Corneal endothelial cells play a crucial role in maintaining corneal transparency. Corneal clarity requires a net movement of fluid from corneal stroma to aqueous humor [1]. The efficiency of flux depends on undamaged cells and cellular density. Any factor that decreases the cellular density in corneal endothelium may reduce the efficiency of corneal pump function and transparency.

Many antiglaucoma drugs have been developed for the treatment of glaucoma patients. It is now recognized that glaucoma is a chronic disease caused by the death and subsequent loss of ganglion cells and their axons in the optic nerve [2]. Many glaucoma patients need to have antiglaucoma drug administration for a long time until the intraocular pressure is well controlled by laser therapy or surgery such as trabeculectomy. Under this circumstance, the corneal endothelium is continuously soaked in antiglaucoma drugs over a long period of time. The physiologic function of corneal endothelial cells may be changed gradually in the process of drug administration.

Some evidence indicates that loss of corneal endothelial cells occurs with long-term topical epinephrine therapy in glaucoma patients [3]. The mean percentage loss in endothelial cell density from baseline was 3.6%, 4.5% and 4.2%, respectively, for the dorzolamide, timolol and betaxolol treated groups on human
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corneas after 1 year of treatment [4]. Thus, it is crucial to know which commercial antiglaucoma drugs cause corneal endothelial cell damage. Although many antiglaucoma drugs are available for clinical use, the effects of these drugs on corneal endothelial cells have been poorly investigated. In this study, we compared the effects of various commercial antiglaucoma drugs including betaxolol, timolol, levobunolol, carteolol, brimonidine, dipivefrin, dorzolamide, latanoprost, unoprostone, and pilocarpine on cellular cytotoxicity in cultured corneal endothelial cells with assay of medium lactate dehydrogenase (LDH), which is a stable cytosolic enzyme that is released upon cell lysis.

**Materials and Methods**

**Materials**

Culture mediums include trypsin, essential minimal essential medium (EMEM), glutamine, gentamicin, and fetal bovine serum were obtained from GIBCO (Grand Island, NY, USA). Commercially available antiglaucoma drugs such as 0.5% (16.2 mM) betaxolol (Betoptic, Alcon, containing 0.1 mg/mL benzalkonium chloride), 0.25% (5.8 mM) timolol (Timoptol, Merck Sharp & Dohme-Chibret, containing 0.048 mg/mL benzalkonium chloride), 0.5% (17.1 mM) levobunolol (Bunolgan, Allergan, containing 0.04 mg/mL benzalkonium chloride), 1% (68 mM) carteolol (Arteoptic, Otsuka, containing 0.005 mg/mL benzalkonium chloride), 0.2% (6.8 mM) brimonidine (Alphagan, Allergan, containing 0.01 mg/mL benzalkonium chloride), 0.1% (2.8 mM) dipivefrin (Propine, Allergan, containing 0.04 mg/mL benzalkonium chloride), 2% (61.6 mM) dorzolamide (Trusopt, Merck Sharp & Dohme-Chibret, containing 0.075 mg/mL benzalkonium chloride), 1% (26 mM) brinzolamide (Azopt, Alcon, containing 0.1 mg/mL benzalkonium chloride), 0.005% (0.11 mM) latanoprost (Xalatan, Pharmacia & Upjohn, containing 0.08 mg/mL benzalkonium chloride), 0.12% (2.82 mM) unoprostone (Rescula, Fujisawa, containing 0.015 mg/mL benzalkonium chloride), and 1% (40.8 mM) pilocarpine (Spersacarpine, Dispersa, containing 0.1 mg/mL benzalkonium chloride) were diluted with serum-free medium to make three dilutions of 1/100, 1/1,000, and 1/10,000. Benzalkonium chloride was purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals were obtained from Merck (Darmstadt, Germany).

**Culture of bovine corneal endothelial cells**

BCE C/D-1b cells, a stable cell line derived from bovine corneal endothelium, were obtained from American Type Culture Collection (CRL-2048; Rockville, MD, USA). Cells were grown in minimal essential medium (MEM) containing 20% fetal bovine serum, 3.8 mM L-glutamine, and 50 μg/mL gentamicin following previously published procedures [5,6]. The cultures were kept in a humidified chamber at 5% CO₂ and 37°C, and the medium was changed every 2 or 3 days.

