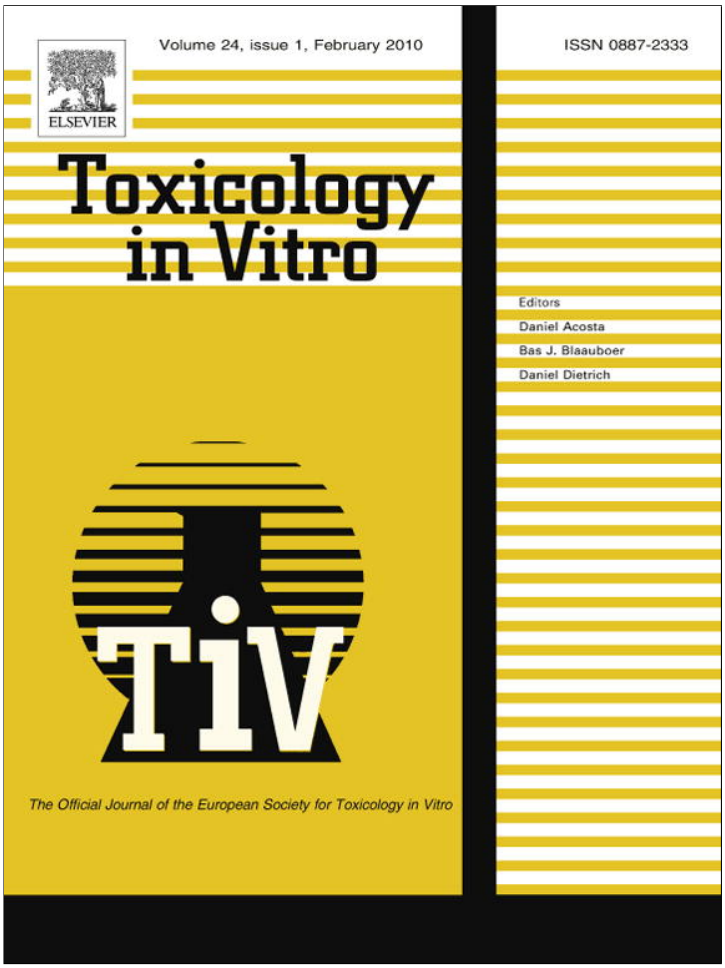

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Occludin gene expression as an early *in vitro* sign for mild eye irritation assessment

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ABSTRACT

Purpose: To test a new multiple endpoint analysis (MEA) including occludin gene expression for screening the ocular irritation potential of tear substitutes on human corneal epithelium (HCE), an *in vitro* model proposed to limit the use of animal testing in pre-clinical studies.
Methods: Four chemically-preserved and two non chemically-preserved tear substitutes were tested after acute (24 h, 24 h + 24 h post incubation) and repeated applications (for 72 h) and compared to the positive control, benzalkonium chloride (BAK) at 0.1% and 0.01%, by assessing complementary parameters. Cellular viability was evaluated using MTT, histomorphologic analysis was performed on H&E stained vertical sections, IL-8 release was measured by ELISA, and occludin gene expression was quantified using qRT-PCR.
Results: Cellular viability was moderately reduced by Perborate and Polyquad-preserved tear substitutes and dramatically reduced by BAK and by Thiomersal® and Oxyd® preserved tear substitutes. Thiomersal® also increased IL-8 release. Occludin expression profiles were modified by the four chemically-preserved tear substitutes and by the mechanically-preserved Comod®, but not by the mechanically-preserved Abak®. The behavior of BAK and tear substitutes led us to propose a prediction model for the classification of different levels of irritants, mainly based on the occludin transcriptional study.
Conclusion: The versatility and sensitivity of the HCE model allowed the modeling of cumulative effects that may approach conditions obtained after long term application of tear substitutes. Thus, the modified MEA proposed in this study represents a valuable tool for *in vitro* eye irritation assessment with the power to detect mild irritants and subclinical eye irritant potential.

1. Introduction

The conventional method used to assess ocular irritation potential (acute ocular toxicity) is the rabbit eye test (Draize test OECD TG 405, 2; EC B.5,3) (Draize et al., 1944). The Draize test has multiple applications: it is used for the hazard identification of severe irritant and corrosive substances (EU classification R41), for testing moderate eye irritants (EU classification R36). The Draize is used also for the safety assessment of ophthalmic formulations, on the contrary it is no longer accepted for testing cosmetics. However this test has limitations including subjectivity of scores, very low inter-laboratory reproducibility (Weil and Scala, 1971). and the pain imposed on animals especially in repeated application protocols. Within the framework of the EU commitment to promote the reduction of the use of animals in pre-clinical studies, in April 2007, the ESAC (Scientific Advisory Committee of ECVAM-European Centre for the Validation of Alternative Methods (to animal experimentation)) endorsed the scientific validity of two alternative tests: the bovine corneal opacity permeability (BCOP) test and the isolated chicken eye (ICE) test (ESAC 2007; SCCP/111/07). In the EU and the USA, these methods may replace the use of animals to identify severe irritants as a screening assay in a tiered approach, although some animal testing will still be required for mild irritants (Balls et al., 1995; Eskes et al., 2005).

The assessment of ocular tolerance is particularly important for ophthalmologic multi-dose formulations intended for long-term applications (i.e. chronic diseases) and for personal care products with modifications to previous ingredients, concentration, technical form or mode of application. Such products often lack early clinical signs of irritation and are defined as non irritants but may determine irritation when applied repeatedly for long periods. Chemically-preserved eye drops, for example, can induce ocular surface inflammation after long-term use as demonstrated by clinical, experimental and *in vitro* studies (Pauly et al., 2007; Guenoun

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et al., 2005a,b; Pisella et al., 2000; De Saint Jean et al., 1999; Baudouin et al., 2004, 2005). In particular, the preservative benzalkonium chloride (BAK) has been demonstrated to decrease cell viability and enhance apoptotic phenomena or oxidative effects (De Saint Jean et al., 1999). The assessment of eye compatibility as opposed to eye irritation requires the determination of early biological endpoints in a relevant biological model and the adaptation of protocols and testing parameters to the type of product. In this context, thanks to modern techniques and considering that toxicogenomic responses *in vitro* and *in vivo* are comparable (Boverhof and Zacharewsky, 2006; Daston, 2007), it is possible to have access to a vastly increased amounts of biological information .Gene expression signatures might be indicative of the start of a toxic responses, are characteristic of chemical modes of action, and in some cases, they can be causally related if there is knowledge of mechanism and serve the diagnosis of pathologies.

In the research of alternatives to eye irritation testing is crucial to take into account the role of corneal epithelium as the first line of defence against many types of injury, trauma and infection and because it contributes to the maintenance of corneal transparency and rigidity. *In vitro* 3D models of corneal epithelium, based on air-lift technology, have been developed in the last ten years (Nguyen et al., 2003; Powers et al., 2007; Van Goethem et al., 2006). These models may support basic research investigation; they are versatile for the set-up of modified protocols and allow objective and reproducible quantification of complementary testing parameters. Compared to *in vivo* studies on animals, which are time-consuming, often invasive and may lack suitable sensitive tools for detecting infra-clinical reactions, the *in vitro* tissue is an easy-to-handle model of human origin that more closely resembles human epithelial physiology than conventional monolayer models. Despite having a strong morphological similarity, *in vitro* tissue however has increased permeability compared to the *in vivo* tissue (Reichl, 2008; Becker et al., 2008) but this could be considered as a further advantage in designing an experimental model with an increased sensitivity, allowing early detection of the effect of sub-toxic doses. An important application of one of these *in vitro* reconstituted tissues, the human corneal epithelium (HCE) model, is the assessment of non classified products; the use of MTT test for the assessment of cellular viability has been proposed as a major endpoint. In the testing of finished personal care products, a linear correlation has been demonstrated between *in vivo* (MMAS Draize test scores) and *in vitro* data (calculated using the cell viability as a toxicity marker) (Doucet et al., 1999, 2006). However, when used as a single endpoint, the MTT test has some limitations, in particular the exclusive contribution of the basal layer of the 3D construct to the cell viability results led to the underestimation of the cellular events occurring at the superficial level. To overcome this limitation, the multiple endpoints analysis (MEA) that assesses complementary parameters was proposed several years ago: the cellular viability at the basal epithelial layer was evaluated using the MTT test, while the histo-morphological analysis allowed the detection of both superficial and deeper morphological modifications and helped to confirm the biochemical investigations (Meloni et al., 2002). Finally, the release of soluble mediators such as IL-8 appeared to be a sensitive, mechanistically-based and reliable endpoint for the prediction of human eye tolerance consistent with studies showing a good correlation between clinical and *in vitro* data for mildly irritant products (Debbasch et al., 2005).Compared to human clinical data IL-8 scores were correlated with itching, burning and bulbar conjunctival redness and positively associated with increased severity of clinical signs :the results confirmed IL-8 as an useful marker for evaluation of human ocular discomfort.

