Session I-8: Safety testing for chemically-induced eye injuries: Recent Three Rs advances

Session I-8: Oral presentations

I-8-673

Chemical injuries and the corneal response

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Chemical injuries represent one of the most difficult clinical entities to treat due to the variety of chemicals and their actions on corneal tissue. The risk is of course the loss of vision which may result in corneal blindness. The most frequent injuries are from encounters with extreme pH conditions (<4 or >10); however, there are also unusual or exotic chemicals used in industrial solutions and processing for which the response would not be well understood. As the chemical penetrates into the corneal tissue it has access to other ocular tissues, such as the iris and lens. Damage to the endothelium is one of the most damaging effects as the endothelial cells in humans do not regenerate and a corneal graft is the only realistic therapeutic approach. Chemical splashes also will take in the conjunctival tissue surrounding the cornea and may damage the stem cell for the cornea at the limbus. This type of injury can be approached by the use of regenerative limbal or conjunctival stem cell transplants, or by oral epithelium transplants or by use of amniotic membrane patches. A common outcome of chemical injuries is also the invasion of blood vessels into the cornea with a subsequent loss of visual acuity. Additionally, damage to the conjunctiva can lead to exteme dry eye. The access of chemicals to the cells and cellular structure is critical to understand for both awareness and avoidance of potential injury and to understand the outcome for restoration of vision.

I-8-444

Human eye exposure to surfactant solutions; *in silico* determination

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For the last three decades we have been dedicated to the development and use of *in vitro* alternatives to replace and reduce animal testing. The trans-epithelial permeability (TEP) assay has been validated internally as an alternative to the Draize rabbit eye test and has been used as part of our safety assessment program for 20 years. Over the last two decades we have compiled a large data set of human ocular testing with end points of eye stinging, bulbar conjunctive redness, palpebral conjunctive redness, and lacrimation. This data set includes ocular testing on approximately 20,000 human eyes with 170,000 end points.

From this data set, ingredient effects of surfactant solution are modeled, and human clinical results are predicted.

With over 19,000 ingredients available in personal care, a structure-activity relationship-type approach is used to compress the many ingredients into fewer compound families. The experimental clinical data sets are also augmented with surfactant theory and high throughput physical chemistry testing to create a structural model of the ingredient effects on clinical results. This modeling of clinical results based on the constituent ingredients creates insight into the effects of different ingredient types (e.g., anionic surfactants create harsher surfactant systems, non-ionic surfactants make systems milder). This large data set enables the evaluation of variations in ocular response among subject populations; we observe differences in the age dependence of ocular redness between males and females. Also, the neuronal eye sting response is not observed to be correlated with eye redness.

I-8-363

Measuring Depth of Injury (DoI) in the Bovine Corneal Opacity and Permeability (BCOP) Assay

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Past studies have shown that the severity of ocular irritation in the Low-Volume Eye Test is dependent on DoI regardless of chemical type, and that DoI measuring biomarkers for cell death and viability can be used to assess irritation potential in an *ex vivo* rabbit eye test. An ongoing project in the COLIPA eye irritation program builds on this earlier DoI work using the standard BCOP assay. This study is evaluating the ability of DoI to discriminate between different Globally Harmonised System (GHS) categories in the BCOP test. Bovine eyes were obtained from a slaughterhouse and processed for standard BCOP protocol (minimum 3 eyes/irritant) using chemicals with different irritation potential having GHS (Draize) classifications of Category 1 (7 severe irritants), Category 2 (5 irritants) and Not Classified (4 non-irritants) compared to water as control. All corneas received 10 minute exposure and 3 h recovery and were then fixed in 2% paraformaldehyde, frozen in liquid nitrogen, sectioned and evaluated by fluorescent staining with phalloidin. DoI was then assessed using an epifluorescence microscope to measure dead and viable corneal epithelial and stromal thickness. Percent DoI for epithelium, stroma and cornea were then calculated. Results indicate that stromal DoI was different between different GHS Classifications with Category 1 producing >50% compared to Category 2 (0-40%) and Not Classified (0%). These preliminary data suggest that measuring DoI in the BCOP assay can correctly identify Category 1 from Category 2 ocular irritants.

I-8-055

A procedure for application of eye irritation alternative methods to cosmetic ingredients

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Alternative methods to replace the Draize rabbit eye test for evaluation of eye irritation by cosmetic ingredients have been studied by many in the cosmetics industry and independent organizations in several countries for many years, such as ECVAM, ICCVAM, IRAG, etc. Alternative assays have been extensively researched, and some have undergone formal validation. However, to date, there is no single *in vitro* assay that has been validated as a full replacement for the Draize rabbit eye test. The isolated rabbit eye test (IRE) and the hen's egg test-chorioallantoic membrane (HET-CAM) assays are accepted for specific and limited regulatory purposes. Both have already been validated by ICCVAM. In light of the deadlines established in the EU Cosmetics Directive for cessation of animal testing for cosmetic ingredients and the regulation for the least amount of animal testing for safety assessment on chemicals in the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), we evaluated the applicability and predictive capacity of IRE and HET-CAM, and established the prediction model with both of the alternative methods by discriminant analysis. Furthermore, we proposed and evaluated a procedure for application of eye irritation alternative methods (PEIAM) to cosmetic ingredients. The PEIAM had a good predictive capacity when compared to the results of animal tests, indicating potential for *in vitro* screening of chemicals for eye irritation.

