ASSESSMENT OF CORNEAL SURFACE IMPAIRMENT AND EARLY SIGNS OF EYE IRRITATION ON RECONSTRUCTED HUMAN CORNEAL EPITHELIUM

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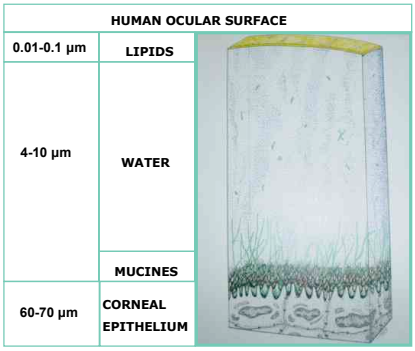
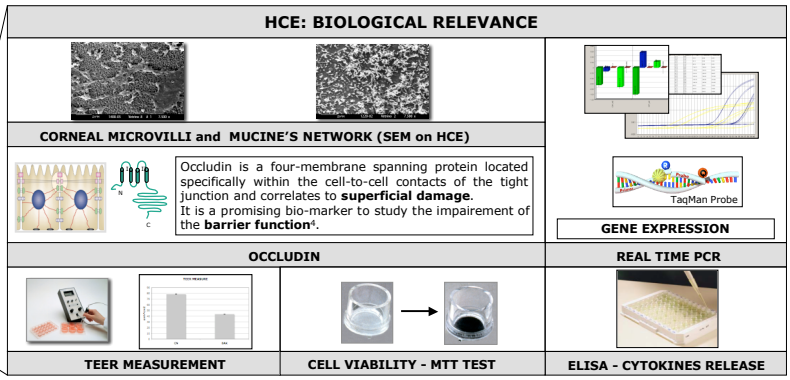
BACKGROUND

The pre-corneal tear film and associated lipids, the corneal epithelium, the mucine's network and the conjunctiva represent the target of Eye Compatibility assessment that concerns very low, low or even non irritant (Non Classified-NC) chemicals or products able to generate early toxicity without clinical signs at eye level as discomfort or itching. Beside the regulatory requirements the assessment of eye compatibility represents a real need for Pharmaceutical and Cosmetic industries: cosmetics that strongly differ for ingredient's type and concentration, technical form, mode of application and long term ophtalmological treatments involving repeated application, represent the applicability domain of test methods proposed in this area of toxicology. The investigation of relevant endpoints at corneal epithelium level and associated structural components allow to set-up sensitive and predictive testing strategies in the area of NC and mild chemicals and products.

EXPERIMENTAL APPROACH





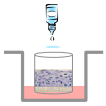
The Multiple Endpoints Analysis **(MEA)** Protocol[1] based on the assesment of complementary parameters (cell viability by MTT test, tissue morphology and the release of cytokines) has been modified by including transcriptional regulation of a structural component of the epithelial barrier as an early marker of the effects of sub toxic doses: OCCLUDIN gene expression has been investigated by quantitative RT-PCR by using Taqman® technology - Real Time.

MEA has also been structured on 3 time points: 24h, 24h followed by product wash and by a post incubation period of 24h, and repeated application (twice a day) for a continuous exposure of 72h. The repeated application procedure tested for the first time on HCE model represents a new approach of the assessment of potential non eye irritant compounds by reproducing the long term application and cumulative effects.

BAK (Benzalkonium Chloride) as a well known eye irritant has been tested at toxic (0,1 %) and non toxic doses (0,01%) previously defined after acute application of 24h.

HUMAN CORNEAL EPITHELIAL MODEL (HCE)

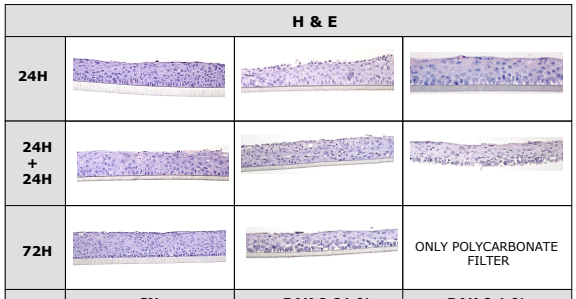
The reconstructed Human Corneal Epithelium model (HCE) by SkinEthic Laboratories (Nice, France) has been used. HCE model has been recognized as a succesful tool for irritation and compability testing[2,3] and it is currently used within cosmetic and pharmaceutic industries for hazard identification and internal risk assessment.



