AOP and IATA applied to ocular surface toxicity

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REVIEW

An Overview of Current Alternative Models in the Context of Ocular Surface Toxicity

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Abstract

The 21st century has seen a steadily increasing social awareness of animal suffering, with increased attention to ethical considerations. Developing new integrated approaches to testing and assessment (IATA) strategies is an Organisation for Economic Co-operation and Development (OECD) goal to reduce animal testing. Currently, there is a lack of alternative models to test for ocular surface toxicity (aside from irritation) in lieu of the Draize eye irritation test (OECD guideline No. 405) performed in rabbits. Five alternative *in vitro* or *ex vivo* methods have been validated to replace this reference test, but only in combination. However, pathologies like Toxicity-Induced Dry Eye (TIDE), cataract, glaucoma and neuropathic pain can occur after exposure to a pharmaceutical product or chemical and therefore need to be anticipated. To do so, new models of lacrimal glands, lens, neurons innervating epithelia are required. These models must take into account real life exposure (dose, time, and tear film clearance). The scientific community is working hard to develop new, robust, alternative, *in silico* and *in vitro* models, while attempting to balance ethics and availability of biological materials. This review provides a broad overview of the validated methods for analysing ocular irritation and those still used by some industries, as well as promising models that need to be optimized according to the OECD. Finally, we give an

overview of recently developed innovative models which could become new tools in the evaluation of ocular surface toxicity within the scope of IATAs.

Short abstract

Until now, the Draize test in rabbits has been the only test performed to anticipate ocular toxicity of pharmaceutical compounds, mainly irritation. However, in the field of alternative approaches, new models must be developed and validated. This review aims to give an overview of the OECD validated methods and of innovative models, which could become new tools in the evaluation of ocular surface toxicity.

Key words: Draize Eye Test; OECD guidelines; Ocular Surface; In Silico; 3D Multicellular; Cornea-on-a-chip; Organoids

Introduction

Since the beginning of the 21st century, modern toxicology has been focusing on the 3R principle, "Reduce, Refine, Replace", established in 1959 by Russell and Burch, stipulating that the use of laboratory animals should be only a last resort. Since 2013, in Europe, the cosmetic industry has been confronted with strict prohibition of evaluating its products on animals. Integrated approaches of testing and assessment (IATA), promoted by the OECD (Organisation for Economic Cooperation and Development) might enable validation of new compounds in this sector (Canavez *et al.* 2021).

To date, validated alternative models have been available only for the evaluation of potential ocular surface irritation. Models to predict Toxicity-Induced Dry Eye (TIDE), anterior segment neuropathies or other ocular surface changes are still in the stage of basic science research. Furthermore, classification of ocular irritants is based on the United Nations Organization (UNO) system, *i.e.* the GHS "Globally Harmonized System of Classification and Labelling of Chemicals" (Luechtefeld *et al.* 2016). This international system distinguishes severe irritants (Category 1), moderate irritants (Category 2A), mild irritants (Category 2B) and non-irritants (No Category). However, unlike the Draize test, the *in vivo* reference model in rabbits, current alternative models for ocular irritation cannot distinguish Category 2A from 2B irritants. These irritants are usually differentiated based on the kinetics

of the reversibility of damage. Of note, the lack of reproducibility of the *in vivo* test of reference, the Draize test, complicates the validation of alternative models by the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods)(OECD Webinar 2019a).

In its first section, this review presents updates in the latest methodology for evaluation of ocular irritation, first presenting the five *in vitro* or *ex vivo* models validated by the OECD, in combination, to replace the Draize test (Guideline (GL) 405)(OECD, 2020a): Reconstructed human Cornea-like Epithelium (RhCE) viability tests (GL 492)(OECD, 2019a), Bovine Corneal Opacity and Permeability (BCOP) test (GL 437)(OECD, 2020b), Isolated Chicken Eye (ICE) test (GL 438)(OECD, 2018a), Fluorescein Leakage (GL 460)(OECD, 2017), and Short Time Exposure assay (STE, GL 491)(OECD, 2020c). The ocular irritation IATA indicates the combination of tests that should be considered depending on whether the product is suspected to be an irritant ('top-down' approach) or is thought to be in the non-irritant category ('bottom-up' approach). This review also presents models used by some cosmetic companies that either were or still are under evaluation by the OECD, such as the Isolated Rabbit Eye (IRE) test, Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM). Characteristics and protocol details of the models for ocular irritation are summarized in Table I.

Next, promising models mentioned in the OECD Guidance Document No. 263 (OECD, 2019b) are presented. These models, if optimized and validated, might represent a major asset in classifying new compounds into Categories 2A and 2B: PorCORA (Porcine Ocular Cornea Opacity/Reversibility Assay), EVEIT (Ex Vivo Eye Irritation Test), 3D Hemi-Cornea and SMI (Slug Mucosal Irritation assay).

Finally, in order to prevent complex toxicities as TIDE, glaucoma, cataract, some of which are rare topical side effects, new models presented in the literature could be validated and incorporated into new IATAs, taking into account real-life exposure, pharmacokinetics and knowledge already reported in the literature. Therefore, this final section provides an overview of *in silico* and *in vitro* models which could, in combination, enable complete evaluation of ocular surface toxicities within the framework of IATAs.

Alternative models to the Draize test according to OECD GL 405

Reconstructed human Cornea-like Epithelium (RhCE)

Since the last update of GL 492 in 2019 (OECD, 2019a), four models of RhCE are now available to evaluate ocular surface irritation, two of which are considered Validated Reference Methods (VRM): EpiOcular™ (VRM1), SkinEthic™ HCE (VRM2), LabCyte CORNEA-MODEL24 and MCTT HCE™. These RhCE models mimic human corneal epithelium morphologically, histologically, biochemically and physiologically and can be used first in a 'bottom-up' approach to identify non-irritant substances. Even though cellular damage can occur through several mechanisms, only cytotoxicity measurements are carried out. Indeed, cell viability is considered to be proportional to the severity of damage and representative of the global response of the ocular surface: mild irritants with low transcorneal penetration alter only the superficial corneal epithelium, whereas moderate or severe irritants can penetrate more deeply, reaching the corneal stroma and sometimes endothelium. This global response would be a correct representation of the damage that could occur in humans after toxic exposure, no matter the cellular mechanisms involved, ranging from slight conjunctival erythema or edema to severe changes such as corneal opacification.

While there may be differences between RhCE models (Table II), mainly concerning the cell types used and duration of epithelium culture, the testing method is similar: direct application of the tested compounds on the 3D epithelium and viability cytotoxicity assays reflecting the mitochondrial metabolic ability of viable cells. If corneal viability diminishes to below the fixed threshold (specific to each RhCE, see Table II), this will suggest classification of the compound as an ocular irritant. Above the threshold, the compound will not be classified and must be combined with another validated GL (437, 438, 460, 491 or in last option, if the test compound is not a cosmetic, GL 405 *i.e.* Draize test). Nevertheless, the OECD Guidance Document No. 263 (OECD, 2019b) reports the ongoing OECD evaluation of the EpiOcular™ time-to-toxicity (ET_{50}) assay, a test that could enable the differentiation of category 2A from 2B irritants (Kandarova *et al.* 2018). This new protocol is based on multiple time and concentration exposures. It could represent a major asset in the scope of IATA decision trees, since as of yet, no validated alternative model alone can distinguish between all the categories of irritants.

Another limitation is that GL 492 can only be used for solids, semi-solids, liquids and waxes, since gases and aerosols have not undergone validation procedures. Nonetheless, this aspect should be investigated, since many accidental ocular exposures are caused by volatile compounds (OECD 2019a).

Bovine Corneal Opacity and Permeability assay (BCOP)

Recommended as the first step of a 'top-down' strategy, the organotypic BCOP model described in GL 437 (OECD, 2020b) enables differentiation between severe irritants applied to isolated bovine cornea from slaughterhouses. It can also identify non-irritants in a 'bottom-up' approach. The eyeballs are kept *ex vivo* for a brief period, during which physiological and biochemical functions remain unaltered. After excision, corneas are anchored on a corneal holder composed of two chambers, both filled with preservation medium. Briefly, the endothelial surface of the cornea is placed on the *o-ring* in the posterior chamber, while the epithelial surface is positioned in the anterior chamber.

Two application methods, adapted to the type of compound being tested, are described in the GL, but an important parameter is verification that the product covers the entire epithelial surface and that the washing step is sufficient to retrieve all of the compound. Irritancy potential is then measured through the *In Vitro* Irritancy Score (IVIS), which combines the diminution of light transmission capacity (corneal opacity, measured with an opacimeter) and the increase in fluorescein sodium passage (permeability, *i.e.* the amount of dye dropped in the anterior chamber and that crosses the corneal thickness). Of note, fluorescein sodium is an anionic compound, not retained by a healthy, negatively charged epithelium. A substance will be categorized as a severe irritant if the IVIS is greater than 55 and as a non-irritant if the IVIS is less than 3. However, if the IVIS is between 3 and 55, additional tests will be required to distinguish category 2 irritants. It is also possible to complement these results with a histologic analysis of the cornea, which procedure is described in the Guidance Document No. 160 (OECD, 2018b).

Of note, since the last guideline update in June 2020, a second opacimeter can be used (LLBO), requiring adaptation of the IVIS equation and decision criteria, but the performance is comparable to OP-KIT, the first opacimeter validated.

Isolated Chicken Eye (ICE)

Like the BCOP model, the ICE aims to discriminate Category 1 GHS substances in a 'top-down' strategy but can also be included in a 'bottom-up' approach to identify non-irritants. The ICE is regulated by OECD GL 438, last updated in 2018 (OECD, 2018a). This test uses enucleated eyes of chickens for human consumption. In this assay, corneas are not excised. The whole eye is placed in a stainless-steel clamp with the cornea positioned vertically. The clamp is placed in a superfusion chamber to nourish the cornea. At the start of the test, the

clamp is retrieved from the chamber and the cornea positioned horizontally in order to apply the tested compounds. A qualitative and quantitative evaluation of the cornea is conducted to establish potential opacities, epithelial morphological alterations (detected by fluorescein sodium retention) and edema. As for BCOP, corneal opacity and fluorescein retention are scored, and are associated to a morphological evaluation which is "subjective according to the interpretation of the investigator" (GL 438). The combination of these scores enables the GHS classification of test compounds. For instance, with three scores of I, the substance is considered a non-irritant.