I-8-503

Development of a non-animal testing strategy for ocular hazard labeling of some specific EPA-regulated products

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Changes in toxicological testing strategies within a regulatory agency can occur through several pathways. In 2009, the EPA's Office of Pesticide Programs instituted a pilot program which utilizes results from specific non-animal testing methods for assessment of eye irritation potential, as long as the testing methods and testing results are deemed by EPA to be adequate and appropriate to support labeling decisions. Prior to this, cleaning products making an anti-microbial claim (and thus considered pesticides) normally had to be tested using the Draize rabbit eye irritation test to provide data for hazard labeling. However, several years ago a suggestion was made by the Pesticide Program Dialog Committee (a diverse group of stakeholders selected by the EPA to provide feedback to the pesticide program on various pesticide regulatory, policy and program implementation issues) that non-animal methods should be investigated for their ability to provide satisfactory information to determine an EPA hazard category for eye irritation. Subsequently seven AMCP manufacturers agreed to share data from non-animal, *in vitro* ocular studies (along with historic animal data) which they had individually generated. This allowed the creation of an extensive database describing the performance of several *in vitro* methods for assigning eye irritation hazard categories for AMCPs, relative to that of the traditional rabbit eye test. A testing strategy involving up to three *in vitro* methods (Bovine Cornea Opacity and Permeability assay, EpiOcular™ tissue (MatTek Corp., Ashland, MA), and the Cytosensor Microphysiometer (Molecular Devices, Sunnyvale, CA) was devised and subsequently evaluated by EPA staff. It was determined that this *in vitro* strategy for labeling of ocular hazard was sufficient to propose its further prospective evaluation in a pilot program. Cooperation among the seven companies was essential for this success, since it is unlikely that the amount of information that any one company possessed would have been sufficient to allow the EPA to initiate the pilot program.

Session I-8: Poster presentations

I-8-057

Comparative study of five *in vitro* tests as an alternative method for eye irritation testing

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The purpose of this study was to evaluate the applicability and predictive capacity by comparing five *in vitro* tests for eye irritation, to try to find better testing methods for eye irritation of cosmetics. 27 cosmetic products were assessed in the study, they were tested by five alternative methods for eye irritation tests, i.e. HET-CAM, CAM-TBS, FLT, 3T3-NRU cytotoxicity assay and red blood cell (RBC) haemolysis assay. The cosmetics were tested *in vivo* by the Draize test. *In vivo* and *in vitro* test results were compared and analyzed with SPSS software. It was shown that ranking correlation and class concordance existed between the five alternative methods and the Draize test by applying 27 cosmetic products, the relationship between HET-CAM, CAM-TBS, FLT and MMAS is better, a predictive model was developed of Y from X, subject to the maximum possible correlation between the MMAS and HET-CAM, CAM-TBS, FLT from X. It is suggested that the three *in vitro* assays, HET-CAM, CAM-TBS and FLT have good predictive capacity, reproducibility and reliability when compared with the Draize test. In addition, a predictive model has valuable application as a screening test in cosmetics safety evaluation.

I-8-091

Extracellular acidification and changes in bioimpedance of L929 cells

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Monitoring of extracellular acidification from L929 mouse fibroblasts with the cytosensor microphysiometer is a non-animal method to identify non-irritant water-soluble substances in the field of eye irritation (Hartung et al., 2010). No other non-animal methods exist for this category. However, the cytosensor microphysiometer is no longer commercially available. Other instruments (e.g. from Bionas GmbH or cellasys GmbH) for determining extracellular acidification rate may fill the gap (Scott et al., 2010).

Besides the extracellular acidification, cellular respiration and changes in bioimpedance have the potential to classify substances correctly. In this work, extracellular acidification was compared with changes in bioimpedance of L929 fibroblasts using the IMOLA-IVD technology which was developed in the group of Prof. B. Wolf at the Technische Universität München (Wiest et al., 2006). The results show that changes in bioimpedance are also useful to investigate toxicological effects. However the mechanistic background is so far not completely understood. A prediction model has to be established to allow bioimpedance measurement to enter the validation process.

References

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I-8-127

In vitro eye irritation assessment using the SkinEthic HCE test method applied to ingredients used in cosmetics

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To comply with the EU Cosmetics Directive, predictive alternative methods are required to evaluate eye damage potential of chemicals. Linked to the complexity of both eye irritation mechanisms and the diversity of chemicals, the *in vitro* assessment of eye irritation potential is a complicated issue. The corneal epithelium is crucial and represents the first line of defense against injury. 3D *in vitro* tissues sustained by adapted technologies allow the testing of substances in conditions similar to *in vivo* exposure. In this study, we have used the standardized SkinEthic reconstructed human corneal epithelial (HCE) model to evaluate *in vitro* eye irritancy. A specific protocol was developed aiming to match chemicals' properties with adapted exposure steps (1 h exposure, 16 h post exposure incubation) in particular for cosmetic ingredient families. Analyses were performed according to the Globally Harmonized System (GHS) classification. The Prediction Model, using a 50% viability cut-off, allowed the drawing up of 2 categories: Irritants (grouping Cat1 and 2) and No Category. Applied to a broad set of 435 cosmetic substances the SkinEthic HCE test method showed good and balanced prediction performances (81% sensitivity; 82% specificity). Furthermore by using appropriate controls the applicability domain of the method can be extended to the MTT reducers and/or dye substances by using additional controls. Severe or irreversible irritant chemicals were not specifically differentiated from reversible and mild irritants. This test method is part of the ongoing ECVAM eye irritation validation. It could be part of a specific tiered test strategy for hazard assessment of test substances in regulatory schemes.

