



Letter

Comparison of cytotoxicity between fixed- and unfixed-combination ophthalmic solutions *in vitro*

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ABSTRACT — In the therapy of ocular diseases, different active pharmaceutical ingredients are often instilled concomitantly as different ophthalmic solutions at an appropriate interval (unfixed-combination therapy) or simultaneously as one formulation (fixed-combination therapy). In this study, we aimed to compare the *in vitro* cytotoxicity of fixed- and unfixed-combination ophthalmic solutions using the glaucoma therapeutic agents, timolol and brimonidine. Cultured human corneal epithelial cell line was used as a test system. Exposure period was set at 5 min. Compared with the fixed-combination treatment, the unfixed-combination treatment (timolol followed by brimonidine, and vice versa) led to a significant reduction in the percentage of cell viability. In conclusion, it was suggested that a fixed-combination ophthalmic solution was expected to cause less cellular damage to the ocular surface, compared to an unfixed-combination ophthalmic solution.

Key words: Glaucoma, Ophthalmic solution, Benzalkonium chloride, Human corneal epithelial cells, Cytotoxicity

INTRODUCTION

Ophthalmic solutions are directly administered on the surface of the eyes, causing extremely high drug concentration just after their instillation. In many cases, ophthalmic solutions are instilled repeatedly within one day or during the treatment period. In addition, ophthalmic solutions are administered concomitantly with other ophthalmic solutions (unfixed-combination therapy) or administered as a formulated combination (fixed-combination therapy) to improve the therapeutic effects (Gross, 2014).

In this point, anti-glaucoma therapy with ophthalmic solutions is one typical case. Ophthalmic solutions have been approved as anti-glaucoma therapies with different mechanisms, *e.g.* prostaglandin analogs, beta-blockers, Rho-kinase inhibitors, alpha2-adrenoceptor agonists, and carbonate anhydrase inhibitors, *etc.* (Schmidl *et al.*,

2015). Ophthalmic solutions for anti-glaucoma therapy are administered as a fixed or unfixed combination, which have different mechanisms (Gross, 2014). Among these solutions, the beta-blocker timolol is frequently employed as an agent in a fixed or unfixed combination with other types of anti-glaucoma drugs (Radcliffe, 2014).

In determining the safety of ophthalmic solutions, another important factor for consideration is the components of the formulation; for example, antibacterial preservatives. The most popular preservative for ophthalmic solutions, including anti-glaucoma drugs, is benzalkonium chloride (BAC). It is widely used because of its high antimicrobial efficiency. There are many reports indicating that BAC induces cytotoxic and inflammatory changes in the ocular surface (Asiedu and Abu, 2019). A fixed-combination therapy is expected to reduce repeated exposure of cells on the ocular surface to artificial solutions, includ-

ing BAC.

This study aimed to evaluate the cytotoxicity of fixed- and unfixed-combination ophthalmic solutions using the human corneal epithelial cell line *in vitro*. In this study, we used brimonidine tartrate/timolol fixed combination ophthalmic solution (SJP-0135) as a fixed-combination treatment, and AIPHAGAN® and TIMOPTOL® as an unfixed-combination treatment.

MATERIALS AND METHODS

Cell culture

An SV40-immortalized human corneal epithelial cell line (HCE-T) (RCB2280; RIKEN BRC, Tsukuba, Japan) (Araki-Sasaki *et al.*, 1995) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific, MA, USA) supplemented with 5% inactivated fetal bovine serum (Thermo Fisher Scientific), 10 ng/mL human recombinant epidermal growth factor (Thermo Fisher Scientific), 5 µg/mL insulin (FUJIFILM Wako, Osaka, Japan), and 40 µg/mL gentamicin (Thermo Fisher Scientific) in 5% CO₂ at 37°C. The cells were seeded in a 96-well culture plate at a density of 20,000 cells/well. After approximately 24 hr of incubation, the cells were used for cell viability assay.

Anti-glaucoma drugs

We evaluated the cytotoxicity of the following anti-glaucoma drugs in HCE-T cells: 0.1% brimonidine tartrate ophthalmic solution with sodium chloride as a preservative (AIPHAGAN®; Senju Pharmaceutical Co., Ltd., Osaka, Japan), 0.5% timolol ophthalmic solution with 0.005% BAC (TIMOPTOL®; Santen Pharmaceutical Co., Ltd., Osaka, Japan), and 0.1% brimonidine tartrate/0.5% timolol fixed combination ophthalmic solution with 0.002% BAC (SJP-0135; Senju Pharmaceutical Co., Ltd.). We diluted these commercial ophthalmic solutions to 1/2 concentration with serum-free medium.