**Measurement of LDH release from cells**

To evaluate cellular damage in the presence of drug treatment, LDH release from cells into the medium was assayed following the manufacturer’s guidelines included in the kit (CytoTox 96R Non-Radioactive Cytotoxicity Assay; Promega Corp.). Cultured corneal endothelial cells with 4,000 cells/well were seeded in 96-well culture plates and allowed to settle for 24 hours to form a monolayer. The cells in each well were exposed to 150 μL of serum-free MEM containing various antiglaucoma drugs at three dilutions of 1/100, 1/1,000, and 1/10,000 with serum-free medium for 100 minutes at 37°C. After drug exposure, 50 μL of supernatant from each well was transferred to new 96-well culture plates. Then, supernatant was incubated with 50 μL of substrate mix solution at room temperature for 30 minutes and kept away from light. After the addition of 50 μL stop solution, the optical density of the fluid in each well was read at 490 nm in a multiwell spectrophotometer (Titertek Multiscan, Flow Lab, Scotland, UK). LDH release was calculated based on the difference in optical density between the treatment and control groups with the value of serum-free medium subtracted. The values obtained from control cells were recorded as 100%. Results are expressed as a percentage of those in corresponding control cells.

**Statistical analysis**

All data were analyzed with ANOVA followed by comparison with Dunnett’s test. The values were presented as mean ± SEM from three experiments.
with triplicate determinations. The values were considered to be significantly different from corresponding controls when $p < 0.05$. Control values were set as 100% response.

**RESULTS**

To determine the effects of antiglaucoma drugs on cellular cytotoxicity, LDH release was assayed after corneal endothelial cells were exposed to various antiglaucoma drugs in three dilutions of 1/100, 1/1,000, and 1/10,000 for 100 minutes. Drug-induced LDH release in the medium was increased in the presence of the 1/100 dilution of six drugs: betaxolol, brimonidine, dorzolamide, dipivefrin, latanoprost and unoprostone. Following pretreatment of cells with selective $\beta_1$-adrenergic antagonist betaxolol at 1.62, 16.2, and 162 $\mu$M for 100 minutes, the value of LDH in the medium significantly increased only at 162 $\mu$M to 130±5% when compared with the value of the corresponding control (Figure 1). After addition of $\alpha_2$-agonist brimonidine at 0.68, 6.8, and 68.0 $\mu$M for 100 minutes, the LDH value in medium was significantly increased only at the concentration of 68.0 $\mu$M to 123±4% in comparison with the control group (Figure 2). After treatment of the cells with three dilutions of dorzolamide at 6.16, 61.6, and 616 $\mu$M for 100 minutes, the LDH value in the medium significantly increased only at 616 $\mu$M to 145±5% in comparison with the control group (Figure 3). For the sympathomimetic drug dipivefrin, only the 1/100

**Figure 1.** Effects of betaxolol on lactate dehydrogenase (LDH) release from cultured bovine corneal endothelial cells. Cells were exposed to betaxolol at concentrations of 162, 16.2 and 1.62 $\mu$M for 100 minutes. All data are presented as percentage of control from three separate experiments. Individual experiments were carried out in triplicate. Data are mean ± SEM. *$p < 0.05$ compared with control.

**Figure 2.** Effects of brimonidine on lactate dehydrogenase (LDH) release from cultured bovine corneal endothelial cells. Cells were exposed to brimonidine at concentrations of 68, 6.8 and 0.68 $\mu$M for 100 minutes. All data are presented as percentage of control from three separate experiments. Individual experiments were carried out in triplicate. Data are mean ± SEM. *$p < 0.05$ compared with control.

**Figure 3.** Effects of dorzolamide on lactate dehydrogenase (LDH) release from cultured bovine corneal endothelial cells. Cells were exposed to dorzolamide at concentrations of 616, 61.6 and 6.16 $\mu$M for 100 minutes. All data are presented as percentage of control from three separate experiments. Individual experiments were carried out in triplicate. Data are mean ± SEM. *$p < 0.05$ compared with control.
dilution (28 μM) increased LDH release significantly to 157 ± 4% compared to the corresponding controls (Figure 4). Of the three concentrations of latanoprost (1.1, 0.11, 0.01 μM), only 1.1 μM increased LDH release significantly to 128 ± 5% in comparison with controls (Figure 5). When cells were exposed to the synthetic docosanoid unoprostone for 100 minutes, the LDH value in the medium significantly increased only at 31 μM to 237 ± 8% compared with corresponding controls (Figure 6). In this study, we found that LDH values were not induced to increase by the other drugs (timolol, levobunolol, carteolol, brinzolamide, pilocarpine) at any dilution (data not shown).

