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Medical devices biocompatibility assessment on HCE: Evidences of delayed cytotoxicity of preserved compared to preservative free eye drops

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ABSTRACT

A multiple endpoint analysis (MEA) approach on human reconstructed corneal epithelium (HCE) model has been applied to assess the biocompatibility (cytotoxicity and irritation potential) of medical devices (MD): ophthalmology literature clearly shows the need to better assess these products to exclude any potential chronic damage to the ocular surface. Preserved eye drops (Artelac Multidose, Optive multidose and Artelac Rebalance Multidose) and the same without preservative (Artelac Edo, Optive Unidose, Artelac Rebalance Unidose) and Thealoz Duo were tested after acute (24 h + 16 h post incubation) and repeated (2 applications/day for 72 h) exposure using BAK 0.01% as positive control on HCE. Cellular viability, trans-epithelial electrical resistance measurements, LDH release and occludin gene expression were evaluated for each product to discriminate the potential toxicity of preservatives. The BAK 0.01% toxicity on HCE was confirmed following both exposures. The analysis of the same parameters reveals that the 72 h exposure was suitable to identify toxicity and damages to the ocular surface even for 'soft' preserved MD. The results confirm the reliability, sensitivity and predictivity of the MEA on HCE in detecting subclinical signs of cellular toxicity: 'soft' preservatives resulted toxics suggesting that delayed toxicity should be integral part of the biocompatibility assessment of ophthalmic formulations intended for long-term use.

1. Introduction

Alternative methods for assessing eye irritation have been developed, validated and are available for the assessment of chemical inducing serious eye damage (classified according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) as UN GHS Category 1) and the identification of chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No category).

The process that led to the replacement of the Draize rabbit test with alternative non animal approaches such as Integrated Approaches on Testing and Assessment (IATA) for Serious Eye Damage and Eye irritation (OECD, 2017) has taken almost 20 years and has clearly shown that *in vitro* mechanistic approaches based on a body of evidences corresponds to a sustainable and robust strategy. These integrated approaches for regulatory purposes are currently used to assess chemicals and cosmetic ingredients, are accepted and promoted by their respective legislations (REACH and Regulation 1223/2009) and are sustainable with respect to the Directive 2010/63 to which also Medical Device Regulation EU n. 2017/745 (MDR) refers (*cosiderandum* 73). A set of *in vitro* methods have been validated to cover the classification requirement: OECD Test Guidelines (TGs) 437, 438, 460, 491 which address the human health endpoint serious eye damage (UN GHS Cat. 1). All these methods with the addition of TG 492 can also be used to identify chemicals not requiring classification (UN GHS No category).

OECD TG 492 uses 3D human reconstructed tissues models as biological system for the Eye Irritation Test (EIT): the SkinEthic™ Human Corneal Epithelium (HCE), the EpiOcular™ EIT and the LabCyte CORNEA-MODEL24. For the SkinEthic™ EIT one protocol for liquids and one for solids have been defined to assess the hazard potential of a test chemical after acute exposure (Alépée et al., 2016a, 2016b). Both protocols are based on the evaluation of cytotoxicity induction after 30min-exposure for liquids and 4 h-exposure for solids measured by the MTT assay.

The use of a pool of *in vitro* methods for the evaluation of eye irritancy of eye drops has been recently reported by Yun et al. who assessed the irritation potential of representative contact lenses using the Bovine Corneal Opacity and Permeability test (BCOP) (OECD TG 437) and OECD TG 492 (Yun et al., 2016). These studies have suggested that the combination of *in vitro* tests produce similar results to the *in vivo* findings and that such integrated testing strategies could be used to replace conventional *in vivo* testing in the evaluation of eye irritancy of

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Abbreviations

BAK	Benzalkonium chloride
BCOP	Bovine Corneal Opacity and Permeability Test
EIT	Eye Irritation Test
HCE	Human Corneal Epithelium
IATA	Integrated Approaches on Testing and Assessment
ISO	International Organization for Standardization
ITS	Integrated Testing Strategy
LDH	Lactate dehydrogenase
MD	Medical Device
MDR	Medical Device Regulation EU n. 2017/745
MEA	Multiple Endpoint Analysis
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
OCLN	Occludin
OECD	Organization for Economic Co-operation and Development
OSD	Ocular surface disease
PF	Preservative-free
qRT-PCR	Quantitative Real Time Polymerase chain reaction
SD	Standard Deviation
TEER	Trans-Epithelial-Electrical-Resistance
TG	Test Guideline
TJs	Tight junctions
UN GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals

ophthalmic medical devices (e.g. ISO 10993–10). It is important to underline that corneal modifications are extremely important and relevant in the assessment of eye irritation potential (they represent the 30% of the score used to evaluate eye irritation/corrosion in the Draize Rabbit Eye test) thus indicating the human 3D reconstructed corneal models as the closer model to humans, predictive and biologically relevant.

Preservatives are currently used to protect multidose eye drop formulations from bacterial contamination during their use (Baudouin, 2010). However, this represents a well known risk factor to patients because of their potential toxicity that includes cellular and molecular damages that are not clinically evident after short term exposure but that slowly accumulates leading to chronic or irreversible damage of clinical relevance, particularly in long-term therapies. They are used in multi-dose eye-drops to prevent from any risk of bacterial contamination of the product. Long-term application of preservatives has been shown to be responsible for discomfort, early sub-clinical reactions and then severe and chronic damages at corneal epithelium level (e. g. Dry Eye Syndrome) (Baudouin, 2008; Vaede et al., 2010; Skalicky et al., 2012; Rossi et al., 2012; Coroi et al., 2015).

