Session 5.5
Advancements and needs for developing and validating alternatives for ocular irritancy and corrosivity testing

Poster

Performance of the Isolated Chicken Eye (ICE) test method in detecting ocular corrosives and severe irritants

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Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop *in vitro* alternatives for the current *in vivo* rabbit eye test. NICEATM evaluated four *in vitro* test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, ICE, is an organotypic model that provides short-term maintenance of the chicken eye in an isolated system. The ability of ICE to correctly identify ocular corrosives and severe irritants using available ICE and corresponding *in vivo* rabbit eye test data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System of Classification and Labeling of Chemicals. Based on an interim analysis, ICE appears useful (with the exception of testing alcohols, surfactants, and solids) in a weight-of-evidence tiered testing strategy. Accordingly, positive results could be used to classify and label a substance, while substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of ICE for the detection of ocular corrosives and severe irritants.

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Poster

Proposed reference substances for optimisation and validation studies with *in vitro* ocular test methods

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NICEATM evaluated four *in vitro* ocular test methods (BCOP, ICE, IRE, and HET-CAM) for their ability to identify substances that cause severe irritation or corrosion. During these evaluations, a proposed list of reference substances for future optimisation and validation studies of these and other alternative test methods intended to detect ocular corrosives/severe irritants was developed. Based on the ICCVAM Submission Guidelines (ICCVAM 2003; http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm), substances included in this list are intended to: 1) represent the range of ocular responses (i.e., corrosive, severe irritant, non-severe irritant, non-corrosive) that is expected to be detected; 2) represent the classes of chemicals that are expected to be tested; 3) have produced high quality results in the Draize *in vivo* rabbit eye test and/or in humans; 4) have well-defined chemical composition; 5) be readily available; and 6) not be associated with excessive hazard or costs (purchase and/or disposal). Following completion of any optimisation and validation studies for each test method, reference substances from this list could be selected for inclusion in performance standards and for proficiency testing. This proposed list of substances is intended to represent the minimum number of substances that should be used to evaluate the accuracy and reliability of an *in vitro* ocular test method proposed for the detection of ocular corrosives and severe irritants. Testing the complete list of all reference substances will facilitate future validation efforts and comparison of performance among different test methods and protocols.

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Poster

A human corneal epithelial cell-based tissue model for assessment of ocular irritancy

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We have developed an *in vitro* tissue model comprising human corneal epithelial cells in a physiologically relevant environment. Primary corneal epithelial cells were isolated from human corneas and transfected with Human Papilloma Virus (HPV) type 16 E6 and E7 genes. These cells were seeded onto microporous membrane inserts and grown to confluence under standard submerged culture conditions. The inserts were then cultured at the air-liquid interface in Corneal Epithelial Model Differentiation Medium to induce stratification and differentiation of the cells. Cell growth and culture parameters were tightly regulated to ensure normal cell behaviour and morphology. Routine quality control characterisation included Trans Epithelial Electrical Resistance (TEER), histological analysis, and time-to-toxicity (ET_{50}) for reference compounds using the MTT cytotoxicity assay. The culture models exhibited a histological profile similar to that observed with in vivo corneal epithelium. Furthermore, the cytotoxic response of the system to known irritants shows good correlation with *in vivo* Draize data for several different irritant categories. Based on these results, the human corneal epithelial culture model described here presents a promising *in vitro* system for the assessment of ocular toxicity and irritancy.

Poster

Comparison of tissue viability and histological changes in EpiOcular™ human cell construct following exposure to surfactants

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The ability of the EpiOcular™ construct to predict the eye irritation potential of surfactants has been the subject of a formal validation program. EpiOcular™ correlates a test article's potential for ocular irritation with the time it takes to reduce tissue viability by 50% (ET_{50}) as measured by MTT. This study investigated whether the histological changes following exposure to the surfactants are in agreement with the MTT results. Eight surfactants were selected and applied to the EpiOcular tissue using exposure times that bracketed the ET_{50} values established in the validation study. Upon completion of the exposure half of the tissues (2/timepoint) were set aside for histological examination while the viability of the remaining tissues was assessed. For all surfactants, the results showed a good relationship between the degree of histological damage with changes in tissue viability. An increase in the depth and severity of tissue damage was associated with a decrease in tissue viability. Histological changes ranged from subtle cellular changes such as vacuolisation and punctuate chromatin condensation to overt tissue loss and cell necrosis. Loss of or damage to the surface squamous epithelium was associated with <20% decrease in viability, while the degree of damage to the central squamous epithelium was directly related to a 20-80% decrease in viability. In conclusion, the nature and severity of the histological changes were in agreement with the MTT results. Understanding the progression and types of cellular changes associated with tissue damage may be able to help distinguish the degrees of ocular irritation.

