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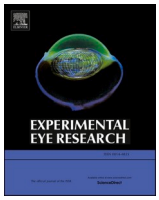

Research article

Characterization and functional performance of a commercial human conjunctival epithelial cell line

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ABSTRACT

The conjunctiva is a complex tissue that covers the eye beginning at the corneal limbus and extending over the inner surfaces of the eyelids. Due to its important functions in maintaining the health of the ocular surface, adequate *in vitro* models of conjunctival structure and function are essential to understand its roll in different pathologies. Because there is scarcity of human conjunctival tissue that can be used in research, cell lines are often the only option for initial studies. An immortalized human conjunctival epithelial cell (IM-HConEpiC) line is now commercially available; however, it is not very well characterized. In this study, we have developed a new protocol to culture these cells without the use of collagen-coated culture surfaces, but with a defined cell culture medium. We characterized IM-HConEpiCs cultured under these conditions and corroborated that the cells maintained a conjunctival epithelial phenotype, including acidic and neutral mucins, junctional proteins E-cadherin and zonula occludens 1, and expression of CK8 and CK19, among others. In addition, we analyzed the response to oxidative stress and inflammatory stimuli and found that IM-HConEpiCs respond as expected for conjunctival epithelial tissue. For instance, cells exposed to oxidative stress increased the production of reactive oxygen species, and that increase was blocked in the presence of an antioxidant agent. In addition, after stimulation with TNF-α, IM-HConEpiCs significantly increased the production of IL-1β, IL-6, IL-8, and IP-10. Therefore, with this study we conclude that IM-HConEpiCs can be a useful tool in functional studies to determine the response of the conjunctiva to pathological conditions and/or to test new therapeutic strategies.

1. Introduction

The conjunctiva is the mucosal tissue that covers the eye beginning at the corneal limbus (bulbar conjunctiva) and reflects back onto the inner surfaces of the upper and lower eyelids (tarsal conjunctiva). It is a complex tissue with many important functions that maintain the health of the ocular surface. Structurally, the conjunctiva is composed of a stratified squamous epithelium with a loose stroma underneath. Morphologically, there are five different types of conjunctival epithelial cells (Steuhl, 1989), but functionally, they are classified in just two types: goblet cells (type I) and stratified squamous cells (types 2–5) (Dartt, 2002).

Conjunctival epithelial cells are essential in the maintenance of a healthy ocular surface. They can secrete water, electrolytes, and different types of mucins; thus contributing to the mucous-aqueous layer of the tear film (Dartt, 2002). Although all conjunctival epithelial cells can secrete mucins, only goblet cells produce and secrete the large gel-forming mucin MUC5AC. Stratified squamous epithelial cells express membrane-spanning mucins such as MUC1, MUC4, and MUC16 (Gipson, 2004).

Conjunctival epithelial cells can also respond to different stimuli by producing cytokines and other factors that regulate the ocular response to insults; thus, many of the epithelial cells have important immunomodulatory roles (Zhan et al., 2003). Because they are in contact with the external environment, they are also affected by pathogens, external conditions, and ophthalmic topical drugs.

For those reasons, it is essential to study conjunctival epithelial cells in the pathophysiology of ocular diseases and in ocular drug development (Hosoya et al., 2005). Thus, different *in vitro* models utilizing conjunctival epithelial cells have been described. Frequently, the first screening of drugs is performed in immortalized cell lines. Although results should be taken with caution, cell lines are an important tool for

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initial studies because they are easy to use and able to proliferate nearly without limit. Unfortunately, there are only a few human conjunctival cell lines available. We recently reviewed them (Diebold and García--Posadas, 2021) and found that until now, the most widely used conjunctival cell lines are the Wong-Kilbourne derivative of Chang cells (ChWK) and the spontaneously immortalized epithelial cell line from normal human conjunctiva (IOBA-NHC) cells (Diebold et al., 2003). Much information regarding conjunctival epithelium functioning has been revealed thanks to these cell lines. Sadly, both lines are currently presenting important problems that may negatively influence their usefulness in future studies. For instance, ChWK cells express different markers than primary cultured epithelial cells (Tong et al., 2009), respond differentially to inflammatory cytokines (De Saint Jean et al., 2004), and, in addition, they are cross-contaminated with HeLa cells (Lavappa, 1978). IOBA-NHC cells appear to better resemble primary conjunctival epithelial cells (Tong et al., 2009) and have been widely used to study the conjunctival inflammatory response (Diebold et al., 2003; Enríquez-de-Salamanca et al., 2008). However, recently the line has shown signs of senescence, which has limited its use. Another conjunctival epithelial cell line is ConjEp-1/p53DD/cdk4R/TERT, commonly known as HCjE (Gipson et al., 2003) or, more recently, HCjE-Gi. These cells were derived from human supero-temporal bulbar conjunctiva and were immortalized through transduction with human telomerase reverse transcriptase. HCjE cells consist of both stratified squamous cells as well as some goblet cells that can limit the use of this line in some studies. Their short tandem repeat (STR) profile was recently tested in cells obtained from three different laboratories and, although no cross-contamination was detected, small differences in STR profiles were found (McDermott et al., 2018). Bearing all this in mind, it is extremely important to develop new cell lines that allow researchers to continue with conjunctival research.

Currently, another immortalized human conjunctival epithelial cell line, designated as the "IM-HConEpiC" line, is commercially available. However, the information disclosed by the company is limited, and the characteristics and functional properties of the cell line are not currently available. Thus, the purpose of this study was to optimize the cell culture conditions and protocols for this cell line and to develop easily performed functional studies that characterize its properties.

2. Material and methods

2.1. Human conjunctival epithelial cell line

The IM-HConEpiC line was acquired from Innoprot (Derio, Spain. Ref. P10870-IM). According to the manufacturer, these cells were developed by immortalizing primary human conjunctival cells with the SV40 large T antigen.

2.2. Cell culture conditions

A vial containing more than 1 million IM-HConEpiCs in passage 3 was thawed and cultured in IM-Conjunctival epithelial cell medium (Innoprot) following all of the manufacturer's protocols. The cells were then expanded in collagen I-coated T75 flasks (Corning, Kennebunk, ME, USA), and subcultured after isolation with 0.25% trypsin/EDTA (Gibco, Waltham, MA, USA). After obtaining enough cells, they were cultured on different surfaces and culture media as described below.

First, we tested the capacity of cells to attach to Nunc® EasYFlask™ culture flasks without collagen coating, designated here as "uncoated flasks", obtained from ThermoFisher Scientific (Waltham, MA, USA), and compared the outcomes with the cells cultured in collagen-coated flasks. Then, we evaluated culture performance using 8 different culture media (Table 1). Except for Innoprot's medium, for which the composition is not known, the other formulations were all based on DMEM/F12 + GlutaMax base medium (Invitrogen-Gibco, Inchinnan, UK), supplemented with 10% fetal bovine serum (FBS, Gibco) and 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco). Some, but not all culture media contained insulin (Sigma-Aldrich, St. Louis, MO, USA), hydrocortisone (HC, Sigma-Aldrich), and/or epidermal growth factor (EGF, Invitrogen) (Table 1).

Table 1
Culture media composition.

Name	Base medium	FBS (%)	Pen/Strep (U/ml)/(μg/ml)	EGF (ng/ml)	INS (μg/ml)	HC (μg/ml)
Innoprot	Unknown	10%	50/50	Unknown	Unknown	Unknown
CM + EGF + INS + HC	DMEM/F12 + Glutamax	10%	50/50	2	1	0.5
CM + EGF_{enr} + INS + HC	DMEM/F12 + Glutamax	10%	50/50	10	1	0.5
CM + EGF + HC	DMEM/F12 + Glutamax	10%	50/50	2	–	0.5
CM + EGF_{enr} + HC	DMEM/F12 + Glutamax	10%	50/50	10	–	0.5
CM + EGF + INS	DMEM/F12 + Glutamax	10%	50/50	2	1	–
CM + EGF_{enr} + INS	DMEM/F12 + Glutamax	10%	50/50	10	1	–
CM + EGF_{enr}	DMEM/F12 + Glutamax	10%	50/50	10	–	–

FBS: Fetal bovine serum; Pen/Strep: penicillin/streptomycin; EGF: epidermal growth factor; CM: conjunctival medium; EGF_{enr}: medium enriched with epidermal growth factor (10 ng/ml); INS: insulin; HC: hydrocortisone; DMEM: Dulbecco's modified Eagle's medium.

