Comparative Study of *In Vitro* Ocular Surface Cytotoxicity of a Fixed Combination of 0.5% Timolol/1% Dorzolamide Eyedrop and Its Components with 0.005% Benzalkonium Chloride

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We evaluated the cytotoxicity of antiglaucoma ophthalmic solutions preserved with the same concentration of benzalkonium chloride (BAK) in four cultured corneal and conjunctival cell lines. The viability of cell cultures was determined following the exposure of cells to timolol maleate, dorzolamide, and their fixed combination, Kosoputo® (MSD, a Japanese formulation of Cosopt® (Merck)), and two commercially available eyedrop solutions, 0.5% Timpotol® (containing 0.5% timolol maleate, MSD) and 1% Trusopt® (containing 1% dorzolamide, MSD) for varying exposure times and at various dilutions using the MTT and neutral red assays. All the three commercially available eyedrop solutions tested in this study were preserved with 0.005% BAK. The toxicity of each solution was compared using the % cell viability score (CVS). Cell viability was also subjected to statistical analysis using ANOVA, Dunnett’s multiple comparison tests and a chi-square test. %CVS50/50CVS40/80s for the tested solutions were 53/-13 for 0.5% Timpotol®, 100/88 for preservative-free 0.5% timolol maleate, 50/-10 for 1% Trusopt®, 72/100 for preservative-free 1% dorzolamide, and 44/-17 for Kosoputo®. The results of statistical analysis were consistent to them. In conclusion, Kosoputo® had greater cytotoxicity than each component; however, in actual use it may have the advantages of reduced toxicity (side effect) due to reduced instillation frequency, and better patient adherence to the treatment regimen as well as a comparable pressure reduction effect.

*Key words*: Benzalkonium chloride/Fixed combination/Cell viability score/Glaucoma/Cornea.

The use of glaucoma medications continues for a long time in most cases and side effects and patient adherence to the treatment regimen (Okeke et al., 2009; Nordstrom et al., 2005) as well as pressure reduction are the major issues. Toxicity (as a side effect) is often associated with intolerance and poor patient adherence resulting in the failure of effective treatment. Benzalkonium chloride (BAK) (Boudouin et al., 2010) is a major ingredient involved in the cytotoxicity in eyedrops, and new formulations with no or less BAK such as travoprost (Kahook et al., 2008), tafluprost, and bimatoprost have been launched.

Recently fixed combination drugs have been introduced for better patient adherence to the treatment regimen and more pressure reduction in a single dose. Cosopt® (Merck and Co., Whitehouse Station,
NJ, USA) is a fixed combination of 0.5% timolol/2% dorzolamide and is equally (Hutzelmann et al., 1998) or more (Francis et al., 2004; Boyle et al., 1998) effective than the concomitant use of timolol maleate and dorzolamide, although Strohmaier et al. (1998) reported that it was less effective than concomitant administration. The pressure reduction effect of Cosopt® has been reported to be comparable with Xalatan®, a latanoprost-containing first-line drug in glaucoma treatment (Miglior et al., 2010; Fechner et al., 2004; Konstas et al., 2008), indicating that Cosopt® could be an alternative single medication for patients suffering from side effects of a prostanagin analogue. Another expected advantage of a fixed combination drug is there are less toxic side effects because it is accompanied with a reduced frequency of instillation. Only clinical manifestations have been noted for the toxicity of Cosopt® and patients treated with Cosopt® had a significantly greater sense of burning and taste perversion than those treated with Timoptic® (Merck) (Boyle et al., 1998). Other adverse effects included blurred vision, foreign body sensation, and itching.

The fixed combination of 0.5% timolol maleate and 1% dorzolamide has only become available only in Japan since 2010 as Kosoputo® (Santen pharmaceutical Co., Ltd., Osaka, Japan and Merck Sharp & Dohme, Osaka, Japan). The single-dose and fixed combination solutions are preserved with 0.005% BAK and the cytotoxicity of each is of clinical interest in terms of switching or add on medical therapy. We examined the cytotoxicity of Kosoputo® and that of its components to discuss the combined effects presented by the fixed combination drug in the aspect of cytotoxicity.

