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Research paper

QobuR – A new in vitro human corneal epithelial model for preclinical drug screening

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ABSTRACT

A new in vitro human corneal epithelial model (QobuR) obtained from normal limbal tissue has been developed to study ocular irritancy of different ophthalmic compounded drugs. Phenotypical characterization and trans-epithelial electrical resistance (TEER) of QobuR revealed essential similarities compared with a native human cornea, displaying functional markers and TEER values near 1500 Ωcm^2 at day 7th of cellular differentiation. Using this model, ocular irritancy and barrier integrity alterations were evaluated using MTT reaction and variations in TEER. We found that some of the Non-Irritant products evaluated still damage the corneal epithelial integrity and current protocols for ocular irritancy should therefore include a barrier integrity evaluation. Moreover, in order to comprehensively evaluate corneal permeability of the active ingredients, we propose the use of QobuR as an all-in-one alternative method for evaluating ocular irritancy, barrier disruptions and permeability rates of topically applied ocular drugs to improve current in vitro drug testing procedures.

1. Introduction

In order to guarantee the safety of the general public, every pharmaceutical or cosmetic product must be evaluated and classified according to its potential to damage the ocular surface according to the Globally Harmonised System (GHS) proposed by the United Nations [1]. This classification defines four different categories based on the results obtained in the in vivo Draize Rabbit Eye Test [2]: Category 1 (irreversible damage to eyes), Category 2A (irritating to eyes), Category 2B (mildly irritating to eyes) and No Category (non-irritating). The European Union has officially adopted GHS classification into its legislation with the only difference being combining GHS categories 2A and 2B into a single Category 2 (irritating to eyes) [3].

Following the European Directive 2010/63/EU on the protection of animals used for scientific purposes [4], toxicity studies using animal models are being replaced by non-animal tests. To date, several alternative methods have been validated and regulatory accepted as OECD Test Guidelines (TG) for the prediction of ocular irritation. These alternative methods can be grouped into 3 categories:

1- Ex vivo models: Bovine Corneal Opacity and Permeability (BCOP) test method (OECD TG 437) and Isolated Chicken Eye (ICE) test method (OECD TG 438).
2- In vitro monolayer models: Short Time Exposure (STE) test method (OECD TG 491) and Fluorescein Leakage (FL) test method (OECD TG 460).
3- In vitro 3D models: Reconstructed human Cornea-like Epithelium (RhCE) test method (OECD TG 492).

Of these alternative methods, ex vivo animal models and in vitro monolayer models have substantial differences in comparison with a native human cornea. Interspecies anatomical differences may lead to erroneous interpretations, while monolayer cell culture does not reflect the real microstructure of a normal epithelium [5]. In vitro 3D models of corneal epithelium most accurately represent the cellular architecture of the human ocular surface. In the OECD TG 492, the use of two commercially available cornea models are described: EpiOcular™ (MatTek, MA, USA) and SkinEthic HCE (SkinEthic, Lyon, France). However, the use of non-corneal cells (EpiOcular™ [6]) and

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immortalized cell lines (SkinEthic HCE [7]) for the construction of these models, may represent a certain degree of difference with native human corneal tissue. To solve these issues, different 3D cornea models using primary cultures of human corneal cells have been developed [8,9]. However, none of them have yet received regulatory acceptance.

All these methods are used to predict ocular irritancy and base prediction on the measure of cell viability as an end point after exposing test materials. However, the barrier integrity of corneal epithelium represents the first mechanical and chemical barrier of the ocular system and is the main limiting factor in the effective absorption for topically applied ocular drugs [10]. Therefore, barrier disruptions must be evaluated in order to quantify the interactions that could exist after substance exposure and that could lead to increased permeability rates of ocular drugs.

The development of new drugs for clinical use involves sophisticated approaches for dissolution, solubilization and preservation of the active pharmaceuticals [11]. Moreover, it requires drug absorption studies to determine drug concentration for an appropriate therapeutic effect. Current models for toxicity prediction may not be useful to determine permeability rates since their barrier integrity does not mimic the native state of a human cornea [12]. Therefore, additional are being developed models for preclinical drug and formulation studies [13,14].

In this study, we developed a new reconstructed human corneal epithelial model, obtained from normal human limbal tissue, to improve common in vitro drug testing procedures, being a two-in-one model to determine ocular irritancy and barrier integrity. Furthermore, trans-epithelial permeability was evaluated upon application of different products using this model.

2. Material & methods

2.1. Human tissues

Human donor corneal tissues were handled in accordance with the Declaration of Helsinki. Sixteen (16) corneoscleral rings (age range: 40–76 years; mean age: 63 years) from corneas previously used for penetrating keratoplasty were obtained from the Instituto Oftalmológico Fernández-Vega (Oviedo, Asturias, Spain). Additionally 5 human corneas discarded by the Centro Comunitario de Sangre y Tejidos (Oviedo, Asturias, Spain) due to low endothelial cell density were used for TEER measurements. All tissues were maintained at 4 °C in Eusol-C storage medium (Alchimia, Ponte S.Nicolò, Italy) for less than 10 days before the study.