Exposure procedure

At 2 hr before exposure, the cell medium was replaced with serum-free medium. For the fixed-combination treatment, the cells were exposed to SJP-0135 (100 µL/well) at 37°C for 5 min. The solution was aspirated and gently washed once with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific). For the unfixed-combination treatment (TIMOPTOL® followed by AIPHAGAN®, and vice versa), the cells were exposed to the first ophthalmic solution in the same way as in the fixed combination treatment, washed with Dulbecco's phosphate-buffered

saline, and then exposed to the second ophthalmic solution in the same way as in the fixed combination treatment. For both the fixed- and unfixed-combination treatments, the controls were subjected to the same methods using serum-free medium instead of ophthalmic solution.

Cell viability assay

Cell viability was tested using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories Co., Ltd., Kumamoto, Japan) according to the manufacturer's instructions. CCK-8 solution (10 µL) was added to each well containing the cells in 100 µL serum-free medium, which were incubated at 37°C for 2 hr. Next, absorbance was measured at 450 nm using a microplate reader (SH-1100R Lab; Corona Electric Co., Ltd., Ibaraki, Japan). Cell viability in ophthalmic solutions was calculated as a percentage of control cell viability in serum-free medium. All experiments were performed in triplicate.

Statistical analysis

Statistical significance was assessed using Bartlett's test, one-way analysis of variance, followed by Dunnett's multiple comparison test using the INATOX-DP system (Ina Research Inc., Nagano, Japan). Statistical significance was indicated at the level of $P < 0.05$ (one-sided test).

RESULTS AND DISCUSSION

Figure 1 shows the viability of HCE-T cells after exposure to fixed or unfixed combination of anti-glaucoma drugs. Compared with after the fixed-combination treatment, significant reductions in the percentages of cell viability were observed after the unfixed-combination treatments (TIMOPTOL® to AIPHAGAN®, and vice versa). Cell viabilities were comparable between the unfixed-combination treatment groups.

One possible reason of this difference in cell viability was the repeated exposure of cells to ophthalmic solutions in the unfixed-combination treatments. However, the repeated exposure might not be the sole cause of this difference because cell viability in ophthalmic solutions was calculated as a percentage of each control in serum-free medium. This difference might be related to the components of the ophthalmic solutions. In this respect, BAC is well known to induce cytotoxicity to HCE-T cells in a concentration- and time-dependent manner (Hakkarainen *et al.*, 2016; Ammar *et al.*, 2010; Nagai *et al.*, 2011). In addition, following exposure of HCE-T cells to AIPHAGAN®, which does not contain BAC, at 37°C for 5 min, no cytotoxicity was observed (cell viability: 101.7%; data

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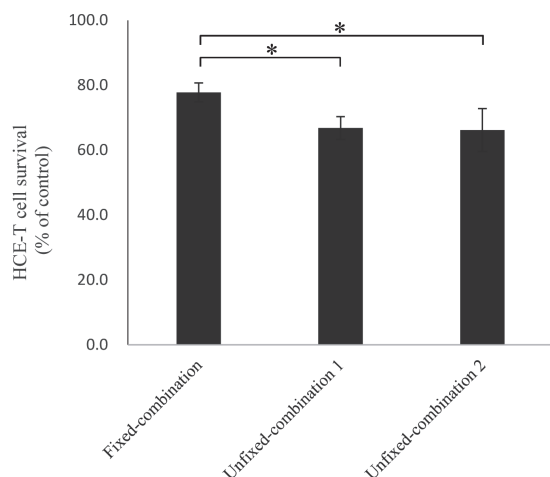


Fig. 1. Comparison of cell viability of HCE-T cells after exposure to fixed- or unfixed-combination ophthalmic solutions. The data are presented as mean \pm SD ($n = 3$). Fixed combination: SJP-0135; unfixed combination 1: AIPHAGAN[®] followed by TIMOPTOL[®]; unfixed combination 2: TIMOPTOL[®] followed by AIPHAGAN[®]. * $P < 0.05$ versus fixed-combination.

not shown). Therefore, it might be considered that the high cytotoxicity of the unfixed-combination treatments was mainly caused by the higher BAC concentration in TIMOPTOL[®] than in SJP-0135.

Regarding cytotoxicity to the ocular surface, an *in vitro* test method named the short time exposure *in vitro* test method is available to assess eye irritation (OECD, 2015). This short time exposure *in vitro* test method has been established for cytotoxicity testing of chemicals. In addition, most of the volume of an ophthalmic solution is eliminated from the conjunctival sac within several minutes (Motose, 1984; Regnier, 2013). The method employed in this study to evaluate the cytotoxicity of ophthalmic solutions was modified in terms of the cell line used (HCE-T cells) and combination of ophthalmic solutions with cell medium. Some reports (Mori *et al.*, 2017; Ueda *et al.*, 2010) showed that the initial concentration of an ophthalmic solution on the surface of the cornea is diluted twice by tear fluid; therefore, we diluted the ophthalmic solutions to 1/2 concentration with cell medium.

In conclusion, the present study suggested that compared with an unfixed-combination therapy, a fixed-combination therapy may cause less ocular surface damage due to its chronic use in patients with glaucoma, and may exert beneficial pharmacological effects with different mechanisms from different ophthalmic solutions.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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