

Two-dimensional cell based assays

Cell function based assays make use of the observation that some materials that are damaging to the eye appear also to be cytotoxic to a number of cell types. Cell damage can occur by coagulation of macromolecules, cell membrane lysis, saponification of lipids and alkylation or other covalent interactions with macromolecules (Scott et al., 2010; Malkinson, 1978; Klaassen, 2013). Cell function based assays are simple, quick and have low evaluation costs and thus allow high throughput screening (Tab. 1).

The cytosensor microphysiometer (CM) is a cell function based assay that measures the potential to cause ocular irritation by investigating the metabolic activity of treated L929 mouse fibroblasts. Cells are exposed to increasing concentrations of the test material, rinsed and analyzed with regard to metabolic activity by measuring extracellular acidification. The CM assay is recommended for the identification of GHS no category and GHS category 1 substances (OECD, 2012b). The test is applicable for GHS category 1 test materials that are soluble or form a stable solution. For GHS no category test substances, it may also be used for surfactants that are water soluble or form a soluble solution. However, a high false positive rate was found for no category substances. Since the CM assay is non-invasive, the recovery of the cells from the treatment also may be measured. In 2012, an OECD test guideline on the CM was drafted and is currently under review.

The neutral red release assay uses the capacity of vital cells to incorporate and bind Neutral Red dye in lysosomes. Thus, if a test compound has a cytotoxic effect, the uptake of the dye is inhibited. The test material should be soluble in water and have only limited acid or alkaline properties. The assay was successfully employed in combination with the Het-CAM and IRE test to identify severely eye irritating materials (Spielmann et al., 1996, 1998a,b; Spielmann, 1996; Pape et al., 1987).

For the red blood cell hemolysis test, red blood cells are treated with the test substance. If the test substance has a cytotoxic potential, the cell membrane is disrupted and hemoglobin leaks. The amount of hemoglobin can then be measured photometrically. The assay is used for surfactants and is applicable for water soluble and water dispersible substances (Pape and Hoppe, 1991; Lewis et al., 1993; Pape et al., 1987).

The fluorescein leakage (FL) assay is an *in vitro* assay that may be used for identifying water-soluble ocular corrosives and severe irritants or chemicals that form a stable suspension. Madin Darby Canine Kidney CB 997 tubular epithelial cells are cultured on a transwell insert in a confluent monolayer. The permeability of the monolayer is increased if the test substance has the potential to impair the tight junctions. Cells are exposed for 1 min to the test material followed by a 30 min incubation with fluorescein. The amount of fluorescein leaking through the cell layer can be measured quantitatively. The assay was recommended to identify severe irritants (GHS category 1) but not GHS category 2 and non-irritants (OECD, 2012c). The test guideline on fluorescein leakage was adopted as OECD TG 460 in 2012. It is not suitable for colored and viscous chemicals, for strong acids and bases, cell fixatives and highly volatile chemicals.

In the short time exposure assay a confluent layer of Statens Seruminstitut Rabbit Cornea cells is exposed to the test materials in a concentration of 5% and 0.05% in physiological saline for five minutes. Cytotoxicity is measured with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Takahashi et al., 2008). The test is validated and recommended for identifying GHS category 1 and GHS no category. A draft for an OECD guideline is under discussion. However, experimental data show that high false negative rates occur for volatile chemicals and that the test is not applicable to non-soluble substances and substances that do not form stable suspensions (OECD, 2014b).

Between May 2006 and October 2008 a retrospective validation study of four cytotoxicity and cell function based assays was conducted. The CM and FL were scientifically validated as an initial step within a top-down approach to identify ocular corrosives and severe irritants from all other classes for water soluble chemicals. Moreover, the CM was considered to be scientifically valid and to be ready for consideration for regulatory use as an initial step within the bottom up approach to identify non-irritants for water soluble surfactants and water soluble surfactants containing mixtures. In 2012, an OECD guideline (OECD, 2012c) on the FL assay was adopted and an OECD guideline on the CM was drafted.

Three-dimensional corneal epithelial models

Organotypic methods and cell based tests are promising methods but lack comparability to the human eye. Interspecies differences caused by using organotypic models might lead to over- and underprediction of irritative effects. Two-dimensional cell cultures used for cell based tests do not emulate natural tissues, in which cells are located in a complex three-dimensional microenvironment. The unnatural rigid and flat surrounding of two-dimensional cell culture surfaces can alter cell metabolism and reduce functionality (Antoni et al., 2015). Furthermore, two-dimensional cell cultures are restricted to testing water-soluble test substances whereas three-dimensional epithelial models also allow testing of hydrophobic liquid and solid substances as they allow direct topical exposure. Generally three-dimensional tissues withstand mechanical or chemical damage to a greater extent than two-dimensional cell layers (Sun et al., 2006). Additionally, epithelial tissue equivalents are able to mimic the barrier function of these tissues, which is a vital aspect of irritation testing *in vitro* as it limits the penetration of toxic substances to the target cells (Spielmann et al., 2007).