2.2. Development of QobuR human corneal epithelial model

QobuR models (named after the oak tree *Quercus robur*, the symbolic tree of Oviedo – Asturias) were developed using human corneal epithelial cells obtained from individual donors and expanded in the absence of feeder cells with a fully defined medium (CnT-Prime; CellNTec, Bern, Switzerland) optimized for the expansion of epithelial cells without fetal bovine serum. Cells from different donor were not pooled together. Briefly, limbal tissues were dissected under a dissecting stereomicroscope, cut into small pieces and digested with trypsin/EDTA 0.25% (Sigma-Aldrich, MO, USA) for 90 min at 37 °C. The loosened cells were centrifuged using an Eppendorf 5702R centrifuge (Eppendorf, Hamburg, Germany) at 0.4 rcf for 10 min and the supernatant was removed. Fresh medium was added and the cells were seeded on a culture plate (25 cm^2). Once the culture was confluent, cells were digested with accutase (Sigma-Aldrich, MO, USA), centrifuged and reseeded on ten 1.12 cm^2, 0.4 µm pore size Transwell® inserts (Corning, NY, USA) in a 12-well culture plate (50,000 cells/insert). Cells were cultured until confluence with medium changes 3 times per week. To promote 3D differentiation, culture media was changed to CnT-PR-3D (CellNTech) and QobuR model was achieved by cultivating confluent human limbal epithelial cells at the air-liquid interface for 7 days.

2.3. Characterization of QobuR human corneal epithelial model

2.3.1. Phenotypical characterization

Deparaffinized tissue sections (5 µm thick) of differentiated formalin-fixed QobuR were rinsed with PBS solution twice for 10 min and permeabilized for 5 min in PBS-0.3 %Triton-X100. Following several washes in PBS, sections were incubated overnight at 4 °C with 1:100 dilutions of primary antibodies p63, cytokeratin 3, connexin-43, zonula occludens-1 and β-catenin (Abcam, Cambridge, UK) containing 10% Normal Goat Serum as a blocking agent. After several washes in PBS, sections were incubated with corresponding secondary antibodies (Life Tecnologies, CA, USA) for 2 h at room temperature, and stained with 4́,6-diamidino-2-phenylindole (DAPI; Abcam). All the samples were examined on a Leica DM6000B fluorescence microscope (Leica, Wetzlar, Germany).

2.3.2. Characterization of barrier function: trans-epithelial electrical resistance (TEER)

A pair of Ag/AgCl probes and a Millicell-ERS2 volt-ohm meter (Millipore, MA, USA) were used according to the manufacturer guidelines to evaluate variations in the barrier function of the models during epithelial stratification in a daily basis. TEER value (Ωcm^2) was calculated using the following equation:

$$TEER = (R_{sample} - R_{blank}) \times effective\ \ area$$

R_{sample}: resistance value of the models.
$R_{blank:}$ resistance value of the inserts without cultured cells.
Effective area: 1.12 cm^2.

All measurements were carried out in duplicate and the mean value and the standard error of mean (SEM) for 10 batches consisting in 10 models each were calculated. TEER values of human corneas (n = 5) were obtained using a bicameral Ussing chamber in which the cornea was placed between the two cameras with a circular opening of 0.88 cm^2.

Table 1
Selected test chemicals. GHS: Globally Harmonized System.

Chemical name	CAS Number	Organic Functional Group	GHS	Supplier
Methyl thioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	Category 1	Sigma Aldrich
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	Category 1	Sigma Aldrich
2,4,11,13-Tetraazatetradecane-diimidamide, N,N“-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20% aqueous)	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	Category 2A	Sigma Aldrich
Diethyl toluamide	134-62-3	Benzamide	Category 2B	Sigma Aldrich
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	No Category	Sigma Aldrich
Octane, 1,1′-oxybis-	629-82-3	Alkoxy; Ether	No Category	Sigma Aldrich
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	No Category	Sigma Aldrich

2.3.3. Proficiency test of QobuR ocular irritancy and barrier integrity test

All liquid test chemicals on the list of proficiency chemicals on the OECD guideline TG-492 [15] were selected for this study. Details on the selected chemicals together with their eye irritation classification are shown in Table 1.

Test protocol for ocular irritancy and barrier integrity was based on EpiOcular Eye Irritation Test (OCL-200_EIT) [16] with some modifications. Each test chemical was evaluated in duplicates in 4 different assays using different batches. Negative (culture media) and positive control (100% Triton X-100) cultures were run in each assay to monitor intralaboratory variation and to provide the necessary data for defining quality control criteria.

Prior to conducting assays, the ability of each test chemical to directly reduce MTT was assessed. 100 µL of each compound was added to 1 mL of a 0.5 mg/mL MTT solution and the mixture was incubated at 37 °C for 1 h. If the MTT solution turned purple, the test chemical was classified as MTT reducer and freeze-killed QobuR were included in the test protocol for ocular irritation and barrier integrity, as described in OECD TG 492. Briefly, freeze-killed QobuR were dosed, rinsed, and exposed to MTT in the same way as was done for the viable QobuR. Freeze-killed models were prepared by placing QobuR in a −20 °C freezer overnight, thawed to room temperature, and then refrozen and stored at −20 °C until required. Net viability percentage was obtained by subtracting freeze-killed viability from the test article viability obtained from the irritation assay.

Trans-epithelial electrical resistance (TEER) was evaluated in each model prior to testing compound dosage. Next, inserts (1.12 cm^2) were placed in 500 µL fresh culture medium and dosed topically with 100 µL of each test chemical for 30 min at room temperature. Following incubation, test chemicals were decanted from the inserts and models were extensively rinsed in PBS. After rinsing, models were post-soaked for 10 min in warmed culture medium to remove any test chemical absorbed into the tissue. At the end of the post-soak, each model was transferred to a culture plate containing 1.5 mL warm culture medium. Each insert was filled with 500 µL warm culture medium and culture plates were incubated for an additional 2 h at 37 °C. After post-incubation, TEER was revaluated in each model and one of the duplicates was transferred to a freshly prepared MTT solution (0.5 mg/mL; 1 mL). MTT is a yellow tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that is reduced to a purple formazan by mitochondrial succinate dehydrogenase in viable cells [17]. Culture plates were incubated in MTT solution for 1 h at 37 °C. After incubation, MTT solution was removed; inserts were blotted on absorbent material and transferred to a 12-well plate containing 500 µL DMSO. An additional 500 µL of DMSO was added on top of each insert and plates were shaken for 5 min at room temperature to extract the reduced MTT out of the models. Three 100 µL aliquots of the extracted MTT solution were transferred to a 96-well plate and the optical density (OD) of each well was measured at 570 nm with a VICTOR Multilabel Plate Reader (PerkinElmer, MA, USA).

