

Comparative assessment of the cytotoxicity of six anti-inflammatory eyedrops in four cultured ocular surface cell lines, as determined by cell viability scores

Masahiko Ayaki¹
Atsuo Iwasawa²
Yoshimi Niwano³

¹Department of Ophthalmology, International University of Health and Welfare, Mita Hospital, Tokyo,

²Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, ³Laboratory for Redox Regulation, Tohoku University Graduate School of Dentistry, Sendai, Japan

Purpose: Anti-inflammatory eyedrops are often used in the treatment of corneal epithelial disorders. In the present study, we evaluated the cytotoxicity of six anti-inflammatory eyedrops in four ocular surface cell lines.

Methods: The cytotoxicity of six commercially available anti-inflammatory ophthalmic solutions (ie, diclofenac, bromfenac, pranoprofen, betamethasone, and fluoromethorone) was assessed in three corneal cell lines and one conjunctival cell line. Cell viability was determined by the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide and neutral red assays after exposing the cells to 10, 30, and 60 minutes of onefold, twofold, and tenfold dilutions of the drugs. Cytotoxicity was compared using the cell viability score (CVS), an integrated cytotoxic parameter that takes various factors into account, such as dilution by tear fluid or concentration by evaporation, drug exposure time, and ocular surface cell type.

Results: Based on the CVS scores, the order of the anti-inflammatory eyedrops tested from least to most cytotoxic, with the active ingredient %CVS₅₀, and %CVS_{40/80} for each solution given in parentheses, was as follows: Rinderon® (betamethasone, 100%, 100%) > 0.02% Flumethoron® (fluoromethorone, 68%, 22%) = 0.1% Flumethoron® (fluoromethorone, 76%, 22%) > Bronuck® (0.1% bromfenac, 53%, -8%) = Diclod® (0.1% diclofenac, 44%, -15%) = Niflan® (pranoprofen, 50%, -19%). Rinderon® exhibited the least toxicity of all the anti-inflammatory eyedrops tested. Eyedrops containing non-steroidal anti-inflammatory drugs exhibited greater cytotoxicity than those containing steroids with benzalkonium at comparable concentrations. Concentration was the most significant factor affecting cell viability.

Conclusion: The cytotoxicity of the anti-inflammatory eyedrops evaluated in the present study depended on both the pharmaceutical components and preservatives. The CVS is a concise indicator of drug cytotoxicity.

Keywords: benzalkonium chloride, toxicity, cornea, cell viability score, anti-inflammatory drug

Introduction

Anti-inflammatory eyedrops are routinely used postoperatively, as well as to treat inflammatory eye diseases. A previous study examining cytotoxicity after 48 hours exposure of ocular cell lines to anti-inflammatory eyedrops in the presence of culture medium indicated that eyedrops containing benzalkonium chloride (BAK) exhibited considerable cytotoxicity.¹ However, a 48-hour period of exposure to eyedrops is not an accurate reflection of the actual situation in which eyedrops are generally applied several times a day. In addition, patients' ocular surface cells come in direct contact with

Correspondence: Masahiko Ayaki
Department of Ophthalmology,
International University of Health and
Welfare, Mita Hospital, 1-4-3 Mita,
Minatoku, Tokyo 1088329, Japan
Tel +81 433 451 81 21
Fax +81 334 540 067
Email mayaki@olive.ocn.ne.jp

eyedrop formulations. Thus, to reflect the actual situation, we have tried to improve cytotoxicity assays for ocular cells.²⁻⁶ In these studies, ocular surface cells came into direct contact with drug formulations in the absence of culture medium. Based on our studies, we proposed the use of a cell viability score (CVS) as a simple parameter to express the cytotoxic potential of eyedrops.

Originally, we defined the CVS₅₀ as the number of cell lines with $\geq 50\%$ viability in the presence of a tenfold dilution of the drug (eg, when four cell lines are used, the score should be 0, 1, 2, 3, or 4).¹ However, in recent studies we have improved the CVS such that the CVS₅₀ is now defined as the number of measurements showing $\geq 50\%$ cell viability and is expressed as a percentage over the range 0%–100%.^{2,3} Using this improved method, the total number of measurements is 72 (three concentrations, three exposure times, four cell lines, and two assays).^{2,3} Because topically applied eyedrops can be affected by various factors, such as dilution by lacrimal fluid or concentration by moisture loss, and since they can come into contact with different cell types (eg, corneal epithelial and conjunctival cells) on the corneal surface for varying amounts of time, the cytotoxicity of eyedrops needs to be evaluated in a comprehensive manner. As such, the CVS has been modified based on the number of cytotoxicity measurements in all situations, taking into account concentration, exposure time, and cell type.