All of the commercial antiglaucoma drugs tested contain benzalkonium chloride as a preservative. To estimate the effect of benzalkonium chloride on corneal endothelial cells, we also tested various concentrations of benzalkonium chloride, 0.001, 0.0001 and 0.00001 mg/mL, on LDH release. The results demonstrated that no statistical difference was found between the three concentrations of benzalkonium chloride and the corresponding controls on LDH release (data not shown).

**DISCUSSION**

Although many antiglaucoma drugs have been administered in glaucoma patients with the distinct mechanism of lowering intraocular pressure, the comparison of the cytotoxic effects of the various antiglaucoma drugs is not well characterized. In this study, we...
compared the cellular cytotoxicity of various commercial antiglaucoma drugs on corneal endothelial cells under similar culture conditions. We found that 1/100 dilution of betaxolol, brimonidine, dipivefrin, dorzolamide, latanoprost and unoprostone can cause corneal endothelial cellular damage after 100 minutes of incubation. The most potent effect of drug-induced LDH release was displayed by unoprostone.

Corneal endothelial cells play a crucial role in maintaining corneal transparency [1]. Under conditions such as surgery or disease, excessive cellular damage may impair the ability of the endothelium to act effectively as a barrier to fluid flow from the aqueous humor to the stroma [7,8]. This loss of endothelial function may bring about corneal edema and decrease in visual acuity. There is evidence to show that some antiglaucoma drugs may damage or interfere with the normal physiologic functions of corneal endothelium [3]. After the administration of dorzolamide, timolol and betaxolol for 1 year, the loss in endothelial cell density was 3.6%, 4.5% and 4.2%, respectively, compared with cell density at baseline [4]. Dipivefrin has been shown to inhibit the growth of corneal endothelial cells [9]. As to normal function, timolol and betaxolol have been shown to inhibit the activity of Na⁺K⁺-ATPase in corneal endothelial cells [10]. Dorzolamide also significantly inhibits carbonic anhydrase activity in corneal endothelial cells [11]. Although we found that the six drugs betaxolol, brimonidine, dorzolamide, dipivefrin, latanoprost and unoprostone induced cellular LDH release at 1/100 dilution, the identification of various drugs on cellular target sites in corneal endothelial cells remains to be investigated.

According to the pharmacokinetic assay of latanoprost, the maximum concentration of active latanoprost is detected in the aqueous humor within 1–2 hours amounting to approximately 10⁻⁷ M after topical administration of the clinical dose of 10⁻⁴ M in humans [12]. The elimination half-life of latanoprost in the cornea has been determined to be 1.8 hours in rabbits [13]. Thus, we estimated the effect of antiglaucoma drugs on corneal endothelial cells with concentrations starting from 1/100 dilution of the original commercial concentrations for an exposure of 100 minutes.

In summary, the results reported here provide evidence to indicate that some antiglaucoma drugs may cause cytotoxicity to corneal endothelial cells and gradually interfere with normal corneal function. Moreover, after intraocular surgery, the damaged corneal endothelium in glaucoma patients who have been treated with cytotoxic antiglaucoma drugs may take more time to recover to normal corneal clarity compared to that in glaucoma patients who have been treated with nontoxic drugs. Thus, the long-term use of these drugs may need to be carefully monitored for changes in corneal endothelium.

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抗青光眼藥水對角膜內皮細胞毒性之影響

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2高雄醫學大學附設醫院 眼科

本實驗主要是研究不同的抗青光眼藥包括 betaxolol、timolol、levobunolol、
carteolol、brimonidine、dipivefrin、dorzolamide、brinzolamide、
latanoprost、unoprostone 及 pilocarpine 對體外培養牛角膜內皮細胞毒性的影
響。細胞以 1/100、1/1,000 及 1/10,000 稀釋的上述眼藥水培養 100 分鐘後，以
lactate dehydrogenase (LDH) 的釋出量測試不同的眼藥水對細胞的毒性作用。結果
發現細胞經過 1/100 稀釋的 betaxolol、brimonidine、dorzolamide、
dipivefrin、latanoprost 及 unoprostone 培養 100 分鐘後會明顯增加細胞釋放
lactate dehydrogenase 的量到控制組的 130%、123%、145%、157%、128% 及
237%，而且細胞經 0.001、0.0001 及 0.00001 mg/mL 防腐劑 benzalkonium
chloride 培養 100 分鐘後並不會改變角膜內皮細胞釋放 lactate dehydrogenase
的量。因此本實驗顯示高濃度的抗青光眼藥水可能會對角膜內皮細胞產生毒性作用。

關鍵字：抗青光眼藥，細胞毒性，角膜內皮細胞，lactate dehydrogenase

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