Toxicity of Ophthalmic Solutions, Benzalkonium Chloride and UV Radiation on Human Corneal Epithelial Cells *in vitro*

by

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Author’s Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

I would like to acknowledge my co-authors, Dr. Jacob Sivak and Dr. David McCanna, for their contributions to this thesis.

Authors' contributions

Author	Concept/Design	Data Collection	Data Analysis	Article Writing	Article Editing
Xu	✓	✓	✓	✓	✓
Sivak	✓				✓
McCanna	✓				✓

Abstract

The purpose of this work was to develop a sensitive *in vitro* method for ocular toxicity testing; and to use this method to assess the individual and combined effects of ophthalmic solutions, benzalkonium chloride (BAK), and ultraviolet (UV) radiation on the eye; as well as to investigate the detoxification of BAK *in vitro*.

Using an *in vitro* test battery with immortalized human corneal epithelial cells (HCEC) and fluorescent dyes, the toxicity of differently preserved ophthalmic solutions was assessed in the first chapter of the thesis. The BAK-preserved solutions demonstrated the greatest adverse effect on HCEC, followed by the solutions with other preservatives. The preservative-free ophthalmic solution had the least adverse effect. The *in vitro* test battery demonstrated high sensitivity and good correlation with *in vivo* and clinical studies.

PrestoBlue is a new resazurin-based reagent for assessing cell metabolic activity and cytotoxicity. The second chapter compared PrestoBlue, alamarBlue, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide (MTT) in assessing cell viability of HCEC. The result showed that PrestoBlue was more sensitive than MTT, but similar to alamarBlue. The plate color, reading mode and plate storage up to 7 days did not affect the performance of the PrestoBlue assay.

Using the *in vitro* test battery with the new reagent PrestoBlue, the third chapter assessed the individual and combined toxicity of UV radiation (UV A and UVB) and BAK on HCEC. The combined effects of UV plus BAK were either greater than (synergistic) or equal to

(additive) the sum of individual effects. The synergistic effects occurred between a low dose UV radiation (0.1719 J/cm ^{2}) and low concentrations (0.001%, 0.002%, 0.003% and 0.004%) of BAK.

The last chapter investigated the detoxification of BAK by UVC radiation. The result showed that BAK toxicity on HCEC can be neutralized by an appropriate dose of UVC radiation. After complete neutralization, the toxicity of BAK was similar to phosphate buffered saline. While, the antimicrobial effect of BAK against *Pseudomonas aeruginosa* was reduced at the same time.

The results of this work suggest that BAK-free, especially preservative-free ophthalmic solutions are safer alternatives to BAK-preserved ones. The combined toxic effects of BAK and solar radiation should be taken into consideration in the risk assessment of BAK-preserved ophthalmic solutions. UVC radiation can be used to neutralize BAK toxicity. This detoxification may be of great value in utilizing the antimicrobial efficacy of BAK while minimizing its potential hazards. The *in vitro* test battery with cultured HCEC used for this research may provide a sensitive and meaningful approach for evaluating both individual and combined ocular toxicity of UV radiation and chemicals.

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I would like to thank my supervisor, Dr. Jacob Sivak, for his great guidance, valuable advice and support. Numerous times, I was impressed by how fast Dr. Sivak reviewed my work, even after hours and during holidays, he often replied to me within couple hours with great suggestions. During the last 4 years, he has truly been my ideal supervisor, and I cannot thank him enough for that.

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Dedication

This work is dedicated to my family:

My mother and my father: Thank you for allowing me to pursue my PhD degree abroad. Thank you for your unconditional love, understanding and support. I could not have achieved this without you.

My husband, Yaning Hu: Thank you for your unhesitating confidence in me, and for your complete support for my career goals. You come into my life and light up my dreams; you accompany me through the hard times; you are always here whenever I need you. What else I can ask for?

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List of Abbreviations

annexin V	annexin V-Alexa Fluor 647 conjugate
BAK	benzalkonium chloride
CFU	colony forming units
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid disodium salt
EthD-1	ethidium homodimer-1
HCEC	human corneal epithelial cells
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide
OD	optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PAPB	polyaminopropyl biguanide
PBS	phosphate buffered saline
PS	phosphatidylserine
RCF	relative centrifugal force
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
TSA	Tryptic Soy Agar
UV	ultraviolet
ZO-1	zonula occludens-1

General Introduction

The main goal of toxicological science is to protect humans from possible damage caused by a wide variety of substances. Human eyes are exposed to different insults throughout the day, such as solar ultraviolet (UV) radiation, air pollutants, personal hygiene products, skin care products, makeups, microorganisms, and ocular medications. Toxicity testing should be able to determine both the individual and combined effects of these insults to the eye.

It is well known that the *in vivo* Draize test is the standard method of ocular toxicity testing accepted worldwide.[1] However, in addition to ethical concerns, this test has been criticized increasingly on the basis of poor repeatability, poor sensitivity, and lack of objectivity. In order to reduce the use of animals in such experiments and to improve the ocular toxicity testing, there is a need to develop *in vitro* tests to replace the Draize test. Immortalized human corneal epithelial cell lines have been shown to be useful in the development of *in vitro* models for assessing ocular toxicity.[2-6] Human corneal epithelial cells (HCEC) with the measurements of cell viability, membrane integrity, and cell growth are some of the assays that have been proposed as an alternative to the Draize test.[7,8] However, at present, no standard *in vitro* ocular toxicity method has been established.

Eye drops are commonly used for the treatment of dry eye, ocular allergies, glaucoma and ocular infections. The inclusion of chemical preservative in these solutions is necessary to prevent contamination by microorganisms. However, ocular preservatives have been showed to cause damage to the eye.[9-11] BAK, the most commonly used preservative, is a

well-known example. Numerous clinical studies have shown that BAK-preserved ophthalmic solutions can cause adverse effects on the eye.[12-14] Extensive investigations involving clinical studies with patients,[12-14] *in vivo* experiments with different animals,[15-17] and *in vitro* tests using various cell culture systems[18-21] have confirmed BAK's toxicity to corneas and cultured cells at concentrations used clinically. Due to the toxic effect of BAK, new preservatives with less toxicity and similar antimicrobial capability are being developed and introduced into the market. Preservative-free ophthalmic solutions are also available. To assess the potential adverse effects of ophthalmic solutions on the eye, an *in vitro* test battery using HCEC and fluorescent dyes was developed. The validity of this test battery can be evaluated by its ability to predict toxicity as demonstrated from *in vivo* toxicology studies and clinical investigations.

The assessment of cell viability plays an important role in toxicity testing. A wide range of assays based on various cell functions are available for cell viability detection. It is essential to choose the appropriate assay for the cell model and the type of toxic agents under evaluation. For example, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide (MTT) and alamarBlue are two viability reagents that have been widely used in cell proliferation and cytotoxicity assessments. MTT mainly measures the mitochondrial dehydrogenate activity;[22] while almarBlue is based on resazurin that functions as a cell viability indicator.[23,24] Recently, a new reagent, PrestoBlue, has been brought into the market. It has the advantage of being water soluble, and non-toxic to cells;[24,25] it is also claimed to be a fast and highly sensitive assay. At present, there is no report on the use of

PrestoBlue in cultures of human epithelial cells. To improve the sensitivity and efficiency of the *in vitro* test battery used in this work, this thesis compared PrestoBlue, alamarBlue, and MTT in assessing the cell viability of our cell model (HCEC), and investigated the effect of working conditions (plate color, reading mode, and plate storage) on the performance of the PrestoBlue assay.

Human eyes are exposed to sunlight on a daily basis. Solar radiation includes UV radiation (100-400 nm), visible light (400-700 nm) and infrared radiation (700-10,000 nm).[26] UV radiation is subdivided into UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm).[27] The solar radiation at sea level consists of UVA and a small amount of UVB.[28] However, due to ozone depletion, the amount of hazardous UVB radiation reaching the Earth's surface has increased in recent years, posing a higher risk for UV damage to the eye.[29] UV radiation can damage DNA directly,[30,31] decrease mitochondrial function,[32] and induce apoptosis;[33] it can also cause lipid peroxydation of cellular membranes through generation of free radicals.[34] Increasing evidence has shown that UV exposure to the eye is associated with photokeratitis, pterygia, cataracts, and macular degeneration.[35]

Many ophthalmic solutions containing preservatives are applied directly into eyes that are then exposed to sunlight; thus, a co-exposure of the cornea to ocular preservatives and solar radiation can occur. Such co-exposures may generate a toxic effect to the eye that is much greater than the sum of their individual effects. However, most risk assessments are based on the toxicity of individual compound or product formulations without a full consideration of the possible risks due to additional exposures to other toxic agents. Little attention has been

paid to the combined effects of ocular preservatives (such as BAK) and solar UV radiation on the eye.

Mitigating the effect of toxic agents is of great importance in protecting humans from possible hazards. BAK is not only used as a preservative in ophthalmic solutions, but also widely used in various disinfectant formulations in the health care and food processing industries. As an ocular preservative, BAK causes adverse effects on the eye;[12-14] similarly, as a disinfectant, BAK residue is toxic to humans and the environment,[36-39] and can also contribute to bacterial disinfectant resistance.[40] Some chemical compounds, such as high molecular weight hyaluronan,[41] sodium hyaluronate,[42] polyoxyethylene hydrogenated castor oil 40 (HCO-40) and polysorbate 80 (PS-80),[43] have been proposed as BAK toxicity reducers in ophthalmic solutions. Nevertheless, little research has been done on eliminating the toxic effect of BAK and its residues in other applications. To the best of my knowledge, there is no report that UVC radiation can reduce the toxicity of BAK and its residues.

The hypotheses of this dissertation include: 1) a sensitive *in vitro* method using HCEC can be developed for ocular toxicity testing; 2) this method developed can then be used to evaluate both the individual and combined effects of ophthalmic solutions, BAK and UV radiation on the eye; 3) the toxicity of BAK can be reduced by UVC radiation.

To test these hypotheses, an *in vitro* test battery using HCEC with different bioassays was developed and used in evaluating the effects of ophthalmic solutions on the eye. The performances of three viability reagents on HCEC were compared, and the *in vitro* test

battery was confirmed by including a reagent that showed high sensitivity and efficiency. Using this *in vitro* method, the individual and combined effects of BAK and UV radiation on the eye were assessed; the detoxification of BAK using UVC radiation was also investigated.

A detailed review of the literature dealing with the combined ocular toxicity of chemical mixtures and chemicals plus UV radiation was carried out as part of a graduate course (BIOL 681, with Prof. D.G. Dixon), and is included as appendix A.

Chapter 1

Comparison of the Effects of Ophthalmic Solutions on Human Corneal Epithelial Cells Using Fluorescent Dyes

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Authors' contributions

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Xu	✓	✓	✓	✓	✓
Sivak	✓				✓
McCanna	✓				✓

1.1 Overview

Purpose: To investigate the effect of differently preserved ophthalmic solutions on the viability and barrier function of human corneal epithelial cells (HCEC) using fluorescent dyes.

Methods: HCEC monolayers were exposed to the ophthalmic solutions containing benzalkonium chloride (BAK), edetate disodium (EDTA), polyquad, stabilized oxychloro complex (Purite), sodium perborate or sorbic acid for 5 min, 15 min, and 1 h. At 24 hours after exposure, the cultures were assessed for metabolic activity using alamarBlue. The enzyme activity, membrane integrity and apoptosis were evaluated using confocal microscopy. Barrier function was assessed using sodium fluorescein.

Results: The metabolic assay showed that the BAK-preserved ophthalmic solutions significantly reduced cell viability after 5 min exposure compared to the phosphate buffered saline treated control ($p < 0.05$). Using confocal microscopy, the micrographs showed that BAK-preserved solutions caused a reduction in enzyme activity, increased membrane permeability and decreased the number of viable cells. Ophthalmic solutions with new preservatives had varying time-dependent adverse effects on cell viability, and the preservative-free solution had the least effect on HCEC. Sodium fluorescein permeability showed that HCEC monolayers treated with BAK-preserved solutions were more permeable to sodium fluorescein than those treated by the other ophthalmic solutions ($p < 0.05$).

Conclusions: BAK-preserved solutions had greater adverse effect on metabolic activity, enzyme activity, membrane integrity, cell viability and barrier function than the solutions that were not preserved with BAK. Our study suggests that BAK-free, especially preservative-free ophthalmic solutions are safer alternatives to BAK-preserved ones.

1.2 Introduction

The inclusion of preservatives in multiple-dose ophthalmic solutions is needed to prevent contamination by microorganisms. However, preservatives in ocular medications can cause damage to the corneal epithelium, and the long-term use of some preserved ophthalmic products has been associated with the occurrence of adverse changes to the ocular surface.[1-4] Benzalkonium chloride (BAK), the most commonly used preservative in ophthalmic solutions has been shown to cause ocular toxicity since 1944.[5] In animal studies, BAK has been shown to cause damage to corneal epithelial cells. BAK caused swelling and desquamation of the rabbit corneal epithelial cells at a concentration of 0.005%.[6] BAK also caused a progressive increase in damage to rabbit and cat corneas at concentrations between 0.001% and 0.01%.[7] In clinical studies, BAK-preserved ophthalmic solutions were also shown to cause tear film instability (0.005% BAK),[8] disruption of corneal barrier function (0.005% BAK),[9] decrease of corneal sensitivity and reduction of the density of superficial epithelial cells (0.01% BAK).[10] Increasing evidence revealed that BAK is highly toxic, in a time- and dose-dependent manner, with a threshold of toxicity as low as 0.005%.[4,11-14]

Due to the toxicity of BAK, considerable effort has been made to discover and develop new preservatives which have less toxicity but have similar capabilities of inhibiting microorganism growth in multiple-dose containers. Some new preservatives are commercially available, such as stabilized oxychloro complex (Purite), polyquad, sodium perborate and sorbic acid. Single-dose preservative-free ophthalmic solutions are also now available.

Several *in vivo* studies have compared the newer preservatives (Purite, polyquad, and sodium perborate) with BAK. Studies that investigated the effects of glaucoma medications on the cornea demonstrated that Purite-preserved eye drops were significantly more comfortable and showed less damage to the cornea than the BAK-preserved products.[4,15,16] Bernal and Ubels found that BAK containing eye drops caused rabbit corneas to become significantly more permeable to carboxyfluorescein than corneas treated with polyquad-preserved eye drops.[17] Labbe et al. also showed that polyquad 0.5% did not cause significant changes in the ocular surface of rats compared to saline.[18] Garcia-Valenzuela et al. studied the use of corneal lubricants during the course of vitrectomy surgery and determined that the sodium perborate maintained corneal clarity and epithelial integrity better than the lubricant that contained BAK.[19]

In order to assess the potential damaging effects of the ophthalmic solutions on the cornea, an *in vitro* test battery using fluorescent dyes can be used. The validity of the test battery can be shown by its ability to predict toxicity as demonstrated from *in vivo* toxicology studies and clinical investigations. Due to the extensive data available on the harmful effects of BAK on the cornea in animal studies and in humans, the concentrations of 0.01%, 0.005%, and 0.001% were used as the positive controls in our investigation. Also, newer preservatives that have been shown to be less toxic from *in vivo* studies were used within our study as comparative controls due to the fact that they should show less toxicity to human corneal epithelial cells than BAK. The aim of this study was to compare the relative toxicity of

commonly used eye drops and eye washes, and to demonstrate the utility of this *in vitro* battery for assessing the toxicity of ophthalmic products.

1.3 Methods

1.3.1 Chemicals and solutions

Nine ophthalmic solutions including six dry eye drops and three eye washes were evaluated in this study. The solutions were purchased from commercial sources and were used within their labeled expiration dates. One solution (Visine) is preservative-free; five solutions (Systane, Refresh, GenTeal, ReNu, and Rite Aid) contain new preservatives; and three solutions (Akwa, Collyrium, and Optrex) contain BAK. Table 1-1 shows all the test solutions and their preservatives. Phosphate buffered saline (PBS) (Lonza, Walkersville, MD) was used as a negative control, and BAK 0.01%, BAK 0.005%, BAK 0.001% (CAS No. 63449-41-2, Sigma-Aldrich St. Louis, MO) were used as positive controls.

Table 1-1. Test solutions and their preservatives

Name of the Solution Full name (Abbreviation)	Supplier	Preservative
Visine® for dry eye Enduring Moisture™ (Visine)	Johnson & Johnson Inc, Markham, ON	None
Systane® Ultra Lubricant Eye Drops (Systane)	Alcon, Fort Worth, TX	Polyquad 0.001%
Refresh Tears® Lubricant Eye drops (Refresh)	Allergan, Irvine, CA	Stabilized oxychloro complex (Purite)
GenTeal® Artificial Tears(Genteal)	Novartis Mississauga, ON	Sodium Perborate (GenAqua)
ReNu Multiplus® Lubricating Rewetting Drops (ReNu)	Bausch & Lomb, Rochester, NY	Edetate disodium 0.1% and sorbic acid 0.1%
Rite Aid Eye Wash (Rite Aid)	Rite Aid corporation, Camp Hill, PA	Edetate disodium 0.025% and sorbic acid 0.1%
Akwa Tears® Lubricant Eye Drops (Akwa)	Akorn. Inc., Lake Forest, IL	BAK 0.005% and edetate disodium
Soothing Collyrium for FRESH EYES Eye Wash (Collyrium)	Bausch & Lomb, Rochester, NY	BAK 0.01%
Optrex® Eye Wash (Optrex)	Reckitt Benckiser, Mississauga, ON	BAK

BAK: benzalkonium chloride

1.3.2 Cell culture

Human corneal epithelial cells (HCEC) were obtained from RIKEN BioResource Center, Tsukuba, Japan (#RCB 2280). It is a SV40-transformed human corneal epithelial cell line. The present study was performed with this cell line because it has good growth characteristics, shows a cobble-stone like appearance, develops good tight junctions, and it is free of infectious virus particles.[20-22] The monolayers of the HCEC were prepared, with cultures that were less than 30 passages.

The HCEC were cultured in DMEM/F-12 Ham Medium (Gibco Invitrogen, Grand Island, NY), with 10% fetal bovine serum (Gibco Invitrogen), and penicillin/streptomycin (Gibco

Invitrogen) in a humidified environment at 37 ℃ with 5% CO_2. These cultures were maintained with weekly subculture using the Tryple Express (stable trypsin replacement; Gibco Invitrogen) and fed every 2 to 3 days.

1.3.3 Assessment of metabolic activity

A cell suspension (1 mL) containing 10^5 cells was seeded in 24-well plates (BD Falcon, Franklin Lakes, NJ) and grown to approximately 75% to 80% confluent at 37 ℃ with 5% CO_2. The cells were then exposed to test solution for 5 min, 15 min and 1 h. After exposure, the test solutions were removed and the cultures were rinsed with 1mL PBS. The cultures were then incubated for another 24 h in new culture medium at 37 ℃ with 5% CO_2. Following incubation, the medium was removed, and the well was rinsed with 1 mL PBS again. One mL of 10% alamarBlue (Invitrogen, Carlsbad, CA) prepared in medium without serum and phenol red was then added to each well and the cultures were incubated at 37 ℃ for 4 h. After incubation, the change in the fluorescence of the alamarBlue reagent (resazurin to resorufin) was measured using a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) with the excitation/emission wavelengths set at 530/590 nm. The few cells that detached from the plate surface after exposure to the test solution and PBS rinses were collected and assessed for viability using trypan blue. All experiments were done in triplicate.

1.3.4 Confocal microscopy study with fluorescent viability dyes

Confocal microscopy (Carl Zeiss LSM) with fluorescent viability dyes (calcein AM, ethidium homodimer-1 (EthD-1), and annexin V-Alexa Fluor 647 conjugate (annexin V); Invitrogen) were used to visualize live, dead, and apoptotic cells after exposure. Calcein changes to a fluorescent green molecule after reaction with intracellular enzymes in live cells; EthD-1 penetrates the compromised membranes and stains the nuclei of dead or dying cells; annexin V binds to phosphatidylserine (PS) on the outer surface of the cell membrane in apoptotic cells, and can stain PS in the interior of the cell membrane in dead cells.

HCEC, 4×10^5 cells in 1 mL of culture medium, were transferred into collagen coated glass bottom culture Petri dishes (MatTek Corp., Ashland, MA), and grown to confluence at 37 ℃ with 5% CO_2 for 2 days. The cultures were then exposed to test solution for 5 min, 15 min, and 1 h. After exposure, the test solutions were removed, and the cultures were rinsed with 1mL PBS. New medium was then added into the dishes and the cultures were then incubated for another 24 h at 37 ℃ with 5% CO_2. After incubation, the medium was removed from each Petri dish, and the dish was rinsed with 1 mL PBS again. The cells were then stained with annexin V (10 μl in 500 μl buffer), calcein AM (2 μM), and EthD-1 (4 μM) for 20 min at 37 ℃. After staining, the fluorescence of the three dyes was then visualized with an Axiovert 100 microscope with a Zeiss confocal laser scanning microscope (CLSM) 510 system. The excitation/emission wavelengths for calcein AM, EthD-1, and annexin V, were 495/515 nm, 528/617nm, and 650/665 nm, respectively. The natural color of annexin V fluorescence was red. However, in order not to confuse with the color of EthD-1 (which was

also red), we set the confocal software to present annexin V as yellow. We distinguished the cells according to the colors and locations of the three dyes. Live cells stain with calcein (green), and exclude annexin V (yellow) and EthD-1(red); dead cells stain with EthD-1 (red) in the nuclei and can stain with annexin V (yellow) in the interior of the cell membrane; apoptotic cells stain with annexin V (yellow) on the outer surface of the membrane and exclude EthD-1 (red).The percentage of live, dead and apoptotic cells in the confocal micrographs was calculated and compared. All experiments were done in triplicate.

1.3.5 Assessment of barrier function

A cell suspension (0.5 mL) containing 10^5cells was seeded in Millicell HA 13-mm inserts (Millipore, Bedford, MA). The inserts were then transferred into 24-well plates containing 0.5 mL of growth medium per well and incubated at 37 ℃ with 5% CO_2 for 7 days. Medium was changed every day during that period. On day 7, each insert was gently rinsed three times with 1 mL of PBS using a 5-mL syringe without a needle and placed in a fresh 24-well plate. Then the cells were exposed to test solutions for 5 min, 15 min and 1 h. After exposure, the test solutions were removed and the inserts were rinsed with 3 mL PBS and placed in a fresh 24-well plate containing 0.5 mL of growth medium per well and fresh medium (0.5mL) was also added to the each cell insert. The cultures were incubated for another 24 h at 37 ℃ with 5% CO_2. After incubation, each insert was individually rinsed three times with 1mL of PBS and placed in a fresh 24-well plates containing 0.5mL of PBS in each well. Sodium fluorescein (Sigma-Aldrich, St. Louis, MO; 0.5 mL; 3 mg/100 mL in PBS) was added to each insert. After a 20 minute incubation period the inserts were removed

from the wells and the amount of sodium fluorescein that penetrated through the cell monolayer was measured with a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) at 485 nm excitation and 530 nm emission. All experiments were done in triplicate. Each series of triplicate samples was handled sequentially to allow the exact timing of the treatment and subsequent steps.

1.3.6 Statistical analysis

One-way ANOVA was used in the data analysis of this study. Pairwise multiple comparison procedures were performed using the Bonferroni post hoc test for the results of confocal microscopy study and the assessment of barrier function. The Games-Howell post hoc test was used for the results of metabolic activity tests because of the unequal variance of the data. The criterion of statistical significance was set to be $p < 0.05$.

1.4 Results

1.4.1 Measurement time points

The toxicity of the test solutions was measured both immediately after exposure and at 24 hours after exposure. However, the data at 24 hours after exposure was chosen because the toxicity increased with increasing concentrations of BAK and exposure time, which correlated well with *in vivo* data. Some other *in vitro* studies also used 24 hours post-exposure time for ocular toxicity and irritation tests, and showed good correlations to *in vivo* studies.[23-25] Therefore, this paper only shows the data at 24 hours after exposure.

1.4.2 Assessment of metabolic activity

The effect of the test solutions on the cell viability of HCEC was measured using alamarBlue. The metabolic activity of the cells at 24 hours after exposure is shown in Figure 1-1. The BAK-preserved ophthalmic solutions reduced cell viability to no more than 3% (compared to PBS) after 5 min and 15 min exposFures, which was significantly lower than all the other tested solutions without BAK ($p < 0.05$), and similar to BAK 0.01% and BAK 0.005%. The BAK-free solutions had varying time-dependent adverse effects on cell viability after 5 min, 15 min and 1 h exposures. The preservative-free eye drop reduced cell viability significantly less than all the other products after 15 min, and 1 h exposures ($p < 0.05$). No significant differences were observed in the cell viability between Visine and the PBS control ($p = 0.77$) after 5 min exposure. Trypan blue exclusion test with manual cell count showed that less than 10 % of the cells being washed away were live cells (Data not shown).

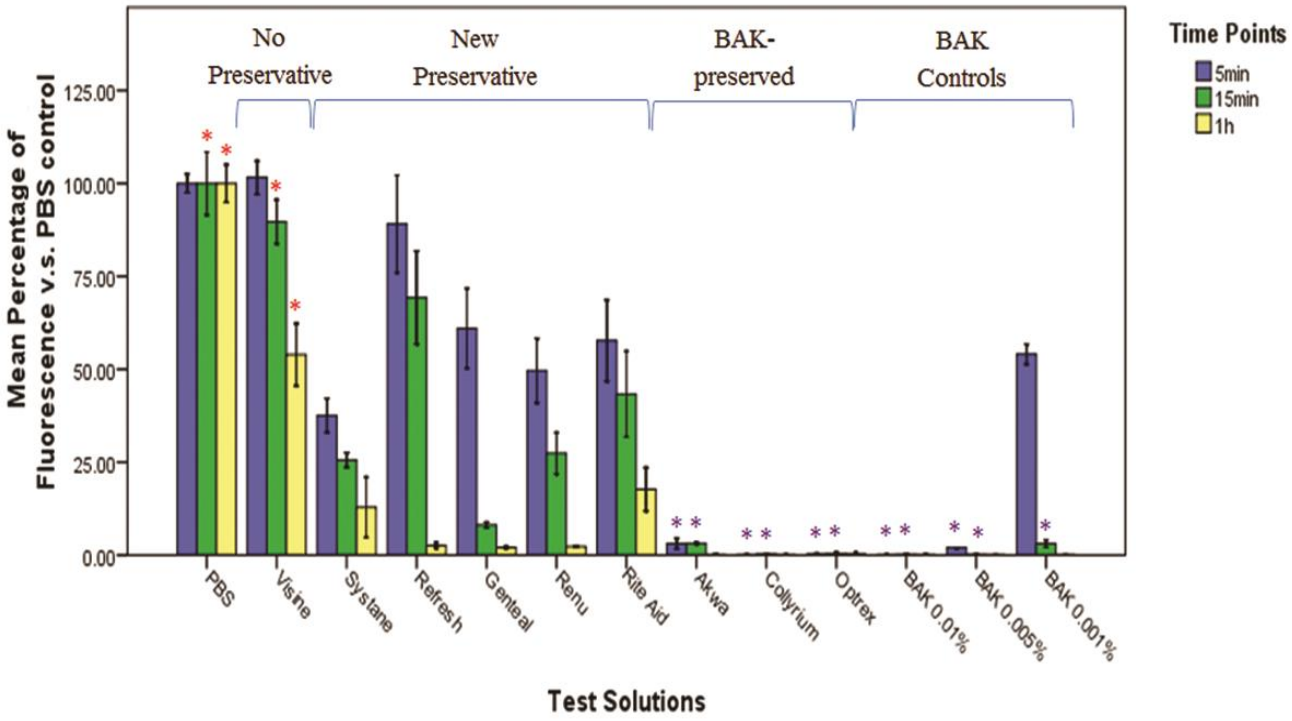

Figure 1-1. Viability of HCEC using alamarBlue.

Cell viability at 24 hours after exposure to the test solutions was measured using alamarBlue. Significantly different from all the preserved ophthalmic solutions ($p < 0.05$) is indicated by a red asterisk (*). Significantly different from all BAK-free ophthalmic solutions after 5 min and 15 min exposure ($p < 0.05$) is indicated by a purple asterisk (*). Error bars: ± SD.

1.4.3 Confocal microscopy study with fluorescent viability dyes

The confocal microscopy with fluorescent viability dyes showed the cell viability of the cultures at 24 hours after exposure to the test solutions. Figure 1-2, Figure 1-3, and Figure 1-4 are representative confocal laser scanning micrographs. Cell size may vary depending on whether the cells adhered to the culture dish or not. The counts of live, dead and apoptotic cells in confocal laser scanning micrographs are shown in Figure 1-5. Mean counts were made using 3 separate cultures that are treated with each solution. In all the time points, BAK-preserved solutions showed significantly more cell death compared to the BAK-free solutions and the PBS control (Figure 1-2, 1-3, 1-4, and 1-5, $p < 0.05$). After 5 min exposure, Akwa had the least cell toxicity among the solutions with BAK (Figure 1-2 and 1-5, $p < 0.05$). After 1 h exposure, the preservative-free solution caused the least amount of cell death among all the test solutions ($p < 0.05$); Refresh reduced the cell viability the most among all the solutions with new preservatives ($p < 0.05$); and the BAK-preserved solutions caused 100% cell death (Figure 1-4 and 1-5).

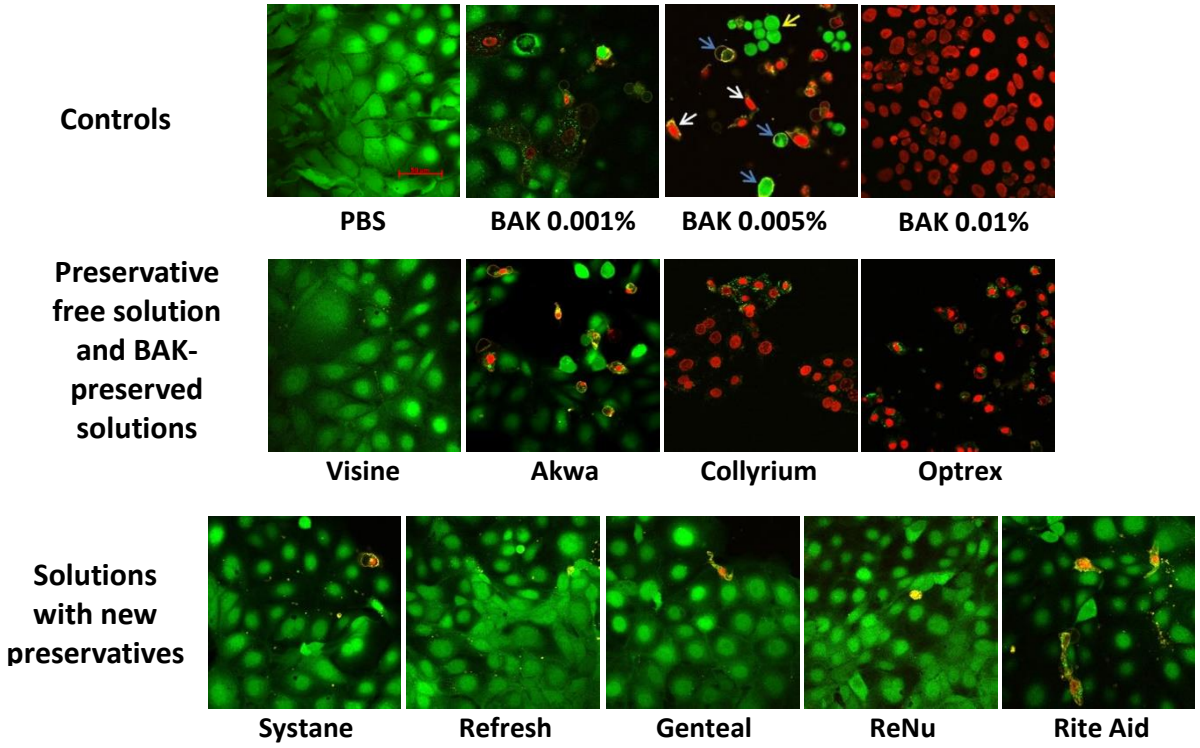

Figure 1-2. Representative confocal laser scanning micrographs of HCEC at 24 hours after 5 min exposure.

The effect of the test solutions is shown on the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein (green) - positive, annexin V (yellow) - negative, and EthD-1(red) – negative; dead cells are EthD-1 (red) - positive; apoptotic cells are annexin V (yellow) - positive and EthD-1 (red) – negative. In the first row are the controls; in the second row are the Preservative-free solution (Visine) and the BAK-preserved solutions; and in the third row are the solutions which contain new preservatives. The yellow arrow indicates live cells; the blue arrows show cells in apoptosis; the purple arrows indicate dead cells. Bar = 50 μm. All the micrographs use the same magnification.

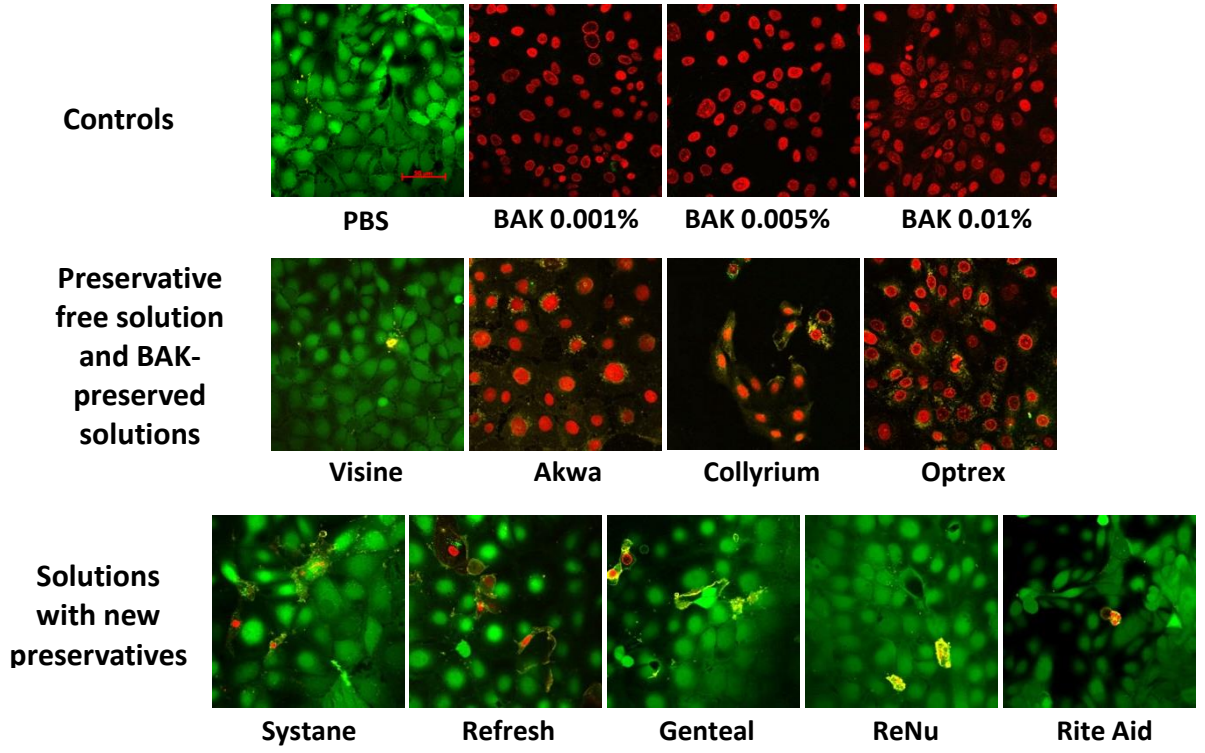

Figure 1-3. Representative confocal laser scanning micrographs of HCEC at 24 hours after 15 min exposure.

The effect of the test solutions is shown on the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein (green) - positive, annexin V (yellow) - negative, and EthD-1(red) – negative; dead cells are EthD-1 (red) - positive; apoptotic cells are annexin V (yellow) - positive and EthD-1 (red) – negative. In the first row are the controls; in the second row are the Preservative-free solution (Visine) and the BAK-preserved solutions; and in the third row are the solutions which contain new preservatives. Bar = 50 μm. All the micrographs use the same magnification.