Increasing awareness of the toxicity and potential chronic damages of any ophthalmic preservatives has led to an increasing variety of preservatives (Tu, 2014; Aihara et al., 2012; Baudouin et al., 2010; Gado and Macky, 2011; Katz et al., 2010; Rouland et al., 2013). Up to date, extensive research has been conducted to discover and develop less toxic preservatives than BAK. However, since a preservative must be a potent antimicrobial agent while not being cytotoxic, only very few agents have been proposed and are commercially available for glaucoma patients (Freeman and Kahook, 2009; Tu, 2014): Purite® as antioxidant preservative (Mundorf et al., 2003), Sofzia® as ionic buffer system (Henry et al., 2008; Horsley et al., 2009; Kahook and Noecker, 2008; Lipener C 2009; Rolando et al., 2011; Brignole-Baudouin et al., 2011; Ammar et al., 2011; Gandolfi et al., 2012; Labbé, 2006; Peace et al., 2015). Because of their lower cytotoxic effects, these alternative preservatives have been recently envisaged to address iatrogenic dry eye disease (DED); however, their possible effects on the tear film and tolerance in dry eye patients have not been fully investigated (Gomes et al., 2017).

Many of the eye drops on the market are certified as Medical Device (MD), a sector which covers a large heterogeneity of products. As reported in the MDR, a general requirement is that devices shall be safe and effective and shall not compromise the clinical condition or the safety of patients or other users provided that any risks associated with their use is acceptable when weighed against the benefits to the patient (Chapter I of Annex 1 of the MDR). Accordingly, the compatibility between the materials and substances used and biological tissues, cells and body fluids, should be investigated taking into account the intended purpose of the device (Chapter II of Annex 1 of the MDR).

Medical devices can be classified according to their duration of use as transient (intended for continuous use for less than 60 min), short term (intended for continuous use for between 60 min and 30 days) and long term (intended for continuous use for more than 30 days) devices.

Thus, in the assessment of the eye biocompatibility of eye drops, all the three application conditions should be included: transient (acute), short term (daily repeated) and long term (daily repeated for months). However, there are not suggested protocols to assess this endpoint in current status of ISO standards available without using animals. Furthermore, the *in vivo* approach currently used as reported in the ISO 10993–10 is clearly not in agreement with the requirement of EU 2010/63 to reduce and replace animal testing.

Part 10 of the ISO 10993 introduces how to assess possible contact hazards from chemicals released from medical devices, including substances that may produce eye irritation when come into contact with eye and eyelid (ISO 10993–10:2010). According to this standard, the Draize rabbit irritation assay (OECD TG 405) is required to identify weak irritants or the reversibility of the damage. Repeated exposure can also be conducted after the acute exposure test. However, the ethical concern, scientific limitation and poor predictivity of this approach is nowadays recognized and furthermore animal studies have been criticized because of the variability, low sensitivity and low predictiveness *vs* human response (Spielmann et al., 1996; Doucet et al., 2006; McNamee et al., 2009; Baudouin et al., 2010; Hartung et al., 2010; Adriens et al., 2014; Wilson et al., 2015; Meigs et al., 2018).

The Multiple Endpoint Analysis (MEA) approach on the human reconstructed corneal model HCE after acute and repeated exposure has been developed (Meloni et al., 2010) to provide the pharmaceutical industry with a predictive tool to assess acute and long-term eye irritation potential: in particular, more than 120 MD intended for ophthalmological use have been tested (data not shown) to assess the biocompatibility to the eye taking into account the clinical posology of the test items. The MEA has already been reported in literature as a robust tool to identify toxicity potential of eye-drops and to mirror the chronic toxicity effects observed clinically on the ocular surface by mild eye irritant allowing a mechanistic understanding of the sign of low or very low irritation potential (Baudouin, 2008; Pauly et al., 2009; Meloni et al. 2010, 2012; Liang et al., 2011; Meloni and Ranzini, 2017).

The aim of this work is to apply the MEA approach on the HCE to assess the biocompatibility of commercially available MD as eye-drops: each preserved or unpreserved formulation has been tested according to the two exposures relevant to assess their biocompatibility for transient and long-term use:

- Acute exposure: 24 h + followed by 16 h post incubation to identify a possible recovery from the induced acute damage
- Repeated exposure: 72 h (3 days with 2 applications/day) to assess long term tolerance.

2. Materials and methods

2.1. Test items and controls

The list of medical devices tested for their eye irritation potential is reported in Table 1 together with their respective preservative systems. Saline solution (0,9% NaCl) (Eurospital) was used as negative control for its known neutral action on tissues. Benzalkonium chloride (BAK) (Sigma) was used as positive control at the standard concentration used in eye drops (0.01%). At this concentration, it is known to be a mild irritant after acute exposure (Baudouin, 2008; Pauly et al., 2009; Meloni et al., 2010; Liang et al., 2011; Postnikoff et al., 2014).