Poster

Performance of the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) test method in detecting ocular corrosives and severe irritants

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Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop *in vitro* alternatives for the current *in vivo* rabbit eye test. NICEATM evaluated four *in vitro* test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, HET-CAM, was developed to mimic the mucosal eye tissues. The ability of HET-CAM to correctly identify ocular corrosives and severe irritants using available HET-CAM and corresponding *in vivo* rabbit eye test data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System. Results from an interim analysis suggest that HET-CAM has a high false positive rate. However, the assay may still be useful in a weight-of-evidence tiered testing strategy, where a positive substance could be either re-tested with a modified method to confirm the result or used to classify and label a substance. Substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of HET-CAM for the detection of ocular corrosives and severe irritants.

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Poster

A proposal for classifying shampoos as irritant or non irritant using the Neutral Red Uptake assay

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The use of rabbits for the eye irritation test has been severely criticised since the end of 1970's. Brazil is still using animals for assessing the safety of shampoos. In order to be updated with world tendency, we are studying some *in vitro* assays to be applied to the regulatory quality control. Since literature data do not present a cut off to establish when a product is considered irritant or not, we compared the results of Neutral Red Uptake assay (NRU) with those obtained from the routine rabbit assays.

Twenty-three shampoos and eight surfactants were studied. a) *In vivo*: Five rabbits were used for each products. 100 ml were instilled in one eye and the alterations were graded using the Draize scale. b) *In vitro*: SIRC cells were used. Products were kept in contact during 24 hours and the cells stained with Neutral Red. The stain was removed from cells and read by photocolorimetry.

The IC_{50} was calculated and the results compared with those from rabbits. Comparison between rabbits and SIRC presented good linear correlation (r=0.81 for shampoos and r=0.97 for surfactants). We found that products dilution that present IC_{50} value below 1 mg/ml were considered irritant by rabbit test, while IC_{50} values greater than 1 mg/ml referred to non irritant products.

Conclusion: The NRU assay may be used for classifying products. When IC_{50} value is lower than 1 mg/ml, it means that the product is irritant, otherwise, it should be considered as non irritant.

Lecture

ECVAM progress in evaluating *in vitro* test methods for identifying mild and moderate ocular irritants

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European legislation calls for alternatives to animal testing, especially in the area of cosmetics where animal replacement is required. Major validation and evaluation studies took place in the 90's to replace the Draize test for eye irritation. The studies showed good reproducibility and reliability of some alternative methods, but no single method was able to replace the Draize rabbit eye test for regulatory purposes. In order to advance towards validation of *in vitro* alternatives, ECVAM is working on 3 parallel fronts. First, existing data on the Draize rabbit eye test are being reviewed to gain a better understanding of its current uses and limitations. Secondly, the most promising *in vitro* models for identifying mild and moderate ocular irritants are being evaluated, based on an in-depth review of the existing data and on the application of the Modular Approach to Validation. These test methods comprise organotypic models, reconstituted human tissue models, cell cytotoxicity assays and cell function assays. Finally, the potential use of testing strategies that utilise the strengths of particular *in vitro* assay systems to address required ranges of irritation potential and/or chemical classes is being assessed. The latest progress of these on-going evaluations will be presented with focus for test methods for mild and moderate ocular irritants.

Poster

Tissue engineering: HET-CAM test evaluation at day 10

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Aim: The HET-CAM (Hen Egg Test – Chorioallantoic Membrane) angiogenesis test system, originally conceived as alternative method for toxicity and irritation studies, has for some time been suggested for tissue engineering tasks, biocompatibility testings and also as cell transplantation model. Published works show that in most of these cases incubation of the eggs was performed for up to 15 days. This time-point is way past neural tube fusion at ~day 11, resulting in possibility of embryo pain conduction. Therefore rating as actual animal testing has been discussed previously. Aim of the work was to show the feasibility of altering existing CAM-test protocols by cancelling experiments at incubation day 10 for reasons of achieving satisfactory data quality while following animal welfare considerations.