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

A 100-μL aliquot of culture medium containing approximately 2 × 10⁶ cells was seeded onto a 96 well microplate (NUNC™ 167008, Thermo Fisher Scientific, Denmark) and cells were incubated for 2 days. The various ophthalmic solutions to be tested were diluted with physiological saline to make concentrations equivalent to 1-, 2-, and 10-fold dilutions, and a 20-μL aliquot of each diluted drug solution was added to the cells following the removal of culture medium. After 10, 30, and 60 min exposure of cells to the test drug solutions, the cells in each well were washed with the culture medium and a 100-μL aliquot of culture medium was added to determine cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (SigmaAldrich, Tokyo, Japan) assay and with the neutral red (NR) assay after the culture medium was replaced with fresh medium and cells were incubated for a further 48 h for recovery. At the conclusion of the assays, absorbance was read on a spectrophotometer (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) and the cell viability in test solutions was calculated as a percentage of the control cell viability (in medium only). Experiments were repeated eight times and the results are presented as the mean value ± SD.

Antiglaucoma solutions evaluated in the present study were 0.5% Timoptic® (0.5% timolol maleate with 0.005% BAK, Merck Sharp & Dohme), preservative-free 0.5% timolol maleate, 1% Trusopt® (1% dorzolamide with 0.005% BAK, Merck Sharp & Dohme), preservative-free 1% dorzolamide, and Kosoputo® (fixed combination of 0.5% timolol maleate/1.0% dorzolamide with 0.005% BAK, Merck Sharp & Dohme; a unique formula available only in Japan, using the same brand name as Cosopt® (fixed combination 0.5% timolol maleate/2% dorzolamide with 0.0075% BAK available in the other countries). Preservative-free 0.5% timolol maleate and preservative-free 1% dorzolamide were prepared for the present investigation only.

The cell viability obtained from each assay was subjected to statistical analysis using analysis of variance (ANOVA). Then, the cell viability of each cell line exposed to 2-fold diluted drug formulations was further analyzed using Dunnett’s multiple comparison test to determine whether the viability of the cells exposed to the Kosoputo® were significantly different from that exposed to the other two products composing Kosoputo®. To assess if the measured cell viability values for each drug are significantly different, a 2x2 chi-square test for independence was applied by counting the total number of plots over or below 50% in each drug. P values of <0.05 were considered to be significant.

The cell viability score (CVS) (Ayaki et al., 2011a) was used to compare the toxicity of the test solutions. This concept is similar to that of LD₅₀ (lethal dose for 50% death of animals) and MIC₅₀ (minimum inhibitory concentration for 50% growth inhibition of a pathogen). We set the reference point of cell viability at 50% for CVS50 and 40% and 80% for CVS40/80.
Fifty% is a simple application of LD₅₀ and MIC₅₀. The 80% viability can be recognized as a “non toxic level” and 40% as a “significantly toxic level” according to our previous experiments with thousands of assays (Ayaki et al., 2009; Ayaki et al., 2010; Ayaki et al., 2011a; Ayaki et al., 2011b). Since a series of assays under multiple conditions including different drug concentrations, different exposure times, and different cell lines gives a useful but staggering volume of information, the methodology is required to summarize the information to be plain and simple. For this purpose, we have proposed the idea of CVS, which enables us to compare the cell viability simply by counting the number of plots beyond or under a reference point. The CVS50 was determined by the number of measurements for viability ≥ 50% of control. The CVS40/80 was calculated as follows: 

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\text{CVS40/80} = \left( \frac{\text{the number of measurements for a viability value >80%}}{\text{the number of measurements for a viability value <40%}} \right)
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The total number of measurements was 72 (3 concentrations, 3 exposure times, 4 cell lines, 2 assays). Results were expressed as % of total measurements (%CVS).

The interaction between a drug formulation and either the cell line or dilution rate was proved to be significant by ANOVA, and the cell viability seemed to decrease with an increase in exposure time. Thus, the cell viability after 60 min exposed to Kosoputo® at each dilution was evaluated in terms of significant differences from that to the other solutions by using Dunnett’s multiple comparison test as shown in Figs. 1 and 2. The cell viability in BAK-containing solutions seemed to be extremely low in undiluted solutions and extremely high in 10-fold diluted solutions, suggesting that the cell viability in 2-fold diluted solution is well-suited for statistical analysis comparing the cytotoxicity of Kosoputo® with that of either 0.5% Timoptol® or 1% Trusopt®. Thus, Dunnett’s multiple comparison test was conducted to assess the cell viability in the 2-fold diluted solutions of Kosoputo® in comparison with that of other drugs.