Moreover, one duplicate of each test chemical was fixed in 10% v/v formalin solution and embedded in paraffin for histological analysis.

2.4. QobuR ocular irritancy and barrier integrity test on ophthalmic compounded drugs

Seven ophthalmic compounded drugs were evaluated by predicting ocular irritancy and barrier disruptive potential. Concentration, pH, osmolarity, solvents and suppliers of selected drugs are shown in Table 2. Drugs were compounded from powder in the Pharmacy Service of the Instituto Oftalmológico Fernández-Vega.

Ophthalmic compounded drugs were evaluated in duplicates in 2 different assays using different Qobur batches. Test protocol was performed accordingly to the previous section.

2.5. Permeability studies

Trans-epithelial permeability using QobuR was evaluated upon application of hydrophilic dye sodium fluorescein (20 mg/mL; Bausch & Lomb, NY, USA), lipophilic dye rhodamine B (50 µg/mL) and macromolecule FD-4 (2.5 mg/mL; Sigma-Aldrich, MO, USA). Moreover, permeability of previously used compounded drugs of phenylephrine (100 mg/mL), tetracaine (5 mg/mL) and ceftazidime (50 mg/mL) was evaluated. Permeability studies using QobuR were performed directly in the Transwell® insert in triplicates.

Parameters of permeability experiments were fixed as follows: acceptor volume: 500 µL; donor volume: 100 µL, dimensions of the permeation surface: 1.12 cm^2 and exposure time: 1800 s. All experiments were conducted at room temperature.

After 30 min exposure, samples were taken from the acceptor compartment and the amount of sodium fluorescein, rhodamine B and FD-4 were quantified using a VICTOR Multilabel Plate Reader (PerkinElmer, MA, USA), with an excitation and emission filter of 485 and 535 nm respectively for sodium fluorescein and FD-4, and 530 and 595 nm respectively for rhodamine B. Amount of phenylephrine, tetracaine and ceftazidime was determined by an electrochemical measure using a DRP-110 electrode (DropSens, Asturias, Spain). Calibration standards were prepared from the stock solutions, diluted into culture media, in the range of 0.25–2 µg/mL for sodium fluorescein, 0.125–3 µg/mL for FD-4 and 0.1–1 µg/mL for rhodamine B, 1.5–15 µg/mL for phenylephrine, 2.5–250 µg/mL for tetracaine and 5.5–55 µg/mL for ceftazidime resulting in a correlation coefficient $r^2 > 0.99$.

2.6. Data analysis and prediction model

All calculations were performed using Microsoft Office Excel version 2010.

2.6.1. Ocular irritancy and barrier integrity

Relative tissue viability was calculated as a percentage relative to the MTT optical density of the negative controls (culture medium). Barrier integrity disruptions were calculated as a percentage relative to the initial TEER value of each model. Mean values of identically treated tissues were used to classify chemicals and ophthalmic compounded drugs according to the prediction model.

The prediction model for ocular irritation and barrier integrity using QobuR was set to 60% of relative viability and 60% of initial TEER as the threshold of irritancy and barrier disruption prediction respectively. If the % viability was > 60%, the substance was predicted as Non-irritant (GHS classification: No Category). If the % viability was ≤60%, the substance was predicted as Irritant (GHS classification: Category 1 or Category 2). Moreover, if the % barrier integrity was ≤60%, the substance was classified as barrier disruptive.

2.6.2. Corneal permeability

The permeation coefficients [P_{app} (cm/s)] for all compounds were calculated using the following equation:

$$P_{app} = \frac{V}{A \times \delta t} \times \frac{\delta C}{C_0}$$

where V is the acceptor volume (cm^3), A is the permeation area (cm^2), δt is the exposure time (s), δC is the quantity of the substance that passed through the QobuR and C_0 is the initial concentration of the donor substance.

3. Results

3.1. Control values for QobuR

Control data for the barrier function, tissue viability and barrier integrity of each QobuR batch is summarized in Table 3. The

Table 2
Selected ophthalmic compounded drugs.

Name	Supplier	Concentration	pH	mOsm/kg	Solvent	Solvent supplier	Excipients
Phenylefrine	Novartis	10%	5.76	861	H_2O	–	Thiomersal 0.01%
Tetracaine	Fagron	0.5%	6.84	306	Hyaline	LCA pharma	–
Ceftazidime	Normon	5%	4.72	695	NaCl 0.9%	Braun	–
Tacrolimus (A)	Guinama	0.03%	6.94	201	Liquifilm	Allergan	–
Tacrolimus (B)	Astellas	0.03%	7.04	224	Liquifilm	Allergan	Lactose 0.74%
Chlorhexidine	Fagron	0.05%	5.7	249	H_2O	–	Sodium acetate 4.6%, glacial acetic acid 0.2%
Mydriatic solution	Fagron	Phenylefrine 1.5%; Cyclopentolate 0.1%; Lidocaine 2%	6.25	362	Balanced salt solution	Braun	–

mean ± SEM of barrier function (Ωcm^2) of all batches was 1540 ± 200. The mean ± SEM of tissue viability (OD) from the evaluation of all batches of QobuR were 0.278 ± 0.010 (negative control-culture medium) and 0.018 ± 0.008 (positive control-100% Triton X-100). The mean ± SEM of barrier integrity (%TEER) from the evaluation of all batches of QobuR were 88.30 ± 3.92 (negative control-culture media) and 1.56 ± 0.24 (positive control-100% Triton X-100).