Cell cultures were maintained at 37 °C and 5% CO_2, and the media were changed every other day. Cell viability after thawing and after each passage was measured using the Trypan blue dye exclusion assay.

2.3. Determination of cell doubling time

To determine the cell doubling time (CDT), cells from passages 3 to 7 were seeded at defined densities and cultivated in collagen-coated or uncoated flasks until reaching 90% confluence. Cells were then trypsinized, harvested, and counted using a TC20™ Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA). The CDT was calculated using the following formula: $CDT = [t \times \ln(2)]/[\ln(Fc) - \ln(Ic)]$, where t = time between plating and passaging, Fc = final concentration of cells, and Ic = initial concentration of cells. At least 4 independent experiments were used for each condition.

2.4. Cell proliferation assay

To test the efficacy of the different culture media, we used three different seeding densities: 2500 cells/cm^2 (low density), 5000 cells/cm^2 (medium density), and 10,000 cells/cm^2 (high density).

Cell proliferation was measured using the AlamarBlue proliferation assay at three time points (24, 48, and 72 h). Cells were incubated with 10% AlamarBlue reagent (BioRad Laboratories) for 3 h, and the fluorescence was then measured at 560 nm excitation and 590 nm emission wavelengths, using the SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). Three independent experiments were performed in triplicates.

2.5. Alcian blue (AB) and periodic acid Schiff (PAS) staining

IM-HConEpiC cells were cultured with CM + EGF_{enr} + INS culture medium on glass slides, fixed with acetone (7 min at room temperature [RT]), and stained with a combination of AB and PAS to identify acidic (stained in blue by AB) and neutral (stained in magenta by PAS) mucins. Briefly, fixed slides were washed in distilled water and then immersed in 3% acetic acid for 3 min. The slides were incubated in AB solution (pH 2.5; Sigma-Aldrich) for 15 min. After that, they were immersed in 0.5% periodic acid for 5 min, rinsed with distilled water, and placed in Schiff's solution (Millipore, Billerica, MA, USA) for 15 min. The slides were then rinsed in running tap water for 10 min, counterstained with Mayer's Hematoxylin (Millipore) for 5 min, and finally rinsed, dehydrated, cleared, and mounted with cover slips.

2.6. Colloidal iron staining

To identify acidic mucins, cells of the IM-HConEpiC line were cultured with CM + EGF_{enr} + INS culture medium on slides and stained with colloidal iron using a commercial kit (Bio-Optica Milano S.p.A., Milano, Italy). In this technique, sialo and sulphated mucins (acid mucins) form a stable complex with trivalent iron that appears as precipitates of Prussian blue. Staining was performed following manufacturer's instructions.

2.7. Immunofluorescent staining

Characteristic markers that are expressed by human conjunctival epithelial cells, as well as the proliferation marker Ki67, were assessed by immunofluorescence microscopy. The selected markers were cytokeratin (CK) 19, CK8+18, mucin MUC4, adherens junction protein E-cadherin, and tight junction protein zonula occludens 1 (ZO-1). For Ki67, cells from passages 6–8 were cultured in 8-well multichamber Permanox slides (Nunc, Roskilde, Denmark) with the different culture media (Table 1). After 48 h of culture, the cells were fixed in cold methanol for 10 min. For the other markers, cells were grown in CM + EGF_{enr} + INS medium and fixed in cold methanol when they reached confluence. After fixation, the cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) for 10 min at RT. Slides were blocked with PBS +5% donkey serum for 1 h at RT, and then incubated with primary antibodies (Table 2) in blocking buffer. After that, the cells were washed three times with PBS, and AlexaFluor plus 488 conjugated secondary antibody (Invitrogen) was applied for 1 h at RT. Cell nuclei were counterstained with Hoechst dye. The preparations were viewed under an epifluorescence microscope (Leica DMI 6000B; Leica Microsystems, Wetzlar, Germany). Negative controls included the omission of primary antibodies. All antibodies had previously been tested in adequate controls. At least three independent experiments were done for each antibody.

2.8. Western blotting

The expressions of cell markers CK19, CK7, E-cadherin, and MUC4 were analyzed by electrophoresis and Western blotting, as previously described (García-Posadas et al., 2013).

Confluent IM-HConEpiCs from passages 14 to 17 cultured with CM + EGF_{enr} + INS medium were homogenized in ice cold radioimmunoprecipitation assay (RIPA) buffer supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (100 μL/ml), aprotinin (6 μL/ml), and sodium orthovanadate (100 nM), all from Sigma-Aldrich. After incubation on ice for 30 min, samples were centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatants were collected, and the protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Lysates were diluted in Laemmli buffer (1:1) and heated at 70 °C for 10 min. Proteins (10 μg) were loaded in 10% polyacrylamide gels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (70 V, 20 min; 140 V, 55 min). They were then transferred to nitrocellulose membranes in a tank at 4 °C for 1.5 h at 350 mA. After blocking the membranes in tris-buffered saline (TBS) with 0.05% Tween-20 (TBST), 5% powder milk, 5% BSA for 1 h at RT, they were incubated with primary antibodies for CK19 (at 1:500 dilution), CK7 (1:500), E-cadherin (1:10000), and MUC4 (1:500) at 4 °C, overnight. Then, the membranes were washed with TBST and incubated with the secondary antibody goat anti-mouse IgG (HRP) (Abcam, Cambridge, UK) for 1 h at RT. Immunoreactive bands were visualized by chemiluminescence detection with the SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an endogenous control. Results were analyzed in the ChemiDoc system (Bio-Rad Laboratories) with the Quantity One software (Bio-Rad Laboratories). Three independent experiments were performed.

2.9. Reverse transcript – polymerase chain reaction (RT-PCR)

Total RNA from cells cultured with CM + EGF_{enr} + INS medium (passages 7, 12, and 20) was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and reverse transcribed to complementary DNA (cDNA) with the SuperScript Vilo cDNA Kit (Invitrogen). Then, RT-PCR for CK19, MUC4, MUC5AC, and GAPDH genes was conducted with 12.5 μl cDNA AmpliTools MasterMix (Biotools, Madrid, Spain), 5 μl of 10 μM primers (Table 3, custom-made by ThermoFisher), and 2 μl cDNA (at 5 ng/μl) in a total volume of 25 μl using the MyGene™ L Series Peltier Thermal Cycler (LongGene® Scientific Instruments Co., Ltd., Hangzhou, China). The protocol consisted of an initial cycle of 2 min at 95 °C, then 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 40 s at 72 °C, followed by a final cycle of 10 min at 72 °C. PCR products were revealed in 2% agarose gels, and 100 bp ladder (Biotools) was used to determine PCR product size. Results were visualized in the ChemiDoc system. The levels of GAPDH for each sample were used as endogenous controls. Non-template controls included the omission of cDNA.

Table 2
Antibodies used in IF and WB experiments.

Antibody	Source	Dilution for IF	Incubation protocol for IF	Dilution for WB	Incubation protocol for WB
Ki67	Dako	1:50	1 h, 37 °C	–	–
CK7	Invitrogen	–	–	1:500	Overnight, 4 °C
CK19	Abcam	1:50	Overnight, 4 °C	1: 500	Overnight, 4 °C
E-Cadherin	BD Transduction Lab	1:100	Overnight, 4 °C	1:10,000	Overnight, 4 °C
MUC4	Invitrogen	1:50	Overnight, 4 °C	1:500	Overnight, 4 °C
Ck8+CK18	Abcam	1:50	1 h, 37 °C	–	–
ZO-1	Invitrogen	1:100	1 h, 37 °C	–	–
GAPDH	Santa Cruz Biotechnology Inc.	–	–	1:500	Overnight, 4 °C

IF, immunofluorescence microscopy; WB, Western blotting; CK, cytokeratin; MUC, mucin; ZO-1, zonula occludens 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Table 3
Primers used in PCR.

