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A Comparison of the Effects of Ocular Preservatives on Mammalian and Microbial ATP and Glutathione Levels

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The aim of this study was to investigate the mechanism of action of the preservative sodium chlorite ($NaClO_2$), and the relationship with intracellular glutathione depletion. A detailed comparison of the dose responses of two cultured ocular epithelial cell types and four species of microorganism was carried out, and comparisons were also made with the quaternary ammonium compound benzalkonium chloride (BAK), and the oxidant hydrogen peroxide (H_2O_2). The viability of mammalian and microbial cells was assessed in the same way, by the measurement of intracellular ATP using a bioluminescence method. Intracellular total glutathione was measured by reaction with 5,5′-dithiobis-2-nitrobenzoic acid in a glutathione reductase-dependent recycling assay. BAK and H_2O_2 caused complete toxicity to conjunctival and corneal epithelial cells at ~25 ppm, in contrast to $NaClO_2$, where >100 ppm was required. The fungi *Candida albicans* and *Alternaria alternata* had a higher resistance to $NaClO_2$ than the bacteria *Staphyloccus aureus* and *Pseudomonas aeruginosa*, but the bacteria were extremely resistant to H_2O_2. $NaClO_2$ caused substantial depletion of intracellular glutathione in all cell types, at concentrations ranging from <10 ppm in *Pseudomonas*, 25–100 ppm in epithelial cells, to >500 ppm in fungal cells. The mechanisms of cytotoxicity of $NaClO_2$, H_2O_2 and BAK all appeared to differ. $NaClO_2$ was found to have the best balance of high antibacterial toxicity with low ocular toxicity. The lower toxicity of $NaClO_2$ to the ocular cells, compared with BAK and H_2O_2, is in agreement with fewer reported adverse effects of application in the eye.

Keywords: Glutathione; Oxidative stress; Corneal epithelial cells; Conjunctival epithelial cells

Abbreviations: BAK, benzalkonium chloride; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GSH, reduced glutathione; GSSG, glutathione disulfide; RCE, rabbit corneal epithelial cells; WKD, human conjunctival epithelial cells

INTRODUCTION

Since the decisions by the FDA and U.S. Pharmacopoeia implementing the addition of preservatives in all multidose ophthalmic preparations, the potential benefits of inhibiting microbial contamination and the drawbacks of adverse patient reactions have been often reported.[1–3] A wide range of preservatives is available and these are routinely used to maintain acceptable levels of microbial contamination in a variety of multidose ocular applications, including glaucoma and dry eye treatments. One of the most commonly used groups of preservatives is the quaternary ammoniums, which includes the compound benzalkonium chloride (BAK). However, there are increasing concerns over the effects of long term ocular application of BAK, following many observations over the last two decades of chronic inflammation or toxicity in eyes treated with BAK-preserved ocular solutions.[4–9] There are also many reports that cultured ocular cells, including conjunctival, trabecular, and lens epithelial cells are also susceptible to damage by this compound.[11–13] These preservatives exhibit surfactant-like properties, and their cytotoxic action was previously assumed to be due mainly to a physical mechanism involving membrane disruption. While this may be

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true for microorganisms, evidence is emerging that the cytotoxic mechanism in ocular cells is considerably more complex.[14] As a result of the adverse reactions encountered with BAK-preserved solutions, there is considerable interest in alternative preservatives, particularly those which act by an oxidative mechanism and are perceived to have milder effects on the eye.

Oxidative preservatives are usually small molecules that can penetrate cell membranes and interfere with cellular function,[15] causing oxidative stress. Examples include hydrogen peroxide (H_2O_2) and oxychloro compounds such as sodium chlorite ($NaClO_2$) and chlorine dioxide (ClO_2). These oxidizing compounds appear to cause few problems of chronic sensitivity, at least in studies to date.[16] The effects of H_2O_2 and other peroxides on both mammalian cells and microorganisms have been extensively studied in a variety of contexts, and it has been reported that antioxidants such as catalase, ascorbate, glutathione, and the thiol-cycling enzymes are important protectants in mammalian ocular cells.[17–19] The effects of $NaClO_2$, by contrast, are less well established. Its low toxicity to animals has long been established, owing to its use in water purification in some countries, and some studies of its antimicrobial effectiveness in relation to oral infections,[20] teat disinfection[21] and reducing bacteria on food surfaces[22] have been reported. However, its mechanism of action is not well understood and to date very few biochemical studies have been carried out on cells relevant to the eye.

We have shown previously that $NaClO_2$ reacts very readily with glutathione, a thiol-containing antioxidant, both *in vitro* and in cultured cells or microorganisms, whereas attack on phospholipids only occurs at very high concentrations.[23] In that study, the effect of two concentrations of $NaClO_2$ was compared with BAK and various oxidizing compounds over a 24 h timecourse. It was found that $NaClO_2$ had relatively low toxicity to ocular epithelial cells, and a possible role for intracellular glutathione in protecting both mammalian and microbial cells against damage by chlorite was suggested.

In view of the increasing use of $NaClO_2$ for preserving ocular solutions, direct comparisons with other preservatives and an improved understanding of its mechanism is essential. The current study aims to address this issue by determining the cellular dose responses to these compounds using a method that would allow a direct comparison between mammalian cells and various microorganisms, in order to assess the relative toxicity and antimicrobial effectiveness of these preservatives. For this purpose, a bioluminescence method was used to measure cytosolic ATP levels, which has been reported to be a sensitive indicator of cytotoxicity in both microorganisms and mammalian ocular cells.[24–27] Such a comparative study of these preservatives has not previously been conducted. We also wished to investigate the importance of glutathione in toxicity or cellular resistance of the different cell types, by comparison of the glutathione depletion response with the ATP depletion response.

MATERIALS AND METHODS

Materials

The rabbit corneal epithelial cell line (RCE) and human conjunctival epithelial cell line (WKD, a Wong–Kilbourne derivative of Chang conjunctival cells) were obtained from ECACC (No. 95081046 and No. 88021103, respectively). *Candida albicans* was donated by Dr C. Gemmel at Glasgow Royal Infirmary and *Alternaria alternata* species group (IMI 389319, CABI Bioscience UK, Egham, UK) was provided by Allergan, California, USA. Yeast extract and bactopeptone were purchased from Difco, Michigan USA, and tryptone soya broth was from Oxoid, Hampshire, UK.

