Cytotoxicity of Topical Medications Used for Infection and Inflammation Control after Cataract Surgery in Cultured Corneal Endothelial Cells

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Postoperative vision-threatening corneal edema sometimes occurs after eye surgery, and corneal endothelial damage may be caused or exacerbated by drug toxicity. A range of commercially available antibiotic and anti-inflammatory ophthalmic solutions used postoperatively, namely levofloxacin, moxifloxacin, gatifloxacin, cefmenoxime, diclofenac, bromfenac, pranoprofen, betamethasone, and fluoromethorone, were assessed by using human corneal endothelial cells (HCECs). Propylparaxoxybenzoate and methylparaxoxybenzoate were also examined. Cell survival after 48 h exposure to the drugs was evaluated using the WST assay. Cefmenoxime and betamethasone were the least toxic antibiotic and anti-inflammatory drug, respectively. Cell survival was concentration dependent and increased markedly to ≥ 80% with dilutions of 100-fold or more. Two preservatives seemed to cause minimal cytotoxicity among those tested. Antibiotic cytotoxicity to HCEC was ranked as cefmenoxime < levofloxacin = gatifloxacin < moxifloxacin, while the toxicity of anti-inflammatory drugs was dependent on benzalkonium chloride and polysorbate. These drugs are unlikely to cause HCEC damage at the concentrations used under the usual conditions. Preservatives are essential ingredients in ophthalmic solutions to control postoperative infection and inflammation and we should be aware of their toxicity as well as efficacy.

Key words: Benzalkonium chloride / Toxicity / Ophthalmic solution / Preservative / Cornea.

Cataract surgery is the most commonly performed surgery in medicine and more than 1,000,000 operations are performed every year in Japan. Unexplained persistent corneal edema leading to considerable visual loss occurs postoperatively in some cases of cataract surgery. Corneal endothelial cells dictate water transport in the cornea and their decrease or dysfunction leads to severe corneal edema and vision loss. Postoperative corneal edema is caused mostly by surgical trauma to the corneal endothelium and other causative factors (Liu et al., 2001). In addition, it could be related to drug toxicity. Ocular toxic damage, termed toxic anterior segment syndrome (TASS), has been attributed to various factors including the inadequate sterilization of surgical instruments (Mamalis et al., 2006; Breebaart et al., 1990). Ophthalmic solutions are another possible source of toxicity in such cases because they are applied directly to the postoperative ocular surface. The corneal endothelium is composed of hexagonal columnar cells located on the inner side of the cornea. They have little mitotic activity, and excessive loss or damage leads to irreversible corneal edema.

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requiring corneal transplantation. Therefore, protective or toxic factors for the corneal endothelium are very important considerations in eye surgery.

Special attention should be paid to postoperative topical medications in two aspects: (1) control of infection and inflammation is a minimum requirement in modern eye surgery since early visual recovery is expected for most cases, (2) ophthalmic solutions contain preservatives that act as infection control and cytotoxic reagents. Although the guidelines of the American Academy of Ophthalmology (American Academy of Ophthalmology, 2006) do not stipulate specific regimens or treatment periods, topical medications are used after surgery for up to 3-6 months. Epithelial complications are easily detected during this treatment period by routine examination, while endothelial damage can be evaluated only with a special instrument, the specular microscope (Sakurai et al., 2006), and little is known about the potential endothelial toxicity of ophthalmic solutions (Kaji et al., 2007; Ayaki et al., 2008). To address this issue, we investigated the cytotoxicity of topical medications used postoperatively on human corneal endothelial cells (HCECs).

