Investigating The Impact of Multipurpose Solutions Released From Silicone Hydrogel Lenses on Corneal Epithelial Cells, *in vitro*

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

Cytotoxicity of Multi-Purpose Solutions (MPS) is commonly tested on cells using diluted MPS or extracts from MPS soaked contact lenses. There is evidence that lens type will affect uptake and release of compounds contained in MPS. To assess the cytotoxicity of agents contained in MPS that would be released by contact lens, an in vitro "onlay" model was used, whereby MPS soaked silicone hydrogel lenses were directly set onto a confluent monolayer of corneal cells. Chapter 4 describes the impact of MPS released from contact lenses on immortalized human corneal epithelial cells. MPS-soaked lens interactions with cells were characterized by studying cell viability, cell adhesion and caspase assays. In Chapter 5, mechanisms of cell death induced by exposure to MPS from contact lenses were determined through evaluation of apoptotic markers, such as activation of caspase 3 and 9. In Chapter 6, the impact of the physical properties of silicone hydrogel lenses, specifically surface treatments, on cytotoxicity of MPS were investigated. The development of methods for characterizing the release of MPS from lenses, using absorbance spectra, is also described.

The results indicate that exposure to contact lenses soaked in Opti-Free Express (OFX) and ReNu not only induces cell death in vitro, but also has an adverse effect on adhesion phenotype, suggesting that the remaining cells may have a compromised epithelial structure. Borate- buffered MPS were found to be more cytotoxic than phosphate-buffered base solutions. Investigation of the mechanisms of cell death revealed that ReNu and OFX induced corneal epithelial cell death in vitro using different pathways, whereby ReNu induced a necrotic pathway while OFX-induced cell death was mediated by the intrinsic pathway of apoptosis. The in vitro model was also able to identify differences between silicone hydrogels with different surface

treatments: the different surface treatments and chemistries of silicone hydrogels lens will affect the release profile of MPS and hence their potential cytotoxicity.

By investigating the induction of cell death processes by solution-lens combinations in vitro, we aim to prevent potential adverse effects in the cornea, which may ultimately compromise various visual and barrier functions. The findings indicate the wealth of information in vitro cytotoxicity testing can provide when evaluating the toxicological profile of MPS.

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Over the past two years, my experience at the University of Waterloo has far surpassed my expectations. It has been a challenging life experience, and I have achieved many academic and personal goals. I owe many thanks to the wonderful people who supported me during this time.

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Dedication

To myself.

Nil Satis Nisi Optimum.

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List of Abbreviations

µg	micrograms
µl	microlitre
ANOVA	Analysis of Variance
AMO	Abbott Medical Optics
APAF-1	Apoptotic Protease Activating Factor-1
BA	Balafilcon A
BAK	Benzalkonium Chloride
BCOP	Bovine Corneal Opacity and Permeability Assay
BEC	Basal Epithelial Cells
BSA	Bovine serum albumin
BVD	Back Vertex Distance
CA	Comfilcon A
Cyt C	Cytochrome C
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic
EVOM	Electrovoltohmeter
FDA	Food and Drug Administration
GA	Galyfilcon A
HBSS	Hanks' balanced salt solution
HCC	Human Conjunctival Cells
HCEC	Human Corneal Epithelial Cells
ICAD	Inhibitor of caspase-activated deoxyribonuclease
LA	Lotrafilcon A
LB	Lotrafilcon B
LDH	Lactate dehydrogenase
MAPK	Mitogen Activated Protein Kinase
MDCK	Madin-Darby Canine Kidney Cells
MPS	Multipurpose Solution
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium Bromide
mg	milligram
ml	millilitre
NRR	Neutral Red Release
NS	Not Significant
NUMA	Nuclear/Mitotic Apparatus Protein
OFX	Opti-Free Express
OFR	Opti-Free RepleniSH
PBS	Phosphate-Buffered Saline
PHMB	Polyhexamethylene Biguanide
PI	Propidium Iodide
PQ	Polyquad, Polyquaternium-1
PS	Phosphatidylserine
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SEC	Superficial Epithelial Cells
SEM	Scanning Electron Microscopy

SV-40	Simian Vacuoloting Viris 40
Tris	Tris (hydroxymethyl) aminomethane
TNF	Tumor Necrosis Factor
α	Alpha
β	Beta
° C	degree Celsius

CHAPTER 1

INTRODUCTION

Corneal epithelial cell culture has been increasingly accepted as a valid tool for toxicology and biocompatibility studies.[1-3] Many laboratories currently employ this model for drug screening and safety and for assessing ocular irritation.[1-3] Most recently, cell culture models have been used to screen for the potential cytotoxicity of multipurpose solutions (MPS).

MPS are single solutions, that are used to rinse, clean, disinfect, rewet and store contact lenses.[4-7] MPS contain many different components to enhance disinfection and preservative properties. The disinfecting properties of MPS are conferred by the active biocide, which are commonly a polyquaternium, biguanide or hydrogen peroxide agent.[8,9] The preservatives in MPS, such as Polyquad®, Aldox® and Polyhexamethylene biguanide (PHMB), are intended to breach cell walls of microbes, but may have the potential to cause corneal epithelial cell membrane toxicity.[5,9-10] MPS also contain a buffering solution to maintain the pH of the solution, which is typically either borate or phosphate-based.[9]

Silicone hydrogel (SH) lenses are the newest generation of contact lens materials, which incorporate siloxane moieties to increase the oxygen permeability to the cornea.[11,12] The physical properties of siloxane allow for the potential adsorption and absorption of active components onto lenses while they are soaked in the cleaning solution, which then can be released onto the corneal surface during lens wear.[8] While various surface modifications and proprietary chemistry are employed to reduce the hydrophobicity of the SH lens surface and the potential interaction of the lens with tear film components and active components of MPS[11-13], it has been shown that

certain combinations of MPS and silicone hydrogel lenses can lead to a cytotoxic effect in vitro,[5-9, 15-17] and certain combinations (not necessarily the same as those that demonstrate problems in vitro) have the potential to exhibit corneal staining in vivo.[14] With these observations, many biocompatibility studies have been undertaken to examine the effect of ophthalmic solutions, including contact lens packaging solutions and multipurpose solutions, on corneal and conjunctival cells.[15-19] Many of these biocompatibility studies have used extracts or dilutions of the solutions to evaluate the corneal effect, or have used non corneal cell types as a proxy. These studies were able to evaluate the potential cytotoxic effect of ophthalmic and multipurpose solutions in vitro, and while this is valuable research, there is currently no information on the effect of the direct release of solutions from silicone hydrogel lenses on human corneal epithelial cells.

This study was undertaken to determine the effect of the direct release of multipurpose solutions from silicone hydrogel contact lenses on corneal cell adhesion phenotype and viability. There is evidence that lens type will affect uptake and release of compounds contained in MPS.[11] Thus, to assess the cytotoxicity of agents contained in multipurpose solutions that would be released by contact lenses, a model whereby the contact lens was directly set onto a monolayer of corneal cells, was used.

HYPOTHESIS

The hypothesis of this research project is that borate buffer-based multipurpose solutions have a greater cytotoxic potential and induce more apoptosis in HCEC, compared to phosphate buffer-based solutions. We acknowledge from the literature that solution-lens incompatibilities

exist; thus we also hypothesize that lens properties affecting the release profile will also have an effect on the cytotoxicty of a solution.

This thesis reports the results of in vitro experiments that were undertaken to verify our hypothesis. Before presenting the results, the structure and physiology of the cornea and mechanisms of wound healing and cell death are introduced in Chapter 2. Chapter 3 presents a review on models and methodologies used to evaluate the cytotoxicity potential of multipurpose solutions. Chapters 4 to 6 report and discuss the experimental results for cell viability and adhesion phenotype (Chapter 4), mechanisms of apoptosis (Chapter 5) and lens effect (Chapter 6). Conclusions and recommendations for future work are presented in Chapter 7.

CHAPTER 2

CORNEAL PHYSIOLOGY, WOUND HEALING AND CELL DEATH

INTRODUCTION

Preservation of vital corneal functions is essential to maintaining good ocular health. A compromised corneal epithelium could decrease the defence systems of the ocular surface, and increase the risk of serious infection.[1,2] A "wounded" surface can also disrupt the refractive property of the cornea, compromising vision.[3] The presence of cell adhesion molecules and the wound healing process in the cornea are essential to maintaining a healthy cornea when cell death and wounding occurs.

STRUCTURE AND PHYSIOLOGY

The cornea is a transparent avascular, non-keratinized epithelial structure, forming one-sixth of the area of the outer wall of the eye.[4-6] It represents the optical interface between the eye and external environment and functions as an optical element and protective barrier.[3] Together with the lens, the primary function of cornea is to refract light to focus an image on the retina; therefore, they must maintain their transparency, optical physiology and structure.[3] The corneal epithelium and the lens both originate from the surface ectoderm during embryonic development.[3,7]

The normal human cornea is 500 µm thick and consists of 5 layers: corneal epithelium, Bowman's layer, stroma, Descemet's membrane and the corneal endothelial monolayer.[8] The corneal epithelium is a stratified structure, 50 µm thick, consisting of a single layer of squamous superficial epithelial cells (SEC) several layers of intermediate wing cells, and a single layer of

columnar basal epithelial cells (BEC) (Figure 2.1).[3,8,9] SECs range from 20-30 µm in length and are 5µm thick, while BECs are 10-15µm in diameter; wing cells can vary in size.[8] Superficial corneal cells provide a substrate for the precorneal tear film, which acts as the primary refracting surface of the eye.[10]

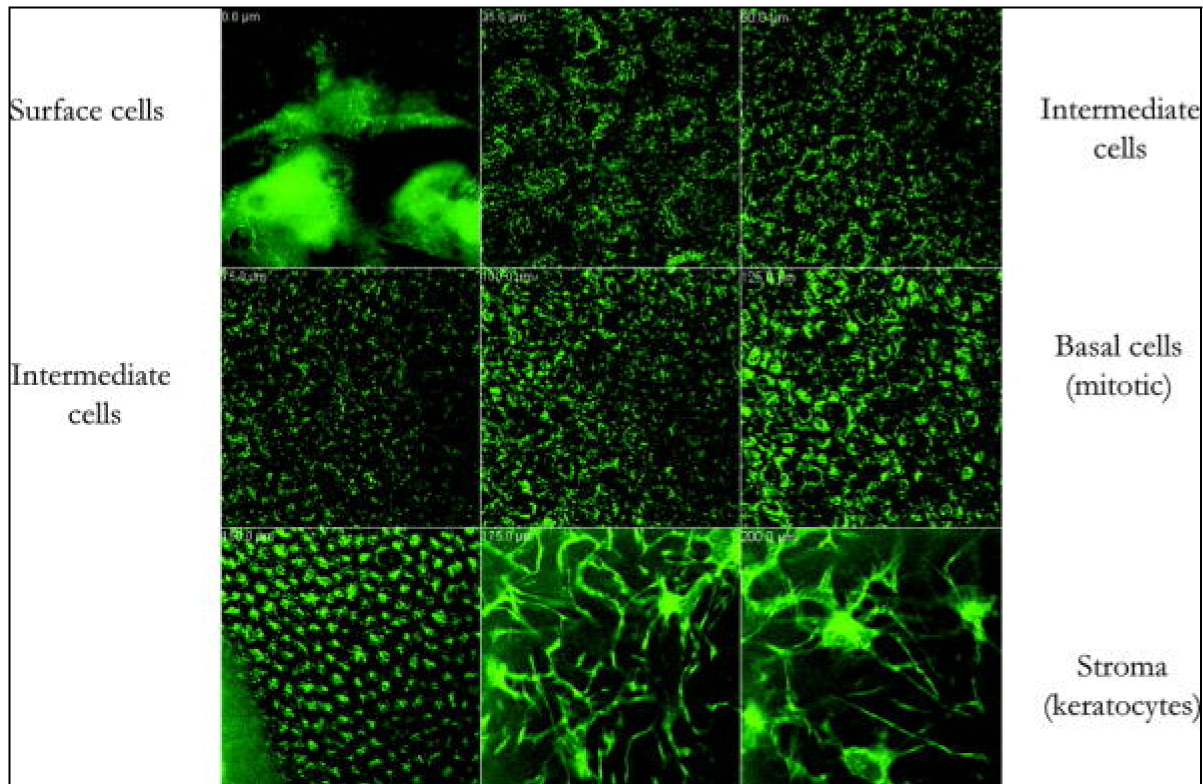

Figure 2.1: In vivo confocal images of the normal corneal epithelium.[11]

Reprinted from Eye & Contact Lens. Bantseev V, McCanna DJ, Driot JY, et al . Biocompatibility of contact lens solutions using confocal laser scanning microscopy and the in vitro bovine cornea. *Eye Contact Lens.* 2007; 33: 308-316, with permission from Wolters Kluwer Health.

The cornea is well protected from pathogens and the external environment by tight junctions and the constant self-renewal, lacrimation and blinking, antimicrobial enzymes in tears, and nearby antigens, cytokines, inflammatory mediators or leukocytes that enter the cornea via limbic and or ciliary body vessels.[5,6,13] The population of epithelial cells is maintained by the

balance between cell divisions at the limbus and basal layers and cell loss or sloughing at the surface.[6] After divisions, mature cells will migrate both centripetally and anteriorly, and flatten as they approach the surface. As older cells slough off into the tear film, the newly matured cells can be exposed.[6] The epithelial cell turnover rate was found to be approximately 7 days in normal human corneas.[14] Similarly, the turnover rate in mice is 6-7 days.[15] The rapid epithelial renewal rate and continuous shedding of surface cells reduces the time and opportunity for mechanical stresses and potentially adherent and infectious organisms to contact the corneal surface.[15]

The presence of tight junctions in the corneal epithelial layer plays a vital role in the barrier function of the cornea, protecting intraocular structures against diffusion of substances from the tears, transport of ionic or polar molecules, microbial infections and other environmental stresses.[10,12] Tight junctions (Figure 2.2) are formed from two integral transmembrane proteins, occluding and claudins, and several membrane associated proteins, ZO-1, ZO-2 and ZO-3. ZO-1 is localized in the apical region of SECs cell-cell junctions.[2] In rabbits, however, ZO-1 is distributed in the BEC and wing cells.[2]

Like tight junctions, gap junctions occur between adjacent cells (Figure 2.2). They are membrane channels that allow for the flow of ions and small molecules between adjacent cells.[16] These hexametric channels are composed of connexons, which can attach to connexons in the plasma membrane of an adjacent cell.[16]

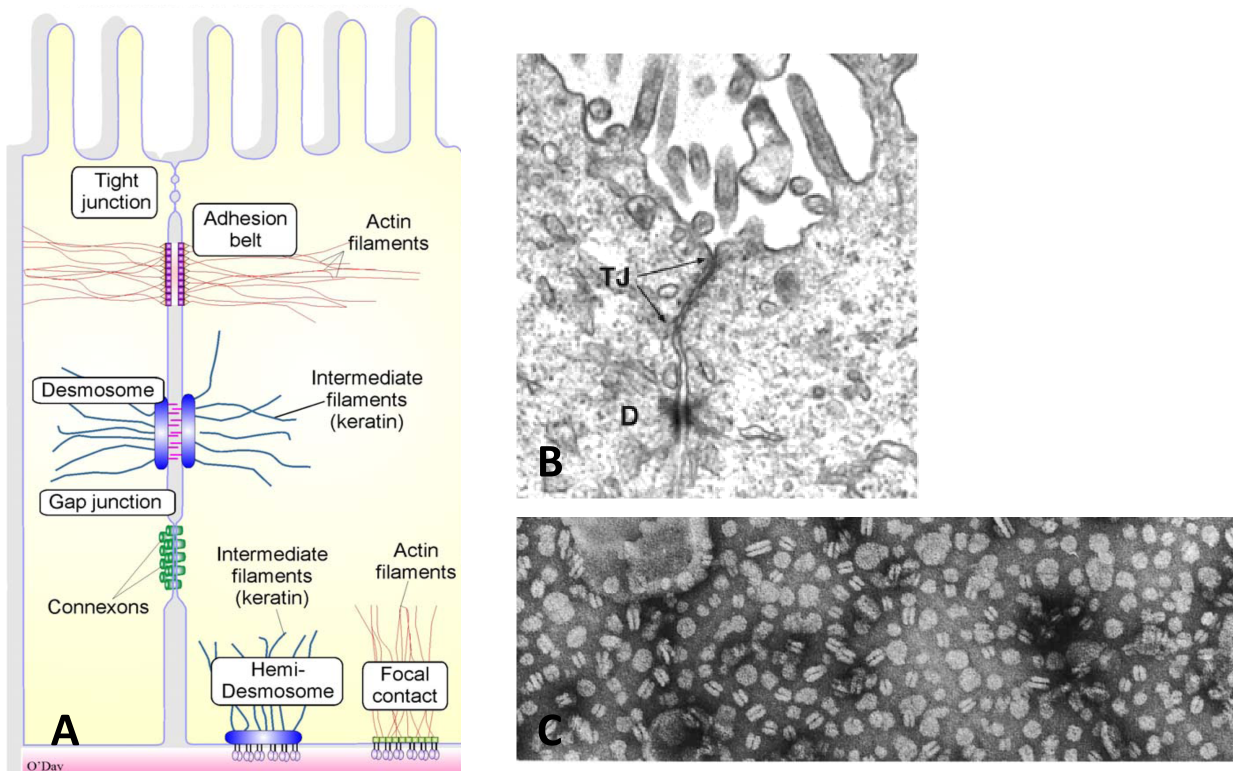

Figure 2.2: Types of cell-cell and cell-ECM junctional complexes, which facilitate adhesion and communication in corneal cells. A. Diagrammatic representation of tight junctions, gap junctions and other cell adhesion complexes. B. Electron micrograph of tight junctions (TJ) and desmosomes (D) in an ultrathin section of Epon-embedded MDCK cells.[17] C. Electron micrograph of self assembled gap junctions.[18]

A. Reprinted with permission from Dr. Danton O'Day.
B. Reprinted from Invasion and Metastasis. Huber D, Balda MS, Matter K. Transepithelial migration of neutrophils. *Invasion Metastasis.* 1998; 18: 70-80, with permission from S. Karger AG, Basel.
C. Reprinted from Microscopy and Research Techniques. Kistler J, Evans C, Donaldson P, et al. Ocular lens gap junctions: protein expression, assembly, and structure-function analysis. *Microsc Res Tech.* 1995; 31: 347-56, with permission from John Wiley & Sons.

The cornea also contains integrins, which are a family of cell surface receptors.[19,20] There are at least 9 unique beta subunits and 24 alpha subunits, which can associate non-covalently in multiple combinations as a heterodimer.[19,20] They are characterized by a large extracellular domain, an alpha-helical transmembrane domain and a cytoplasmic domain (Figure 2.3).[19,20] Integrins serve many functions, including cell adhesion, migration, formation of adhesion

structures and maintenance of tissue integrity.[19-22] The association of integrins with various extracellular matrix proteins, including fibronectin, vitronectin or laminin, as a ligand, allows integrins to carry out these functions, such as in cell migration where integrins transmit forces from the matrix to the cytoskeleton and regulate changes in cytoskeletal organization.[19,20] Many adhesive proteins, such as fibronectin and vitronectin, contains an RGD sequence, which serves as a cell attachment site where many integrin families and extracellular matrix molecules can bind to.[19,23]

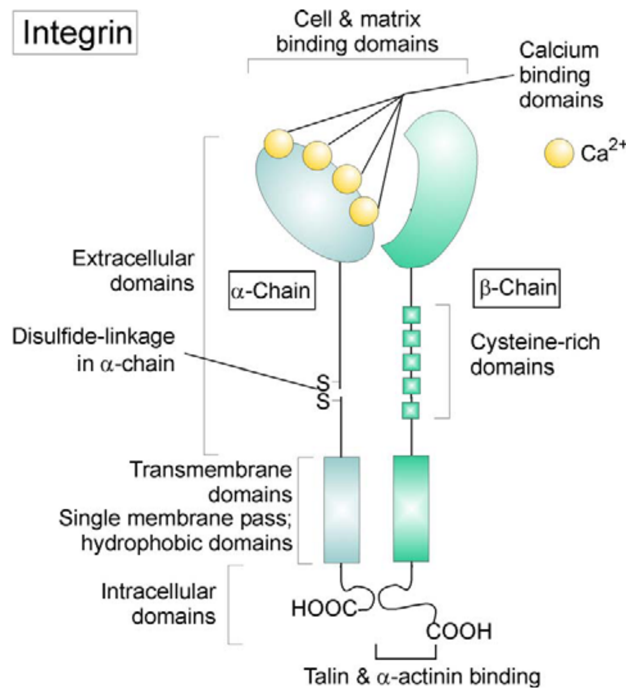

Figure 2.3: Basic structure of integrin heterodimer on cell surface.

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There are several different integrins key to cell adhesion and wound healing processes of the cornea. The most relevant are described below and Table 2.1 lists all ocular integrins, which play a role in cell adhesion, cell cycle regulation and wound healing in the cornea.

- **α3β1** is important in cell migration as it regulates the expression of matrix metalloproteinases responsible for cell movement and spreading. It is also important in the maintnance of cell-cell junctions and hemidesmosome stability.[19,21]
- **α6β4** is a cell survival factor.[11] Like α3β1, it regulates cell cycle progression.[19] During cell migration, this integrin is phosphorylated to prevent premature reassembly of hemidesmosomes.[19] It is also a strong component of hemidesmosomes.[19-21] Expression of this integrin is crucial for stable adhesion of the epithelium to the basement membrane.[19] Knockout studies in mice have found that the absence of β4 results in defects in epidermal adhesion and the assembly of the basement membrane.[19,24] Low levels of these important integrins results in a disruption of cell adhesion.[19,24]
- **α5β1** is an important part of the wound healing process.[25] Healthy, actively migrating cells will express α5β1, which will increase the sensitivity to fibronectin, which can act as a chemotatic migratory stimulus.[25]
- **α9β1** is localized only in the corneal epithelium and also plays an important part in the wound healing process.[19] α9 regulates the association of proteins with the cytoskeleton to aid in cell migration.[19] It is especially important in sustaining cell migration once it has been inititated.[19]
- **αvβ6** is another important integrin in the wound healing process and in cell migration.[19] The expression of this integrin during wound healing helps to maintain the integrity of the tissue.[19] It also is a regulator of E-Cadherin expression.[19] Loss of this integrin leads to loss of adherens junctions.[19]

Table 2.1: Ocular integrins, which are integral in cell adhesion and the wound healing process of the cornea.

Subunit	Subunit	Known Ligands	Pattern of Expression		Functions
			Eye	Cornea	
β1	α1	FN	Anterior chamber, choroid, conjunctiva, cornea, iris and retina		Cell adhesion
	α2	CN,FN	Anterior chamber, choroid, conjunctiva, cornea, iris, lens, limbus, optic nerve and retina	Keratocytes in situ, Fibroblasts, Epithelium incl. Limbal basal cells, Nerve	Wound healing
	α3	CN,FN,LN	Anterior chamber, choroid, conjunctiva, cornea, iris, lens, limbus, and retina	Fibroblasts, Epithelium, incl. Limbal basal cells, Nerve	Regulate cell cycle, cell migration, secretion of MMPs, cell-cell junctions
	α4	FN-IIIA	Anterior chamber, choroid, conjunctiva, cornea, iris and retina	Fibroblasts	Immune response, inflammation, wound healing and cell adhesion
	α5	CN,LN,FN-RGD	Anterior chamber, choroid, conjunctiva, cornea, lens and retina	Fibroblasts	Wound healing
	α6	CN,FN,LN	Anterior chamber, choroid, conjunctiva, cornea, iris, lens and retina	Keratocytes in situ, Fibroblasts, Epithelium, incl. Limbal basal cells, Nerve	Cell adhesion: hemidesmosomes, cell-basement membrane junction
	α9	FN-EIIIA, TNC,FBN,FVIII, VEGF-C	Conjunctiva, cornea, and limbus	Epithelium incl. Limbal basal cells	Later stages of wound healing, sustains cell migration
	αV	LN,FN-RGD	Anterior chamber, conjunctiva, cornea, lens, limbus, optic nerve and retina	Keratocytes in situ, Fibroblasts, Epithelium, incl. Limbal basal cells, Nerve	Cell adhesion
β3		FN,VN,Fbn	Anterior Chamber, cornea, retina	Endothelium, Keratocytes, Fibroblasts	Neovascularization
β6		VN,FN-RGD	Cornea	Epithelium	Cell migration, maintain E-cadherin expression, maintain sheet integrity
β4	α6	CN,FN,LN-5	Conjunctiva, cornea, retina, and limbus	Epithelium	Regulate cell cycle , hemidesmosome component

The abbreviations used for the ligands are as follows: CN, collagens; FVIII, Factor VIII; Fbn, Fibrinogen; FN, Fibronectin; FN-EIIIA, alternatively spliced FN; LN, Laminin; TNC, Tenascin; VN, Vitronectin.

MECHANISM OF WOUND HEALING

Corneal wound healing occurs in three phases.[26-29] Upon epithelial injury, such as corneal lesions caused by continued inappropriate contact lens use, fibronectin will accumulate at the injury site from dilated conjunctival vessels in the first, slow phase of wound healing.[25-29] Healthy cells 50-70 μm from the wound edge will start to disassemble their hemidesmosomes.[19,20] The presence of fibronectin, peaking between 3 and 12 hours post injury, is found to be both chemotatic and haptotactic, and will act as a migratory stimulus for these newly detached cells.[19,26,28] Fibrin together with fibronectin will fill the wound bed and act as a hemostatic plug to prevent any further blood loss from deep scrape wounds.[30] The actively migrating cells will express α5β1, which increases the sensitivity to fibronectin.[28,29] Chemotactic signals allow the healthy cells to migrate in a "sliding motion" into the provisional matrix (Figure 2.4) and will enter the rapid, second phase of wound healing upon proliferation.[20,27-30] During this phase, cell number increases and thickness of epithelium returns to normal.[29] The third phase is the consolidating phase.[27] Differentiation occurs to establish a well layered epithelium.[29] New matrix proteins are synthesized to replace what was lost or damaged.[30]

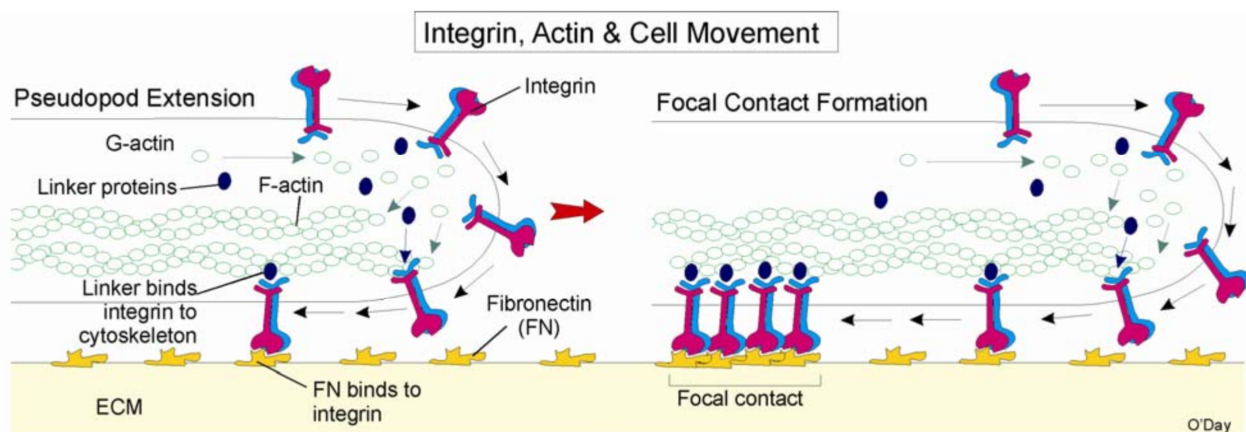


Figure 2.4: Migration of epithelial cells in wound healing, mediated by cell extension by actin polymerization and integrins binding to fibronectin. Reprinted with permission from Dr. Danton O'Day.

MECHANISMS OF CELL DEATH

Apoptosis is a programmed form of cell death that has been evolutionarily conserved among multicellular organisms.[31-34] It plays a role in several normal physiological process such as embryonic development, tissue homeostasis, and self-organizational processes in the immune system and central nervous system.[33-35] It also serves as a defence mechanism in its role of removal of damaged cells.[33-35] This form of cell death has several morphologically recognizable features, including cytoplasmic shrinkage, plasma membrane blebbing, compaction of nuclear chromatin formation of vesicles and apoptotic bodies, and rapid cleavage of DNA into 180bp fragments, corresponding to internucleosomal spacing.[33,34] There are two, possibly three, distinct molecular signalling pathways that lead to apoptotic cell death: the extrinsic or extracellularly activated pathway and the intrinsic, mitochondrial mediated pathway (Figure 2.5).[32] Both signalling pathways involve cysteine aspartate proteases (caspase), which act as mediators for initiating cellular disassembly.[32,34] Caspases are initially translated in an inactive precursor form, containing two subunits (one large and one small) and a prodomain.[33,34] In order to become active and cause apoptosis, they must be cleaved at aspartate residues and assembled into heterotetramers.[34,36-38] Upon activation, caspases will cleave select substrates at aspartate residues to enhance or inhibit activity of pro-apoptotic or anti-apoptotic proteins and enzymes.[34] The ability to cleave at aspartate residues suggests that caspases act on themselves or other caspases in a hierarchal cascade.[33] Activated effector caspases can act on substrates to induce apoptosis specific morphological and biochemical changes.