In the early 80's, Bell et al. published the first development of a reconstructed skin equivalent which involved epithelial cells exposed to the air-liquid interface. These reconstructed realistic epithelial equivalents show histological features similar to those found *in vivo* (Bell et al., 1981).

Great efforts were put into generating three-dimensional reconstructed human corneal epithelium (RCE) in order to overcome drawbacks of two-dimensional cell cultures (McConnell et al., 1992). Stratified epithelium, such as corneal epithelium, is characterized by its multilayered morphology with a significant barrier function. The corneal epithelium comes into direct contact with substances entering the eye. It is the first and also the main barrier to ocular penetration. Reconstructed human corneal epithelium (RCE) models could be used for eye irritation testing, assessment of the barrier function, drug transport, cell physiology, metabolism and the development of delivery systems (Pepic et al., 2014; Reichl et al., 2004; Kaluzhny et al., 2011).

In the field of skin toxicology *in vitro* test methods based on reconstructed human epidermis have been reviewed and adopted by the OECD in two test guidelines (OECD, 2014a, 2013b). In these guidelines corrosive and irritating effects are determined using tissue viability as a readout (EpiSkin (SM), EpiDerm™ SIT (EPI-200), SkinEthic™ RHE, LabCyte EPI-MODEL24 SIT) (Spielmann et al., 2007; Alepee et al., 2010; Kojima et al., 2014). The measurement of viability to predict the correct classification is based on the assumption that all chemicals inducing serious irritation will induce a cytotoxic effect in the epithelium. A similar approach employing RCE to predict eye irritation seems to be viable. In comparison to the reconstructed human epidermis, RCE has no cornified layer and shows a different morphology of viable cell layers (Katoh et al., 2013; Poumay et al., 2004).

The various models described to date differ mainly in the cell type used. RCE models are comprised of corneal or non-corneal cells, including primary human skin keratinocytes (e.g., EpiOcular™ , MatTek Corporation, USA), primary human corneal cells (e.g., LabCyte CORNEA-MODEL, Japan Tissue Engineering Co., Ltd., Japan or the MCTT- human corneal epithelium (HCE) model, (Jung et al., 2011)), or immortalized human corneal epithelial cells (e.g., SkinEthic™ HCE, EpiSkin, France; Vitrigel-HCE (Yamaguchi et al., 2013)). The corresponding eye irritation tests are based on the depth of injury model, where initial injury is determined by biochemical measurements to predict the outcome of ocular irritation (Maurer et al., 2002). The depth of injury model suggests that the potential of surfactants to induce ocular irritation can be correlated to the area and depth of cell death.

Three RCE models are commercially available (EpiOcular™ , SkinEthic™ HCE, LabCyte CORNEA-MODEL) and two of them entered an eye irritation prevalidation study in 2008. This

Tab. 2: Alternative test methods validated for the UN GHS categories

GHS – No Category	GHS – Category 2	GHS – Category 1
BCOP ICE CM EIT HCE	No validated stand alone alternative method available	BCOP ICE CM FL

full prospective validation study was jointly sponsored by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) and Cosmetics Europe and aimed to assess the scientific proficiency to predict eye irritation with the "SkinEthic™ Human Corneal Epithelium" test method and the EpiOcular™ eye irritation test (EIT) (ECVAM, 2015).

The EpiOcular™ RCE is a non-keratinized epithelium prepared from non-transformed primary human epidermal skin keratinocytes. Using specially designed culture conditions the skin derived keratinocytes form a three-dimensional model, which mimics the corneal epithelium. The model consists of 5-8 cell layers with an upper and central layer of squamous cells and a lower layer of rounded cells. The EpiOcular™ EIT protocol differs between solids and liquids in the exposure time and post-exposure period allowing for development of cytotoxic effects (for liquids 30 min exposure followed by 2 h post-exposure and for solids 90 min exposure followed by 18 h post-exposure). If the tissue viability is less or equal 60%, measured by MTT assay, the compound is classified as an irritant in comparison to the negative control, which is set to 100%. A higher viability leads to a classification as non-irritant. The EpiOcular™ EIT differentiates those materials that are non-irritants from those that would require labeling as either GHS category 1 or category 2, but the assay is not able to distinguish between GHS category 1 and category 2 (Pfannenbecker et al., 2013; Kaluzhny et al., 2011).