Eyedrop cytotoxicity is a contentious issue because once an eyedrop is applied to the ocular surface, its concentration and drug penetration can change very rapidly. Therefore, in the present study we reevaluated the cytotoxicity of anti-inflammatory eyedrops by conducting comprehensive investigations covering a variety of concentrations and treatment times based on the modified CVS system.

Material and methods

Six commercially available anti-inflammatory eyedrops were tested in the present study. Their trade names, active components, inactive components (preservatives), and manufacturers are listed in Table 1. The chemical structures of the active components have been published elsewhere.⁴

The methods for cell culture, the cytotoxicity assays, and data evaluation have been described in detail elsewhere.² Briefly, the following commercially available cell lines were used: SIRC (rabbit corneal epithelium; ATCC CCL-60; American Type Culture Collection [ATCC], Manassas, VA); BCE C/D-1b (bovine corneal epithelial cells; JCRB-9129; Health Science Research Resource Bank, Osaka, Japan); RC-1 (rabbit corneal epithelium; JCRB-0246; Health Science Research Resource Bank); and Chang conjunctiva (human conjunctival cells; ATCC CCL-20.2; ATCC). After cells had reached confluence, the culture medium was replaced with undiluted solution or twofold and tenfold dilutions of the different test solutions, and cell monolayers were incubated in the presence of these solutions for 10, 30, or 60 minutes.

Sterile physiological saline was used as the diluent for each eyedrop, and it was confirmed that exposure of each cell line to saline for 60 minutes had no significant effect on cell viability compared with the corresponding control. After 10, 30, or 60 minutes of incubation, the ophthalmic solutions were replaced with fresh culture medium and the cells were incubated for a further 48 hours. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Co, St Louis, MO) and neutral red (NR; Wako Pure Chemical Industries, Osaka, Japan) assays. Cell viability in test solutions was calculated as a percentage of control cell

Table 1 Anti-inflammatory ophthalmic solutions evaluated in the present study

	Active component	Preservatives	Manufacturer
Rinderon®	0.1% Betamethasone	0.05% methyl paraoxybenzoate 0.02% propyl paraoxybenzoate	Shionogi and Co, Ltd, Osaka, Japan
Flumethoron® 0.1%	0.1% Fluoromethorone	0.005% BAK Polysorbate 80	Santen Pharmaceutical Co, Ltd, Osaka, Japan
Flumethoron® 0.02%	0.02% Fluoromethorone	0.005% BAK Polysorbate 80	Santen Pharmaceutical Co, Ltd, Osaka, Japan
Diclod®	0.1% Diclofenac	Chlorobutanol Polysorbate 80	Wakamoto Co, Ltd, Tokyo, Japan
Bronuck®	0.1% Bromfenac	Boric acid 0.005% BAK Polysorbate 80	Senju Pharmaceutical Co, Ltd, Osaka, Japan
Niflan®	0.1% Pranoprofen	0.007% BAK Polysorbate 80 Boric acid	Senju Pharmaceutical Co, Ltd, Osaka, Japan

Abbreviation: BAK, benzalkonium chloride.

viability in medium only. Experiments were repeated eight times, and the results are presented as the mean \pm SD.

To evaluate the cytotoxicity of anti-inflammatory eyedrops, mean values for 1/2 concentrations were compared with those for Rinderon® (Shionogi and Co, Ltd, Osaka, Japan), betamethasone-containing eyedrops, using Dunnett's multiple comparison test, with $P < 0.05$ set as the level of significance. In addition, the effects of the type of eyedrop, cell line, exposure time, concentration, and assay method on cell viability were evaluated using analysis of variance for bivalent parameters, with $P < 0.05$ taken to indicate statistical significance.