Primer	Forward	Reverse	Product size
CK19	CCAAGGCAGGACCCATCTCTC	GAGTCACAACCCCCTCTCCCTAT	502
MUC4	CCTAATGCTCCACTGCTGCCC	CAGCTGAGGCCTTGCCTTGA	549
MUC5AC	CCACTGGTTCTATGGCAACACC	GCCGAAGTCCAGGCTGTGCG	313
GAPDH	GAACGTGAAGGTCGGAGTCAAC	CGTGAAGATGGTGATGGGATTTC	230

2.10. Measurement of ascorbic acid antioxidant activity

The production of reactive oxygen species (ROS) is the cause of oxidative stress that is involved in different pathologies affecting the conjunctiva. Oxidative stress can be inhibited by using different substances with antioxidant activity, such as ascorbic acid (vitamin C). The ability of ascorbic acid to scavenge ROS in IM-HConEpiCs was assessed by H_2DCF-DA (Sigma-Aldrich), which passively diffuses into the cells and indicates ROS upon conversion to the fluorescent metabolite dichlorofluorescein (DCF).

IM-HConEpiCs in passages 14–17 were seeded into 24-well plates (60,000 cells/well) and cultured in CM + EGF_{enr} + INS medium for 24 h until the cells reached 90% confluence. After this, the medium was changed to serum-free medium, and the cells were incubated for an additional 24 h. Then, the cells were pre-treated with 25–500 μM ascorbic acid at 37 °C for 1 h. The supernatants were then discarded, and 10 μM H_2DCF-DA was added and incubated for 30 min. The H2DCF-DA was then removed, and the cells were treated again with ascorbic acid at the same concentrations as before and exposed to 8-W UV-B lamp for 15 s. Control cultures were not irradiated. After UV-B exposure, the cells were cultured for 1 h. Fluorescence intensity was measured at 488 nm excitation and 522 nm emission wavelengths. The data obtained from the fluorescence measurements were normalized by the total protein content, which was measured in adherent cells by the BCA assay. Three independent experiments were performed in triplicates.

2.11. Induction and measurement of inflammatory response

To induce an inflammatory response, the cells were seeded in 24-well plates (550,000 cells/cm^2) and grown in CM + EGF_{enr} + INS culture medium until they reached 90% confluence. The medium was then replaced by DMEM/F12 + GlutaMax without supplements. After 24 h, the cells were stimulated with 25 ng/ml TNF-α for 24 h, at 37 °C. Non-stimulated cells were used as controls. After incubation, the culture medium was collected and centrifuged at 18,000 g for 10 min. The supernatant was stored at −80 °C until use. Adhered cells were used for protein extraction, to normalized results. Three independent experiments were performed in duplicates.

IL-1β, IL-6, IL-8, IL-17A, and IP-10 cytokines and chemokines were measured in the collected supernatants by a multiplex bead-based array, using a commercial Milliplex 5-plex Human Cytokine/Chemokine immunobead-based kit (HCYTOMAG-60K), as previously described (Enríquez-de-Salamanca et al., 2008). Briefly, 25 μL of each supernatant was incubated with antibody-immobilized magnetic beads at 4 °C overnight. The beads were then washed, and a detection antibody solution (biotinylated cytokine/chemokine antibodies) was added to the plate and incubated for 1 h at RT. After that, a streptavidin-phycoerythrin solution was added and incubated for 30 min at RT. Finally, the beads were washed and resuspended in sheath fluid for transportation to the instrument's optics. The plate was read in a Luminex 100-IS (Luminex Corporation, Austin, TX, USA). Calibration curves of each human cytokine/chemokine standard were performed, converting fluorescence to cytokine/chemokine concentration units (pg/mL) using the BeadView Software (Upstate-Millipore Corporation, Watford, UK). The cytokine concentration data were normalized to the corresponding protein content of each well, determined by the BCA protein assay. Three independent experiments were performed in duplicates.

2.12. Statistical analysis

For quantitative results, data were expressed as means ± standard deviation (SD). The *t*-test was performed when comparing two groups. To compare more than two groups, one-way analysis of variance (ANOVA) was used. Then, post-hoc comparisons were performed with Tukey's or Games-Howell tests. P-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Morphological description, cell attachment, and growth

Cultured cells were rounded when thawed, but they adhered quickly to the culture surface. Most adhered cells were small and polygonal in shape, although a minority of cells had a more elongate shape. Each cell had a single nucleus, and there were no apparent large vacuoles in the cytoplasm (Fig. 1).

A comparison of cultures in collagen-coated flasks (Fig. 1A) and uncoated flasks (Fig. 1B) revealed no differences in cell morphology or in CDT (40.33 ± 5.67 h in collagen-coated flasks *vs* 41.88 ± 4.97 h in uncoated flasks, P = 0.64). Because of the simplicity of using uncoated flasks and to avoid the risk of introducing variability through the use of collagen coatings, we continued our experiments with uncoated flasks.

3.2. Cell proliferation with different culture media

The first expansion of the IM-HConEpiCs was done with the culture medium recommended by the manufacturer. However, the main inconvenience of this medium is that the full composition was not disclosed by the manufacturer. Because a lot of the research performed using conjunctival cell lines is devoted to testing new drugs, it is paramount to know the exact composition of each medium or supplement used during cell culture. For that reason, different culture media were tested.

IM-HConEpiCs were successfully cultured with all tested media. The cells expanded well, with no evident morphological changes. While the expansion of cells in the Innoprot medium was the highest, the proliferation was not significantly different from the other media at any time period tested, regardless of the initial plating cell density (Fig. 2). At medium cell density (5000 cells/cm^2), CM + EGF_{enr} + INS culture medium showed very similar proliferation rates, specially at 24 h and 72 h.

In addition, we analyzed the expression of the proliferation marker Ki67 by immunofluorescent staining (Fig. 3). Many cells were positive for Ki67 in all different conditions, as expected for an immortalized cell line. There were no differences between the percentage of Ki67 positive cells when IM-HConEpiCs were cultured with Innoprot medium (58.3 ± 5.3%) or with the other culture media. Although not significantly different, CM + EGF_{enr} + INS culture medium showed the highest percentage of positive cells (78.9 ± 10.5%).

Bearing all this in mind, CM + EGF_{enr} + INS culture medium was selected to culture IM-HConEpiCs, since it showed adequate proliferation and Ki67 expression rates.

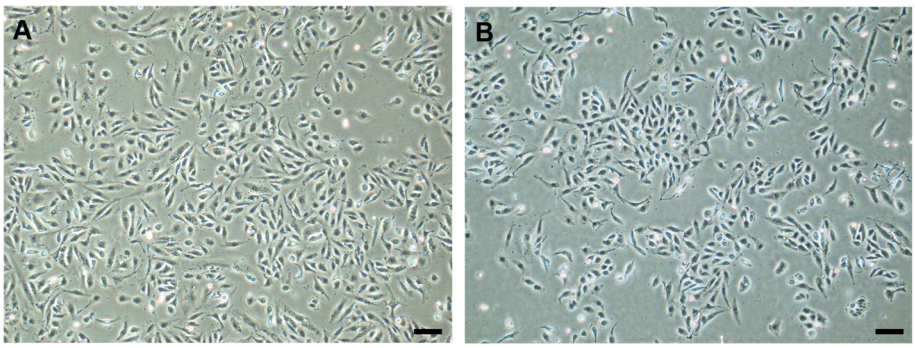

Fig. 1. Morphology of IM-HConEpiCs after 48 h in culture. A) Cells grown in collagen coated flasks. B) Cells grown in uncoated flasks. Bar = 100 μm.