In the present study, the MEA protocol was further modified by introducing two additional testing procedures in order to enlarge the field of application to a wide range of formulations and to increase the test's power to discriminate between products. In addition to the classical 24 h acute exposure, a 24 h exposure followed by a post-incubation time as well as a repeated application procedure for 72 h were used. Furthermore, reconstituted HCE tissues compared to conventional cell culture offered the opportunity to investigate early and superficial modifications induced on structural components of the epithelial surface barrier. The most apical part of the lateral membrane in the superficial epithelial cells contains the junctional complex, including tight junctions which thus directly contribute to the first line of defence of cornea. Tight junctions regulate passive movement of fluid, electrolytes, macromolecules and cells through the paracellular pathway. In the present study, the role of occludin, a 60-kDa tetraspan membrane protein associated to tight junctions, has been particularly focused because it appears to play a regulatory rather than a structural function in tight junctions and could be an early marker of physical disorder and damage (McCarthy et al., 1996; Matter and Balda, 2007; Schneeberger and Lynch, 2004; Ajani et al., 2007; Ban et al., 2003). It has been already showed (Pauly et al., 2009) the dose-dependent effects of BAK on its gene expression, suggesting that occludin may be an early and predictive marker of sub-toxic doses and could predict the intensity of tissue damage and recovery. In the present study, the objective was to validate the relevant markers and exposure protocols of the modified MEA for screening the eye irritation potential of commercially available multi-dose tear substitutes *in vitro* using the HCE model in controlled and reproducible experimental conditions. We hypothesized that the detection of the barrier structure modification using occludin mRNA expression by quantitative RT-PCR (Taqman® technology), in conjunction with the measurement of IL-8 release, the classical evaluation of cell viability by the MTT test and the histological analyses, would allow identification of sub-toxic doses and early damage to the corneal epithelium, thus helping to predict infra-clinical reactions at the corneal epithelium level.

2. Material and methods

2.1. Biological model

The reconstructed human corneal epithelium model (HCE) supplied by SkinEthic® Laboratories (Nice, France) consisted of immortalized human corneal epithelial cells cultured on an inert, permeable polycarbonate filter of 0.5 cm^2 for 5 days at the air liquid interface in a supplemented chemically-defined medium (modified MCDB 153). The overall morphology of HCE model is similar to that of the human corneal epithelium with a layer of non keratinized superficial cells flattened. At the intermediate cell layer the cells displayed more lateral cytoplasmatic extension than those in the basal layer, similar to the wing cells. The basal layer presents regular column cell. At the ultrastructure level the basal membrane reveals mature hemidesmosomes and associated anchoring filaments that form *in vivo* the complex for the attachment of the epithelium to the stroma.The resulting 3D construct showed the morphology of the stratified cellular organization of HCE and has been characterized for different relevant markers (Nguyen et al., 2003). The HCE were shipped on day 5: upon arrival they were aseptically removed and placed in a 6-well culture plate (Falcon) with 1 ml of chemically-defined maintenance medium supplied by SkinEthic which was changed every 24 h. Different batches of HCE were used with an average thickness of 70 μm as reported in the quality data sheet of each batch. The variability of positive controls (BAK at 0.1% and 0.01% and SDS 0.25%) in different batches over 5 years of use in the laboratory did not exceed 15% in terms of cell viability measured by MTT test.

2.2. Products, controls and treatments

The test items were the routine long-term treatments for dry eye symptoms, mainly based on hyaluronic acid, and containing active ingredients of different origins (botanical, biotechnology, and chemical). These products can be considered as potentially non toxic and may constitute a good model to study delayed toxicity reactions. Tear substitutes contain different categories of chemical and physical preservative systems (Table 1). Comod® and Abak® are multi-dose containers for eye drops which make the addition of chemical preservatives unnecessary. The preservation is mechanically achieved by an airless pump system (Comod®) or by a 0.2 μm filter (Abak®).

As a negative control, saline solution was chosen for its neutral effect on the epithelium surface. In order to validate the experimental approach, the reference standard benzalkonium chloride (BAK) was tested as a positive control. Two concentrations were tested on the basis of previous internal data on HCE: 0.01% – defined as a non toxic dose by the MTT test after 24 h exposure; and 0.1% – definitely cytotoxic after acute 24 h exposure. The test items (30 μl) undiluted were directly applied to, and gently spread over, the whole epithelium surface. The test was performed on duplicate cultures for MTT and IL-8 quantification, single cultures for histology and duplicate cultures for the gene expression study with the following treatment procedures:

- 24 h acute application in incubator at 37 °C with 5% of CO_2 followed by a gentle wash with Phosphate Buffer Saline solution (PBS) (3 times) – standard exposure for *in vitro* toxicological studies.
- 24 h acute application followed by a gentle PBS wash (3 times) and by 24 post incubation in incubator at 37 °C with 5% of CO_2 – to take into account the recovery and to increase the discriminating power.
- 72 h by performing twice daily applications of the product for 3 days in order to evaluate the cumulative effect. The PBS wash was only performed at the end of the experiment. The test has been done keeping the HCE in incubator at 37 °C with 5% of CO_2.

2.3. MTT test

The Mossman[10] modified test was performed on HCEs (duplicate) following different exposure times: HCEs were rinsed 3 times with PBS solution and transferred to 24-well plates containing 300 μl of MTT solution (diluted at 0.5 mg/ml in maintenance medium). After 3 h of incubation at 37 °C, the HCE were placed for 1 h at room temperature in a 6-well plate containing 1 ml of isopropanol for extraction of MTT crystals and optical density was measured at 570 nm (Microplate Autoreader Infinite® M-200, Tecan). Results were expressed as percentages of viability compared to negative control (CN). The Accuracy of the spectro-photometrical measurements ranged from 0 to 2 OD (optical density) : < ± (1% + 10 m OD).

2.4. ELISA assay

The medium of the HCE used for the MTT test was collected immediately after the end of the exposure or after the post-incubation time and conserved at −20 °C in plastic cryovials. Before the test, the medium was stored at room temperature. The release of IL-8 was quantified in 200 μl of HCE medium, at the same recovery times as the MTT test, by specific high sensitivity enzyme-linked immunosorbent essay (ELISA) (Kit Quantikine® D80 IL-8; R&D systems SPACE, Milan, Italy). The O.D. of the end products was measured at 450 nm. Results were expressed in pg/ml and then normalized to 100% of cell viability results, so as to avoid any bias due to cytotoxic effects induced by the products.

2.5. Histology

At the end of the experiments the single HCE was removed from the insert using a sharp scalpel, and fixed in 10% formalin solution (Sigma). After embedding in paraffin, vertical sections of 4 μm were cut and hematoxylin and eosin (H&E, Merck) stained following internal procedures. The histological samples were analyzed under light microscopy. The overall morphology and its modifications were analyzed and compared to the negative controls.