Furthermore, since the last GL update, histological features after paraffin embedding can be analyzed notably for detergents and surfactant irritants (OECD, 2019b). Indeed, there should be a correlation between erosion, vacuole formation in the inferior area of the epithelium, presence of pycnotic nuclei and irreversibility of the damage. GL 438 proposes another table to score those parameters.

Fluorescein Leakage (FL)

The FL test follows GL 460 (OECD, 2017). The FL method is performed *in vitro* on a semi-permeable membrane (insert) leading to a single-layer culture of renal tubular cells of Madin-Darby Canin (MDCK CB997). This is a well described cell line, known to form tight junctions and desmosomes. Its organization is similar to the non-proliferative apical corneal epithelium. Furthermore, permeabilization of corneal epithelium is known to be one of the first phenomena occurring in toxicity-induced ocular irritation.

Changes in tight junctions and desmosomes are proportional to the quantity of fluorescein sodium that diffuses into the basal chamber, evaluated though $FL_{20\%}$, that is to say the concentration of the tested compound that leads to an FL of 20% compared to negative controls (single layer of cells not exposed and insert without cells). The substance tested is categorized as a severe irritant on the GHS ocular irritation classification if the $FL_{20\%} \leq 100$ mg/mL.

Integrated into a 'top-down' strategy, this simple method enables distinguishing Category 1 chemicals without additional data. However, unlike the previous methods presented, this method can only be used with water-soluble compounds or mixtures. Indeed, solids in suspension will precipitate. In addition, it is not applicable to strong bases or acids, volatile compounds or cellular fixatives, because the toxic mechanisms for these types of compounds (such as protein coagulation or saponification) cannot be evaluated by FL. Finally, colored or

viscous compounds should be tested with other methods, since their complete washout required before the fluorescent measurement is complicated. Of note, a compound with a strong affinity for the insert membrane can lead to the same problem. Therefore, this affinity must be tested, as described in the GL, before beginning the assay. While reversibility of the epithelial changes cannot yet be evaluated, this will be considered in the next update of the GL, with the possibility of using FL to separate Categories 2A and 2B of the GHS classification.

Short Time Exposure Assay (STE)

The STE, GL 491 (OECD, 2020c), can be considered in 'bottom-up' and 'top-down' strategies. STE enables the evaluation of all types of chemicals except volatile compounds with a vapor pressure above 6 kPa[1] and solid non-surfactants (not water-soluble after at least 5 min in NaCl). This *in vitro* model consists of a confluent monolayer culture of rabbit corneal fibroblasts (several cell lines are possible, such as CCL60 or SIRC). If cell viability (MTT assay, see Table 2) is less than 70% with both concentrations, the substance is placed in Category 1 without any additional assay. If cell viability is above 70% with at least one concentration, additional tests are required.

Other ocular irritation models evaluated by the OECD

Vitrigel-Eye Irritancy Test (EIT) method

While not mentioned in GL 405 as an alternative method, the OECD introduces this method in its Guidance Document n°263 (OECD, 2019b) and in the 2019 GL 494 (OECD, 2019c), establishing the protocol for the Vitrigel EIT method. This method can be used only in a 'bottom-up' approach to identify non-irritants. It evaluates the barrier function of a human corneal epithelium reconstructed on a Vitrigel matrix (ECh-T immortalized cell line ; collagen gel obtained by rehydration of a hydrogel that has undergone a vitrification process)(Takezawa *et al.* 2004; Yamaguchi *et al.* 2016) with a Transepithelial Electrical Resistance (TEER) measurement. Of note, this ohmmeter analysis is sensitive to the number of cell passages and to room temperature (Srinivasan *et al.* 2015). This measurement is characterized by three parameters: time lag, intensity and plateau level. A non-irritant product is identified by a time lag > 180 seconds, an intensity < 0.05% and a plateau level ≤ 5.0%. If one of these criteria differs, additional studies are required to classify the product. A

limitation of this method is its small range of application, being limited to liquids or semi-liquids with a pH > 5. However, unlike previous methods described, it can be used for volatile compounds and products that interfere with the detection of formazan in the MTT assay.

Ocular irritection®

Ocular Irritection® is an *in vitro* macromolecular test which is the subject of a GL drafted in 2019 (OECD, 2019d). It is suitable either in a 'bottom-up' or 'top-down' strategy for solids and liquids with a pH between 4 and 9. It is an acellular system composed of proteins, glycoproteins, carbohydrates, lipids and low molecular weight compounds. Ocular Irritection® test aims to mimic the organized and transparent structure of the cornea after rehydration (Eskes *et al.* 2014). This enables specific detection of protein coagulation or lipid saponification mechanisms. Nevertheless, since the system is devoid of cells, cytotoxicity cannot be evaluated. The matrix and the testing principle are presented in figure 1. Any change in the matrix organization leads to a modification of the turbidity and reflects the irritative capacity of the test compound. However, like many alternative models, this testing method alone is unable to distinguish mild irritants.

Cytosensor Microphysiometer (CM)

Because of the lack of commercial availability of the Cytosensor Microphysiometer technology, the preliminary GL version released in 2012 (OECD, 2012) for the evaluation of water-soluble compounds, solids, viscous substances or homogenous suspensions has seen its development discontinued in 2016 (European Commission 2020a). Nevertheless, it could be integrated into a 'bottom-up' or 'top-down' approach if similar instruments were to come to market. It consists of an adherent, confluent, single layer of mice fibroblasts (cell line L929) cultured on a polycarbonate insert. These cells are designed to represent conjunctival and corneal epithelia. The test endpoint is the Metabolic Rate Decrement of 50% (MRD_{50}), that is to say the concentration that reduces the acidification rate by 50%. This measurement reveals irritation potential, since damaged cells will produce less acidic metabolites in the culture medium. On the one hand, if the $MRD_{50} \leq 2$ mg/mL, the product is considered to be a severe irritant in a 'top-down' approach. On the other hand, if the $MRD_{50} > 10$ mg/mL, the test compound is classified as a non-irritant in a 'bottom-up' strategy. Of note, the GL mentions that this testing method could evaluate reversibility if optimized.

Neutral Red Release (NRR)

266 The NRR test evaluates cytotoxicity on a single layer fibroblast or keratinocyte culture loaded
267 with neutral red 3 hours before the exposure to test compounds (OECD, 2019b). This vital
268 dye incorporates itself into lysosomes of viable cells. Several protocols have been proposed in
269 the literature (Zuang 2001), such as the FRAME protocol based on mice embryonic
270 fibroblasts (3T3-L1 cell line) or the *Clonetics Corporation* protocol using human
271 keratinocytes. In both cases, the endpoint is the NRR_{50}, that is to say the test compound
272 concentration that releases, in the culture medium, 50% of the neutral red incorporated by
273 lysosomes. The more toxic a substance is, the more cellular membranes, including lysosomal
274 membranes, are altered, leading to leakage of intracellular compounds such as neutral red.
275 Validated by internal procedures in many industries, the ICCVAM is requesting
276 supplementary data on inter-laboratory reproducibility before publishing a GL on the Neutral
277 Red Release assay (OECD, 2019b). It is also being considered for use in combination with the
278 EpiOcular time-to-toxicity assay on RhCE.
279
280 *Red Blood Cell test (RBC)*
281 The RBC test evaluates the ability of test compounds to disrupt red cell membranes (relation
282 between hemolysis and oxyhemoglobin denaturation) and in this way, to classify products
283 into GHS Categories 1 or non-classified (OECD, 2019b). RBC test can be conducted on red
284 blood cells from various species (pig, sheep, rabbit) (Lewis *et al.* 1993; Mehling *et al.* 2007;
285 Pape *et al.* 1987; Pape 1990). The irritant potential score corresponds to the ratio between the
286 leakage of red blood cell hemoglobin in the supernatant (H_{50} concentration inducing a red cell
287 hemolysis of 50%) and oxyhemoglobin (denaturation index, DI). If $H_{50}/DI > 100$, the
288 substance is considered a non-irritant, between 10 and 100 the substance is categorized as a
289 mild irritant (Category 2), between 1-10 as a moderate irritant, and if the $H_{50}/DI < 1$, the
290 compound is classified in Category 1 (severe irritant).
291 An application of this method on 12 shampoos and 7 conditioners was proposed by Alves *et*
292 *al.* (2008), attesting to a 91.6% sensitivity and 100% specificity of the method. However, in
293 the Guidance Document n°263 (OECD, 2019b), the OECD underscores the necessity for
294 more data on the types of compound that can be tested, in other words, the method's range of
295 application. Indeed, while the literature reports other studies on surfactants, mixtures
296 (Mehling *et al.* 2007) and eyedrops (Martins *et al.* 2012), the chemical and physical properties
297 of test compounds must be further investigated.
298

299 *Isolated Rabbit Eye (IRE)*

300 Although the IRE test is similar to the ICE (compound exposure time, endpoints ; see Table
301 1), this organotypic model on the enucleated rabbit eye has not been validated by the
302 ICCVAM since its 2010 evaluation, due to the lack of a standardized protocol, the lack of
303 data on decision criteria, and the fact that rabbit eyes come from experimental animals and not
304 from slaughterhouses as with BCOP or ICE (Lee *et al.* 2017; Prinsen and Koëter 1993).
305 Nevertheless, the IRE is accepted in the European Union for distinguishing severe irritants
306 (except alcohols, solids and surfactants, for which there are too many false negatives)
307 (ICCVAM 2010).

308

309 *Hen's Egg Test on Chorioallantoic Membrane (HET-CAM)*

310 The HET-CAM is an alternative model developed by Luepke in 1985 and modified to classify
311 irritant compounds. Indeed, the chorioallantoic membrane of the egg is considered to be a
312 reasonable facsimile of the conjunctiva and its vasculature. Of note, from this model was
313 derived another model, the Chorioallantoic Membrane Vascular Assay (CAMVA). The main
314 nuance between the two (see figure 2) could enable differentiation of non-irritants from mild
315 or moderate irritants.
316 The main advantages of using embryonated eggs are their accessibility, low cost and rapid
317 growth. These eggs can be kept in an incubator for up to 13 days of maturation. After 14 days
318 of growth, the development of the embryo is advanced, and the model is then considered an *in*
319 *vivo* model (Kue *et al.* 2015).
320 While the ICCVAM did not validate this testing method for distinguishing severe irritants
321 (ICCVAM 2010), this method is still used by some industrials in their internal weight of
322 evidence WoE, these methods being recognized in the European Union. The procedure for
323 opening the eggs without breaking the vascular membrane is described in figure 2.
324 However, one should bear in mind that this testing method has been increasingly criticized,
325 being considered an *in vivo* model even in the first days of embryonic development.

