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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.