2.2. Test system

Reconstructed Human Corneal Epithelium (HCE). HCE/S/5 (Reconstructed Human Epidermis, Small, age day 5, 0,5 cm^2) was purchased from EPISKIN (Lyon France). It consists of immortalized HCE cells grown vertically on a 0.5-cm^2 inert permeable polycarbonate filter and cultivated for 5 days at the air–liquid interface in a supplemented, chemically defined medium. All the handling before incubation was performed under a sterile air flow. The EPISKIN tissues were removed from nutrient agar immediately after delivery, transferred into six-well plates filled with the manufacturer's grow media, then the tissues were equilibrated in an incubator at 37 °C and 5% CO_2 before experiments were conducted.

2.3. Exposures

Acute exposure: Thirty microliters of saline solution, BAK at 0.01%, and of medical devices were applied on the apical surface of HCE tissues for 24 h followed by product removal by washing with 3 mL of saline solution. A post incubation of 16 h was adopted as recovery period. The post-incubation period was chosen based on previous data (Meloni et al., 2010) as the most predictive to assess the capacity of the corneal tissue to recover a toxic damage and thus to identify a reversible damage.

Repeated exposure: Thirty microliters, twice a day without washing, of saline solution, BAK at 0.01%, and of products were applied on the apical surface of HCE tissues for 72 h to mimic daily exposure allowing to investigate the long-term compatibility. 3 days repeated exposure on this 3D model has shown to mimic the damages induced by BAK in long-term clinical application (Meloni et al. 2010, 2012).

2.4. MTT test: cell viability quantification

Cell viability was evaluated by the MTT assay: this colorimetric assay measures the activity of the mitochondrial enzyme succinyl dehydrogenase, which is expressed in living cells and the relevance of the parameter to conclude about the eye irritation potential is described in the OECD TG 492. In this study the modified procedure on HCE originally described by A. Pauly (Pauly et al., 2009) has been adopted. Experiments were conducted on two HCE tissues and results are expressed as mean ± SD. The results are expressed as a percentage of cell viability compared with the negative control OD.

2.5. Trans-epithelial electrical resistance (TEER)

TEER was measured before application, after product removal, and after the recovery period using the Millicell-ERS instrument (range 0–20 kΩ). To perform the measurement (TEER), 0.5 mL of saline solution were directly applied on the tissue placed in a 6 well plate containing 5 mL of saline solution as well. The Millicell-ERS instrument was placed with the electrodes in the two chambers. Three measurements for each tissue were done. Experiments were conducted on five tissues and results are expressed as mean ± SD.

2.6. Lactate dehydrogenase (LDH) quantification

Membrane integrity was determined by measuring lactate dehydrogenase (LDH) in the extracellular medium using a commercially available kit (Cytotoxicity Detection KIT-LDH, Roche). A standard curve using different concentrations of LDH was used for LDH determination: 125; 62,5; 31,25; 15,6; 7,8; 3,91; 1,95 mU/mL. The biological replicate OD was interpolated in the concentration-OD curve of LDH standards to calculate the LDH concentration in the samples. Experiments were conducted on three HCE tissues and results are expressed as mean ± SD.

2.7. Real time PCR

Transcriptional studies of occludin mRNA suing qRT-PCR are described in our previous publications (Meloni et al., 2010).

3. Results

3.1. Cell viability by MTT test

The results of MTT test on HCE following both acute and repeated exposure are presented in Fig. 1 and Table 2.

For both exposures, 100% cell viability has been assigned to the negative control (saline solution) (NC).

The positive control BAK 0.01% has significantly reduced the cellular viability after acute exposure (44.81 ± 0.1%) and in the repeated treatment (0.9 ± 0.1%) as expected based on published data. According to OECD TG 492, there is an irritation potential if tissue viability after exposure to liquid substances and post-exposure incubation is ≤ 60%. Thus, in both the adopted experimental conditions BAK 0.01% can be considered as irritant for the eye.

Overall, the products have not significantly reduced the cellular viability and can be considered as not toxic after 24 h exposure (none of them has induced a viability reduction > 40%) but they have differently modified the viability according to the type of preservation system: a residual viability around 60% was quantified for products including “soft preservatives”: Artelac Rebalance Multidose (61.3 ± 2.3%), Optive Multidose (64.2 ± 2.4%) and Artelac Multidose (68.4 ± 0.7%).

A residual viability > 70% and up to of 85% have been quantified for unpreserved products: Optive Unidose (69.1 ± 0.0%), Artelac Rebalance Unidose (77.5 ± 2.8%), Thealoz Duo (78.1 ± 1.8%) and Artelac Edo (84,1 ± 3.4%).

After twice daily exposure during 3 days, all the “soft preserved” eye drops have reduced severely the cellular viability: Artelac Multidose (16.6 ± 0.7%), Artelac Rebalance Multidose (22.3 ± 0.7%) and Optive Multidose (30,2 ± 1.2%), comparable to the BAK control.

Differences in absolute residual % viability have been quantified within the preservative free eye drops and respectively: Optive Unidose

Table 1
List of tested products and their respective preservative system. These medical devices have been selected as they were commercially available with and without preservative systems (based on the same formulation), their preservatives are considered as soft preservatives suitable for dry eye condition. Thealoz Duo is only available as unpreserved.