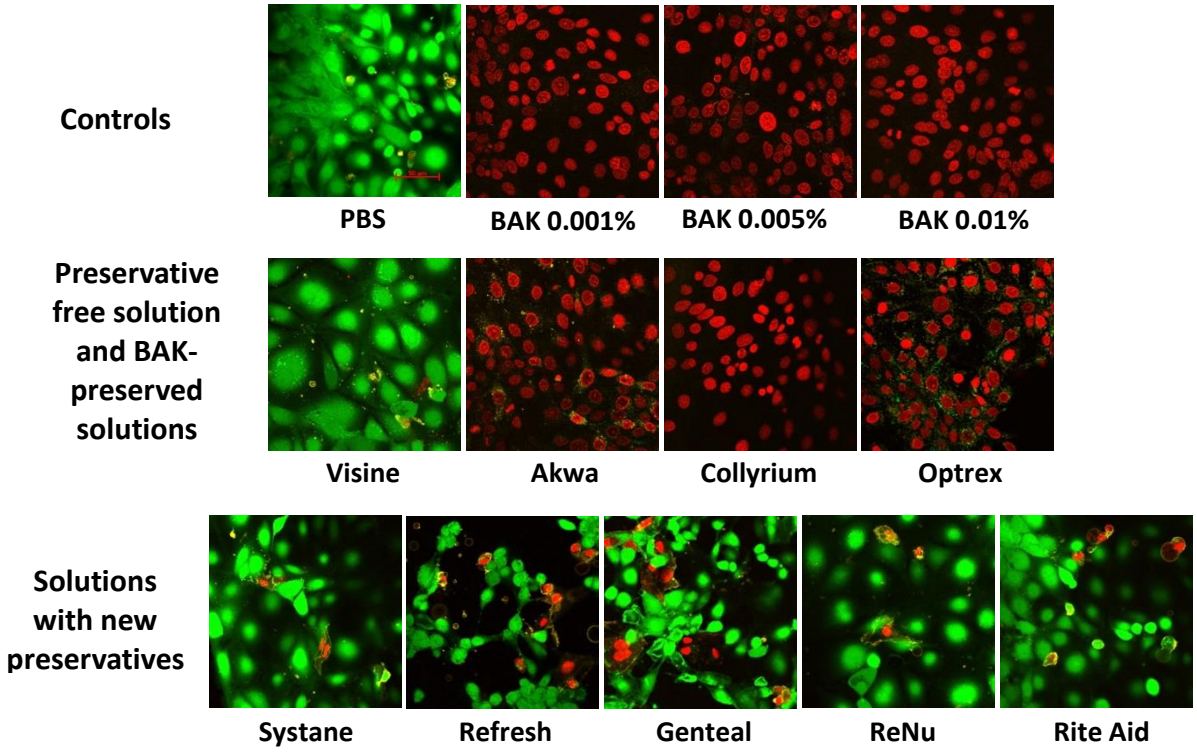

Figure 1-4. Representative confocal laser scanning micrographs of HCEC at 24 hours after 1 h exposure.

The effect of the test solutions is shown on the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein (green) - positive, annexin V (yellow) - negative, and EthD-1(red) – negative; dead cells are EthD-1 (red) - positive; apoptotic cells are annexin V (yellow) - positive and EthD-1 (red) – negative. In the first row are the controls; in the second row are the Preservative-free solution (Visine) and the BAK-preserved solutions; and in the third row are the solutions which contain new preservatives. Bar = 50 μm. All the micrographs use the same magnification.

Figure 5

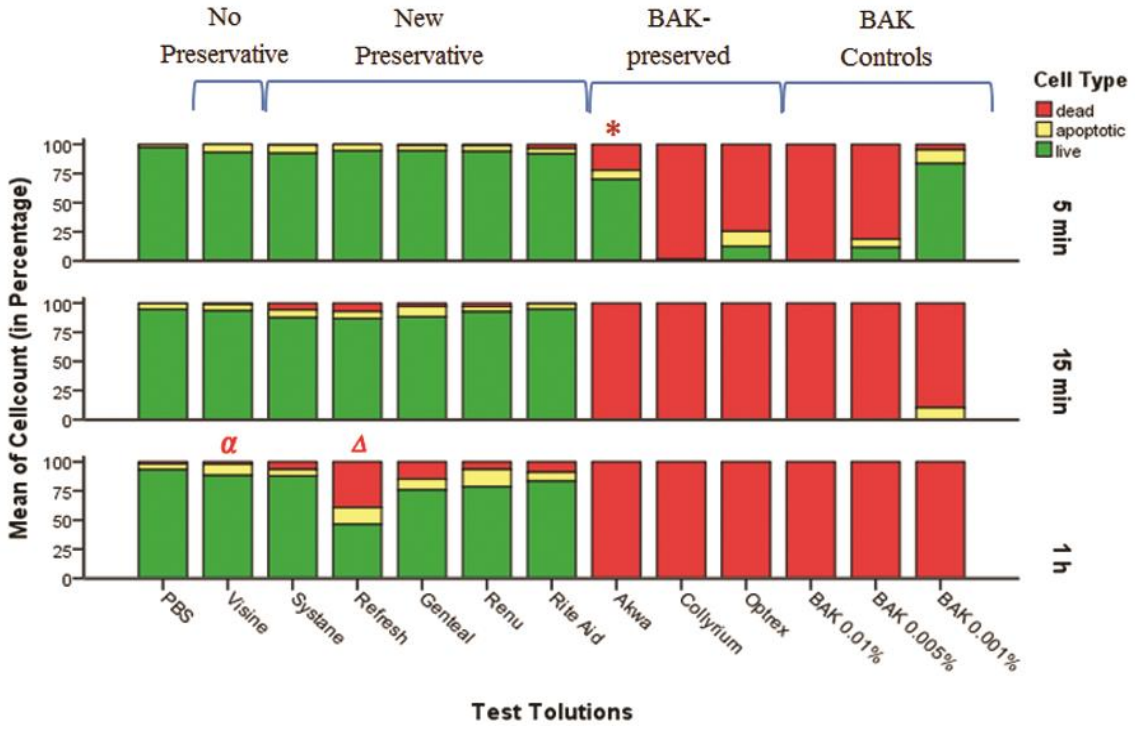

Figure 1-5. The counts of cells in the confocal laser scanning micrographs.

The results are expressed as a percentage of the cells. The percentages of live cells in the BAK-preserved solutions were significantly lower than those in the BAK-free solutions after 5 min, 15 min and 1 h exposures ($p < 0.05$). *: significantly different from the other BAK-preserved solutions ($p < 0.05$); α: significantly different from the BAK-preserved solutions and the solutions with new preservatives ($p < 0.05$); Δ: significantly different from all the other solutions with new preservatives ($p<0.05$).

1.4.4 Assessment of barrier function

The effect of the test solutions on corneal tight junctional integrity was assessed using sodium fluorescein. Figure 1-6 shows the results for all the test solutions at 24 hours after exposure. There was no significant difference in fluorescein permeability between PBS control and BAK-free ophthalmic solutions after 5 min, 15 min and 1 h exposure. However, BAK-preserved solutions acted differently. Collyrium Eye Wash (contains BAK 0.01%) showed significantly greater fluorescein permeability than all the other products and the PBS control after 15 min exposure ($p < 0.013$). All BAK-preserved products showed remarkably greater fluorescein permeability than the PBS control and all the BAK-free products after 1 h exposure ($p < 0.001$) (Figure 1-6).

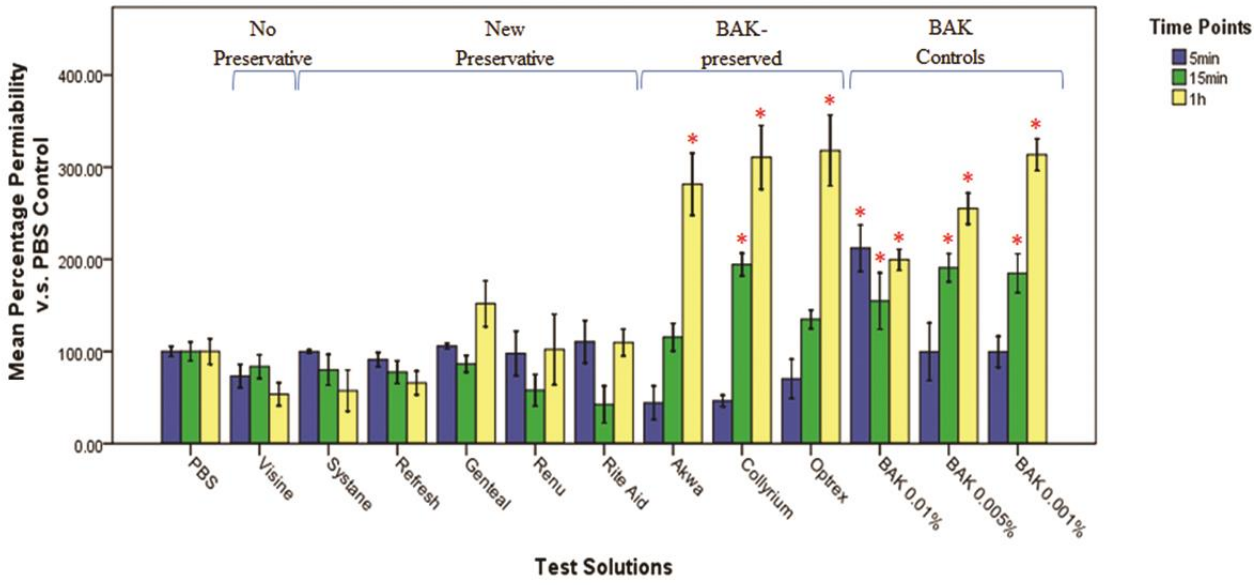

Figure 1-6. Barrier function of HCEC at 24 hours after 5 min, 15 min, and 1 h exposures using the sodium fluorescein permeability measurement.

The results are expressed as a percentage of the permeability of the PBS control. The greater percentage of permeability compared to the PBS control represents the more sever loss of integrity of the culture. Significantly greater than PBS control ($p < 0.05$) is indicated by an asterisk (*). Error bars: ± SD.

1.5 Discussion

The results of this study demonstrate that BAK-preserved solutions had significantly greater adverse effect on cell viability and tight junctions than the eye drops and eye washes that do not contain BAK. The *in vitro* test battery correlated well with *in vivo* studies and clinical studies which showed that BAK-preserved eye drops caused ocular toxicity at 0.01% and 0.005% concentrations,[4,6,8-11,13,14,18,26,27] and with *in vivo* comparison studies showing that the products without preservatives or with newer preservative caused less damage to the ocular surface than BAK-preserved products.[4,18]

BAK has been the most commonly used preservative for topical ophthalmic medications used by clinicians. Its use is aimed at preventing contamination of multiple-dose containers. However, BAK does have adverse effects, which may be more apparent under conditions of long term use. It has been consistently shown to be toxic to ocular tissue in clinical and experimental studies.[1,4,8-14,28-35] To develop less toxic alternatives to BAK, new preservatives such as polyquad, stabilized oxychloro complex (Purite), sodium perborate and sorbic acid have been proposed and are commercially available. A few studies have evaluated the cytotoxicity of these new preservatives.[4,15,16,18,36] Our results show that the preservatives polyquad, Purite, sodium perborate and sorbic acid did not cause obvious cell toxicity after short exposure (5 min, and 15 min), and were significantly less toxic than the solutions with BAK, which is consistent with the previous *in vivo* and clinical studies.[4,15,18] Nevertheless, we also found that the solutions with new preservative had a time-dependent toxicity to cultured HCEC. They caused mild to moderate degrees of toxicity after 1 h exposures. This was not

shown in the previous *in vivo* toxicity or clinical studies. In addition, compared to the products with BAK and new preservative, the preservative-free solution consistently showed least toxicity in our study and in all the previous studies.[8,9,28,35,37,38]

AlamarBlue is designed to quantitatively measure the proliferation of various human and animal cell lines. It incorporates a fluorescent growth indicator based on the detection of metabolic activity. The innate metabolic activity of the viable cells results in a chemical reduction of alamarBlue (also called resazurin), which causes it to change from oxidized form (non-fluorescent, blue) to reduced form (fluorescent, red).[39] AlamarBlue is non-toxic to cells. A decrease in the alamarBlue fluorescence readings indicates the decrease of metabolic activity (the viability) of the cultured cells. In the present study, cells exposed to BAK-preserved ophthalmic solutions showed a significant reduction in metabolic activity compared to the PBS treated control, and the BAK-free solutions (Figure 1-1). Preservative-free eye drops caused the least reduction of metabolic activity in the tested ophthalmic solutions. This suggests that among the ophthalmic solutions used in this study, the BAK-preserved solutions are the most, and preservative-free solution is the least toxic to cultured HCEC. In addition, according to our results, the assessment of metabolic activity of HCEC with alamarBlue is very sensitive to the toxicity of preservative. It showed remarkable cell toxicity of the BAK-preserved ophthalmic solutions after only 5 min exposure, and showed differences between the solutions with and without BAK, as well as with and without preservative after only 15 min exposure.

Cell viability after exposure was also analyzed in this study using confocal microscopy with fluorescent viability dyes. The dyes are calcein AM, EthD-1, and annexin V for detecting live, dead and apoptotic cells, respectively. Calcein AM can penetrate live cells. Because of the intracellular esterase activity of the live cells, the nonfluorescent calcein AM is then converted to the intensely fluorescent calcein, which stains the intracellular cytoplasm and produce an intense uniform green fluorescence in live cells.[40] EthD-1 is excluded by the intact plasma membrane of live cells. However, nonfluorescent EthD-1 enters cells with damaged membranes, and binds to the nucleic acids, which enhances the fluorescence and produces a bright red fluorescence in dead cells.[40] Annexin V is a phospholipid-binding protein that has a high affinity for phosphatidylserine (PS). In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. However in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V can bind to PS exposed on the outer leaflet and then the fluorescence can be detected.[41,42] Annexin V can also penetrate the compromised membranes of dead cells and stain PS in the interior of the cell. The three fluorescent dyes calcein AM, EthD-1, and annexin V can be used together due to the non-overlapping emission spectra and different binding sites of these molecules. Thus, we can show the distributions of live, dead and apoptotic cells of the culture in the same micrograph of the culture. In the present study, the results were similar to alamarBlue study. After 5 min exposure to the BAK-preserved ophthalmic solutions, the cultures showed an obvious increase of apoptotic cells and dead cells as well as cell loss. On the contrary, the cultures of the BAK-free solutions looked similar to the PBS control after 5 min exposure

(Figure 1-2 and 1-5). As the exposure time increased from 5 min to 1 h, the amount of apoptotic cells and dead cells increased in all the cultures except the PBS controls: the most in the solutions with BAK (100% cell death), and the least in the preservative-free solution (Figures 1-3, 1-4 and 1-5). This confirmed the result of the alamarBlue evaluation: BAK-preserved solutions are the most toxic to the cultured HCEC, followed by the solutions with new preservative, and the preservative-free solution is the least toxic. In addition, the comparison of the cell counts in the confocal micrographs indicated that, among the three BAK-preserved solutions, Akaw was the least toxic at 5 min exposure, and among the five solutions with new preservatives, Refresh was the most toxic at 1 h exposure.

Tight junctions are an important characteristic of healthy human corneal epithelium. Sodium fluorescein permeability can be used to detect the loss of tight junctions and defects of integrity in corneal epithelium. It measures sodium fluorescein leakage. The more fluorescein leakage indicates the more loss of tight junctions and the more defects of integrity. This method has been used in many studies to measure damage to corneal tissue.[43-46] Our study also used it to detect damage of the barrier function caused by ophthalmic solutions. Again, the BAK-preserved solutions were shown to cause the greatest loss of integrity of the culture, followed by the solutions with new preservative. The preservative-free solution caused the least damage to the barrier function. This assay was not as sensitive as the alamarBlue assay and confocal microscopy study with fluorescent viability dyes. We didn't find a significant difference in fluorescein permeability between PBS control and BAK-free ophthalmic solutions after 5 min, 15 min and 1 h exposures (Figure 1-6). This

may be due to the age of the culture (1 - 2 days for the alamarBlue and confocal microscope study vs. 7 days in the fluorescein permeability assessment). Also, because the cells in the fluorescein permeability assessment were grown for 7 days, the cells may have been more tightly opposed to each other and therefore the surface area exposed to the chemical was less than the other two studies where the cells were not as dense.

In order to see the potential toxicity of all the test solutions, we use three different time points. In our results, the difference between the solutions with and without BAK was shown after 5 min exposure in the metabolic assay and confocal microscopy study with fluorescent viability dyes (Figures 1-1,1- 2 and 1-5); and after 1 h exposure in the measurement of barrier function (Figure 1-6). The difference between the preservative-free product and those with new preservatives was shown after 15 min exposure in the metabolic assay (Figure 1-1) and after 1 h exposure in confocal microscopy study with fluorescent viability dyes (Figure 1-4 and 1-5), but was not shown in the measurement of barrier function. The dis-concordance among the metabolic assay, confocal microscopy study and measurement of barrier function indicates that the toxicity which causes a reduction in the cell metabolic activity may not cause cell death; and the effect on cell viability may not cause the disruption of tight junctions at low toxicity levels. Therefore, in order to have a better understanding and obtain a better assessment of the toxicity of an agent, we need to combine several assays together, and analyze the toxic effect from different aspects.

One limitation of this study is that we evaluated the toxic effect of whole commercial ophthalmic solutions instead of their components. Typical formulations contain active

components and additives such as preservatives and buffers. We could not identify the individual effects of the components in this study. We know the toxicity is mainly caused by the preservative, but the other components may increase or decrease the effect. Further investigations of individual components are necessary to fully understand the toxicity of the ophthalmic solutions.

Dry eye is a common disease that affects a patient's quality of life and may require long-term treatment with eye drops. BAK in eye drops may aggravate dry eye disease. It has been shown that BAK can cause goblet cells loss, meibomian gland disruption and tear film instability.[8,27] Symptoms of BAK toxicity that include irritation, dry eye, foreign body sensations and blurred vision have been shown to decrease significantly by switching from a BAK-preserved formulation to a preservative-free one.[8,9,38] Due to the increased sensitivity of corneal epithelial cells in dry eye patients from the reduced flow of tears, preservatives such as BAK in eye drops may present a considerable problem for long-term use by patients with dry eyes. We recommend that clinicians consider solutions without preservative, or at least without BAK, especially for the patients who have compromised ocular surface where damage of corneal epithelium by preservatives is suspected. Because of single-dose packaging, it may be costly to use preservative-free ophthalmic solutions. However it will provide significant benefit to patients, especially those with a compromised ocular surface and a need for the long term use of topical medications.

In summary, the present study clearly revealed that the tested commercial ophthalmic solutions are less cytotoxic with new preservative than with BAK; and without preservative

than with new preservative. Our study suggests that BAK-free and preservative-free ophthalmic solutions are safer alternatives to BAK-preserved ones. The results of this study also demonstrated that cytotoxicity of the ophthalmic solutions can be evaluated using cultured HCEC with three different assays: the metabolic assay using alamarBlue, confocal microscope study with fluorescent viability dyes, and measurement of barrier function using sodium fluorescein.

The next chapter compared the performances of three reagents – PrestoBlue, alamarBlue and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide (MTT), in assessing cell viability of human corneal epithelial cells *in vitro*, and investigated the effect of working conditions (plate color, reading mode, and plate storage) on the performance of the PrestoBlue assay.

This is a methodology study witch was done to investigate whether PrestoBlue is an appropriated reagent for the cell model used in this thesis work.

Chapter 2

Use of the Viability Reagent PrestoBlue in Comparison with Alamarblue and MTT to Assess the Viability of Human Corneal Epithelial Cells

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Authors' contributions

Author	Concept/Design	Data Collection	Data Analysis	Article Writing	Article Editing
Xu	✓	✓	✓	✓	✓
McCanna	✓				✓
Sivak	✓				✓

2.1 Overview

Introduction: PrestoBlue is a new resazurin-based reagent to assess cell viability and cytotoxicity. It is claimed to be a fast and highly sensitive assay. Here, we compared PrestoBlue, alamarBlue, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide (MTT) in assessing cell viability of human corneal epithelial cells (HCEC), and investigated the effect of plate color, reading mode, and plate storage on the performance of PrestoBlue assay.

Methods: The viability of different numbers of healthy HCEC and the toxicity of various chemicals on HCEC were evaluated using PrestoBlue (fluorescence), alamarBlue (fluorescence), and MTT (absorbance). The sensitivities of the three assays were compared. In the PrestoBlue assay, three plate colors and two reading modes were used and compared in assessing the toxic effect of SDS. The PrestoBlue solutions after reaction were stored and measured on day 1, 2, 3, 5, and 7. The fluorescence readings obtained on different days were then compared.

Results: Both PrestoBlue and alamarBlue were able to detect 5000 healthy cells after 30 min incubation and 1000 cells after 1h, 2h, and 4h incubation; while MTT was able to detect 5000 cells after 3h incubation. In the assessment of the toxicity of various chemicals, PrestoBlue and alamarBlue performed similarly. There was no significant difference between the results obtained by these two reagents. All the three plate colors and two reading modes

showed similar results in the PrestoBlue assay in assessing the toxicity of SDS. Plate storage up to 7 days did not affect the result of the PrestoBlue assay.

Conclusion: Our study suggests that in evaluating the viability of HCEC, PrestoBlue is more sensitive than MTT, but similar to alamarBlue. The plate color, reading mode and plate storage up to 7 days did not affect the performance of the PrestoBlue assay.

2.2 Introduction

The measurement of cell viability plays an essential role in the toxicity testing. It is a fundamental tool for screening new drugs and chemicals, and provides initial data prior to performing *in vivo* and clinical studies. A wide range of assays are available for cell viability detection. They are based on various cell functions such as mitochondrial enzyme activity, cell membrane permeability, ATP production, and cellular uptake activity. The viability assays chosen for assessment in our study are based on mitochondrial enzyme activity. Three reagents were compared here: PrestoBlue, alamarBlue, and MTT.

The MTT assay has been widely used in cell viability and cytotoxicity tests since it was first introduced by Mosmann in 1983.[1-5] This assay relies on a reductive coloring reagent (tetrazolium salt) and the mitochondrial dehydrogenase activities to determine cell viability with a colorimetric method.[3] In viable cells, MTT is reduced mainly by NADH to an insoluble purple formazan, which forms crystals in cells. After crystal formation, an organic solvent is used to dissolve the purple crystals and absorbance is measured to assess the viability of target cells.

Both almarBlue and PrestoBlue are based on resazurin which functions as a cell viability indicator.[6,7] In viable cells, resazurin is reduced to resorufin in cellular respiration by accepting electrons from NADPH, FADH, FMNH, NADH and cytochromes.[8] This reduction causes PrestoBlue and alamarBlue to change from non-fluorescent form to strong fluorescent form.[9] The conversion is proportional to the number of metabolically active cells and can be evaluated quantitatively using fluorescence or absorbance measurements.[10,11] Both alamarBlue and PrestoBlue are water soluble, non-toxic to cells and hence are less likely to interfere with normal metabolism and allow further usage of the cells in subsequent test.[11] Although both reagents are resazurinbased, PrestoBlue is a newer product and is claimed to have several advantages over alamarBlue and other viability reagents. According to the manufacturer supplied data, PrestoBlue measured cell viability in an incubation time as short as 10 min, and was able to detect 12 Jurkat cells after 16 h incubation using fluorescence measurement, which means that it is a faster assay with higher sensitivity. However, there is no information available on how PrestoBlue differs from alamarBlue in terms of the mechanism or chemical reaction. The only information we were able to obtain from the manufacturer is that the buffers in these two reagents are different, while the details of the difference, how it affects the chemical reaction and performance of the reagent are proprietary.

Since the PrestoBlue assay is a highly sensitive assay, in order to obtain the optimum result, selecting the proper plate and the correct reading mode is of great importance. Different plates have different reflective properties. Black plates absorb light, reduce

background fluorescence and minimize well-to-well crosstalk. White plates reflect light and maximize output signal. Clear plates are favorable in cell culturing studies because they enable microscopy applications to examine the cells during the experiments, and allow top and bottom reading capabilities. Top reading offers better signal-to-noise ratios for solution-based assays; while bottom reading provides higher fluorescence signal and can preserve the sterility of the well contents by allowing the cover to be left on the plate during reading. What kind of plate (black, white or clear) and reading mode (top or bottom reading) gives better result for the PresotBlue assay? How does the plate color and reading mode affect the performance of the assay? To the best of our knowledge, no study has been done to address these questions.

Not all investigators have fluorescence plate reader. It may be difficult to read plates right after experiments. According to the manufacturer of PrestoBlue reagent, assay plates can be wrapped in foil, stored at 4 ℃, and read within 1-3 days. However, will a longer storage of the plates affect the result of the PrestoBlue assay? Is there a new way to store the plates for delayed measurements without affecting the result? As far as we know, there is no report on these questions.

To date, there are three studies that compared PrestoBlue with other similar viability reagents in assessing the viability of different parasites[12,13] and microorganisms.[14] However, for the application in human cells, only one study conducted by Boncler compared PrestoBlue and MTT in evaluating the viability of human endothelial cells.[15] The aim of this study was to compare the sensitivity of PrestoBlue, alamarBlue, and MTT in assessing cell

viability of human corneal epithelial cells (HCEC), and to investigate the effect of plate color, reading mode and plate storage on the performance of the PrestoBlue assay.

For these purposes we used PrestoBlue, alamarBlue and MTT to assess the viability of different numbers of healthy HCEC and to evaluate the toxicity of various chemicals on HCEC. In the fluorescence measurement of the PrestoBlue assay, three plate colors and two reading modes were used, and the plates were stored and measured up to 7 days.

2.3 Methods

2.3.1 Chemicals and solutions

Four chemicals with different concentrations were tested in this study: benzalkonium chloride (BAK) (Sigma-Aldrich, St. Louis, MO), Ethylenediaminetetraacetic acid disodium salt (EDTA) (Sigma-Aldrich, St. Louis, MO), Polyaminopropyl biguanide (PAPB) (Lotioncrafter, Olga, WA), and sodium dodecyl sulfate (SDS) (Sigma-Aldrich, St. Louis, MO). PrestoBlue and alamarBlue were purchased from Invitrogen, Carlsbad, CA. MTT was purchased from Sigma-Aldrich, St. Louis, MO.

2.3.2 Cell culture

Human corneal epithelial cells (HCEC) were obtained from RIKEN BioResource Center, Tsukuba, Japan (#RCB 2280). It is a SV40-transformed human corneal epithelial cell line. The monolayers of the HCEC were prepared with cultures that were less than 10 passages to ensure the consistency among experiments. The HCEC were cultured in DMEM/F-12 1:1 Media (Hylcone, Thermo Scientific, South Logan, Utah), with 10% fetal bovine serum

(Gibco Invitrogen), and 1% penicillin/streptomycin (Gibco Invitrogen) in a humidified environment at 37 ℃ with 5% CO_2. These cultures were maintained with weekly subculture using the Tryple Express (stable trypsin replacement; Gibco Invitrogen) and fed every 2 to 3 days.

2.3.3 MTT assay

The assay was carried out in 96-well plates (BD Falcon, Franklin Lakes, NJ). Each well contained the cells to be tested with cultured medium or rinsing solution removed. 100 ul MTT solution (1mg/ml in clear medium without serum and phenol red) was added to each well and the plates were incubated at 37 ℃ for 3 hours. During the incubation, the active enzymes of the viable cells transformed the yellow MTT into purple formazan crystals. The top medium was then removed and isopropanol was added to each well to dissolve the formazan crystals. The absorbance of the solution was determined at 570nm by a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA).

2.3.4 AlamarBlue assay and PrestoBlue assay

The assays were carried out in either 96-well or 24-well plates (BD Falcon, Franklin Lakes, NJ). Each well contained the cells to be tested with cultured medium or rinsing solution removed. 100 ul (for 96-well plates) or 1 ml (for 24-well plates) alamarBlue or PrestoBlue solution (10% in medium without serum and phenol red) was added to each well and the plates were incubated at 37 ℃ for a specified time period. After incubation, 100 ul of the alamarBlue or PrestoBlue solution from each well of the assay plates (96-well plates or

24-well plates) was transferred to a new well in 96-well plate, and the change in the fluorescence of the test reagent (resazurin to resorufin) was measured in the new plate using a SpectraMax fluorescence multi-well plate reader with the excitation/emission wavelengths set at 530/590 nm for alamarBlue and at 560/590 nm for PrestoBlue.

2.3.5 Assessment of healthy cells

Cell suspension (100ul) containing different numbers of healthy HCEC ranging from 20 to 50,000 was seeded in 96-well plates and allowed to attach and stabilize overnight at 37 ℃ with 5% CO_2. Then the cell viability was tested using either (a) MTT with 3 h incubation, or (b) alamarBlue with 4 different incubation times: 30 min, 1 h, 2 h, and 4 h, or (c) PrestoBlue with 4 different incubation times: 30 min, 1 h, 2 h, and 4 h. The ability of the three assays in detecting the viability of healthy HCEC was evaluated and compared. Another test was carried out to see whether the cell numbers at the time of measurement are different from the cell numbers seeded. Different numbers of cells (50, 100 and 500, n=8 in each group) were seeded in a 96-well plate and incubated overnight. The numbers of the cells adhered in each well were then counted the next morning (the same time that the measurements were carried out). One sample T-test was done to compare the actual cell counts to the cell numbers seeded.

2.3.6 Assessment of the toxicity of different chemicals

Four chemicals with different concentrations were tested: BAK 0.001%, BAK 0.01%, EDTA 0.01%, EDTA 0.02%, PAPB 0.0001%, PAPB 0.001%, SDS 0.01%, and SDS 0.05%.

1 ml of cell suspension with 10^5cells was seeded in 24-well plates and grown to approximately 80% confluent at 37 ℃ with 5% CO_2. The cells were then exposed to test solution for 5 min. After exposure, the test solutions were removed, the cultures were rinsed with 1 ml PBS and then the cell viability of the culture was tested using alamarBlue and PrestoBlue with 1 h incubation. The ability of the two assays in differentiating the cytotoxicity of various chemicals was compared.

An additional test was done to further assess the ability of PrestoBlue and almarBlue in evaluating the toxicity of EDTA on HCEC. The viability of HCEC cultures after exposure to 5 concentrations of EDTA (0.01%, 0.05%, 0.1%, 0.5% and 1%) was tested using PrestoBlue and alamarBlue with the same method described in the previous paragraph.

2.3.7 Effect of plate color and reading mode on the performance of PrestoBlue assay

One milliliter of cell suspension with 10^5cells was seeded in 24-well plates and grown to approximately 80% confluent at 37 ℃ with 5% CO_2. The cells were then exposed to 6 concentrations of SDS (0.5%, 0.1%, 0.05%, 0.02%, 0.01% and 0.005%) for 5 min. After exposure, the SDS solutions were removed and the cultures were rinsed with 1 ml PBS. 1 ml PrestoBlue solution (10% in medium without serum and phenol red) was then added to each well and the plates were incubated at 37 ℃ for 1 h. After incubation, 300 μl of PrestoBlue solution from each well of the assay plates was transferred and divided equally into 3 wells (100 μl/well) in 3 types of 96-well plates (white, black, and clear plate). The change in the fluorescence of the test reagent was measured in the 3 types of plates, with 2 reading modes

in the clear plates (top reading and bottom reading). The excitation/emission wavelengths were set at 560/590 nm. The measurements obtained by different plates and reading modes were compared.

2.3.8 Effect of plate storage on the measurement of PrestoBlue assay

In order to prevent unwanted reduction of PrestoBlue reagent (resazurin) by the cells during the plate storage, we separated the PrestoBlue solutions from the cells by transferring the solutions to new 96-well plates after incubation. The plates were then fully covered to prevent evaporation, wrapped in foil to protected from light, and kept at 2 - 8 ℃ for 7 days. The fluorescence of the plates was measured before storage on day 1, and during the storage on day 2, 3, 5, and 7. All the measurements were made at room temperature (22 ℃) and were compared to the reading on day 1.

2.3.9 Statistical analysis

All experiments were done in quadruplicate. One-way ANOVA followed by the Bonferroni post hoc test was used in the data analysis in the assessment of healthy cells. In the assessment of the toxicity of different chemicals and the comparison of different plates and reading modes, one-way ANOVA and the Games-Howell post hoc test were performed because of the unequal variance of the data. Pearson's correlation was used in analyzing the effect of plate storage on measurements. The criterion of statistical significance was set to be $p < 0.05$.

2.4 Results

2.4.1 Assessment of healthy cells

Our result showed no significant difference between the actual cell counts at the time of measurement and the cell numbers seeded (52.05 ± 8.18 vs. 50, p = 0.501; 98.96 ± 4.93 vs. 100, p = 0.281; and 498.44 ± 16.46 vs. 500, p = 0.797). Therefore, the cell numbers seeded represented the cell counts at the time of measurements.

The number of viable, healthy HCEC cells was measured using PrestoBlue, alamarBlue and MTT. The results are shown in Figure 2-1 (PrestoBlue), Figure 2-2 (alamarBlue) and Figure 2-3 (MTT). All of the 3 assays showed good dose response of fluorescent/absorbance value to the cell numbers. As the cells number increased, the fluorescence/absorbance increased. In both the PrestoBlue and alamarBlue assays (Figure 2-1 and Figure 2-2), significantly higher fluorescent value compared to the control with no cells was detected in ≥5000 cells after 30 min incubation and in ≥1000 cells after 1 h, 2 h, and 4 h incubation ($p \leq 0.027$). The MTT assay was not able to detect 1000 cells at 3 hour incubation ($p = 0.171$). The MTT assay only showed significantly higher absorbance for ≥5000 cells vs the control with no cells after 3 h incubation ($p < 0.001$) (Figure 2-3).

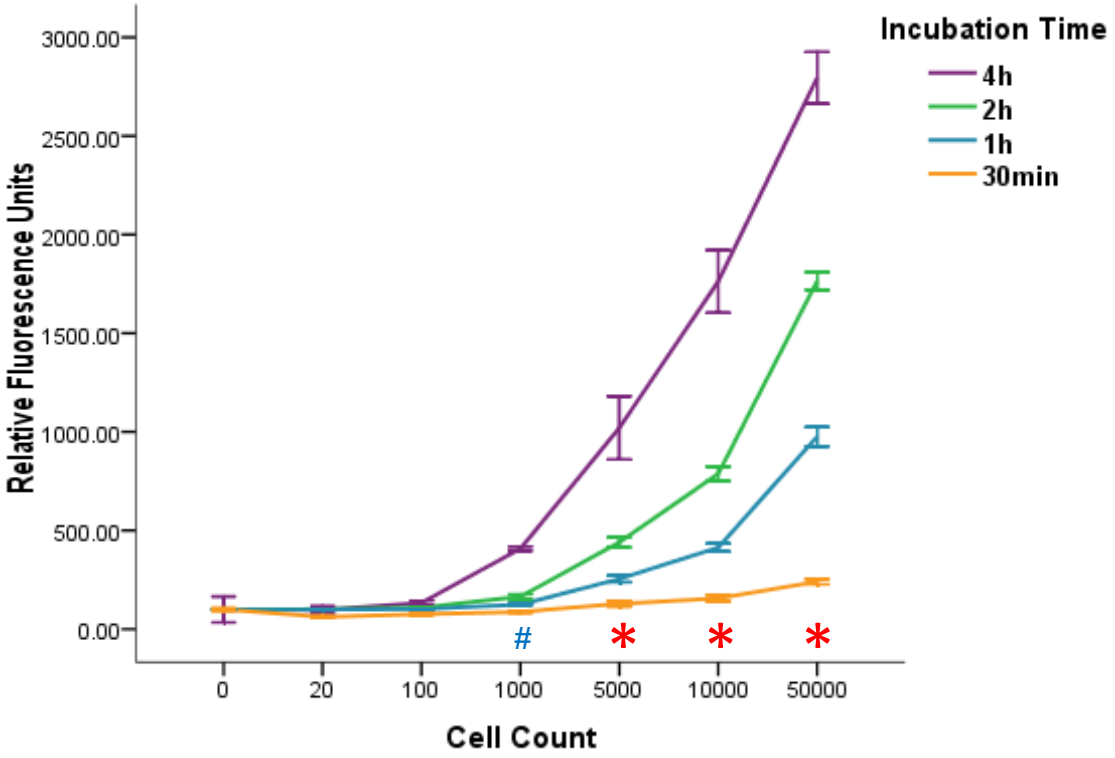

Figure 2-1. Viability of healthy HCEC measured by PrestoBlue.

Error bars: ±SD. *Significantly different from the control with no cells after 30 min, 1 h, 2 h, and 4 h incubation, $p \leq 0.018$. # Significantly different from the control with no cells after 1 h, 2 h and 4 h incubation, $p \leq 0.023$.

