**Note**

**In vitro** Assessment of the Cytotoxicity of Six Topical Antibiotics to Four Cultured Ocular Surface Cell Lines

MASAHIKO AYAKI¹, ATSUO IWASAWA², AND YOSHIMI NIWANO³

¹Director of Ophthalmology, Mita Hospital, International University of Health and Welfare  
²Department of Bioengineering, Graduate School of Bioscience and biotechnology  
Tokyo Institute of Technology  
³Laboratory for Redox Regulation, Tohoku University Graduate School of Dentistry

Received 25 October, 2011/Accepted 16 December, 2011

To determine the cytotoxicity of antibiotic eyedrops to ocular surface cells using a semi-quantitative method, a range of commercially available antibiotic eyedrops were assessed by using three corneal cell lines and one conjunctival cell line. All antibiotic solutions were free of benzalkonium chloride. Cell viability was determined by the MTT assay and neutral red assay following the exposure of cells to the undiluted, 2- and 10-fold diluted drugs for 10, 30, and 60 min. Toxicity was compared using % cell viability score (%CVS). The tested eyedrops and values of %CVS50 and %CVS40/80 were Bestron® (cefmenoxime, 100, 94), Panimycin® (dibekacin, 86, 58), Norflo® (norfloxacin, 90, 50), Cravit® (levofloxacin, 86, 46), Tosulfon® (tosufloxacin, 57, -3), and Vigamox® (moxifloxacin, 57, -6). Cell viability markedly increased after dilution. For instance, cell viability assayed by MTT was > 80% for all the measurements in antibiotics diluted 10-fold, and the rate of the measurements showing > 80% cell viability decreased to 43% (31 out of 72 measurements) in the solutions diluted 2-fold. Of the drugs tested, Bestron® containing cefmenoxime showed the weakest toxicity. Vigamox® containing moxifloxacin and Tosulfon® containing tosufloxacin were more toxic when compared with the other antibiotics. CVS was useful for the comparison of the cytotoxicity of the drugs.

**Key words**: Antibiotics/Cornea/Toxicity/Cell viability score.

Antibiotics are often used for the treatment of the compromised ocular surface in cases such as keratitis, dry eye, postoperative eye or traumatized eye, and they should have no harmful effect on ocular surface structures. Antibiotic eyedrops without benzalkonium chloride (BAK) are available in Japan and we are able to determine only the cytotoxicity of their active ingredient. Since there has been no standard method for evaluating the cytotoxicity of eyedrops, the aim of this study was to quantify the cytotoxicity of common antibiotic eyedrops using a cell viability score (CVS) (Ayaki et al., 2010a).

The methods of the cell culture, cytotoxicity assay, and data evaluation were identical to those described previously (Ayaki et al., 2010a; Ayaki et al., 2011).

*Corresponding author. Tel : +81-3-3451-8121, Fax : +81-3-3454-0067, E-mail:ayaki(a)ruhw.ac.jp

Briefly, 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). The culture media for the confluent monolayers of cells were replaced with undiluted or, 2-fold, and 10-fold diluted doses of test ophthalmic solutions, and the cells were incubated for 10, 30, or 60 minutes. Fresh culture media was then substituted and the cells were further incubated for 48 h. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, St. Louis, MO) assay and the NR (neutral red, Wako Pure Chemical Industries,
Osaka, Japan) assay. Cell viability in test solutions was calculated as a percentage of the control cell viability in the media only. The experiments were repeated 8 times and results are presented as the average +/- the standard deviation. To evaluate the cytotoxicity of fluoroquinolone-containing eyedrops, mean values of the cell viability for each concentration were compared with those of Bestron®, cefmenoxime (a cepham antibiotic)-containing eyedrops, by Dunnett’s multiple comparison test with p<0.05 as the level of significance.

The CVS was used to compare the toxicity of test solutions. The CVS50 was determined by the number of measurements showing a viability ≥ 50%. The CVS40/80 was calculated as follows: CVS40/80 = (the number of measurements showing viability >80%) - (the number of measurements showing viability <40%). The total number of measurements was 72 (3 concentrations, 3 exposure times, 4 cell lines, 2 assays). Results were expressed as % of the total measurements (%CVS). Table 1 lists the antibiotic drugs evaluated in the present study. The chemical structures were shown previously (Ayaki et al., 2010b).

Cell viability results are summarized in Figs. 1-4, showing that cell viability markedly increased dependently on the dilution rate. For instance, the cell viability assayed by MTT was > 80% for all measurements in antibiotics diluted 10-fold, and the rate of the measurements showing > 80% cell viability decreased to 43% (31 out of 72 measurements consisting of 6 eyedrops x 3 exposure times x 4 cell lines) in the solutions diluted 2-fold. When the discussion is confined to the fluoroquinolone-containing eyedrops, cytotoxicity to all the cell lines tested became higher as the dilution rate increased in the following way. That is, the rates of the measurements showing > 80% cell viability assayed by MTT were 100% (48/48), 25% (12/48) and 4% (2/48) in the 10-fold diluted, 2-fold diluted and undiluted solutions, respectively.