Mammalian Cell Culture

RCE cells were cultured in DMEM:Ham's F12 medium supplemented with 2 mM glutamine, 5 μg/ml insulin, 10 ng/ml EGF and 15% foetal calf serum. WKD cells were cultured in Medium 199 (Hanks) supplemented with 2 mM glutamine and 10% foetal calf serum.

Growth of Microorganisms

C. albicans and *A. alternata* were grown aerobically in an orbital incubator for 18 h in YEPD broth containing 1% yeast extract, 2% bactopeptone and 2% glucose. The *Candida* cultures grown at 37°C, whereas *Alternaria* cultures were grown at 26°C. The bacterial species *Staphylococcus aureus* and *Pseudomonas aeruginosa* were incubated aerobically in 3% tryptone soya broth at 37°C for 18 h.

Preservative Treatments

The preservatives tested were H_2O_2, BAK and $NaClO_2$ at final concentrations of 0, 5, 25, 50, 100, 250, 500, 750, 1500, 3000 and 6000 ppm. Mammalian cells were treated at concentrations up to 750 ppm, whereas higher concentrations were used for some treatments of the microorganisms. An incubation period of 6 h was chosen as previous studies found that loss of viability at intermediate to high treatment concentrations (e.g. 50–200 ppm BAK; 2000 ppm $NaClO_2$) was observed for all microbes between

4 and 24 h [23 and Ingram *et al.*, unpublished data], and also because this is the first criteria application point in the European Pharmacopoeia PET method. Individual dose response experiments (6–10 concentrations) were carried out on different days for each of the six cell types, three preservative treatments and both analyses (ATP and glutathione), and each of these 36 different experiments was repeated four times, also on separate days.

Concentrations were expressed in ppm, as these (or %) are the units most commonly given for commercial therapeutic preparations. Moreover, BAK is a mixture of alkyl chain lengths and therefore doesn't have a single formula weight. However, as the formula weights for $NaClO_2$ and H_2O_2 are significantly different, the molarities of the treatments were calculated (Table I) and used for a further comparison on a molecular basis. For this purpose an average formula weight of 343.1 was used for BAK.

Mammalian Cell Treatments for Measurement of Glutathione Changes

The ocular epithelial cells were grown to 90–100% confluence and then washed with sterile PBS. Fresh, serum free culture media, containing the preservative or oxidant test solution at the concentrations indicated above, was added to the cells in tissue culture flasks, and these were incubated in the dark for 6 h at 37°C. At the end of the treatment cells were harvested using Acutase (icT, California, USA), and washed twice with PBS. The wet cell weight was recorded for each pellet. The cells were extracted by the addition of 5% trichloroacetic acid (TCA) to precipitate cellular protein, followed by freeze thawing and centrifugation (10,000*g* for 1 min) to remove the cell debris. The supernatants were stored at 4°C and assayed for total glutathione within 2 h. Treatments were carried out in the dark to prevent decomposition of H_2O_2 and $NaClO_2$, which are somewhat light sensitive. Under the conditions used, in the absence of serum and at physiological pH, all the preservatives used are essentially stable for at least the duration of the treatment.

Analysis of Total Glutathione

Total intracellular glutathione concentration was measured in TCA extracts using a spectrophotometric assay based on the modified DTNB-GSSG recycling method described previously.[28] The assay mixture contained 770 μl of 200 mM sodium phosphate buffer pH 7.0 containing 8 mM EDTA, 100 μl of 6 mM DTNB, 100 μl of 1.9 mM NADPH, 25 μl of sample or glutathione standard, and the reaction was started by the addition of 5 μl of glutathione reductase (340 units/ml). GSH standards (20–100μM) were prepared in equivalent concentrations of TCA to the samples.

Mammalian Cell Treatments for Measurement of ATP

The WKD and RCE cells were seeded at 1×10^4 cells per well in 100μl of media (i.e. 1×10^5 cells/ml stock) on 96 well luminometer plates (Greiner, UK) and allowed to adhere overnight. Additional wells on the plate were seeded with 100μl of serial (1/2) dilutions of the 1×10^5 cells per ml stock, to generate a calibration curve. The culture medium was removed and cells were washed with PBS. A 100 μl of serum free medium containing the preservatives was added to the wells, and the plate was incubated for 6 h at 37°C in the dark. After this the medium was removed and 100μl of fresh serum-free media was placed in each well. The well contents were assayed for intracellular ATP levels using a CellTiter-Glo™ assay kit (Promega, UK) and a LUMIstar Galaxy (BMG, UK) microplate luminometer. The protocol followed the "Instructions for Use and Notes" specifically written for the LUMIstar Galaxy machine.[29]

Microbial Cell Treatments for Measurement of ATP

Pseudomonas, *Staphylococcus*, *Alternaria* and *Candida* were harvested by centrifugation after 18 h growth, washed twice in PBS and resuspended at 0.01 g/ml in preservative treatments prepared in PBS. The microorganisms were incubated in the dark for 6 h at their original culture temperature. At the end of incubation, 100 μl of treated culture were placed in wells of a 96 well luminometer 0.2 μm filter plate (Millipore, UK). Additional wells on the plate were seeded with 100 μl of serial dilutions of the control cells, to generate a calibration curve. Media was removed and cells washed with PBS by vacuum manifold (Pall, UK) filtration. A 100 μl of PBS was placed in each well to re-suspend the cells.

TABLE I Conversion of treatment concentrations in ppm or percentage to millimolar

	0	5	25	50	100	250	500	750	1500	3000	6000
ppm											
(%)	0	0.0005	0.0025	0.005	0.01	0.025	0.05	0.075	0.15	0.3	0.6
H_2O_2 (mM)	0	0.147	0.74	1.5	2.9	7.4	14.7	22.1	44.1	88	176
Chlorite (mM)	0	0.055	0.28	0.55	1.1	2.8	5.5	8.3	16.6	33.1	66
BAK (mM)	0	0.015	0.07	0.15	0.3	0.7	1.5	2.2	4.4	8.7	17.5

The well contents were assayed for cellular ATP levels using a ViaLight MDA (Cambrex, UK) assay kit on a LUMIstar Galaxy (BMG, UK) microplate luminometer. Bactolyse (100 μl) was injected into the wells and plate incubated for 5 min with shaking of the plate. ATP monitoring reagent (20 μl) was then injected into the wells and the luminescence measured for three 1 s readings after a 2 s delay.