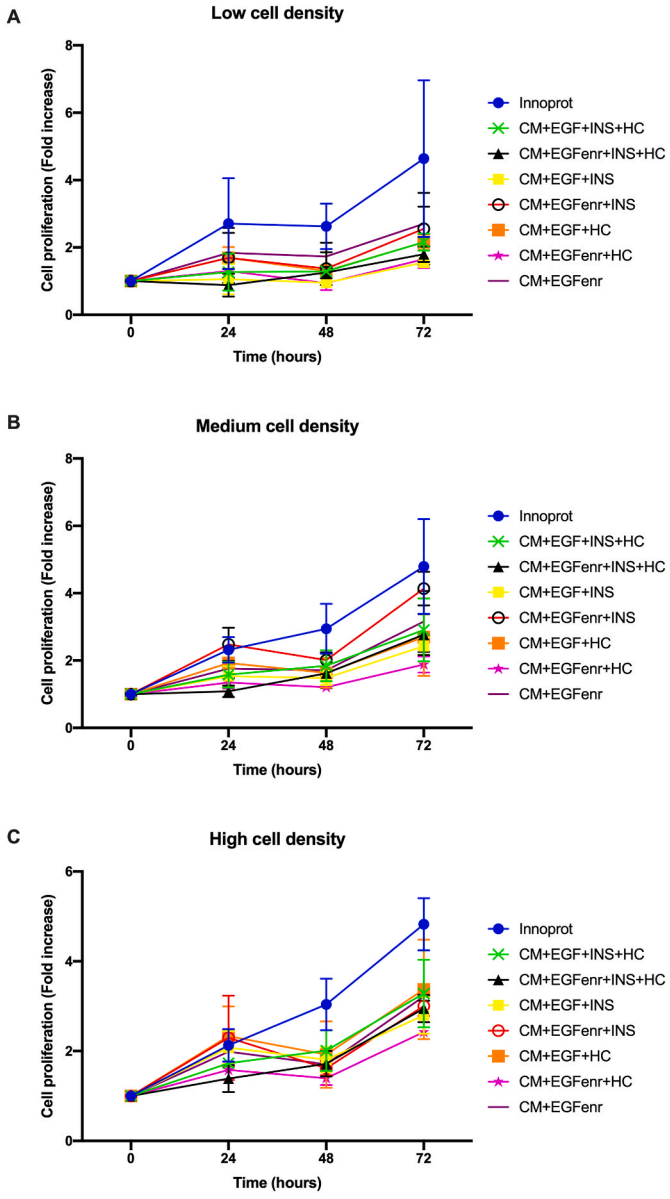

Fig. 2. IM-HConEpiC proliferation with different culture media. Cells were seeded at three different cell densities: A) low density (2500 cells/cm^2), B) medium density (5000 cells/cm^2), and C) high density (10,000 cells/cm^2). CM: conjunctival medium; EGF: epidermal growth factor (2 ng/ml); EGF_{enr}: medium enriched with epidermal growth factor (10 ng/ml); INS: insulin (1 μg/ml); HC: hydrocortisone (0.5 μg/ml).

3.3. Mucin production

Conjunctival epithelial cells produce mucins that can be detected by histochemical methods. AB/PAS staining showed that the IM-HConEpiCs produced mucins (Fig. 4A). Most of the cells were stained in magenta, indicating the presence of neutral mucins. The granules were clearly identified in the cytoplasm. A minority of cells were stained purple, indicating a mixture of acidic and neutral mucins. None of the cells were stained exclusively with AB. To corroborate the presence of acidic mucins, staining with colloidal iron staining revealed the presence of Prussian blue granules in the cytoplasm (Fig. 4B).

3.4. IM-HConEpiCs cultured with CM + EGF_{enr} + INS medium expressed conjunctival epithelial cell markers

To confirm that cells cultured in our selected conditions (uncoated flasks and CM + EGF_{enr} + INS medium) maintained the conjunctival epithelial phenotype, expressions of different cell marker proteins were analyzed by immunofluorescent detection and by electrophoresis and western blotting. Immunofluorescent staining revealed expression of CK8+CK18, CK19, MUC4, E-cadherin, and ZO-1 (Fig. 5). In addition, the expressions of CK7, CK19, mucin MUC4 and the adherens junction protein E-cadherin were confirmed in western blots (Fig. 6).

3.5. Gene expression

Gene expression of CK19 was analyzed to determine if the cultured cells were of conjunctival epithelial origin. IM-HConEpiCs in passages 7, 12, and 20 (Fig. 7A) cultured with CM + EGF_{enr} + INS medium expressed an intense band of approximately 250 bp, indicating the presence of GAPDH PCR products in the agarose gel. The equivalence of the three bands indicated that the same amount of cDNA was loaded onto the gel. For the same samples, a single band of approximately 500 bp was present in the CK19 PCR product lanes. The expected size of the product was 502 bp. Therefore, we confirmed CK19 gene expression in IM-HConEpiC cells at passages 7, 12, and 20, indicating the conjunctival epithelial origin of the cells grown with CM + EGF_{enr} + INS culture medium.

Using a similar approach for the expression of conjunctival mucins at passages 12 and 20 (Fig. 7B), three different bands of MUC4 were detected, indicating the presence of stratified squamous epithelial cells. In contrast, MUC5AC was not detected, suggesting that no goblet cells were present in the IM-HConEpiC cultures (Fig. 7B).

3.6. Intracellular antioxidant capacity of ascorbic acid in IM-HConEpiCs

The eye is exposed to the external environment, and of particular interest regarding the conjunctiva is UV light that can generate ROS. For that reason, we analyzed the production of ROS within IM-HConEpiCs in response to UV light. To confirm that IM-HConEpiCs respond to