2.6. Transcriptional study of occludin mRNA using qRT-PCR

After each treatment timepoint, total RNA was extracted from HCE using the RNAqueous kit according to the manufacturer's protocol (Ambion-Applied Biosystems, Monza, Italy). The cDNAs were then synthesized using 2 μg of RNA template in a 20-μL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Ten microlitre RNA was added to the master mix, then subjected to reverse transcription in a thermal cycler (Applied Biosystems ABI PRISM 7500 Real Time PCR System) under the following conditions: 25 °C for 10 min, 37 °C for 60 min, 85 °C for 5 s. Real Time Polymerase Chain Reaction was then performed in triplicate in a 25 μl final reaction volume using the Applied Biosystems ABI PRISM 7500 Real Time PCR System instrument with TaqMan® assay (Applied Biosystems).The cDNA was amplified using TaqMan Universal PCR Master Mix and TaqMan gene expression assay provided as a 20× Assay mix (Human Occludin: Taqman probe OCLN Hs00170162_m1 and Human GAPDH, as the calibrator gene: Taqman probe GAPDH Hs99999905_m1). The PCR conditions were 95 °C for 10 min (AmpliTaq Gold DNA Polymerase Activation) followed by 40 amplification cycles (95 °C for 15 s; 60 °C for 1 min).

The Relative Quantification (RQ) minimum and RQ maximum define statistical boundaries for Relative Quantification, based upon a 95%-specified RQ Min/Max confidence setting. A confidence setting of 95% means that the user can expect the true RQ value to fall within the RQ Min/Max range with a 95% confidence. The RQ Min/Max is calculated using the following equation:

$$2^{-(\Delta\Delta C_{T(s,t)} \pm T \times VAB(C_{T(s,t)}))} \quad (1)$$

Table 1
Positive and negative controls and identification of multi-dose tear substitutes.

Identification code	Active ingredient	Preservation system
Negative control	NaCl 0.9%	Sterile
Positive control BAK	Benzalkonium chloride	BAK 0.01% and BAK 0.1%
Perborate	Hydropropylmethyl cellulose	KCl perborate
Polyquad®	Hydroxypropyl guar	Polydronium chloride 0.001%
Thiomersal	Sodium hyaluronate 0.2%	Thiomersal
Oxyd®	Sodium hyaluronate 0.15%	OXYD® 0.06%
Comod®	Sodium hyaluronate 0.1%	Patented device + Ag^+
Abak®	Sodium hyaluronate 0.15%	Patented device

where $\Delta\Delta C_{T(s,t)} = \Delta C_{T(s,t)} - \Delta C_{T(\text{calibrator},t)}$.

s = sample name , t = target detector, T = student's T value at the selected confident setting using a degree of freedom that is associated with the test sample $\Delta C_{T(s,t)}$, VAB is the Applied Biosystems' variability function for calculating the variability of the test sample ΔC_T statistic. Calculations of relative gene expression used relative differences in the threshold cycle C_T (the cycle of PCR at which the fluorescence reaches a given value or threshold that is in the log-linear range of amplification). Samples containing higher levels of expression of a particular gene reach the threshold value at lower cycle numbers during PCR than samples containing lower levels of expression of the same gene. Because each cycle in the PCR reaction corresponds to a 2-fold increase in PCR product, a difference of one in threshold cycle number represents a 2-fold difference in the expression of a particular gene or internal control target sequence and can be considered as significant. Duplicate cultures were analyzed and the data were represented as Relative Quantification data (RQ) ± standard deviation.

The software integrated in the Instrument within this laboratory directly set a confidence level upon 95% that allow to work in a real range of significance for biological samples.

2.7. Occludin immunolocalization on frozen sections

The HCE construct was transferred to a Petri dish containing 500 µl of PBS and cut into two pieces under the binocular glass. Each piece was embedded in OCT® (Tissue-Tek, Miles Inc., Elkhart, IN) and frozen at −80 °C. Vertical sections (10 µm thick) were cut with a cryotom (Leica CM 3050 s, Leica Microsystems AG, Wetzlar, Germany) and stored at −20 °C until staining. Sections were subjected to immunofluorescence staining with antibodies against occludin. Briefly, sections were fixed with 4% paraformaldehyde (PFA) for 5 min and then permeabilized with 0.01%-diluted Triton X100® (Sigma Chemical®) for 5 min. Then they were rinsed with 1% Bovine Serum Albumin (BSA, Sigma) diluted in PBS and incubated for 1 h with the rabbit anti-occludin IgG1 (1:50, R&D Systems Europe, Lille, France or the Mouse monoclonal clone C-3F10 (1:50 Zymed)) or its isotypic control rabbit IgG1. Then sections were rinsed twice in 1% BSA-PBS and incubated again for 1 h in the dark with Alexa 488 conjugated-goat anti-rabbit IgG at a 1:500 dilution or FITC (Invitrogen, Molecular Probes, Cergy Pontoise, France). The nuclei were labeled with propidium iodide or DAPI, mounted in Vectashield (Vector Laboratories, AbCys, Paris, France) and analyzed under a laser confocal microscope Nikon PCM 2000 or using light microscopy (LEICA DMLB + SSC DC Sony).

2.8. Statistical analyses

Statistical comparisons of controls with treatment groups for MTT and qRT-PCR data were performed using the t-test (Statview V for Windows; SAS Institute, Cary, NC, USA). Statistical analyses of IL-8 results were not performed because IL-8 concentrations were considered to be dependent on viability levels and were thus normalized to the corresponding 100% of cell viability.

3. Results

3.1. Cell viability measured by MTT test

Products inducing a reduction of cellular viability % under 50% are identified as toxic and potentially irritant for the eye.

As expected, BAK induced a dose-dependent and protocol-dependent decrease of cellular viability (Fig. 1). BAK at 0.01% was found to be non toxic after acute 24 h application, inducing a reduction of viability (43%) after the post-incubation protocol, but it was clearly toxic in the repeated application procedure. At 0.1%, BAK reduced the cell viability to zero at all exposures. Following treatment with the tear substitutes (Fig. 1), no significant toxicity was detected after acute exposure for 24 h. The 24 h exposure followed by the 24 h post-incubation period and the repeated application for 72 h allowed to differentiate between products that were identically identified as to eye irritancy after acute exposure; at 24 h + 24 h the Oxyd® decreased the cell viability to 71%. At 72 h the Perborate, the Polyquad®, the Thiomersal and the Oxyd® decreased the cellular viability to 75%, 70%, 1% and 4.5% respectively; for the other products tested, the reduction was not significant.

