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Cellular fluorescein hyperfluorescence is dynamin-dependent and increased by Tetronic 1107 treatment

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ABSTRACT

Sodium fluorescein ('fluorescein') staining of the ocular surface is frequently an indicator of compromised ocular health, and increases in the presence of certain contact lens multi-purpose solutions (MPS), a phenomenon known as solution induced corneal staining (SICS). The mechanism(s) underpinning fluorescein hyperfluorescence are uncertain, though may reflect increased cellular uptake of fluorescein by corneal epithelial cells. We have developed an *in vitro* model to study fluorescein uptake in both 'generic' mammalian cells (murine fibroblasts) and human corneal cells. Fluorescein hyperfluorescence increased after treatment with two MPS associated with clinical corneal fluorescein staining, yet there was no cellular hyperfluorescence for two MPS that do not cause this staining. Increased fluorescein uptake did not correlate with presence of a necrotic or an apoptotic marker (propidium iodide and caspase-3 respectively). Incubation of MPS-treated cells with dynasore (an inhibitor of dynamin, implicated in endocytic pathways) reduced fluorescein uptake irrespective of MPS treatment. The non-ionic surfactant Tetronic 1107 (present in both MPS associated with corneal fluorescein staining) increased uptake of fluorescein for both cell types, whereas an unrelated surfactant (Triton X-100) did not. We conclude that the clinical hyperfluorescence profile observed after exposure to four MPS can be reproduced using a simple model of cellular fluorescein uptake, suggesting this is the biological basis for SICS. Fluorescein entry does not correlate with necrosis or apoptosis, but instead involves a dynamin-dependent active process. Moreover the surfactant Tetronic 1107 appears to be a key MPS constituent triggering increased fluorescein entry, and may be the major factor responsible for SICS.

1. Introduction

Sodium fluorescein ('fluorescein') is frequently used in cell biological research, and as a diagnostic tool in ophthalmology and optometry, where it indicates the integrity of the corneal and conjunctival surfaces. When hyperfluorescence (staining) is observed, it is assumed to indicate dead or damaged cells (G. Wilson et al., 1995; Tabery, 1992; Tabery, 2003a; Tabery, 2003b). However, the cellular basis for this staining is uncertain, and may reflect physiological changes unrelated to cellular damage (Bandamwar et al., 2014; Bakkar et al., 2014). Corneal staining is typically observed with pre-existing conditions such as keratoconjunctivitis sicca (dry eye) (Jie et al., 2008; McMonnies, 2007), following trauma such as alkali burns or corneal abrasions (Wipperman and Dorsch, 2013; Fante and Trobe, 2014; S. A. Wilson and Last, 2004; Smally et al., 1992; Scarlett and Gee, 2007), contact lens wear, and with the use of certain contact lens care solutions (Andrasko and Ryen, 2008; Carnt et al., 2007b). It remains unclear whether increased fluorescein intensity is localised on the corneal surface, within or between epithelial cells, or within the stroma. It has been suggested that fluorescein can be internalised by epithelial cells (Mokhtarzadeh et al., 2011; Bandamwar et al., 2014) and that it is the increased localisation of fluorescein within the cell that is observed as hyperfluorescence.

Many recent reports have described the phenomenon of solution induced corneal staining (SICS) associated with soft contact lens wear (Efron, 2013; Andrasko and Ryen, 2008; Carnt et al., 2007b; Carnt et al., 2009; Carnt et al., 2007a; Morgan and Maldonado-Codina, 2009; Ward, 2008). SICS presents as superficial corneal and conjunctival staining, consistent with single (or clusters of) cells appearing brighter than adjacent cells (Maldonado-Codina et al., 2013). The intensity is greatest 1–2 h after exposure to certain soft lens/multipurpose solution (MPS) combinations (Bandamwar et al., 2010; Garofalo et al., 2005;

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Andrasko and Ryen, 2008; Lebow and Schachet, 2003). MPS is formulated to both clean and disinfect re-useable contact lenses and characteristically contains a surfactant cleaner (*e.g.* a poloxamine block copolymer such as Tetronic 1107), a biocide such as polyhexamethylene biguanide (PHMB) or polyquaternium-1 (PQ1) and a buffering agent (*e.g.* citrate, phosphate, or borate).

