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Spectrofluorometry assays for oxidative stress and apoptosis, with cell viability on the same microplates: A multiparametric analysis and quality control

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ABSTRACT

Our objective was to compare neutral red (NR) tests performed separately and on the same microplates as hydroethidine, H2DCF-DA and YO-PRO®-1 assays, and to compare toxicity ratios calculated with the two methods. Human trabecular meshwork cells (HTM-3) were exposed to different concentrations of benzalkonium chloride (BAC), to PBS and to diluted solutions of latanoprost and bimatoprost. Microplate fluorescence spectrophotometry assays were performed: NR uptake for cell viability evaluation, hydroethidine and H2DCF-DA for reactive oxygen species (ROS), and YO-PRO®-1 for apoptosis. The four NR assays presented identical toxicological profiles and correlation coefficients between them were close to one. There was no difference between toxicity ratios for ROS assays calculated by the two methods, with high correlation coefficients. However, in the apoptosis assay, ratios calculated with the double staining technique were more accurate. Thus, it is possible and recommended to perform NR assays on the same microplates as the other assays. This double staining procedure could constitute a quality control procedure and would allow multiparametric analysis.

1. Introduction

Fluorometric analysis has multiple applications in the field of toxicology as it helps to understand the mechanisms of cellular responses induced by toxic agents and to compare different drugs in order to determine their potential toxic or cytoprotective effects. This technique could be applied at the level of single suspended cells as in flow cytometry, with a potential for a multiparametric analysis using different probes simultaneously on the same cell (Herrera et al., 2007). However, in case of adherent cells, cell suspension implies a quite stressful step for cell detachment that might interfere with metabolic analysis. This can be overcome using microplate fluorescence spectrophotometry that allows direct analysis on living and adherent cultured cells. After being exposed to toxic agents, the metabolic response is analyzed for the whole cell population but without a multiparametric approach.

In ophthalmology, and particularly in ocular surface toxicology, microplate fluorescence spectrophotometry, as well as flow cytometry and in vivo experimentation, have proven their usefulness in the study of eye drop toxic effects (Baudouin et al., 2007; Pisella et al., 2004). These techniques confirmed that deleterious effects on conjunctival epithelial cells were caused by the preservative present in the solution, namely benzalkonium chloride (BAC) (Baudouin et al., 2010, 1999; De Saint Jean et al., 1999). This cytotoxicity was mediated by reactive oxygen species (ROS) production, particularly superoxide anions $O_2^{\cdot -}$, inducing a decrease in cell viability and an increase in apoptosis (Brasnu et al., 2008; Debbasch et al., 2001). Different assays and probes could be used to assess the underlying toxicological mechanisms. Neutral Red (NR) assay is used to evaluate cell viability and lysosomal membrane integrity as it is concentrated in functional lysosomes of living cells. This assay is validated by the European Centre for the Validation of Alternative Methods (ECVAM) (Zuang, 2001) and is considered as a good estimation of the number of viable cells (Borenfreund et al., 1990; De Saint Jean et al., 2000; Rat et al., 1994). Hydroethidine probe is highly reactive, and relatively specific, to superoxide radicals $O_2^{\cdot -}$ and it is a major tool in oxidative stress evaluation. Other free radicals, like $CO_3^{\cdot -}$ and $NO_2^{\cdot}$, may be detected by 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA or H2DCF-DA) (Soh, 2006; Wardman, 2007). Finally, apoptosis may be assessed by using the YO-PRO®-1 probe which enters apoptotic cells through membrane pores mediated by P2X(7) receptors (Dutot et al., 2006; Gibbons et al., 2001).

Further eye drop evaluations on conjunctival epithelial and on trabecular meshwork cell lines showed that observed toxic effects

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of BAC could be reduced by active compounds used for glaucoma treatment. Betaxolol, timolol and especially prostaglandin $PGF_{2\alpha}$ analogs showed a relative protective effect, though the current mechanism of this effect remains not understood (Guenoun et al., 2005; Hamard et al., 2003). For this purpose, ROS production and apoptosis needed to be reported relatively to cell viability in order to get a more accurate evaluation of toxicity in the remaining viable cells. However, these assays were performed on separate microplates and ratios were calculated over mean values of NR used as estimators of remaining viable cells.

The aim of our study was to perform the NR assays on the same microplates as the other assays in order to get an assessment of the number of viable cells in the same well and to compare the results with those obtained when NR was performed separately. This would prepare the field for a multiparametric analysis with microplate fluorescence spectrophotometry and for a more accurate estimation of the toxicity ratio per well. For this purpose, we tested a set of antiglaucomatous ophthalmic solutions because they are instilled over a long period of time and their potential toxic effects might thus have important clinical consequences. The experiments were performed on a trabecular meshwork cell line and toxic effects were evaluated by NR, hydroethidine, H2DCF-DA and YO-PRO®-1 assays.

2. Materials and methods

2.1. Trabecular meshwork cell line

HTM-3 cells, a line established from human trabecular meshwork of a glaucomatous donor, were cultured under standard conditions (humidified atmosphere of 5% CO_2 at 37 °C) in Dulbecco's minimum essential medium +4.5 g/l glucose (DMEM; Eurobio, Les Ulis, France) supplemented with 10% heat-treated fetal calf serum, 1% L-glutamine, 50 IU/ml penicillin, and 50 IU/ml streptomycin (Eurobio). Cells from passages 18 through 29 were used in all experiments. Normal culture development was assessed on daily basis by phase-contrast microscopy (Leica, DMIRB, Wetzlar, Germany).