3.2. Characterization of QobuR

3.2.1. Phenotypical characterization

QobuR human corneal epithelial models, prepared using primary cultures of human limbal epithelial cells, showed a human cornea-like morphology where basal cells, transitional wing cells and superficial squamous cells could be observed. Expression of phenotypical marker p63, a progenitor cell marker, could be observed in the basal layer while differentiation marker cytokeratin 3 was only found in the most apical layer of QobuR models. Intercellular junction maker connexin-43 and tight junction marker zonula occludens-1 were observed in the suprabasal layer while β-catenin was observed in all epithelial layers (Fig. 1).

3.2.2. Barrier function: Trans-epithelial electrical resistance (TEER)

TEER values of QobuR models gradually rose as the culture period under air-lift conditions increased, reaching a mean value of 1,536.07 ± 205.29 Ωcm^2 on the 7th day (Fig. 2). In comparison, mean TEER values of human corneas displayed values of 1443.99 ± 47.32 Ωcm^2.

3.2.3. Proficiency test of QobuR ocular irritancy and barrier integrity test

Of the 7 liquid proficiency test chemicals recommended by the OECD TG 492, methyl thioglycolate was found to reduce MTT. Therefore, corrected values of viability using freeze-killed QobuR were obtained. As shown in Table 4, all chemicals were predicted according to its GHS classification when the criterion for ocular irritation potential was set to 60% of cell viability. In addition, all irritant chemicals were found to alter barrier integrity as set by a %TEER less than 60% of initial values. However, 1-ethyl-3-methylimidazolium ethylsulphate (classified as No Category) was classified as a barrier disruptive chemical since %TEER resulted in 38.60 ± 7.69% of its initial values.

Histological analysis of QobuR models treated with irritant test chemicals showed marked alterations in hematoxylin-eosin staining. Moreover, significantly altered epithelial structure was observed in 1-ethyl-3-methylimidazolium ethylsulphate although being classified as non-irritant under our experimental conditions (Fig. 3).

3.3. QobuR ocular irritancy and barrier integrity test on ophthalmic compounded drugs

Cell viability and barrier integrity of 7 ophthalmic compounded drugs are presented in Table 5. Each tested formulation produced concordant results in the two independent runs. Cellular viability values were overall higher than 90%, therefore, all ophthalmic formulations were classified as Non-Irritant. However, phenylephrine and chlorhexidine formulations were found to alter barrier integrity of QobuR model with values of %TEER of 42.27 ± 1.74 and 53.00 ± 5.49 respectively.

Histological analysis revealed a slightly alteration in the basal layer of the model exposed to chlorhexidine. No other remarkable alterations were found in any of the models (Fig. 4).

3.4. Permeability studies

Hydrophilic and lipophilic markers sodium fluorescein and rhodamine B displayed a permeability coefficient $P_{app} = 1.36 \pm 0.61 \times 10^{-7}$ cm/s and $1.21 \pm 0.32 \times 10^{-7}$ cm/s respectively. High molecular weight marker FD-4 demonstrated the lowest permeability with $P_{app} = 3.5 \pm 1.04 \times 10^{-9}$ cm/s.

Ophthalmic formulations displayed a permeability coefficient $P_{app} = 1.62 \pm 0.05 \times 10^{-6}$ cm/s for phenylephrine, $P_{app} = 2.84 \pm 0.30 \times 10^{-5}$ cm/s for tetracaine and $P_{app} = 1.24 \pm 0.15 \times 10^{-4}$ cm/s for ceftazidime.

4. Discussion

The need of new ophthalmic compound drugs to cover all pathologies is leading to an major demand for ocular irritation and absorption studies during the early phases of drug development [18]. Nowadays, different in vitro alternative methods that resemble a native cornea are adopted as OECD guidelines to determine ocular toxicity [15].

Table 3
Barrier function, cellular viability and barrier integrity of evaluated QobuR batches. OD: Optical density. Negative control: culture media. Positive control: 100% Triton X-100.

	Barrier function	Viability		Barrier integrity	
	Ωcm^2	OD Negative control	OD Positive control	%TEER Negative control	%TEER Positive control
Mean ± SEM	1,536.07 ± 205.29	0.278 ± 0.010	0.018 ± 0.008	88.30 ± 3.92	1.56 ± 0.24
Range					
Minimal batch	630.11 ± 67.27	0.238 ± 0.001	4.44E-04 ± 2.96E-04	72.23 ± 4.94	1.07 ± 0,09
Maximal batch	2.654.29 ± 82.69	0.385 ± 0.002	0.050 ± 3.57E-04	99.44 ± 7.27	2.04 ± 0.07

