Workshop 5.11
Mechanisms of chemically-induced ocular injury and recovery: Current understanding and research needs

Poster

The assessment of the oral irritation potency of dentifrices with and without sodium lauryl sulphate as evaluated with the Slug Mucosal Irritation assay

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Ingredients such as sodium lauryl sulfate often used in dentifrices can induce oral irritation. In this study the mucosal irritation potency of dentifrices with and without SLS was assessed using the Slug Mucosal Irritation test. The concentration-response effect of 5 OTC dentifrices (A, B: containing no SLS; C, D, E: containing SLS) on the mucosal tissue was evaluated by placing the slugs two times for 60 min each on the diluted dentifrice (1%, 3%, 10% and 30% w/v in PBS). The mucus produced during each 60 min treatment is a measure for irritation. After the 60 min treatments, the protein and enzyme release (LDH, ALP) from the slug mucosa was measured. Concentrations up to 10% of dentifrice A and B resulted in a mucus production (< 2%) and protein release (<50 µg/ml.g) that was comparable with the negative controls (PBS). However, the 30% dilutions resulted in significantly increased mucus production. Formulation B induced an increased protein and LDH release whereas formulation A induced no tissue damage. The SLS (C, D, E) containing dentifrices induced an increased mucus production already at 3% dilutions. Higher concentrations induced mild to moderate tissue damage as was detected by the increased protein (>100 µg/ml.g) and LDH release (>1 U/l.g). None of the dentifrices induced ALP release. According to the SMI assay the following rank order of increasing irritation potency was established: A<B<<C~D<E. These results are consistent with other *in vitro* data and confirm clinical inflammatory effects of SLS in oral care products reported in the literature.

Lecture

Ocular toxicology *in vitro* – cell based assays

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Background and aims: Interactions between the three cell types in the cornea control cell differentiation and responses to stimuli. We have sequentially added cell types in a 3-dimensional construct to assess the minimal requirements for a representative model of the human cornea to be used in toxicology tests.

Methods: We used single applications of toxicants from 3 different classes on cell cultures in defined medium. For a number of human corneal epithelial cell lines we assessed whether stratification modifies responses to toxicants. Primary corneal stromal cells were grown in collagen gels, keeping activation to a minimum. We assessed the influence of three-dimensional co-culture of the two cell types on cell differentiation, cytokine production and recovery from exposure to the chosen toxicants. This process was repeated after the addition of an endothelial layer.

Results: Stratification of epithelium, compared to monolayer cultures, did not modify responses to toxicants probed by classical toxicology assays. Co-cultured cell types displayed patterns of cytokines different from the single cell-type 3D models, suggesting interactions between the different cells of the construct. Following exposure to toxicants there were marked changes in cytokine profiles, that could be related to the toxicant used. These changes were, however, markedly influenced by the epithelial cell-line used.

Conclusions: To rationalise the choice of cell lines for complex corneal constructs, their steady-state immune signal molecule patterns should be compared to those observed in normal human preocular fluid.

Supported by Colipa.

Lecture

Can toxicogenomics be used to identify chemicals which cause ocular injury?

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Chemical injury to the eye can be severe, moderate or mild dependent on the type of chemical (e.g. acid, alkali, surfactant), concentration, duration of exposure and the ability of the eye to repair itself. Severe chemical injuries to the cornea can result in tissue coagulation, degradation of the stroma, acute inflammation, angiogenesis, fibrosis, and recurrent corneal ulcers. By contrast, mild chemical injury can present as irritation, pain, inflammation, red eye and cell loss, which usually subsides following treatment and tissue repair. The Draize test in rabbits is currently the "gold standard" for identifying ocular hazards associated with chemicals and a variety of household formulations (e.g. cleaners and cosmetics). However, political necessity and public concern require an alternative to animal testing. One option is the toxicogenomic approach, whereby a gene fingerprint directory can be used to identify mild/moderate chemical preparations, which are toxic to the eye. This can be achieved by gene expression profiling of bioengineered human corneas using microarray analysis. Proof of principle is confirmed by exposure to generic chemicals/preparations with well documented ocular injury characteristics. Clustering and pathway analysis of the gene profiles will lead to the development of diagnostic arrays to identify key genes/pathways differentially regulated by various chemicals. The diagnostic arrays can then be used for high throughput testing of new chemical preparations. Thus, this two-pronged approach; the combination of human bio-engineered corneas and microarray analysis will provide a cheap, effective and rigorous alternative to the Draize test.