I-8-150

Assessment of eye irritation potential using the reconstructed human corneal tissue LabCyte CORNEA-MODEL

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In vitro eye irritation testing alternative to animal testing such as Draize eye test using rabbits is required from an animal welfare standpoint. We have developed the new reconstructed human corneal epithelium model, LabCyte CORNEA-MODEL, and we investigated a test method to evaluate eye irritation using this model. Claudin-1, a component of tight junction; E-cadherin, a component of adherence junction; and Desmogrein-3, a component of desmosome, were strongly expressed in all layers of LabCyte CORNEA-MODEL. Mucin-1 and Mucin-16 were expressed in the super-facial layer. These results suggested that this model was correlated with the tissue structure of normal human corneal epithelium. New methods were also established for eye irritation testing using LabCyte CORNEA-MODEL. The application period of chemicals was set to 1 min for liquids and 24 h for solids, and the post-incubation period was set to 24 h for liquid or none for solid. If the viability was less than 50%, the chemical was judged to be an eye irritant. Sixty-one chemical substances were applied to this new *in vitro* eye irritation test and the correlation between *in vivo* class and the *in vitro* prediction of eye irritation was evaluated. Since *in vitro* results using LabCyte CORNEA-MODEL were highly correlated with *in vivo* eye irritation (sensitivity 100%, specificity 80.0%, and accuracy 91.8%), it is suggested that the eye irritation test using this model could be useful for a variety of chemicals with irritant potency as an alternative method to the Draize eye test.

I-8-151

A tiered approach combining the STE test, the EpiOcular assay, the HET-CAM assay and the BCOP assay for predicting eye irritation potential of chemicals

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The predictive potential of a tiered approach using the results from the STE test, the EpiOcular assay, the HET-CAM assay and the BCOP assay, was examined for assessing GHS eye irritation. Around 50 chemicals with a balance of GHS eye irritation categories and a wide range of chemical classes were selected. The chemicals were evaluated in either the STE test or the EpiOcular assay. In addition, we adopted the evaluated classification of the HET-CAM data and BCOP data from the background review document (BRD). The first step in our approach was to evaluate the chemicals in the STE test, depending on chemical solubility. For non-soluble chemicals, EpiOcular results or adopted HET-CAM data for tested chemicals from the BRD were used. If the chemical was classified as a "non-irritant" by first phase tests, it was considered to have a GHS ranking of "not classified." If the chemical was classified as an "irritant" in first level tests, the classification was subsequently confirmed by reviewing the results from the BCOP data adopted from the BRD. If the classification was "severe", the chemical was considered a GHS "Cat.1". For those chemicals classified as "non-severe", these were considered to be GHS "Cat.2". The tiered (bottom-up) approach combining either the STE test or the EpiOcular assay with the HET-CAM assay and then the BCOP assay allowed the evaluation of GHS eye irritation category with an accuracy of more than 73%.

I-8-180

Inter-laboratory phase II validation study of *in vitro* eye irritation test; Short Time Exposure (STE) test

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The Short Time Exposure (STE) test is an easy-to-use *in vitro* eye irritation test using the cell viability of SIRC (rabbit corneal cell line) cells as an end point following one 5 min treatment. The Executive Committee of the Japanese Society for Alternatives to Animal Experiments conducted a validation study with five laboratories from 2008 to 2009. These data showed good transferability. Assignment of 25 blinded chemicals to the STE irritation categories "Non-Irritant" (NI) or "Irritant" (I) showed good inter-laboratory reproducibility and predictive capacity for predicting the GHS category of "Not classified" (NC) or I (category 1 or category 2). In this study, we selected 40 additional blinded chemicals to optimize the balance of GHS category and re-evaluate the predictive capacity of the STE test.

The results showed that the STE test was not only easy to acquire and implement among three laboratories, but it also had a high intra- and inter-laboratory reproducibility, and a high ability to predict the GHS category (NC or I). However, a predictive ability of the STE rank for predicting GHS categories was not good compared with that of STE irritation categories (NI or I). Therefore, the STE test can assess not only the severe/corrosive ocular irritant (correspond to GHS category 1) but also the mild or moderate ocular irritants (correspond to GHS category 2). However, a predictive ability of the STE rank was insufficient for identification of GHS categories. The STE test was recommended for use as part of a tiered testing strategy for regulatory classification.

I-8-302

Development of IRR-IS®, an Episkin® based model for quantifying chemical irritation potency using an algorithm based on analysis of magnitude of gene expression of selected biomarkers

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Although a number of assays for irritation potency classification using 3D reconstructed epidermitis and MTT measurement have been developed, their ability to quantify irritation potency is limited. To formulate and address new and future regulatory demands, the ability to measure and quantify the skin irritation potential of chemicals without animals is of high importance. Moreover the ability to quantify skin irritation can be valuable information when measuring eye irritation. We developed IRR-IS®, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®). The selection of biomarkers was done by analysis expression profiles in 3D reconstructed epidermis with several irritants. Test chemicals were applied for 30 minutes then washed and the tissues were further incubated for 6 h. Tissues were teased, total RNA purified with Trizol and expression of genes measured by quantitative PCR after reverse transcription. We selected 25 biomarkers and developed an algorithm based on analysis of magnitude of gene expression. We will present here the results of these studies and will show the quantitative capacity of this approach on a set of 40 chemicals

I-8-314

Comparative studies for three *in vitro* methods to evaluate the eye irritation potential of disinfectants

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In Brazil, the health legislation requires the Draize test for registering household products. However, the global trend has been the replacement of *in vivo* tests by *in vitro* assays. Thus, there is a need for studying *in vitro* methods to develop effective predictive models to detect the irritation potential. This study aims to compare three *in vitro* assays to the method described by Draize to evaluate the ocular irritation potential. Ten disinfectants were tested: seven pure and three diluted to 30% (v/v). The following tests were performed *in vitro*: i) Red Blood Cells test (RBC) according to the protocol INVITTOX 37, ii) chorionic allantoic membrane with trypan blue staining (CAM-TBS) assay, protocol INVITTOX 108, and iii) hen's egg test-chorionic allantoic membrane (HET-CAM) was performed according to the method described in the Journal Officiel de La Republique Française. The *in vivo* data were obtained from the INCQS database. The results were arranged in contingency tables to determine the accuracy, specificity and sensitivity of each method to *in vivo* the test. In *in vivo* test 1 product has been classified as non-irritant (NI), 5 weak irritant (WI) and 4 moderate irritant (MI). In the RBC test 1 product was classified MI, 4 severe irritant (SI) and 5 maximum irritant (Mx). In CAM-TBS 2 MI and 8 severe irritant and HET-CAM all products were classified as SI. The three *in vitro* methods showed five false positive results, implying low specificity. But the three methods showed good sensitivity.