326

327 **Models requiring optimization according to the OECD**

328

329 The models introduced in the following section are models mentioned in the OECD Guidance
330 Document n°263 as interesting models, if optimized, for evaluation of reversibility of ocular

331 irritation/corrosion, which may thus be able to distinguish between all GHS categories,
332 including category 2 compounds.
333
334 *Porcine Ocular Cornea Opacity/Reversibility Assay (PorCORA)*
335 PorCORA is an organotypic model similar to BCOP, since it is based on the maintenance *ex*
336 *vivo* of porcine cornea obtained from slaughterhouses. Its added value lies in the air interface
337 preservation allowing maintenance for 21 days (same as in the Draize reference test, the
338 amount of time needed to evaluate reversibility of damage). Several steps are required to
339 prepare the excised corneas (Vij *et al.* 2017). First, the corneas are placed in a 24-well plate,
340 with the epithelium facing the bottom of the well. A mixture of agar/gelatin/medium is poured
341 onto the corneas, which are then placed in Petri dishes after gelification. The test compounds
342 are applied directly to the corneal surface (10 µL for liquids, 20 mg for solids) for 5 minutes.
343 The corneas are then washed with PBS (Piehl *et al.* 2011). Corneal alterations and their
344 reversibility are then estimated and scored based on the area of staining with fluorescein
345 sodium over the course of 1, 2, 3, 7, 10, 14 and 21 days after compound exposure (European
346 Commission 2020b).
347 In this way, Piehl *et al.* demonstrated in 2011 that this method gave similar results to the
348 Draize reference test (correlation coefficient of 0.98) with reproducible results for the five
349 control test substances: phosphate buffered saline (PBS), absolute ethanol (EtOH), 3%
350 sodium dodecyl sulfate (SDS), 1% benzalkonium chloride (BAK), and 10% sodium
351 hydroxide (NaOH). Furthermore, in this study, PorCORA identified reversible and
352 irreversible effects. By establishing a PorCORA score, it could be possible to distinguish
353 GHS category 1 products (irreversible alterations before 21 days) from category 2 products
354 (reversible damages before 21 days, with a score returning to 0).
355 Nevertheless, additional data is needed. Indeed, in this study, Piehl *et al.* found that the
356 method was too sensitive for surfactants. Similarly, in another study conducted on shampoos
357 and hair dyes, PorCORA overestimated the irritant potential (Donahue *et al.* 2011). Finally, a
358 drawback of this model is the progressive opacification of the cornea due to the gel that
359 prevents the endothelium from correctly regulating corneal stromal fluids (Spöler *et al.* 2015).
360
361 *Ex Vivo Eye Irritation Test (EVEIT)*
362 The EVEIT is an air-liquid interface culture system, enabling maintenance of excised rabbit
363 corneas (from slaughterhouses) for 72 hours following compound application. Briefly,
364 corneas with a scleral ring are removed and anchored in a chamber filled with a minimal

volume of medium to maintain hydrostatic pressure. This *ex vivo* model reflects the biochemical activity of corneal epithelium and endothelium. Its advantage compared to the PorCORA system presented above is that the EVEIT does not lead to corneal opacification during culture (Spöler *et al.* 2015). Decision criteria are evaluated four times over 72 hours enabling differentiation of non-irritants from category 2A irritants (OECD, 2019b; Spöler *et al.* 2015): macroscopic observation of corneal opacity, fluorescein sodium diffusion, corneal thickness and structural changes measured by optical coherence tomography. Each measurement results in a score, similar to those used in the ICE or Draize tests, which were described by Spöler *et al.* in 2015. If preservation time of the corneas *ex vivo* could be improved, this testing method could enable differentiation of all category 2 products. Of note, this method was used by Schrage *et al.* in 2012 to evaluate the effect of artificial tears on corneal epithelial repair after mechanical damage. This study highlights the fact that the models presented in this section could serve equally well for toxicity studies as for pharmacological studies for the development of ophthalmic treatments.

3D Hemi-Cornea

The first *in vitro* system that may potentially discriminate GHS categories 1 and 2 alone, the 3D Hemi-Cornea combines, in an insert, a corneal human epithelium reconstituted from an immortalized cell line with human corneal immortalized keratinocytes which represent stromal cells (Bartok *et al.* 2015; Engelke *et al.* 2013; Zorn-Kruppa *et al.* 2014). The two cell types are separated by a collagen membrane allowing evaluation of the two cell lines independently after a 60 min-exposure of the chemical (Zorn-Kruppa *et al.* 2014). This model is adapted for liquids as well as solids but is constraining since it has to be cultured during 7 days with a daily change of medium. The endpoint measured is metabolic activity and the cytotoxicity MTT test. The distinction between GHS categories non-irritant, 1 and 2 could be observed though the extension and/or localisation of corneal changes (Tandon *et al.* 2015). Moderate irritants lead to a loss of viability of the corneal epithelium and can affect the stroma, whereas severe irritants lead to severe corneal epithelial and stromal alterations. As a result, this system properly classifies category 1 compounds and 80% of category 2 compounds, but only 50% of non-irritant substances, with an overestimation of their irritant potential. A hypothesis to explain these last, disappointing results is that the compounds in this category were frequently viscous and difficult to remove during the washing steps, leading to the deterioration of some epithelial layers (Bartok *et al.* 2015). Furthermore, in

398 another study, the irritation potential of compounds with extreme pH were again
399 overestimated, as in other *in vitro* tests, possibly because of the absence of the mucinous layer
400 of the tear film, which has a buffer effect *in vivo* (Zorn-Kruppa *et al.* 2014).
401 Nonetheless, this test quoted in the Guidance Document n°263 of OECD seems to be an
402 option for the evaluation of surfactants, alcohols, ketones, and volatile compounds, in other
403 words, compounds that, in many other alternative models, lead to false positives results. This
404 3D hemi-cornea could at the same time allow the evaluation of compound diffusion, since the
405 test substances need to cross an aqueous collagen membrane.
406
407 *Slug Mucosal Irritation* (SMI) *assay*
408 Described in the literature for the evaluation of reversible or irreversible ocular (Lenoir *et al.*
409 2011a) and nasal (Lenoir *et al.* 2013) stinging, itching and/or burning (SIB), the SMI test
410 measures the liberation of mucus proteins from *Arion lusitanicus* slugs. This method can
411 screen for ocular discomfort generated by isolated ingredients or final products. As presented
412 in the schematic protocol Figure 3, the slug's weight is compared before and after every
413 contact period (CP)(Lenoir *et al.* 2009, 2011a, 2013; Cutuli *et al.* 2021).
414 Developed by Lenoir *et al.*, this test was used to evaluate shampoos and artificial tears. The
415 results were correlated with a clinical study (Spearman's Rank correlation of 0.986, $p <$
416 0.001)(Lenoir *et al.* 2011b). Similarly, Petit *et al.* 2017 was able to reproduce this alternative
417 model in 2017 to evaluate veterinary products. Recently, a new SMI alternative model, using
418 a "Yellow slug", was reported to evaluate surface disinfectants used against SARS-CoV-2
419 (Cutuli *et al.* 2021).
420 Since it can distinguish category 2 irritant products, optimization and validation of this test is
421 mentioned to be of interest in the OECD Guidance Document n°263. However, depending on
422 national regulations, this test might be considered animal experimentation (OECD, 2019b).
423
424 **New innovative models for ocular surface toxicity evaluation**
425
426 Mimicking ocular structures *in vitro* is challenging (lacrimal glands, conjunctiva, innervation,
427 lens, …). New models are being developed in basic science research, notably using fluidic
428 and three-dimensional approaches. These technologies of organ-on-a-chip originate from the
429 area of pharmaceutical research and development (Wilson *et al.* 2015). In addition, this
430 review will focus on *in silico* approaches, which are required to understand real-life exposure

431 and thus aid in design of the *in vitro* strategy, reducing time and costs of development.
432 Organoid models will be described in the final part of this section, even though these new
433 cellular structures are mainly studied for the purpose of replacing deficient patient structures.
434 Table III proposes an overview of the selected models.
435
436 *In silico models*
437 *In silico* approaches, using computer and mathematical tools, aim to simulate *in vivo*
438 biological processes, mimicking a multicellular organ crossed by biological flows and
439 connected to other structures of the organism. Inspired by the "PB-PK", *Physiologically*
440 *Based Pharmacokinetics,* approach (predicting absorption, distribution, metabolism and
441 elimination), these *in silico* methods try to improve toxicological evaluation, taking into
442 account local metabolism, barriers, …., and to estimate a toxic dose (Knudsen *et al.* 2015).
443 Once the organ is modelled, multiple scenarios can be tested by changing dose, time, method
444 of exposure and other parameters that could influence the risk of toxicity (for instance,
445 enzyme polymorphisms, pregnant women or pediatric differences in metabolism)(Jones *et al.*
446 2015).
447 For each product tested, an exhaustive knowledge of its physicochemical properties must
448 come through computerized channels (Brochot *et al.* 2014). To this end, other *in silico* tools
449 can contribute to the information in the literature: *Qualitative and Quantitative Structure*
450 *Activity Relationship* (QSAR) models that can predict biological properties such as affinity,
451 protein binding, based on chemical structure. These models are available as free access or
452 commercial software (ECHA 2019). To encourage regulatory acceptance of these QSAR
453 models, the OECD released Guidance Document n°69 and created a free access toolbox with
454 some QSAR models (OECD 2020d). First developed for conception of possible
455 pharmaceuticals, some QSAR models aim to predict ocular irritation and damage based on the
456 compound's toxicodynamic properties: acidity, electrophilicity, chemical reactivity, surfactant
457 effect (OECD, 2019b). For instance, Kulkarni *et al.* (2001) examined membrane interactions
458 of compounds with the stratified lipophilic corneal epithelium to determine the irritant
459 potential of substances already classified by the Draize test *in vivo*.
460 For local ocular toxicity, it is essential to mimic three main factors that influence ocular
461 surface penetration and distribution: static barriers with different transport systems (claudins,
462 zonula occludens), dynamic clearance (lacrimal fluids, Schlemm's canal drainage) and
463 metabolic factors (enzymes, efflux pumps, receptors). In 2018, Pak *et al.* applied these

principles to develop an *in silico* rabbit cornea model (epithelium, stroma, endothelium), the Quasi-3D CoBi (Computational Biology) model which includes passive transport (paracellular, transcellular) through the corneal epithelium (barrier to the passage of hydrophilic compounds), transport though the stroma (barrier to the passage of lipophilic compounds) and protein binding (such as glycosaminoglycans which can retain hydrophilic compounds). To do so, the research team created a precise geometric representation of the multilamellar corneal structure, applying complex mathematic equations to reflect the various flows. Nevertheless, this *in silico* model should be elaborated by adding all of the ocular structures (such as conjunctiva, tear film, neurons, retina) and should be based on human data to improve the predictions made through these models. The lack of human data on barriers, thickness and porosity of layers, local metabolism, physical constants, ..., remains to this day a barrier to the development of *in silico* models and use as a high throughput tool. Implementation of this work would be extremely time-consuming and would require a great deal of computing power but would improve extrapolations.