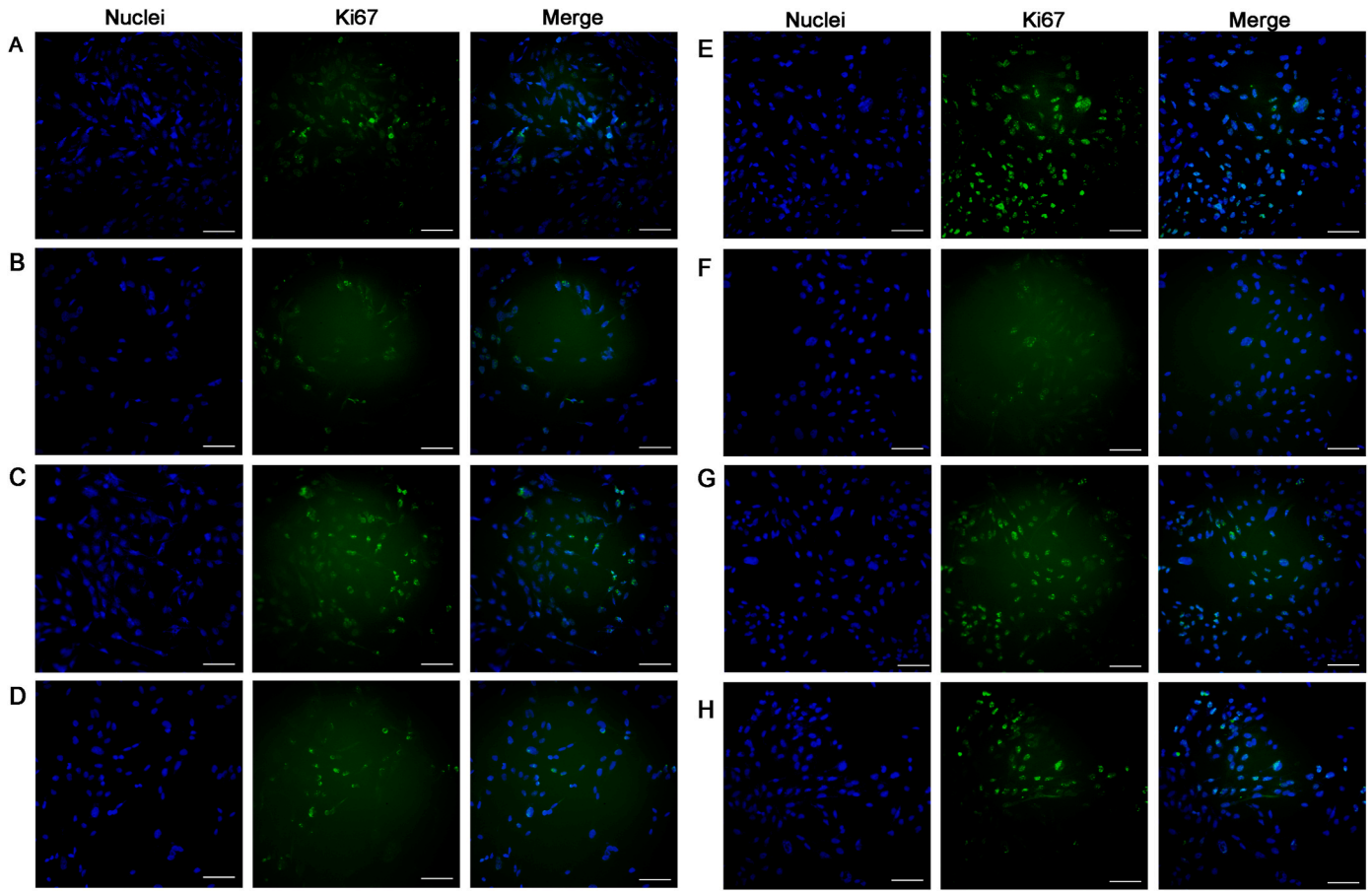

Fig. 3. Expression of Ki67 in IM-HConEpiCs grown with different culture media. A) Innoprot medium. B) CM + EGF + INS + HC medium. C) CM + EGF_{enr} + INS + HC medium. D) CM + EGF + INS medium. E) CM + EGF_{enr} + INS medium. F) CM + EGF + HC medium. G) CM + EGF_{enr} + HC medium. H) CM + EGF_{enr} medium. The amount of Ki67 expression was similar among all of the culture media. Bar = 100 μm. CM: conjunctival medium; EGF: epidermal growth factor (2 ng/ml); EGF_{enr}: medium enriched with epidermal growth factor (10 ng/ml); INS: insulin (1 μg/ml); HC: hydrocortisone (0.5 μg/ml).

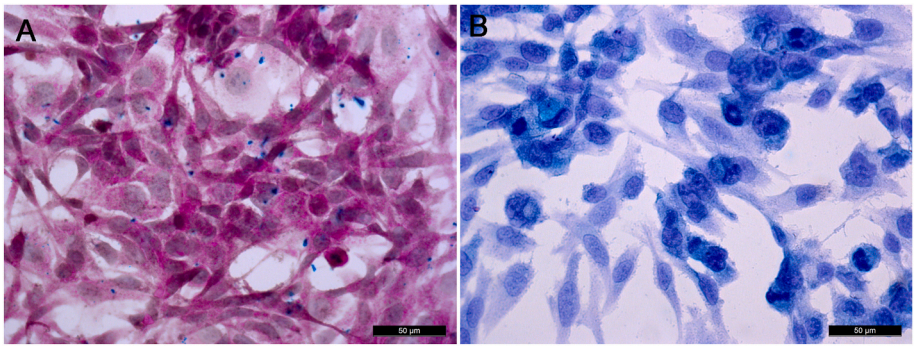

Fig. 4. IM-HConEpiCs stained with AB/PAS and colloidal iron. A) Neutral mucous granules were stained magenta by PAS reactivity. A minority of cells were stained purple by combination of AB and PAS staining, indicating a mixture of acidic and neutral mucins. B) Sialo and sulphated mucin acidic granules were indicated by the presence of intense blue following staining with colloidal iron. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

oxidative stress, we used ascorbic acid as the antioxidant control. When the IM-HConEpiCs were exposed to UV-B, they responded by increasing the production of ROS (Fig. 8). When these cells were pre-treated with ascorbic acid, ROS production was significantly decreased in a dose-dependent manner.

3.7. Inflammatory response of IM-HConEpiCs

Conjunctival epithelial cells respond to inflammatory stimuli by secreting different cytokines and chemokines. Stimulation of the IM-HConEpiCs with 25 ng/ml TNF-α increased the secretion of IL-6, IL-8, IP-10, and IL-1β ($P < 0.001$ for all, Fig. 9); however it did not increase the secretion of IL-17.

4. Discussion

In this study, we analyzed the morphological and functional characteristics of a commercially available human conjunctival epithelial cell line, IM-HConEpiC. In addition, we described a new protocol for culturing the cells in flasks that were not coated with collagen and for using cell culture media that were different from the one recommended by the company.

Coating culture dishes with collagen promotes adhesion of cells (Kleinman et al., 1981; Somaiah et al., 2015). However, the use of

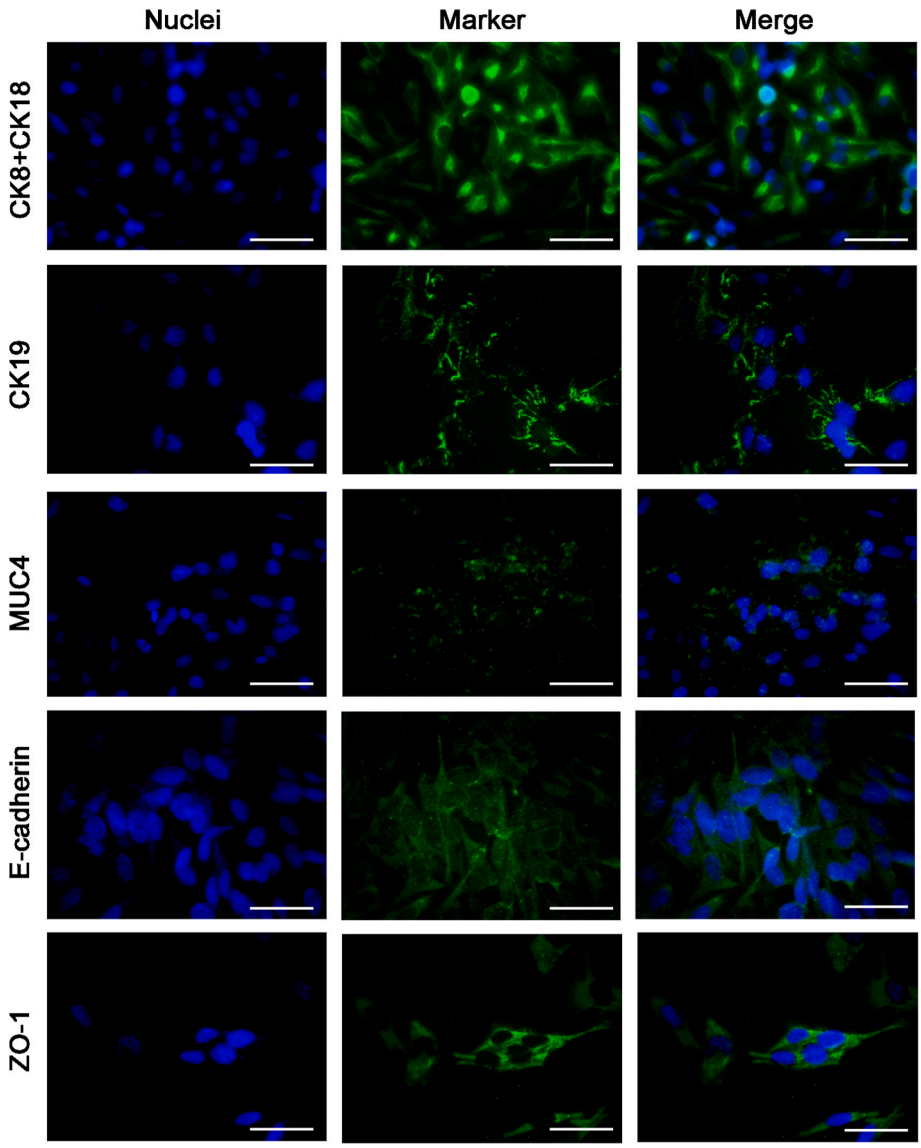

Fig. 5. Characterization of cultured IM-HConEpiCs by immunofluorescence microscopy. The cells expressed CK8+CK18, CK19, MUC4, E-cadherin, and ZO-1. Bar = 50 μm.