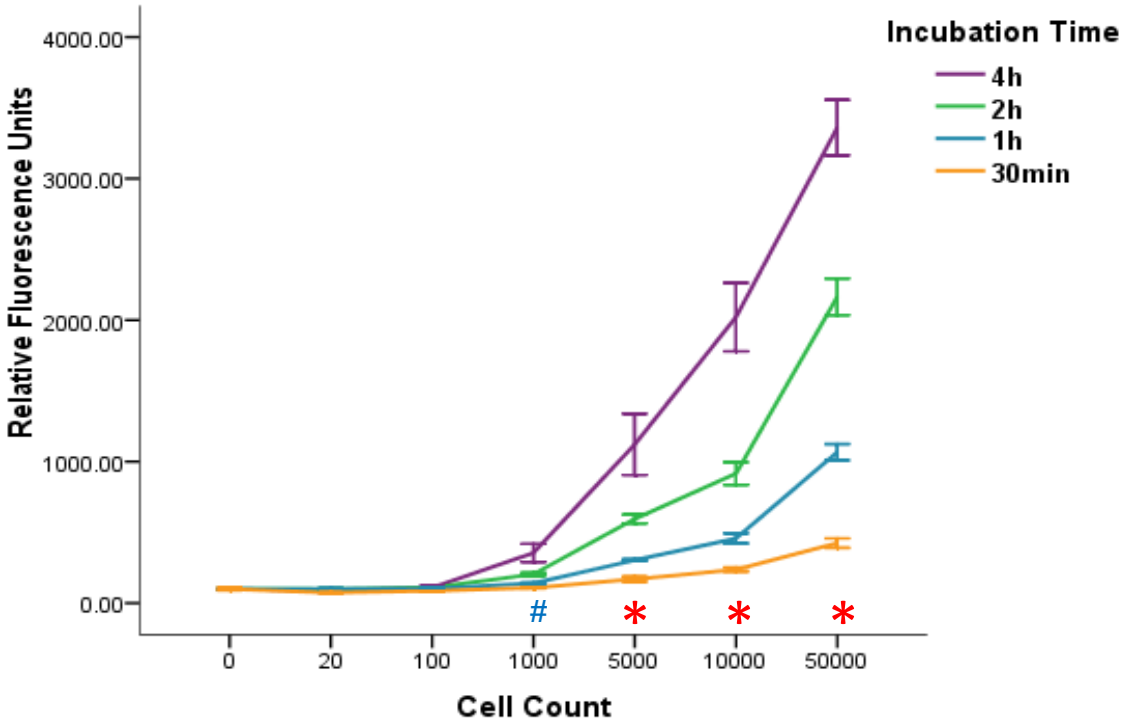

Figure 2-2. Viability of healthy HCEC measured by alamarBlue.

Error bars: ±SD. *Significantly different from the control with no cells after 30 min, 1 h, 2 h, and 4 h incubation, $p \leq 0.027$. # Significantly different from the control with no cells after 1 h, 2 h and 4 h incubation, $p \leq 0.021$.

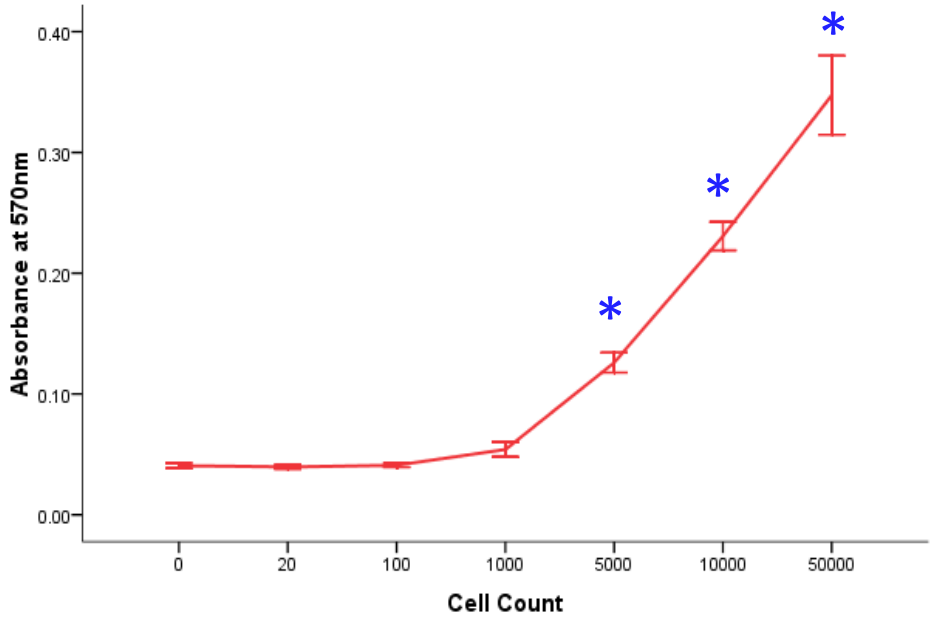

Figure 2-3. Viability of healthy HCEC measured by MTT.

Error bars: ±SD. *Significantly different from the control with no cells after 3 h incubation, $p < 0.001$.

2.4.2 Assessment of the toxicity of different chemicals

Based on the results in the assessment of healthy cells, 1 h, 2 h, and 4 h incubations had the same sensitivity in the Prestoblue and alamarblue assays. Therefore, we used 1h incubation in our following tests. MTT was shown to be less sensitive and hence we only compared PrestoBlue and alamarBlue.

The toxicity of four different chemicals was assessed using PrestoBlue and alamarBlue. Results are shown in Figure 2-4 and Figure 2-5. Again, these two assays performed similarly.

According to the results of both assays, BAK, PAPB and SDS showed dose-dependent cytotoxicity on HCEC. SDS 0.05% was the most toxic, followed by BAK 0.01%, SDS 0.01%, BAK 0.001% and PAPB 0.001%. All of them significantly reduced the cell viability compared to the medium control ($p \leq 0.012$.). There is only one difference between the two assays. PrestoBlue was able to show significant difference between EDTA (both 0.01% and 0.02%) and the medium control (p = 0.026 and 0.035, respectively), while alamarBlue did not (p = 0.335 and 0.126, respectively).

An additional test was done to further assess the ability of PrestoBlue and almarBlue in evaluating the toxicity of EDTA on HCEC. In the additional test, PrestoBlue and alamarBlue also performed similarly in evaluating the toxicity of EDTA 0.01%, 0.05%, 0.1%, 0.5% and 1% (Figure 2-6 and Table 2-1). The cell viabilities obtained by the two reagents were in good agreement with each other. Both assays showed a dose response on EDTA toxicity, with the highest cell viability obtained in EDTA 0.01% (85.40 ± 2.90 with PrestoBlue and 86.28 ± 3.88 with alamarBlue) and the lowest obtained in EDTA 1% (71.26 ± 2.09 with PrestoBlue and 70.70 ± 3.30 with alamarBlue). All the five concentrations of EDTA (0.01%, 0.05%, 0.1%, 0.5% and 1%) significantly reduced the cell viability compared to the medium control (p = 0.001~0.005). There was no significant difference between the results of the two assays (p = 0.730).

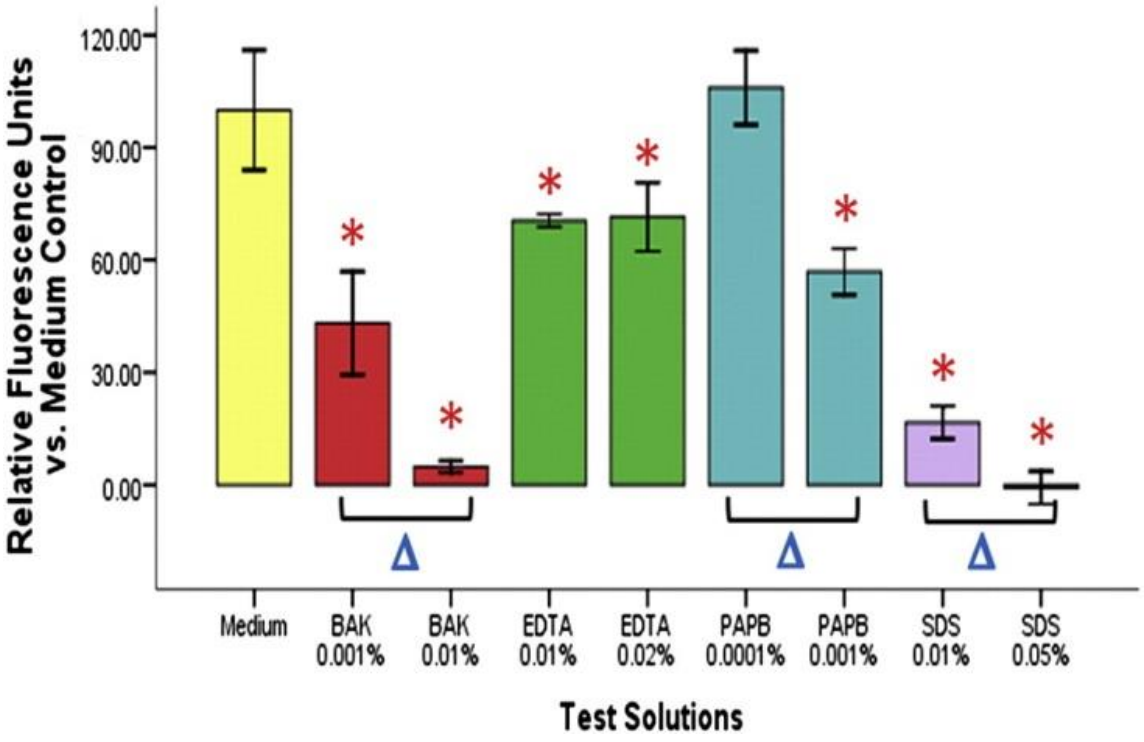

Figure 2-4. Viability of HCEC after 5-min exposure to different chemicals measured by PrestoBlue.

Error bars: ±SD. *Significantly different from the medium control, $p \leq 0.035$. Δ Significantly different from each other: BAK 0.001% vs. BAK0.01%; PAPB 0.0001% vs. PAPB 0.001% and SDS 0.01% vs. SDS 0.05%, $p \leq 0.007$.

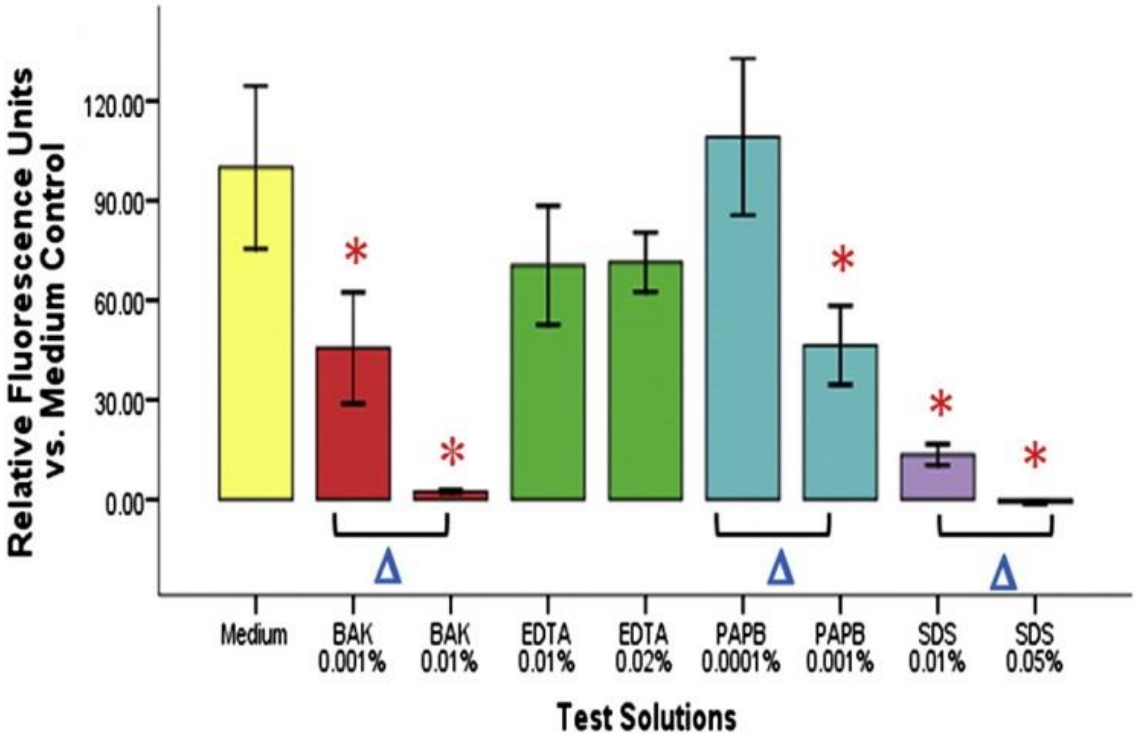

Figure 2-5. Viability of HCEC after 5-min exposure to different chemicals measured by alamarBlue.

Error bars: ±SD. *Significantly different from the medium control, $p \leq 0.012$. ΔSignificantly different from each other: BAK 0.001% vs. BAK 0.01%; PAPB 0.0001% vs. PAPB 0.001% and SDS 0.01% vs. SDS 0.05%, $p \leq 0.021$.

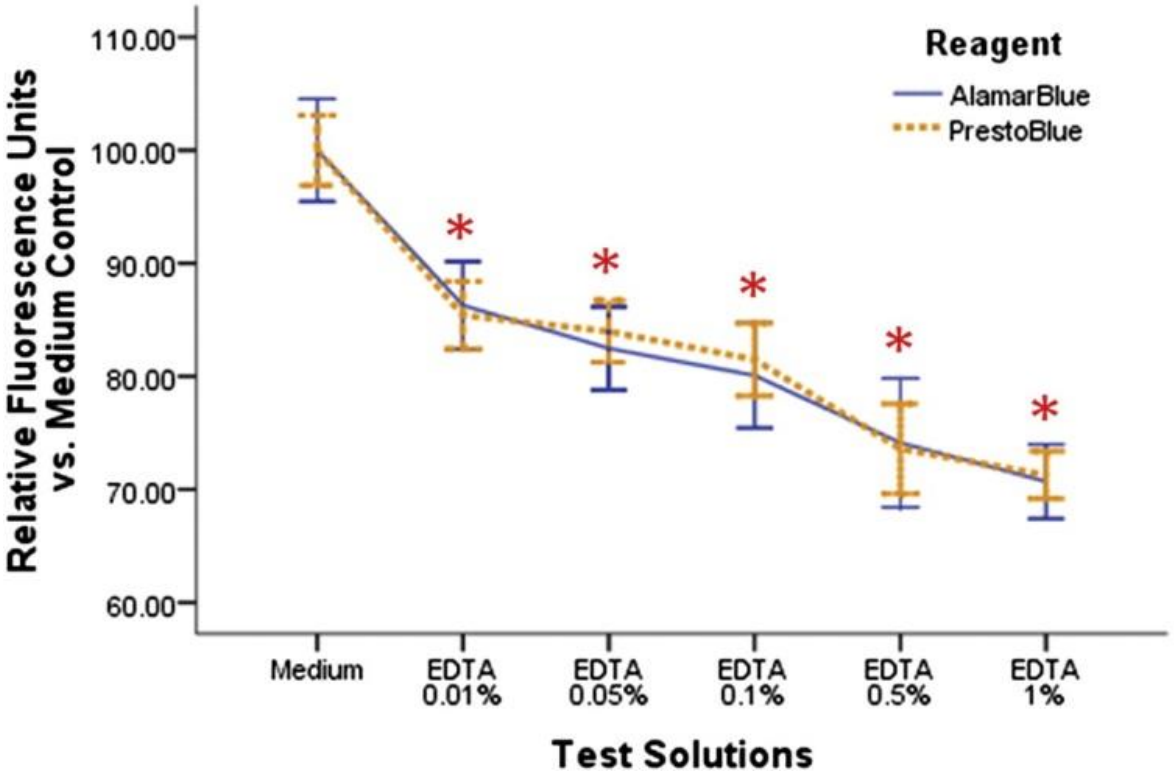

Figure 2-6. Viability of HCEC following EDTA treatment measured by PrestoBlue and alamarBlue.

Error bars: ±SD. *Significantly different from the medium control, p = 0.001~0.005.

Table 2-1. Viability of HCEC after exposure to EDTA mesured by PrestoBlue and alamarBlue.

	EDTA Concentration				
Reagent	0.01%	0.05%	0.10%	0.50%	1%
PrestoBlue	85.40 ± 2.90	83.97 ± 2.72	81.48 ± 3.23	73.59 ± 3.98	71.26 ± 2.09
AlamarBlue	86.28 ± 3.88	82.47 ± 3.68	80.08 ± 4.65	74.12 ± 5.71	70.70 ± 3.30

Results are calculated in percentage of medium control and expressed as Mean ± SD.

EDTA, Ethylenediaminetetraacetic acid disodium salt; HCEC, human corneal epithelial cells.

2.4.3 Effect of plate color and reading mode on the performance of PrestoBlue assay

The viability of HCEC after 5-min exposure to 6 concentrations of SDS was evaluated using PrestoBlue with three different colors of plates (white, black, and clear) and two reading modes in the clear plates (top reading and bottom reading). Figure 2-7 A shows the raw fluorescence reading of the four different test conditions. White plate with top reading mode had significantly higher raw fluorescence readings compared to all the other test conditions (all $p < 0.001$). There was no significant difference among clear plate with bottom reading, clear plate with top reading and back plate with top reading (all $p > 0.05$). For a further comparison, we subtracted the blank and calculated the percentage of cell viability compared to the medium control. The result is shown in Figure 2-7 B. In all the test conditions, all of the 6 concentrations of SDS (0.02%, 0.05%, 0.1%, 0.5%, 0.005% and 0.01%) were significantly different from the medium control ($p \leq 0.017$); both SDS 0.005% and 0.01% had significantly higher cell viability compared to SDS 0.02%, 0.05%, 0.1% and 0.5% ($p < 0.001$). There was no significant difference in among the cell viability obtained in the four test conditions (all $p > 0.05$).

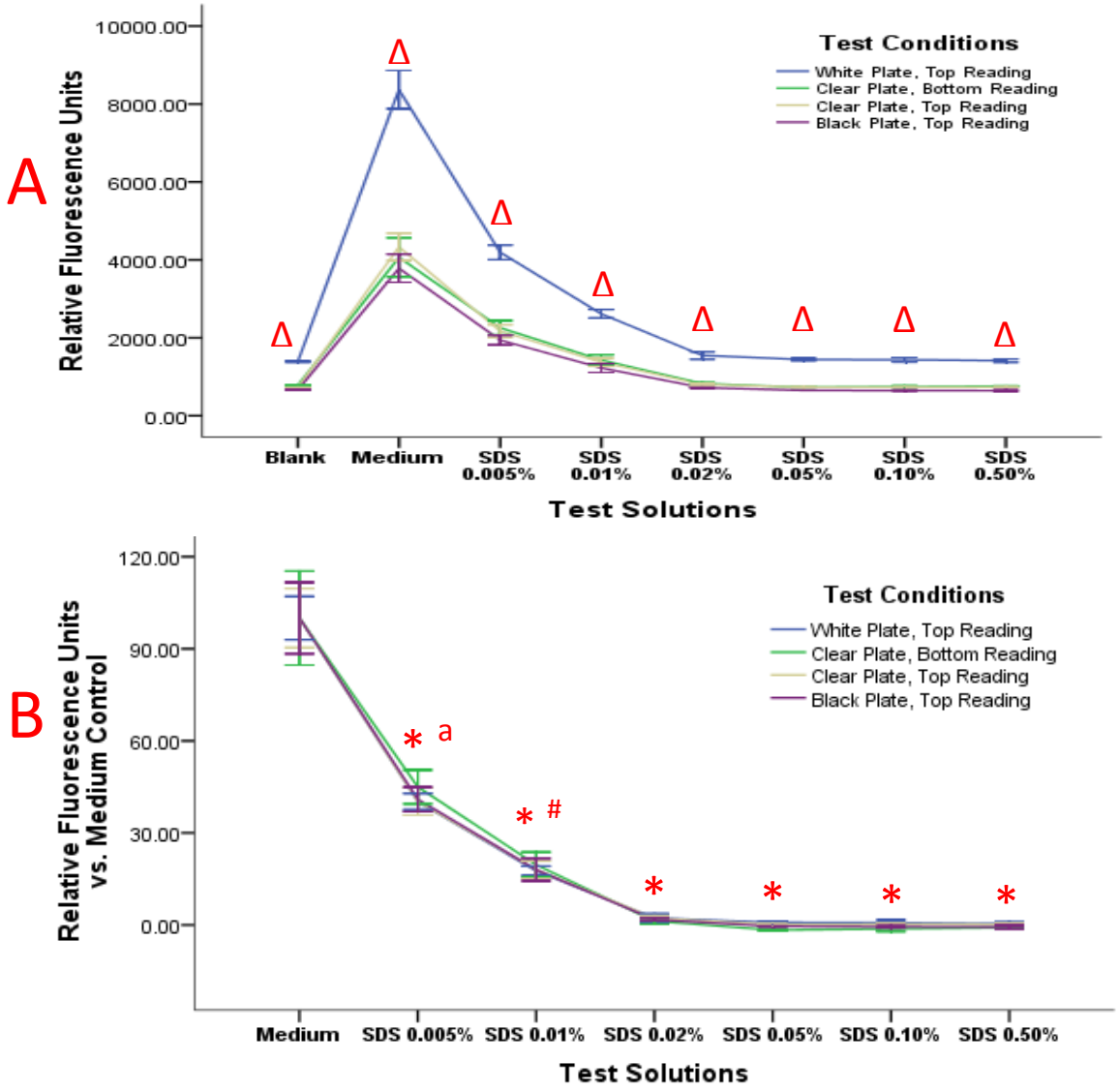

Figure 2-7. Effect of plate color and reading mode on the measurement of PrestoBlue assay.

The viability of HCEC after 5-min exposure to 6 concentrations of SDS was tested using PrestoBlue with 3 types of plates and 2 reading modes. Figure 2-7 A shows the raw fluorescence readings. Figure 2-7 B shows the result after the readings were subtracted by the

blank and compared to the medium control. Error bars: ±SD. ΔSignificantly higher than the corresponding readings in the other test conditions, $p < 0.001$. * Significantly different from the medium control in all test conditions, $p < 0.017$. a: Significantly different from SDS 0.01%, SDS 0.02%, SDS 0.05%, SDS 0.1% and SDS 0.5% in all test conditions, $p < 0.001$. #: Significantly different from SDS 0.02%, SDS 0.05%, SDS 0.1% and SDS 0.5% in all test conditions, $p < 0.001$.

2.4.4 Effect of plate storage on the measurement of PrestoBlue assay

In the PrestoBlue assay, the fluorescence readings obtained on day 2, 3, 5, and 7 after sampling were compared to the reading obtained on day 1. Pearson's correlation analysis showed great linear correlations between the reading on day 1 and all the readings on day 2, 3, 5 and 7 (Figure 2-8, $R^2 = 0.998 \sim 0.999$, all $p < 0.001$).

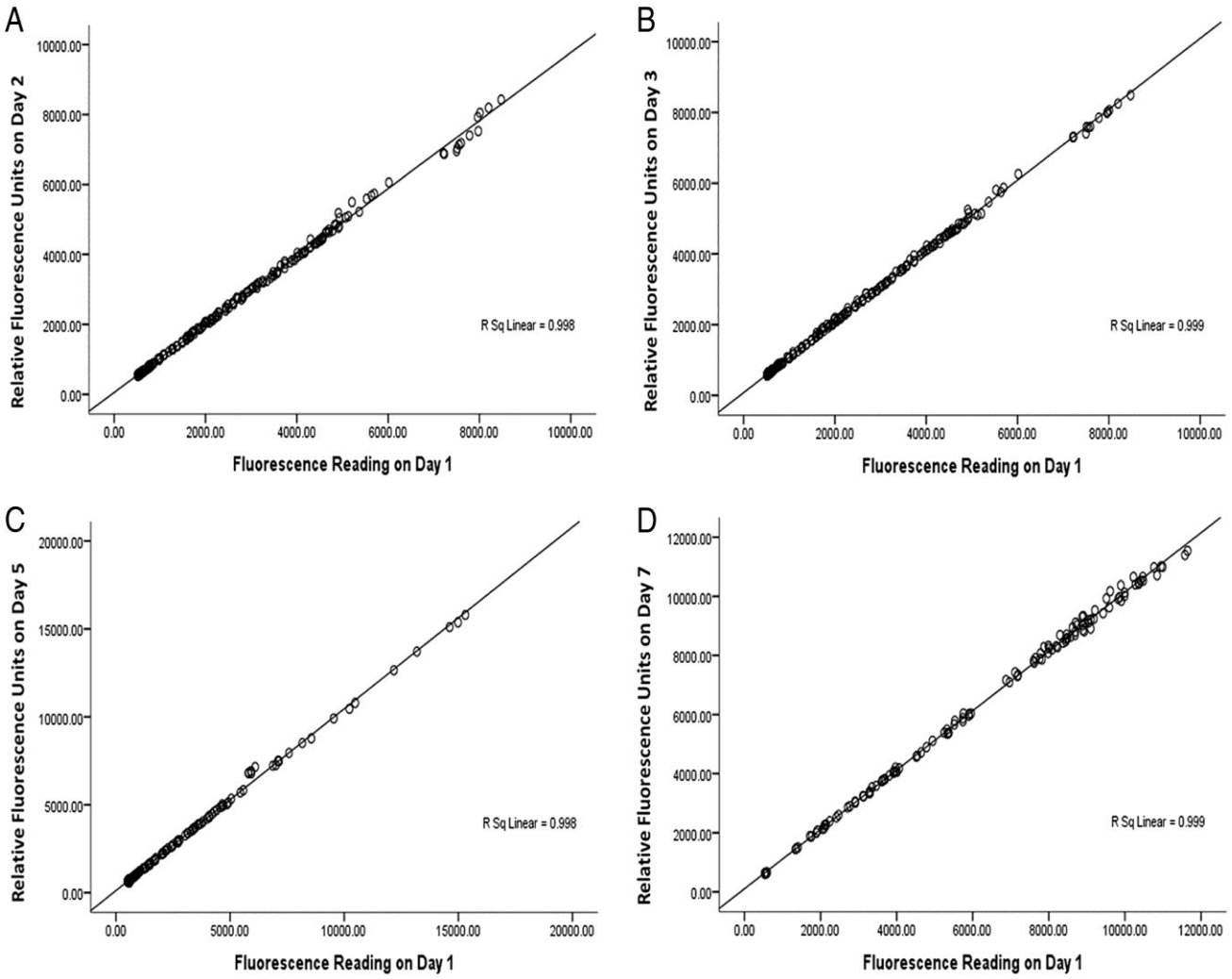

Figure 2-8. Effect of Plate Storage on the Measurement of PrestoBlue Assay.

Pearson's correlation between the fluorescence readings on day 1 and day 2, 3, 5, and 7. A: Day 2 vs. Day 1; B: Day 3 vs. Day 1; C: Day 5 vs. Day 1; D: Day 7 vs. Day 1. $R^2 = 0.998 \sim 0.999$, all $p < 0.001$.

2.5 Discussion

MTT, alamarBlue and PrestoBlue are all viability reagents based on mitochondrial enzyme activity. The first two reagents have been used widely in cell proliferation and

cytotoxicity assessments.[16-20] PrestoBlue is a new reagent recently brought into the market. Similar to alamarBlue, it is resazurin-based, water soluble, and non-toxic to cells.[7,15] However, it is claimed to be a fast and highly sensitive assay. According to the manufacturer supplied data, it could assess cell viability with 10 min incubation, and was able to detect as few as 12 Jurkat cells. So far, there are four studies which validated the use of PrestoBlue in different types of cells, one in microorganisms,[14] two in parasites,[12,13] and one in human endothelial cells.[15] To the best of our knowledge, there is no report on the use of PrestoBlue in cultures of human epithelial cells; neither there is any study on the effect of plate color, reading mode and plate storage on the performance of the PrestoBlue assay.

In our study, the sensitivity of MTT, PrestoBlue and alamarBlue in evaluating the viability of healthy HCEC was compared. Since PrestoBlue was claimed by the manufacturer to be able to detect as few as 12 cells, a wide range of cell numbers (from 20 to 50,000) was used in our test. Not only did it cover the smallest magnitude of cells that PrestoBlue was claimed to detect, it also included the magnitude of cells that are commonly used in cell viability evaluation. This enabled us to better explore the sensitivity of the three assays.

When tested in 96-well plates, PrestoBlue and alamarBlue were able to detect 5000 cells after 30 min incubation and 1000 cells after 1 h, 2 h, and 4 h incubation; while MTT was only able to detect 5000 cells after 3 h incubation. This indicates that PrestoBlue and alamarBlue are more sensitive than MTT, or when the same sensitivity is achieved (detecting 5000 cells), PrestoBlue and alamarBlue are faster assays (30 min incubation vs. 3 h incubation). There are two possible reasons. First, MTT mainly measures the mitochondrial

dehydrogenate activity,[3] and is reduced primarily by oxidoreductase enzymes, the majority of which utilize NADH.[21] On the other hand, PrestoBlue and alamarBlue involve more mitochondrial enzymes and accept electrons from NADPH, FADH, FMNH, NADH, and also from cytochromes.[8] Besides, MTT formazan is insoluble in cell culture media and forms purple needle-shaped crystals in cells. The culture media has to be removed and an organic solvent is required to dissolve the crystals before measuring the absorbance.[3] However, it is difficult to remove the culture media due to many floating cells with MTT formazan needles. This gives significant well-to-well error, increases the standard deviation, and eventually decreases the assay sensitivity. On the contrary, PrestoBlue and alamarBlue are soluble in culture media.[6] No solubilizing process is required, and hence this simplifies the assays and reduces error. What is more, PrestoBlue and alamarBlue are non-toxic to cells, which allows the assayed cells to be used in subsequent test;[6,7] while MTT assay can only be used as an end-point procedure because it kills the cells by forming crystals.[3] Based on the reasons above, PrestoBlue and alamarBlue are considered superior to MTT.

Our result is in contrast to a study by Boncler et al.[15] They compared PrestoBlue and MTT in assessing the anti-proliferative effects of plant extracts on confluent human vein endothelial cells and found that MTT assay had lower inter-assay variability and higher signal-to-noise ratio compared to PrestoBlue assay.[15] Vascular endothelial cells are particularly sensitive to cell contacts and undergo rapid inhibition of cell proliferation by cellular confluence.[22,23] As a result, there was a notable difference in the cell proliferation between the cells tested in Boncler's study and our study (confluent vascular endothelial cells

vs. non-confluent corneal epithelial cells), which would affect the results of PrestoBlue and MTT assays.

Although PrestoBlue is claimed to be a faster assay with higher sensitivity, it performed similarly to alamarBlue in evaluating the viability of healthy HCEC. In order to further compare these two assays, a second test (assessment of the toxicity of different chemicals on HCEC) was conducted. Again, these two reagents performed similarly. Therefore, an additional test was carried out to further assess these two assays in evaluating the toxicity EDTA on HCEC. Once more, PrestoBlue and alamarBlue performed similarly. There was no significant difference between the results obtained by the two assays ($p = 0.730$). This suggests that in evaluating the viability of HCEC, PrestoBlue and alamarBlue are comparable. This is not surprising. Similar result was reported by one recent study. Carmen et al. compared PrestoBlue and alamarBlue directly using absorbance and fluorescence measurements and concluded that these two reagents were equally useful in evaluating the viability of Acanthamoeba.[13] Nevertheless, Lall et al. used PrestoBlue for antimicrobial analysis against different microorganisms and found that it was faster than alamarBlue as a viability indicator for *Streptococcus mutans*, *Prevotella intermedia,* and *Mycobacterium* tuberculosis.[14] The different results obtained in different studies indicate that in evaluating different organisms and cell lines, PrestoBlue performed differently.

In the current study, we also investigated the effect of plate color and reading mode on the performance of the PrestoBlue assay using fluorescence measurement. Four test conditions were compared: white plate with top reading, black plate with top reading, clear

plate with top reading, and clear plate with bottom reading. They were judged based on their performances in assessing the cytotoxicity of six concentration of SDS on HCEC. As expected, white plate with top reading mode had significantly higher raw fluorescence readings compared to all the other test conditions (all $p < 0.05$) (Figure 2-7 A). However, top reading mode and bottom reading mode in clear plate had very similar raw fluorescence signals. The possible reason is that the particular clear bottom plastic in this type of clear plate gave very little autofluorescence at the emission wavelength (590nm) used in this experiment. More unexpectedly, after subtracting the blank control and calculating the percentage of viability compared to the medium control, all four test conditions showed very similar results (Figure 2-7 B). There was no significant difference in the sensitivity among the four different test conditions. This suggests that with the proper controls (blank and negative/positive controls) to minimize the effect of background noise, the three different plates and two reading modes perform similarly in the PrestoBlue assay in assessing the cell viability of HCEC. This may broaden the plate selection for PrestoBlue.

Sometimes, it is difficult to read a plate right after an experiment is performed. It would be helpful if plates can be stored and read a few days later without affecting the result. It is recommended by the manufacturer that plates can be refrigerated, wrapped in foil and read within 1-3 days. They also suggest stopping and stabilizing the reaction by adding 3% SDS to the cells for an end-point assay.[10] However, there is a disadvantage in storing plates with cells under test. The metabolic activity of the cells can reduce resazurin during the storage. With high cell numbers and prolonged storage, over-reduction of resazurin may occur and

produce an uncolored, nonfluorescent product (hydroresorufin), leading to artefact results.[24] In our study, one more procedure was added to improve the result of the stored plates. We separated the PrestoBlue solutions from the cells by transferring the solution to new 96-well plates which were then stored for further measurements. This prevented unwanted reduction of PrestoBlue reagent (resazurin) during the plate storage, and what is more, saved the cells for further culturing and subsequent test. In the current study, the plates without cells were stored up to 7 days and fluorescence readings were obtained on day 1, 2, 3, 5, and 7 after sampling. There were great linear correlations between the readings on day 1 and all the readings on day 2, 3, 5 and 7 (Figure 2-8, $R^2 = 0.998 - 0.999$, all $p < 0.001$). This is a promising finding. It indicates that plates without cells can be saved up to 7 days without affecting the result. It increases the flexibility of the PrestoBlue assay and offers more convenience to the investigators, especially for those who share one fluorescence plate reader with many people. There is one thing to keep in mind while taking measurements with stored plates. Because fluorescence measurements are influenced by temperature, the plates should be warm to the temperature at which the readings are normally taken.

In summary, our study suggests that in evaluating the viability of HCEC, PrestoBlue is more sensitive than MTT, but similar to alamarBlue. When fluorescence measurement was used, the plate color, reading mode and plate storage up to 7 days did not affect the performance of the PrestoBlue assay.

According to the results of Chapter 2, PrestoBlue is a fast and sensitive viability reagent that also offers flexibility and great convenience to investigators. This finding confirms the use of PrestoBlue in the *in vitro* cell model.

Using this cell model, the next chapter assessed the individual and combined toxicity of ultraviolet radiation and BAK on human corneal epithelial cells.

Chapter 3

Synergistic Toxicity of UV Radiation and Benzalkonium Chloride on Cultured Human Corneal Epithelial Cells

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Authors' contributions

Author	Concept/Design	Data Collection	Data Analysis	Article Writing	Article Editing
Xu	✓	✓	✓	✓	✓
McCanna	✓				✓
Sivak	✓				✓

3.1 Overview

Purpose: To investigate the combined effect of benzalkonium chloride (BAK) and ultraviolet (UV) radiation on cultured human corneal epithelial cells (HCEC), and to analyze whether there is any additive or synergistic effect between UV radiation and BAK.

Methods: Cultured HCEC were exposed to BAK alone, UV alone, or BAK plus UV. The exposure of UV plus BAK was evaluated using three different protocols to evaluate the possible effect of the exposure order: a) UV and BAK simultaneously, b) UV 1st and BAK 2nd, c) BAK 1st and UV 2nd. After exposure, cell metabolic activity was measured with PrestoBlue, and cell viability was determined using confocal microscopy with viability dyes. To test for photoreactivity, BAK solutions were treated by UV radiation, and the cell toxicity of the UV-treated BAK on HCEC was measured with PrestoBlue. Phosphate buffered saline (PBS) was used as a negative control.

