Development of Sensitive In Vitro Assays to Assess the Ocular Toxicity Potential of Chemicals and Ophthalmic Products

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The utilization of *in vitro* tests with a tiered testing strategy for detection of mild ocular irritants can reduce the use of animals for testing, provide mechanistic data on toxic effects, and reduce the uncertainty associated with dose selection for clinical trials. The first section of this thesis describes how *in vitro* methods can be used to improve the prediction of the toxicity of chemicals and ophthalmic products. The proper utilization of *in vitro* methods can accurately predict toxic threshold levels and reduce animal use in product development. Sections two, three and four describe the development of new sensitive *in vitro* methods for predicting ocular toxicity. Maintaining the barrier function of the cornea is critical for the prevention of the penetration of infections microorganisms and irritating chemicals into the eye. Chapter 2 describes the development of a method for assessing the effects of chemicals on tight junctions using a human corneal epithelial and canine kidney epithelial cell line. In Chapter 3 a method that uses a primary organ culture for assessing single instillation and multiple instillation toxic effects is described. The ScanTox system was shown to be an ideal system to monitor the toxic effects over time as multiple readings can be taken of treated bovine lenses using the nondestructive method of assessing for the lens optical quality. Confirmations of toxic effects were made with the utilization of the viability dye alamarBlue. Chapter 4 describes the development of sensitive *in vitro* assays for detecting ocular toxicity by measuring the effects of chemicals on the mitochondrial integrity of bovine cornea, bovine lens epithelium and corneal epithelial cells, using fluorescent dyes.

The goal of this research was to develop an *in vitro* test battery that can be used to accurately predict the ocular toxicity of new chemicals and ophthalmic formulations. By comparing the toxicity seen *in vivo* animals and humans with the toxicity response in these new *in vitro* methods, it was demonstrated that these *in vitro* methods can be utilized in a tiered testing strategy in the development of new chemicals and ophthalmic formulations.

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List of Abbreviations

ADME	Absorption, Distribution Metabolism and Excretion
ANOVA	Analysis of Variance
AMO	Advanced Medical Optics
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BVD	Back Vertex Distance Variability
BAK	Benzalkonium Chloride
BCOP	Bovine Corneal Opacity and Permeability Assay
CCCP	Carbonyl Cyanide M-Chloro-Phenylhydrazone
EDTA	Ethylenediaminetetraacetic Acid
DNA	Deoxyribonucleic Acid
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative methods
EVOM	Electrovoltohmeter
EPA	United States Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
GHS	Global Harmonized System
HA	Hyaluronic Acid
HBSS	Hanks' balanced salt solution
HCEC	Human Corneal Epithelial Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HET-CAM	Hen's Egg Test Chorioallantoic Membrane assay
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative methods
ICH	International Conference on Harmonisation
Il-8	Interleukin-8
ICE	Isolated Chicken Eye Test Method
ISO	International Organization for Standardization

JaCVAM	Japanese Center for the Validation of Alternative Methods
LDH	Lactate dehydrogenase
LOAEL	Lowest Observable Adverse Effect Level
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Methods
M199	Medium described by Morgan, Morton and Parker (1950)
MEM	Minimum Essential Medium
MHLW	Japanese Ministry of Health Labor and Welfare
MAS	Draize Maximal Average Scores
MMAS	Draize Modified Maximum Average Scores
MDCK	Madin-Darby Canine Kidney Cells
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium B romide
NOAEL	No Observable Adverse Effect
PBS	Phosphate-Buffered Saline
PHMB	Polyhexamethylene Biguanide
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive Oxygen Species
RGP	Rigid Gas Permeable
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscopy, Standard Error of the Mean
SDS	Sodium Dodecyl Sulphate
SV-40	Simian Vacuoloting Viris 40
TNF	Tumour Necrosis Factor
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XTT	Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazoium-5-carbxanilide
ZO-1	Zonula Occludens-1
ZO-2	Zonula Occludens-2
ZO-3	Zonula Occludens-3

Chapter 1

The use of Sensitive Cell and Molecular Toxicity Models in Developing Risk Asessments for Chemicals Used in New Ophthalmic Formulations

1.1 Introduction

In vitro test methods are being developed to improve the prediction of chemical and product toxicity. Live animal test systems are increasingly being replaced by *in vitro* methods. These test methods are used to understand the underlying toxic mechanisms and to find chemical toxicity thresholds. Regulatory agencies throughout the world are actively involved in reviewing new testing methodologies and determining if adequate *in vitro* replacements for animal tests can be recommended. In September of 2004, the European Union 7th Amendment to the Cosmetic Directive prohibited the animal testing of finished cosmetic products in the EU. Beginning March 11, 2009 there will be a ban on animal testing of cosmetic ingredients and final product formulations (Rossignol, 2005). Currently, there are an estimated 30,000 chemicals in use for which toxicology information has never been registered in Europe. In June of 2007 the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation was adopted that requires registration of these chemicals in an 11-year period with appropriate toxicological characterization. In article one of this legislation the use of alternative methods to animal testing is encouraged, with animal testing carried out only as a last resort (Lilienblum *et al*., 2008; Grindon *et al.*, 2008).

Because of the increased requirements for toxicological characterization of chemicals, concerns about animal welfare and the need for understanding underlying toxicological mechanisms for risk assessment, organizations have been established for reviewing new *in vitro* alternative methods. The validation organization in the United States is the Interagency Coordinating Committee on the Validation of Alternative methods (ICCVAM), administered by the National

Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM). In Europe the validation organization is the European Centre for the Validation of Alternative methods (ECVAM) and in Japan the organization is the Japanese Center for the Validation of Alternative Methods (JaCVAM). These organizations coordinate validation studies and make recommendation to regulatory agencies on the use of the proposed new methods.

Regulatory agencies have adopted a number of new *in vitro* methods. The US government Interagency Coordinating Committee on the Validation of Alternative Methods announced (June 23, 2008) regulatory acceptance of two new *in vitro* ocular safety assays by the US FDA, EPA and Consumer Product Safety Commissions (Mackar, 2008). The committee approved the use of the bovine corneal opacity and permeability assay (BCOP) and the isolated chicken eye (ICE) test method for the assessment of corrosives or severe ocular irritants. These assays are used to establish hazard warnings on chemicals that may cause severe or permanent eye damage without the need for rabbit ocular irritation testing. The adoption of these two *in vitro* assays will reduce significantly the number of animals that will have to be tested in order to satisfy the EU REACH legislation.

1.2 Approval of New In Vitro Assays

The approval of the BCOP and ICE test for hazard identification of corrosives and severe ocular irritants demonstrates the use of *in vitro* tests in a tiered testing strategy for establishing risk. Approval of the test methods was based on validation studies which showed that the accuracy for predicting corrosive or severe ocular irritants for the BCOP was 79% to 81% with an overall

false positive rate of 19 to 21% and a false negative rate of 16 to 25% when compared to *in vivo* rabbit ocular irritation data classified according to EPA, EU and GHS systems. For the ICE test the accuracy was 83% to 87% with a false positive rate of 6% to 8% and overall false negative rate of 41% to 50% (ICCVAM, 2006a; ICCVAM, 2006b). With a tiered testing strategy an assay does not have to be 100% accurate in order for it to be adopted for use to assess toxic risk. For testing severe ocular irritants, if the test is positive, no further testing in animals is required. There is acceptance that some chemicals may be mislabelled as severe irritants, but the rate is low enough to be considered acceptable. False negatives, however, are usually not acceptable, a chemical inappropriately identified as non-corrosive or non-severe could cause harm if handled inappropriately. If the response is negative the product is then tested in animals to confirm that it cannot cause irreversible damage.

In addition to the correct labelling of corrosive or severe ocular irritants, it is hoped that *in vitro* tests will also be used to minimize the use of animals for other product categories. These include drops for dry eye, pharmaceutical drops for ocular diseases, contact lens rewetting drops, contact lens care solutions and contact lenses. In order to adopt the tiered testing strategy for these products the threshold of toxicity would need to be identified for each product category. For consumer products the threshold would likely be very low, as even low grade irritation would be unacceptable. For pharmaceutical products the threshold of toxicity could be higher as the toxic side effects of the drugs would have to be weighed against the severity of the disease that is being mitigated by the drug application. In order to adopt new *in vitro* methods that predict these toxicity thresholds, as was the case with the BCOP and ICE, a test does not have to be 100%

accurate to be adopted. There may be some concerns by industry that in a tiered testing program that utilizes an *in vitro* assay false positives may eliminate a few promising chemicals from potential development. This concern should be considered in validating an assay for each product category. An expectation of an assay having 100% accuracy with no false positives is unreasonable and would inhibit the progression of the use of *in vitro* assays to replace animal tests. Industry representatives and the reviewers at the regulatory agencies will need to come to a consensus for the acceptable number of false positives in these new *in vitro* assays.

1.3 Required Animal Tests for Regulatory Approval of Ophthalmic Products

Currently the use of animal testing is mandatory in the US for the evaluation of contact lens, contact lens care solutions, contact lens rewetting drops and new drugs (FDA, 1994; FDA, 1997; Ukelis *et al.* 2008). To assess ocular toxicity single, repeat instillation and contact lens wear studies in the rabbit are required. Sensitization testing is preformed using the mouse local lymph node assay or guinea pig sensitization assay. Systemic toxicity testing of contact lens extracts in mice is required to assess the toxic potential of leachable chemical constituents. Systemic adsorption of drugs occurs through adsorption into the conjunctiva and cornea. At least 50% of systemic adsorption of topically applied ophthalmic drugs occurs through the nasal pathway after passage of the drop into the nasolacrimal drainage system (Lee *et. al.*, 1993). Drainage from the nasolacrimal duct to the nasal cavity eventually enters the pharynx and can come into contact with the secretions of the upper respiratory and gastrointestinal tracts (Urtti and Salminen, 1993). To evaluate for oral toxicity, contact lens care products are dosed orally at 15 g per Kg body weight (FDA, 1997). If 80% of the animals survive, the test material is not considered orally

toxic. For drug products, oral toxicity LD50 (median lethal dose, dose producing lethality in 50% of the animals) was discontinued in 1991 (Ukelis *et al*., 2008). Single dose (acute) toxicity for a pharmaceutical is performed in two mammalian species prior to the first human exposure (ICH, 2000). Typically doses used are at or above the expected clinical doses in animal acute toxicity studies. For drug products, animal testing is used for repeat dose oral toxicity studies with a duration usually equal to or greater than the duration of the clinical trial (ICH, 2000). Animal testing is also required for assessments of reproductive toxicity, carcinogenicity, safety pharmacology, immunotoxicty and toxicokinetic and pharmacokinetic studies (ICH, 1997; ICH, 1994a; ICH, 1994b; ICH, 1993; ICH, 2000; ICH, 2005).

1.4 Current Use of In Vitro Assays

Currently *in vitro* tests are evaluated for ophthalmic products but are not used in a tiered testing strategy designed to reduce the use of animals in testing. Instead, these *in vitro* tests are used to gain additional information on the potential toxicity of the product that may not be identified by the *in vivo* tests or by the clinical evaluations. *In vitro* tests may at times be more sensitive than the *in vivo* tests and thus may identify sub-clinical toxicity that may present itself in the patient as a pathology after chronic use of the product. The use of *in vitro* tests is particularly important for understanding mechanisms of toxicity. When *in vitro* tests are identified as showing sensitivity greater than the *in vivo* animal and clinical tests, the *in vitro* test can be used to establish the threshold of toxicity.

The cytoxicity tests required for contact lens solutions, contact lens rewetting drops, and contact lenses include the agarose overlay test (also known as the agarose diffusion test), direct contact test, elution test and inhibition of growth assays. (FDA 1997, FDA, 1994). The International Standards Organization has written a standard for cytotoxicity testing for medical devices (ISO, 1994; ISO, 1999). Cytotoxicity testing of contact lenses and contact lens solution products are preformed based on the methodologies described in these ISO documents. Studies performed using the methods described by ISO have typically utilized cell lines that are not of ocular origin. These cell lines include L929 mouse fibroblast cells and V79 Chinese hamster cells. Applications include testing the product in direct contact with the cell lines or indirectly by assessing the toxicity of device extracts (Tsuchiya, 1994).

Only a few limited validation studies have assessed the use of these cytotoxicity methods for the prediction of the ocular irritation potential of ophthalmic products. A validation study evaluated 16 cosmetic products using the agarose diffusion method (Wallin *et al*., 1987). Chemicals were initially tested in the Draize eye test and were classified as either irritants or non-irritants. Comparing the *in vitro* score with the *in vivo* results Wallen *et al.* found that 80% (4/5) of the samples that tested positive in the Draize also tested positive in the agarose diffusion method. Also, 82% (9/11) of samples that tested negative in the Draize tested negative by this method. In another evaluation by the same investigators 22 cosmetic products were tested (Jackson *et al*., 1988). There was a 100% correlation between the *in vitro* and *in vivo* Draize with no false negative or false positive results.

The apparent success of this assay in predicting the eye irritation potential of cosmetic products was later put into doubt by an evaluation of 25 surfactant based formulations (Gettings *et al*., 1996). In this study the assay had a false positive rate of 86% and a false negative rate of 0%. In another investigation both L929 mouse fibroblast cells and rabbit corneal cells were tested by the agar diffusion assay to assess 22 cosmetic products (Milstein and Hume, 1991). A high degree of correlation (r = 0.991) was found between the L929 and the rabbit corneal cell line results. This indicated that cell lines other than L929 could potentially be utilized in the agarose diffusion test. The rabbit corneal cell line was ultimately used to assess the validity of the agar diffusion method (Reboulet *et al*., 1994). A three level ocular classification scheme based on *in vivo* Draize scores was developed. Chemicals were classified as non-irritants, irritants or severe irritants. In this analysis nine compounds were classified as severe irritants, in both the *in vivo* and *in vitro* tests. The false positive rate was 0% and the false negative rate was 7%. Other than the testing performed for the agarose diffusion test, no formal validation studies have been published on the direct contact test, elution test required by the FDA and the International Standards Organization or the V79 colony assay required by the Japanese Ministry of Health, Labor and Welfare.

Although the required *in vitro* tests for regulatory product submissions have not been accepted as alternatives for ocular irritation tests by ICCVAM, ECVAM or JaCVaM, the studies are still useful for assessing potential toxicity by using positive controls. The International Organization for Standardization has set forth ISO-10993-5 "Tests for *in vitro* cytotoxicity" (ISO, 1999). The methods describe general principles for performing cytotoxicity assays, but no one method or

test endpoint is mandated. The key component of each assay used is the comparison of the test sample to the positive and negative controls used in the assays. The positive controls include organo-tin stabilized poly(vinylchloride) for solid materials and extracts, and dilutions of phenol, zinc diethyldithiocarbonate (ZDEC) and zinc dibutyldithio-carbamate (ZDBC) polyurethanes for extract testing (ISO, 1999).

The interpretation of the cytotoxicity potential of medical devices and their extracts consist of establishing a scoring system in which the test articles' effect on the cell cultures are compared to the negative and positive controls. Also, comparisons of the results of the new test article to similar medical devices or materials are suggested. Comparison of the test article to the positive and negative controls allows for the determination of the relative toxicity of the test article to these known toxic materials. Also, since these methods have been required by regulatory agencies for decades, most companies have a database of the results of development formulations and current products for means of comparison, in addition to comparison to the positive controls.

The ISO guidelines indicate a preference for established cell lines in testing but do not require use of any particular cell line or exposure time. The use of primary cell culture, and organo-typic cultures obtained directly from living tissues are allowed, if the reproducibility and accuracy of these tests are demonstrated (ISO, 1999). The exposure time is established by the toxicologist based on the use of the material. The US FDA and the Japanese Ministry of Health Labor (MHLW) and Welfare reference cytotoxicity protocols that are more specific than the tests

recommended by ISO 10993-5. The basic principles are the same in that a test article is compared to established positive controls, although the FDA and the MHLW recommend testing using specific cell lines and exposure times (Anand, 2000). The use of established regulatory cytotoxicity assays for determining the risk of new ophthalmic products can be criticised for the use of cell lines that are not relevant to the eye, exposure times that may exceed the time of exposure of an ophthalmic product in the eye, and the use of positive controls that may not have been established for assessing ocular toxicity. It is for these reasons that new *in vitro* assays using ocular cells, relevant exposure times and control materials that are ocular toxins, have been proposed for determining ocular irritation potential.

1.5 Validation of New In Vitro Assays

The relevance of a new *in vitro* assay for predicting ocular irritation is important for the establishment of a cell culture method in addition to evaluating the method's reproducibility. There are a number of ways in which to measure the relevance of an *in vitro* assay. Since there is limited data on the irritation potential of chemicals in human eyes, *in vitro* test data has traditionally been compared to the Draize maximal average scores (MAS) used for assessment of ocular irritation in the rabbit eye. One method of comparing the *in vitro* data with the *in viv*o data is to assign chemicals to specific irritation classes at various concentrations. The classification scheme can be as simple as categorizing the test sample as either a non-irritant or an irritant, or the scheme could be more complex using multiple categories such as non-irritant, mild irritant, moderate irritant and severe irritant. The ratio of irritants correctly predicted by the *in vitro* test to the total number of irritants identified *in vivo* is then determined. This is

defined as the sensitivity of the test system; the higher the sensitivity, the less likely the occurrence of false negative results. The ratio of non-irritants correctly predicted *in vitro* to the total number of non-irritants identified *in vivo*, defined as the specificity of the test system, is also determined. A higher specificity lowers the likelihood of the occurrence of a false positive response.

Another way to assess the accurancy of an *in vitro* alternative method is to perform a correlation analysis by directly comparing the *in vitro* data endpoints to the *in vivo* MAS scores. If there is a reasonable correlation then a prediction model can be established. A subsequent analysis can then be performed using additional chemicals to test the validity of the assay's prediction model. Using chemicals of known ocular toxicity, the relevance of the method for assessing ocular irritation can be determined.

Recent validation studies have attempted to determine the relevance of *in vitro* test models to rabbit Draize ocular irritation testing. There are two major problems with this validation strategy: the Draize test is not validated for predicting ocular irritation testing in humans nor is it sufficiently sensitive to detect low dose effects of ophthalmic chemicals. In an investigation of intra- and interlaboratory variability of the Draize test, Wiel and Scala (1971) showed variable results both between and within laboratories. The Draize test was found to be particularly variable in the middle range of irritancy (Prinsen, 2006). The variability of the Draize test has been attributed to natural animal variability, variable retention of the test article on the corneal surface, the low numbers of animals used, and variability in scores due to the subjective scoring

system used (York and Steiling, 1998). Beckley *et al.* (1969) compared the ocular irritation response of a 5% soap solution in monkeys, rabbits and humans using slit-lamp biomicroscopy, fluorescein, and the Draize visual observation method. Instillation of a 5 % soap solution in rabbit eyes caused almost no corneal epithelial effect whereas corneal epithelial damage occurred in both monkeys and humans. These lesions were not visible without the use of slit-lamp biomicroscopy and/or fluorescein. The fact that corneal epithelial damage did not occur in the rabbit and that microscopy and/or fluorescein were required to detect damage in the other species shows that the rabbit may not be the best animal model for detecting human ocular response. Since Draize scoring does not require biomicroscopy or use of fluorescein the method may not be sensitive enough to detect corneal epithelial damage. Since the ocular irritation database that most *in vitro* methods are compared to is Draize scoring without biomicroscopy or use of fluorescein (Bagley, 1999) this *in vivo* database may not be accurate, especially for chemicals that cause mild ocular damage.

A recent validation study of product formulations tried to rectify this problem by evaluating mildly irritating formulations that produce eye discomfort in the absence of macroscopic clinical signs in the human against two *in vitro* models without the use of Draize scoring (Debbasch *et al.*, 2005). Make-up removers were applied 12 times to the exterior surface of the eyelids of human subjects. Tears were collected twenty seconds after application to confirm that the product reached the eyes. Severity of irritation was determined by sensation descriptions of burning, stinging, itching, lacrymation, dryness, blurred vision and foreign body sensation on a 5 point scale of severity. Minimal bulbar conjunctival redness occurred with application of 6 of

the 12 products. Results from two *in vitro* assays for eye irritation, the HET-CAM (Hen's Egg Test Chorioallantoic Membrane assay) and the BCOP assay, did not correlate to the clinical discomfort and adverse clinical signs. Measurements using a human corneal epithelial cell line for cytotoxicity and cytokine release of IL-8 showed a lack of correlation between the cytotoxicity measurements used in this study and clinical discomfort, but there was a good correlation of clinical signs with IL-8 release.

There is a dilemma when validating new *in vitro* methods for mild ocular irritation. If *in vitro* results are compared to the Draize database, you are comparing to data that are inaccurate and not sensitive enough to detect mild ocular irritation. Directly validating to human studies is difficult as it is unethical to carry out a large scale tests in humans with ocular irritants that may cause irreversible eye damage. Testing chemicals for ocular irritation in the monkey and adding biomicroscopic and fluorescein evaluations may correlate better to human irritation, but additional human studies would have to be performed to demonstrate that the monkey model is predictive. Also, there would not be public support for performing large scale animal validation studies in primates. Dogs are used to assess the ocular irritation potential of pharmaceuticals, but the results from these studies are rarely published so the utility of these data in validation studies is limited. ICCVAM has recognized the inadequacy of the current database for ocular irritants and has requested the submission of data from substances tested for ocular irritancy in humans, rabbits, and/or *in vitro* systems to develop the best database possible for assessing chemicals that cause mild ocular irritation (Federal Register Notice, 2007).

A standard procedure for identifying safe doses of chemicals is to determine the maximum dose for which there is no observable adverse effect (NOAEL). The NOAEL is then divided by safety (uncertainty) factors to derive acceptable exposure levels (Gaylor *et al*., 1998). The need for the factor is based on the fact that extrapolation from a toxicity model, extrapolation from acute data to chronic use, and/or use of data from tests of unknown accuracy or reliability makes the calculated NOAEL uncertain; the greater the uncertainty the larger the factor. The use of an uncertainty factor of 100, derived from a factor of 10 to account for inter-species differences and a factor of 10 to account for inter-individual differences (Combes, 2005), is typical. The inter-individual differences can be subdivided into factors that allow for differences in the fate of a chemical in the body (toxicokinetics) and differences in tissue sensitivities (toxicodynamics). If there is good understanding of the inter-individual differences of a specific chemical the 10-fold safety factor can be reduced (Renwick and Laxarus, 1998). Ritter *et al.* (2007) described other uncertainties that may exist in the data which can necessitate the application of additional safety factors. These additional factors include having a lowest observable adverse effect level (LOAEL) instead of a NOAEL, extrapolating from incomplete studies, extrapolating from subchronic studies to chronic product use and possible interaction with other chemicals. Risk assessment when applied to drugs also includes assessments to determine if the benefits of drug therapy exceed the risks (Gaylor *et al.*, 1998). Prior to starting a human clinical trial smaller safety factors might be used when toxicities produced by the therapeutic are easily monitored, reversible, predictable, and exhibit a moderate to shallow dose-response relationship with toxicities that are consistent across the tested species (FDA, 2002). This is also true if the

NOAEL was determined based on toxicity studies of longer duration compared to the proposed clinical schedule in healthy volunteers (FDA, 2002).

In order to use sensitive cell and molecular toxicity models for risk assessment of ophthalmic chemicals and formulations, it has to be demonstrated that the model can predict the toxicity of chemicals demonstrated to be toxic to the human eye. Although the number of chemicals tested in human eyes is small, the toxicity of one chemical, (benzalkonium chloride, BAK), has been well characterized. BAK is a widely used preservative in ophthalmic products. In topical multidose solutions it is normally present at an average concentration of 0.01% (range 0.004%–0.02%) (Noecker, 2004). Validation studies that use various concentrations of BAK as positive controls would show relevance to human ocular toxicity in the mild range of irritation. Comparison of the toxicity of new chemicals and product formulations to the effects of BAK can form the basis for assessing the potential risk for human ocular damage and irritation.

Thresholds of BAK toxicity have been determined using a number of sensitive systems and endpoints. Using scanning electron microscopy (SEM) of rabbit corneas treated with drops containing 0.01%, BAK was found to be very injurious (Pfister and Burnstein, 1976). After thirty minutes of exposure most of the top layer of cells desquamated; microvilli were lost in the lower layers. Burnstein (1980) applied BAK to rabbit and cat corneal epithelium at concentrations between 0.001% and 0.01% and the corneas were evaluated by scanning electron microscopy. Initial toxicity was detected at 0.0025%; corneas exposed to higher concentrations showed increased toxic effect. Using *in vivo* confocal microscopy Kovoor (2004) evaluated

fluoroquinolone antibiotics containing 0.005% BAK instilled at least 4 times a day for 7 days in the rabbit eye and demonstrated a significant decrease in corneal epithelial thickness after 7 days of exposure. A recent study by Ly *et al.* (2006) showed that a fourth generation fluoroquinilone with 0.005% BAK compromised the tight junctions of the rabbit cornea whereas another fluoroquinilone that did not contain BAK left the tight junctions intact. Ichijima *et al.* (1992) evaluated *in vivo* rabbit corneas after exposure to BAK drops at 0.02%, 0.01% and 0.005% using Tandem scanning confocal microscopy and SEM. The application of 0.005% BAK caused superficial epithelial cells to swell and desquamate. In a study that evaluated 0.004% BAK and 0.005% BAK instilled in rabbit eyes both as a solution and with use as a RGP contact lens care solution there were significant increases in desquamation of the superficial corneal epithelium and tear LDH activity compared with control eyes after 3 weeks of treatment (Imayasu, 1994). These measurements of ocular toxicity are significantly more sensitive than the response seen in the Draize test which exhibited no toxicity (Score of 0) even at 0.1% BAK levels (Klausner et al, 2003).

In human clinical studies biopsies of conjunctivae and trabeculums of patients who were treated with eye drops containing 0.01% BAK were abnormally infiltrated by cells expressing inflammatory or fibroblastic markers (Baudouin, 1999). Chronic users of glaucoma drops containing BAK showed increased secretion of pro-inflammatory cytokines by conjunctival cells (Malvitte, 2007). Exposure to timolol preserved with 0.005% BAK caused an unstable pre-corneal tear film and disrupted epithelial barrier function to a greater degree than the unpreserved control solution in human patients (Takeshi, 2003). Dry eye patients treated with polyvinyl

pyrrolidone preserved with BAK at 0.005% showed an increase in corneal epithelial permeability (Gobbels and Spitznas, 1992). Overall these studies suggest that the toxicity threshold for BAK in humans is approximately 0.005%.

To our knowledge, there are no studies planned for identifying the toxic potential of other ocular-application chemicals in humans. Human data from clinical studies which failed due to mild irritation is a possible source for establishing toxicity of additional chemicals. However, industry rarely publishes these studies so the data are not available to researchers. Only a few studies have been published that evaluated human toxicity of mild ocular irritants other than BAK (Debbasch *et al.*, 2005, Roggeband *et al.*, 2000, Beckley, 1965, Beckley *et al.*, 1969, Burnstein, 1984).

The absence of multiple-chemical human data for mild ocular irritation presents a challenge for validation of *in vitro* models for predicting that endpoint; comparisons are largely limited to BAK alone. Since the ocular toxicity of BAK is well characterized, assessing the effect of BAK in proposed *in vitro* models can establish the relevance of the new assay for predicting ocular irritation. Chemicals that have been approved for sale can be used as additional controls. Since marketed product has been assessed for toxicity in human clinical trials the extent that the new assay predicts the safety of currently marketed products can be assessed in a validation study. If the new assay accurately predicts toxicity of BAK at the concentration of 0.005% and above and does not overestimate the toxicity of currently marketed product, the relevancy of the new assay for predicting ocular irritation can be determined.

In vitro assays are currently available for assessing various physiological changes to cells after chemical exposure. These assays can assess changes to mitochondria (Bantseev *et al*., 2003; Bantseev *et al*., 2007), metabolic activity (McCanna *et al*., 2008, Debbasch *et al*. 2001, Hallet, 2005; Oriowo, 2006; Dutot *et al*., 2008; Dracopoulos *et al*., 2007), tight junctions (Tchao *et al*., 2002; McCanna *et al*., 2008; Imayasu *et al*., 2008; Chuang *et al*., 2008), optical quality (Banseev *et al*., 2003; Bantseev *et al*., 2008; Oriowo, 2006; Ho *et al*., 2008), reactive oxygen species (Debbasch *et al*., 2001), and cell membrane integrity (Debbasch *et al*., 2001; De Saint Jean *et al*., 2002). Evaluating BAK and currently marketed ophthalmic products can establish the baseline levels of toxicity and response that correlate to human toxicity thresholds. Once these baselines are established new chemicals can be evaluated within these assays to determine if the effect on the cells is above or below the established threshold.

New cell and molecular technologies are being developed that show promise for use as alternatives to animal testing. Recently a microarray chip for gene expression was developed for rabbit ocular research (Popp *et al.*, 2007). Gene expression of messenger RNA showed that when comparing an eye that underwent glaucoma filtration surgery to untreated control eyes, there were 315 genes that were expressed that were altered by glaucoma therapy. Microarrays have been used to evaluate the expression of 207 stress genes of rat primary hepatocytes that were exposed to the cytotoxic chemical cadmium (Badisa *et al.*, 2008). Of the 207 genes evaluated, 32 genes were either upregulated or down regulated by cadmium cytotoxicity. Proteomics using mass spectrometric analyses of proteins enables detailed assessments of chemical changes in

cells after chemical exposure. Bhattacharya *et al.* (2007) describes how proteomic studies can be performed to assess ocular changes after chemical exposures. The characterization of the human proteome and correlations to proteomic studies in cultured cells showed promise for understanding the mechanisms of toxic injury and the means of devising intervention strategies for the mitigation of ocular toxicity. In the future the development of microarrays and advances in the analysis of protein expression will enhance the use of *in vitro* methods for toxicology testing by assessing the details of gene expression after toxic exposure.

What would be the final criteria for establishing that a new assay is valid for use and how could the new assay be used to assess the risk of new ophthalmic chemicals? A large number of currently marketed ophthalmic products would have to be tested using the new assay and assessed for prediction of irritation. If a currently used ophthalmic product is identified as being a mild irritant, it can be used as a mild irritant positive control. Various concentrations of BAK can be tested from 0.005% to 0.1% to evaluate the new *in vitro* assay's ability to discriminate between chemical doses that cause mild and moderate toxicity. Testing marketed product formulations that are not considered to be ocular irritants assesses the assay's ability to correctly identify safe non-irritating chemical formulations. Using this validation strategy, new *in vitro* methods that correctly identify mild irritants and non-irritants can then be utilized. If a new chemical exceeds the established toxicity threshold, the chemical can be identified as toxic and eliminated from further development. Alternatively, the concentration can be lowered to the concentration that is below the established threshold. The validation and use of new methods in this manner reduces the number of animals required for product development.

In addition to the reduction of the use of animals for product development, *in vitro* assays reduce the uncertainty in the prediction of the safe doses for human clinical studies. Prior to starting human clinical trials, smaller safety factors might be used when toxicities indicate a moderate to shallow dose-response relationship with toxicities that are consistent across the tested species (FDA, 2002). Decreasing the uncertainty in the prediction of the safe clinical dose is critical for selecting the appropriate dose that is effective without causing toxicity. Demonstrating that the prediction of the NOAEL for the *in vitro* assays is consistent with tested animal species adds to the weight of evidence showing that the correct dose has been selected for clinical trial.

1.6 Criticisms for the Use of In Vitro Assays for Product Development

In vitro systems have been criticised for lacking the normal pathways for absorption, distribution metabolism and excretion (ADME) found in live animals and for lacking intact immune, endocrine and nervous systems (Combes, 2005). Chemical exposure times may be different *in vivo* than an *in vitro* system due to differences in the rate of absorption into the cornea or conjunctiva. The *in vivo* cornea may provide a larger surface area for absorption than *in vitro* cells due to the presence of microvilli or absorption may be blocked by the presence of tear film components on the corneal surface. A chemical instilled in the eye may be diluted faster than the *in vitro* system due to the larger mass of cells available to absorb the chemical, both on the cornea and the conjunctiva, and by dilution due to the flushing of the eye by tears. Cornea and conjunctival cells may be at a different physiological state than *in vitro* cells and thus metabolise certain chemicals faster or slower. Removal of the chemical from the cells can be dependent on

the presence of capillary beds in the conjunctiva and diffusion and active transport in the cornea. *In vitro* systems do not have the mass of tissue for dilution or capillary beds for chemical removal. Immune responses are complex interactions between different cell types. Cell cultures can model single immune responses such as a release of an inflammatory cytokines, but cannot model the entire immune response. Neurotoxins may not affect cells in a corneal epithelial *in vitro* model, but may have severe detrimental effects in the eye.

1.7 Utilization of In Vitro Assays to Reduce the Use of Animals for Product Developement

These fundamental differences between *in vitro* systems and *in vivo* systems support the use of a tiered testing strategy for the testing of ophthalmic chemicals. If a chemical is identified as being toxic in the *in vitro* test system it could be argued that the chemical may not be toxic *in vivo* due to differences in the ADME of an *in vivo* system vs. an *in vitro* system. ADME may help minimize the toxicity of a chemical. However, would a chemical that has a greater toxicity than BAK be considered for testing in animals due to the possibility of mitigation of this toxicity by ADME? The uncertainty due to inter-individual variability in toxicokinetics has been given an uncertainty factor of 3.16 but may be adjusted for different routes of elimination and different groups within the population (Renwick and Lazarus, 1998). Inter-individual differences related to toxicokinetics would cause uncertainty as to whether certain individuals could eliminate a toxic chemical before it exhibited its damaging effects. Thus, toxic chemicals, regardless of the ADME differences with *in vitro* models, should be eliminated from consideration prior to animal testing due to the variability between individuals in their abilities to eliminate chemicals from the tissue. In a tiered testing strategy chemicals that are determined to be nontoxic *in vitro* are then

sent for animal testing. Therefore, the fact that an *in vitro* system does not have the complexity of the immune system or test for neurotoxicity is not an issue as this aspect of toxicity would be evaluated *in vivo*.

The utilization of *in vitro* tests with a tiered testing strategy for detection of mild ocular irritants can reduce the use of animals for testing, provide mechanistic data on toxic effects, and reduce the uncertainty associated with dose selection for clinical trials. Future development of new *in vitro* assays and their appropriate utilization can allow for the humane development of new ophthalmic products and provide tools for faster development of safe and effective products.

The goal of this research was to develop an *in vitro* test battery that can be used to accurately predict the ocular toxicity of new chemicals and ophthalmic formulations. By comparing the toxicity seen *in vivo* animals and humans with the toxicity response in these new *in vitro* methods, it was demonstrated that these *in vitro* methods can be utilized in a tiered testing strategy in the development of new chemicals and ophthalmic formulations.

Chapter 2

Sensitive Measures of Detecting the Effects of Chemicals and Contact Lens Care Solutions on Tight Junctions

This work describes the use and development of an *in vitro* model to investigate the effects contact lens care solutions have on the barrier function of the cornea. The work was performed in conjunction with Dr. Ruy Tchao at the Philadelphia College of Pharmacy and was published in two articles (McCanna *et al*., 2008; Tchao, McCanna *et al*., 2002) and was presented as posters at three international meetings (McCanna *et al.* 2005: McCanna *et al.* 2006; McCanna *et al.* 2007). I conducted the studies relating to the effect of BAK on the MDCK cells which were presented at the 4th Annual University of Waterloo Graduate conference.

2.1 Introduction

The mammalian eye has adapted mechanisms that protect the eye from damage. Humans before the modern age lived in environments that contained numerous hazards. Dry environments blew dust or sand into the eyes causing irritation. Forest environments pose the continuous hazard of branches scraping the eye during travel through the brush and thicket. Swimming in lakes and rivers could expose the eye to pathogenic organisms. Microorganisms on the skin could be transferred to the eyes causing infection. These hazards were present throughout the evolution of humans and anatomical and biochemical adaptations occurred to counteract these threats to sight. Anatomical features for protection include the eye lids, the epithelial barrier of the cornea consisting of tight junctions, exfoliation (sloughing) of damaged cells, a protective tear film and the washing of the eyes by tears. At the ocular surface the eye has adapted to combat invading microorganisms (Evans *et al.*, 2007). There are molecules in the tear film that contain bactericidal agents, mucins that inhibit bacterial binding, and molecules for leukocyte recruitment at the site of infection (Haxlett, 2005).

Modern day society has created additional hazards that can pose risks to the eye. In a recent study of the effects of rinsing the eyes with water containing chlorine, it was determined that chlorine was potentially harmful to the corneal epithelial barrier as measured by a significant increase in corneal fluorescein uptake in exposed eyes (Ishioka *et al*., 2008). Cosmetic products or shampoos that could wash into the eyes by accident can cause breaks in the epithelial barrier

of the cornea and cause irritation (Cottin and Zanvit, 1997). Contact lens cases can become contaminated by biofilms of microorganisms even when contact lens disinfecting solutions are used (Zhang and Ahearn, 2006; Pens *et al.*, 2008; Boost *et al.*, 2008). These organisms can then be transferred to the eye via the contact lens and cause infection. Contact lenses have been shown to adhere to microorganisms. These adhered organisms can then invade the cornea causing serious eye infections (Ahearn *et al.*, 2007; Imamura *et al.*, 2008; Anger and Lally, 2008; Shoff *et al.*, 2008, Patel and Hammersmith, 2008; Pinna *et al.*, 2008; Margolis and Whitcher, 2006) Preservatives used in eye drops and disinfectants used in to disinfect contact lenses can cause breaks in the epithelial tight junctions (McCanna *et al.*, 2008; Tchao *et al.*, 2002; Imayasu *et al.*, 2008; Chuang *et al*., 2008; Uematau *et al*., 2007; McCarey and Edelhauser, 2007). Corneal toxicity can also occur from topically applied and systemic medications (Nakamura *et al.* 2007, Fraunfelder, 2006; Ly *et al.*, 2006).

The presence of microorganisms on the surface of the eye will not necessary lead to corneal infection. In a study of the microbial flora of the eye of soft contact lens wearers it was found that positive cultures from the conjunctival cul-de-sacs of patients occurred that ranged from 14.3% to 30.9% over the 6 month period of the study (McBride, 1979). The microflora of the eye resembled the microflora on the skin, indicating that the eye can be populated by microorganisms from the skin. Whether microorganisms enter the eye from the skin or from a contact lens, the defence mechanisms in the eye generally protect the cornea from infection (Levy and Orsborn, 2008). However, microbial keratitis due to contact lens wear occurs. In a recent study of microbial keratitis in Austrialia, the risk of infection with daily wear silicone

hydrogel contact lens was 11.9 per annualized incidence per 10000 lens wears and 1.9 for daily wear soft contact lens wearers (Stapeton *et al.*, 2008). In the United States one in 2500 daily wear contact lens users develop bacterial keratitis each year (Hazlett, 2005).

Microbial keratitis can result from infection with bacteria, fungi or amoebaes. The pathogenesis of *Pseudomonas aeruginasa* has been described and multiple risk factors and defence mechanisms are involved in this process (Evans *et al*., 2007; Angus *et al.*, 2008; Fleiszig, 2006; Fleiszig, 2003). In the recent outbreaks of infections related to contact lens products the cause of the *Fusarium* infections related to ReNu MoistureLoc® was attributed to a lack of efficacy of the solution due to absorption of the antimicrobial component from the lens (Rosenthal *et al*., 2006) and growth and survival of *Fusarium* in dried MoistureLoc® films in lens cases (Zhang *et al*., 2006). Imayasu *et al.* evaluated ReNu with MoistureLoc® and three other contact lens disinfecting products on their effects on tight junctions (Imayasu *et al.*, 2008). There was no correlation between infection and solution effects on tight junctions. After a 60 minute exposure, ReNu MultiPlus® and Optifree® Express® showed widely opened junctions whereas cultures exposed to ReNu MoistureLoc® showed only partially opened junctions, among other junctions that were tightly closed. The *Acanthamoeba* infections attributed to the use of Complete® MoisturePlus was possibly due to the ingredient propylene glycol which induced *Acanthamoeba* encystment and which made the organism more resistant to the disinfectants in the contact lens care product (Kilvington *et al.,* 2008). The effect of Complete® MoisturePlus on the corneal surface barrier function does not appear to be a risk factor in the *Acanthamoeba* infections. After a 15 minute exposure to human corneal epithelial cells, Complete® MoisturePlus did not cause

breaks in the tight junctions (McCanna *et al.*, 2008). In a recent article by Dutot *et al.* (2008) survey of the number of contact lens wears, type of infection and lens care solution used by patients was conducted. For contact lens wearers 59% used multipurpose solutions and 35% used oxidative products such as peroxide. Of the contact lens wearers that had keratitis 80% used multipurpose solutions. Whether total antimicrobial effectiveness of the multipurpose solutions against microorganisms compared to peroxide or effects of these solutions on the barrier function were the cause of increased infections with multipurpose solutions was not determined.