PRODUCT NAME	PRESERVATIVE SYSTEM
OPTIVE MULTIDOSE	PURITE®
OPTIVE UNIDOSE	N/A
ARTELAC MULTIDOSE	CETRIMIDE
ARTELAC EDO (unidose)	N/A
ARTELAC REBALANCE MULTIDOSE	OXYD®
ARTELAC REBALANCE UNIDOSE	N/A
THEALOZ DUO (Multi- and unidose)	N/A

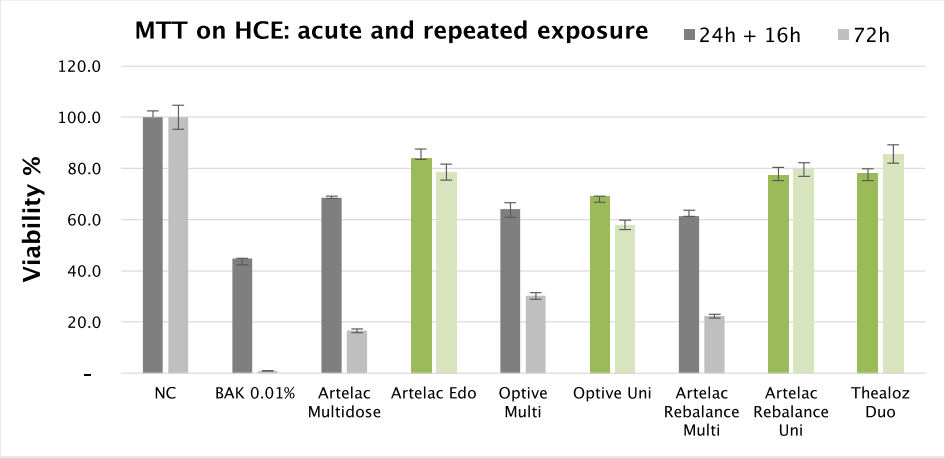

Fig. 1. Cellular viability data after acute exposure (24 h treatment + 16 h post incubation period) and repeated exposure (72 h – double treatment every day for 3 days). In light green are indicated preservative free MD. Experiments were conducted on two HCE tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Cellular viability data after acute exposure (24 h treatment + 16 h post incubation period) and repeated exposure (72 h – double treatment every day for 3 days). Experiments were conducted on two HCE tissues and expressed as mean cell viability % ± SD. Statistical analysis was performed by Student-T test.

	% viability			
	24 h + 16 h	p value	72 h	p value
NC	100.0 ± 2.4		100.0 ± 4.7	
BAK 0.01%	44.8 ± 0.1	$p < 0.01$	0.9 ± 0.1	$p < 0.01$
Artelac Multidose	68.4 ± 0.7	$p < 0.01$	16.6 ± 0.7	$p < 0.01$
Artelac Edo	84.1 ± 3.4	$p < 0.05$	78.5 ± 3.2	$p < 0.01$
Optive Multi	64.2 ± 2.4	$p < 0.01$	30.2 ± 1.2	$p < 0.01$
Optive Uni	69.1 ± 0.0	$p < 0.01$	58.0 ± 1.9	$p < 0.01$
Artelac Rebalance Multi	61.3 ± 2.3	$p < 0.01$	22.3 ± 0.7	$p < 0.01$
Artelac Rebalance Uni	77.5 ± 2.8	$p < 0.05$	79.6 ± 2.7	$p < 0.05$
Thealoz Duo	78.1 ± 1.8	$p < 0.01$	85.6 ± 3.6	Not significant

(58.0 ± 1.9%) (borderline for irritants) Artelac Edo (78.5 ± 3.2%) and Artelac Rebalance Unidose (79.6 ± 2.7%). Thealoz duo has shown a residual cellular viability closer to the negative control (85.6 ± 3.6%).

3.2. Barrier properties by TEER measurements

The mean value of transepithelial electrical resistance (TEER) recorded before treatment (t = 0 h) for the HCE tissues employed for testing test items and controls (N = 45) was 88.7 ± 3.3 $\Omega^{*}cm^{2}$ and indicates the good reproducibility of tissue barrier properties reflecting the global resistance of the barrier resulting from both the integrity of tight junctions' (TJs) structure and the epithelial thickness. Mean TEER values at t = 0 for each test item and control series were used as baseline value and considered as 100%. The TEER values from the different exposures (24 h + 16 h recovery and 72 h treatment) were expressed as % of the values of the basal level (Table 3) and as difference in percentage to the baseline value in Fig. 2.

The increased TEER values of the negative control at 24 h + 16 h and 72 h are due to the increased epithelial thickness during the culturing.

Since a TEER decrease correlates with increased permeability of ions and water through the paracellular pathway associated with changes in the barrier properties at TJs levels, TEER reduction observed at 24 + 16 h recovery (23.3 ± 4.1%) and at 72 h (41.8 ± 7.4%) confirms the toxic action of the preservative BAK at 0.01%.

The % TEER values observed for all the products tested after 24 h + 16 h of post-incubation were not different from the basal value (measure at t = 0 h) indicating that the limited exposure did not alter the epithelial permeability by either preserved or preservative free eye drops. These results suggest an absence of barrier function impairment at corneal epithelium level.