Methods: Example of published testing schemes of biomaterial testing and hetero/autologous cell transplantation using the CAM angiogenesis onset were performed but truncated at day 10. Histological/elektronmicroscopical analysis was conducted.

Results: The authors believe resulting data matched that from original experiments in means of quality and reproducibility sufficiently, proofing the suitability of the altered time-schedule.

Conclusion(s): The CAM-angiogenesis-assay can be used also for innovative experiments in the fields of biomedical engineering, even with a total duration of experiments of just 10 days. Thereby its quality as alternative method to animal testing remains intact without undue minor quality of results.

Poster

Comparative evaluation of benzalkonium chloride on *in vitro* rabbit and human corneas

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Purpose: To develop an *in vitro* technique for comparison of corneal toxicity of surfactants in isolated rabbit and human corneas.

Methods: Rabbit eyes were obtained from Pel-Freeze and human corneas, stored in Optisol GS™, were obtained from the Georgia Eye Bank. Corneas were isolated and mounted for endothelial perfusion in an *in vitro* specular microscope. Benzalkonium Chloride (BAC) was applied to corneas with and without an intact epithelium during which time the corneal endothelium was continuously perfused with a balanced salt solution. The effects of 0.005%, 0.01%, 0.1% and 1% BAC on the rabbit corneal tissue were evaluated after 3, 9 and 18 min exposures and on the human corneal tissue after 3 min. Following exposure, BAC was removed and the corneas were perfused with the balanced salt solution, the corneal thickness was measured half hourly for 3 hours. The corneas were fixed for histological evaluation by light and electron microscopy.

Results: In the rabbit cornea, BAC showed time and concentration dependent corneal swelling and structural changes with associated damage to the endothelial layer. Human corneas exposed to the BAC for 3 min showed the similar corneal alterations as observed in the rabbit corneas.

Conclusion: This study demonstrated that this technique can use commercially available rabbit corneas to examine early BAC-related changes in histopathology. Furthermore, the pattern of damage seen in the human cornea was similar to that of the rabbit.

Poster

Estimated under- and over-classification rates for a 1-3 rabbit sequential Draize rabbit eye test

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ICCVAM is evaluating four *in vitro* assays for their ability to detect ocular corrosives/severe irritants in a weight-of-evidence tiered testing strategy. Ideally, this analysis would evaluate the ability of alternative assays and the Draize eye test to each correctly predict human ocular toxicity. However, the lack of appropriate human data only allows for a determination of how well alternative assays predict the rabbit response. In assessing the performance of alternative assays, information on the Draize eye test reliability would be useful but the paucity of repeat test data precludes an accurate estimate of inter- and intra-laboratory reproducibility. However, Draize eye test results can be used to estimate the likelihood of under-classifying a positive substance or over-classifying a negative substance using the current 1-3 rabbit sequential test. Data from Draize eye testing using 3-6 animals was obtained for ~900 substances from U.S. Federal regulatory agencies, published studies, and scientists and organisations. Ocular irritation categories were assigned based on the 2003 UN Globally Harmonized System of Classification and Labeling of Chemicals. Assuming either a homogeneous or a heterogeneous response among rabbits within a classification category, the distribution of individual rabbit responses was used to estimate the likelihood of under- and over-classification for a 1-3 rabbit sequential testing strategy. The estimated under-classification rate for corrosives/severe irritants that induced any severe response was <10%; rates were higher when this classification was based on only lesion persistence at day 21. Estimated over-classification rates will also be presented.

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Poster

Validation of the BCOP assay for the evaluation of ocular irritation of various petrochemical products

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Relatively few alternative studies have been conducted with products of interest to the petrochemical industry. Therefore, an in-house program was implemented to develop/validate new alternative *in vitro* test methodologies for the prediction of ocular irritation and acute systemic toxicity of petrochemical products.

