IN VITRO ASSESSMENT OF LONG TERM EYE TOXICITY OF PRESERVED OR PRESERVATIVE FREE TIMOLOL EYE DROPS AND EYE GEL FORMULATIONS

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ABSTRACT

PURPOSE

To assess eye irritation potential and long term compatibility of marketed ophthalmological timolol preparations by using a biologically relevant and sensitive 3D model of human corneal epithelium (HCE) and Multiple Endpoint Analysis (MEA).

METHODS

Protocol for MEA was structured on 4 time points: 24h after single application, 24h followed by product wash and by a post incubation period, 72h after repeated application (twice daily) and 72h followed by a 72h recovery. After application of Timolabak® (preservative free eye drops), Timoptol® (BAK preserved eye drops) , Nyogel® (BAK preserved eye gel), Timogel® (preservative free eye gel) and BAK 0.01% as positive control, the following parameters were quantified: Cellular viability, Histological analysis (H&E staining), IL-1 and IL-8 passive release by ELISA and the OCCLUDIN gene expression by quantitative real time-PCR.

RESULTS

Cell viability measured at basal level of HCE model by MTT test method was reduced under the 50%cut-off value after acute exposure (24h and 24h +24h) by BAK 0.01%, Timoptol, and Nyogel. Even after repeated application (72h and 72h+72h) Timolabak and Timogel never reduced under 50 % viability after all the exposures. Morphological results were consistent with the viability data, Timolabak and Timogel didn't significantly modify the HCE morphology after different exposures. Release of the pro-inflammatory cytokine IL-1 has been quantified higher than the negative control (>20pg/ml) for BAK 0.01%, Nyogel and Timoptol treatments and it was unchanged after treatment with Timolabak and Timogel. A prediction model based on occludin mRNA allowed to classify the products as Non irritant (Timolabak), Borderline (between Non irritants and Extremely mild, Nyogel and Timogel), Slightly mild (Timoptol and BAK 0.01%)(3).

CONCLUSIONS

By introducing a molecular endpoint such as the mRNA expression of occludin into the MEA, we added an early, sensitive and quantitative biomarker able to reveal significant effects often neglected as the early and superficial signs of toxicity.

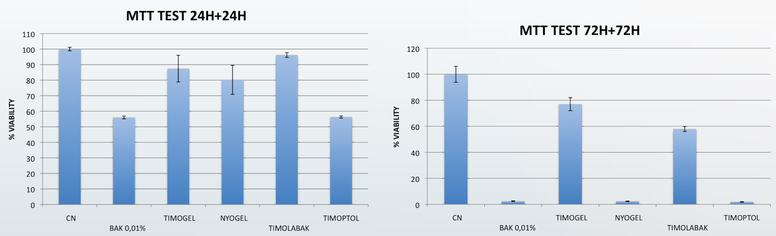
INTRODUCTION

The assessment of ocular tolerance is particularly important for antiglaucoma eye drops, since eye irritation is a very common condition in glaucomatous patients with a negative impact on treatment compliance. Ocular surface irritation was showed higher with preserved than with preservative-free eye drops (1) and in particular benzalkonium chloride (BAK), the most commonly used preservative was demonstrated to have adverse effects on the cornea and conjunctiva (2) and it may induce cell toxicity and ocular damage in a dose-dependent manner. We have already demonstrated that a repeated application procedure tested on the Human Corneal Epithelium 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 (3). The results showed how this new procedure, the modified Multiple Endpoint Analysis (modified MEA) was able to better discriminate between tears subtitutes eye drops that initially appeared to be identical after acute application. The modified MEA protocol include cellular viability by the MTT test (a recognised international standard for cytotoxicity testing), histological analysis, the passive release of Interleukins and the Occludin gene expression (by quantitative real time –PCR). Occludin gene expression in particular may be an early and predictive marker of subtoxic doses and could predict the intensity of tissue damage and recovery. (4) To discriminate the eye irritation potential of the preservative (BAK) in antiglaucoma eye drops, we compared four registred timolol ophtalmological formulations : Timolabak® (preservative free eye drops), Timoptol® (BAK preserved eye drops), Nyogel® (BAK preserved eye gel)and Timogel® (preservative free eye gel) , using the modified MEA protocol