In SIRC (Figs. 1 and 2), the viability of the cells exposed to any of the 2-fold diluted preservative free drugs (0.5% timolol maleate and 1% dorzolamide) was more than 80% irrespective of the dilution rate and cytotoxicity assay, while that exposed to any of the 2-fold diluted drugs containing BAK (0.5% Timoptol®, 1% Trusopt® and Kosoputo®) decreased with the dilution rate. Of the 2-fold diluted drugs containing BAK, the viability of the cells exposed to Kosoputo® was the lowest, and significant differences were obtained in comparison with that of cells exposed to 0.5% timolol maleate and 1% dorzolamide irrespective of the assays. Similar results were obtained in Chang conjunctiva. That is, the viability of the cells exposed to any of the 2-fold dilutions of preservative free drugs was more than 80%, and the lowest viability was obtained in the cells exposed to 2-fold dilutions of Kosoputo®.

On the other hand, BCE seemed to be less tolerable to the preservative-free drugs than other cell lines since the viability of the cells exposed to 2-fold dilution of preservative-free 0.5% timolol maleate was less than 80% in both the assays. Regarding expo-

**FIG. 1.** Effects of anti-glaucoma solutions diluted 1-, 2-, and 10-fold on the viability of cultured ocular surface cells (RC-1, SIRC, BCE C/D-1b, and Chang conjunctiva) after 60 minutes exposure using the MTT assay. Data are means ± SD. *P < 0.05 (vs Kosoputo®, Dunnett’s multiple comparison test) BCE, BCE C/D-1b; Chang, Chang conjunctiva
FIG. 2. Effects of anti-glaucoma solutions diluted 1-, 2-, and 10-fold on the viability of cultured ocular surface cells (RC-1, SIRC, BCE C/D-1b, and Chang conjunctiva) after 60 minutes exposure using the neutral red (NR) assay. Data are means ± SD. *P < 0.05 (vs Kosoputo®, Dunnett’s multiple comparison test) BCE, BCE C/D-1b; Chang, Chang conjunctiva.

sure to the drugs containing BAK, the viability of cells exposed to 2-fold dilution of 0.5% Timoptol® was significantly lower than that exposed to 2-fold dilution of Kosoputo® when assayed by MTT whilst the viability of cells exposed to each of the three drugs was comparable to each other when assayed by NR. In RC-1, the viability of the cells exposed to any of the 2-fold dilutions of preservative-free drugs was more than 90%, and that exposed to any of the 2-fold dilutions of the three drugs containing BAK was less than 40%. Of the 2-fold dilutions of the three drugs containing BAK, 0.5% Timoptol® exerted the least cytotoxic effect with significant differences from that of Kosoputo® in both types of assay, and little differences were found in the viability of cells treated with a 2-fold dilution of Kosoputo® treated cells and that of 1% Trusopt® treated cells.

Percent CVS50 and %CVS40/80 for tested solution were 53 and -13 for 0.5% Timoptol®, 100 and 88 for preservative-free 0.5% timolol maleate, 50 and -16 for 1% Trusopt®, 100 and 100 for preservative-free 1% dorzolamide, and 44 and -17 for Kosoputo®. Preservative-free timolol maleate and preservative-free dorzolamide had low toxicity. Both %CVSs of Kosoputo® were lower than those of 0.5% Timoptol® and 1% Trusopt®.

Scatter diagrams for the cell viability of all the cell lines exposed to Kosoputo®, 0.5% Timoptol®, and 1% Trusopt® are shown in Fig. 3, where results of %CVS values are easily overlooked since distribution of plots are slightly lower in Kosoputo® than in other two drugs. As shown in figure, cell viability with 2-fold diluted Kosoputo® was mostly below 50%, while many of values with 2-fold diluted treatments of the other two drugs were over 50%.

The calculated chi square values for the total number of plots over or below 50% in each drug are 5.552 (p<0.05) for Kosoputo® vs 0.5% Timoptol®, 4.807 (p<0.05) for Kosoputo® vs 1% Trusopt®, and 0.028 (not significant) for 0.5% Timoptol® vs 1% Trusopt®, respectively, indicating that cell viability values in the 1% Trusopt® group and 0.5% Timoptol® group are significantly independent of those in the Kosoputo® group.