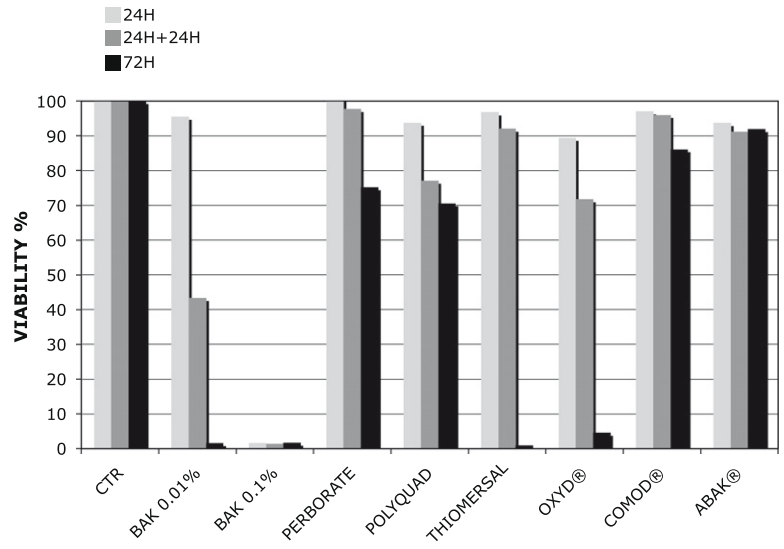


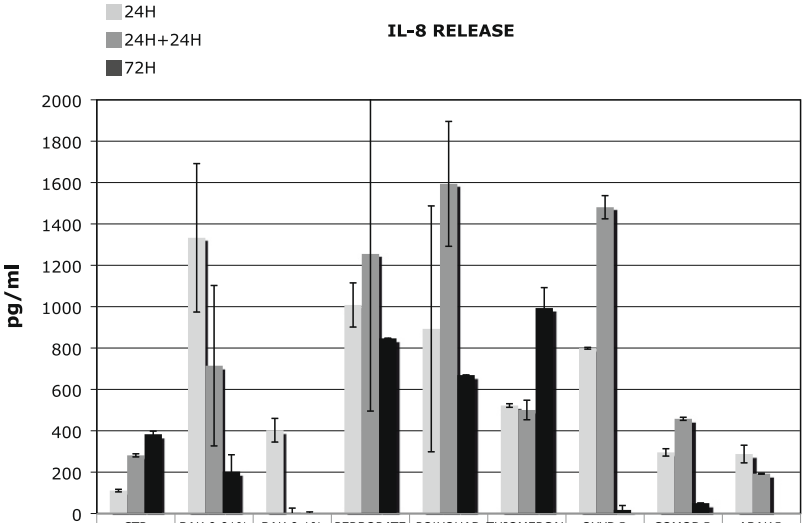
Fig. 1. Cellular viability measured by the MTT test after treatment of the 3D-HCE with saline control solution (CTR), BAK at 0.01%, BAK at 0.1% and multi-dose tear substitutes (Perborate, Polyquad®, Thiomersal, Oxyd®, Comod® and Abak®) for 24 h, 24 h followed by a 24 h post-incubation, and 72 h. BAK at 0.01% induced a 43% reduction of viability after the post-incubation protocol and was clearly toxic in the repeated application procedure. With the 0.1% concentration, cell viability was zero whatever the duration of exposure. No significant toxicity was detected after 24 h treatment with all tear substitutes tested. The 24 h exposure followed by the post-incubation period and the repeated application for 72 h allowed the differentiation between products that were classified as identical after acute exposure.

3.2. IL-8 release

Chemokine release was increased after treatment with Perborate, Polyquad® and Oxyd® with a peak induced at the 24 h timepoint followed by the post-incubation (Fig. 2A). After treatment with Thiomersal, IL-8 release reached a maximum after the repeated application procedure for 72 h. The lowest IL-8 levels were observed with Comod® and Abak®, with a range of variations similar to that of the control and comprised between 0 and 500 pg/ml. The low release of IL-8 observed for BAK 0.1% at 72 h was related to the severe cytotoxicity induced by the chemical. Because any cytotoxic effect may indirectly modify the IL-8 concentration in culture supernatants by decreasing the number of viable cells, the IL-8 release values were normalized to 100% of cellular viability as shown in Fig. 2B. BAK 0.01% and BAK 0.1% shown a different behavior, the former induces a significant release after the repeated application procedure at 72 h and the latter after 24 h. Furthermore these data underline the unique behavior of Thiomersal within the tears substitutes after the repeated application procedure for 72 h.

3.3. Histological analysis

The pictures and the comments on the overall morphology after treatment of HCE are shown in Fig. 3. BAK at 0.1% induced necrosis after only 24 h of incubation. Early superficial signs of toxicity were detected after treatment with 0.01% BAK, Polyquad®, Oxyd® and Perborate although they were not found to be cytotoxic with the MTT test at 24 h and 24 h + 24 h post incubation. At 72 h, these



	CTR	BAK 0.01%	BAK 0.1%	PERBORATE	POLYQUAD	THIOMERSAL	OXYD®	COMOD®	ABAK®
24H	0.111	1.333	0.403	1.008	0.893	0.522	0.799	0.296	0.288
24H+24H	0.281	0.715	0.003	1.255	1.594	0.501	1.481	0.458	0.193
72H	0.383	0.203	-0.033	0.847	0.669	0.993	0.017	0.050	-0.157

Fig. 2A. ELISA quantification of IL-8 release in the culture media after different durations of exposure (24 h – 24 h + 24 h – 72 h) to 0.01% and 0.1% BAK and multi-dose tear substitutes compared to control (saline solution) values. Comod® and Abak® induced IL-8 release in the range of the control. IL-8 release was increased after treatment with Perborate, Polyquad® and Oxyd® with a peak induced at 24 h + 24 h. BAK at 0.01% and Thiomersal induced the maximum release at 24 h and at 72 h respectively. BAK 0.1% induced low levels of IL-8 release, probably due to the high levels of BAK-induced cytotoxicity.

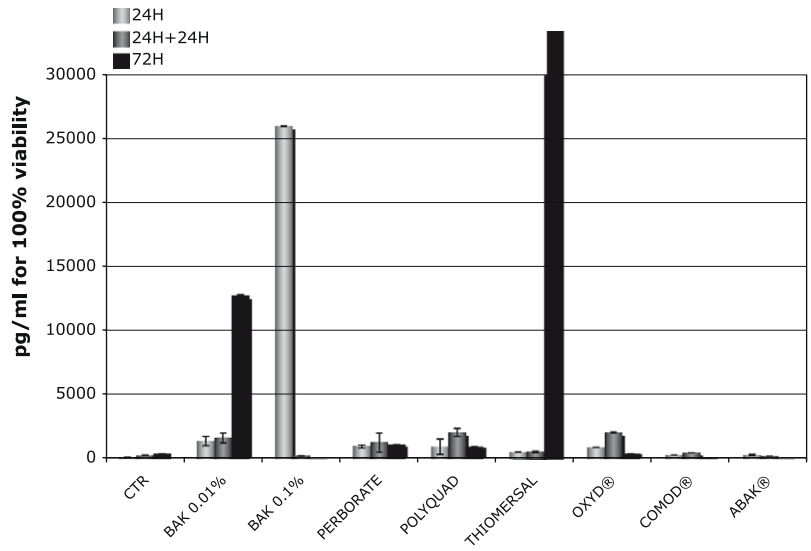


Fig. 2B. Values of IL-8 quantification at the different times after normalization to the corresponding 100% of cellular viability: the highest IL-8 levels were observed at 24 h for BAK 0.1% and at 72 h for BAK 0.01% and for Thiomersal.

four products, as well as the Thiomersal, induced epithelial damage that was consistent with the corresponding MTT results.

3.4. Transcriptional study of occludin mRNA by qRT-PCR

In the first group, Abak® induced an occludin gene expression not significantly different from that of the control at all time points tested (Fig. 4). In the second group, Perborate and Polyquad® induced a significant up-regulation at all time points even after repeated applications for 72 h. In the third group, Comod®, Oxyd® and 0.01% BAK induced a significant down-regulation of occludin gene expression at 72 h, and in some cases increased occludin gene expression at 24 h and 24 h + 24 h. In the fourth group, Thiomersal induced an up-regulation of occludin expression after 24 h exposure that was weaker after the post incubation and a highly significant down-regulation at 72 h. In the last group, the 0.1% BAK treatment induced a significant down-regulation of occludin gene expression at 24 h + 24 h and at 72 h, while increasing this expression at 24 h.

3.5. Immunofluorescence analysis of occludin distribution

At 24 h Occludin was expressed constitutively by control HCEs in almost all cellular layers but the labeling was stronger in the most superficial layers (Fig. 5). BAK at 0.01% induced the disappearance of occludin labeling in the most superficial layers but the protein was still present in the basal layers. BAK at 0.1% induced the complete disappearance of occludin labeling in all cellular layers of HCE . After 24 h of treatment with Comod®, Perborate, Polyquad® and Thiomersal, the occludin distribution was not greatly modified, the protein being mostly distributed in the superficial layers of HCE. The treatment with Abak® shows an homogenous distribution in the whole tissue. However, after treatment with Oxyd®, HCE showed a damaged, thinned epithelium, with an abnormal occludin distribution.