The SkinEthic™ HCE model is a standardized epithelial model reconstructed with an immortalized human corneal cell line. The reconstructed epithelial tissue lacks a stratum corneum and the morphology is similar to that of human corneal epithelium. The HCE model is suitable to detect corneal repair and recovery *in vitro* as well as other *in vitro* indicators, particularly lactate dehydrogenase release, quantification of cytokine release, and gene expression. The SkinEthic™ assay divides chemicals into two groups based on initial assessment of reactivity using an eye peptide reactivity assay. Test substances are incubated with cysteine- or lysine-containing peptides and reactivity with nucleophilic residues is evaluated by high performance liquid chromatography (HPLC). Due to the reactivity test, reactive chemicals are used within the short exposure procedure (10 min exposure and no post-exposure) and for non-reactive chemicals the long exposure time protocol is used (60 min exposure followed by 16 hours post-exposure period). Tissue viability is determined by MTT assay. According to the prediction model, chemicals that reduce the relative tissue viability to less than 50% are classed as potential irritants, while those reducing viability by less than 50% are categorized as non-irritants (Alepee et al., 2013).

The formal EURL ECVAM validation announcement of the EpiOcular™ EIT was published in 2014 and the associated OECD guideline 492 was published in 2015 (OECD, 2015). However, the SkinEthic™ HCE protocol was found to require comprehensive optimization. Due to the estimated optimization time this should be conducted outside the sponsored study (ECVAM, 2014).

Neither EpiSkin nor MatTek use primary human corneal epithelial cells to reconstruct the human corneal epithelium for testing eye irritative potential of chemicals. The application of cell lines or cells of a different origin might lead to an altered cell behavior, e.g., owing to an absence of marker expression (Schoop et al., 1999). Two models based on normal human corneal epithelial cells were recently developed, namely the LabCyte CORNEA-MODEL and the MCTT-HCE model, of which the first is already commercially available (Kojima et al., 2012; Jung et al., 2011; Katoh et al., 2013). Additionally MatTek introduced a corneal epithelium model (EpiCorneal Tissue Model) based on normal human corneal epithelial cells. However, the applicability of primary human cornea cells for eye irritation testing is not officially validated yet.

The LabCyte CORNEA-MODEL is generated from corneal epithelial cells originating from normal human cornea tissue. The culture on a synthetic membrane results in reconstructed tissue similar to the *in vivo* cornea epithelium with a verifiable expression of corneal epithelium marker (Kojima et al., 2012). An eye irritation testing protocol similar to the EpiOcular™ EIT was developed for the prediction of eye irritants. Differences between the two protocols mainly arise from the different exposure and post-exposure times (liquids 1 min exposure and 24 h post-exposure, solids 24 h exposure and no post-exposure). However, with a threshold of 50% viability the prediction model is comparable to the SkinEthic™ assay (Katoh et al., 2013).

Most of the established RCE use synthetic scaffolds such as a polycarbonate membrane. Takezawa and coworkers have developed a RCE on a special collagen membrane, which is composed of high-density collagen fibrils, called collagen vitrigel membrane (Takezawa et al., 2010). Using this Vitrigel-HCE model an eye irritancy test method called "Vitrigel-EIT method" that uses time-dependent relative changes of the transepithelial electrical resistance for the prediction of irritative effects (Yamaguchi et al., 2013) was developed.

All described eye irritation tests showed a sensitivity and specificity in the range of 75% to 100% and therefore predict irritants and non-irritants with high accuracy. Vitrigel-, LabCyte and EpiOcular™ eye irritation test could predict irritancy potential without false negative classification (sensitivity of 100%). However, the specificity of these tests was lower than that of others with values between 75% and 80%. Eye irritation tests employing the SkinEthic™ model achieved a sensi-

tivity around 85% but the assay showed a lower rate of false positive predictions and therefore higher specificity than the other tests.