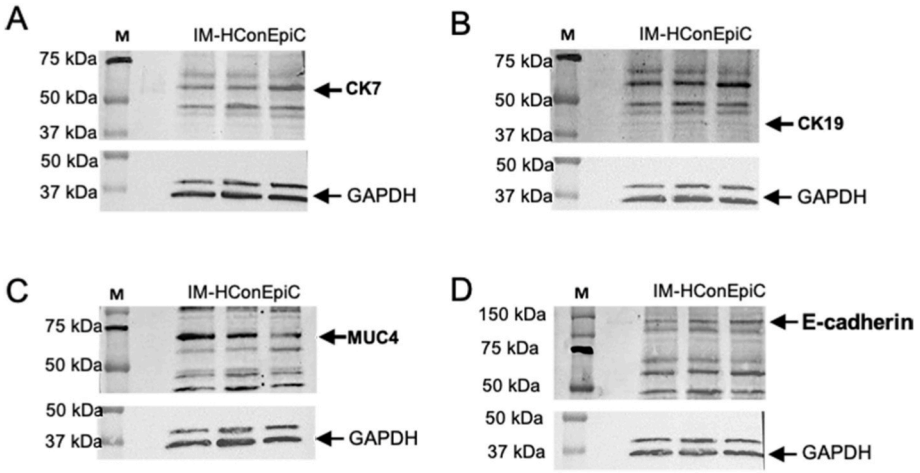

Fig. 6. Characterization of cultured IM-HConEpiCs by electrophoresis and Western blotting. A) Expression of CK7 is observed at 51 KDa. B) Expression of CK19 at 40 KDa. C) Expression of MUC4 at 73 KDa. D) Expression of E-cadherin at 120 KDa n = 3.

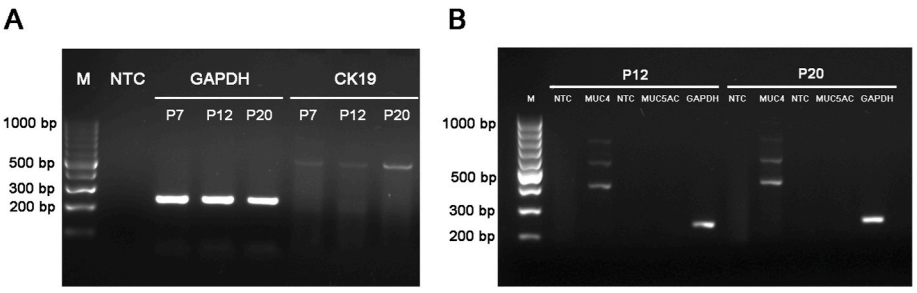

Fig. 7. Characterization of cultured IM-HConEpiCs by PCR. A) PCR showing GAPDH and CK19 expression in cultured IM-HConEpiCs in passages 7, 12, and 20. For all cell passages, a single band was present for GAPDH at approximately 250 bp, and for CK19 at approximately 500 bp. B) PCR showing MUC4, MUC5AC, and GAPDH gene expression. Several bands were present for MUC4, whereas no expression was detected for MUC5AC. The GAPDH band was present at 250 bp. Bp: base pairs. M: base pair marker. NTC: non-template control. P: cell passage.

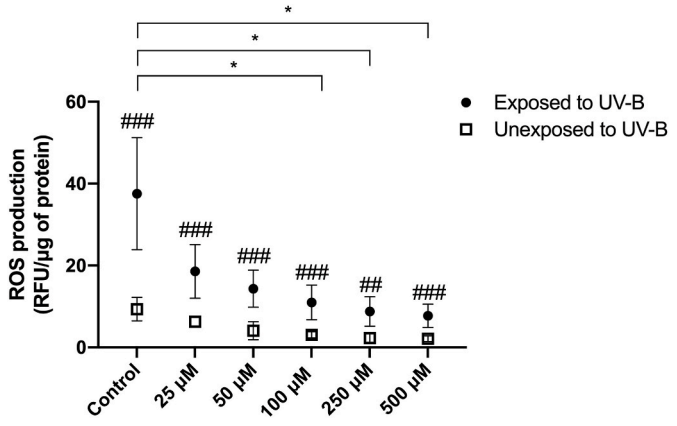

Fig. 8. Antioxidant activity of ascorbic acid in UV-B exposed and unexposed IM-HConEpiCs. *P < 0.05, **P < 0.01, ***P < 0.001 in comparison to the control (cells treated with medium alone) and then exposed to UV-B; #P < 0.05, ##P < 0.01, ###p < 0.001 intergroup comparison to cells not exposed to UV-B. ROS, reactive oxygen species; RFU, relative fluorescence units.

collagen in this way has some important drawbacks. First, the collagen, typically derived from rat tail tendon, is unsuitable for cultures that must exclude animal products. A second important limitation is the high batch-to-batch variability that occurs whether it is derived from animal or non-synthetic origins (Caliari and Burdick, 2016). This variability can result in unnecessary and distracting variations in experimental outcomes. In our first experiments, we observed that cells of the IM-HConEpiC line adhered equally well to collagen-coated flasks and uncoated flasks. After 7 days in culture with the Innoprot medium, there were no differences between the cells growing in collagen coated flasks and uncoated flasks with respect to cell morphology or CDT. For all of those reasons as well as the savings in time and cost, we decided to culture our cells in uncoated flasks for the remaining experiments.

Cell culture medium is a mixture of different nutrients and growth factors with a composition that is essential for adequate cell growth (Price, 2017). Each cell type has different requirements, and therefore, culture media should be selected accordingly. IM-HConEpiCs are commercialized with a suggested culture medium (referred to as Innoprot medium in this study). However, the composition of that medium has not been made available to researchers. Most of our research is devoted to understanding the responsiveness of the conjunctival epithelium to changes in the external environment. Thus, for the experiments reported here, it is essential that we know the exact composition of that environment, i.e., the culture medium. For that reason, we analyzed the effects of different culture media. All of them used DMEM/F12 + GlutaMax as the basal media because it is the one usually employed for culturing conjunctival epithelial cells, either primary cultures (Diebold et al., 1997; García-Posadas et al., 2013; Schrader et al., 2009) or other conjunctival epithelial cell lines, such as IOBA-NHC (Diebold et al., 2003). While higher proliferation rates were obtained with the Innoprot medium, they were not significantly different to those obtained with the other culture media. Proliferation with the CM + EGF_{enr} + INS medium was very similar to the Innoprot medium, especially at 5000 cell/cm^2 cell density. In this medium, in addition to antibiotics and FBS (added to all of the cell culture media), we included insulin and EGF as supplements. Insulin is frequently added at high concentrations to culture media. At those concentrations, which are higher than physiological values, it acts as a growth factor, with mitogenic and anti-apoptotic effects (Hill and Milner, 1985). EGF is also regularly used in mammalian cell culture due to its important mitogenic activity along with a role in suppressing cellular senescence (Alexander et al., 2015). It is noteworthy that there were no differences in Ki67 expression in cells cultured with the different media, indicating that the proliferative capacity was maintained with all of the tested media. However, it is important to maintain a balance between proliferation and differentiation (Ruijtenberg and van den Heuvel, 2016). For that reason, we continued the rest of our experiments using the CM + EGF_{enr} + INS medium.

Conjunctival epithelial cells produce mucins (Dartt, 2002; Gipson and Argüeso, 2003). We corroborated that IM-HConEpiCs produced mucins by applying AB/PAS and colloidal iron stains. PAS reactivity stained the cells magenta, indicating the production of neutral mucins. A minority of cells were stained purple, indicating a mixture of acidic and neutral mucins. Secondary support for the production of acid mucins was provided by colloidal iron staining. In addition, the expression of the transmembrane mucin MUC4, typical of conjunctival stratified squamous epithelial cells, was confirmed by Western blot and

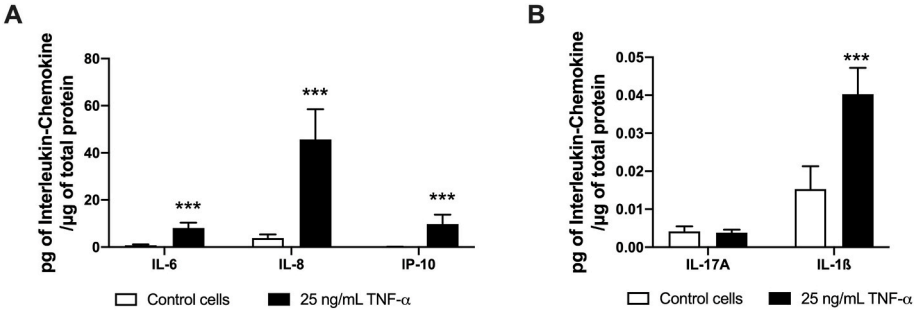

Fig. 9. TNF-α-induced cytokine release model of cultured IM-HConEpiCs. Cells were treated with cell culture medium (control cells, white bars) or 25 ng/ml TNF-α for 24 h (stimulated cells, black bars). A) A statistically significant increase of IL-6, IL-8, and IP-10 secretion occurred following TNF-α stimulation. B) IL-1β secretion was also significantly increased after TNF-α stimulation. However, TNF-α failed to stimulate IL-17A secretion. Normalized cytokine/chemokine secretion, picograms (pg) per total protein (pg/µg). Mean ± SD; n = 3. ***P < 0.001, compared to control cells.

immunofluorescence microscopy. Finally, MUC5AC expression was tested by PCR, but it was not detected, suggesting that the IM-HConEpiCs were typical of conjunctival squamous stratified epithelial cells and not goblet cells.

