Cell Viability of Four Corneocconjunctival Cell Lines Exposed to Five Preservatives and a Surfactant Used for Infection Control in Eyedrops

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The aim of this study was to evaluate the cytotoxicity of six ingredients used in eyedrops with regard to four corneocconjunctival cell lines. Cells were treated with the undiluted solution, and 2-fold, and 10-fold dilutions of each solution for 10, 30, and 60 min and cell viability was measured with the neutral red assay and the MTT assay. The degree of toxicity was based on the cell viability score (CVS). The CVS50 was determined by the number of measurements with a viability ≥ 50% of control. The CVS40/80 was calculated as follows: CVS40/80 = (number of measurements with a viability value >80%) - (number of measurements with a viability value <40%). Results were expressed as % of total measurements (%CVS). The results of each ingredient for %CVS50, and %CVS40/80 were 0.01% benzalkonium chloride (51, -13), 1% boric acid (100, 99), 0.4% methyl paraoxybenzoate (100, 100), 0.4% propyl paraoxybenzoate (100, 100), 1.0% polysorbate 80 (68, 18), and 0.5% chlorobutanol (100, 100). The use of benzalkonium chloride led to apparently low cell viability compared to the other five solutions.

Key words: Benzalkonium chloride/Cytotoxicity/Preservative/Eyedrop/Cell viability score.

Recent major clinical issues in ophthalmology include the safety of the preservatives used in eyedrops especially since numerous new products have been developed for better intraocular pressure control in glaucoma patients. Approximately 4% of adult Japanese have glaucoma (Iwase et al., 2006), and topical medication is the first choice in treatments. Consequently, patients and physicians are concerned about the immediate effects of these medications as well as their possible long term adverse effects.

Benzalkonium chloride (BAK) has been recognized as a major toxic factor in eyedrops (Pfister and Burstein, 1976; Burstein and Klyce, 1977; Furrer et al. 2002; Baudouin, 2010), however, little is known about the toxicity of other preservatives (Hubbard, 1988; Senoo, et al., 2000; Epstein, et al., 2009) although they are supposed to be essential components of effective, safe, and stable eyedrop formulations. We previously examined the toxicity of preservatives with regard to corneal endothelial cells, SIRC, and the Chang conjunctiva and reported that BAK, polysorbate, and propyl paraoxybenzoate were toxic, whereas methyl paraoxybenzoate and chlorobutanol were not (Ayaki et al., 2008). We compared the cytotoxicity of many eyedrops by using a cell culture bioassay system with a semiquantitative method, namely, CVS (cell viability score), aiming for the comprehensive evaluation of drug toxicity (Ayaki and Iwasawa, 2010a; Ayaki et al., 2010b). The experiments for CVS include multiple cell lines, two
bioassays, and a series of concentrations and exposure times. The results from experiments conducted under multiple conditions can produce more generalizable outcomes in comparison with experiments under only in limited conditions. Standardized experiments using CVS have shown correlations between concentration and exposure times for BAK at 11 concentrations (Ayaki and Iwasawa, 2011a). We carried out toxicity assays for five preservatives and a surfactant used in eyedrops to explore their cytotoxicity to ocular surface cells.

The five preservatives, tested at concentrations commonly used in Japanese eyedrop formulations included 0.01% benzalkonium chloride (Wako Pure Chemical Industries, Osaka, Japan), 1% boric acid (Wako Pure Chemical Industries, Osaka, Japan), 0.4% methyl paraxoxybenzoate (Wako Pure Chemical Industries), 0.4% propyl paraxxybenzoate (Wako Pure Chemical Industries), and 0.5% chlorobutanol (Wako Pure Chemical Industries). A surfactant, 1.0% polysorbate 80 (Tween® 20, ICN Biomedicals Inc, Aurora, Ohio, USA), was also examined. Their chemical structures are shown in Fig1.

The following commercially available cell lines were used in this study: SIRC (rabbit corneal epithelium, ATCC CCL-60, distributed by American Type Culture Collection, Manassas, VA), BCE C/D-1b (bovine corneal epithelial cells, JCRB-9129, distributed by Health Science Research Resource Bank, Osaka, Japan), RC-1 (rabbit corneal epithelium, JCRB-0246), and Chang conjunctiva (human conjunctival cells, ATCC CCL-20.2). All cell lines were cultured according to standard protocols provided by the distributors.

Aliquots of media (100 μl) containing approximately 2 x 10⁴ cells were harvested from culture wells and incubated for two days. The culture media were then replaced with 100 μl of undiluted, 2-fold, and 10-fold diluted doses of various test solutions, and the cells were incubated for 10, 30, or 60 minutes. Fresh culture media were then substituted and the cells were incubated for a further 48 h. Cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, St. Louis, MO) assay and the neutral red (Wako Pure Chemical Industries) assay. The assay results were measured spectrophotometrically (Benchmark microplate reader, BIO-RAD, Hercules, CA). Cell viability in test solutions was calculated as a percentage of control cell viability in media only. The experiments were repeated 8 times and results are presented as the average +/- standard deviation. Mean values for each concentration were analyzed with a one-way ANOVA test followed by the Bonferroni test using a statistics software program (ORIGIN 7.0, Light Stone, Tokyo, Japan); the level of significance was fixed at 0.01.