Microbial Cell Treatments for Measurement of Glutathione Changes

Candida, *Psuedomonas*, and *Alternaria* were grown, treated, and incubated in the dark with preservatives in the same manner described for ATP analysis. After incubation cells were centrifuged and washed twice in PBS. The pellet was resuspended in 1.5 ml of PBS and the cells were extracted by cell membrane disruption (Constant Systems) at 20 kPsi. A 0.25 ml aliquot of 20% TCA solution was added to 0.75 ml of pressed cells to precipitate cellular protein. The cell debris was removed by centrifugation and total glutathione was measured in the supernatants as described above.

Analysis of $NaClO_2$ Depletion

The medium remaining after the cells had been harvested for analysis of glutathione was assayed to determine the concentration of $NaClO_2$ remaining. A 50 μl aliquot of sample (diluted as required) was mixed with 50 μl of 2% KI and 1.5 ml of 50 mM HCl, and the formation of iodine was detected at spectrophotometrically at 350 nm. $NaClO_2$ concentration in the samples was calculated from a standard curve in the concentration range 1–100 ppm.

Statistical Analysis

Statistical analysis of the data was performed in Minitab using Mann–Whitney *U* tests. Each dose response curve was carried out at least four times on separate occasions, and the data shown are averages ± the standard error of the mean.

RESULTS

Analogous experiments were carried out on the mammalian cells and microbial cells investigated in this study; the cells were incubated with a range of concentrations of the preservatives for 6 h before analysis either of cellular ATP level or intracellular glutathione levels. The measurement of intracellular ATP by bioluminescence was chosen as the most sensitive method of determining cytotoxicity that could be used with all six cell types. The CellTiter-Glo™ assay kit (Promega, UK) was used for mammalian cell ATP determination, and the ViaLight™ MDA kit (Cambrex, UK) was used for the microorganisms; these kits are marketed by the companies specifically for the determination of cell viability, and there are extensive reports in the literature that this technique correlates well with other measurements for determining cytotoxicity of applied treatments, and is a very sensitive method.[24–27,30,31] In view of this, depletion of cellular ATP was interpreted as equating to loss of cell viability.

Figure 1 shows comparisons of the effect of the preservatives on ATP levels in each of the cell types tested, with the data presented as the percent (%) ATP compared to control treated cells (treated with PBS only). Panels a and b show the results for conjunctival and corneal epithelial cells, respectively, which were tested to a maximum concentration of 750 ppm for all three compounds. For both mammalian cells it is clear that $NaClO_2$ was the least deleterious preservative, with a noticeable depletion in cellular ATP occurring only above 100 ppm (1.1 mM) for RCE and 50 ppm (0.55 mM) for WKD. Both H_2O_2 and BAK caused severe depletion of ATP at 25 ppm (0.74 and 0.07 mM, respectively), and had similar effects on both cells. RCE cells were slightly more resistant to all the preservatives than WKD cells. There was a significant difference between the responses of the mammalian cells to $NaClO_2$ and BAK or H_2O_2. For example, in RCE cells the viability was significantly higher with $NaClO_2$ treatments at 50–250 ppm than with these concentrations of H_2O_2 or BAK ($p < 0.05$). With WKD cells, the viability following $NaClO_2$ treatments of 50 and 100 ppm was significantly higher than for treatments with these concentrations of the other preservatives ($p < 0.05$). This was true regardless of whether the concentrations were considered in ppm or molarity, and is in agreement with results reported previously,[23] which also indicated that $NaClO_2$ had lower toxicity to corneal and conjunctival cells.

Panels c and d show the results obtained with the yeast *C. albicans* and mould *Alternaria*. The main difference in the profile of these fungal cells compared to the mammalian cells is that H_2O_2 was much less toxic. Surprisingly, the fungal cells were as sensitive to BAK as the mammalian cells. On the other hand, $NaClO_2$ was not particularly effective in its antimicrobial action against the fungi: in *Candida* significant ATP depletion was observed at concentrations similar to H_2O_2, while with *Alternaria* it caused a small depletion of ATP at concentrations from 50 to 500 ppm (0.55–5.5 mM), which was not observed with H_2O_2, but had less effect than H_2O_2 above 750 ppm. Together, this suggests that the fungi both have good protection against the oxidative preservatives, but are very susceptible to membrane disrupting agents.