I-8-324

Proposal of a mechanism-based selection of reference chemicals for development / evaluation of *in vitro* eye irritation methods

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The COLIPA (European Cosmetics Association) Task Force Eye Irritation is actively involved in the development of *in vitro* methods to replace the Draize rabbit eye test, now banned for evaluating ingredients used in the cosmetic industry. One of its key projects is focused on a validation study of optimized existing Reconstructed human Tissue (RhT) test methods (MatTek EpiOcular EIT® and SkinEthic® Human Corneal Epithelium (HCE)). After the evaluation of numerous chemicals and completion of pre-validation datasets, a prospective ECVAM validation study is now in progress to reliably discriminate non classified chemicals from all classes of eye irritants. In order to further develop *in vitro* assays that accurately identify the eye irritation potential, selection of appropriate test chemicals is of critical importance.

Here, we provide a set of 49 chemicals which: 1) are single chemical entities; 2) are supported by high quality *in vivo* data; 3) cover the whole range of irritant effects/potencies; 4) cover different chemical classes/physical states and 5) are readily available. All chemicals have been tested in at least one RhT assay and in the Bovine Corneal Opacity and Permeability test method. The use of such a reference list would facilitate early assessment of new method performance with respect to existing tests and its possible contribution to a tiered testing strategy. This poster provides a detailed analysis of a chemical dataset still under refinement, proposed by Colipa for use in the development and evaluation of *in vitro* methods for eye irritation testing.

I-8-345

A novel rapid assay useful for eye irritation testing using a human corneal epithelium model reconstructed in a collagen vitrigel membrane chamber

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A collagen vitrigel membrane (CVM) is composed of high density collagen fibrils equivalent to connective tissues *in vivo*. Also, it possesses excellent transparency and permeability of protein with high molecular weight. Recently, we made a novel CVM chamber adapted to three-dimensional culture. In this study we reconstructed a human corneal epithelium model in the chamber, investigated its histology and barrier function, and evaluated its utility in an eye irritation test.

HCE-T cells (a human corneal epithelium-derived cell line) were cultured in a CVM chamber for 2 days to form a confluent monolayer. Subsequently, a corneal epithelium model was reconstructed by culturing it in the air-liquid interface for 7 days. Histology and barrier function during the process of reconstructing the model were analyzed by fluorescent stain and TEER (transepithelial electrical resistance) measurement, respectively. More than 20 reference chemicals known as eye irritants were used to challenge the model and were evaluated regarding time-dependent changes of TEER.

Histological observations revealed that the reconstructed human corneal epithelium model was composed of around five cell layers and its outer 2-3 layers strongly expressed tight and gap junctions-related proteins. The decreasing ratio of TEER at 10 seconds after exposing the model to each chemical was well correlated with the eye irritancy previously reported as Draize score and/or GHS classifications. These results suggest that the human corneal epithelium model reconstructed in the CVM chamber provides a novel rapid assay useful for eye irritation testing.

I-8-388

In-house validation of the EpiOcular™ eye irritation test (EpiOcular-EIT) with 60 test substances and its implementation into the tiered testing strategy for assessment of ocular irritation according to the GHS

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The bovine corneal opacity and permeability (BCOP, OECD TG 437) test is regulatorily accepted for the identification of corrosive and severe ocular irritants (GHS category 1). However, there is currently no regulatorily accepted *in vitro* test available for the differentiation of ocular irritants and non-irritants (GHS category 2 or no category).

Human reconstructed tissue models have been suggested for incorporation in a tiered test strategy to ultimately replace the Draize eye irritation test (OECD TG 405). In this model, cell death induced by slight or moderate irritants is determined by the MTT assay. We established and evaluated the EpiOcular™ assay to discriminate ocular irritants from non-irritants. Test substances that decreased viability to ≤ 60% (compared to control) are considered eye irritants (GHS cat 1 or cat 2) and test substances with less effect on viability are considered non-irritants.

The tests were performed with 60 test substances including a broad variety of chemicals and formulations for which *in vivo* data (Draize eye irritation test) and BCOP data were available: 18 severe irritants/corrosives (GHS category 1), 21 irritants (GHS category 2), and 21 non irritants (no GHS category). For the assessed data set the EpiOcular™ assay provided sensitivity (cat1+cat 2) >90% and specificity (no cat) >70% resulting in overall accuracy of >80%. Applying an alternative viability threshold (50% instead of 60%) resulted in sensitivity, specificity, and accuracy >80%. The tiered testing strategy combining BCOP and EpiOcular into a "top-down" and "bottom-up" concept was evaluated and results will be presented.

I-8-394

COLIPA Eye Irritation Task Force strategy and programme for development of *in vitro* methods: continued developments and status

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The COLIPA (European Cosmetics Association) eye irritation programme for development of *in vitro* methods is focused on identification of new *in vitro* test systems/endpoints that use the understanding of mechanisms of eye injury/recovery to predict human ocular responses to chemical exposure. The core approach to achieve this is based on optimisation of existing *in vitro* assays through applied research and method refinement. Applied research focuses on models using Depth of Injury (DoI) as a mechanistic basis for eye irritation. An ongoing project builds on the standard BCOP assay to correlate DoI consistent with the degree of irritancy observed *in vivo*, using phalloidin staining to demonstrate loss of F-Actin in cells that have been killed. This approach could result in new/improved *in vitro* methods that would proceed to formal validation.