3.6. Eye irritation potential classification based on occludin gene expression

Based on the quantification of occludin gene expression and the different behaviors observed with tear substitutes, we proposed a model for the classification of the tears substitutes with respect to their ocular irritation potential (Table 2). We postulated that each class of products had a specific pattern of occludin gene expression with given RQ levels found at the different time points tested (24 h, 24 h + 24 h and 72 h). By convention, RQ = 1 represented an occludin gene expression not significantly different from that of the control, RQ = 2 represented any over-expression of occludin gene expression and RQ $\leqslant$ 0.5 any down-regulation of this expression, compared to the control. Five levels of increasing irritation levels within non classified products were thus defined, from level 1 corresponding to non irritant to level 5 corresponding to BAK 0.1% reported as a mild irritant (Frentz et al., 2008). Class 1 included the non irritants that did not induce any effect on occludin gene expression compared to the control calibrator (untreated tissue at the same time point). Class 2 included the extremely mild products that induced a significant occludin up-regulation at 24 h, 24 h + 24 h and/or 72 h; it is noteworthy that no occludin down-regulation was observed at any time point tested with this class. Class 3 included very mild products that induce or not an occludin up-regulation at 24 h and/or 24 h + 24 h; but consistently paired with a dramatic occludin down-regulation after repeated application for 72 h. Class 4 included slightly mild products that induced an occludin up-regulation at 24 h that decreased to RQ level = 1 at 24 h + 24 h; at 72 h, a down-regulation was observed. Finally, class 5 included mild irritants as BAK 0.1% that induced or not an occludin up-regulation at 24 h that decreased to RQ level $\leqslant$ 0.5 at 24 h + 24 h and at 72 h. Globally it appears that class 1, 2, 3 and 4 could correspond to the NC (non classified) in the EU classification and class 5 is between NC and mild irritant). In conclusion, the occludin gene expression is unaffected by non irritant NC products, increased as from extremely mild irritant NC products and the earlier and more important the decrease of occludin gene expression is observed, the more irritant the test product is.

4. Discussion

The *in vitro* assessment of eye irritation potential or eye tolerance of non classified irritants is a research topic that still needs more robust and sensitive endpoints to be assessed in relevant, reproducible, sensitive and versatile biological models. In this framework, the establishment of adapted protocols is critical in order to increase the prediction power of pre-clinical studies. The assessment of complementary parameters, defined as the multiple endpoint analysis (MEA), represents a promising approach to this need and was proposed several years ago by using a biologically relevant and sensitive 3D model of human corneal epithelium (HCE). This approach seemed to be a more useful strategy instead of investigating similar parameters in different biological models and it was shown to contribute to the prediction of the irritation potential, allowing improved classification of mild irritants (Doucet et al., 1999, 2006). Although HCE lack the lachrymal component involved in the dilution and wash-out of topically applied products, it allows us to achieve the challenging goal of modeling long-term exposure for eye irritation hazard identification.

In this study, we focused on the set-up of a new testing protocol, an approach that has often been underestimated, although it has a crucial role in assessing finished products. We also introduced quantification of the occludin mRNA expression as a supplementary biological marker for the determination of eye irritation potential. The resulting modified protocol for MEA was structured on the following three time points: 24 h, 24 h followed by product wash and by a post-incubation period, and repeated application for a continuous exposure of 72 h; thus assessing not only the acute reaction but also a possible recovery as well as mimicking the potential cumulative effects associated with long-term applications. The repeated application procedure tested for the first time on the HCE model represents a new approach for the assessment of eye irritation potential and reflects in some aspects the chronic toxicity effects on a sensitive reconstituted tissue. This protocol was applied to the study of potentially non toxic products: commercially available tear substitutes. The results reported in this study showed how this new procedure was able to better discriminate between products that initially appeared to be identical after acute application. The cellular viability results enabled us to differentiate products that did not induce toxicity at the cellular level (Comod® and Abak®) from products that induced moderate cytotoxic effects after repeated application for 72 h (Perborate and Polyquad®), and from products that had low cytotoxicity after acute exposure but caused cell death after repeated application for 72 h (Thiomersal and Oxyd®). It is noteworthy that, although the histological analysis was able to show some differences occurring in the apical part of the epithelium with the presence of several necrotic cells after an exposure for 24 h, neither the tear substitutes, nor BAK at 0.01%. This underlines the weakness of the MTT test that takes into account the effects at the basal epithelial layer only and is thus better adapted to the prediction of moderate irritants. It also showed how the 72 h repeated application increased the power of prediction when using the MTT test and correlated well with the histology at earlier time points. The IL-8 results were in line with the other results, Perborate, Polyquad®