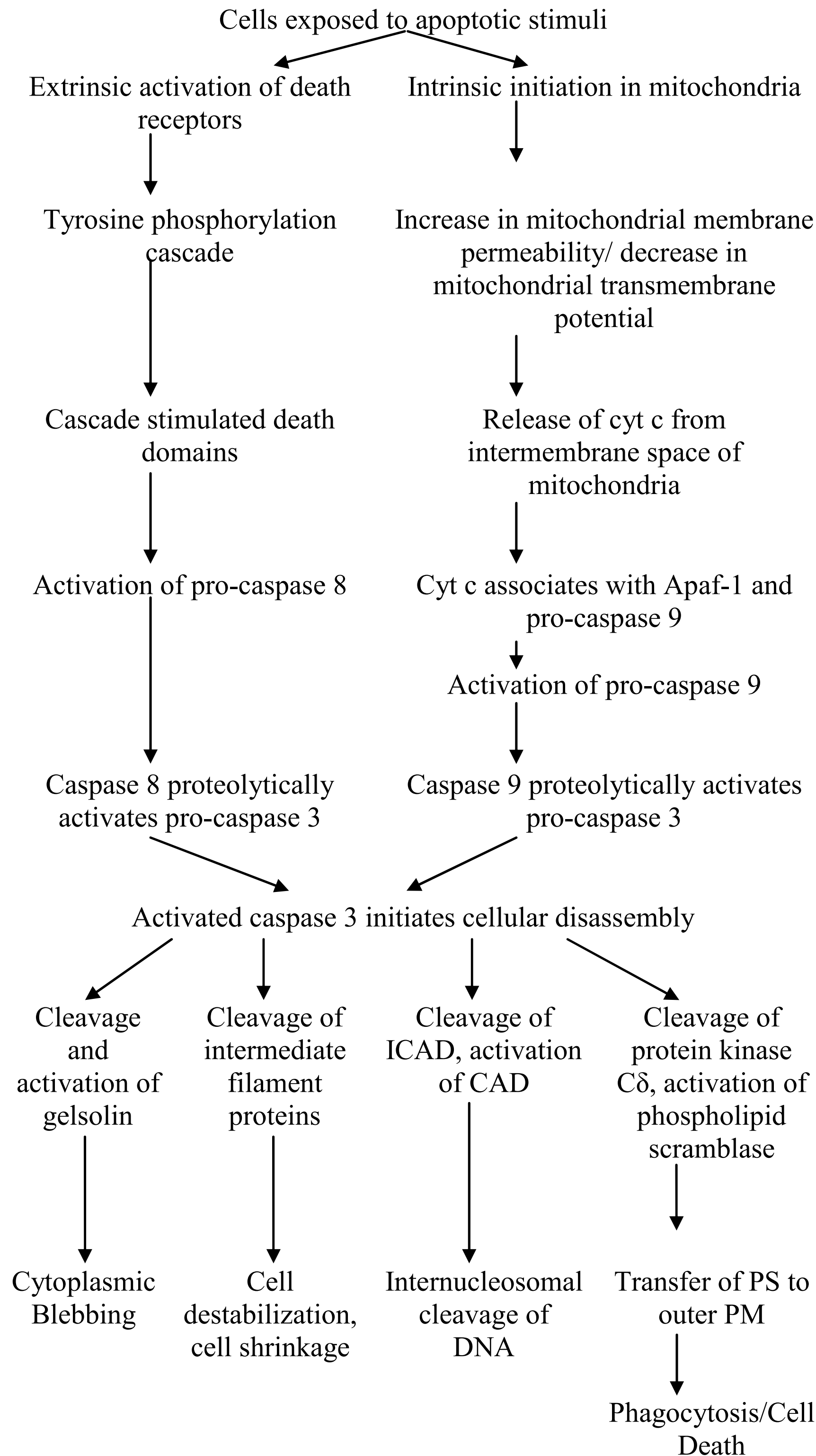


Figure 2.5: Flow chart of apoptotic events in the extrinsic and intrinsic pathways.

The extrinsic pathway differs from the intrinsic pathway, in that it involves the binding of ligands, such as TNF-α or Fas, or viruses, to death receptors on the cell surface to initiate a tyrosine phosphorylation cascade.[32,35] The cascade can stimulate death domains and trigger the activation of procaspase-8.[32]

The intrinsic pathway is activated within the cell in response to cell stress or cell damage, such as UV radiation, osmotic shock, deprivation of growth factors, or exposure to toxic agents (i.e. chemotherapeutic drugs or cytotoxic components of multipurpose solutions released from contact lenses).[35] One of the early events of the intrinsic pathway of apoptosis is alteration of mitochondrial transmembrane potential.[32,35,39,40] When apoptotic stimuli disrupt the mitochondrial transmembrane potential, it triggers the release of cytotchrome c (cyt c) from the intermembrane space, leading to activation of C9 and intrinsic activation of apoptosis.[34,35,39,40] Cyt c in the cytoplasm can bind Apaf-1 (apoptotic protease activating factor-1), a scaffolding molecule key to the formation of the apoptosome.[34,41] Cyt c will induce a nucleoside triphosphate-dependent conformational change, allowing for the binding of procaspase-9.[42-47] Procaspase-9 is the initiator caspase of the intrinsic pathway of apoptosis.[48,49] It is a monomer with misaligned catalytic cysteine and histidine residues and an inactive catalytic pocket.[48,49] The complex of cyt c, Apaf-1 and procaspase-9 forms the apoptosome, which leads to the activation of C9 by conformational changes that open the catalytic pocket and align the catalytic residues. At this point, the intrinsic and extrinsic pathways merge, as both C8 and 9 have the ability to proteolytically activate C3.[32]

As one of the effector caspase, C3 is responsible for mediating characteristic morphological and biochemical changes, by cleavage of several hundred different substrates.[37,38]

Cleavage of these substrates will either favour pro-apoptotic activity or inhibit anti-apoptotic activity.[37] Cytoplasmic blebbing is caused by cleavage and activation of gelsolin, which severs actin filaments.[37,38] C3 also cleaves several intermediate filament proteins, including lamin B, nuclear/mitotic apparatus protein (NuMA) and cytokeratins.[37,38] These proteins in their normal state are responsible for the maintenance of cell shape, and cleavage contributes to destabilization of the cell structure and additional apoptotic morphological changes.[37,38] In the late phase of apoptosis, C3 cleaves the nuclease inhibitor, ICAD (inhibitor of caspase-activated deoxyribonuclease), resulting in the internucleosomal cleavage of DNA, by the enzyme CAD.[50-52] Inactivation of ICAD by C3 cleavage leads to DNA fragmentation.[50-52] C3 also activates a phospholipid scramblase, by cleavage of protein kinase Cδ.[53-57] The activated phospholipid scramblase catalyzes the transfer of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane.[53-57] This provides a signal that can be recognized by the adaptor molecule MFG-E8 on adjacent cells and/or a PS receptor present on macrophages, facilitating phagocytosis of the dying cells.[53-56]

Necrosis is a form of cell death considered to be accidental or inappropriate, which occurs under extremely unfavourable conditions.[58] It is an uncontrollable, irreversible form of cell death characterized by cell swelling, membrane deformation and organelle breakdown.[58] It has not been determined whether signalling pathways mediate necrotic cell death.[58]

Conclusion

Understanding the mechanisms of wound healing and physiology of the cornea help emphasize the importance of preventing corneal cell death. By investigating the induction of cell death processes by toxic agents, we strive to prevent adverse effects in the cornea, which may

compromise visual and barrier functions. Research in this field must consider these mechanisms when developing relevant models and test series to assess ocular toxicity.

CHAPTER 3

OCULAR CYTOTOXICITY TESTING METHODS OF MULTIPURPOSE SOLUTIONS

1. INTRODUCTION

When ocular complications caused by 'Lash Lure,' a cosmetic eyelash dye, were made public, it became apparent that a rigorous procedure for ocular testing was needed to evaluate the irritant or corrosive properties of a substance before marketing it as a product.[1] In the early 1940's, John Draize started to develop a system, which would quantitatively assess the toxicity of topical compounds for cutaneous and ocular use.[1] His methodology involved assessing the acute, intermediate and chronic exposure, by applying compounds to the skin, penis and eyes of rabbits. The biological responses were graded according to an established descriptive scoring scale, to determine the effects on the eye and skin. Draize described his methods as a 'toxicologic appraisal of a given compound or preparation intended for therapeutic, cosmetic or other topical use'.[1] With observations from the Draize test, it was possible to estimate the amount or concentration and frequency of use of application that would be safe to and tolerable by man.[1] These methods soon became considered, by the FDA, as one of the most valuable and reliable methods for evaluating the hazard and safety of a substance introduced into or around the human eye.[1]

One such substance is contact lens care disinfection systems. Before and after lens wear, contact lenses must be disinfected to prevent growth of harmful bacteria that may be present on the lens and remove lipid and/or protein deposits on lenses.[2-5] One of the most commonly prescribed disinfection systems is multi-purpose solutions (MPS).[4-6] MPS are single solutions, that contain a preservative (antimicrobial agent) and other components, that are used to rinse,

clean, disinfect, rewet and store contact lenses.[2-5] The ideal solution must be efficacious enough against a wide spectrum of microbial flora, but gentle enough to not cause adverse effects on the corneal surface, as some of the solution will be exposed to the corneal surface and remain in contact with the epithelium until washed away by the tear film.[4-6] The preservatives in MPS, such as Polyquad®, Aldox® and Polyhexamethylene biguanide (PHMB), are intended to breach cell walls of microbes, but may have the potential to cause corneal epithelial cell membrane toxicity.[3,7] It is thought that the disinfecting agents cannot penetrate the surface of the lens, due to their high molecular weight, but one cannot ignore the potential of the lens to adsorb or form a complex with components of MPS and release them onto the corneal surface post insertion.[4] Because of the potential exposure to the cornea and the high frequency of use, MPS must also be subject to ocular cytotoxicity testing.[8,9]

The cornea is an avascular, multilayered structure, which serves as the optical interface and protective barrier between the eye and external environment (physiology and mechanisms of cell death were described in Chapter 2).[10-12] A disturbed surface can disrupt the refractive property of the cornea, compromising vision.[13] A compromised corneal epithelium could also decrease the defence systems of the ocular surface, and increase the risk of serious infection[14,15] and it is thus important to assess the potential cytotoxicity of contact lens solutions.

Over the years, many methods of testing for ocular toxicity have emerged as modifications or alternatives to the Draize test. While whole animal testing still exists, isolated tissue and tissue culture models have been widely used to screen for ocular toxicity. This review will discuss the whole animal studies used to assess the cytotoxicity potential of MPS, and the isolated tissue and tissue culture approaches, and assays, developed as more ethical, objective and sensitive ocular

toxicity screening procedures. Table 3.1 lists the all of the marketed contact lens solutions tested in the studies discussed in this review, their chemical composition and highlights the evolution of MPS formulation. Table 3.2 lists the family of surfactant agents commonly found in multipurpose solutions

Table 3.1: Chemical composition of multipurpose solutions.

Manufacturer	Solution	Main Disinfecting Agent(s)	Buffers, isotonic agents, surfactants
Advanced Medical Optics	Complete Comfort Plus	0.0001% PHMB	Poloxamer 237 (Pluronic F87), sodium phosphate, sodium chloride, potassium chloride, EDTA
	Complete Moisture Plus No Rub†	0.0001% PHMB	Poloxamer 237 (Pluronic F87), sodium phosphate, sodium chloride, potassium chloride, propylene glycol, taurine, 0.15% HPMC and 0.01% EDTA.
	Complete Easy Rub*	0.0001% PHMB	Poloxamer 237 (Pluronic F87), sodium phosphate, sodium chloride, 0.02% EDTA, and purified water.
Alcon	Opti-Free Disinfecting, Rinsing & Storage Solution	0.001% Polyquad®	Citrate, sodium chloride, 0.05% EDTA
	Opti-Free Express (formerly Opti-Free Disinfecting, Rinsing & Storage Solution)	0.001% Polyquad®, 0.0005% Aldox®	Borate buffer, citrate, sodium chloride, polysorbate 21, poloxamine (Tetronic 1304), 0.05% EDTA
	Opti-Free RepleniSH* (formerly Opti-Free Express)	0.001% Polyquad®, 0.0005% Aldox®	Boric acid, citrate, poloxamine (Tetronic 1304), non-anoyl ethylene-diaminetriacetic acid
Bausch & Lomb	ReNu Sensitive Eyes	0.00005% PHMB	Sodium borate, boric acid, sodium chloride, 0.1% EDTA, poloxamine (Tetronic 1107).
	ReNu MultiPlus No Rub* (formerly Sensitive Eyes)	0.0001% PHMB	Sodium borate, boric acid, hydroxyalkylphosphonate (Hydranate™), 0.1% EDTA, and poloxamine (Tetronic 1107).
	ReNu MoistureLoc	0.00045% Alexidine	Poloxamer 407 (Pluronic F127), poloxamine (Tetronic 1107), boric acid, sodium tetraborate and sodium chloride.
CIBA Vision	AOSept	3% Hydrogen Peroxide	Phosphates

	SoloCare Aqua*	0.0001% PHMB	Tris, sorbitol, 0.025% EDTA, dexpanthenol (provitamin B5), and Poloxamer 407 (Pluronic F127).
	Aquify 5 minute*	0.0001% PHMB	Sodium phosphate dihydrogen, sorbitol, 0.025% EDTA, tromethamine, Poloxamer 407 (Pluronic F127), dexpanthenol
Eurexpan	ContaClair	Disodium edetate	Boric acid, sodium borate, EDTA
Menicon	MeniCare Soft	0.0001% PHMB	Macrogolglycerol hydroxystearate 60, glycine, glycolic acid, AMPD and propylene glycol
Sauflon Pharmaceuticals	Sauflon All in One	0.0005% PHMB	Boric acid, poloxamine, 0.3% EDTA
Titmus	Titmus	Catalase	Disodium hydrogen phosphate, Na-dihydrogenphosphate, hydroxypropylmethylcellulose, disodium EDTA

Notes:

* indicate solutions currently available in North America.
†Complete Moisture Plus No Rub was removed from the market in 2007, and is no longer available.

Table 3.2: Family of surfactant agents commonly found in multipurpose solutions.[44]

Surfactants		Solution
Poloxamer	Poloxamer 237 (Pluronic F87)	Complete Comfort Plus, Complete Moisture Plus, Complete Easy Rub
	Poloxamer 407 (Pluronic F127)	ReNu MoistureLoc, SoloCare Aqua. Aquify
Poloxamine	Tetronic 1107	ReNu Sensitive Eyes, ReNu MultiPlus, ReNu MoistureLoc
	Tetronic 1304	Opti-Free Express, Opti-Free RepleniSH

WHOLE ANIMAL STUDIES

Applications of the Draize test have been used to study the cytotoxicity potential of commercially available contact lens solutions. Many studies have quantified the effects of soft contact lens solutions on rabbit corneal epithelium. In these studies, NZW rabbits were fitted with contact lenses, which were soaked in test solutions. Assays were performed immediately following lens removal. In some studies, the nictitating membrane was partially excised 1 week prior to the start of the experiment.[8] The nictitating membrane is a transparent third eyelid found in birds, reptiles, fish and some mammals, including rabbits.[16] It functions primarily for protection and to moisten the eye. In contrast to human eyelids, the nictitating membrane is drawn across the ocular surface horizontally.[16] In removing this membrane, the results were more generalizable to humans.[1]

Begley et al[2] were one of the first groups to use the Draize test to screen for ocular irritancy. They examined 3 different solutions: ReNu multipurpose disinfecting solution (0.00005% PHMB), Opti-Free rinsing, disinfecting and storage solution (0.001% Polyquad®), and AOSEPT (dilute hydrogen peroxide). Results from Scanning Electron Microscopy (SEM) demonstrated corneal alterations induced by all three solutions. AOSEPT and Opti-Free showed an increase in both the number of wrinkled and peeling cells. Peeling cells refers to cells that have lost their desmosomal attachments, therefore, appearing to peel away from epithelial surface.[2] Exposure to ReNu caused the greatest increase in wrinkled and peeling cells.[2] The intact cells that remained showed few holes and flattened microplicae, indicating exposure of the underlying layer. All 3 experimental solutions showed a significant treatment effect compared to the saline controls, which indicated that it was likely the preservative, residual unneutralized hydrogen peroxide, or

other additives that were responsible for the surface effects.[2] It was suggested by the authors that the increased toxic effect of ReNu may be attributed to the preservative, PHMB. These results suggested that PHMB was not only cytotoxic, causing cell death, but also had the potential to cause exfoliation or peeling of the superficial epithelial layers.[2]

Interestingly, another rabbit study did not find as strong of a cytotoxic effect with ReNu, but still could attribute epithelial damage to PHMB. Chang et al evaluated the corneal response using in vivo confocal microscopy and tear lactate dehydrogenase (LDH).[8] In vivo confocal microscopy was able to provide a non-invasive assessment of the cornea at the cellular level.[8] A specific technique, confocal microscopy through focusing, captured an extended focus, with continuous, confocal optical sectioning of the cornea in the coronal plane through the entire thickness.[8] This approach provided both a 3D view of the cornea and accurate, and repeatable measurements of the corneal epithelial and stromal thickness.[8] To have more precise measurements of the epithelial thickness, several scans through just the corneal epithelium were taken. Tear LDH activity, an indicator of epithelial cell damage,[17] was determined using spectrophotometry, with a modified rate assay. The optical density of the reaction mixture was read at 340nm at 1 minute intervals, on a UV spectrophotometer. Confocal microscopy revealed that epithelial thickness showed a tendency to decrease with contact lens wear.[8] All-in-One showed a significant decrease in the central cornea, while Opti-Free showed a significant decrease in the peripheral cornea. All-in-One also showed a significant decrease in epithelial cell area, in the central cornea. Unisol significantly decreased the epithelial thickness in all areas of the cornea. All-in-One and Unisol also had a significant effect on LDH activity; a 152% and 308% change over time, respectively, was observed. Opti-Free and ReNu did not show any significant change over time.[8] The preservatives were discussed as being the agent responsible

for the solution difference.[8] While previous studies reported that PHMB at 0.00005% had no effect on cytokinetic movement or mitotic activity of cultured HCEC, the effect of PHMB here may be due to the 10 fold increase in concentration. All-in-One, which significantly affected 4 of the 7 parameters evaluated, has a PHMB concentration of 0.0005%. ReNu, with a PHMB concentration of 0.00005% (10x lower than All-in-One), did not significantly affect any of the parameters tested. The increased concentration of PHMB may be the source of the difference between All-in-One and ReNu, and account for the corneal damage.[8] Opti-Free only significantly affected one parameter, indicating that the preservative, 0.001% Polyquad®, did not have a significant effect on the cornea at this concentration.

Researchers using a rat animal model also made similar conclusions about the Opti-Free preservative, Polyquad®, and suggested it as an alternative preservative to Benzalkonium chloride (BAK).[7] Male Lewis rats received eye drops of 0.1% or 0.5% Polyquad twice daily for 11 days. A higher than normal concentration of Polyquad® was used, as lower concentrations did not yield detectable effects on the rat corneo-conjunctival surface in preliminary studies.[7] The results reported that 0.1% and 0.5% Polyquad® produced slight, but not significant, decrease in tear production (as measured by the phenol red thread test), whereas 0.5% BAK did have a significant effect.[7] Polyquad® induced occasional punctate staining, but the slit lamp examination revealed no abnormalities. Confocal microscopy and impression cytology revealed cells exposed to 0.1% Polyquad® had good sheets of normal, polygonal cells, but did observe a significant decrease in goblet cells when exposed to 0.5% Polyquad®, compared to control eyes. Goblet cells are responsible for producing soluble mucins that form the mucous layer of the tear film.[7] The loss of goblet cells is considered an indicator of ocular surface diseases, not limited to dry eye syndromes. The authors conclude that Polyquad® showed little toxicity, especially in

comparison to BAK and that the rat model was able to reproduce the mechanism of toxicity due to preservatives.[7]

Overall, several trends have emerged from animal cytotoxicity testing. Opti-Free (Polyquad®) generally showed little cytotoxicity, and was one of the safest solutions studied. Increasing concentrations of PHMB were correlated to increasingly adverse effects on the corneal surface. The scale of toxicity derived from these studies is generally that OFX < ReNu < All-in-One (Polyquad® < PHMB 0.00005% < PHMB 0.0005%).

While animal studies are still the model of choice, one must consider that numerous physiological factors, such as lower tear production and ocular surface sensitivity, and anatomical and biochemical factors, such as nictitating membranes, genus-specific tear film components, and thinner corneas lacking a Bowman's layer, limit its relevance to humans.[1] In fact, only a few studies have used rabbit models to screen for ocular toxicity. Many of these studies, including the Draize test, have been opposed because of ethical concerns arising over the pain and discomfort associated with the potentially harmful materials being placed directly on the ocular surface.[1, 13, 17,18] Animal studies have also been criticized due to poor repeatability, poor sensitivity and lack of objectivity.[1, 13, 17, 18] This has prompted the development of in vitro assays using intact organs (here to be referred as isolated tissue) or tissue culture techniques to test for potential ocular irritation.

2. Isolated Tissue

a. Bovine Lens Culture System

Bovine eyes can be obtained from an abattoir after animals are killed for human use (i.e. meat) or have reached the end of their useful life (i.e. those raised for milk production).[19] The use

of (abattoir supplied) cultured bovine crystalline lenses was one of the first in vitro alternatives to the Draize test.[20] It is a good proxy for the cornea due to a number of common features such as the common origin from surface ectoderm, gene sharing and common appearance of crystallins and water soluble proteins.[13, 21] When the lens is excised and cultured, it is able to maintain its cellular makeup and refractive function, whereas corneal epithelial cells do not.[13] The advantage of the lens culture system is that the intact lens retains repair mechanisms, an important aspect of the Draize test that has been difficult to model using in vitro systems.[13]

In the lens culture system, one can evaluate the toxicity of a substance based on how it affects the optical quality of the lens. One of the parameters that can be measured is the variability in the Back Vertex Distance (BVD). BVD refers to the ability to have a sharp focus; as there is more variability in the BVD, focus is lost (Figure 3.1).[13] BVD variability has been correlated to corneal opacification.[13] Following treatment with a toxic agent, mitochondria can be affected, making the lens more permeable to oxygen. As oxygen starts to permeate to the core of the lens, opacifications can form, altering the optical quality of the lens, thereby affecting BVD.[13] An automated laser scanner can be used to optically assess the lens integrity.

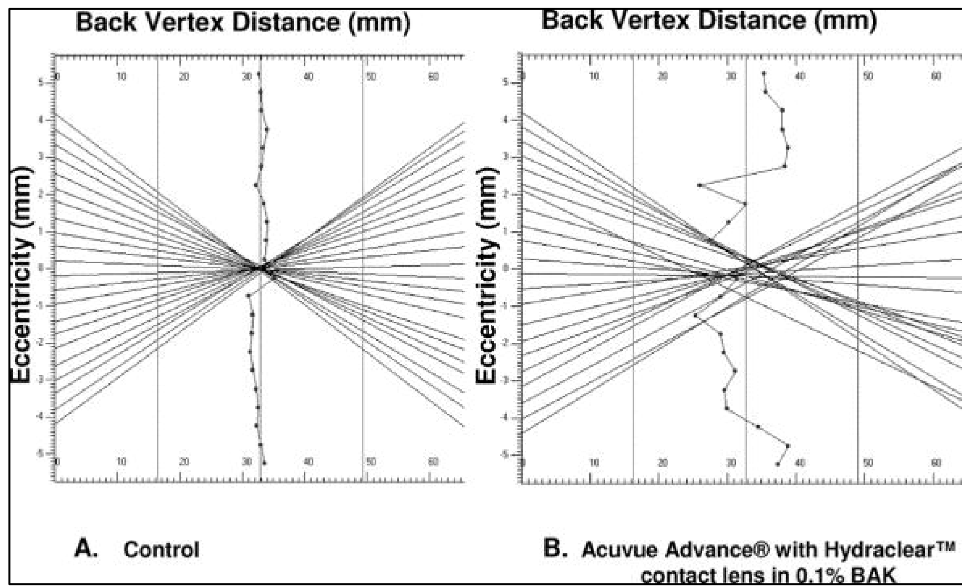


Figure 3.1: Graphic profile of the lens focal variation as seen in an untreated bovine lens (A) and a bovine lens treated with benzalkonium chloride extract (B).[22]

Reprinted from Eye & Contact Lens. Dracopoulos A, Dixon DG, Jones LW, et al. In vitro assessment of medical device toxicity: interactions of benzalkonium chloride with silicone containing and p-HEMA-containing hydrogel contact lens materials. *Eye Contact Lens.* 2007; 33: 26-37, with permission from Wolters Kluwer Health.

In looking at rigid contact lens solutions, Sivak et al[18] were able to determine that the lens culture system was sensitive enough to detect toxicity potential. The study tested several contact lens solutions, which were added directly to the culture, with enough to expose both surfaces of the lens. After the exposure time, a scan was performed to measure focal length, to evaluate focal variability (BVD) and optical function. Toxicity in this study was again attributed to the preservative PHMB.[18] The least toxic solution, the newest formulation of Boston Advance Conditioning solution at the time, had a 3 fold decrease in PHMB concentration compared to the most toxic solution.

While the Sivak study looked at rigid contact lens solutions, the lens culture system has also been used to evaluate toxicity potential of soft contact lens care solutions. In a 2006 study, by Oriowo et al,[4] bovine lenses were exposed to undiluted MPS, Opti-Free Express, ReNu MultiPlus or Complete Comfort Plus, for 3 hours. The optical changes of the lens were measured using a resazurin (alamarBlue) assay. Resazurin (alamarBlue) dye quantifies living cells using resazurin and resorfin, as a fluormetric-colorimetric indicator that fluoresces and changes colour in response to reduction.[4] The oxidation-reduction reaction involving resazurin and resorfin indicates cell metabolism; therefore, viability can be quantified. Fluorescence was measured at baseline and at several other time points post exposure, up to 96 hours. The results found that Complete did not show any changes in fluorescence profile during the time course. At 6 hours, Opti-Free and ReNu showed significant cytosensitive effects. Opti-Free had a significant recovery at 12 hours, but again had a significant toxic effect at 24 hours, before recovery once more at 96 hours. ReNu, however, showed no recovery at 12 hours, but had a gradual recovery to baseline levels by the end of the study. From the results, the authors determined a scale of cytosensitivity where, Complete < ReNu < Opti-Free (0.0001% PHMB < 0.001% Polyquad®, 0.0005% Aldox®).[4]

This trend is different than that observed in the animal studies. While the preservative is still considered the cytotoxic agent, PHMB which was previously considered a toxic preservative, showed little to no toxicity in ReNu and Complete. While both solutions use PHMB, the molecular weight of PHMB may differ between the solutions. Cytotoxicity may be accounted for by this potential difference, as cells of different ocular origin may be susceptible to polymers in a particular size or molecular weight range. Contact lenses may also interact differently with

polymers of different molecular weight, which may affect the amount adsorbed and subsequently released onto the corneal surface.

The Polyquad®-containing Opti-Free solution was the most cytotoxic.[4] Polyquad® was hypothesized as the source of the cytotoxicty, where previously it had been found in the rat model to be much safer than BAK, and proposed as a safe preservative to replace BAK.[4,7] One must consider the different model systems (rat whole animal vs. isolated bovine culture) as a potential source for the contrasting observations about Opti-Free and Polyquad®.

b. WHOLE CORNEA EXPLANTS

Bovine corneas have been shown to have a similar appearance to rabbit and human corneas, after a rigorous washing procedure.[19] While it has more epithelial layers compared to a human cornea, bovine corneas show the same organization as mammalian corneas.[19] This culture system, in addition to the lens culture system, is a good alternative to the use of laboratory rabbits. One of the more recent MPS toxicity studies used corneal buttons obtained from bovine eyes to assess the effects of two contact lens solutions, ReNu and Opti-Free Express, using confocal laser scanning microscopy.[21] Intact corneas were exposed to 2mL of undiluted test solution for 30 minutes at room temperature. Corneal buttons were excised and stained with rhodamine 123, a mitochondrial specific dye, and the nuclear specific dyes, YO-PRO-1, which aided in the visualization of the nuclei and identified apoptotic-related alterations in the nuclear membrane, and propidium iodide, which identified necrotic cells. Confocal microscopy was used to visualize specific dye fluorescence and a MatLab program was used to characterize properties of the mitochondria. Following treatment with Opti-Free Express, a significant decrease was observed in the number of mitochondria in the superficial and intermediate epithelium when

compared to cornea exposed HBSS control or ReNu. ReNu also showed a decrease in number of mitochondria in the superficial epithelium. The observed change in mitochondria suggested that treated cells were undergoing a metabolic change in cell function.[21] The authors proposed that the assay used in this study can be used as a screening tool to compare the depth of formula penetration and detect early cellular changes that may indicate ocular irritation. Opti-Free can be considered as having a greater depth of formula penetration compared to ReNu and HBSS controls.[21]

Porcine corneal models have also been recently proposed as a means to evaluate MPS cytotoxicty.[6] A 2009 study used both isolated tissue and tissue culture approaches to evaluate the corneal response to Opti-Free RepleniSH, ReNu and Complete. [6] Fluorescein staining and flow cytometry were used to evaluate the potential toxic effects. Excised globes were directly immersed in one of the test solutions for 5 or 10 minutes. Integrity of the corneas was graded on the Efron scale, from 0 (no staining) to 4 (severe staining), after instillation of fluorescein.[6] The globe was then carefully dissected, to dissociate corneal cells for flow cytometry. There was only a significant increase in grading scores at the longer time point compared to controls, and fluorescein staining did not show differences between solutions. In contrast, cytotoxic effects were detected by flow cytometry in as little as 5 minutes with Opti-Free RepleniSH. Similar results were observed with the tissue culture approach. Cell suspensions were incubated with one of the test solutions, and it was observed that the greatest amount of fluorescein staining, and evidence of early and late necrosis, was in cells suspended in Opti-Free RepleniSH. The use of both flow cytometry and fluorescein staining approaches allows for the direct comparison of these two assays as accurate measures of cytotoxicity. As shown in Figure 2, it was found that fluorescein staining in this model was not as sensitive to detect corneal damage, as flow

cytometry.[6] The results from this study indicate that fluorescein staining does not correlate well with cytotoxicity (Figure 3.2).[6]

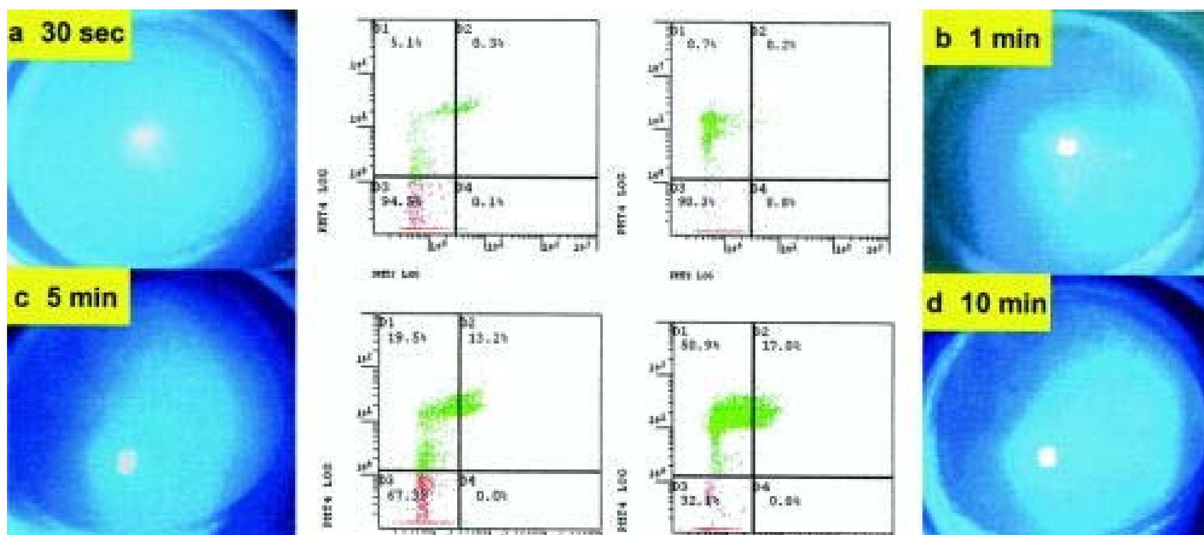


Figure 3.2: Cytotoxicity effect on (a) 30 s, (b) 1 min, (c) 5 min, and (d) 10 min soaked in Opti-Free RepleniSH using FCM and fluorescein staining. (D1: amount of 7-AAD stained cells—early necrosis; D2: amount of Annexin V-FITC + 7-AAD stained cells—late necrosis; D3: amount of non-stained cells—healthy cells; D4: amount of Annexin V-FITC stained cells—apoptosis.[6]

Reprinted from Optometry and Vision Science. Choy CK, Cho P, Boost, MV, et al. Do multipurpose solutions damage porcine corneal epithelial cells? *Optom Vis Sci.* 2009; 86: 1-7, with permission from Wolters Kluwer Health.