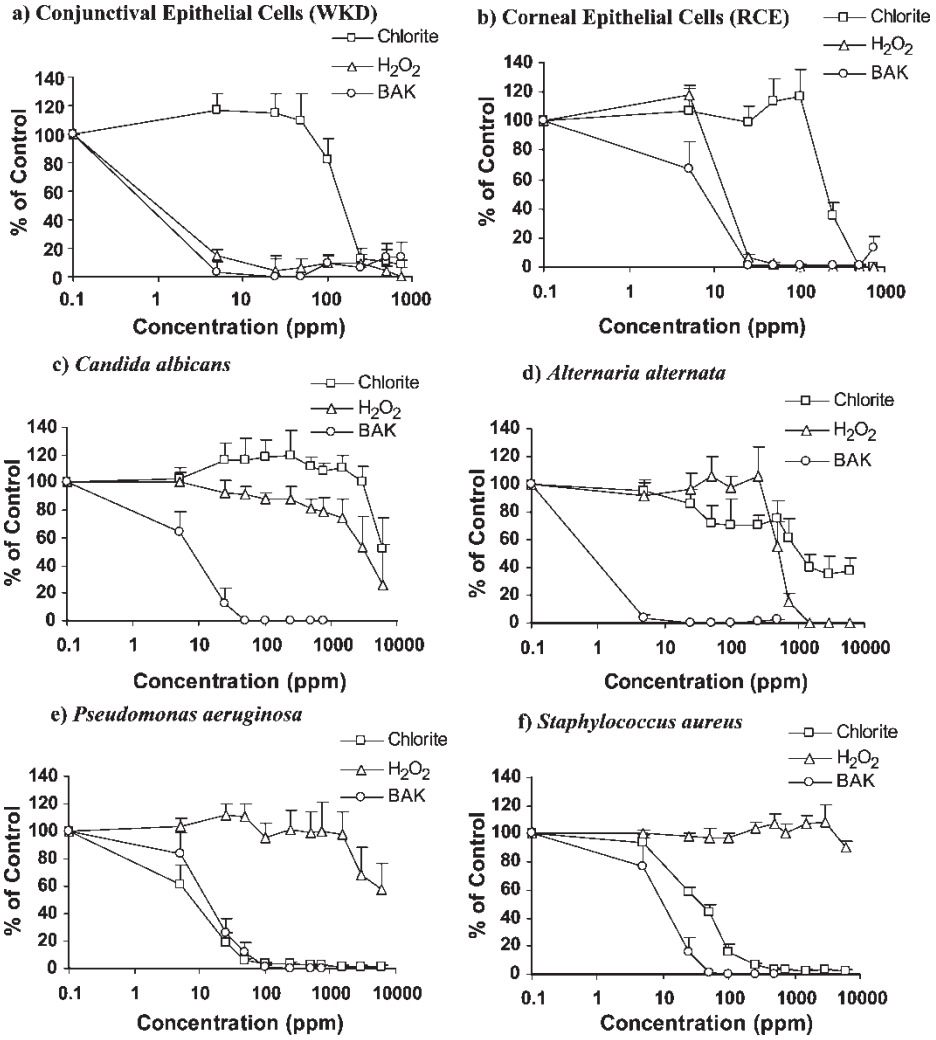

FIGURE 1 *The effect of preservative treatment on intracellular ATP levels*. Six different cell types were treated with the preservatives hydrogen peroxide (H_2O_2), sodium chlorite (chlorite) and benzalkonium chloride (BAK) at a wide range of concentrations for 6 h. After washing to remove the treatments, the intracellular ATP was measured by a bioluminescent assay to monitor cell viability. The results are presented as the percent (%) of control ATP remaining after treatment, where control treatment corresponded to incubation in the absence of preservatives. The error bars correspond to 1 standard error of the mean, and $n \geq 4$ in all experiments.

The profile of the bacterial cells was different to both the fungi and mammalian cells, as shown in panels e and f of Fig. 1. Both *Pseudomonas* and *S. aureus* demonstrated considerable resistance to H_2O_2, which had no effect on ATP levels in the latter even at the highest concentration tested (6000 ppm; 176 mM). $NaClO_2$ showed very good antibacterial activity, and was almost as effective as BAK in reducing the viability of *Pseudomonas*. Thus it appears that the bacteria are very good at detoxifying H_2O_2, but are unable to detoxify $NaClO_2$ to the same extent.

In treatments with $NaClO_2$, the concentration of preservative remaining at the end of the 6 h incubation was analysed, to determine whether $NaClO_2$ was removed from the medium by interaction with the cells. The assay used detects both chlorite and ClO_2, which is important because solutions of $NaClO_2$ usually contain a small amount of ClO_2 in equilibrium, and this is generally considered to be the active antimicrobial ingredient. It was found that the degree of depletion that occurs varies between the different types of cell investigated (Fig. 2). *C. albicans* clearly showed the largest depletion of $NaClO_2$ from the medium (panel c), and thus its response was rather different to the other cell types. The mammalian cells also showed depletion of $NaClO_2$ but the effect observed with RCE cells (panel b) was larger than that of WKD cells (panel a). In contrast, little depletion of $NaClO_2$ was observed with *Alternaria* or the bacterial cells (panels d, e and f) at high treatment levels, although a small depletion occurred at concentrations of 25–750 ppm.

Of the six different cell types investigated, all except *S. aureus* were found to contain detectable concentrations of the antioxidant glutathione.