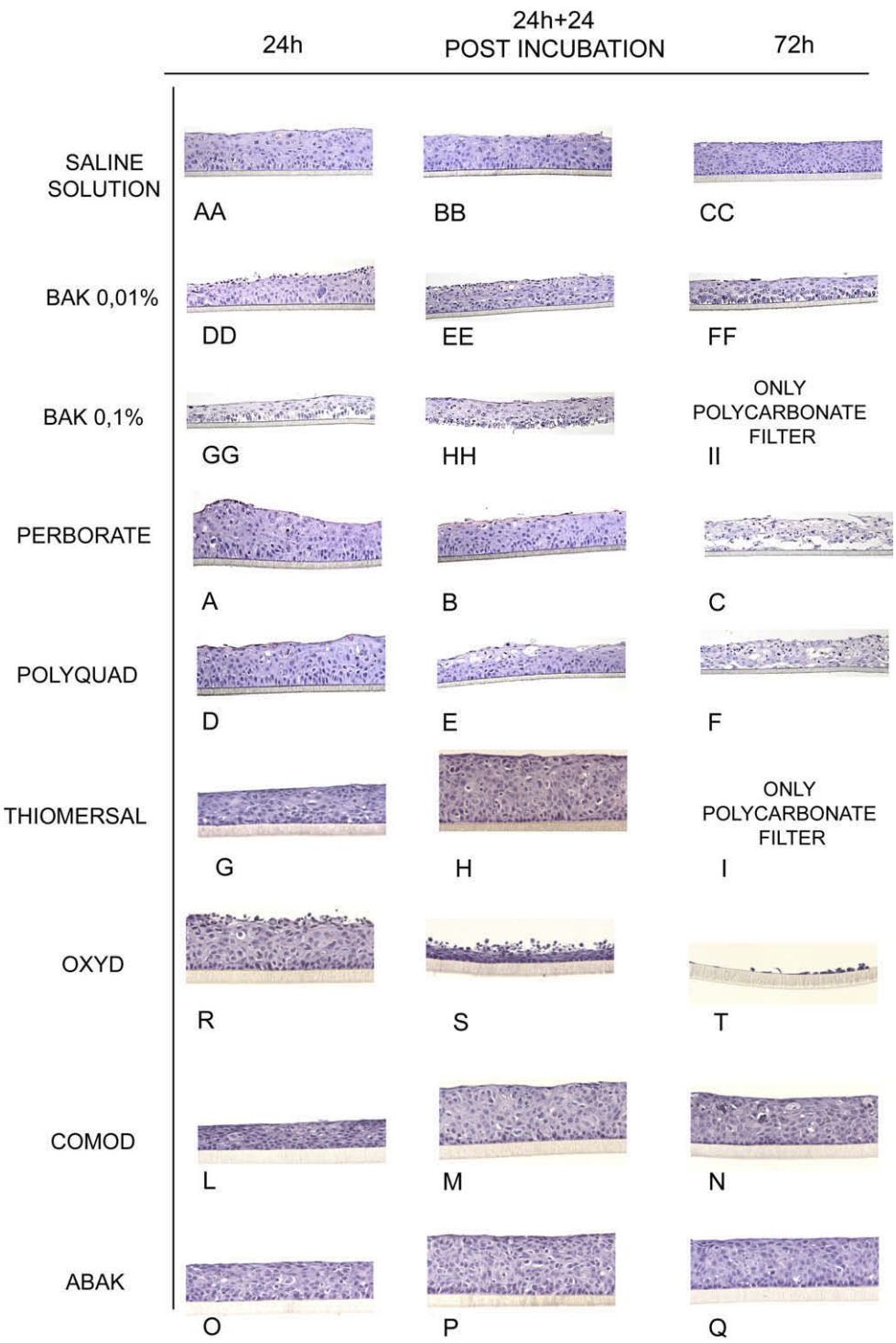


Fig. 3. Histo-morphological analysis of 3D-HCE after 24 h of treatment, 24 h of treatment followed by a 24 h post-incubation, and 72 h of treatment with saline, BAK at 0.01% and 0.1% and tear substitutes. Normal HCE morphology was observed at all the three time points considered (GG–HH–II) with the saline solution: a layer of non keratinized and flattened superficial cells; an intermediate cell layer with cells displayed lateral cytoplasmatic extension similar to the wing cells and a basal layer presenting regular column cuboidal cells was observed. After 24 h treatment, BAK at 0.01% (AA) induced modifications on the continuity of superficial film morphology with the appearance of few necrotic cells and a reduced thickness compared to the control (GG). On the contrary, at 24 h, BAK at 0.1% (DD) produced tissue necrosis, the loss of specific staining on all layers and the detachment of the tissue from the polycarbonate filter (EE–FF). The morphology after repeated application of 0.01% BAK for 72 h was severely damaged with a tissue necrosis observed at basal cells that were however still attached to the polycarbonate filter (CC). Normal morphology of HCE after treatment with multi-dose tear substitutes was observed at all time points for the products Abak® and Comod® (L–M–N–O–P–Q); superficial signs of damage with few isolated necrotic cells were observed at 24 h + 24 h with Perborate and Polyquad® (B–E) and, at 72 h, all the cell layers were deeply modified with a loss of continuity between cells and vacuolisation (C–F). Oxyd® produced early toxicity (24 h treatment) (R) and necrosis after 24 h post incubation (S). Thiomersal induced tissue necrosis only at 72 h and the residual cells were detached from the polycarbonate filter (I). Magnification 20×.

and Oxyd® inducing an increased IL-8 release with a peak at 24 h + 24 h and Thiomersal inducing significant IL-8 release after repeated application for 72 h. Other papers have already reported the use of this endpoint for the detection of early signs of toxicity (Debbasch et al., 2005).Our results confirmed the interest of using IL-8 release quantification as a complementary endpoint in MEA.

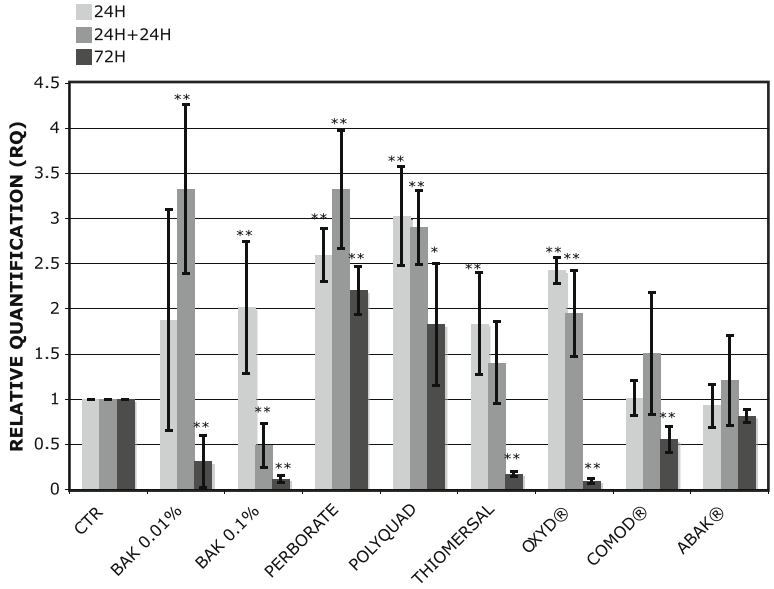


Fig. 4. Quantitative analysis of occludin mRNA expression by qRT-PCR (Taqman assay) in the control non-treated HCE and the HCE treated with tear substitutes and 0.01% and 0.1% benzalkonium chloride solutions after acute (24 h with and without the 24 h post-incubation period) and repeated application (72 h). The CTR (untreated control) for each time point was used as a calibrator. Significantly up-regulation of occludin gene expression was observed after 0.01% and 0.1% BAK treatment at 24 h. A significant up-regulation with 0.01% BAK and down-regulation with 0.1% BAK were observed at 24 h + 24 h. In the long-term treatment, the occludin was down regulated by both concentrations of BAK. The tear substitute treatments resulted in three different behaviors of occludin expression and permitted a differentiation among irritants. A two-fold change of Relative Quantification (RQ) is usually considered significant in comparison to the calibrator. A two-tailed Student's t-test was used to determine statistical significance for real-time PCR. Data were considered significant at $p < 0.05$ (*) and at $p < 0.01$ (**).

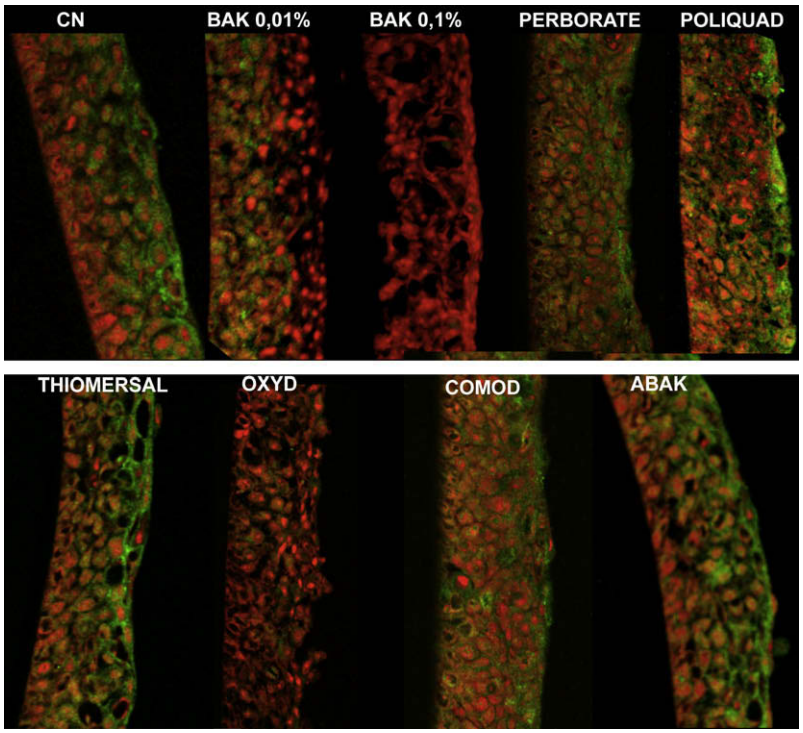


Fig. 5. Immunofluorescent analysis of HCE without treatment or following 24 h treatment with BAK and tear substitutes. Immunofluorescent analysis of the non-treated HCE showing occludin (green) mostly distributed in the superficial layers (red nuclei stained with propidium iodide) of 3D-HCE; Immunofluorescent analysis of HCE following 24 h treatment; 0.01% BAK showing a diffuse occludin staining in the basal layers and the disappearance of occludin in the most superficial layers of 3D-HCE; 0.1% BAK showing the disappearance of occludin in almost all layers of the epithelium; Perborate showing a diffuse occludin staining in the superficial layers of HCE; Polyquad® showing occludin staining mostly distributed in the superficial layers; Thiomersal showing occludin mostly distributed in the superficial layers; Oxyd® showing a damaged, thinned epithelium, with abnormal occludin distribution and a weaker protein labeling; Comod® showing a diffuse occludin staining in the superficial layers of HCE; Abak® showing an occludin staining mostly distributed in the superficial layers of HCE.