Key projects on method refinement focus on Reconstructed human Tissue (RhT) assays using MatTek EpiOcular® and SkinEthic® HCE human corneal models. We have, working with test method developers, completed pre-validation datasets to enable entry into a prospective ECVAM validation study for discrimination of non-classified chemicals from eye irritants (all classes). This ECVAM validation study is in its experimental phase (from mid-2010). A further project integrating use of HPLC/UPLC into RhT assays addresses a known limitation of possible interference with absorbance measurement of MTT by photometry for intrinsically coloured test materials. We continue to work in collaboration with external organisations such as ECVAM, academia and regulatory authorities to achieve validated alternatives.

This poster provides a detailed overview and update of the COLIPA Eye Irritation programme strategy and content.

I-8-408

Eye irritation of eye make-up removers assessed by *in vitro* methods and a clinical study

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Safety assessment of cosmetics intended for use in the eye area requires evaluation of ocular irritancy exclusively using *in vitro* tests due to recent legislative restrictions within the EU. Ten commercially available eye-make up removers were subjected to testing of eye irritation hazard using a battery of *in vitro* tests, including the bovine corneal opacity and permeability (BCOP) test, EpiOcular™ tissue construct assay, cytotoxicity tests using Balb/c 3T3 fibroblasts (Neutral Red Uptake and Neutral Red Release) and Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) assay. Results of the *in vitro* tests were compared to the outcome of a clinical in-use test under ophthalmological control after application of the products to the external eyelid. Individual alternative assays predictions for mild irritant formulations were not entirely consistent in terms of rank ordering relative to the human reactions respecting the foreseen conditions of use. The EpiOcular assay provided the most concordant results with human reactions. Negative HET-CAM results were in agreement with the mildest clinical symptoms recorded in the clinical study. However, the severity of clinical symptoms was not related to the irritation score obtained using the HET-CAM assay. The NRR assay seems to provide relevant additional results to EpiOcular and HET-CAM assays. The results confirm that a battery of *in vitro* tests with different endpoints might be required for reliable assessment of eye irritation; however, not all of the currently used assays seem to be correlated sufficiently with adverse clinical signs *in vivo*.

Supported by Ministry of Health (project No. NS9648-4/2008).

I-8-428

Inter-laboratory validation of the alternative HET-CAM test

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The purpose of this study was to perform the HET-CAM test for inter-laboratory validation between TRIDSKIN and NATURA. Three independent assays were performed to evaluate the potential ocular irritation using the HET-CAM method. Besides the positive results (SDS) and negative ones (saline) three samples were tested, i.e. two bi-phasic oils and one sample powder. The average values of IS in the three assays for the powder sample was 1.0 according to the table of category of irritation, classified as slightly irritant. The only event observed was a discrete hyperemia and, in some cases, a discrete hemorrhage. As for the bi-phasic oils, a test for the inferior and superior phases was needed, once these samples obtained inconsistent results in the three essays. The superior phases of the bi-phasic oils showed IS equal to "0" being, therefore, classified as a non-irritant. However, the inferior phases of bi-phasic oils showed IS values between 3.0 and 3.91, being, therefore, classified as slightly irritant. The only event observed was a light hyperemia and, in some cases, a discrete hemorrhage. The positive control (SDS 1%) showed IS equal to 9.17 while the negative control (saline) showed IS equal to "0", therefore these samples are classified as severe and non-irritants, respectively.

I-8-433

Development of a new opacitometer for the Bovine Corneal Opacity and Permeability (BCOP) assay

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The BCOP assay is generally accepted as a valid alternative method to the Draize eye irritation test to detect corrosive and severe eye irritants. Routinely, opacity is measured by an OP-KIT opacitometer, which provides a centre-weighted reading of light transmission by measuring changes in voltage when the transmission of white light through the cornea alters. However, this may underestimate opacity that develops as spots or heterogeneous opaque areas on the periphery of isolated corneas. In addition, the BCOP test has not proven sensitive in distinguishing among mild and moderate eye irritants.

A prototype of a new laser light-based opacitometer allowing better measurement of opacities was developed by Van Goethem and colleagues. This new device showed improved sensitivity to detect subtle changes in corneal transparency. Furthermore, the new opacitometer allowed the analysis of the complete corneal surface and was able to detect more efficiently opaque spots located along the sides of the excised corneas. CARDAM, in co-operation with Peira Scientific Instruments (Beerse, Belgium), will construct a copy of this prototype and improve further the equipment for opacity measurements by 1) using the laser light-based opacitometer in combination with a camera and 2) modifying treatment conditions of the corneas in the cornea holders in order to better mimic the *in vivo* situation. A set of reference compounds with irritancy potencies, especially in the mild and moderate range, will be tested. These modifications of the classical BCOP assay should allow a more accurate definition of the eye irritating potential of compounds.

This research project is sponsored by the Stavros Niarchos Foundation (Greece).

I-8-438

Validation study on the Ocular Irritection® assay

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A validation study was undertaken to obtain additional prospective data and assess the relevance and reliability of the Ocular Irritection® assay according to the OECD principles for validation and the ECVAM modular approach. The primary goal of the study is to evaluate the ability of the Ocular Irritection® test to reliably discriminate non-classified substances from classified ocular irritants (categories 1 and 2) as defined by the UN Globally Harmonized System for classification.