Results: BAK alone reduced the metabolic activity and cell viability of HCEC in a dose- and time-dependent manner. UV alone at a low dose (0.1719 J/cm^2) had little toxicity on HCEC and showed similar effect to the PBS control. However, when the cultures were treated with UV plus BAK, the combined effects were either greater than (synergistic) or equal to (additive) the sum of individual effects. The synergistic effects occurred between the low dose UV radiation and low concentrations (0.001%, 0.002%, 0.003% and 0.004%) of BAK. In the test for photoreactivity, UV-treated BAK had similar or slightly lower cell toxicity than the untreated BAK.

Conclusions: Our study indicates that co-exposure to UV radiation and BAK can cause synergistic and additive effects on human corneal epithelial cells. This finding highlights the importance of considering the combined toxic effects of BAK and solar radiation in the risk assessment of BAK-preserved ophthalmic solutions.

3.2 Introduction

Chemical preservatives are used in a variety of ophthalmic solutions to prevent contamination by microorganisms, and many ophthalmic solutions containing preservatives are instilled directly into eyes that are then exposed to sunlight. Therefore, co-exposure of the cornea to preservatives and solar radiation can occur. Such exposure might result in greater toxicity to the cornea. A combination of two toxic agents may produce synergistic or additive effects. A synergistic effect occurs when the combined effect of two agents is greater than the sum of their individual effect (e.g., 1+1=10); while, an additive effect takes place when the combined effect is equal to the sum of the effects of each agent given alone (e.g., 1+1=2).

Benzalkonium chloride (BAK), a quaternary ammonium cationic surfactant, is the most commonly used preservative in ophthalmic solutions. Numerous clinical studies have shown that BAK-preserved ophthalmic solutions can cause damage to the eye, such as tear film instability,[1] disruption of corneal barrier function,[2] and reduction of corneal sensitivity.[3] In addition, *in vivo* studies with animals and *in vitro* studies with various cell lines have also demonstrated that BAK alone is toxic to animal corneas and cultured cells at concentrations

used clinically.[4-9] Based on *in vivo* and *in vitro* studies, BAK is highly toxic in a time- and dose-dependent manner, with a toxicity threshold as low as 0.005%.[10-14]

The ambient UV radiation at the Earth's surface consists mainly of UVA (315–400 nm) radiation and a small amount of UVB (280–315 nm) radiation.[15] However, due to the ozone depletion, the amount of hazardous UVB radiation reaching the Earth's surface has increased recently,[16] posing a higher risk for UV damage to the eye. There is some evidence that the ozone layer is recovering due to the reduction in ozone depleting chemicals; however, it is estimated that a complete recovery will not occur until the year 2100.[17] Strong evidence has revealed that acute high dose exposure to UV radiation causes photokeratitis and photoconjunctivitis.[18,19] Also, considerable epidemiological and experimental evidence indicates that chronic exposure to UV radiation is a major risk factor for cataract, pterygium, and age-related macular degeneration.[18-20]

Co-exposure to preservatives and UV radiation may generate a toxic effect to the eye that is much greater than the sum of their individual effects. Withrow et al.[21] tested the mutagenic potential of four ocular preservatives in mouse lymphoma cells with and without UVA radiation. They reported that chlorhexidine alone had little mutagenic potential, but when combined with UVA, there was approximately a threefold increase over background in the number of mutants; also, the mutagenic activity of thimerosal was significantly enhanced in combination with UVA. Another study conducted by Lovely et al. investigated the combined effect of preservatives and visible light in bacterial systems. In this study, thimerosal was found to cause DNA damage only under conditions of illumination; while BAK showed

genetic toxicity in darkness, and this toxicity was enhanced in conjunction with exposure to visible light.[22] Although BAK has been shown to be synergistic with gentamicin in causing disruption to corneal epithelial cells,[23] to the best of my knowledge, no studies have reported any synergistic effect between BAK and UV radiation on human ocular cells.

The aim of this study was to investigate the combined toxicity of BAK and UV radiation (UVA and UVB) on human cornea epithelial cells and to analyze whether there is any additive or synergistic effect between BAK and UV radiation. For this purpose, the effect of BAK alone, UV alone, and BAK plus UV on cultured human corneal epithelial cells (HCEC) was assessed and compared. The toxicity of different treatments was measured using two *in vitro* assays including PrestoBlue and confocal microscopy with viability dyes.

3.3 Materials and Methods

3.3.1 Materials

Human corneal epithelial cells (HCEC) were purchased from RIKEN BioResource Center, Tsukuba, Japan (#RCB 2280). This cell line was chosen for this study because it has good growth characteristics, shows good tight junctions, and is free of infectious virus particles.[24-26] DMEM/F-12 Ham Medium, fetal bovine serum, penicillin/streptomycin, Tryple Express, PrestoBlue, calcein AM, ethidium homodimer-1 (EthD-1) and annexin V-Alexa Fluor 647 conjugate (annexin V) were obtained from Life Technologies Inc. (Burlington, ON). Cell culture plates (24-well plates) were purchased from BD Falcon (Franklin Lakes, NJ). Collagen coated glass bottom culture Petri dishes were from MatTek Corp. (Ashland,

MA). Phosphate buffered saline (PBS) was obtained from Lonza (Walkersville, MD). Benzalkonium chloride (BAK, 10%) was purchased from Sigma-Aldrich (St. Louis, MO), and was diluted in PBS into five lower concentrations (0.001%, 0.002%, 0.003%, 0.004% and 0.005%) for the use of this study.

3.3.2 Cell culture

The HCEC were grown in DMEM/F-12 Ham Medium with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified environment at 37 ℃ with 5% CO_2. These cultures were maintained with weekly subculture using Tryple Express and fed every 2 to 3 days. The monolayers of HCEC were prepared with cultures that were less than 10 passages in order to ensure consistency among experiments.

3.3.3 Cell treatments and measurement time points

There were three different treatments in this study: 1) UV alone, 2) BAK alone, 3) UV plus BAK. The treatment of UV plus BAK was evaluated using three different protocols for the order of the addition of UV and BAK: a) UV and BAK simultaneously, b) UV 1^{st} and BAK 2^{nd}, c) BAK 1^{st} and UV 2^{nd}.

The cultures were assessed for metabolic activity using PrestoBlue immediately after treatment (0h), as well as at 1 h, 2 h, 4 h and 8 h after treatment. Cell viability was examined using confocal microscopy with viability dyes at 2 h after exposure. In order to test for the photoreactivity of BAK, one additional experiment was conducted. In this experiment, BAK solutions were pre-treated with UV radiation for 5 min. Then cell cultures were exposed to

the UV-treated BAK for 10 min. At 0 h, 1 h, 2 h, 4 h and 8 h after exposure, the cultures were tested for metabolic activity with PrestoBlue. In all the experiments, PBS was used as a negative control.

3.3.4 UV irradiation

UV exposure was conducted in a custom designed UV irradiation unit at 37 ℃ with 5% CO_2. The UV source used in this study was two UV fluorescence tubes (Microlites Scientific, Toronto, ON) that emit broadband UVA and UVB (280-400 nm). Before irradiation, the irradiance of the UV source was measured with an USB 2000+ fiber optic spectrometer (Ocean Optics, Inc. Dunedin, FL). The result is shown in Figure 3-1. The calculated irradiance was 5.73 W/m^2. Samples were exposed to UV radiation at a distance of 30 cm from the light source for 5 min (the corresponding dose was 0.1719 J/cm^2). During UV radiation, the samples were covered with quartz to prevent evaporation; a thin layer of solution (1.5 mm) was left above the cells to minimize absorption of the radiation by the solution. When the culture was exposed to UV and BAK simultaneously, the thin layer of solution was BAK solution; otherwise, it was PBS solution. In the photoreactivity test, a thin layer (1.5 mm) of BAK solution was exposed to UV without cells. The solutions were covered with quartz during UV radiation.

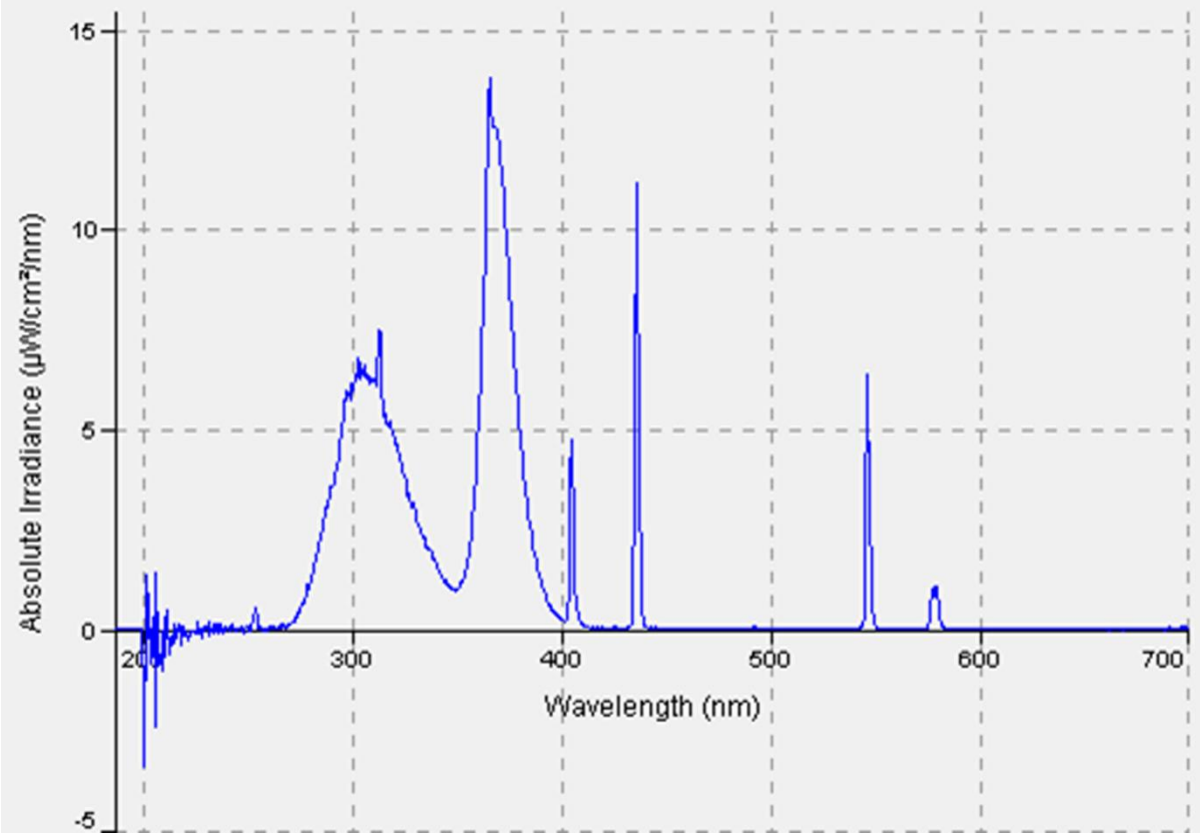

Figure 3-1. Spectrum of the UV source measured with an USB 2000+ fiber optic spectrometer. The calculated irradiance was 5.73 W/m^2.

3.3.5 BAK exposure

BAK (10%) purchased from Sigma-Aldrich was diluted in PBS into lower concentrations for the use of this study. Cell cultures were exposed to various concentrations of BAK with or without UV radiation. Based on the concentrations of BAK commonly used in ophthalmic solutions and on the results of our preliminary tests (data no shown), five concentrations of BAK were used in this study: 0.001%, 0.002%, 0.003%, 0.004% and 0.005%. After

removing growth medium from the cultures, a thin layer of BAK solution (1.5 mm) was placed above the cells. The cultures were then incubated at 37 ℃ with 5% CO_2 for 10 min. Following the incubation, BAK solutions were removed and the cultures were rinsed with 1 ml of PBS.

3.3.6 PrestoBlue assay

One ml of cell suspension containing 10^5 cells was seeded in 24-well plates and grown to approximately 75% to 80% confluent at 37 ℃ with 5% CO_2. The cells were then exposed to various treatments. Cell metabolic activity was assessed with PrestoBlue immediately after treatment, as well as at 1 h, 2 h, 4 h, and 8 h after treatment. At every time point, each well contained the cells to be tested with cultured medium or rinsing solutions removed. One ml of 10% PrestoBlue reagent prepared in medium without serum and phenol red was added to each well and the cultures were incubated at 37 ℃ for 1 h. Following incubation, the change in the fluorescence of the PrestoBlue reagent (resazurin to resorufin) was measured using a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) with the excitation/emission wavelengths set at 560/590 nm. Between time points, PrestoBlue was removed; the cultures were rinsed with 1 ml of PBS and incubated with fresh culture medium at 37 ℃ with 5% CO_2. Experiments were run in triplicate tests with four replicates in each test.

3.3.7 Confocal microscopy study with fluorescent viability dyes

Three fluorescent viability dyes were used with confocal microscopy (Carl Zeiss LSM) to visualize live, dead, and apoptotic cells. The three dyes were: calcein AM, EthD-1, and annexin V. Calcein AM produces an intense green fluorescence in live cells after reaction with intracellular enzymes;[27] EthD-1 binds to the nuclei of dead and dying cells and forms bright red fluorescence in the cells;[27] annexin V stains the phosphatidylserine (PS) of apoptotic cells and dead cells.[28] The natural color of annexin V fluorescence is red. However, in order to distinguish it from EthD-1 (which was also red), we set the confocal software to present annexin V as yellow.

One ml of culture medium containing 4×10^5 cells was transferred into collagen coated glass bottom culture Petri dishes and grown to confluence at 37 ℃ with 5% CO_2 for 2 days. The cultures were then exposed to various treatments. After treatment, the cells were incubated with fresh culture medium at 37 ℃ with 5% CO_2 for 2 h. After the incubation, the cells were stained with Annexin V (10 μl in 500 μl buffer), calcein AM (2 μM), and EthD-1 (4 μM) for 20 min at 37 ℃. Following staining, the fluorescence of the three dyes was visualized with an Axiovert 100 microscope with a Zeiss confocal laser scanning microscope (CLSM) 510 system. The excitation/emission wavelengths for calcein AM, EthD-1, and annexin V, were 495/515 nm, 528/617nm, and 650/665 nm, respectively. The experiments were performed with at least three replicates.

3.3.8 Statistical analysis

The combined effects of UV radiation and BAK on HCEC in each treatment were evaluated by applying the statistical method reported by Ince et al.[29] The method was based on testing the null hypothesis of "additive effect" at 95% confidence level. Specifically, the interaction of UV and BAK was assessed by comparing the observed combined toxicity with the value of the null hypothesis, defined as ''the sum of the toxic effects of each agent given alone''. The combined effects were called ''additive'', or ''synergistic'' according to the statistical significance and the sign of the difference between the calculated hypothesized value and the observed value.

The calculation of the hypothesized values on metabolic activity (MA_H) and cell viability (PCL_H) was based on the following two equations.

H0 Metabolic Activity: $MA_{H(UV + BAK)i} = (MA_{UV}) * (MA_{BAK})i/100$ (1)

H0 Cell Viability: $PLC_{H(UV + BAK)i} = (PLC_{UV}) * (PLC_{BAK})i/100$ (2)

In the equations, $_{(UV + BAK)i}$ was the combination of UV and BAK at i concentration; (MA_{UV}) and (MA_{BAK})i were the metabolic activity values (as % of control) for UV alone and BAK alone at i concentration. (PLC_{UV}) and (PLC_{BAK})i were the percentages of live cells (as % of control) for UV alone and BAK alone at i concentration.

One-way ANOVA was used in the data analysis of this study. The Bonferroni *post hoc* test was performed for the results of the PrestoBlue assay. The Games-Howell *post hoc test*

was used to analyze the cell counts in the confocal images because of the unequal variance of the data. The criterion for statistical significance was set to be $p < 0.05$.

3.4 Results

3.4.1 Metabolic activity

The metabolic activity of HCEC after exposure to different treatments was measured with PrestoBlue. The results are shown in Figures 3-2, 3-3 and 3-4. The type of interaction between UV and BAK in each treatment was evaluated by applying a statistical analysis reported by Ince et al.[29] The results are shown in Tables 3-1, 3-2 and 3-3. Figure 3-2 and Table 3-1 demonstrate the toxic effect of UV only, BAK only and UV plus BAK simultaneously. BAK alone reduced the cell metabolic activity of HCEC in a dose- and time-dependent manner. UV alone showed little cell toxicity and was not significantly different from the PBS control ($p \geq 0.561$). However, when the cultures were exposed to UV plus BAK simultaneously, the toxicity of the treatments was significantly enhanced. Synergistic effects were observed in the followings: UV plus BAK 0.001% at 1 h, 2 h, 4 h and 8 h; UV plus BAK 0.002% and 0.003% at all the time points; and UV plus BAK 0.004% at 0 h and 2 h. Additive effects were determined for all the rest of combinations tested (Table 3-1). Similar results were observed in the other two exposure orders: UV 1^{st} + BAK 2^{nd}, and BAK 1^{st} + UV 2^{nd} (Figures 3-3 and 3-4, Tables 3-2 and 3-3).

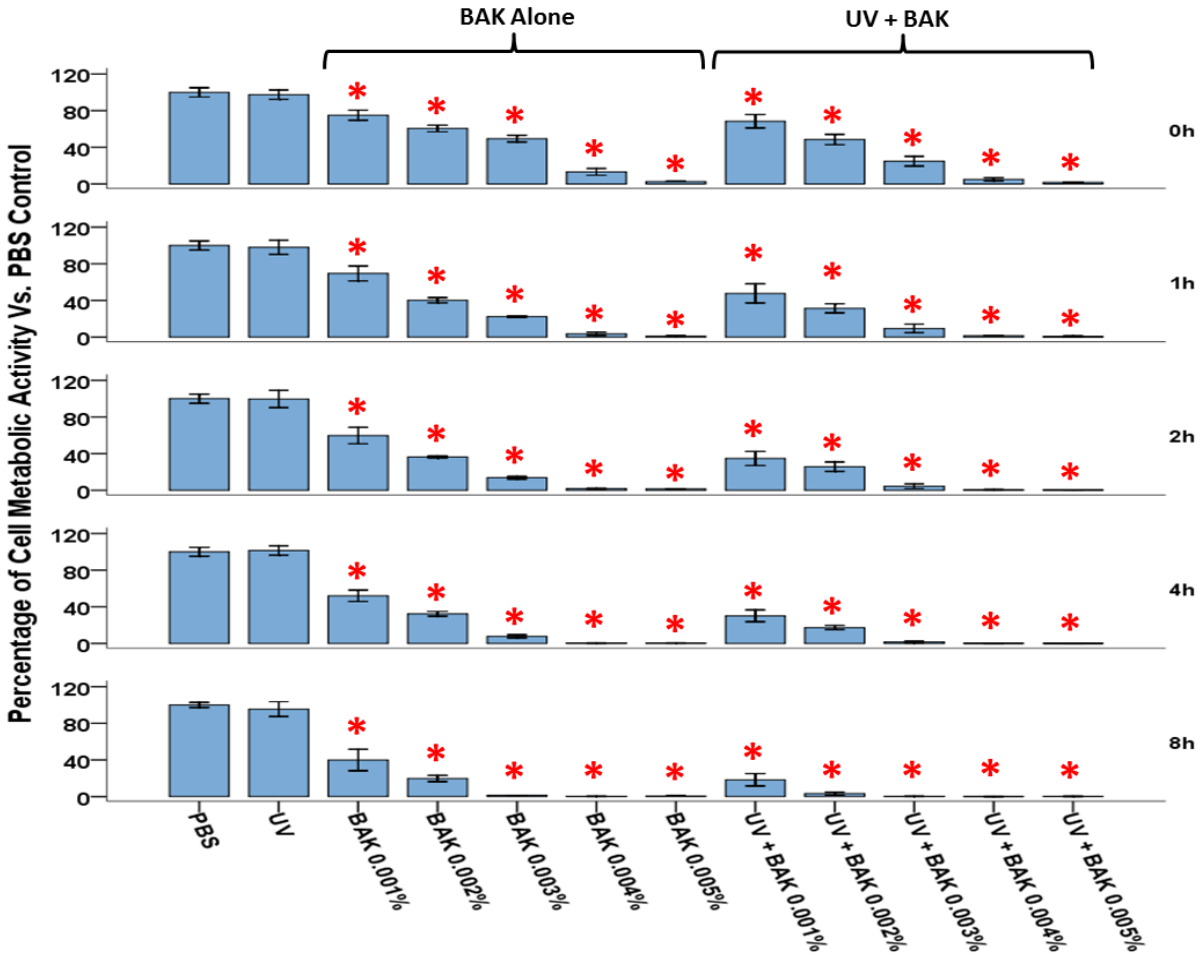

Figure 3-2. Cell metabolic activity measured with PrestoBlue_ UV and BAK simultaneously.

The treatment of UV + BAK was carried out by exposing the cultures to UV and BAK simultaneously. The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. Cell metabolic activity of HCEC was measured at various time points after exposure (0 h, 1 h, 2 h, 4 h, and 8 h). The results are presented in percentage of cell metabolic activity compared to the PBS control. Error Bars: ± SD. * represents: significantly different from the PBS control ($p < 0.05$). The results are based on the average of three experiments with four samples in each experiment.

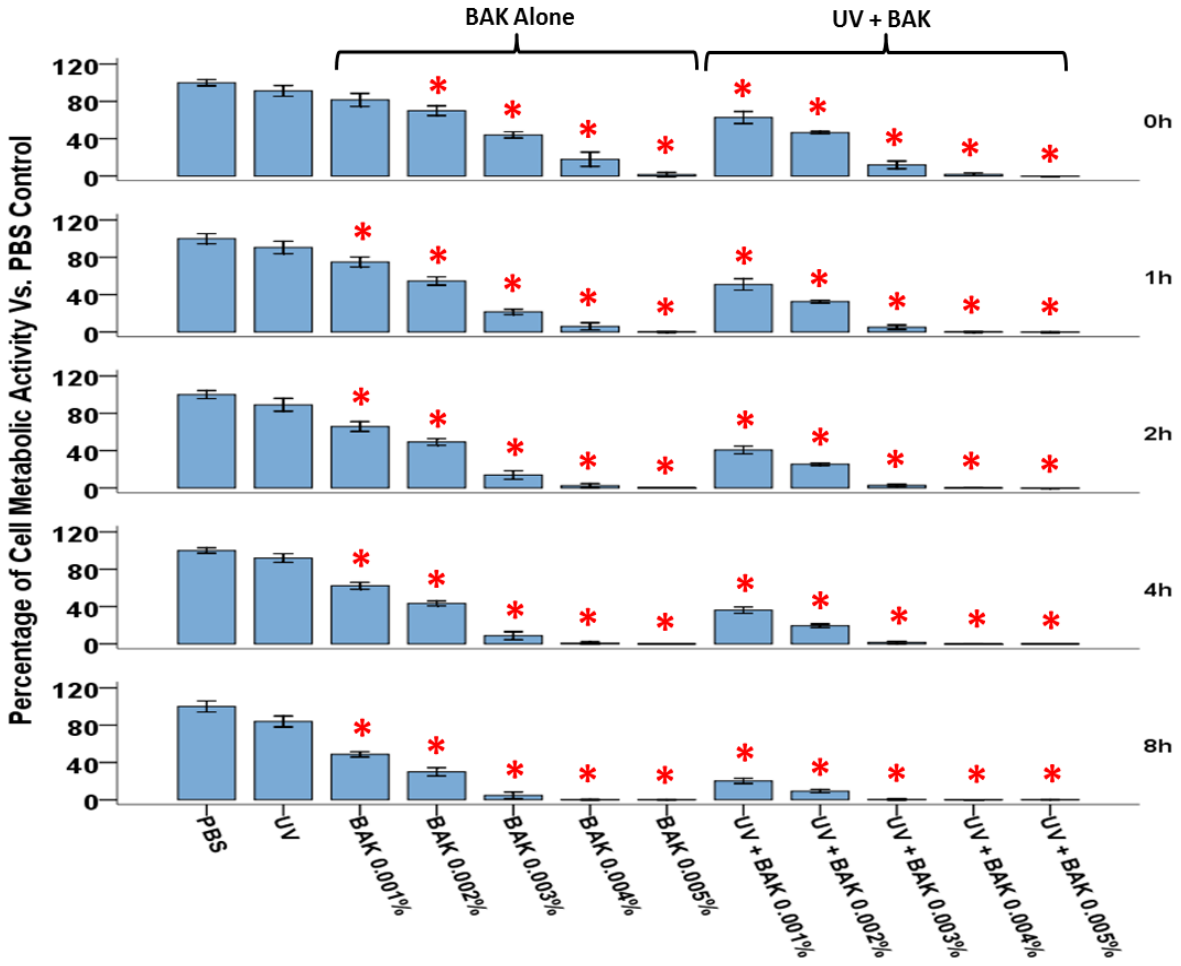

Figure 3-3. Cell metabolic activity measured with PrestoBlue_ UV 1st and BAK 2nd.

The treatment of UV + BAK was carried out by exposing the cultures to UV 1st and BAK 2nd. The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. Cell metabolic activity of HCEC was measured at various time points after exposure (0 h, 1 h, 2 h, 4 h, and 8 h). The results are presented in percentage of cell metabolic activity compared to the PBS control. Error Bars: ± SD. * represents: significantly different from the PBS control ($p < 0.05$). The results are based on the average of three experiments with four samples in each experiment.

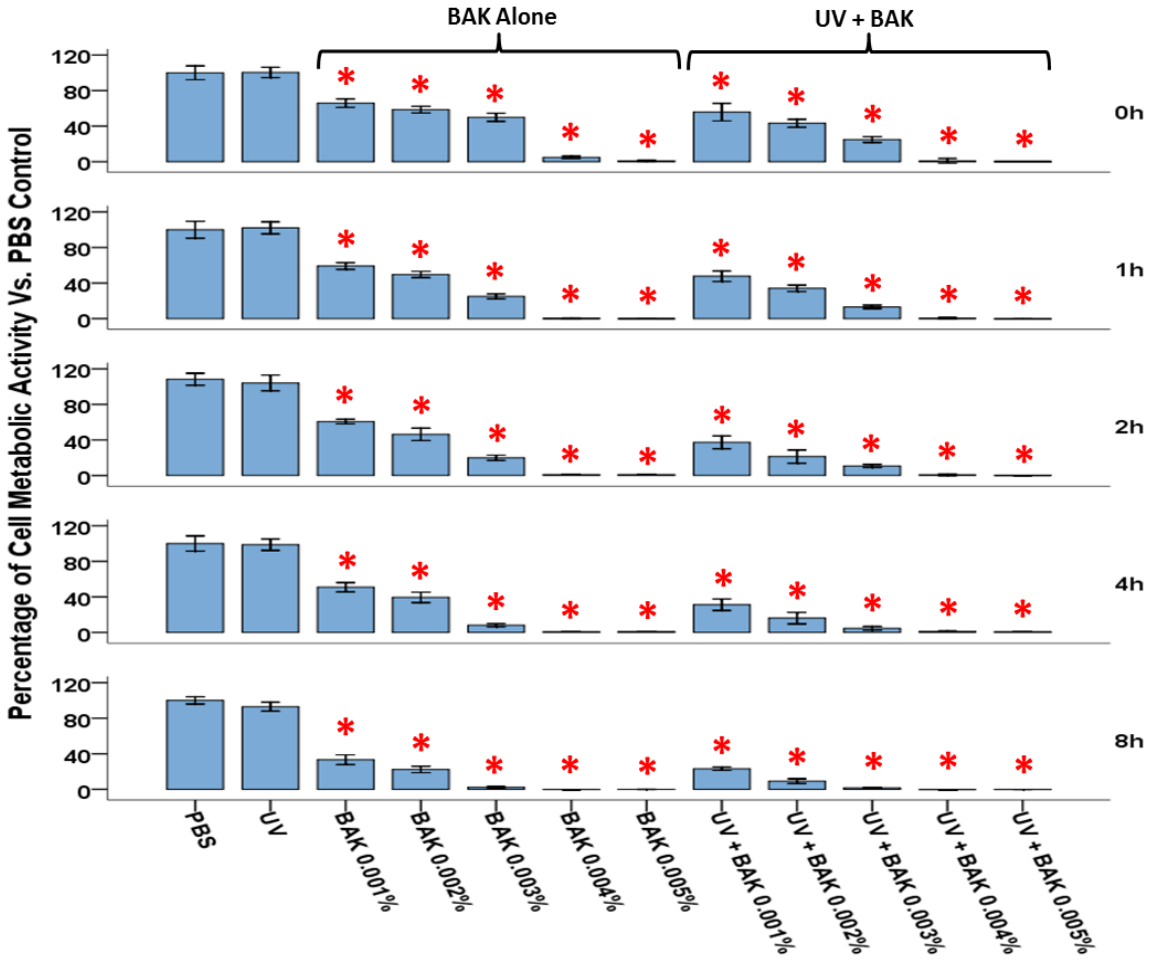

Figure 3-4. Cell metabolic activity measured with PrestoBlue_ BAK 1st and UV 2nd.

The treatment of UV + BAK was carried out by exposing the cultures to BAK 1st and UV 2nd. The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. Cell metabolic activity of HCEC was measured at various time points after exposure (0 h, 1 h, 2 h, 4 h, and 8 h). The results are presented in percentage of cell metabolic activity compared to the PBS control. Error Bars: ± SD. * represents: significantly different from the PBS control ($p < 0.05$). The results are based on the average of three experiments with four samples in each experiment.

Table 3-1. Observed and calculated combined effects of UV and BAK in PrestoBlue assay --- simultaneous exposure

BAK Concentration	UV Dose (J/cm²)	Test Time Point	Observed Metabolic Activity vs. Control (MA_{obs})		Calculated Metabolic Activity vs. Control (MA_{cal})		Difference (MA_{obs}- MA_{cal})	Significance ($p<0.05$)	Interactive Effect
			mean	SD	mean	SD			
0.001%	0.1719	0 h	68.40	7.32	73.09	6.55	-4.69	No	additive
	0.1719	1 h	47.71	10.50	68.06	9.68	-20.35	Yes	synergistic
	0.1719	2 h	34.70	7.72	59.47	10.54	-24.77	Yes	synergistic
	0.1719	4 h	30.09	6.45	52.76	6.86	-22.67	Yes	synergistic
	0.1719	8 h	18.35	6.78	38.21	11.68	-19.86	Yes	synergistic
0.002%	0.1719	0 h	48.47	5.59	59.04	4.65	-10.57	Yes	synergistic
	0.1719	1 h	31.38	4.95	42.45	4.14	-11.07	Yes	synergistic
	0.1719	2 h	25.71	5.11	36.19	3.64	-10.48	Yes	synergistic
	0.1719	4 h	17.34	2.33	32.83	3.08	-15.49	Yes	synergistic
	0.1719	8 h	3.24	1.54	18.83	3.62	-15.59	Yes	synergistic
0.003%	0.1719	0 h	24.78	5.32	48.16	4.30	-23.38	Yes	synergistic
	0.1719	1 h	9.46	4.74	21.99	1.94	-12.53	Yes	synergistic
	0.1719	2 h	4.47	2.56	13.71	2.03	-9.24	Yes	synergistic
	0.1719	4 h	1.60	1.26	7.86	1.76	-6.26	Yes	synergistic
	0.1719	8 h	0.40	0.20	1.28	0.17	-0.88	Yes	synergistic
0.004%	0.1719	0 h	4.95	1.81	12.77	3.65	-7.82	Yes	synergistic
	0.1719	1 h	1.56	0.60	3.47	1.88	-1.91	No	additive
	0.1719	2 h	0.71	0.29	1.73	0.55	-1.02	Yes	synergistic
	0.1719	4 h	0.26	0.25	0.5	0.24	-0.24	No	additive
	0.1719	8 h	0.16	0.27	0.38	0.24	-0.22	No	additive
0.005%	0.1719	0 h	1.69	0.29	2.06	0.18	-0.37	No	additive
	0.1719	1 h	0.78	0.64	1.03	0.48	-0.25	No	additive
	0.1719	2 h	0.57	0.18	1.1	0.38	-0.53	No	additive
	0.1719	4 h	0.12	0.05	0.58	0.45	-0.46	No	additive
	0.1719	8 h	0.33	0.27	0.59	0.18	-0.26	No	additive

Table 3-2. Observed and calculated combined effects of UV and BAK in PrestoBlue assay --- UV 1st and BAK 2nd

BAK Concentration	UV Dose (J/cm²)	Test Time Point	Observed Metabolic Activity vs. Control (MA_{obs})		Calculated Metabolic Activity vs. Control (MA_{cal})		Difference (MA_{obs}- MA_{cal})	Significance ($p<0.05$)	Interactive Effect
			mean	SD	mean	SD			
0.001%	0.1719	0 h	62.66	6.51	74.41	7.90	-11.75	No	additive
	0.1719	1 h	50.94	6.06	67.89	6.94	-16.95	Yes	synergistic
	0.1719	2 h	40.67	4.15	58.63	6.50	-17.96	Yes	synergistic
	0.1719	4 h	36.13	3.41	57.27	4.54	-21.14	Yes	synergistic
	0.1719	8 h	20.18	2.83	40.93	3.73	-20.75	Yes	synergistic
0.002%	0.1719	0 h	46.54	1.32	63.86	6.26	-17.32	Yes	synergistic
	0.1719	1 h	32.59	1.50	49.50	5.43	-16.91	Yes	synergistic
	0.1719	2 h	25.35	1.39	43.83	4.65	-18.48	Yes	synergistic
	0.1719	4 h	19.36	1.89	39.92	3.16	-20.56	Yes	synergistic
	0.1719	8 h	9.29	1.83	25.2	4.10	-15.91	Yes	synergistic
0.003%	0.1719	0 h	11.94	4.09	40.19	3.98	-28.25	Yes	synergistic
	0.1719	1 h	5.08	2.37	19.48	3.00	-14.40	Yes	synergistic
	0.1719	2 h	2.65	1.21	12.23	4.20	-9.58	Yes	synergistic
	0.1719	4 h	1.36	1.24	7.98	4.00	-6.62	Yes	synergistic
	0.1719	8 h	0.46	0.65	3.86	1.62	-3.40	Yes	synergistic
0.004%	0.1719	0 h	1.77	1.62	16.39	7.02	-14.62	Yes	synergistic
	0.1719	1 h	0.00	0.66	5.48	3.49	-5.48	Yes	synergistic
	0.1719	2 h	0.04	0.36	2.14	1.00	-2.10	Yes	synergistic
	0.1719	4 h	0.22	0.11	0.81	1.22	-0.59	No	additive
	0.1719	8 h	0.03	0.28	0.14	0.38	-0.11	No	additive
0.005%	0.1719	0 h	0.37	0.12	1.39	2.12	-1.02	No	additive
	0.1719	1 h	0.47	0.46	0.02	-0.68	0.45	No	additive
	0.1719	2 h	0.00	0.11	0.42	0.33	-0.42	No	additive
	0.1719	4 h	0.07	0.07	0.08	0.21	-0.01	No	additive
	0.1719	8 h	0.25	0.24	0.14	0.29	0.11	No	additive

Table 3-3. Observed and calculated combined effects of UV and BAK in PrestoBlue assay --- BAK 1st and UV 2nd

BAK Concentration	UV Dose (J/cm²)	Test Time Point	Observed Metabolic Activity vs. Control (MA_{obs})		Calculated Metabolic Activity vs. Control (MA_{cal})		Difference (MA_{obs}- MA_{cal})	Significance ($p<0.05$)	Interactive Effect
			mean	SD	mean	SD			
0.001%	0.1719	0 h	63.75	9.88	71.34	6.44	-7.59	No	additive
	0.1719	1 h	56.71	5.86	69.53	5.83	-12.82	Yes	synergistic
	0.1719	2 h	54.38	7.20	73.57	6.25	-19.19	Yes	synergistic
	0.1719	4 h	46.20	6.37	60.89	6.79	-14.69	Yes	synergistic
	0.1719	8 h	23.19	1.98	30.99	5.39	-7.80	Yes	synergistic
0.002%	0.1719	0 h	51.19	4.49	63.52	5.28	-12.33	Yes	synergistic
	0.1719	1 h	46.09	3.64	56.68	5.30	-10.59	Yes	synergistic
	0.1719	2 h	38.30	7.38	56.11	9.43	-17.81	Yes	synergistic
	0.1719	4 h	31.11	6.51	44.85	6.17	-13.74	Yes	synergistic
	0.1719	8 h	13.18	2.63	20.77	3.51	-7.59	Yes	synergistic
0.003%	0.1719	0 h	17.91	3.35	54.17	5.68	-36.26	Yes	synergistic
	0.1719	1 h	13.19	1.96	28.54	3.61	-15.35	Yes	synergistic
	0.1719	2 h	10.73	1.70	24.24	4.01	-13.51	Yes	synergistic
	0.1719	4 h	5.12	2.05	9.91	2.11	-4.79	Yes	synergistic
	0.1719	8 h	1.60	0.74	2.09	0.83	-0.49	No	additive
0.004%	0.1719	0 h	4.94	2.64	10.35	1.49	-5.41	Yes	synergistic
	0.1719	1 h	1.37	1.07	0.22	0.55	1.15	No	additive
	0.1719	2 h	0.70	0.94	2.50	0.52	-1.80	Yes	synergistic
	0.1719	4 h	1.12	0.67	0.95	0.24	0.17	No	additive
	0.1719	8 h	0.29	0.03	0.42	0.16	-0.13	No	additive
0.005%	0.1719	0 h	0.35	0.29	0.98	0.53	-0.63	No	additive
	0.1719	1 h	0.20	0.15	0.50	0.28	-0.30	No	additive
	0.1719	2 h	0.58	0.31	1.00	0.28	-0.42	No	additive
	0.1719	4 h	0.81	0.31	1.03	0.31	-0.22	No	additive
	0.1719	8 h	0.05	0.09	0.14	0.10	-0.09	No	additive

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3.4.2 Confocal microscopy study with fluorescent viability dyes

Cell viability of the cultures after various treatments was assessed using confocal microscopy with fluorescent viability dyes. The toxicity of treatments was illustrated by the distribution of live, dead and apoptotic cells in the cultures. Live healthy cells only stained with calcein AM (green); dead and necrotic cells were EthD-1 (red) - positive; apoptotic cells stained with both annexin V (yellow) and calcein AM (green). Figures 3-5 ~ 3-7 are representative confocal micrographs showing effects of the different treatments in various exposure orders. Similar results were observed in all the three exposure orders. BAK alone demonstrated a dose-dependent cell toxicity: the lowest concentration (0.001%) caused little cell death, while higher concentrations (0.004% and 0.005%) caused significant cell death (read) with obvious cell loss. UV alone had little effect on HCEC, showing similar cell viability compared to the PBS control. However, when combined with BAK, UV enhanced the toxicity of BAK, and together they caused significantly more cell death than BAK alone.