Regardless of the assay methods, when cells were exposed to test solutions for 60 min, all of the undiluted fluoroquinolone-containing eyedrops showed significantly higher cytotoxicity to each of the cell lines than did Bestron®. Of the four fluoroquinolone-containing eyedrops, the cytotoxicity of Vigamox® seemed to be the severest. For instance, viability of all the cell lines exposed to undiluted Vigamox® was low even though the exposure time was as short as 10 min. Panimycin®, a reference eydorp solution containing dibekacin (an aminoglycoside antibiotic), was highly cytotoxic to the Chang human conjunctival cell line, comparable to Vigamox®. However, to the other three corneal epithelial cell lines, the cytotoxicity of Panimycin® was relatively low. In contrast, vulnerability of Chang to the fluoroquinolone-containing eyedrops tested seemed to be similar to that of other three cell lines.

The CVS values were also calculated to estimate the cytotoxicity of fluoroquinolone-containing eyedrops in comparison with that of reference drugs. As shown in Table 1, tested antibiotic eyedrops with %CVS50 and %CVS40/80 were Bestron® (100, 94), Panimycin® (86, 58), Norfloxacin® (90, 50), Cravit® (86, 46), Tosufloxacin® (57, -3), and Vigamox® (57, -6). CVS50 is a rough index for cytotoxicity of the test substance and CVS40/80 is an index reflecting cytotoxicity more finely than CVS50. Eighty percent of 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 and Iwasawa, 2011a; Ayaki and Iwasawa, 2011b). For instance, scatter diagrams for cell viability of all the cell lines exposed to Bestron® and Cravit® are shown in Figs. 5 and 6, where %CVS50

<table>
<thead>
<tr>
<th>Table 1. List of drugs tested and their %CVS values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product name</td>
</tr>
<tr>
<td>Bestron®</td>
</tr>
<tr>
<td>Panimycin®</td>
</tr>
<tr>
<td>Norfloxacin®</td>
</tr>
<tr>
<td>Cravit®</td>
</tr>
<tr>
<td>Vigamox®</td>
</tr>
<tr>
<td>Tosufloxacin®</td>
</tr>
</tbody>
</table>

*Fluoroquinolone antibiotic
EDTA= ethylene-diaminetetraacetic acid (edetic acid). Chemical structures are shown in our previous report (Ayaki et al., 2010b).
FIG. 1. Effects of antibiotic eyedrops diluted 1-, 2-, and 10-fold on the viability of cultured rabbit corneal epithelial cells (RC-1) after exposure for 10, 30, or 60 minutes using the MTT (left) and neutral red (right) assay. Bestron® shows the greatest cell viability. Fluoroquinolones (unshaded circles) result in lower cell viability than other drugs (shaded circles). Data are means ± SD. *P < 0.05 (vs. Bestron®, Dunnett’s multiple comparison test). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

FIG. 2. Effects of antibiotic eyedrops diluted 1-, 2-, and 10-fold on the viability of cultured rabbit corneal epithelial cells (SIRC) after exposure for 10, 30, or 60 minutes using the MTT (left) and neutral red (right) assay. Data are means ± SD. *P < 0.05 (vs. Bestron®, Dunnett’s multiple comparison test).

and %CVS40/80 values are summarized, respectively. As shown in the figures, cell viability with Bestron® treatment was kept at a high level, whilst that with Cravit® fluctuated dependently on the dilution rate and exposure time. To assess if the CVS values in two groups are significantly different, a chi-square test for independence can be applied. For instance, the calculated χ² values for %CVS50 and %CVS40/80 as shown in Figs. 5 and 6 are 10.746 and 14.733, respectively, indicating that either %CVS50 or %CVS40/80 values in the Bestron® and Cravit® groups are significantly independent of each other (P<0.01).

As stated earlier, in the case of a test substance
FIG. 3. Effects of antibiotic eyedrops diluted 1-, 2-, and 10-fold on the viability of cultured bovine corneal epithelial cells (BCE) after exposure for 10, 30, or 60 minutes using the MTT (left) and neutral red (right) assay. Data are means ± SD. *P < 0.05 (vs. Bestron®, Dunnett’s multiple comparison test).

FIG. 4. Effects of antibiotic eyedrops diluted 1-, 2-, and 10-fold on the viability of cultured human conjunctival cells (Chang) after exposure for 10, 30, or 60 minutes using the MTT (left) and neutral red (right) assay. Bestron® shows the greatest cell viability. Panimycin® results in lower cell viability in this cell line than in the other cell lines (Figs 1-3). Data are means ± SD. *P < 0.05 (vs. Bestron®, Dunnett’s multiple comparison test).

showing that a longer exposure time or a higher drug concentration causes severer cytotoxicity, the %CVS40/80 value becomes lower even though the CVS50 value is relatively high. Furthermore, %CVS values of interest in two groups can be statistically assessed by the chi-square test as described above. Based on the CVS values, the order of toxicity was estimated to be Bestron® < Panimycin® = Noflo® = Cravit® < Tosuflo® = Vigamox®. Bestron® was the least toxic. Vigamox® and Tosuflo® were more toxic than the others.