By enabling the identification of target structures, these models could guide the first steps of the AOP (adverse outcome pathway), which are currently the subject of toxicological development as supports for implantation of IATA, limiting unnecessary *in vitro* studies. However, one should bear in mind that if an important metabolic pathway used by the compound tested is missed in the model, the predictions will not be accurate.

3D multicellular models

Numerous 3D models are described in the literature, improving the phenotype of the epithelium formed. Nevertheless, many of them neglect the tear film, which covers the epithelia of the ocular surface, as well as the innervation of the ocular surface, which are, however, two central structures in understanding and anticipating TIDE and anterior segment neuropathies. Thus, this section describes three models that could become assets in the development of IATAs for ocular surface toxicity: the first model presented would allow evaluation of tear film thickness and composition, while the following ones would permit analysis of the toxic impact on neurons interacting with corneal cells.

- Conjunctiva and lacrimal gland coculture

The literature is rich in alternative corneal models but delves less into conjunctival and lacrimal gland toxicity, although these structures which are essential for production of the tear

film, a fundamental structure to be evaluated for the anticipation of TIDE. Nevertheless, in 2017, Lu *et al.* proposed a coculture between rabbit primary epithelial conjunctival cells and spheroids of rabbit primary lacrimal acinar cells. To our knowledge, this is the first *in vitro* 3D model capable of producing aqueous and mucinous layers of the tear film.

After testing several configurations, direct contact between the two cell types, as presented figure 4, was found to present the best configuration, with optimal epithelial morphology, permeability, phenotype and lacrimal fluid production, even though direct contact is not the most physiological configuration (no direct contact in humans between these types of cells). To highlight the usefulness of their model, they demonstrated the protective effect of dexamethasone, a corticosteroid known to reduce inflammation of the ocular surface in TIDE, after exposure to pro-inflammatory IL-1β. This effect could not be seen on a simple monoculture of conjunctival cells. While this model does not allow the formation of a complete tear film with a lipid layer, it remains an interesting advance for the *in vitro* anticipation of TIDE. Further studies could be conducted by adding meibocytes in the culture, to obtain a complete tear film.

- 3D model of nerve-stroma interactions

To date, only a few models consider corneal innervation in a toxic response. Sharif *et al.* (2018) explored the corneal stroma-neuron interaction in depth by proposing a 3D coculture on an insert between HCF (*human primary corneal fibroblasts*) and SH-SY5Y neurons, a well-characterized human neuroblastoma cell line derived from bone marrow. This model is based on the *de novo* production of extracellular matrix by fibroblast cells and tries to mimic the *in vivo* nerve-stroma interaction in the cornea, improving the comprehension and anticipation of corneal cell damage as well as pathways of neuronal regeneration.

However, further studies are needed to characterize the neuronal phenotype of this model and therefore the ability of this model to mimic toxicity affecting the ocular surface. Indeed, SH-SY5Y neurons do not have the same phenotype as primary sensory neurons from trigeminal nerves, the main innervation of the ocular surface (Mélik-Parsadaniantz *et al.* 2018), since they can develop two distinct phenotypes (neuroblastic or epithelial-like). SH-SY5Y includes adherent cells but also floating viable cells whose biological significance is not yet understood. Also, neuroblastic SH-SY5Y cells express tyrosine hydroxylase and dopamine-β-hydroxylase, two catecholaminergic markers, which are not characteristic of trigeminal neurons, which are primarily sensory neurons (Kovalevich and Langford 2013).

530 Nevertheless, transfected SH-SY5Y could be considered to study certain ocular surface
531 symptoms such as stinging or itching. This was the objective of the NociOcular test based on
532 a 2D model of SH-SY5Y expressing the transient receptor potential cation channel subfamily
533 V member TRPV1, known to be implicated in these ocular surface phenomena (Dua *et al.*
534 2018). Using this test, Forsby *et al.* (2012) completed an ocular tolerability study of 19
535 shampoos, resulting in only one false negative and two false positives compared to a clinical
536 evaluation. NociOcular measures, by fluorescence, the intracellular calcium flux mediated by
537 the activation of TRPV1 and correlated to ocular discomfort. A similar study was conducted
538 by Narda *et al.* in 2019) on the ocular tolerance of sunscreens, confirming the need to evaluate
539 disturbances in neuronal transmission and not just damage to the ocular surface epithelial cell
540 in a comprehensive study.
541
542 - Triculture of neuronal, epithelial and stromal cells
543 Wang *et al.* 2017 proposed an air liquid interface (ALI) triculture between human primary
544 corneal cells, human corneal stromal stem cells and Chicken Dorsal Root Ganglion (DRG)
545 neurons, supported by silk proteins. The use of silk proteins aims to mimic the mechanical
546 properties of the cornea, so as to favour neuronal development. Figure 5 explains the cellular
547 organization of the model. Through this set up, Wang *et al.* obtained optimized axonal
548 development as well as a better epithelium / stromal phenotype and viability. At the moment,
549 corneal tissue models are limited to one or two weeks of culture and do not include the
550 nervous component. This ALI culture, integrating corneal interactions with neurons while
551 conserving its integrity for 28 days, enables to evaluate toxic induced alterations of phenotype
552 and viability. This model represents a progress in tissue engineering, promoting the
553 importance of cell types interactions for better differentiation and maturation.
554
555 *Cornea-On-a-Chip models*
556 The focus of much attention in recent decades, organs-on-a-chip seek to miniaturize an organ,
557 facilitate the assembly of cell types and recreate the dynamics of an organ (Mandenius 2018).
558 These chips are mainly based on microfluidic technics, using biocompatible polymers such as
559 polydimethylsiloxane (PDMS), a transparent, flexible and gas impermeable organomineral
560 material. The advantage of these systems lies in the small amount of biological material
561 needed, while improving the representation of dynamic *in vivo* parameters compared to a
562 classic 2D cell culture. Nevertheless, protocols have not yet been standardized, scale-up

563 remains unfeasible for routine experimentation, and the analytical challenge (because of the
564 small quantity of cells) remains to be solved (Sosa-Hernández *et al.* 2018).
565 Because of the complexity of multicompartmental and multi-layered ocular structures,
566 establishing an eye-on-a-chip is a hard task. If we focus on the anterior segment, some
567 corneas-on-a-chip are described in the literature and attempt to include ocular surface flow
568 (blinking of the eyelids, tear secretion, shear stress). Furthermore, microfluidics and
569 compartmentalization on a chip are also being considered to improve the mimicry of ocular
570 surface innervation, taking into account the fact that only nerve endings can be directly
571 exposed to a topically applied toxicant.
572
573 - Cornea-on-a-chip, ocular flows and shear stress
574 A current limitation of corneal barrier models is the lack of flow to mimic the shear stress
575 caused on the epithelium by eyelid blinking, which is responsible for tear film movement, and
576 as a result of drug or toxicant distribution and its effects on the ocular surface. Of note, this is
577 also a limitation of the Draize test when attempting to most closely approximate human
578 physiology, since rabbits blink less frequently than humans, resulting in a longer exposure
579 time (Maurice 1995).
580 In 2018, to study passage through the corneal barrier, Bennet *et al.* 2018 proposed a cornea-
581 on-a-chip with a pulsatile flow to represent blinking or a continuous flow for tear secretion. A
582 confluent epithelium of 5 to 7 layers with a stable phenotype and permeability was obtained
583 on a PDMS chip with a fibronectin coated membrane (mimicking Bowman's layer) and
584 immortalized human corneal epithelial cells. In this system, eyedrop pharmacokinetics and
585 toxicity can be evaluated by applying either the continuous or pulsatile flow for 5 hours. After
586 this experimentation time, 98% of the compounds were found to be eliminated; compared to a
587 static model, it improves the evaluation of absorption, bioavailability and toxicity.
588 Nevertheless, additional studies are required to understand the impact of the two types of
589 flow, since compound penetration appeared more significant with the pulsatile flow.
590 Similarly in 2020, Abdalkader and Kamei published a four chamber microfluidic model with
591 uni- and bi-directional flow to study the impact of shear stress on corneal epithelium barrier
592 phenotype. This PDMS system, composed of human corneal epithelial cells on a porous
593 membrane, aims to simulate human cornea, with an apical side in contact with lacrimal fluid
594 (bidirectional flow for eye blinking) and a proximal side with the aqueous humor
595 (unidirectional flow mimicking drainage through Schlemm's canal). After having obtained a