The assay is based on a macromolecular reagent that, when rehydrated, forms an ordered matrix mimicking the highly ordered structure of the transparent cornea. Irritant substances produce a turbidity of the reagent by changes in protein conformation and degree of hydration that mimics the disruptive effects irritants may have on the corneal proteins and carbohydrates. Because of its nature, Ocular Irritection® presents the advantage of having long shelf-life (years) and is easily shipped around the world.

An international Validation Management Group was formed to manage and oversee the study comprising an independent chairman, co-chair, biostatistician and chair of the chemicals selection, in addition to the sponsor representatives (INT.E.G.RA and InVitro International) and the lead laboratory representative. A challenging set of 60 coded substances for which *in vivo* data are available were selected in collaboration with ECVAM and will be tested in 3 independent laboratories from Europe and the USA, including one naïve laboratory. The testing phase is planned from May to September 2011 after the training and transferability of the test method takes place.

I-8-532

Considerations for demonstrating the inter-laboratory reliability of Chorioallantoic Membrane Vascular Assay (CAMVA) and the Bovine Corneal Opacity and Permeability Assay (BCOP)

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In vitro assays evaluating ocular irritation potential are routinely used by personal care companies. Two of these *in vitro* assays include the Chorioallantoic Membrane Vascular Assay (CAMVA) and the Bovine Corneal Opacity and Permeability Assay (BCOP). These assays do not require the use of live animals, provide reliable predictive data and are rapid to conduct. The BCOP uses excised bovine corneas to predict ocular irritation. The CAMVA uses the vascular network of fertilized chicken eggs as a conjunctival model to predict eye irritation. Both BCOP and CAMVA have been used for over fifteen years for product development, worker safety, and safety claims substantiation.

This poster describes procedures and considerations for demonstrating the inter-laboratory reliability of the BCOP and CAMVA. It is important to have a valid assay that can be implemented consistently at several different laboratories. For Kao Brands Company, a large BCOP and CAMVA database exists that covers multiple consumer product categories such as hair shampoos, skin cleansers, and hair styling sprays (containing ethanol). Therefore, a proper review of candidate laboratories is important for seamlessly generating consistent results that can be used for assessing potential ocular irritation of new products. First, a candidate laboratory should be audited for proper facility operation and personnel training. Second, the laboratory's use of Good Laboratory Practices (GLPs) should be reviewed. Third, reference materials with known BCOP and CAMVA data (one irritant and two non-irritants for initial assessment) should be tested at each new laboratory for verification of proper assay performance.

I-8-541

Prospective validation study of reconstructed human tissue models for eye irritation testing

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A prospective validation study of two *in vitro* test methods using Reconstructed human Tissue (RhT) models (EpiOcular™ and SkinEthic™ Human Corneal Epithelium (HCE)) for the detection of eye irritation effects by chemicals is currently being conducted by ECVAM and COLIPA. These methods are being validated for their usefulness to identify chemicals as either not classified for eye irritation (NC) or irritant (Cat. 1 and Cat. 2) within the United Nations Globally Harmonised System (UN GHS), for inclusion into a Bottom-Up/Top-Down test strategy, with the ultimate goal of replacing the *in vivo* Draize eye irritation test. They are not intended to differentiate between mild/moderate (Cat. 2) and severe (Cat. 1) irritants, which would be left to another tier of the test strategy.

Pre-validation studies indicated that both methods predict eye irritant properties of chemicals with high accuracy (~80%). In the current validation study 104 chemicals are being tested with each method in three independent runs by three laboratories. Selected chemicals cover the full irritancy range, represent a wide range of chemical classes/functionalities, and are also balanced in physical state and chemical reactivity.

The Validation Management Group has organised Quality Assurance audits on each RhT production site to guarantee quality of supplied tissues and has developed guidance on study conduct for laboratories as well as performance criteria for assessing validity of the test methods. The participating laboratories have successfully completed transfer and transferability studies and are expected to finalise the testing phase in mid-2011. This poster presents all latest updates of the validation study.

I-8-576

Usefulness and limitations of the Cytosensor® Microphysiometer (CM) test method for ocular safety testing

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ICCVAM evaluated CM as a potential replacement for the rabbit eye test for identifying ocular hazards. CM is restricted to water-soluble substances. ICCVAM concluded that substances within a limited applicability domain (water-soluble surfactants, surfactant-containing formulations, non-surfactants) that are positive for severe effects in CM can be classified as ocular corrosives/severe irritants (EPA Category I, EU R41, GHS Category 1). False positive rates ranged from 0% (0/17, 0/18) to 10% (3/29) and false negative rates ranged from 9% (2/23) to 50% (6/12) depending on the hazard classification system used. ICCVAM also concluded that substances within an even more restricted applicability domain (water-soluble surfactant chemicals and certain types of surfactant-containing formulations, but not non-surfactants) would not require ocular hazard labeling (EPA Category IV, EU Not Labeled, FHSA Not Labeled) without any further testing if they are negative in CM. Although false positive rates were high (50% [3/6] to 69% [18/26]), false negative rates ranged from 0% (0/27, 0/28, or 0/40) to 2% (1/46 or 1/47) depending on the hazard classification system used. CM is not considered valid for identification of mild or moderate ocular irritants (EPA Categories II/III; EU R36; GHS Categories 2A/2B); these substances would require additional testing. ICCVAM also recommended a standardized CM protocol and future studies to expand the applicability domain. Some ICCVAM agencies have endorsed these recommendations, making CM the first *in vitro* test method available in the US for identifying a subset of substances that do not require ocular hazard labeling. A draft OECD TG for CM is currently being considered.