The observed cytotoxicity was attributed to the presence of Polyquad in Opti-Free RepleniSH. It was also mentioned that other constituents have been shown to have cytotoxic potential, but since this study tested the whole solution, and not individual components, the contributions of these agents could not be assessed.[6]

While this study only evaluated the effects of direct exposure to the solutions, the authors discuss the implications of the interaction between the solutions and lens materials. While disinfecting agents are not able to penetrate lens surfaces, evidence has demonstrated the ability of silicone hydrogel lenses to adsorb components, such as Polyquad® and PHMB, and

subsequently release these agents onto the corneal surface post-insertion.[6] Evidence has also shown that certain lens-solution combinations can lead to different effects on the cornea. The authors discuss the importance of rinsing lenses, prior to insertion, to remove surface deposits of adsorbed disinfecting agents, but this also suggests that moving forward in this field, lenses should be included as part of the model for evaluating cytotoxicity.[6]

3. Tissue Culture

a. Animal cell culture models

One of the earliest tissue cell culture models developed for ocular irritancy screening was developed using a non ocular cell source. The Madin Darby Canine Kidney (MDCK) cell line is an epithelial cell line, with tight junction characteristics similar to the outermost layer of the cornea.[22] Since chemical irritants can weaken the tight junctions and permeabilize the corneal epithelium, allowing the toxic agent access to underlying tissue, this property is important in assessing the cytotoxic potential of solutions.[23] Several assays, neutral red release (NRR) and fluorescein leakage, have been designed to take advantage of this property and evaluate the cell-cell attachments.[23] The NRR assay evaluates cells that have been preloaded with neutral red dye. The assay can then assess the potency of a test chemical based on the ability of the solution to cause the preloaded cells to release the neutral red dye.[23]

Following treatment with test solution, amount of fluorescein diffusing across the epithelium can be evaluated using a plate reader. This assay was used to evaluate the effect on the integrity of the corneal epithelium following treatment with undiluted ReNu MPS, ReNu MultiPlus, Opti-Free, Opti-Free Express (with Aldox), Solo Care and Complete Comfort Plus for 20 minutes.

Opti-Free Express exhibited the greatest permeability, as indicated by the greatest fluorescence, which was significantly different from controls and all other solutions (Figure 3.3). Opti-Free was also significantly different from controls and all other solutions.

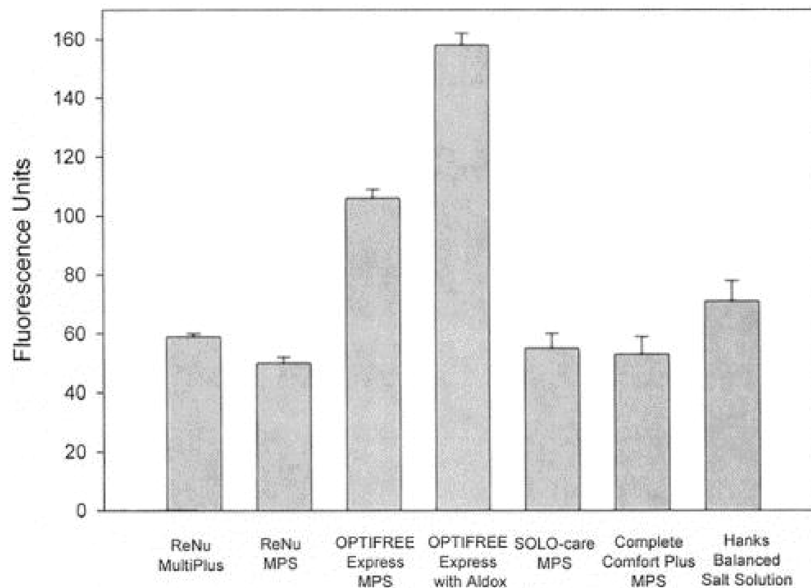


Figure 3.3: The effect of contact lens multipurpose solutions on MDCK epithelial permeability.[24]

Reprinted from Contact Lens Association of Ophthalmologists. Tchao R, McCanna DJ, Miller MJ. Comparison of contact lens multipurpose solutions by in vitro sodium fluorescein permeability assay. *CLAO J.* 2002; 28: 151-156, with permission from Wolters Kluwer Health.

Scanning electron microscopy (SEM) revealed that cells exposed to Opti-Free Express showed a loss of tight junctions and microvilli, and damaged cell membranes, including membrane blebbing and folding. Similar loss of tight junctions and cell membrane damage was observed in Opti-Free. All other solutions showed tight junctions and intact cell membranes, similar to that of controls. The SEM confirmation of compromised tight junctions and damaged membranes, allowed for correlation of the increased fluorescein permeability, by both Opti-Free solutions, to physiological damage to the MDCK cultures (Figure 3.4).[23] The authors postulate a new possible agent responsible for the toxicity and cell damage resulting in permeability. Both

Opti-Free solutions contain citrate buffer. It is possible that the presence of citrate buffer may cause a depletion of extracellular calcium, leading to the breakdown of tight junctions due to the disruption of the normal localization and incorporation of ZO-1, an essential tight junction protein, to the plasma membrane.[23]

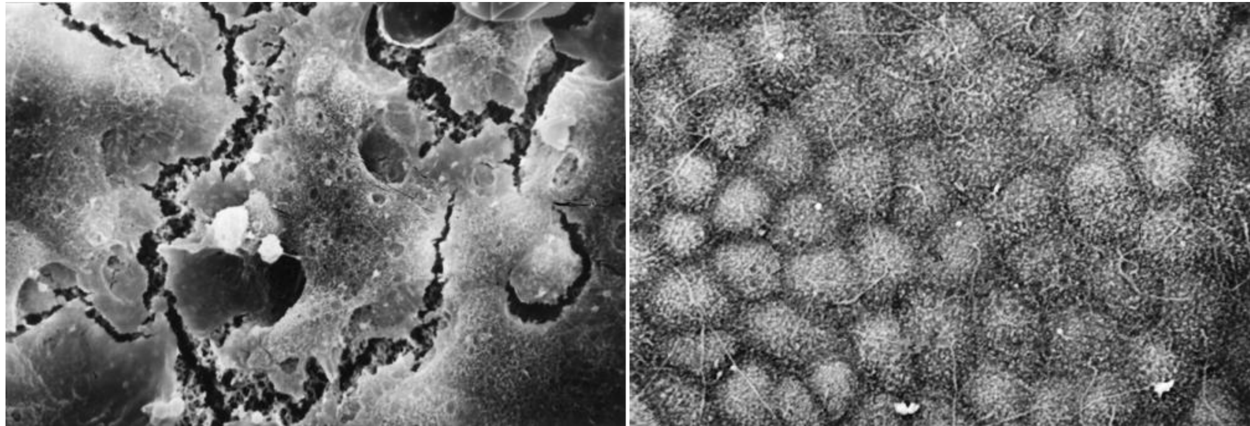


Figure 3.4: Scanning electron micrograph (SEM) view of MDCK cell monolayer culture exposed to OFX, Hanks' balanced salt solution (HBSS) (magnification × 1000). [24]

Reprinted from Contact Lens Association of Ophthalmologists. Tchao R, McCanna DJ, Miller MJ. Comparison of contact lens multipurpose solutions by in vitro sodium fluorescein permeability assay. *CLAO J.* 2002; 28: 151-156, with permission from Wolters Kluwer Health.

Several in vitro studies have also compared the cytotoxicty of MPS to that of contact lenses. It is important to not only consider the cytotoxicity of liquids (i.e. MPS), but also the toxicity of solids (i.e. contact lenses).[25] It is also essential to consider that the interaction between the solids and the liquids must be tested for cytotoxicity as well (i.e. contact lens – MPS combinations). Vaughan et al developed a cytotoxicity assay for use with contact lenses, where cells are placed in direct contact with the contact lens and the contact lens solution.[25] With minor modifications, this assay can also provide kinetic data to assess mechanism and rate of toxicity. A mouse fibroblast cell line was used; harvested cells were divided into two equal volumes, to allow for

the parallel testing of solutions and lens-solution combinations.[25] One portion of cells was further subdivided into enough tubes to resuspend cells in all test solutions. For the lens test run in parallel, the 2nd volume of the cell suspension were placed into the cup formed by a contact lens, and placed in a well of a culture plate. The polymacon contact lenses were soaked for 4 hours in the test solution, and blotted dry prior to use. The cells were incubated in the tubes and lenses for 15 – 240 minutes. A portion of the cells were removed and mixed with acridine orange and propidium iodide, to assess effect on viability.

The MPS containing 0.005% chlorhexidine, and 0.0005% chlorhexidine plus 0.001% thimerosal were all significantly toxic as solutions, but lenses soaked in these solutions were relatively safe. This indicates that lenses soaked in these solutions should be rinsed prior to lens insertion, to prevent any residual solution adsorbed on the lens surface from contacting the ocular surface.[25] Both Polyquad® 0.001% and PHMB 0.00005% did not show a cytotoxic effect in either of the assays. While Polyquad® was found to be relatively safe in this study,[25] many other studies disagree.[4,24,26,28,30] The discrepancy could be due to the difference in incubation times with test solutions. This study had shorter time points, and the assay may not be sensitive enough to detect differences at these earlier time points. What is more interesting is the result that the combination of a lens with the Polyquad® solution is also non-toxic, which is consistent with whole animal in vivo studies. One must consider that clinically, MPS only comes into contact with the corneal surface by deposits, which were adsorbed onto the contact lens during the overnight disinfecting cycle. It is important for in vitro models to model the clinical condition as close as possible to accurate predict toxicity, such as evaluating the effect of MPS release from a contact lens. This method is useful in that it can detect both the cytotoxic potential of

solutions alone, and solution-lens combinations, and thus aids in establishing the toxicity profile of a solution-lens combination.

While Polyquad® solutions were considered non-cytotoxic in the previous study,[25] another study using solution-lens combinations and direct contact in a mouse fibroblast cell culture model had a different result.[26] The study evaluated the cytotoxicity potential of AOSept, Solo-Care, Opti-Free Express (with Aldox®), ReNu Multi-purpose solution, ReNu MultiPlus and Complete, using the USP direct contact test and three modifications to the USP elution test: trypan blue uptake method, cell regrowth method and quantification of viable cells. While the previous study looked at lens contact with a cell suspension,[25] this study examined the effect of lens contact with a confluent monolayer.[26] Cells were scored for biological reactivity (cellular degeneration, cellular malformation, and trypan blue uptake by non-viable cells) and size of the zone of reactivity, where grades of 2 or less met the test requirements and were not considered cytotoxic, whereas grades 3 and 4 were considered cytotoxic.[26] Opti-Free Express was the only solution having a grade of 2 or higher on all tests, and was therefore, in contrast to the previous study, deemed cytotoxic. However, while both ReNu solutions did not score higher than 2 on any of the grading scales, which is in accordance to results from the previous study,[25] viability as determined by the cell count was similar to that of Opti-Free Express, at less than 50% of the saline control. This result indicated that while exposure does not damage cell membranes, the exposure did inhibit cell growth.[26]

Similar to using cell regrowth as a method of assessing cytotoxicity, colony-forming rate can also be used as a measure of a solutions potential toxicity.[5] Chinese hamster lung fibroblast V79 cell line is another accepted cell line used for testing of ocular toxicity.[5] Cultured fibroblasts

were exposed to diluted MPS at concentrations ranging from 1.25% to 10%. After 6 days of culture, cells were fixed with ethanol and stained with 2% Giemsa solution. Numbers of colonies, containing 50 or more cells, were counted and the colony forming rate was calculated for each test solution as a fraction of the negative control group. Of the solutions tested, only Complete and Menicare Soft did not significantly affect colony forming rate at any concentration, and were therefore considered non-cytotoxic. Solo and Opti-Free Express showed little to no cytotoxicity at 1.25%, but showed a dose-dependent increase in cytotoxicity. ReNu MultiPlus was highly cytotoxic at concentrations of 2.5% and higher, while ReNu MoistureLoc was cytotoxic at all concentrations tested. From their results, a scale of cyotoxicity was derived where Complete ≤ Menicare < Solo ≤ OFX < ReNu MultiPlus < ReNu MoistureLoc.[5] The study also looked at the effect that surfactants, buffering and isotonic agents had on the colony-forming rate, in an attempt to explain the scale of cytotoxicty. Of the surfactants tested, poloxamer 407 and Tetronic 1107 were found to be cytotoxic. These surfactants are components of Solo and ReNu MultiPlus, respectively, and may have contributed to their cytotoxicity. ReNu MoistureLoc contains both of these surfactants, which may account for the increased cytotoxicity compared to all other solutions and ReNu MultiPlus.[5] Of the buffering and isotonic agents, boric acid and 2-amino-2-methyl-1-propanol (AMP) were cytotoxic at concentrations of 0.1%. The presence of these agents in the ReNu solutions (boric acid only) and Opti-Free Express (both agents), may help to explain their cytotoxicity.[5] Previously, the presence of citrate buffer was hypothesized as the cytotoxic agent of Opti-Free Express, as it could disrupt tight junction proteins.[24] While citrate is still present, the addition of borate replaces citrate as the primary buffering agent. Other studies have also attributed the preservative Polyquad® as being the cytotoxic agent.[3,4,6,9,21,26-28] Neither of these components were included in this study,[5] so at the

present time, we are unable to compare the cytotoxicity potential of citrate or Polyquad®, nor conclude that any particular one is responsible for the cytotoxicity of OFX.

There has also been extensive debate regarding PHMB, which is the preservative in ReNu.While some assays find it to be cytotoxic[2,4,5,8], others do not.[24, 27-29] Both ReNu and Complete contain the same preservative, but very often have markedly different effects on cells. While it has been postulated that the different chemical composition of the two solutions may account for the difference, no study has addressed this issue. The Santodomingo study is the first study[5] that not only postulated that other constituents, such as buffering agents and surfactants, may be the cause of ReNu-induced cytotoxicity, but also evaluated the individual components. Like Polyquad®, PHMB was not evaluated in this study[5] and cannot be compared to boric acid, Tetronic 1107 and poloxamer 407.

Another indicator of cell growth (cell differentiation and tissue homeostasis) is gap junctional intercellular communication (GJIC).[29] This assay was first used in an earlier study[31] using V79 cells, which reported that the concentration which suppresses cell growth to 50% of control assays (IC50) was found to be the lowest for ReNu at 1.8 vol%, whereas both Complete and Opti-Free had values greater than 10%, indicating no inhibitory action on GJIC. This indicated that much lower concentrations of ReNu have greater cytotoxic effects on cultured V79 cells, compared to the other solutions tested, which are only cytotoxic at higher concentrations. Since the main disinfecting and surfactant agents (Table 3.2) of ReNu are PHMB and Tetronic 1107, these constituents were evaluated individually. It was found that Tetronic 1107 had the lowest IC50, at 0.25%; this data suggests that Tetronic 1107 is the component of ReNu

responsible for inhibition of GJIC. Poloxamer 407, another type of surfactant (Table 3.2), also had the same observed inhibitory effect as Tetronic 1107.[31]

Following this study, Sumide et al,[30] used a rabbit keratocyte cell culture model to assess the effects of MPS on GJIC using a Scrape Loading and Dye Transfer (SLDT) test. Confluent keratocytes were treated in a 35mm^2 dish, with diluted test solutions. After the incubation period with MPS, 10 scrapes were made in the cell monolayer with a steel blade scalpel, and 0.2% Lucifer yellow dye was added. The distance that the Lucifer yellow travelled was measured; if there was an adverse effect on the cells, the distance of the dye transfer would decrease due to inhibition of GJIC by the MPS (Figure 3.6).[30] The study evaluated the effect of both concentration and time. In the rabbit keratocyte cell culture model, ReNu again showed the greatest disruption of GJIC at concentrations from 0.3125% to 10%.[30] Inhibition only occurred at much higher concentrations of Complete and Opti-Free, at 2.5% and 10% respectively. The relevance of inhibition induced by these concentrations was not discussed. Western blot analysis revealed that in normal, untreated cells, 3 bands were present for connexin-43, which is a major gap junctional protein found in rabbit keratocytes. The results corresponded to the degree of phosphorylation. After 30 minute exposure to ReNu at 0.625%, the total level of connexin-43 was reduced, and further analysis revealed that there was a decrease in P_2, the highly phosphorylated form. After a 24 hour recovery period, total levels, including P_2, had recovered. The inhibitory action on GJIC may be related to the change from phosphorylated connexin to the dephosphorylated form, as phosphorylated connexin is important for accurate assembly and distribution of gap junctions.[30]

Further evaluation with PHMB, Tetronic 1107 and poloxamer 407 showed that Tetronic 1107 had the greatest inhibition at concentrations from 0.0313% to 10%, while inhibition was seen with 1% PHMB. This assay was sensitive enough to detect differences between Tetronic 1107 and poloxamer 407. Poloxamer 407 was similar to PHMB in that inhibiton of dye migration was only significant at a concentration of 1%.[30] While the authors conclude that the differences observed in poloxamer 407 is due to the increased sensitivity of the SLDT assay, it is difficult to conclude whether it is due to the assay or the cell line. The results from the V79 Sumide study[30] are in agreement with the work of Santodomingo et al[5] whose findings with the same cell line hypothesized that poloxamer 407 was the cytotoxic agent in SoloCare MPS. These results indicate the importance of cell source and type on the relevance of cytotoxicity testing.

Primary bovine corneal epithelial cultures are also commonly employed to characterize toxicity profiles of MPS. Pham and Huff[9] tested four solutions, ContaClair, Opti-Free Disinfecting, Rinsing and Storage Solution, ReNu Sensitive Eyes MPS and Lens Plus Saline, in a bovine model. Three assays were used to assess cytotoxicity following exposure to increasing concentrations of test solutions for 4 days. Lactate production assay was used to assess cell glycolytic activity or anaerobic profile.[9] An increase in absorbance was directly proportional to concentration of lactate.[9] AlamarBlue is a cell viability assay, previously described as an assay used to quantify cells based on reduction potential. Kenacid blue assay evaluates the total protein biomass. The binding of kenacid blue dye is directly related to protein content, and as such can also quantify cell number.[9] This battery of tests were all performed on the same well, and was very useful in being able to provide a wealth of information from a single well. The data was reported as log concentration curves, and the authors looked at the IC50, to compare the relative potency of test solutions. For all solutions, Alamar blue and kenacid blue were consistent in their

assessment of IC50. The lactate production assay was consistently higher, but only significantly higher in Opti-Free. All 3 MPS showed similar log concentration curves, in sharp contrast to the Lens Plus saline solution, which was not toxic to cell metabolism or proliferation at any of the concentrations investigated. For lactate production and cell respiration, Opti-Free had a significantly higher IC50, indicating that a higher concentration was needed to elicit the same toxic response observed in lower concentrations of Contaclair and ReNu (Figure 3.5).[9] The cytotoxicity ranking determined from this battery of tests was Lens Plus Saline << Opti-Free ≤ Contaclair ≤ ReNu.[9]

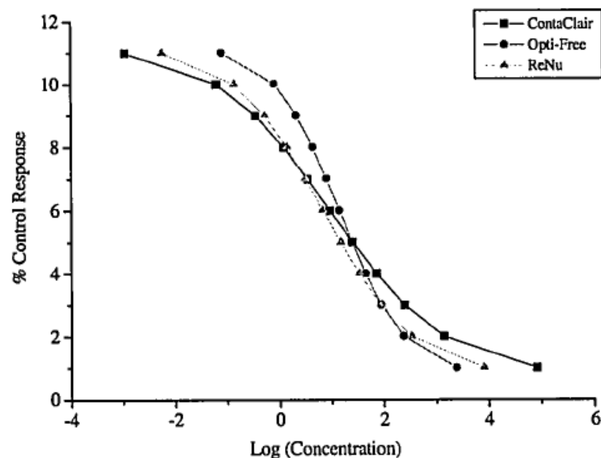


Figure 3.5: Log concentration response curves for contact lens solutions determined by the lactate production assay. [9]

Reprinted from Contact Lens Association of Ophthalmologists. Pham XT, Huff JW. Cytotoxicity evaluation of multipurpose contact lens solution using an in vitro test battery. *CLAO J.* 1999; 25: 28 – 35, with permission from Wolters Kluwer Health.

The cytotoxicity of ReNu and Contaclair may be contributed to by the presence of both disodium edetate (EDTA) and boric/borate buffer.[9] The toxic potential of borate has been demonstrated before,[5,31] but EDTA is a component of MPS that has not been investigated as thoroughly. EDTA can chelate Ca^{2+} and Mg^{2+} and may compromise normal cellular functions, by

inducing cell rounding, leading to detachment.[9] While EDTA is common among most MPS, ReNu has the highest concentration of EDTA compared to any given solution currently on the market.[9] At 0.1%, the concentration of EDTA in ReNu is 2 times that in OFX, 4 times that in Solo, and 10 times that of Complete.[9] EDTA needs to be more thoroughly researched, as it is one component of ReNu and other PHMB based solutions which is highly variable. PHMB was also presumed a cause of the metabolic and structural damaged that inhibited cell proliferation.[9] The results with Opti-Free were consistent with in vivo observations by Begley et al,[2] but in disagreement with other tissue culture models discussed previously.[4,24,26,28,30] The explanation may be due to a change in formulation. The Opti-Free solution used in this study[9] and the Begley study[2] contains citrate buffer and PolyQuad. More recent studies use a new formulation of Opti-Free that contain borate buffer as the primary buffering agent, which has largely been speculated as a cytotoxic agent, as well as Aldox and other new surfactants (such as TearGlyde). Solutions themselves should not solely be deemed cytotoxic, but rather the specific formulation or constituents. The change from a citrate only solution to a solution with both citrate and borate buffer may be responsible for the changing trend of Opti-Free cytotoxicity observed in other studies.

A study by Cavet et al, also used an in vitro bovine corneal epithelial model, with the newly formulated Opti-Free Express and RepleniSH.[29] The primary focus of this study was in an immortalized human cell line, which is discussed in the next section, but the primary bovine cultures demonstrated results in stark contrast to the previous study, but consistent with the observations of the newly formulated Opti-Free solutions as made by several studies.[15,17,33] A significant reduction in ATP content of 40% or more was observed with both Opti-Free Express and RepleniSH. An LDH release assay assessing membrane integrity also detected a significant

increase in release from cells exposed to both Opti-Free solutions.[29] The data suggested that the evolution in the formulation of Opti-Free solutions has contributed to its increasing cytotoxicity potential. Table 3.1 (page 25) highlights the changes in formulation and the solutions, which have replaced older generation MPS.

b. Immortalized human cell culture models

Animal cell line and primary cell cultures are valuable for assessing morphological changes, cell degeneration, cell death and other parameters of ocular irritation and inflammation, but there can be considerable variation in donor material and primary cultures, low reproducibility and a short lifespan.[33-35]With most animal cell lines, there is often little relevance to humans due to differences in ocular anatomy, physiology and biochemistry. In primary cultures or isolated tissue models, cells can become senescent after several passages, therefore, having a restricted lifespan.[33-35] The availability of donor material for such models is uncertain.[33]An immortalized human cell line, that retains features of the original tissue, would be an ideal system to model the human ocular surface in vitro and study mechanisms underlying ocular toxicity.[1] There are many advantages to an immortalized human system: continual passability, storage and revitalization, ease of handling, reproducibility, among others.[33-35] These cell lines can be acquired by transfection or infection of primary cultures with oncogenic DNA viruses or essential fragments of host DNA.[33-35]

Between 1993 and 1999, Kahn, Araki-Sasaki and Offord,[33-35] all developed immortalized human corneal epithelial cell lines, with the intention of modelling the ocular surface for toxicity and irritation studies. These lines have been used extensively in ocular toxicity work, specifically evaluating MPS. The cell lines have been characterized based on differentiation and

metabolism.[33-35] In knowing that the cell lines can stably express a broad spectrum of metabolic enzymes, cytokines and growth factors, a battery of tests can be performed to not only assess toxicity and inflammation, but also investigate mechanisms.

Araki-Sasaki cells were used in a study to evaluate the effect of MPS on cell viability, apoptosis rates, and integrity of tight junctions.[3] Test solutions included Complete EasyRub, Opti-Free Express, Opti-Free RepleniSH and ReNu MultiPlus diluted with culture medium. To assess cell viability, an MTT assay was used. The MTT assay is a colorimetric assay based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).[36] It can be bioreduced in metabolically active cells into a purple formazan product.[36] Only functional mitochondria can convert MTT into this product, and the changes in absorbance related to the formation of formazan can be detected using a micro plate reader.[36] Apoptosis rates were evaluated using a Tunel (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) DNA fragmentation assay. This assay used an antidigoxigen antibody conjugated to a fluorescein reporter molecule to identify changes in DNA fragmentation. Cells were exposed to test solution for 30 minutes and Tunel assay was performed using ApopTag fluorescein in situ apoptosis detection kit. The cells were fixed and incubated with TdT enzyme. Positive and negative cells were counted manually at 100x magnification. Fluorescein permeability and tight junction protein staining were used to evaluate the effect of MPS on tight junctions. Like the MDCK assay[23], cells were grown in filters and the amount of fluorescein that permeated the cell monolayer following treatment was quantified fluorometrically. Tight junction protein staining was performed following 2 and 6 hour incubation with the test solution (Figure 3.6). The MTT assay revealed significant differences in viability after 6 hour exposure to Opti-Free Express, Opti-Free RepleniSH and ReNu. OFX and ReNu showed a time dependent decrease in viability.

Tunel assay revealed that OFX, OFR and ReNu also had a significant increase in apoptotic cells, at 16.5%, 12.6% and 10.2% respectively. ReNu and OFX had the greatest amount of permeability, with OFR showing a lesser degree of permeability. In the histochemical analysis, all 3 solutions also showed significant disruption of ZO-1 and occludin tight junction proteins.

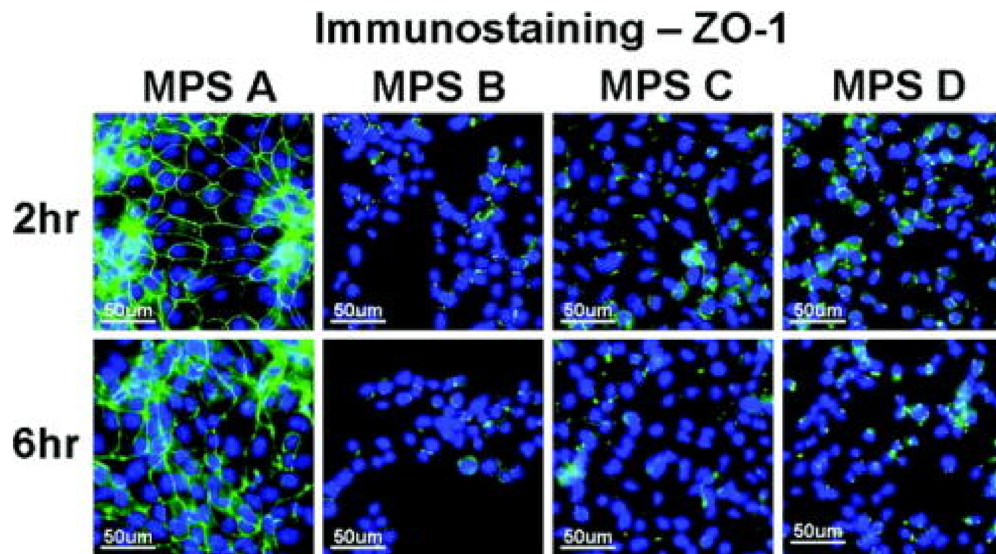


Figure 3.6: Representative images of immunofluorescent staining for ZO-1 (green) in human corneal epithelial cells after 2 and 6 hrs exposure to each of the 4 MPS studied. Strong green staining between the cells indicates intact ZO-1 tight junction proteins while the weak or negative staining shows disrupted junctions. Hoechst 33342 (blue) was used for cell nuclear counterstaining.[3]

Reprinted from Eye & Contact Lens. Chuang EY, Li DQ, Bian F et al. Effects of contact lens multipurpose solutions on human corneal epithelial survival and barrier function. *Eye Contact Lens.* 2008; 34: 281-286, with permission from Wolters Kluwer Health.

Cell membrane integrity can also be evaluated using a NRR assay, as previously described in its use in MDCK cells.[24] This assay was consistent with a previous study,[27] in finding that 24 and 48 hour exposure of transformed HCEC to ReNu and Opti-Free Express significantly affected cell membrane integrity, at concentrations of 5 and 10ppm. Solo and Complete did not have a significant adverse effect at any time point or concentration.[27]

Using SV-40 immortalized HCEC to evaluate tight junctions yielded a different response. Opti-Free Express again showed the greatest amount of permeability, a loss of tight junctions, decreased metabolic activity (Figure 3.7), and the appearance of damaged cell membranes, including membrane blebbing and folding.[28] In this study, ReNu did not have adverse effects on the HCEC monolayer. The results from the McCanna study[28] are consistent with the MDCK study,[24] and more relevant given the use of human cells, but the finding that ReNu did not have an adverse effect is in disagreement with previous studies.[3,5,9,26]

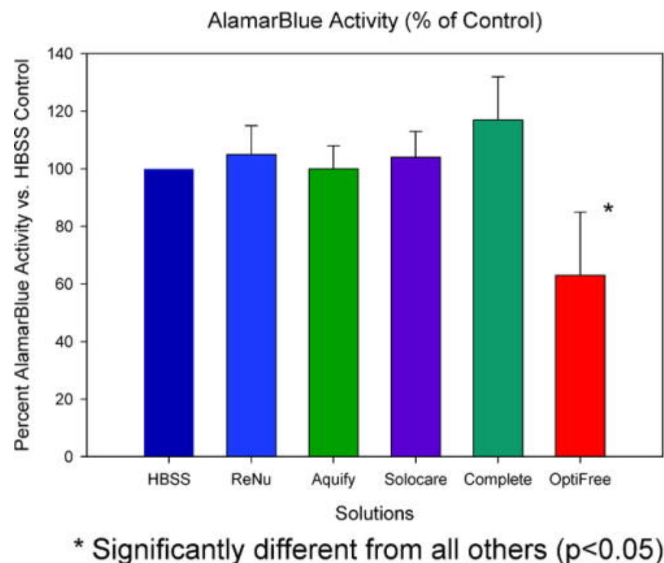


Figure 3.7: The results of alamarBlue assay after treating the cultures with various solutions. The results represent three separate experiments and are expressed as a percentage of the Hank's balanced salt solution activity. A reduced percentage, as seen with the Opti-Free Express–treated cultures, represents a toxic effect on cells.[28]

Reprinted from Eye & Contact Lens. McCanna DJ, Harrington KL, Driot JY, et al. Use of a human corneal epithelial cell line for screening the safety of contact lens care solutions in vitro. *Eye Contact Lens* 2008; 34: 6-12, with permission from Wolters Kluwer Health.

The difference may potentially be accounted for by the difference in exposure time. For the previous studies, by Chuang et al[3] and Wright et al,[27] cells were exposed to test solutions for 6 hours, and 24 – 48 hours, respectively. In contrast, the McCanna study[28] treated cells with the

test solutions for only 15 minutes. The short time period may not have been long enough for the solution to have an effect. Additionally, the assays may not have been sensitive enough to detect smaller differences at such short time periods. In the Wright study, they note that their assays were more sensitive at the longer time point of 48 hours.[27] It can be argued that shorter treatment periods are justified by the fact that blinking and the constant regeneration of the tear film may dilute the effects of the biocides in vivo. However, it should be considered that the uptake and slow release by the contact lens will increase the exposure time to the cornea, which further warrants the importance of evaluating longer exposure to test solutions and solution release from a contact lens in vitro, possibly at lower concentrations than that typically evaluated. Characterization of the lens release profile would provide information about rate of release and concentration of released biocides, which would then provide experimenters with the ideal concentration to evaluate.