596 stratified (2-3 layers), permeable (evaluation by fluorescein diffusion), phenotyped
597 (expression of tight junction proteins such as the zonula occludens proteins), they applied
598 both flows for 24 hours and observed that shear stress did not alter cellular adhesion and
599 improved the expression of cytokeratins, which are important proteins for flexibility, cellular
600 elasticity and maintaining corneal barrier integrity. In addition, this model could take into
601 account the compound real time of remanence in the tissue.
602 Nevertheless, these two models are limited in their representation of the cornea, since they
603 lack formation of the stromal and endothelial layers, corneal elements that are notably
604 essential for aqueous humor flow. This limitation is addressed by Bai *et al*. (2020) with their
605 cornea-on-a-chip, a PDMS compartmentalized chip using primary murine corneas; they
606 simultaneously isolate both epithelial and endothelial corneal cells and plant them into two
607 separate compartments with a collagen membrane to mimic Bowman's layer.
608
609 - Cornea and conjunctiva-on-a-chip
610 Another approach to the 3D ocular model on-a-chip was designed in 2019 by Seo *et al*.,
611 combining human primary corneal epithelial cells and immortalized conjunctival cells
612 (epithelial and glandular cells), cultured in an ALI system. The primary corneal cells are
613 incorporated into a collagen matrix which mimics the stromal layer. A perfusion system
614 mimics tear flow, while a biomimetic system recreates blinking of the eyelids. Their
615 complementary data gives a better representation of this complex model. Seo *et al.* obtained a
616 pluristratified epithelium with 7 to 8 layers like human cornea, expressing specific markers
617 (*ex.* cytokeratins 3, 19) and producing a "tear film" of 6 µm comparable to the *in vivo*
618 thickness. Like the previous models, they proved that shear stress induced cellular
619 differentiation and limited pro-inflammatory cytokine production. To attest to the utility of
620 their model, they demonstrated the anti-inflammatory action of lubricin, a protein-like mucin.
621 While this model does not include the vasculature or immune cells normally present in the
622 conjunctiva nor the nerve endings of the ocular surface which participate in tear secretion, this
623 chip represents a major improvement for pharmacological and toxicological compound
624 evaluation, especially for a TIDE IATA.
625
626 - Corneal innervation compartmentalization
627 Currently, most ocular surface models, like the flow systems just discussed, neglect toxic
628 effects on ocular surface innervation, whereas during a toxic exposure, trigeminal nerve

629 endings can be altered, with an indirect impact on neuronal cell bodies. Therefore, stimulating
630 primary cell cultures of neurons directly does not mimic real life exposure, and, as a result,
631 mechanisms of toxicity are impossible to analyze properly. In order to improve anatomical
632 representation of the ocular surface innervation, Sarkar *et al.* (2012) used a Campenot device
633 to evaluate morphological alterations (neurite fragmentation, axon breaks, lack of
634 regeneration) of mice primary trigeminal neurons after exposition to BAK, preservative
635 contained in many eyedrops. With this model, they highlighted a dose-dependent toxicity of
636 BAK on neurites. Campenot devices were the first systems to allow neuronal
637 compartimentalization but new microfluidic organ-on-a-chip devices could be considered.
638 Indeed, these microchips can be precisely designed to optimized axonal guidance of
639 trigeminal neurons (Courte *et al.* 2018). This innovative system also allows to analyze
640 separately nerve ending and cell body responses. Finally, this model could be improved by
641 adding corneal epithelial cells in the distal compartment to allow interaction between these
642 cells and the nerve endings, coming even closer to corneal physiology. It could provide a
643 better understanding of toxic mechanisms and facilitate establishment of TIDE AOPs and
644 screening of new therapeutic agents (anti-inflammatory, axonal regeneration,
645 neuroprotection). Nevertheless, a limitation of this model is the use of primary murine cells,
646 which does not entirely respect the 3R rule to "Reduce, Replace, Refine," central in IATA
647 development. Even if primary cells are a better representation of a peripheral neuronal
648 phenotype, in the framework of alternative methods, induced Pluripotent Stem Cells should
649 be considered, as in the organoid models described below.
650
651 *Organoid models of the anterior segment of the eye*
652 While the definition can vary between authors, organoids are 3D structures, derived from
653 embryonic stems cells or induced Pluripotent Stem Cells (iPs), capable of self-organization on
654 their framework (such as porous membrane and hydrogel) (Duboule 2019). A Pubmed search
655 with "eye organoid" as keywords reports mostly retinal organoids or organoids destined to be
656 transplanted in humans to replace deficient structures. Few articles address anterior segment
657 organoids for *in vitro* evaluation of pathologic or toxic pathways. However, some of the
658 organoids described could be adapted for toxicological studies.
659
660 - Corneal organoids

661 In 2017, Foster *et al.* presented a corneal organoid derived from an IMR90.4 iPs cell line
662 (Foster *et al.* 2017) and published their precise methodology in 2020 (Foster *et al.* 2020).
663 Mature transparent organoids are obtained after 120 days of cellular sequential selection,
664 forced aggregation and differentiation. Their lamellar structure is composed of epithelial,
665 stromal and endothelial layers and expresses specific corneal markers (cytokeratins 3, 14,
666 collagen of type I, V, VII). Even if any toxicological study has already been conducted, this
667 model could be further optimized to evaluate the impact of toxic compounds on the
668 interactions between the three main corneal layers (epithelium, stroma, and endothelium).
669 Nevertheless, cell differentiation sometimes appears incomplete, leading to the presence of
670 some retinal cells within the corneal organoid. Other protocols presented to obtain corneal
671 organoids for transplantation seem to result in pure corneal organoids, such as that of
672 Susaimanickam *et al.* (2017), but additional studies are needed to evaluate the reproducibility
673 of these models.
674
675 - Lens organoids
676 In 2018, Murphy *et al.* addressed the unsolved problem of obtaining pure lens cells from
677 human embryonic pluripotent stem cells (CA-1 cell line). Their objective was to elaborate a
678 simple, reproducible method to study lens pathologies and anticipate toxicity-induced
679 cataracts. To this end, they put in place a complex, semi-automated selection protocol based
680 on knowledge of embryonic development, with successive inhibition and activation of the
681 FGF, TGF-β and Wnt pathways (Yang et al. 2010) and magnetic selection of ROR1+
682 expressing cells (orphan receptor expressed on epithelial lens cells). These organoids remain
683 viable for 42 days, expressing, among others, α and β crystallins, present *in vivo* in lens fibers
684 and necessary for focusing of light. In this study, they proved the ability of these microlenses
685 to evaluate the toxic potential of a drug candidate, Vx-770, tested in 2016 for cystic fibrosis.
686 This compound, which has induced toxic cataracts in rats, also altered the lens organoids'
687 ability to focus light. To summarize, after reproducibility and intra-laboratory transferability
688 is addressed, this innovative model could be used routinely for the evaluation of mechanisms
689 of toxicity-induced cataract, which still remain poorly understood, as well as the efficacy of
690 new treatments.
691
692 **Conclusion**

693 The 21st century has seen an increase in the movement toward alternative methods to animal
694 testing, especially since the complete ban of animal experimentation in cosmetics. Ocular
695 toxicity studies are no exception, and studies still need to be conducted for new compounds.
696 Indeed, the alternative models to the Draize reference test present similar disadvantages,
697 among which figure the absence of detection of conjunctival or iris damage, the absence of
698 evaluation of systemic toxicity that can occur after ocular exposure and the possibility of false
699 negatives or false positives. Furthermore, none of them alone is able to identify all of the GHS
700 ocular irritant categories, and reversibility of damage is still difficult to evaluate, explaining
701 the impetus of the OECD to optimize some other models. In recent decades, toxicology
702 procedures have aimed to develop IATAs to circumvent these limitations of the alternative
703 methods. Putting aside Draize reference test, known for its lack of reproducibility which
704 complexifies the validation of alternative models by the ICCVAM (OECD Webinar 2019a),
705 and constructing new models, from scratch, based on established AOPs, might be necessary to
706 improve the robustness of the toxicology approaches and results for human use. Indeed, we
707 need to break free from Draize eye irritation test and its poor quality of result to improve
708 inter-laboratory validation of new models (Spielmann 2014) that could enable the
709 identification of a new category of compounds, very low irritants, which requires finer
710 sensitivity methods. This validation step is essential to develop robust alternative approaches
711 to animal testing in the ocular surface field, as it has been done for skin sensitization. Indeed,
712 in June 2021, OECD released GL 497 on "Defined Approaches for Skin Sensitisation",
713 describing the integrated testing strategy and combination of tests that can be used in
714 toxicology studies in replacement of the reference test on rabbits, the Local Lymph Node
715 Assay (OECD 2021).
716 In the field of ophthalmology, IATAs should extend the assessment of toxicity to pathologies
717 other than irritation, especially Toxicity-Induced Dry Eye (TIDE), that can occur after chronic
718 exposure to very low concentrations (Bonneau *et al.* in press). While much less frequent, a
719 toxic compound can also lead to, cataract, glaucoma or ocular surface neuropathies after local
720 exposure. These effects should be considered, taking into account real-life exposure to the
721 compound, determined through literature searches and *in silico* models. As a result, new
722 drugs, cosmetic compounds, or other chemicals, should be investigated for acute irritation
723 and/or for chronic adverse events, depending on real-life use, requiring the development and
724 validation of models and tests with short and/or repeated exposures.

Establishing integrated decision trees for these newly considered adverse events will require a precise understanding of toxic mechanisms, with the development of Adverse Outcome Pathways (AOP), a concept also promoted by the OECD with the establishment of new collaborative tools such as AOP wiki, Effectopedia and the e.AOP.Portal (OECD Webinar 2019b). The innovative models presented in the last section of this review could, after assessment of robustness and regulatory validation, be included in IATAs. They could be a key asset to understanding molecular mechanisms and establishing AOPs. Validation of new models will be a lengthy process, since they should be developed in such a way as to be as cost-effective and least constraining as possible (ethics and supply logistics).