For the eye irritation evaluation, the Bovine Corneal Opacity and Permeability (BCOP) assay was used to differentiate 16 petrochemical products (e.g. lubricant additive packages, base stocks, cutting fluids, solvents). When compared to the 10-minute exposure results, the BCOP assay correctly classified the 14 out of 16 products and produced 2 false negatives. For the assessment of potential acute systemic toxicity, the same 16 test articles were examined in the 3T3 Neutral Red Uptake (NRU) bioassay. The low solubility of the petrochemical products resulting in the inability of cells to be exposed to concentrations below desired concentrations was reflected in a poor predictability of the 3T3 NRU assay (less than 60%). Despite the poor predictability of the 3T3 NRU assay (*vs. in vivo* data), results did indicate its potential if the procedure is modified to suit this class of chemicals. These results suggested a good potential of the *in vitro* BCOP assay in predicting eye irritation for certain petrochemicals: it may be a screening tool before *in vivo* testing (refinement) or an alternative stand alone *in vitro* assay (replacement) for assessing hazard. Ultimately, the validation and acceptance of alternative testing methodologies will benefit animal welfare through the reduction, replacement, and more-humane use of laboratory animals.

Lecture

An industry perspective – needs, strategies and development programmes for ocular irritancy

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The standard regulatory approved test to evaluate eye irritation is the Draize test. Success in fully replacing it with *in vitro* methods has not occurred. It has been concluded (COLIPA Workshop 1997, ECVAM Workshop, 1998) that reasons for this are multiple and include lack of understanding for the physiological mechanisms of eye irritation, variability of *in vivo* Draize test data and ability of the Draize test to reliably predict the human response.

Though not formally validated, some of the currently available *in vitro/ex vivo* methods are used in industry by raw material suppliers and cosmetic companies for in-house routine screening purposes in the development of new products or in integrated testing strategies. Although reduction/refinement methods/approaches are available today, there remains a clearly identified need to define *in vitro* methods that reliably predict the human eye response to chemicals exposure and can replace the *in vivo* test. As such, a fundamental understanding of what is needed to fill the knowledge gaps is essential to continued progress. Certainly, the key focus area for current/future research that emerged is the need for mechanistic understanding of eye injury resulting from chemical exposure.

This presentation provides an overview of: 1) why eye irritation evaluation is needed; 2) current industry use of *in vitro* assays; 3) previous development/validation efforts and what was lacking; 4) what is new today (e.g. new approaches, technology advances, changes in regulatory environment e.g. 7th Amendment, REACH) and 5) current industry efforts/development programmes/collaborations that will aid success.

Lecture

ICCVAM progress in evaluating *in vitro* test methods for identifying severe ocular irritants/corrosives

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ICCVAM, with the assistance of NICEATM and in response to a nomination by the US Environmental Protection Agency, recently initiated a review of the validation status of four *in vitro* ocular toxicity test methods with potential for use in screening chemicals for severe eye irritation or corrosion. The four test methods are the Bovine Corneal Opacity and Permeability (BCOP) assay, the Hen's Eegg Test – Chorioallantoic Membrane (HET-CAM) assay, the Isolated Chicken Eye (ICE) assay, and the Isolated Rabbit Eye (IRE) assay. NICEATM compiled available data and information for the four methods and prepared a Background Review Document (BRD) for each. An expert panel was then convened to evaluate the usefulness and limitations of the *in vitro* tests based on information in the BRDs. The conclusion of the panel was that each of the four test methods could be used in a tiered testing strategy to identify severe eye irritants and corrosives, with specific limitations and caveats. Several modifications to optimise the standardised protocols were proposed by the panel and it was also recommended that additional data be requested from test method users. Subsequently, additional data received in response to a Federal Register Notice were included in accuracy and reliability analyses for the ICE, IRE and HET-CAM assays. The panel also recommended the inclusion of known human severe eye irritants as reference chemicals. Collaborative interactions between ICCVAM, NICEATM, and ECVAM to review the validation status of other available methods for assessing ocular corrosion or irritancy will also be discussed.

Poster

Evaluation of two alternative methods for assessing the ocular irritancy of hair-care products

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Introduction: In recent years, various approaches for assessing eye irritation potential by using *in vitro* test systems have been developed for replacing the Draize rabbit eye irritation test. The purpose of this study was to compare the HET-CAM and RBC tests for predicting the ocular irritancy of hair-care products, with a view to determine which alternative method may represent a suitable alternative to the *in vivo* assay.