The CVS was used to compare the toxicity of test solutions. The CVS₅₀ was calculated as the number of measurements indicating $\geq 50\%$ viability compared with control. The CVS_{40/80} was calculated as: (the number of measurements indicating $> 80\%$ viability) – (the number of measurements indicating $< 40\%$ viability). The total number of measurements was 72 (three concentrations, three exposure times, four cell lines, and two assays). Results are expressed as a percentage of all measurements (%CVS).

Results

For all cell lines tested, cell viability after exposure to Rinderon® was $\geq 80\%$ (Figures 1–4). Cell viability after exposure of the cells to the other eyedrops tested was concentration-dependent.

The CVS for the anti-inflammatory eyedrops tested in the present study are summarized in Table 2. Based on the CVS, the order of the anti-inflammatory eyedrops tested from least to most cytotoxic, with the active ingredient, %CVS₅₀, and %CVS_{40/80} for each solution given in parentheses, was

as follows: Rinderon® (betamethasone, 100%, 100%) $> 0.02\%$ Flumethoron® (Santen Pharmaceutical Co, Ltd, Osaka, Japan; fluoromethorone, 68%, 22%) = 0.1% Flumethoron® (Santen Pharmaceutical Co, Ltd; fluoromethorone, 76%, 22%) $>$ Bronuck® (Senju Pharmaceutical Co Ltd, Osaka, Japan; 0.1% bromfenac, 53%, –8%) = Diclod® (Wakamoto Co, Ltd, Tokyo, Japan; 0.1% diclofenac, 44%, –15%) = Niflan® (Senju Pharmaceutical Co, Ltd; pranoprofen, 50%, –19%). Rinderon® exhibited the least toxicity of all the anti-inflammatory eyedrops tested. Microscopy revealed that the morphology of all Rinderon®-treated cell lines was similar to that of the control cells (data not shown). Overall, eyedrops containing non-steroidal anti-inflammatory drugs exhibited greater toxicity than those containing steroids.

Figure 5 shows cell viability according to drug, cell line, exposure time, drug concentration, and assay method. All these factors had a significant effect on cell viability, as determined by analysis of variance for bivalent parameters. Drug concentration, followed by exposure time, had the most significant effects on cell viability. Of the cell lines tested, Chang conjunctival and RC-1 cells seemed to be slightly more sensitive to drug exposure when compared with the other two cell lines, with the MTT assay appearing to be slightly more sensitive than the NR assay for evaluating cell viability. The type of drug was a pivotal factor affecting cell viability and, of the different ophthalmic solutions tested, Rinderon® (which contains the synthetic steroid betamethasone as the active ingredient) was least cytotoxic, followed by 0.02% Flumethoron® and 0.1% Flumethoron®, both of which contain the synthetic steroid fluoromethorone as the active ingredient. The viability of cells exposed to the

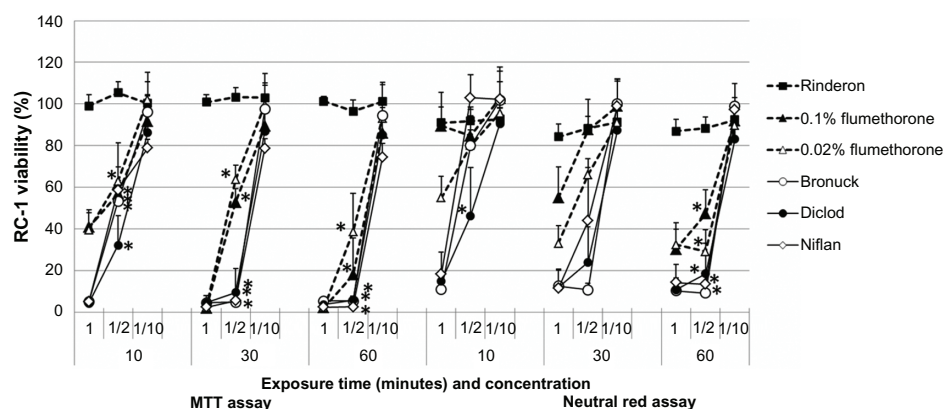


Figure 1 Effects of anti-inflammatory eyedrops, diluted onefold, twofold, and tenfold, on the viability of cultured rabbit corneal epithelial cells (RC-1) after 10, 30, or 60 minutes of exposure, as determined by the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and NR assays.