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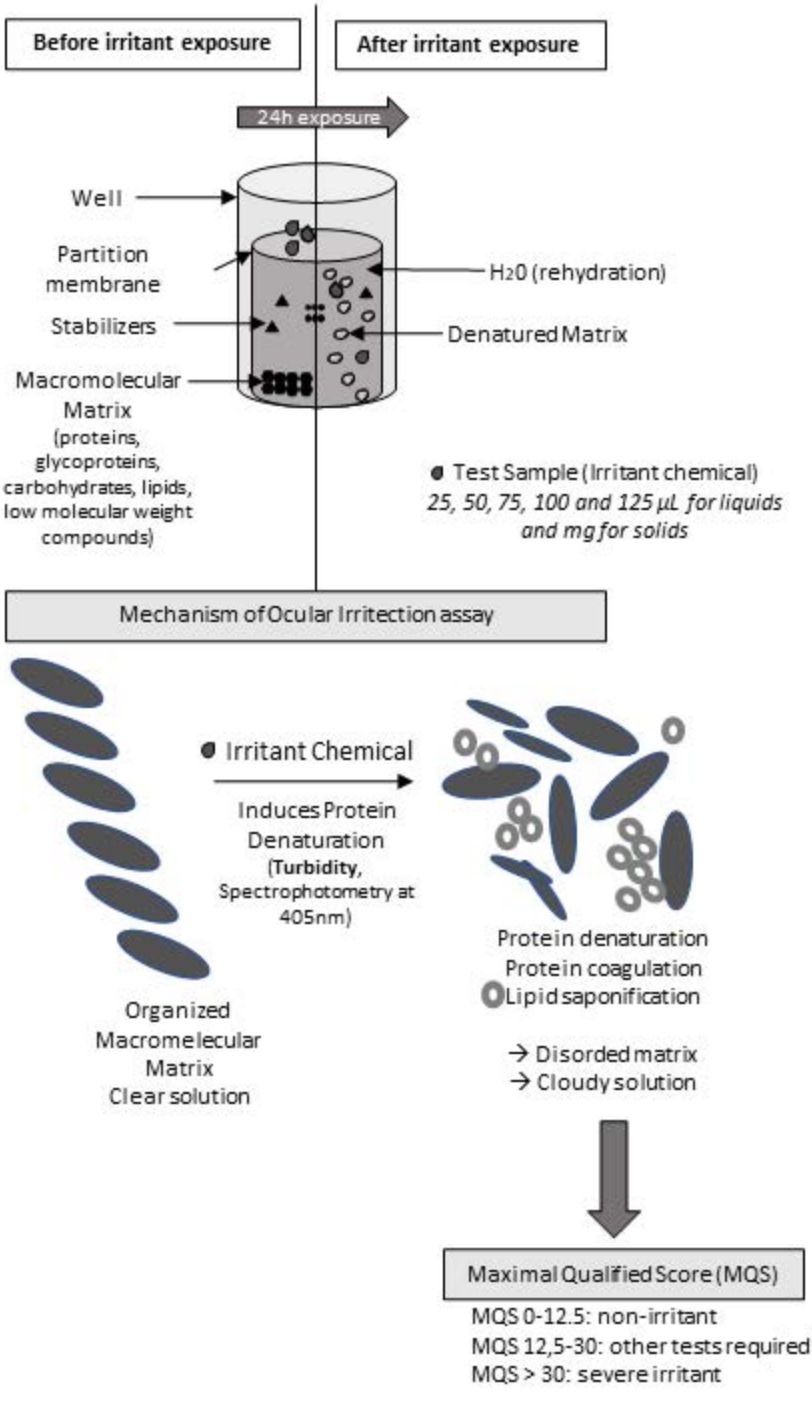
1021 **Tables**

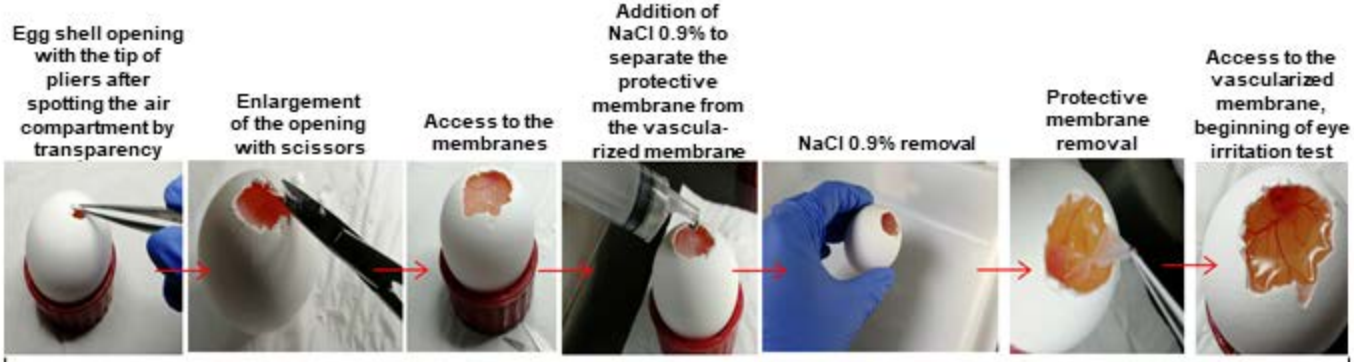
1022 **Table I. Summary of models validated or under evaluation by the OECD. ***Values given
1023 in OECD GL to identify Category 1 or not-classified substances (depending on assay
1024 applicability) in comparison to the Draize eye irritation test.

1025 **Table II. Summary of validated RhCE models for ocular irritation according to OECD**
1026 **GL 492.**
1027 **Table III – Summary of innovative models with potential for evaluation of ocular**
1028 **surface toxicity.**
1029

1030 **Figures**

1031 **Figure 1. Schematic presentation of the matrix created in the Red Blood Cell test and**
1032 **the principle of denaturation** (modified from OECD Webinar 2019a)
1033 **Figure 2. Procedure to prepare the embryonated egg in the HET-CAM testing method**
1034 (personal images, not published)
1035 **Figure 3. Schematic evaluation protocol for ocular discomfort in the slug irritation**
1036 **model** (modified from Lenoir *et al.* 2011)**.** *CP: Contact Period; SIB: Stinging, Itching and*
1037 *Burning*
1038 **Figure 4. Schematic representation of the coculture established** between conjunctival
1039 epithelial cells and lacrimal spheroids (modified from Lu *et al.* 2017)**.**
1040 **Figure 5. Schematic comparison of human corneal structure with 3D triculture model**
1041 **structure** (modified from Wang *et al.* 2017)**.**
1042


Before irritant exposure
After irritant exposure
24h exposure
Well
Partition membrane
Stabilizers
Macromolecular Matrix
(proteins, glycoproteins, carbohydrates, lipids, low molecular weight compounds)
H20 (rehydration)
Denatured Matrix
Test Sample (Irritant chemical)
25, 50, 75, 100 and 125 µL for liquids and mg for solids
Mechanism of Ocular Irritection assay
Irritant Chemical
Induces Protein Denaturation
(Turbidity, Spectrophotometry at 405nm)
Organized Macromelecular Matrix
Clear solution
Protein denaturation
Protein coagulation
Lipid saponification
→ Disorded matrix
→ Cloudy solution
Maximal Qualified Score (MQS)
MQS 0-12.5: non-irritant
MQS 12,5-30: other tests required
MQS > 30: severe irritant