Certain lens/MPS combinations induce notable cytotoxic effects *in vitro* (Choy et al., 2012; Wright and Mowrey-McKee, 2005; Dutot et al., 2008; Dutot et al., 2010), although there is little evidence linking this to fluorescein hyperfluorescence. More broadly it is uncertain whether any cellular damage has occurred when hyperfluorescence is observed *in vivo*. For example, exposure to PHMB-based MPS, most commonly associated with SICS resulted in no histological change when observed using scanning electron microscopy (SEM) (Tchao et al., 2002). Conversely, after exposure to PQ-1-based MPS, typically *not* associated with increased hyperfluorescence, both membrane disruption and increased membrane permeability was observed (Tchao et al., 2002). It has been suggested that SICS is a benign phenomenon which is not indicative of dead or damaged cells (Maldonado-Codina et al., 2013). It is noteworthy that studies of MPS-associated hyperfluorescence predominately focus on the effects of biocides, and there is little exploration of the potential hyperfluorescence-inducing effects of other MPS components.

An *in vitro* model of SICS potentially provides a useful tool to investigate a) the biological basis of the interaction of epithelial cells with fluorescein and b) a basic understanding of the clinical SICS response. A convincing laboratory model of SICS should exhibit similar hyperfluorescence responses to those observed clinically. Our laboratory has previously developed such models utilising multiple cell types and has shown that fluorescein is taken into cells resulting in hyperfluorescence in response to treatment with a single MPS (Bakkar et al., 2014). We also demonstrated that internalisation and efflux of fluorescein are both energy dependent processes (Bakkar et al., 2014).

The present study aimed to test this model further by confirming that the presence or absence of hyperfluorescence associated with clinical use of four different MPS is also found in our model system. We additionally aimed to clarify the likely mechanisms underlying SICS by investigating whether any hyperfluorescence caused by these solutions is associated with increased apoptosis or endocytosis. There are numerous endocytic pathways that involve dynamin (a GTPase protein) and the actin cytoskeleton. These components in particular are integral to the invagination of the plasma membrane and the movement of endocytic vesicles (that contain extracellular materials) into the cell (Jeng and Welch, 2001). Dynamin is a GTPase implicated in several endocytic pathways, and has also been linked to actin cytoskeleton activity (Jeng and Welch, 2001; Doherty and McMahon, 2009). Both processes are integral to vesicle formation and uptake of extracellular materials into the cell body. Dynasore is a well-known inhibitor of dynamin (Tsai et al., 2009; Barrias et al., 2010; Yamada et al., 2009; Macia et al., 2006), and so this was used to investigate dynamin-associated endocytic pathways as a potential mechanism for fluorescein uptake. Finally, we aimed to identify the component(s) of MPS responsible for inducing hyperfluorescence.

2. Materials and methods

2.1. Cell culture

Human corneal (HC) cells were obtained from human corneal explants provided by Manchester Eye Bank. Briefly, HC cells were grown from segmented sections of corneal explants in wells pre-coated for 1 h with 0.01% calf-skin type I collagen (Sigma-Aldrich, UK) prepared in Dulbecco's PBS (PBS) (ThermoFisher, UK) (adapted from Kahn et al., 1993). Murine fibroblasts (L929 cells) (ECACC, UK) and HC cells were maintained at 37 °C and 5% CO_2 with Dulbecco's Modified Eagle Medium (DMEM) containing 4500mg^{-L} glucose (Sigma-Aldrich, UK)

Table 1
MPS formulations. MPS are referred to by biocide and surfactant combinations. P, PQ1, Aldox, and Alex refer to the biocides PHMB, Polyquaternium-1, Aldox and Alexidine dihydrochloride respectively. 1107, 1304 and 904 refer to surfactants Tetronic 1107, Tetronic 1304 and Tetronic 904 respectively.