The number of live, dead, and apoptotic cells in each culture were counted and the percentage of live cells in each treatment was shown in Figure 3-8. The combination of UV and BAK caused significantly more cell death than UV and BAK individually ($p < 0.05$). The type of interaction existing between these two agents was evaluated and shown in Table 3-4. In all the three exposure orders, synergistic effects were found between UV and BAK 0.002%, 0.003%, and 0.004%; whereas, additive effects were shown between UV and BAK 0.001% and 0.005%.

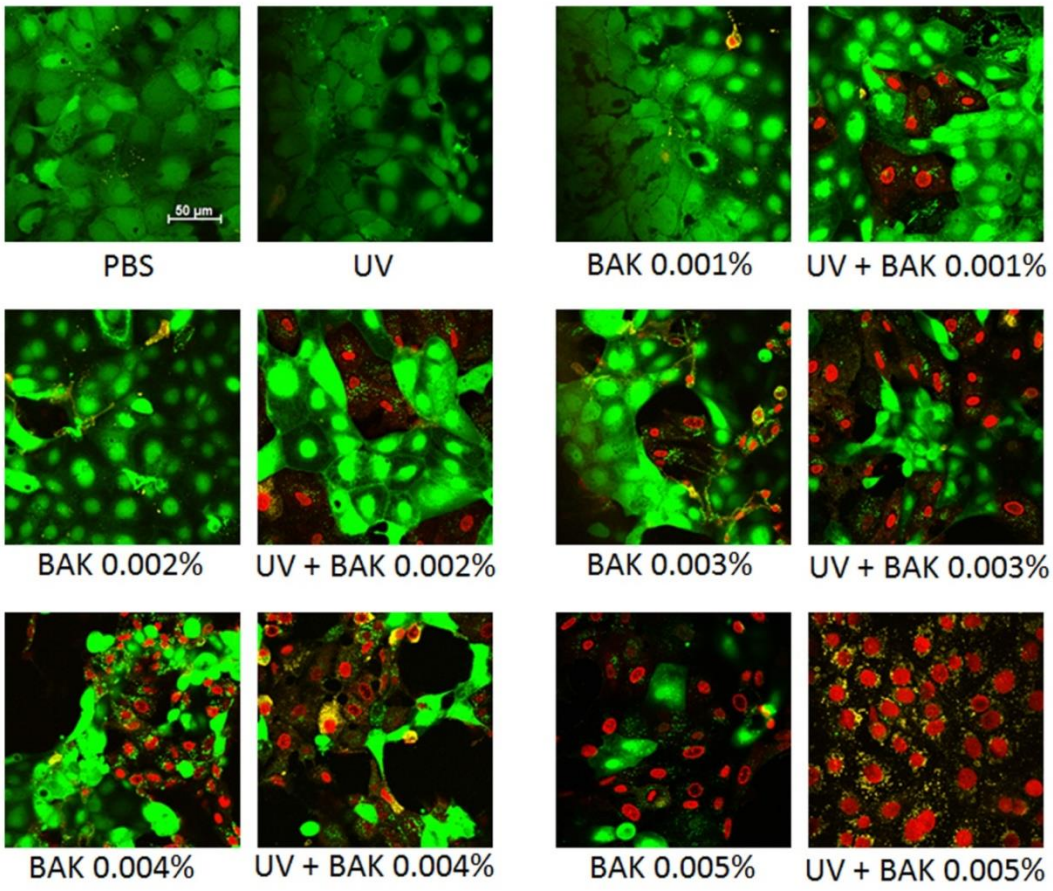

Figure 3-5. Representative confocal laser scanning micrographs of HCEC at 2 h after exposure_ UV and BAK simultaneously.

The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. The treatment of UV + BAK was carried out by exposing the cultures to UV and BAK simultaneously. The cell viability of HCEC after treatment is illustrated by the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein AM (green)-positive, annexin V (yellow)-negative, and EthD-1(red)-negative; dead cells are EthD-1 (red)-positive; apoptotic cells are annexin V (yellow)-positive and EthD-1 (red)-negative. All the micrographs were taken at the same magnification.

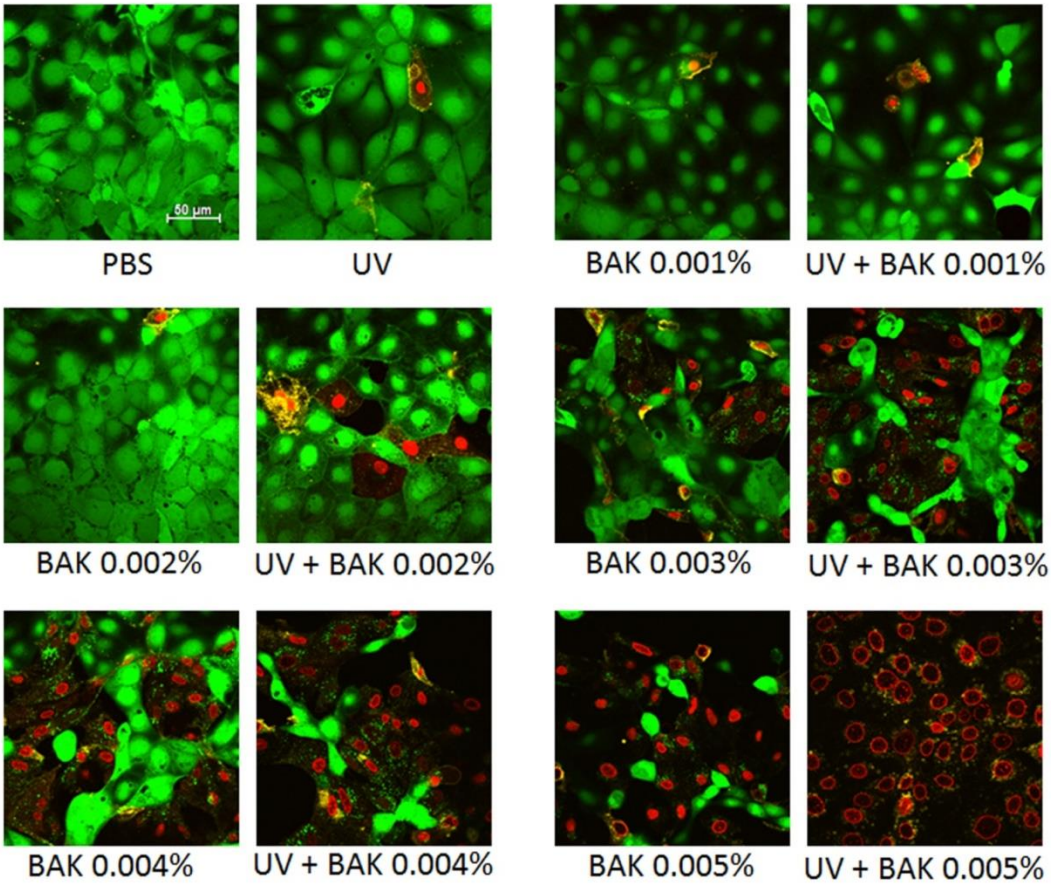

Figure 3-6. Representative confocal laser scanning micrographs of HCEC at 2 h after exposure_ UV 1st and BAK 2nd.

The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. The treatment of UV + BAK was carried out by exposing the cultures to UV 1st and BAK 2nd. The cell viability of HCEC after treatment is illustrated by the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein AM (green)-positive, annexin V (yellow)-negative, and EthD-1(red)-negative; dead cells are EthD-1 (red)-positive; apoptotic cells are annexin V (yellow)-positive and EthD-1 (red)-negative. All the micrographs were taken at the same magnification.

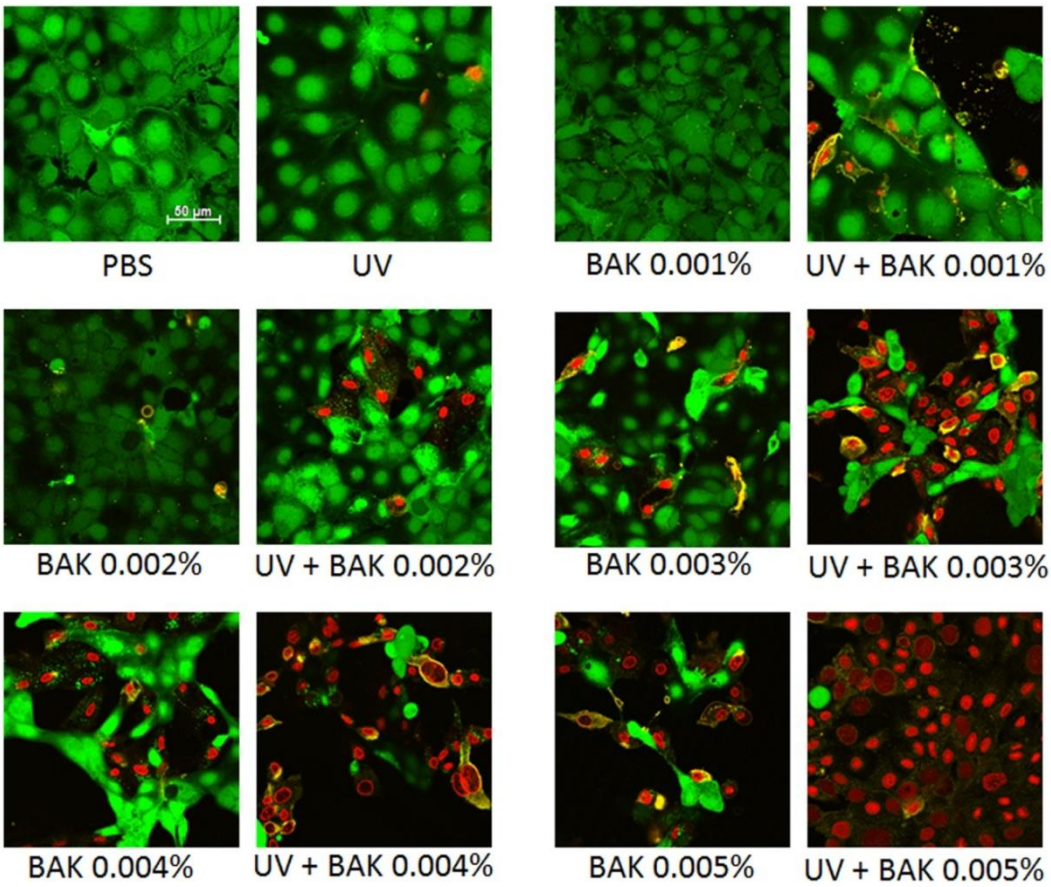

Figure 3-7. Representative confocal laser scanning micrographs of HCEC at 2 h after exposure_ BAK 1st and UV 2nd.

The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. The treatment of UV + BAK was carried out by exposing the cultures to BAK 1st and UV 2nd. The cell viability of HCEC after treatment is illustrated by the distributions of live, dead, and apoptotic cells in the culture. Live cells are calcein AM (green)-positive, annexin V (yellow)-negative, and EthD-1(red)-negative; dead cells are EthD-1 (red)-positive; apoptotic cells are annexin V (yellow)-positive and EthD-1 (red)-negative. All the micrographs were taken at the same magnification.

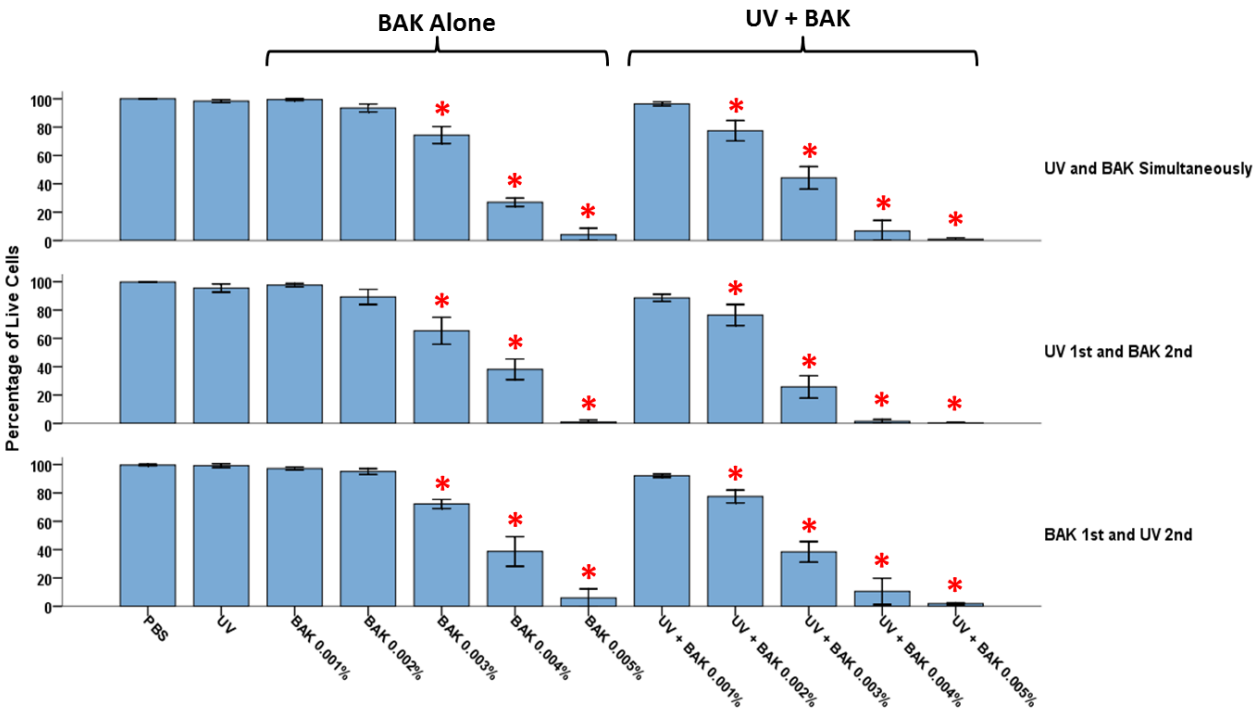

Figure 3-8. Percentage of live cells in the confocal laser scanning micrographs.

The UV dose used was 0.1719 J/cm ², and the BAK exposure time was 10 min. * represents: significantly different from the PBS control ($p < 0.05$). The results are based on the average of at least three experiments.

Table 3-4. Observed and calculated combined effects of UV and BAK in confocal microscopy study

Exposure Order	UV Dose (J/cm²)	BAK Concentration	Observed Percentage of Live Cells vs. Control (PLC_{obs})		Calculated Percentage of Live Cells vs. Control (PLC_{cal})		Difference (PLC_{obs}- PLC_{cal})	Significance ($p<0.05$)	Interactive Effect
			mean	SD	mean	SD			
UV and BAK simultaneously	0.1719	0.001%	96.39	1.46	97.81	1.34	-1.42	No	additive
	0.1719	0.002%	77.45	7.20	91.93	2.90	-14.48	Yes	synergistic
	0.1719	0.003%	44.21	7.92	73.16	5.94	-28.95	Yes	synergistic
	0.1719	0.004%	6.70	7.54	26.56	2.96	-19.86	Yes	synergistic
	0.1719	0.005%	0.80	1.12	4.06	4.48	-3.26	No	additive
UV 1st and BAK 2nd	0.1719	0.001%	88.59	2.53	93.19	3.03	-4.60	No	additive
	0.1719	0.002%	76.47	7.44	85.27	5.72	-8.80	Yes	synergistic
	0.1719	0.003%	25.78	7.91	62.41	9.29	-36.63	Yes	synergistic
	0.1719	0.004%	1.48	1.30	36.44	7.07	-34.96	Yes	synergistic
	0.1719	0.005%	0.00	0.00	0.86	1.49	-0.86	No	additive
BAK 1st and UV 2nd	0.1719	0.001%	92.13	1.40	96.49	2.95	-4.36	No	additive
	0.1719	0.002%	77.42	4.56	94.34	2.37	-16.92	Yes	synergistic
	0.1719	0.003%	38.43	7.22	71.64	3.32	-33.21	Yes	synergistic
	0.1719	0.004%	10.56	9.27	38.39	10.40	-27.83	Yes	synergistic
	0.1719	0.005%	1.91	0.62	5.87	6.27	-3.96	No	additive

3.4.3 Photoreactivity test

A photoreactivity test was conducted to see whether UV radiation acts on BAK and increases or decreases the cell toxicity of BAK. The cultures exposed to untreated and UV-treated BAK were measured for metabolic activity with PrestoBlue. The result is shown in Figure 3-9. In most of the concentrations at most of the time points, there was no significant difference in the cell toxicity between untreated BAK and UV-treated BAK. Whereas, UV-treated BAK showed slightly lower cell toxicity compared to untreated BAK in the following concentrations and time points: 0.001% and 0.002% at 8 h; 0.003% at 0 h, 1 h and 4 h; and 0.004% at 0 h and 1 h. The differences were small but statistically significant ($p = 0.001 \sim 0.016$).

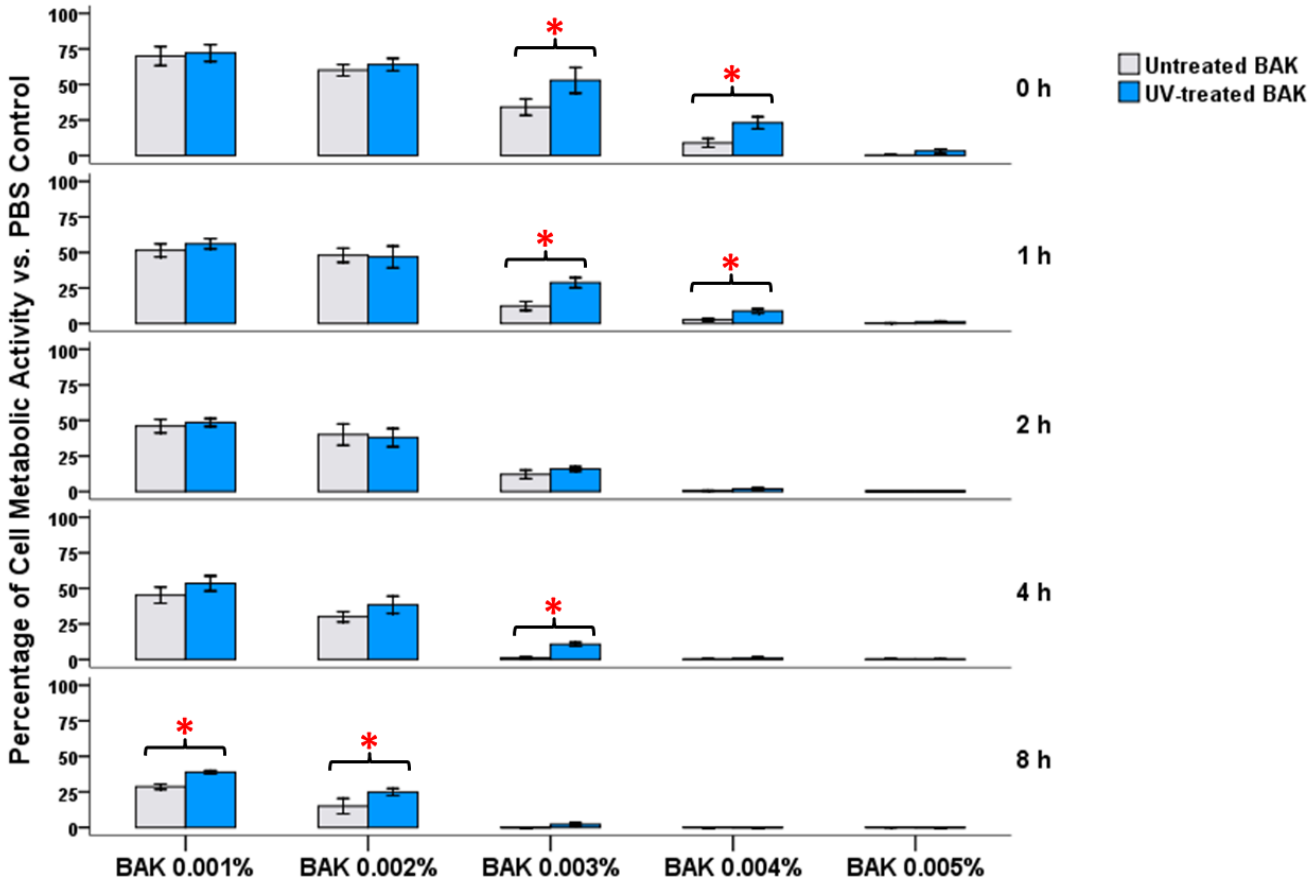

Figure 3-9. Comparison of untreated BAK and UV-treated BAK using PrestoBlue.

The UV dose used to treat BAK solutions was 0.1719 J/cm ². The effect of untreated and UV-treated BAK solutions on the metabolic activity of HCEC was measured at various time points after exposure (0 h, 1 h, 2 h, 4 h, and 8 h). The results are presented in percentage of cell metabolic activity vs. PBS control. Error Bars: ± SD. * represents: significantly different from each other (p = 0.001 ~ 0.016). The results are based on the average of three experiments.

3.5 Discussion

3.5.1 Background

In daily life, human eyes are exposed to sunlight and various chemicals, such as personal hygiene products, ocular and systemic mediations, air pollutants, make-up, and skin care products. The ocular toxicity of these potential toxic agents is dependent on the dose and time of exposure. During ocular safety testing the effect of each individual chemical is assessed and the additive or synergistic effects are measured if the chemicals are added into product formulations. As noted in the introduction, an additive effect occurs when the combined effect is equal to the sum of the individual effect; while a synergistic effect takes place when the combined effect is greater than the sum of the effects of each agent given alone. In most cases, FDA approved products are safe (below toxicity thresholds) when used as directed. However, product safety testing does not always include toxic reactions due to non-compliance to the product use procedures, contra-indications with other drugs used, or additional exposure to environmental conditions (such as UV exposure). It is important to assess the potential impact of co-exposures to multiple toxic agents, as the co-exposure may enhance individual toxicity and produce an effect that exceeds the threshold level. The eye is exposed to solar UV radiation in a daily basis; as yet, little attention has been paid to the combined effects of UV radiation and other toxic agents on the eye.

3.5.2 Combined toxicity of UV radiation and BAK

The present study investigated the combined toxicity of UV radiation and BAK on cultured HCEC. Our results indicated that synergistic effects can occur between a low dose of UV radiation (0.1719 J/cm^2) and low concentrations of BAK (0.001%-0.004%). This is the first demonstration of the synergistic effect between UV and BAK in human cells. In addition, additive effects between UV and BAK were also observed in our study. This is in accordance with one previous experiment, in which Withrow et al. described that the combined effect of UVA and BAK on mouse lymphoma cells appeared to be additive.[21]

3.5.3 Comparison of the results of PrestoBlue assay and confocal microscopy

In our study, the combined toxicity was evaluated with two different *in vitro* assays. In the PrestoBlue assay, the synergistic effects were mainly shown between UV and the lower concentrations of BAK: 0.001%, 0.002% and 0.003%. BAK 0.004% and 0.005% demonstrated extremely high individual toxic effects and reduced cell metabolic activity to no more than 5%; hence, it was difficult to observe any synergistic effects in these two concentrations. All three exposure orders (UV and BAK simultaneously, UV 1st and BAK 2nd, BAK 1st and UV 2nd) showed similar results, suggesting that the synergistic effects were independent of the exposure order. This independence was confirmed in the second assay: confocal microscopy with cell viability dyes. However, in the second assay, the synergistic effects were observed between UV and the higher concentrations of BAK 0.002%, 0.003% and 0.004%. One possible reason for this difference between two assays is that the PrestoBlue assay is more sensitive than the confocal microscopy. The PrestoBlue assay

measures cell metabolic activity;[30] whereas the confocal microscopy demonstrates cell viability (live, dead and apoptotic).[27,28,31] Low levels of toxicity decrease cell metabolic activity but do not cause cell death or apoptosis.

3.5.4 Photoreactivity test

A photoreactivity test was conducted to further investigate whether UV radiation acts on BAK and increases or decreases BAK toxicity. The result showed that, in most of the concentrations and most time points, the cell toxicities of UV-treated BAK and untreated BAK were similar. This finding, in conjunction with the results of the other two experiments in this study, suggested that very little interaction occurred between UV and BAK. This is not surprising, because the absorption of UV radiation by BAK is essential for any interaction to occur. BAK does not absorb at wavelengths > 290 nm,[21,32] while, the UV radiation source used in our study mainly emitted UVA and UVB (280 – 400 nm). As a result, the absorption of UV radiation by BAK was likely small. Nevertheless, in a few concentrations at some specific time points, UV-treated BAK showed slightly less cell toxicity compared to untreated BAK. The differences were small but statistically significant. It is likely that a photochemical reaction occurred along with the small absorption of UV radiation, and turned BAK into a less toxic substance.

3.5.5 Potential mechanism

Our study revealed that UV radiation and BAK can have a synergistic effect on HCEC. However, the mechanism for this effect is not clear. One possibility is a photosensitization

mechanism, as many chemicals are shown to have certain or potential photosensitizing effects on the eye.[33-36] These chemicals are photoreactive and can cause damage to the eye on the presence of light. Nevertheless, the following points would argue against this mechanism. First, if the photosensitization occurred, exposing the cells to BAK and UV simultaneously would generate stronger toxic effect than the exposures conducted in sequence. However, similar synergistic effects were observed in all the three different exposure orders. Also, in the photoreactivity test, UV-treated BAK had similar or slightly lower cell toxicity than untreated BAK, suggesting that UV reduced the toxicity of BAK instead of increasing it. Moreover, the absorption of UV radiation by BAK is essential for photosensitization to occur;[37] but as discussed above, the absorption was likely inconsequential. Thus, this mechanism is not well supported.

It is well known that BAK induces cell death through membrane destruction.[38,39] Also, BAK has been shown to cause DNA damage and cell apoptosis via the introduction of reactive oxygen species (ROS).[40,41] UV radiation is associated with photochemical damage to ocular tissues. UVB damages DNA directly, leading to the formation of pyrimidine dimers;[42] while UVA indirectly damages DNA through the production of oxygen radical species.[42] Experimental evidence also showed that UV radiation can decrease mitochondrial function[43] and induce apoptosis.[44] It is possible that UV and BAK enhanced each other's toxic effect through the combination of their different mechanisms. Further study is required to investigate the mechanism involved, as well as to confirm the significance of the synergistic effect *in vivo*.

3.5.6 Conclusion

In summary, our study indicates that co-exposure to UV radiation and BAK can cause synergistic and additive effects on human corneal epithelial cells. This finding highlights the importance of considering the combined ocular toxicity of solar radiation and BAK in the risk assessment of BAK-preserved ophthalmic solutions.

As noted in Chapter 3, UV (UVA and UVB)-irradiated BAK had similar or slightly lower cell toxicity than untreated BAK. Since BAK absorbs wavelengths < 290 nm, which falls mainly in UVC region, an appropriate dose of UVC radiation may be able to reduce the toxicity of BAK completely. The next chapter investigated whether the BAK toxicity on HCEC can be neutralized by UVC radiation.

Chapter 4

Neutralization of Benzalkonium Chloride Using UVC Radiation

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This chapter has been submitted for publication.

Authors' contributions

Author	Concept/Design	Data Collection	Data Analysis	Article Writing	Article Editing
Xu	✓	✓	✓	✓	✓
McCanna	✓				✓
Sivak	✓				✓

4.1 Overview

Purpose: To investigate the detoxification of benzalkonium chloride (BAK) by ultraviolet C (UVC) radiation.

Methods: BAK solutions were irradiated with a germicidal UVC lamp at various doses. Human corneal epithelial cells (HCEC) were then exposed to the UVC-irradiated BAK solutions for 5 minutes. After exposure, the cultures were assessed for metabolic activity using PrestoBlue; cell viability using confocal microscopy with viability dyes; and membrane integrity using immunofluorescence staining for zonula occludens (ZO)-1. The antimicrobial efficacy of UVC-irradiated BAK against *Pseudomonas aeruginosa (P. aeruginosa)* was also assessed. Phosphate buffered saline (PBS) was used as a negative control.

Results: BAK toxicity on cell metabolic activity was reduced by UVC radiation in a dose-dependent manner. When the solution depth of BAK was 1.7 millimeter, the UVC doses needed to completely neutralize the toxicity of BAK 0.005% and 0.01% were 2.093 J/cm^2 and 8.374 J/cm^2, respectively. Cell viability of the cultures treated with UVC-neutralized BAK was similar to the cultures treated with PBS. After exposure to PBS and UVC-neutralized BAK, the tight junction proteins ZO-1 were well maintained. In contrast, the expression of ZO-1 was greatly disturbed by untreated BAK. The antimicrobial effect of BAK against *P. aeruginosa* was reduced after UVC irradiation.

Conclusions: The cell toxicity of BAK can be neutralized by UVC irradiation. This finding provides a unique way of detoxifying BAK, and may be of great value in utilizing the antimicrobial efficacy of BAK while minimizing its potential hazards to human health and the environment.

4.2 Introduction

Benzalkonium chloride (BAK) is a group of quaternary ammonium compounds (QACs) that have an extremely wide range of applications, such as biocides, disinfectants, food additives, as well as preservatives in pharmaceutical and cosmetic products. Benzalkonium is composed by a positively charged nitrogen atom linked to a long alkyl chain (ranging from 8 to 18 carbon atoms) on one side, and a benzyl group on the other. The two last positions are occupied by methyl groups (Figure 4-1).[1] The key mechanism of BAK's antimicrobial action is membrane destruction, which is effective against bacteria, enveloped viruses, and some fungi, yeasts and protozoa.[2] Because of BAK's efficiency, stability and low cost, it has become a major non-alcohol-based active ingredient used for hospitals, food processing plants, and domestic household biocides.[3,4]

However, BAK has its limitations. Its toxicity has garnered much attention with widespread studies revealing its hazardous effects. It has been shown to cause allergic contact dermatitis,[5] eye irritations,[6] and ototoxicity in humans.[7] It is also a suspected genotoxicant due to its ability in causing DNA changes in respiratory epithelial cells *in vitro*.[8] With regard to ecological risk, BAK is categorized as highly toxic to fish, very highly toxic

to aquatic invertebrates, and moderately toxic to birds on an acute basis.[9] As a result, the potential hazards of BAK and its residues are causing human health and environmental concerns.

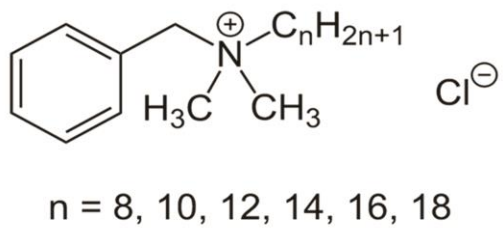

Figure 4-1. Molecular structure of benzalkonium chloride.

From Wikimedia Commons, the free media repository. File:Benzalkonium Chloride Structure V.1.svg

Due to the extensive applications of BAK and the accumulating evidence on its toxicity, considerable effort has been put into the development of methods to minimize BAK's toxicity. High molecular weight hyaluronan,[10] sodium hyaluronate (SH),[11] polyoxyethylene hydrogenated castor oil 40 (HCO-40) and polysorbate 80 (PS-80)[12] are a few of the chemicals that have been reported to have some protective effects against the ocular toxicity of BAK in ophthalmic solutions. Very little is reported in the literature on ways to minimize the toxicity of BAK in other applications such as cleaners and disinfectants in the healthcare and food processing industries. Ultraviolet (UV) radiation, as opposed to the chemical disinfectants, uses a physical process to inactivate microorganisms and does not generate any hazardous residues.[13] To the best of our knowledge, there is no report on the use of UVC radiation in mitigating the toxic effect of BAK.

The aim of this study was to investigate the detoxification of BAK using an *in vitro* toxicity model. For this purpose, BAK solutions were exposed to different doses of UVC radiation. Then the toxic effect of the UVC-irradiated BAK on HCEC was assessed with *in vitro* assays that measure cell metabolic activity, cell viability and tight junction proteins. The antimicrobial efficacy of UVC-neutralized BAK against *Pseudomonas aeruginosa (P. aeruginosa)* was also assessed.

4.3 Materials and Methods

4.3.1 Materials

Human corneal epithelial cells (HCEC) were obtained from RIKEN BioResource Center, Tsukuba, Japan (#RCB 2280). It is a SV40-transformed human corneal epithelial cell line that has good growth characteristics, develops good tight junctions, and is free of infectious virus particles.[14-16] DMEM/F-12 Ham Medium, fetal bovine serum, penicillin/streptomycin, Tryple Express, PrestoBlue, calcein AM, ethidium homodimer-1 (EthD-1), annexin V-Alexa Fluor 647 conjugate (annexin V), ZO-1 mouse monoclonal antibody, and Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) antibody were all purchased from Life Technologies Inc. (Burlington, ON). Cell culture plates (24-well plates) were obtained from BD Falcon (Franklin Lakes, NJ). Collagen coated glass bottom culture Petri dishes were from MatTek Corp (Ashland, MA). Polystyrene and polypropylene wells were purchased from VWR (Mississauga, ON).

P. aeruginosa was obtained from ATCC Rockville, MD, USA (ATCC 9027). Tryptic Soy Agar (TSA) was produced by Fisher Scientific (Pittsburgh, PA). Blood agar was from VWR (Mississauga, ON).

Benzalkonium chloride (BAK), 100% methanol, and Dey-Engley Neutralizing Broth were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was obtained from Lonza (Walkersville, MD).

4.3.2 Cell culture

HCEC were grown in DMEM/F-12 Ham Medium with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified environment at 37 ℃ with 5% CO_2. These cultures were maintained with weekly subculture using Tryple Express and fed every 2 to 3 days. To ensure consistency among experiments, the monolayers of HCEC were prepared with cultures that were less than 10 passages.

4.3.3 UV exposure

UV exposure was produced by one commercial germicidal UVC lamp (Light Spectrum Enterprises, Inc., Philadelphia, PA) in a custom designed UV irradiation unit at room temperature (22 ℃). Before irradiation, the spectrum and irradiance of UVC source was measured with an USB 2000+ fiber optic spectrometer (Ocean Optics, Inc. Dunedin, FL). The result is shown in Figure 4-2. The central wavelength was 253.7 nanometers (nm) and the calculated irradiance level was 2.9075 watts per square meter (W/m^2).

BAK solutions (0.005% and 0.01% in PBS) were transferred into sterile, flat bottom wells and irradiated for 10 min, 30 min, 1 h, 2 h, 4 h, and 8 h. The corresponding doses were 0.175, 0.523, 1.047, 2.093, 4.187 and 8.374 J/cm^2. The wells were covered with quartz to prevent evaporation during UV irradiation. The amount of BAK solution in each well was measured accordingly to achieve a specified solution depth. The solution depths used in our study were relatively thin in order to allow maximum interaction between UV radiation and BAK.