Levy and Orsborn evaluated the clinical risks of contact lens care products (Levy and Orsborn, 2008). Levy argues that "there is only one sight-threatening event related to contact lens wear – infectious keratitis". They also cited evidence that epidemiological studies conducted on lens-related corneal infections 10 years apart show the same rates of infection. Over this 10 year period in the market the use of PHMB products increased with no increase in the incidence of microbial infection. The two products that were recalled from the market for increasing the incident rate of infection contained unique ingredients that reduced the efficacy of the Alexidine and PHMB to the *Fusarium solani* and *Acanthamoeba* and was not attributed to solution cytotoxicity.

As contact lens care manufactures increase the efficacy of their products against pathogenic microorganisms due to concerns of the lack of efficacy against *Fusarium solani* and *Acanthamoeba,* increased toxicity to the eye caused by these products could occur due to the use of higher concentrations of disinfectants or more potent antimicrobials that have deleterious

effects on biological membranes and cell organelles. Increased toxicity could cause enhanced breaks in tight junctions and lead to infection. Yi *et al.* (2000) determined that lippolysaccharides in gram negative organisms such as *Pseudomonas aeruginasa* induce the breakdown in the epithelial barrier of human corneal epithelial cells by targeting the ZO-1 and ZO-2 proteins. Also, Fleiszig *et al.* (1997) found that corneal epithelial cells with intact tight junctions were significantly less susceptible to *Pseudomonas aeruginosa* infection than corneal epithelial cells with disrupted tight junctions. Thus, breaks in tight junctions may be precursors to invasion by virulent *Pseudomonas* into the cornea. Kinnear evaluated the relative susceptibility of human keratocyte and corneal epithelial cells to *Acanthamoeba castellani* (Kinnear, 2004). For both cell types the first observable sign of cell damage was cell shrinkage, with the formation of retraction of fibres and gaps. Moore *et al.* (1991) evaluated the *in vitro* penetration of human corneal epithelium by *Acanthamoeba castellanii* and found that after initial adhesion the tight junctions break down and *Acanthamoeba* then penetrated the cell layer. An animal model evaluated the infection of the abraded corneas with *Acanthamoeba*-laden contact lenses (Van Klink *et al.*, 1993). This study determined that corneal abrasion was necessary for the induction *of Acanthamoeba* keratitis in hamsters infected with contaminated contact lenses. Thus, resistance of the cornea to *Acanthamoeba* may be due to presence of tight junctions between cells and that breaks in tight junctions caused by contact lens disinfecting products could allow for *Acanthamoeba* to penetrate the cornea and cause keratitis.

In addition to protection from infection, tight junctions also function to create a semi-permeable barrier preventing harmful chemicals from penetrating into the cornea. The molecules that make

up the tight junctions sealing the cells together are the tansmembrane proteins claudin and occludin. The tight junction complex (zonula occludens) also contains membrane associated proteins ZO-1, ZO-1 and ZO-3 (Ban *et al.*, 2003). These tight junctions form in the most superficial layer of the cornea. Saitau *et al.* (1998) discovered no significant differences in number or morphology of tight junctional strands between wild-type and occludin-deficient epithelial cells. Saitau hypothesised that there were other transmembrane proteins responsible for tight junctions. In 1998 Furuse *et al.* (1998) discovered a second class of transmembrane proteins and they were named claudins. It is now believed that the claudins are the structural component of the tight junctions and the occludins are involved in a regulator function of these junctions (Ban *et al.*, 2003). The ZO-1, Z0-2, and Z0-3 proteins bind to the occludins or the claudins and also bind to the intracellular actin cytoskeleton (Ban *et al.*, 2003). Thus the actin filaments of adjacent cells are bridged together through the transmembrane proteins occludin or claudin with anchors to actin by the Z0-1, Z0-2 or Z0-3 proteins. The interaction of these proteins with the actin cytoskeleton stabilizes the junctional structure and tightens the epithelial barrier (Ivanov, 2008).

Chemicals that enter into the tear film do not always penetrate the cornea due to the tight junctional barrier. Damage to epithelial cells can occur at the surface. Because the surface cells are undergoing desquamation, damage to only surface cells may not cause major disruptions in corneal function. If however the cytotoxicity is high enough to kill the cells or disrupt the tight junctions, these toxic chemicals would be able to penetrate into the cornea causing damage to deeper cells. These cells would include epithelial wing cells that migrate to the epithelial

surface, basal cells that are undergoing mitosis, keratocytes in the stroma and endothelial cells. Disrupting the normal physiology of these cells could cause corneal damage.

If tight junctions remain intact harmful chemicals or drugs can enter the cornea only if they have the chemical structure to pass through the epithelial barrier. Hydrophilicity/lipophilicity ratio and molecular size are important properties that can determine penetration into the cornea. Chemicals can have two potential paths for penetration. Passage is through the cell body (transcellular) or between the cell junctions (paracellular). Lipophilic molecules preferentially pass via the transcellular route whereas hydrophilic molecules pass via the paracellular route (Zhang *et al.*, 2004). In human eyes, bioavailability was predicted by Zhang *et al.* (2004) to range between 1% and 5% for topically applied lipophilic molecules and to be less than 0.5% for hydrophilic molecules. The size limitation for the paracellular pathway is about 60 angstroms (6 nm) which is the molecular size of glycerol (Lee, 1990). Permeation enhancers increase corneal uptake by drugs by modifiying the molecules in the tight junctional complex of the epithelium. Benzalkonium chloride (BAK) and ethylenediaminetetraacetic acid (EDTA), common ingredients in ophthalmic formulations, have shown a significant enhancement in corneal drug absorption (Audus *et al.*, 1990). Both chemicals have been shown to break down the tight junctions. BAK has been shown break tight junctions (Nakamura *et al*., 2007) and EDTA chelates calcium which loosens the tight junctions (Lee, 1990).

In vitro models have been developed for detecting breaks in tight junctions caused by chemicals. *In vitro* models have employed the use of cell cultures on membranes as media, drugs and

chemicals can be exchanged from both the apical and basal membranes, whereas cells grown on plastic dishes exchange occurs only at the apical surfaces (Audus *et al*., 1990). Using cell membrane inserts and a monolayer culture of MDCK cells, Tchao developed a cell culture model for detecting chemical damage to tight junctions (Tchao, 1988). This model was proposed as an alternative to the Draize rabbit ocular irritation test. This *in vitro* assay has been used by several researchers and in industry to evaluate the ocular irritation potential of chemicals and product formulations (Tchao, 1988; Balls and Clothier, 1992; O'Connor *et al.*, 1991; Shaw *et al.*, 1990; Shaw *et al.*, 1991; Martin and Stott, 1992). Validation studies evaluated the feasibility of using this method for predicting the ocular irritation of chemicals and found that it was useful for testing the irritation potential of cosmetic products containing surfactants (Gautheron *et al*., 1994; Botham *et al.*, 1997).

A potential limitation of the using MDCK cells to measure damage to the corneal epithelium is that the cells used are of canine kidney origin, rather than human cornea. These cells may not have identical membrane and cytoskeletal proteins as those expressed by the corneal epithelial cells. Therefore, it was desirable to develop an assay using human corneal epithelial cells. Although several cytotoxicity studies have used primary or transfected human corneal epithelial cells (Balls *et al*., 1995; Neville *et al*., 1996; Tripathi *et al.*, 1992; Kahn *et al.*, 1993; Kruszewski *et al*., 1997; Offord *et al.*, 1999; Huhtala *et al.*, 2002) these assays have not been based on the functional characteristic of the corneal epithelium, namely the barrier function. To develop the assay an immortalized human corneal epithelial cell line had to be found that formed tight junctions. The human corneal epithelial cell line deposited in the ATCC culture collection

(Kahn *et al.*, 1993; Hornof *et al.*, 2005) has been determined to be limited for cytotoxicity assays due to virus shedding (Hornof *et al*., 2005). Three additional immortalized human corneal epithelial cell lines have been developed. These three cell lines are a human corneal epithelial cell line developed by SkinEthic, another developed by Clonetics and a third developed by Araki-Sasaki *et al* .(Araki-Sasaki *et al*., 1995 ; Becker *et al.*, 2008). Using transepithelial resistance and transmission electron microscopy Toropainen *et al.* (2001) established that the Araki-Sasaki cell line could form tight junctions in culture.

Using a sub-clone of the Araki-Sasaki cell line that formed good tight junctions we were able to develop a human corneal epithelial culture model that could be used to assess the effects of chemicals and product formulations on the barrier function. In a recent article by Becker *et al.* (2008) the SkinEthic human corneal cell line did not form tight junctions whereas the cell line from Clonetics shows promise for use as it formed tight junctions in culture.

This work describes the use and development of an *in vitro* model to investigate the effect contact lens care solutions have on the barrier function of the cornea. MDCK and HCEC lines were exposed to contact lens solutions. Using sodium fluorescein permeability and SEM the effect of these solutions on the tight junctions was determined. A study was also performed using the known ocular toxins SDS and BAK. The breakdown in the tight junctions due to these chemicals was assessed after exposure of the cell monolayers to various chemical concentrations and exposure times. The cultures were assessed immediately after exposure and after 24 hours of recovery in growth media. To determine the relevance of these *in vitro* results a contact lens

wear study was performed in rabbits and the tight junctions of the corneas were evaluated using scanning electron microscopy.

2.2 Materials and Methods

2.2.1 Comparison of Contact Lens Multipurpose Solutions by In Vitro Sodium Fluorescein Permeability Assay Using MDCK Cells

All lens care solutions were purchased from commercial sources and were used within their labeled expiration dates. Madin-Darby canine kidney cells were obtained from American Type Culture Collection (Manassas, VA), ATCC#CCL34, and maintained in minimum essential medium (MEM) (BioWhittaker, Walkersville,MD) (MEM) supplemented with 10% bovine calf serum with iron supplementation.

2.2.1.1 Sodium Fluorescein Permeability Assay

A 0.5-mL cell suspension containing $2x10^5$ cells was seeded in Millicell HA 13-mm inserts (Millipore, Bedford, MA). The inserts were transferred into 24-well plates containing 0.5 mL of MEM per well. The plates were then incubated at 37°C with 5% CO_2 for 6 days. Fresh media was added to the wells on days 2 through 6. On day 6 the inserts were used for the permeability assay.

Each insert was gently rinsed three times with 1 mL of Hanks' balanced salt solution (HBSS) using a 10-mL syringe, without a needle. An amount of 0.5 mL of test solution was added to separate inserts that had been placed in a fresh 24-well plate. Triplicate inserts were used for each test solution. The inserts were incubated in a 100% humidified chamber at 37°C.

ReNu MultiPlus® (Bausch & Lomb, Rochester, NY), SOLO-care® (CIBA Vision), Complete® Comfort Plus (Allergan, Irvine, CA), OPTIFREE® Express® with Aldox (Alcon, Ft. Worth, TX) solutions were incubated for 20 minutes.

Boston® SimPlus Multi-Action Solution, Alcon Unique® pH Multi-Purpose Solution, Optimum® by Lobob were incubated for 15 minutes.

Sodium Dodecyl Sulphate (SDS) and Benzalkonium chloride (BAK) controls were tested at various exposure times and temperatures. SDS and BAK were purchased from Sigma Chemical Co. (St. Louis MO).

Each series of triplicate samples was handled sequentially to allow exact timing of the treatment and subsequent steps. After incubation, each insert was individually rinsed five times with 1 mL HBSS using a 10-mL syringe (without a needle), and then placed in a fresh 24-well plate containing 0.5 mL HBSS in each well. To each insert was added 0.5 mL of sodium fluorescein (3 mg/100 mL in HBSS). The inserts were incubated at room temperature for 20 minutes and removed from the wells, and the amount of sodium fluorescein was measured using a

fluorometer at 485 nm excitation and 530 nm emission. Triplicate negative controls (HBSS solution) and positive controls consisting of sodium dodecyl sulphate (SDS) in water (50-1000 µg/mL were included during these evaluations.

Boston® SimPlus Multi-Action Solution, Alcon Unique® pH Multi-Purpose Solution, Optimum® by Lobob, Sodium Dodecyl Sulphate (SDS) and Benzalkonium chloride (BAK) controls were evaluated for 24 hour recovery. After reading in the fluorometer as indicated above the inserts were rinsed with HBSS and were transferred into 24-well plates containing 0.5 mL of MEM per well and 0.5 mL of MEM was placed on top of each well. The plates were then incubated at 37°C with 5% CO_2 for 24 hours. At 24 hours the inserts were rinsed with HBSS and 0.5 mL of sodium fluorescein (3 mg/100 mL in HBSS) was added to each insert. The inserts were incubated at room temperature for 20 minutes, removed from the wells, and the amount of sodium fluorescein was measured using a fluorometer at 485 nm excitation and 530 nm emission.

2.2.1.2 Scanning Electron Microscopy

The MDCK cell monolayers used in permeability assays were fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) for 2 hours at room temperature. The inserts were then transferred to PBS and kept at 4°C until processed for dehydration. After dehydration with graded ethanol, from 50% to 100%, the inserts were immersed in hexamethyldisylazane for 10 minutes, removed, and then air dried in a fume hood. Samples were sputter-coated with gold and examined with a Hitachi (San Jose, CA) S530 scanning electron microscope.

2.2.2 Use of a Human Corneal Epithelial Cell Line for Screening the Safety of Contact Lens Care Solutions *In Vitro*

2.2.2.1 Chemicals and Solutions.

The lens care solutions OPTIFREE® Express® Multi-Purpose Disinfecting Solution (Alcon, Ft. Worth, TX), ReNu MultiPlus® Multi-Purpose Solution (Bausch & Lomb, Rochester, NY), SOLO-care® Plus with Aqualube Multi-Purpose Solution (CIBA Vision, Duluth, GA), Complete® Moisture Plus™ Multi-Purpose Solution (AMO, Santa Ana, CA), and Aquify® 5 Minute Multi-Purpose Solution (CIBA Vision, Duluth, GA) were purchased from commercial sources and were used within their labeled expiration dates. Other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.2.2.2 Culture Conditions.

The medium used to culture human corneal epithelial cells (HCEC) cells is as follows: 50/50 Ham's F12/Dulbecco' modified Eagle's medium(Mediatech, Inc, Herndon, VA), 10% heat-inactivated fetal bovine serum, (Atlanta Biological, Lawrenceville, GA), 5 μg/ml insulin, 0.1 μg/ml cholera toxin, 10 ng/ml epidermal growth factor, and 50 μg/ml gentamycin. Cells were incubated at humidified 37°C and 5% CO_2. Cultures were maintained with weekly subculture using trypsin/EDTA at a ratio of 1:6 and fed every 2-3 days.

2.2.2.3 HCEC Cloning.

The original human corneal epithelial cell line shows a heterogeneous population of cells. A suspension of low density of cells was seeded in several plastic Petri dishes. After cell adhesion, several single cells were identified and isolated with cloning cylinders. The isolated cells were observed daily and those that developed into colonies with typical epithelial morphology were selected for feeding and removal with trypsin/EDTA and cultured in separate T-25 flasks. A clone with epithelial morphology was again cloned and used as subsequent culture for further studies.

2.2.2.4 Transepithelial Resistance.

A cell suspension (0.5 ml) containing $2x10^5$ cells was seeded in Millicell HA 13-mm inserts (Millipore, Bedford, MA). The inserts were transferred into 24-well plates containing 0.5 ml of growth medium per well. The plates were then incubated at 37°C with 5% CO_2. Transepithelial resistance was measured using the EVOM instrument (World Precision Instruments, Sarasota, FL).

2.2.2.5 Transmission (TEM) and Scanning Electron Microscopy (SEM).

Cells grown on plastic cover slips were fixed in freshly prepared phosphate-buffered saline (PBS)-buffered 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 2 hours at room temperature and transferred to PBS and kept at 4°C. For TEM, the specimens were post-stained with osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA).

After standard graded dehydration with ethanol, the specimens for SEM were placed in hexamethyldisilazane for 10 minutes and then air-dried overnight. For TEM, the specimens were embedded in epon and sectioned at 0.1-micron thickness and floated onto Formvar^{TM}-coated grids, and stained with uranyl acetate, dehydrated in graded ethanol, and viewed with a Zeiss EM10 electron microscope. For SEM, the dried specimens were sputter-coated with gold and viewed with a Hitachi S530 scanning electron microscope. Based on preliminary observations under light microscopy, the cultures were fixed for SEM at 10 minutes after treatment of cultures with various solutions.

2.2.2.6 Sodium Fluorescein Permeability Assay.

A cell suspension (0.5 ml) containing $2x10^5$ cells was seeded in Millicell HA 13-mm inserts (Millipore, Bedford, MA). The inserts were transferred into 24-well plates containing 0.5 ml of growth medium per well. The plates were then incubated at 37°C with 5% CO_2 for six days. Fresh media was added to the wells on days two through six. On day six the inserts were used for the permeability assay. Each insert was gently rinsed three times with 1 ml of HBSS using a 10-ml syringe without a needle. Test solution (0.5 ml) was added to each individual insert which had been placed in a fresh 24-well plate. Triplicate inserts were used for each test solution. The inserts were incubated in a 100% humidified chamber at 37^o C for 15 minutes. Each series of triplicate samples was handled sequentially to allow exact timing of the treatment and subsequent steps. After incubation, each insert was individually rinsed five times with 1 ml HBSS using a 10-ml syringe (without a needle), and placed in a fresh 24-well plate containing 0.5 ml HBSS in each well. Sodium fluorescein (0.5 ml; 3 mg/100 ml in HBSS) was added to

each insert. The inserts were incubated at room temperature for 20 minutes, removed from the wells, and the amount of sodium fluorescein was measured using a fluorometer (Perkin Elmer, Norwalk, CT) at 485 nm excitation and 530 nm emission. Triplicate negative controls (HBSS solution) were included during these evaluations.

2.2.2.7 Statistical Analysis.

Statistical procedures were performed using analysis of variance.

2.2.3 The Effect of Two Contact Lens Care Solutions on the Ocular Surface

The rabbits were treated at a contract research facility. The fixed eyes were then sent to the Philadelphia College of Pharmacy for scanning electron microscopy evaluation.

New Zealand white rabbits wore balafilcon A contact lenses for 2 hours that were soaked overnight in OptiFree® Express® or ReNu MultiPlus® contact lens disinfecting solution. Rabbits' eyes that were not treated with contact lenses were used as controls. Two rabbits were evaluated for the each test solution and the controls. The lenses were removed from the eyes after 2 hours and the eyes were fixed with 2% glutaraldehyde in PBS for Scanning Electron Microscopy. The corneas were removed and processed through graded ethanol, air dried, sputter coated with gold and examined in a Hitachi S530 Scanning Electron Microscope.

2.3 Results

2.3.1 Comparison of Contact Lens Multipurpose Solutions by In Vitro Sodium Fluorescein Permeability Assay Using MDCK cells

The effect of SDS and HBSS on an MDCK epithelial cell monolayer is shown in Figure 1. An increase in sodium fluorescein permeability was observed after the monolayer was exposed to SDS at concentrations greater than 50 ug/mL. Physiologic changes in these epithelial monolayers were observed using scanning electron microscopy (SEM) when compared with a HBSS control. A monolayer exposed to HBSS remained intact, the epithelial cells were closely apposed to each other, and they exhibited tight junctions (Fig. 2). The cell surface exposed to HBSS had numerous microvilli, which indicated a healthy epithelial cell monolayer. Monolayers exposed to SDS demonstrated a breakdown of tight junctions and cell membrane damage, as the concentration increased from 50 to 1000 µg/mL (Figs. 3–9). After a SDS treatment of 50 µg/mL (Fig. 3), the monolayer and the cells appeared similar to the HBSS control. At 75 µg/mL concentration (Fig. 4), the monolayer began to detach in small groups, and gaps between cells were observed. At 125 µg/mL (Fig. 5) and 150 µg/mL (Fig. 6), there was an increase in the number of gaps between the cells and marked damage to the cell membranes. At 200 µg/mL concentration (Fig. 7) debris from lysed cells was observed on the support membrane. At 500 ug/mL (Fig. 8), a greater number of cells were detached from the support membrane; furthermore, in areas where there were attached cells, the cells were predominately separated from each other. At 1000 µg/mL (Fig. 9), the support membrane was covered with a layer of dead cells and cell debris. A comparison of the effect of contact lens care products on sodium fluorescein permeability of a MDCK epithelial cell monolayer is presented in Figure 10. There is no significant difference ($P > 0.05$) between ReNu and ReNu MultiPlus (Bausch & Lomb, Rochester, NY), SOLO-care (CIBA Vision), and Complete Comfort Plus (Allergan, Irvine, CA) solutions. Additionally, sodium fluorescein permeability associated with these solutions was not

significantly different from the HBSS negative control ($P > 0.05$). OPTIFREE Express with Aldox (Alcon, Ft. Worth, TX) solution was significantly more damaging to the MDCK epithelium ($P < 0.05$) than all of the other lens-care solutions and the negative HBSS control. OPTIFREE Express Mutli-Purpose Solution was significantly more damaging to the MDCK epithelium ($P< 0.05$) than ReNu Multiplus, and ReNu multipurpose solution (Bausch & Lomb, Rochester, NY), SOLO-care (CIBA Vision, Duluth, GA) and Complete Comfort Plus (Allergan, Irvine, CA) multipurpose solutions. The physiologic effect of each contact lens care solution on tight junctions and the MDCK epithelial cell membranes, as observed by SEM, are shown in Figures 11 to 16. Cells cultures exposed to OPTIFREE Express with Aldox showed a definite loss of tight junctions, damaged cell membranes, loss of microvilli, and the appearance of membrane blebs and folds (Fig. 11). OPTIFREE Express solution showed loss of tight junctions and cell membrane damage (Fig. 12). Cell cultures exposed to ReNu (Fig. 13), ReNu MultiPlus (Fig. 14), SOLO-care (Fig. 15), and Complete Comfort Plus (Fig. 16) appear to have similar tight junctions and intact cell membranes, when compared to the HBSS negative control (Fig. 2).

2.3.2 Comparison of the Safety of Rigid Gas Permeable Contact Lens Solutions

The effect of Boston® SimPlus Multi-Action Solution, Alcon Unique® pH Multi-Purpose Solution, Optimum® by Lobob, SDS and HBSS on an MDCK epithelial cell monolayer is shown in Figure 17. An increase in sodium fluorescein permeability was observed after the monolayer was exposed to the Alcon Unique® pH and the Optimum® by Lobob solutions $p < 0.05$. At 24 hours recovery the cultures exposed to Optimum® by Lobob did not recover. Using SEM the

damage caused by the Alcon Unique® pH and the Optimum® by Lobob solutions could be seen Figure 18. Optimum by Lobob caused significantly more damage to the monolayer than the other solutions tested.

2.3.3 Evaluation of the Effect of Chemical Concentration, Exposure Time, Temperature and Dissolution Vehicles Have on the Permeability of an Epithelium

The sodium fluorescein permeability assay showed dose response up to 100 ppm (0.01%) BAK after the 30 minute exposure and a dose response for 24 hour recovery at all concentrations (Figure 19). The drop in the values between 100 ppm and 200 ppm is likely due to the binding of BAK to the filter and blocking the penetration of fluorescein. When cultures were exposed to SDS the effects increasing the time of exposure resulted in higher permeability at 24 hours of recovery after initial exposure (Figure 20 and Figure 21). Cultures exposed to 0.025% SDS at 37 degrees after 24 hours of recovery showed significantly greater permeability to sodium fluorescein than the cultures exposed at room temperature (Figure 22). Cultures exposed to 0.025% SDS in HBSS were more permeable to sodium fluorescein than cultures exposed to 0.025% SDS in water or saline Figure 23.

2.3.4 Use of a Human Corneal Epithelial Cell Line for Screening the Safety of Contact Lens Care Solutions *In Vitro*

To verify appropriate corneal structure of the newly cloned human corneal epithelial cell line, scanning electron microscopy was used. The original colony of cells showed heterogeneity, without a consistent cornea like structure (Fig. 24A). However, by using the techniques

described earlier, substantially greater culture homogeneity was achieved (Fig. 24B); this human corneal epithelial cell line was used in all subsequent studies and forms the basis for the screening assay described herein. As shown by transmission electron microscopy, these cells possessed well-developed organelles and microvilli at the apical surface (Fig. 25A). There was some degree of cell overlapping and multilayering of cells. Furthermore, tight junctions were clearly present at the apical membranes of adjacent cells (Fig. 25B). Under the scanning electron microscope (Fig. 26), the cells showed close juxtaposition and ridged membranes, further showing the presence of tight junctions. Finally, as anticipated, these cells showed the development of electrical resistance after several days of culture (Fig. 27), providing further evidence of the generation of functional tight junctions. Using this characterized cell line, preliminary experiments were next conducted with various marketed contact lens care solutions to explore their effects on the culture system. By using phase microscopy, the effect of 15 minutes of solution exposure to the cells was evaluated. HBSS treatment resulted in normal cell structure and no apparent visual effect on the integrity of the cellular monolayer (Fig. 28A). Likewise, treatment with ReNu MultiPlus resulted in microscopic images (Fig. 28B) that were indistinguishable from the HBSS-treated cultures, with normal cellular structure. In sharp contrast, 15 minutes of exposure to OPTI-FREE Express (Fig. 28C) resulted in a distinct separation of the cells that was readily visible in all cultures, along with a clustering of some cells.

The effect of these products on corneal tight junctional integrity was also assessed by using the sodium fluorescein permeability assay (Fig. 29). There was no significant difference in

fluorescein permeability among HBSS-treated controls, ReNu MultiPlus, SOLO-care Plus With Aqualube, AQuify 5 Minute, and Complete Moisture Plus. However, OPTI-FREE Express was significantly more damaging to the human corneal epithelial cells, with significantly greater sodium fluorescein permeability (approximately twice the control values) compared to all other products tested. To verify the structural correlates of these biochemical changes in the assay system, the physiologic effect of each contact lens care solution on tight junctions and the human corneal epithelial cell membranes, as observed by scanning electron microscopy, was determined (Fig. 30). Cell cultures exposed to OPTIFREE Express showed a definite loss of tight junctions, damaged cell membranes, loss of microvilli, and the appearance of membrane blebs and folds (Fig. 30A). In contrast, cell cultures exposed to ReNu MultiPlus (Fig. 30B), SOLO-care Plus With Aqualube (Fig. 30C), Complete Moisture Plus (Fig. 30D), and AQuify 5 Minute (Fig. 30E) appeared to have similar tight junctions and intact cell membranes when compared to the HBSS negative control (Fig. 30F). Cultures treated with Complete Moisture Plus (Fig. 30D) and AQuify 5 Minute (Fig. 30E) showed slight breaks in the monolayer, which were insignificant compared to those seen in cultures treated with OPTI-FREE Express (Fig. 30A).

2.3.5 The Effect of Two Contact Lens Care Solutions on the Ocular Surface (In vivo Rabbit Cornea).

Untreated Control: The corneal epithelial cells appear very flat of squamous morphology (Figure 31). There were some cells with lighter appearance, representing more superficial cells. The center of the photo shows debris, possibly a sloughed off cell. At higher magnification (Figure

32) it shows that the superficial cells have "lesions" on the cell membrane. These "lesions" (Figure 33) appear to be cell membrane blebs, not a "hole" in the membrane.

ReNu MultiPlus®-lens treatment: The cornea (Figure 35) shows morphology very similar to the untreated control cornea, compare with photo (Figure 31). There are very occasional sloughing of superficial cells revealing the underlying epithelium (Figure 36) and (Figure 37), compare with photo (Figure 32). Higher magnification (Figure 34) shows detail of the cell membrane with microvilli and tight junctions.

OptiFree® Express®-lens treatment: Shows distinct sloughing of large patches of cells in (Figure 38). The left side of the picture shows flat squamous cells and as the cells slough off, the appearance is that on the right side of the picture. The exposed underlying cells show prominent cell nuclei and cell junctions. However, in some areas, the cell junctions may not be as "tight" as shown in picture (Figure 39). Cells in the upper middle show detachment from their neighbors. The loss of cell junctions is very clear at higher magnification as in picture (Figure 40).

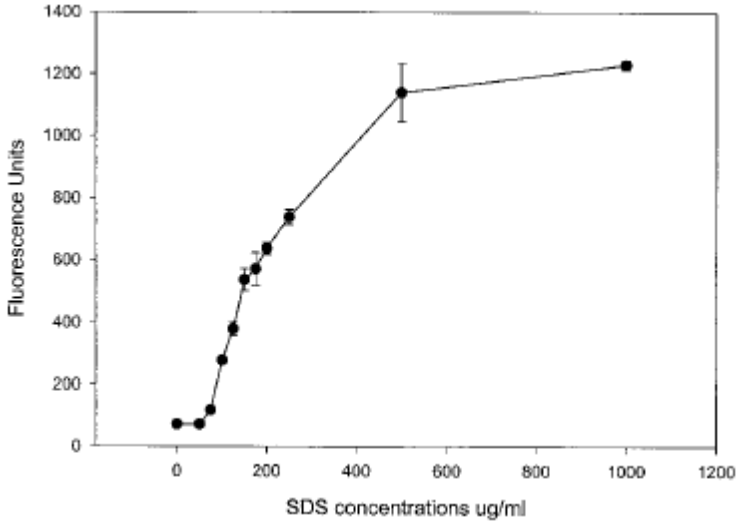

FIG. 1. The effect of sodium dodecyl sulfate (SDS) on a Madin-Darby canine kidney (MDCK) cell epithelial cell monolayer culture (n_3) (magnification x 1000).

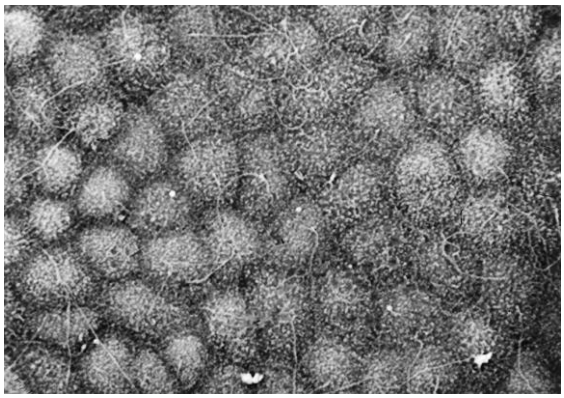

FIG. 2. Scanning electron micrograph (SEM) view of MDCK cellmonolayer culture exposed to Hanks' balanced salt solution (HBSS) (magnification X 1000).

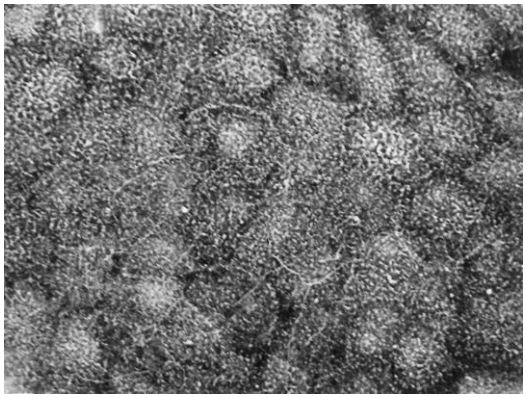

FIG. 3. SEM of MDCK cell monolayer culture exposed to 50 µg/mL sodium dodecyl sulfate (SDS) (magnification X 1000).

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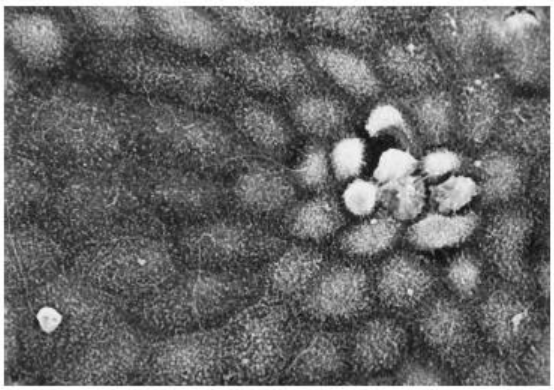

FIG. 4. SEM of MDCK cell monolayer culture exposed to 75 μg/mL SDS (magnification × 1000).

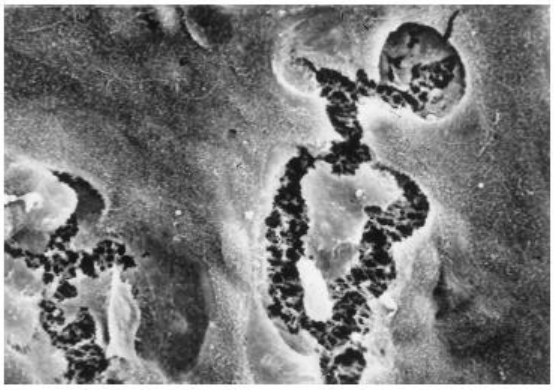

FIG. 5. SEM of MDCK cell monolayer culture exposed to 125 μg/mL SDS (magnification × 1000).

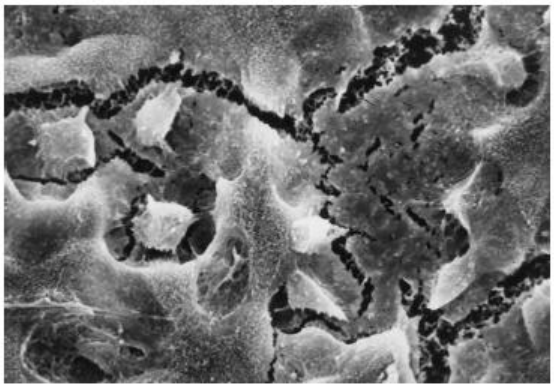

FIG. 6. SEM of MDCK cell monolayer culture exposed to 150 μg/mL SDS (magnification × 1000).

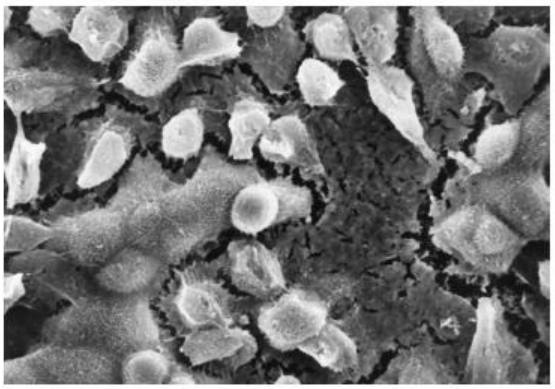

FIG. 7. SEM of MDCK cell monolayer culture exposed to 200 μg/mL SDS (magnification × 1000).

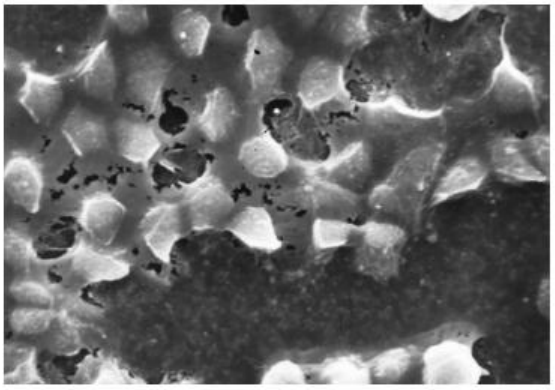

FIG. 8. SEM of MDCK cell monolayer culture exposed to 500 μg/mL SDS (magnification × 1000).

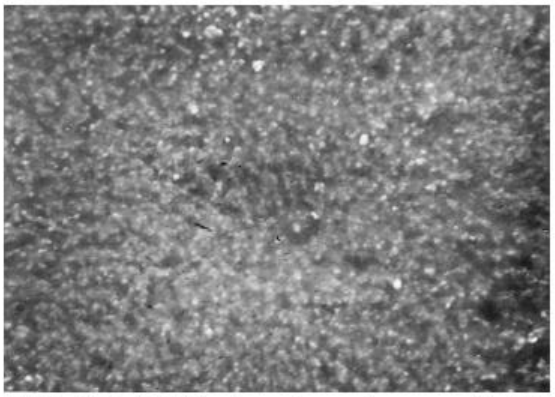

FIG. 9. SEM of MDCK cell monolayer culture exposed to 1000 μg/mL SDS (magnification × 1000).

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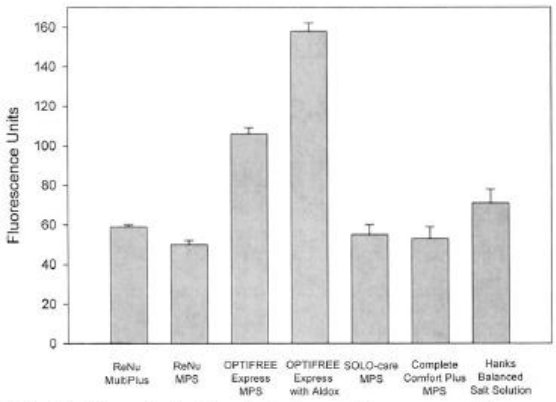

FIG. 10. The effect of contact lens multipurpose solutions on MDCK epithelial permeability.

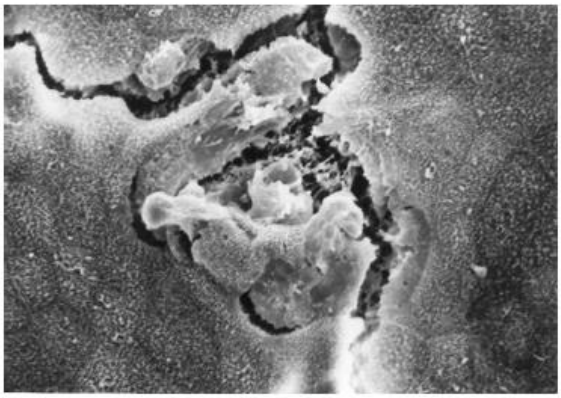

FIG. 12. SEM of MDCK cell monolayer culture exposed to OPTI-FREE Express multipurpose solution (magnification × 1000).

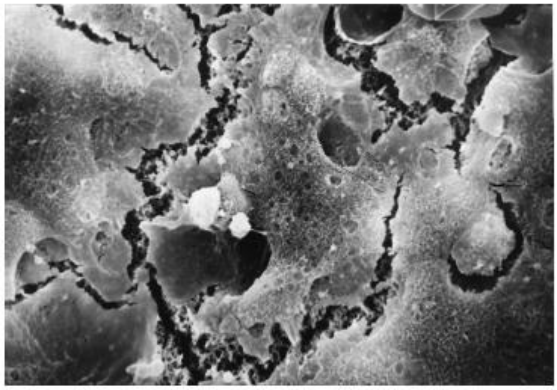

FIG. 11. SEM of MDCK cell monolayer culture exposed to OPTI-FREE Express solution with Aldox (magnification × 1000).