The Institutional Review Board of Showa University Fujigaoka Rehabilitation Hospital approved the present study. A primary human corneal endothelial cell (HCEC) line was established as described previously (Ayaki et al., 2007) from human eye bank (SightLife, Seattle, WA) tissue. A 100-μl aliquot of the culture containing approximately 2×10⁴ cells was harvested from the culture wells. The various drugs and agents to be tested were diluted 10, 20, 100, 1000 and, when necessary, 10,000-fold for the final concentration, and then added to the cells in culture. Cell survival was determined after a 48-h incubation in growth media using the 4-[3-(4-i odo phenyl)-2-(4-nitrophenyl)]-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Dojindo Laboratories, Kumamoto, Japan) assay to give a quantitative colorimetric measures of mitochondrial activity as an index of cell survival and proliferation. It detects living cells only and the signal generated is directly proportional to the number of live cells. At the completion of the assays, absorbance was read (Benchmark microplate reader; Bio-Rad, Hercules, CA, USA), and cell survival was calculated for each agent relative to the levels in cells incubated in growth media alone. Experiments were repeated 8-16 times with the results presented as means ± SD. Statistical analysis (student t test) was performed using Microsoft Excel (Microsoft, Tokyo, Japan).

Table 1 lists the antibiotic and anti-inflammatory drugs evaluated in the present study. Ophthalmic solution ingredients were also tested at commonly used concentrations and included 0.4% methyl paraoxybenzoate (Wako Pure Chemical Industries, Osaka, Japan) and 0.4% propyl paraoxybenzoate.

### TABLE 1

#### 1a Antibiotic ophthalmic solutions evaluated in the present study

<table>
<thead>
<tr>
<th>Active component</th>
<th>Trade name</th>
<th>Preservative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin (0.5%)</td>
<td>Cravit (Santen, Osaka, Japan)/Quixin (in US; Vistakon, Jacksonville, FL, USA)</td>
<td>No</td>
</tr>
<tr>
<td>Moxifloxacin (0.5%)</td>
<td>Vigamox (Alcon, Fort Worth, TX, USA)</td>
<td>No</td>
</tr>
<tr>
<td>Gatifloxacin (0.3%)</td>
<td>Gatiflo (Senju, Osaka, Japan)/Zymar (in US; Allergan, Irvine, CA, USA)</td>
<td>No</td>
</tr>
<tr>
<td>Cefmenoxime (0.5%)</td>
<td>Bestron (Kaken, Tokyo, Japan)</td>
<td>0.026% methyl paraoxybenzoate, 0.014% propyl paraoxybenzoate, EDTA, boric acid</td>
</tr>
</tbody>
</table>

#### 1b Anti-inflammatory ophthalmic solutions evaluated in the present study

<table>
<thead>
<tr>
<th>Active component</th>
<th>Trade name</th>
<th>Preservative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betamethasone (0.1%)</td>
<td>Rinderon (Shinogi, Osaka, Japan)</td>
<td>0.05% methyl paraoxybenzoate, 0.02% propyl paraoxybenzoate</td>
</tr>
<tr>
<td>Fluoromethonone (0.1%)</td>
<td>Flumethoron 0.1% (Santen, Osaka, Japan)</td>
<td>0.005% BAC, polysorbate 80, EDTA Chorobutanol, polysorbate 80, boric acid</td>
</tr>
<tr>
<td>Diclofenac (0.1%)</td>
<td>Diclod (Wakamoto, Tokyo, Japan)/Volten Ophthalmic Solution (in US; Novartis, East Hanover, NJ)</td>
<td></td>
</tr>
<tr>
<td>Bromfenac (0.1%)</td>
<td>Bronuck (Senju, Osaka, Japan)/Xibrom (in US; ISTA Pharmaceuticals, Irvine, CA)</td>
<td>0.005% BAC, polysorbate 80</td>
</tr>
<tr>
<td>Pranoprofen (0.1%)</td>
<td>Nifian (Senju, Osaka, Japan)</td>
<td>0.007% BAC, polysorbate 80, boric acid, EDTA</td>
</tr>
</tbody>
</table>

BAC= benzalkonium chloride, EDTA= ethylene-diaminetetraacetic acid (edetic acid).
FIG. 1. Chemical structure of tested ophthalmic solutions
(Wako Pure Chemical Industries). 0.01% benzalkonium chloride, 0.5% chlorobutanol, and 1.0% polysorbate 80 were examined previously (Ayaki, et al., 2007). The concentrations of these chemicals were determined according to the usual concentrations contained in commercial drugs.

