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Ocular safety comparison of non-steroidal anti-inflammatory eye drops used in pseudophakic cystoid macular edema prevention

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ABSTRACT

Non-steroidal anti-inflammatory drug (NSAID) eye drops are widely used to treat ocular inflammatory conditions related to ophthalmic surgical procedures, such as pseudophakic cystoid macular edema, and they have been used for off-label treatments. The most commonly used NSAIDs are diclofenac and ketorolac and the new molecules bromfenac and nepafenac have also been used. We used primary human keratocytes in cell culture in combination with a novel technology that evaluates dynamic real-time cytotoxicity through impedance analysis. This study also included classic cell viability tests (WST-1® and AlamarBlue®), wound healing assay, Hen's Egg Test and an ex vivo histopathological assay. NSAIDs were shown to have important cytotoxicities and to retard the healing response. Furthermore, the new eye drops containing bromfenac and nepafenac were more cytotoxic than the more classical eye drops. Nevertheless, no immuno-histochemical changes or acute irritation processes were observed after the administration of any eye drops tested. Due to cytotoxicity and the total absence of discomfort and observable injuries after the administration of these drugs, significant corneal alterations, such as corneal melts, can develop without any previous warning signs of toxicity.

1. Introduction

Ophthalmic surgical procedures are often associated with inflammatory reactions, which can have serious consequences (Roberts and Thum, 2014). Cataract surgery is one of the most common ocular procedures, and pseudophakic cystoid macular oedema (PCME) can happen as a result (Yonekawa and Kim, 2012). Non-steroidal anti-inflammatory drug (NSAID) eye drops are widely used to treat PCME and to treat other conditions off-label (Kim et al., 2010).

NSAIDs inhibit cyclooxygenase, which correlates with their affinity for the enzyme, and this results in a decline in the production of pro-inflammatory products (Warner and Mitchell,

Abbreviations: PCME, pseudophakic cystoid macular edema; NSAID, non-steroidal anti-inflammatory drug; WST-1, water soluble tetrazolium salts assay; RTCA, xCELLigence Real-Time Cell Analyzer System; HET-CAM, Hen's Egg Test-chorioallantoic membrane; CAM, vasculated chorioallantoic membranes; KCH, human corneal keratocytes; CI, cell index; NCI, normal cell index; WHA, wound healing assay; IS, irritation score; OCT, original concentration of eye drops.

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2004). For this reason, NSAIDs reduce swelling and pain (Quintana et al., 2014).

The most commonly used NSAIDs in ophthalmology are diclofenac (Voltaren®, Thea) and ketorolac tromethamine (Acular®, Allergan) ophthalmic solutions. However, an increase in the use of two novel NSAIDs, bromfenac (Yellox®, Baush&Lomb) and nepafenac (Nevanac®, Alcon), has been recently occurred.

The safety of any medication is determined by regulatory agencies, but post-marketing studies are necessary to further support the safety of medications over the long-term (Bennett et al., 2005). Ophthalmic NSAIDs have been proven to be safe and effective. However, they have also been shown to be associated with several side effects (Kessel et al., 2014). The most commonly reported side effects of topical NSAIDs include superficial punctate keratitis, stinging corneal infiltrates and corneal melts (Flach, 2001). It is important to note that the majority of these side effects occur when the drugs are used off-label (Turner et al., 1999).

Several authors have hypothesized that these side effects are related to the inhibition of cyclooxygenase, as it is important for cellular integrity (Lu et al., 1995). Other publications have suggested that the mechanism of NSAID toxicity is related to the up-regulation of the metalloproteinase matrix, which is crucial for the remodelling of the cornea (Reviglio et al., 2003).

This toxic effect was described previously in a pharmacokinetics analysis (Ahuja et al., 2008), in ex vivo histopathological studies (Hsu et al., 2003) and in corneal cell viability experiments (Xu et al., 2011). The majority of studies on ocular cytotoxicity are based on classic methods, such as cell proliferation reagent WST-1® uptake, which uses water soluble tetrazolium salts (Ayaki et al., 2010a), and 3H-thymidine uptake (Wu et al., 1999). These classical studies show variability on the time of sample measurement, cell type, cell density and drug concentrations tested. Therefore, the results obtained using these methods can vary between studies and be contradictory (Ayaki et al., 2010b).

xCELLigence Real-Time Cell Analyzer System (RTCA) impedance analysis is a novel technique with several advantages over the classical methods, as it does not interfere with dyes, is non-invasive, does not require a single time point measurement and does not require the use of labels. Furthermore, it is a high-throughput technique that allows acquisition of a multitude of data and is able to capture and reflect these data in a dynamic real-time manner (Otero-González et al., 2012).