Another recent study using immortalized human cell lines also looked at the effect of MPS on brief time courses.[29] Cells were exposed directly to 100% ReNu MultiPlus, Opti-Free Express, Aquify and Opti-Free RepleniSH over a 2 hour time course, or to increasing concentrations, diluted in medium, between 20-100% for 2 hours. ATP quantitation and resazurin reduction were employed as measures of cell viability, using luminescence and fluorescence to estimate cell number. Both assays indicated that exposure to Opti-Free Express and Opti-Free RepleniSH reduced viability, in comparison to medium control for all concentrations, and to all test solutions for concentrations greater than 40%. ATP content was only significantly reduced by ReNu and Aquify at 100% concentration, but effects were still minimal compared to Opti-Free products. In examining that effect over time, Opti-Free Express and RepleniSH had a significant decrease in viability at 15 minutes. ReNu and Aquify did not

differ from medium controls at any timepoint. This study also used primary bovine cultures to confirm that the results obtained from the immortalized cell line were not limited to transformed cells.[29] The Opti-Free products showed a greater adverse effect compared to ReNu, which the authors concluded is supported by the literature, but there is a large body of industry and non-industry sponsored studies that demonstrates the cytotoxicity of ReNu is comparable and in some cases greater than that of Opti-Free.[3,5, 9,15,30] Like the McCanna study,[28] the cells were exposed to the test solutions for a very short time, and may not be accurately predicting the full cytotoxic potential.[29]

A study by Imayasu et al[15] also looked at the effect of MPS on structure and barrier function of epithelial tight junctions, while specifically comparing borate buffer based MPS; ReNu MultiPlus, ReNu MoistureLoc and Opti-Free Express, to unbuffered Menicare Soft.[15] Integrity of tight junctions was assessed by immunohistochemistry, transmission electron microscopy (TEM) and transepithelial electrical resistance (TER). Cells were exposed directly to test solutions for 60 minutes, prior to incubation with ZO-1 antibody. For TEM, cells were only exposed to test solutions for 15 minutes before being fixed. Similar to NaF permeability studies, cells were grown in filters in wells to measure the TER. The test solution was added to each filter, while the well remained full of serum free medium. TER readings were measured, at time 0, 30, 60 and 120 minutes. Confocal analysis of tight junctions found that all three buffered solutions showed partially destructed ZO-1 structure, with discontinuous, wide spreading intercellular spaces. Menicare showed a normal, continuous distribution of ZO-1 along cell-cell borders. By TEM, it was also observed widely opened junctions after exposure to ReNu MultiPlus and Opti-Free Express; whereas ReNu MoistureLoc had both partially opened and tightly closed tight junctions. The quantitative results from the TER assay also confirmed these findings. Menicare

and ReNu MoistureLoc showed no difference in electrical resistance over time. ReNu MultiPlus and Opti-Free Express showed a significant reduction in resistance, and on collagen membranes, lost all electrical resistance after 120 minutes. The results suggest that ReNu MultiPlus and Opti-Free Express, and to a lesser extent ReNu MositureLoc, have the potential to cause destruction of barrier function. The authors hypothesize that the adverse effect is likely mediated through the MAPK pathway.[15] Activation of this pathway could lead to destruction of tight junction structure and epithelial barrier function,[15] but this pathway needs to be specifically studied before making any definitive conclusions.

The adverse effect of ReNu MultiPlus, Opti-Free Express and ReNu MoistureLoc may be due to the concurrent use of boric acid as a buffer and the poloxamine family of surfactants.[15] The authors hypothesized that these components may be responsible for the adverse effect on tight junctions. Their hypothesis was further supported by work by Santodomingo et al,[5] who demonstrated the cytotoxic potential of various buffers, surfactants and isotonic agents found in MPS, as discussed previously. The authors tested their hypothesis using 1% diluted poloxamine and boric acid (Figure 3.8). While poloxamine showed almost no effects on tight junctions (Figure 3.8A), the boric acid showed the same discontinuous partially destructed ZO-1 structure, which was observed with the three buffered solutions (Figure 3.8B). While the evidence from ReNu MultiPlus and Opti-Free Express strongly suggests that boric acid may be responsible for the adverse effects on tight junctions, more quantitative work is necessary. In addition, viability assays need to be included in this series of cytotoxicity testing, to be able to correlate whether destruction of tight junctions and TER is associated with cell death.

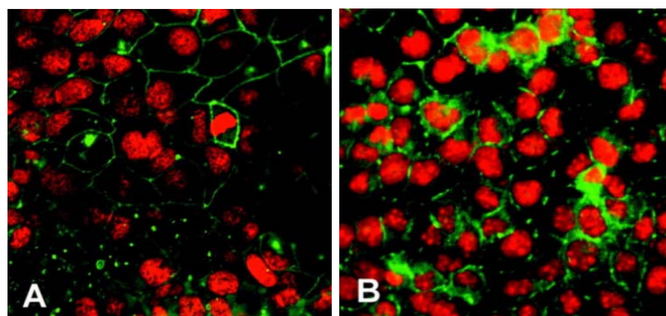


Figure 3.8: Confocal laser scanning micrographs of ingredient-treated human corneal epithelial cells stained with ZO-1 antibody and counterstaining with propidium iodide (original magnification, 400×). (A) Treatment with 1% poloxamine for 60 minutes. (B) Treatment with 1% boric acid for 60 minutes.[15]

Reprinted from Eye & Contact Lens. Imayasu M, Shiraishi A, Ohashi Y, et al. Effect of Multipurpose Solutions on Corneal Epithelial Tight Junctions. *Eye Contact Lens.* 2008; 34: 50-55, with permission from Wolters Kluwer Health.

Recently, the use of stratified cultures has been used to more closely model the multilayered cornea.[37] The study evaluated the validity of in vitro toxicity testing measures, by comparing monolayer and stratified HCEC cultures.[37] The authors hypothesized that monolayer and stratified culture models will yield different results, using traditional cytotoxicity assays. While the results suggested that monolayer cultures appeared to be more sensitive to MPS exposure, there was only a direct comparison of the two models on the basis of morphology and viability by fluorescence microscopy, which were both analyzed subjectively by imaging software. Without a direct comparison of monolayer and stratified cultures using objective assays, it is difficult to call into question the validity of monolayer in vitro modelling systems.

Contact lens wear and use of contact lens solutions can also cause reactions on the conjunctival surface.[38] Several recent studies have been undertaken to examine whether MPS can also damage human conjunctival cells. The earliest of these studies looked at the effects of Opti-Free, ReNu, SoloCare and Titmus.[38] The primary conjunctival fibroblast cultures were treated for 24 hours with varying concentrations of test solutions. The effect of the solution was

evaluated using the MTT assay and a cell analysis was performed using the CASY-1 system to obtain an absolute cell count. At 1%, Opti-Free and Titmus demonstrated a siginificant reduction of mitochondrial activity, as detected by the MTT assay. At 5%, all solutions exhibited a significant effect. No significant effect was detected at concentrations lower than 1%. Similar trends were seen with the absolute cell count, where cell number decreased with increasing concentration, although this data was not significant.

A similar study was conducted using an immortalized human conjunctival cell (HCC) line. They also evaluated cell viability, but in this study membrane integrity (neutral red test) and intracellular redox status (alamar blue test) were used as measures of conjunctival viability. In addition, the study evaluated oxidative stress, by production of reactive oxygen species (ROS) and superoxide anions. A focus was also placed on changes to the mitochondrial mass and membrane potential, and activation of the cell death receptor P2X7. Mitochondrial mass was assessed using the NonylAcridine Orange test, which uses a dye to stain lipids found specifically in the mitochondrial inner membrane. Mitochondrial potential was measured using the JC-1 test. Red fluorescent J aggregates are detected when mitochondrial activity is high; green fluorescent J monomers are detected when mitochondrial activity is low.[14] Activation of P2X7 cell death receptor was detected using YO-PRO-1, which was previously used in an isolated tissue model to visualize the nuclei and identify apoptotic-related alterations in the nuclear membrane by confocal microscopy.[21] This fluorescent DNA probe can enter the apoptotic cells via activated P2X7 pores, and can quantify apoptotic cells in a population by flow cytotmetry. [39] P2X7 receptors are non-selective cation channels, known to induce apoptosis.[14] They facilitate the influx of extracellular calcium, which can contribute to the generation of ROS.[14] The results found that at 15 minutes only OFX induced a significant loss of membrane integrity and decrease

of intracellular redox potential. It was also demonstrated that OFX could stimulate an overproduction of ROS and anion superoxides. At the 3 hour time point, OFX was even more cytotoxic in terms of these parameters. ReNu and Solo also induced a significant decrease in cell viability and redox potential, at 3 hours only. At 3 hours, Solo induced an overproduction in ROS, while Complete and ReNu induced a decrease. For both time points, mitochondrial mass was found to increase with OFX exposure, and decrease with ReNu and Solo exposure. Increased mitochondrial size, forming megamitochondria, is an indicator of oxidative stress. OFX caused a decrease in mitochondrial potential, and induced activation of the P2X7 receptor, adding support to the hypothesis that oxidative stress can lead to apoptosis. The authors hypothesize that the concentration of the biocide is responsible for the differences in oxidative stress and apoptosis. The concentration of preservatives such as Polyquad® in OFX (0.001%) is 10 times that of PHMB in ReNu, Solo and Complete (0.0001%).[14] Solo also caused P2X7 receptor activation, and further study into mechanism of Solo induced apoptosis was necessary. While Solo has the same preservative as ReNu and Complete, the difference in cytotoxicity may be due to the difference in buffers, EDTA concentration or PHMB polymer size as previously discussed.[14]

Similarly, the same cell line was used to evaluate caspase 3 activity and chromatin condensation, induced by OFX and Solo. Hoescht 33342 and propidium iodide (PI) were used to image chromatin condensation, a morphological change characteristic of apoptosis.[40] Hoescht can enter living and apoptotic cells, whereas PI can enter necrotic cells.[40] Both OFX and Solo significantly induced caspase 3 activity, with OFX inducing levels similar to the apoptosis inducer, camptothecin. Both OFX and Solo also caused a significant increase in chromatin

condensation, 8.8x and 2.6x, and activated P2X7 cell death receptors, 9.8x and 1.9x, respectively.

The finding that Solo also induced caspase 3 activity is contrary to results from our own laboratory work with HCEC, where only OFX activated caspase 3. The difference may be accounted for by two reasons; cell type or concentration. While conjunctival cells may come into contact with residual solution on lenses post insertion, the main contact is with the corneal epithelium.[3] The different cell types may have different physiological responses to toxic solutions, and/or conjunctival cells may be more sensitive to solutions compared to epithelial cells. If such is the case, conjunctival cells are an inappropriate cell line to model the mechanisms of toxicity in epithelial cells. This is not to say that the effect on conjunctival cells is to be dismissed, only that drawing conclusions about epithelial cells from conjunctival models may not be entirely accurate. Another key difference between the studies is the concentration of test solution exposed to the cell cultures. The Dutot study used the undiluted solution directly, whereas the study from our laboratory looked at the impact of solution released from silicone hydrogel contact lenses. The concentration of solution released from contact lenses is very likely to be much less than 100%, so the use of undiluted solution directly overestimates the concentration the ocular surface would normally be exposed to and may account for the increased activity caspase 3 induced by Solo.

4. Conclusions (Tiered Testing Strategies)

The literature reviewed demonstrates a wealth of knowledge in the field of ocular toxicity testing, using whole animal, isolated tissue and tissue culture models. Table 3.3 summarizes the models and assays used to assess ocular toxicity and biocompatibility of MPS. While there are

still many differences between the findings from in vivo and in vitro testing strategies, in vitro testing, using tissue culture, is becoming increasingly accepted as a valid tool for toxicology and biocompatibility studies.[34,41-43] As discussed, many laboratories currently employ this model to screen for the potential cytotoxicity of multipurpose solutions.

Currently, there is no single in vitro test that has been accepted as an alternative to the Draize test and the prospect of eliminating whole animal testing is a challenge.[1] A stepwise system employing all three models may help to address the criticisms and concerns surrounding the Draize test. The first step would involve a detailed literature review of the physiochemical and biotoxicological data of the proposed compounds, and related substances.[1] Skin irritancy would be assessed in the next step for compounds found by the literature review to not be toxic.[1] Following dermal irritation testing, an in vitro test battery can be performed with compounds found to be non-corrosive to the skin, using whole tissue or tissue culture models.[1] Specifically, cultured corneal epithelial cells are advantageous for irritancy screening.[34,41-43] CEC are potentially more predictive of ocular irritancy responses compared to other cell lines.[9] These cultures can be used to study mechanisms of cytotoxicity, which cannot be investigated easily in vivo, or clinically.[9] In vitro models are reproducible, sensitive, relatively fast, easy to execute, cost effective, and have standardized endpoints for easy comparisons.[1,9, 27,29] Compounds, which display non-serious irritancy can then be tested on one animal, using the Draize test, before repeating with a large scale in vivo study.[1] Use of the stepwise system reduces sample size in whole animal studies based on information acquired in the previous tier or step; in vitro cell culture and isolated tissue systems can also provide mechanistic data on toxic effects.[1] In using all three systems collectively, we can acquire a wealth of knowledge about the safety and efficacy of new ocular drugs or MPS.

Table 3.3: Measures for assessing ocular toxicity and biocompatibility of MPS (Adapted from Wilhemus, 2001)

Measures for Assessing Ocular Toxicity and Biocompatibility of Multipurpose Solutions			
System	Endpoint	Assays	Studies
Whole Animal	Morphology	Observational scoring of eye irritation	Begley CG et al, 1994
		Corneal epithelial dye staining	Labbe A et al, 2006
		Corneal dye permeability	Labbe A et al, 2006
		Epithelial thickness	Chang JH et al, 1999
		Stromal thickness	Chang JH et al, 1999
		Surface epithelial cell size	Chang JH et al, 1999
		Confocal microscopy	Chang JH et al, 1999; Labbe A et al, 2006
		Impression and exfoliative cytology	Labbe A et al, 2006
	Physiology	Corneal epithelial regeneration rate	
		Tear flow	Chang JH et al, 1999; Labbe A et al, 2006
		Tear constituents	Chang JH et al, 1999
Isolated tissue (eye, cornea, lens)	Morphology	Observational scoring	Choy KMC et al, 2009
		Corneal dye permeability	Choy KMC et al, 2009
		Confocal microscopy	Bantseev V et al, 2007
		Lens optics	Sivak JG et al, 1995; Oriowo et al, 2006
		Nuclear size, shape and appearance	Bansteev V et al, 2007
	Physiology	Mitochondrial function (e.g., resazurin/alamarBlue reduction)	Oriowo et al, 2006
Cell culture (tissue-cultured cells, artificial cornea; primary and immortalized)	Morphology	Cell density	
		Cell size and shape	Tchao R et al, 2002; Mowrey-McKee M et al, 2002
		Cell-cell contacts (e.g., ZO-1)	Chuang EY et al, 2008; Imayasu M et al 2008
		Nuclear size, shape and appearance	
	Viability	Dye (e.g., acridine orange) uptake	Vaughan JS et al, 1993
		Dye (e.g., trypan blue) exclusion	Mowrey-McKee M et al, 2002
		Cell count	Mowrey-McKee M et al, 2002; Horwath-Winter J et al, 2004
		Mitochondrial function (e.g., MTT dye reduction, alamar blue/resazurin reduction, etc.)	Tanti NC et al, 2009; Pham XT et al, 1999; Horwath-Winter J et al, 2004; Chuang EY et al,

		2008; McCanna DJ et al, 2008; Cavet ME et al, 2009
Adhesion	Cell-substrate attachment (e.g. CD29, CD49c, CD104, etc.)	Tanti NC et al, 2009
	Cell-cell adhesion (e.g. ZO-1, occludin)	Chuang EY et al, 2008, Imayasu M et al, 2008
	Gap junction efficiency (e.g. lucifer yellow)	Sumide T et al, 2002
Proliferation	Colony formation and efficiency	Santodomingo-Rubido J et al, 2006; Pham XT et al, 1999
	Cell regrowth following exposure	Mowrey-McKee M et al, 2002
	DNA and RNA changes	Chuang EY et al, 2008
Membrane Integrity	Dye (e.g., fluorescein and neutral red) leakage	Shaw AJ et al, 1991; Tchao R et al, 2002; Chuang EY et al, 2008; McCanna DJ et al, 2008
	Chemical (e.g., ethidium bromide) uptake	
	Transepithelial electrical resistance	Imayasu M et al, 2008
	Phospholipid symmetry (e.g. annexin-V)	Choy KMC et al, 2009; Tanti NC et al, 2009
Metabolism	Intracellular protein content	Pham XT et al, 1999
	Chemical (e.g., neutral red, etc.) uptake	
	Chemical (e.g., glucose, calcium, etc.) utilization	
	Rate of normal molecular (e.g., protein) synthesis	
	Output of normal metabolic products (e.g., ATP, NADPH, ions, plasminogen activator, enzymes, etc.)	Cavet ME et al, 2009
	Output of abnormal metabolic products (e.g., C-reactive protein, lactate dehydrogenase, reactive oxygen species, etc.)	Pham XT et al, 1999; Dutot M et al, 2008; Cavet ME et al, 2009
	Mitochondrial function (e.g., MTT dye reduction, alamar blue reduction, etc.)	Tanti NC et al, 2009; Pham XT et al, 1999; Horwath-Winter J et al, 2004; Chaung EY et al, 2008; McCanna DJ et al, 2008; Cavet ME et al, 2009; Epstein SP et al, 2009
	Mitochondrial physical properties (e.g. mass,	Dutot M et al, 2008; Tanti NC

		membrane potential, etc.)	et al, 2009
	Gene expression	Intercellular communication proteins (e.g., connexin-43)	Sumide T et al, 2002
		Apoptosis proteins (e.g., procaspases, caspases, etc.)	Dutot M et al, 2009; Tanti NC et al, 2009
	Apoptosis	Caspase activation	Dutot M et al, 2009; Tanti NC et al, 2009
		Cell death receptor activation (e.g., P2X7)	Dutot M et al, 2008
		Phospholipid membrane symmetry (e.g. annexin-V)	Choy KMC et al, 2009; Tanti NC et al, 2009
		DNA fragmentation	Chuang EY et al, 2008

CHAPTER 4

THE IMPACT OF MULTIPURPOSE SOLUTIONS RELEASED FROM SILICONE HYDROGEL LENSES ON CORNEAL EPITHELIAL CELL VIABILITY, ADHESION PHENOTYPE AND APOPTOTIC PATHWAYS *IN VITRO*

INTRODUCTION

Ocular toxicology and biocompatibility studies have been increasingly conducted using corneal epithelial cell culture models and many laboratories currently employ this model for screening of new compounds and assessing potential safety and ocular irritation issues.[1-3] Recently, cell culture models have been used to screen for the potential cytotoxicity of multipurpose solutions used to clean and disinfect contact lenses.[4-7]

The disinfecting properties of multipurpose solutions (MPS) are conferred by the active biocide, which are commonly a polyquaternium, biguanide or hydrogen peroxide agent.[8] MPS also contain a buffering solution, which is typically either borate or phosphate-based.[8] The physical properties of the chemical polymers used in hydrogel and siloxane-based contact lens materials allow for the adsorption and absorption of components of these care regimens during the overnight disinfection cycle, followed by potential release onto the corneal surface during lens wear. Certain combinations of MPS and silicone hydrogel lenses can lead to a cytotoxic effect in vitro[4-6] and some combinations have the potential to exhibit excessive corneal staining in vivo.[8-15] As a result of such observations, many biocompatibility studies have been undertaken to examine the effect of ophthalmic solutions, including contact lens packaging solutions and multipurpose solutions, on corneal and conjunctival cells.[5-7, 16, 17] Many of these biocompatibility

studies have used extracts or dilutions of the care regimens to evaluate the potential corneal effect, or have used non-corneal cell types as a proxy. These studies were able to evaluate the potential cytotoxic effect of ophthalmic and multipurpose solutions in vitro, and while this is valuable research, there is currently limited information on the effect of the direct release of solutions from silicone hydrogel lenses on human corneal epithelial cells. Previous studies have shown that lens type will affect uptake and release of compounds contained in MPS,[18] and this may also have an impact on any subsequent cytotoxic responses observed. Thus, to assess the cytotoxicity of agents contained in multipurpose solutions that would be released by contact lenses, a model in which the contact lens was directly set onto a monolayer of corneal cells was developed and corneal cell viability, adhesion phenotype and caspase activation were assessed following exposure to the care-regimen soaked lens materials.

MATERIALS AND METHODS

Reagents and antibodies

Keratinocyte serum free medium, growth supplement (Bovine Pituitary extract) and pen-strep solution were purchased from ScienCell (Carlsbad, CA, USA). All other cell culture reagents, Dulbecco's minimum essential medium, fetal bovine serum, phosphate buffer saline and TriplExpress were purchased from Invitrogen (Burlington, Ontario, Canada). Monoclonal antibodies to β_1 integrin (CD29, Immunotech-Coulter, Marseilles, France) and β_4 integrin (CD104, Serotec, Mississauga, Canada) were fluorescein isothiocyanate (FITC) conjugates. The monoclonal antibody against α_3 integrin (CD49c, Serotec, Mississauga, Ontario, Canada) was a R-phycoerythrin (PE) conjugate. Parafomaldehyde was purchased from Fisher Scientific (Ottawa, Ontario, Canada) and all other chemicals used to prepare Hepes Tyrode Buffer were of analytical or reagent grade.

Contact lenses and multipurpose solutions

Two silicone hydrogel lens materials were tested: lotrafilcon A (LA from CIBA Vision, Duluth, GA, USA) and balafilcon A (BA from Bausch & Lomb, Rochester, NY, USA). All lenses were purchased in their original packaging, had a diameter between 14.0 and 14.2 mm and a curvature of 8.5 to 8.7mm. Four polyquaternium and biguanide preserved multipurpose solutions were tested and their composition is described in Table 4.1.

Table 4.1: Disclosed composition of the MPS used in the study.

Manufacturer	Brand (abbreviation)	Disinfecting Agent	Buffer	Other reported agents (surfactants and chelating agents)[19]
Alcon	Opti-Free Express (OFX)	Polyquad 0.001%, Aldox 0.0005%	Borate	Sorbitol; citrate (citric acid), 0.05% EDTA; poloxamine (Tetronic 1304)
AMO	Complete Moisture Plus (Complete)	PHMB 0.0001%	Phosphate	Taurine; 0.01% EDTA; Poloxamer 237 (Pluronic F87); HPMC 0.15%; propylene glycol
Bausch & Lomb	ReNu MultiPlus (ReNu)	PHMB 0.0001%	Borate	Sodium borate; Hydroxyalkylphosphonate (Hydranata); 0.1% EDTA; Poloxamine (Tetronic 1107)
CIBA Vision	SoloCare Aqua (Solo)	PHMB 0.0001%	Tris	Sorbitol; 0.025% EDTA; dexpanthenol (provitamin B5); Pluronic F127 (poloxamer 407)

PHMB: polyhexamethylene biguanide (also known as polyhexanide, Dymed, polyhexadine, and polyaminopropyl biguanide)[19]

In vitro cell culture

Immortalized human corneal epithelial cells (HCEC)

SV40-immortalized human corneal epithelial cells were cultured in keratinocyte serum free medium supplemented with bovine pituitary extract, recombinant epidermal growth factor and pen-strep (KSFM). Fresh medium was added every other day and cells were grown to 90% confluency in tissue culture treated flasks. Adherent cells were removed using a dissociation solution, TriplExpress (Invitrogen, Burlington, Ontario, Canada). Cells were routinely observed for any morphological changes.

In vitro model

A direct contact in vitro model was used.[7] HCEC were seeded onto a 24 well tissue culture treated polystyrene (TCPS) plate at 10^5 cells per well. Cells were left to adhere overnight

(18-24 hours) in a humid CO_2 incubator, which resulted in the formation of a monolayer of HCEC. Simultaneously, 2 mL of MPS was added to the wells of a sterile 12-well polystyrene plate (BD Falcon, Mississauga, ON, Canada). Using sterile tweezers, SH lenses were gently removed from their blister pack and added to the wells containing MPS. SH were totally immersed and left to soak for 18 – 24 hours. All lens-solution soaking combinations were performed under sterile conditions.

The next day, supernatant was removed from the cells and fresh serum-free medium (700 μL) was added. MPS-soaked SH lenses were placed gently on top of the monolayer, face-down, with the concave surface facing upwards and incubated for 8 and 24 hours at 37°C (5% CO_2 in a humid incubator). Lenses were totally immersed in medium. After 8 and 24 hours, lenses were carefully removed from wells. The lenses did not adhere to the HCEC monolayer. Lenses were also routinely observed for the presence of adherent cells on their surface and no HCEC proliferation was observed.

Cellular viability

To measure cytotoxicity of the products released from the contact lenses, the MTT cellular viability assay was performed. After a gentle rinse in sterile PBS, cells were incubated with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT at 1mg/mL in KSFM medium). After 3 hours at 37°C, cells were lysed with DMSO and absorbance read at 595nm (Thermo MultiSkan Spectrum Photometer, Fischer Scientific, Ottawa, ON, Canada). All results are expressed as relative viability compared to cells grown in the absence of a contact lens.

Precycling of Lens Cases

Cellular viability was also assessed to determine the difference between using precycled lens cases and tissue culture polystyrene wells. This methodology, which was developed by Alcon Laboratories, involved preconditioning the lens cases with their appropriate solutions for six 8-hour periods. The aim of preconditioning was to eliminate interactions between the solution and polymers of the lens case.[9,10] Lenses were then soaked in the cases or sterile tissue culture polystyrene wells overnight before being placed on the monolayer. The MTT assay was repeated as described previously.

Cellular activation

To determine cellular activation by a change in integrin expression or caspase activation induced by MPS release from contact lens, HCEC were removed from the wells with TriplExpress (Invitrogen, Burlington, Ontario, Canada), following a gentle wash in PBS. Cells were washed and resuspended in DMEM/FBS.

For integrin expression, small aliquots (30μL) of HCEC, diluted in DMEM-FBS, were incubated with saturating concentration of fluorescently-labeled antibodies for 1 hour at 4°C. Samples were then diluted in Hepes Tyrode Buffer, fixed in 1% paraformaldehyde (final concentration) and analysed by flow cytometry within 5 days.

To determine caspase activation, small aliquots of HCEC, diluted in DMEM-FBS, were incubated with a fluorescently-labelled pan caspase inhibitor (FITC-VAD-FMK, Calbiochem, San Diego, California) for 1 hour at 37°C. Samples were washed and resuspended in wash buffer, before immediate analysis by flow cytometry.

All samples were acquired on Becton Dickinson FACSVantage flow cytometer (Mountain View, CA, USA) using CELLQuest Software. Appropriate isotype controls were used with each experiment. Analysis was also performed using FACSExpress post data acquisition.

Statistical analysis

All results are reported as means ± standard deviation (SD). To evaluate the significance of the differences in cell viability and cell activation, an ANOVA was carried out followed by a post hoc Bonferroni test using Statistica V8 (StatSoft, Tulsa, OK, USA). A p value of less than 0.05 was required for statistical significance. The number of experiments was equal to or greater than three with different cell passages.

RESULTS

Cell viability

Cell viability was assessed after incubation periods of 8 and 24 hours. For both time and lens types, there was no significant difference in cell viability between cells incubated in the absence of a lens and cells incubated with a PBS-soaked lens ($p = 1.000$). At 8 hours, the borate buffer-based solutions (OFX and ReNu) resulted in a significant decrease in viability for LA and BA. A 20% reduction or more was seen at 8 hours with LA soaked in OFX and ReNu ($p < 0.001$) as well as with LA soaked in Solo ($p = 0.047$) (Figure 4.1). Incubation for 8 hours in the presence of lenses soaked in Complete and PBS did not significantly reduce viability. As illustrated in Figure 4.2, BA lenses soaked in OFX and ReNu also significantly reduced cell viability after 8 hours of incubation ($p < 0.025$). Further analysis comparing viability results and lens type showed that there was no significant effect of lens type at 8 hours ($p > 0.05$).

After 24 hours, further decrease in viability was observed with MPS-soaked lenses. As opposed to 8 hours, OFX and ReNu-soaked LA lenses led to different levels of viability (Figure 1). The increased exposure time to OFX-soaked LA lenses significantly decreased cell viability down to 49% ($p = 0.0001$), while viability of cells exposed to ReNu-soaked LA only decreased slightly and remained around 65% viability. Cells exposed to LA lenses soaked in Solo and Complete had levels similar to viability at 8 hours. A significant decrease in viability was observed in BA lenses, with OFX, Solo, and ReNu, as compared to controls at 24 hours ($p < 0.002$).

At 24 hours, lens type did have a significant effect on viability. While there were similar trends in viability with both lenses, there was a significant difference in the viability of cells

exposed to OFX at 24 hours. LA-OFX had significantly lower viability compared to BA lenses soaked in the same solution ($p = 0.022$). Unlike LA-OFX, increased exposure time to OFX-soaked BA lenses did not significantly reduce viability further when compared to the 8-hour time point.