Moxifloxacin was the most toxic and other antibiotics had weak toxicity (Fig. 2a). Antibiotic cytotoxicity was ranked as cefmenoxime < levofloxacin = gatifloxacin < moxifloxacin. Cell survival was concentration-dependent, with ≥80% survival observed with dilutions of 100-fold or more. Of the anti-inflammatory drugs, betamethasone without BAC or polysorbate showed minimal toxicity (Fig. 2b). The toxicity of the other drugs preserved with BAC and/or polysorbate was marked and clearly dependent on concentration, although survival also increased to ≥80% when the drugs were diluted 100-fold or more. The difference in the strength of the anti-inflammatory effect of the steroid or the difference between the steroid and non-steroid did not affect the HCEC toxicity. Methyl paraoxybenzoate and propyl paraoxybenzoate did not exhibit significant toxicity (Fig. 2c).

Fluoroquinolones are the most commonly used drugs for pre- and postoperative infection control for eye surgery. The present results are consistent with previous reports of corneal toxicity resulting from use of these agents (Kaji et al., 2007; Sakurai et al., 2006). Although the 48-h exposure used here may not be directly applicable to safety evaluations of the intraocular injection of antibiotics at the conclusion of cataract surgery (Endophthalmitis Study Group, European Society of Cataract & Refractive Surgeons, 2007; O’Brien et al., 2007; Arbisser et al., 2008), our data suggested that toxicity levels would be acceptable since HCEC survival increased remarkably with dilution. We reported previously that preservatives also play a dominant role in drug toxicity for HCECs (Ayaki et al., 2007). For example, HCEC survival rates in 10, 20, 100, and 1,000-fold diluted preservatives were 22.4%, 20.7%, 76.7%, and 93.6% for 0.01% BAC, 19.7%, 43.1%, 98.4%, and 94.7% for 1%

**FIG. 2a** (left panel) Effects of antibiotic ophthalmic solutions on the survival of cultured human corneal endothelial cells after a 48-h exposure. Data are means ± SD. Moxifloxacin was the most toxic and cefmenoxime was the least. *P < 0.01 (t test) for cefmenoxime versus other three solutions, **P < 0.01 for moxifloxacin versus other three solutions.

**FIG. 2b** (center panel) Effects of anti-inflammatory ophthalmic solutions on the survival of cultured human corneal endothelial cells after 48-h exposure. Data are means ± SD. Betamethasone without BAC and polysorbate were the least toxic. The toxicity of other drugs was concentration-dependent. *P < 0.01 (t test) for betamethasone versus other four solutions.

**FIG. 2c** (right panel) Cell survival of cultured human corneal endothelium as a percentage of the control. Test solutions were preservatives in ophthalmic solutions: 0.4% methyl paraoxybenzoate and 0.4% propyl paraoxybenzoate. Other common preservatives contained in tested drugs including 0.01% benzalkonium chloride, 1% polysorbate, and 0.5% chlorobutanol were examined previously (Ayaki et al., 2007).
polysorbate, 98.7%, 88.9%, 86.6%, 90.2% for 0.5% chlorobutanol, respectively, which is almost consistent with the present data for preservative-containing drugs. The corneal toxicity of diclofenac was already established (Hsu et al., 2003) and bromfenac and pranoprofen preserved with BAC exhibited cytotoxicity levels comparable to that seen for diclofenac.