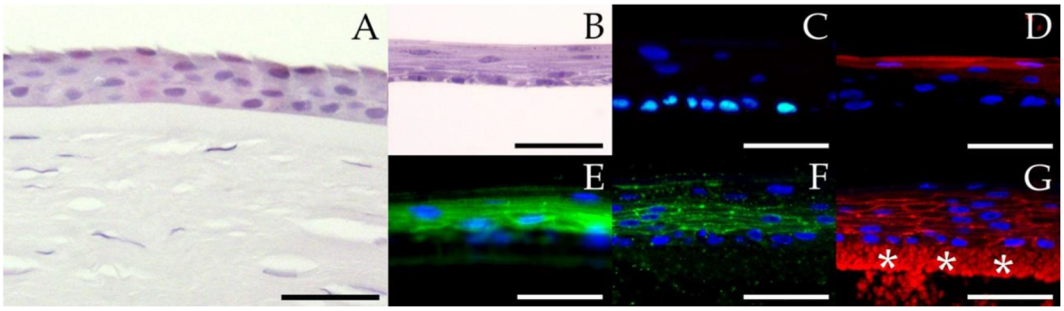

Fig. 1. Histological analysis. (A): Human cornea; (B): QobuR; (C): p63 (green); (D): Cytokeratin 3 (red); (E): Zonula occludens-1 (green); (F): Connexin-43 (green); (G): β-catenin (red). Scale bar 50 μm. Nuclei stained in blue (DAPI). (*): Unspecific background stain of Transwell membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

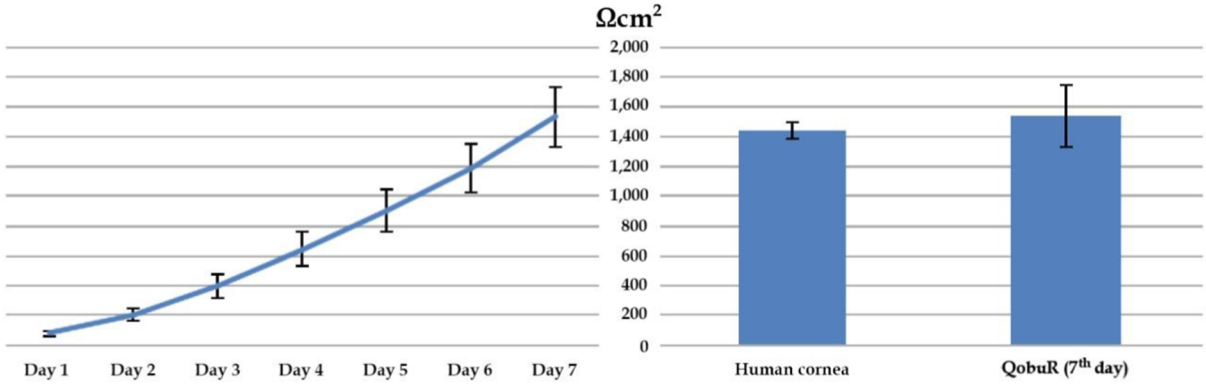

Fig. 2. Left: TEER values of QobuR models during epithelial stratification; n = 10 different production batches consisting in 10 QobuR models each. Right: Comparison of TEER values between human corneas and QobuR models at the 7th day of stratification.

Table 4
Barrier integrity and cell viability of proficiency chemicals suggested by OECD TG 492 in the ocular irritancy and barrier integrity test using QobuR. I: irritant; NI: non-irritant. GHS: Globally Harmonized System.

Chemical No.	Run	% TEER	% Viability	Barrier alterations	QobuR classification	GHS
Methyl thioglycolate	1	7.79	8.12	YES	I	1
	2	6.09	0.00	YES	I	
	3	7.53	0.00	YES	I	
	4	5.78	6.87	YES	I	
Hydroxyethyl acrylate	1	12.94	40.46	YES	I	1
	2	10.39	32.34	YES	I	
	3	19.15	34.56	YES	I	
	4	4.06	34.02	YES	I	
2,4,11,13-Tetraazatetradecane-diimidamide, N,N“-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20% aqueous)	1	2.20	1.18	YES	I	2A
	2	1.00	0.96	YES	I	
	3	0.68	1.54	YES	I	
	4	1.34	2.48	YES	I	
Diethyl toluamide	1	11.49	2.16	YES	I	2B
	2	5.71	8.52	YES	I	
	3	4.88	18.37	YES	I	
	4	3.11	5.51	YES	I	
1-Ethyl-3-methylimidazolium ethylsulphate	1	22.01	79.87	YES	NI	NC
	2	52.83	73.59	YES	NI	
	3	50.47	67.63	YES	NI	
	4	29.06	91.21	YES	NI	
Octane, 1,1′-oxybis-	1	78.17	98.61	NO	NI	NC
	2	89.75	96.94	NO	NI	
	3	90.45	96.51	NO	NI	
	4	90.74	96.34	NO	NI	
Piperonyl butoxide	1	87.59	104.23	NO	NI	NC
	2	72.23	104.03	NO	NI	
	3	87.59	107.70	NO	NI	
	4	110.29	108.98	NO	NI	