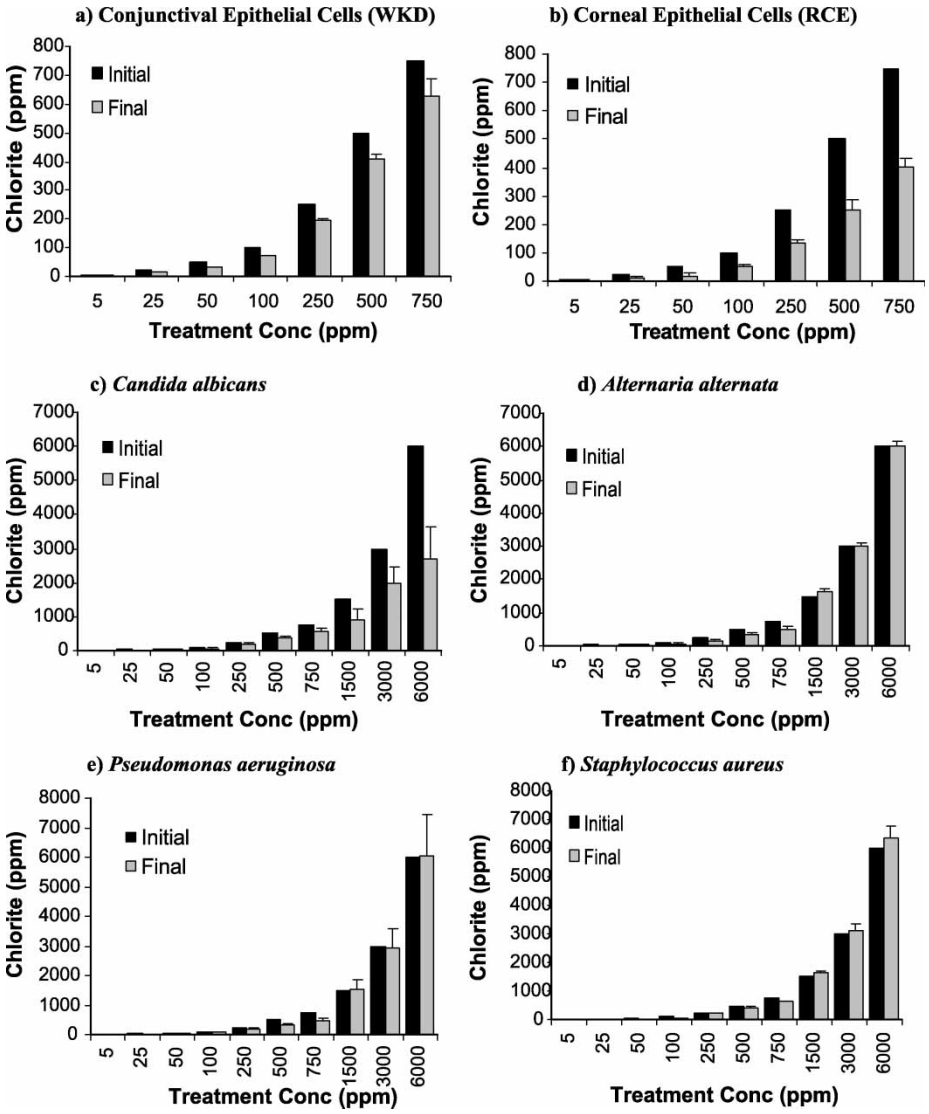

FIGURE 2 *The depletion of sodium chlorite from treatment medium following incubation with cells*. Six different cell types were treated sodium chlorite as described in Fig. 1, and at the end of the 6 h treatment the concentration of sodium chlorite remaining in the medium was determined by reaction with potassium iodide and spectrophotometric assay. The results are compared to the starting concentration at each different dose tested, to indicate the depletion of sodium chlorite by the cells. The error bars correspond to 1 standard error of the mean, and $n = 4$ in all experiments.

The lack of detectable glutathione in *S. aureus* is in agreement with previous reports that most Gram-positive bacteria lack this thiol compound.[32,33] These five cell types were then used to study the effect of the preservatives on intracellular glutathione levels, over the same dose range as tested for the viability studies, and the different cellular responses were compared. Figure 3 shows the effects of $NaClO_2$ on total glutathione concentration in mammalian cells, fungal cells and *Pseudomonas*. Panels a and b shows the results from ocular conjunctival and corneal cells, respectively, and it can be seen that the responses of these two cell types were very similar, with a steady depletion of glutathione occurring between 5 and 250 ppm (0.055–2.8 mM). The fungal cells (panels c and d) were able to resist glutathione depletion up to $NaClO_2$ concentrations of 100 ppm (1.1 mM), with *Candida* showing slightly greater ability to maintain intracellular glutathione levels. In contrast, *Pseudomonas* was unable to prevent loss of glutathione even at the lowest $NaClO_2$ concentration tested (panel e). Interestingly, $NaClO_2$ at low concentrations increased the intracellular glutathione content of *Alternaria*, and to a lesser extent that of *Candida*. When the glutathione dose response is compared with the ATP dose response, it can be seen that in the ocular cells that the glutathione decrease appears to occur at lower levels of stress than the loss of viability. It is possible that this is also true for *Pseudomonas*, but more low doses would need to be tested to confirm this. The effect is unclear in the fungi, owing to the increase in glutathione level that was observed at sublethal stresses.

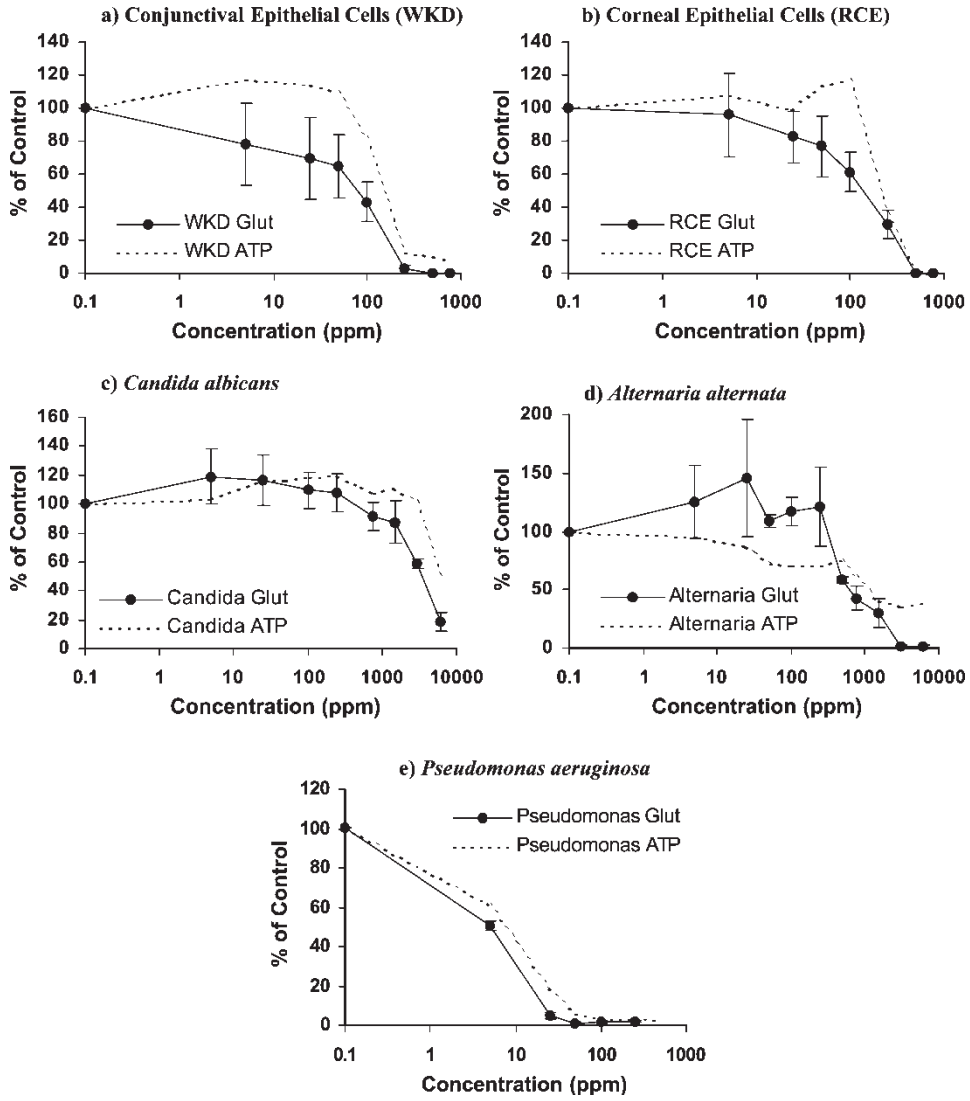

FIGURE 3 *The effect of sodium chlorite treatment on intracellular glutathione concentration.* Five different cell types were treated with a range of sodium chlorite concentrations as described in Fig. 1, and the total intracellular glutathione (GSH + GSSG) was then measured using the DTNB-glutathione reductase recycling assay. The results are presented as the percent (%) of control glutathione remaining after treatment. The error bars correspond to 1 standard error of the mean, and $n \geq 4$ in all experiments.