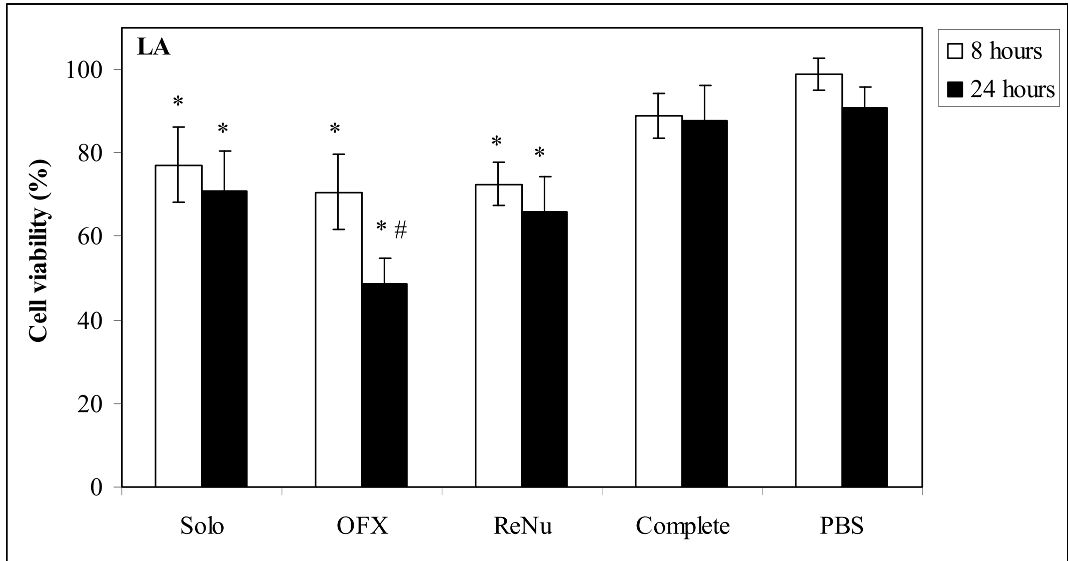


Figure 4.1: HCEC viability after 8 and 24 hour contact with Lotrafilcon A lenses (LA) soaked in various MPS. Viability was measured by MTT assay and is expressed as a percentage relative to cells grown in the absence of lenses. N = 3 to 6, * Significantly different from cells grown in the absence of a lens ($p < 0.04$), # Significantly different from 8 hours ($p < 0.03$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

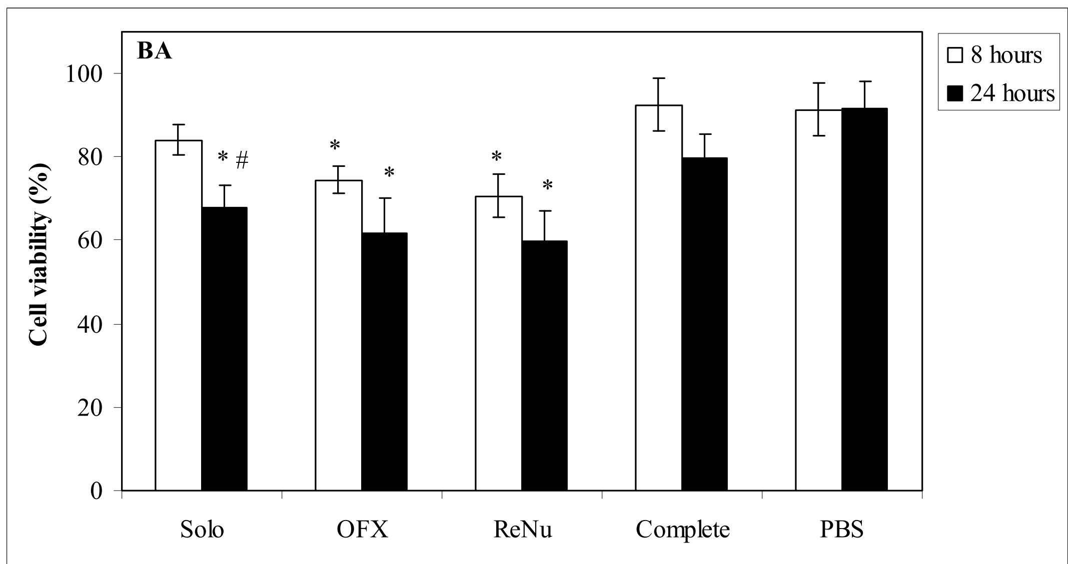


Figure 4.2: HCEC viability after 8 and 24 hour contact with Balafilcon A (BA) lenses soaked in various MPS. Viability was measured by MTT assay and is expressed as a percentage relative to cells grown in the absence of a lens. N = 3 to 6, * Significantly different from cells grown in the absence of lenses ($p < 0.04$), # Significantly different from 8 hours ($p < 0.03$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Precycling of Lens Cases

The MTT assay was also used to determine whether there was a difference in viability between lenses soaked in cycled lenses case, with the appropriate MPS, or in a sterile 12-well polystyrene plate. Results demonstrated that there was no difference in HCEC viability due to the different lens soaking strategies (Figure 4.3); therefore, from this point onwards, all studies used sterile polystyrene plates for lens soaking to prevent potential contaminations.

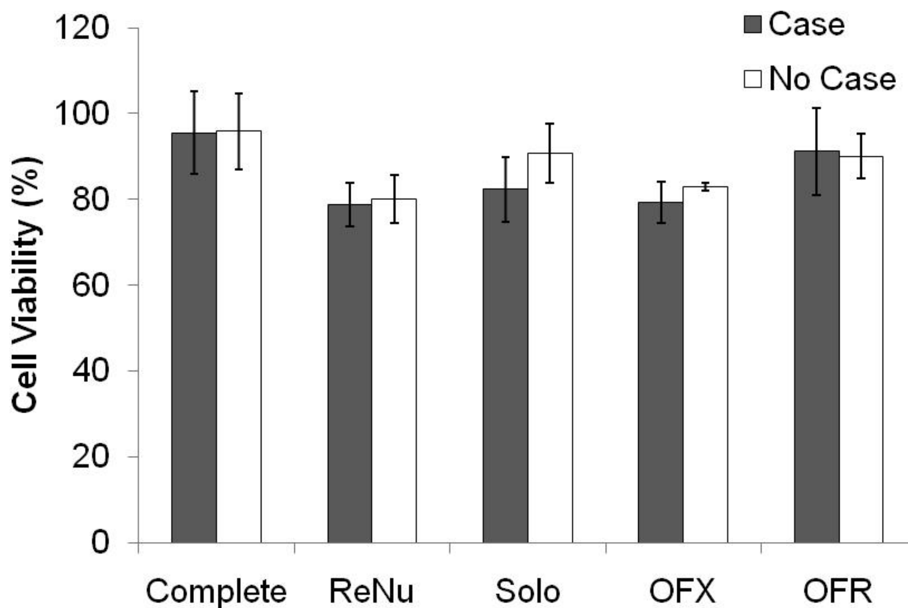


Figure 4.3: HCEC viability after 24 hour contact with Balafilcon A (BA) lenses soaked in various MPS in cycled lens cases and sterile polystyrene wells. Viability was measured by MTT assay and is expressed as a percentage relative to cells grown in the absence of a lens. N = 3. Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Cell Integrin Expression

To assess the cell adhesion phenotype, flow cytometric studies were performed on adherent cells following 8 and 24 hour contact with MPS-soaked LA and BA lenses. As illustrated in Figure 4.4, after 24 hour contact, significant downregulation of α_3 (CD49c) was observed with OFX and ReNu-soaked lenses.

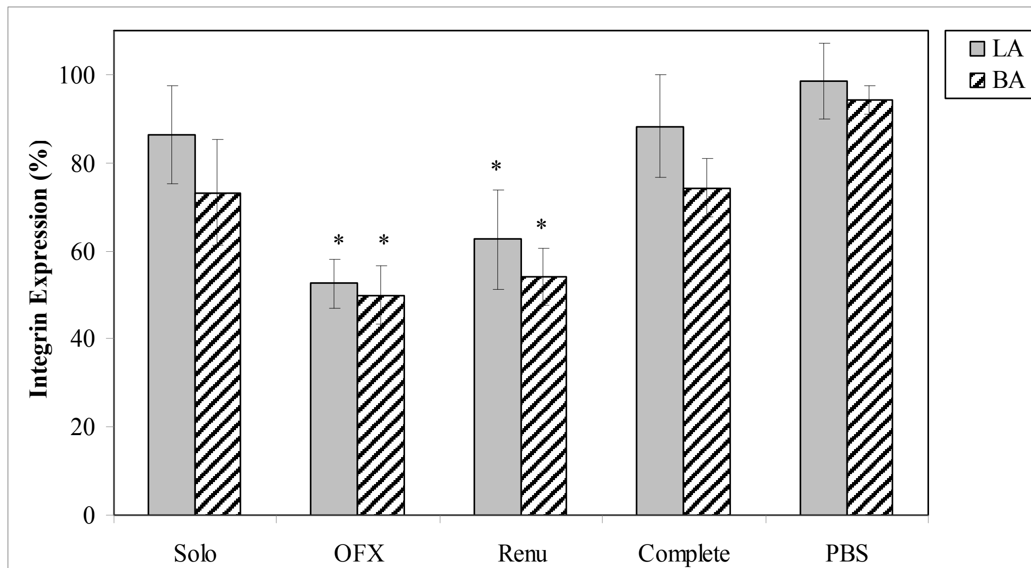


Figure 4.4: HCEC α_3 expression after 24 hour contact with Lotrafilcon A (LA) and Balafilcon A (BA) lenses soaked in various MPS. Integrin expression was measured by flow cytometry and is expressed as a percentage relative to cells grown in the absence of a lens.
N = 4 to 5, * Significantly different from cells grown in the absence of lenses ($p \leq 0.004$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

As opposed to LA lenses, BA lenses soaked in Solo also led to a downregulation of β_1 (Table 4.2) at 24 hours. Significant decrease in the expression of β_1 (CD29) and β_4 (CD104) were also observed with OFX and ReNu-soaked lenses ($p < 0.04$, Table 4.2). Similar to the viability results, a further decrease was seen in integrin expression after a 24-hour incubation time ($p < 0.04$, Table 4.2). The observed downregulation of integrins suggest that HCEC

adhesion phenotype was compromised in the presence of lenses soaked in the borate buffer based solutions.

Table 4.2: Down regulation of integrin expression induced by contact with MPS-soaked lenses after 8 and 24 hours. β1 and β4 expression were measured by flow cytometry and are expressed as a percentage relative to control (cells incubated in the absence of a lens). N = 4 to 5.

		β1		**β4**	
Lens	**Solution**	**8 hrs**	**24hrs**	**8hrs**	**24 hrs**
LA	**Solo**	88 ± 4	88 ± 8	96 ± 5	98 ± 4
	OFX	79 ± 4*	58 ± 4*	91 ± 6	67 ± 5* #
	Renu	77 ± 5*	63 ± 10*	93 ± 4	76 ± 5* #
	Complete	91 ± 3	88 ± 7	99 ± 5	98 ± 9
	PBS	98 ± 5	94 ± 4	101 ± 5	99 ± 3
BA	**Solo**	88 ± 4	73 ± 6*	98 ± 5	92 ± 6
	OFX	78 ± 3*	57 ± 6* #	88 ± 5	68 ± 8* #
	Renu	79 ± 4*	56 ± 5* #	87 ± 5	70 ± 4* #
	Complete	99 ± 3	79 ± 6	95 ± 3	95 ± 4
	PBS	103 ± 6	97 ± 4	98 ± 2	97 ± 2

* Significantly different from cells incubated with PBS-soaked lens ($p < 0.003$). # Significantly different from 8hrs-soaked lens value ($p \leq 0.02$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Caspase Activation

Activated caspases were detected by flow cytometry with the fluorescently-labelled pan caspase inhibitor, FITC-VAD-FMK.[20] Increased fluorescence intensity can be observed in the presence of activated caspases, resulting in a bimodal distribution of fluorescence (as observed in Figure 4.5). No significant caspase activation was detected prior to 24 hours (data not shown). Caspase activation was observed at 24 hours with LA lenses soaked in OFX by detecting a significant amount of cells staining positive for caspases ($p < 0.012$) (Figure 4.6). The caspase activation detected in cells exposed to OFX-BA was significantly lower than OFX-LA ($p < 0.015$). While the cell viability data showed reduced viability with ReNu-soaked lenses, suggesting the potential for inducing cell apoptosis, no significant increase in caspase positive cells was detected at 24 hours.

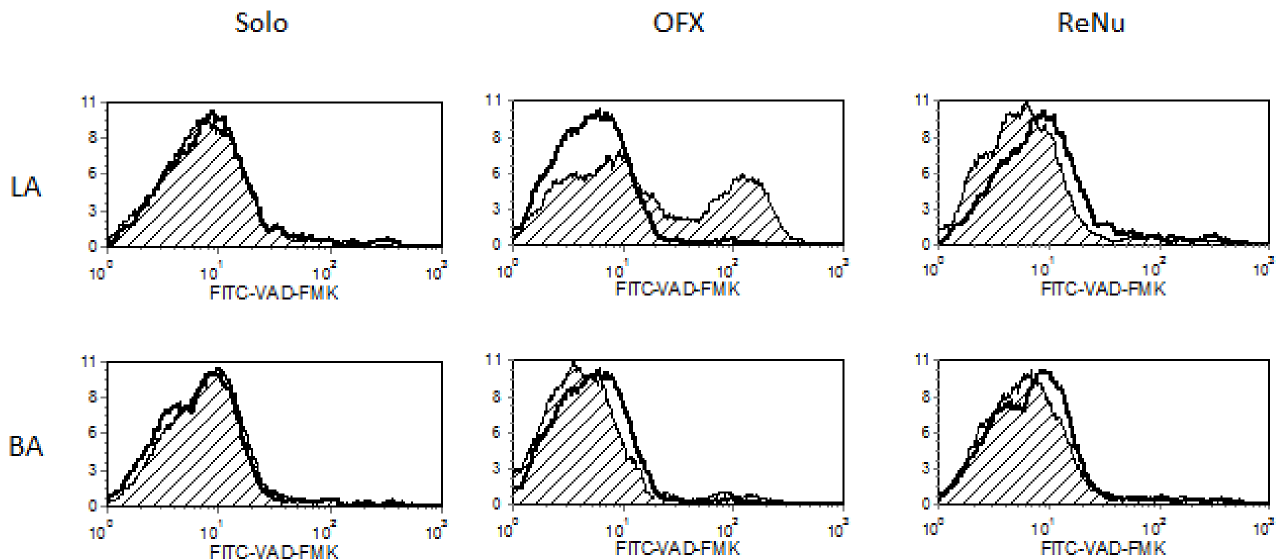


Figure 4.5: Histograms of HCEC caspase activation induced by MPS-soaked contact lenses. FITC-VAD-FMK fluorescent intensity was measured by flow cytometry after 24 hour exposure to Lotrafilcon A (LA) and Balafilcon A (BA) lenses soaked in MPS. Cross-hatched curve represents the fluoresecent intensity observed with MPS-soaked lens and overlaid dark line represents the negative control, PBS-soaked lens. Histograms are representative of all experiments performed (N = 3 to 4).

OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

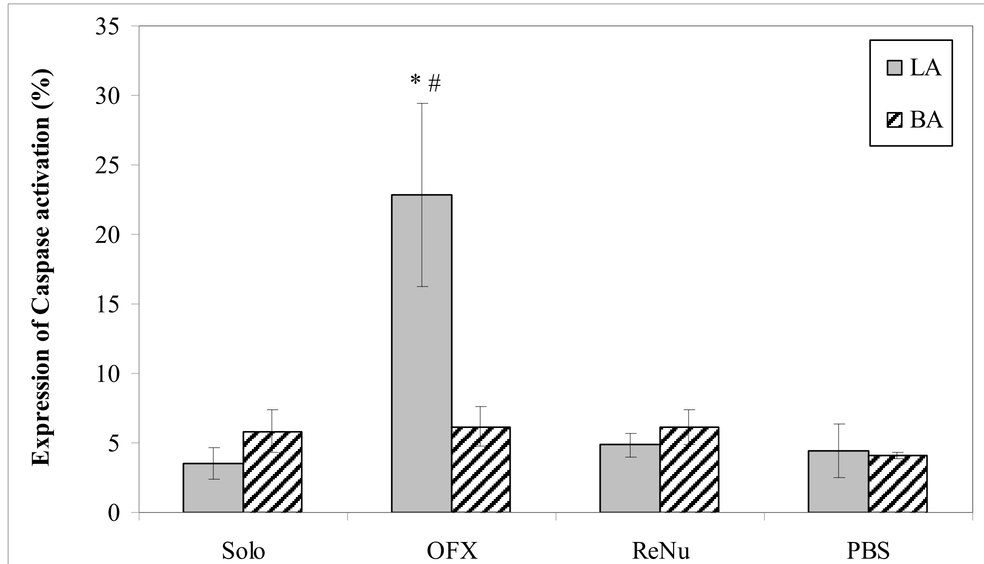


Figure 4.6: HCEC caspase activation after 24 hour contact with Lotrafilcon A (LA) and Balafilcon A (BA) lenses soaked in various MPS. Activation was measured by flow cytometry and the percentage of cells staining positive for caspase activation is reported. N = 3 to 4, * Significantly different from cells grown in the absence of a lens ($p < 0.012$), # Significantly different from BA ($p < 0.015$).
OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

DISCUSSION

The in vitro contact lens "onlay" model reported in this thesis appears to be a valuable tool to study the direct release of multipurpose solutions on corneal epithelial cells. While blinking and the constant regeneration of the tear film may dilute the effects of the biocides in vivo, the uptake and slow release of MPS by the lens will increase the exposure time to the cornea, which further warrants the importance of evaluating solution release from a contact lens in vitro. Table 4.3 summarizes the results obtained in this study.

In this study, the results indicate that a scale of cytotoxicity can be derived among the commercially available MPS tested. The results obtained from cell contact with lenses soaked in phosphate buffer demonstrated near 100% viability and no change in cell adhesion phenotype and thus confirmed the validity of our in vitro model. Complete MPS demonstrated results similar to PBS, while OFX, ReNu and Solo demonstrated a significant adverse effect on viability. With the two SH lenses tested in this study, in vitro results demonstrated the following scale of corneal cell cytotoxicity: PBS < Complete < Solo < ReNu < OFX. Our cytotoxicity scale, based on MPS solution released from contact lenses, is in accordance with previous studies where MPS was added directly to a monolayer of corneal cells.[5,6]

As a consequence of being exposed to cytotoxic MPS, we hypothesized that this may lead to a compromised corneal monolayer in vitro. The flow cytometry results confirmed this concept. The integrins examined have strong roles in epithelial cell adhesion. α_3, which heterodimerizes exclusively with β_1, is important in the maintenance of cell-cell junctions.[21] It is also involved in cell spreading and hemidesmosome stability.[21-22] β_4 is also an important component of hemidesmosomes.[21-22] Expression of this integrin is crucial for stable adhesion of epithelium to the basement membrane.[21] Significant reduction in integrin expression were

observed with lenses soaked in OFX and ReNu, suggesting that HCEC adhesion phenotype was being disrupted. Our results are in agreement with a recent in vitro study showing that exposure to MPS, including OFX, caused a disruption in the structure of corneal epithelial tight junctions in vitro.[29]

As previous studies have suggested, the disinfection agent present in the care regimen may be responsible for the observed cytotoxic effect on cells.[16, 17, 24-28] However, both OFX and ReNu led to reduced cell viability and integrin expression, but their disinfecting agent is different (Polyquad®/Aldox® versus PHMB). Moreover, Complete also uses PHMB as a disinfecting agent, but viability and adhesion phenotype were not significantly affected by the solution when LA lenses were used. Complete is buffered with a phosphate solution, while both ReNu and OFX are buffered with a borate solution, suggesting the buffering agent may have a significant effect on the biocompatibility property of MPS. The findings that borate buffer has a significant effect on corneal cells are consistent with previous work in our laboratory, which found a significant decrease in viability, β_1 and α_3 with borate buffered contact lens packaging solutions.[7] In addition, Imayasu et al, also found that borate buffer had a negative effect on corneal tight junctions.[29] Further studies in which various individual ingredients in MPS are investigated to determine their potential cytotoxic effects in the presence of borate versus phosphate buffers would be worthy.

Most in vitro studies with MPS have been limited to studying the effect of solutions on cells.[4-6, 16,17, 27-29] A difference in cell viability observed at 24 hours with lenses soaked in OFX emphasizes the importance of testing solution-lens combinations in vitro. The difference in viability and adhesion phenotype may be attributed to the different physical properties of the

lenses. BA and LA lenses have different surface treatments: LA lenses are plasma-coated while BA lenses undergo plasma oxidation. This difference, which may affect the uptake and/or release of biocides from the lens and the actual concentration of MPS the cells are exposed to, had a significant effect on cells. Further studies are currently underway to better understand the interaction between MPS solution, surface treatment on silicone hydrogel lenses and the effect on cells.

Based on the findings from the viability and cell adhesion assays, it was hypothesized that the borate solutions were capable of inducing apoptosis. Apoptosis is a highly regulated, programmed cell death pathway, mediated in part by the actions of caspases.[30] Caspases are cysteine proteases, normally found in the inactive, pro-caspase form. Initiation of apoptosis occurs upon activation of initiator caspases by intrinsic or extrinsic factors, which can subsequently activate downstream effector caspases. Apoptosis is characterized by several morphological features, such as membrane blebbing, cell shrinkage and chromatin condensation, which are the results of activated effector caspases.[30,31] It was observed that OFX-soaked LA lenses caused an increase in the number of cells staining positive for active caspases ($p < 0.04$), but the same was not true for OFX-soaked BA or ReNu-soaked LA lenses. Several hypotheses can be formed from this latter result. ReNu may be causing necrosis in HCECs, but since the integrin expression was shown to be adversely affected by ReNu, it was expected that the cells exposed to both borate solutions were undergoing an apoptotic pathway. It is plausible that ReNu is inducing a caspase-independent apoptotic pathway, or that the induction of apoptotic pathways by ReNu is on a different time-scale compared to OFX. If ReNu induces a more rapid apoptotic pathway, HCEC would have entered secondary necrosis. If ReNu induces a slower apoptotic pathway, HCEC may still be in the early phase of apoptosis, prior to caspase

activation. Both scenarios would explain the absence of activated caspases at 24 hours. The fact that the OFX-soaked BA lens did not induce significant caspase activation further indicates MPS interaction with lens material and how uptake and release of MPS from SH lens may affect mechanism of cell activation. Our caspase results further emphasize the importance of testing MPS-lens combinations when determining the biocompatibility of MPS.

Our OFX results with LA lens support the findings of Dutot et al,[17] showing that OFX induced apoptosis in conjunctival cells by upregulation of caspase 3. While other studies[32] have correlated their findings to the OFX observation, this study is the first to specifically assess caspase activation in corneal cells exposed to MPS in vitro. DNA fragmentation assays cannot differentiate between apoptosis and necrosis and thus, while previous studies suggest a cytotoxic effect, it is difficult to infer the potential role for a caspase-mediated apoptosis pathway with such an assay. Dutot et al also demonstrated the upregulation of caspase 3 in conjunctival cells by Solo. Such a result was not observed in our in vitro model with corneal epithelial cells.This suggests that corneal cells and conjunctival cells may have different physiological responses to MPS-induced toxicity and that the release from a lens versus direct solution test may also contribute to potential differences in cellular mechanisms.

In summary, the results from this study shows that for MPS-released from a contact lens, a scale for in vitro corneal cell cytotoxicity can be derived, where the borate based MPS are the most cytotoxic and the phosphate based MPS are least cytotoxic. As demonstrated by the absence of an effect on cells exposed to a PBS-soaked lens, our results indicate that it is not the presence of the lens that affects cell viability and phenotype, but what is being released from the lens. The in vitro model also demonstrated a lens effect in the mechanism of MPS-induced

cytotoxicity. The differences in physical properties of BA and LA lenses, which affect the uptake and/or release of the various ingredients in MPS, had a significant effect on viability, adhesion phenotype and caspase activation. The caspase results indicate that ReNu may induce apoptosis through a caspase-independent pathway, whereas OFX-induced apoptosis is caspase dependent. Further studies are under way to gain a better understanding of the preferential adsorption or release profile of certain compounds by silicone hydrogel lenses with different physical properties and surface treatments.

Table 4.3: Summary of the impact of lens-solution interaction on corneal epithelial cells in vitro

	SoloCare PHMB *Tris Buffer*			**ReNu** PHMB *Borate Buffer*			**Complete** PHMB *Phosphate Buffer*			**OptiFree Express** Polyquad/Aldox *Borate Buffer*		
	Viability	Integrin expression	Caspase activation	Viability	Integrin expression	Caspase activation	Viability	Integrin expression	Caspase activation	Viability	Integrin expression	Caspase activation
LA	↓	—	—	↓	↓↓	—	—	—	ND	↓↓	↓↓	↑↑
BA	↓	↓	—	↓	↓↓	—	—	—	ND	↓	↓↓	—

— No significant change compared to controls (cells grown in the absence of a lens or contact with PBS-soaked lens)

↓ Decrease compared to controls (cells grown in the absence of a lens or contact with PBS-soaked lens)

↑ Increase compared to controls (cells grown in the absence of a lens or contact with PBS-soaked lens)

ND: Not determined

CHAPTER 5

INVESTIGATING MECHANISMS OF SILICONE HYDROGEL LENSES SOAKED IN MULTIPURPOSE SOLUTION INDUCED APOPTOTIC PATHWAYS IN HUMAN CORNEAL EPITHELIAL CELLS, *IN VITRO*

INTRODUCTION

Evidence has shown that exposure to MPS, and certain lens-solution combinations, can cause cell death in vitro.[1-5] It is has been hypothesized that the cell death pathway is a caspase-mediated apoptotic pathway.[5,6] The use of DNA fragmentation assays is common[7], but this assay cannot differentiate between apoptosis and necrosis and thus; while previous studies suggest a cytotoxic effect, it is difficult to infer the potential role for a caspase-mediated apoptosis pathway with such an assay.

Apoptosis is a programmed form of cell death that has been evolutionarily conserved among multicellular organisms.[8-11] It plays a role in several normal physiological process such as embryonic development, tissue homeostasis, and self-organizational processes in the immune system and central nervous system.[10-12] It also serves as a defense mechanism in its role of removal of damaged cells.[10-12] This form of cell death has several morphologically recognizable features, including cytoplasmic shrinkage, plasma membrane blebbing, compaction of nuclear chromatin formation of vesicles and apoptotic bodies, and rapid cleavage of DNA into 180bp fragments, corresponding to internucleosomal spacing.[8,10,11] There are two main distinct molecular signaling pathways that lead to apoptotic cell death: the extrinsic or death receptor activated pathway and the intrinsic, mitochondrial mediated

pathway (Figure 5.1).[9] Both signaling pathways involve cysteine aspartate proteases (caspase) as mediators for initiating cellular disassembly.[9,11]

Necrosis is a form of cell death considered to be accidental or inappropriate, which occurs under extremely unfavorable conditions. It is an uncontrollable, irreversible form of cell death characterized by cell swelling, membrane deformation and organelle breakdown. It has not been determined whether signaling pathways mediate necrotic cell death.[13]

Thus, to investigate the mechanisms of cell death induced by multipurpose solutions released from contact lenses, a model in which the contact lens was directly set onto a monolayer of HCEC was used and corneal cell viability, mitochondrial transmembrane potential, caspase 3 and 9 activity and Annexin-V/Propidium Iodide (PI) staining were assessed following exposure to the care-regimen soaked lens materials.

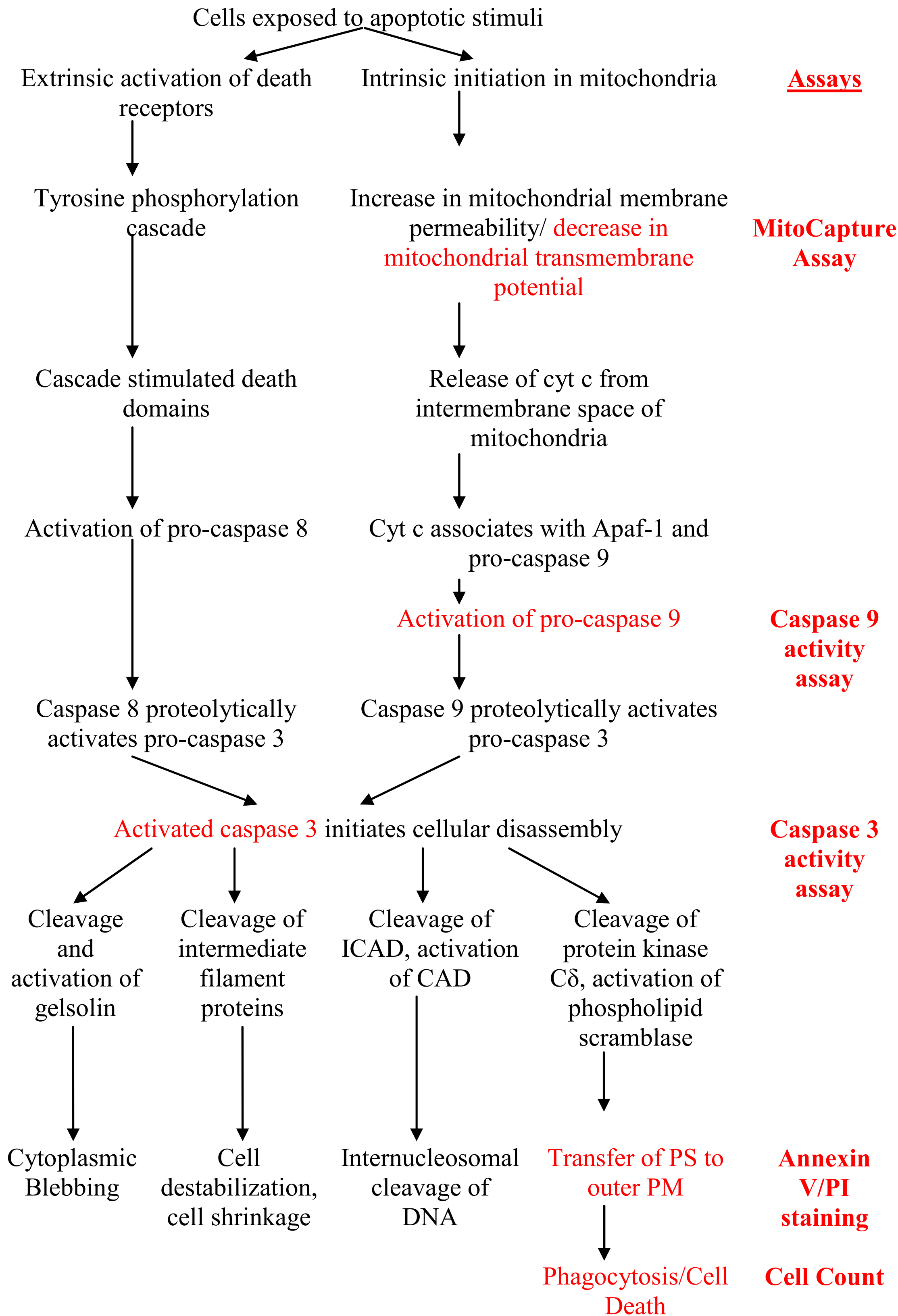


Figure 5.1: Flow chart of apoptotic events in the extrinsic and intrinsic pathways. Highlighted are the assays used to evaluate different benchmarks in these pathways.

MATERIALS AND METHODS

Reagents and antibodies

Keratinocyte serum free medium, growth supplement (Bovine Pituitary extract) and pen-strep solution were purchased from ScienCell. All other cell culture reagents, Dulbecco's minimum essential medium, fetal bovine serum, phosphate buffer saline and TriplExpress were purchased from Invitrogen (Burlington, Ontario, Canada). Antibodies for annexin V and propidium iodide (PI) were obtained as part of the Vybrant Apoptosis Assay Kit also purchased from Invitrogen.

Contact lenses and multipurpose solutions

Three silicone hydrogel lens materials were tested: Lotrafilcon A (LA; CIBA Vision; Duluth, GA, USA), Lotrafilcon B (LB; CIBA Vision; Duluth, GA, USA), and Balafilcon A (BA; Bausch & Lomb, Rochester, NY, USA). All lenses were purchased in their original packaging, had a diameter between 14.0 and 14.2 mm and a curvature of 8.5 to 8.7mm. Four polyquaternium and biguanide preserved multipurpose solutions were tested and their composition is described in Table 5.1.