Poster

Evaluating the eye irritancy of solvents in a simple fragrance mixture with the Bovine Corneal Opacity and Permeability (BCOP) assay

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Fragrances are complex mixtures used in many consumer products. Organic solvents, such as ethanol, are major components of fragrance formulations functioning mainly as solubilisers and fragrance delivery mechanisms. The BCOP assay and primary eye irritation study (EPA-OPPTS 870.2400) were conducted using simple fragrance mixtures containing six commonly used solvents. The corneal depth of injury was assessed histologically both *in vitro* and *in vivo*. In the BCOP assay, corneas were exposed for 3 minutes, rinsed and incubated for 2, 4 and 20 hours before the opacity and permeability endpoints were assessed. Thus, the time course of lesion development was determined. The early lesions (2 and 4 hours after exposure) were compared to damage observed after 20+ hours *in vitro* and *in vivo*. *In vivo*, animals were scored at 1, 4, and 24 hours and then the eyes taken for histology. Individual solvents in both assays impacted the level of irritation of these formulations. *In vivo*, certain solvents increased the rate of lesion development but not the overall intensity or duration compared to the fragrance alone. Other solvents decreased the overall intensity and duration. The BCOP assay showed a generally similar pattern of lesion development as seen *in vivo*. Those combinations that showed opacity at 4 hours *in vivo*, showed epithelial and stromal lesion in the BCOP by 4 hours post-exposure. Fragrance alone was slower to develop opacity *in vivo* and required the 20 hour post-exposure to produce appreciable lesions *in vitro*. These data suggest that the standard post-exposure (2 hour) can be predictive of irritation potential of fragrance/solvent mixtures.

Lecture

In vitro models for ocular injury: Current and potential biomarkers

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Numerous *in vitro/ex vivo* methods for eye irritation have been developed and are currently being used within industry for specific purposes. *In vitro* model systems for eye irritation can be divided into four major categories: organotypic models, human corneal epithelium models, cell cytotoxicity assays and cell function assays. The biomarkers and mechanisms usually addressed range from simple cytotoxicity to more complex functional endpoints such as corneal light transmission and barrier functions. However, the range of criteria for injury, inflammation and reversibility covered by the Draize rabbit eye test was found to be unlikely to be replaced by a single *in vitro* test. One of the recommendations to achieve full animal replacement is to support the development of mechanistically-based models in order to address the mechanisms not currently covered by the existing assays. During the ICCVAM-NICEATM-ECVAM symposium on Mechanisms of Chemically-Induced Ocular Injury and Recovery (May 11-12, 2005), novel and existing biomarkers were identified that may allow further mechanistic insight into the ocular irritancy potential of a test substance. Discussions addressed the potential *in vitro* test systems and biomarkers that may allow adequate prediction of the mechanisms of chemically-induced ocular injury and lesion persistence *versus* reversibility. Finally, novel *in vitro* biomarkers or test systems were identified where further research and development is recommended to investigate the correlation with the *in vivo* test.

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Poster

Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay

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The BCOP assay was developed by Pierre Gautheron and Joe Sina to address ocular irritation potential of pharmaceutical intermediates and is now widely applied across industries and chemical/formulation classes. For many, if not most, of these chemical/formulation classes, the mode of action(s) of the test material is known. Membrane lysis, protein coagulation, and saponification are common modes of action that lead to ocular irritation. In our experience, the opacity and permeability endpoints (generally combined into an "*in vitro* score") have been able to identify the epithelial and stromal changes associated with this type of damage. However, chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets, that do not lead to immediate loss of cellular integrity or protein precipitation, have proven more difficult to identify without the addition of histological evaluation of the treated corneas. Histological evaluation is performed on the epithelial, stromal and endothelial layers of the cornea and can identify lesions not revealed by opacity or permeability. It also provides a direct measure of the depth of injury which Maurer et al. (2002) have shown to be predictive of the degree and duration of eye irritation. Thus, understanding the depth of injury to the treated corneas (especially relative to the injury from known benchmark materials) through histological evaluation of the bovine corneal tissue, can be crucial to interpreting the actual ocular irritation potential of novel materials or formulations. Data from reference compounds will be used to illustrate the approach.