The effects of H_2O_2 treatment on the intracellular glutathione were rather different (Fig. 4). With the exception of the corneal cell line (panel b), H_2O_2 was less effective at depleting intracellular glutathione than $NaClO_2$ in all the cells tested, with *Candida* (c) and *Pseudomonas* (d) showing particularly high resistance. It was noted that *Candida* showed an increased glutathione levels in response to H_2O_2 concentrations between 5 and 1000 ppm (0.15–30 mM), whereas *Alternaria* did not appear to be able to respond to H_2O_2 induced stress by raising glutathione levels. In all except the corneal cells (where glutathione and ATP depletion profiles were very similar), loss of viability was evident at lower H_2O_2 concentrations than the depletion of glutathione. A similar observation was made for the effect of a range of BAK concentrations on intracellular glutathione (Fig. 5), again with the exception of corneal cells. However, the response to BAK was different in that glutathione depletion occurred in mammalian cells and micro-organisms at low doses, with 100 ppm (0.3 mM) BAK causing almost complete loss of the anti-oxidant. The glutathione response in the two mammalian cells (panels a and b) to BAK was very similar, and the microorganisms were only slightly more resistant.

It was also noted that neither of the mammalian cells showed any up-regulation of glutathione metabolism in response to either H_2O_2 or $NaClO_2$, but instead there was an increase in ATP levels (probably suggesting increased proliferation) at sub-lethal preservative levels (Fig. 1a and b). This may reflect fundamental differences in the behaviour of mammalian and fungal cells when subjected to oxidative stress.

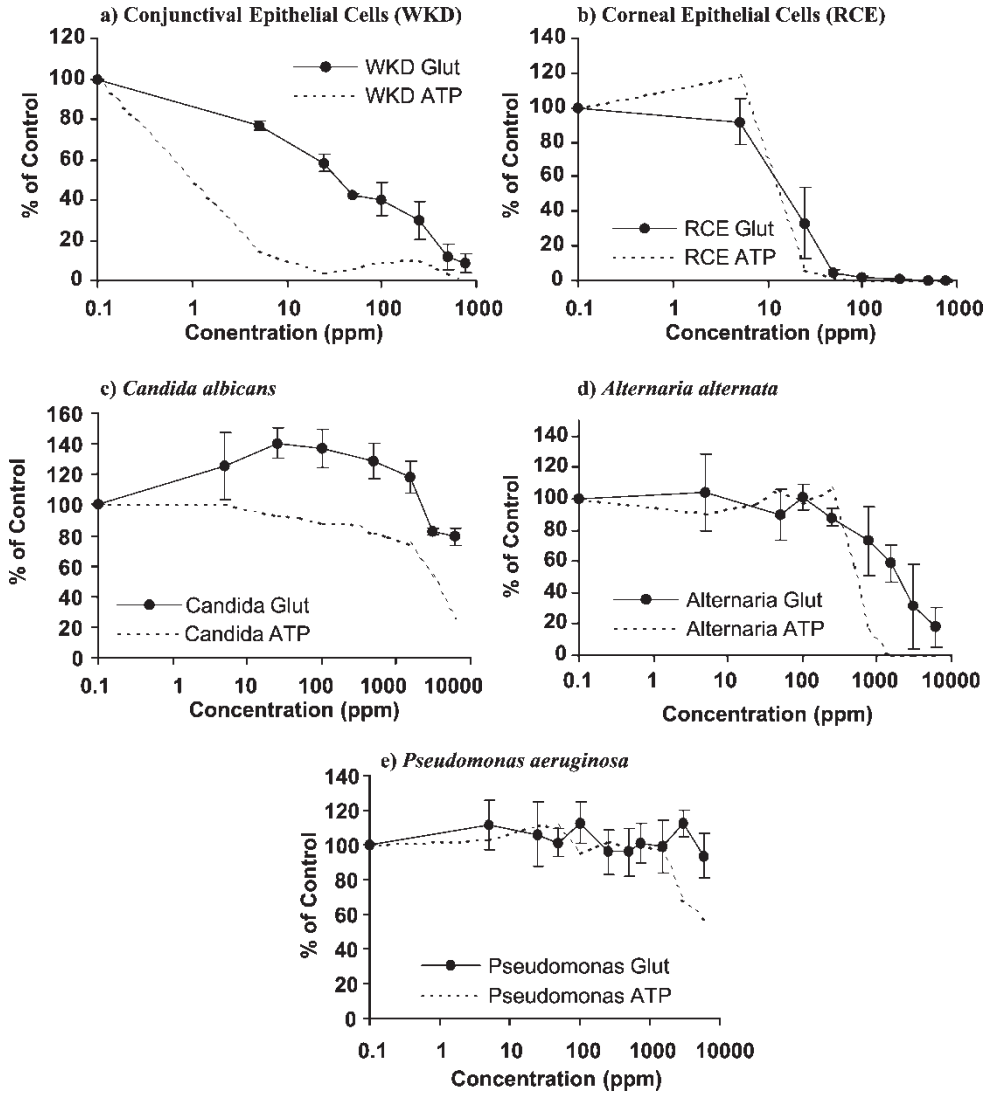

FIGURE 4 *The effect of hydrogen peroxide (H_2O_2) treatment on intracellular glutathione concentration.* Five different cell types were treated with a range of H_2O_2 concentrations before extraction and analysis of total intracellular glutathione as described in Fig. 3. The error bars correspond to 1 standard error of the mean, and $n \geq 4$ in all experiments.

DISCUSSION

All solutions for multidose ocular application contain preservatives to prevent microbial contamination. BAK is a commonly used preservative in treatments for glaucoma at concentrations varying from 0.004 to 0.02% (e.g. Alphagan [0.005%], Azopt [0.01%], Rescula [0.015%], Xalatan [0.02%]) and dry eye (e.g. HypoTears [0.01%]), but long-term application often leads to adverse pathohistological and inflammatory effects.[3] Two alternative preservatives used in artificial tears are 0.001% polyquaternium-1 (Tears Naturale II) and sodium perborate, which releases 60 ppm H_2O_2 in solution (GenTeal). More recently, ocular therapies preserved with stabilized oxychloro compound (i.e. $NaClO_2$) have been developed, such as Refresh Tears P for dry eye and Alphagan P for glaucoma (both contain 0.005% preservative). So far, these formulations have been well received, with equivalent therapeutic effectiveness and minimal adverse effects on the eye reported.[16] Although the low general toxicity of $NaClO_2$ has long been established, little work on the molecular interactions of $NaClO_2$ with cells (either mammalian or microbial) has been carried out, and research into its mechanism of preservative action is needed.