However, none of these tests is suitable to distinguish between category 1 and 2 in the GHS classification. Thus, until now, no single *in vitro* assay has been developed and validated as a full replacement for the Draize eye test (Tab. 2). Due to the lack of a single *in vitro* assay as a full regulatory replacement for the Draize eye test, a EURL ECVAM Expert Meeting (February 2005) suggested a tiered-testing approach to correctly predict the different eye irritation categories. The combination of different *in vitro* tests in a top-down or a bottom-up approach, proposed in the meeting, could be used to develop an eye irritation testing strategy to reduce animal studies (Scott et al., 2010). For example, the integrated testing strategy for classification of eye irritation potential of antimicrobial cleaning products developed by the Institute for *In Vitro* Sciences (IIVS) and validated by US Environmental Protection Agency (US EPA). This strategy combines the BCOP, EpiOcular™ and the CM assay. In the first step, it is determined whether the test material has oxidizing chemistry or is to be expected to be a moderate or severe irritant. If yes, the BCOP assay can be used. If not, the EpiOcular™ or the CM assay can be employed. These assays are more sensitive to small amounts of damage, while the BCOP assay can still highlight differences between moderate and severe irritants. Taken together the benefits of all assays help to improve the risk assessment (US EPA, 2015).

To differentiate between moderately and severely irritating effects in a single test, the presence of a stroma in addition to the epithelium is required, because severity of eye irritation is correlated to the depth of injury (Scott et al., 2010). However, the described corneal RCE mimic the human corneal epithelium, but lack the deeper corneal layers of stroma and endothelium. Therefore, the development and a following validation of full thickness cornea models to assess eye irritation potential for all GHS classifications without the help of a tiered-testing strategy is recommended.

5 Complex cornea models

In order to enhance predictability and work towards a standalone test, which can cover all GHS classes of eye irritation, more complexity might be necessary in the existing models to mimic the physiological environment of the cornea. This would allow more mechanisms of eye irritation to be addressed in one model. The main features to include are the different cell types and the highly organized stroma.

Currently available complex models containing the three main cell types of the epithelium, stroma and endothelium were established using cell lines (Griffith et al., 1999), animal cells (Alaminos et al., 2006) or primary human cells (Proulx et al., 2010). Models based on cell lines are easy to handle and can be created in large quantities. One of the first models to use immortalized cells from the epithelium, stroma and endothelium aiming for risk assessment was established by Griffith et al. (1999). Immortalized cells were generated from human corneal cells. The cell lines were selected to correlate best with their primary equivalents regarding gross histology and electrophysiology. Additionally, different chemicals tested on the model showed similar impact on transparency and cell death as observed in human and rabbit corneas. Nevertheless, transformed cells are altered, proliferate in an unrestrained manner and show significantly changed protein expression patterns as seen in the gene expression comparison (Griffith et al., 1999). Therefore, cell line based models, might be more suitable for an initial screening at the beginning of an integrated testing strategy.

In vitro models created with primary animal cells have the benefit of being accessible in large quantities from slaughterhouse waste and still have their physiological features. Many models were established from different species. One of the full thickness models was created with the three main cell types: Epithelial, stromal and endothelial cells from rabbit cornea on a fibrin-agarose scaffold. The model shows cornea epithelium specific Keratin 3 marker and ultrastructural features such as tight junctions (Alaminos et al., 2006). Yet, there are still differences between animal and human physiology concerning, for example, the regenerative potential of rabbit endothelial cells. The model published by Proulx et al. (2010) is one of the most advanced models to incorporate all three cell types from human cornea and a self-assembled matrix from fibroblasts. Furthermore, the model is characterized with regard to channel expression such as sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase) and adherence junctions, e.g., cadherins (Giasson et al., 2014). However, the model also has limitations, i.e., a thin stroma and a significantly reduced transparency in comparison to a human cornea. Test substances have yet to be evaluated with this model.

The stroma is still a significant challenge due to its highly organized ultra-structure and the resulting mechanical properties. Scott and Maurer emphasize the importance of the stroma for eye irritation by linking the regenerative power to the depth of the stromal injury (Maurer et al., 2002; Scott et al., 2010). Different approaches from artificial scaffolds using polymers, protein carrier or biological matrices are undertaken to mimic the biomechanical properties of the cornea. The most physiological approach at the moment is the acellular cornea stroma. In 2008, for instance, an acellular matrix from porcine corneas was developed, which could be transplanted into rabbit eyes (Xu et al., 2008). While these scaffolds could be interesting for clinical implants, the species differences and a rather costly preparation might push the field towards artificial scaffolds, which can be produced cost efficiently and in large quantities. The most commonly used material is collagen as the main component of the cornea. Bovine dermal collagen for example was used for rabbit corneal epithelial expansion and the acellular scaffold could be implanted into rabbit eyes (Geggel et al., 1985). In order to circumvent animal impurities and batch-to-batch variations recombinant human collagen can be used (Fagerholm et al., 2014; Griffith et al., 1999). Collagen hydrogels still bring with them mechanical problems such as stability, transparency and cell mediated contraction (Bell et al., 1979). Different methods to