The reason that Bestron® appeared to be least cytotoxic is probably due to the low cytotoxicity of its active ingredient cefmenoxime. The fluoroquinolones,
which are active ingredients of other antibiotic eye drops except Panimycin®, are broad-spectrum antibiotics commonly used for ocular infection control. The cytotoxicity of the fluoroquinolone products containing a preservative to ocular cells has been reported (Walter et al., 2006; Dutot et al., 2006) and the present study indicated that cytotoxicity of the preservative-free products was still generally greater than that of reference antibiotic products. The present results were consistent with not only those of previous studies using human corneal endothelial cells (Bezwada et al., 2008; Ayaki et al., 2010b) but also those of other investigators using ocular surface cells (Sakurai et al., 2006; Kim et al., 2007; Sosa et al., 2008).

Cytotoxicity to keratocytes in the corneal stroma has been evaluated (Leonardi et al., 2006; Bezwada et al., 2008) since cases with corneal perforation during treatment with fluoroquinolones have been reported (Gangopadhyay et al., 2000; Mallari et al.,
While the present study discusses the cytotoxicity of fluoroquinolone-containing eyedrops, underlying mechanism by which fluoroquinolones cause cytotoxic effects has not yet been clarified. The antibacterial targets of fluoroquinolones are DNA gyrase (topoisomerase II) and topoisomerase IV (Hooper, 2002), and it was suggested in a study examining the structure-activity relationship of fluoroquinolones was examined that increased inhibition of human topoisomerase II results in mammalian cellular cytotoxicity (Lawrence, 2001). Thus, cytotoxicity induced by fluoroquinolones might be associated with the interactive effect on the human topoisomerase II.

Furthermore, one of the major adverse events caused by fluoroquinolones is phototoxicity (Domagala, 1994; Arata et al., 1998; Oliveira et al., 2000). In the present study, however, the cells were exposed to the test drugs in an incubator in which no light was radiated, indicating that phototoxic effect was not associated with the in vitro cytotoxicity induced by fluoroquinolones. In real situations, eyes are exposed to light from various sources so that the possible involvement of the phototoxic properties in fluoroquinolone-induced cytotoxicity should be further evaluated.

Intraocular antibiotic toxicity is one of the major concerns in clinical ophthalmology in terms of prevention of endophthalmitis by intraocular injection of antibiotics especially since intracameral cefuroxime has been proven effective by a large multicenter study (Endophthalmitis Study Group, 2007). Moxifloxacin was reported to be safe for intraocular injection and 0.05% moxifloxacin was not toxic to human corneal endothelial cells after 30 days of treatment (Kernt et al., 2009). In the cornea of living subjects, recent reports of a clinical study on levofloxacin (Bai et al., 2010) and an animal study on a series of fluoroquinolones including moxifloxacin and norfloxacin (Sharma et al, 2011) concluded that they were safe. Thus, we speculated that the cytotoxicity of fluoroquinolone-containing eyedrops tested in the present study would not be so severe as to cause adverse effects in clinical treatments.

The values of %CVS50 and %CVS40/80 for Cravit®, Norflo®, and also a non-quinolone antibiotic Panimycin® were equivalent to those for 0.005%BAK (%CVS50 = 79, %CVS40/80 = 39), and those for Vigamox® and Tosufl® were equivalent to those for 0.01%BAK (%CVS50 = 53, %CVS40/80 = -15) according to our recent study with the same protocol using 0.0003%-0.02%BAK (Ayaki and Iwasawa, 2011a). Since these concentrations of BAK are commonly used as a preserved for eyedrops and the %CVS values of these BAK solutions are comparable to those of the fluoroquinolone-containing eyedrops tested in the present study, it is suggested that the tested antibiotic eyedrops are probably tolerable to ocular surface cells.

The present study is unique in that only the cytotoxicity bioassays were used under various conditions. The evaluation method for eyedrop cytotoxicity has not been standardized yet since eyedrop concentrations after instillation change rapidly due to blinking and tears. The cytotoxic effects of drugs need to be evaluated and compared quantitatively and comprehensively under a wide range of concentrations and exposure times. In the present study, we attempted to address these issues by evaluating the effects under various conditions. We were able to compare the cytotoxic effects of the drugs easily using the CVS as well as graphically by plotting cell viability.

In conclusion, the toxicity of BAK-free antibiotic eyedrops was evaluated. Because sufficient data regarding cell viability were available, the results of the MTT assay and NR assay were expressed as CVS to enable a simple comparison of drug effects. Tested antibiotic eyelid products appeared to be tolerable to ocular surface cells.

REFERENCES