Cells were grown in 75 cm^2 sterile cell culture flasks. Confluent cultures were removed by gentle trypsin incubation, and the cells were counted and diluted to a concentration of 90.000 cells/ml. They were then seeded into 96-well culture plates (Corning BV, Schiphol-Rijk, The Netherlands) for microtitration analyses (200 µl/well). Cultures were kept at 37 °C for 24 h. After subconfluence was attained (culture surface covering nearly 90%), cells were exposed to the different formulations.

2.2. Cell treatments

We tested six different preparations at a 1:10 dilution, according to previously validated protocols (Guenoun et al., 2005). The preservative, BAC (Théa, Clermont-Ferrand, France) was used at three different concentrations, i.e. 0.005%, 0.015% and 0.02%. Toxic effects of BAC are well reported and well known (Brasnu et al., 2008; De Saint Jean et al., 1999; Debbasch et al., 2001), and they were used as landmarks in our experiment. Two commercialized prostaglandin PGF2α analogs were chosen for their high and low BAC concentrations: latanoprost 0.005% containing 0.02% BAC (Xalatan®; Pfizer, New York, NY) and bimatoprost 0.003% containing 0.005% BAC (Lumigan®; Allergan, Irvine, CA). Phosphate buffered saline (PBS, Eurobio, Les Ulis, France) was used as negative control. The cultured cells were exposed to these different solutions (200 µl/well) for 30 min at 37 °C, according to previously validated protocols (Guenoun et al., 2005). Afterward, toxic effects were evaluated with the different assays.

2.3. Equipment

Microplate fluorescence spectrophotometry assays were performed on 96-well microplates using Safire™ monochromator-based microplate reader (Tecan Group Ltd., Männedorf, Switzerland). This technique is characterized by high sensitivity and specificity, with an adjustable excitation wavelength (230–850 nm) and a wide range of emission wavelength detection (280–850 nm). Wavelength accuracy and precision are ±1 nm. For all assays, excitation and emission bandwidths were 5.0 nm, and fluorescence was measured using the bottom reading mode and an optimal gain (Rat et al., 1994).

2.4. NR assay

Neutral red uptake was used to assess lysosomal membrane integrity, a technique validated for the evaluation of cell viability in vitro by spectrophotometry. It is considered as a good estimation of the number of viable cells. Neutral red (Fluka, Ronkonkoma, NY) was used at 50 µg/ml according to a previously validated protocol (Borenfreund et al., 1990; De Saint Jean et al., 2000). In the microplates that were not tested with the other assays, cells were incubated with PBS for 20 min, in the dark and at room temperature. This maneuver was intended to simulate the other assays performed before NR assay and to eliminate any bias. After cell treatment for 30 min and washing with PBS, 200 µl of medium containing neutral red were added to living cells in each well, and the microplates were incubated for 3 h at 37 °C in atmosphere with 5% CO_2. Cells were then washed with PBS, elution medium (EtOH/AcCOOH, 50%/1%) was added and plaques were gently shacked for 10 min. The fluorescence was measured with an excitation wavelength of 535 nm and an emission wavelength of 600 nm (Dubrovsky et al., 2006).

2.5. Hydroethidine assay

Dihydroethidium (hydroethidine) (Molecular Probes, Eugene, OR) is a highly reactive and specific probe to superoxide radicals $O_2^{\cdot -}$ (Soh, 2006; Wardman, 2007). It exhibits blue-fluorescence in the cytosol until oxidized into 2-hydroxyethidium. The resulting product intercalates within nuclear DNA enhancing a fluorescent red staining. The initial solution (5 mM in Dimethyl Sulfoxide, DMSO) was diluted to 1:1000 in PBS. Before the distribution of the tested treatments, cells were pre-incubated in the presence of 200 µl/well of the diluted hydroethidine solution (5 µM), for 20 min, in the dark and at room temperature. Then the probe solution was removed and the cells were incubated in presence of the six treatments for 30 min, in the dark at 37 °C. Afterward, fluorescence was measured with Safire™ (excitation, 485 nm; emission, 600 nm) (Budd et al., 1997), then cells were washed once with PBS and the NR assay was performed on the same microplates.

2.6. H2DCF-DA assay

2′,7′-Dichlorofluorescein diacetate (H2DCF-DA; Molecular Probes, Eugene, OR) is a nonfluorescent cell-permeable compound used as a marker of oxidative stress. Once inside the cell, it is cleaved by endogenous esterases to H2DCF thus preventing the back-diffusion of the dye into the extracellular space. The de-esterified product becomes the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) on oxidation by ROS. H2DCF-DA has been widely used for the detection of H_2O_2, however it is considered more specific of the free radicals $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ (Soh, 2006; Wardman, 2007). The initial solution (0.1 M in DMSO) was diluted to 1:5000. Cells were pre-incubated for 20 min, in the dark and at room temperature. Then the probe solution was removed and the