I-8-598

The impact of US adoption of the UN Globally Harmonized System on the use of *in vitro* methods for ocular and dermal irritation and corrosion

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Endorsed by the United Nations in 2003, the UN Globally Harmonized System for classification and labeling is intended to harmonize hazard classification and labeling criteria throughout the world for human health and ecotoxicity endpoints. While GHS was designed to correlate with existing classification systems and the European Union, Canada, and United States have committed in principle to adopting GHS in place of their own national classification systems, differences among classification systems have delayed adoption of GHS by various agencies. Harmonization with GHS impacts the replacement, reduction, and refinement of animals in testing, since *in vitro* methods for skin and eye irritation have been and are currently being validated according to GHS classification. This poster compares US EPA, US OSHA and GHS classifications for skin and eye irritation as they relate to validated *in vitro* methods for skin and eye irritation and discusses methods to harmonize these classification systems. The methods include: The Bovine Corneal Opacity and Permeability test method, the Isolated Chicken Eye test method, the Cytosensor Microphysiometer (CM) test method, and the Fluorescein Leakage test method for eye irritation, and Reconstructed Human Epidermis and barrier models for skin irritation. Widespread adoption of GHS will help speed harmonized adoption of existing and new *in vitro* methods for relevant endpoints.

I-8-629

The eyes have it: Calf versus adult eyes in the Bovine Corneal Opacity and Permeability (BCOP) assay

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The Bovine Corneal Opacity and Permeability (BCOP) assay uses excised bovine corneas obtained from cattle slaughtered for food use. Consequently, the age of the cattle is generally not provided with the corneas. This introduces a variable at the start of the assay, which can be reduced by using eyes from cattle at a defined age.

The OECD guidelines for the testing of Chemicals No. 437 recommends the use of eyes from cattle 6 to 12 months of age but encourages investigators to report the estimated age and/or weight of the animals providing the corneas. In a commercial operation capable of killing large numbers of cattle, it may not be possible to determine age and weight of animals as they are received from a number of suppliers. Additionally, random-source animals typically have not been raised in constant environment and are subject to numerous environmental variables. This study looked at the effect of the cattle's age on the BCOP assay. Five-month-old calf eyes used in this study were obtained from a well-managed barn-raised herd, which had weekly veterinary monitoring and controlled feed and medication.

In this study we compared the *In Vitro* Scores (IVS) from random source cattle corneas with those of corneas from a well-managed calf herd 5 months of age. Thirty over the counter cleaners, hair dyes, hair sprays, deodorants and moisturizers, which had IVS scores obtained from random-age animals were used in the evaluation. The use of five-month old calf eyes resulted in a highly successful correlation with data from adult eyes except with products with a high colorant content or with hydrogen peroxide containing materials. A second *in vitro* assay helped predict the potential ocular irritation.

I-8-630

PorCORA ocular reversibility assay testing with personal care products

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To ensure consumer safety, ocular irritation testing is routinely performed on personal care products. Two alternative ocular toxicity tests, the Chorioallantoic Membrane Vascular Assay (CAMVA) and Bovine Corneal Opacity and Permeability Assay (BCOP), are widely used in the cosmetic industry since they do not require the use of animals. These assays provide reliable data predicting ocular irritation and have high predictive value when compared to rabbit or human eye results. To complement the CAMVA/BCOP assays, the Porcine Corneal Opacity Reversibility Assay (PorCORA) was developed using an *ex vivo* model to predict reversibility of ocular damage caused by potential irritants. In the current study, three commercially a consumer products (a shampoo, a hair color glaze, and a 12% hydrogen peroxide product) were tested in the PorCORA for ocular damage and reversibility. The PorCORA indicates that under the exaggerated *in vitro* study conditions the surfactant-based shampoo may cause irreversible ocular damage: histological changes occurred in the squamous-cell layer of the corneas and mild to moderate changes in the basal-cell layer. However, scientific literature contradicts these results, and ocular damage reversibility does occur *in vivo* following exposure to shampoo. Furthermore, the PorCORA predicts that under the same study conditions used for the shampoo, ocular damage caused by a hair color glaze and a 12% hydrogen peroxide product are fully reversible with histology reporting only minimal or mild microscopic effects to the superficial squamous-cell layer. Like the shampoo, the scientific literature also indicates that ocular damage is reversible *in vivo* following exposure to hydrogen peroxide. In summary, the PorCORA assay, in conjunction with other alternative toxicology ocular irritation assays is a valuable and predictive method to determine the extent of ocular damage and reversibility that products may cause following consumer eye exposure.

I-8-631

Porcine Corneal Ocular Reversibility Assay (PorCORA) predicts EU R41 and GHS Category 1

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Currently, there is no alternative (non-"*in vivo*") ocular irritation assay that can measure corneal tissue damage and reversibility. With the support of two Colgate-Palmolive Grants for Alternative Research, we have developed an alternative assay: Porcine Corneal Opacity Reversibility Assay (PorCORA). PorCORA measures corneal damage and recovery over extended time periods using porcine corneas excised from by-product abattoir eyes. Test articles (liquid and solid) are dosed directly onto the corneal surface, and tissue damage and recovery are assessed by sodium fluorescein (NaFL) retention in the same corneas over time (up to 21 days). We have confirmed NaFL retention results and corneal recovery in the PorCORA system via several approaches. Both fluorescence and reflective confocal microscopy confirm damage repair indicated by fluorescein retention in the cultured corneas. In addition, we have shown histological evidence that also correlates well with NaFL staining in the PorCORA assay. Here we report the results of a 32-reference chemical validation including chemicals from the following classes: acetates, acids, alcohols, alkalis, esters, hydrocarbons, inorganics, ketones, surfactants, and several solid compounds. To determine if the PorCORA system can predict R41 or GHS Category 1 we considered corneas that retained NaFL at 21 days post-dose to be R41 and GHS Category 1. ECETOC historical rabbit eye data was used to classify EU and GHS eye irritation for the 32 compounds tested. PorCORA predicted 11/11 compounds classified as R41 and 12/13 compounds classified as GHS Category 1. Since PorCORA can predict these categories, compounds that cause damage that is reversible in the PorCORA system may be considered R36 or Category 2. Thus PorCORA is a highly predictive method to distinguish between ocular irritancy classifications R36 or R41 and Category 1 or 2 without the use of live animals.