To assess how solution depth and well material affects the neutralization of BAK by UVC, two solution depths (1.7 mm and 3.4 mm) and two well materials (polystyrene and polypropylene) were tested. The treated BAK solutions were then tested on cells using PrestoBlue. To assess whether BAK toxicity changes depending on the time of assessment after UV exposure, each BAK solution was divided into two aliquots. The first aliquot was tested for its toxicity immediately (at 0 h) after UV exposure; the second aliquot was placed in the dark for 24 h after UV exposure, and then tested for its toxicity.

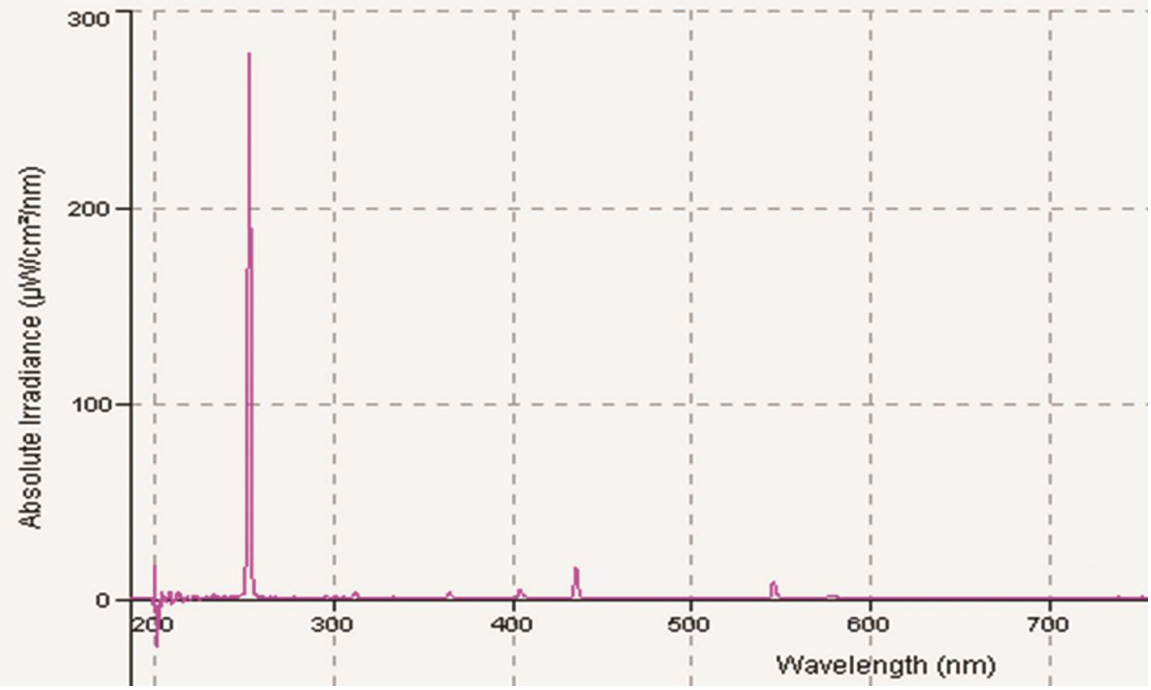

Figure 4-2. Spectrum of the UVC lamp measured with an USB 2000+ fiber optic spectrometer.

The UVC source used in our experiments was one commercial germicidal UVC lamp. The central wavelength was 253.7 nm; the irradiance was 2.9075 W/m ².

4.3.4 PrestoBlue assay

One milliliter (ml) of cell suspension containing 10^5 cells was seeded in 24-well plates and grown to approximately 75% to 80% confluent at 37 ℃ with 5% CO_2. The cells were then exposed to test solutions for 5 min. After exposure, the test solutions were removed and the cultures were rinsed with 1ml of PBS. One ml of 10% PrestoBlue reagent prepared in medium without serum and phenol red was then added to each well and the cultures were

incubated at 37 ℃ for 1 h. After incubation, the change in the fluorescence of PrestoBlue reagent (resazurin to resorufin) was measured using a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) with the excitation/emission wavelengths set at 560/590 nm.

4.3.5 Confocal microscopy study with fluorescent viability dyes

Three fluorescent viability dyes were used with confocal microscopy (Carl Zeiss LSM) to visualize live, dead, and apoptotic cells. They were calcein AM, EthD-1, and annexin V. Calcein AM produces an intense green fluorescence in live cells after reaction with intracellular enzymes; EthD-1 binds to the nuclei of dead and dying cells; annexin V stains the phosphatidylserine (PS) of apoptotic cells and dead cells.

One ml of culture medium containing 4×10^5 cells was transferred into collagen coated glass bottom culture Petri dishes and grown to confluence at 37 ℃ with 5% CO_2 for 2 days. The cultures were then exposed to test solution for 5 min. After exposure, the test solutions were removed, and the cultures were rinsed with 1ml of PBS. The cells were then stained with Annexin V [10 microliter (μl) in 500 μl buffer], calcein AM [2 micromolar (μM)], and EthD-1 (4 μM) for 20 min at 37 ℃. After staining, the fluorescence of the three dyes was visualized with an Axiovert 100 microscope with a Zeiss confocal laser scanning microscope (CLSM) 510 system. The excitation/emission wavelengths for calcein AM, EthD-1, and annexin V were 495/515 nm, 528/617nm, and 650/665 nm, respectively.

4.3.6 ZO-1 and Hoechst fluorescence staining

The distribution of tight junction protein zonula occludens-1 (ZO-1) was assessed by immunofluorescence staining with confocal scanning laser microscopy. ZO-1 mouse monoclonal antibody and Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) antibody were used as primary and secondary antibody, respectively. Hoechst 33342 was used for nuclear counterstaining.

One ml of culture medium containing 4×10^5 cells was transferred into collagen coated glass bottom culture Petri dishes and grown to confluence at 37 ℃ with 5% CO_2 for 2 days. The cultures were then exposed to test solution for 5 min. After exposure, the test solutions were removed, and the cultures were rinsed with 1ml of PBS. The cultures were then fixed with 100% methanol for 20 min at -20 ℃ and rinsed 4 times with PBS at room temperature (RT). Nonspecific antibody binding was blocked with 2% bovine serum albumin (BSA) in PBS (1 h, RT). For ZO-1 staining, the monolayers were first incubated with primary ZO-1 antibody (5 μg/ml in 2% BSA, 1 h, 37 ℃) followed by 4 rinses with PBS, and then incubated with secondary antibody Alexa Fluor® 488 Goat Anti-Mouse antibody (2 μg/ml in 2% BSA, 1h, 37 ℃) and rinsed 4 times with PBS again. For nuclear counterstaining, the cultures were incubated with Hoechst 33342 (10 μg/ml in clear medium, 15 min, 37 ℃) and washed twice with PBS. After staining, the samples were examined using an Axiovert 100 microscope with a Zeiss confocal laser scanning microscope (CLSM) 510 system. The excitation/emission wavelengths for Alexa Fluor® 488 Goat Anti-Mouse antibody and Hoechst 33342 were 495/520 nm and 355/460 nm, respectively.

4.3.7 Antimicrobial efficacy of BAK against *P. aeruginosa*

Preparation of bacterial suspensions: *P. aeruginosa* was regrown from frozen stocks onto a flask containing blood agar at 37 ℃ for 24 hours. The organism was harvested with 10 ml of PBS, centrifuged for 5 min at 490 relative centrifugal force (RCF) to a pellet, and re-suspended in PBS. A cell concentration of 1.0×10^8 colony forming units (CFU) per ml was used. To ensure a good degree of reproducibility, a Den-1 spectrophotometer (Grant Instruments, Cambridge, UK) was used with an optical density (OD) of 0.3 being equivalent to a bacterial concentration of 1.0×10^8 CFU/ml.

Measurement of antibacterial activity of BAK: An aliquot of 0.03 ml of bacterial suspension (1.0×10^8 CFU/ml) was added to each tube containing 3 ml of test solution. This produced a bacterial concentration of approximately 1.0×10^6 CFU/ml. The tube was vortexed to mix well and left in room temperature for 5 min. After the 5 min exposure, 1 ml of the tube solution was added to 1 ml of Dey-Engley Neutralizing Broth (DEB), mixed well and incubated for 15 min for neutralization. A continuous series of decimal dilutions of the neutralized solution were made in PBS until the concentration of bacteria reached the level of 1.0 CFU/ml. The viable count of bacteria in all the dilutions was assessed by preparation of triplicate plates of Tryptic Soy Agar (TSA). The plates were incubated at 37 ℃ for 72 h and then counted manually for CFU.

4.3.8 Statistical analysis

The experiments were performed with four replicates. One-way ANOVA was used in the data analysis of this study. Pairwise multiple comparison procedures were performed using the Bonferroni *post hoc* test. The criterion of statistical significance was set to be $p < 0.05$.

4.4 Results

4.4.1 Metabolic activity

The metabolic activity of HCEC after exposure to UVC-irradiated and untreated BAK was measured using PrestoBlue. The result is shown in Figure 4-3. This experiment was conducted with a solution depth of BAK set at 1.7 mm during UVC irradiation. The untreated BAK 0.005% and 0.01% reduced metabolic activity of HCEC to 20% and 0%, respectively, demonstrating significant cell toxicity after 5 min exposure. UVC irradiation lowered the toxicity of BAK in a dose-dependent manner. As the UVC dose increased, the toxicity of UVC-irradiated BAK decreased. The cultures treated by 2h UVC-irradiated BAK 0.005% and 8 h UVC-irradiated BAK 0.01% showed similar metabolic activity compared to those treated with the PBS control ($p = 1.000$ and 0.806, respectively). This suggests that when the solution depth of BAK was 1.7 mm, complete neutralization of BAK toxicity was achieved within 2 hours of UVC radiation (2.093 J/cm^2) for BAK 0.005% and within 8 hours of UVC radiation (8.374 J/cm^2) for BAK 0.01%. Therefore, under this condition, we refer to the 2 hour UVC-irradiated BAK 0.005% and the 8 hour UVC-irradiated BAK 0.01% as UVC-neutralized BAK.

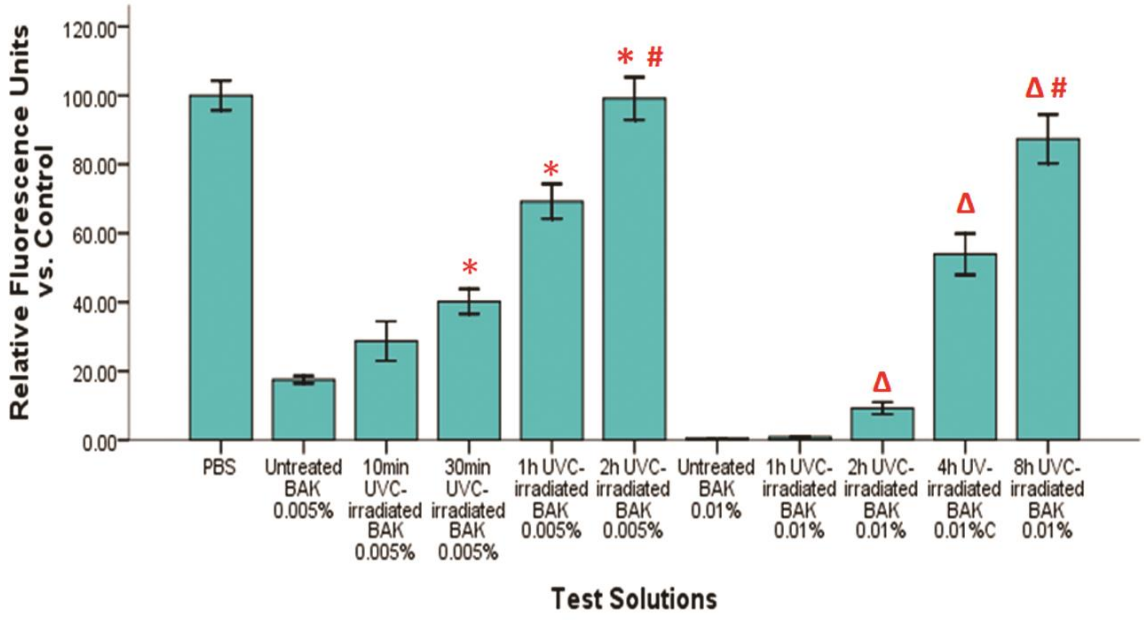

Figure 4-3. Effect of the test solutions on the metabolic activity of HCEC after 5 min exposure measured by PrestoBlue.

The BAK solutions were positioned in polystyrene wells with a solution depth of 1.7 mm while exposed to UVC radiation. The toxicity of the solutions was evaluated right after UVC irradiation. The results are presented in percentage of cell metabolic activity compared to the PBS control. Error Bars: ± SD. * Indicates significantly different from untreated BAK 0.005% ($p = 0.001 \sim 0.006$). Δ Indicates significantly different from untreated BAK 0.01% ($p = 0.001 \sim 0.017$). # Indicates not significantly different from the PBS control ($p = 1.000$ and 0.806, respectively).

To investigate the effect of solution depth of BAK, well material, and time of assessment on the UVC neutralization effect, two solution depths (1.7 mm and 3.4 mm), two well materials (polystyrene and polypropylene), and two assessment times (0 h and 24 h after UVC irradiation) were tested and compared in our study (Figure 4-4). When the solution depth of BAK increased from 1.7 mm to 3.4 mm, the cell toxicity of UVC-irradiated BAK 0.01% increased significantly ($p < 0.01$), while the cell toxicity of UVC-irradiated BAK 0.005% was not changed ($p = 0.922$) (Figure 4-4_Solution Depth). The results obtained with polystyrene wells and polypropylene wells were similar (Figure 4-4_Well Material, $p \geq 0.712$). Also, there was no significant difference between the cell toxicity tested at 0 h after UVC irradiation and that tested at 24 h after UVC irradiation (Figure 4-4_Measure Time, $p \geq 0.761$).

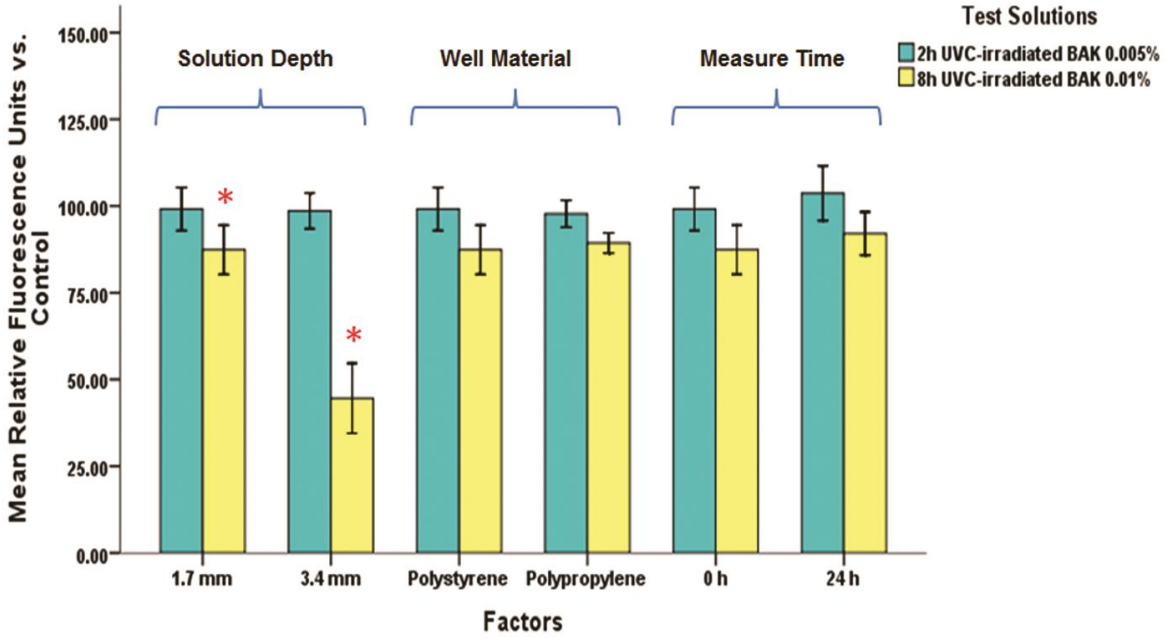

Figure 4-4. Toxicity of UVC-irradiated BAK under various UVC irradiation conditions and at different times of measurement.

The toxicity of the test solutions on HCEC was assessed with the PrestoBlue assay. BAK 0.005% and 0.01% were irradiated by UVC at two solution depths (1.7mm and 3.4 mm) with two well materials (polystyrene and polypropylene), and the toxicity of UVC-irradiated BAK was measured at 0 h and 24 h after UV irradiation. PBS was used as a negative control. The results are presented in percentage of cell metabolic activity compared to the control. Error Bars: ± SD. * Indicates significantly different from each other ($P < 0.001$).

The following experiments were based on the result of metabolic activity shown in Figure 4-3. In these experiments, we further investigated the effect of UVC-neutralized BAK on cell viability and tight junctions of HCEC, as well as its antimicrobial activity against *P. aeruginosa*.

4.4.2 Confocal microscopy study with fluorescent viability dyes

Confocal microscopy with fluorescent viability dyes (calcein AM, EthD-1 and annexin V) illustrated the cell viability of cultures by showing the distribution of live, dead, and apoptotic cells in the cultures. Figure 4-5 shows the representative confocal laser scanning micrographs of HCEC after 5 min exposure to the test solutions. The culture treated by PBS control showed approximately 100% cell alive (green). The untreated BAK demonstrated significant cell toxicity in a dose-dependent manner, with the untreated BAK 0.01% causing 100% cell death (red). The cells exposed to UVC-neutralized BAK 0.005% and 0.01% had similar cell viability to those exposed to PBS control, showing very few apoptotic cells (yellow) in the live cell (green) background.

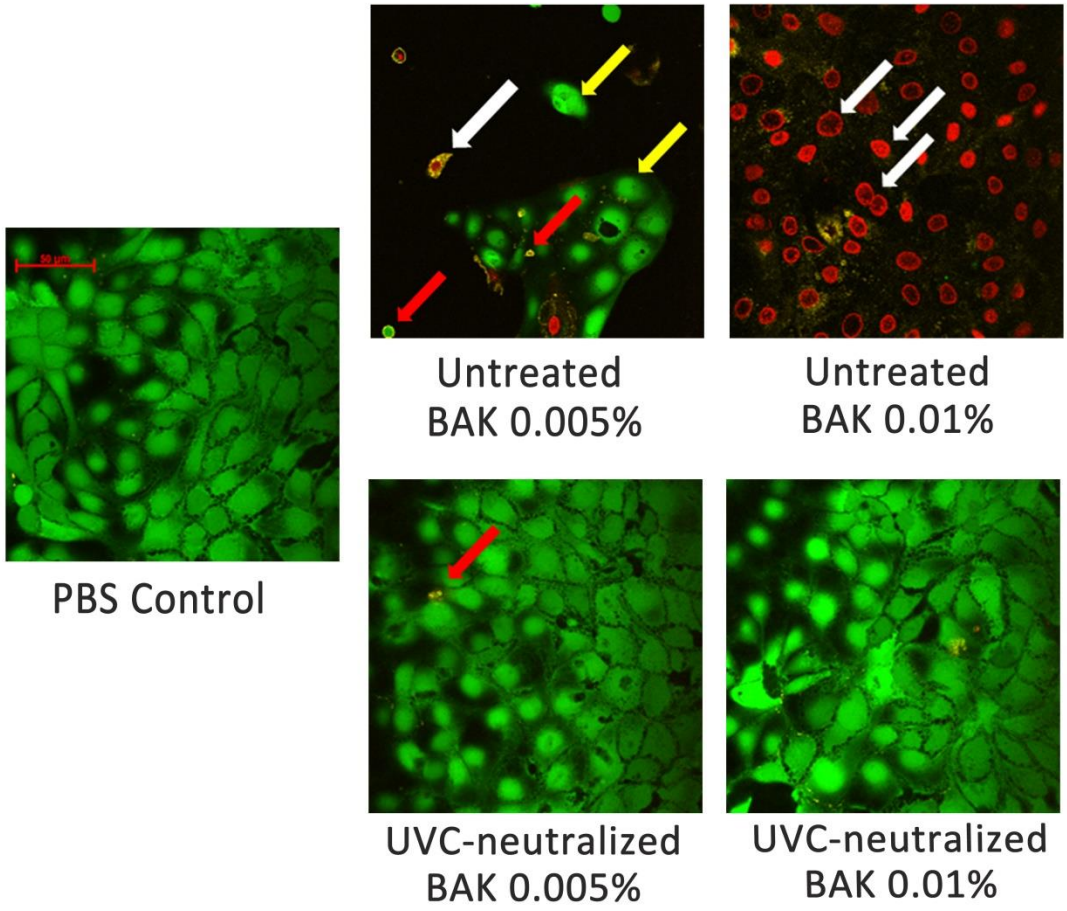

Figure 4-5. Effect of the test solutions on the cell viability of HCEC after 5 min exposure assessed by confocal microscopy with viability dyes.

Live cells are calcein AM (green)-positive, annexin V (yellow)-negative, and EthD-1(red)-negative; dead cells are EthD-1 (red)-positive; apoptotic cells are annexin V (yellow)-positive and EthD-1 (red)-negative. The yellow arrows indicate live cells; the red arrows show cells in apoptosis; the white arrows indicate dead cells. Bar = 50 μm. All the micrographs were taken at the same magnification. The UVC-neutralized BAK 0.005% and 0.01% were irradiated by UVC for 2 h and 8 h, respectively. The corresponding UVC doses were 2.093 J/cm^2 and 8.374 J/cm^2.

4.4.3 ZO-1 and Hoechst fluorescence staining

The effect of UVC-neutralized BAK on HCEC tight junctions was investigated by confocal microscopy with ZO-1 and Hoechst fluorescence staining. The distribution of tight junction proteins ZO-1 after 5 min exposure to the test solutions is shown in Figure 4-6. The culture exposed to PBS control showed intact cell junctions with regular distributions of ZO-1 on the cell borders. However, the distribution of ZO-1 proteins was significantly disturbed by the untreated BAK 0.005%, and was almost completely destroyed by the untreated BAK 0.01%. In contrast, the cultures treated by UVC-neutralized BAK exhibited a normal, continuous linear pattern of ZO-1 proteins along the cell-cell junctions, which was similar to the culture exposed to PBS control.

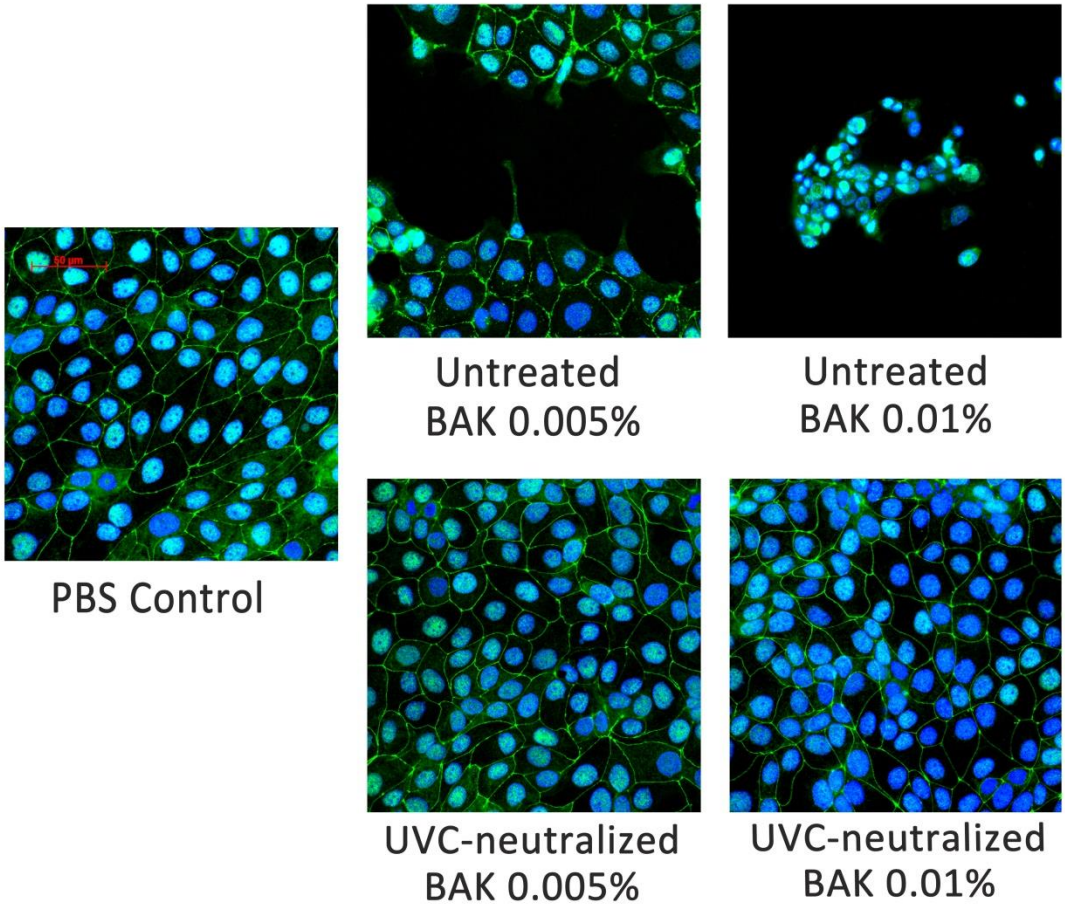

Figure 4-6. Effect of the test solutions on the distribution of tight junction proteins ZO-1 of HCEC after 5 min exposure.

ZO-1 proteins were labeled with Alexa Fluor 488 (green); nuclei were stained with Hoechst 33342 (blue). Bar = 50 μm. All the micrographs were taken at the same magnification. The UVC-neutralized BAK 0.005% and 0.01% were irradiated by UVC for 2 h and 8 h, respectively. The corresponding UVC doses were 2.093 J/cm^2 and 8.374 J/cm^2.

4.4.4 Antimicrobial efficacy against *P. aeruginosa*

The antimicrobial efficacy of UVC-neutralized BAK and untreated BAK against *P. aeruginosa* is shown in Figure 4-7. In a 5-min disinfection time, the untreated BAK 0.005% and 0.01% killed all the *P. aeruginosa.* In contrast, UVC- neutralized BAK 0.005% and 0.01% showed little antimicrobial efficacy with a great number of bacterial survivors. There was no significant difference in the number of bacterial survivors between UVC- neutralized BAK 0.005% and the PBS control (p = 1.000).

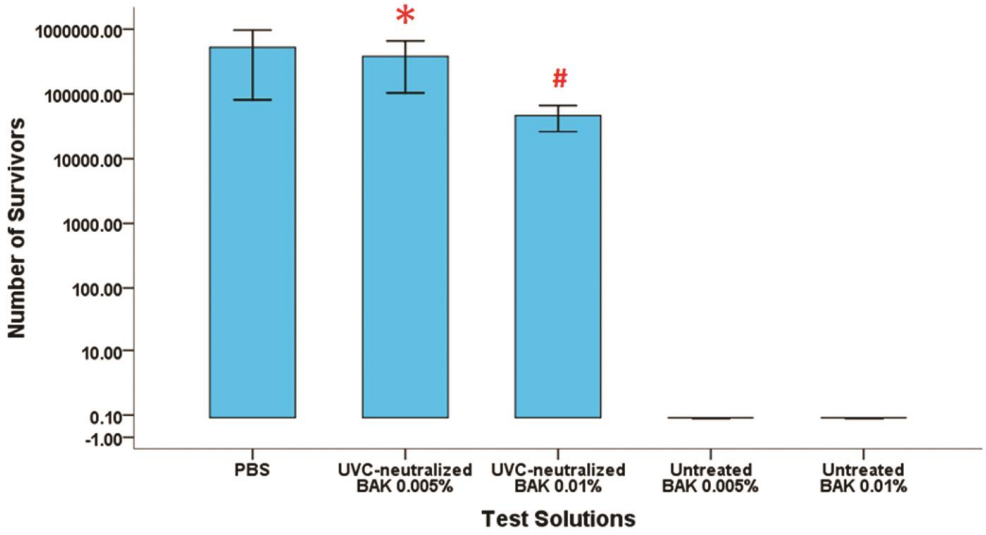

Figure 4-7. Number of survivors of *P. aeruginosa* after 5 min exposure to the test solutions.

Error Bars: ± SD. * Indicates significantly different from untreated BAK 0.005% and 0.01% (p < 0.001), but not significantly different from the PBS control (p = 1.000); # Indicates significantly different from the PBS control and untreated BAK 0.005% and 0.01% (p < 0.001). The UVC-neutralized BAK 0.005% and 0.01% were irradiated by UVC for 2 h and 8 h, respectively. The corresponding UVC doses were 2.093 J/cm^2 and 8.374 J/cm^2.

4.5 Discussion

Benzalkonium chloride (BAK) is extensively used in different disinfectant formulations; however, it is highly toxic to humans and the environment.[5-8,17] After being released to the environment, BAK residue is stable to microbial degradation, hydrolysis and direct photolysis by sunlight.[18] Therefore, substantial effort has been put into minimizing the potential hazards of BAK and its residues.

Considerable progress has been made in mitigating BAK toxicity in ophthalmic solutions. High molecular weight hyaluronan was found to significantly decrease oxidative stress, apoptosis and necrosis induced by BAK in human epithelial cells.[10] Latanoprost and travoprost were reported to protect conjunctiva-derived epithelial cells against BAK toxicity *in vitro*.[19] Polyoxyethylene hydrogenated castor oil 40 (HCO-40) and polysorbate 80 (PS-80) were shown to alleviate the corneal toxicity of BAK.[12] Topical application of sodium hyaluronate (SH) was proposed to reduce ocular toxicity induced by BAK-preserved latanoprost.[11]

Nevertheless, little research has been done on eliminating the toxic effect of BAK and its residues in other applications, such as disinfectants and cleaners in the health care and food processing industries. To the best of my knowledge, there is no report on utilizing UVC radiation to reduce the toxicity of BAK and its residues. The present study assessed the effect of UVC radiation on the toxicity and antimicrobial efficacy of BAK. The results demonstrated that BAK toxicity on HCEC can be completely eliminated with a proper dose

of UVC radiation, while the antimicrobial efficacy of BAK against *P. aeruginosa was* reduced simultaneously.

PrestoBlue assay is a fast and highly sensitive assay in evaluating cell metabolic activity.[20] The results of this assay demonstrated that the untreated BAK 0.01% and 0.005% were highly toxic to HCEC, and this toxicity was reduced by UVC radiation in a dose-dependent manner. Several factors (the solution depth of BAK, well material, and time of assessment) that may affect the neutralization effect were also assessed. The neutralization effect on BAK 0.01% was significantly reduced by increasing the solution depth of BAK from 1.7 mm to 3.4 mm; whereas, this did not happen to BAK 0.005%. On possible reason for this difference is: there were more BAK molecules at 0.01% in 3.4 mm depth, and thus a greater amount of UVC was needed for a complete neutralization. This indicates that the concentration and the solution depth of BAK should be taken into consideration when determining the dose of UVC radiation to be used. Different well materials (polystyrene vs. polypropylene) and different time of measurement (0 h vs. 24 h after UVC radiation) showed similar neutralization effect. This result suggests that the neutralization effect is not affected by well material, and the neutralized BAK solutions do not regain their toxicity at 24 hours after UVC radiation.

Based on the results of PrestoBlue assay, we further assessed the effect of UVC-neutralized BAK on the cell viability of HCEC using confocal microscopy with fluorescent viability dyes. The cultures exposed to untreated BAK showed significant decrease in cell viability in a dose-dependent manner. In contrast, the cultures treated by UVC-neutralized

BAK demonstrated similar cell viability to the PBS control. This result suggests that the toxic effect of BAK on the cell viability of HCEC was also neutralized.

Besides causing cell death, BAK is also known to destroy the barrier function of human corneal epithelium.[20-22] A complete neutralization of BAK toxicity should also preserve the barrier function of HCEC. Tight junctions are intercellular junctional structures that are essential for epithelial barrier function. The components of tight junctions include transmembrane proteins (occluding and claudin), junctional adhesion molecules, and cytoplasmic proteins [such as zonula occludens (ZO)-1, ZO-2, and ZO-3].[23] ZO-1 was the first protein localized to tight junctions[24] and has been used extensively as a measurement of tight junctions.[25,26] In the present study, we used immunofluorescence staining for ZO-1 to detect the damage of the barrier function caused by the test solutions. Intact cell junctions with regular distributions of ZO-1 were observed in the cultures exposed to PBS control and UVC-neutralized BAK 0.005% and 0.01%. In contrast, the expression of ZO-1 was greatly disturbed by the untreated BAK. This result further confirmed the neutralization effect observed in the assessments of metabolic activity and cell viability.

It is suspected that UVC radiation may simultaneously reduce the antimicrobial activity of BAK. Thus, the antimicrobial activity of UVC-neutralized BAK against *P. aeruginosa* was also tested. The result showed the UVC- neutralized BAK 0.005% and 0.01% had little antimicrobial efficacy against *P. aeruginosa* in a 5 min disinfection time. This indicates that UVC radiation reduces BAK activity in both corneal epithelium and bacteria.

Based on the results of this study, the toxicity of BAK can be completely neutralized by appropriate doses of UVC radiation. The mechanism of this neutralization is not clear. It is likely that BAK absorbs the energy from UVC and undergoes a photochemical reaction that turns BAK into a non-toxic substance. The photochemical reaction is a chemical reaction that initiated by the absorption of energy in the form of electromagnetic radiation and ends with the formation of stable products that are different from the original chemical(s). In a photochemical reaction, a molecule absorbs energy from electromagnetic radiation and is promoted to an exited electronic state, thereby initiating a sequence of events that continuously lower the energy of the excited molecule. These events can include a number of bond-breaking and/or bond-making processes.[27] The structure of BAK contains a benzyl group and a long alkyl chain (ranging from 8 to 18 carbon atoms).[28] Several possible processes may occur to BAK upon absorption of the UV radiation: fragmentation of the long alkyl chain, cycloadditions of unsaturated benzene ring, and substitution reactions of the benzyl group. In these processes, the excited BAK species can fall apart, change to new structures, combine with each other or other molecules, and hence result in the formation of new chemicals (stable final products). In the current study, the final products formed in the photochemical reaction were stable and non-toxic. Further studies are needed to analyze the final products and to understand the mechanisms involved in this neutralization effect.

BAK is particularly used by the food industry and hospitals for disinfecting surfaces and equipment.[29,30] In the food industry, residues of BAK have been recently reported in a broad variety of fruits and vegetables, exceeding the default statutory maximum residue level

(MRL) of 0.01 milligrams per kilogram (mg/kg) in many cases.[31] Conventional and organic produce were affected likewise. The residues might be caused by using BAK-containing disinfection products in food processing like washing, packaging and cleaning surfaces. In hospitals, BAK residues are also problematic. A few case reports have documented occupational asthma as a result of exposure to BAK.[32-34] Allergic contact dermatitis to BAK in health care workers have also been reported.[5] An even worse effect of BAK residues in hospitals is the development of bacterial resistance. A recent study demonstrated that the presence of BAK residues in polyethylene surfaces increased the bacteria's ability to adhere to the surfaces and to develop biofilms, thereby could enhance resistance to sanitation attempts.[35] Moreover, the concentration of BAK in hospital effluents and its associated environmental risk cannot be overlooked. Kummerer et al. analyzed the BAK in complex effluent samples from different hospitals and reported concentrations of BAK up to 6.03 mg/L.[30] An increasing numbers of articles that emphasize the environmental concern of BAK have been found in the literature.[28,36,37]

Based on the finding of this study, a disinfecting procedure using BAK and UVC in the food processing industries and hospitals may be of great value. BAK is a fast-acting biocidal agent that acts through membrane destruction.[2,38] UVC is a non-chemical process which is environmental friendly. Its germicidal effects involve photochemical damage to ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)[39] by dimerization of pyrimidine molecules.[40] With the combination of BAK and UVC, two different antimicrobial mechanisms are utilized to maximize the disinfection effect; simultaneously, UVC neutralizes BAK residues at the

end of the disinfecting procedure and thus eliminates BAK's potential hazards to human health and the environment. Therefore, this disinfecting procedure has a great potential to reduce the BAK residues in food products as well as to lower the bacterial disinfectant/drug resistance in hospitals.