Lecture

An overview of the COLIPA eye irritation research programme

Penny Jones [1], Dan Bagley [2], Claudine Faller [3], Beatrice Le Varlet [4], Pauline McNamee [5], Irene Manou [6], Wolfgang Pape [7], Christine Van den Berghe [8] and Freddy Van Goethem [9]

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The COLIPA eye irritation programme for the development of *in vitro* methods for eye irritation incorporates integrated research projects and collaborative activities with external partners. The integrated research projects focus on understanding mechanisms of eye injury and identification of new *in vitro* endpoints that are more predictive of the *in vivo* human response to chemical injury. This is expected to result in new or improved *in vitro* methods that would proceed to formal validation. There are three projects: 1) investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea *in vitro* can predict a chemical's potential to damage the eye with a focus on recovery; 2) identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-dimensional human conjunctival and corneal constructs and 3) a genomics project using a pattern recognition approach to identify new endpoints for injury and repair that build on corneal models being evaluated in projects 1 and 2 for potential use in current/future *in vitro* assays. Equally important to achieve validated *in vitro* methods is collaboration of industry, academia, external scientific organisations and regulators. COLIPA is working with ECVAM by actively participating in its Eye Irritation Task Force and providing support for post-hoc statistical analysis of current *in vitro* methods. The presentation describes these different projects and activities and how they are combined into the overall COLIPA strategy to address the development of *in vitro* alternatives for eye irritation.

Poster

The COLIPA *strategy* for the development of *in vitro* alternatives: Eye irritation

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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. This is in part attributed to lack of understanding of underlying physiological mechanisms of eye irritation.

To address this, COLIPA research is focused on understanding mechanisms of eye injury and identification of new *in vitro* endpoints that are more predictive of the human response to chemical injury resulting in new or improved *in vitro* methods that would proceed to formal validation. The programme has three integrated projects: 1) investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea *in vitro* can predict a chemical's potential to damage the eye with a focus on recovery; 2) identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-dimensional human conjunctival and corneal constructs and 3) a genomics project using a pattern recognition approach to identify new endpoints for injury and repair that build on corneal models being evaluated in projects 1 and 2 for potential use in current/future *in vitro* assays.

Equally important to achieve validated *in vitro* methods is collaboration of industry, academia, external scientific organisations and regulators. COLIPA is working with ECVAM by actively participating in its Eye Irritation Task Force and providing support for post-hoc statistical analysis of current *in vitro* methods.

This poster provides a detailed overview of the integrated elements of the COLIPA eye irritation programme.

Poster

The cytokine response of a wounded corneal model

Marcus Radburn-Smith and Monica Berry

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Background and aims: Interactions between epithelial cells and keratocytes maintain a healthy cornea and its ability to respond to an insult. Following chemical insults, we assessed cytokine production, as an indication of intercellular communication, on addition of a stromal construct to stratified corneal epithelia.

Methods: Immortalised human corneal epithelial cell models were built and stratified at the air-liquid interface in a fully defined medium. Models were also built with the epithelium stratified on top of a collagen gel seeded with primary human keratocytes. The stratified constructs were treated for 10 min with NaOH, sodium dodecyl sulfate and Tomadol™ 45-7 at a concentration of 0.66%. Cytokine production was assessed as well as fluorescein leakage, LDH release, protein and metabolic assays.

Results: IL-8 and IL-6 were detected in the Araki-Sasaki cell line, whilst IL-10, 8, 6 and 12p70 was produced by the USA line. The cytokine responses were different with different toxicants. The stromal constructs did not produce any measurable cytokines. In co-cultures of stroma and USA epithelium IL-8 production increased ten-fold, whilst there was a four-fold increase in IL-6. On stratified epithelia the non-ionic surfactant caused an increase in IL8, while SLS and NaOH did not. In epithelium-stroma models it is the latter which cause an increase in IL8 and trigger IL6 production, whilst the non-ionic surfactant did not.

Conclusion: Upon addition of further layers to the constructs cytokine patterns altered, implying communication between the layers.

Supported by Colipa.

Poster

Effect of environment on signaling profiles of corneal constructs

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Background and aims: Keratocytes play a major role in wound healing, transforming from a quiescent state to an activated fibroblast or myofibroblast phenotype that acts to contract the wound and release inflammatory mediators. Cytokines are such mediators, allowing interactions to occur between all cell types in the cornea. Understanding the factors that influence this communication is essential for building a bioengineered model that can be used to study corneal physiology.

Methods: Primary fibroblasts, immortalised corneal epithelia and immortalised endothelial cells were grown in either serum enhanced or serum free media. Three dimensional cultures were grown within type I collagen gels.

Results: Production of IL-6 and IL-8 decreased with time after transfer to serum-free medium. IL-12p70, TNF, IL-10 and IL-1 were not detected. Ratios of IL-6/IL-8 are different in fibroblasts grown on different substrates. Constructs were stable for 34 days in KGM with some low production of IL-6. Addition of endothelium caused slight increase in IL-6 and production of IL-8. A marked increase in both cytokines was observed after epithelial addition. Continuity or contiguity of the two cell types influence the cytokine profile. Contraction of the gel was accompanied by a striking increase in IL6 and IL8.