Methods: Twenty-two shampoos and eight conditioner formulations were selected for this study which covered a range of irritant categories (non-irritant/mild/moderate/severe). The Red Blood Cell assay was a modification of that described by Pape et al. (1987) and HET-CAM procedure was as described by Luepke (1985).

Results: Results of each assay were compared with MAS of the Draize test. The frequency of agreement of the HET-CAM test and MAS was 91% sensibility, 86% specificity and a precision value of 90%. The performance of the RBC assay presented 87% sensibility, 100% specificity and 90% precision. The rank correlation coefficients for HET-CAM and RBC assays in relation to Draize test were 0.799 and 0.814 respectively.

Discussion: Observations of the performance of the HET-CAM test for the assessment of ocular irritancy of shampoos presented an over-prediction of *in vivo* effects and appears less reliable to identify mild irritant. In contrast, the Red Blood Cell presented an under-prediction of the ocular irritancy, but was able to identify mild irritants. These results indicate that both assays should be used together in order to assess the eye irritancy.

Poster

Optimisation of an *in vitro* long term corneal culture assay

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The long-term culture of corneas has been proposed as an *in vitro* model to evaluate potential eye irritation and post-treatment recovery following chemical exposures. Porcine eyes were obtained within 24 hours of sacrifice and disinfected prior to excising corneas. Corneas were filled with an agar/gelatin gel in M199 medium to support the corneas, and were cultured at 37°C, 5% CO_2, 90% RH in M199 medium to the limbus. The corneas were moistened by brief immersion in medium every 2.7 hours using a modified plate rocker. Corneas were treated with either SLS, EtOH, or H_2O (controls). The corneas were rinsed with PBS, cultured for a pre-determined post exposure time, and fixed in buffered formalin. H&E-stained control corneas showed normal morphology after 4 days, similar to excised/immediately fixed corneas. Controls were characterised by an intact epithelium with viable squamous, wing, and basal cells. The stroma showed minimal swelling with frequent viable keratocytes. The endothelium was typically intact. Some stromal swelling near the sclera was noted after 5 to 7 days. Corneas treated with 3% SLS or EtOH showed complete epithelial cell damage or loss 24 hours after treatment, as well as loss of viable keratocytes in the upper stroma. After 48 hours, epithelial cell sheet migration was observed into the damaged zone. After 120 hours, the regeneration of a stratified epithelium was observed. These results confirm the ability to culture porcine corneas for at least 120 hours, as well as demonstrate the potential for further optimisation of evaluating recovery of damaged corneas.

Poster

In vitro methods for assessing ocular irritancy of cosmetics

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Varieties of *in vitro* test protocols serve as replacement for the Draize test to assess ocular irritancy. Five protocols (BCOP, HET/CAM, ICE, CEET and IRE) are validated and accepted by legal authorities. For the EpiOcular assay validation is under its way. Additionally, the RBC test is a widespread method to assess the irritating potential of surfactants.

We compared the feasibility of common *in vitro* methods to assess ocular irritancy of cosmetics. Also, the RBC assay is often used, it is only suitable for anionic surfactants and susceptible to interfering elements from the test material. For cell culture tests a high enough solubility of test materials in the watery test system is a basic prerequisite. Therefore, testing of ready formulations is limited. The HET/CAM test proved to be practicable for cosmetics and shows good correlation with the recognition of highly irritant and non-irritant substances. Nevertheless, it is based on a subjective rating and thus missing an objective endpoint. Similarly, the EpiOcular assay is suitable for various test products, but has the advantage that test materials can be analysed directly on human tissues. It proofed to be a valuable tool in the routine analysis of formulations and single substances. Simultaneously with measuring tissue viability additionally parameters e.g. LDH, PGE-2 and IL-1α can be quantified to obtain further information about the irritation potential and possible inflammatory processes.

In conclusion, the HET/CAM test is a cost-effective alternative whereas testing on human tissues equivalents enables an objective distinctive classification for assessing ocular irritancy of cosmetics.