Furthermore when normalized to 100% of viability, IL-8 concentrations underlined the unique behavior of Thiomersal, which is consistent with its known sensitization effects (Mondino et al., 1982; Tosti and Tosti, 1988). IL-8 is involved in sensitization reaction also in skin (Coquette et al., 2003; Mitjans et al., 2008). Studies on occludin localization revealed that the associated tight junction

Table 2
Classification into different irritation levels according to occludin transcriptional levels.

Class	Definition	Relative Quantification by qRT-PCR		
		24 h	24 h + 24 h Post incubation	72 h
1	Non irritant	1	1	1
2	Extremely mild	2	2	2
3	Very mild	2	2	0.5
4	Slightly mild	2	1	0.5
5	Mild irritant	1	0.5	0.5

Transcriptional study data on occludin mRNA expression (qRT-PCR) for tear substitutes were used for the construction of a prediction model based on irritation levels classes. We postulated that each class of irritants had a specific pattern of occludin expression. Irritation levels were considered from level 1 to level 5 according to the occludin expression levels RQ (RQ = 1: similar to control, RQ = 2: over-expression, RQ $\leqslant$ 0.5: down-regulation) at different time points (24 h - 24 h + 24 h–72 h). Class 1 – non irritants did not induce any effect on occludin gene expression compared to the control calibrator (untreated tissue at the same time point); class 2 – extremely mild did not affect occludin gene expression or induced a significant occludin up-regulation at 24 h, 24 h + 24 h and 72 h. No occludin down-regulation was observed at any time point tested; class 3 – very mild did not modify occludin gene expression or induced an occludin up-regulation at 24 h and 24 h + 24 h. A dramatic occludin down-regulation was observed after repeated application for 72 h; class 4 slightly mild induced an occludin up-regulation at 24 h that decreased to RQ level = 1 at 24 h + 24 h. At 72 h, a down-regulation was observed; and class 5 induced an occludin up-regulation at 24 h that decreased to RQ level $\leqslant$ 0.5 at 24 h + 24 h and at 72 h.

protein tends to disappear from the apical superficial layer as a function of the toxic action. In the mouse cornea, the occludin distribution pattern has already been described as being altered by a detergent treatment (Triton X100) using immunohistochemistry (Sosnová-Netuková et al., 2007). Recently, the effects of contact lens multipurpose solutions were assessed *in vitro* on the basis of barrier function evaluation by performing fluorescein permeability assay and immunofluorescent staining for tight junctions proteins (ZO-1 and occludin) on immortalized corneal epithelial cells (Chuang et al., 2008). Recently, we showed that the immunolocalization of occludin through the HCE reconstituted tissue contributes to the overall comprehension of BAK effects on cornea and in this work (Pauly et al., 2009) we showed that occludin mRNA expression was correlated to BAK very early toxic effects (6 h exposure) that were not detected by the others techniques used. This underlined the advantage of using toxico-genomics information that is quantitative for the assessment of eye irritation potential. It was recently described that the loss of corneal epithelial barrier function during ocular inflammation may be associated to the IL-1β-induced redistribution of ZO-1 and occludin from TJs of simian virus 40-transformed human corneal epithelial cells (Kimura et al., 2009). These morphological and functional studies highlight the interest of occludin study as a protein involved in toxic effects but they cannot be deemed as a discriminating tool for a refined eye irritation assessment. By introducing a molecular endpoint such as the mRNA expression of occludin into the MEA, we added an early, sensitive and quantitative marker, able to reveal significant effects before any modification of cell viability or any cytokine release could be observed. Interestingly, effects on occludin gene expression were obtained for substances that were not expected to be irritants, which is particularly interesting for the prediction of the long-term effects of sub-toxic doses or ophthalmic formulations. Such early signs of toxicity should not be neglected, especially when considering the treatment of suffering, reactive ocular surfaces found in dry eye patients. We also demonstrated time-dependent occludin expression profiles that could help for product classification: using different time exposures, it was possible to distinguish between different levels of irritation. We hypothesized that the occludin gene expression was a biomarker of irritation, with an up-regulation of occludin expression (RQ = 2) observed for all irritants even if extremely mild, eventually followed by the decrease of this over-expression ($1 \leqslant RQ < 2$) and/or the down-regulation of the gene expression (RQ < 0.5) for stronger irritants. The stronger the irritant, the earlier the down-regulation occurred reflecting the degradation of the tissue. On the contrary, no down-regulation was observed with the non and extremely mild irritants because they always enabled the tissue to recover. The up-regulation of the occludin gene expression appears to be associated with the early signs of cell defense and recovery potential. It is worthwhile to underline that in the case of very damaged or necrotic tissues, the observed occludin gene expression decrease is probably directly associated to the tissue exfoliation. Among the endpoints used in the new MEA, extremely mild irritant potential can be predicted only by the occludin mRNA endpoint increase. By using the proposed prediction model, the tear substitutes could be easily ranked as follows: BAK 0.1% (mild irritant), Thiomersal (slightly mild), 0.01% BAK, Oxyd® and Comod® (very mild), Perborate and Polyquad® (extremely mild) and Abak® (non irritant).

The occludin mRNA quantification demonstrated its relevance in detecting early corneal epithelial modifications and its usefulness in discriminating extremely mild to mild irritants, in conjunction with the evaluation of cellular viability, the histological analysis and the quantification of IL-8 release. Severe irritants and corrosives (R41) were not assessed in this study because validated replacement alternatives already exist for their hazard identification (i.e. BCOP and ICE). In future studies, it would be interesting to test more ingredients and products with a low eye irritation potential to better characterize the discrimination power of the proposed model based on occludin gene expression. The HCE model succeeded in discriminating irritant from non classified chemicals in a prevalidation study based on MTT endpoint and the occludin gene expression might be useful to extend the applicability domain of the human reconstituted tissues in a tiered testing for eye irritation. We previously showed that a very early analysis time point at 6 h allows to increase the sensitivity of the proposed prediction model (Pauly et al., 2009) and the limitations of the classification into different irritation levels according to occludin transcriptional levels will be explored in further studies. The morphological relevance and sensitivity of the HCE model allowed the modeling of cumulative effects that may approach conditions obtained after long term application of tear substitutes. Thus, the modified MEA proposed in this study represents a valuable and promising tool for *in vitro* eye irritation assessment with the power to detect mild irritants and subclinical eye irritant potential.

Conflict of interest

The authors state no conflict of interest.

References

Ajani, G., Sato, N., Mack, J.A., Maytin, E.V., 2007. Cellular responses to disruption of the permeability barrier in a three-dimensional organotypic epidermal model. Experimental Cell Research 313, 3005–3015.

Balls, M., Botham, P.A., Brunner, L.H., Spielmann, H., 1995. The EC/HO international validation study on alternatives to Draize eye irritation test. Toxicology In Vitro 9, 871–929.

Ban, Y., Cooper, L.J., Fullwood, N.J., Nakamura, T., Tsuzuki, M., Koizumi, N., Dota, A., Mochida, C., Kinoshita, S., 2003. Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting. Experimental Eye Research 76, 735–743.

Baudouin, C., Hamard, P., Liang, H., Creuzot-Garcher, C., Bensoussan, L., Brignole, F., 2004. Conjunctival epithelial cell expression of interleukins and inflammatory markers in glaucoma patients treated over the long term. Ophthalmology 111 (12), 2186–2192.

Baudouin, C., Liang, H., Bremond-Gignac, D., Hamard, P., Hreiche, R., Creuzot-Garcher, C., Warnet, J.M., Brignole-Baudouin, F., 2005. CCR 4 and CCR 5 expression in conjunctival specimens as differential markers of T(H)1/T(H)2 in ocular surface disorders. Journal of Allergy and Clinical Immunology 116 (3), 614–619.

Becker, U., Ehrhardt, C., Schneider, M., Muys, L., Gross, D., Eschmann, K., Schaefer, U.F., Lehr, C.M., 2008. A comparative evaluation of corneal epithelial cell cultures for assessing ocular permeability. Alternatives to Laboratory Animals 36, 33–44.