The dose response curves performed allow a direct comparison of the balance of microbial and ocular cell toxicity of $NaClO_2$ with that of BAK and H_2O_2, which can also be used as a preservative at low concentrations.[34] As expected, it was found that BAK caused deleterious effects at the lowest concentrations tested in both ocular cell lines, in agreement with a number of previous reports that BAK is toxic to a variety of cultured ocular cells.[10–13] However, BAK was also highly effective at killing the microbial cells, with *Alternaria* showing susceptibility comparable to

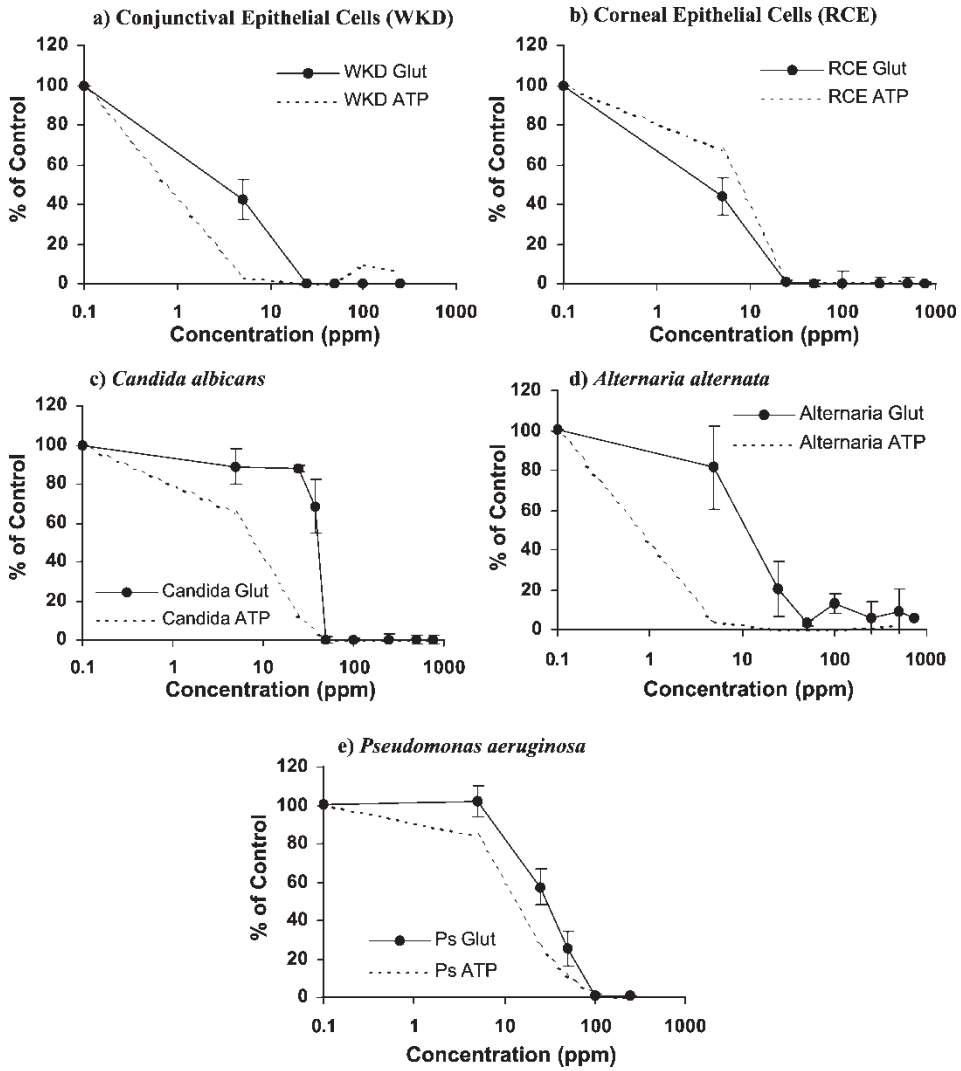

FIGURE 5 *The effect of benzalkonium chloride (BAK) treatment on intracellular glutathione concentration*. Five different cell types were treated with a range of BAK concentrations, otherwise the experimental details were the same as in Fig. 3.

conjunctival cells, and overall the responses of mammalian, fungal and bacterial cells to BAK were very tightly clustered, suggesting that the mechanism of toxicity of BAK is not one that readily can be avoided by cellular detoxification defence. H_2O_2 was also found to have very severe effects on the conjunctival and corneal epithelial cells, but in contrast had relatively little effect on the fungal cells, and the bacteria were highly resistant to it. H_2O_2 levels within normal lens and aqueous humour are <1 ppm, although levels may increase up to 5-fold in cataract patients,[35] and concentrations of 1–10 ppm have been shown to be toxic to lens epithelial cells,[35,36] so the cytotoxicity to corneal and conjunctival epithelial cells observed in this study is in good agreement with previous findings in ocular cells. The presence of efficient antioxidant defences are likely to be responsible for the high resistance of the microorganisms to H_2O_2: *P. aeruginosa* and *C. albicans* are known to contain high activities of catalase, as do many strains of *S. aureus*.[37–39] Many microorganisms also contain alkyl hydroperoxide reductases that are able to detoxify H_2O_2.[40,41] The profile of $NaClO_2$ toxicity was found to be different to that of both BAK and H_2O_2, in that it had little deleterious effect on mammalian cells up to approximately 100 ppm, and was almost as toxic as BAK to the bacteria, although the fungal cells showed considerable resistance. Overall, $NaClO_2$ appeared to be the compound with the best preservative action, in combining low ocular cell toxicity at the concentration used in ocular formulations (50 ppm) with good antibacterial effectiveness. It should also be taken into account that microbial contaminants in the multi-dose container would be likely to be in contact with the preservative for a period of several hours, whereas the preservative would be expected to clear from the ocular surface rather faster than this following dispensing of the formulation. However, it is important to point out that, although Chang conjunctival cells in particular have been used in numerous studies,[10,14,45] there

could potentially be inherent differences in response between immortalized cell lines and primary ocular cells.