In conclusion, the present study revealed that BAK toxicity can be eliminated with a proper dose of UVC radiation. This finding provides a unique way of detoxifying BAK, and may be of great value in utilizing the antimicrobial efficacy of BAK with minimal potential hazard to human health and the environment. Further studies are needed to understand the mechanisms involved in this neutralization effect, and to develop practical protocols for its potential applications.

General conclusions

As an alternative to animal ocular toxicity testing, an *in vitro* cell model using immortalized human corneal epithelial cells (HCEC) with different assays was developed. These assays include measurement of metabolic activity using PrestoBlue or alamarBlue, assessment of cell viability using confocal microscopy with fluorescent viability dyes, measurement of barrier function using sodium fluorescein, evaluation of tight junction proteins using immunofluorescence staining for zonula occludens (ZO)-1, and nuclear counterstaining with Hoechst 33342.

The results presented in chapter 1 clearly revealed that the tested commercial ophthalmic solutions were less cytotoxic with new preservative than with BAK; and without preservative than with new preservative. This finding suggests that BAK-free, especially preservative-free ophthalmic solutions are safer alternatives to BAK-preserved ones. This study also demonstrated that the *in vitro* test battery had high sensitivity and correlated well with *in vivo* and clinical studies.

The performances of three reagents – PrestoBlue, alamarBlue and MTT, in assessing cell viability of HCEC were compared in chapter 2. The result showed that PrestoBlue is more sensitive than MTT, but similar to alamarBlue. When fluorescence measurement was used, the plate color, reading mode and plate storage up to 7 days did not affect the performance of the PrestoBlue assay. This finding increases the flexibility of the PrestoBlue assay and offers more convenience to investigators.

Using the *in vitro* test battery with PrestoBlue, chapter 3 assessed the individual and combined toxicity of UV radiation and BAK on HCEC. The result proved that co-exposure to UV radiation and BAK can cause synergistic and additive effects on HCEC. This finding highlights the importance of considering the combined ocular toxicity of solar radiation and BAK in the risk assessment of BAK-preserved ophthalmic solutions.

Mitigating the effect of toxic agents is of great importance in protecting humans from possible hazards. Chapter 4 demonstrated that the cell toxicity of BAK can be completely neutralized by appropriate doses of UVC radiation. This finding provides a unique way of detoxifying BAK, and may be of great value in utilizing the antimicrobial efficacy of BAK while minimizing its potential hazards to human health and the environment.

In summary, the *in vitro* cell model developed in this research proved to be useful for assessing the corneal epithelial cell damage induced by ophthalmic solutions, BAK, and UV radiation; as well as for investigating the detoxification of BAK *in vitro*. This model may provide a sensitive and meaningful approach for evaluating both individual and combined ocular toxicity of UV and chemicals.

References

References to General Introduction

1. Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther*. 1944;82(3):377-390.

2. Youn HY, McCanna DJ, Sivak JG, Jones LW. In vitro ultraviolet-induced damage in human corneal, lens, and retinal pigment epithelial cells. *Mol Vis*. 2011;17:237-246.

3. Hoffman HM, Choi JH, Clousing DP, Ubels JL, McCarey BE, Edelhauser HF. Corneal epithelial testing strategies for safety evaluation of ophthalmic formulations. *Cutaneous Ocul Toxicol*. 2007;26(4):311-327.

4. Konynenbelt BJ, Mlnarik DS, Ubels JL. Effects of peroxide-based contact lens-disinfecting systems on human corneal epithelial cells in vitro. *Eye Contact Lens*. 2011;37(5):286-297.

5. Lim MJ, Hurst RK, Konynenbelt BJ, Ubels JL. Cytotoxicity testing of multipurpose contact lens solutions using monolayer and stratified cultures of human corneal epithelial cells. *Eye Contact Lens*. 2009;35(6):287-296.

6. Ward SL, Walker TL, Dimitrijevich SD. Evaluation of chemically induced toxicity using an in vitro model of human corneal epithelium. *TOXICOL VITRO*. 1997;11(1-2):121-139.

7. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36(3):614-621.

8. Offord EA, Sharif NA, Mace K, et al. Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. *Invest Ophthalmol Vis Sci*. 1999;40(6):1091-1101.

9. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: The good, the bad and the ugly. *Prog Retin Eye Res*. 2010;29(4):312-334.

10. Freeman PD, Kahook MY. Preservatives in topical ophthalmic medications: Historical and clinical perspectives. *Expert Rev Ophthalmol*. 2009;4(1):59-64.

11. Hommer A. A review of preserved and preservative-free prostaglandin analogues for the treatment of open-angle glaucoma and ocular hypertension. *Drugs Today*. 2010;46(6):409-416.

12. Baudouin C, de Lunardo C. Short term comparative study of topical 2% carteolol with and without benzalkonium chloride in healthy volunteers. *Br J Ophthalmol*. 1998;82(1):39-42.

13. Ishibashi T, Yokoi N, Kinoshita S. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. *J Glaucoma*. 2003;12(6):486.

14. Martone G, Frezzotti P, Tosi GM, et al. An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol*. 2009;147(4):725-735. e1.

15. Pfister RR, Burstein N. The effects of ophthalmic drugs, vehicles, and preservatives on corneal epithelium: A scanning electron microscope study. *Invest Ophthalmol*. 1976;15(4):246-259.

16. Freeman PD, Kahook MY. Preservatives in topical ophthalmic medications: Historical and clinical perspectives. *Expert Rev Ophthalmol*. 2009;4(1):59-64.

17. Burstein NL. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. *Invest Ophthalmol Vis Sci*. 1980;19(3):308-313.

18. Xu M, Sivak JG, McCanna DJ. Comparison of the effects of ophthalmic solutions on human corneal epithelial cells using fluorescent dyes. *J Ocul Pharmacol Ther*. 2013;29(9):794-802.

19. Ammar DA, Noecker RJ, Kahook MY. Effects of benzalkonium chloride- and polyquad-preserved combination glaucoma medications on cultured human ocular surface cells. *Adv Ther*. 2011;28(6):501-510.

20. Epstein SP, Ahdoot M, Marcus E, Asbell PA. Comparative toxicity of preservatives on immortalized corneal and conjunctival epithelial cells. *Journal of ocular pharmacology and therapeutics*. 2009;25(2):113-119.

21. Ayaki M, Iwasawa A. Cell viability of four corneoconjunctival cell lines exposed to five preservatives and a surfactant used for infection control in eyedrops. *Biocontrol Sci*. 2011;16(3):117-121.

22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1-2):55-63.

23. Ansar Ahmed S, Gogal Jr. RM, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [3H]thymidine incorporation assay. *J Immunol Methods*. 1994;170(2):211-224.

24. Byth H, Mchunu BI, Dubery IA, Bornman L. Assessment of a simple, non-toxic alamar blue cell survival assay to monitor tomato cell viability. *Phytochem Anal*. 2001;12(5):340-346.

25. Boncler M, Różalski M, Krajewska U, Podswdek A, Watala C. Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *J Pharmacol Toxicol Methods*. 2014;69(1):9-16.

26. Roberts JE. Ocular photoxicity. *J Photochem Photobiol B Biol*. 2001;64(2-3):136-143.

27. McCarty CA, Taylor HR. Recent developments in vision research: Light damage in cataract. *Invest Ophthalmol Vis Sci*. 1996;37(9):1720-1723.

28. Diffey BL. Sources and measurement of ultraviolet radiation. *Methods*. 2002;28(1):4-13.

29. Solomon KR. Effects of ozone depletion and UV-B radiation on humans and the environment. *Atmos Ocean*. 2008;46(1):185-202.

30. Cadet J, Berger M, Douki T, et al. Effects of UV and visible radiation on DNA - final base damage. *Biol Chem*. 1997;378(11):1275-1286.

31. Ravanat JL, Douki T, Cadet J. Direct and indirect effects of UV radiation on DNA and its components. *J Photochem Photobiol B Biol*. 2001;63(1-3):88-102.

32. Jou MJ, Peng TI, Hsu LF, et al. Visualization of melatonin's multiple mitochondrial levels of protection against mitochondrial ca 2+-mediated permeability transition and beyond in rat brain astrocytes. *J Pineal Res*. 2010;48(1):20-38.

33. Clydesdale GJ, Dandie GW, Muller HK. Ultraviolet light induced injury: Immunological and inflammatory effects. *Immunol Cell Biol*. 2001;79(6):547-568.

34. Halliwell B. Antioxidant defence mechanisms: From the beginning to the end (of the beginning). *Free Radic Res*. 1999;31(4):261-272.

35. Cullen AP. Ozone depletion and solar ultraviolet radiation: Ocular effects, a united nations environment programme perspective. *Eye Contact Lens*. 2011;37(4):185-190.

36. Suneja T, Belsito DV. Occupational dermatoses in health care workers evaluated for suspected allergic contact dermatitis. *Contact Derm*. 2008;58(5):285-290.

37. Hong J, Bielory L. Allergy to ophthalmic preservatives. *Current opinion in allergy and clinical immunology*. 2009;9(5):447-453.

38. Rizzo JÂ, Medeiros D, Silva AR, Sarinho E. Benzalkonium chloride and nasal mucociliary clearance: A randomized, placebo-controlled, crossover, double-blind trial. *Am J Rhinol*. 2006;20(3):243-247.

39. EPA Environmental Protection Agency. Reregistration eligibility decision for alkyl dimethyl benzyl ammonium chloride (ADBAC). prevention, pesticides and toxic substances (7510C). 2006;EPA 739-R-06-009.

40. Machado I, Gra ça J, Sousa AM, Lopes SP, Pereira MO. Effect of antimicrobial residues on early adhesion and biofilm formation by wild-type and benzalkonium chloride-adapted pseudomonas aeruginosa. *Biofouling*. 2011;27(10):1151-1159.

41. Pauloin T, Dutot M, Warnet JM, Rat P. In vitro modulation of preservative toxicity: High molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. *Eur J Pharm Sci*. 2008;34(4-5):263-273.

42. Yu F, Liu X, Zhong Y, et al. Sodium hyaluronate decreases ocular surface toxicity induced by benzalkonium chloride-preserved latanoprost: An in vivo study. *Invest Ophthalmol Vis Sci*. 2013;54(5):3385-3393.

43. Onizuka N, Uematsu M, Kusano M, Sasaki H, Suzuma K, Kitaoka T. Influence of different additives and their concentrations on corneal toxicity and antimicrobial effect of benzalkonium chloride. *Cornea*. 2014;33(5):521-526.

References to Chapter 1

1. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: The good, the bad and the ugly. *Prog Retin Eye Res*. 2010;29(4):312-334.

2. Noecker R, Miller KV. Benzalkonium chloride in glaucoma medications. *Ocul Surf*. 2011;9(3):159-162.

3. Noecker RJ, Herrygers LA, Anwaruddin R. Corneal and conjunctival changes caused by commonly used glaucoma medications. *Cornea*. 2004;23(5):490-496.

4. Noecker RJ, Herrygers LA, Anwaruddin R. Corneal and conjunctival changes caused by commonly used glaucoma medications. *Cornea*. 2004;23(5):490.

5. Swan KC. Reactivity of the ocular tissues to wetting agents. *Am J Ophthalmol*. 1944;27(10 PART 1):1118-1122.

6. Ichijima H, Petroll WM, Jester JV, Cavanagh HD. Confocal microscopic studies of living rabbit cornea treated with benzalkonium chloride. *Cornea*. 1992;11(3):221-225.

7. Burstein NL. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. *Invest Ophthalmol Vis Sci*. 1980;19(3):308-313.

8. Baudouin C, de Lunardo C. Short term comparative study of topical 2% carteolol with and without benzalkonium chloride in healthy volunteers. *Br J Ophthalmol*. 1998;82(1):39-42.

9. Ishibashi T, Yokoi N, Kinoshita S. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. *J Glaucoma*. 2003;12(6):486.

10. Martone G, Frezzotti P, Tosi GM, et al. An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol*. 2009;147(4):725-735. e1.

11. Debbasch C, Pisella PJ, De Saint Jean M, Rat P, Warnet JM, Baudouin C. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on chang conjunctival cells. *Invest Ophthalmol Vis Sci*. 2001;42(11):2525-2533.

12. Guenoun JM, Baudouin C, Rat P, Pauly A, Warnet JM, Brignole-Baudouin F. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46(12):4594-4599.

13. Pauly A, Brignole-Baudouin F, Guenoun JM, et al. Comparative study of topical anti-allergic eye drops on human conjunctiva-derived cells: Responses to histamine and IFNγ and toxicological profiles. *Graefe's Archive for Clinical and Experimental Ophthalmology*. 2007;245(4):534-546.

14. Pisella PJ, Debbasch C, Hamard P, et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: An ex vivo and in vitro study. *Invest Ophthalmol Vis Sci*. 2004;45(5):1360-1368.

15. Mundorf T, Wilcox KA, Ousler GW, Welch D, Abelson MB. Evaluation of the comfort of alphagan® p compared with alphagan® in irritated eyes. *Adv Ther*. 2003;20(6):329-336.

16. Dong JQ, Babusis DM, Welty DF, Acheampong AA, Tang-Liu D, Whitcup SM. Effects of the preservative purite® on the bioavailability of brimonidine in the aqueous humor of rabbits. *Journal of ocular pharmacology and therapeutics*. 2004;20(4):285-292.

17. Bernal DL, Ubels JL. Quantitative evaluation of the corneal epithelial barrier: Effect of artificial tears and preservatives. *Curr Eye Res*. 1991;10(7):645-656.

18. Labbe A, Pauly A, Liang H, et al. Comparison of toxicological profiles of benzalkonium chloride and polyquaternium-1: An experimental study. *J Ocul Pharmacol Ther*. 2006;22(4):267-278.

19. Garcia-Valenzuela E, Abdelsalam A, Eliott D, et al. Reduced need for corneal epithelial debridement during vitreo-retinal surgery using two different viscous surface lubricants. *Am J Ophthalmol*. 2003;136(6):1062-1066.

20. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36(3):614-621.

21. Kimura K, Teranishi S, Fukuda K, Kawamoto K, Nishida T. Delayed disruption of barrier function in cultured human corneal epithelial cells induced by tumor necrosis factor-α in a manner dependent on NF-kB. *Invest Ophthalmol Vis Sci*. 2008;49(2):565-571.

22. Toropainen E, Ranta VP, Talvitte A, Suhonen P, Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci*. 2001;42(12):2942-2948.

23. Kruszewski FH, Walker TL, DiPasquale LC. Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation. *Fundam Appl Toxicol*. 1997;36(2):130-140.

24. Pauly A, Meloni M, Brignole-Baudouin F, Warnet J, Baudouin C. Multiple endpoint analysis of the 3D-reconstituted corneal epithelium after treatment with benzalkonium chloride: Early detection of toxic damage. *Invest Ophthalmol Vis Sci*. 2009;50(4):1644-1652.

25. Youn HY, McCanna DJ, Sivak JG, Jones LW. In vitro ultraviolet-induced damage in human corneal, lens, and retinal pigment epithelial cells. *Mol Vis*. 2011;17:237-246.

26. Collin HB. Ultrastructural changes to corneal stromal cells due to ophthalmic preservatives. *Acta Ophthalmol*. 1986;64(1):72-78.

27. Herreras JM, Pastor JC, Calonge M, Asensio VM. Ocular surface alteration after long-term treatment with an antiglaucomatous drug. *Ophthalmology*. 1992;99(7):1082-1088.

28. Ayaki M, Iwasawa A, Inoue Y. Toxicity of antiglaucoma drugs with and without benzalkonium chloride to cultured human corneal endothelial cells. *Clinical Ophthalmology (Auckland, NZ)*. 2010;4:1217.

29. Brasnu E, Brignole-Baudouin F, Riancho L, Warnet JM, Baudouin C. Comparative study on the cytotoxic effects of benzalkonium chloride on the wong-kilbourne derivative of chang conjunctival and IOBA-NHC cell lines. *Molecular vision*. 2008;14:394.

30. Brignole-Baudouin F, Riancho L, Liang H, Baudouin C. Comparative in vitro toxicology study of travoprost polyquad-preserved, travoprost BAK-preserved, and latanoprost BAK-preserved ophthalmic solutions on human conjunctival epithelial cells. *Curr Eye Res*. 2011;36(11):979-988.

31. Eleftheriadis H, Cheong M, Sandeman S, et al. Corneal toxicity secondary to inadvertent use of benzalkonium chloride preserved viscoelastic material in cataract surgery. *Br J Ophthalmol*. 2002;86(3):299-305.

32. Epstein SP, Ahdoot M, Marcus E, Asbell PA. Comparative toxicity of preservatives on immortalized corneal and conjunctival epithelial cells. *Journal of ocular pharmacology and therapeutics*. 2009;25(2):113-119.

33. Jaenen N, Baudouin C, Pouliquen P, Manni G, Figueiredo A, Zeyen T. Ocular symptoms and signs with preserved and preservative-free glaucoma medications. *Eur J Ophthalmol*. 2007;17(3):341.

34. Pisella P, Pouliquen P, Baudouin C. Prevalence of ocular symptoms and signs with preserved and preservative free glaucoma medication. *Br J Ophthalmol*. 2002;86(4):418-423.

35. Yee RW, Norcom EG, Zhao XC. Comparison of the relative toxicity of travoprost 0.004% without benzalkonium chloride and latanoprost 0.005% in an immortalized human cornea epithelial cell culture system. *Adv Ther*. 2006;23(4):511-518.

36. Lipener C. A randomized clinical comparison of OPTI-FREE EXPRESS and ReNu MultiPLUS multipurpose lens care solutions. *Adv Ther*. 2009;26(4):435-446.

37. Ayaki M, Yaguchi S, Iwasawa A, Koide R. Cytotoxicity of ophthalmic solutions with and without preservatives to human corneal endothelial cells, epithelial cells and conjunctival epithelial cells. *Clin Experiment Ophthalmol*. 2008;36(6):553-559.

38. Bron A, Chiambaretta F, Pouliquen P, Rigal D, Rouland JF. Efficacy and safety of substituting a twice-daily regimen of timolol with a single daily instillation of nonpreserved beta-blocker in patients with chronic glaucoma or ocular hypertension. *J Fr Ophtalmol*. 2003;26(7):668-674.

39. Fields RD, Lancaster MV. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. *Am Biotechnol Lab*. 1993;11(4):48-50.

40. Poole CA, Brookes NH, Clover GM. Keratocyte networks visualised in the living cornea using vital dyes. *J Cell Sci*. 1993;106(2):685-691.

41. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ*. 1998;5(7):551.

42. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J Immunol Methods*. 1995;184(1):39-51.

43. Chuang EY, Li DQ, Bian F, Zheng X, Pflugfelder SC. Effects of contact lens multipurpose solutions on human corneal epithelial survival and barrier function. *Eye & contact lens*. 2008;34(5):281.

44. McCanna DJ, Harrington KL, Driot JY, Ward KW, Tchao R. Use of a human corneal epithelial cell line for screening the safety of contact lens care solutions in vitro. *Eye & contact lens*. 2008;34(1):6.

45. Tchao R, McCanna DJ, Miller MJ. Comparison of contact lens multipurpose solutions by in vitro sodium fluorescein permeability assay. *Eye & Contact Lens*. 2002;28(3):151.

46. Ward S, Walker T, Dimitrijevich S. Evaluation of chemically induced toxicity using an in vitro model of human corneal epithelium. *Toxicology in vitro*. 1997;11(1-2):121-129.

References to Chapter 2

1. Freire V, Andollo N, Etxebarria J, Durán JA, Morales M. In vitro effects of three blood derivatives on human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2012;53(9):5571-5578.

2. Jung KM, Lee SH, Ryu YH, et al. A new 3D reconstituted human corneal epithelium model as an alternative method for the eye irritation test. *Toxicol Vitro*. 2011;25(1):403-410.

3. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1-2):55-63.

4. Ubels JL, Clousing DP, Van Haitsma TA, et al. Pre-clinical investigation of the efficacy of an artificial tear solution containing hydroxypropyl-guar as a gelling agent. *Curr Eye Res*. 2004;28(6):437-444.

5. Van Goethem F, Adriaens E, Alépée N, et al. Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. *Toxicol Vitro*. 2006;20(1):1-17.

6. Ansar Ahmed S, Gogal Jr. RM, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [3H]thymidine incorporation assay. *J Immunol Methods*. 1994;170(2):211-224.

7. Byth H, Mchunu BI, Dubery IA, Bornman L. Assessment of a simple, non-toxic alamar blue cell survival assay to monitor tomato cell viability. *Phytochem Anal*. 2001;12(5):340-346.

8. Al-Nasiry S, Hanssens M, Luyten C, Pijnenborg R. The use of alamar blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod*. 2007;22(5):1304-1309.

9. Fields RD, Lancaster MV. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. *Am Biotechnol Lab*. 1993;11(4):48-50.

10. Lancaster MV, Fields RD, inventors*Antibiotic and Cytotoxic Drug Susceptibility Assays using Resazurin and Poising Agents.*. patent 5,501,959. 1996.

11. Yu HG, Chung H, Yu YS, Seo JM, Heo JW. A new rapid and non-radioactive assay for monitoring and determining the proliferation of retinal pigment epithelial cells. *Korean J Ophthalmol*. 2003;17(1):29-34.

12. Heredero-Bermejo I, Copa-Patiño JL, Soliveri J, Gómez R, De La Mata FJ, Pérez-Serrano J. In vitro comparative assessment of different viability assays in acanthamoeba castellanii and acanthamoeba polyphaga trophozoites. *Parasitol Res*. 2013;112(12):4087-4095.

13. Martín-Navarro CM, López-Arencibia A, Sifaoui I, et al. PrestoBlue® and AlamarBlue® are equally useful as agents to determine the viability of acanthamoeba trophozoites. *Exp Parasitol*. 2014;http://www.sciencedirect.com/science/article/pii/S0014489414000745:(Epub ahead of print).

14. Lall N, Henley-Smith CJ, De Canha MN, Oosthuizen CB, Berrington D. Viability reagent, prestoblue, in comparison with other available reagents, utilized in cytotoxicity and antimicrobial assays. *Int J Microbiol*. 2013:DOI: 10.1155/2013/420601.

15. Boncler M, Różalski M, Krajewska U, Podswdek A, Watala C. Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *J Pharmacol Toxicol Methods*. 2014;69(1):9-16.

16. Youn HY, McCanna DJ, Sivak JG, Jones LW. In vitro ultraviolet-induced damage in human corneal, lens, and retinal pigment epithelial cells. *Mol Vision*. 2011;17:237-246.

17. Xu M, Sivak JG, McCanna DJ. Comparison of the effects of ophthalmic solutions on human corneal epithelial cells using fluorescent dyes. *J Ocul Pharmacol Ther*. 2013;29(9):794-802.

18. Idziak M, Pedzisz P, Burdzinska A, Gala K, Paczek L. Uremic toxins impair human bone marrow-derived mesenchymal stem cells functionality in vitro. *Exp Toxicol Pathol*. 2014;66(4):187-194.

19. Tomankova K, Kolarova H, Pizova K, et al. Cytotoxicity and antioxidative effects of herbal and fruit extracts in vitro. *Food Biophys*. 2014;9(3):267-276.

20. McCanna DJ, Harrington KL, Driot JY, Ward KW, Tchao R. Use of a human corneal epithelial cell line for screening the safety of contact lens care solutions in vitro. *Eye Contact Lens*. 2008;34(1):6-12.

21. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnol Annu Rev*. 2005;11(SUPPL.):127-152.

22. D'Amore PA. Mechanisms of endothelial growth control. *Am J Respir Cell Mol Biol*. 1992;6(1):1-8.

23. Lampugnani MG, Dejana E. Interendothelial junctions: Structure, signalling and functional roles. *Curr Opin Cell Biol*. 1997;9(5):674-682.

24. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*. 2000;267(17):5421-5426.

References to Chapter 3

1. Baudouin C, de Lunardo C. Short term comparative study of topical 2% carteolol with and without benzalkonium chloride in healthy volunteers. *Br J Ophthalmol*. 1998;82(1):39-42.

2. Ishibashi T, Yokoi N, Kinoshita S. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. *J Glaucoma*. 2003;12(6):486.

3. Martone G, Frezzotti P, Tosi GM, et al. An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol*. 2009;147(4):725-735. e1.

4. Ichijima H, Petroll WM, Jester JV, Cavanagh HD. Confocal microscopic studies of living rabbit cornea treated with benzalkonium chloride. *Cornea*. 1992;11(3):221-225.

5. Burstein NL. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. *Invest Ophthalmol Vis Sci*. 1980;19(3):308-313.

6. Ammar DA, Noecker RJ, Kahook MY. Effects of benzalkonium chloride- and polyquad-preserved combination glaucoma medications on cultured human ocular surface cells. *Adv Ther*. 2011;28(6):501-510.

7. Epstein SP, Ahdoot M, Marcus E, Asbell PA. Comparative toxicity of preservatives on immortalized corneal and conjunctival epithelial cells. *J Ocul Pharmacol Ther*. 2009;25(2):113-119.

8. Ayaki M, Iwasawa A. Cell viability of four corneoconjunctival cell lines exposed to five preservatives and a surfactant used for infection control in eyedrops. *Biocontrol Sci*. 2011;16(3):117-121.

9. Liang H, Pauly A, Riancho L, Baudouin C, Brignole-Baudouin F. Toxicological evaluation of preservative-containing and preservative-free topical prostaglandin analogues on a three-dimensional-reconstituted corneal epithelium system. *Br J Ophthalmol*. 2011;95(6):869-875.

10. Noecker RJ, Herrygers LA, Anwaruddin R. Corneal and conjunctival changes caused by commonly used glaucoma medications. *Cornea*. 2004;23(5):490.

11. Debbasch C, Pisella PJ, De Saint Jean, et al. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on chang conjunctival cells. *Invest.Oph-thalmol.Vis.Sci.42*. 2001;42:2525-2533.

12. Guenoun JM, Baudouin C, Rat P, Pauly A, Warnet JM, Brignole-Baudouin F. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46(12):4594-4599.

13. Pauly A, Brignole-Baudouin F, Guenoun JM, et al. Comparative study of topical anti-allergic eye drops on human conjunctiva-derived cells: Responses to histamine and IFNγ and toxicological profiles. *Graefe's Archive for Clinical and Experimental Ophthalmology*. 2007;245(4):534-546.

14. Pisella P-, Debbasch C, Hamard P, et al. Conjunctival proinflammatory and proapoptic effects of latanoprost and preserved and unpreserved timolol: An ex vivo and in vivo study. *Invest Ophthalmol Vis Sci*. 2004;45(5):1360-1368.

15. Diffey BL. Sources and measurement of ultraviolet radiation. *Methods*. 2002;28(1):4-13.

16. Solomon KR. Effects of ozone depletion and UV-B radiation on humans and the environment. *Atmos Ocean*. 2008;46(1):185-202.

17. United Nations Environment Programme, Environmental Effects Assessment Panel. Environmental effects of ozone depletion and its interactions with climate change: Progress report, 2011. *Photochem Photobiol Sci*. 2012;11(1):13-27.

18. Cullen AP. Ozone depletion and solar ultraviolet radiation: Ocular effects, a united nations environment programme perspective. *Eye Contact Lens*. 2011;37(4):185-190.

19. Norval M, Cullen AP, De Gruijl FR, et al. The effects on human health from stratospheric ozone depletion and its interactions with climate change. *Photochem Photobiol Sci*. 2007;6(3):232-251.

20. Norval M, Lucas RM, Cullen AP, et al. The human health effects of ozone depletion and interactions with climate change. *Photochem Photobiol Sci*. 2011;10(2):199-225.

21. Withrow TJ, Brown NT, Hitchins VM, Strickland AG. Cytotoxicity and mutagenicity of ophthalmic solution preservatives and UVA radiation in L5178Y cells. *Photochem Photobiol*. 1989;50(3):385-389.

22. Lovely TJ, Levin DE, Klekowski E. Light-induced genetic toxicity of thimerosal and benzalkonium chloride in commercial contact lens solutions. *Mutat Res Genet Toxicol*. 1982;101(1):11-18.

23. Chang SW, Chi RF, Wu CC, Su MJ. Benzalkonium chloride and gentamicin cause a leak in corneal epithelial cell membrane. *Exp Eye Res*. 2000;71(1):3-10.

24. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36(3):614-621.

25. Kimura K, Teranishi S, Fukuda K, Kawamoto K, Nishida T. Delayed disruption of barrier function in cultured human corneal epithelial cells induced by tumor necrosis factor-α in a manner dependent on NF-kB. *Invest Ophthalmol Vis Sci*. 2008;49(2):565-571.

26. Toropainen E, Ranta VP, Talvitte A, Suhonen P, Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci*. 2001;42(12):2942-2948.

27. Poole CA, Brookes NH, Clover GM. Keratocyte networks visualised in the living cornea using vital dyes. *J Cell Sci*. 1993;106(2):685-691.

28. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ*. 1998;5(7):551.

29. Ince NH, Dirilgen N, Apikyan IG, Tezcanli G, Üstün B. Assessment of toxic interactions of heavy metals in binary mixtures: A statistical approach. *Arch Environ Contam Toxicol*. 1999;36(4):365-372.

30. Fields RD, Lancaster MV. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. *Am Biotechnol Lab*. 1993;11(4):48-50.

31. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J Immunol Methods*. 1995;184(1):39-51.

32. Lyman WJ, Reehl WF, Rosenblatt DH. *Handbook of chemical property estimation methods: Environmental behavior of organic compounds.* Washington, DC: American Chemical Society; 1990:8-12. http://www.osti.gov/scitech/biblio/6902382.

33. Dwivedi A, Mujtaba SF, Kushwaha HN, et al. Photosensitizing mechanism and identification of levofloxacin photoproducts at ambient UV radiation. *Photochem Photobiol*. 2012;88(2):344-355.

34. Hull DS, Csukas S, Green K. Rose bengal induced corneal swelling: Relation to inciting wavelength. *Curr Eye Res*. 1981;1(8):487-490.

35. Allen JE. Drug-induced photosensitivity. *Clin Pharm*. 1993;12(8):580-587.

36. Bagheri H, Lhiaubet V, Montastruc JL, Chouini-Lalanne N. Photosensitivity to ketoprofen. mechanisms and pharmacoepidemiological data. *Drug Saf*. 2000;22(5):339-349.

37. Dawe RS, Ibbotson SH. Drug-induced photosensitivity. *Dermatol Clin*. 2014;32(3):363-368.

38. Fazlara A, Ekhtelat M. The disinfectant effects of benzalkonium chloride on some important foodborne pathogens. *Am Eurasian J Agric Environ Sci*. 2012;12(1):23-29.

39. Russell AD. Antibiotic and biocide resistance in bacteria: Comments and conclusions. *J Appl Microbiol Symp Suppl*. 2002;92(1):171S-173S.

40. Wu H, Zhang H, Wang C, et al. Genoprotective effect of hyaluronic acid against benzalkonium chloride-induced DNA damage in human corneal epithelial cells. *Mol Vision*. 2011;17:3364-3370.

41. Debbasch C, Brignole F, Pisella P-, Warnet J-, Rat P, Baudouin C. Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on chang conjunctival cells. *Invest Ophthalmol Vis Sci*. 2001;42(3):642-652.

42. Cadet J, Douki T, Pouget JP, Ravanat JL, Sauvaigo S. Effects of UV and visible radiations on cellular DNA. *Curr Probl Dermatol*. 2001;29:62-73.

43. Jou MJ, Peng TI, Hsu LF, et al. Visualization of melatonin's multiple mitochondrial levels of protection against mitochondrial ca 2+-mediated permeability transition and beyond in rat brain astrocytes. *J Pineal Res*. 2010;48(1):20-38.

44. Clydesdale GJ, Dandie GW, Muller HK. Ultraviolet light induced injury: Immunological and inflammatory effects. *Immunol Cell Biol*. 2001;79(6):547-568.

References to Chapter 4

1. File:Benzalkonium chloride Structure V.1.svg. Wikimedia Commons Website. http://commons.wikimedia.org/wiki/File:Benzalkonium_chloride_Structure_V.1.svg Published November 19, 2013. Accessed January 3, 2015.

2. Fazlara A, Ekhtelat M. The disinfectant effects of benzalkonium chloride on some important foodborne pathogens. *Am Eurasian J Agric Environ Sci*. 2012;12(1):23-29.

3. Kampf G, Kramer A. Epidemiologic background of hand hygiene and evaluation of the most important agents for scrubs and rubs. *Clin Microbiol Rev*. 2004;17(4):863-893.

4. Mangalappalli-Illathu AK, Korber DR. Adaptive resistance and differential protein expression of salmonella enterica serovar enteritidis biofilms exposed to benzalkonium chloride. *Antimicrob Agents Chemother*. 2006;50(11):3588-3596.

5. Suneja T, Belsito DV. Occupational dermatoses in health care workers evaluated for suspected allergic contact dermatitis. *Contact Derm*. 2008;58(5):285-290.

6. Hong J, Bielory L. Allergy to ophthalmic preservatives. *Current opinion in allergy and clinical immunology*. 2009;9(5):447-453.

7. Rizzo JÂ, Medeiros D, Silva AR, Sarinho E. Benzalkonium chloride and nasal mucociliary clearance: A randomized, placebo-controlled, crossover, double-blind trial. *Am J Rhinol*. 2006;20(3):243-247.

8. Deutschle T, Porkert U, Reiter R, Keck T, Riechelmann H. In vitro genotoxicity and cytotoxicity of benzalkonium chloride. *Toxicology in vitro*. 2006;20(8):1472-1477.

9. EPA Environmental Protection Agency. Reregistration eligibility decision for alkyl dimethyl benzyl ammonium chloride (ADBAC). prevention, pesticides and toxic substances (7510C). . 2006;EPA 739-R-06-009.

10. Pauloin T, Dutot M, Warnet JM, Rat P. In vitro modulation of preservative toxicity: High molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. *Eur J Pharm Sci*. 2008;34(4-5):263-273.

11. Yu F, Liu X, Zhong Y, et al. Sodium hyaluronate decreases ocular surface toxicity induced by benzalkonium chloride-preserved latanoprost: An in vivo study. *Invest Ophthalmol Vis Sci*. 2013;54(5):3385-3393.

12. Onizuka N, Uematsu M, Kusano M, Sasaki H, Suzuma K, Kitaoka T. Influence of different additives and their concentrations on corneal toxicity and antimicrobial effect of benzalkonium chloride. *Cornea*. 2014;33(5):521-526.

13. Sobotka J. The efficiency of water treatment and disinfection by means of ultraviolet radiation. *Water Sci Technol*. 1993;27(3-4):343-346.

14. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36(3):614-621.

15. Kimura K, Teranishi S, Fukuda K, Kawamoto K, Nishida T. Delayed disruption of barrier function in cultured human corneal epithelial cells induced by tumor necrosis factor-α in a manner dependent on NF-kB. *Invest Ophthalmol Vis Sci*. 2008;49(2):565-571.

16. Toropainen E, Ranta VP, Talvitte A, Suhonen P, Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci*. 2001;42(12):2942-2948.

17. Baffa LdP, Ricardo, Jose Reinaldo da Silva, Dias AC, et al. Tear film and ocular surface alterations in chronic users of antiglaucoma medications. *Arq Bras Oftalmol*. 2008;71(1):18-21.

18. Lyman W, Reehl W, Rosenblatt D. Environmental behavior of organic compounds. In: WJ Lyman, WF Reehl, DH Rosenblatt, eds. *Handbook of chemical property estimation methods.* Washington, DC: American Chemical Society; 1990:8-12. http://hero.epa.gov/index.cfm/reference/details/reference_id/4237.

19. Guenoun JM, Baudouin C, Rat P, Pauly A, Warnet JM, Brignole-Baudouin F. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46(12):4594-4599.

20. Xu M, McCanna DJ, Sivak JG. Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells. *J Pharmacol Toxicol Methods*. 2015;71:1-7.

21. Xu M, Sivak JG, McCanna DJ. Comparison of the effects of ophthalmic solutions on human corneal epithelial cells using fluorescent dyes. *J Ocul Pharmacol Ther*. 2013;29(9):794-802.

22. Pauly A, Brasnu E, Riancho L, Brignole-Baudouin F, Baudouin C. Multiple endpoint analysis of BAC-preserved and unpreserved antiallergic eye drops on a 3D-reconstituted corneal epithelial model. *Mol Vision*. 2011;17:745-755.

23. Stevenson BR, Anderson JM, Bullivant S. The epithelial tight junction: Structure, function and preliminary biochemical characterization. *Mol Cell Biochem*. 1988;83(2):129-145.

24. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: A high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol*. 1986;103(3):755-766.