The CVS was used to compare the toxicity of test solutions (Ayaki and Iwasawa, 2010a). The CVS50 was determined as the number of measurements with a viability ≥ 50% of control. The CVS40/80 was calculated as follows: CVS40/80 = (number of measurements with a viability value >80%) - (number of measurements with a viability value <40%). The total number of measurements was 72 (3 concentrations, 3 exposure times, 4 cell lines, 2 assays) in the present study. Results were expressed as % of total measurements (%CVS).

FIG. 1. Chemical structures of tested reagents.
The viability of all four cell lines exposed to the different test solutions is shown in Figures 2-5. BAK and polysorbate treatments resulted in low cell viability compared to the other four solutions. After 10 minutes exposure in 10-fold dilution, every cell viability measurement for BAK and polysorbate exhibited greater than 80%. The cell viability with the other four preservatives was greater than 80% for all 288 measurements (4 preservatives, 3 concentrations, 3 exposure times, 2 assays, and 4 cell lines) but one for boric acid. The results of each ingredient for %CVS50, and %CVS40/80 were: 0.01% benzalkonium chloride (51, -13), 1% boric acid (100, 99), 0.4% methyl paraxoxybenzoate (100, 100), 0.4% propyl paraxoxybenzoate (100, 100), 1.0% polysorbate 80 (68, 18), and 0.5% chlorobutanol (100, 100).

The preservatives and surfactant tested in the present study are very commonly found in eyedrop formulations in Japan, but we found BAK and polysorbate had considerable toxicity. BAK is the most frequently used preservative in prescription eyedrops and boric acid is the second (Ohashi, et al., 2004). Polysorbate 80 (Tween® 20) is a surfactant
FIG. 4. Effects of ingredients on the viability of cultured bovine corneal epithelial cells (BCE) using the undiluted solution, and 2-fold and 10-fold dilutions after 10, 30, or 60 minutes exposure using the MTT (left) and neutral red (right) assay. *P < 0.01 (vs control, one-way ANOVA test followed by Bonferroni test). Data are means ± SD.

FIG. 5. Effects of ingredients on the viability of cultured human conjunctival cells (Chang) using the undiluted solution, and 2-fold and 10-fold dilutions after 10, 30, or 60 minutes exposure using the MTT (left) and neutral red (right) assay. *P < 0.01 (vs control, one-way ANOVA test followed by Bonferroni test). Data are means ± SD.

used for resolving pharmaceutical ingredients and for the facilitation of drug penetration (Tinguchii et al., 1988). Although it has significant ocular toxicity (Quiroga and Klintworth, 1967), this has been rarely documented until intraocular administration has become one of the major strategies to deal with macular edema (Zhengyu et al., 2009). The preservatives other than BAK had little toxicity and may be safe for patients' ocular surfaces (Hubbard, 1988).

Epstein et al. (2009) examined the toxicity of BAK, edetic acid, chlorobutanol, methyl paraoxybenzoate, and boric acid in various concentrations after human corneal epithelial cells and human conjunctival cells received a one-hour exposure. They found very low toxicity for boric acid and edetic acid, and significant toxicity for the other preservatives at concentrations used in eyedrops. The dose dependant cytotoxicity of BAK was demonstrated in concentrations higher than 0.002% (Ayaki and Iwasawa, 2011a). However, a very popular eyedrop (Sancoba®, Santen pharmaceutical, Osaka, Japan) containing 0.01% BAK tested in the present study has been used safely by a huge number of ophthalmic patients for more than 40 years in Japan ( Ayaki and Iwasawa, 2011b). BAK exhibits toxicity very rapidly and the results of the present study using an exaggerated-use protocol can be applied to various clinical conditions.

The apparent inconsistency between cell culture results and clinical facts may be due to a protective barrier mechanism involving the immediate dilution
with a preconal tear film (Mishima et al., 1966; Namamura and Nishida, 1994) and tear turnover due to the pumping effect of blinking (Doane, 1981; Tsubota, 1998) at human ocular surfaces. Drug toxicity may become serious problem only in cases of severe ocular surface disorders (Burnstein, 1985; Leung et al., 2008). Moreover, the differences in drug toxicity results in basic experiments seem often subclinical since even a comparative study among the drugs with extremely different preservative systems (e.g. 0.02% BAK versus no BAK) showed conflicting results (Horsley and Kahook, 2009; Townley and Reilly, 2009). Despite the well-known cytotoxicity of BAK, an infection control system is still necessary for eye drops because higher safety is required for eye disease patients who are mostly aged and visually or physically impaired and may not be able to use eye drops properly (Solomon et al., 2003).

REFERENCES