MEA MODIFIED PROTOCOL	EXPOSURE	END POINTS
	BAK 0,01% - 0,1 % : 30 µL	• CELL VIABILITY • H&E • IMMUNO-HISTOCHEMISTRY • OCCLUDIN GENE EXPRESSION
ACUTE APPLICATION	24H ACUTE	
ACUTE APPLICATION WITH HIGHER DISCRIMINATING POTENTIAL	24H ACUTE + 24H POST INCUBATION	
LONG TERM APPLICATION AND CUMULATIVE EFFECT	72H REPEATED APPLICATION (3 DAYS)	

RESULTS

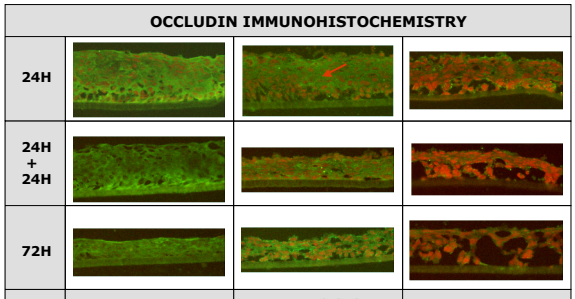
H & E



Histological analysis confirms the time and dose dependent effect with the two BAK concentrations: an increasing number of necrotic cells from the apical to the basal layers is observed with BAK 0,01% at 24h associated to a morphological impairment of the upper layers that became a necrotic zone after the post incubation period without sign of recovery. After 72h the tissue morphology is still observed even in presence on necrosis.

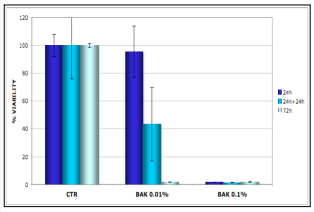
BAK 0,1% induce an early necrosis in the whole tissue.

OCCLUDIN IMMUNOHISTOCHEMISTRY



Occludin membrane staining is detected in the basal and apical layers of the control tissues.
BAK 0,01% shows a different localization of occludin in the upper apical part of the tissue.
Occludin staining disappeared with BAK 0,1% early after 24h treatment[5]

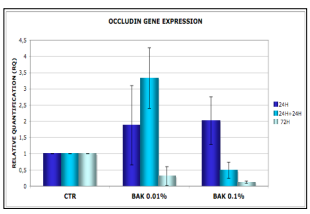
VIABILITY - MTT TEST



BAK 0.01% has induced a dose and protocol dependent decrease of cellular viability: it has been defined as non toxic after acute 24h application, inducing a 60% reduction of viability after the post incubation protocol and it is definitely toxic in the repeated application procedure.

BAK 0.1% is toxic at all different exposures.

OCCLUDIN GENE EXPRESSION



For both BAK concentrations after 24h a significant up-regulation is observed.

The treatment of 24h + post incubation has significantly up-regulated the occludin expression at the dose of 0.01% (viability 40%) and on then contrary the higher dose of 0.1% (viability 0%) has significantly down-regulated the occludin expression.

In the long term treatment (both cytotoxic) the occludin is down regulated by both concentrations of BAK where both viability results have shown any residual cellular viability.

CONCLUSION

The use of the MEA modified protocol may enlarge the scale for discriminating between NC and mild and very mild chemicals and products. MEA approach has been confirmed as a useful tool for preclinical irritation screening of new ophthalmological or cosmetic products. The study of Occludin regulation allows a better prediction of the effects of sub toxic doses of chemicals treatment: these are mostly superficial and modify the penetration of the toxicant without inducing a toxic effect at basal level. Occludin gene expression seems to be a promising marker to investigate superficial damages at corneal epithelium level and to overcome some limits of MTT test. Thanks to different procedures it is possible to use this new bio-marker to distinguish between a strong irritant that is responsible of the degradation of the tissue (down regulation and early up regulation) and a low irritant able to allows tissue recovery (up regulation followed by any down regulation) or finally to distinguish between different levels of irritation (constant up-regulation).

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2. Doucet O. *et al.* Toxicol In Vitro. 2006 Jun;20(4):499-512
3. Van Goethem F. *et al.* Toxicology in vitro. 2006; 20, 1-17
4. Ban Y *et al. Exp Eye Res* 2003; 76: 663-9
5. Meloni M. Proceedings EVER Congress - October, 2007 Portoroz, Slovenia

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