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RE-EPITHELIZING EFFECT OF XANTHAN GUM IN A MODEL OF MECHANICAL INJURY PERFORMED ON CULTURED HUMAN CORNEAL EPITHELIUM (HCE)

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INTRODUCTION

• Damage to the corneal epithelium caused by chemical, physical or microbial insults results in swelling of the stroma, activation of stromal fibroblasts and infiltration of various inflammatory cells, leading to the loss of corneal transparency and integrity. Therefore, it is important to repair corneal epithelial damage as rapidly as possible. Most corneal epithelial wounds are indeed repaired promptly. However, under certain clinical conditions, resurfacing of the corneal epithelium is delayed. Knowledge of the mechanisms that underlie the healing of corneal epithelial wounds is crucial for the development of new treatments.

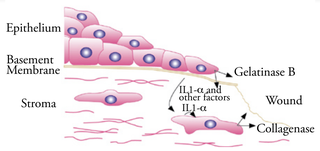
Resurfacing of corneal epithelial defects occurs in three phases: 1) immediately after the wounding of epithelial cells, the neighboring intact epithelial cells begin to migrate over the affected area until they cover it with a cell monolayer. 2) This is followed by the proliferation of the migrated epithalial cells, resulting in the restoration of the normal epithelial thickness. 3) A few weeks after the wounding occurred, the epithelial cells begin to differentiate, the surface of the healed region becomes smooth and a well-layered structure is restored. The three phases of corneal epithelial wound healing are thus characterized by migration, cell proliferation, and cell differentiation, respectively.(1)

Two different types of epithelial movement during the covering process of the exposed area of the cornea have been observed as shown in figure 1: epithelial abrasion (upper panel) is followed by an initial sheet-like movement of individual epithelial cells (middle panel) and a subsequent landslide-like movement of the remaining epithelium en masse (lower panel).

Fig. 1: Hepitilial movment

• The matrix metalloproeinases (MMPs) are a family of proteolytic enzymes whose function is to maintain and remodel the tissue architecture. They have been implicated in a wide range of processes and diseases and their substrates comprise a variety of extracellular matrix components, secreted cytokines and cell surface molecules. MMPs including MMP-9 (gelatinase B), -2 (gelatinase A), and -1 (collagenase) have been reported to be involved in the pathogenesis of ulceration[2] and pterygia[3] of the cornea. Animal model studies have demonstrated the presence of MMP-9 in migrating epithelial cells after injury[4]. MMPs expression during corneal healing displays both a long-term and a short-term response. MMP-9 is secreted in the short-term by the basal epithelial cells migrating to close a wound, while collagenase (MMP-1) expression is upregulated in wound fibroblasts by an Il 1-α autocrine loop for long term remodelling (fig.2).[5]

Fig. 2: MMPs in Corneal Woun Healing



• In this study we used an experimental model of mechanical injury on multilayer human corneal epithelium (HCE) to mimic the conditions of the wound healing process and to evaluate the re-epithelizing effect induced by xanthan gum formulations at 1% or 0.2% and by an hydrogel containing 1% xanthan gum plus 0.15% sodium hyaluronate. The epithelium surface morphology and the evolution of the repair process were evaluated by histology and by scanning electron microscopy (SEM). Moreover, the expression of MMP-9 was measured by real time RT-PCR.

MATERIALS AND METHODS

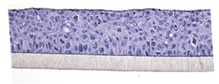
BIOLOGICAL MODEL

Commercially available 3D HCE multilayer of 0.5 cm^2 (SkinEthic® LAB–Nice-F) reconstituted from immortalized Human corneal epithelial cells was used for the experiment.

Epithelial corneal cells were deposited on a polycarbonate filter and cultured at the air-liquid interface for 7 days in a chemically defined medium (modified MCDB 153) in order to form a structured epithelium.

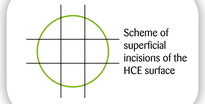
This model has been fully characterized and it presents the same features and morphology of the human corneal epithelium *in vivo* (60-70 µ thickness)[6] (Fig.3).

Fig. 3: HCE multilayer



Injury and treatments:

Reproducible superficial incisions were obtained by using a sterile thin glass edge and by making 4 cross sections on the middle of the HCE surface.



HCEs were placed on 6 well plates with culture medium and, immediately after the injury, all the HCEs were placed in a CO_2 incubator (5%) at 37 ° C. The maintenance medium, modified MCDB 153 without added serum, was supplied by SkinEthic and it was changed every 24 h.

Thirty minutes following mechanical injury, HCE samples were treated with 1% or 0.2% xanthan gum formulations, or with 1% xanthan gum plus 0.15% sodium hyaluronate gel and were monitored at the following time points: 6, 24, 48 and 72 h.

Desamethasone was tested as reference product counteracting a quick re-epithelization.

Treatments (50 µl) were applied directly on the HCEs surface with a micropipette. At the time points defined for the study, samples were processed for histology; scanning electron microscopy and real-time polymerase chain reaction analises.

Histology

At different times, using a sharper scalpel, the single HCE was removed from the insert and fixed in 10% formalin solution. After paraffin embedding, vertical sections of 4 µ were cutted and stained with H&E following classical procedures.