Egg opening procedure

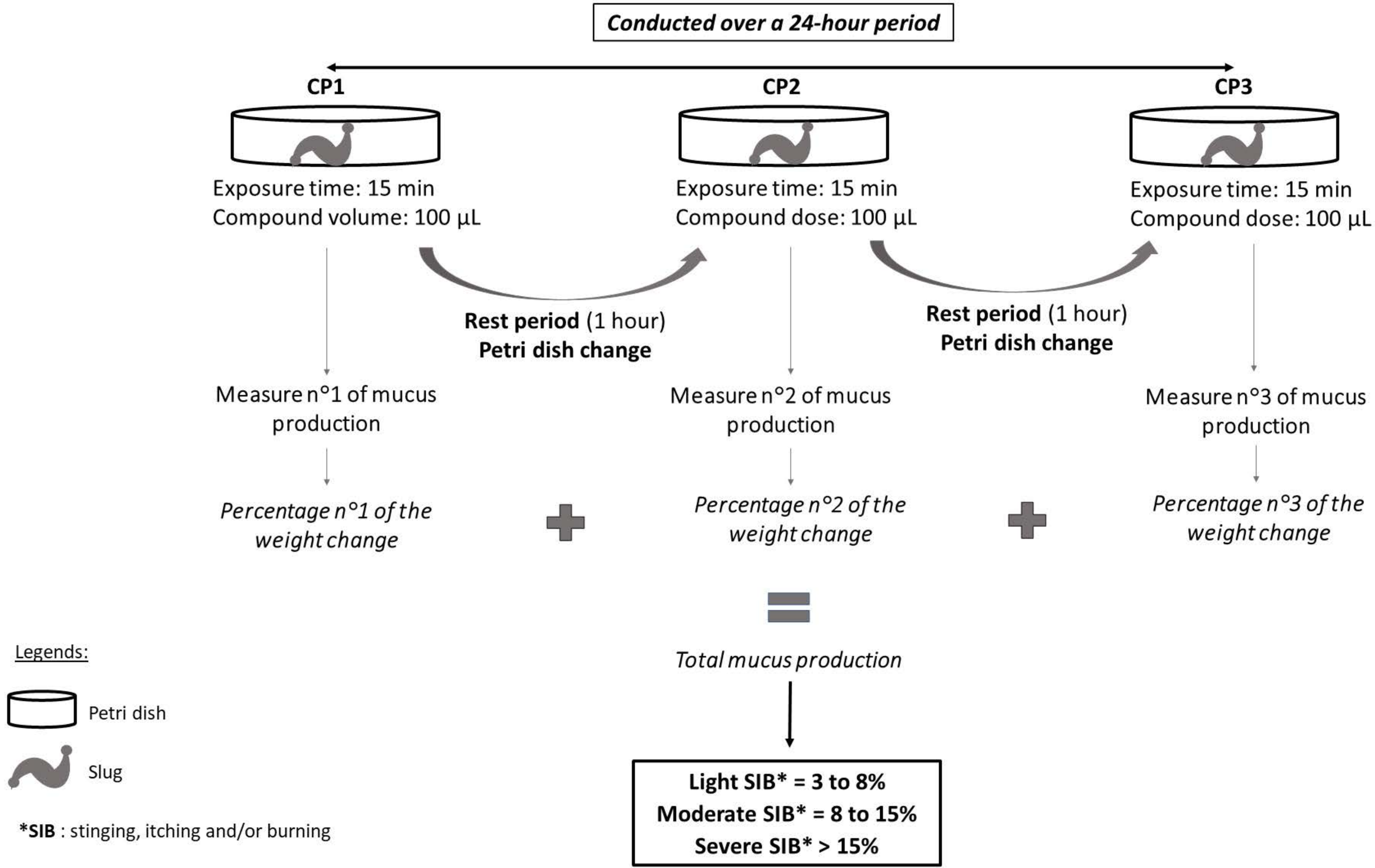
Exposure to test compound

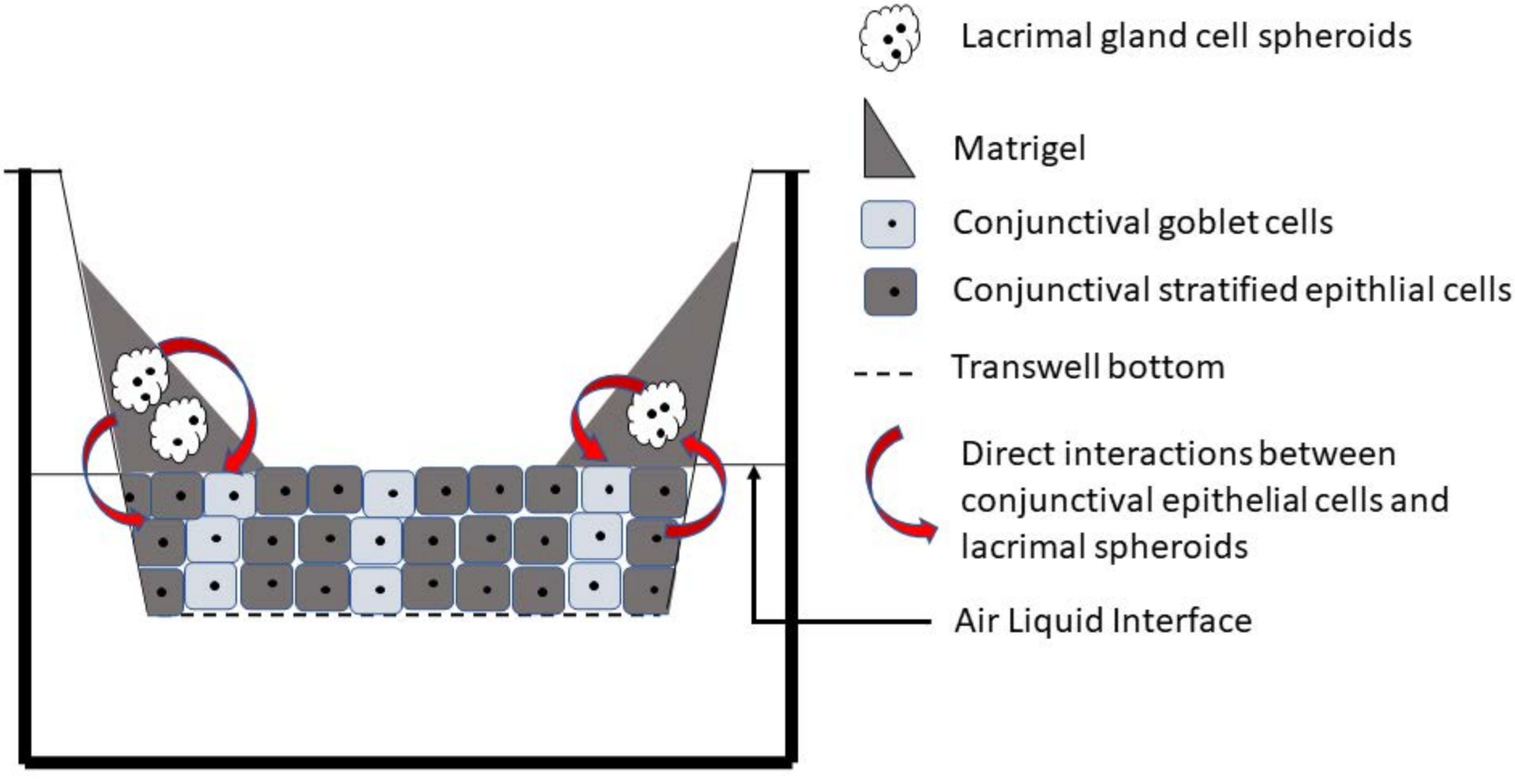
HET-CAM: 30 sec, 2, 5 min

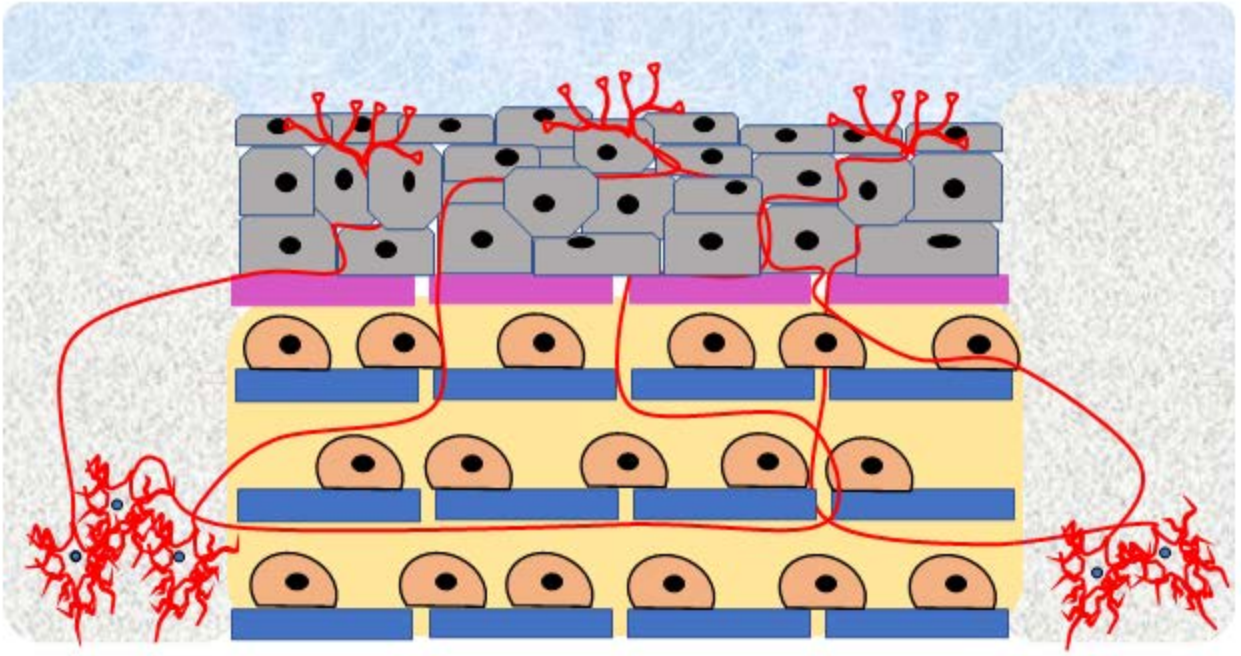
CAMVA: 30 min

Irritation Score, IS

- hemorrhage appearance time,
- vessel lysis appearance time,
- protein coagulation apparition time


Conducted over a 24-hour period
CP1
CP2
CP3
Exposure time: 15 min
Compound volume: 100 µL
Exposure time: 15 min
Compound dose: 100 µL
Exposure time: 15 min
Compound dose: 100 µL
Rest period (1 hour)
Petri dish change
Rest period (1 hour)
Petri dish change
Measure n°1 of mucus production
Measure n°2 of mucus production
Measure n°3 of mucus production
Percentage n°1 of the weight change
Percentage n°2 of the weight change
Percentage n°3 of the weight change
Total mucus production
Light SIB* = 3 to 8%
Moderate SIB* = 8 to 15%
Severe SIB* > 15%
Legends:
Petri dish
Slug
*SIB : stinging, itching and/or burning


Lacrimal gland cell spheroids
Matrigel
Conjunctival goblet cells
Conjunctival stratified epithlial cells
Transwell bottom
Direct interactions between conjunctival epithelial cells and lacrimal spheroids
Air Liquid Interface



Legends

stamped porous silk film for epithelium and stroma development

porous patterned silk films for neuronal growth stimulation

silk sponge for corneal rigidity

collagen hydrogel (containing NGF) for neuronal growth stimulation

air liquid interface (ALI) to improve triculture phenotype

chicken Dorsal Root Ganglion (DRG) cell body

DRG axons

DRG nerve ending

human primary corneal cells

human corneal stromal stem cells

Ocular irritation tests	Draize test	RhCE	BCOP	ICE	Fluorescein Leakage	STE	Vitrigel EIT	Ocular Irritection®	IRE	HET-CAM / CAMVA	CM	NRR	RBC
OECD guideline (last update)	OECD GL 405 (2020)	OECD GL 492 (2019)	OECD GL 437 (2020)	OECD GL 438 (2018)	OECD GL 460 (2017)	OECD GL 491 (2020)	OECD GL 494 (2019)	OECD GL 496 (2019)	Not validated	Not validated	Temporary version released in 2012 (development discontinued in 2016)	Not validated	Not validated
Model	*In vivo*, albino rabbit	*In vitro*, 3D human reconstructed epithelium	*Ex Vivo*, isolated bovine cornea	*Ex Vivo*, enucleated chicken eye	*In vitro*, tubular kidney MDCK CB997 cell line, monolayer, semi-permeable membrane	*In vitro*, monolayer confluent rabbit corneal fibro-blasts (ex. CCL60 cell line)	*In vitro*, human reconstructed epithelium (Vitrigel matrix)	*In vitro*, acellular system, macro-molecular matrix (proteins, lipids, carbohydrates,...)	*Ex vivo*, enucleated rabbit eye	Chorioallantoic Membrane of chicken embryo egg	*In vitro*, mono-layer mice fibro-blasts from L929 cell line cultiva-ted on a polycar-bonate insert	*In vitro*, mono-layer of 3T3-L1 fibroblasts or NHEK human keratinocytes (FRAME/ Clonetic protocol)	Isolated red blood cells
Recommended strategy	Last resort (forbidden for cosmetics)	Bottom-Up	Bottom-Up, Top-Down	Bottom-Up, Top-Down	Top-Down	Bottom-Up, Top-Down	Bottom-Up	Bottom-Up, Top-Down	Not recommended	Not recommended	(If validation of GL: Bottom-Up, Top-Down)	Not recommended (supplementary data required)	Not recommended (supplementary data required)
Field of applicability	Liquids, solids, aerosols	Liquids, semi-solids, solids, waxes	Liquids, semi-solids, creams, waxes (including surfactants)	Substances and mixtures	Water-soluble substances and mixtures	All types of products (except volatile substances, non surfactant products)	Chemical products with pH > 5, inclu-ding volatile or coloured compounds (excluding solids)	Solids and liquids with $4 \leq pH \leq 9$	Substances and mixtures	Substances and mixtures	Water-soluble compounds (including mix-tures), solids/ viscous substan-ces / uniform suspensions	Water-soluble substances	Substances and mixtures
GSH categories	1, 2A, 2B, not-classified	Not-classified (in process of validation to distinguish 1, 2A et 2B with EpiOcular® time-to-toxicity assay)	1, not-classified	1, not-classified	1	1, not-classified	not-classified	1, not-classified	(accepted in European Union for category 1)	HET-CAM accepted in European Union for category 1	1, not-classified	not-classified	1, not-classified
Compound exposure time	21 days	See Table 2	10 min *(other exposure times if scientific rationale)*	10 sec *(rinsing removal)*	1 min *(followed by a 30min incubation of fluorescein)*	5 min *(two concentrations, 0.5% and 0.05%)*	3 min	24h *(5 concentrations, 25, 50, 75, 100, 125 µL or µg)*	10 sec *(rinsing removal)*	30 sec, 2, 5 min (HET-CAM) / 30 min (CAMVA)	810 sec *(= 13, 5min, followed by a 6 min wash out cycle)*	1 or 5 min (FRAME or Clonetic protocol)	10 min to 1 hour at room temperature *(under continuous stirring)*
Endpoints	**Conjunctiva** (chemosis, red-ness, tearing), **Corneal** opacifi-cation, **Iris** (swelling, light reactivity)	Mitochondrial metabolic capacity	Corneal opacity; Fluorescein retention	Corneal opacity; Fluorescein retention; Morphological alteration *(evaluated after 30 min, 1, 2, 3, and 4 hours of product retrieval)*	Fluorescein diffusion *(spec-trophotometry at 530 nm)*	Mitochondrial metabolic capacity	TEER *(measured every 10 s during 3 min)*	Turbidity variations *(spec-trophotometry at 405 nm)*	Corneal opaci-ty, edema; Fluorescein penetration; Epithelial changes *(evaluated after 30 min, 1, 2, 3, and 4 hours of product retrieval)*	Hemorrhage / vessel lysis / protein coagulation apparition times	Dose-response study, pH changes evaluation over time	Dose-response study, Release of preloaded neutral red, 3 hours before exposure *(spectrophotome-try at 546-550 nm)*	Hemoglobin leakage *(photometry at 540 nm);* Oxyhemoglobin denaturation *(spectrophotometr y at 575 nm)*
Threshold or Score	Maximal ocular irritation (**Max.O.I**)	MTT or WST threshold (see table 2)	*In Vitro* Irritancy Score (**IVIS**)	Addition of scores for each endpoint graded from I to IV	Fluorescein Leakage of 20% (**$FL_{20\%}$**)	MTT threshold	Score that combines time lag, intensity and plateau level	Maximal Qualified Score (**MQS**)	Addition of scores for each endpoint	Irritation Score (**IS**)	Metabolic Rate Decrement of 50% (**MRD_{50}**)	Neutral Red Release of 50% (**NRR_{50}**)	ratio concentra-tion inducing a red cell hemo-lysis of 50% / Denaturation index (**H_{50}/DI**)