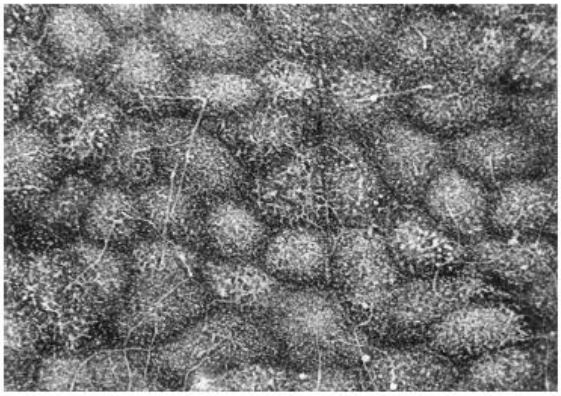

FIG. 13. SEM of MDCK cell monolayer culture exposed to ReNu multipurpose solution (magnification × 1000).

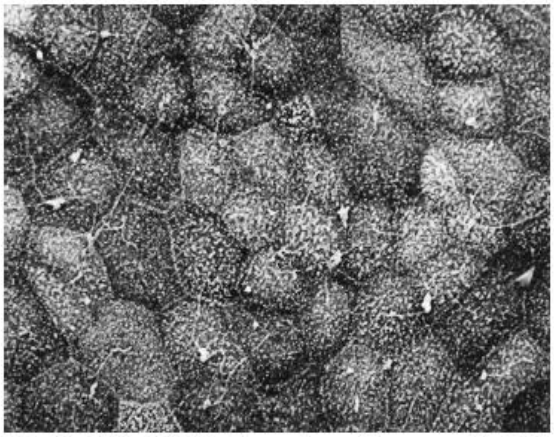

FIG. 14. SEM of MDCK cell monolayer culture exposed to ReNu MultiPlus multipurpose solution (magnification × 1000).

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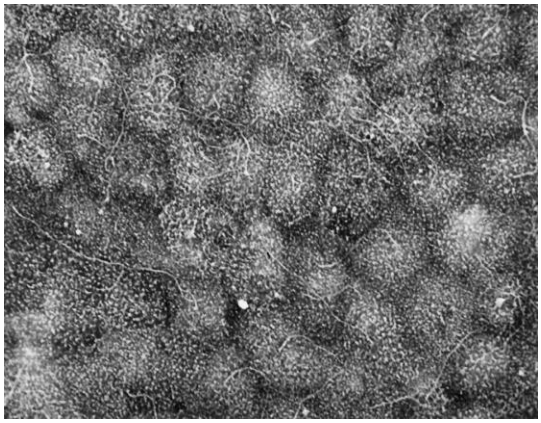

Fig. 15. SEM of MDCK cell monolayer culture exposed to SOLO-care solution (magnification X 1000

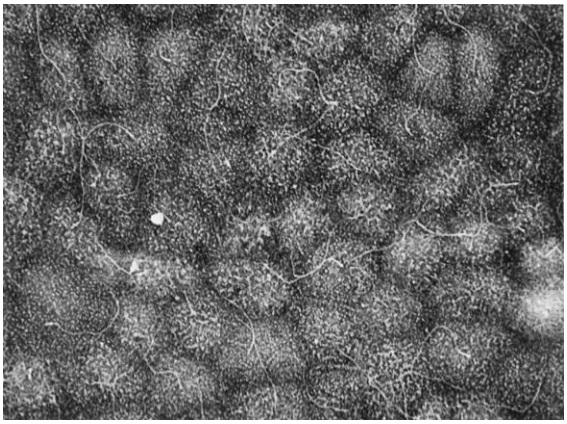

Fig. 16. SEM of MDCK cell monolayer culture exposed to Complete Comfort Plus solution (magnification X 1000)

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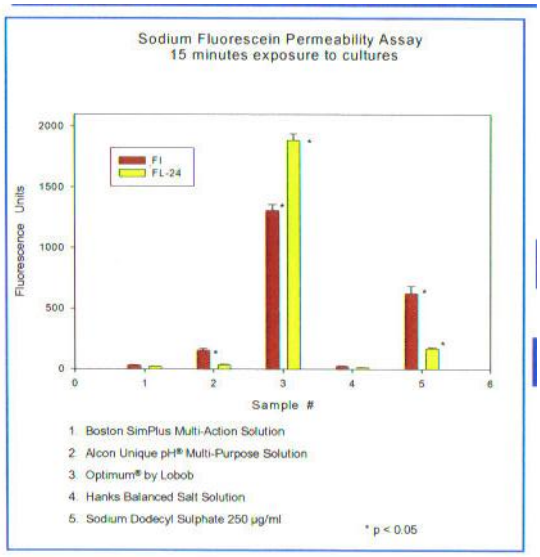


Figure 17. The effect of Boston® SimPlus Multi-Action Solution, Alcon Unique® pH Multi-Purpose Solution, Optimum® by Lobob SDS and HBSS on an MDCK epithelial cell monolayer using the fluorescein permeability assay after a 15 minute exposure. * indicates significant differences as compared to control ($p < 0.05$).

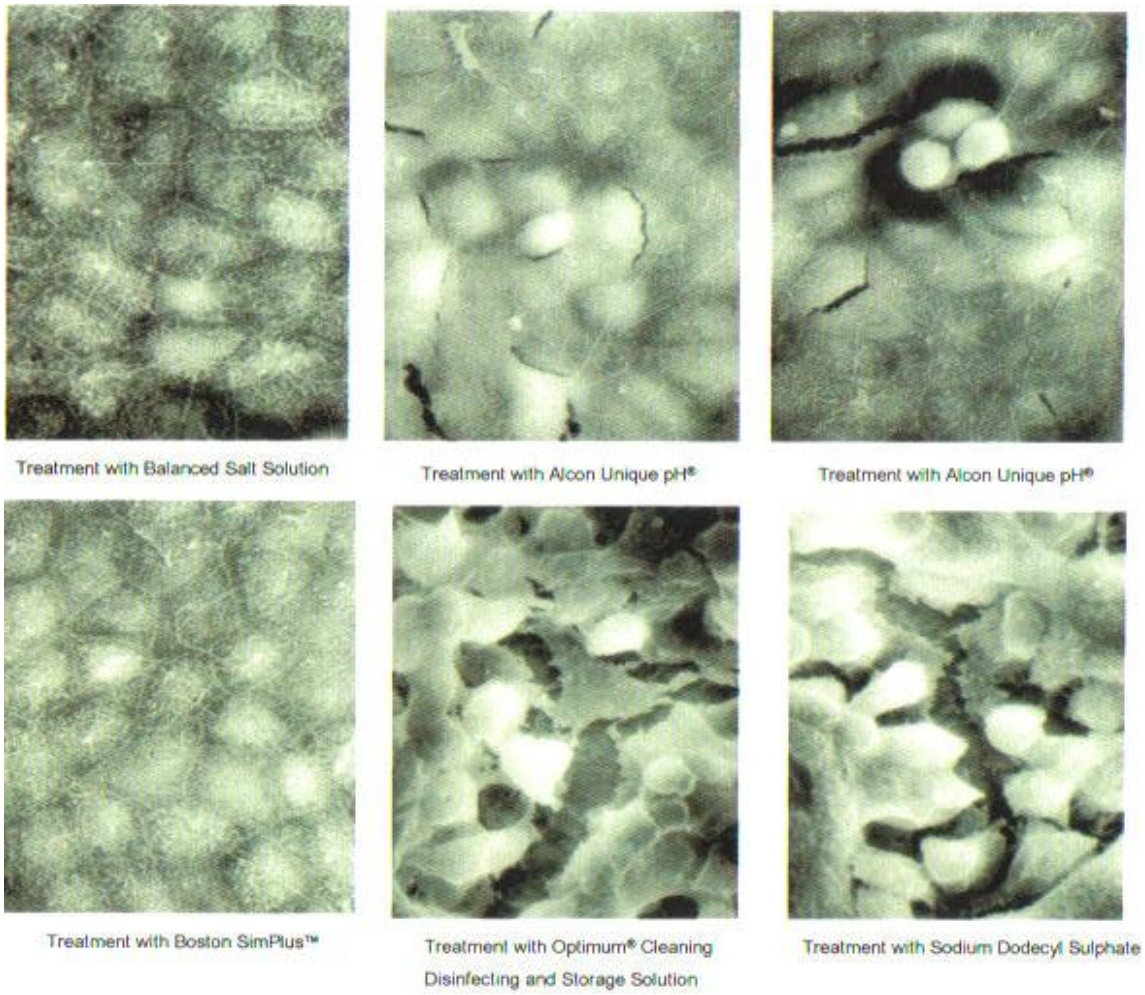


Figure 18. SEM of MDCK cell monolayers after exposure to Boston® SimPlus Multi-Action Solution, Alcon Unique® pH Multi-Purpose Solution, Optimum® by Lobob SDS and HBSS for 15 minutes. (magnification X 1000)

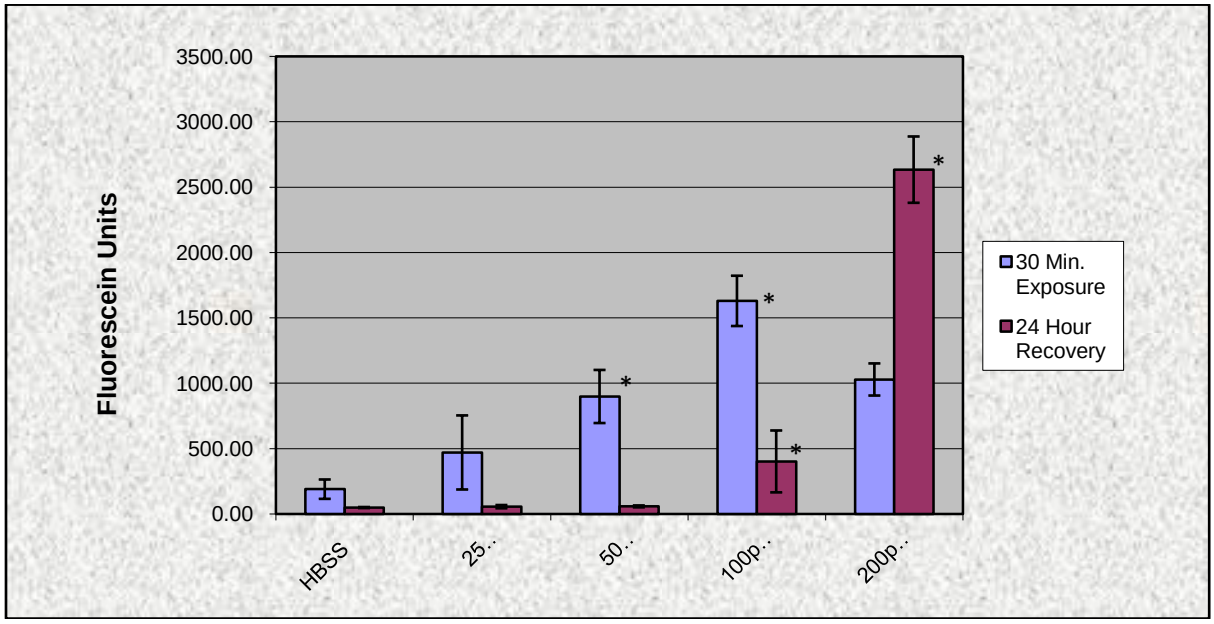


Figure 19. The effects of various concentrations of BAK in BS on a MDCK monolayer after 30 min exposure and 24 hours of recovery. 100 ppm = 0.01%. * indicates significant differences as compared preceding dose ($p < 0.05$)

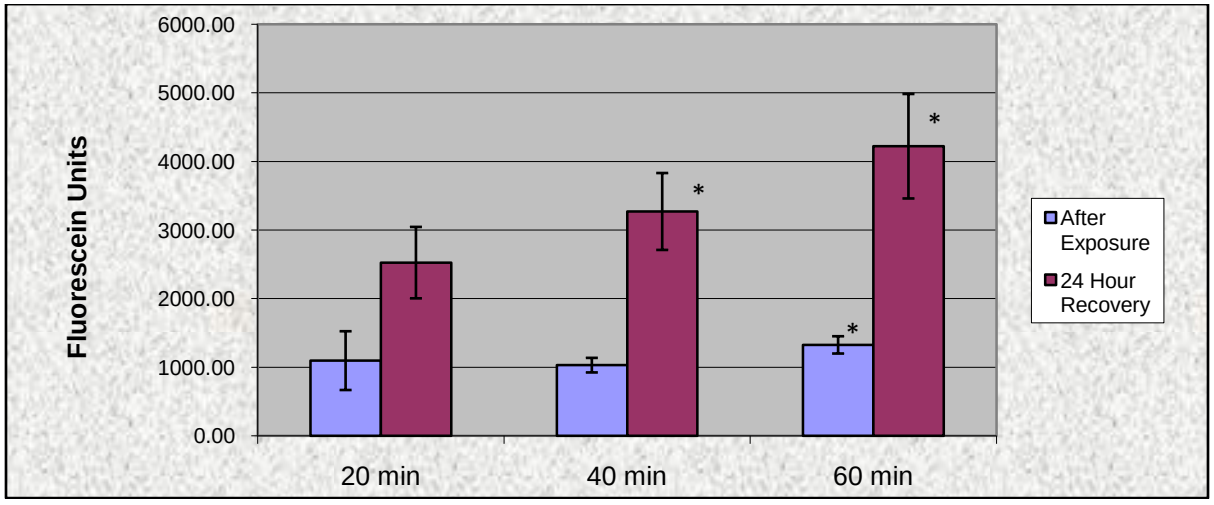


Figure 20. The effect of exposure to 0.05% SDS in HBSS on a MDCK monolayer at different exposure times. * indicates significant differences as compared to preceding exposure time.

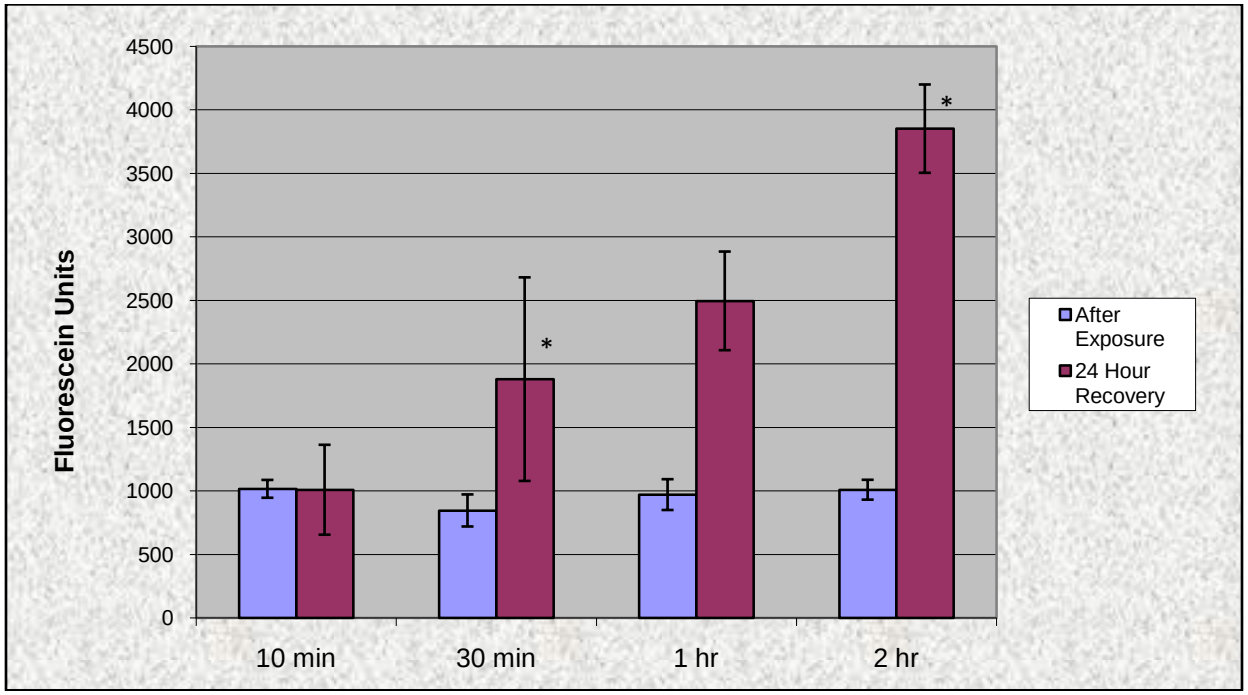


Figure 21. . The effect of exposure to 0.05% SDS in HBSS on a MDCK monolayer at different exposure times. * indicates significant differences as compared to preceding exposure time.

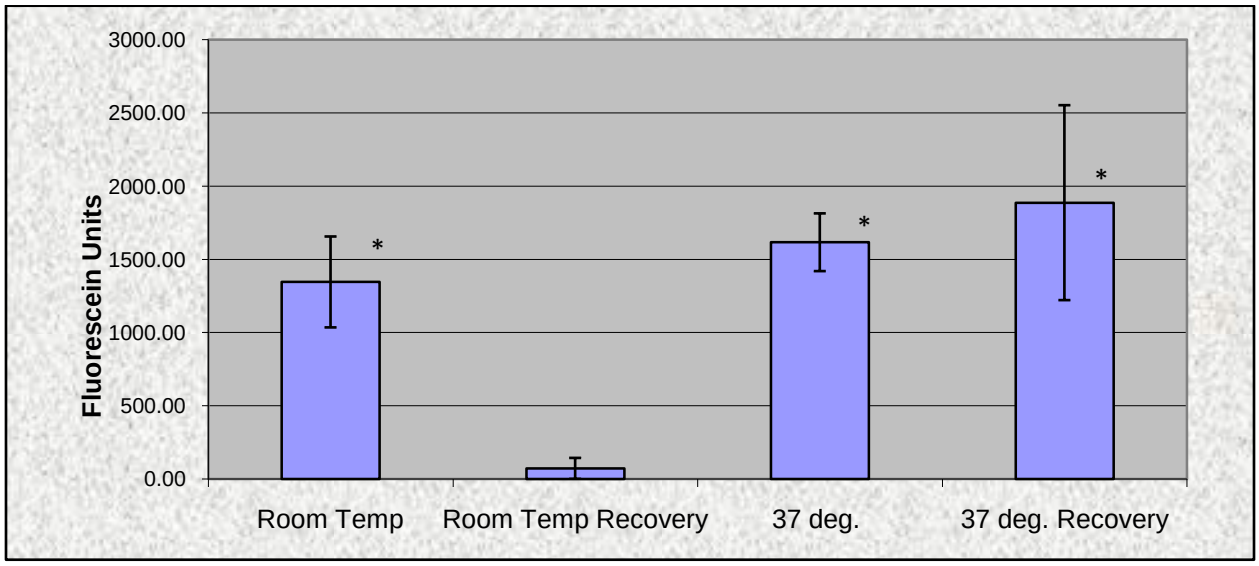


Figure 22. The effect of temperature on sodium fluorescein permeability. MDCK monolayers were exposed to 0.025% SDS in HBSS for 30 min. Readings were taken after exposure and after recovery (24 hours later). * indicates significant differences as compared to room temp recovery.

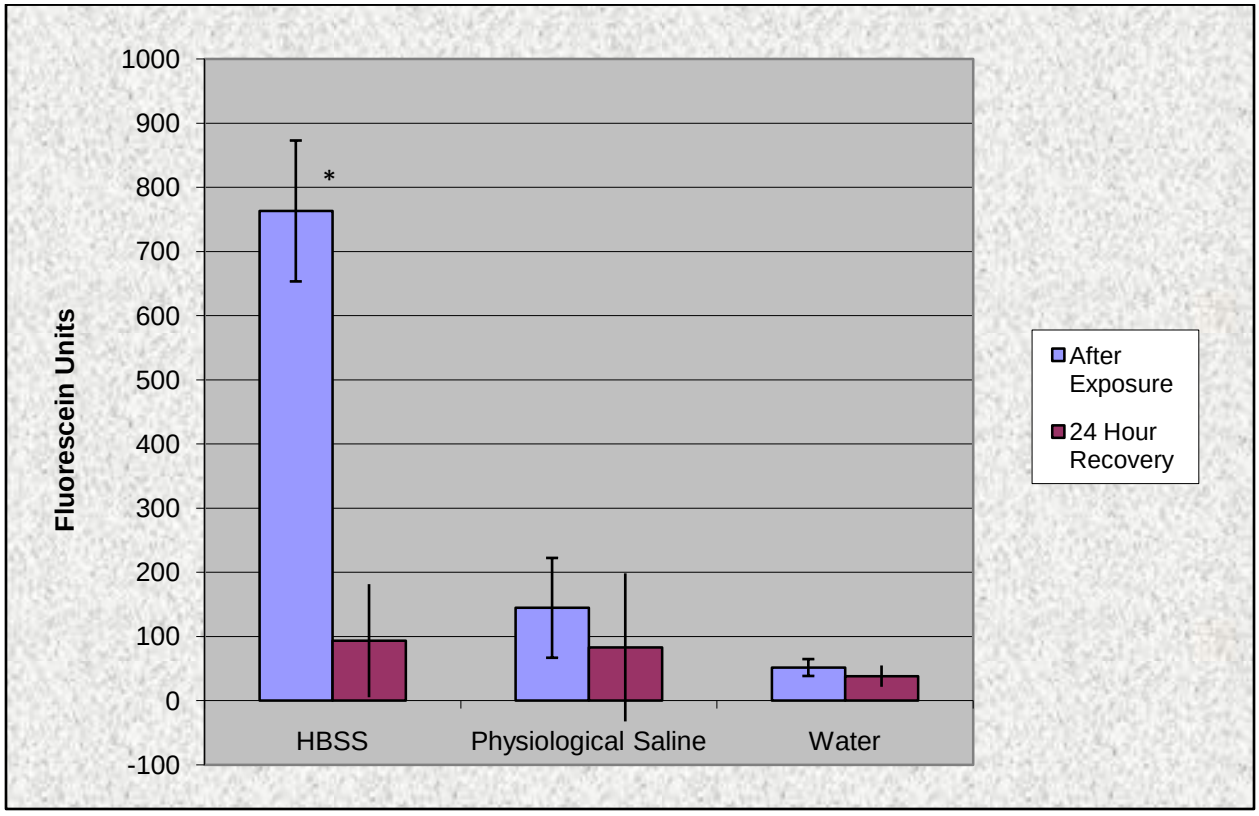


Figure 23. The effect 0.025% SDS prepared in different diluents on the MDCK monolayer after 20 min. exposure. * indicates significant differences as compared to other diluents.

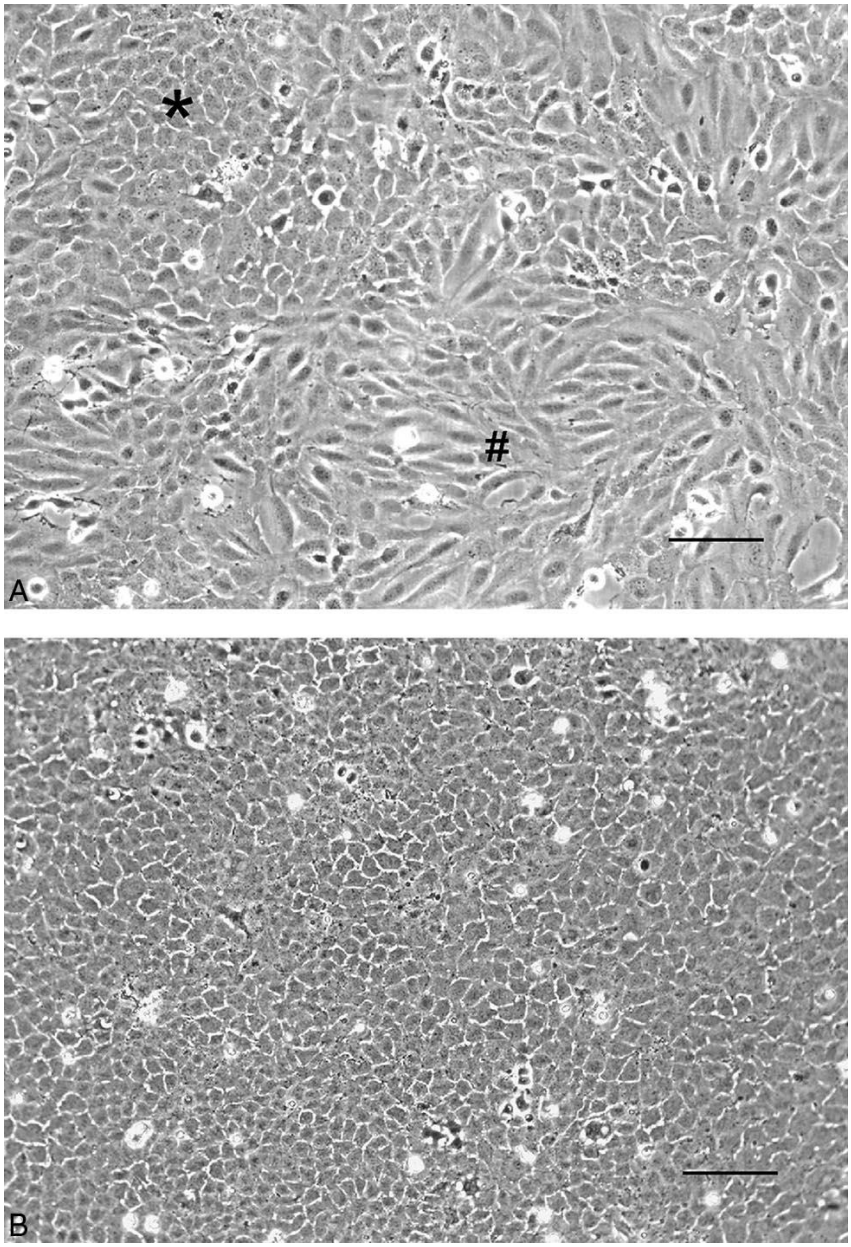


Figure 24. Phase contrast light microscopy (A) The original human corneal epithelial cells show two distinct populations: epithelial cells (*) and fibroblast cells (#). On the upper left are cells showing an epithelial structure, whereas the center and lower right portions show a fibroblastic structure. The bar represents 24 µm. (B) After cloning to select the epithelial cell type, the culture showed a uniform characteristic epithelial structure. The bar represents 24 µm. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

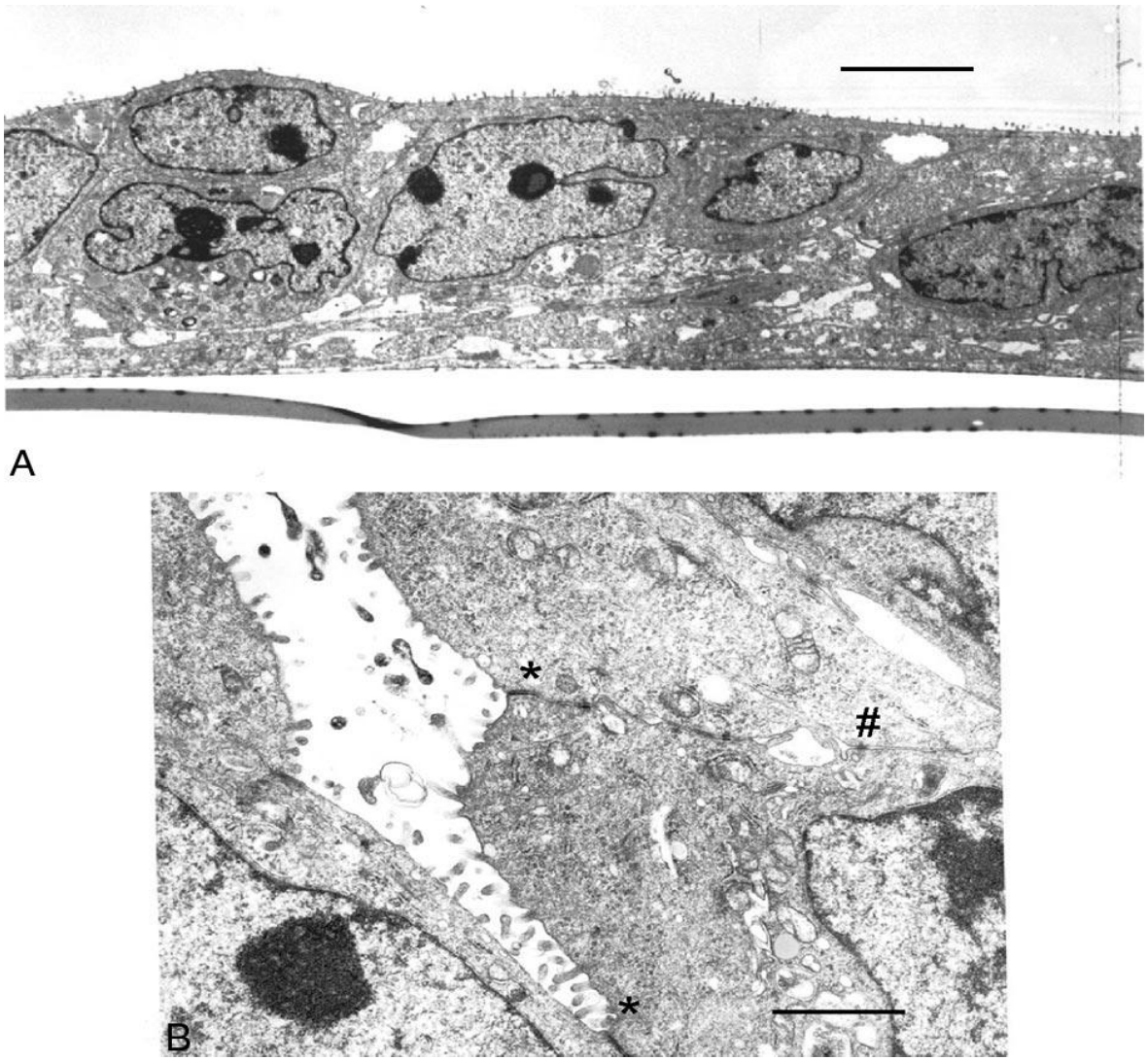


Figure 25. (A) Transmission electron microscopy of cross-sections of the human corneal epithelial cells. Some degree of stratification of cells is seen. These cells have short microvilli at the apical surface and evidence of tight apposition of the membranes. The cell nucleus often contains several darkly stained nucleoli. The bar represents 4µm. (B) Higher magnification shows the presence of tight junctions (*) at the apical surface of cells. In addition, the cells contain desmosomal and adherent junctions (#), which are typical of epithelial cells. The bar represents 0.8µm. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

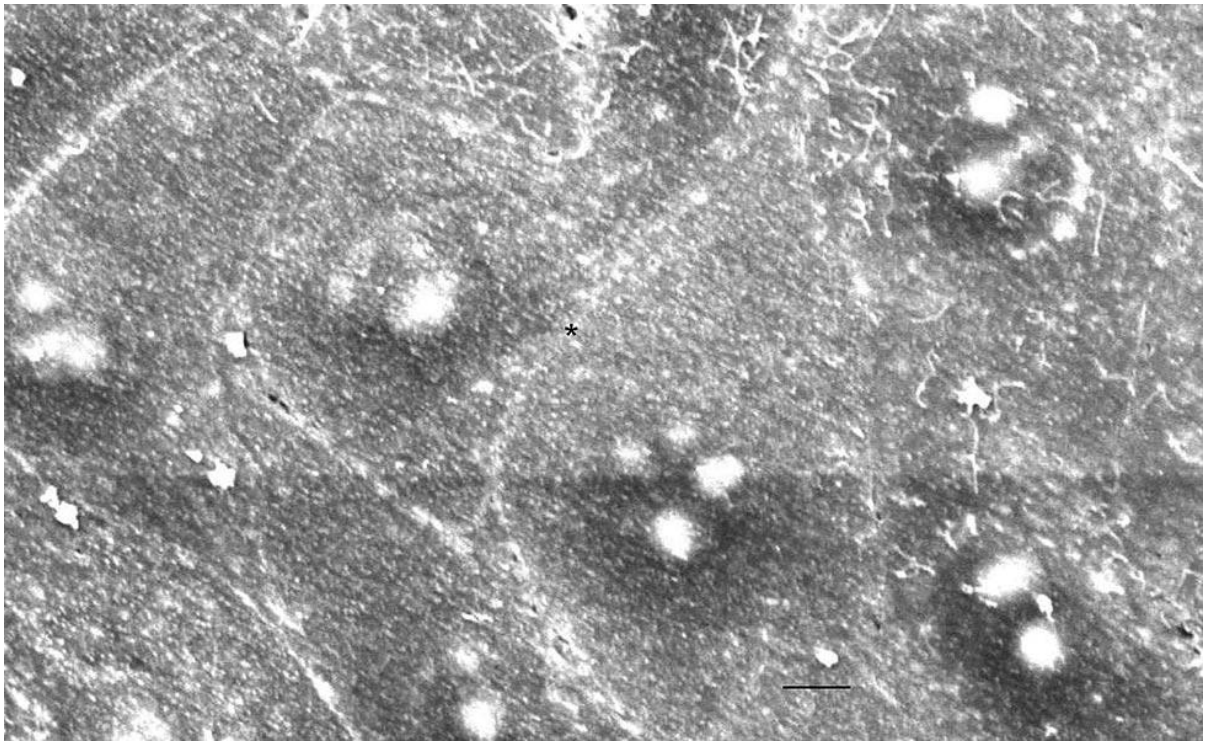


Figure 26. Scanning electron microscopy of human corneal epithelial cells. These cells are closely juxtaposed with each other, and the ridges between cells represent tight junctions. The bar represents 6 µm. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

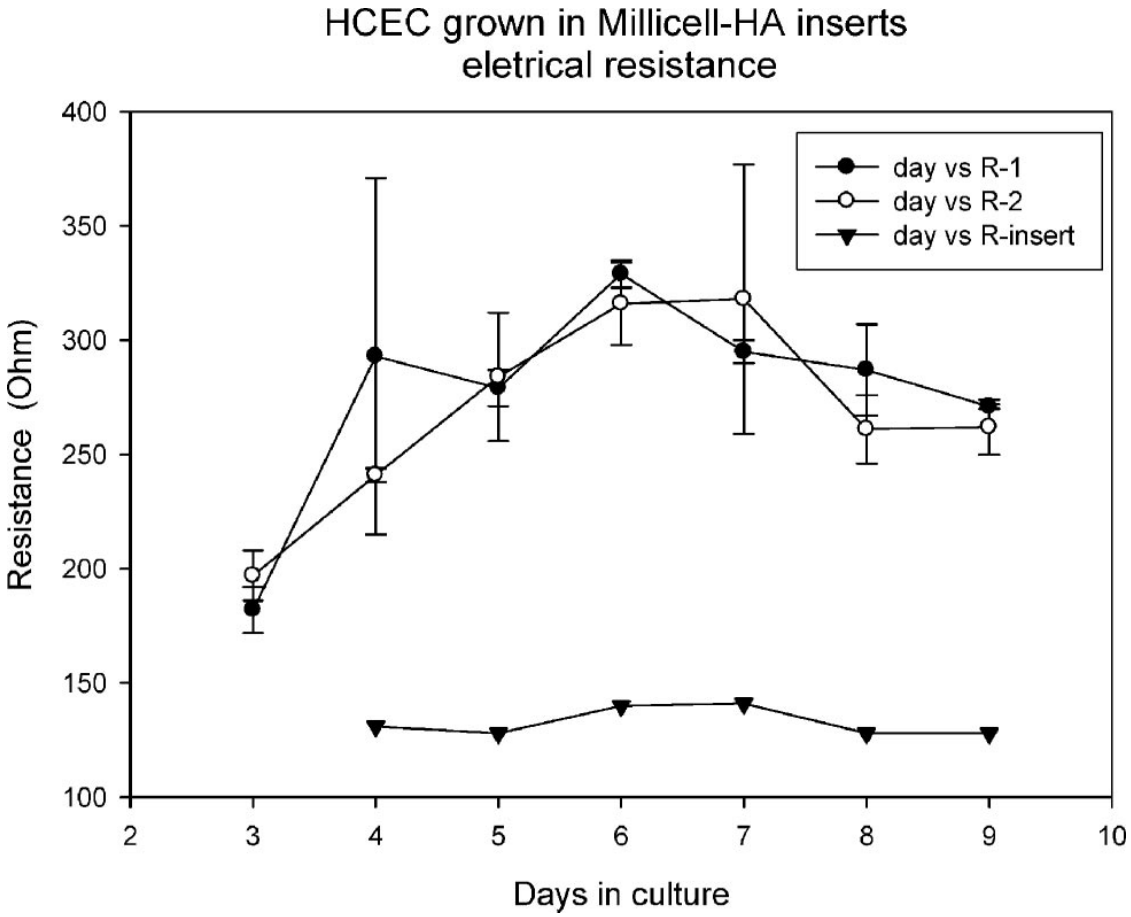


Figure 27. The development of resistance by human corneal epithelial cells cultured in Millicell HA inserts, measured daily from the third day after seeding the inserts with 0.5 mL of 2 X 10^5/mL cells. The open and closed circles represent two separate sets of cultures of four inserts each (R1 and R2). The inverted triangles represent the resistance of blank inserts without cells. The error bars represent the standard deviations of resistance measurements from four inserts. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

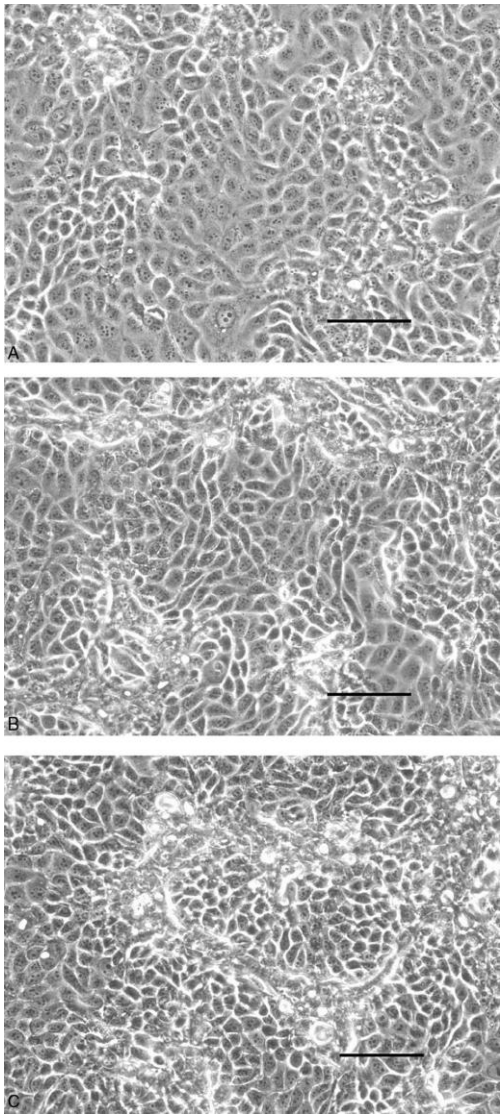


Figure 28. Phase contrast light microscopy. Incubation of human corneal epithelial cells for 15 minutes with Hank's Balanced salt solution (A), ReNu MultiPlus (B), and OPTI-FREE Express (C). Parts A and B are similar to each other, suggesting no damaging effect of ReNu MultiPlus on cells. Part C shows cell separation and clustering, indicating some damage to the cells. The bar in each part represents 24 µm. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

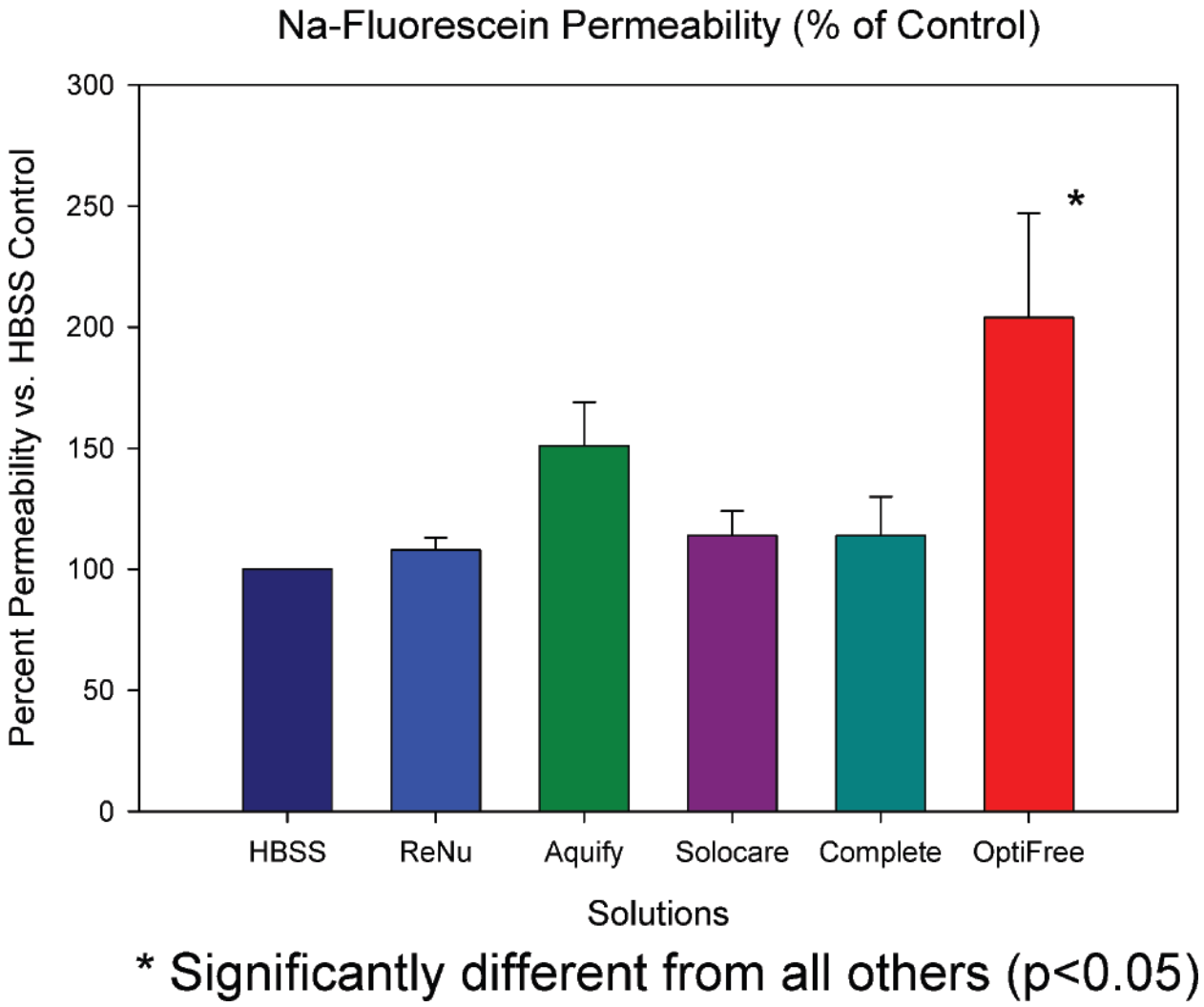


Figure 29. The results of sodium fluorescein permeability measurements on cultures after 15 minute treatment of human corneal epithelial cells with various solutions. The results represent three separate experiments and are expressed as a percentage of the permeability of cultures in Hank's balanced salt solution. The greater percentages of permeability compared to Hank's balanced salt solution represent the loss of integrity of the corneal epithelium. The highest effect was shown by incubating the cultures with OPTI-FREE Express, suggesting toxicity to the cells. Reprinted with Permission McCanna *et al. Eye and Contact Lens.* 2008.

