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Development and characterization of a bioengineered conjunctiva model on the basis of immortalized cells.

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INTRODUCTION

Until today none of the existing *ex vivo* or *in vitro* methods has proved fully satisfactory to completely replace the Draize Rabbit Eye Irritation Test. Although damages to the cornea are the most influential drivers of eye irritation for all classes within the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), also conjunctival damages gain more importance particularly as a driver of irritation, especially for category 2A and 2B classification. However, none of the already validated *in vitro* methods for eye irritation testing sufficiently addresses the conjunctival involvement. Therefore it was our aim to develop a bioengineered conjunctiva model for application in eye irritation studies.

EXPERIMENTAL SETUP

Construction of the human conjunctival constructs: To construct the conjunctiva epithelial models hTert immortalised HCjE cells [1] were seeded on permeable polycarbonate membrane filters (Millicell, 0,4µm). For construction of full thickness conjunctiva models suspensions of SV40 immortalised HCK-Ca cells [2] in collagen gels were cast on permeable polycarbonate filters (Nunc, 3,0µm). Subsequently HCjE cells were seeded onto this stromal equivalents (Fig 1A). To induce multilayered growth and cell differentiation filters were lifted to the air-liquid interface (ALI) for 7 days after 2 days of submerse cultivation and followed by 1-2 days incubation in serum/EGF-containing stratification-medium.

Characterization:*TER-measurements:* The influence of varying cultivation conditions on the barrier function i.e. on the transepithelial electrical resistance (TER) was investigated by using an EVOM and Endohm chamber as described in [3] *Immunofluorescence Microscopy:* For transverse images, paraffin sections (6 µm) of formaldehyde-fixed tissues were subjected to immunohystochemistry as decribed in [4]. For en-face images, cultured cells grown on membrane inserts were fixed in ice-cold methanol/acetone and processed as described in [4]. Primary antibodies against Cldn-1 (1:3000); ZO-1 (1:100), Occl (1:20), CK13 (1: 80) were obtained from Zymed/ Santa Cruz. Alexa Fluor 488 Fab (1:600; Life Technologies) was used as secondary antibody.

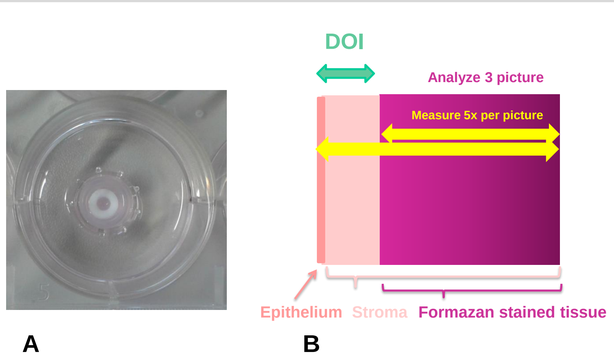


Fig. 1: A: conjunctiva model with fixed Teflon ring to avoid spreading of the test substances, B: scheme showing the principle of depth of injury calculation. DOI was calculated by subtracting Formazan-stained length from total cross sectional length.

Analysis of tissue viability in MTT-stained cryosections. Tissues were exposed to different test substances using a limiting Teflon ring (Fig 1A). A method was developed to analyse the initial depth of conjunctival injury (DOI) in tissue models by combining the MTT viability assay with standard cryosectioning procedures. For quantification images of transverse sections were transmitted to FiJi software program. The straight line tool was used to define (i) Formazan-stained length and (ii) total cross sectional length. DOI was calculated by subtracting Formazan-stained length from total cross sectional length (Fig.1B).