Conclusion: Cytokine patterns were influenced by the culture medium, the substrate and the presence and contact with other cell types.

Supported by Colipa.

Poster

Inflammation mediator detection in the *ex vivo* Eye Irritation Test (EVEIT)

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Objective: Eye irritations can be simulated during cultivation of isolated rabbit corneas in the EVEIT. We found early epithelial healing of the cornea after abrasion. Irritaton was give proof by opacificaton and epithelial defect. We examine the immunological response of the isolated cornea on specific irritations.

Methods: In an *ex vivo* culture we subjected each 8 corneas to epithelial abrasio of 34 +-3% surface, a corneal burn with 2n NaOH in (LVET) and another 8 cultured corneas served as negative control. We examined at different time points perfusion medium and supernatants on the content of IL 1 alpha and beta, IL 8 and FGF by means of ELISA technique.

Results: We found high contents of FGF 6 h after exposition towards NaOH in supernatants but not in perfusates. FGF concentrations were elevated in supernatants of corneas with epithelial abrasion at day 1 significantly. The levels of FGF in negative controls remained low. In sodiumhydroxide exposure IL 8 decreased within the medium and a rise from 280 +- 220 pg/ml to 780 +- 270 pg/ml in supernatants. In abraded corneas 290 +- 30 pg/ml rose to 375 +- 50 pg/ml *post expositionem*. Negative controls reamined stable. IL-1 alpha and beta were measured high after sodiumhydroxide expositon and less in case of corneal abrasion.

Conclusions: We conclude that the EVEIT model gives reliable biochemical reactions in irritation and recovery. The mediators released from the isolated cornea are causative for inflammation, leucocyte recruitment and ulceration. There is strong evidence that the system might replace animal experiments in future.

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Poster

Replacement of the Draize test by a new system of *ex vivo* cornea culture

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Objective: By a new construction of *ex vivo* corneal culture we try to simulate irritation, recovery and healing on cultured animal corneas to improve predictive results on chemicals for human eyes.

Methods: Rabbit corneas freshly prepared from abattoir are mounted in a perfusion system and pre-incubated for 32 hours. After this time we expose the corneas with a modified Low Volume Eye Irritation Test (LVET) or to mechanical abrasion. We monitor the vitality of the corneas by means of continuous glucose lactate measurements in medium and supernatants, microscopic and macroscopic examination of the erosion, endothelial damage and opacification.

Results: Each 16 corneas were exposed to abrasion, no touch or 2n NaOH for 20 sec. Corneas without any touch showed stable epithelium with small rough zones of 2 +- 2% of fluorescein positive staining. Defined corneal abrasions of 34 +- 3% healed within 5 days completely and expositons to sodium hydroxid resulted in persistent corneal erosion of 45 +- 12%. All 36 corneas showed considerable consumption of glucose and production of lactate. The supernatants showed less lactate in case of epithelial damage.

Conclusions: With the presented system we are able to simulate the two main criteria of eye irritation in animal experiments, the acute damage and its regeneration or healing. The system is close to the natural cornea and is ophthalmological evaluated and proofed to replace eye irritation in animals. Additional parameters of tissue repair and inflammation are available in the sampled media.

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Lecture

Predictive eye irritation test ex *vivo* system on rabbit corneas

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The rabbit cornea is the mostly spread *in vivo* test system used in Draize test but also in ophthalmological experiments. Thereby the rabbit eye has been well described and parallel diseases and features especially in eye burns and irritation been found to be predictive for humans. This was the reason for us to search for an *ex vivo* rabbit eye irritation test. We derived knowledge from cornea banking and storage and built an *ex vivo* system for rabbit corneas. In several experiments we were able to maintain the cornea for more than 20 days in culture exposed to air with the epithelium. Perfusatees of an artificial anterior chamber and supernatant fluids were incubated and analysed on Glucose and lactate and pH and proinflammatory factors and growth factors. We were able to demonstrate stability and healing of epithelium of the cornea and found differential healing dependent on the amount and severity of irritative substances.

We found differential images of reactions concerning healing, expression of VEGF triggered by mechanical abrasion and hydrogen peroxide exposure as well as IL 8 releas in early phase of exposure.

We believe that the *ex vivo* rabbit cornea test provides the possibility to replace Draize test by an as close to nature system as possible based on the huge amount of existing data on humans and rabbits.