Following 72 h treatment, a significant reduction of TEER values has been found for Artelac Rebalance Multidose (43.1 ± 2.8%) and Artelac Multidose (37.6 ± 2.7%) and to a lesser extent for Optive Multidose (24.3 ± 2%). TEER values for soft preserved products resulted in the same range of BAK control indicating a significant modification of epithelial permeability.

After exposure to preservative-free (PF) products (Optive Unidose, Artelac Edo, Artelac Rebalance Unidose and Thealoz Duo) TEER values were not found different from the basal values. These data allow to conclude that after repeated exposure the corneal epithelium surface

Table 3
TEER values expressed as % of the values measured at t = 0 h (basal level) considered as 100% for acute (24 h + 16 h) and repeated (72 h) exposure. Measures were conducted on five HCE tissues. Statistical analysis was performed by Student-T test.

	% of the basal level (t = 0 h)			
	24 h + 16 h	p value (24 h + 16 h)	72 h	p value (72 h)
NC	130.7 ± 4.1		163.3 ± 7.4	
BAK 0,01%	76.7 ± 1.5	$p < 0.01$	58.2 ± 4.0	$p < 0.01$
Artelac Multidose	100.3 ± 2.2	$p < 0.01$	62.4 ± 2.7	$p < 0.05$
Artelac Edo	106.6 ± 3.7	$p < 0.05$	96.4 ± 3.7	$p < 0.05$
Optive Multi	109.3 ± 2.4	$p < 0.01$	75.7 ± 2.0	$p < 0.05$
Optive Uni	103.5 ± 3.5	$p < 0.01$	93.7 ± 4.2	$p < 0.05$
Artelac Rebalance Multi	103.0 ± 1.6	$p < 0.01$	56.9 ± 2.8	$p < 0.05$
Artelac Rebalance Uni	109.1 ± 2.0	$p < 0.05$	105.4 ± 6.0	$p < 0.05$
Thealoz Duo	99.1 ± 3.1	$p < 0.01$	103.4 ± 3.0	$p < 0.05$

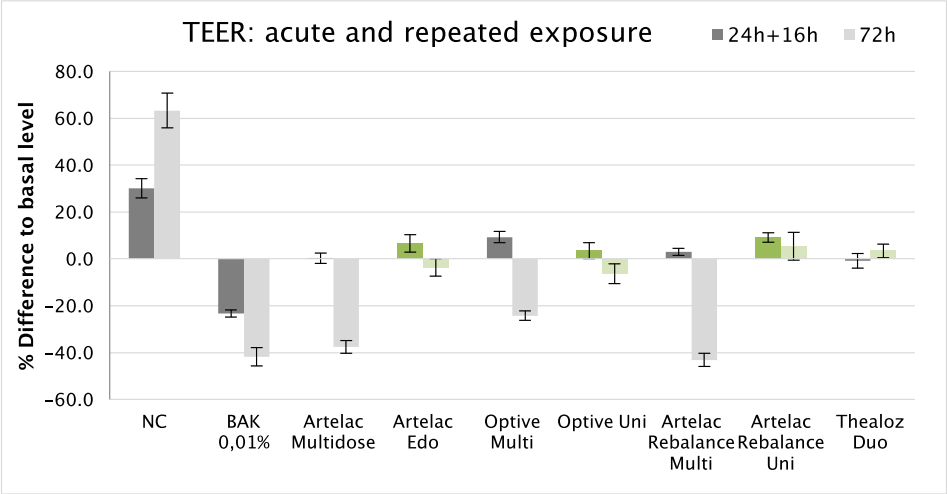

Fig. 2. Difference in percentage to the values measured at basal level (t = 0 h) considered as 100% for acute (24 h + 16 h) and repeated (72 h) exposure. In light green are indicated preservative free MD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was preserved (no significant TEER alteration) exclusively by unpreserved eye drops.

3.3. Membrane integrity by LDH release

LDH release in the medium has been quantified after 24 h + 16 h recovery (acute exposure) and after 72 h treatment. The results are reported as cumulative value during the 3 days indicating the relative amount of LDH release each day corresponding to medium change every 24 h (Fig. 3, Table 4).

As expected BAK 0,01% during the repeated exposure procedure has induced the higher release of LDH in the extracellular medium (cumulative release of 3600 mU/mL) which corresponds to a 6.7 fold increase compared to the NC (537 mU/mL). This release correlates to a damage at cellular membrane level and confirms its cytotoxic action on corneal tissue.

Within the preserved Eye drops, Oxyd® preservative system (in Artelac Rebalance Multidose) has determined a release of LDH comparable to BAK 0.01%, with a cumulative release of 3502 mU/mL, while Artelac Multidose and Optive Multidose presented a lower cumulative LDH release of 2732 and 2400 mU/mL, respectively.

All the preservative free eye drops have shown in 3 days a cumulative LDH release < of 2000 mU/mL, that is substantially higher to the negative control but lower to the BAK 0.01%. LDH measure reflects cellular membrane modifications occurring in the whole thickness and does not have a direct correlation with a toxic effect. Considering the viability and barrier permeability data, these results represent a basal level of tissue response to the treatment and confirm MTT assay that it has not induced a significant cytotoxic damage.