RESULTS

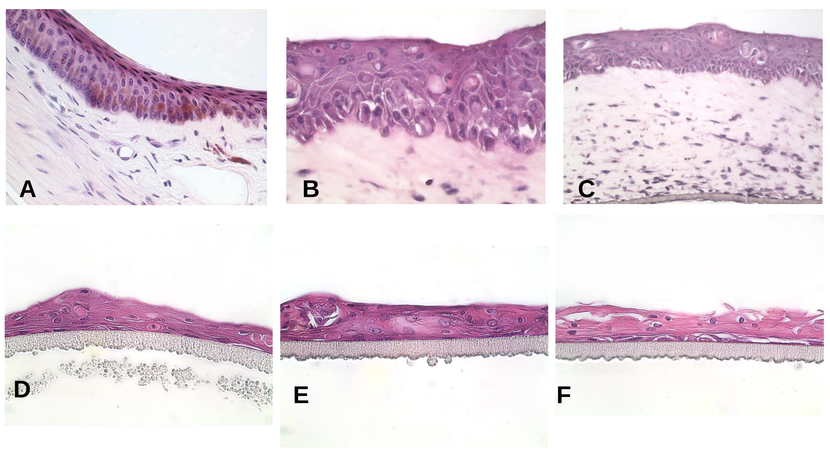


Figure 2: Hematoxylin and eosin stain (H&E stain) of native bulbar conjunctiva tissue (A) and of different conjunctiva models (B-F). B and C show conjunctiva full thickness models with stratified epithelial layer on top of the stromal matrix with embedded fibroblasts in 20-fold and 40-fold magnification. D-F show conjunctiva epithelial models after 1 day (D), 2 days (E) and 5 days (F) of final incubation in serum/EGF-containing stratification-medium (40-magnification). *Note improved stratification on top of the collagen matrix.*

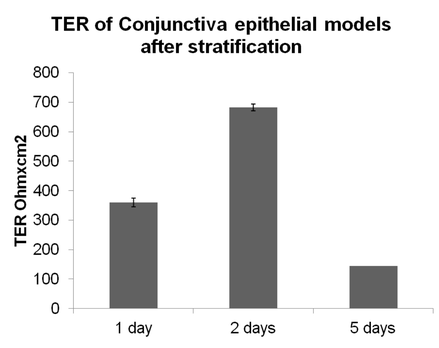


Figure 3: Transepithelial electrical resistance (TER) values of conjunctiva epithelial models after 1, 2 and 5 days of stratification. *Note: 2 days of stratification induce tight barrier.*

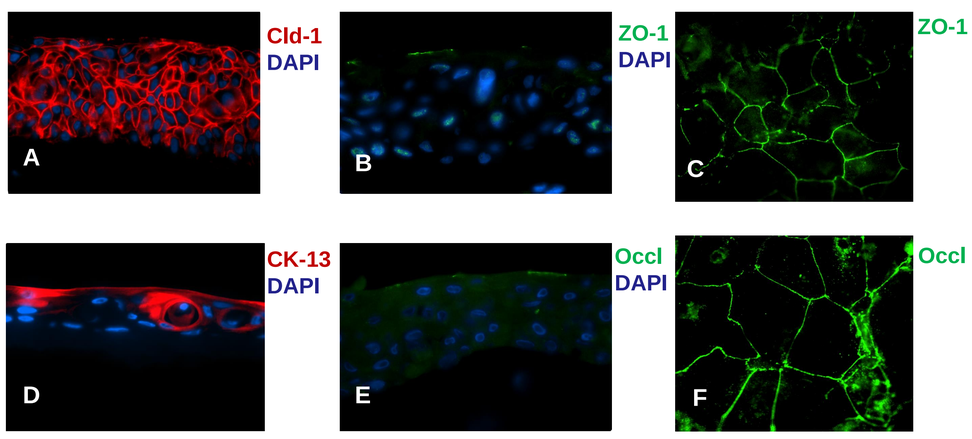
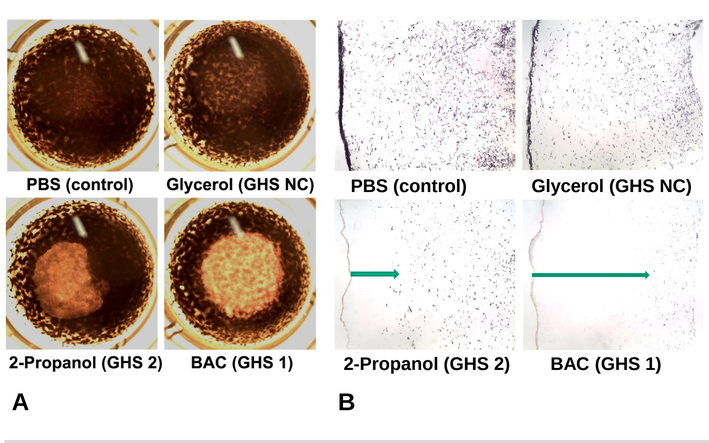
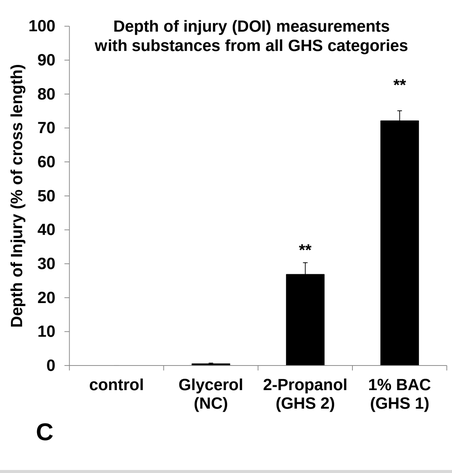