Boverhof, D.R., Zacharewsky, T.R., 2006. Toxicogenomics in risk assessment: applications and needs. Toxicological Sciences 89 (2), 352–360.

Chuang, E.Y., Li, D.Q., Bian, F., Zheng, X., Pflugfelder, S.C., 2008. Effects of contact lens multipurpose solutions on human cornea epithelial survival and barrier function. Eye & Contact Lens 34 (5), 281–286.

Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1 and interleukin-8 expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization. Toxicology In Vitro 17, 311–321.

Daston, G.P., 2007. Genomics and development of risk assessment. Birth Defects Research Part A Clinical and Molecular Teratology 79, 1–7.

De Saint Jean, M., Brignole, F., Bringuier, A.F., Bauchet, A., Feldmann, G., Baudouin, C., 1999. Effects of benzalkonium chloride on growth and survival of change conjunctival cells. Investigative Ophthalmology & Visual Science 40, 619–630.

Debbasch, C., Ebenhahn, C., Dami, N., Pericoi, M., Van den Berghe, C., Cottin, M., Nohynek, G.J., 2005. Eye irritation of low irritant cosmetic formulations: correlation of in vitro results with clinical data and product composition. Food & Chemical Toxicology 43, 155–165.

Doucet, O., Lanvin, M., Zastrow, L., 1999. Comparison on three in vitro methods for the assessment of the eye irritating potential of formulated cosmetic products. In Vitro and Molecular Toxicology 12, 63–76.

Doucet, O., Lanvin, M., Thillou, C., Linossier, C., Pupat, C., Merlin, B., Zastrow, L., 2006. Reconstituted human corneal epithelium: a new alternative to the Draize eye test for the assessment of the eye irritation potential of chemicals and cosmetic products. Toxicology In Vitro 20 (4), 499–512.

Draize, J.H., Woodard, G., Calvery, H.O., 1944. Methods for study of irritation and toxicity of substances applied topically to the skin and mucous membranes. The Journal of Pharmacology and Experimental Therapeutics 82, 377–390.

ESAC, 2007. ESAC Statement on the conclusions of the ICCVAM retrospective study on organotypic in vitro assays as screening tests to identify potential ocular corrosives and severe irritants as determined by US EPA, EU (R41) and UN GHS classifications in a tiered testing strategy, as part of a weight of evidence approach. Website: Joint Research Center (accessed 28.09.07).

Eskes, C., Bessou, S., Bruner, L., Curren, R., Harbell, J., Jones, P., Kreiling, R., Liebsch, M., McNamee, P., Pape, W., Prinsen, M., Seidle, T., Vanparys, P., Worth, A. & Zuang, V., 2005. Eye irritation. In: Eskes C., Zuang V. (Eds.), Alternative (Non-Animal) Methods for Cosmetics Testing: Current Status and Future Prospects. Alternatives to Laboratory Animals 33 (1), 47–81 (Subchapter 3.3).

Frentz, M., Goss, M., Reim, M., Schrage, N.F., 2008. Repeated exposure to benzalkonium chloride in the ex vivo eye irritation test (EVEIT): observation of isolated corneal damage and healing. Alternatives to Laboratory Animals 36, 25–32.

Guenoun, J.M., Baudouin, C., Rat, P., Pauly, A., Warnet, J.M., Brignole-Baudouin, F., 2005a. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. Investigative Ophthalmology & Visual Science 46, 4594–4599.

Guenoun, J.M., Baudouin, C., Rat, P., Pauly, A., Warnet, J.M., Brignole-Baudouin, F., 2005b. In vitro study of inflammatory potential and toxicity profile of latanoprost, travoprost, and bimatoprost in conjunctiva-derived epithelial cells. Investigative Ophthalmology & Visual Science 46, 2444–2450.

Kimura, K., Teranishi, S., Nishida, T., 2009. Interleukin-1beta-induced disruption of barrier function in cultured human corneal epithelial cells. Investigative Ophthalmology & Visual Science 50 (2), 597–603.

Matter, K., Balda, M.S., 2007. Epithelial tight junctions, gene expression and nucleo-junctional interplay. Journal of Cell Science 120 (Pt. 9), 1505–1511.

McCarthy, K.M., Skare, I.B., Stankewich, M.C., Furuse, M., Tsukita, S., Rogers, R.A., Lynch, R.D., Schneeberger, E.E., 1996. Occludin is a functional component of the tight junction. Journal of Cell Science 109 (Pt. 9), 2287–2298.

Meloni, M., Dalla Valle, P., Cappadoro, M., De Wever, B., 2002. The importance of multiple endpoint analysis (MEA) using reconstituted human tissue models for irritation and biocompatibility assay. Invitox Congress Proceedings 4, 7.

Mitjans, M., Viviani, B., Lucchi, L., Galli, C.L., Marinovich, M., Corsini, E., 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in na THP1 cells. Toxicology In Vitro 22, 386–395.

Mondino, B.J., Salamon, S.M., Zaidman, G.W., 1982. Allergic and toxic reactions of soft contact lens wearers. Survey of Ophthalmology 26 (6), 337–344.

Nguyen, D.H., Beuerman, R.W., De Wever, B., Rosdy, M., 2003. Three-dimensional construct of the human corneal epithelium for *in vitro* toxicology. In: Salem, H., Katz, S.A. (Eds.), Alternatives Toxicological Methods. CRC Press, pp. 147–159.

Pauly, A., Brignole-Baudouin, F., Labbé, A., Liang, H., Warnet, J.M., Baudouin, C., 2007. New tools for the evaluation of toxic ocular surface changes in the rat. Investigative Ophthalmology & Visual Science 48, 5473–5483.

Pauly, A., Meloni, M., Brignole-Baudouin, F., Warnet, J.M., Baudouin, C., 2009. Multiple endpoint analysis of the 3D-reconstituted corneal epithelium after treatment with benzalkonium chloride: early detection of toxic damage. Investigative Ophthalmology & Visual Science 50 (4), 1644–1652.

Pisella, P.J., Fillacier, K., Elena, P.P., Debbasch, C., Baudouin, C., 2000. Comparison of the effects of preserved and unpreserved formulations of timolol on the ocular surface of albino rabbits. Ophthalmic Research 32, 3–8.

Powers, M.J., Amenuvor, L., Rodin, R., Darner, J., 2007. An *in vitro* Human Corneal Epithelial Model for Evaluation of Ocular Response to Xenobiotic Compounds. Investigative Ophthalmology & Visual Science 48, 790 (E-Abstract).

Reichl, S., 2008. Cell culture models of the human cornea – a comparative evaluation of their usefulness to determine ocular drug absorption in vitro. Journal of Pharmacy and Pharmacology 60, 299–307.

SCCP/111/07. European commission directorate general health & consumer protection: memorandum on actual status of alternative method on the use of experimental animals in the safety assessment of cosmetic ingredients in the European Union. Opinion adopted at 12th plenary session, 19 June, 2007.

Schneeberger, E.E., Lynch, R.D., 2004. The tight junction: a multifunctional complex. American Journal of Physiology–Cell Physiology 286, C1213–C1228.

Sosnová-Netuková, M., Kuchynka, P., Forrester, J.V., 2007. The suprabasal layer of cornea epithelial cells represents the major barrier site to the passive movement of small molecules and trafficking leukocytes. British Journal of Ophthalmology 91, 372–378.

Tosti, A., Tosti, G., 1988. Thimerosal: a hidden allergen in ophthalmology. Contact Dermatitis 18 (5), 268–273.

Van Goethem, F., Adriaens, E., Alépée, N., Straube, F., De Wever, B., Cappadoro, M., Catoire, S., Hansen, E., Wolf, A., Vanparys, P., 2006. Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. Toxicology In Vitro 20, 1–17.

Weil, C.S., Scala, R.A., 1971. Study of intra- and interlaboratory variability in the results of rabbit eye and skin irritation tests. Toxicology and Applied Pharmacology 19, 276–360.