Notes: Cell survival was greatest after exposure to Rinderon®, with $> 80\%$ cell viability noted in all experiments. Eyedrops containing nonsteroidal anti-inflammatory drugs (solid lines) resulted in lower cell viability than eyedrops containing steroids (dashed lines). Similar findings were obtained for all cell lines (Figures 2–4). Data are the mean \pm SD ($n = 8$). $*P < 0.05$ compared with Rinderon® at twofold dilution (Dunnett's multiple comparison test).

Abbreviations: MTT, 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; NR, neutral red.

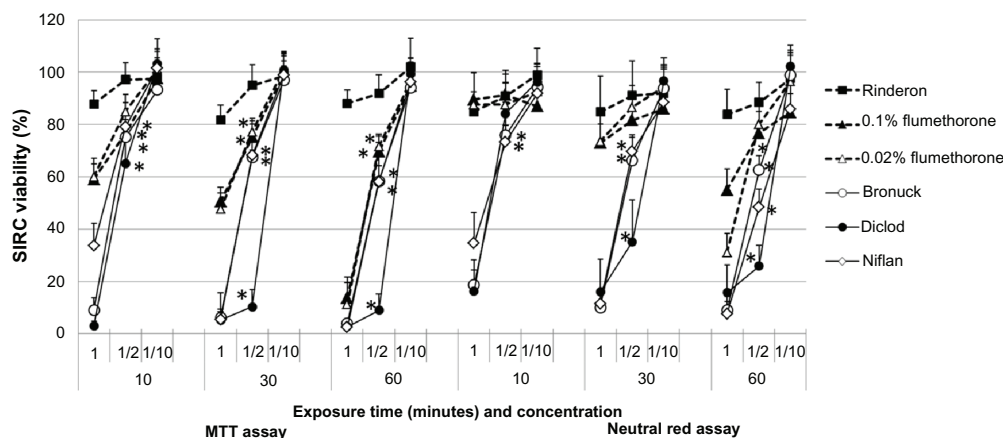


Figure 2 Effects of anti-inflammatory eyedrops, diluted onefold, twofold, and tenfold, on the viability of cultured rabbit corneal epithelial cells (SIRC) after 10, 30, or 60 minutes of exposure, as determined by the MTT and NR assays.

Notes: Data are the mean \pm SD ($n = 8$). * $P < 0.05$ compared with Rinderon® at twofold dilution (Dunnett's multiple comparison test).

Abbreviations: MTT, 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; NR, neutral red.

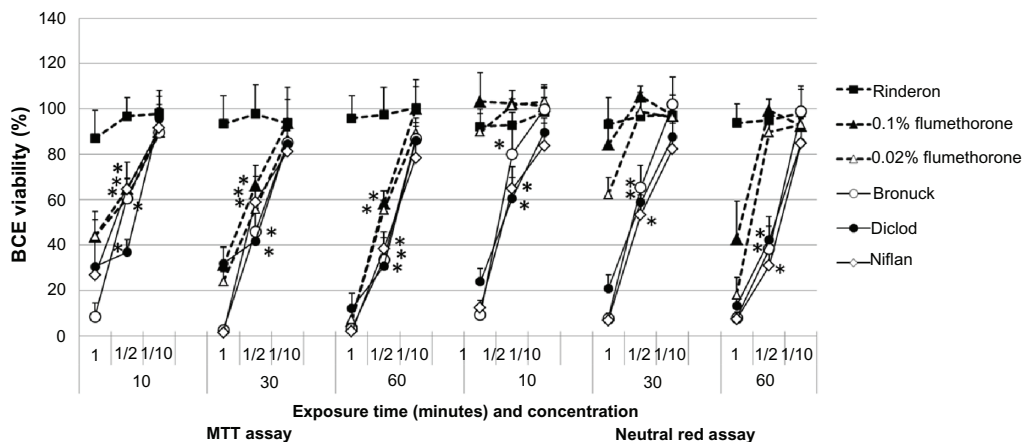


Figure 3 Effects of anti-inflammatory eyedrops, diluted onefold, twofold, and tenfold, on the viability of cultured BCE cells after 10, 30, or 60 minutes of exposure, as determined by the MTT and NR assays.