The efficacy of NSAIDs depends on their penetration of the aqueous humour, which is directly related to their solubility in the stroma. The corneal stroma plays an important role in corneal integrity, as it forms 85% of the thickness of the cornea (Patel et al., 2001), and human corneal keratocytes (KCH) are the main cells responsible for corneal healing (Majmudar et al., 2000). For this reason we studied the viability and response of these cells under NSAID treatment. In addition, previous work has shown that this cell type is appropriate for testing the toxicities of specific drugs (Zorn-Kruppa et al., 2004).

For the above reasons, and due to the increasing reports of side effects of NSAIDs (Wolf et al., 2007), we developed a comparative toxicological study of four marketed eye drops containing the NSAIDs diclofenac, ketorolac tromethamine, bromfenac and nepafenac. We performed one ex vivo inmunohistochemical study, one organotypic in vitro assay called the Hen's Egg Test-chorioallantoic membrane (HET-CAM) and two methods developed in KCH, the classical wound healing migration assay and the novel RTCA assay.

To the best of our knowledge, there is no previous report in the literature on the use of this methodology to evaluate the cytotoxicity of this therapeutic group. Therefore, this is the first in vitro demonstration of the effect of NSAIDS on behaviour and the true viability of KCH using the RTCA system.

2. Materials and methods

2.1. Materials

We performed an experimental study to evaluate the safety of the four most popular NSAIDs (Voltaren®, Acular®, Nevanac® and Yellox®).

2.2. Isolation of human keratocytes

The cells were cultured from remaining corneal tissues after transplantation in accordance with ethical regulations. We performed the study according to the modified Ramke method (Ramke et al., 2013). The cornea was incubated for 10 min in trypsin at 37 °C, followed by mechanical removal of the endothelium and epithelium. The corneal stroma was cut into 2-mm sections and immersed in Dulbecco's Modified Eagle's/Ham's F-12 medium supplemented with 10% foetal bovine serum, containing 2 mM L-glutamine and antibiotics (100 IU penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin). After the keratocytes had proliferated at 37 °C and 5% CO_2, the tissue pieces were removed, and a cell pass of KCH was performed by trypsinization. All experiments were conducted between passages 4 and 10.

2.3. Keratocyte characterization

Corneas were fixed in 10% buffered formalin for 24 h and routinely embedded in paraffin. Sections of 4 μm in thickness were mounted on coated glass slides (Dako, Glostrup, Denmark). The immunohistochemical technique was automatically performed in an AutostainerLink 48 (Dako). The FLEX ready to use primary antibodies vimentin (clone V9) and cytokeratin (clone AE1/AE3) (Dako) were employed. Epitope retrieval was performed in PT Link at 97 °C for 20 min using low pH (for vimentin) and high pH (for cytokeratin) target retrieval solutions. We used the detection system EnVision FLEX/HRP (Dako). Sections were evaluated and photographed using a BX51 microscope equipped with a DP70 digital camera (Olympus, Tokyo, Japan). To characterize the keratocytes in culture and to ensure that the isolated cells were purely stromal, we performed an immunohistochemical analysis using antibodies against vimentin and cytokeratin AE1-AE3. Briefly, keratocytes were grown to 95% confluence on slides that had been fixed in ethanol 96° for 1 h. The immunohistochemical study was performed as indicated above, except for the epitope retrieval, which was performed in a microwave at 750 W for 20 min.

2.4. RTCA system

Cell experiments were performed using a cellular impedance biosensor system xCELLigence Real-Time Cell Analyser System (RTCA) (ACEA Biosciences, San Diego, CA). The RTCA system has been used in previous studies by other authors with the same objective (Xing et al., 2005). Briefly, this system uses electronic microchips that measure changes in impedance between the electrodes and the solution. When the cell is adhered to the well (E-plate 16 Acea Bioscence), the resistance increases and, consequently, increases the impedance. Using a mathematical algorithm, impedance values are transformed into a parameter called the cell index (CI). A lower CI represents a low number of cells binding to the microelectrode, while a high CI can be attributed to an increased cell number, increased cell adhesion or increased area of cell contact with the electrode. Thus, any cell change is reflected by CI number (Xing et al., 2006). The data are presented as dynamic changes in NCI (normalized CI at the time