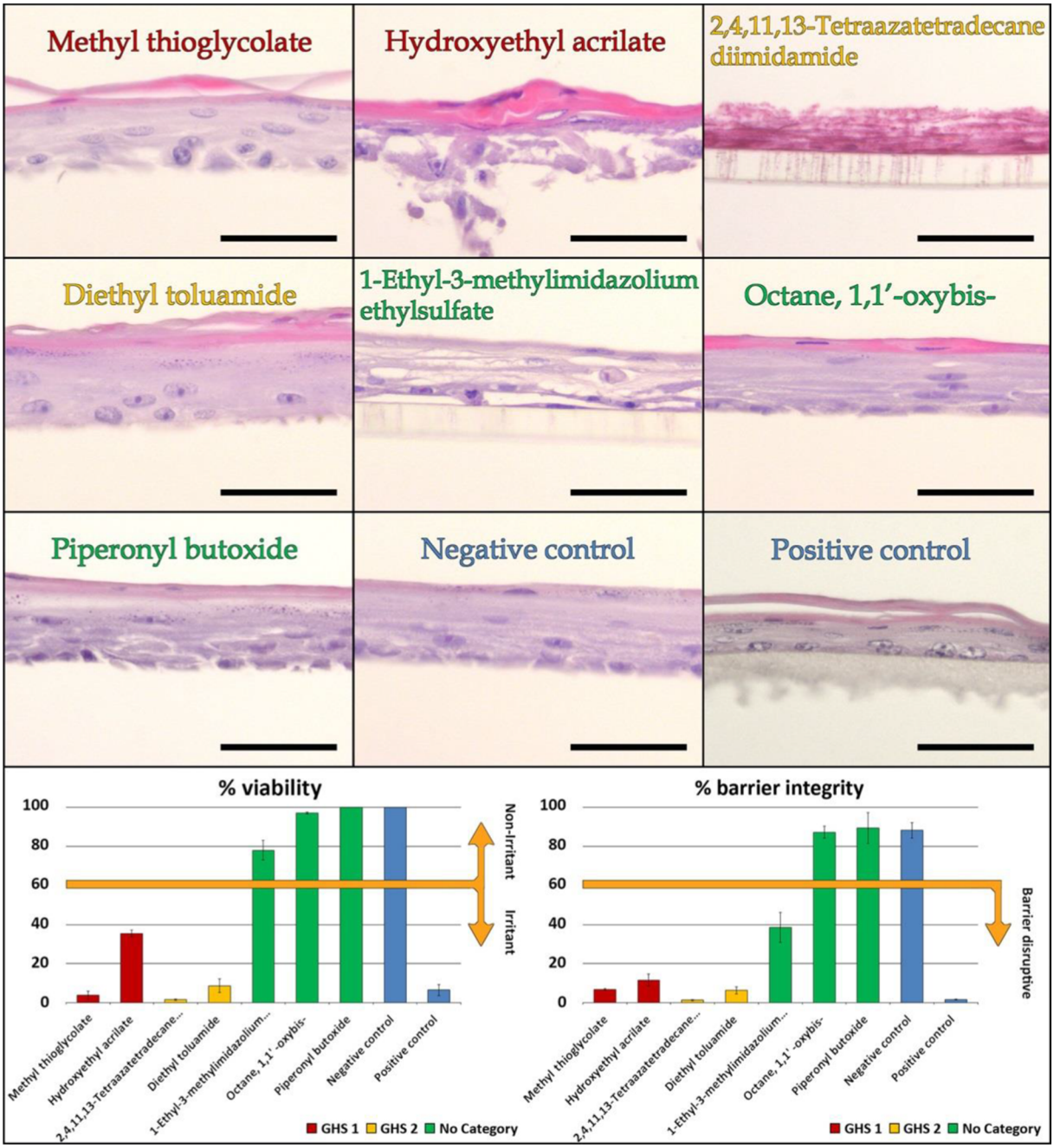

Fig. 3. Histological analysis of QobuR after exposure to chemicals suggested by OECD TG 492 in the ocular irritation on barrier integrity test. Scale bar 50 µm. (Negative control): culture medium; (Positive control): 100% Triton-X100.

However, these methods are only focused on cell viability while corneal barrier integrity, the main limiting factor for corneal permeability and consequently for bioavailability of ocular drugs, is generally ignored.

In this study, we developed and characterized a new reconstructed human corneal model produced with primary human corneal epithelial cells that mimics the structure and the barrier integrity of a native human corneal epithelium as proven by phenotypical markers and a barrier function of nearly 1500 Ωcm^2. Using this model, we were able to classify test substances, between irritant and non-irritant, based on cellular viability via MTT reduction as is the internationally accepted methodology for these types of studies [6,7,15,19,20]. Moreover, we also explored the changes in TEER after test substance exposure, defining a non-evaluated property of test substances: their barrier disruptive potential. Finally, we explored the use of the same model in a proof-of-concept permeability assay, so the model could be used to characterize three different properties at once: ocular irritancy, barrier integrity and permeability rates.

Structural morphology of the QobuR models resembles a native human corneal epithelium, where the basal epithelial layer maintains an undifferentiated phenotype while the uppermost layer is terminally differentiated. This is shown by the expression of p63 and cytokeratin 3 respectively, and is in line with current models developed using primary limbal cells that differentiate into cornea [9]. In QobuR, we found that representative markers of three different junction protein families were expressed. β-catenin, an adherent junction protein that regulates actin organization and provides strong mechanical attachment [21], was expressed across all the epithelium, while connexin-43, a component of gap junctions that allow the transfer of cytosolic components from cell to cell, was only present in the suprabasal layer in accordance with other studies [22]. Moreover, zonula occludens-1, a component of

Table 5
Barrier integrity and cell viability of ophthalmic compounded drug in the ocular irritancy and barrier integrity test using QobuR. NI: non-irritant.

Formulation name	Run	% TEER	% Viability	Barrier alterations	QobuR classification
Phenylefrine	1	44	97.5	YES	NI
	2	40.53	100.48	YES	NI
Tetracaine	1	64.94	93.68	NO	NI
	2	61.36	94.22	NO	NI
Ceftazidime	1	68.62	88.21	NO	NI
	2	76.71	98.82	NO	NI
Tacrolimus (A)	1	69.33	93.91	NO	NI
	2	74.80	92.61	NO	NI
Tacrolimus (B)	1	72.46	90.18	NO	NI
	2	82.26	91.05	NO	NI
Chlorhexidine	1	58.49	96.78	YES	NI
	2	47.51	88.92	YES	NI
Mydriatic solution	1	73.43	97.24	NO	NI
	2	74.31	88.92	NO	NI

tight junctions is positive particularly between superficial cells, which is in concordance with TEER results since it is known that barrier function relies critically on the distribution of tight junction proteins [23].