Scanning electron microscopy (SEM)

HCE samples, were fixed in 2% glutaraldehyde in 0.1 M PBS for 2h at 4°C and then post-fixed for 1h at 4°C in 1% osmium tetroxide in the same buffer. After thorough washing with PBS, samples were dehydrated in a series of ethanol dilutions (30-100%). Specimens were mounted on aluminium stubs with conductive carbon cement, allowed to dry and then coated with a gold film. Samples were observed with an S-400 scanning microscope (Hitachi).

Real-time polymerase chain reaction (RT-PCR)

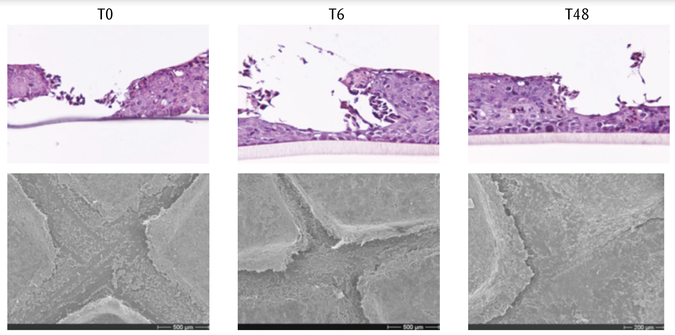
Samples for RT–PCR were placed in Lysis buffer and stored at -80 °C . Applied Biosystems 7500 real time PCR and the TaqMan assay were used. Briefly, pre-designed gene specific primers and probes specific for the target sequence, containing a fluorescence reporter and a non fluorescent quencer with a minor groove binder, provide quantitative detection of nucleic acid sequences using real-time analysis. The progress of the reaction is monitored as it occurs. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. Data are collected throughout the PCR process.

• RNAqueous kit (AMBION) was used for RNA extraction
• High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used for cDNA reverse-transcription.

RESULTS

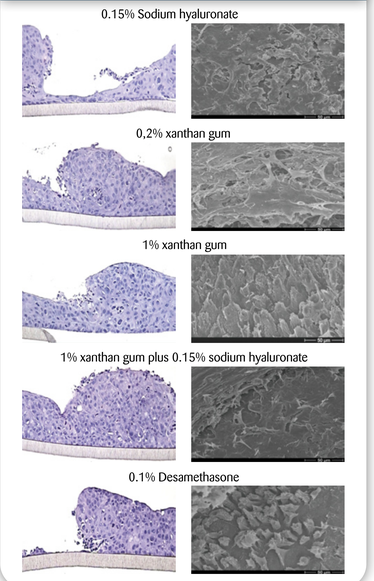
Histological and SEM analyses showed that during the first 24h the epithelium underwent a proliferation phase involving the basal cells. In the next 48-72h an evident cellular migration and the repopulation of the injured area were observed, characterized by proliferation and remodelling of the multilayer (Fig 4).

Fig. 4: Mechanical lesion



The proliferation and cellular migration occurred earlier when the cells were treated with xanthan gum at both the concentrations used. In fact, at 48h xanthan gum treated cells showed the typical landslide-like movement (Fig 5). Conversely, at the same time point cells treated with 0.15% sodium hyaluronate alone were shown to be in a preliminary phase of the sheet-like movement (Fig 5). After 48h, the application of 0.1% dexamethasone retarded the corneal epithelial healing, in this experimental model of mechanical injury on multilayer HCE, as reported in *in vivo* models of wound healing (Fig 5) [7].

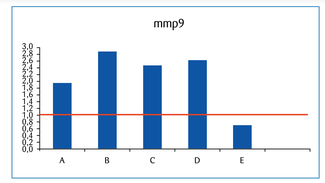
Fig. 5: Treatments T 48 h



MMP-9, the primary MMP synthesized and secreted by basal corneal epithelial cells during the wound healing process, was shown to be upregulated in xanthan gum treatment groups.

At 72 h the levels of expression of MMP-9 in the hyaluronic acid treated group were doubled compared to the positive control (dexametasone), while a 3-times increase was observed in the xanthan gum 1%, xanthan gum 0.2% and xanthan gum 1% plus hyaluronic acid groups (Fig 6)

Fig. 6: Expression of MMP9 (RT-PCR) T 72 h



A: Hyaluronic acid 0.15%; B : Hyaluronic acid + xanthan gum 1%; C: xanthan gum 1%; D: xanthan gum 0.2%; E: Desametasone; Red line, lesion

CONCLUSIONS

With this experimental *in vitro* model of mechanical injury on HCE multilayer, it was possible to monitor the wound healing process and to investigate the effect of treatments. The optimal time point to highlight differences among treatments was 48 hour. The proliferation and cellular migration took place earlier when the cells were treated with xanthan gum. The tissue of best quality was obtained when the combination xanthan gum plus sodium hyaluronate was used, confirming the data obtained in *in vivo* studies[8]. The xanthan gum probably improves and speeds up the healing process by promoting MMP-9 expression, known to be released by HCE to remodel the matrix behind the leading migratory front[9].

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