Table 5.1: Disclosed composition of the MPS used in the study.[14]

Manufacturer	Brand (abbreviation)	Disinfecting Agent	Buffer	Other reported agents (surfactants and chelating agents)
Alcon	Opti-Free Express (OFX)	Polyquad® 0.001%, Aldox® 0.0005%	Borate	Sorbitol; citrate (citric acid), 0.05% EDTA; poloxamine (Tetronic 1304)
	Opti-Free RepleniSH (OFR)	Polyquad® 0.001%, Aldox® 0.0005%	Borate	Citrate, poloxamine (Tetronic 1304), non-anoyl ethylene-diaminetriacetic acid
AMO	Complete Moisture Plus (Complete)	PHMB 0.0001%	Phosphate	Taurine; 0.01% EDTA; Poloxamer 237 (Pluronic F87); HPMC 0.15%; propylene glycol
Bausch & Lomb	ReNu MultiPlus (ReNu)	PHMB 0.0001%	Borate	Sodium borate; Hydroxyalkylphosphonate (Hydranate™); 0.1% EDTA; Poloxamine (Tetronic 1107)
CIBA Vision	SoloCare Aqua (Solo)	PHMB 0.0001%	Tris	Sorbitol; 0.025% EDTA; dexpanthenol (provitamin B5); Pluronic F127 (poloxamer 407)

PHMB: polyhexamethylene biguanide (also known as polyhexanide, Dymed, polyhexadine, and polyaminopropyl biguanide)

In vitro cell culture

Immortalized human corneal epithelial cells (HCEC)

SV40-immortalized human corneal epithelial cells were cultured in keratinocyte serum free medium supplemented with bovine pituitary extract, recombinant epidermal growth factor and pen-strep (KSFM). Fresh medium was added every other day and cells were grown to 90% confluency in tissue culture treated flasks. Adherent cells were removed using a dissociation solution, TriplExpress. Cells were routinely observed for any morphological changes.

In vitro model

A direct contact in vitro model was used.[5,15] HCEC were seeded onto a 24 well tissue culture treated polystyrene (TCPS) plate at 10^5 cells per well. Cells were left to adhere for 18-24 hours in a humid CO_2 incubator, which resulted in the formation of a monolayer of HCEC. Simultaneously, 2 mL of MPS was added to the wells of a sterile 12-well polystyrene plate (BD Falcon, Mississauga, ON, Canada). Using sterile tweezers, lenses were gently removed from their blister package and added to the wells containing MPS. Lenses were totally immersed and left to soak for 18 – 24 hours. All lens-solution soaking combinations were performed under sterile conditions.

The next day, supernatant was removed from the cells and fresh serum-free medium (700 µL) was added. MPS-soaked SH lenses were placed gently on top of the monolayer, face-down, with the concave surface facing upwards and incubated for up to 24 hours at 37°C (5% CO_2 in a humid incubator). Lenses were totally immersed in medium. After 24 hours, lenses were carefully removed from wells. The lenses did not adhere to the HCEC monolayer. Lenses were also routinely observed for the presence of adherent cells on their surface and no HCEC proliferation was observed.

Cellular Loss (Cell Count)

HCEC were harvested and resuspended in 200 µL of fresh serum-free medium. A 50 µL aliquot of cells was diluted in 5 mL of ISOTON II Diluent (Beckman Coulter Inc., Fullerton, CA, USA). Cell counts were determined using a Z2 Coulter Particle Counter and Size Analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Healthy cells were counted in the range of 5.896µm – 20.85µm; dead/dying cells were counted below this range. The percentage of cell viability was calculated for each test solution and is expressed as relative viability compared to cells grown in the absence of a contact lens.

MitoCapture Assay

To determine mitochondrial transmembrane potential, MitoCapture Apoptosis Detection kit (Calbiochem, La Jolla, CA, USA) was used. Harvested cells, resuspended in MitoCapture reagent, were incubated for 20 minutes, at 37^oC (5% CO_2 in a humid incubator). Samples were then centrifuged, resuspended in 500 µL of pre-warmed incubation buffer and analysed immediately by flow cytometry. All samples were acquired on BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using BD CELLQuest Pro Software. Appropriate isotype controls were used with each experiment.

Caspase Proteolytic Enzyme Activity

Proteolytic enzyme activity of caspase-3 (C3), and caspase-9 (C9) were determined in HCEC culture using the substrates, Ac-DEVD-AMC (Alexis Biochemicals, Burlington, ON, Canada), and Ac-LEHD-AMC (Alexis Biochemicals, Burlington, ON, Canada), respectively. Following treatment, HCEC were harvested and stored at -70^0C in the absence of growth medium, prior to use in the assay. Briefly, cultured HCEC was homogenized in ice-cold lysis buffer (20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl, 1 mM DTT, 20% glycerol and 0.1% Triton X100; pH 7.4) not containing protease

inhibitors and centrifuged at 1000 x *g* for 10 min at 4°C. Cell supernatants were then incubated with the appropriate substrate at room temperature for 1 hour. Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 360 nm and 440 nm, respectively. As a positive control for caspase proteolytic activity, the appropriate substrate was incubated with human recombinant active C3 (Alexis Biochemicals, Burlington, ON, Canada) or C9 (Alexis Biochemicals, Burlington, ON, Canada). In all cases, a strong fluorescent signal was obtained (*data not shown*). Furthermore, control experiments using the C3, and C9 inhibitors, Ac-DEVD-CHO (Alexis Biochemicals, Burlington, ON, Canada), and Ac-LEHD-CHO (Alexis Biochemicals, Burlington, ON, Canada), respectively, completely inhibited the fluorescent signal observed from both the active recombinant enzymes as well as HCEC (*data not shown*). Caspase activity was normalized to total protein content and expressed as mean fluorescence intensity in AU per mg protein.

Annexin V-FITC/Propidium Iodide Staining

To evaluate plasma membrane expression of phosphatidylserine, and subsequently apoptosis, harvested cells were also used for flow cytometry, which was performed according to the Vybrant Apoptosis Kit, FITC annexin V/propidium iodide (Invitrogen, Burlington, ON, Canada). In brief, each cell suspension was washed with cold PBS and centrifuged at 1000 rpm for 5 min at 24°C. The cell pellet was resuspended in 100 µL of binding buffer. 5 µL of Annexin V-FITC solution and 1 µL of PI were added to each suspension. The suspension was incubated at room temperature for 15 minutes in the dark, and then diluted with 400 µL of binding buffer. Cell suspensions were analyzed by flow cytometry immediately. All samples were acquired on BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using BD CELLQuest Pro Software. Appropriate isotype controls were used with each experiment.

Statistical analysis

All results are reported as means ± standard deviation (SD). Analysis was performed using FCSExpress post data acquisition. To evaluate the significance of the differences in cell viability, mitochondrial transmembrane potential, caspase activity and Annexin V-FITC/PI staining, an ANOVA was carried out followed by a post hoc Bonferroni test using Statistica. A p value of less than 0.05 was required for statistical significance. The number of experiments was equal to or greater than three with different cell passages.

RESULTS

Cell Loss (Cell Count)

Cell viability was assessed after incubation periods of 24 hours. There was no significant difference in cell count between cells incubated in the absence of a lens and cells incubated with a PBS-soaked lens ($p = 1.000$). The borate buffer-based solutions (OFX and ReNu) resulted in a significant decrease in viability for LA lenses. A 50% reduction was seen with LA soaked in OFX ($p = 0.0002$), while LA-ReNu led to a 30% reduction, which was significantly different from the positive PBS control ($p < 0.05$) (Figure 5.2). LA lenses soaked in OFX and ReNu also demonstrated the greatest number of dead/dying cells (Figure 5.3), as indicated by the number of cells counted in the size range below 11.38 μm. Incubation in the presence of lenses soaked in Complete, Solo and PBS did not significantly reduce viability. As illustrated in Figure 2, BA lenses soaked in OFX and ReNu also reduced cell count, at 63% and 71%, respectively ($p < 0.05$).

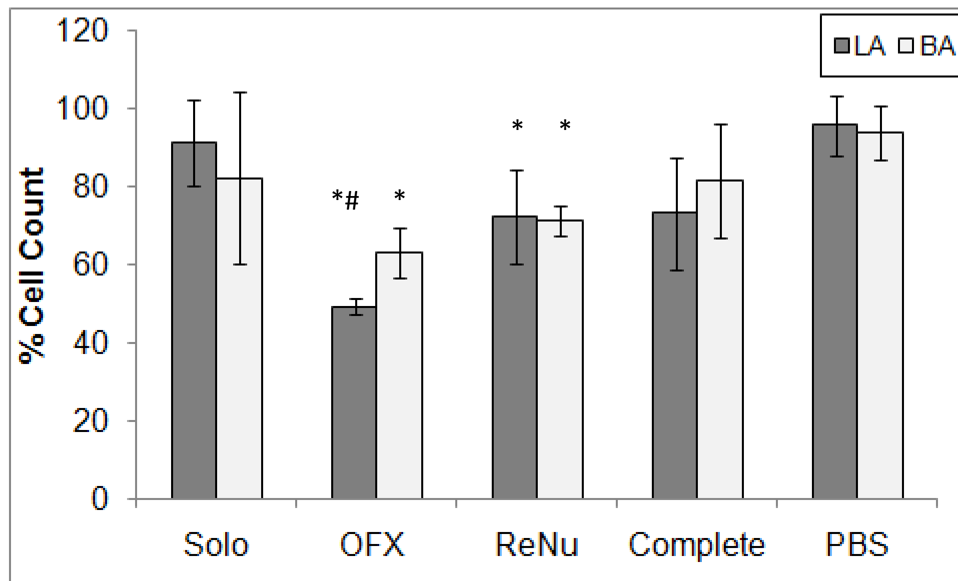


Figure 5.2: HCEC count of healthy cells after 24 hour contact with Lotrafilcon A and Balafilcon A lenses soaked in various MPS. Viability was measured by cell count and is expressed as a percentage relative to cells grown in the absence of lenses. N = 3 to 4, * Significantly different from cells grown in the absence of lenses ($p < 0.05$), # Significantly different from Balafilcon A ($p < 0.0006$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

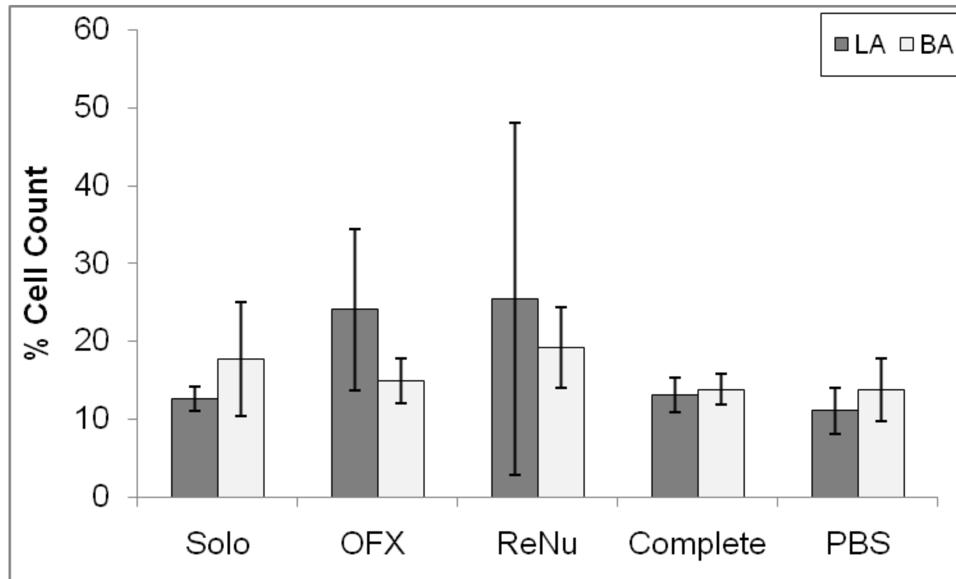


Figure 5.3: HCEC count of dying cells after 24 hour contact with Lotrafilcon A and Balafilcon A lenses soaked in various MPS. Viability was measured by cell count and is expressed as a percentage relative to cells grown in the absence of lenses. N = 2 to 3.
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Similar results were obtained using an MTT assay, as previously described in Chapter 4, with the exception of Solo, which previously was found to have significant lower viability compared to controls at 24 hours. The cell count did not detect a significant cell loss for LA or BA lenses soaked in Solo. The greatest reduction of cell viability was from LA-OFX at 49%, while both LA and BA soaked in Complete had levels similar to controls, at 88% and 79% respectively.

Lens type did have a significant effect on viability. While there were similar trends in viability with both lenses, there was a significant difference in the viability of cells exposed to OFX at 24 hours. LA-OFX had significantly lower viability compared to BA lenses soaked in the same solution ($p = 0.0006$).

Mitochondrial membrane potential

Alterations in the mitochondrial membrane potential were detected by flow cytometry with the fluorescent MitoCapture reagent. Red fluorescence can be detected in healthy mitochondria containing

aggregates of the MitoCapture reagent, which can be detected using the FL2 or FL3 channel.[16] In apoptotic cells, the MitoCapture reagent cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential, and thus it remains in its fluorescent green monomeric form, to be detected using the FL1 channel (Figure 5.3).[16] The resulting changes in membrane potential are reported as the mean ratio of red:green cells in Table 5.2. While LA-OFX had the lowest red:green ratio, the observed loss of mitochondrial membrane potential were not statistically significant.

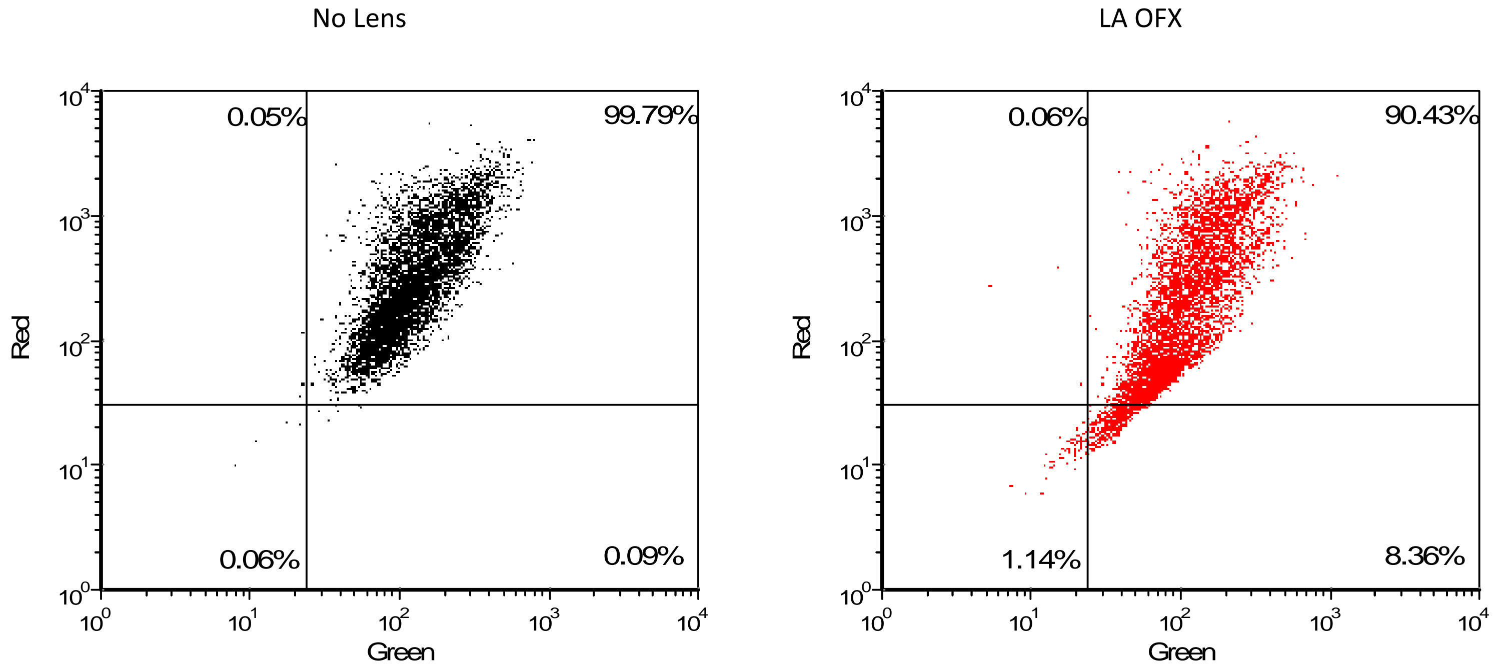


Figure 5.3: Changes in HCEC mitochondrial transmembrane potential by MPS-soaked contact lenses. Figures represent one trial, after 24 hour exposure to Lotrafilcon A soaked in Opti-Free Express (right) and no lens (left). Red staining represents living cells, with healthy mitochondria; green staining represents apoptotic cells, with altered mitochondrial transmembrane potential.

Table 5.2: Ratio of MitoCapture reagent fluorescence representative of alterations of the mitochondrial transmembrane potential after 24 hours, as detected by flow cytometry. N = 3 to 4.

Lens	Solution	Red:Green	SD
LA	Solo	2.45	0.91
	OFX	1.6	0.51
	ReNu	2.57	0.86
	OFR	2.11	0.62
	PBS	2.07	0.84
BA	Solo	2.27	0.5
	OFX	2.12	0.58
	ReNu	2.33	0.74
	OFR	1.96	0.88
	PBS	2.04	0.75
No Lens		2.75	0.44

Caspase Proteolytic Enzyme Activity

Activated caspases were detected by fluorescence with the substrates, Ac-DEVD-AMC and Ac-LEHD-AMC. These fluorogenic substrates are weakly fluorescent but yield highly fluorescent products following proteolytic cleavage by their corresponding active caspase enzyme. Increased fluorescence intensity can be observed in the presence of activated caspases.

C3 activation was observed at 24 hours with LA lenses soaked in OFX by detecting a significant increase in fluorescence at 29.57AU per mg protein (Figure 5.4), resulting in a 315% increase relative to cells grown in the absence of a lens ($p = 0.00002$) and all other solution-lens combinations ($p < 0.02$). The C3 activation detected in cells exposed to BA-OFX was significantly lower than LA-OFX ($p = 0.0199$). No significant upregulation of C3 was detected with any other solution-lens combination. In addition, significant levels of C9 were also not detected (Table 5.3).

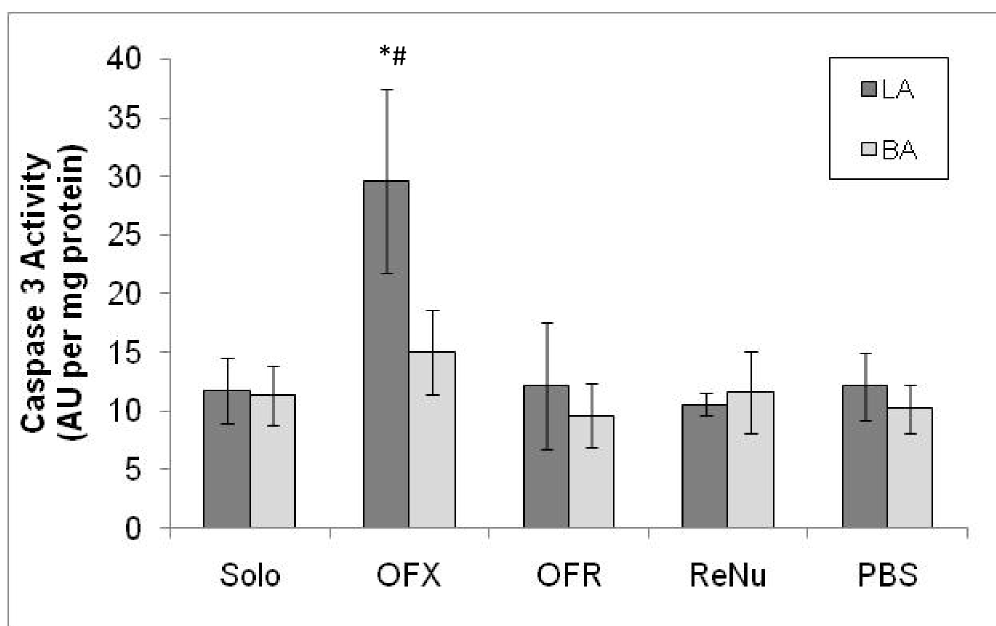


Figure 5.4: Caspase 3 activity after 24 hour contact with Lotrafilcon A and Balafilcon A lenses soaked in various MPS. Caspase activity was normalized to total protein content and expressed as mean fluorescence intensity in AU per mg protein. N = 3 to 4, * Significantly different from cells grown in the absence of lenses ($p < 0.05$), # Significantly different from Balafilcon A ($p < 0.02$).
OFR, Opti-Free RepleniSH, OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Table 5.3: Caspase 9 (C9) activity after 24 hour exposure to lens solution treatments, as measured by fluorescence. Caspase activity was normalized to total protein content and expressed as mean fluorescence intensity in AU per mg protein. N = 3 to 4.

Lens	Solution	C9 Activity (AU per mg protein)	SD
LA	Solo	8.66	3.1
	OFX	11.62	8.5
	OFR	5.82	6.2
	ReNu	9.49	3.7
	PBS	14.42	7.2
BA	Solo	7.07	4.4
	OFX	11.41	6.8
	OFR	4.30	4.7
	ReNu	10.59	6.0
	PBS	11.48	6.3
No Lens		7.28	3.1

Annexin V-FITC/Propidium Iodide Staining

To assess apoptosis and necrosis, Annexin V-FITC/PI staining was detected using flow cytometry. Post analysis using FCSExpress was used to determine the percentage of healthy cells (not stained), apoptotic cells (stained with Annexin V-FITC only), early necrotic cells (stained with PI only), and secondary necrotic cells (stained with both Annexin V-FITC and PI) (Figure 5.5). When cells were grown in the absence of a lens, 0.4, 2.2 and 1.2% of cells were stained with Annexin V-FITC, PI and both, respectively. This indicated that 96.2% of cells remained healthy, as the cells stained neither with annexin V nor PI. Exposure to OFX resulted in significant populations of apoptotic and early necrotic cells (Table 5.4). LA-OFX had 14.4% early necrotic cells, and 2.1% apoptotic cells ($p < 0.013$). LA-OFR had similar results, with 7.6% of cells in early necrosis and 2.1% of cells in apoptosis, but only the apoptotic population was significantly different from control cells grown in the absence of a lens ($p = 0.0002$). While both LA-OFX and OFR induced 4% of the cell population to enter secondary necrosis, this value was not significant. No solution combination with BA lenses yielded a significant effect. LB lenses were also tested to further compare the effects of lens material and surface treatment. LB-OFX also demonstrated similar significant levels of apoptotic cells, at 2.9% ($p = 0.0043$).

Table 5.4: Apoptotic effects of lens-solution treatments as detected by flow cytometry (mean ± SD, n = 3 to 7).

Treatment		No staining (healthy cells)	Annexin V-FITC staining (apoptotic cells)	PI staining (cells in early necrosis)	Annexin V-FITC and PI staining (cells in secondary necrosis)
LA	Solo	96.4 ± 1.7	0.5 ± 0.2	1.8 ± 1.3	1.3 ± 0.5
	OFX	79.5 ± 8.7*	2.1 ± 1.4*	14.4 ± 9.6*	4.0 ± 0.5
	OFR	85.7 ± 6.4*	2.6 ± 1.8*	7.6 ± 3.4	4.1 ± 3.3
	ReNu	95.5 ± 2.0	0.5 ± 0.3	2.6 ± 0.9	1.5 ± 0.9
	PBS	97.1 ± 1.1	0.4 ± 0.2	1.4 ± 0.7	1.1 ± 0.2
BA	Solo	96.0 ± 2.3	0.5 ± 0.4	2.4 ± 2.2	1.1 ± 0.6
	OFX	91.5 ± 2.8	1.4 ± 1.2	4.2 ± 1.9	2.9 ± 2.3
	OFR	94.4 ± 1.3	0.9 ± 0.5	3.3 ± 1.0	1.4 ± 0.4
	ReNu	96.6 ± 1.2	0.3 ± 0.1	1.9 ± 0.6	1.2 ± 0.8
	PBS	97.1 ± 1.3	0.5 ± 0.2	1.3 ± 0.6	1.2 ± 0.6
LB	Solo	91.8 ± 4.8	0.8 ± 0.6	3.5 ± 1.0	3.9 ± 4.1
	OFX	83.9 ± 2.3	2.9 ± 2.4*	7.8 ± 5.4	5.4 ± 4.5
	OFR	91.9 ± 1.5	0.7 ± 0.2	6.4 ± 1.2	1.1 ± 0.1
	ReNu	90.0 ± 2.5	0.4 ± 0.4	8.2 ± 2.9	1.3 ± 0.1
	PBS	90.2 ± 4.3	0.4 ± 0.5	5.9 ± 1.2	3.5 ± 3.9
10% dilution	OFX	91.0 ± 2.5	0.9 ± 0.5	4.6 ± 1.9	3.5 ± 1.1
	OFR	92.4 ± 3.2	0.7 ± 0.7	4.3 ± 2.6	2.6 ± 2.6
	ReNu	91.6 ± 4.3	0.3 ± 0.2	6.9 ± 4.4	1.2 ± 0.4
Controls	No Lens	96.2 ± 1.5	0.4 ± 0.3	2.2 ± 1.4	1.2 ± 0.4
	Alcohol	65.3 ± 7.5*	1.4 ± 0.7	22.1 ± 4.6*	11.2 ± 4.5

* Significantly different from cells grown in the absence of a lens, $p < 0.047$.

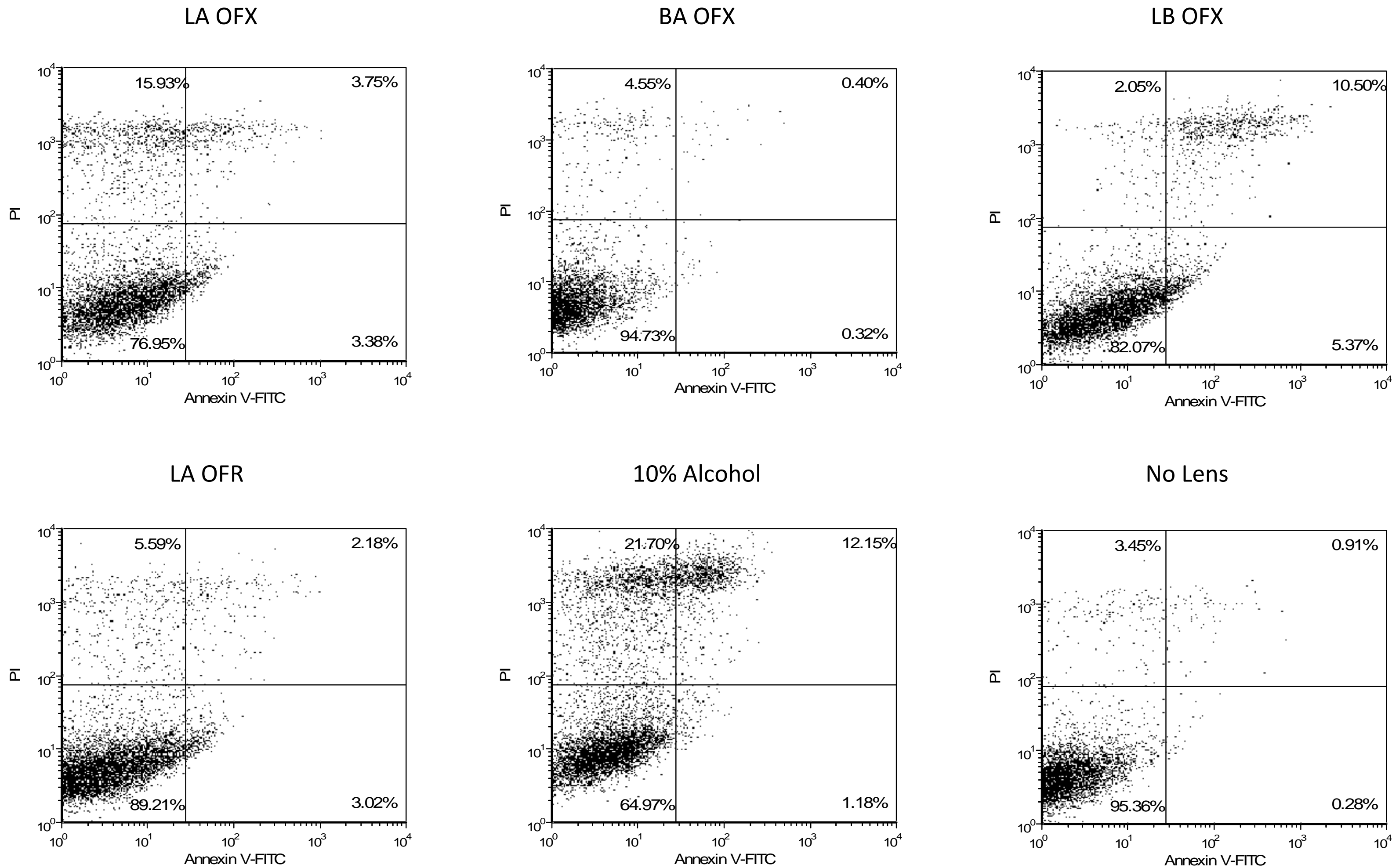


Figure 5.5: Annexin V-FITC/PI staining induced by MPS-soaked contact lenses. Figures represent one trial, after 24 hour exposure to Lotrafilcon A, Lotrafilcon B and Balafilcon A lenses soaked in MPS. Cell populations in the lower left quadrant are healthy cells (not stained), lower right quadrant are apoptotic cells (stained with Annexin V-FITC only), upper left quadrant are early necrotic cells (stained with PI only), and upper right quadrant are late/secondary necrotic cells (stained with both Annexin V-FITC and PI). Cells exposed to 10% alcohol represent positive control; no lens treatment represents negative staining control.OFX, Opti-Free Express; OFR, Opti-Free RepleniSH; PI, Propidium Iodide

Discussion

The results obtained from cell contact with lenses soaked in phosphate buffer demonstrated near 100% viability and no change in caspase activation or annexin V staining and thus confirmed the validity of our in vitro model. The cell count results are in agreement with previously published cellular viability results, obtained from an MTT assay. While there were no significant differences in viability between the assays, the MTT assay appeared to be more sensitive in detecting changes in mitochondrial function rather than cell loss, as it detected a significant decrease in cells exposed to both lenses soaked in ReNu and Solo, at 24 hours.

One of the early events of the intrinsic pathway of apoptosis is alteration of mitochondrial transmembrane potential.[9,12,17,18] While the MitoCapture assay did not demonstrate a significant change in mitochondrial transmembrane potential, the trend was that more cytotoxic solution-lens combinations, such as LA-OFX, as determined by the cell count and previous cytotoxicity testing, had the most reduction in membrane potential. When apoptotic stimuli disrupt the mitochondrial transmembrane potential, it triggers the release of cytotchrome c (cyt c) from the intermembrane space, leading to activation of C9 and intrinsic activation of apoptosis.[11,12,17,18] Cyt c in the cytoplasm can bind Apaf-1 (apoptotic protease activating factor-1), a scaffolding molecule key to the formation of the apoptosome.[11,19] Cyt c will induce a nucleoside triphosphate-dependent conformational change, allowing for the binding of procaspase-9.[20-25] Procaspase-9 is the initiator caspase of the intrinsic pathway of apoptosis.[26,27] It is a monomer with misaligned catalytic cysteine and histidine residues and an inactive catalytic pocket.[26,27] The complex of cyt c, Apaf-1 and procaspase-9 forms the apoptosome, which leads to the activation of C9 by conformational changes that open the catalytic pocket and align the catalytic residues.[27] While the results of the C9 activity assay were also not significant, the trend was still consistent that the most cytotoxic solutions had the highest activity of C9. All assays were

performed at the same time point of 24 hours, which appears to correlate better with the mid phase apoptotic events. The alteration of mitochondrial membrane potential and activation of C9 are early phase apoptotic events[9]; if evaluated at earlier time points, mitochondrial potential and C9 activity may be significant.