Lecture

Use of *in vitro* data for classifying eye irritating chemicals in the EU – experience at the BfR

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The New REACH Chemicals Policy of the EU suggests to replace animal tests for the assessment of acute human health hazards by using both information based on (Q)SARs and *in vitro* test results to reduce costs and duration of testing and the number of test animals employed.

At the BfR the authors have compiled a regulatory database using data on chemicals with a purity >95%, which were submitted within the current notification procedure for New Chemicals of the EU. We have used this database to develop (Q)SAR rules for the prediction of acute local lesions on eye and skin within the new REACH system of the EU.

From these data, (Q)SARs for the prediction of local irritation/corrosion were developed and published. These (Q)SARs and an expert system supporting their use was submitted for official validation and application within regulatory hazard assessment strategies to European Chemicals Bureau ECB.

Main features of the BfR database are: a decision support system (DSS) for the prediction of skin and/or eye lesion potential built from information extracted from our database. This DSS combines SARs defining reactive chemical substructures relevant for local lesions to be classified, and (Q)SARs for the prediction of the absence of such a potential.

The impact of the use of (Q)SARs and physico-chemical data on current EU testing strategies for eye irritation testing of chemicals will be discussed.

Poster

Safety assessment of ocular contact lens solutions

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Introduction: Today there are millions of contact lens wearers. One out of every 20 contact lens wearers develops a lens-related complication each year.

Methods: The safety of ocular contact lens solution MULTISON (M) (Latvia) has been tested on a monolayer culture of NIH 3T3 cells (ECACC), i.e. embryonic murine fibroblasts. Solution M, the solution in which the lenses are stored (K-) (USA), and the positive control 50 mg/ml phenol (K+) were used.

The testing was performed according to the Guidance Document from ICCVAM and NICEATM. The irritation effect of M was also tested on 3 albino rabbits (EN ISO 10993-10). The cornea, area of cornea involved, iris, conjunctiva and chemosis discharge were assessed.

Results and discussion: Solution M displayed a slight toxicity with an IC_{50} of 37 µl. When solution M was diluted 20-fold, it lost its toxic effect. Solution M (50.0 and 15.8 µl) caused the death of cells, but did not change their morphology. The toxicity of M corresponds to its disinfectant feature. The solution's leftovers on the lenses cannot do any harm to the eye, as the solution is naturally diluted in the eye fluid. These results were confirmed with the rabbit experiment – during the experiment there was no state of lacrimation, photophobia, swelling of lids, sap and pain in the eyes, opacity of cornea, or other inflammatory signs of the eyes or the conjunctiva.

Solution K- was not toxic. The IC_{50} for K+ was 0.6 mg/ml and K+ causes the death of cells and changes their morphology.

Lecture

Minimising pain and distress in ocular safety testing: Current best practices and research needs

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Rabbit eye testing has been used effectively for over 60 years to safeguard human health by identifying and classifying chemicals and products that have the potential to cause temporary or permanent eye damage. However, pain and distress may occur in such testing from the initial application of the test substance and from subsequent chemically-induced damage to ocular tissues. Current test guidelines attempt to avoid and minimise animal pain and distress by allowing ocular hazard decisions to be made in some situations without the use of animals, and requiring only 1-3 animals in most situations where animals must be used. Current guidelines also seek to minimise pain and distress by allowing the use of pre-application treatment with topical anaesthetics. However, regulatory guidelines state that such agents should not interfere with the outcome of the study. The lack of information about potential interference has precluded the routine use of pre- and post-application analgesics and topical anaesthetics. Current guidelines also seek to reduce the duration of pain and distress by allowing for humane euthanasia of animals that develop severe ocular lesions or that exhibit severe and enduring signs of pain and distress. If identified, predictive biomarkers could serve as humane endpoints for terminating studies in order to avoid potential pain and distress. Additional research is needed to support the identification and use of humane endpoints, analgesics, and topical anaesthetics that will further minimise or eliminate pain and distress in routine ocular toxicity testing. Related recommendations from a recent ICCVAM-NICEATM-ECVAM symposium will be discussed.