enhance the mechanical properties of collagen carriers by cross-linking collagen (Liu et al., 2006), vitrification (Calderon-Colon et al., 2012) or plastic compression (Mi et al., 2010) are being investigated. Cross linked collagen could also be used as an implant in humans (Fagerholm et al., 2014). Other materials such as polycaprolactone (PCL), a biocompatible polyester, are being investigated, which have the benefit of being already medically approved for pharmaceutical products and have been used as scaffolds for skin and bone regeneration. Moreover, it could be shown that conjunctival epithelial cells can be expanded *in vitro* on the polyester (Ang et al., 2006).

One of the most promising models for eye irritation testing includes human corneal keratinocytes grown on a collagen scaffold with human corneal keratocytes. First tests of the so called hemi-cornea showed the ability to distinguish substances that cause severe irritation (GHS category 1) from substances which are mildly irritant (GHS category 2) (Engelke et al., 2013; Bartok et al., 2015).

Further development aims to include other cell types such as immune cells and cells of the nervous system, which can add new features of the physiological milieu, e.g., debris clearing. Also, the crosstalk between cells could be an important readout for different aspects of eye irritation, which basic models cannot deliver. Suuronen et al. (2004) developed a cornea model incorporating nerve growth, showing a stimulation of tissue growth and a possible interaction between the neurites and epithelial cells through substance P. However, for a repetitive toxicological test it has to be evaluated if these features are necessary in eye irritation.

Another challenge is the long-term culture. In the Draize eye test effects are monitored over a period of 21 days for the categorization of substances of the category 2. The vital importance of persisting effects for the classification of eye irritants was presented in a retrospective study by Adriaens and coworkers (2014) that showed that 35% of all category 1 chemicals listed in the reference chemicals databases are categorized as category 1 due to persistence effects only. For category 1 substances within the European new chemicals database this value was as high as 65% (Adriaens et al., 2014). Only the organotypic PorCORA assay using porcine cornea achieved an observation time of 21 days. The PorCORA test assesses the fluorescein staining retention of the corneal epithelium and could predict 28 out of 32 GHS category 1 and 2 compounds correctly (Piehl et al., 2010, 2011). Most *in vitro* models cannot be cultured over such a period, yet many substances probably need this time to be correctly classified.

One approach to prolong the life span of primary cells could be optimized culture conditions. Other possibilities to prolong the lifetime that have been introduced in regenerative medicine are bioreactor systems, which can enhance viability and functionality in a vascularized liver system. Additionally, those systems could include a mechanical eyelid movement (Linke et al., 2007; Hansmann et al., 2013). Still, it has to be determined if the 21 day period of the Draize eye test is needed to predict all GHS categories for new *in vitro* test methods based on human cornea models.

To date there is no single test method validated for all GHS categories. Although, the tiered testing strategies comprising several tests to differentiate between GHS categories might be an applicable method to reduce animal testing for eye irritation, the complicated nature of this procedure and the costs make it unappealing for industrial use. The main problem in developing a test that can predict all categories is the lack of characterization, especially concerning toxicological pathways, biomarkers and standardization of the complex models to be used or validated. An important step was established by classifying the modes of action and the importance of the stroma for identifying reversible effects (Maurer et al., 2002; Jester, 2006; Scott et al., 2010). This should give new input and help to design a model that can evaluate the toxicological effect of a substance in one test, thereby creating a cost efficient and easy to use alternative method for eye irritation.

6 Conclusion

In the last years significant progress has been made to replace the *in vivo* Draize eye test by alternative *ex vivo* or *in vitro* methods. Especially *in vitro* methods that employ RCE have the potential to replace animal experimentation without being dependent on explanted animal tissues. Similar to reconstructed skin models the first method, namely the EpiOcular™ test method, has demonstrated scientific proficiency and is now available as an OECD guideline. However, in contrast to skin, no available test method is able to assess the full spectrum of the GHS categories and thus no full replacement of the Draize eye test is available yet. To circumvent this pitfall integrated testing strategies have been proposed that combine different test methods in top-down or bottom-up approaches. In addition to the combination of multiple test methods that require significant numbers of test models, advanced *in vitro* models that mimic the full thickness of the cornea might be suitable to predict all GHS categories in one model.

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Conflict of interest

The authors declare no conflict of interest.

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