Figure. 4: Immunofluorescence expression and localization of Tight junction components Claudin-1 (Cldn-1, A), Zona occludens-1 (ZO-1, B, C), Occludin (Occl, E, F), and of conjunctiva-specific Cytokeratin-13 (CK-13, D). *Note: that images show conjunctiva–typical expression of the different proteins in transversal section of conjunctiva tissue (A, B, E) or conjunctiva epithelial tissue (D), as well as in en-face sections of tissues grown on membrane filters [5,6].*





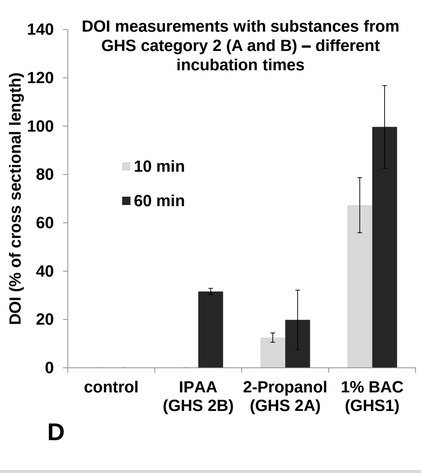


Figure 5 Quantitative analysis of tissue injury in conjunctiva models by combining the MTT viability assay with cryosectioning procedures. 10µl of test substances were applied on top of the tissue for 10 or 60 min. The test material included covers the whole range of GHS classification for ocular irritation based on draize data having nonirritant (Glycerol, GHS NC), slight/moderate irritant (Isopropylacetoacetat,, IPAA, GHS 2B/ 2-Propanol, GHS 2A;) as well as severe irritant substances (1% BAC, GHS1).
A and B show MTT-stained tissues from top-view (A) and the corresponding transversal cryosections.(B), wherat dark staining indicates viable tissue area. C and D show two examples of quantitative analysis of DOI in conjunctival models after 10 min (C, D) and 60 min (D) exposition time. *Note that the tissue penetration and injury of tested compounds is in correlation with their Draize eye irritating potential.*

DISCUSSION & CONCLUSION

3D conjunctiva equivalents have been developed from immortalized cells originally derived from the human eye. The 3D models comprise multi-layered epithelia with or without a subjacent stromal matrices of collagen-embedded fibroblasts. We established standard operating procedures for the construction and cultivation of the bioengineered models and for its use in an eye irritation toxicity assay under serum-free conditions.
The conjunctiva model was characterized by H&E-staining, immunohistology as well as barrier function analysis by means of transepithelial electrical resistance studies (TER). In addition, a modified MTT method for the determination of tissue viability in cryosections was applied to the conjunctiva models to assess the tissue damage induced by topically applied chemicals. By using this method we can clearly distinguish the reference substances by means of their depths of injury.
Therefore, the 3D conjunctiva model represents a promising non-animal *in vitro* alternative and offers a well-defined and standardized system for assessing the conjunctival effects in an eye irritation reaction.

References:

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