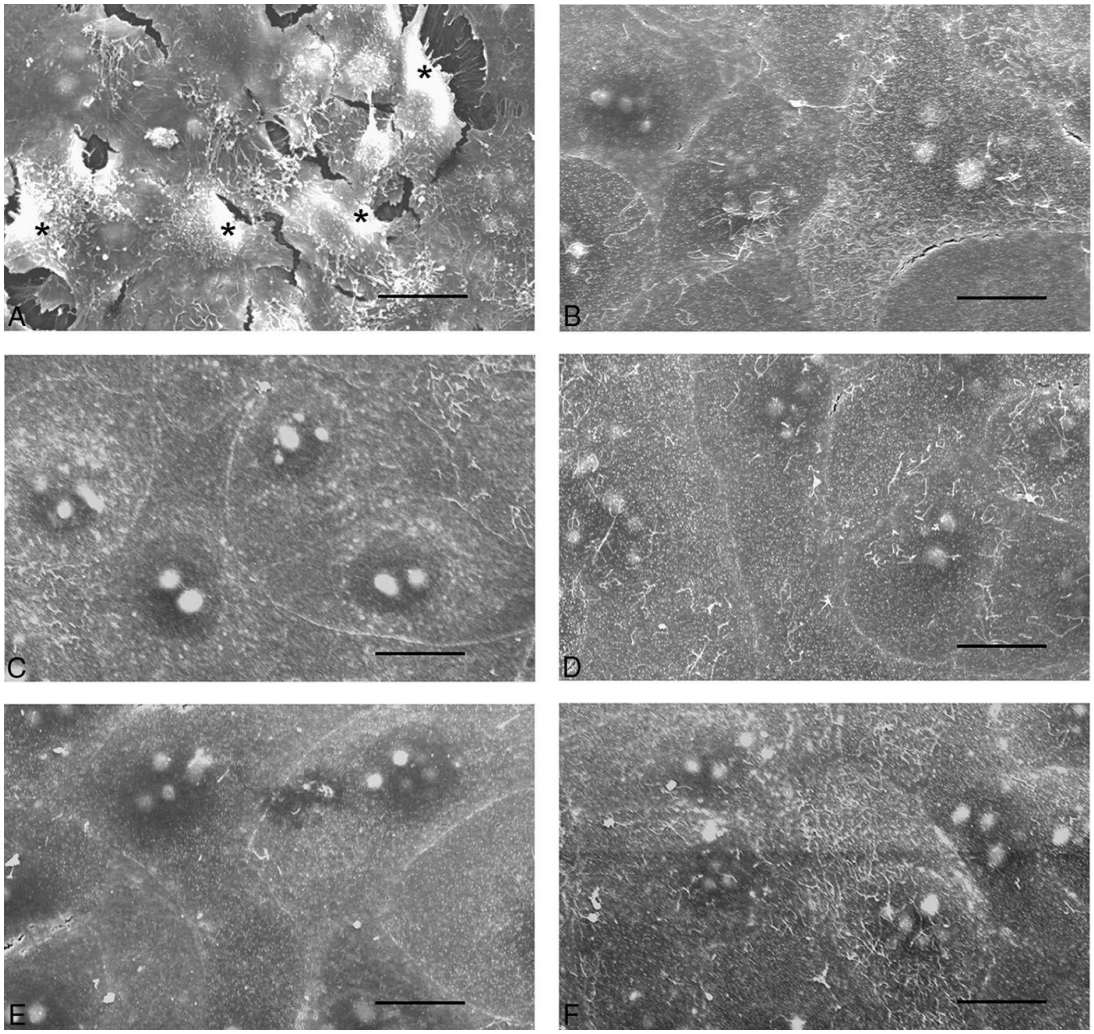


Figure 30. Treatment of human corneal epithelial cells (A) Scanning electron micrographs of OPTI-FREE Express-treated cells. Loss of microvilli and development of clusters on cell membranes are seen in many cells. There are also fissures among the apposing cells in the monolayer, suggesting the loss of tight junctions. Areas marked with an asterisk show that adjacent cells have actually separated from the monolayer, with some intercellular adhesion bridges remaining. The bar in each figure represents 18µm. (B) Scanning electron micrograph of ReNu MultiPlus-treated cultures. The cells show essentially a similar structure to Hank's balanced salt solution-treated cultures. (C) Scanning electron micrograph of SOLO-care Plus with Aqualube-treated cultures; similarity to part F is noticeable. (D) Scanning electron micrograph of Complete Moisture Plus Multi-Purpose Solution treated cultures; similarity to part F is noticeable. (E) Scanning electron micrograph of Aquify 5 minute-treated cultures; similarity to part F is noticeable. (F) Scanning electron micrograph of Hank's balanced salt solution-treated cultures, representing a negative control without treatment. The cells show close adherence to each other, and tight junctions are seen as raised ridges between two juxtaposed cells. The microvilli are distinct on the cells' surfaces. Reprinted with Permission McCanna *et al. Eye and Contact Lens*. 2008.

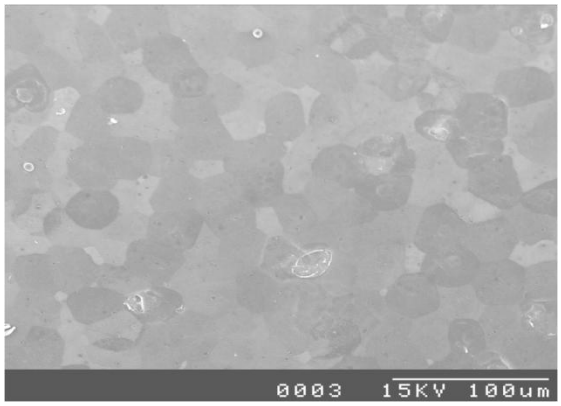


Figure 31. SEM Untreated Control. Rabbit cornea.

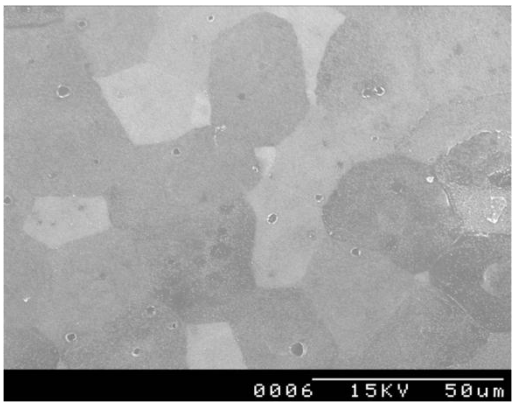


Figure 32. SEM Untreated Control. Rabbit cornea.

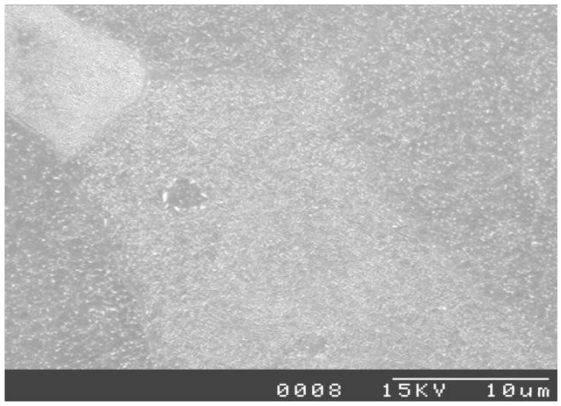


Figure 33. SEM Untreated Control. Rabbit cornea.

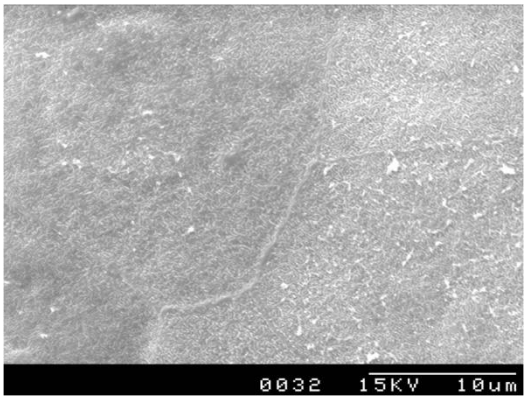


Figure 34. SEM ReNu MultiPlus-lens ment cornea. Rabbit cornea.

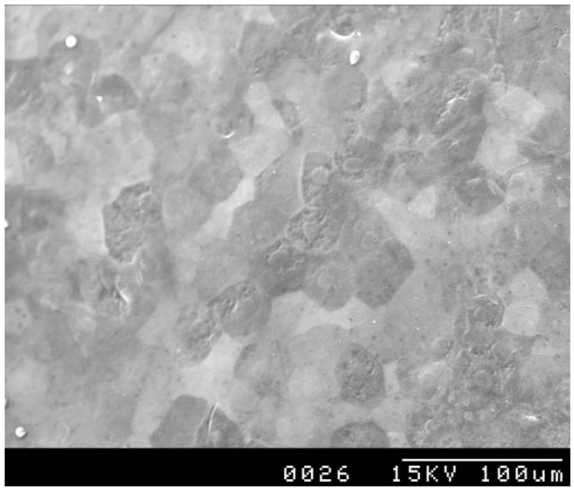


Figure 35. SEM ReNu MultiPlus-lens treatment. Rabbit cornea.

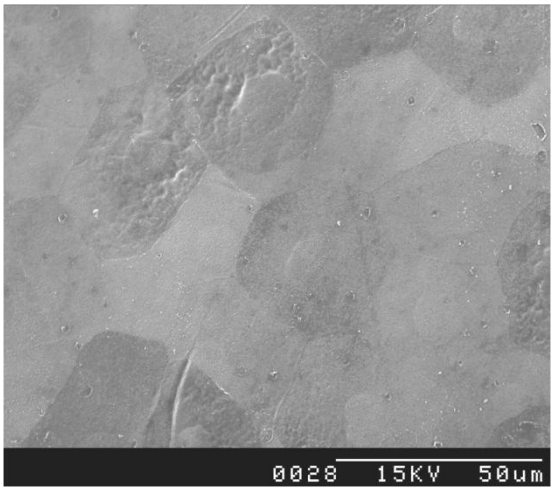


Figure 36. SEM ReNu MultiPlus-lens treatment. Rabbit cornea.

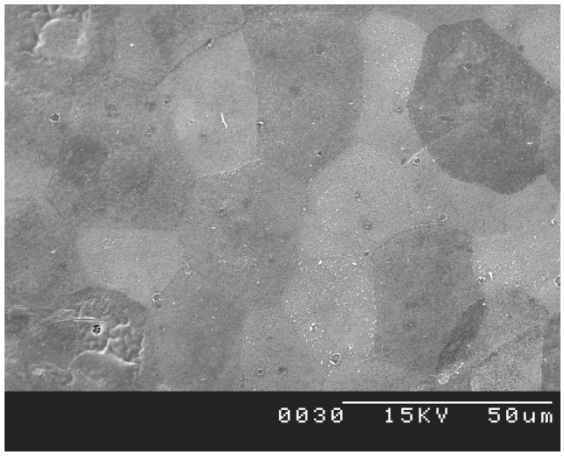


Figure 37. SEM ReNu MultiPlus-lens treatment. Rabbit cornea.

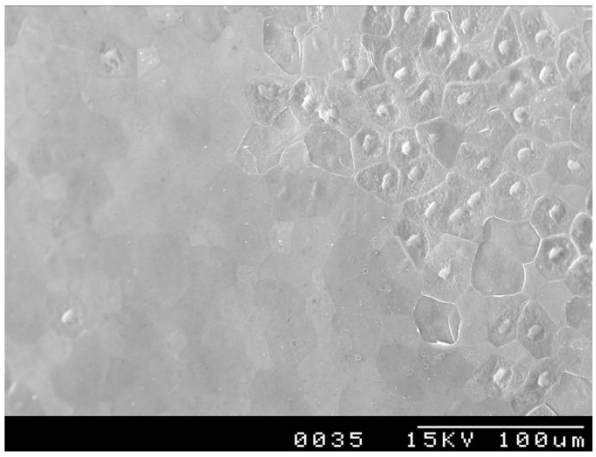


Figure 38. SEM Optifree Express-lens treatment. Rabbit cornea.

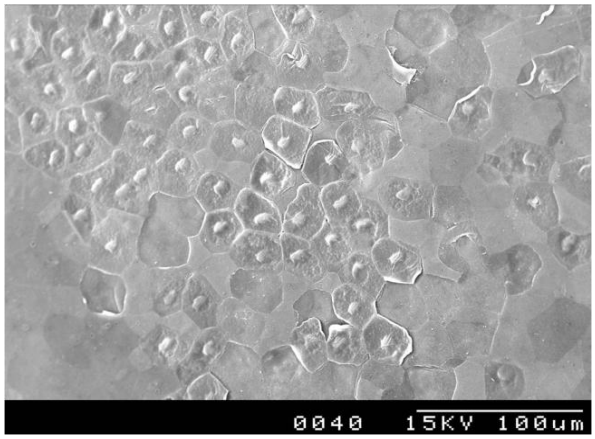


Figure 39. SEM Optifree Express-lens treatment. Rabbit cornea.

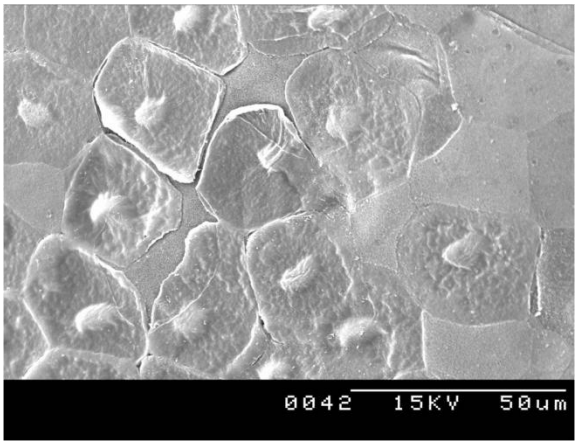


Figure 40. SEM Optifree Express-lens treatment. Rabbit cornea.

2.4 Discussion

The sodium fluorescein permeability assay using either Madin-Darby canine kidney cell line or a human corneal epithelial cell line can be used to detect the loss of tight junctions caused by chemicals or product formulations. Using scanning electron microscopy this work confirmed that there is a correlation between sodium fluorescein permeability and disruption in the tight junctions between the epithelial cells exposed to sodium dodecyl sulphate and contact lens care disinfecting products. Using BAK we showed that evaluating the monolayers 24 hours after recovery will detect potential delayed effects on the cells and is helpful in assessing high concentrations of chemicals as it may take time for viscous chemicals to pass through a membrane filter. Light microscopy also showed disruption in the cell monolayers. To verify the relevance of the *in vitro* results we evaluated the corneal surface of rabbit cornea after exposure to contact lenses soaked in contact lens care solutions. The contact lens care solution that showed the most permeability to sodium fluorescein also showed the most damage to the rabbit cornea.

There are other methods that are being used by researchers to detect disruptions in the tight junctions of the cornea. *In vivo,* the integrity of this corneal barrier can be assessed by measuring the electrical resistance across the tissue (Uematau *et al.,* 2007; Klyce, 1972) by evaluating its permeability to sodium fluorescein (Maurice, 1967), or caboxyfluorescein (McCarey and Edelhauser, 2007), and by assessing breaks in corneal junctions using scanning electron microscopy of fixed tissue (Uematau *et al.*, 2007, Tonjum, 1975, Burstein, 1980). Confocal microscopy of fixed cornea with stained immune-fluorescent secondary antibody to

monoclonal anti-ZO-1 was done to evaluate the toxicity of fourth-generation fluoroquinolone antibiotic solutions on the rabbit corneal epithelium (Ly *et al*., 2006). Two recent reviews of clinical confocal microscopy (Patel and McGhee, 2007; Zhivov *et al.* 2006) do not mention the use of this technology for assessing the tight junctions of the cornea. Two articles were published in 2008 in addition to McCanna *et al.* that also assessed the effect of contact lens care solutions on tight junctions. Both investigations used the Araki-Sasaki cell line but did not clone the cell line to select for epithelial structure. The cultures used would have contained a mixture of both epithelial-like and fibroblastic-like cells. Imayasu *et al.* (2008) evaluated tight junction integrity with transepithelial electrical resistance and Z0-1 labelling of the monolayer and confocal microscopy. Chuang *et al.* (2008) evaluated tight junction integrity by fluorescein permeability and ZO-1 and occludin labelling. These studies showed that contact lens care solutions can break the cell junctions. In the investigations (McCanna *et al*., 2008; Tchao *et al*., 2002) described here, shorter exposure times were used than those by the prior investigators. The relevance of exposure time depends on the duration of exposure to the contact lens care solution after lens insertion.

It was desirable to develop a sodium fluorescein permeability assay using human corneal epithelial cells. This study carried out and described above showed the successful isolation and usefulness of a clone of human corneal epithelial cells with a typical and homogeneous epithelial cell structure from a heterogeneous cell line originated by using SV-40 transfection to immortalize primary cultures of human corneal epithelial cells. Ryeom *et al.* (2000) showed the presence of tight junctions in the original heterogeneous culture system. Tight junction

characteristics were also shown in the clone derived in the current study and when cultured in Millicell inserts, develop electrical resistance. Therefore, this cell line maintains an important characteristic of healthy human corneal epithelial cells. In previous studies, multilayered epithelium of human corneal epithelial cells has been cultured to form at the air–liquid interface (Kruszewski *et al*., 1997) or as organoid cultures (Zorn-Kruppa *et al*., 2004). A organotypic culture of human corneal epithelial cells that includes the trigeminal nerve has also been produced (Suuronen *et al.*, 2004). These cultures have been developed to mimic the cornea, but the endpoints used are still cell viability. Furthermore, these cultures are expensive, complicated, difficult to maintain, and variable. Therefore, for the purpose of determining the function of an epithelium and screening agents for their effect on the cornea, a simplified monolayer, such as that developed in the current study, may be preferable.

Human corneal epithelial cells have been used in several *in vitro* cytotoxicity assays from as early as 1986 (Nevelle *et al.*, 1986). These previously described methods involve endpoints of cell viability that may represent downstream effects after corneal damage or loss of function. In terms of the cornea, an essential function of the outermost layer of the squamous epithelium is the barrier function. Loss of barrier function may or may not involve loss of cell viability. The loss of tight junctions in the corneal epithelium could certainly lead to the sensation of irritation or provide an opening for virulent micro-organisms to establish and infection. Therefore the development of a corneal assay with an endpoint of cellular permeability represents a sensitive and relevant assay, particularly for characterizing compounds with low toxicity.

By using this cell line and assay conditions, various contact lens care solutions were tested. The culture of these cells use fetal bovine serum in a conventional medium, so these cells may represent metabolic states different from those of the *in vivo* human corneal epithelial cells. However, testing of solutions was performed in the absence of culture medium, so the response observed at the cell membrane may represent the *in vivo* conditions. The loss of tight junction functions *in vitro,* as determined by the sodium fluorescein permeability, represents the effect of test agents on a cell monolayer rather than a stratified corneal epithelium consisting of several layers of epithelial cells. However, it has been shown *in vivo* that the integrity of the outermost epithelium is important in determining the irritancy of chemicals in the eye (Klyce, 1972; Ban *et al.*, 2003; Marsh and Maurice, 1971; Throft and Friend, 1975). As described earlier, most of the solution regimens produced innocuous effects on cellular structure and tight junction integrity in this test system. However, the OPTI-FREE Express solution stood out as being significantly more damaging to human corneal epithelial cells. These data are consistent with previous observations using MDCK cells (Tchao *et al.,* 2002), but the relevance of these observations is even more significant given the improvements in the assay conditions and the correlation to the effects on the tight junctions that occurred when contact lenses soaked in OPTI-FREE were applied to the corneas of rabbits *in vivo*.

Chapter 3

Using the Optical Quality of the Bovine Lens as a Sensitive Measure of Assessing the Ocular Toxicity of Chemicals

This work describes the use of an *in vitro* model that evaluates toxicity thresholds evaluating changes in the organs functionality. It was performed partly in conjunction with Dr. Sivak and Dr. Bantseev and was published in an article and poster presentation. However, the descriptions and interpretations concerning this approach as given here are my own. In addition I carried out the multiple instillation study.

3.1 Introduction

In vitro models for determining the ocular irritation potential of chemicals and product formulations have been proposed for use with immortalized cell lines, primary cells or organ cultures. These models detect toxicity by evaluating the effects either at the molecular level by assessing changes in cell physiology, or by evaluating effects on the functionality of the cell layers or organs. Determining the effects chemical have on cell organelles or chemical reactions within the cell can be helpful in assessing the relative toxicity of chemicals. However, there is uncertainty regarding the relevance of these molecular toxicity endpoints as predictors of toxicity *in vivo*. Toxicity will occur if the concentration of a chemical is high enough for a long enough duration to cause damage. Because of the capacity of cells and organs to heal through biochemical detoxification, cellular repair, cell division and cell replacement, toxicity will only occur if the concentration and duration of contact exceed the body's capacity for detoxification and repair.

There are two types of *in vitro* assays: assays that evaluate the change in the molecules or organelles of a cell or, assays that evaluate effects on an organ function. *In vitro* assays that measure effects at the molecular level in cells can detect toxic effects from chemicals at various concentrations. Because cells and tissues have the capacity for repair, the relevance of these effects need to be assessed. One way to assess the relevance of molecular *in vitro* assays is to test chemicals at concentrations of known toxicity in animals and in humans and determine if the new chemicals have toxic responses that are equal to the magnitude of these chemicals. If the

tested concentration of the new chemical has effects that are equal to or greater than that of a known toxin, this is grounds for concern and should be considered in the safety assessment. Reducing the concentration of the chemical to a non-toxic dose would be required.

Another way to assess relevance is to use an *in vitro* model that evaluates the effect of the toxin on the functionality of an organ. If the tested chemical does not impact the organ's functionality, then the concentration of the chemical used did not exceed the organs capacity to repair itself. If however, the functionality of the organ does change, this is an indication that the molecular damage exceeded the capacity for repair and thus a reduction in the concentration to a dose below the level that affects the functionality of the organ would be required.

An *in vitro* assay that can be used to reduce the level of uncertainty in predicting the safe concentrations of new chemicals in product formulations is the Scantox™ assay that measures the effect of toxic chemicals on the ability of the bovine lens to focus light. The use of a scanner to measure optical quality of a cultured lens was first described by Sivak in 1983 (Sivak and Dovrat, 1983). Sivak developed an updated laser system with software for measuring the change in the light focus (Sivak *et al.*, 1986). The modern system is called Scantox™and has been used to measure the toxicity of chemicals and product formulations (Dovrat and Sivak, 2005). The optical quality (focus or lack of focus) is measured using a scanning laser beam and video monitor operated by computer software.

The use of the ScanTox™ system has advantages over cell culture and other assays with endpoints based on organ functionality. This assay utilizes the bovine lens. Many cell culture assays utilize immortalized human cells since primary human corneal epithelial cells only undergo a limited number of cell divisions. Immortalized cells may not retain the same physiology of the initial cultured cells as the process of immortalization resulting from DNA insertions may affect some cellular processes. Cultured corneas are also utilized but culturing corneas for extended periods of time is difficult to do and can lead to significant degradation in the organs functionality implying that the culturing alone is causing enough damage at a molecular level to cause irreparable toxicity. The bovine lens however retains its functionality in culture, thus it is uniquely a primary organ culture that is relatively unaffected by dissection and culturing conditions. The measurement made by the ScanTox is non-destructive in that laser does not damage the tissue. Other assays typically utilize dyes or reagents that are toxic to the tissue and prevent further analysis after a reading is obtained. Also because of the non-destructive nature of the reading using the ScanTox and the ability to culture the bovine lens for weeks, the toxicity of a chemical can be measured for delayed effects and the organ can be monitored over time for recovery from toxic injury. In addition, multiple instillation studies can be performed with the bovine lens as the lens tissue can be monitored after each instillation as ScanTox allows for non-invasive measurements to be taken.

A potential limitation of evaluating the bovine lens is that these cells may not have identical membrane proteins, cytoskeletal proteins, metabolism, or cell life cycle as human *in vivo* corneal epithelial cells. This limitation however also exists for human primary or immortalized cell

cultures and cultured animals cells. *In vitro* test cultures will have some differences in physiology than *in vivo* human corneal cells due to culturing conditions, changes in the DNA from the natural state and difference between the physiology of human and animal corneas. The corneas of *in vivo* animals also are not physiologically identical to human corneas. Thus all toxicology models have a degree of uncertainty of prediction of toxicity due to these physiological differences. When choosing toxicity models it is the goal of the toxicologist to choose the test system that minimizes the potential physiological differences between the test tissue and human physiology. The rationale for choosing bovine lens is that the cells are from ocular origin and the cells are primary cells that have not been modified from the initial *in vivo* condition. Embrylogically and physiologically, the epithelium of the lens is similar to the epithelium of the cornea. Also, the culturing conditions for the bovine lens do not change its functional state. Bovine lenses are cultured in an M199 medium instead of aqueous humour. However, there is no change in the optical quality of the bovine lens cultured in the M199 medium (Sivak *et al*., 1990). The optical quality of the lens can be maintained up to 37 days indicating that the cells are under a low stress condition where cellular viability is maintained (Sivak *et al*., 1990).

This investigation evaluated the use of the ScanTox for assessing the ocular irritation of ophthalmic chemicals. The ScanTox™ system is the only *in vitro* assay that can measure toxicity over an extended period of time without the use of viability dyes. By utilizing the laser scanner the effect of a toxin can be assessed multiple times over the course of weeks. This

technology enables the development of an *in vitro* assay that can assess for recovery from injury and assess the toxic effects of multiple daily instillations of ophthalmic formulations.

3.2 Materials and Methods

3.2.1 Bovine Lens as an In Vitro Model of Ocular Toxicity single and multiple instillation

3.2.1.1 Chemicals and reagents.

Culture medium (M199), sodium bicarbonate, agarose, L-glutamine, NaCl, SDS, BAK, Hydrogen Peroxide and NaOH were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES, penicillin, streptomycin, and dialyzed fetal bovine serum were obtained from Gibco-BRL (Burlington, ON, Canada).

3.2.1.2 Eye dissection.

Bovine eyes obtained from a local abattoir were opened under sterile conditions and the lenses were removed. To minimize physical handling of lenses (i.e., transfer from the culture plate and then to the chamber for scanning) they were immediately placed into a three-part chamber (see Analysis of the Lens Optical Properties below) containing 25 ml of culture medium (M199) supplemented with 21 mM HEPES, 26 mM sodium bicarbonate,0.7 mM L-glutamine, 7 mM of NaOH, 100,000 units penicillin and 100 mg streptomycin, and 3% dialyzed fetal bovine serum and incubated at 37°C with 4–5% CO_2. After 24 h lenses exhibiting mechanical damage during dissection, as evaluated by the visible opacities, were discarded.

3.2.1.3 Treatment Sodium Dodecyl Sulftate

Lenses were exposed to SDS (0.1 to 0.00625%) for 30 min. by submerging the lens in 10 mL solution in a 50 mL polypropylene conical tube, rinsed with saline (0.9% NaCl), placed in fresh M199 and incubated at 37°C and 4–5% CO_2. Scanning was performed before exposure, immediately, 4, 8, and 24 h after the treatment.

3.2.1.4 Hydrogen Peroxide

Bovine lenses were exposed to H_2O_2 (3.0, 0.3, 03%) for 15 minutes by submerging the lens in 40 ml of solution in a 50 mL polypropylene conical tube, rinsed with saline (0.9% NaCl) and M199 and incubated in culture medium at 37°C and 4–5% CO_2. Scanning was performed 4, 8 and 24 hours after treatment. Additional scans were performed daily until the 8^{th} day of incubation.

3.2.1.5 BAK Multiple Instillation

Lenses were exposed to BAK (0.01%) for 15 min. submerging the lens in 40 ml of solution in a 50 mL polypropylene conical tube, rinsed with saline (0.9% NaCl), placed in fresh M199 and incubated at 37°C and 4–5% CO_2. The single instillation group was maintained in culture medium after initial exposure. The multiple instillation group was exposed for 15 min. immediately after the 24, 48, 72 hour scans. Scanning was performed 4, 8 and 24, 48 and 72 hours after initial instillation. AlamarBlue reading of the lenses occurred 24 hours after the 72 hour exposure time.

3.2.1.6 Measurement of lens optical properties.

The optical quality (Back vertex distance variability) was assessed using the Scantox™ system. The Scantox™ system consists of a collimated laser that projects the beam to a plain mirror mounted at 45° on a carriage assembly. This mirror reflects the laser beam directly up through the scanner table surface and through the lens under examination. A digital camera views the position of the laser beam. The data is analysed and is used to calculate the back vertex distance variability (BVD) for each position and the difference in that measurement between beams. Lenses were placed in 25 ml of M199 into a specially designed three-part chamber made of 70 mm tall square glass tube, silicone rubber insert, and a metal base (modified from Weerheim and Sivak, 1992) and suspended within the chamber on a 14-mm inner diameter bevelled washer designed to support the lens at the equatorial rim. A series of 22 laser beams were passed through the lens at specified increments of 0.5 mm for a total range of 11 mm.

3.2.1.7 AlamarBlue Assay

Lenses were exposed to BAK (0.01%) for 15 min submerging the lens in 40 ml of solution in a 50 mL polypropylene conical tube, rinsed with saline (0.9% NaCl), placed in fresh M199 and incubated at 37°C and 4–5% CO_2. The single instillation group was maintained in culture medium after initial exposure. For the multiple instillation group exposure was repeated at 24, 48 and 72 hours after the initial exposure. After 24 hours of recovery from the third exposure to BAK the lenses were removed from M199 and rinsed with 0.9% saline. Each lens was then placed anterior side down into 1mL of 8% alamarBlue solution in M199 made without serum in a well of a 12 well plate. The lenses were then incubated at 37°C and 4–5% CO_2 for 2 hours.

After incubation the lenses were removed and fluorescence of the solution was read at 530 excitation and 590 emmision using a CytoFluor II fluorescence multi-well plate reader (PerSeptive Biosystems Inc., Framingham, MA, USA)

.

3.2.1.8 Statistical analysis.

Statistical calculations were completed for SDS treatment using a two-way repeated measures ANOVA or one-way ANOVA. A probability value of less than or equal to 0.05 was considered significant. Statistical calculations for the H_20_2 and BAK treatments were completed using a one-way ANOVA.

3.3 Results

3.3.1 Bovine Lens as an In Vitro Model of Ocular Toxicity

3.3.1.1 SDS Treatment

The optical quality as measured by back vertex distance variability did not change for the control lenses. However, there was a significant increase in the BVD variability associated with SDS treatment. A 2-fold increase in BVD variability was evident in the 0.1% SDS group lenses as early as the 0 h (Immediately after treatment) scan point. The 0.1% SDS treated lenses showed a BVD variability that was statistically different ($p < 0.0001$) at all timepoints. The 0.05%, 0.025% and 0.0125% were statistically different from the control for at least two timepoints ($p <$

0.05) . The lowest concentration of SDS 0.00625% was statistically different from the controls at the 24 hour reading ($p < 0.05$) (Table 1, Figure 1).

3.3.1.2 Optical Properties after in Vitro Hydrogen Peroxide treatments

Bovine lens treatment with H_2O_2 resulted in a concentration and time dependent loss of sharp focus (Fig. 2). The optical quality of the bovine lenses treated with 3.0% H_2O_2 showed a substantial increase in BVD variability compared to control lenses after 4 hours. An increase in BVD variability did not occur for the 0.3 % H_2O_2 treated lenses until 24 hours after treatment. Lenses treated with 0.03% hydrogen peroxide showed only a slight increase in BVD variability after 7 days of incubation.

3.3.1.3 Optical Properties and metabolic activity after in Vitro BAK treatments

The optical quality measured by BVD variability of lenses treated with a single treatment and multiple treatments of 0.01% BAK were different from control lenses after 24 hours (Fig. 3). Lenses treated with multiple treatment showed a greater average BVD variability than lenses that only received a single treatment (48 and 72 hours). The lenses were incubated another 24 hours in M199 and a cell viability measure was taken using alamarBlue. There was a significant difference in the viability between the lenses that received multiple treatments with 0.01% BAK and the lenses that received only a single treatment (Fig. 4) in that the alamarBlue fluorescence of the single treatment lenses were much greater than the alamarBlue fluorescence obtained from the lenses that were treated with multiple treatments.

TABLE 1

List of Back Vertex Distance Variability (Loss of Sharp Focus, BVD Variability, mm) ± SEM Bovine Lenses Treated with Different SDS Concentrations Over Time

SDS treatment (%)

Scan Point	0.1 (n = 14)	0.05 (n = 15)	0.025 (n =16)	0.0125 (n = 11)	0.00625 (n = 11)	Control (n = 17)
Initial	0.42 ± 0.05	0.30 ± 0.02	0.39 ± 0.03	0.39 ± 0.03	0.40 ± 0.05	0.33 ± 0.02
0 h	0.85 ±0.16**	0.42 ± 0.04	0.50 ± 0.05	0.35 ± 0.03	0.45 ± 0.05	0.34 ± 0.04
4 h	0.93 ± 0.23**	0.51 ± 0.05*	0.48 ± 0.05	0.61 ± 0.05*	0.43 ± 0.03	0.34 ± 0.03
8 h	0.947 ± 0.13**	0.47 ± 0.03	0.60 ± 0.05*	0.53 ± 0.05*	0.43 ± 0.05	0.33 ± 0.02
24 h	0.952 ± 0.14**	0.64 ± 0.04*	0.75 ± 0.10**	0.92 ± 0.09**	0.66 ± 0.06*	0.34 ± 0.02

*Indicates significant loss of sharp focus at $p \leq 0.05$ as compared to controls
** Indicates significant loss of sharp focus at $p < 0.0001$ as compared to controls
0h is immediately after treatment, Reprinted with Permission Bantseev, McCanna *et al. Toxicological Sciences.* 2003.