According to ANOVA, it is indicated that the combination of drug formulation and dilution rate affected prominently the cell viability. From this point of view, Dunnett’s multiple comparison test was performed to evaluate the cytotoxicity of Kosoputo® eyedrops in comparison with its components. As a result, it was clearly demonstrated that the two preservative-free solutions, 0.5% timolol maleate and 1% dorzolamide, were less cytotoxic in all cell lines than any of BAK-containing eye drops including Kosoputo®. Of the three drugs containing BAK, the cytotoxicity of Kosoputo® was comparable to or slightly more potent than that of other two drugs, 0.5% Timoptol® and 1% Trusopt®. Especially in both SIRC and Chang conjunctiva, the viability of the cells exposed to 2-fold dilution of Kosoputo® was the lowest with some significant differences in comparison with that exposed to 2-fold dilutions of 0.5% Timoptol® and of 1% Trusopt®.

In the present study, the cytotoxicity of Kosoputo®
was also assessed by %CVS50 and %CVS40/80. It is indicated that the values of both %CV50 and %CVS40/80 well reflected the cytotoxicity of the drugs tested in the present study. That is, the values for the two preservative-free drugs are prominently higher than those of the drugs containing BAK. Meanwhile, the values of both parameters for Kosoputo® were lower than those of 0.5% Timoptol® and 1% Trusopt®, which is coincident with the cell viability data of the 2-fold dilutions. It was further confirmed statistically by a chi square test. The %CVS50 and %CVS40/80 of 0.005% BAK from the same protocol as the present study was 79 and 39, respectively (Ayaki and Iwasawa, 2011b), and it implies that the reduction of %CVS may represent the effects of active ingredients or the interaction between BAK and active ingredients. In a cytotoxicity assay as in the present study where many factors such as drug type, cell line, dilution rate, and exposure time are involved, the interpretation of the data is supposed to be complicated. Since the values of both %CVS50 and %CVS40/80 reflect well the cytotoxicity as evaluated by cell viability as shown in Figs. 1 and 2, it is suggested that the %CVS could be a concise indicator for cytotoxicity of chemicals and medicinal products even though the cytotoxicity is multilaterally and intricately assessed.

The present results for preservative free-0.5% timolol maleate, preservative-free 1% dorzolamide, 0.5% Timoptol® and 1% Trusopt® were consistent with those for corneal endothelial cells (Ayaki et al., 2009; Ayaki et al., 2010). The toxicity of Kosoputo® was slightly greater than that of 1% Trusopt® and 0.5% Timoptol®. Since Kosoputo® is instilled less frequently than in the case of combined use of each component, 0.5% timolol and 1% dorzolamide, Kosoputo® (Japanese formulation of Cosopt®) may have a reduced toxicity (side effect), even though it has greater cytotoxicity than each component.

Ocular signs or symptoms of adverse effects may depend on individual factors as well as the eyedrop solution’s pH, pharmaceutical components, and ingredients including preservatives. We also need to know if the concentration of BAK in commercially available eyedrops is different with each manufacturer. Since the other commercially available eyedrops with similar brand names to those of the three tested eyedrop solutions, Cosopt® (Merck), 0.5% Timoptic® (Merck), and 2% Trusopt® (Merck), contain concentrations of BAK other than 0.005%, a comparative study in terms of cytotoxicity should be conducted in the future.

BAK is a known irritant and disturbs ocular surface. Clinical and animal studies (Ishibashi et al., 2003; Lass et al., 1998; Pisella et al., 2000; Wilson et al., 1975) revealed that timolol with BAK showed more intolerance or toxic effects compared to timolol without BAK. Assay by a single layer cell culture is a sensitive method and some detected toxicity may be subclinical. Due to individual factors among patients and rapid turnover of tear film, clinical manifestations may not always correlate to the difference in
cytotoxicity indicated by basic experiments. The results from bioassays may be applied especially to the cases with ocular surface pathology requiring special attention.

In conclusion, Kosoputo® (Japanese formulation of Cosopt®) had greater cytotoxicity than each component; however, it may have the advantages of reduced toxicity (side effect) due to a reduced instillation frequency, and better patient adherence to the treatment regimen as well as comparable pressure-reduction effect.

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REFERENCES