The barrier function of the QobuR models displayed a progressive increase in TEER during air-lift culture reaching values of 1540 Ωcm^2 after 7 days. These values are higher than those shown by other models during epithelial stratification [24–26], possibly because these models use immortalized cell lines instead of native human corneal cells, and are highly similar to the 1450 Ωcm^2 value of the human corneas evaluated in this study. Due to the poor availability of intact tissues, there are not many studies that evaluate normal human corneal barriers. Becker et al [27] described in 2008, TEER values of human corneas in the range of 500 Ωcm^2, a value much lower than that found in our study. This variance in the results of human corneal TEER could be due to damages in epithelium during enucleation, loss of epithelial integrity during organ storage or differences between time of death and experimental measures between both studies.

When our data on ocular toxicity was compared with the reported data for the 7 reference liquid chemicals, a 100% sensitivity, specificity and accuracy was achieved, demonstrating optimal performance in comparison with the data reported for the validated methods EpiOcular™ and SkinEthic HCE [15]. However, since only a small subset of chemicals was evaluated, this study sets the first step towards a predictive model and its correlation with Draize test results and corneal irritancy would require a bigger set of study in order to fully develop a predictive model. Nevertheless, due to the TEER evaluation after substance exposure, a prediction in non-deadly alterations that compromise the integrity of the corneal epithelium after substance exposure could be described, and could help categorize irritant products more accurately. Although not entirely new [28], the study of TEER alterations has been described in corneal epithelial models that lack a resemblance with a physiological corneal barrier. In our study, we developed a model that mimics human corneal barrier integrity, so TEER alterations could reliably correlate with human physiology. That was the case of 1-ethyl-3-methylimidazolium ethylsulphate, a test chemical that displayed cell viability values of 78.08% using QobuR, and 79.9% and 79.4% using the validated methods SkinEthic HCE and EpiOcular™ respectively [15]. However, our results indicate that after exposure, the barrier integrity of QobuR decreased to 38.59% of its initial values while its histological structure revealed marked alterations. Similar results were found when evaluating ophthalmic formulations. None of them showed a decrease in cellular viability of more than 10%, but application of phenylephrine and chlorhexidine formulations resulted in a barrier integrity decrement to 42.27% and 53.00% of initial TEER values respectively, probably due to the mercury-based thiomersal and glacial acetic acid used as excipients in their compounding. In these cases, the current classification of topical products via cell viability does not cover the full range of alterations that occur when these exogenous substances contact the corneal epithelium, leading to an altered state in the ocular surface. However, further studies will be required to elucidate how long this altered state lasts, since in this study the barrier integrity was evaluated only 2 h after post-incubation in culture medium. Moreover, further studies will also be required to determine the appropriate cut-off value to distinguish between barrier disruptive products; since we established an arbitrary 60% cut-off as that used in the viability studies.

It is known that corneal epithelium is a highly exposed structure that acts as the first mechanical and chemical barrier of the ocular system, playing a key role in corneal permeability by limiting the entrance of exogenous substances into the eye [29]. Permeability studies that used cellular models with low ($\approx 100\ \Omega cm^2$) or high ($> 2000\ \Omega cm^2$) TEER values conclude that low TEER values leads to an increased permeability rate compared to the models with higher TEER values [30]. Therefore, studying TEER alterations in a corneal epithelial model that resembles a native epithelium represents a crucial tool when defining the final formulation of an ocular drug, since cell viability only indicates cell death but does not give any information about the non-deadly alterations that may occur in a normal epithelium.

Barrier integrity is also the main parameter to evaluate transport rates across in vitro cellular models. It has been proposed that corneal epithelial cell cultures with TEER values $> 400\ \Omega cm^2$ are suitable for investigating the transport of hydrophilic and lipophilic drugs [12,31,32]. Over the years, different reconstructed cornea models have been proposed for drug transport studies: primary cultures of rabbit corneal cells [33,34], immortalized rabbit corneal cells [35,36], primary cultures of human epidermal keratinocytes [6], immortalized human corneal cells [27,30,37,38] or normal human corneal cells [9]. However, only a few present reliable data since most of them display inadequate TEER values leading to leaky epithelial models. According to Reichl, only HCE-T (a SV-40 immortalized human corneal epithelial cell line [39]) based models seem appropriate in transcorneal absorption studies due to their TEER values [12]. This is why Reichl and coworkers have developed a promising human cornea construct, using HCE-T cells, that was used in comparative permeation studies revealing a good correlation in permeability rates between their model and human corneal tissue [40], leading to the only model that has already been prevalidated for permeation studies, with excellent results and very low variability [32]. However, HCE-T has been proven to be composed of heterogeneous cell populations with altered genomic content [41] and different expression pattern of transporter proteins [42] leading to different TEER values and permeation rates among different laboratories [43,44] or under different culture conditions [30,37]. Therefore results obtained from this cell line should be used with much caution when predicting permeability rates.

In the present study, a proof-of-concept permeability assay was also included to investigate QobuR limitations and experimental possibilities. However, permeability rates could have been inaccurately determined and should not be taken as a fact. Since only one value in a defined time point was evaluated for the permeated amount of compound, there is a risk of major error when calculating permeation rates. Evaluating drug permeation requires multiple time points in order to avoid the lag phase during permeation of hydrophilic markers and large molecules and such measures will be taken into account in future studies. Nevertheless, preliminary results indicate that there are little differences between hydrophilic and lipophilic markers. This could be explained by the lack of hydrophilic stromal tissue in QobuR, and could be expected that an improved model that includes a stroma-like collagen matrix could improve the permeability rate prediction.