Cytokeratins are intermediate filament cytoskeletal proteins. Because the pattern of cytokeratin expression is characteristic of each tissue, they have been widely used as cell markers. They are also expressed in a differentiation-dependent manner. For that reason, different layers of the same tissue can present a different staining pattern. The datasheet provided by Innoprot, the company that commercializes IM-HConEpiCs, indicates that these cells are positive for CK19 and CK8. CK19 is one of the most widely accepted markers for conjunctival epithelial cells because it is strongly expressed in all layers of conjunctival epithelium (Merjava et al., 2011b). We confirmed the expression of CK19 mRNA and protein in the IM-HConEpiCs cultured with CM + EGF_{enr} + INS medium. The CK8+CK18 pair is typically found in simple epithelia. Nevertheless, several authors have reported its presence in the stratified conjunctival epithelium (Krenzer and Freddo, 1997; Merjava et al., 2011a, 2011b). We confirmed the expression of CK8+CK18 in IM-HConEpiCs when cultured following our protocol using the uncoated flasks and CM + EGF_{enr} + INS medium. Other cytokeratins have also been studied in the conjunctiva. CK7 is a typical cytokeratin in glandular epithelium. However, it was described in the conjunctiva by Krenzer and Fredo in 1997 (Krenzer and Freddo, 1997). They detected CK7 in the superficial layers of the conjunctiva and initially suggested that it may be a marker of conjunctival goblet cells. However, other authors showed that CK7 can be used to detect conjunctival epithelium in general, and not specifically goblet cells (Jirsova et al., 2011). In this study we showed that IM-HConEpiCs express CK7, and along with CK19, confirm the conjunctival phenotype of these cells.

Finally, there are many other proteins apart from cytokeratins that can be used to characterize a particular cell type. We analyzed the expression of the adherens junction transmembrane glycoprotein E-cadherin that mediates cell-cell junctions and is expressed in all layers of conjunctival epithelium (Scott et al., 1997). We also analyzed the presence of the tight junction protein ZO-1 due to its importance in the epithelial barrier function of the ocular surface and its relationship with pathologies such as ocular allergy (Singh et al., 2021). ZO-1 is a peripheral membrane protein that forms part of each tight junction and is important in the maintenance of barrier function and cell polarity. It is also expressed in conjunctival epithelium (Yoshida et al., 2009). We previously demonstrated that E-cadherin and ZO-1 were present in primary cultures of human conjunctival epithelial cells (Laura García-Posadas et al., 2013). In this study, we confirmed that cultured IM-HConEpiCs also express both adhesion proteins.

Oxidative stress represents an imbalance in the generation of free radical species and the counterbalancing anti-oxidative mechanisms. The direct exposure of the ocular surface to the external environment, including UV radiation, is responsible for several ocular diseases that are a consequence of oxidative stress (Dogru et al., 2018; Navel et al., 2022). To test novel therapeutic approaches that are able to counteract oxidative stress, it is important to have a reliable assay. As a first step in designing such an assay, the use of established and distinct conjunctival cell lines is critical. Therefore, our aim was to determine if cultures of IM-HConEpiCs are suitable as a cell model to test antioxidant properties of potential or proven antioxidant molecules. Ascorbic acid was selected as a model of an antioxidant molecule (Njus et al., 2020). The tested concentration range, 25–500 μM, effectively scavenged in a dose dependent manner free radicals that were generated in the cells upon exposure to UV-B light. Our results suggest that cultures of IM-HConEpiCs will provide a suitable *in vitro* model upon which to test the efficacy of novel antioxidative molecules.

The conjunctiva plays a significant role in the immune response of the ocular surface. This is due not only to the presence of conjunctival-associated lymphoid tissue (CALT) (Knop and Knop, 2000), but also to the epithelial cell responses to immunological challenge (Enríquez-de-Salamanca et al., 2008; Gamache et al., 1997). Inflammatory stimuli induce the production and/or secretion of different cytokines, such as IL-6 or IL-8, and others. In an *in vitro* model of conjunctival inflammation developed using the IOBA-NHC cell line, TNF-α induced the greatest effect on cytokine secretion by the epithelial cells (Enríquez-de-Salamanca et al., 2008). Specifically, after 2 h treatment, IL-6, IL-8, and IP-10 were preferentially increased. Those cytokines are increased in inflammatory pathologies affecting the ocular surface (Enríquez-de-Salamanca and Calonge, 2008). In this study we showed that the IM-HConEpiC line responded in a similar way, thus, making it useful to perform inflammatory studies.

In summary, in this study we characterized IM-HConEpiCs and established a new protocol to culture them, using uncoated flasks instead of collagen-coated ones, and using a well-defined culture medium. Our results showed that cells cultured under these conditions proliferate, express specific conjunctival markers, and respond appropriately to oxidative and inflammatory stresses as characteristic of conjunctival epithelium. Therefore, IM-HConEpiC represent a new and useful tool for the initial studies of conjunctival epithelium pathophysiology and drug screening.

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Declaration of competing interest

None. All authors want to make it clear that we have no relationship with Innoprot, the company that commercialized the cell line described in this study.

Data availability

Data will be made available on request.

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References

Alexander, P.B., Yuan, L., Yang, P., Sun, T., Chen, R., Xiang, H., Chen, J., Wu, H., Radiloff, D.R., Wang, X.F., 2015. EGF promotes mammalian cell growth by suppressing cellular senescence. Cell Res. 25, 135–138. https://doi.org/10.1038/cr.2014.141.

Caliari, S.R., Burdick, J.A., 2016. A practical guide to hydrogels for cell culture. Nat. Methods 13, 405–414. https://doi.org/10.1038/nmeth.3839.

Dartt, D.A., 2002. Regulation of mucin and fluid secretion by conjunctival epithelial cells. Prog. Retin. Eye Res. 21, 555–576. https://doi.org/10.1016/S1350-9462(02)00038-1.

De Saint Jean, M., Baudouin, C., Di Nolfo, M., Roman, S., Lozato, P., Warnet, J.M., Brignole, F., 2004. Comparison of morphological and functional characteristics of primary-cultured human conjunctival epithelium and of Wong-Kilbourne derivative of Chang conjunctival cell line. Exp. Eye Res. 78, 257–274. https://doi.org/10.1016/j.exer.2003.10.006.

Diebold, Y., Calonge, M., De Salamanca, A.E., Callejo, S., Corrales, R.M., Sáez, V., Siemasko, K.F., Stern, M.E., 2003. Characterization of a spontaneously immortalized cell line (IOBA-NHC) from normal human conjunctiva. Investig. Ophthalmol. Vis. Sci. 44, 4263–4274. https://doi.org/10.1167/iovs.03-0560.

Diebold, Y., Calonge, M., Fernandez, N., Lázaro, M.C., Callejo, S., Herreras, J.M., Pastor, J.C., 1997. Characterization of epithelial primary cultures from human conjunctiva. Graefe's Arch. Clin. Exp. Ophthalmol. 235, 268–276. https://doi.org/10.1007/BF01739635.

Diebold, Y., García-Posadas, L., 2021. Is the conjunctiva a potential target for advanced therapy medicinal products? Pharmaceutics 13, 1140. https://doi.org/10.3390/pharmaceutics13081140.

Dogru, M., Kojima, T., Simsek, C., Tsubotav, K., 2018. Potential role of oxidative stress in ocular surface inflammation and dry eye disease. Investig. Ophthalmol. Vis. Sci. 59, DES163–DES168. https://doi.org/10.1167/iovs.17-23402.