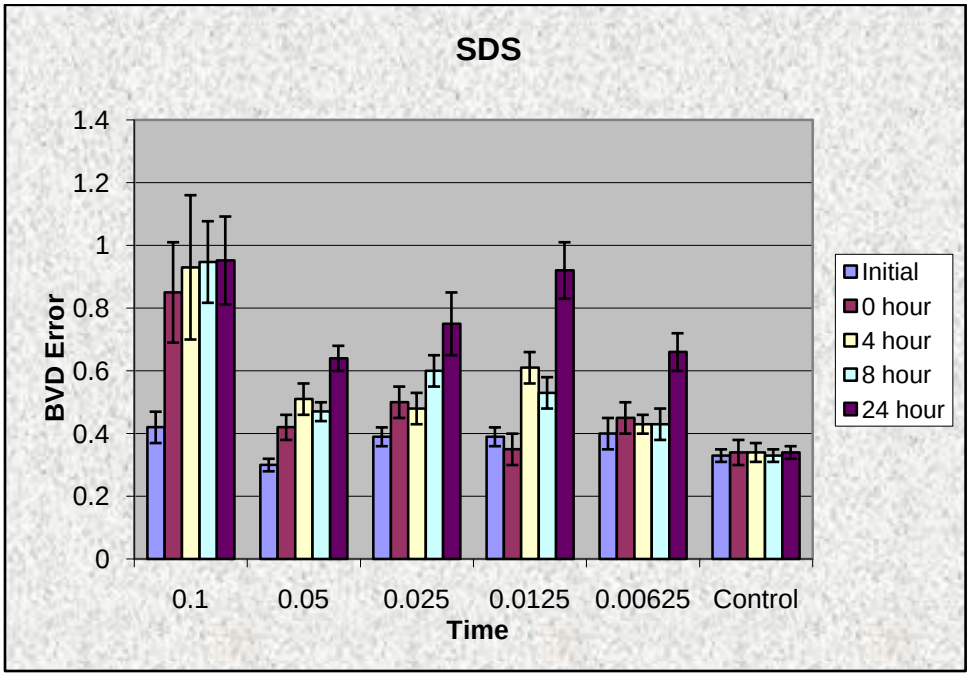


Figure 1. Back vertex distance variability (loss of sharp focus, BVD variability, mm) ± SEM in bovine lenses treated with different SDS concentrations (%) over time (Same data as in table 1)

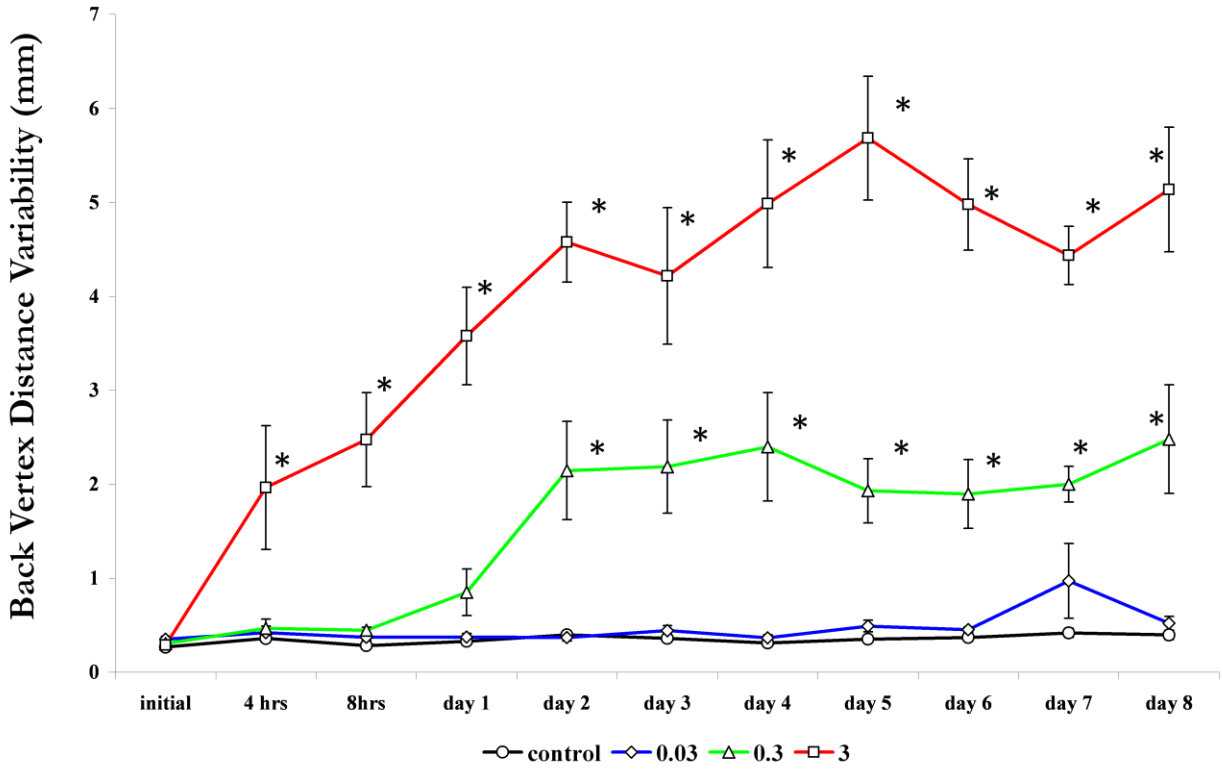


Figure 2. Optical response over time of the cultured bovine lenses following H_20_2 (3, 0.3 and 0.03%) treatment for 15 minutes. * indicates significant differences as compared to the other concentrations and control.

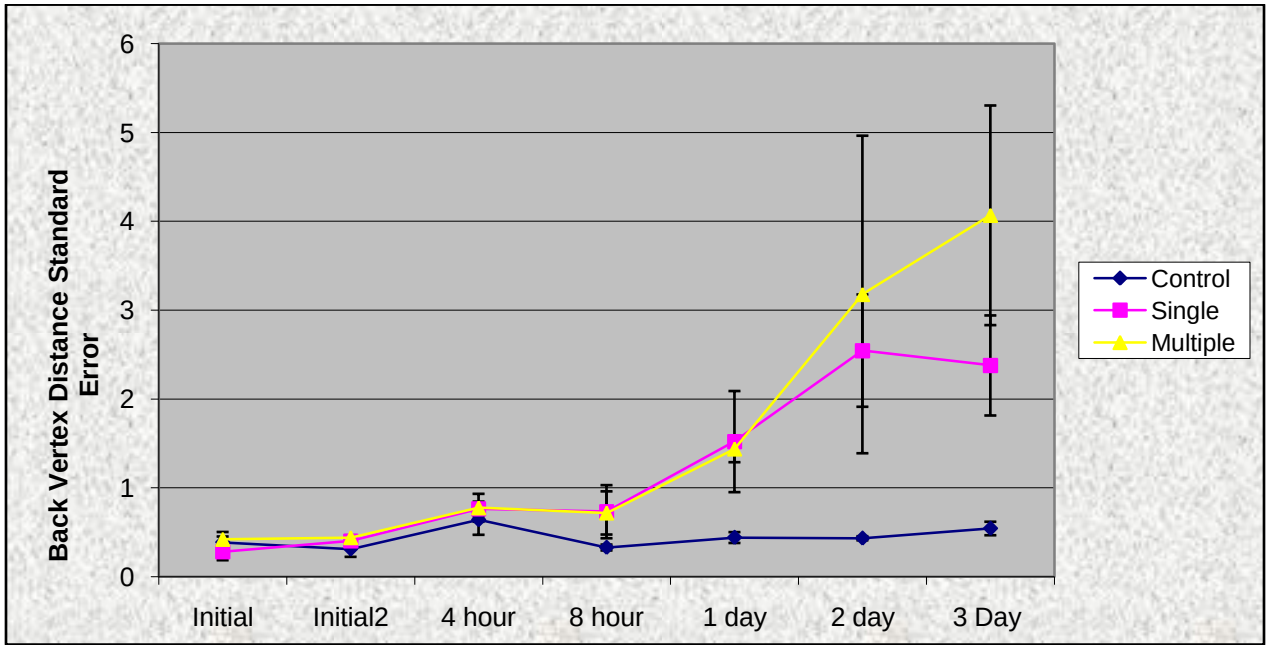


Figure 3. Back vertex distance of bovine lenses after single and multiple instillation of 0.01% BAK.

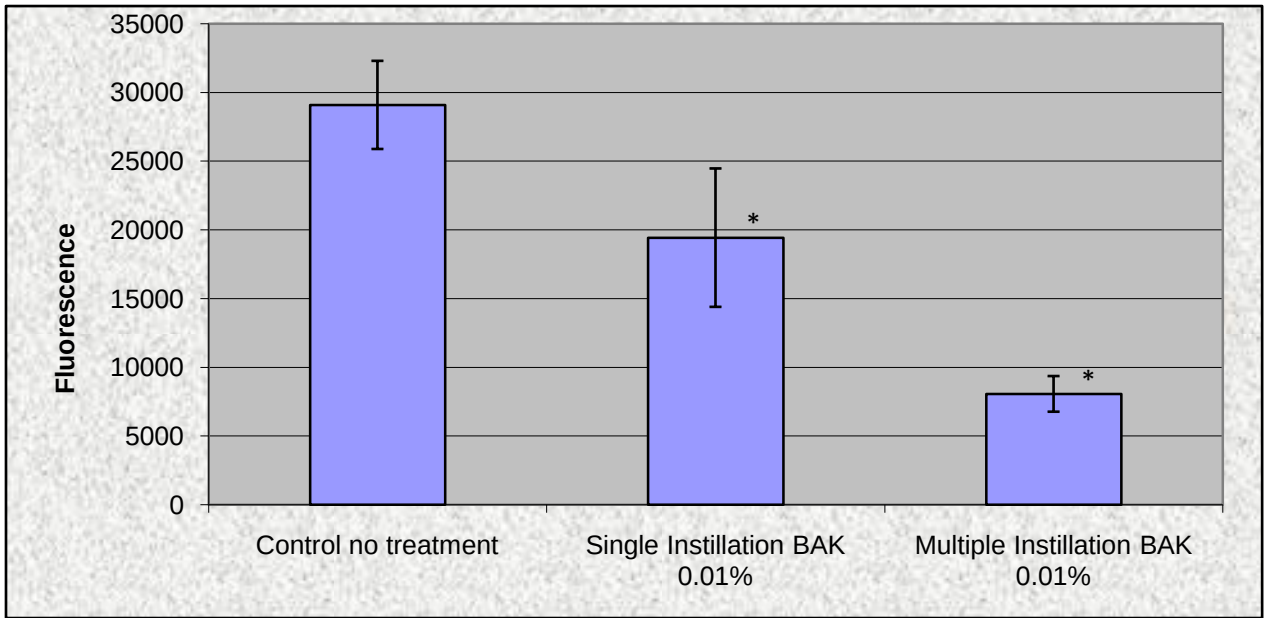


Figure 4. AlamarBlue reading after treatment. This figure shows the fluorescence of bovine lens epithelium after single and multiple instillation of 0.01% BAK. All lenses were tested after 4 days of incubation with the multiple instillation receiving three days of treatments of BAK during this time period. * indicates significant differences as compared to controls ($p < 0.05$).

Table 2. Modified Maximum Average Scores (ECETOC, 1998)

	1 Day	2 Day	3 Day
3% Sodium Dodecyl Sulphate	**16**	**5.6**	**1**
1% Hydrogen Peroxide	**25.8**	**23.25**	**10.5**
15% Sodium Dodecyl Sulphate	**59.1**	**33.6**	**19**
5% BAK	**83.8**	**62.5**	**57.25**

Table 3. Modified Maximum Average Scores (ECETOC, 1998)

	1 hour	1 Day	2 Day	3 Day
100% Methyl Cyanoacetate,	**10**	**27.6**	**22.6**	**21.3**
100% 2,6 Dichlorobenzoyl chloride	**8**	**23.8**	**21.6**	**21.6**

3.4 Discussion

3.4.1 ScanTox™ Assay

In order to assess the toxicity of chemicals and product formulations a model was developed by Sivak using the cultured bovine lens (Sivak and Dovrat, 1983; Sivak *et al*., 1986; Dovrat and Sivak, 2005; Bantseev *et al.*, 2008). Bovine lens cells are ocular cells that originate from the same germinal or embryonic source of the surface ectoderm as the cornea (Ho *et al*., 2008). The lenses are maintained in culture so the cells are in a state of normal homeostasis and are not in an unusually stressed condition. The lenses can be monitored for up to 37 days (Sivak *et al.*, 1990) for optical quality so recovery and delayed toxicity effects can be measured. The laser scanner provides a non-destructive assessment of the health of the bovine lens organ culture so multiple readings can be performed on the same treated lens throughout the duration of the experiment.

An *in vitro* system that can measure for recovery and delayed effects is critical for assessing the toxicity of chemicals. In Draize rabbit ocular irritation studies the measurements of irritation of the cornea, iris and conjunctiva are performed at various time intervals after instillation. Typical readings occur after 1 hour or 4 hours of instillation and at additional intervals of at least 24, 48 and 72 hours. Table 2 lists Draize Modified Maximum Average Scores (MMAS) for 3% Sodium Dodecyl Sulphate, 15 % Sodium Dodecyl Sulfate, 1% Hydrogen Peroxide and 5% BAK at observations after instillation of 1, 2 and 3 days after instillation (ECETOC, 1998). The relative toxicity of these three solutions are 5% BAK > 15% SDS >1% Hydrogen Peroxide > 3% SDS based on the MMAS score and the time of recovery. In addition to recovery from initial injury, delayed toxicity effects also must be identified by an *in vitro* test system. In Table 3 100%

Methyl Cyanoacetate, 100% 2,6 Dichlorobenzoyl chloride exhibited higher MMAS scores at the 1, 2, and 3 day observation periods compared to the 1 hour observation, indicating that the toxicity response to these two chemicals were delayed in that the maximum scores obtained occurred at least a day after the 1 hour observation period (ECETOC, 1998). Toxins with delayed effects were also reported by Sina (Sina, 1994).

The ScanTox™ system was used in this study to evaluate the ocular irritants SDS, hydrogen peroxide and BAK. As already noted, bovine lenses were exposed to SDS (0.1 to 0.00625%) for 30 minutes and to H_2O_2 (0.03%to 3%) and BAK (0.01%) for 15 minutes. Using a laser scanner it was determined that a significant change from the controls occurred for the lenses exposed to 0.1 % SDS for all observation points (0, 4, 8 and 24 hours) and as low as 0.00625% at the 24 hour observation point. Exposure to H_2O_2 at 0.3% and BAK at 0.01% showed toxicity after 24 hours. A study performed by Youn *et al.* (2004) using the ScanTox also showed that 0.01% SDS and 0.01% BAK were toxic to the lens after a 15 min exposure. No toxicity was detected at concentrations of BAK of 0.001% (Youn *et al*., 2004).

3.4.2 In Vitro Cornea Toxicity models

Other than the bovine lens assay the only other assays that evaluate functionality as an endpoint for toxicity for an organ culture of ocular origin are assays that evaluate cultured corneas or enucleated whole eyes. The bovine corneal opacity and permeability assay evaluates the effects of chemicals on the ability of the cornea to maintain clarity and function as a permeability barrier (Gautheron *et al*., 1992). The rabbit enucleated eye test also tests for changes to corneal clarity

and permeability but also measures corneal swelling (Prinsen and Koeter, 1993). Bovine corneas have been shown to be cultured for up to three weeks when the appropriate culturing conditions are made (Foreman *et al.*, 1996). These corneas can be under low stress but the culturing conditions are complex and it is difficult to assure the quality of the corneas obtained from a slaughter house. In one study that used bovine corneas, the damage due to transport of the corneas from the slaughter house was minimized by transporting complete cow heads cooled to 4 degrees C prior to dissection (Frentz *et al.*, 2008). Using a viability dye and confocal microscopy it was determined that the corneal endothelium following 7 days of organ culture demonstrated occasional areas of endothelium devoid of cells together with some dying cells (Foreman *et al.*, 1996). A method that has been used to assess the quality of bovine corneas has been to test for epithelial integrity with fluorescein staining (Frentz *et al.*, 2008). The endothelium appearance is evaluated by visualizing the endothelium appearance from the back of the chamber and lactate production of the cornea is measured. However, in an investigation of the quality of the corneas used in the BCOP assay using the destructive dyes trypan blue and alizarin red it was determined that 20% of the endothelial cells were damaged due to the wrinkling of the corneas when placed in the BCOP holder (Ubels *et al.*, 2000). Thus the damage to the endothelium cells could cause variability in test systems that use bovine corneas. Using the bovine lens however, the quality of the dissected lens can be determined easily using the non-destructive measure of determining the optical quality of the lens. Thus the elimination of the variability due to the use of poor quality of tissue is a major advantage of using the bovine lens over the use of cultured corneas for toxicity studies.

Bovine, porcine and rabbit corneas have tested effects of chemicals on corneal function including effects on maintaining clarity and maintenance of the barrier function. Not knowing if the corneas are damaged prior to starting a study limits its usefulness and difficulty in culturing can limit testing of chemicals for recovery or delayed effects. With these limitations corneas have been tested for toxicity using SDS and BAK with measurements taken up to 4 days, to measure for recovery. In a study using bovine corneas SDS did not cause opacity at concentrations that were considered to be severe irritants (Gautheron *et al.* 1992). After a 30 minute exposure fluorescein permeability was detected at concentrations of 0.5% SDS (Gautheron *et al.* 1992). Porcine corneas were exposed to various concentrations of SDS for 2 minutes (Xu *et al.*, 2004). 1% and 3% SDS caused breaks in the epithelium that recovered after 4 days whereas after treatment with 15% SDS the epithelium did not recover. Abraded bovine corneas were treated with 24 drops of various doses of BAK (0.0001% -0.1% BAK in hyaluronate citrate and calcium containing artificial tears) applied every hour (Foreman *et al*., 1996). Concentrations as low as 0.001% BAK prevented healing from the mechanical abrasions after 4 days and 0.01% BAK caused marked destruction of the epithelium, as measured by staining with fluorescein.

3.4.3 Prediction of Human Toxicity

Acute, sub-chronic and chronic *in vivo* toxicology studies are performed where animals are dosed with progressively higher concentrations in order to determine the threshold of toxicity. The highest dose that does not cause an observable toxicity and the lowest dose where there is an observable toxicity are found. In between the no-observable-effect level (NOAEL) and the lowest-observable effect level (LOAEL) is the threshold of toxicity.

There is a level of uncertainty in the results obtained in animal studies as a prediction of human toxicity thresholds. A standard practice of accounting for this uncertainty is the use of safety factors. Safety factors take into account interspecies variability, intraspecies variability, and additional factors based on the quality of data and duration of testing performed (FDA, 2002, Renwick, 2004). After finding the NOAEL in mg/kg/day the value is divided by appropriate safety factors. For systemic exposures safety a factor of 10 to account for interspecies variability and a factor of 10 to account for intraspecies variability is typical (Renwick, 2004).

Standard safety factors for topically applied drugs, disinfectants or preservatives to the cornea have not been established. However, to support human trials, the US FDA requests that the dosing frequency, drug concentrations and study duration in nonclinical studies should be at least equal and preferably exceed the maximum frequency, concentration and duration of clinical human studies (FDA, 2002). Testing doses higher than clinical dose in animal studies is needed due to the interspecies variability. Appropriate dose response studies are helpful in establishing safe concentrations. Knowing the safety margins make it possible to maximize product formulations by using the most effective dose with acceptable margins of safety.

With the use of safety factors the uncertainty of the toxicity of a chemical can be addressed by using clinical doses that are less concentrated than the NOAEL or LOAEL. For contact lens disinfecting products and preservatives used in ophthalmic products large safety factors can be difficult as the chemicals have to be at sufficient concentrations to kill micro-organisms. Also,

the typical *in vivo* study for FDA approval is only a sub-chronic study with duration of 21 days for contact lens products and 28 days for eye drop products. Using sub-chronic data to predict chronic use requires additional safety factors to account for the uncertainty of extrapolating a 21 day or a 28 day study to the chronic use of eye drops or contact lens care products.

It is clear that due to the interspecies variability, intraspecies variability and the uncertainty of using subchronic testing to predict the toxicity of chronic use, using animal studies alone to assess the potential safety of disinfecting and eye drop products is not sufficient. Clinical studies reduce the uncertainty as the interspecies uncertainty is eliminated from the calculation. However, it is not ethical to use humans as the primary model for determining safe concentrations of chemicals in product formulations and even for critically ill patients the benefits to the patient must seriously be considered before dose escalation studies are preformed (Daugherty, 1999). It is also important to minimize the use of high dose studies in animals as these studies can cause pain and suffering. Therefore, in order to reduce the uncertainty of the safe doses to use in product formulations and to ethically develop novel product formulations *in vitro* testing is essential for determining the chemical concentrations that can be tested in clinical investigations.

Due to uncertainty in correlating animal data to human data as to the NOAEL or LOAEL and the threshold of toxicity in between, what should be the appropriate safety factors used to determine the safe concentration to use in a human clinical trial? Other microscopic techniques have been used to assess toxicity of BAK at the cellular level. Scanning electron microscopy of

treated rabbit corneas to drops containing 0.01% BAK, shown that the BAK is very injurious to the cornea of rabbits (Pfister and Burnstein, 1976). After thirty minutes of exposure most of the top layer of cells desquamate and microvilli are lost on the lower layers. Benzalkonium chloride was applied to rabbit and cat corneal epithelium at concentrations between 0.001% and 0.01% and the corneas were evaluated by scanning electron microscopy (Burnstein, 1980). Initial toxicity was detected at 0.0025% and corneas exposed to higher concentrations showed increased toxic effect. An evaluation of fluoroquinolone antibiotics containing BAK at a concentration of 0.005% instilled at least 4 times a day for 7 days demonstrated that these products showed a significant decrease in epithelial thickness after 7 days of exposure (Kovoor *et al*., 2004). A recent study by Ly *et al.* showed that a fourth generation f*luoroquinilone* with 0.005% BAK compromised the tight junctions of the cornea whereas another f*luoroquinilone* that did not contain BAK left the tight junctions intact (Ly *et al*., 2006). Ichijima *et al.* evaluated *in vivo* rabbit corneas after exposure to BAK drops at 0.02%, 0.01% and 0.005% BAK using tandem scanning confocal microscopy and SEM (Ichijima *et al.*, 1992). The application of 0.005% BAK caused superficial epithelial cells to swell and desquamate. In a study that evaluated 0.004% BAK and 0.005% BAK instilled in rabbit eyes both as a solution and with use as a RGP contact lens care solution, there was significant increases in desquamation of the superficial corneal epithelium and tear lactate dehydrogenase activity compared with control eyes after 3 weeks of treatment (Imayasu *et al.* 1994). Thus, when using SEM or confocal microscopy to study rabbit corneas exposed to BAK, the threshold for toxicity is approximately 0.005%. This is very similar to the toxicity of BAK detected using the ScanTox™ method which

detected BAK toxicity at 0.01% BAK but not at 0.001% BAK. Thus the toxicity threshold for BAK was accurately detected by the ScanTox™ method as being between 0.01% and 0.001%.

Benzalkonium chloride (BAK), is a preservative used in ophthalmic products and is used at an average concentration of 0.01% (range 0.004%–0.02%) in topical multidose solutions (Noecker *et al*., 2004). In human clinical studies biopsies of conjunctivae and trabeculums of patients who were treated with eye drops containing 0.01% BAK were abnormally infiltrated by cells expressing inflammatory or fibroblastic markers (Baudouin *et al.* 1999). Chronic users of glaucoma drops containing BAK showed increased secretion by conjunctival cells of pro-inflammatory cytokines (Malvitte et al., 2007). Exposure to preserved timolol at 0.005% BAK caused an unstable pre-corneal tear film and it disrupted the epithelial barrier function more than the unpreserved control solution in human patients (Takeshi *et al.* 2003). Dry eye patients treated with polyvinyl pyrrolidone preserved with BAK at 0.005% showed an increase in corneal epithelial permeability (Gobbels and Spitznas, 1992). These studies show that the toxicity threshold in humans is approximately 0.005% BAK.

Using available data on the toxicity of BAK we can assign an appropriate safety factor. Because the ScanTox™ assay is a sensitive assay the threshold toxicity values detected were closer to the true toxicity value so a smaller safety factor can be used than that suggested by other methods that are less sensitive. The ScanTox™ assay correlated to toxicity thresholds detected using *in vivo* confocal microscopy and scanning electron microscopy of rabbits exposed to various concentrations of BAK (between 0.001% and 0.01%). Human clinical studies have also detected

toxicity with use of preserved eye drops at a BAK concentration of 0.005%. Using sensitive measures for detecting toxicity using *in vitro, in vivo* animal and in human patients there does not appear to be significant variability in the estimates for toxicity. Therefore the NOAEL *in vitro* and *in vivo* animals of 0.001% BAK should approximate the NOAEL in humans. Because BAK is used in eye drops in the range of 0.004%–0.020%, a concentration of 0.001% BAK, depending on the formulation is a good starting point for a new product formulation. Other factors such as preservative efficacy at 0.001% and adequate drug penetration into the cornea need to be considered. Disruption of the cornea due to BAK toxicity is required in order for some drugs to penetrate through the epithelial barrier (Kaur and Kanwar, 2007). Concentrations of BAK should be as low as possible since sensitive human individuals who already have compromised epithelium due to disease such as dry eye could have their condition exacerbated by BAK in the formulation (Baudouin, 2008).

Chapter 4

Measurements of Mitochondrial Integrity to Determine Early Stage Toxicity of Chemicals and Contact Lens Care Solutions

This chapter is partly based on three articles that describe the use of the confocal microscope to assess toxicity of chemicals (Bantseev, McCanna *et al*., 2003; Bantseev, McCanna *et al*., 2007; Bantseev, McCanna *et al.*, 2008). These articles were a collaborative effort. I contributed to the study design, data interpretation and in the preparation of the manuscript. In addition, I carried out all of the studies related to the use of the bovine lens epithelium and human corneal epithelial cells to evaluate the toxicity of contact lenses that were soaked in contact lens solution products. I also carried out all of the studies that evaluated the use of the human corneal epithelial cell line to assess the toxicity of solutions.

4.1 Introduction

Molecular biology methods for assessing changes to cells after exposure to toxins can be very helpful in determining potential mechanisms of toxicity. These methods are very sensitive and can detect changes at concentrations that may not cause clinically observable pathological findings. Although the chemical concentrations that cause physiological changes in cells may be below the concentration threshold that causes clinical pathological effects, cell and molecular techniques can be used to assess the potential toxicity of chemicals and also estimate clinical toxicity thresholds. In order to correlate molecular assay results to clinically observed thresholds, chemicals with known toxicity thresholds can be run as positive controls. The changes caused by novel chemicals or product formulations at the cell or molecular level can then be compared to these positive controls to determine the relative effects.

Whether a chemical concentration is identified as exhibiting *in vivo* pathological effects can depend on the technique used to measure these effects. In humans the typical clinical assessments using macroscopic observations, patient subjective symptoms and slit-lamp microscopy may not detect damage that is occurring at the sub-clinical level. Although this sub-clinical damage may not initially present itself to the clinician, after repeat use of the product for many days or months the damage may increase to the level where a clinical observation of pathology is detected. Human tests have been developed that are potentially more sensitive than traditional observations of ocular irritation. Assessments of the tear film of patients and *in vivo* clinical confocal microscopy have been used to assess corneal health (Patel *et al*., 2007; Malvitte

et al., 2007). The Draize test for assessing ocular irritation by observing for changes in the cornea, conjunctiva and iris has been used as an *in vivo* (rabbit) test for years (Draize *et al*., 1944). This test has been criticized for its lack of sensitivity (Bantseev *et al*., 2008). More sensitive measures using confocal microscopy and scanning electron microscopy for assessing damage to the ocular tissue have been used (Burnstein N., 1980; Ly *et al*., 2006). A chemical concentration that was determined to be non-toxic using traditional methods for assessing toxicity in human clinical studies or in rabbits using the Draize test may later be determined to be toxic using more sensitive measures for assessing effects or may be identified as toxic after chronic use of the product. Thus, a clinical toxicity threshold for a chemical is the concentration that causes measurable toxicity using the most sensitive measures for detecting toxic effects after chronic use of a product.

One sensitive measure of determining toxicity *in vivo* has been the assessment of the cornea using the *in vivo* confocal microscope. The corneal epithelium, Bowman's layer, corneal stroma, corneal endothelium and the conjunctiva can all be imaged by the *in vivo* confocal microscope (Patel *et al*., 2007; Messmer *et al.*, 2006). Observations of corneas of patients that wore contact lenses include the presence of mucein balls that may penetrate the corneal epithelium (Millar *et al., 2003*), reduced keratocyte density (Efron, 2007), and a greater number of Langerhans cells observed in the layer of the sub-basal nerve plexus (Zhivov *et al*., 2007). These observations were not classified as clinical pathological observations. However, if in the future there is a correlation with later onset of disease these observations would become clinically relevant. Use of the *in vivo* confocal microscope to measure toxicity of a chemical was demonstrated by

Kaufman *et al.* (2006). A comparison of the toxicity of two fourth-generation fluoroquinolones gatifloxacin ophthalmic solution 0.3% and moxifloxacin ophthalmic solution 0.5%, was investigated. It was found that 0.5% moxifloxacin solution caused greater corneal epithelial cell loss than 0.3 % gatifloxacin. The loss of epithelial cells may be an indication of the cytotoxicity of the moxifloxacin fluoroquinolone.

Other sensitive measures for determining toxic or immunoinflammatory effects in humans have been used for confirming threshold levels for benzalkonium chloride (BAK). In human clinical studies biopsies of conjunctivae and trabeculums of patients who were treated with eye drops containing 0.01% BAK were abnormally infiltrated by cells expressing inflammatory or fibroblastic markers (Baudouin *et al.*, 1999). Chronic users of glaucoma drops containing BAK increased secretion by conjunctival cells of pro-inflammatory cytokines (Malvitte *et al*., 2007). Using a tear specular microscope and a fluorophotometer it was determined that exposure to preserved timolol at 0.005% BAK caused an unstable pre-corneal tear film and it disrupted the epithelial barrier function more than the unpreserved control solution in human patients (Takeshi, 2003). Dry eye patients treated with polyvinyl pyrrolidone preserved with BAK at 0.005% showed an increase in corneal epithelial permeability (Gobbels and Spitznas, 1992). These studies show that the toxicity threshold for BAK in humans in the formulations investigated was at or below 0.005%.

There are a few studies that have been published that directly compare the toxicity of a chemical using rabbit and human subjects. Roggeband *et al*. (2000) tested very low concentrations of

surfactants in rabbits and humans to determine the relative prediction value of the rabbit ocular irritation test. The volumes instilled in the rabbit and human was ten-fold lower than the 0.1 ml used in the standard Draize test. Responses in the eye were graded by the Draize scoring scale (Draize *et al*., 1944) and scored using a slit lamp at nine different time intervals throughout a 3 day period. The clinical examinations also included fluorescein corneal staining, conjunctival fluorescein staining, and tear film break uptime. The scores for ocular irritation were greater in the rabbit than the scores obtained from the human subjects. Rabbits also had higher ocular irritation scores than human subjects in an earlier study where 0.1 ml of liquid detergent was instilled (Beckley, 1965). In another study that compared the ocular effects of a soap suspension and a household cleaner in rabbit, monkey and human eyes, it was determined that the rabbit was less sensitive to the soap suspension but more sensitive to the household cleaner than humans (Beckley *et al*., 1969). A fourth study compared cornea fluorescein permeability in rabbits and humans after exposure to 0.01% and 0.02% BAK using a simple fluorophotometer attached to a slit lamp (Burnstein, 1984). In rabbits, 0.01% and 0.02% BAK caused an increase in fluorescein permeability over the controls; whereas, in humans an increase in permeability only occurred after instillation of 0.02% BAK. The human subjects did indicate a stinging sensation 30 minutes after 0.01% and 0.02% BAK instillation when treated with fluorescein. Increasing the concentration of BAK from 0.02% to 0.05% in the rabbit increased sodium fluorescein permeability threefold. In these studies using the objective measures for grading irritation there were some differences in the sensitivity of human and rabbit corneas exposed to various chemicals.

Due to ethical considerations there are few published studies directly comparing rabbit ocular irritation with human clinical effects. Industry does perform clinical studies after Draize rabbit testing has been completed, but few of these studies have been published. Benzalkonium chloride is one chemical that has been used extensively by humans. Toxic effects in humans have occurred after instillation of a concentration of BAK as low as 0.005% (Takeshi, 2003; Gobbels and Spitznas, 1992). Animal studies that examined the effects of BAK on the cornea using scanning electron microscopy and confocal microscopy also show toxicity at the 0.005% concentration of BAK (Pfister and Burnstein, 1976; Burnstein, 1980; Kovoor *et al*., 2004; Ly *et al*., 2006; Ichijima *et al*., 1992; Imayasu *et al*., 1994). Pauly *et al*. (2007) demonstrated the sensitivity of the confocal microscopy by showing that macroscopic and slit lamp examinations revealed signs of ocular irritation only at the 0.25% and 0.5% BAK concentrations; whereas, *in vivo* confocal microscopy revealed epithelial defects at 0.01% and 0.1%. Histopathological techniques detected conjunctival infiltrates (Bequet *et al*., 1998) and corneal thinning (Pauly *et al*., 2007) in rats treated with 0.01% BAK.

The studies performed to evaluate the toxicity of BAK in rabbits and humans indicate that the BAK is toxic at concentrations as low as 0.005%. It would be ideal if other chemicals were investigated as extensively as BAK using sensitive measures of toxicity. In order to determine the toxicity of other chemicals less sensitive measures for assessing toxicity using Draize rabbit test have been performed (Bagley *et al*., 1999). Unlike BAK, many of these chemicals have not been tested in humans. It is difficult to make the comparison between a molecular assay and human toxicity because BAK is the only chemical that has been extensively evaluated for

effects using the more sensitive measures for detecting toxicity. The molecular techniques can be compared to more chemicals if a comparison is made to Draize testing. However, the molecular techniques would likely show toxic effects at lower concentrations due to their sensitivity. The toxicity threshold of BAK has been detected using the most sensitive measures for assessing toxic effect in humans and in animals. Therefore, the accuracy of the molecular models can be investigated using BAK as a positive control.

The focus of this investigation is the effect chemicals and contact lens care solutions have on the mitochondria of cells. There are two major functions carried out by mitochondria relevant to cell cytotoxicity. One is that the mitochondria are the powerhouses of the cell. Using the electron transport chain protons are pumped out of the inner membrane. This electrochemical gradient allows for the coupling of the synthesis of ATP when the protons cross back through the mitochondrial inner membrane (Frey and Mannella, 2000). This ATP is then used to maintain the cell's energy needs. A second function of mitochondria is the regulation of apoptosis.

Mitochondria hold cytochrome c and the release of this molecule can initiate a cascade of caspases that carry out the process of apoptosis (Detmer and Chan, 2007). Other proteins are released by mitochondria such as apoptosis-inducing factor, but only cytochrome c has been determined to be essential for apoptosis (Desagher and Martinou, 2000). Apoptotic cell death is non-inflammatory and necrotic cell death can be inflammatory (Freis *et al*., 1999). Because of the varied responses to the different types of cell death, an understanding of the role of mitochondria as either the trigger for apoptotic cell death or part of the events leading to necrosis

can be critical for understanding the toxicity of chemicals and product formulations. Friers *et al*. (1999) outlined 5 major known processes that can lead to cell death after stimulus of the cell death inducers tumour necrosis factor (TNF) or CD95L. Three of these processes involve the mitochondria. One leads to apoptosis, a second leads to necrosis and a third can cause apoptosis or necrosis. In the first process, cytochrome c and other proteins are released from the intermembrane space. Caspase then activates an apoptotic pathway. The second process involves triggering the mitochondria to produce reactive oxygen species leading to necrosis. The reactive oxygen species cause the cell to swell and then suddenly collapse releasing its intracellular contents. In a third pathway reactive oxygen species are produced that release the caspase initiating the apoptotic pathway but the production of the reactive oxygen species could also lead to necrosis. An interesting aspect of the effects of the reactive oxygen species (ROS) is that the ROS can damage the respiratory chain which causes the mitochondria to produce more ROS. The dramatic increase in ROS by this feedback loop then leads to cell swelling and death by necrosis.

In order to measure the health of the mitochondria of cells after chemical exposure this investigation evaluated the activity of mitochondria of the lens using the metabolic dye alamarBlue and determined mitochondrial integrity using the fluorescent dye rhodamine 123. AlamarBlue, also known as resazuran, changes from a blue nonfluorescent molecule to resorufin which is pink and highly fluorescent (O'Brien *et al*., 2000). Decreases in alamarBlue fluorescence may be the result of lose of viability due to damage to mitochondria. However, it was shown that the cytosolic and microsomal enzymes also reduce alamarBlue (Gonzolas and

Tarloff, 2001). Therefore, using another indicator for determining the cytotoxic effects on mitochondria is helpful for assessing potential mitochondrial damage. Another common viability dye MTT also is reduced by mitochondria, cytosolic and microsomal enzymes (Gonzolas and Tarloff, 2001). Unlike alamarBlue there is a requirement that the cells are killed in order to release the insoluble crystals from the cells that were generated by the reduction of formation to an insoluble product (O'Brien *et al.*, 2000). These crystals may also cause damaged cell membranes (Berridge *et al*., 2005). Other viability dyes such as XTT, WST-1 are water-soluble but have a net negative charge and therefore are mostly cell impermeable. Reductions of these dyes do not occur at the mitochondria but at the plasma membrane (Berridge *et al*., 2005).

The dye Rhodamine 123 is specific for mitochondria thus is an ideal dye for evaluating mitochondrial function. Rhodamine 123 at concentrations of 10 ug /ml for 30 minutes was shown to adequately stain gerbil fibroma cells (Johnson *et al*., 1980). Anaesthetics were shown to inhibit mitochondrial electron transport in isolated mitochondria (Chazootte and Vanderkooi, 1981). Using a fibroblast cell line, Rhodamine 123 was used to detect the disruption of mitochondria by local anaesthetics. Exposure to the anaesthetic bupivacaine caused a reduction in Rhodamine 123 fluorescence and diffusion of the stain into the cytoplasm (Grouselle *et al*., 1990). Rhodamine 123 was used to measure the toxicity of mercuric chloride on Madin-Darby canine kidney cells. Treatment of cells with mercuric chloride caused rhodamine 123 fluorescence to dissipate from the mitochondria into the cytoplasm (Lachowiez *et al*, 1989). Bantseev *et al*. (2003) demonstrated that exposure of lens epithelial mitochondria and the mitochondria of superficial cortical fibre cells to the mitochondrial depolarizing agent carbonyl

cyanide m-chloro-phenylhydrazone (CCCP) caused the mitochondria to become short and swollen. Using the Rhodamine 123 to assess fluorescence reduction and mitochondrial morphology the effect of chemicals on the integrity of mitochondria can be investigated.

The objective of this study is to determine the ocular toxicity potential of chemicals used in ophthalmic products utilizing methods which measure effects on mitochondrial integrity. Using these sensitive measures of toxicity the effects of contact lens solutions and benzalkonium chloride on epithelial cells were determined. The chemicals from contact lens solutions can uptake into contact lenses and the exposure time and concentration of the chemicals will be affected by release from the lens over time. To simulate real use conditions contact lenses were placed on a lens epithelium and toxicity was determined after exposure. The bovine lens can be cultured *in vitro* for extended periods of time so recovery from damage can be investigated. Also the advantage of using a primary culture is that changes in physiology from the natural *in vivo* state due to viral immortalization and sub-culturing are minimized. In order to cover the physiological differences that may exist between bovine and human cells toxicity studies were also performed using a human corneal epithelial cell line.

4.2 Materials and Methods

4.2.1 Chemicals and reagents.

4.2.1.1 Chemicals

Rhodamine 123, was obtained from Molecular Probes (Eugene, OR). AlamarBlue was obtained from Biosource (Camarillo, Ca). Benzalkonium chloride (BAK) solution (Fluka Chemika, *Steinheim*, Germany) and Sodium dodecyl sulphate (SDS) chemical (Sigma, St. Louis, MO) prepared in sterile 0.9% saline solution. ReNu MultiPlus Multi-Purpose Solution (Bausch & Lomb, Rochester, NY), OPTI-FREE Express Multi-Purpose Disinfecting Solution (Alcon, Fort Worth, TX) , OPTI-FREE Replenish, Multi-Purpose Disinfecting Solution (Alcon), SOLOCARE AQUA All-In-One Solution (Ciba Vision, Duluth, GA), COMPLETE Multi-Purpose Solution and Sensitive Eyes Saline Plus (Bausch & Lomb) were purchased from commercial sources and were used with in the labelled expiration dates.

4.2.1.2 Bovine lens culture medium.

M199 modified with Earle's salts, 26 mM sodium bicarbonate, 0.7 mM L-glutamine , 7 mM NaOH were purchased from Sigma Chemical Co. (St. Louis, MO). 21 mM HEPES , 100,000 units penicillin/L, 100 mg streptomycin/L, and 3% dialyzed fetal bovine serum (Invitrogen, Burlington, ON, Canada).

Madin-Darby canine kidney cell culture medium (MEM). Minimum Essential Media with Earle's Salts and L-Glutamine, 100,000 units penicillin/L, 100 mg streptomycin/L, and 10%

dialyzed fetal bovine serum were purchased from Invitrogen Canada Inc (Burlington, ON, Canada).

4.2.1.3 Human corneal epithelium cell culture medium.

The human corneal epithelial cells (HCEC) cells were initially cultured in (F12/DMEM-1) 50/50 Ham's F12/Dulbecco' modified Eagle's medium(Mediatech, Inc, Herndon, VA), 10% heat-inactivated fetal bovine serum, (Atlanta Biological, Lawrenceville, GA), 5 μg/ml insulin, 0.1 μg/ml cholera toxin, 10 ng/ml epidermal growth factor, and 50 μg/ml gentamycin. It was determined that a simplified media could be used to culture the HCEC. Media was changed to simplified medium to (F12/DMEM-2) Dulbecco's Modified Eagle Medium with L-Glutamine and 15 mM HEPES, 100,000 units penicillin/L, 100 mg streptomycin/L and 10% dialyzed fetal bovine serum were purchased from Invitrogen Canada Inc (Burlington, On, Canada).