Ocular irritation tests	Draize test	RhCE	BCOP	ICE	Fluoresceine Leakage	STE	Vitrigel EIT	Ocular Irritection®	IRE	HET-CAM / CAMVA	CM	NRR	RBC
Accuracy *	Reference	EpiOcular™, 80% (96/112) SkinEthic™ HCE, 84% (168/200)	79% (150/191)	83% (142/172)	77% (117/151)	83% (120/140)	78% (73/93)	74% (65/88)	78% (110/141)	69% (41/59)	Data not found	Variable, protocol dependent	96.7% (Alves et al. 2008)
Specificity *	Reference	EpiOcular™, 37% (21/55) SkinEthic™ HCE, 28% (29/103)	25% (32/126)	7% (9/127)	7% (7/103)	1% (1/86)	70% (23/33)	81% (55/68)	6% (4/62)	64% (18/28)	2% (1/48)	Variable, protocol dependent	100% (Alves et al. 2008)
Sensitivity *	Reference	EpiOcular™, 4% (3/57) SkinEthic™ HCE, 5% (5/97)	14% (9/65)	47% (21/45)	56% (27/48)	51% (20/39)	83% (50/60)	50% (10/20)	34% (27/79)	0% (0/31)	20.5% (7/34)	Variable, protocol dependent	91.6% (Alves et al. 2008)
Main limits	- 3R rule ethical problem - Forbidden for cosmetics - Inter/Intra laboratory variability - Over-estimation of toxicities occur-ring in humans	- No toxicity evaluation of eyelid, iris, and other ocular structures - No evaluation of gas and aerosols	- No toxicity evaluation of eyelid, iris, etc - No evaluation of gas and aerosols - Over-estimation for pour alcohols, ketones	- No toxicity evaluation of eyelid, iris, and other ocular structures - No evaluation of gas and aerosols	- No toxicity evaluation of eyelid, iris, and other ocular structures - No evaluation of gas and aerosols	- No toxicity evaluation of eyelid, iris, and other ocular structures - No evaluation of gas and aerosols	- No toxicity evaluation of eyelid, iris, and other ocular structures - No evaluation of gas and aerosols	- No cytotoxicity evaluation - Reduced field of applicability	- No standardized protocol, - No sufficient data on deci-sion criteria and on inter-laboratory reproducibility	- No standardized protocol - Embryo egg can be consi-dered as animal experimenta-tion depending on countries	- No evaluation of gas and aerosols	- No sufficient data on inter-laboratory reproducibility - Reduced field of applicability (supplementary data needed)	- No sufficient data on field of applicability

OECD GL 492		EpiOcular™	SkinEthic™ HCE	LabCyte CORNEA-MODEL24	MCTT HCE™
Cell type		Primary human keratinocytes from human epiderma	Immortalized human corneal epithelial cells	Primary human corneal epithelial cells	Primary human corneal epithelial cells
Field of applicability		Solids, liquids, semi-solids and waxes			
Validated Reference Methods (VRM)		MRV1	MRV2	/	/
3D development*		At least three viable cell layers and of a non keratinized surface	At least four viable cell layers that include basal columnar cells, transitory amplifier cells and squamous superficial cells	At least three viable cell layers and of a non keratinized surface	At least three viable cell layers and of a non keratinized surface
Compound exposure time	**Liquids**	30 min	30 min	1 min	10 min
	Solids *(or liquids non applicable with a pipette)*	6 hours	4 hours	24 hours	3 hours
Cytotoxicity test (Non irritant threshold)**		MTT (> 60 %)	MTT (>50%)	WST-8 (>40%)	WST-8 (> 35% for liquids; > 60% for solids)

* The barrier function of the 3D reconstructed-cornea epithelia must be validated based on their ability to resist penetration by cytotoxic compounds such as Triton X-100 and sodium dodecylsulfate.

** The two colorimetric tests, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and WST (water-soluble tetrazolium salts)-8 tests are similar. In the first one, formazan is formed intracellularly, requiring the step of cell lysis with isopropanol before the absorbance measurement, while in the second one, formazan is present directly in the cell culture medium. This colorimetric measure is proportional to the number of live cells (Pauly et al. 2009).

	Multicellular 3D models			Models on-a-chip				Organoids	
	Coculture conjunctiva / lacrimal glands	Coculture SH-SY5Y neurons / stromal corneal cells on an insert	Tri-culture neurons / epithelial cells / stromal cells	Cornea-on-a-chip, lacrimal flows	Cornea-on-a-chip and shear stress	Coculture on-a-chip cornea – conjunctiva	Compartmentalized corneal neurones	Cornea	Lens
Cell types	• Primary epithelial conjunctival rabbit cells • Primary rabbit acinous lacrimal glands spheroids	• Primary human corneal fibroblasts • Human neuroblastoma SH-SY5Y cell line	• HCE human corneal epithelial cell line • Stromal human stem cells (hCSSCs) • Neuronal cells (DRG)	• HCE human corneal epithelial cell line	• HCE human corneal epithelial cell line **Or** • Primary mice epithelial and endothelial corneal cells	• Primary human corneal epithelial cells • Human immortalized conjunctival cells	• Primary trigeminal ganglion mice cells *(model improvement possible by adding epithelial corneal cell to form a coculure)*	• IMR90.4 iPS cell line	• human pluripotent embryonic stem cells (hESC line CA1)
Advantages	• Production of aqueous and mucinic lacrimal layers	• Production *de novo* of extracellular matrix by fibroblasts • Mimic the interactions of nerves with the stroma	• Air liquid interface culture on silk protein to better mimic mechanical corneal properties and improve neuronal development	• Mimic pulsatile flow generated by eyelid blinking • Mimic continuous flow generated by lacrimal secretion	• Mimic lacrimal flow generated by eye blinking (bidirectional flow) • Mimic aqueous humour evacuation through Schlemm's canal (unidirectional flow)	• Mimic stroma through collagen matrix • Mimic lacrimal flow through a perfusion system • Mimic eyelid blinking through biomimetic system	• Separate nerve endings (distal compartment) from neuronal cell bodies (proximal compartment) to better mimic physiology and independently evaluate the impact of a toxic on nerve endings	• Lamellar structure of the cornea (epithelium, stroma, endothelium) identifiable at 30 days of culture	• Formation of a fibrillary structure characteristic of the lens
Evaluated parameters	• Permeability of tight junctions (conjunctival epithelium) to dextran • Lacrimal fluid thickness • Epithelial gene marker (KRT4) • Mucin gene marker / production (MUC5AC) • Inflammatory gene marker (IL-1β, MMPs)	• Collagen and fibrosis gene markers (alpha-SMA) • Structural changes (transmission electron microscopy) • Neuronal activation markers (cFOS, TRPV1, TRPM8, *etc)*	• Cell viability (LIVE/DEAD Viability/ Cytotoxicity Kit) • Corneal epithelium and stromal phenotype (involucrin, KRT3, connexin 37, ALDH3A1)	• Epithelium thickness • Corneal epithelium phenotype (ZO-1) • Membrane permeability (TEER)	• Epithelium thickness • Epithelial permeability (fluorescein, dextran) • Corneal epithelium phenotype (ZO-1, KRT19, KRT12)	• Epithelium thickness • Corneal epithelium phenotype (p63, KRT19, KRT3) • Lacrimal film thickness • Inflammatory cytokines production (IL-β, TNF-α) and metalloproteinases (MMP-9)	• Inflammatory markers • Cell death markers • Morphological alterations of axons (CFSE coloration)	• Lamellar structure thickness • Corneal epithelium phenotype (KRT3, KRT14, p63α, KERA, type I / V / VIII collagen, LUM) • Organization of collagen fibrils (transmission electron microscopy)	• Lens phenotype (ROR, crystallines α et β, integrins, laminins et collagens) • Light focusing ability
References	Lu *et al.* 2017	Sharif *et al.* 2018	Wang *et al.* 2015 Wang *et al.* 2017	Bennet *et al.* 2018	Abdalkader *and* Kamei 2018 Bai *et al.* 2020	Seo *et al.* 2019	Vitoux *et al.* 2020	Foster *et al. 2017* Susaimanickam *et al.* 2017 Foster *et al.* 2020	Murphy *et al.* 2018