Another aim was to investigate whether the loss of viability with $NaClO_2$ treatment was linked to depletion of intracellular glutathione, a thiol-containing antioxidant found to be present at significant levels in all the cell types tested except *Staphylococcus*. Under normal conditions oxidized glutathione (GSSG) is recycled to the reduced form GSH by the action of glutathione reductase with concomitant utilization of NADPH, but under severe oxidative stress this reaction may not be sufficiently fast to prevent the build up of GSSG in the cell. As this form is toxic, efflux of GSSG occurs, resulting in depletion of the intracellular glutathione pool.[42] Comparison of the dose response curves for viability and intracellular glutathione of cells treated with $NaClO_2$ showed that with this treatment glutathione concentrations in the mammalian cells decreased at lower preservative doses than were needed to induce severe depletion of ATP. The greater sensitivity of glutathione compared to viability in mammalian cells could be compatible with the hypothesis that depletion of intracellular glutathione, as a result of imposed oxidative stress, contributes to loss of viability caused by $NaClO_2$ treatment. Interestingly, it has been suggested in neurodegenerative disorders that oxidative stress and glutathione depletion is instrumental in cell death by both apoptotic and necrotic pathways.[43] However, it was not clear whether a similar effect occurred with the fungi and with *Pseudomonas*. In the fungal cells it was difficult to ascertain because of initial increases in glutathione concentration at low doses, which suggest that an adaptive stress response was occurring; enzymes of glutathione are known to be inducible by oxidative stress in microorganisms.[44] It was also apparent that high initial levels of glutathione do not necessarily prevent high sensitivity to $NaClO_2$: *Pseudomonas* contained high levels of glutathione but had similar (low) resistance to that of *Staphylococcus*, which had undetectable levels of glutathione, and *Alternaria* with low intracellular glutathione had much higher resistance.

When the same comparisons were made for H_2O_2 and BAK treatments it was found that depletion of ATP (corresponding to cytotoxicity) mostly occurred at lower doses in the response profile than depletion of glutathione, suggesting that the loss of glutathione was unlikely to contribute significantly to the loss of viability, and instead was the consequence of membrane damage and cell death. As H_2O_2 is an oxidant that can be detoxified by glutathione peroxidase, this was somewhat unexpected. However, cytosolic glutathione peroxidase (GPx-1) has a relatively low K_m for H_2O_2 (40 μM) compared to catalase, whose reaction is increasingly effective up to millimolar H_2O_2 concentrations, and it has been observed previously in ocular cells that there is redundancy between GPx-1 and catalase.[35] Thus possibly catalase protects the intracellular glutathione pool from H_2O_2, and cell death results from plasma membrane damage, as H_2O_2 is relatively membrane soluble. This is in contrast with $NaClO_2$, which is ionic, and has been found previously not to induce significant lipid oxidation in cells.[23] What is clear is that different mechanisms of cytotoxicity must be occurring with these two compounds.

As BAK is not an oxidant, the loss of intracellular glutathione at higher doses than those which caused loss of viability could be understood simply in terms of the surfactant properties of BAK causing membrane disruption and consequent loss of cellular contents. However, it has been demonstrated that low doses of BAK can cause apoptosis in cultured Chang epithelial cells, and reactive oxygen species (H_2O_2 and superoxide) are produced during this process.[14] There is evidence that these reactive oxygen species (of which O_2^- appears to be most relevant) are released from the mitochondria, following uncoupling of the respiratory chain, and that there was a decrease in cellular GSH levels 24 h after the short treatment, which was thought to be due to glutathione efflux.[45] The actual role of cellular GSH in this apoptosis is unclear; possibly glutathione depletion is merely a secondary event resulting from mitochondrial oxidant generation as an apoptotic signal. In this case our findings in conjunctival epithelial cells, namely that glutathione depletion appeared to be a consequence of BAK cytotoxicity, would be in agreement with this report, as decreases in ATP are thought to occur in apoptosis as well as necrosis in mammalian cells.[46] However, the situation in the corneal epithelial cells appeared to be different, and in the microorganisms, where apoptosis is not a well established process, loss of glutathione as a result of membrane disruption remains the most likely scenario.

It was hypothesized that $NaClO_2$ would be depleted during treatment either by its damaging interaction with biomolecules, or owing to detoxification by antioxidants. It was therefore expected that chlorite depletion might relate either to the levels of antioxidants in the cells, or to the cellular resistance to the oxidant. *Candida* caused the largest depletion of $NaClO_2$, and also had the highest resistance, but resistance and $NaClO_2$ depletion did not show a similar correlation in the other types of cells, and no clear overall relationships between the percent (%) chlorite consumed with intracellular glutathione or ATP levels could be found. Despite the fact that we have shown previously that $NaClO_2$ reacts very readily with glutathione,[23] the large $NaClO_2$ depletion by *Candida* cannot be explained simply by native glutathione levels, as *Pseudomonas* had

the highest glutathione level (3.50 ± 0.81 μmol/g cells) while *Candida* had an intermediate level (1.90 ± 0.49 μmol/g cells). Possibly the fact that *Candida* was the only cell type tested to have significant levels of the ascorbate analogue erythroascorbate[23] may contribute to this difference in behaviour. Alternatively, enzymatic pathways for removal of either $NaClO_2$ or ClO_2 may be involved; for example, it is known that some microorganisms have enzymes that are able to reduce oxychloro compounds.[47,48] Overall, the results suggest that the disappearance of $NaClO_2$ from the treatment medium may relate more to detoxification of the compound by the cells, than to deleterious reactions that lead to cell death.

In summary, the data presented indicate that $NaClO_2$, H_2O_2 and BAK all have different mechanisms of cytotoxicty in the cells tested. Native intracellular glutathione levels do not correlate with cellular resistance to $NaClO_2$, but depletion of intracellular glutathione could be instrumental in the loss of mammalian cell viability that occurred at concentrations above 100 ppm. Overall it appeared that $NaClO_2$ had the best profile for a preservative compound of the three tested, as it was substantially less toxic to ocular epithelial cells than the other two compounds, but was a very effective antibacterial agent. In addition, the importance of developing effective but non-toxic anti-fungal agents to assist in preserving solutions for ocular therapy is apparent.

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