25. Kimura K, Teranishi S, Kawamoto K, Nishida T. Protective effect of dexamethasone against hypoxia-induced disruption of barrier function in human corneal epithelial cells. *Exp Eye Res*. 2011;92(5):388-393.

26. Chuang EY, Li DQ, Bian F, Zheng X, Pflugfelder SC. Effects of contact lens multipurpose solutions on human corneal epithelial survival and barrier function. *Eye & contact lens*. 2008;34(5):281.

27. Turro NJ, Lamola AA. Photochemistry. In: Smith KC, ed. *The science of photobiology*. New York NY: Plenum Press; 1977:63-86.

28. Van De Voorde A, Lorgeoux C, Gromaire MC, Chebbo G. Analysis of quaternary ammonium compounds in urban stormwater samples. *Environ Pollut*. 2012;164:150-157.

29. Kröckel L, Jira W, Wild D. Identification of benzalkonium chloride in food additives and its inefficacy against bacteria in minced meat and raw sausage batters. *Eur Food Res Technol*. 2003;216(5):402-406.

30. Kümmerer K, Eitel A, Braun U, et al. Analysis of benzalkonium chloride in the effluent from european hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. *J Chromatogr A*. 1997;774(1-2):281-286.

31. Hepperle J, Schüle E, Kolberg D, Scherbaum E. Determination of quaternary ammonium compound residues in fruits and vegetables by QuEChERS following LC-MS/MS analysis. . *Online Magazine for Analytical Laboratories*. 2014. http://www.analytic-news.com/papers/detail/435.html.

32. Purohit A, Kopferschmitt-Kubler MC, Moreau C, Popin E, Blaumeiser M, Pauli G. Quaternary ammonium compounds and occupational asthma. *Int Arch Occup Environ Health*. 2000;73(6):423-427.

33. Burge PS, Richardson MN. Occupational asthma due to indirect exposure to lauryl dimethyl benzyl ammonium chloride used in a floor cleaner. *Thorax*. 1994;49(8):842-843.

34. Innocenti A. Occupational asthma due to benzalkonium chloride. *Med Lav*. 1978;69(6):713-715.

35. Machado I, Graça J, Sousa AM, Lopes SP, Pereira MO. Effect of antimicrobial residues on early adhesion and biofilm formation by wild-type and benzalkonium chloride-adapted pseudomonas aeruginosa. *Biofouling*. 2011;27(10):1151-1159.

36. Kümmerer K. Antibiotics in the aquatic environment - A review - part II. *Chemosphere*. 2009;75(4):435-441.

37. Sarkar B, Megharaj M, Xi Y, Krishnamurti GSR, Naidu R. Sorption of quaternary ammonium compounds in soils: Implications to the soil microbial activities. *J Hazard Mater*. 2010;184(1-3):448-456.

38. Russell AD. Antibiotic and biocide resistance in bacteria: Comments and conclusions. *J Appl Microbiol Symp Suppl*. 2002;92(1):171S-173S.

39. Wolfe RL. Ultraviolet disinfection of potable water: Current technology and research needs. *Environ Sci Technol*. 1990;24(6):768-773.

40. Sinha RP, Häder DP. UV-induced DNA damage and repair: A review. *Photochem Photobiol Sci*. 2002;1(4):225-236.

References to Appendix A

1. Loewe S, Muischnek H. Effects of combinations: Mathematical basis of problem. *Archives of Experimental Pathology and Pharmacology*. 1926;114:313-326.

2. Bliss CI. The toxicity of poisons applied jointly. *Annuals of Applied Biology*. 1939;26(3):585-615. doi: DOI: 10.1111/j.1744-7348.1939.tb06990.x.

3. Plackett RL, Hewlett PS. Quantal responses to mixtures of poisons. *Journal of the Royal Statistical Society*. 1952;14(2):141-163.

4. Fraunfelder FW, Fraunfelder FT. Adverse ocular drug reactions recently identified by the national registry of drug-induced ocular side effects. *Ophthalmology*. 2004;111(7):1275-1279.

5. Santaella RM, Fraunfelder FW. Ocular adverse effects associated with systemic medications: Recognition and management. *Drugs*. 2007;67(1):75-93.

6. Shopsis C, Sathe S. Uridine uptake inhibition as a cytotoxicity test: Correlations with the draize test. *Toxicology* [- 3]. 1984;29:195-206.

7. Cometto-Muñiz JE, Cain WS, Hudnell HK. Agonistic sensory effects of airborne chemicals in mixtures: Odor, nasal pungency, and eye irritation. *Percept Psychophys*. 1997;59(5):665-674.

8. Hempel-Jorgensen A, Kjaergaard SK, Molhave L, Hudnell KH. Sensory eye irritation in humans exposed to mixtures of volatile organic compounds. *Arch Environ Health*. 1999;54(6):416-424.

9. Erdinger L, Kirsch F, Sonntag HG. Irritating effects of disinfection by-products in swimming pools. *Zentralbl Hyg Umweltmed*. 1998;200(5-6):491-503.

10. Chang SW, Chi RF, Wu CC, Su MJ. Benzalkonium chloride and gentamicin cause a leak in corneal epithelial cell membrane. *Exp Eye Res*. 2000;71(1):3-10.

11. Kikuchi T, Suzuki M, Kusai A, Iseki K, Sasaki H. Synergistic effect of EDTA and boric acid on corneal penetration of CS-088. *Int J Pharm*. 2005;290(1-2):83-89.

12. Blake DR, Winyard P, Lunec J, et al. Cerebral and ocular toxicity induced by desferrioxamine. *Q J Med*. 1985;56(219):345-355.

13. Durand-Cavagna G, Delort P, Duprat P, Bailly Y, Plazonnet B, Gordon LR. Corneal toxicity studies in rabbits and dogs with hydroxyethyl cellulose and benzalkonium chloride. *Fundam Appl Toxicol*. 1989;13(3):500-508.

14. Qian W, Amin RH, Shichi H. Cytotoxic metabolite of acetaminophen, N-acetyl-p-benzoquinone imine, produces cataract in DBA2 mice. *J Ocul Pharmacol Ther*. 1999;15(6):537-545.

15. Weinstein BI, Gordon GG, Southren AL. Potentiation of glucocorticoid activity by 5 beta-dihydrocortisol: Its role in glaucoma. *Science*. 1983;222(4620):172-173.

16. Mindel JS, Kharlamb AB, Friedman AH, Karam JH, Stone RD, Siegel IM. N-3-pyridylmethyl-N'-p-nitrophenylurea ocular toxicity in man and rabbits. *Br J Ophthalmol*. 1988;72(8):584-590.

17. Bolaji OM, Olabode OO. Modulating effect of aqueous extract of telfairia occidentalis on induced cyanide toxicity in rats. *Niger J Physiol Sci*. 2011;26(2):185-191.

18. Sahin A, Kürsat Cingü A, Kaya S, et al. The protective effects of caffeic acid phenethyl ester in isoniazid and ethambutol-induced ocular toxicity of rats. *Cutaneous Ocul Toxicol*. 2013;32(3):228-233.

19. Ling P. Effect of sodium hyaluronate in reducing adverse reaction of preservative in eye drops. *Chin Ophthalmic Res*. 2006;24(4):438-440.

20. Yu F, Liu X, Zhong Y, et al. Sodium hyaluronate decreases ocular surface toxicity induced by benzalkonium chloride-preserved latanoprost: An in vivo study. *Invest Ophthalmol Vis Sci*. 2013;54(5):3385-3393.

21. Yu FF, Liu X, Zhong YM, et al. Protection effect of sodium hyaluronate on ocular surface toxicity induced by benzalkonium chloride-preserved brimonidine in rabbits. *Zhonghua Yan Ke Za Zhi*. 2013;49(11):973-980.

22. Guenoun JM, Baudouin C, Rat P, Pauly A, Warnet JM, Brignole-Baudouin F. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46(12):4594-4599.

23. Pauloin T, Dutot M, Warnet JM, Rat P. In vitro modulation of preservative toxicity: High molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. *Eur J Pharm Sci*. 2008;34(4-5):263-273.

24. Onizuka N, Uematsu M, Kusano M, Sasaki H, Suzuma K, Kitaoka T. Influence of different additives and their concentrations on corneal toxicity and antimicrobial effect of benzalkonium chloride. *Cornea*. 2014;33(5):521-526.

25. Solomon KR. Effects of ozone depletion and UV-B radiation on humans and the environment. *Atmos Ocean*. 2008;46(1):185-202.

26. Dwivedi A, Mujtaba SF, Kushwaha HN, et al. Photosensitizing mechanism and identification of levofloxacin photoproducts at ambient UV radiation. *Photochem Photobiol*. 2012;88(2):344-355.

27. Glickman RD. Ultraviolet phototoxicity to the retina. *Eye Contact Lens*. 2011;37(4):196-205.

28. Roberts JE. Ocular photoxicity. *J Photochem Photobiol B Biol*. 2001;64(2-3):136-143.

29. Sharma S, Sharma OP, Singh B, Bhat TK. Biotransformation of lantadenes, the pentacyclic triterpenoid hepatotoxins of lantana plant, in guinea pig. *Toxicon*. 2000;38(9):1191-1202.

30. Hull DS, Csukas S, Green K. Rose bengal induced corneal swelling: Relation to inciting wavelength. *Curr Eye Res*. 1981;1(8):487-490.

31. Allen JE. Drug-induced photosensitivity. *Clin Pharm*. 1993;12(8):580-587.

32. Bagheri H, Lhiaubet V, Montastruc JL, Chouini-Lalanne N. Photosensitivity to ketoprofen. mechanisms and pharmacoepidemiological data. *Drug Saf*. 2000;22(5):339-349.

33. To T-H, Townsend JC. Ocular toxicity of systemic medications: A case series. *Optom*. 2000;71(1):29-39.

34. Ehrenshaft M, Roberts JE, Mason RP. Hypericin-mediated photooxidative damage of alpha-crystallin in human lens epithelial cells. *Free Radic Biol Med*. 2013;60:347-354.

35. Lerman S. Ocular phototoxicity and psoralen plus ultraviolet radiation (320-400 nm) therapy: An experimental and clinical evaluation. *J Natl Cancer Inst*. 1982;69(1):287-302.

36. Barker FM, Dayhaw-Barker P, Forbes PD, Davies RE. Ocular effects of treatment with various psoralen derivatives and ultraviolet-A (UVA) radiation in HRA/Skh hairless mice. *Acta Ophthalmol (Copenh)*. 1986;64(4):471-478.

37. Hull DS, Csukas S, Green K. Chlorpromazine-induced corneal endothelial phototoxicity. *Invest Ophthalmol Vis Sci*. 1982;22(4):502-508.

38. Hull DS, Csukas S, Green K. Trifluoperazine: Corneal endothelial phototoxicity. *Photochem Photobiol*. 1983;38(4):425-428.

39. Hull DS, Green K, Hampstead D. Effect of hematoporphyrin derivative on rabbit corneal endothelial cell function and ultrastructure. *Invest Ophthalmol Vis Sci*. 1985;26(11):1465-1474.

40. Laycock NL, Schirmer K, Bols NC, Sivak JG. Optical properties of rainbow trout lenses after in vitro exposure to polycyclic aromatic hydrocarbons in the presence or absence of ultraviolet radiation. *Exp Eye Res*. 2000;70(2):205-214.

41. Withrow TJ, Brown NT, Hitchins VM, Strickland AG. Cytotoxicity and mutagenicity of ophthalmic solution preservatives and UVA radiation in L5178Y cells. *Photochem Photobiol*. 1989;50(3):385-389.

42. Zuclich JA, Griess GA, Harrison JM, Brakefield JC. *Research on the ocular effects of laser radiation.* USAF School of Aerospace Medicine: Technology Inc.; 1979.

43. Jiang Q, Cao C, Zhou C, et al. Quercetin attenuates UV- and H(2)O(2)-induced decrease of collagen type I in cultured human lens epithelial cells. *J Ocul Pharmacol Ther*. 2008;24(2):164-174.

44. Cao X-, Li X-, Bao Y-, Xing N-, Chen Y. Responses of human lens epithelial cells to quercetin and DMSO. *Invest Ophthalmol Visual Sci*. 2007;48(8):3714-3718.

45. Pauloin T, Dutot M, Joly F, Warnet J-, Rat P. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. *Molecular Vision*. 2009;15:577-583.

46. Fursova AZ, Gesarevich OG, Gonchar AM, Trofimova NA, Kolosova NG. Dietary supplementation with bilberry extract prevents macular degeneration and cataracts in senesce-accelerated OXYS rats. *Adv Gerontol*. 2005;16:76-79.

47. Anthrayose CV, Shashidhar S. Studies on protein and taurine in normal, senile and diabetic cataractous human lenses. *Indian J Physiol Pharmacol*. 2004;48(3):357-360.

48. Aydin B, Yagci R, Yilmaz FM, et al. Prevention of selenite-induced cataractogenesis by N-acetylcysteine in rats. *Curr Eye Res*. 2009;34(3):196-201.

Appendix A.
The Combined Ocular Toxicity of Chemical Mixtures and Chemicals plus UV Radiation

Overview

The human eye is often exposed both to sunlight and to variable combinations of different chemical compounds. Co-exposure to various toxic agents may produce a response that is equal, greater to or less than the sum of the individual toxicity. There are four kinds of toxicologic interactions between chemicals: additive effect, synergy, potentiation, and antagonism. Additive ocular toxicity has been shown in the combinations of xenobiotic chemicals with different functional groups. Exposure to disinfectants and their by-products can cause synergistic eye irritation. For example, certain viscosity-enhancing agents in ophthalmic solutions can potentiate benzalkonium chloride (BAK) - induced corneal damage. Antagonism has been used as the basis of many antidotes for poisons. Ozone depletion has increased the amount of solar ultraviolet (UV) radiation reaching the Earth's surface, enhancing the risk of phototoxicity to the eye. When the eye is exposed to chemicals in the presence of UV radiation, some chemicals absorb the light, become excited to reactive states and provoke phototoxicity through a type 1 or type 2 reaction pathway; others do not react with the light but might produce stronger ocular toxicity when combined with UV; and certain agents such as antioxidants can protect the eye from UV damage. Potential additive and synergistic effects on the eye produced by exposure to chemical mixtures, or chemical plus UV radiation should always be considered in risk assessment procedures.

1. Introduction

In daily life, human eyes are exposed to complex and variable combinations of different chemical compounds (i.e. personal hygiene products, air pollutants, make-up, skin care products, ocular and systemic mediations, etc). In most cases, exposures to each compound are below the toxic threshold level. However, interaction among chemicals may modify the magnitude and sometimes the nature of the toxic effect, producing stronger or weaker toxicity to the eye. Chemical interaction might occur in the toxicokinetic phase (such as processes of uptake, distribution, metabolism, biotransformation, and excretion) or in the toxicodynamic phase (such as effects of chemicals on receptors, cellular targets, or organs).

Recently, the amount of solar ultraviolet (UV) radiation reaching the Earth's surface has been increased due to the destruction of the ozone layer, posing an enhanced risk of phototoxicity to the eye, which can lead to impaired vision and even blindness. Some commonly used drugs, such as certain antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) as well as some popular herbal medications, can act as photosensitizers and greatly enhance the ocular toxicity of UV radiation. Non-photosensitizing chemicals can also generate a stronger toxic effect to the eye in the presence of UV radiation.

This article reviews the combined toxic effects of chemical mixtures, and chemicals plus UV radiation on the ocular and visual system. It also covers the basic concepts of the toxicological action of chemical mixtures, and mechanisms of chemical-induced ocular phototoxicity.

2. Combined Toxic Effects of Chemicals

2.1 Basic concepts of combined actions of chemical mixtures

The main goal of risk assessment of chemical combinations is to predict the toxicological effects of the mixture. The effects of co-exposure to different chemicals may produce a response that is equal, greater or less than the expected sum of their individual responses. These basic concepts of toxicological action of chemical mixtures have been defined by Loewe & Muischnek[1], Bliss[2] and Plackett & Hewlett[3].

2.1.1 No interaction

There are two types of combined action without interaction: simple similar action and simple dissimilar action.

Simple similar action: A simple similar action occurs when the compounds act on the same biological site by the same mechanism and do not react to each other. The combined effect is equal to the sum of the effects of each agent given alone.

Simple dissimilar action: In a simple dissimilar action, the chemicals act by different mechanisms, and may act on different biological sites. The presence of one chemical does not influence the effect of another chemical. Therefore, when the dose of each chemical is below the toxic threshold of the individual compounds, the combined toxic effect of all chemicals will also be below the toxic threshold.

2.1.2 Interactions

Interactions among chemicals can occur in a variety of ways. They may occur in the toxicokinetic phase (such as during processes of uptake, distribution, metabolism and excretion) or in the toxicodynamic phase (such as effects of chemicals on receptors, cellular targets or organs). The results of interactions may be additive of their individual responses, or may be stronger (synergistic, potentiated) or weaker (antagonistic) than the sum of individual effects.

Addition: An additive effect occurs when the combined effect of two chemicals is equal to the sum of the effects of each chemical given alone (i.e., 1+1=2).

Synergistic effect: A synergistic effect occurs when the combined effect of two chemicals is greater than the sum of the effects of each chemical given alone (i.e., 1+1=10).

Potentiation: Potentiation occurs when the toxicity of a chemical on a certain organ or system is enhanced when given together with another chemical that alone is not toxic to the same organ or system (i.e., 1+0=5).

Antagonism: Antagonism occurs when the combined effect of two chemicals is less than the sum of the effects of each chemical given alone (i.e. 5+5=7, or 5+0 = 2). Antagonistic effects are the basis of many antidotes.

2.2 Additive effect

The additive effect is the most common mode of combined action of chemicals. Many medications have been reported to have the potential to cause additive ocular adverse

reaction when used in combination. These medications include but are not limited to cetirizine and antihistamines, *Ginkgo biloba* and warfarin or aspirin, vitamin A and other type of retinoids [4,5]. However, there are relatively few studies about the definite additive ocular toxicity of chemical combinations.

Using the uptake of [3H]-uridine by mouse fibroblasts as a measurement, an *in vitro* test evaluated the ocular irritancy of 25 xenobiotic chemicals individually and in combination. The test compounds included alcohols, ethers, esters, ketones, amides, acids and a detergent. The result correlated well with the Draize test and indicated that combinations of agents with differing functional groups produce additive effects on ocular irritancy [6].

The exposure of the eye to surfactants and detergents often causes various deleterious effects on the eye. Some surfactants, like ordinary soap, cause immediate eye irritation with little or no injury. Other surfactants produce corneal oedema and loss of corneal epithelium with no discomfort. Some cationic surfactants, e.g. benzalkonium chloride (BAK) and cetylpyridinium chloride, may cause immediate eye irritation as well as severe delayed effects on the corneal epithelium and stroma. Additive irritant effects on the eye were observed to various degrees in response to different solvents in combination [7].

Airborne chemicals such as volatile organic compounds (VOCs) produce sensory irritation and the combined mixtures of VOCs play an important role in the perception of indoor air quality. Using a visual analogue scale and a comparative scale, one study conducted by Hempel-Jorgensen et al. assessed the ocular irritation caused by mixtures of

VOCs. It was demonstrated that three mixtures of VOCs interacted additively to produce ocular irritation [8].

2.3 Synergy

Many chemical combinations have been shown to cause synergistic toxic effects on the ocular and visual system.

Exposure to a combination of disinfectants and their by-products can greatly enhance eye irritation. A study using the HET-CAM Test (Hens Egg Test at the Chorion Allantois Membrane) to investigate the mucous membrane irritating effect of halogenated carboxyl compounds (HCCs) demonstrated that, when combined with aqueous chlorine, a number of HCCs exhibited significantly enhanced effects. This result suggested that the eye irritating potential of swimming pool water is a consequence of the effects and synergistic action of a number of disinfection by-products in the presence of chlorine [9].

Several surfactants such as BAK, cetylpyridinium chloride, and Ethylenediaminetetraacetic acid (EDTA) can increase corneal permeability and hence produce additive or synergistic effects when administrated together with other chemical compounds. Chang et al. tested the individual and combined toxicity of BAK and gentamicin on rabbit corneal epithelial cell membranes. They found that the presence of BAK synergized the effect of gentamicin on causing a leak in corneal epithelial cell membranes [10]. Kikuchi et al. investigated the effects of EDTA plus boric acid on the corneal penetration of an anti-

glaucoma agent (CS-088) and revealed that EDTA plus boric acid synergistically enhanced the corneal permeability of CS-088 via the transcellular pathway [11].

Systemic medications can also interact synergistically to produce increased ocular adverse effects. A clinical study on patients with rheumatoid disease revealed that desferrioxamine and prochlorperazine were neurologically toxic and produced optic neuropathy and pigmentary retinopathy[12]. Subsequent *in vivo/vitro* studies conducted by the same investigators clearly demonstrated that the neurological effects were due to a synergistic action of desferrioxamine and prochlorperazine[12].

2.4 Potentiation

Potentiation can occur through an alteration in the absorption, distribution, metabolism or excretion of a toxic compound, increasing the concentration and/or duration of the toxic compound in the target tissue or organ.

Chemicals that prolong ocular contact time and increase intraocular drug levels have the potential to enhance the toxicity of other chemicals to the eye. One example is hydroxyethyl cellulose (HEC), a viscosity-enhancing agent in ophthalmic formulations. A previous study has shown that HEC potentiated BAK-induced corneal damage in rabbit eyes, by increasing the viscosity and prolonging the contact time of BAK with the cornea. Formulations with 0.01% BAK and HEC caused corneal epithelial damage, but no damage occurred with the same concentration of BAK or with HEC alone [13].

Potentiation has also been shown to be one of the causes of cataracts and glaucoma. Using a mouse model, a report revealed that acetaminophen did not cause cataracts unless it was metabolized to N-acetyl-p-benzoquinone imine (NAPQI) in the liver, a process potentiated by cytochrome P450 enzymes [14]. Another study showed that 5 beta-Dihydrocortisol potentiated the threshold level (the smallest dose producing a measurable effect) of topically applied cortisol (0.02%) and dexamethasone (0.003%) in raising intraocular pressure. The author concluded that this potentiation may be responsible for the steroid sensitivity and for the ocular hypertension seen in glaucoma [15].

2.5 Antagonism

Antagonism of chemicals often produces beneficial effects in toxicology and is the basis of many antidotes. Extensive studies have demonstrated antagonist effects in reducing or eliminating the toxic effects of poisons, medications, drug additives and preservatives.

N-3-pyridylmethyl-N'-p-nitrophenylurea (PNU) is a rodenticide that is also poisonous to humans. Gentamicin administered orally was shown to be an effective antidote for the ocular toxicity of PNU, with a speculated mechanism involving the killing of gastrointestinal bacteria responsible for transforming PNU into an ocular toxin [16]. Cyanide is another poison which also has ocular toxicity. Bolaji et al. investigated the effect of lyophilized aqueous extract of *Telfairia occidentalis* (TO) on induced cyanide toxicity in rats and concluded that the extract had good potential as a safe antidote for cyanide poisoning [17]. The potential mechanism is that TO extract facilitates the excretion of cyanide by offering a rich source of

sulphur to the mitochondrial enzyme rhodanese, which is responsible for converting cyanide into thiocyanate.

Antagonism has also been used to decrease the ocular adverse effects of medications and preservatives in ophthalmic solutions. Recent work have demonstrated that caffeic acid phenethyl ester treatment may decrease the oxidative stress in the retina and optic nerve of isoniazid- and ethambutol-treated rats, and may prevent retinal ganglion cell (RGC) loss by an interaction with superoxide dismutase (SOD) [18]. In glaucoma medications, sodium hyaluronate (a vehicle with antioxidation, moistening and lubricating properties) [19] has been shown to significantly decrease the ocular surface toxicity induced by BAK-preserved latanoprost and brimonidine in rabbits [20,21].

Due to the extensive use of BAK as a preservative in ophthalmic solutions and its toxicity to the eye, substantial effort has been put into the development of antidotes for BAK toxicity. Latanoprost and travoprost were reported to have significant protective effects against BAK toxicity on conjunctiva-derived epithelial cells *in vitro* [22]. Pauloin et al. investigated the effect of hyaluronan on the toxicity of BAK on two human epithelial cell lines *in vitro*. They found that high molecular weight hyaluronan (1000 kDa) significantly decreased oxidative stress, apoptosis and necrosis induced by BAK, and suggested that high molecular weight hyaluronan is an effective protective agent against BAK [23]. Very recently, the ability of polyoxyethylene hydrogenated castor oil 40 (HCO-40) and polysorbate 80 (PS-80) to alleviate the corneal toxicity of BAK has been reported in an *in vitro* study with rabbit

corneal epithelial cells [24]. However, the antibacterial effect of BAK was suppressed simultaneously.

3. Combined Toxic Effects of Chemicals and UV Radiation

3.1 Introduction

Because of its specialized structure for focusing incoming light, the eye is susceptible to light damage. Specific ocular disorders that can be caused by exposure to sunlight include photokeratitis, pingueculae and pterygia, uveal melanoma, cataracts, and macular degeneration. The spectrum of sunlight includes UV radiation (100-400 nm), the visual spectrum (400-700 nm) and infrared (700-10,000 nm). UV radiation has been subdivided into UVC (100–280 nm), UVB (280–315 nm), and UVA (315–400 nm). As already noted, due to the release of chlorofluorocarbons and nitrogen oxides in the stratosphere, ozone concentration has been reduced significantly in the past 30 years.[25] Ozone depletion increases the amount of UVB solar radiation reaching the Earth's surface, which leads to a higher risk of phototoxicity in the eye.

UV radiation-induced ocular damage can be attributed to two mechanisms; 1) direct process in which the radiation is absorbed by specific naturally occurring chromophores (such as the nucleic acids or aromatic amino acid residues) within the eye; and 2) indirect or photosensitized process when the radiation is absorbed by a photosensitizing agent or other extraneous compound, which then become excited to reactive state and damages the ocular tissues through producing free radicals and reactive oxygen species [26].

Exposing the eye simultaneously or consecutively to a chemical and UV radiation may generate a combined toxic effect, which is greater, less, or equal to the sum of their individual toxicity. Chemicals may serve as photosensitizers to enhance the toxicity of UV (phototoxicity), or act synergistically with UV to produce much greater ocular toxicity, or may protect the eye from UV damage.

3.2 Phototoxicity

3.2.1 Mechanisms

Phototoxicity can be produced by the presence of photosensitizing agents. Chemicals absorb the incident light, become excited to reactive states, and then interact with substrate molecules through two principal reaction pathways, one involving direct free radical reactions (Type 1), and the other involving production of reactive oxygen species (ROS) (Type 2) [27].

In the Type 1 reaction, the chemical is excited by light to a triplet state and undergoes a direct electron or hydrogen exchange with a substrate, creating a free radical intermediate. This free radical reacts with an available substrate to create peroxidation reaction products. In contact, in the Type 2 reaction, the excited chemical directly transfers energy to oxygen and creates reactive singlet oxygen. Singlet oxygen reacts with lipids to create peroxides without a free radical intermediate, or it can react with other substrates to generate reactive free radicals [27]. The free radicals and ROS can cause oxidative damage to lipids, proteins, mitochondria, DNA, and so on.

3.2.2 Photosensitizers

Whether a chemical is capable of producing phototoxicity in the eye depends on several factors, including the chemical's structure, it's absorption spectra, its capability to bind to ocular tissue, and its ability to cross blood-ocular barriers (this last factor applies only to the systemic medications) [28]. Any compound that has a tricyclic, heterocyclic or porphyrin ring structure is a potential photosensitizer. Many commonly used drugs and chemical compounds have known or potential photosensitizing properties on the eye. These drugs include fluoroquinolone antibiotics (Levofloxacin, Lomefloxacin, etc.) [26,29], diagnostic dye [30], antimalarial medication (chloroquine) [31], nonsteroidal anti-inflammatory drugs (NSAIDs) (Ketoprofen, piroxicam, etc.)[32], psoralen [31], and psychotherapeutic agents [31,33], as well as some herbal medicines [34]. The action spectrum for most of them includes UVA and the visual spectrum, with the cornea and lens being their most common potential targets.

3.2.3 Examples of chemical induced phototoxicity

Psoralen has been well known for its photosensitizing property. Photosensitization damage to the lens and retina with psoralen plus UV radiation (320-400 mm) (PUVA) has been demonstrated in experimental animals, as well as in clinical report on patients given PUVA therapy [35]. The effects of various psoralen derivatives and UVA radiation have also been studied [36]. Barker et al. tested the ocular toxicity of acute administration of 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and 3-carbethoxypsoralen (3-CPS) with and without 0.2 or 48 J/cm^2 UVA radiation in a mouse model. Ocular damage

consisting of dense central corneal opacification was seen at significant levels in animals given 8-MOP or 5-MOP and exposed to UVA, but not in animals given derivatives or UVA alone. The mechanisms of damage were not addressed.

Using *in vitro* models, the ocular phototoxicity of diagnostic dye, certain antipsychotic medications and antibiotics have been evaluated. Hull and his colleagues assessed photosensitization of a diagnostic dye and three antipsychotic medications on corneal endothelium cells [30,37-39]. They found that Rose Bengal [30], chlorpromazine [37], trifluoperazine [38] and hematoporphyrin derivative (HPD) [39] were all able to induce corneal endothelium phototoxicity *in vitro*. Endothelium swelling was a manifestation common to all. Persad et al. investigated the cytotoxic effects of UV-visible irradiation on retinal pigment epithelial (RPE) cells in the presence of chlorpromazine. They found that the phototoxic effect of chlorpromazine was due to stable photoproducts formed during light irradiation, and this occurred at low concentrations (5ug/ml) of chlorpromazine. Vitamin E was able to decrease the phototoxicity both in the dark and upon irradiation. Hypericin is one of the bioactive ingredients of a medication for depression in Europe. A recent study investigated the photooxidative damage of hypericin on α-crystallin in human lens epithelial cells and detected extensive polymerization of α-crystallin exposed *in vitro* to hypericin and UVA [34]. Another *in vitro* study tested the photosensitization of Levofloxacin under the ambient environmental intensities of UV-A, UV-B and sunlight exposure and revealed that photosensitized Levofloxacin caused lipid peroxidation, significantly reduced cell viability, and induced upregulation of p21 and Bax/Bcl-2 genes ratio [2639].

Fish eyes are uniquely vulnerable to environmental stress. The effect of polycyclic aromatic hydrocarbons (PAHs) on the optical properties of Rainbow Trout lenses in the presence or absence of UV radiation was evaluated by Laycock and his colleagues [40]. They found that simultaneous exposure to UV and PAHs damaged rainbow trout lens membranes and increased focal length variability (FLV); whereas UV and PAHs alone did not cause this damage. The speculated mechanism is that the PAHs accumulate in lens cells membranes, and upon absorbing radiation, undergo chemical reactions that generate ROS and cause cell membrane disruption.

3.3 Synergistic effect of UV radiation and chemicals

Some chemicals do not absorb UV radiation; however, their toxicity is greatly enhanced when combined with UV radiation.

One *in vitro* study assessed the toxic and mutagenic potential of four commonly used ophthalmic preservatives (BAK, chlorhexidine, thimerosal and ethylenediaminetetraacetic acid) in the presence and absence of UVA radiation. The result demonstrated that UV radiation synergistically enhanced the mutagenic activity of chlorhexidine and thimerosal. Chlorhexidine alone had little effect on the mutation rate, but when combined with UVA exposure, there was approximately a three fold increase over background in the numbers of mutants. Thimerosal by itself appears to be weakly mutagenic. When cells were exposed to thimerosal plus UVA treatment there was about a six-fold increase in the number of mutants over the control value [41]. In another study with primate eyes, Zuclich et al. demonstrated that

eyes that were moistened with a wetting solution containing BAK had lower thresholds for damage by a krypton laser (350-360 nm) than eyes that were not premoistened before exposure to laser radiation[42].

3.4 Protective effect of chemicals against UV radiation

Some chemicals have been found to have protective effect against UV damage to the eye. Quercetin is a well knows antioxidant. Researchers speculate that quercetin may have a role in protecting lens cells against UV-induced damage. In a recent *in vitro* study using human lens cells, Jiang et al. showed that quercetin protected against a UV- and H_2O_2-induced decrease of collagen type I in a dose-dependent manner [43]. Quercetin also protects human lens cells from damage resulting from other toxic chemicals [44]. Hyaluronic acid (HA) is another useful tool to protect the skin and the eye against UV radiation. The ability of HA in protecting the eye from UV radiation has been demonstrated in a recent study [45]. When human corneal cells were exposed to HA before exposure to UVB radiation, hyaluronic acid protected the cells against UVB radiation-induced inflammation and apoptosis. Some nutrients like bilberry's (*Vaccinium myrtillus*) flavonoid components, the amino acid taurine and N-acetyl cysteine are also shown to improve the health of the eye and protect against cataracts formation [46-48].

4. Conclusion

Human eyes are exposed to UV radiation and various chemicals on a daily basis. Many chemical combinations have been shown to cause enhanced ocular toxicity through

synergism, addition, or potentiation. With better understanding of the ocular pototoxicity of UV radiation and chemicals, more and more medications and chemical compounds with known or potential ocular photosensitizing property have been identified. However, there are relatively few studies on the combined effect of UV radiation and non-photosensitizing chemicals on the eye. Many of the mechanisms of combined toxicity are still unknown. Most risk assessments are based on individual ocular toxicity. More attention should be paid to the combined effects and to the efforts to understand the mechanisms of co-exposure.

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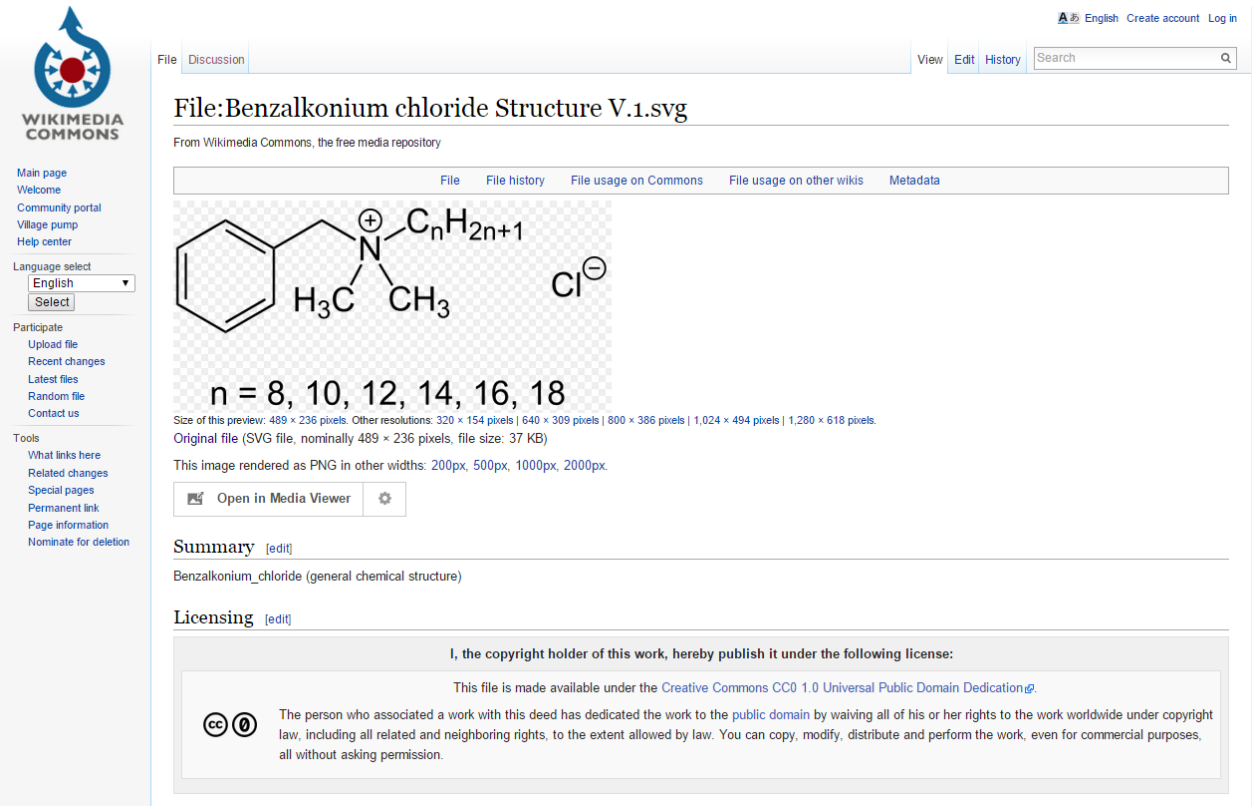

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