Poster

Performance of the Bovine Corneal Opacity and Permeability (BCOP) test method in detecting ocular corrosives and severe irritants

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Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop *in vitro* alternatives for the current *in vivo* rabbit eye test. NICEATM evaluated four *in vitro* test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, BCOP, is an organotypic model that provides short-term maintenance of the cornea in an isolated system. The ability of BCOP to correctly and reproducibly identify ocular corrosives/severe irritants using available BCOP and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System of Classification and Labeling of Chemicals. Based on an interim analysis, BCOP appears useful (with the exception of testing alcohols, ketones, and solids) in a weight-of-evidence tiered testing strategy. Accordingly, positive results could be used to classify and label a substance, while substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of BCOP for the detection of ocular corrosives and severe irritants.

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Poster

Performance of the Isolated Rabbit Eye (IRE) test method in detecting ocular corrosives and severe irritants

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Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop *in vitro* alternatives for the current *in vivo* rabbit eye test. NICEATM evaluated four *in vitro* test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, IRE, is an organotypic model that provides short-term maintenance of the rabbit eye in an isolated system. The ability of IRE to correctly identify ocular corrosives and severe irritants using available IRE and corresponding *in vivo* rabbit eye test data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System of Classification and Labeling of Chemicals. Based on an interim analysis, IRE appears useful in a weight-of-evidence tiered testing strategy, pending corroboration of existing data with a larger database. Accordingly, positive results could be used to classify and label a substance, while substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of IRE for the detection of ocular corrosives and severe irritants.

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Poster

The use of corneas from animals of different age in the Bovine Corneal Opacity and Permeability (BCOP) assay

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Recently, the *in vitro* Bovine Corneal Opacity and Permeability (BCOP) assay was considered acceptable by the EU National Regulatory Authorities in order to identify and label severe eye irritants. In addition, the BCOP assay was part of ICCVAM's evaluation on the current status of *in vitro* test methods for detecting ocular corrosives and severe irritants.

Although bovine eyes are widely used in ocular irritancy testing, limited studies are available that address potential sources of variability. For example, in the present literature no detail is provided on the specific age of cattle used as the source of the bovine eyes. Consequently, it is recommended to perform additional studies in order to specify an age range for donor animals.

In order to evaluate the impact of age on the performance of the BCOP test, this study compared the experimental outcome when 20 chemicals were tested both on corneas from young animals (6-8 months) and adult animals (>24 months). Corneas were treated for 10 minutes followed by a 2-hour post-exposure period. Opacity and permeability were determined and the calculated *in vitro* scores for both cornea types were compared with the *in vivo* (EU and GHS) classification.

Results clearly showed that age can impact the outcome of the assay. Although no important differences in opacity were observed, permeability values assessed in calf corneas were clearly decreased (especially for alcohols) when compared with adult corneas. The possible advantages related to the use of corneas from young animals will be discussed.

Poster

Comparative study of the Chorioallantoic Membrane based test and the Red Blood Cell test as alternative to the Draize test to assay surfactants

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The ocular irritation has been studied since 1944 by the Draize *in vivo* test. This method has been very criticised due to their cruelty and the anatomical differences between the human and rabbit eyes.

Different alternative methods have been proposed to replace the Draize test but today no validated method is available. The study of the different methods is of high interest in order to find suitable methods to replace the *in vivo* test.

In the present work we have studied the potential ocular irritation of amino acid-based surfactants by the Red Blood Cell, and the Chorioallantoic Membrane (CAM) based test in order to correlate the results with the *in vivo* study. The surfactants studied were synthesised in our laboratory and compared with commercial ones.

The Red Blood Cell test gives information about the HC_{50} or concentration inducing 50% of haemolysis and the denaturation index as indicative of protein denaturation, similar to corneal irritation (Invittox protocol 37). The endpoints determined by the CAM method are the time of appearance of haemorrhage, coagulation and vasoconstriction (Invittox protocol 15) and also the determination of the amount of trypan blue adsorbed onto the membrane, to reduce the subjectivity of the procedure (Invittox protocol 108). These two last methods are more sensible than the previous and give false positives when we compare with the *in vivo* results.

The Red Blood Cell test is more specific to study the potential ocular irritation of surfactants of different type.