One of the major limitations in our experimental design was the

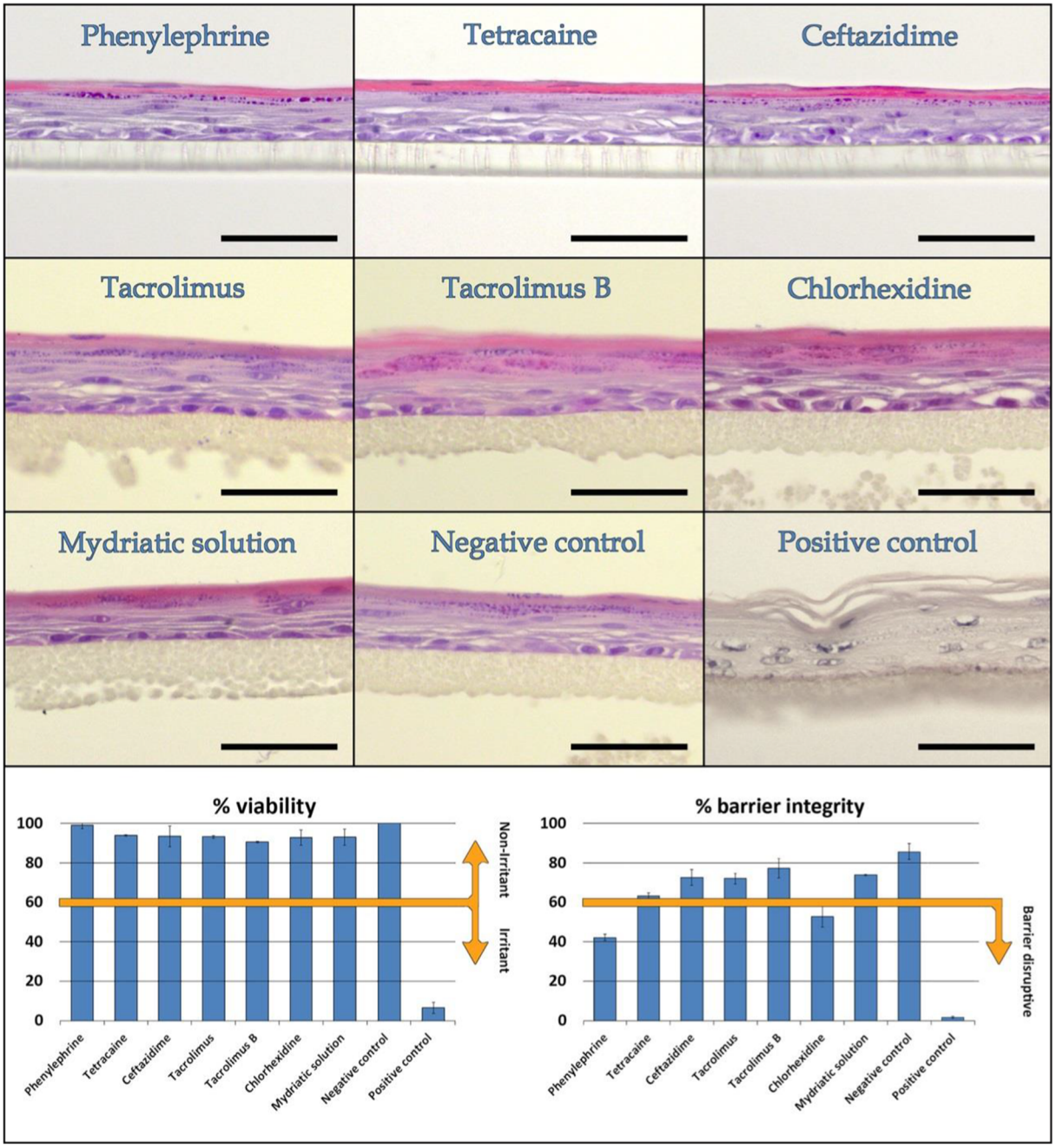

Fig. 4. Histological analysis of QobuR after exposure to selected ophthalmic compounded drugs in the ocular irritation and barrier integrity test. Scale bar 50 µm. (Negative control): culture medium; (Positive control): 100% Triton-X100.

static exposure of compounds. Dynamic exposure conditions reflect a more physiological approach for the evaluation of topical products, and in order to obtain an even better approximation to the in vivo situation, further studies under dynamic conditions should be addressed as already described [14].

Other limitations include the use of human tissue for model production. Although using primary cultures isolated from individual donors may seem as a limitation regarding model reproducibility, we obtained consistent results regarding TEER and histological features among different batches. Moreover, tissue availability for model production could also be pointed out as a critical limitation, and although true, under optimal culture conditions we could stablish up to 60 QobuR models in each production batch.

All in all, the present results strongly suggest that QobuR could be used for predicting drug permeability and therefore it could be a useful alternative method for predicting ocular toxicity, barrier alterations and permeability rates in an all-in-one model to be used in the early development stage of new ocular drugs. However, in order to comprehensively evaluate corneal permeability of the active ingredients, further studies should be conducted using barrier disruptive ocular drugs.

5. Conclusion

QobuR is a new reconstructed cornea model with barrier properties and functional markers that truly resembles a native human cornea. Using QobuR, we set the first step towards a predictive model for ocular irritancy based on cell viability. Moreover, TEER analysis revealed an altered corneal barrier upon application of Non-Irritant products. These results suggest that Non-Irritant products still damage the corneal

epithelial integrity and therefore, toxicity assays may not be focused only on cell viability, and corneal barrier integrity must be evaluated in the early phase of drug development.

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