I-8-632

PorFocal, for your eyes only!

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MB Research Laboratories has developed a procedure for the culturing of excised porcine corneas for a period of up to 21 days. Using these corneas, we have developed a method, PorFocal, to evaluate the effects of multiple doses of low-level irritants. Corneas are excised from enucleated porcine eyes from the abattoir, and can be cultured in a living state for up to 21 days. In order to detect and quantify low-level damage to corneal tissue, this assay was created to assess ocular irritation by measuring cell viability using a dead stain, Ethidium homodimer (EtH), and fluorescence confocal microscopy. For the PorFocal assay eight cultured corneas (4 per test material) were treated with either Dulbeccos's Phosphate Buffered Saline (dPBS), or 0.01% Benzalkonium Chloride (BAK) for a total of 10 doses (2x/day on days 1, 2, 6; 1x/day on days 0, 3, 7, 8) at 50 μl/treatment. On day 8, these corneas were incubated for 30 min with 2 μM EtH dead cell stain and imaged using confocal microscopy. The EtH stained dead cell nuclei were imaged in 6 random 450 μm x 450 μm x 56 µm-deep tissue fields via confocal z-stacks composed of eight 8 μm-thick optical slices. A maximum projection of image z-stacks was created so that no nucleus was counted twice. All dead nuclei (cells) were counted for each tissue field, the counts were summed, and statistical analysis was performed using ANOVA. PBS-treated corneas (n=4) exhibited 1659 dead cells and 0.01% BAK-treated corneas (n=4) exhibited 3591, a 216% increase in cell death, which was statistically significant ($p<0.001$). These data indicate that low-level damage can be detected by using confocal microscopy. Future directions for this project include increasing the amount of replicates to decrease variance. Also, the complimentary component of the staining kit is a live cell stain. This stain could be further developed, and a ratio of live to dead cells in each group could yield higher sensitivity corneal irritation measurements.

I-8-633

Historical data on personal care products over fourteen years using the Chorioallantoic Vascular Membrane Assay (CAMVA) and the Bovine Cornea Opacity/ Permeability Assay (BCOP)

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The Chorioallantoic Membrane Vascular Assay (CAMVA) and Bovine Corneal Opacity and Permeability Assay (BCOP) are two common assays used to determine ocular irritation for consumer-use products. These assays do not require the use of live animals, provide reliable predictive data, provide results similar to *in vivo* models and are rapid and inexpensive to conduct. Data from 321 studies performed from 1995 to 2009 (a total of 345 test materials assessed by CAMVA and/or BCOP) were compiled to determine the feasibility of predicting ocular irritation for various formulations. Review of the data from both assays found that hair shampoos, skin cleansers, and hair styling sprays (containing ethanol) were repeatedly predicted to be ocular irritants. In contrast skin lotions/moisturizers were repeatedly predicted not to be ocular irritants. Based on the findings for these product types, future ocular irritation testing (i.e., CAMVA/ BCOP) can be nearly eliminated as long as formulations are compared to those previously tested. For example, skin cleanser irritation appears to be solely dependent on surfactant species and level in these formulations.

For other product types (e.g., deodorants, make-up removers, hair styling, body sprays) it was concluded that these products should continue to be tested in CAMVA/BCOP for ocular irritation potential because either significant variability exists in the historical data (non-spray hair stylers) or the historical sample size is too small to permit definitive conclusions (deodorants, make-up removers, massage oils, facial masks, body sprays, and hair styling products).

I-8-634

Development of the Replacement Ocular Battery – tiered testing strategy of alternative toxicology tests to replace the need for rabbit eye tests

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Using a series of non-animal assays in a tiered approach, the Replacement Ocular Battery (ROBatt) accurately predicts the categories of acute ocular irritation corresponding to OECD, EPA and GHS guidelines. At present, no single alternative assay has been accepted by regulatory agencies to completely replace the use of live animals. The BCOP (Bovine Cornea Opacity/ Permeability) test has been accepted by OECD as a screen for severe and corrosive materials. EpiOcular™ and other ocular tissue models are in various stages of review or acceptance. The Cytosensor Microphysiometer has been accepted for sub-severe testing but it is only applicable to aqueous-based materials. The ROBatt approach uses a series of up to three non-animal assays to categorize both aqueous and non-aqueous materials.

An FDA-NIH Grant has been awarded to develop the ROBatt decision tree criteria. Initially, screening will use the Chorioallantoic Membrane Vascular Assay (CAMVA) to discriminate slight or non-irritants from moderate to severe irritants. Slight or non-irritating materials will be categorized using the Porcine Cornea Confocal Assay (PorFocal). 3D human reconstructed tissue models and/or the Bovine Cornea Opacity/Permeability test (BCOP) will be used for discriminating between moderate and severe to corrosive materials. Lastly, the Porcine Cornea Opacity Reversibility Assay (PorCORA) will be used to categorize severe irritants and corrosives.

Fifty validation chemicals from the ECETOC database of ocular irritation will be initially tested. Having performed over 6,700 CAMVA, 5,700 BCOPs, 3,000 MatTek EpiOcular™/ SkinEthic HCE™, and nearly 100 PorCORA assays, the researchers are confident of the ability of ROBatt to properly categorize any material to international standards.