MPS	Manufacturer	Biocide(s)	Surfactant
Biotrue P-PQ1-1107	Bausch + Lomb	Polyquaternium-1 (0.0001%), PHMB (0.00013%)	Tetronic® 1107 (1.0%)
ReNu Sensitive P-1107	Bausch + Lomb	PHMB (0.00005%)	Tetronic® 1107 (1.0%)
Opti-free Replenish PQ1-Aldox-1304	Alcon	Polyquaternium-1 (0.001%), ALDOX (0.0005%)	Tetronic® 1304 (0.05%)
Complete Revitalens PQ1-Alex-904	AMO	Polyquaternium-1 (0.0003%), Alexidine dihydrochloride (0.00016%)	Tetronic® 904 (0.1%)

supplemented with 10% v/v fetal bovine serum (FBS) (Sigma-Aldrich, UK) and 4 mM L-glutamine (Gibco by Life Technologies, UK), referred to hereafter as 'growth medium'. For HC cells, all tissue culture plasticware had been pre-coated for 1 h with 0.01% calf-skin type I collagen in PBS. Cells were maintained until they reached approximately 80% confluency, before rinsing with PBS and treating with 0.25% trypsin-EDTA (Life Technologies, UK) for L929 cells or 0.05% trypsin-EDTA in PBS for HC cells, and later neutralisation of trypsin-EDTA with growth medium. For experiments, cells were seeded in 24-well plates (Sigma-Aldrich, UK) at a density of 1×10^5cells/well for fluorescence microscopy, or in 96 well plates (Sigma-Aldrich, UK) at 2.5×10^4cells/well for high content analysis (HCA) or cell viability experiments, and in CELLview™glass bottom dishes (Greiner Bio-One, UK) at 5×10^4 cells per chamber for confocal microscopy.

2.2. Cell treatment with MPS, BKC, or MPS components

MPS formulations were obtained commercially, based on their differential association with SICS, and their main biocide and surfactants components (shown in Table 1). MPS known to cause hyperfluorescence were: ReNu Multi-purpose Solution Sensitive® and Biotrue® (Bausch + Lomb, Kingston up Thames, UK) referred to as P-1107 and P-PQ1-1107 respectively. MPS investigated which do not cause hyperfluorescence were: Complete RevitaLens® (AMO, High Wycombe, UK) referred to as PQ1-Alex-904, and Opti-Free Replenish® (Alcon, Surrey, UK) referred to as PQ1-Aldox-1304. In later experiments a 10% w/v solution of Tetronic 1107 (BASF, New Jersey, USA) was prepared in borate buffer (0.6% boric acid, 0.1% borax); borate buffer was used as this is the buffering system in use in P-PQ1-1107 and P-1107 formulations, and this was diluted to 1% (the concentration used in those MPS) and also 0.1% in borate buffer prior to addition to cells. A 1.25% solution of Triton X-100 (Sigma-Aldrich, UK) was diluted in PBS to produce 0.125%, 0.0125%, and 0.00125% solutions. Finally 0.01% w/v benzalkonium chloride (BKC) (Sigma-Aldrich, UK) was prepared in PBS. For cell treatment media was removed and the various MPS or surfactant solutions were added, before incubation for 2 h at 37 °C. BKC solution was added for 10 min at 37 °C (a sufficient time to induce necrosis). Effects were compared with cells treated with growth medium alone, hereafter referred to as 'media control', or another appropriate control.

2.3. Fluorescein staining

After treatment, test formulations were aspirated and cells incubated with 0.01% w/v fluorescein (sodium fluorescein, Sigma-Aldrich, UK) in PBS for 10 min at 37 °C. Hoecsht 33342, at a final

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