Notes: Data are the mean \pm SD ($n = 8$). * $P < 0.05$ compared with Rinderon® at twofold dilution (Dunnett's multiple comparison test).

Abbreviations: BCE, bovine corneal epithelial; MTT, 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; NR, neutral red.

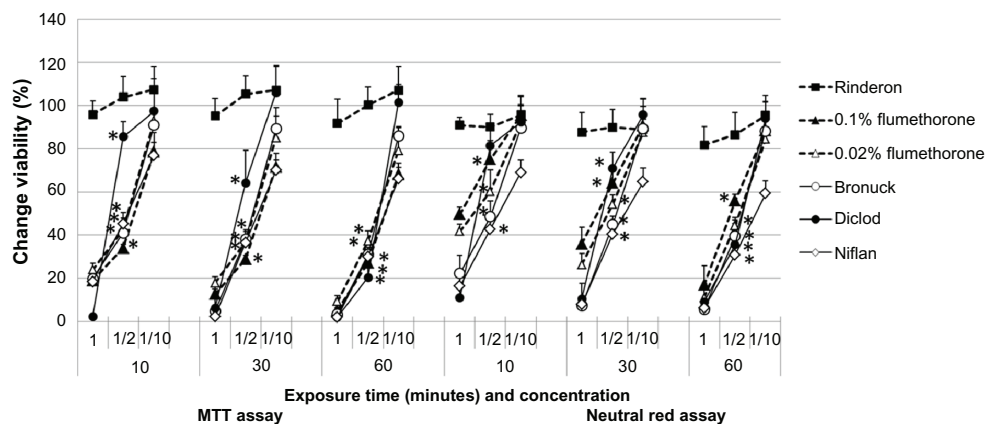


Figure 4 Effects of anti-inflammatory eyedrops, diluted onefold, twofold, and tenfold, on the viability of cultured human conjunctival cells (Chang) after 10, 30, or 60 minutes of exposure, as determined by the MTT and NR assays.

Notes: Data are the mean \pm SD ($n = 8$). * $P < 0.05$ compared with Rinderon® at twofold dilution (Dunnett's multiple comparison test).

Abbreviations: MTT, 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; NR, neutral red.

Table 2 Cell viability scores for the anti-inflammatory ophthalmic solutions evaluated in the present study

Trade name	%CVS ₅₀	%CVS _{40/80}
Rinderon®	100	100
Flumethoron® 0.1%	72	21
Flumethoron® 0.02%	67	19
Diclod®	49	-10
Bronuck®	44	-14
Niflan®	49	-24

Notes: %CVS₅₀, number of measurements indicating $\geq 50\%$ viability compared with control; %CVS_{40/80}, the number of measurements indicating $>80\%$ viability – the number of measurements indicating $<40\%$ viability.

other eyedrops, which are classified as non-steroidal anti-inflammatory drugs, was low. These observations correspond to the %CVS findings described above.

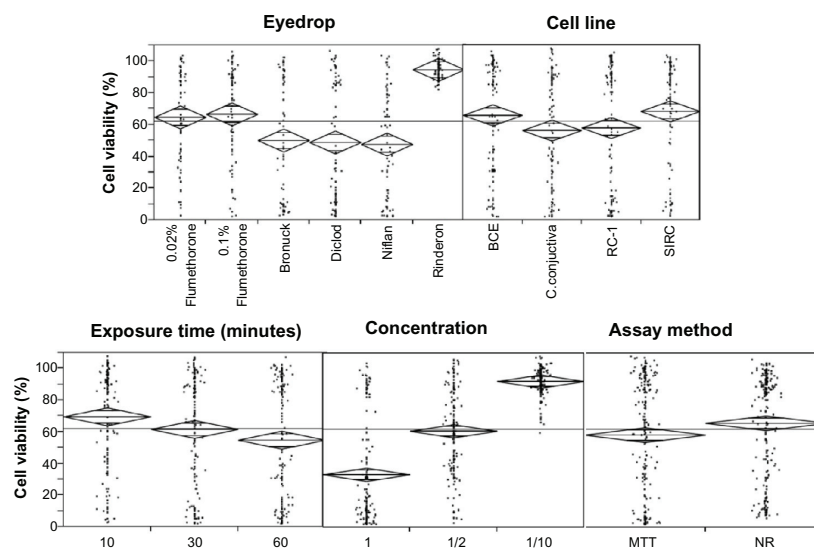
Because cell viability seemed to decrease with increasing exposure time for all eyedrops other than Rinderon®, cell viability in ophthalmic solutions diluted twofold was compared with that following exposure to Rinderon® using Dunnett's multiple comparison test, as shown in Figures 1–4.