Previous work has shown that the combination of LA lenses and OFX solution caused an upregulation of caspases, as detected by a pancaspase assay. Findings from this study confirm that this is primarily due to upregulation of downstream effector C3 at 24 hours. Evidence of initiator C9 activity and altered mitochondrial membrane potential, and absence of activation of Fas death receptors (data not shown), suggests that apoptosis induced by LA-OFX is through the intrinsic pathway. C3 activation was also observed in human conjunctival cells, when exposed to OFX.[6] The study also found that Solo significantly induced C3 activity, which is contrary to the findings of this study.[6] The discrepancy may be due to the concentration of MPS the cells were exposed to, or the difference in cell type. The release of biocides from a contact lens is likely a low concentration, so by using the MPS directly grossly overestimates the concentration the ocular surface would normally be exposed to. Evidence has also shown that certain combinations of MPS and silicone hydrogel lenses can lead to a cytotoxic effect in vitro [1-3] and some combinations have the potential to exhibit excessive corneal staining in vivo, further supporting the importance of using contact lenses in MPS cytotoxicity testing.[39-47] While conjunctival cells also have the potential to exhibit adverse reactions following contact lens wear, the contact lens and released MPS come into the most contact with the corneal epithelium.[48] If the difference is due to cell type, rather than concentration, conjunctival cells should be considered an inappropriate proxy for corneal epithelial cells, when investigating MPS cytotoxicity.

Previously, it was hypothesized that the borate solutions, OFX and ReNu, were capable of inducing apoptosis. It was observed that OFX-soaked LA lenses caused an increase in the number of

cells staining positive for Annexin V-FITC and PI, indicating significant populations of apoptotic and early necrotic cells. The presence of Annexin V staining indicates that the phospholipid scramblase was activated by cleavage of Cδ, and PS is being expressed on the plasma membrane surface as a signal that can be recognized by the adaptor molecule MFG-E8 on adjacent cells and/or a PS receptor present on macrophages, facilitating phagocytosis of the dying cells.[34-38] These findings may be related to recent in vivo observations where LA lenses in combination with Opti-Free MPS was associated with a significantly higher incidence of corneal infiltrative events.[47] Exposure to dilute ReNu showed a greater amount of PI staining compared to Annexin V staining, indicating a pure necrotic response, rather than an apoptotic response. Previously, it was hypothesized that both borate solutions, including ReNu, may be causing apoptosis in HCECs, due to evidence that integrin expression was adversely affected by ReNu. Upon further investigation, the results from this study indicate that apoptosis is not likely to be induced by ReNu. Since both solutions are borate based, borate is also likely to not cause apoptosis. Borate has been shown to cause cell death, and the results from this study indicate a potential role in necrosis.[3,5,15] It is likely that other constituents of OFX are acting as the apoptotic stimuli. Many studies have attributed the cytotoxicity potential of OFX to its active ingredient, Polyquaternium-1 (PQ), a quarternary ammonium, which targets bacterial cytoplasmic and fungal plasma membranes.[1,2,48-50] Studies have shown that after interaction with lenses during the overnight disinfection cycle, OFX (and OFR) solution retains their bactericidal and fungicidal activity; while ReNu (PHMB) does not.[50] While it is ideal to retain this property, it means that residual active disinfecting agents on lenses have the potential to interact adversely with the corneal surface.[50] The added effect of borate in OFX may also contribute to cell death, but through a necrotic pathway rather than apoptosis. ReNu showed a significant decrease in both the activity and PHMB biocide concentration after a 6 hour lens soaking period.[50] It is likely that the residual or adsorbed solution,

containing other active constituents such as borate, is responsible for necrosis. The surfactants in OFX may also be responsible for the cytotoxicity, since the newest Opti-Free solution, RepleniSH, has a new TearGLYDE surfactant system. While OFR still contains Polyquad, the difference is in the surfactant system. Unlike OFX, OFR did not significantly affect C3 activity. In addition, the absence of EDTA in Opti-Free RepleniSH may account for differences between the two Opti-Free solutions.

It was found that lens type also played a role in inducing apoptosis, where only LA soaked in OFX significantly affected C3 activation and Annexin V/PI staining. The difference may be attributed to the different physical properties of the lenses. BA and LA lenses have different surface treatments: LA lenses are plasma coated while BA lenses undergo plasma oxidation.[51] This difference, which may affect the uptake and/or release of biocides from the lens and the actual concentration of MPS the cells are exposed to, had a significant effect on cells. This theory was supported by the Annexin V-FITC staining demonstrated by OFX released from LB lenses, which was similar to levels seen with LA lenses. While they are different materials, LB has the same plasma surface coating which LA lenses possess, indicating that the surface treatment may be one of the lens parameters affecting uptake and release. Levels of PI staining were however much lower with LB-OFX than with LA-OFX suggesting that other lens parameters, such as lens chemistry, can also influence lens adsorption and release profiles and hence will affect cell response. Additionally, one must consider the ability of the lens material to form a complex with constituents of MPS, which would affect the way in which cytotoxic constituents are exposed to the epithelial surface.[52] Further studies are currently underway to better understand the interaction between MPS solution, surface treatment on silicone hydrogel lenses and the effect on cells.

In summary, the results from this study shows that for MPS-released from a contact lens, mechanisms of cell death can be investigated. As demonstrated by the absence of an effect on cells exposed to a PBS-soaked lens, our results indicate that it is not the presence of the lens that affects cell viability and phenotype, but what is being released from the lens. The results indicate that OFX-induced cell death is caspase mediated intrinsic apoptosis pathway, whereas the cell death induced by ReNu is necrosis. The in vitro model also demonstrated a lens effect in the mechanism of MPS-induced cell death pathways. The differences in physical properties of BA and LA lenses, which affect the uptake and/or release of the various ingredients in MPS, had a significant effect on viability, C3 activation and Annexin V/PI staining. Further studies are under way to gain a better understanding of the preferential adsorption or release profile of certain compounds by silicone hydrogel lenses with different physical properties and surface treatments.

CHAPTER 6

IMPACT OF LENS MATERIAL ON CYTOTOXICITY POTENTIAL OF MULTIPURPOSE SOLUTIONS IN HCEC, *IN VITRO*

INTRODUCTION

After contact lens wear, lenses must be disinfected to prevent growth of harmful bacteria that may be present on the lens and remove lipid and/or protein deposits on lenses.[1-4] The most commonly prescribed disinfection systems are multi-purpose solutions (MPS).[3-5] MPS are single solutions, that are used to rinse, clean, disinfect, rewet and store contact lenses.[1-4] MPS contain many different components to enhance disinfection and preservative properties. The disinfecting properties of MPS are conferred by the active biocide, which are commonly a polyquaternium, biguanide or hydrogen peroxide agent.[6,7] The preservatives in MPS, such as Polyquad, Aldox and Polyhexamethylene biguanide (PHMB), are intended to breach cell walls of microbes, but may have the potential to cause corneal epithelial cell membrane toxicity.[2,8] MPS also contain a buffering solution to maintain the pH of the solution, which is typically either borate or phosphate-based.[7] The solution must be efficacious enough against microbial flora, but gentle enough to not cause adverse effects on the corneal surface, as some of the solution will be exposed to the corneal surface and remain in contact with the epithelium until washed away by the post-lens tear film.[3-5] While some have hypothesized that, due to their high molecular weight, the disinfecting agents cannot penetrate the surface of the lens, one cannot ignore the potential of the lens to adsorb or form a complex with components of MPS and release them onto the corneal surface post insertion.

Silicone hydrogel (SH) lenses are the newest generation of contact lens materials, which incorporate siloxane moieties to increase the oxygen permeability to the cornea.[9-11] The physical

properties of the chemical polymers used in hydrogel and siloxane-based contact lens materials allow for the potential adsorption and absorption of components of care regimens during the overnight disinfection cycle, followed by potential release onto the corneal surface during lens wear. Various surface modifications and proprietary chemistry are employed to reduce the hydrophobicity of the SH lens surface and the potential interaction of the lens with tear film components and active components of MPS.[9,10] The surface of Lotrafilcon A (LA) and Lotrafilcon B (LB) lenses are permanently modified with a mixture of trimethyl-silane oxygen and methane, in a gas plasma reactive chamber.[9,10] The resulting coating is continuous ultra-thin, hydrophilic surface. Balafilcon A (BA) lenses are treated in a gas plasma chamber to convert all siloxane components into silicate compounds, making the surface more hydrophilic.[9,10] The transformed areas form ‘glassy islands’, which bridge over the underlying hydrophobic lens material.[9,10] This differs from the surfaces of all other lenses tested, as it is the only lens with a relatively “rough”, discontinuous surface.[9,10] The exposed bulk material makes the lens more hydrophobic in comparison to other SH lenses, which may collect more lipid and protein, and has higher contact angles.[9,10]

Galyfilcon A (GA) and Comfilcon A (CA) are both non-surface treated lenses. In GA lenses, an internal wetting agent, derived from poly(vinylpyrrolidone), have been incorporated in the bulk material to improve hydrophilicity.[9,10] To date, there is very little published on CA lenses. Proprietary chemistry is used to create a highly wettable surface, without specific surface modifications.[9] The surface features are comparable to that of conventional polyHEMA lenses.[9]

Despite modifications aimed to reduce adsorption and release of active biocides onto corneal surface, certain combinations of MPS and SH lenses can lead to a cytotoxic effect in vitro[4,12-14] and some combinations have the potential to exhibit excessive corneal staining in vivo.[6,16-22] With these

observations, many biocompatibility studies have been undertaken to examine the effect of ophthalmic solutions, including contact lens packaging solutions and multipurpose solutions, on corneal and conjunctival cells.[2-5,12,13,23-26] Most in vitro studies with MPS have been limited to studying the effect of solutions on cells, using extracts or dilutions of solutions to evaluate the corneal effect. These studies were able to evaluate the potential cytotoxic effect of ophthalmic and multipurpose solutions in vitro, and while this is valuable research, there is currently no information on the effect of the direct release of solutions from silicone hydrogel lenses on human corneal epithelial cell (HCEC).[2-5,12,13,23-26] Previous studies have shown that lens type will affect uptake and release of compounds contained in MPS[11], and this may also have an impact on any subsequent cytotoxic responses observed. Previously published data from our laboratory, which detected differences in cell viability and caspase activation at 24 hours with lenses soaked in OFX, emphasizes the importance of testing solution-lens combinations in vitro. The difference in viability and adhesion phenotype may be attributed to the different physical properties and surface treatments of the lenses. This difference, which may affect the uptake and/or release of biocides from the lens and the actual concentration of MPS the cells are exposed to, had a significant effect on cells.

This study was undertaken to determine the effect that the surface properties of silicone hydrogel contact lenses have on the direct release of multipurpose solutions and understand the interaction between MPS solution, surface treatment, lens material and HCECs.

Materials and Methods

Reagents

Keratinocyte serum free medium, growth supplement (Bovine Pituitary extract) and pen-strep solution were purchased from ScienCell. All other cell culture reagents, Dulbecco's minimum essential medium, fetal bovine serum, phosphate buffer saline and TriplExpress were purchased from Invitrogen (Burlington, Ontario, Canada).

Contact lenses and multipurpose solutions

Six silicone hydrogel lens materials were tested (Table 6.1): balafilcon A (BA; Bausch & Lomb, Rochester, NY, USA), lotrafilcon A (LA; CIBA Vision; Duluth, GA, USA), lotrafilcon B (LB; CIBA Vision; Duluth, GA, USA), comfilcon A (CA; CooperVision; Fairport, NY, USA) and galyfilcon (GA; Vistakon; Jacksonville, FL, USA). All lenses were purchased in their original packaging, had a diameter between 14.0 and 14.2 mm and a curvature of 8.5 to 8.7mm. Four polyquaternium and biguanide preserved multipurpose solutions were tested (Table 6.2).

Table 6.1: Properties of lenses used in the study[27]

Proprietary Name	PureVision	Focus Night & Day	O_2 Optix	Biofinity	Acuvue Advance
Manufacturer	Bausch & Lomb	CIBA Vision	CIBA Vision	CooperVisi on	Vistakon (Johnson & Johnson)
USAN	Balafilcon A	Lotrafilcon A	Lotrafilcon B	Comfilcon A	Galyfilcon A
Water Content (%)	36	24	33	48	47
Dk	99	140	110	128	60
Charge	Ionic	Non-ionic	Non-ionic	Non-ionic	Non-ionic
Principle Monomers	NVP + TPVC	DMA + TRIS + siloxane macromer	DMA + TRIS + siloxane macromer	Undisclosed	mPDMS + DMA + EGDMA + HEMA + siloxane macromer + PVP
Surface Treatment	Plasma oxidation process	25nm plasma coating	25nm plasma coating	None	None, internal wetting agent

DMA (*N*,*N*-dimethylacrylamide); EGDMA (ethyleneglycol dimethacrylate); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane); NVP (*N*-vinyl pyrrolidone); PVP (polyvinyl pyrrolidone) TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate; TRIS (trimethylsiloxy silane).

Table 6.2: Disclosed composition of the MPS used in the study[28]

Manufacturer	Brand (abbreviation)	Disinfecting Agent	Buffer	Other reported agents (surfactants and chelating agents)
Alcon	Opti-Free Express (OFX)	Polyquad® 0.001%, Aldox® 0.0005%	Borate	Sorbitol; citrate (citric acid), 0.05% EDTA; poloxamine (Tetronic 1304)
	Opti-Free RepleniSH (OFR)	Polyquad® 0.001%, Aldox® 0.0005%	Borate	Citrate, poloxamine (Tetronic 1304), non-anoyl ethylene-diaminetriacetic acid
AMO	Complete Moisture Plus (Complete)	PHMB 0.0001%	Phosphate	Taurine; 0.01% EDTA; Poloxamer 237 (Pluronic F87); HPMC 0.15%; propylene glycol
Bausch & Lomb	ReNu MultiPlus (ReNu)	PHMB 0.0001%	Borate	Sodium borate; Hydroxyalkylphosphonate (Hydranate™); 0.1% EDTA; Poloxamine (Tetronic 1107)
CIBA Vision	SoloCare Aqua (Solo)	PHMB 0.0001%	Tris	Sorbitol; 0.025% EDTA; dexpanthenol (provitamin B5); Pluronic F127 (poloxamer 407)

PHMB: polyhexamethylene biguanide (also known as polyhexanide, Dymed, polyhexadine, and polyaminopropyl biguanide)

In vitro cell culture

Immortalized human corneal epithelial cells (HCEC)

SV40-immortalized human corneal epithelial cells were cultured in keratinocyte serum free medium supplemented with bovine pituitary extract, recombinant epidermal growth factor and pen-strep (KSFM). Fresh medium was added every other day and cells were grown to 90% confluency in tissue culture treated flasks. Adherent cells were removed using a dissociation solution, TriplExpress (Sigma-Aldrich, Oakville, Ontario, Canada). Cells were routinely observed for any morphological changes.

In vitro model

A direct contact in vitro model was used.[7] Briefly, HCEC were seeded onto a 24 well tissue culture treated polystyrene (TCPS) plate at 10^5 cells per well. Cells were left to adhere for 18-24 hours in a humid CO_2 incubator, which resulted in the formation of a monolayer of HCEC. Simultaneously, SH lenses were totally immersed in the four MPS, in a sterile 12-well polystyrene plate and soaked for 18 – 24 hours. All lens-solution soaking combinations were performed under sterile conditions.

Supernatant was then removed and fresh serum-free medium was added. MPS-soaked SH lenses were placed gently on top of the monolayer, face-down, with the concave surface facing upwards and incubated for up to 24 hours at 37^oC (5% CO_2 in a humid incubator). Lenses were totally immersed in medium. After 24 hours, lenses were carefully removed from wells. The lenses did not adhere to the HCEC monolayer. Lenses were also routinely observed for the presence of adherent cells on their surface and no HCEC proliferation on the lens was observed.

Additionally, cells were exposed to dilutions of MPS, between 0.1 – 10%, added to the medium for a 24 hour incubation period.

Cellular viability

To measure cytotoxicity of the products released from the contact lenses, the MTT cellular viability assay was performed. After a gentle rinse in sterile PBS, cells were incubated with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT at 1mg/mL in KSFM medium). After 3 hours at 37°C, cells were lysed with DMSO and absorbance read at 595nm. All results are expressed as relative viability compared to cells grown in the absence of a contact lens.

Caspase activation

To determine cellular activation by change in caspase activation induced by MPS release from contact lens, following a gentle wash in PBS, HCEC were removed from the wells with TriplExpress (Invitrogen, Burlington, Ontario, Canada). Cells were washed and resuspended in DMEM/FBS and were incubated with a fluorescently-labelled pan caspase inhibitor (FITC-VAD-FMK, Calbiochem, San Diego, California) for 1 hour at 37°C. Samples were washed and resuspended in wash buffer, before immediate analysis by flow cytometry.

All samples were acquired on Becton Dickinson FACSVantage flow cytometer (Mountain View, CA, USA) using CELLQuest Software. Appropriate isotype controls were used with each experiment. Analysis was also performed using FCSExpress post data acquisition.

Lens release profile

The release of OFX from contact lenses was characterized by absorbance. OFX – soaked lenses were incubated in 700 μL PBS for 24 hours at 37°C. The lens extracts were stored in glass vials at 4°C until analysis. Samples were transferred to quartz cuvettes, and absorbance was read on a UV spectrophotometer (Thermo MultiSkan Spectrum Photometer, Fischer Scientific, Ottawa, ON, Canada) in the range of 202 – 220 nm. Regression analysis was performed using Microsoft Excel.

Statistical analysis

All results are reported as means ± standard deviation (SD). To evaluate the significance of the differences in cell viability and cell activation, an ANOVA was carried out followed by a post hoc Bonferroni test using Statistica. A p value of less than 0.05 was required for statistical significance. The number of experiments was equal to or greater than three with different cell passages.

RESULTS

Cell viability

Cell viability was assessed after incubation periods of 24 hours. There was no observed difference between cells exposed to PBS-soaked lenses and cells cultured in the absence of a lens, indicating that it was not the presence of a lens that reduced cell viability; but rather the product released from the lens.

For the dilutions tested, it was found that 10% OFX was significantly different from 1% and 0.1% OFX ($p < 0.002$), while 5% OFX was only found to be significantly different from 0.1% ($p = 0.0273$) (Figure 6.1). ReNu also demonstrated a significant concentration - dependent effect, where the 10% dilution was also significantly different from 0.1 and 1% ReNu ($p < 0.00002$). This finding was in contrast to Complete and Solo, which have the same disinfecting agent as ReNu. Both Complete and Solo did not adversely affect viability with increasing concentrations; at the maximum concentration tested, both solutions had around 80% viability.

For the lens-solution combinations tested, LA-OFX was significantly different from all dilutions ($p < 0.041$), suggesting that the released OFX from LA lenses is either greater than 10% or modified in such a way, by LA, to be more cytotoxic to HCEC. OFX soaked GA, BA, CA and LB exhibited levels of viability similar to diluted OFX below 10%. In contrast to its interaction with OFX, ReNu soaked LA was found to have viability levels similar to the ones observed with ReNu at concentration between 5 and 10%. BA and LB also demonstrated viability similar to the same range.

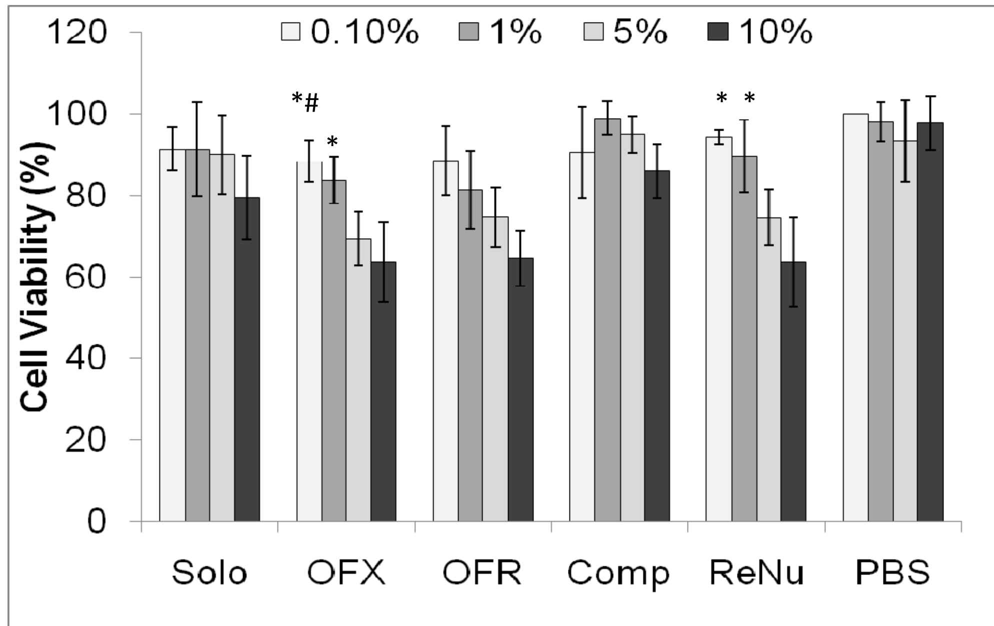


Figure 6.1: HCEC viability after 24 hour contact with various MPS in concentrations between 0.10% and 10%. Viability was measured by MTT assay and is expressed as a percentage relative to cells grown in the absence of lenses. N = 4 to 6. * Significantly different from cells exposed to 10% dilution ($p < 0.002$), # Significantly different from cells exposed to 5% dilution ($p < 0.03$) Complete, Complete Moisture Plus; OFX, Opti-Free Express; OFR, Opti-Free RepleniSH; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

A 50% reduction was seen with LA soaked in OFX ($p = 0.0002$), while LA-ReNu led to more than 30% reduction, which was significantly different from the positive PBS control ($p = 0.00006$) (Figure 6.2). While there were similar trends in viability with all lenses, there was a significant difference in the viability of cells exposed to OFX at 24 hours. LA-OFX had significantly lower viability compared to BA, LB and CA lenses soaked in the same solution ($p < 0.01$). Cells exposed to LB, GA and CA lenses soaked in OFX had levels similar to viability of BA, at 72, 61 and 71% viability after a 24 hour incubation time. These results demonstrate that lens type did have a significant effect on viability ($p = 0.00000$) and a significant interactive effect with solution type ($p = 0.0282$).

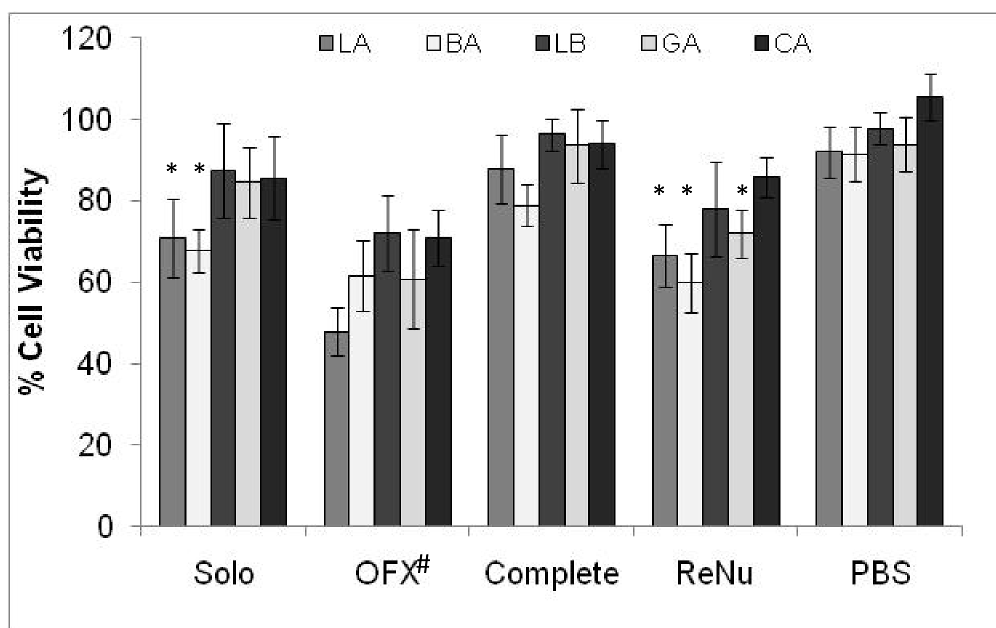


Figure 6.2: HCEC viability after 24 hour contact with Lotrafilcon A (LA), Balafilcon A (BA), Lotrafilcon B (LB), Galyfilcon A (GA) and Comfilcon A (CA) soaked in various MPS. Viability was measured by MTT assay and is expressed as a percentage relative to cells grown in the absence of lenses. N = 4 to 6. # Significantly different for all lenses soaked in OFX from cells grown in the absence of lenses ($p < 0.002$). * Significantly different from cells grown in the absence of lenses ($p < 0.002$).
Complete, Complete Moisture Plus; OFX, Opti-Free Express; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Table 6.3: P values of significance for effect of lens-solution combinations on cell viability.

	Solo	OFX	Complete	ReNu
LA	0.0017	0.000	1.00	0.000
BA	0.00009	0.000	0.93	0.00006
LB	1.00	0.0014	1.00	0.0886
GA	1.00	0.000001	1.00	0.0033
CA	0.0827	0.000001	1.00	0.1048

Caspase activation

Activated caspases were detected by flow cytometry. Cells were incubated with a fluorescently-tagged pan caspase inhibitor, which can bind to any and all activated caspases. The fluorescently-tagged pan caspase inhibitor fluoresced most intensely in cells with active caspases, as indicated by the bimodal distribution of fluorescence. Caspase activation was observed at 24 hours,

with LA lenses soaked in OFX, by detecting a significant amount of cells staining positive for caspases (p = 0.00000) (Figure 6.3). As in the viability results, BA-OFX was significantly better than LA-OFX (p = 0.002) and BA did not induce activation of caspases for any solution. In contrast, LB, GA and CA, had levels of caspase activation similar to LA-OFX (p = 1.000).

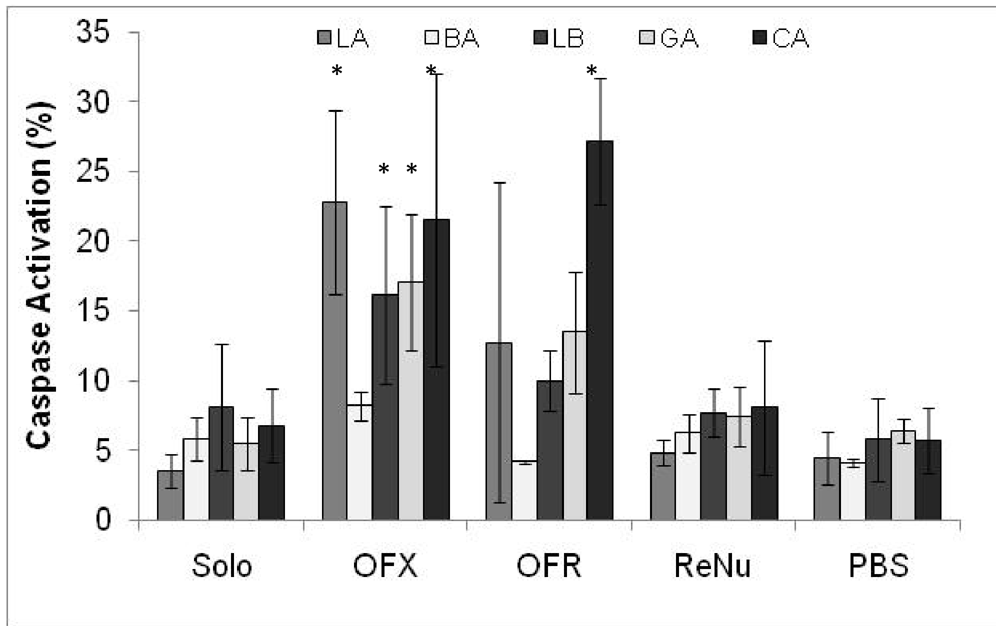


Figure 6.3: HCEC caspase activation viability after 24 hour contact with Lotrafilcon A (LA), Balafilcon A (BA), Lotrafilcon B (LB), Galyfilcon A (GA) and Comfilcon A (CA) soaked in various MPS. Activation was measured by flow cytometry and is expressed as a percentage relative to cells grown in the absence of lenses. N = 4 to 6, * Significantly different from cells grown in the absence of lenses ($p < 0.0006$), # Significantly different from BA ($p < 0.005$).
OFX, Opti-Free Express; OFR, Opti-Free RepleniSH; ReNu, ReNu MultiPlus; Solo, SoloCare Aqua

Table 6.4: P values of significance for effect of lens-solution combinations on caspase activation.

	Solo	OFX	OFR	ReNu
LA	1.00	0.000	0.882	1.00
BA	1.00	1.00	1.00	1.00
LB	1.00	0.0005	1.00	1.00
GA	1.00	0.0006	0.408	1.00
CA	1.00	0.000	0.000	1.00

With LB, GA and CA lenses soaked in OFX, a significant amount of cells staining positive for caspases was also detected, compared to cells grown in the absence of a lens ($p < 0.0006$). In addition, the level of caspase activation by CA-OFX was also significantly different from BA-OFX ($p = 0.0041$). These results demonstrate that lens type also have a significant effect on caspase activation ($p = 0.000005$) and a significant interactive effect with solution type ($p = 0.00007$).

Lens release profile

Both the MTT assay and caspase assay were sensitive enough to detect differences in release profiles of OFX from the tested silicone hydrogel lenses. Figure 6.4 illustrates the effect release of OFX from the various lenses can have on viability and caspase activation.