In details, Artelac Edo presented a cumulative LDH release of 1992 mU/mL, Optive Unidose of 1632 mU/mL and Artelac Rebalance Unidose of 1588 mU/mL. The lowest value has been quantified for Thealoz Duo (1433 mU/mL).

3.4. Occludin gene expression by qRT-PCR

In Fig. 4 are reported the Occludin (OCLN) gene expression results for the 24 + 16 h and 72 h treatments using the negative control (NC) as calibrator sample (RQ = 1).

The occludin has a functional role in repairing the epithelial barrier and restructuring the tight junctions (TJs): the proposed dynamic approach to the gene regulation by considering the two exposure times allows to identify the different levels of interaction and involvement of the corneal epithelium in order to identify the reversibility of the damage.

As expected and confirming our previous findings (Meloni et al.,

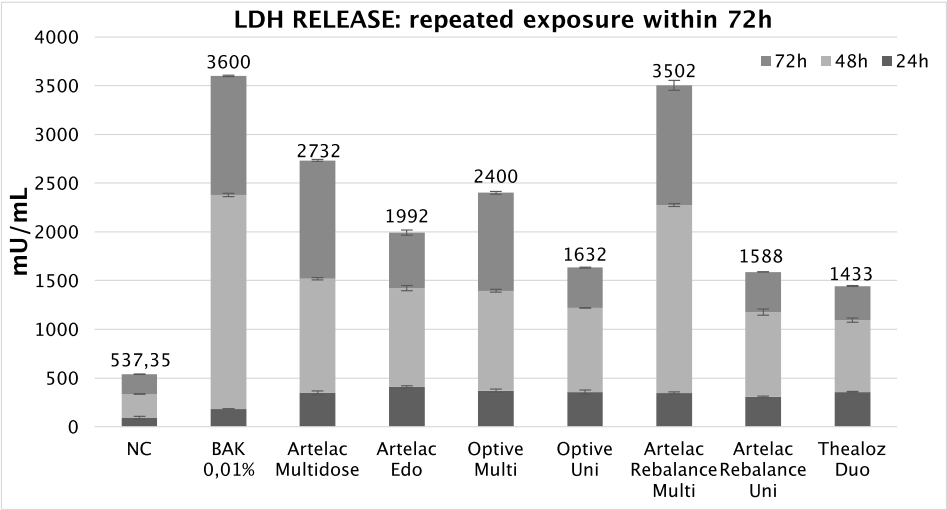

Fig. 3. Cumulative LDH quantification during repeated (72 h) exposure. Measures were conducted on three HCE tissues.

Table 4
LDH quantification indicating the relative amount of LDH release each day corresponding to medium change every 24 h. Measures were conducted on three HCE tissues. Statistical analysis was performed by Student-T test.

	mU/mL					
	24 h	p value (24 h)	48 h	p value (48 h)	72 h	p value (72 h)
NC	93 ± 13		244 ± 10		201 ± 20	
BAK 0,01%	183 ± 10	$p < 0.05$	2197 ± 17	$p < 0.01$	1220 ± 90	$p < 0.01$
Artelac Multidose	350 ± 19	$p < 0.01$	1167 ± 12	$p < 0.01$	1216 ± 11	$p < 0.01$
Artelac Edo	410 ± 80	$p < 0.01$	1012 ± 26	$p < 0.01$	571 ± 27	$p < 0.01$
Optive Multi	369 ± 17	$p < 0.01$	1024 ± 15	$p < 0.01$	1007 ± 14	$p < 0.01$
Optive Uni	356 ± 22	$p < 0.01$	864 ± 10	$p < 0.01$	412 ± 50	$p < 0.01$
Artelac Rebalance Multi	345 ± 11	$p < 0.01$	1929 ± 15	$p < 0.01$	1229 ± 51	$p < 0.01$
Artelac Rebalance Uni	305 ± 11	$p < 0.01$	870 ± 31	$p < 0.01$	413 ± 20	$p < 0.01$
Thealoz Duo	356 ± 70	$p < 0.01$	739 ± 21	$p < 0.01$	348 ± 50	$p < 0.01$

2010; Pauly et al., 2009) significant up-regulation of occludin gene expression was observed following 24 h exposure + 16 h recovery for BAK 0.01% followed by a significant high toxicity, which impacted on the relevance of the expression level in the long-term treatment (72 h); mRNA quality was nevertheless checked and considered as acceptable (data not shown).

The products treatments resulted in different behaviors for occludin gene transcriptional activity and have allowed a differentiation among products:

- not modifications induced at TJs level (neutral action),
- reversible damage,
- irreversible damage of corneal epithelial surface and TJs structure.

Namely, unpreserved eye drops (Thealoz Duo, Artelac Edo and Artelac Rebalance Unidose) did not induce modifications at TJs level (gene expression values closed to 1 for both exposure times). Artelac Multidose and Optive Multidose revealed a reversible damage with OCLN gene up-regulation only at 24 h + 16 h while Artelac Rebalance Multidose and BAK 0.01% showed irreversible damage with OCLN gene (e.g. up-regulation at 24 h + 16 h and close to the down regulation threshold (RQ = 0,5) at 72 h).

According to OCLN gene expression, the medical device Optive Unidose can be considered as a borderline product between products that cause reversible damage and products with a neutral action at epithelial surface level.