Discussion

Anti-inflammatory eyedrops are considered to be a major causative factor of ocular surface disorders during treatment of inflamed eyes, such as postoperatively or for inflammatory eye diseases. Based on the CVS values obtained in the present study, the cytotoxic potential of the anti-inflammatory eyedrops tested was, in order, Niflan® = Diclod® = Bronuck® $>$ 0.02% Flumethoron® = 0.1% Flumethoron® $>$ Rinderon®. In terms

of the active ingredients in each of the eyedrops, the results of the present study confirm our previous findings, namely that steroids are less cytotoxic than non-steroids.^{1,4} Because some studies have demonstrated that dexamethasone, a synthetic steroid, is cytoprotective,^{5–7} betamethasone may have exerted a cytoprotective effect in the present study, explaining why Rinderon® exhibited the lowest cytotoxicity of all the solutions tested. Another reason why eyedrops containing nonsteroidal anti-inflammatory drugs exhibited greater cytotoxicity may be due to the use of BAK as a preservative. Previous studies have reported an inverse relationship between the CVS of various eyedrops and BAK concentrations contained therein.^{1,2,8–10} In our most recent study in which the *in vitro* cytotoxicity of BAK-containing antiglaucoma eyedrops was evaluated, the cytotoxicity of the eyedrops tested was dependent on the BAK concentration.³ In the case of Diclod®, which is BAK free, the active ingredient diclofenac has been used for the control of postoperative inflammation and pain relief but the side effects include severe corneal damage,^{11–14} which suggests considerable ocular surface cytotoxicity. Of the other inactive ingredients, polysorbate is toxic to the eye,¹⁵ and particular caution has been recommended when considering intraocular injections of drugs containing polysorbate.¹⁵ Boric acid, methyl paraoxybenzoate, and chlorobutanol have been reported to exhibit only low toxicity at the most commonly used concentrations.^{16–18}

In the present study, we evaluated the effects of a range of drug concentrations and exposure times in four commercially available cell lines because, in the clinical situation, the eyes

**Figure 5** Scatter diagrams showing cell viability according to drug, cell line, exposure time, drug concentration, and assay method.

Notes: The horizontal line in each diagram indicates the mean cell viability obtained from all the assays. The width, height, and center line of each lozenge indicates the sample size, 95% confidential intervals, and the mean value, respectively. Overall, cell viability was most affected by drug concentration, followed by exposure time.

are exposed to various drug treatments. For example, the drug may be concentrated on the eye due to evaporation or decreased drainage, the drug may be adsorbed on the eye for a prolonged period of time, or the vulnerability to a particular drug may differ among cell types (eg, conjunctival versus corneal epithelial cells). Indeed, the scatter diagrams shown in Figure 5 demonstrate that drug concentration and exposure time significantly affect cell viability.

One of the limitations of the present study is that we only used bioassays to evaluate cytotoxicity. In addition to a conventional monolayer cell culture system, as used in the present study, evaluating cytotoxicity using newly improved ocular models, such as a three-dimensional (3D) model, would have provided useful information. For example, previous studies have used a 3D epithelial model cultivated from human corneal cells as an alternative to the Draize eye test to assess the potential of chemicals to cause eye irritation.^{19,20} In another study, dose-dependent responses to BAK were revealed, with significant toxic effects noted among concentrations as low as 0.005% in a 3D corneal epithelium model.²¹ Accordingly, future studies should use one of these new models, such as the 3D corneal epithelium model, to confirm whether the significant correlations obtained with the CVS system presented here can be replicated in other models. In addition, future studies need to examine correlations between in vitro and in vivo cytotoxicity.

Disclosure

The authors report no conflicts of interest in this work.

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