4.2.2 Confocal Analysis of Lens Mitochondrial Integrity

4.2.2.1 Eye dissection (Lens)

Bovine eyes obtained from a local abattoir. Fresh bovine eyes were between 2 and 3 years of age and were dissected the same day of post mortem. The lenses were carefully dissected making sure the cutting instruments never touched the lens during dissection. The zonular ligaments of the iris were cut as the last step in the dissection removing the lens from all other ocular tissues. After dissection the bovine lens was put into a three-part chamber containing medium and

incubated at 37°C with 4-5% CO_2. After 24 h lenses exhibiting mechanical damage during dissection, as evaluated by the visible existence of opacities, were discarded.

4.2.2.2 SDS Exposure

Lenses were exposed to Sodium dodecyl sulfate (0.1 to 00625%) for 30 min by submerging the lenses into a 50mL conical tube containing 10 mL of SDS solution. The lenses were then rinsed with saline (0.9%), placed in fresh M199 and incubated at 37 ºC at 4-5% CO_2 for 24 hours.

4.2.2.3 Direct Contact Contact Lens/Solution Exposure

AcuVue 2 lenses (Johnson & Johnson) were soaked for 20 hours. A twenty hour soak was choosen to cover an extended overnight soak period. Contact lenses were soaked in a 50 mL polypropylene conical tube (VWR, Westchester PA) containing 10 mL of test solution (BAK 1%, 0.1%, 0.01%, 0.005%, 0.001% in saline or contact lens care solution). Bovine lenses were removed from the culture medium and rinsed by submersing them in 0.9% saline. Contact lenses that soaked overnight in the test solution were then placed onto the anterior surface of the bovine lens (epithelial surface). Each bovine lens was then moved to a well of a 6 well plate containing 5 mL of 0.9% saline so that it covered the bovine lens equator but did not cover the anterior surface. The treated bovine lenses were then placed in a 37 degree C incubator with 4-5 % CO_2 for 19 hours. In a survey of sleep patterns less than 5% reported sleeping less than 5 hours a day (Groeger *et al*., 2004). A 19 hour exposure time was chosen to cover the longest possible lens wear time for 95% of the population. After the 19 hour exposure to the treated lenses, the contact lenses were removed and each bovine lens was rinsed by submersing in 0.9% saline. The

bovine lenses were placed back into growth media and after a 24 hour recovery the mitochondrial integrity of the bovine epithelium was visualized using the confocal microscope.

4.2.2.4 Staining lens cells with Rhodamine 123

Lenses were transferred into 10 ml serum-free M199 in Wheaton-33 sample glass vials (VWR, Mississauga, ON, Canada). Lens mitochondria were stained using 20 µM Rhodamine 123 for 45 min at 37°C for lenses exposed to SDS and 20 min for lenses exposed to the contact lens/solutions. Shorter exposure times for the evaluations with lens/solutions were preformed as shorter exposure times were shown to be effective in assessing bovine lens epithelium for changes in mitochondrial integrity (Bantseev and Youn, 2006). Lenses were rinsed and immobilized on cover glasses, attached over 10 mm holes drilled in the bottom of each well of a six well plate using 1% agarose, previously melted in M199 and cooled to 35°C.

4.2.2.5 Confocal imaging of bovine lens

A Zeiss confocal laser scanning microscope (CLSM) 410 system attached to an Axiovert 100 microscope with a 40x water-immersion C-Apochromat objective was used. The combination of an argon/ krypton laser with a 488 nm excitation laser line, and either a 505 or 590 nm long pass emission filter, were used to observe Rhodamine 123 fluorescence.

4.2.2.6 Statistical analysis

For the lens cells exposed to SDS statistical calculations were completed using a two way repeated measures ANOVA or one-way ANOVA. A probability value of less than or equal to 0.05 was considered significant. For the bovine lenses exposed to the contact lens/solution combinations assessed in comparison to the BAK controls, the difference in the mitochondria integrity between the test and BAK controls were obvious using visual observation. Statistical analysis was not required.

4.2.3 Confocal analysis of Cornea, Human Corneal Epithelial Cells and MDCK cells mitochondrial integrity

4.2.3.1 Eye dissection (Cornea)

Fresh (2–3 hours after death) bovine eyes obtained from a local abattoir were carefully dissected free of extraocular muscles in a sterile laminar flow hood. For control benchmark evaluations, untreated 9.5-mm central corneal buttons were cut with a trephine blade, and the corneal buttons were then placed in glass vials containing different fluorescent markers previously dissolved in 10 mL of Hanks' balanced salt solution (HBSS).

4.2.3.2 Cell culture

Madin-Darby canine kidney cells (MDCK) were obtained from American Type Culture Collection (Manassas, VA), ATCC# CCL34. SV40-Adeno vector transformed immortalized

human corneal epithelial cells (HCEC) were kindly provided by Dr. Sasaki via RIKEN BioResource Center (Japan).

4.2.3.3 Exposure of corneas to contact lens care solutions

For treatments, 20-mm washers coated with petroleum jelly were placed over the central cornea of an intact eye, and the volume inside each washer was filled with approximately 2 mL of OPTI-FREE Express multipurpose disinfecting solution, or ReNu MultiPlus No Rub multipurpose solution for 30 minutes at room temperature. By using a trephine blade 9.5 mm in diameter, central corneal buttons were excised and rinsed twice in HBSS. Control eyes were exposed for 30 minutes to HBSS.

4.2.3.4 Staining of Corneas with Rhodamine 123

After treatment, the corneal buttons were stained with a single mitochondrial-specific fluorescent dye, rhodamine 123. Before confocal analysis, corneal buttons were rinsed twice in HBSS and mounted in 1% agarose, previously dissolved in HBSS, on glass-bottom multiwell plates (MatTek Corp., Ashland, MA).

4.2.4 Exposure of human corneal epithelial and Madin-Darby canine kidney cells to BAK and contact lens care solutions

Human corneal epithelial cells were grown for 7 days and MDCK cells were grown for 4 days in MatTek glass bottom collagen coated culture dishes (Ashland, MA) prior to treatment and

staining. The day of treatment, the culture medium was removed, the cells were rinsed with 1 mL of HBSS, and 2 mL of the test article was added to the wells for 1 hour, 15 minutes or 5 minutes. For contact lens exposure culture media was removed and 2 mL of HBSS was added followed by placing an AcuVue 2 lens (Johnson & Johnson) in the center of the culture well for three hours. The contact lenses were soaked for 20 hours in 50 mL polypropylene conical tube (VWR, Westchester PA) containing 10 mL of solution prior to placing the lens over the human corneal epithelial cells. The cells were rinsed with 1 mL of MEM and then 1 mL of MEM was placed in each culture plate.

4.2.4.1 Staining cells with Rhodamine 123

Cell mitochondria were stained for mitochondria using 160 µM Rhodamine 123 for 20 min at 37°C. After 20 minutes, the Rhodamine 123 was removed, the tissue was rinsed with 1 mL of MEM, and then 1 ml of MEM was added to each well prior to visualizing the mitochondria using the confocal microscope.

4.2.4.2 Confocal imaging of corneas, HCEC, and MDCK cells

The mitochondria of corneas and cells lines were imaged using a Zeiss 510 Meta 18 confocal laser scanning microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) system equipped with an inverted Axiovert 200 mol/L microscope and 40X water-immersion C-Apochromat high numerical aperture objective. High-resolution X, Y (0.22 µm x 0.22 µm) and Z-series (0.44–0.62µm) stacks of cornea epithelial layers were acquired. The images taken for the corneal cell

lines were taken near the center of the cells. The combination of appropriate lasers and emission filters, as suggested by the manufacturer, were used to visualize specific dye fluorescence.

Cornea images were analyzed by semi-automated quantitative image analysis. Lengths and number of mitochondria were taken using the image analysis toolbox of the MatLab software. The minimum mitochondrial area filter specified the minimum area (in pixels) to recognize an object as a mitochondrion. The threshold level establishes the intensity level to separate mitochondria from background. Digital confocal images were processed first by the software to estimate the number and length of the mitochondria and subsequently corrected by an operator using a set of software tracing tools. The operator, an experienced computer user, was masked to the treatment groups.

4.2.4.3 Statistical Analysis

The analysis of the cornea mitochondria (i.e., the number and length of the mitochondria) was carried out by using an analysis of variance over depth, as calculated by SAS 9.1 statistical software (SAS Institute, Inc., Cary, NC). Differences were considered significant at probability levels less than or equal to 0.05. The difference was recorded numerically with all results expressed as mean ± standard error of the mean.

For the cell lines, the difference in the mitochondria integrity between the test and BAK controls were obvious from visual observation. Mitochondrial integrity differences between Optifree

Express and Optifree Replenish versus the other lens care solutions were substantial enough that no additional quantification differences in addition to the visual analysis was required.

4.2.5 AlamarBlue Test of Bovine Lenses and Human Corneal Epithelial Cells

4.2.5.1 Direct Contact -Contact Lens/Solution Exposure Bovine Epithelium

AcuVue 2 lenses (Johnson & Johnson) were soaked for 20 hours in 50 mL polypropylene conical tube (VWR, Westchester PA) containing 10 mL in BAK 1%, 0.1%, 0.01% or a contact lens care solution. Bovine lenses were removed from the culture medium and rinsed by submersing them in 0.9% saline. Contact lenses that soaked overnight in the test solution were then placed onto the anterior surface of the bovine lens (epithelial surface). Each bovine lens was then moved to a well of a 6 well plate containing 5 mL of 0.9% saline so that it covered the bovine lens equator but did not cover the anterior surface. The treated bovine lenses were then placed in a 37 degree C incubator with 4-5 % CO_2 for 19 hours.

After the 19 hour exposure to the treated contact lenses, each bovine lens was rinsed by submersing in 0.9% saline. One group of treated bovine lenses were placed into M199 culture medium and evaluated after a 1 day recovery period, followed by reincubation in culture media and an assessment of viability after an additional 2 days of incubation. A second group of treated bovine lenses were placed into M199 and evaluated after a 3 day recovery period, followed by a reincubation of the lenses and an assessment of viability at 5 days and 7 days following the initial treatment.

The alamarBlue test consisted of placing the anterior side of the bovine lens down into a well of a 12 well plate that contained 1 mL of an 8% alamarBlue solution prepared in M199 culture media without bovine serum. The bovine lenses were incubated in the alamarBlue solution for 2 hours. After 2 hours the bovine lenses were removed from the alamarBlue and the plates were read at 530 excitation and 590 emmision using a CytoFluor II fluorometer (perceptive Biosystems Inc. Framingham, Ma. USA).

4.2.5.2 Treatment of human corneal epithelial cells (cell monolayer)

A cell suspension (1 mL) containing 10^5 cells was seeded in 24-well plates. The plates were then incubated at 37°C with 5% CO2 for 2 days, when the cultures were approximately 75% to 80% confluent (McCanna *et al.*, 2008). The medium was aspirated from each well, and the well was rinsed with 1 mL Hank's balanced salt solution (HBSS). After aspirating the HBSS, the wells were treated with the test solutions for 15 minutes at room temperature. Four replicate wells were used for each solution. After removal of the solutions, the wells were rinsed with 1 mL HBSS, and then 1 mL of 10% alamarBlue prepared in medium without phenol red and serum was added to each well. Some alamarBlue was also added to four blank cell-free wells. The fluorescence of each well was measured by using a fluorescence plate reader at 530 nm excitation and 590 nm emission. The 24-well culture plate was then incubated at 37°C for 4 hours, and then the fluorescence was determined again. The difference of the fluorescence at 4 hours and at time zero represented the metabolic activity of the cells in that well, and the average fluorescence of the four wells was determined after subtracting the fluorescence of the blank wells.

4.2.5.3 Statistical Analysis

For the alamarBlue assay the statistical significance of differences between treatment groups was determined with an ANOVA. The criterion of statistical significance was predetermined to be $P \leq 0.05$.

4.2.6 Treatment of human corneal epithelial cells and MDCK cells(trypsinized cells)

Additional studies were performed to evaluate the effect of contact lens solutions on trypsinized cells. Human corneal epithelial cells from RIKEN Bio Resource Center and MDCK cells were grown in flasks for 4 days in DMEM/F12 media (HCEC) and MEM media (MDCK). The cells were harvested with 0.25% trypsin and counted in a hemocytometer. The cells were then placed into Millicell wells in 1 mL of medium and allowed to settle for 5 minutes. The media was then rinsed out of the insert from the bottom using sterile absorbant gauze pads. One ml of HBSS was added to the well and rinsed out through the bottom of the insert. Two mL of the test solution was added to the top of the well and the wells were placed into a 37 degree incubator for 1 hour. After one hour of incubation, the test article was removed and 5 mL of medium with serum was added to the filter and rinsed through. After rinsing the filters, treated cells were placed into the wells of a 6 well plate that contain 1 mL of 10% alamarBlue in MEM (no serum) per well. Two mL of 10% alamarBlue in MEM was then added to the top of each well and the plates were placed into a 37 degree C incubator for 4 hours. After 4 hours, 1 mL aliquots from the plates were taken and placed into 12 well plate. The plates were read at 530 excitation and 590 emmision using a CytoFluor II fluorometer (perceptive Biosystems Inc. Framingham, Ma. USA).

The HCEC were placed after treatment into 7mL of growth media and evaluated for alamarBlue reduction after 24 hours recovery. The MDCK treated cells that were evaluated immediately after treatment were rinsed and placed into media and then evaluated again for alamarBlue reduction after 24 hours of recovery.

4.3 Results:

4.3.1 Confocal analysis of the bovine lens after exposure to SDS

The toxicity due to SDS exposure could be seen by visualizing the epithelial and fiber cells after 30 minutes exposure and 24 hours of recovery (Bantseev, McCanna *et al*., 2003). No mitochondria were present in the epithelial cells exposed to 0.1%, 0.05% or 0.025% SDS. Mitochondria were present in the epithelial cells exposed to 0.0125% SDA and 0.00625% SDS. Mitochondria were longer and showed a greater relative fluorescence in the lens epithelial cells exposed to the 0.00625% SDS concentration than the 0.0125% SDS concentration (Table 1).

No mitochondria were present in the superficial cortical fiber cells exposed to the 0.1%, 0.05% or 0.025% SDS. The cells exposed to SDS concentrations 0.0125% and 0.00625% were significantly more damaged than the control cells (Table 2). Fiber cells at the equator had shorter mitochondrial length and decreased relative fluorescence when compared to the mitochondria of the control lenses. In the lenses treated with 0.0125% SDS, the mitochondria were absent in the anterior and posterior superficial cortical fiber cells. The mitochondria were also absent in the posterior superficial cortical fiber cells exposed to 0.00625% SDS. The depth below where there is a mitochondria free zone was also measured (Table 2). This zone was

significantly different than the control (anterior 153.90 ± 17.10, equator 205 ± 11.40) for the cells exposed to the 0.0125% concentration (anterior unable to measure, equator 71.35 ± 2.95) whereas the mitochondria free zone was not different than the controls for the cells exposed to 0.00625% SDS (anterior 142.50 ± 5.70, equator 183.10 ± 34.90). At the 0.0125% concentration the mitochondrial free zone could not be measured as no mitochondria were present.

4.3.1.1 Confocal analysis of the bovine lens after exposure contact lens/solution.

Bovine lenses exposed to contact lenses treated with various concentrations of BAK were examined at different magnifications using a 10x and a 40x objective. After a 19 hour exposure and a 24 hour recovery the effects of BAK could be detected after staining with Rhodamine 123. As shown in figure 1, the epithelial layer exposed to an untreated contact lens has cells that are tightly apposed to each other and show visible mitochondria (Fig. 1A-1C). Epithelial cells exposed to an AcuVue lens that was soaked overnight in 0.001% BAK have separated from each other and the Rhodamine dye is dispersed into the cytoplasm of the cell (Fig. 1D-1F). Epithelial cells exposed to an AcuVue lens that was soaked overnight in 0.01% BAK showed a significant loss of epithelial cells from the lens and wider gaps exist between cells (Fig 1G-1I). All but one lens out of thirteen negative controls for this experiment showed no damage after treatment Table 3. The lenses treated with contact lenses soaked in BAK showed progressively more damage as the BAK concentration was increased from 0.0001% to 0.01% (Table 3).

Shorter exposure times of 15 minutes and 1 hour with 24 hour recovery, caused less damage to the epithelial cells than 19 hour exposure. However, the initial stages in the loss of

mitochondrial integrity can be seen (Figure 2). The bovine lens epitheliums exposed to contact lenses soaked in 0.9% saline for 1 hour were not damaged (2A-D). Exposure to a contact lens soaked in 0.01% BAK for 15 minutes caused breaks between the cells and shortened and swollen mitochondria in one of the two lenses tested (2E-2F) The second lens was not damaged (2G, 2H). After a 1 hour exposure to a contact lens soaked in 0.01% BAK, there were breaks in between cells in one of the two tested lenses (Fig. 2I) and both lenses showed some shortened and swollen mitochondria (Fig. 2J, 2L).

Treatment of the epithelium with contact lenses soaked in ReNu MultiPlus or Optifree Express for 19 hours and 24 hour recovery caused little damage (Table 4).

4.3.1.2 AlamarBlue Bovine lens

Bovine lens epitheliums were exposed for 19 hours with 24 hour recovery to contact lenses soaked in different concentrations of BAK. The first experiment evaluated the same lenses for lens epithelial viability using alamarBlue at 24 hours post exposure and 72 hours post exposure (Figure 3). After the initial alamarBlue exposure the lenses were washed in 0.9% saline and incubated in growth medium until the second reading at 72 hours post exposure. In a second experiment, different lenses were prepared and tested separately (Figure 4) at the 72 hour timepoint to determine if previous alamarBlue exposure had an effect on the viability of the lens epithelium. These studies show that there was a reduction in the viability of the lens epithelium as BAK concentrations were increased and that previous exposure to alamarBlue did not have a substantial effect on the outcome of the test. After incubating the lenses for 7 days after

exposure there was a slight reduction in alamarBlue reduction in the control and treated lenses (Figure 5).

Additional lenses were prepared and exposed to contact lenses treated for 19 hours with different concentrations of BAK and contact lens care products. Readings were taken at the 24 hours recovery point (Figure 6) and the lenses were reincubated and read at 72 hours (Figure 7) after initial exposure. The contact lens care solutions did not show a reduction in the viability of the lens epithelium after either 1 day and 3 days of recovery. The 3 day recovery timepoint showed a greater difference between the viability of the various doses of BAK than the 1 day recovery timepoint.

4.3.2 Confocal analysis of Cornea, Human Corneal Epithelial Cells and MDCK cells for mitochondrial integrity

Corneas exposed to Optifree Express Multipurpose solution and ReNu MultiPurpose solution for 30 minutes were evaluated for effects on the mitochondria of cultured bovine corneas (Bantseev, McCanna *et al*., 2007). Treatment with Optifree Express significantly decreased ($P < 0.05$) the number of mitochondria in the superficial epithelium and the intermediate epithelium compared to Hanks' balanced salt solution (HBSS) controls. Treatment with ReNu MultiPlus also significantly decreased ($P<0.05$) the number of mitochondria, but only in the superficial epithelium, compared to HBSS controls. The corneas exposed to Optifree Express had less mitochondria at the depths of 3.74 to 7.49µm, compared to ReNu MultiPlus.

Human corneal epithelium cells exposed to BAK and various contact lens care solutions were evaluated for mitochondria integrity after various exposure times. Exposure of the human corneal epithelial cells to 0.01% BAK for one hour caused significant damage (Figure 8A). Also, there was no recovery after 24 hours (Figure 8B). After 1 hour exposure, three contact lens care products were tested. Compared to Sensitive Eyes Saline Solution, Optifree Replenish and ReNu MultiPlus showed damage to the corneal epithelial cells (Figure 9).

After 15 minutes exposure, human corneal epithelial cells exposed to Optifree Replenish did not exhibit distinct mitochondria, whereas the mitochondria in the cells exposed to ReNu MultiPlus solution were visible (Figure 10). Solocare (Fig. 11A) and Optifree Express (11B) also caused a loss of mitochondrial integrity after 15 minute of exposure; whereas Complete (11C) did not cause damage and the culture was similar to the 0.9% saline control (11D). BAK caused damage to the mitochondria after 15 minute of exposure at 0.01% (11E) but did not affect the mitochondria at the 0.001% concentration (11F).

A 5 minute exposure to Optifree Express, Optifree Replenish and BAK 0.01% still causes damage to the mitochondria of the epithelial cells (Figure 12). The solutions Complete, Solocare, ReNu MultiPlus and BAK 0.001% did not cause damage and the mitochondria looked very similar to the 0.9% saline control (not shown).

Because human tears can dilute solutions after initial instillation into the eye, an assessment was made to determine the dilution in 0.9% saline at which Optifree Express, Optifree Replenish and

BAK do not affect the mitochondrial integrity of human corneal epithelial cells (Figure 13). Optifree Express had to be diluted to a 20% solution and Optifree Replenish to a 25% solution in 0.9% saline before distinct mitochondria were visible. Even at these dilutions, degradation of some of the cells in the cell monolayer were visible. BAK had to be diluted to 0.003% before there was a marked decrease in the number of degraded mitochondria. The cells exposed to a BAK concentration of 0.001% showed mostly distinct mitochondria, although there was an occasional cell that showed loss of mitochondrial integrity.

After establishing the concentrations at which these solutions affect the mitochondria of the lens epithelial monolayer, we investigated whether contact lenses soaked in these solutions could produce similar effects. When a contact lens is placed in the eye there will be some carryover of the solution in the lens case to the eye. In addition, preservatives and other chemicals can also concentrate in the matrix of the lens and be released over time. The effects of the contact lens solution combinations after 3 hours exposure were investigated (Figure 14). The effect of Optifree Express was evaluated at 2 hours, as it had the strongest effect on the cell monolayers when evaluated as a solution (Fig. 14F). The effect of Optifree Express and Optifree Replenish-soaked contacted lenses had on the cells was similar to the effect seen using just the solution alone at shorter exposure times. The cells are damaged and the mitochondria are not easily identified. The other contact lens solutions appear to have mitochondria that are slightly degraded in comparison to the 0.9% saline soaked contact lens control. However, the mitochondria are clearly visible (Fig. 14B-14E). Damage to the human corneal epithelial cells can be seen with lenses soaked in 0.001% and 0.01% BAK (Fig. 14H, 14I).

4.3.3 Confocal evaluation MDCK cells

Madin-Darby Canine kidney cells were evaluated to see if they also could be used to assess toxicity of chemicals. After 15 minute of exposure cultures exposed to 0.9% Saline and BAK were evaluated. Cells were exposed to BAK 0.001%, BAK 0.01%, and 0.9% Saline (Figure 15). Both BAK concentrations disrupted the mitochondrial integrity of the cells. The mitochondria of one of the cells exposed to the 0.001% BAK concentration have round shapes that are very different than mitochondria in the neighboring cells.

4.3.4 AlamarBlue test of human Corneal Epithelial cells.

Monolayers of human corneal epithelial cells were evaluated for viability after 15 minutes exposure to ReNu MultiPlus, Solocare, Complete, Aquify, and Optifree Express (McCanna *et al*., 2008). Optifree Express-treated cultures showed significantly reduced alamarBlue activity when compared to the HBSS control and all other tested lens care products $p < 0.05$. (Figure 16)

HCEC and MDCK trypsinized cells were exposed to contact lens care products for 1 hour. Optifree Express and Optifree Replenish showed reduced alamarBlue activity when compared to HBSS and the other lens care solutions (Figure 17, Figure 18).

Tables and Figures

Table 1

List of average mitochondrial (MT) length (μm) ± SEM and Relative Rhodamine 123 Fluorescence in Epithelial of Bovine Lenses Treated with Different SDS Concentrations 24 h Postexposure and Controls

Measurement	SDS treatment (%) 0.0125 (n = 8)	0.00625 (n = 8)	Control (n =8)
Average MT length in the central epi	2.95 ± 0.72**	7.19 ± 0.29	7.01 ± 0.41
A verage MT length in the intermediate epi	3.98 ± 0.31**	7.37 ± 0.41	7.03 ± 0.24
Average MT length in the epi, at equator	4.55 ± 0.47**	7.49 ± 0.44	7.04 ± 0.25
The ΔΨ, in the centrol epi ± SEM	12,816 ± 1657**	35,485 ± 665**	40,216 ± 766
The ΔΨ, in the intermediate epi ± SEM	11,487 ± 601**	26,978 ± 381**	40,072 ± 1046
The ΔΨ, in the epi at equator ± SEM	14,168 ± 1131**	28,182 ± 1374**	40,976 ± 1457

Note. Relative Rhodamine 123 fluorescence indicates changes in mitochondiral electron chain potential ΔΨ. ** indicates significantly lower ΔΨ, at $p < 0.0001$ as compared to controls.

Table 2

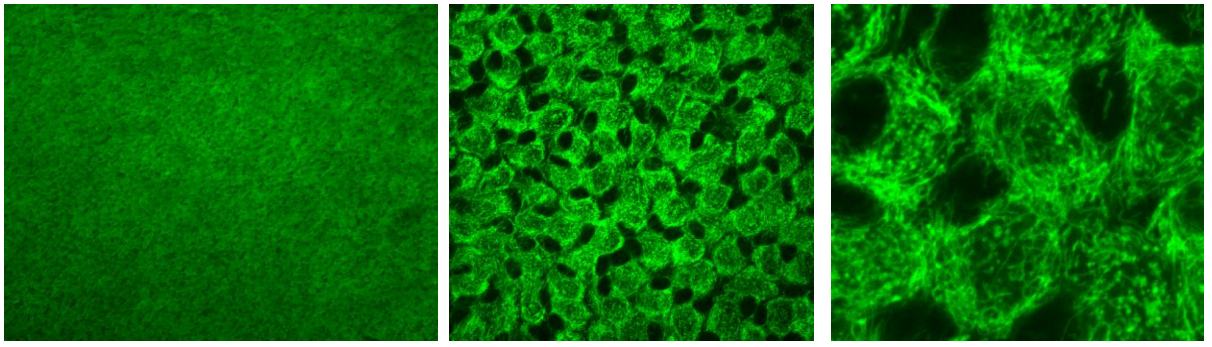
List of the Average Mitochondrial (MT) Length (μm) ± SEM and Relative Rhodamine 123 Fluorescence in Epithelial and Superficial Cortical Fiber Cells and the Depth Below Which the Mitochondria Free Zone Starts (MFZ below a depth, μm) in Bovine Lenses Treated with Different SDS Concentrations 24 h Postexposure as Compared to Controls

SDS treatment (%)

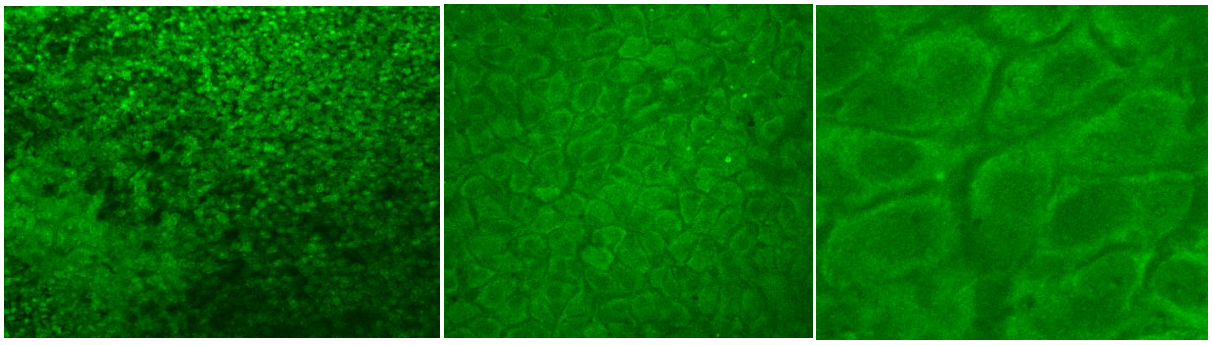
Measurement	0.0125 (n = 8)	0.00625 (n = 8)	Control (n =8)
Average MT length in the central fib	Unable to measure	19.55 ± 1.41	18.37 ± 1.22
Average MT length in the fib at equator	17.42 ± 2.30**	23.00 ± 1.78 **	41.16 ± 2.24
Average MT length in the posterior fib	Unable to measure	Unable to measure	17.22 ± 1.22
The $\Delta\Psi$, in the fib at the equator ± SEM	151,369 ± 24,424 **	292,836 ± 42,774*	446.390 ± 59,100
The $\Delta\Psi$, in the anterior fib. ± SEM	Unable to measure	255,575 ± 22,786*	361,329 ± 16,115
Anterior MFZ ± SEM	Unable to measure	142.50 ± 5.70	153.90 ± 17.10
MFZ at equator ± SEM	71.35 ± 2.95**	183.10 ± 34.90	205.20

Note. Relative Rhodamine 123 fluorescence indicates changes in mitochondiral electron chain potential $\Delta\Psi$. * indicates significantly lower $\Delta\Psi$, at $p < 0.05$ as compared to controls.* *indicates significantly lower $\Delta\Psi$, at $p < 0.0001$ as compared to controls.

Table 1 and Table 2. Reprinted with Permission Bantseev, McCanna *et al. Toxicological Sciences.* 2003.



1A 1B 1C



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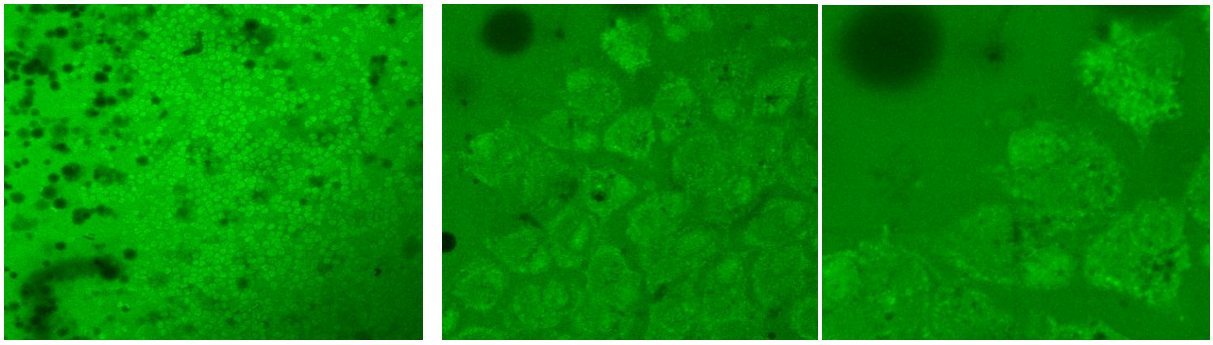
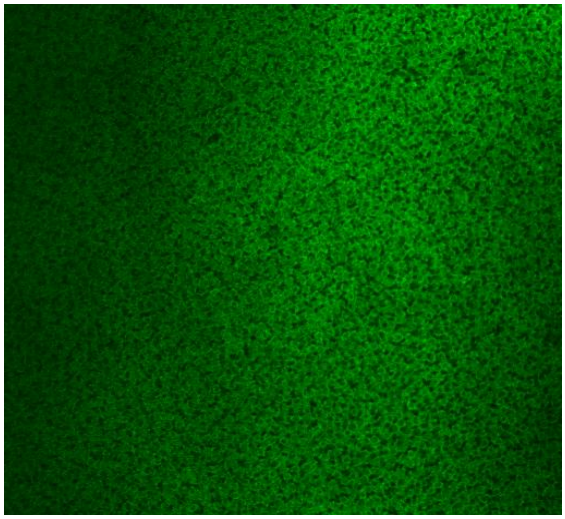


1G 1H 1I

Figure 1: Bovine lens epithelium after a 19 hour exposure and a 24 hour recovery. Untreated contact lens treated cells are tightly apposed to each other and show visible mitochondria (Fig. 1A-1C). Exposure to an AcuVue II lens that was soaked overnight in 0.001% BAK (Fig 1D-1F). Cells have separated from each other and the Rhodamine dye is dispersed into the cytoplasm of the cells. Exposed to an AcuVue II lens soaked overnight in 0.01% BAK showed a significant loss of epithelial cells from the lens and show wide gaps between cells (Fig 1G-1I).

Table 3: Bovine lens epithelium after 19 hours exposure and 24 hour recovery to AcuVue II contact lenses soaked in various solutions. Healthy bovine lens epithelium/number of lenses tested.

	Contact Lens Treated	Saline Control No Treatment	Sensitive Eyes Saline treated contact lens	0.9% Saline Treated Contact Lenses	Negative Control Total	0.0001% BAK Treated Contact Lenses	0.0005% BAK Treated Contact Lenses	0.001% BAK Treated Contact Lenses	0.01% BAK Treated Contact Lenses
Cells tightly apposed to each other	4/4	2/2	2/2	4/5	12/13	2/3	2/3	1/6	0/3
Mito-chondria present	4/4	2/2	2/2	5/5	13/13	3/3	2/3	2/6	0/3



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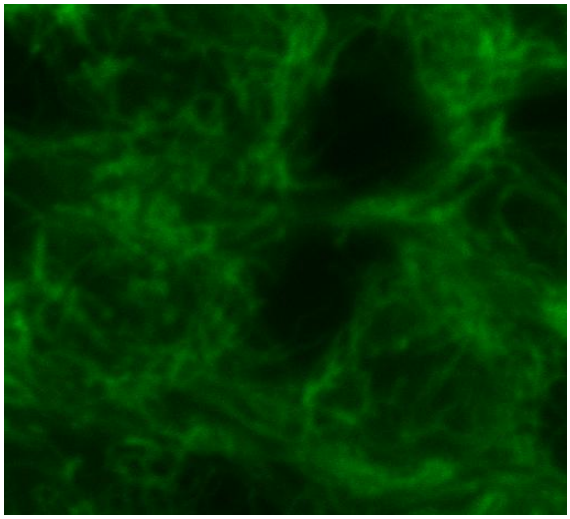

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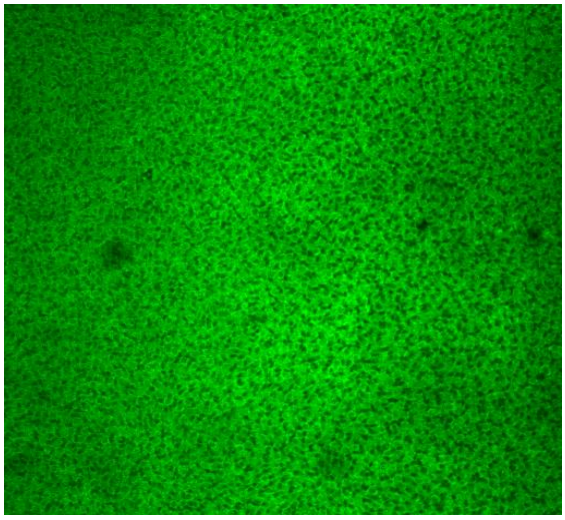

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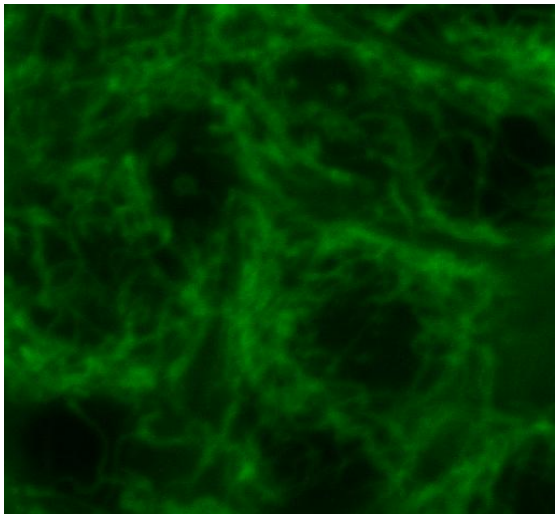
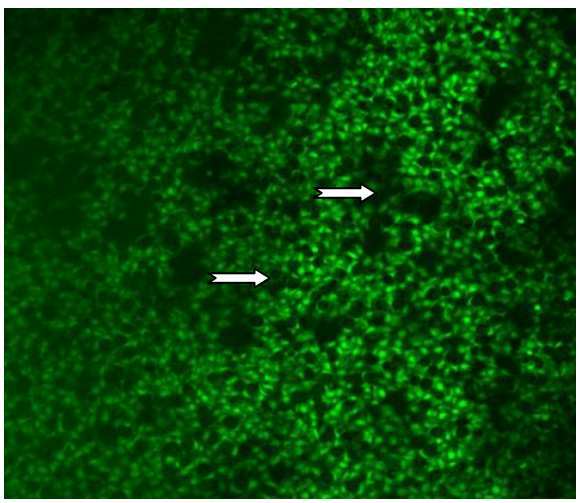
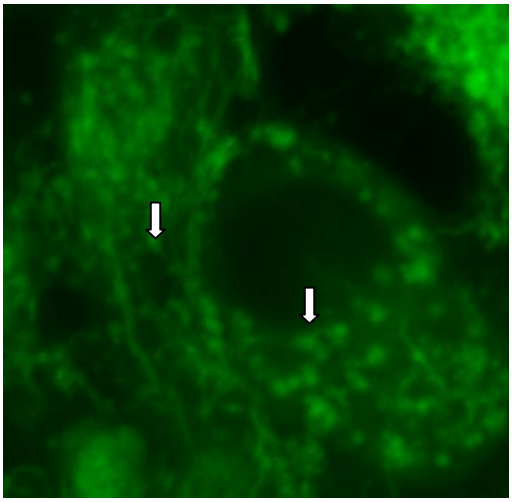


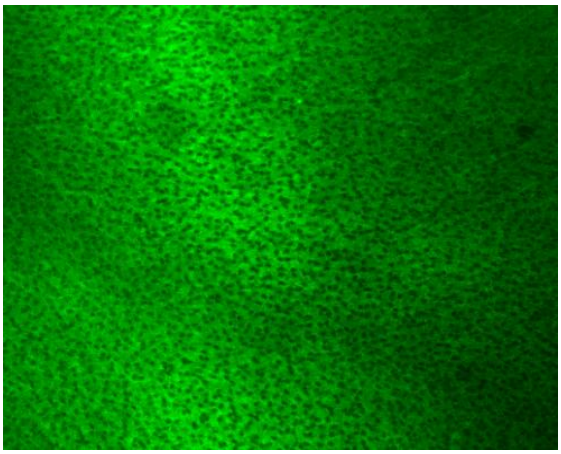
2D

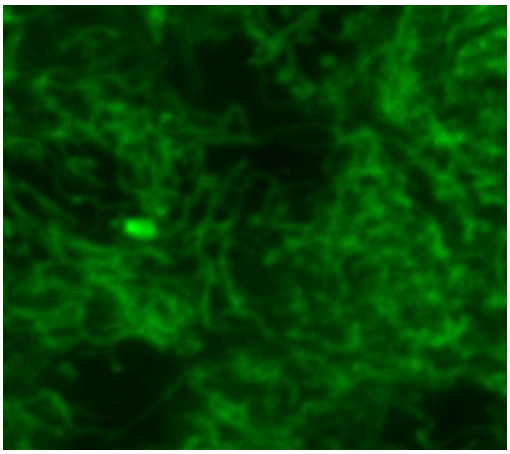
Figure 2 (A-D): Bovine lens epithelium after 1 hour exposure with 24 hour recovery to AcuVue II contact lenses soaked in 0.9% saline. The bovine epitheliums exposed to contact lenses soaked in 0.9% saline for 1 hour were not damaged (2A-2D).