Uematsu et al. (2007) and Nakashima et al. (2008) reported that BAC could disrupt the barrier function of epithelial and endothelial layers. Surgical trauma to the eye caused by surgical procedures, irrigating solutions (Edelhauser et al., 1975; Glasser et al., 1985), and inflammation (Oishi et al., 1979) might therefore increase the penetration of topical medications into the anterior chamber compared with non-postoperative applications. For example, the intraocular penetration of micromycin into the cornea and intraocular fluid (aqueous humor) 30 min after instillation increased 200- and 163-fold, respectively, in rabbit eyes inflamed by sodium hydralate compared with untreated eyes (Oishi et al., 1979). Endothelial cells in the eye could similarly be exposed to greater concentrations of the components of topical medications during postoperative care than under normal treatment conditions. Other factors implicated in the cytotoxicity of ophthalmic solutions include decreased lacrimal secretion and tear drainage (Burnstein et al., 1985; Tomii et al., 1990), with a 100-fold higher concentration of cefmenoxime found in the conjunctival sac of dry-eye patients compared to normal subjects (Tomii et al., 1990).

Nevertheless, based on actual drug concentrations in the anterior chamber at 500-30,000-fold dilutions according to the literature (Levine et al., 2004; Fukuda et al., 2004; Fukuda et al., 2006; Yamada et al., 2003; Kim et al., 2005; Ellis et al., 1994; Isaka et al., 1999) (Table 2) and the turnover of aqueous humor, we speculate that ocular topical medications used postoperatively will not cause endothelial cell damage unless unusual conditions prevail that facilitate intraocular transportation of the drug, such as wound dehiscence, thinning of the ocular wall, prolonged extensive destruction of the barrier effect of the ocular surface, or deterioration of the drainage system in the conjunctival sac. In addition, the present results indicated that cell survival increases to ≥80% following the dilution of ophthalmic solutions to 100-fold or more.

Limitations of the present study include using only bioassays to evaluate cytotoxicity, the relatively long exposure time, and using commercially available ophthalmic solutions. The cytotoxic or ocular toxic effects of the drugs clearly need to be evaluated more comprehensively, such as by morphological evaluations and testing their effects on membrane integrity (Hockley et al., 1986), metabolic activity, protein synthesis, and apoptosis. We approached this aim here by evaluating a range of drug concentrations in primary HCEC cultures.

With regard to the exposure time used in the present cell culture study, optimal times have not been established. Steroid levels in the cornea are known to decrease by 50% within 1 h after application (Short et al., 1966). In addition, the rapid turnover of tear film makes it difficult to determine optimal exposure times for cell culture experiments. A 48-h exposure is routine for in vitro cytotoxicity assays and probably most relevant for HCEC, which have very slow mitotic activity.

The use of commercial ophthalmic solutions does not allow the effects of individual components in the solution to be defined, thus interactions between active and adjunct components of the ophthalmic solu-

<table>
<thead>
<tr>
<th>Drug</th>
<th>Maximum concentration in aqueous humor (μg/ml) (Dilution-fold)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Human study</td>
</tr>
<tr>
<td>0.5% Levofloxacin</td>
<td>0.60 (8333)§</td>
</tr>
<tr>
<td>0.5% Moxifloxin</td>
<td>2.10 (2381)§, 1.80 (2778)§</td>
</tr>
<tr>
<td>0.3% Gatifloxin</td>
<td>0.40 (7500)§, 0.48 (6250)§</td>
</tr>
<tr>
<td>0.1% Diclofenac</td>
<td>0.082 (12195)††</td>
</tr>
<tr>
<td>0.1% Bromfenac</td>
<td></td>
</tr>
</tbody>
</table>

* = Levine et al., 2004, ** = Fukuda et al., 2004, *** = Fukuda et al., 2006, § = Yamada et al., 2003, §§ = Kim et al., 2005, §§§ = Ellis et al., 1994, † = Isaka et al., 1999.
tion might confound results. Moreover, the concentration of each component in the ophthalmic solutions is not disclosed in the product information. Nevertheless, these commercial solutions are used routinely in patients and so we believe our results do have clinical relevance.

In conclusion, preservatives are essential ingredients in ophthalmic solutions to control postoperative infection and inflammation and we should be aware of their toxicity as well as efficacy.

REFERENCES