Lecture

In vivo models of ocular injury and recovery: Current and potential biomarkers to support development and validation of predictive *in vitro* models

Bill Stokes [1], Wiley Chambers [2], Meta Bonner [3], David Allen [4], Raymond Tice [4], Neepa Choksi [4], Chantra Eskes [5], Jill Merrill [2] and Leonard Schechtman [2]

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Significant efforts have been made during the past 25 years to develop and validate *in vitro* test methods to replace the rabbit ocular irritancy and corrosivity test. Despite these efforts, there still is no scientifically valid test method or battery of test methods capable of completely replacing the *in vivo* test for the assessment of novel substances. Critical reviews of this issue by various expert groups have generally concluded that greater emphasis on mechanism-based data from *in vivo* and *in vitro* test methods is essential for future meaningful progress. However, observations in the current rabbit eye test remain unchanged since the establishment of this test over 60 years ago. These consist of visual observations and subjective numerical scores for damage to the cornea, iris, and conjunctiva. In contrast, ophthalmological assessments of human ocular injuries have evolved to routinely include slit lamp biomicroscopy, confocal microscopy, fluorescein staining, and other measures of inflammation and injury. Accordingly, experts have suggested that animal ocular tests, when such tests are necessary, should include similar assessments as part of a new set of standard observations that can be used to maximise the comparability of animal and human data. The routine use of objective quantitative endpoints and biomarkers to assess human and animal chemically-induced ocular injuries is expected to provide mechanistic insights that will support the development and validation of more predictive *in vitro* methods, and improve the accuracy and reliability of ocular hazard assessments. Related recommendations from a recent ICCVAM-NICEATM-ECVAM scientific symposium will be discussed.

Poster

Mechanisms of chemically-induced ocular injury and recovery: Current understanding and knowledge gaps

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A two-day Scientific Symposium on Mechanisms of Chemically-Induced Ocular Injury and Recovery was held in May 11-12, 2005 in the USA. The symposium was organised and sponsored by NICEATM, ICCVAM and ECVAM, with additional support from COLIPA. A major goal of the symposium was to identify research needed to advance the development of test systems necessary to meet regulatory testing requirements that provide for human health protection while reducing, refining (less pain and distress), and/or replacing the use of animals. Three consecutive talks will summarise the symposium discussions. After a brief overview of the symposium, this talk will focus on the part of the meeting dealing with issues related to the present understanding of currently known mechanisms and modes of action of chemical-related ocular injury, persistence and recovery. Areas pertinent to this theme included injury type, ocular cellular and tissue responses to chemical injury in humans and animals, and the role of histopathology and depth of injury in evaluating ocular injury onset, extent, severity, and recovery potential. A summary of expert speaker and panel discussions will be presented on these topics and additional aspects, such as, the relevance of species, dose, and toxicokinetics to a better understanding of chemical-induced ocular injury-related mechanism and response, and, a possible future role for toxicogenomics in elucidating processes involved in the characterisation of ocular injury and *sequelae*. Knowledge gaps and areas identified for further investigation at the symposium will be highlighted.

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Poster

Comparative *in vitro* cytotoxicity of lens care products with three cell lines and two assay methods

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Purpose: Compare cytotoxicity potential of contact lens care products (LCP) according to the Neutral Red Uptake and Release assay (NRUR), using immortalised human corneal epithelial (HCE-T) and murine fibroblastic cells (L929), with cytotoxicity using the Fluorescein Leakage assay with Madin-Darby canine kidney cells (MDCK).

Methods: NRUR assay: Serially dilute SOLOcare® AQUA and OPTI-FREE® Express® with Aldox™ solutions on 96 well plates to final test concentrations of 12.5%, 25 % and 50% for the NRUR assay for 24 hours cell exposure. Positive control: 10 ppm BAC. Negative control: DPBS. MDCK assay: MDCK inserts exposed to neat solutions for 30 min and observed post 24 hour recovery for fluorescein leakage. Negative control: HBSS. Positive control: 300 µg/mL SDS.

Results: SOLOcare® AQUA was non-cytotoxic at all concentrations with both cell lines for the NRUR and for the MDCK assay. The following solutions were considered cytotoxic in comparison to the negative control for the NRUR assay: OPTI-FREE® Express® (50 & 25%) and BAC. The following solutions were significantly different in comparison to the negative control using the MDCK assay: OPTI-FREE® Express® and SDS. Cytotoxicity was determined by an ED5024 for NRUR.

Conclusions: The cytotoxicity results for LCPs determined using the NRUR assay are similar to results obtained using the MDCK fluorescein leakage assay. The LCP cytotoxic by the NRUR assay was cytotoxic by the MDCK assay and the LCP noncytotoxic by the NRUR assay was noncytotoxic by the MDCK assay. Both of these assays help differentiate subtle differences in LCPs.