2E 2F

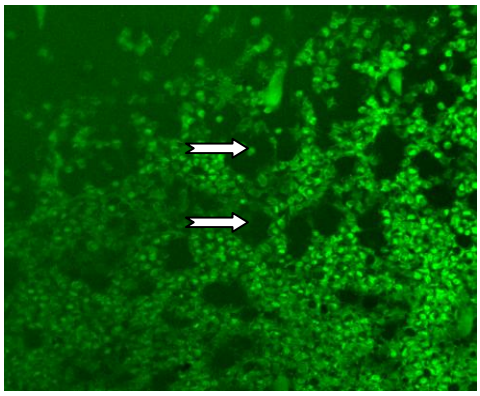




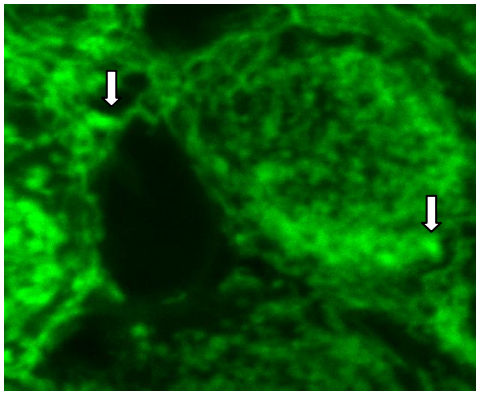
2G 2H

Breaks = ⟹ Swollen mitochondria = ⇩

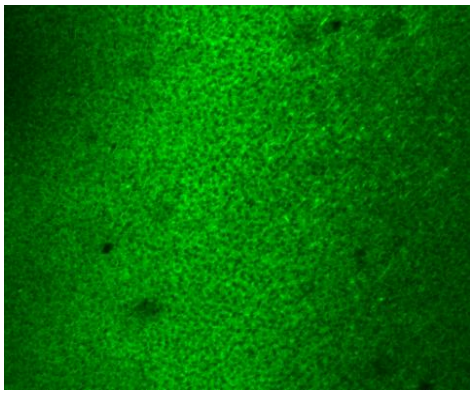
Figure 2 (E-H): Bovine lens epithelium after 15 minute with 24 hour recovery to AcuVue II contact lenses soaked in 0.01% BAK. Exposure to a contact lens soaked in 0.01% BAK for 15 minutes caused breaks in between the cells and shortened and swollen mitochondria in one of the two lenses tested (2E, 2F) The second lens was not damaged (2G, 2H).



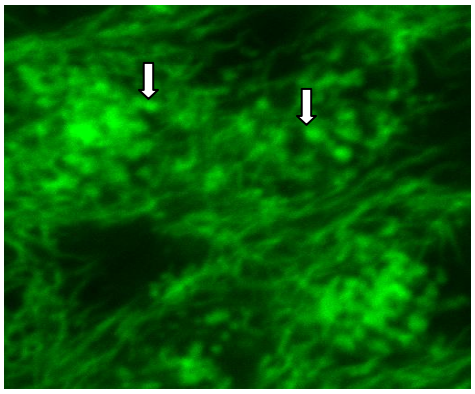
2I



2 J



2K



2L

Breaks = ⟹ Swollen mitochondria = ⇩

Figure 2 (I-L): Bovine lens epithelium after 1 hour exposure with 24 hour recovery to AcuVue II contact lenses soaked in 0.01% BAK. After a 1 hour exposure to a contact lens soaked in 0.01% BAK there were breaks in between cells in one of the two tested lenses (Fig. 2I). The other epithelium there was no breaks between cells (Fig. 2K). Both epitheliums exposed a contact lens soaked in 0.01% BAK showed some shortened and swollen mitochondria (Fig. 2J, 2L).

Table 4: Bovine lens epithelium after 19 hours exposure and 24 hour recovery to AcuVue II contact lenses soaked in various solutions. Healthy Bovine Lens Epithelium/Number of Lenses tested.

	ReNu Multiplus	Optifree Express	Negative Control Total	0.001% BAK Treated Contact Lenses	0.01% BAK Treated Contact Lenses
Cells tightly apposed to each other	4/5	3/3	12/13	1/6	0/3
Mitochondria present	4/5	3/3	13/13	2/6	0/3

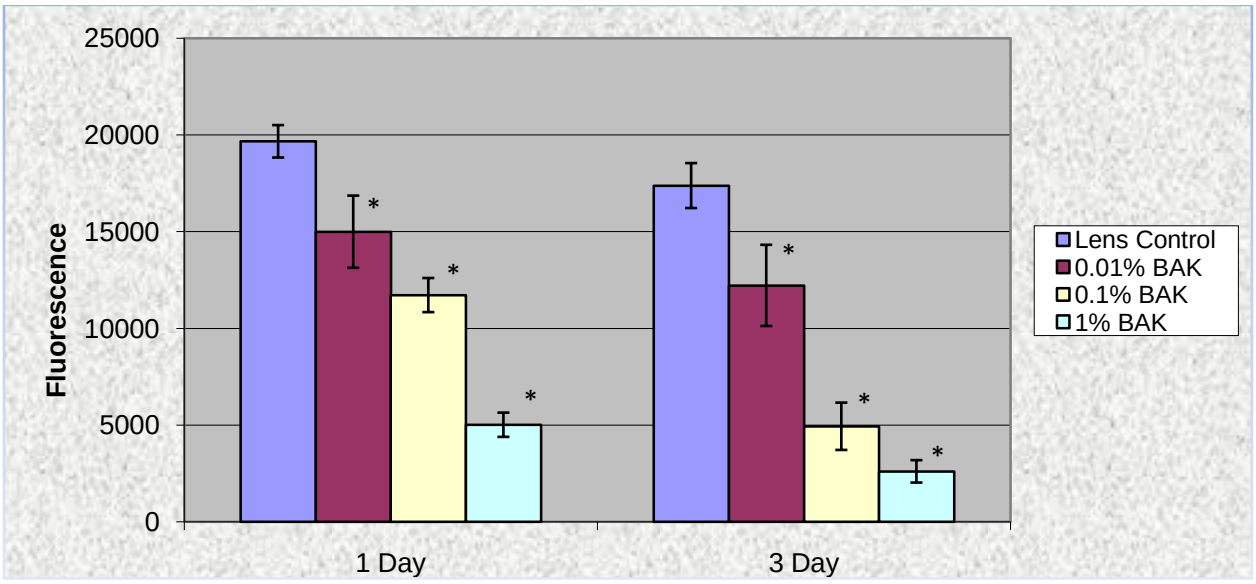


Figure 3: AlamarBlue reduction. Exposure of lens epithelium to AcuVue II contact lenses soaked in various concentrations of BAK for 19 hours with 1 and 3 day recovery. The same lenses were used for both timepoints. * indicates significant differences as compared preceding dose ($p < 0.05$)

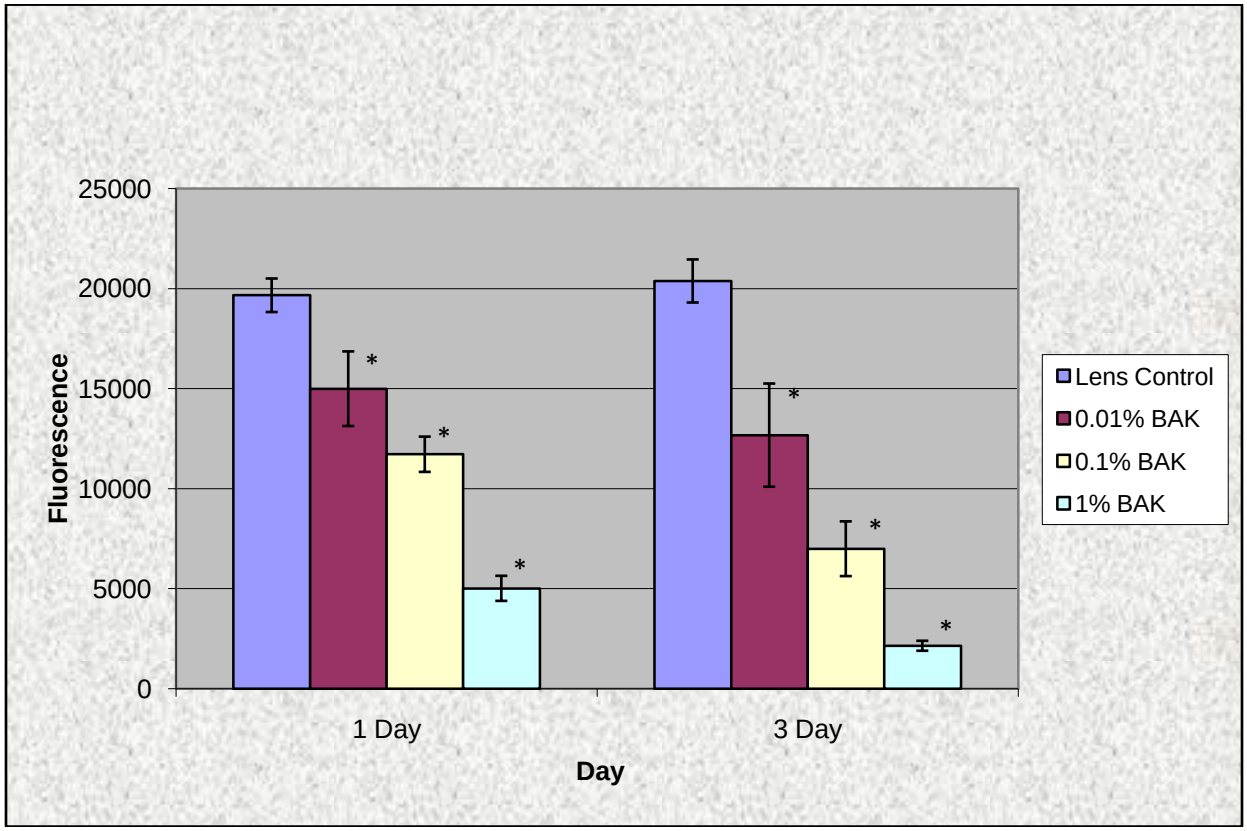


Figure 4: AlamarBlue reduction. Exposure of lens epithelium to AcuVue II contact lenses soaked in various concentrations of BAK for 19 hours with 1 and 3 day recovery. Different lenses were used for each timepoint for comparison with the study where the same lens was used for each recovery timepoint (figure 3). This was done to see if previous alamarBlue exposure could effect the recovery of the lenses from exposure to BAK. * indicates significant differences as compared preceding dose ($p < 0.05$)

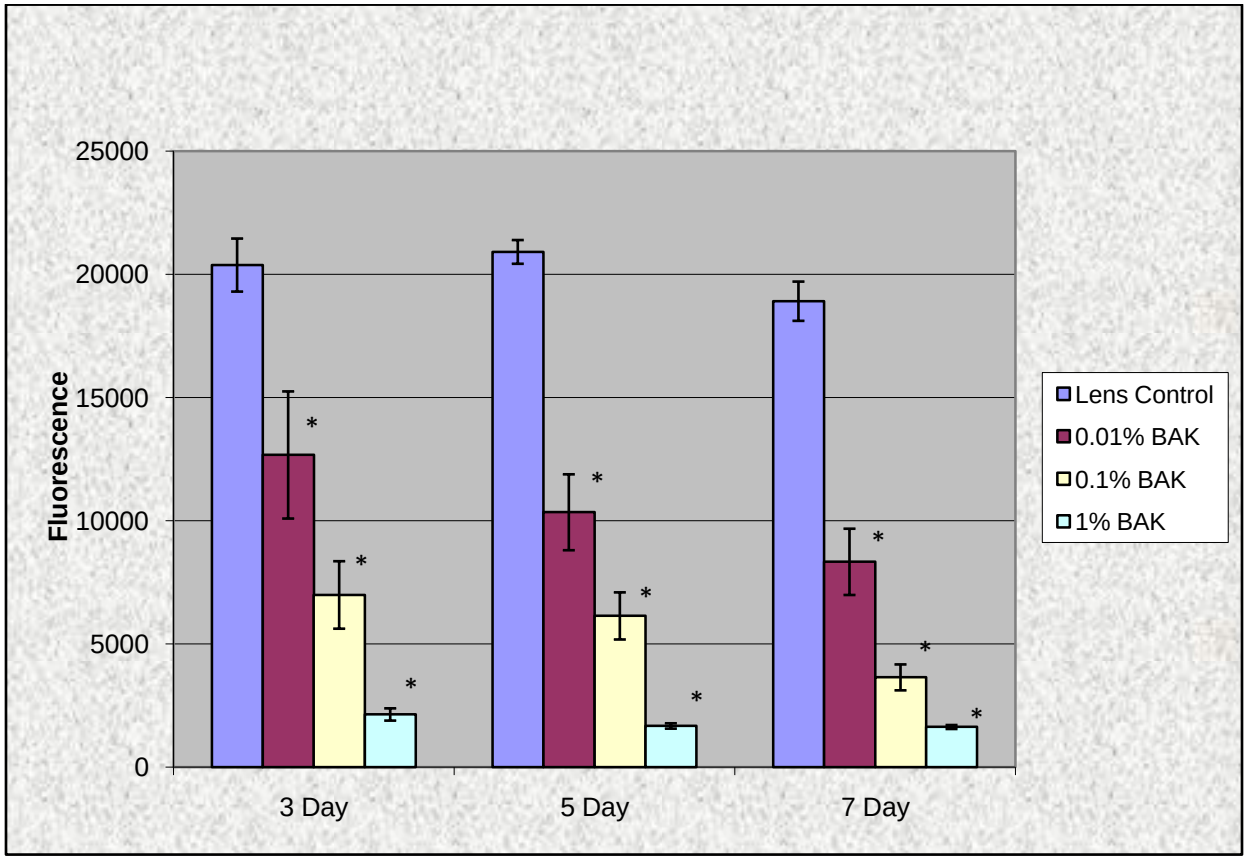


Figure 5: AlamarBlue reduction. Exposure of lens epithelium to AcuVue II contact lenses soaked in various concentrations of BAK for 19 hours with 3, 5 and 7 day recovery. The same lenses were used for each timepoint. * indicates significant differences as compared preceding dose ($p < 0.05$).

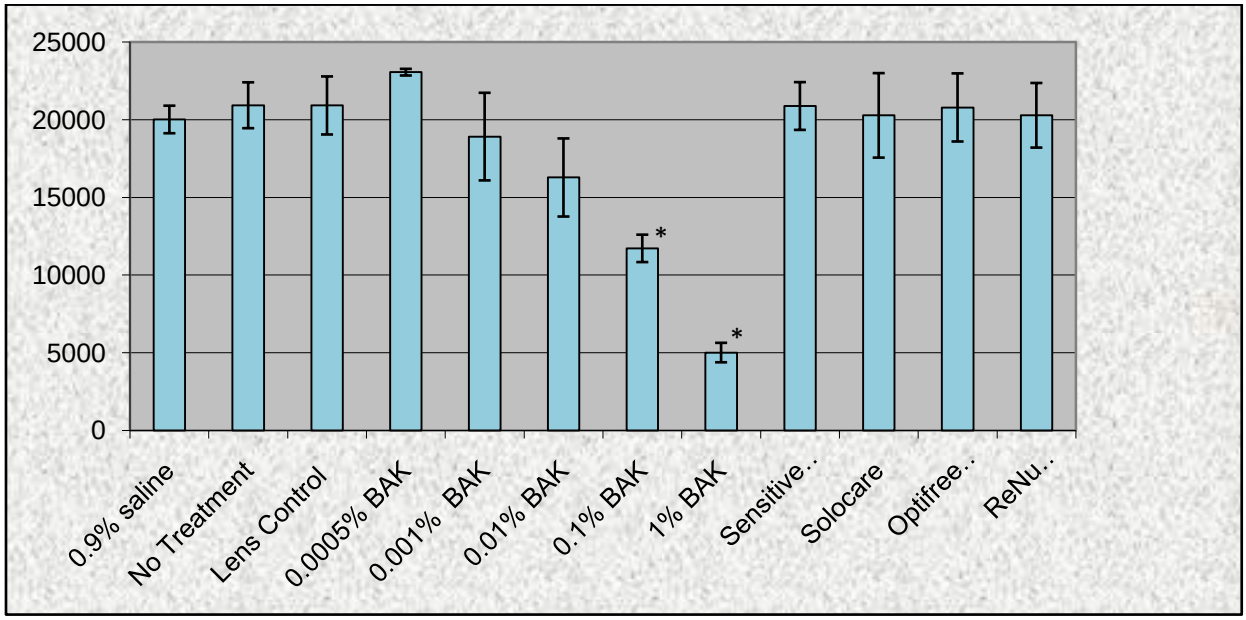


Figure 6: AlamarBlue reduction. Exposure of lens epithelium to AcuVue II contact lenses soaked in various solutions for 19 hours, after 1 day of recovery. * indicates significant differences as compared preceding dose ($p < 0.05$).

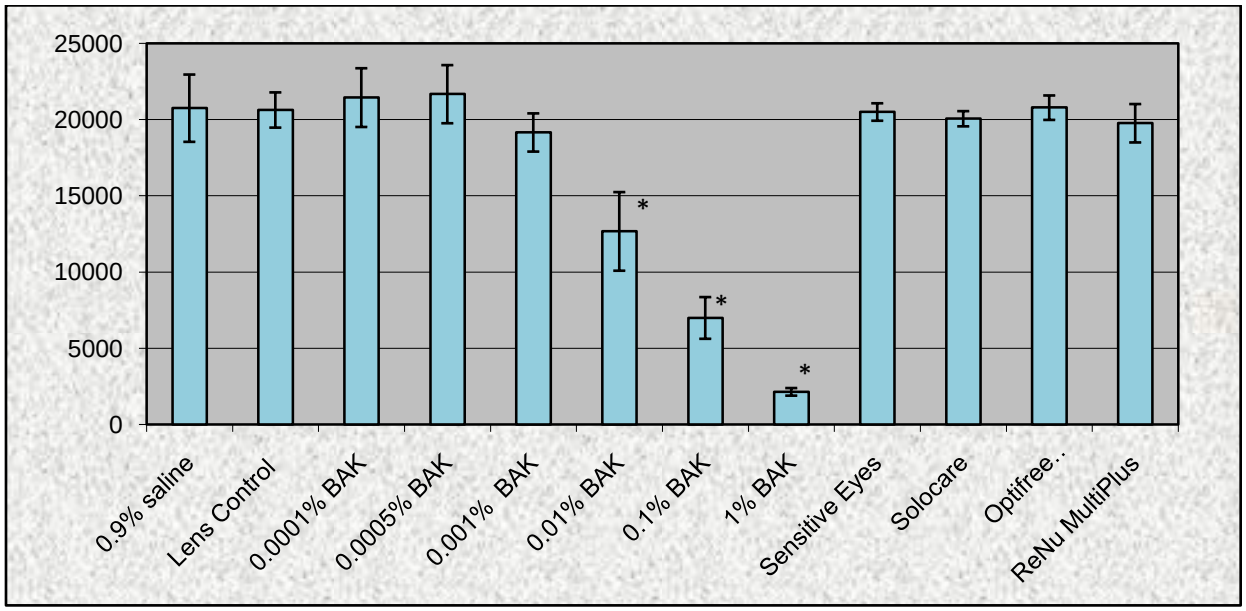
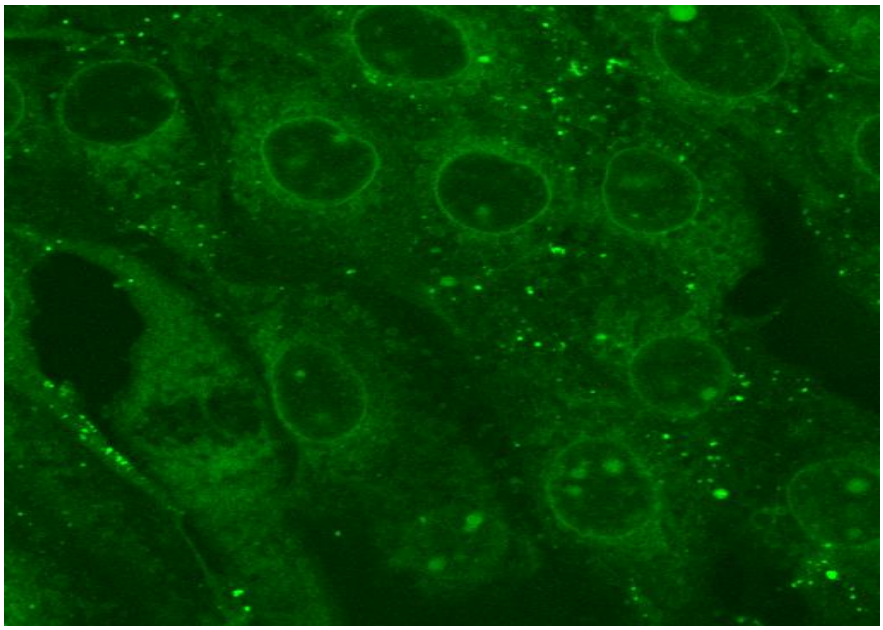
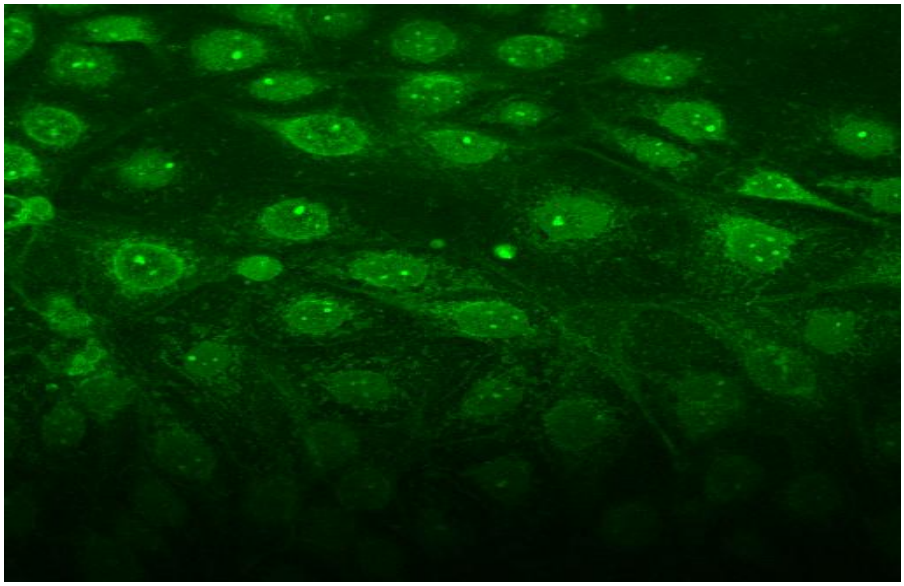


Figure 7: AlamarBlue reduction. Exposure of lens epithelium to AcuVue II contact lenses soaked in various solutions for 19 hours after, 3 days of recovery. * indicates significant differences as compared preceding dose ($p < 0.05$)

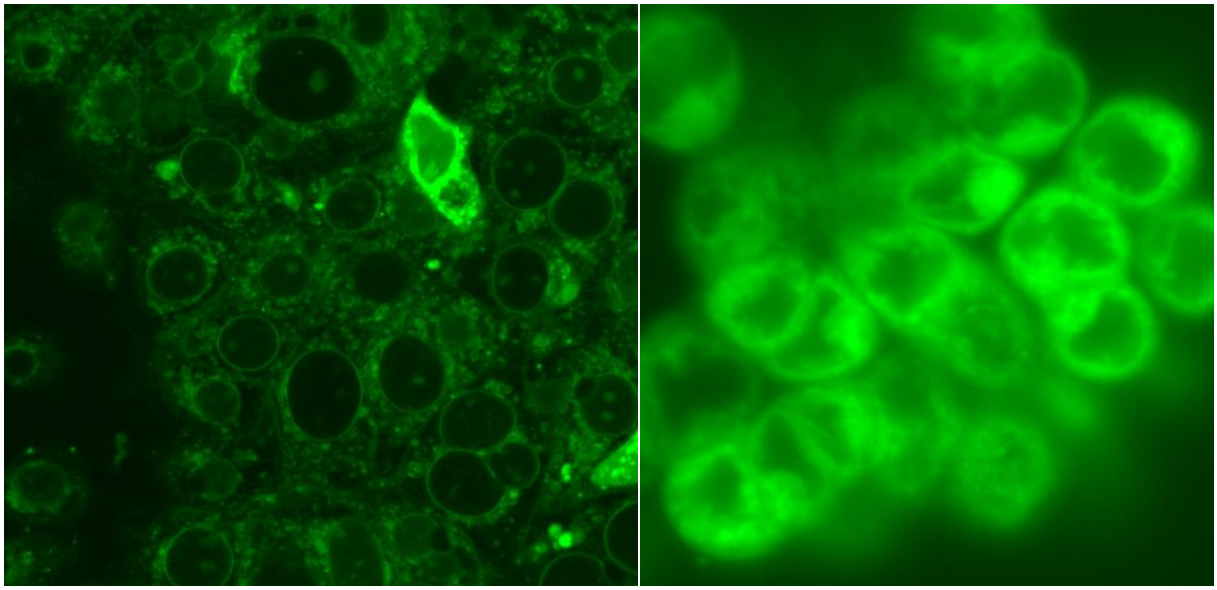


8A

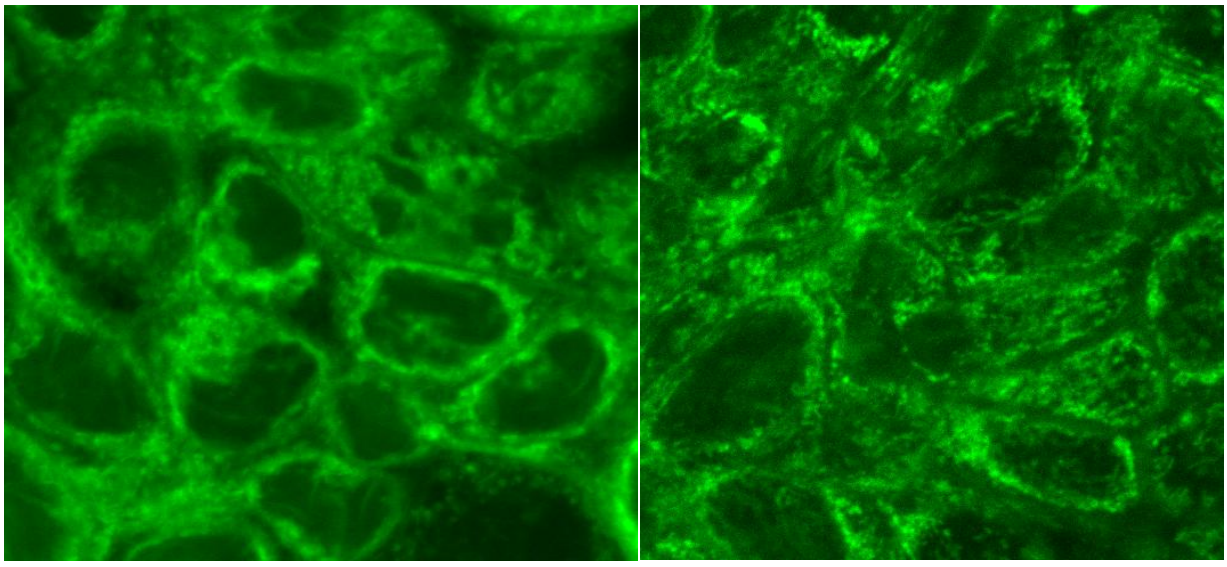


8B

Figure 8: Human corneal epithelial cells exposed to 0.01% BAK after 1 hour exposure (8A) and 24 hours later after recovery (8B) in culture media.

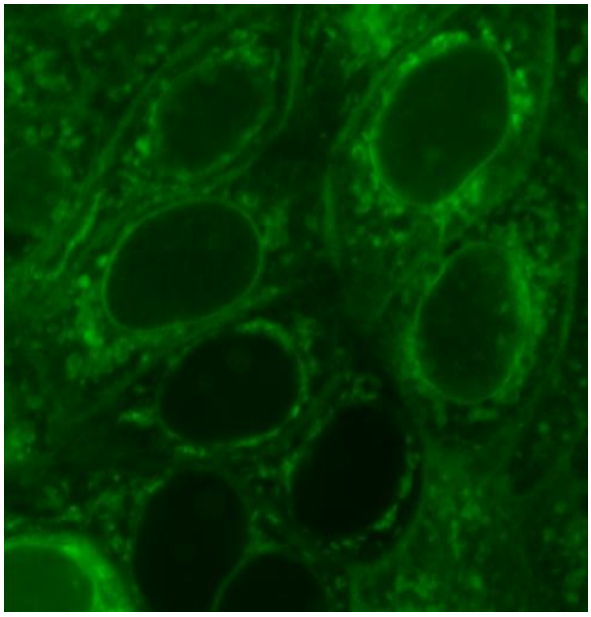


9A 9B

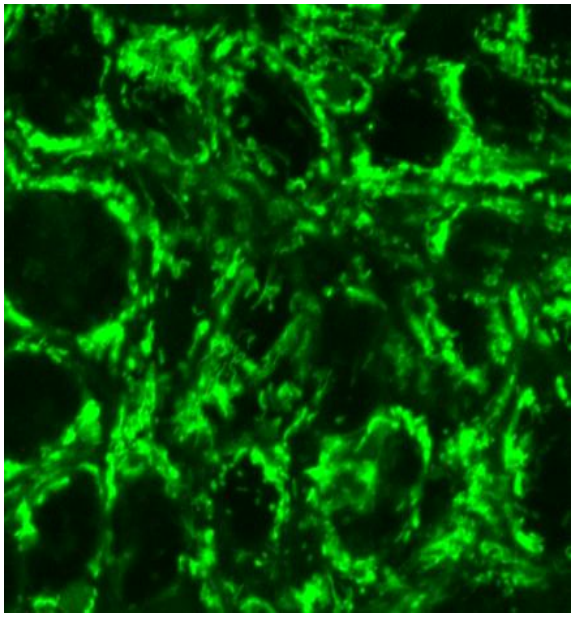


9C 9D

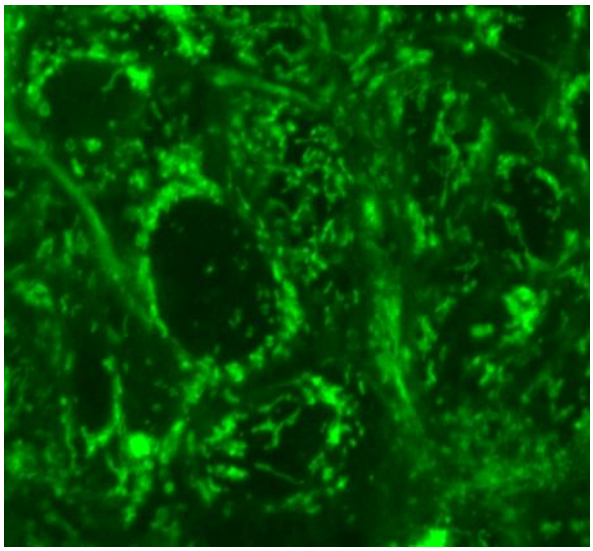
Figure 9: Human corneal epithelial cells exposed to Optifree Replenish (A), ReNu MultiPlus (B) and Sensitive Eyes Saline Solution (C), for 1 hour. The images of Optifree Replenish and ReNu MultiPlus exposed epithelial cells showed damage to the corneal epithelial cells. Untreated control (D) (Figure 9).



10A

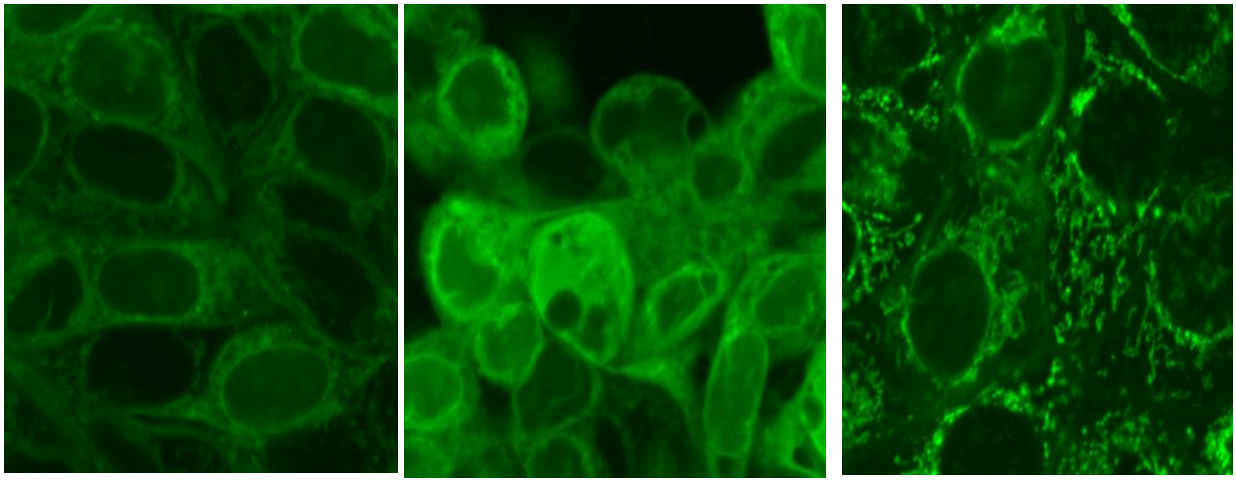


10B

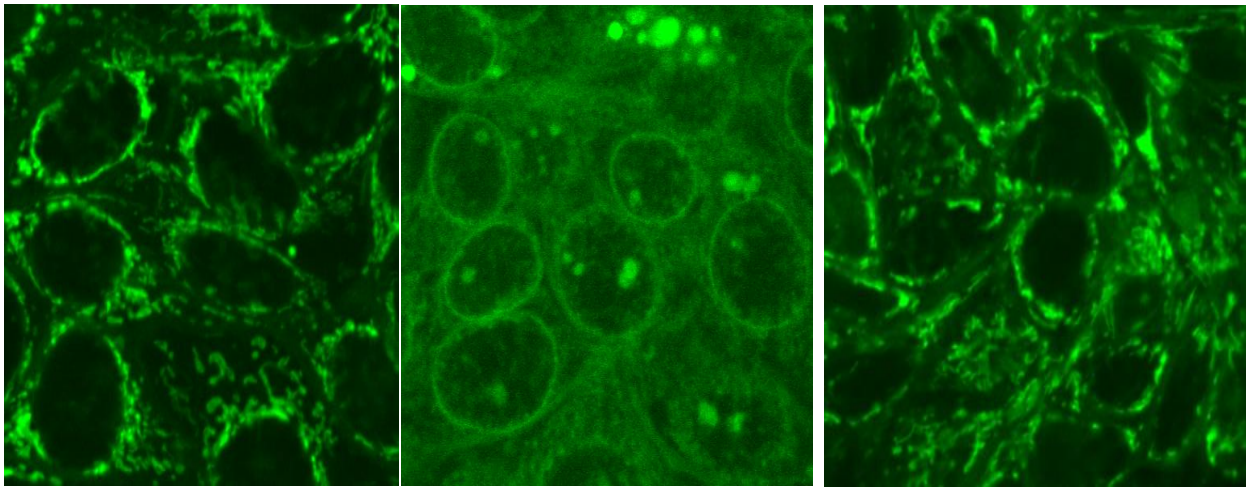


10C

Figure 10: Human corneal epithelial cells exposed to Optifree Replenish (A), ReNu MultiPlus (B) and Sensitive Eyes Saline Solution (C), for 15 minutes . Cells to Optifree Replenish did not exhibit distinquishable mitochondria whereas cells exposed to ReNu MultiPlus and Sensitive eyes saline maintained their mitochondrial integrity.

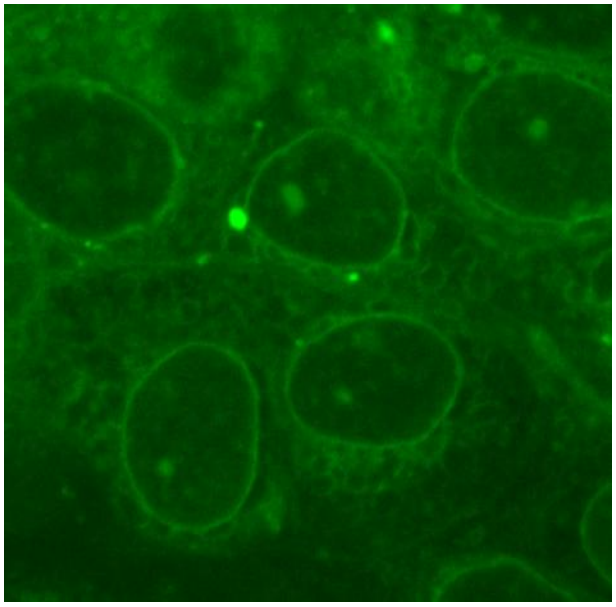


11A 11B 11C

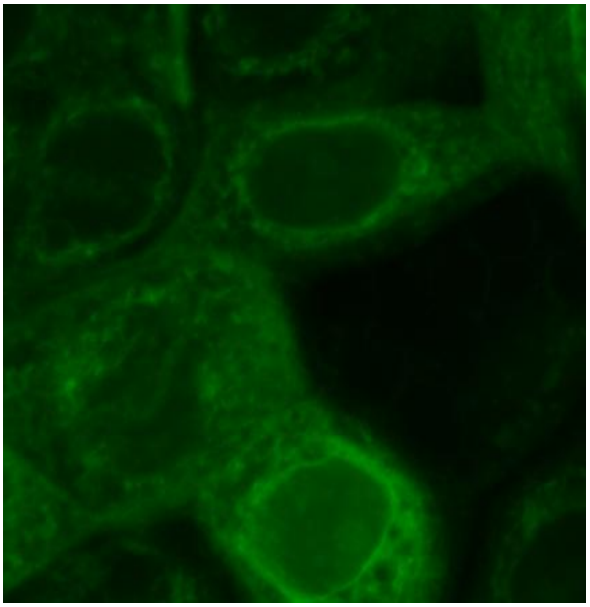


11D 11E 11F

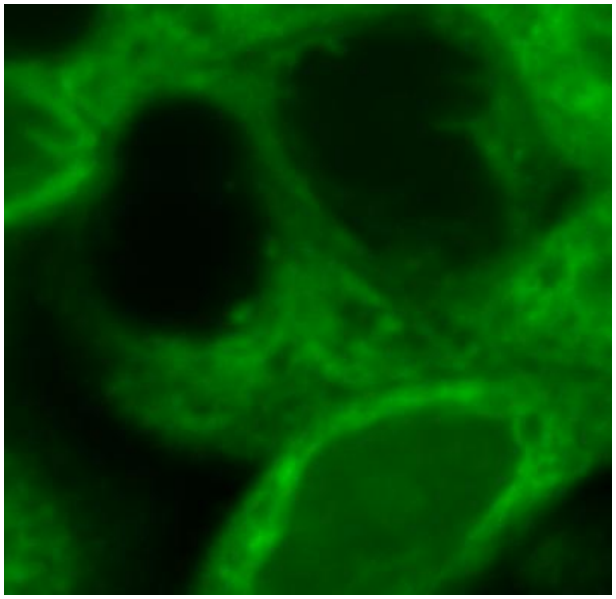
Figure 11: Human corneal epithelial cells exposed for 15 minutes to Solocare (Fig. 11A), Optifree Express (11B), Complete (11C), 0.9% saline control (11D), BAK 0.01%(11E) and 0.001% BAK concentration (11F). Solocare, Optifree Express and BAK 0.01% treated cells have lost mitochondrial integrity whereas the mitochondria of cells treated with Complete and 0.001% BAK were similar cells treated with 0.9% saline control (11D).