Enríquez-de-Salamanca, A., Calder, V., Gao, J., Galatowicz, G., García-Vázquez, C., Fernández, I., Stern, M.E., Diebold, Y., Calonge, M., 2008. Cytokine responses by conjunctival epithelial cells: an in vitro model of ocular inflammation. Cytokine 44, 160–167. https://doi.org/10.1016/j.cyto.2008.07.007.

Enríquez-de-Salamanca, A., Calonge, M., 2008. Cytokines and chemokines in immune-based ocular surface inflammation. Expet Rev. Clin. Immunol. 4, 457–467. https://doi.org/10.1586/1744666X.4.4.457.

Gamache, D.A., Dimitrijevich, S.D., Weimer, L.K., Lang, L.S., Spellman, J.M., Graff, G., Yanni, J.M., 1997. Secretion of proinflammatory cytokines by human conjunctival epithelial cells. Ocul. Immunol. Inflamm. 5, 117–128. https://doi.org/10.3109/09273949709085060.

García-Posadas, L., Arranz-Valsero, I., López-García, A., Soriano-Romaní, L., Diebold, Y., 2013. A new human primary epithelial cell culture model to study conjunctival inflammation. Investig. Ophthalmol. Vis. Sci. Vis. Sci. 54, 7143–7152. https://doi.org/10.1167/iovs.13-12866.

García-Posadas, Laura, Arranz-Valsero, I., López-García, A., Soriano-Romaní, L., Diebold, Y., 2013. A new human primary epithelial cell culture model to study conjunctival inflammation. Invest. Ophthalmol. Vis. Sci. 54, 7143–7152. https://doi.org/10.1167/iovs.13-12866.

Gipson, I.K., 2004. Distribution of mucins at the ocular surface. Exp. Eye Res. 78, 379–388. https://doi.org/10.1016/S0014-4835(03)00204-5.

Gipson, I.K., Argüeso, P., 2003. Role of mucins in the function of the corneal and conjunctival epithelia. Int. Rev. Cytol. 231, 1–49. https://doi.org/10.1016/S0074-7696(03)31001-0.

Gipson, I.K., Spurr-Michaud, S., Argüeso, P., Tisdale, A., Ng, T.F., Russo, C.L., 2003. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. Investig. Ophthalmol. Vis. Sci. 44, 2496–2506. https://doi.org/10.1167/iovs.02-0851.

Hill, D.J., Milner, R.D.G., 1985. Insulin as a growth factor. Pediatr. Res. 19, 879–886. https://doi.org/10.1203/00006450-198509000-00001.

Hosoya, K.I., Lee, V.H.L., Kim, K.J., 2005. Roles of the conjunctiva in ocular drug delivery: a review of conjunctival transport mechanisms and their regulation. In: European Journal of Pharmaceutics and Biopharmaceutics, pp. 227–240. https://doi.org/10.1016/j.ejpb.2004.12.007.

Jirsova, K., Dudakova, L., Kalasova, S., Vesela, V., Merjava, S., 2011. The OV-TL 12/30 clone of anti-cytokeratin 7 antibody as a new marker of corneal conjunctivalization in patients with limbal stem cell deficiency. Investig. Ophthalmol. Vis. Sci. 52, 5892–5898. https://doi.org/10.1167/iovs.10-6748.

Kleinman, H.K., Klebe, R.J., Martin, G.R., 1981. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88 (3), 473–485. https://doi.org/10.1083/jcb.88.3.473.

Knop, N., Knop, E., 2000. Conjunctiva-associated lymphoid tissue in the human eye. Investig. Ophthalmol. Vis. Sci. 41, 1270–1279.

Krenzer, K.L., Freddo, T.F., 1997. Cytokeratin expression in normal human bulbar conjunctiva obtained by impression cytology. Investig. Ophthalmol. Vis. Sci. 38, 142–152.

Lavappa, K.S., 1978. Survey of ATCC stocks of human cell lines for hela contamination. In Vitro 14, 469–475. https://doi.org/10.1007/BF02616110.

McDermott, A.M., Baidouri, H., Woodward, A.M., Kam, W.R., Liu, Y., Chen, X., Ziemanski, J.F., Vistisen, K., Hazlett, L.D., Nichols, K.K., Argüeso, P., Sullivan, D.A., 2018. Short tandem repeat (STR) profiles of commonly used human ocular surface cell lines. Curr. Eye Res. 43, 1097–1101. https://doi.org/10.1080/02713683.2018.1480043.

Merjava, S., Brejchova, K., Vernon, A., Daniels, J.T., Jirsova, K., 2011a. Cytokeratin 8 is expressed in human corneoconjunctival epithelium, particularly in limbal epithelial cells. Investig. Ophthalmol. Vis. Sci. 52, 787–794. https://doi.org/10.1167/iovs.10-5489.

Merjava, S., Neuwirth, A., Tanzerova, M., Jirsova, K., 2011b. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. Histol. Histopathol. 26, 323–331. https://doi.org/10.14670/HH-26.323.

Navel, V., Sapin, V., Henrioux, F., Blanchon, L., Labbé, A., Chiambaretta, F., Baudouin, C., Dutheil, F., 2022. Oxidative and antioxidative stress markers in dry eye disease: a systematic review and meta-analysis. Acta Ophthalmol. 100, 45–57. https://doi.org/10.1111/aos.14892.

Njus, D., Kelley, P.M., Tu, Y.J., Schlegel, H.B., 2020. Ascorbic acid: the chemistry underlying its antioxidant properties. Free Radic. Biol. Med. 159, 37–43. https://doi.org/10.1016/j.freeradbiomed.2020.07.013.

Price, P.J., 2017. Best practices for media selection for mammalian cells. Vitro Anim. Cell Dev. Biol. 53, 673–681. https://doi.org/10.1007/s11626-017-0186-6.

Ruijtenberg, S., van den Heuvel, S., 2016. Coordinating cell proliferation and differentiation: antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle 15, 196–212. https://doi.org/10.1080/15384101.2015.1120925.

Schrader, S., Notara, M., Beaconsfield, M., Tuft, S., Geerling, G., Daniels, J.T., 2009. Conjunctival epithelial cells maintain stem cell properties after long-term culture and cryopreservation. Regen. Med. 4, 677–687. https://doi.org/10.2217/rme.09.39.

Scott, R.A.H., Lauweryns, B., Snead, D.M.J., Haynes, R.J., Mahida, Y., Dua, H.S., 1997. E-cadherin distribution and epithelial basement membrane characteristics of the normal human conjunctiva and cornea. Eye 11, 607–612. https://doi.org/10.1038/eye.1997.163.

Singh, N., Diebold, Y., Sahu, S.K., Leonardi, A., 2021. Epithelial barrier dysfunction in ocular allergy. Allergy Eur. J. Allergy Clin. Immunol. https://doi.org/10.1111/all.15174.

Somaiah, C., Kumar, A., Mawrie, D., Sharma, A., Patil, S.D., Bhattacharyya, J., Swaminathan, R., Jaganathan, B.G., 2015. Collagen promotes higher adhesion, survival and proliferation of mesenchymal stem cells. PLoS One 10, e0145068. https://doi.org/10.1371/journal.pone.0145068.

Steuhl, K.P., 1989. Ultrastructure of the conjunctival epithelium. Dev. Ophthalmol. 19, 1–104.

Tong, L., Diebold, Y., Calonge, M., Gao, J., Stern, M.E., Beuerman, R.W., 2009. Comparison of gene expression profiles of conjunctival cell lines with primary cultured conjunctival epithelial cells and human conjunctival tissue. Gene Expr. 14, 265–278. https://doi.org/10.3727/105221609788681231.

Yoshida, Y., Ban, Y., Kinoshita, S., 2009. Tight junction transmembrane protein claudin subtype expression and distribution in human corneal and conjunctival epithelium. Investig. Ophthalmol. Vis. Sci. 50, 2103–2108. https://doi.org/10.1167/iovs.08-3046.

Zhan, H., Towler, H.M.A.T., Calder, V.L., 2003. The immunomodulatory role of human conjunctival epithelial cells. Investig. Ophthalmol. Vis. Sci. 44, 3906–3910. https://doi.org/10.1167/iovs.02-0665.