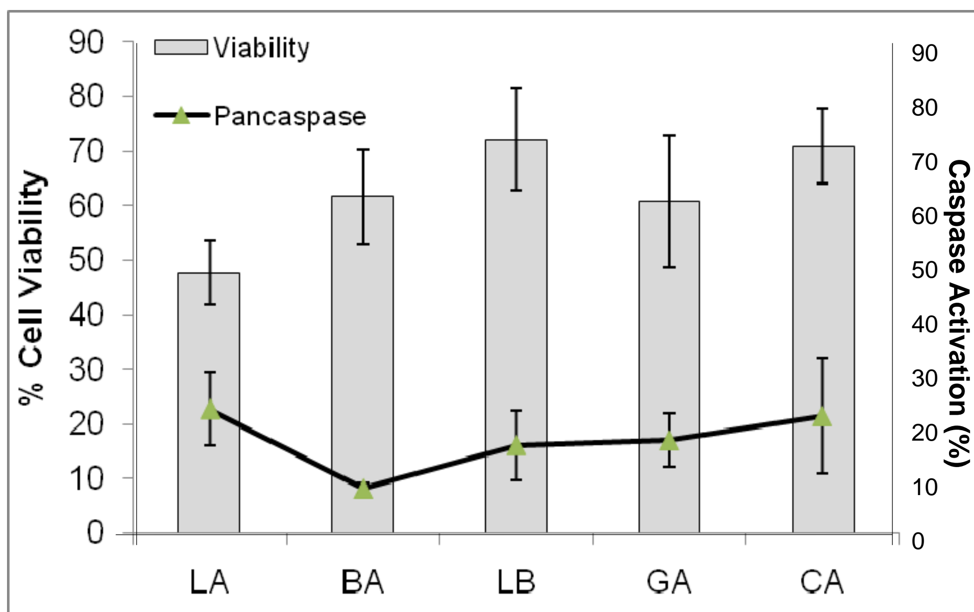
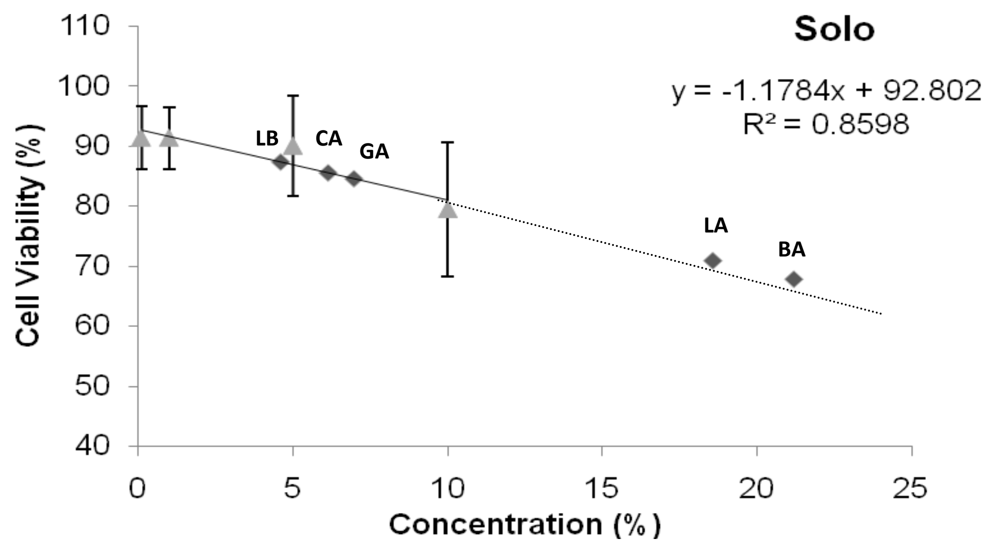
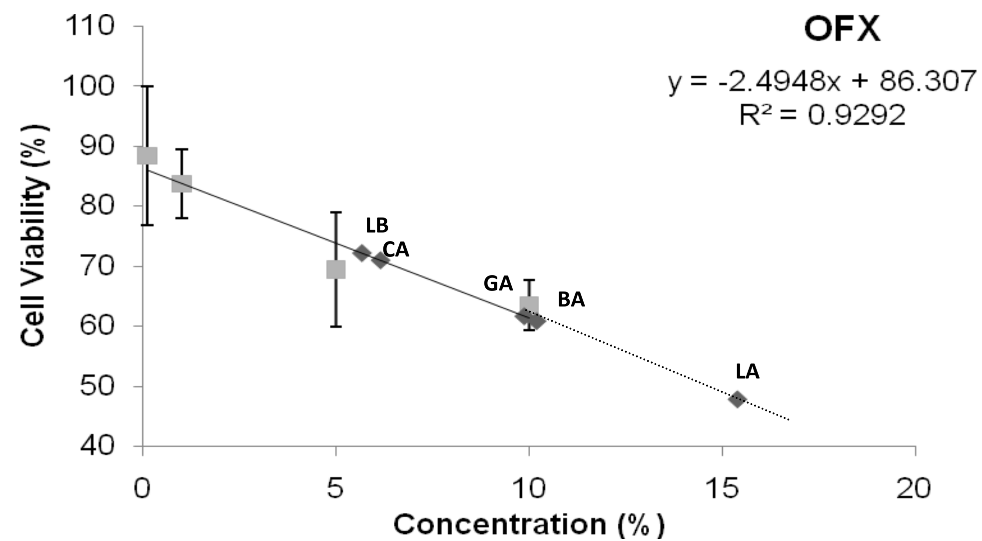


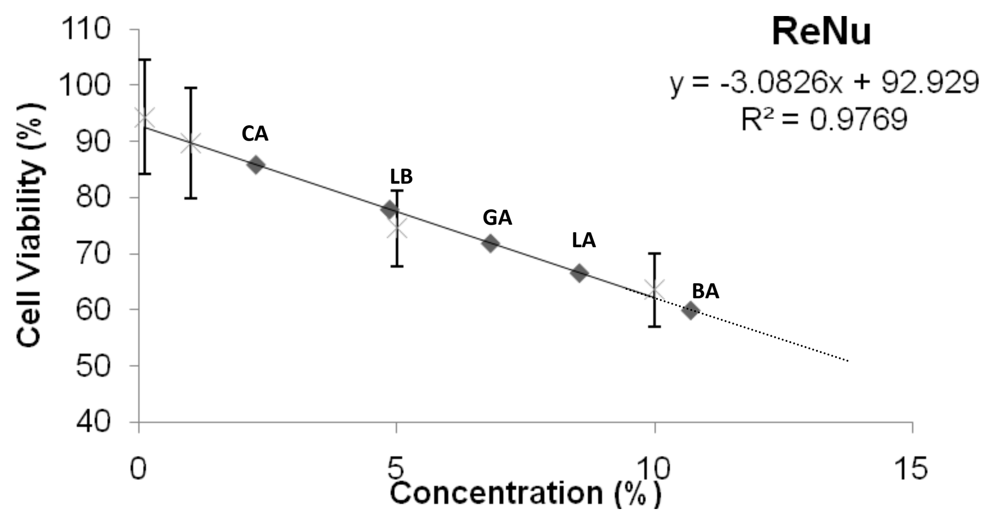
Figure 6.4: HCEC caspase activation and viability after 24 hour contact with Opti-Free Express released from Lotrafilcon A (LA), Balafilcon A (BA), Lotrafilcon B (LB), Galyfilcon A (GA) and Comfilcon A (CA). Viability was measured by MTT assay and activation was measured by flow cytometry. Both are expressed as a percentage relative to cells grown in the absence of lenses. N = 4 to 6.

The differences in cell viability and caspase activation may be due to the concentration of MPS released from the contact lens. A study on dilutions demonstrated that with increasing concentration of MPS, a greater adverse effect on HCEC viability was observed (Figure 6.1). In investigating the effect of dilutions on viability, one can estimate the potential release of MPS from contact lenses (Figure 6.5). Using the dilution data for each solution, a regression line was fitted to the data. The formula of the generated trendline was used to estimate the concentration of MPS released from contact lenses, given the viability observed using the MTT assay (Table 6.5). This method was able to estimate that LA-OFX, which has demonstrated decreased viability and increased apoptotic markers (previously discussed in Chapter 5), had released the equivalent of approximately 16% dilution onto the monolayer. All other lenses released concentrations in the range of 5-10%. While the regressions for Solo and Complete generated concentrations in a similar range as LA-OFX, one must consider that the R-squared values are lower and statistical analysis revealed that there was no significant effect of concentration on viability.

Integrin data also indicates that the concentration of OFX released from LA lenses is greater than 10%. It was observed that the expression of α3, β1 and β4 after exposure to LA-OFX was significantly lower than 10% OFX ($p < 0.05$), suggesting that LA lenses are releasing more than 10%. The integrin data also suggests that for all other lens-solution combinations with LA and BA, the release is less than 10%. Findings with the pancaspase assay for LA-OFX also found that the release is likely to be greater than 10%. Results demonstrated that caspase activation was also concentration dependent, and showed an increase in cells staining positive for caspases, with increasing concentration of OFX. LA-OFX activated more caspases than both 5 and 10% OFX, further supporting the approximation of MPS release from the regression analysis (Table 6.5).







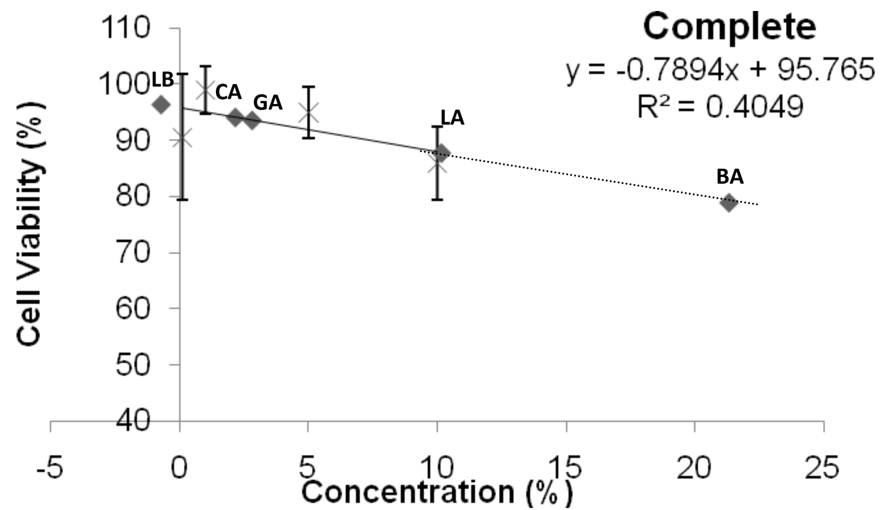


Figure 6.5: Concentration-dependent effect on viability. Regression analysis yielded trendline and formula for each solution tested and was used to approximate concentration released from contact lenses, given the viability.

Table 6.5: Approximation of concentration (%) released from contact lenses, given the viability.

MTT Viability (%)				
	Solo	OFX	ReNu	Complete
LA	70.91	47.87	66.60	87.75
Conc. %	18.58	15.41	8.54	10.16
BA	67.81	61.66	59.94	78.93
Conc. %	21.21	9.88	10.70	21.33
LB	87.40	72.15	77.95	96.35
Conc. %	4.59	5.67	4.86	-0.74
GA	84.60	60.86	71.92	93.56
Conc. %	6.96	10.20	6.82	2.80
CA	85.59	70.96	85.95	94.07
Conc. %	6.12	6.15	2.26	2.15
R squared	0.8598	0.9292	0.9769	0.4049

A standard curve was also generated from the absorbance spectra of OFX dilutions. A peak in absorbance was observed at 206nm for all concentrations (Figure 6.6), therefore the standard curve was derived from the relative absorbance at this wavelength (Figure 6.7). A strong correlation was found, as indicated by the R-squared value of 0.9961.

Lens extracts, diluted to 50%, were used to determine the concentration of MPS released (Figure 6.8); the given absorbance at 206nm was used in the regression analysis. In contrast to the previous predictions of OFX release, the lens release profile study using absorbance determined that the release of OFX from LA lenses is less than 2% (Table 6.6). For all other OFX combinations, the release characterized by absorbance spectra was found to be much less that the concentration approximated by the viability standard curve.

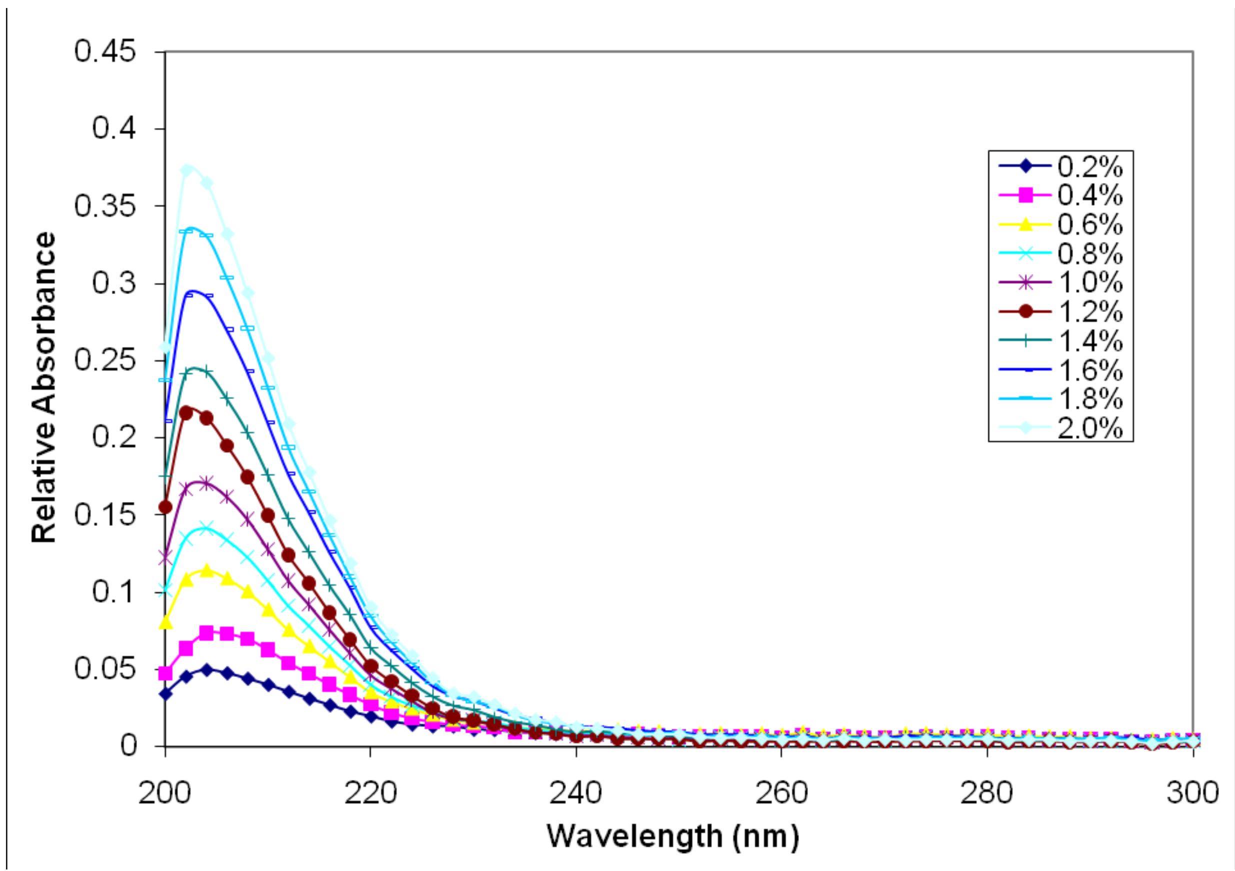


Figure 6.6: UV Absorption spectra of OFX diluted in PBS.

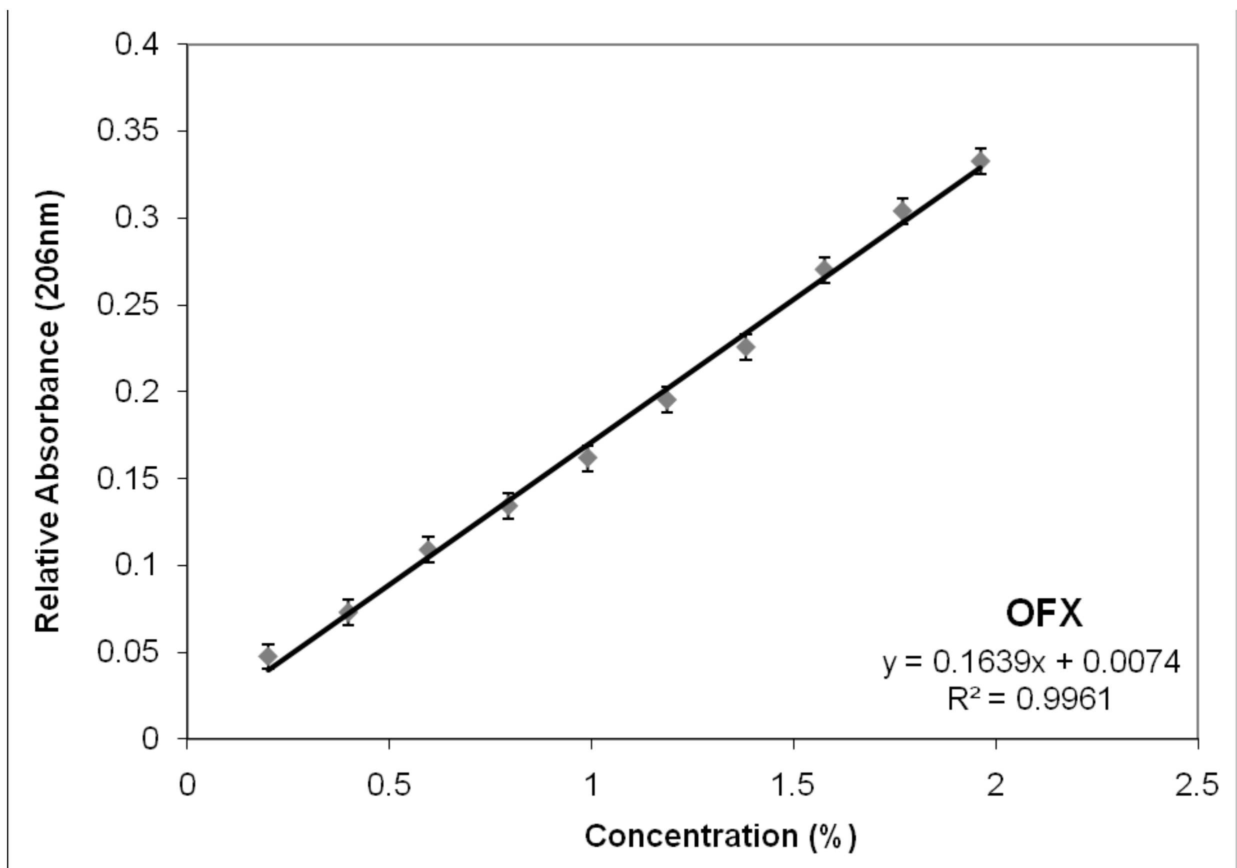


Figure 6.7: Absorbance-concentration curve of OFX, at 206nm. Regression analysis yielded trendline and formula and was used to approximate OFX concentration released from contact lenses, given the absorbance at 206nm.

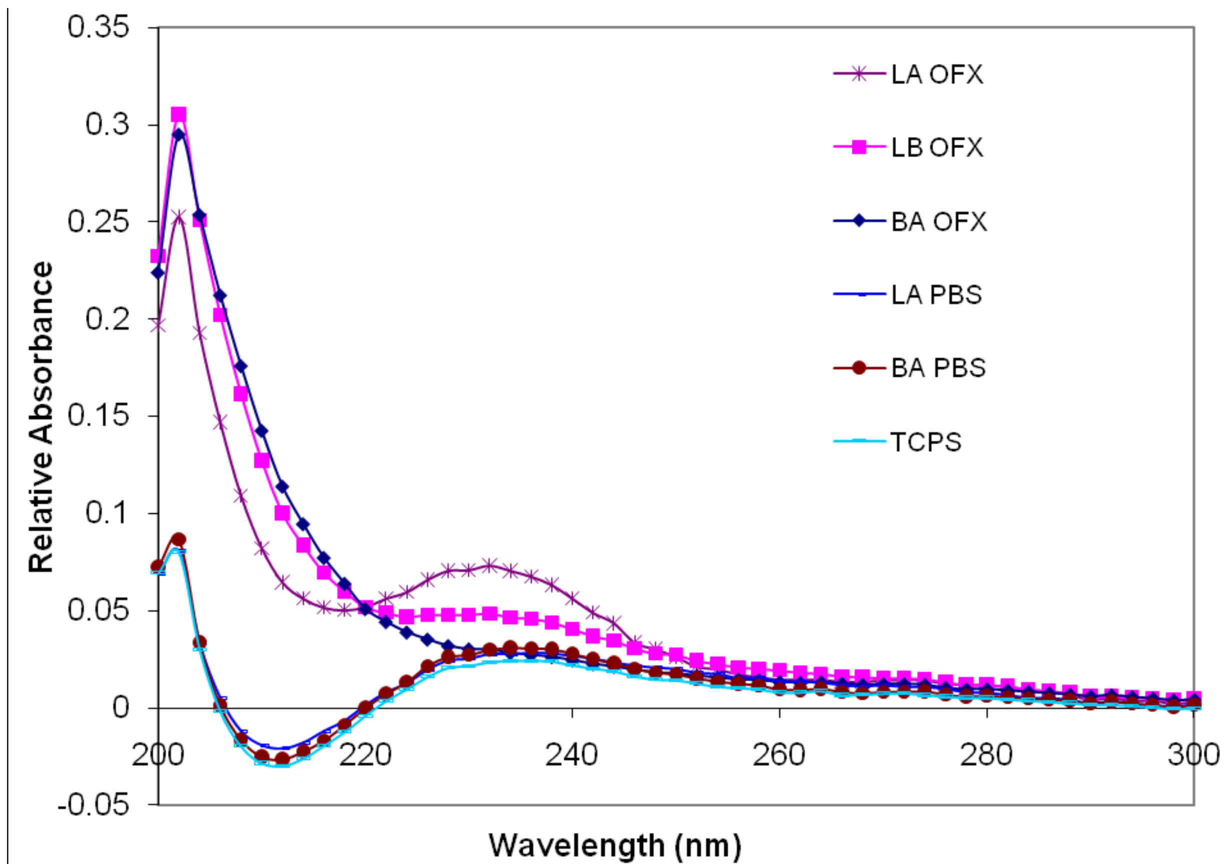


Figure 6.8: Absorption spectra of lens extracts, diluted to 50%, after 24 hour incubation.

Table 6.6: Approximation of concentration released from contact lenses, given the relative absorbance at 206nm.

Relative Absorbance (206nm)	
	OFX
LA	0.147
Conc. %	1.7
BA	0.212
Conc. %	2.5
LB	0.171
Conc. %	2.0

Discussion

The results obtained from cell contact with lenses soaked in phosphate buffer demonstrated near 100% viability and no change in caspase activation and thus confirmed the validity of our in vitro model. The major difference observed with the various lens-solution combinations was with BA-OFX. While it was similar in viability to LB, GA and CA, it was significantly different in terms of caspase activation. The BA-OFX combination was the only OFX combination that did not cause a significant increase in activated capases. The findings of this study can be explained in part by adsorption and release studies by Powell et al.[11] They compared the sorption and release of Aldox, the active constituent of OFX and OFR, and PHMB, the active component of ReNu, Solo and Complete from various lenses. For both agents, LA and BA lenses had the greatest amount sorbed, but there was a much greater uptake of Aldox than PHMB. They also noted that Senofilcon A (not tested here), a silicone hydrogel with similar chemistry to GA, demonstrated high sorption rates. While the sorption rates were similar between these three lenses, the release profiles differed. Aldox was released rapidly from all lenses, with most release coming from LA lenses. This indicates that the high amount of sorbed Aldox on LA lenses was not tightly bound, thus releasing faster and to a greater degree than all other lenses.[11] Conversely, BA lenses did not release Aldox in quantities as high as LA. Perhaps due to the surface heterogeneity and surface roughness of BA lenses[9,10], Aldox was able to be sorbed tightly to the exposed hydrophobic material, thus only releasing to a lesser degree. The variable release of Aldox, a potentially cytotoxic agent, from SH lenses can explain the differences in viability and caspase activation demonstrated in this study. A greater release will expose cells to a higher concentration of the disinfecting agent, causing greater adverse events as demonstrated by the concentration dependent effect of OFX and ReNu. The most significant effects were induced by the combination of LA and OFX.

One must also consider the properties of the solutions, when looking at uptake and release profiles of contact lenses. In a recent drug uptake and release study,[28] the authors concluded that if the uptake and release of a particular drug was only affected by material properties, then one would expect to see similar trends irrespective of drug or MPS tested. By demonstrating different trends in the Karlgard study,[28] Powell study[11] and the present study, it suggests that drug and MPS properties also can have an effect on the mechanism of uptake and release. While differences between Aldox and PHMB based solutions may be accounted for by differences in release profiles, a recent study[30] has shown that, unlike PHMB solutions, after interaction with lenses during the disinfection cycle OFX (and OFR) solution retains their bactericidal and fungicidal activity.[30] While it is ideal to retain this property, it means that residual active disinfecting agents on lenses have the potential to interact adversely with the corneal surface. Additionally, the effect of dilutions also helps to illustrate this point. It was found that soluthe PHMB solutions, ReNu, Complete and Solo, had different effects with increasing concentrations, where ReNu had a significant concentration dependent effect whereas Complete and Solo did not. These findings likely indicate that there is not only the potential for interaction of the disinfecting agent with the lens, but also with other solution components. This additional interaction may account for the increased cytotoxicity in solutions with the same disinfecting agent.

Several methods were used to characterize the release profile of lenses, to determine whether the concentration released could account for the difference between BA-OFX and all other combinations. Using dilutions, viability data was obtained for several concentrations and used to create a standard curve. Regression analysis was used to generate a trendline, which could determine the concentration released, given the viability as determined by MTT. Similarly, absorption spectra of dilutions were also used to create a standard curve. Given the absorption of a

lens extract, the MPS concentration was determined. Contrasting results were obtained in the different methods used to approximate the release of MPS from contact lenses. The viability derived standard curve appeared to overestimate the release of MPS compared to the absorption spectra. It is possible that preferential adsorption and release by contact lenses may account for the difference. While it was determined by absorbance, that LA released less than 2% OFX, the composition of the solution being released is unknown. Since a greater effect on the cells is being observed, it would be expected that the lens extract would be largely composed of cytotoxic agents, due to the potential preferential adsorption or release. As previously discussed, the Powell study[11] found that Aldox was released rapidly from LA lenses. While the cytotoxicity of Aldox is not known, this experiment demonstrates the ability of a lens to preferentially release certain agents from MPS after the disinfection cycle. Another possible explanation may be that the interaction of the lens may modify components of the solution to make them more cytotoxic. This would account for a greater cytotoxic effect at a lower concentration of release. This hypothesis is supported by the presence of an additional peak for LA-OFX around 230nm, which was not present the absorbance spectra of the diluted solution. This suggests there is an interaction of the lens and solution, which may result in modifications to the original components.

In conclusion, the results from this study shows that for MPS-released from a contact lens, lens release profile can be investigated. As demonstrated by the absence of an effect on cells exposed to a PBS-soaked lens, our results indicate that it is not the presence of the lens that affects cell viability and phenotype, but what is being released from the lens. The in vitro model also demonstrated a lens effect in the mechanism of MPS-induced cell death pathways. The results indicate that OFX-induced cell death may be influenced by the surface treatment of certain SH lenses. The differences in physical properties of lenses, which affect the uptake and/or release of

the various ingredients in MPS, had a significant effect on viability and caspase activation. Further studies are underway to gain a better understanding of the preferential adsorption or release profile of certain compounds by silicone hydrogel lenses with different physical properties and surface treatments. The use of FTIR is currently being investigated as a means to characterize release of known components.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The in vitro contact lens "onlay" model appears to be a valuable tool to study the direct release of multipurpose solutions on corneal epithelial cells. In this thesis, the results indicate that a scale of cytotoxicity can be derived, among the commercially available MPS. The results obtained from cell contact with lenses stored in phosphate buffered packaging solution demonstrated 100% or near 100% viability and confirmed the validity of our in vitro model. As demonstrated by the absence of an effect on cells exposed to a PBS-soaked lens, our results indicate that it is not the presence of the lens that affects cell viability and phenotype, but what is being released from the lens.

From the first chapter, Complete MPS demonstrated results similar to PBS, while OFX, ReNu and Solo demonstrated a significant adverse effect on viability and cell adhesion. Additionally, OFX demonstrated significant levels of caspase activation, indicating the possibility of a caspase-mediated cell death pathway. These in vitro studies demonstrated the following scale of corneal cell cytotoxicity: PBS < Complete < Solo < ReNu < OFX. Our cytotoxicity scale is based on our results from MPS solutions released from contact lenses and is in accordance with previous studies where MPS was added directly to a monolayer of corneal cells.

Studies on the mechanisms of cell death demonstrated that MPS-released from a contact lens has the potential to induce apoptosis. The results indicated that cell death induced by the borate

solutions, OFX and ReNu, were induced by different mechanisms. OFX-induced cell death is a caspase mediated intrinsic apoptosis pathway, whereas the cell death induced by ReNu is necrosis. The in vitro model also demonstrated a lens effect in the mechanism of MPS-induced cell death pathways. The differences in physical properties of BA and LA lenses, which affect the uptake and/or release of the various ingredients in MPS, had a significant effect on viability, C3 activation and Annexin V/PI staining.

The results from the lens material study showed that for MPS-released from a contact lens, a solution release profile from the lenses under examination can be determined. The in vitro model demonstrated a lens effect in cell viability and the mechanism of MPS-induced cell death pathways. The results indicate that OFX-induced cell death is affected by lenses with homogeneous surfaces. The differences in physical properties of lenses, which affect the uptake and/or release of the various ingredients in MPS, had a significant effect on viability and caspase activation. Further studies are under way to gain a better understanding of the preferential adsorption or release profile of certain compounds by silicone hydrogel lenses with different physical properties and surface treatments.

Future work should focus on identifying the components of MPS suspected of being cytotoxic agents. This can be accomplished through the use of custom multipurpose solutions, where the concentrations of the preservatives can be varied, to determine concentration dependent effect and custom combinations of preservatives and buffering agents, which are not commercially available (i.e. Polyquad®/Aldox® with phosphate buffer compared to borate buffer). This would help to isolate the agent of MPS responsible for inducing apoptotic cell death and determine if there is an interactive effect of the components commonly found in MPS. Additionally, the use of BAK as

a control should be considered, given its presence as a preservative in a wide range of ocular durgs and solutions.

Further work is also required to characterize the lens/solution release profile. Different methods, such as FTIR analysis, are currently being considered for use in identifying and quantifying different components of MPS in lens extracts. These methods will also aid in determining whether components of MPS are being modified after interaction with contact lenses in the overnight disinfection cycle. Additionally, the use of stratified cultures on curved surfaces can aid in making the in vitro model more generalizable to in vivo findings.

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Dear Silvia or Tatjana,

My name is Nicole Tanti and I am a graduate student at the University of Waterloo. I am currently finishing my MSc thesis, and I would like to request the use of a figure from your journal to complement one of my chapters. This figure will only be used in my thesis.
The figure is an Electron micrograph of tight junctions (TJ) and desmosomes (D) in an ultrathin section of Epon-embedded MDCK cells. It comes from the following paper published in Invasion and Metastasis:

Huber D, Balda MS, Matter K. Transepithelial migration of neutrophils. Invasion Metastasis. 1998; 18: 70-80.

I would appreciate if you could grant me the permission to use this figure in my thesis and I thank you in advance for your efforts.

Regards,
Nicole Tanti

Hi Nicole, It was great to hear from you and that you are completing your MSc at Waterloo! Of course it's alwasy nice to get compliments so how could I refuse sharing my figures (actually I'd send them to you anyway). I'm attaching the three I think you want. Let me know if they are the right ones or if you'd like the colour figures to be sent in grayscale. Two are jpg the intergrin is a tiff (hi res). I can send you the colour version of integrin if you want. I also wasn't sure if you wanted the JAMs pic I'm sending or the one with the adhesion molecules listed. Anyway, good luck with the writing and defense, Dr. O'Day

ntanti@sciborg.uwaterloo.ca wrote:

Dear Dr. O'Day,

My name is Nicole Tanti, and I am a graduate of the biology program at UTM. During my time at UTM, I had the priviledge of taking BIO 315 and 380 with you, in the Fall of 2005 and 2006, respectively. Currently, I am finishing my MSc degree in Vision Science at the School of Optometry, University of Waterloo. Part of my thesis deals with cell adhesion, integrins and wound healing, and many of the wonderful figures you have from your lectures would complement this chapter very nicely. I would like to request your permission to use 3 of your figures in my thesis from your BIO 315 lectures:

Figure of Integrin Structure
Figure of Junctional Complexes
Figure of Integrin, Actin and Cell Movement

I would very much appreciate if you would grant me permissions to use these figures, and of course all credit will be appropriately given to you.

I look forward to hearing back from you, and I hope all is well with your lab, as I know many of your grad students, who were colleagues of mine while I was at UTM.

Thank you in advance,

Nicole Tanti