12 A

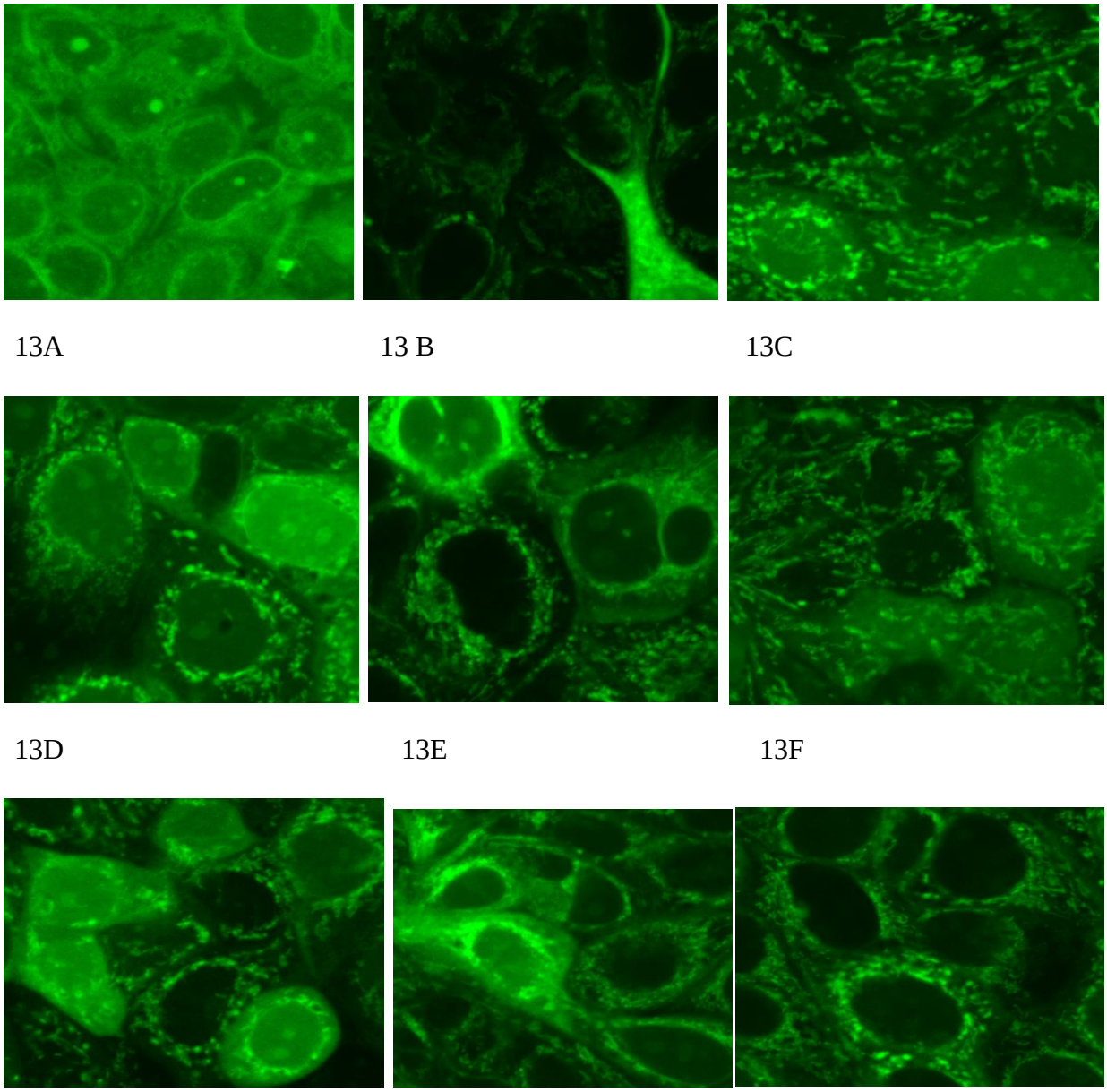


12B



12C

Figure 12: Human corneal epithelial cells exposed for 5 minutes to 0.01% BAK (12A), Optifree Express (12B), and Optifree Replenish (12C), caused a loss of mitochondrial integrety. The solutions Complete, Solocare, ReNu MultiPlus and BAK 0.001% did not cause damage and the mitochondria looked very similar to the 0.9% saline treated control (not shown).

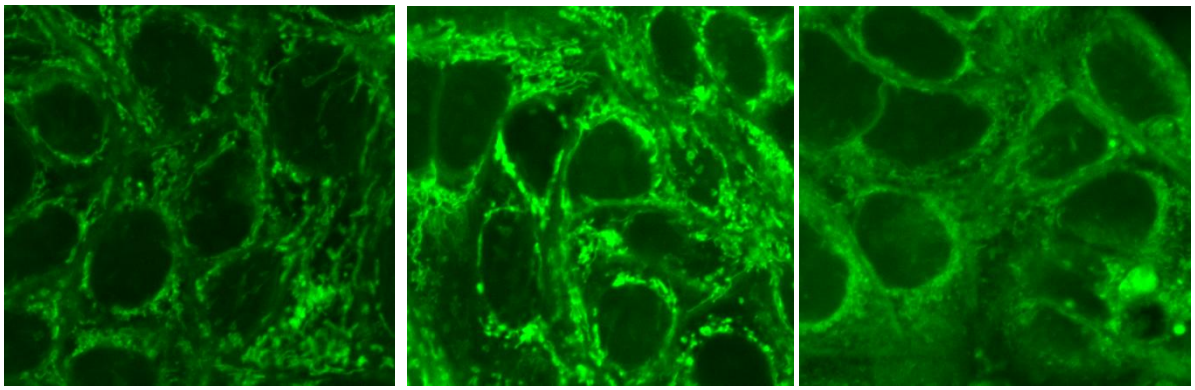


13A 13 B 13C

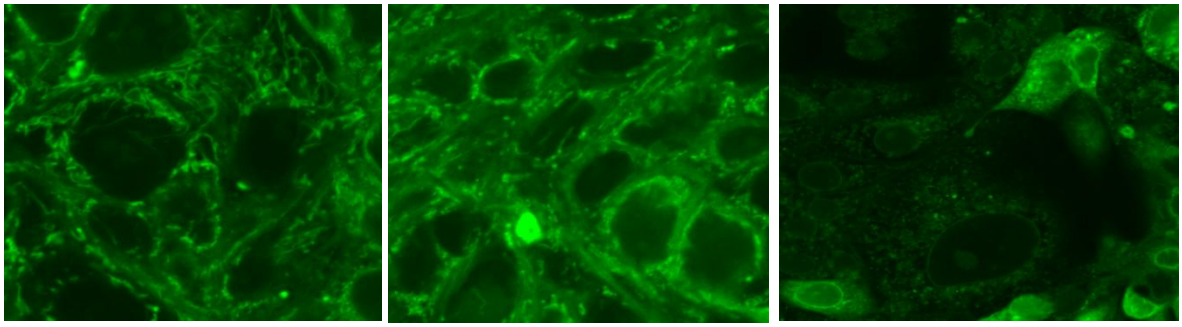
13D 13E 13F

13G 13H 13I

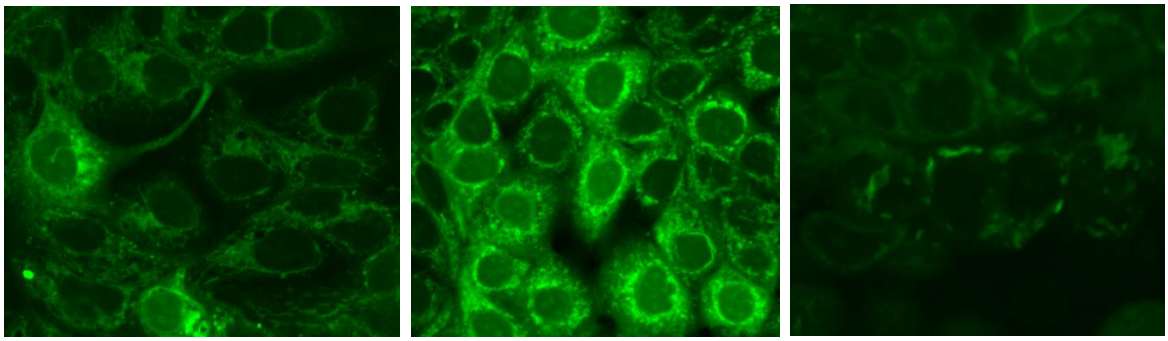
Figure 13: Human corneal epithelial cells exposed for 5 minutes to various dilutions of solutions in 0.9% saline. 25% dilution Optifree Express (13A), 20% dilution of Optifree Express (13B), 10% dilution of Optifree Express(13C), 25% dilution of Optifree Replenish (13D), 20% dilution of Optifree Replenish (13E), 10% dilution of Optifree Replenish(13F), BAK 0.003%(13G) , BAK 0.002% (13H), BAK 0.001% (13I).



14A 14B 14C

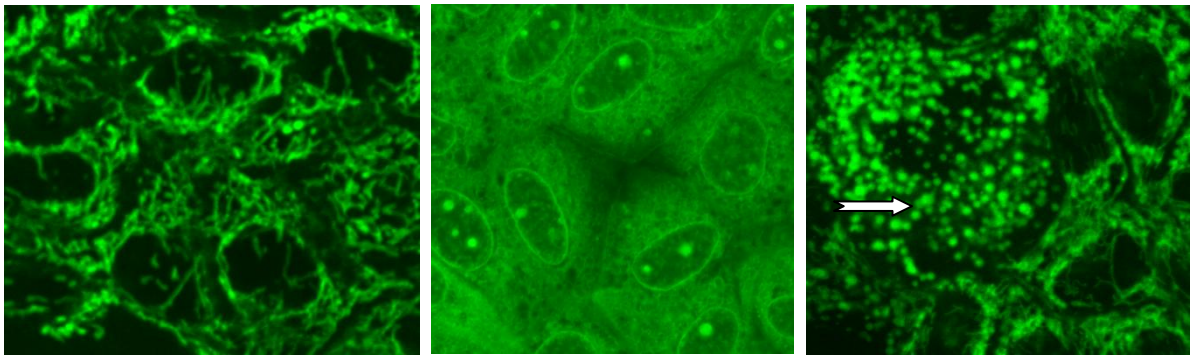


14D 14E 14F



14G 14H 14I

Figure 14: Human corneal epithelial cells exposed to AcuVue II contact lenses soaked in various contact lens care solutions for 3 hours (Optifree Express 2 hours). AcuVue lenses soaked in 0.9% saline control (14A), Sensitive Eyes Saline (14B), Solocare (14C), ReNu MultiPlus (14D), Complete (14E), Optifree Express (14F), Optifree Replenish (14G), BAK 0.001% (14H), BAK 0.01% (14I). Optifree Express and Optifree Replenish treated human corneal epethelial cells showed less mitochondireal integrity than the cells exposed to the other contact lens care solutions.



15A 15B 15C

Figure 15: Madin-Darby Canine kidney cells exposed to BAK 0.001% (15C) and 0.01% (15B) for 15 minutes. Both BAK concentrations disrupted the mitochondria integrity of the cells. The mitochondria of one of the cells exposed to the 0.001% BAK concentration had a round shape that is very different than the neighboring mitochondria. Cells exposed to 0.9% saline are shown (15A).

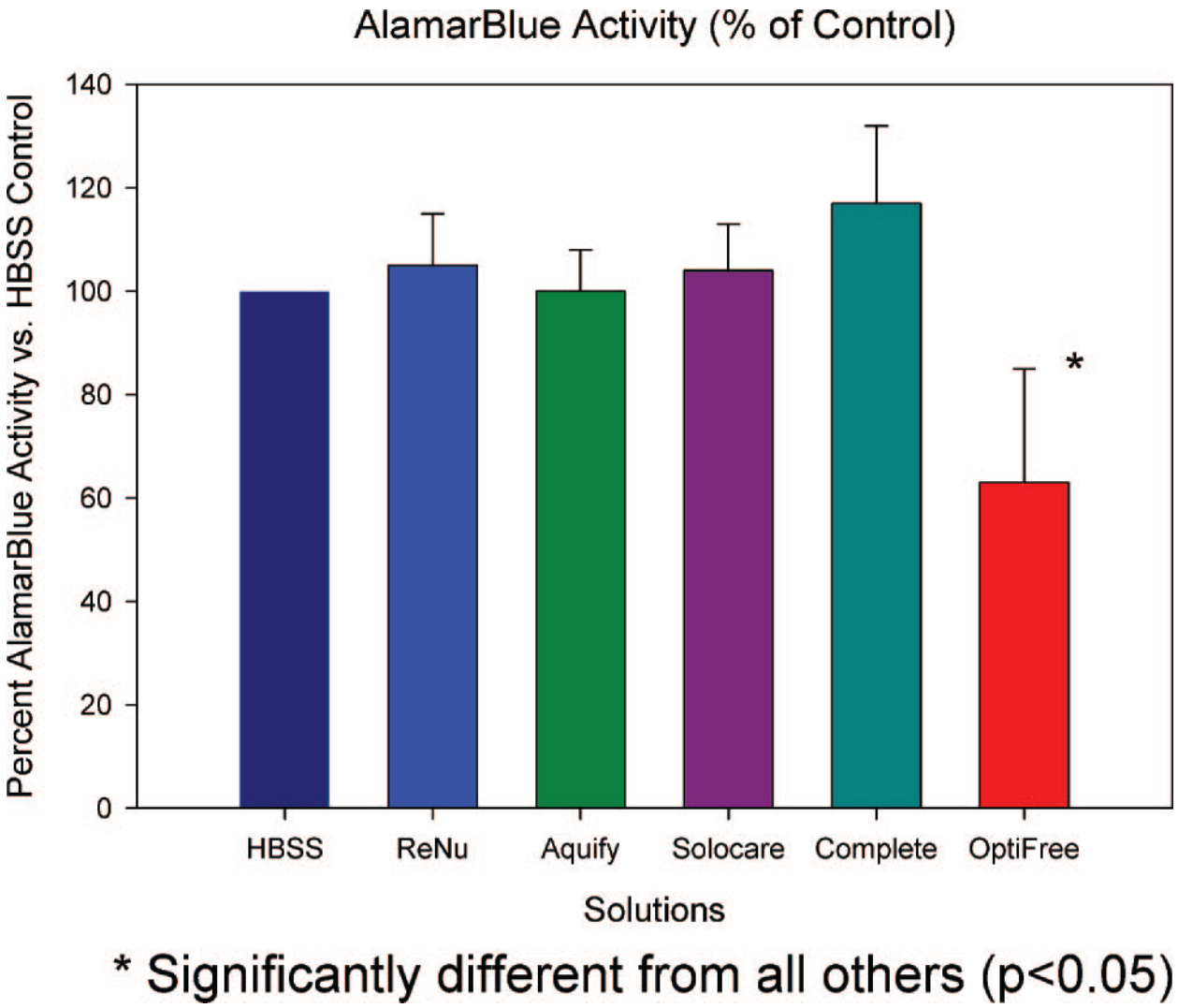


Figure 16. The results of alamarBlue assay after treating the cultures with various solutions. The results represent three separate experiments and are expressed as a percentage of the Hank's balanced salt solution activity. A reduced percentage, as seen with the OPTI-FREE Express–treated cultures, represents a toxic effect on cells.

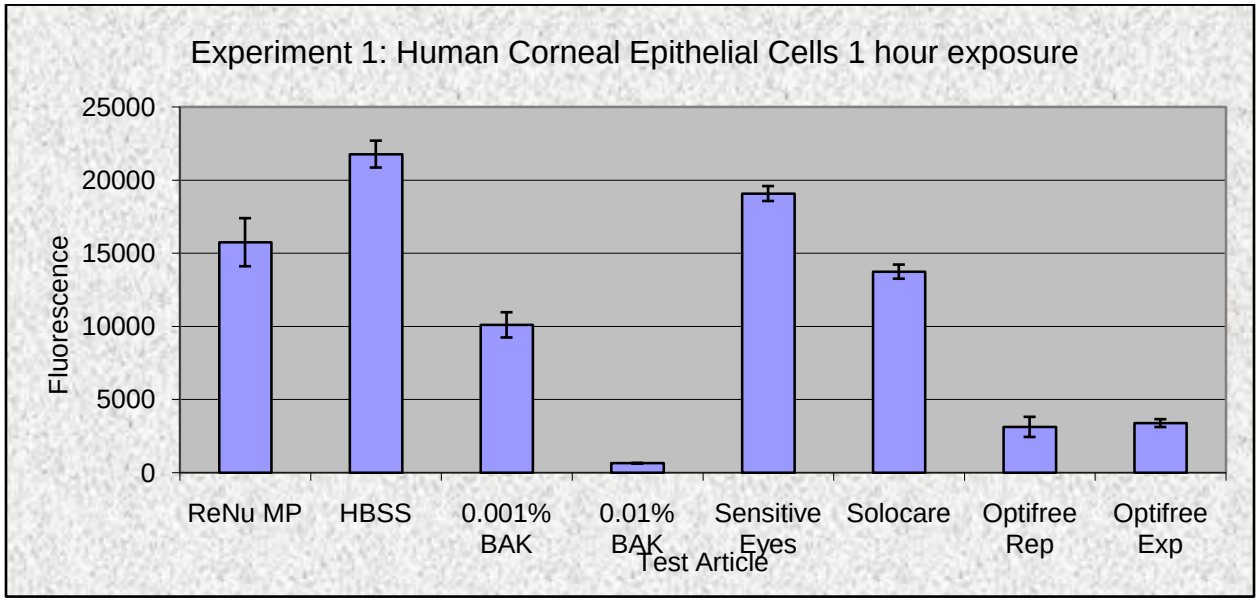


Figure 17. HCEC trypsinized cells were exposed to contact lens care products for 1 hour and evaluated for viability after 24 hour recovery. Optifree Express and Optifree Replenish showed reduced alamarBlue activity when compared to HBSS and the other lens care solutions.

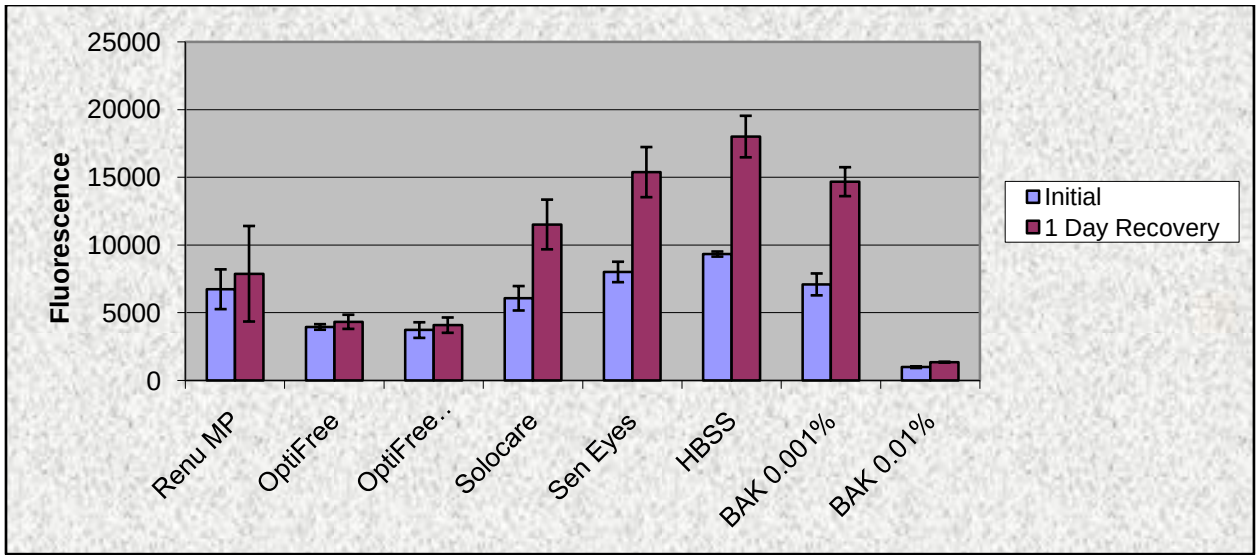


Figure 18. . MDCK trypsinized cells were exposed to contact lens care products for 1 hour and were assessed for viability immediately after exposure and after 24 hour recovery. Optifree Express and Optifree Replenish showed reduced alamarBlue activity when compared to HBSS and the other lens care solutions.

4.4 Discussion

4.4.1 BAK Toxicity

Sensitive measures for determining toxic effects in humans have shown that benzalkonium chloride that can cause toxic effects at concentration ≥ 0.005% (Baudouin *et al*., 1999; Malvitte *et al*., 2007; Takeshi, 2003; Gobbels and Spitznas, 1992). Animal studies that investigated the ocular toxicty of BAK using sensitive measurements of assessing damage also determined that BAK can be toxic to the eye at concentration ≥ 0.005% (Pfister and Burnstein 1976; Burnstein, 1980; Kovoor *et al*., 2004; Ly *et al*., 2006; Ichijima *et al*., 1992; Imayasu *et al*., 1994). The confirmation of the ocular toxicity threshold for BAK in humans and in animal makes BAK an ideal chemical for investigating the accuracy of *in vitro* toxicity models.

Many molecular techniques have been proposed for assessing toxicity at the molecular level. Debbasch *et al*. (2001) investigated the toxicity of quaternary ammonium compounds by evaluating cell membrane integrity of human conjunctival cells using the neutral red test, mitochondrial activity using flow cytometry with Rhodamine 123, DNA condensation test, and measuring for the production of reactive oxygen species after 15 minute exposure and at 24 hours of recovery. After 15 minutes of treatment BAK caused a significant change in membrane integrity and mitochondrial activity at the 0.005% level. Production of reactive oxygen species was detected as low as 0.00001% and chromatin condensation, which measures apoptosis, was detected as low as 0.001%. After 24 hours of recovery, membrane integrity decreased in the 0.001% treatment group and chromatin condensation did not show signs of recovery. De Saint

Jean *et al*. (2002) exposed human conjunctival cells for 10 minutes and measured recovery after 24, 48, and 72 hours. A change in membrane integrity occurred as low as 0.0001% and above at 24, 48 and 72 hours of recovery. These cells were additionally measured for expression of the apoptotic marker Apo 2.7. Cells treated with concentrations of BAK as low as 0.0001% showed expression of Apo 2.7. Membrane integrity decreased after recovery and apoptosis occurred at a concentration that is at least ten-fold lower that the dose of BAK that has been shown to be toxic in the eye. Thus, delayed effects on membrane integrity and the onset of apoptosis, although present, may not be severe enough at the BAK concentrations of 0.0001% to 0.001% to cause pathology in humans. The initiation of cell death in a cell population due to the effect of a preservative may initially present itself as apoptosis at low concentrations. These cells are removed from the body through the normal process of removal of naturally occurring apoptotic cells and may not cause pathology. As the damage increases due to increasing the concentration the process of cell death may change from apoptosis to necrosis resulting in the increased level of damage caused by inflammation and damage from necrotic cell death. BAK was shown by De Saint Jean *et al.* (1999) to cause necrosis at high concentration of 0.1% and 0.05% and delayed death by apoptosis at concentrations between 0.01% and 0.001%.

Various other cell types have been evaluated for cytotoxicity after exposure to BAK. Using Acridine orange and propidium iodide to assess live and dead cells Vaughan and Porter (1993) determined that the threshold for cell death from BAK using mouse L-929 mouse fibroblast cells exposed for 2 hours to BAK was 0.01%. Hallet (2005) showed toxicity to Deruminstitut rabbit corneal cells using the metabolic dye MTT after a 15 minute treatment to

0.01% BAK. In both of these studies cells exposed to BAK at a concentration of 0.001% did not show significant reduction in the number of viable cells, compared to a buffered saline control.

This current investigation determined the effect of BAK on the mitochondrial integrity of epithelial cells using confocal microscopy with the fluorescent dye Rhodamine 123 and the viability dye alamarBlue. Even at a short exposure time of 5 minutes BAK at 0.001% caused some human corneal epithelial cells to lose mitochondrial integrity. Increasing the time of exposure and the BAK concentrations caused increased detrimental effects on the mitochondrial. BAK concentrations between 0.001% and 0.01% showed substantial effects on mitochondrial integrity. Measurements of mitochondrial integrity are relevant endpoints for predicting human ocular toxicity, as the levels of BAK that cause toxicity in humans ($\geq$ 0.005%) had substantial effects on the mitochondria. Other chemicals and product formulations can be assessed for toxicity by evaluating effects on mitochondrial integrity and relative toxicity to BAK can be determined.

4.4.2 Cell Culture

In vitro cell culture tests establish the safety of new chemicals and product formulations by determining whether there is a disruption in the normal physiology of cells after exposure. A disruption in the cells physiology may not result in a change in the cells functionality or lead to cell death. The cells of the corneal epithelium are responsible for maintaining a barrier function through the formation of tight junctions (Barar *et al*., 2008) and maintenance of tear film stability by the projections of microvilli (Collin and Collin, 2006). The lacrimal gland, the meibomian

glands, conjunctival goblet cells, conjunctival cells, accessory lacrimal glands, the surrounding blood vessels in the conjunctiva and aquaporin-controlled water channels in the cornea and conjunctiva all contribute molecules needed for maintaining a healthy tear film. (Ramamoorthy *et al*., 2008; Tiffany, 2008, Verkman *et al*., 2008). Normal cell function within these tissues will lead to a healthy tear film that functions to provide oxygen, protection, elimination of debris and antibacterial and immune functions (Tiffany, 2008). The energy required by the cornea comes from the diffusion of glucose from the aqueous humor and to a lesser extent from the limbal vessels (Myung *et al*., 2006). In order for a change in the functionality of the cornea or conjunctiva to occur, the physiology of enough cells would need to change above the threshold for tolerance. The cornea is a complex tissue that turns over approximately every 7 to 14 days from the division of the stem cells to the ultimate loss of the cells to the tear film after apoptosis in the outer layer of the corneal epithelium (Hanna *et al*. 1961; Chang *et al*., 2008; Thoft 1983). The cells in the conjunctiva also migrate and are replaced by new cells over time (Zajicek *et al*. 1995; Chan *et al*., 2008; Secker and Daniels, 2008). Thus it is a normal renewal process for the cells of the cornea and conjunctiva. Toxicity would result only if the rate of cell death was increased or the toxicity impaired normal cell functionality.

The level at which a change in cell physiology could result in a pathology is difficult to assess. Chemicals that have shown toxic effects *in vivo* can be used as measures of comparison to assess if new chemicals and product formulations change the physiological of cells to the level of these known toxins. If the physiological changes are equal to or greater than those of known toxins then the chemicals used in these formulations would be considered potential safety hazards.

Ideally, since there are many physiological parameters that can be assessed in cells, many different physiological assessments should be made for determining whether chemicals are potentially hazardous. A battery of *in vitro* studies can be performed to look at the most relevant physiological changes in cells that could lead to toxicity. A number of tests that looked at different endpoints to assess the effect of contact lens care solutions on cells have been investigated (McCanna *et al*., 2008; Bantseev *et al*., 2007; Tchao *et al*., 2002; Bantseev *et al*., 2008; Dutot *et al*., 2008; Oriowo, 2006; Santodomingo-Rubido and Mori, 2006; Imayasu *et al*. 2008; Horwath-Winter *et al.*, 2004; Wright and Mowrey-McKee, 2005) . These studies assessed the physiological changes caused by contact lens care solutions measuring the effects on metabolism, epithelial tight junctions, production of reactive oxygen species, and activation of the P2X7 cell death receptor, mitochondrial integrity, optical quality and cell membrane integrity.

4.4.3 Contact Lens Care Solutions and their effect on the epithelium of the lens

Antimicrobials in contact lens care solutions and active drugs in ophthalmic eye drop products have been shown to uptake into contact lenses (Dracopoulos *et al*., 2007, Kargard *et al*., 2003, Rosenthal *et al*., 2006; Chapman *et al*., 1990). The concentration of chemicals on the contact lens surface and in the fluid phase within the contact lenses can influence the concentration and exposure time to the chemicals in these products. Evaluating the interactions between the contact lens solution and contact lenses provides additional information that can be utilized in assessing product safety. There are four FDA contact lens groups. Each group is based on the percent water within the matrix of the lens and the ionic nature of the polymers. Karlgard *et al*.

(2003) found that ophthalmic drugs will have variable uptake times depending on the chemical nature of the contact lens material. The release of the chemical is also dependent on the interaction between the lens material and the drug tested. The uptake of BAK by a soft contact lens after 7 days of continuous exposure was shown to be between 30 to 56 micrograms/mg of lens using a radioactive tracer (Chapman et al., 1990). Dracopoulous *et al*. (2007) studied the effects of extracts from four different contact lens products soaked in 1%, 0.1%, 0.01%, 0.001% BAK for 24 hours on the bovine lens epithelium. After a 15 minute exposure to these extracts, mitochondrial integrity and lens optical properties are degraded as the concentration of BAK soaking solution increased.

The toxicity of a chemical on the eye is dependent on the time of exposure and concentration (Liu, 1981). The effect a contact lens care solution bound to a contact lens will have on the cornea will depend on the concentration of the chemicals in the lens and on the time of release of the chemicals from the contact lens. The tear film will interact with the chemicals within the contact lens and affect the release over time. Also, tears will flush the eye draining chemicals into the nasal lacrimal duct, absorbing them into the mucosal lining and also releasing them into the nose (Mochizuki *et al.*, 2008; Zhu and Chauhan, 2008). In addition, the presence of capillary beds allow for the removal of chemicals to the general circulation, minimizing the concentration and time of exposure of the chemical to this tissue (Barar *et al*., 2008). Thus, the exposure time on the cornea depends on the release rate from the contact lens, the washing out of the chemical by the tears and the chemical's removal by adsorption into the tissue and removal by the fluid flow in the capillaries.

Many *in vitro* studies have been performed to evaluate the cytotoxicity potential of contact lens care formulations. Investigations have shown that contact lens care formulations will have some effects on the cell cultures that are different than the untreated controls. The studies evaluated various aspects of cellular physiology and functionality. Cytotoxicity can also be defined as the degree to which something is toxic to living cells. Toxic, as it pertains to cells, is defined as capable of causing injury or death. A substance that causes cell death would be toxic to the eye if the number of dead cells exceeds the tolerability threshold. If there is a physiological change in a cell due to the presence of a chemical, it is important to know if the physiological change is harmful to the cell, or is it just a change from normal that will not result in cell death or a change in cell functionality. Since cell death or injury is the result of a large physiological change in the cell it is important to note if a chemical causes a deviation from the normal state and design follow up experiments should determine if this change could result in toxicity.

A number of studies have been performed that evaluated the acute cytotoxicity potential of currently marketed contact lens care products. These products have been approved by regulatory agencies and have been evaluated in long term clinical studies demonstrating their safety. The goal of testing currently marketed products is not to question the conclusions of these clinical studies but to highlight the physiological changes that may occur to the cells when the corneas are exposed to these products. Possibly, cells that are altered physiologically may be more susceptible to other potential insults to the eye. Although long term clinical studies have demonstrated safety to a certain cohort in a narrowly defined modality use of contact lenses and

contact lens care products, the real life use of the products, or the conditions of the patient's eyes, may be very different than the clinical conditions tested.

A contact lens wearer can have additional stresses on their corneas that have not been evaluated in a clinical setting. If a person experiences dry eye they may add a lubricating eye drop to their daily lens care regimen. These eye drops may contain preservatives that will cause additional physiological changes to the cornea. If a cornea already has experienced a physiological change from normal due to the effect of a contact lens care product, this additional change may result in cytotoxicity. Also, individuals can expect physiological changes to their cornea due to everyday challenges to their eyes. Typical insults to the eye can result from soap entering the eye while showering, chlorine exposure during swimming or chemical exposure to makeup or household aerosols. Because clinical studies do not investigate cohorts that have these additional stresses to their corneas, understanding the physiological state of the cells in the cornea after exposure is relevant for assessing potential overall health risk to a contact lens wearer. Corneal cells that are in a physiological state that is an extreme deviation from the normal, will die or lose their functionality. Dry eye, lose of endothelial cells, reduced immune response or breaks in the barrier function of the cornea can all result in conditions that put the contact lens wearer at risk for ocular damage. Thus, knowing the physiological changes to cells that occur after exposure to chemicals such as contact lens care disinfecting solutions is important for understanding potential health risks.

Optifree Express and Optifree Replenish caused a greater disruption of the mitochondrial integrity of human corneal epithelial cells than the other lens care solution. Optifree Express also was shown to decrease the number of mitochondria in bovine corneas to a greater extent than ReNu MultiPlus. However, the degree of disruption in the bovine cornea was not as great as the disruption seen using a monolayer of human corneal epithelial cells. Bovine corneas were exposed to Optifree express and ReNu Multiplus for 30 minutes and mitochondria could be counted. Human corneal epithelial cells exposed to Optifree Express for 5 minutes showed complete loss of mitochondrial integrity. A possible explanation for this difference is related to the mass of the tissue being evaluated. Since the cornea contains a six cell layer epithelium, a stroma and an endothelium, there is a large mass of tissue that the contact lens solution will dilute into. When using a monolayer, a dilution of the solution into tissue is not possible. Cell monolayers are useful to evaluate the relative toxicity of various chemicals. However, dilution of the chemical into tissue and the transport of the chemical through the tissue should be considered in an assessment of potential risk. Additionally, the differences seen in the toxicity of Optifree in the bovine cornea and the cell culture may be due to physiological differences between the cornea primary culture and immortalized human corneal epithelial culture impacting the effect of this solution on the cells (Yamasaki *et al.,* 2009).

The use of the bovine lens in addition to the use of human corneal epithelial cells can help characterize the toxicity of a solution to a multilayered tissue. AcuVue II lenses soaked in Optifree Express did not have a substantial effect on the mitochondrial integrity of the bovine epithelial cells after a 19 hour exposure and recovery; whereas BAK treated (0.01% and 0.001%)

AcuVue II treated lenses did. In the eye, there will be a dilution of the contact lens care solution in the cornea, conjunctiva and other ocular tissue. Dilution and retention time in the cells is a relevant consideration when identifying the potential risk of the chemical. Neither *in vitro* corneas nor bovine lenses contain a circulatory system for removal of toxic chemicals. However, the cultured bovine lens and the cultured cornea do have a potential sink for the chemical as the lens core and the corneal stroma are tissues that a chemical may be able to move into over time. The movement of Optifree Express into this sink versus the lack of movement of BAK through the tissue may explain the difference between the effects of these solutions on the bovine epithelium. BAK could have had a longer exposure time in the bovine epithelial cells than Optifree express, causing increased cytotoxicity.

In vitro molecular biology methods can be used to predict the potential risk of chemicals and contact lens solution products. Use of the bovine lens and human corneal epithelial cells show significant toxicity with the known toxin BAK Assessments of potential toxicity using human corneal epithelial cells were sensitive enough to show differences in the effects of contact lens solutions on mitochondrial activity. Evaluations using bovine lenses demonstrated substantial differences between a currently used product, Optifree Express, and the toxin BAK. This investigation has shown that determining the effects of solutions on the mitochondrial integrity of both bovine lens and human corneal epithelial cells can determine the potential toxicity of these chemicals and product formulations.

Summary and General Conclusions

The utilization of *in vitro* tests with a tiered testing strategy for detection of mild ocular irritants can reduce the use of animals for testing, provide mechanistic data on toxic effects, and reduce the uncertainty associated with dose selection for clinical trials. This thesis described the development of *in vitro* assays for assessing the toxicity of chemicals and ophthalmic products using measurements of effects on tight junctions, organ functionality, and mitochondrial integrity. The first chapter of this thesis described how *in vitro* methods can be used to improve the prediction of the toxicity of chemicals and ophthalmic products. Chapters two, three and four demonstrated the sensitivity and relevance of the new *in vitro* assays.

All of the *in vitro* assays described in this thesis showed the sensitivity and relevance necessary for use in risk assessment. These *in vitro* tests were significantly more sensitive than the Draize rabbit ocular irritation test. As shown in table 1, BAK at 0.1% and SDS at 0.3% give scores of 0 in the Draize rabbit ocular irritation test. The Draize rabbit test showed some mild scores when 0.3% BAK and at 1 % SDS solutions were instilled into the rabbits eyes (Klausner et al. 2003). The assays for measuring tight junctions, organ functionality and effects on mitochondrial acitivity showed toxic effects at substantially lower concentrations than the concentrations that caused irritation in the Draize test. Also these *in vitro* assays showed effects at the levels of BAK that were shown to be toxic using sensitive *in vivo* animal studies utilizing SEM and confocal microscopy. The *in vitro* studies also showed effects at concentrations of BAK that showed toxicity in humans (0.005% -0.01%) BAK.

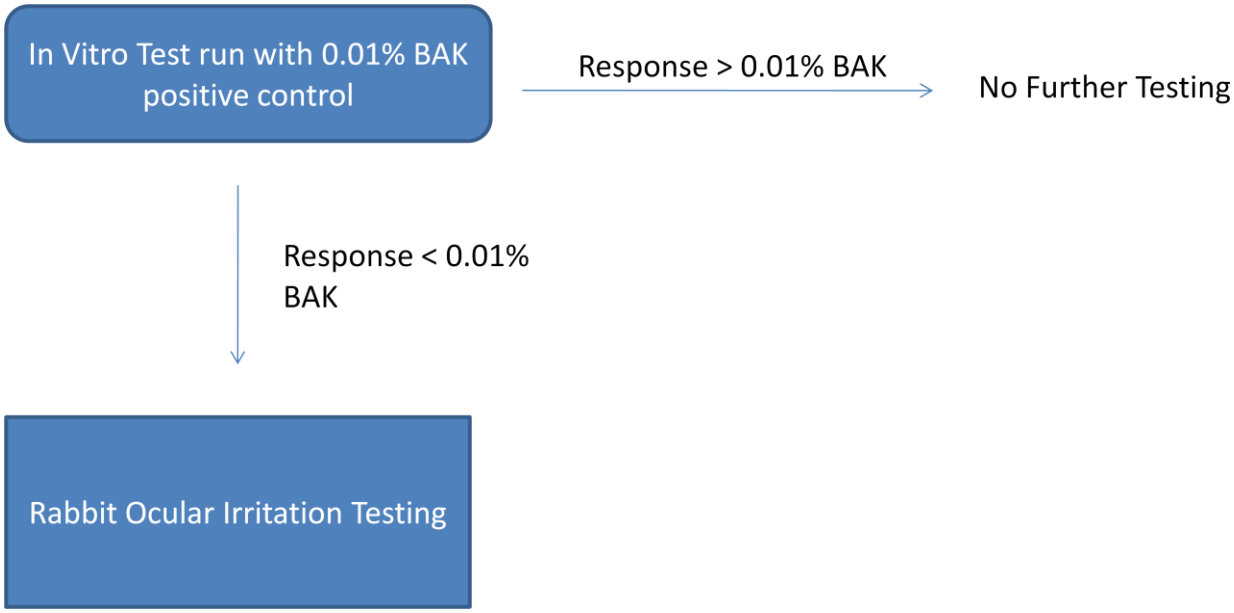
It is the goal of researchers who work on alternatives to animal tests to Refine (decrease the severity of the toxicity seen in animal studies), Reduce (Lower the number of animals used in these studies), and Replace (replace animal studies with *in vitro* alternative methods). By utilizing the tiered testing approach that is outlined below in figure 1 the severity of the response used in animals will be lowered as chemicals with toxic effects greater than 0.01% BAK will no longer be tested in rabbits thus lowering the severity and pain in the animal. Also the number of animals used in testing will be reduced as the *in vitro* tests will be used to screen out toxic chemicals and thus reduce the number solutions submiited for animal tests.

The proper utilization of *in vitro* methods can accurately predict toxic threshold levels and reduce animal use in product development. The goal of this research was to develop an *in vitro* test battery that can be used to accurately predict the ocular toxicity of new chemicals and ophthalmic formulations. By comparing the toxicity seen *in vivo* animals and humans with the toxicity response in these new *in vitro* methods, it was demonstrated that these *in vitro* methods can be utilized in a tiered testing strategy in the development of new chemicals and ophthalmic formulations reducing the severity of the toxic response in animals and reducing the number of animals that need to be utilized in product development.

Table 1: Draize Rabbit MMAS scores

Concentraton	10.00%	5.00%	1.00%	0.30%	0.10%	0.03%
Benzalkonium chloride	108.0	83.8	45.3	8.67	0	0
Concentration	30.00%	15.00%	3.00%	1.00%	0.30%	0.10%
Sodium Dodecy sulfate	60.5	59.2	16.0	0.67	0	0

Figure 1


In Vitro Test run with 0.01% BAK positive control
Response > 0.01% BAK
No Further Testing
Response < 0.01% BAK
Rabbit Ocular Irritation Testing

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