4. Discussion

The assessment of the long-term irritation potential of eye-drops is an issue since animal models have been proved not to be sensitive enough to assess weak irritants and patients' compliance, in clinical studies/practices, is complicated to evaluate over such long periods of treatments. Alternative methods based on the use of 3D reconstructed human corneal tissues are emerging to give a predictive and clinically relevant information about the biological impact of eye-drops as requested in the safety assessment of medical devices taking into account the intended purpose of the device, the part of the body where the device act and the duration of the contact.

The MEA approach on the HCE model was used to test preserved and unpreserved eye drop with different treatment times (acute and repeated exposure) in order to assess their irritation potential. The MEA provides not only one results but a body of evidences that together better asses the biocompatibility requirement in the context of a risk assessment taking into account the intended exposure scenario and increasing the relevance, sensitivity and predictivity *versus* humans.

As these eye drops are used for long term treatment, according to ISO 10993, these are qualified to have a permanent contact time (> 30 days treatment). This new approach could represent a necessary tool, bringing a more refine discriminatory test to highlight long-term irritancy potential and replacing *in vivo* irritation testing, required in biocompatibility testing of eye drops classified as medical devices.

Globally data on LDH release and TEER measurements confirmed the MTT results giving more sensitive information about the overall tissue response at ocular surface level with respect to the cell viability

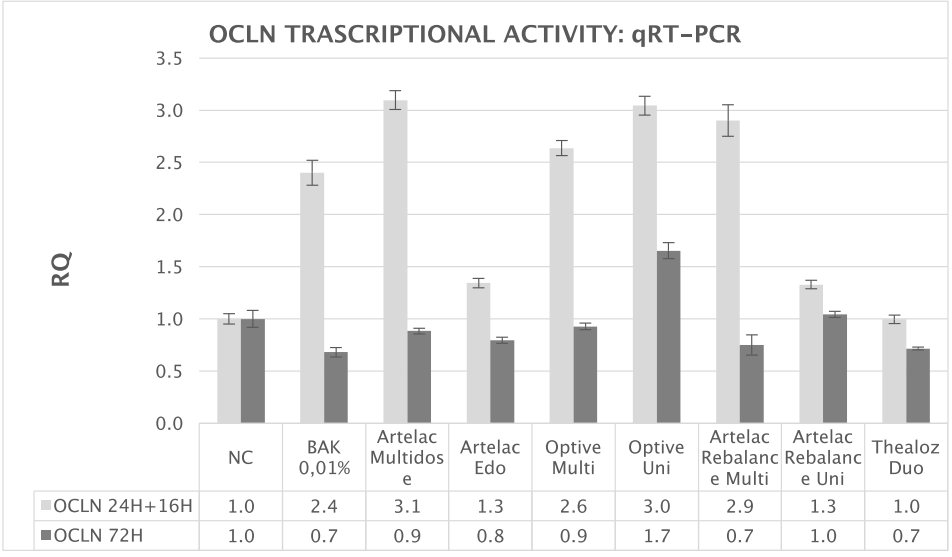

Fig. 4. Relative quantification (RQ) compared to negative control (RQ = 1) after 24 h treatment + 16 h recovery and after 72 h treatment. RQ = 1 corresponds to the same transcriptional activity of the control; RQ = 2 refers to significant up-regulation and RQ = 0.5 corresponds to significant down regulation. A value was accepted as significant based on 95% of confidence level when the gene is "one fold" up (RQ > 2) or down regulated (RQ < 0.5) compared to the calibrator sample (RQ = 1). Experiments were conducted on two HCE tissues.

data alone. In addition, mechanistic information derived from occludin gene expression analysis has been integrated into the MEA approach as a second level investigation and not for classification purpose.

The predictive value of this approach has been validated by the results obtained on BAK tested at 0.01% that corresponds to the dose usually present in eye drops. The BAK can be considered as a good reference of chemical irritant for the eye (Liang et al., 2011; Postnikoff et al., 2014; Yun et al., 2016) and its toxicity mechanism seems to not involve extracellular matrix degradation but rely on apoptotic mechanisms (Baudouin et al., 2010; Debbasch et al., 2001) and mitochondrial dysfunction (Sandipan et al., 2017).

Knowing that BAK still remains one of the main preservative system in eye drops on the market, it is therefore used as reference in this model for validating our approach for a robust and predictive biocompatibility assessment of eye drop medical device:

I. The toxicity of BAK 0.01% has been confirmed in our study with both protocols by a reduction of cell viability (< 50%), reduction of TEER values of 23.3% for acute and 41.8% for repeated exposure, increased release of LDH (2,3 and 6,7 fold for acute and repeated exposure, respectively) and up-regulation of occludin gene expression.
II. This is in agreement with our previous publication where BAK 0.01% resulted not toxic after 24 h treatment but cytotoxic after 24 h exposure + 16 h-24 h recovery and 72 h exposures (Meloni et al., 2010).

After 24 h acute exposure followed by 16 h post incubation, none of the selected preserved and unpreserved test items reveal signs of toxicity as compared to the negative control. On the contrary, after repeated exposure (72 h), the results obtained for cellular viability, membrane integrity (LDH release), barrier function (TEER measurements) and expression of occludin gene have demonstrated that repeated treatments induce different degree of corneal damages according to the presence/absence of preservatives. On the other hand, preservative free product such as Thealoz Duo is extremely well tolerated and does not affect corneal epithelium.