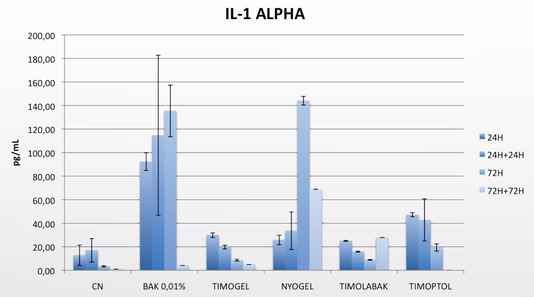
METHODS

The reconstructed human corneal epithelium model (HCE) supplied by SkinEthic Laboratories® consisted in immortalized human corneal epithelial cells cultured on a polycarbonate filter at the air-liquid interface for 7 days in a chemically defined medium in order to form a structured epithelium (60 µ thickness). The test products Timolabak®, Timoptol® , Nyogel®, Timogel®, in addition to saline solution, as a negative control, and benzalkonium chloride (BAK) non toxic dose 0.01% as a positive control were directly applied (30 µl volume) to the whole epithelium surface. Modified MEA protocol (cellular viability by the MTT test, histological analysis, release of IL-1 and IL-8 by Elisa and Occludin gene expression by quantitative Real Time PCR) was structured on 4 time points: 24h after single application, 24h followed by product wash and by a post incubation period, 72h after repeated application (twice daily) and 72h followed by a 72h recovery. The test has been performed on duplicate cultures for MTT and IL-1a and IL-8 quantification, single cultures for histology and duplicate cultures for occludin mRNA expression study

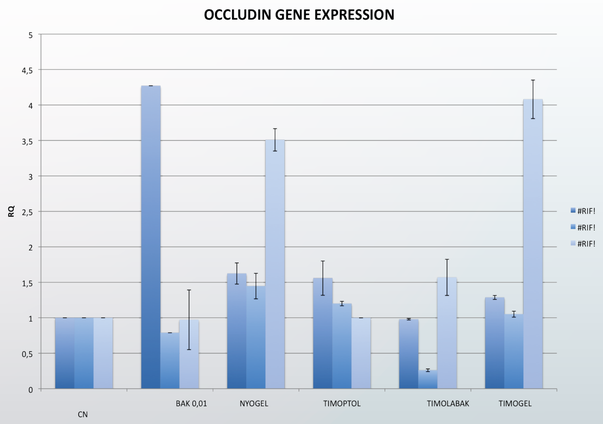
RESULTS



The cell viability measured at basal level of the HCE by the MTT test method was reduced under the 50 % cut-off value after acute exposure (24h and 24h +24h) by BAK0.01%, Timoptol and Nyogel. Cell viability was dramatically reduced under 10% after repeated application (72h and 72h+72h) by BAK 0.01%, Nyogel and Timoptol. Timolabak and Timogel never reduced under 50 % the viability after all the exposures.



BAK 0.01% induced a significant release of the pro-inflammatory citokine confirming the well known eye irritation potential of the molecule. Only Timogel and Timolabak never induced a significant release of the citokine for all the time points defined.



Occludin gene is an early marker of barrier function impairment by detecting early sign of toxicity at the epithelium surface before that the cellular viability could be involved. The occludin expression is a new and early biomarker to distinguish between an irritant that is responsible of the degradation of the tissue (down regulation) and a low irritant able to allows tissue recovery (up regulation followed by any regulation) or finally to distinguish between different levels of mild irritation (constant up-regulation). By using the prediction model previously defined (3) based on occludin mRNA expression it is possible to classify the tested products as: Non irritant (Timolabak), Borderline (between non irritants and extremely mild, Nyogel and Timogel), Slightly mild irritant (Timoptol and BAK 0.01%).

CONCLUSIONS

By introducing a molecular endpoint such as the mRNA expression of occludin into the MEA, we added an early, sensitive and quantitative biomarker able to reveal significant effects often neglected as the early and superficial signs of toxicity at corneal epithelium level. The modified MEA on reconstituted in vitro Human Corneal Epithelium represents a valuable and promising tool pre-clinical studies of eye irritation assessment with the power to detect mild irritants and subclinical eye irritant potential. The results allow to classify the tested products according to their irritation potential : higher for TIMOPTOL and NYOGEL, lower for TIMOGEL and TIMOLABAK. Results are in line with clinical evidence of the better tolerabilty of preservative-free antiglaucoma drops, to be preferred in clinical practice to preserved formulations.

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