Poster

The concepts of systems biology to validate an *in vitro* ocular test battery: Why new Draize eye data is not relevant

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Validation has been defined as "the process of determining how well one system replicates properties of some other system." However, a predictive model can only be validated by judgement, since a model may fit past data without being predictive. The use of judgement is the critical criterion missing from current validation guidelines. Historical experience with the Draize test has shown human eye hazard can be estimated by clinical signs of chemical injury to the rabbit eye. The large variability in historical Draize data confirms the futility in using these data in rigorous *in vitro/in vivo* comparisons. Even when new animal testing has been conducted, correlative results have not fit well within 95% confidence intervals – a stringent comparator for biological assays. Ophthalmic experts have agreed that: 1) human data should be the gold standard, and 2) ocular chemical injuries can almost exclusively be evaluated as corneal responses with the corneal epithelium being the first tissue injured, and the overall degree of epithelial/corneal injury correlating well with severity and recovery. The identification of the appropriate biologically-relevant *in vitro* models, and a battery of mechanistically-based endpoints in each model that accurately assesses the degree of injury from different chemical and product classes will provide predictive data for human hazard assessment. This "perfect battery" will not pass the scrutiny of current validation criteria without the concurrent use of "judgement." The concepts of systems biology will be used to explain how to identify and validate this test battery.

Poster

Evaluation of the effects on viability and barrier function prolonged surfactant exposure has on a human corneal epithelial cell line

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In this study we aim to generate an *in vitro* model to investigate chronic corneal damage. Initially, the viability and barrier function of SV40 human corneal epithelial cells (donated by Dr Araki-Sasaki, Japan, J-HCET) were monitored during prolonged exposure to subcytotoxic concentrations of representative surfactants.

J-HCET were grown to confluency (n=4) on 24 well plate culture inserts in a defined media containing subcytotoxic concentrations of tween 20 (T20: 25 µgml-1), sodium dodecyl sulphate (SDS: 4 µgml-1), benzalkonium chloride (BAK: 0.0025 µgml-1) or cocamidopropylbetaine (CAPB: 3 µgml-1). Cell viability and barrier function was assayed repeatedly from prior to chronic exposure and subsequently at 72 hour intervals over 504 hours, using the combined resazurin/fluorescein leakage assay. Chronic exposure effects were determined and compared statistically (repeat measures ANOVA) with non- chronically exposed cultures. Total cell number was assessed by the Kenacid Blue protein assay. The location of adhesion molecules Zonula Occludins 1 (ZO-1) and E-Cadherin were assessed using immunostaining.

At all time-points the level of fluorescein leakage across the J-HCET monolayer, the production of resorufin (µgm-1) and the total protein content (µgml-1) were unaltered in surfactant exposed cultures compared to the unexposed control. ZO-1 localisation was disrupted in BAK pre-exposed cultures, whilst E-Cadherin expression remained unaltered during exposure to all test surfactants. Modulation of J-HCET viability and barrier function was not detected compared to control cultures. Assessment of ZO-1 and E-cadherin expression indicted surfactant specific effects. This work was funded by the sponsors of the FRAME research programme.

Poster

A tissue engineered human corneal model for the prediction of ocular irritation

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Introduction: Over the last 60 years the Draize rabbit eye test has been used in regulatory safety testing. Apart from ethical considerations, this test has often been criticised for its lack of objectivity, reproducibility and for over-predicting human responses. Despite intensive efforts to replace the Draize test by alternative methods, acute local eye irritation is still tested on animals, because existing non-animal methods are considered to be only suitable for the prediction of severe lesions. Since organotypic models are supposed to give rise to several advantages, it was the aim of our study to develop a cornea equivalent model composed of immortalised cell types derived from the natural human tissue.

Methods: Human corneal keratocytes were immortalised via SV40-transfection. The cytotoxic response towards different surfactants was measured by MTT test. Cornea equivalents were constructed in cell culture inserts using immortalised human epithelial and endothelial cells and keratocytes embedded in collagen. For morphological estimation sections were stained and analysed by light microscopy.

Results and discussion: We established a new human keratocyte cell line with cytotoxic sensitivities towards different surfactants comparable to primary keratocytes. Therefore, the new cell line represents an appropriate model for the prediction of keratocyte-specific toxicity. A stromal matrix, built up with these cells, displayed morphological accordance to the natural human stroma and serves as a biomatrix for corneal epithelial and endothelial cells. Consequently, we report a case of construction of a whole corneal equivalent from immortalised cells. Application of this model may be useful in regulatory eye irritation testing.