The new generation of so called “soft preservatives” have been shown to induce long-term potential cytotoxic effects based on preclinical and clinical data (Mundorf et al., 2003; Henry et al., 2008; Horsley et al., 2009; Kahook and Noecker, 2008; Lipener C 2009; Rolando et al., 2011; Brignole-Baudouin et al., 2011; Ammar et al., 2011; Gandolfi et al., 2012; Labbé, 2006; Peace et al., 2015). This has been confirmed by recent ophthalmological literature providing clinically relevant evidences that glaucoma patient satisfaction with preservative-free latanoprost was improved compared to patients using preserved treatments (Rouland et al., 2013; Aihara et al., 2012; Pauly et al., 2012; Negrete et al., 2017). The same good compliance and absence of chronic damages when using preservative free eye drops in the long-term use has been reported for patients with dry eye disease (DED) (Nasser and Navas, 2018).

Since ocular long-term medications can cause DED due to their allergic, toxic and immuno-inflammatory effects on the ocular surface and the presence of preservatives may further aggravate DED, the development and screening of less toxic drugs (both systemic and topical) and alternative preservative molecules remain an open field of research.

Furthermore, eye-drops are used also to protect the epithelial surface from allergenic compounds (Pauly et al., 2011; Guzman-Aranguez et al., 2014) and to assess the interference of new artificial tear formulations on corneal healing and permeation properties (Guzman-Aranguez et al., 2014; Pinheiro et al., 2015; Dutescu et al., 2017) and these treatments need to be performed without any risk to induce damages to the ocular surface.

The 3D human corneal epithelium model and the protocol based on acute and repeated exposures used in this study are of high interest because this approach was able to identify toxicity signals even for ‘soft’ preserved MD which were not detectable by performing the acute treatment.

The MEA approach includes different types of complementary parameters: cell toxicity (MTT), membrane damage (LDH), barrier impairment (TEER) and as additional read out a molecular parameter, the occludin gene transcriptional activity for 2 different exposures (acute 24 h + 16 h post-incubation relevant for the acute exposure toxicity prediction and 72 h relevant for the assessment after repeated exposures).

The overall advantages in using the HCE corneal model in the biological evaluation of a MD are numerous as 3D models possess a multilayered structure which takes into account penetration and buffering properties, display functionality close to the *in vivo* mechanisms and are predictive of human corneal epithelium responses either at cellular (MTT, LDH), functional (TEER) and molecular (occludin gene expression) levels.

The overall interpretation of these results, which is here proposed as a tentative mechanism based-prediction model, should consider as biocompatible a test item showing the following features:

- Not significant (< 50%) toxicity after acute and repeated exposure (MTT)
- Not significant release of LDH compared to the negative control after acute and repeated exposure
- Not significant or reversible reduction of TEER

The approach proposed in this study should be taken into consideration as reliable, predictive and sensitive alternative approach when investigating the biocompatibility and eye irritation potential of MD such as ophthalmic formulations intended for daily and long-term use with the following advantages: identification and discrimination within slightly irritant and mild products (sub-clinical signs) and identification of chemical structure related toxicity pathways.

5. Conclusion

In this study, the use of the repeated exposure protocol associated with the advanced MEA on HCE has allowed to identify reversible and irreversible damage at the corneal epithelium level associated with the application of products containing preservatives. The results suggest that even so-called ‘soft’ preservatives can cause direct toxicity but also ocular surface impairment and be as toxic as BAK 0.01% after chronic exposure. Unlike preservative free product as Thealoz Duo, even ‘soft’ preservatives are not completely safe, thus all preserved eye-drops should be avoided wherever possible in chronic conditions and clinicians should recommend the use of PF products for patients with established ocular surface disease (OSD) and for patients on multiple medications (Steven et al., 2018). The results presented confirm the sensitivity and predictivity of the MEA approach in detecting subclinical signs of cellular toxicity and the mechanisms of the toxic action that are responsible of the adverse reactions associated with long-term use of ophthalmic products.

The interest to perform a risk assessment based the MEA approach rely on different advantages: from a regulatory point of view it allows to perform the biological evaluation of substance based MD for ophthalmologic use with the actual dose and in the realistic exposure conditions providing a complete and predictive assessment of biocompatibility either after acute and repeated exposures as requested by the new MDR. Furthermore, the cytotoxicity and irritation potential evaluation are addressed in the same assay. From ethical point of view it allows to be compliant to the EU 2010/63 and at last but not least from a scientific point of view overcomes the well-known limitations of cell monolayers and *in vivo* approaches.

The applicability of 3D tissue models to MD biocompatibility testing has also being actively explored for the biological evaluation of MD, in

particular for the assessment of skin irritation and sensitization potential (McKim et al., 2012; Casas et al., 2013; Coleman et al., 2015; De Jong et al., 2018).

Currently, a new ISO 10993-23 standard for *in vitro* testing of skin irritation potential of medical devices which takes into account also eye and vaginal irritation is under development (ISO 10993–23 Determination of Skin Irritation of Medical Device Extracts using Reconstructed human Epidermis).

Conflicts of interest

The authors have no conflicts of interest to disclose and VitroScreen has no financial interest in the experimental models described in this manuscript.

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