Viability Profile of *ex vivo* Corneal Epithelial Cell Samples

by

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AUTHOR'S DECLARATION

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

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ABSTRACT

The corneal epithelium is a vital tissue which must retain its integrity to preserve vision and protect against harmful bacterial infections and other insults. Corneal disease represents the second most common cause of world blindness after cataract.[1] Examination of this tissue is therefore important in any ophthalmic routine, and in particular in contact lens practice where an increased number of factors, such as lens material, lens fit, care solution and contamination may directly affect its integrity. The ocular surface cell collection apparatus (OSCCA) allows safe and efficacious collection of human corneal epithelial cells[2] and may provide the ability to examine cytological changes to the human cornea during lens wear. The overall objective of this project was to demonstrate the efficacy and reliability of the OSCCA as a tool to collect human corneal epithelial cells and examine cytological changes to the human cornea. This was achieved by characterizing the phenotype and viability status of cells collected from the ocular surface using the OSCCA and by comparing the obtained results with samples collected using other non-invasive techniques.

There was a high level of uncertainty whether or not the cells collected were in fact corneal or conjunctival epithelial cells. Chapter 2 and 3 showed the Hoechst and PI were not optimal stains to measure the viability status of cells collected with the OSCCA because there was an unanticipated overlap of the fluorescence from PI+ nucleated cells into the blue spectrum and the Hoechst stained both live and dead cells. Chapter 4 looked at other cytological stains and concluded that the LIVE/DEAD® Viability/Cytotoxicity Kit (calcein AM/ethidium homodimer-1) was the most appropriate stain to use with the OSCCA collected cells due to the lack of overlap between stains. Chapter 3 showed that cells that stained with

sodium fluorescein stained with only Hoechst and not PI. Since Hoechst stains live and early apoptotic cells and PI stains cells that are late stage apoptotic, necrotic and dead cells, we can conclude that sodium fluorescein stains live and early apoptotic cells. Similarly in chapter 5 it was found that cells that stained with sodium fluorescein stained exclusively with calcein blue AM and not ethidium homodimer-1.

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DEDICATION

To my family and friends!

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As one of the supervisors left for Australia during the term of this MASc thesis, the contributions of the supervisors (Maud Gorbet: MG; Rachael Peterson: RP and Gina Sorbora: GS) and graduate student (Dan Cira: DC) in the various aspects of this work are being identified.

2 CHARACTERIZATION OF COLLECTED CELL PHENOTYPE
DC Generation of research question, ex vivo and in vitro experimental design, methodology procedures, data analysis
MG Generation of research question, methodology procedures, and in vitro experimental design, data analysis
RP Generation of research question, equipment design, methodology procedures, ex vivo experimental design

3 INVESTIGATIONS OF DIAGNOSTIC DYES FLUORESCEIN AND LISSAMINE GREEN ON HUMAN CORNEAL EPITHELIAL CELLS
DC Experimental design, in vitro experimental design, methodology procedures, data analysis
MG Generation of research question, methodology procedures, data analysis
RP Generation of research question, equipment design, methodology procedures, ex vivo experimental design, data analysis

4 DEVELOPMENT OF VIABILITY STAINING PROTOCOL
DC Generation of research question Experimental design, in vitro experimental design, methodology procedures, data analysis
MG Generation of research question Experimental design, in vitro experimental design, methodology procedures, data analysis
RP Generation of research question

5 INVESTIGATIONS OF BENZALKONIUM CHLORIDE AND SODIUM FLUORESCEIN ON HUMAN CORNEAL EPITHELIAL CELLS

5.1 IN VITRO

5.2 EX VIVO

DC Generation of research question, ex vivo and in vitro experimental design, methodology procedures, data analysis

MG Generation of research question, methodology procedures, ex vivo and in vitro experimental design, data analysis

RP Generation of research question, equipment design, ex vivo experimental design

GS Methodology procedures, ex vivo experimental design

LIST OF ABBREVIATIONS

µg	Micrograms
µl	Microliter
µm	Micrometer
µM	Micromolar
BAK	Benzalkonium Chloride
CCLR	Centre for Contact Lens Research
FBS	Fetal bovine serum
HCEC	Human corneal epithelial cells
HConjEC	Human conjunctival epithelial cells
LG	Lissamine Green
Mg	Milligram
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
MPS	Multi-purpose solution
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium Bromide
NC-CIC	Noncontact corneal irrigation chamber
NaFl	Sodium Fluorescein
Nm	Nanometer
OSCCA	Ocular surface cell collection apparatus
PATH	preservative-associated transient hyperfluorescence
PBS	Phosphate-Buffered Saline
PHMB	Polyhexamethylene Biguanide
PI	Propidium Iodide
SICS	Solution induced corneal staining
˚C	Degree Celsius

1 INTRODUCTION

1.1 THE OCULAR SURFACE

1.1.1 Anatomy of the Ocular Surface

The ocular surface, including the cornea and conjunctiva form a functional unit. The cornea is a transparent avascular, non-keratinized epithelial structure, forming one-sixth of the area of the outer wall of the eye.[4,5] The transparency of the cornea is due to its uniform structure, avascularity and deturgescence. The corneal surface represents the optical interface between the eye and the external environment and functions as an optical element and protective barrier.[6] Together with the lens, the primary function of the cornea is to refract light to focus an image on the retina; therefore, the cornea must maintain its transparency, optical physiology and structure.[6] The corneal epithelium and the lens both originate from the surface ectoderm during embryonic development.[6,7] The corneal epithelial stem cells reside in the basal layer of the peripheral cornea in the limbal zone. These cells have superior proliferative capacity compared to the central corneal epithelial cells; therefore, they provide the potential for rescue or reconstruction of the damaged corneal epithelium.[8] The human cornea is 500 µm thick and is composed of five layers which are the corneal epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium.[9] (Figure 1) The corneal epithelium is approximately 50 µm thick and is a stratified structure consisting of a single layer of squamous superficial epithelial cells, several layers of intermediate wing cells, and a single layer of columnar basal epithelial cells.[9] The superficial epithelial cells range from 20-30 µm in length and are 5 µm thick, while the columnar basal epithelial cells are 10-15 µm in length and the wing cells can vary in size.[9] Superficial corneal cells provide

a substrate for the precorneal tear film, which acts as the primary refracting surface of the eye.[10]

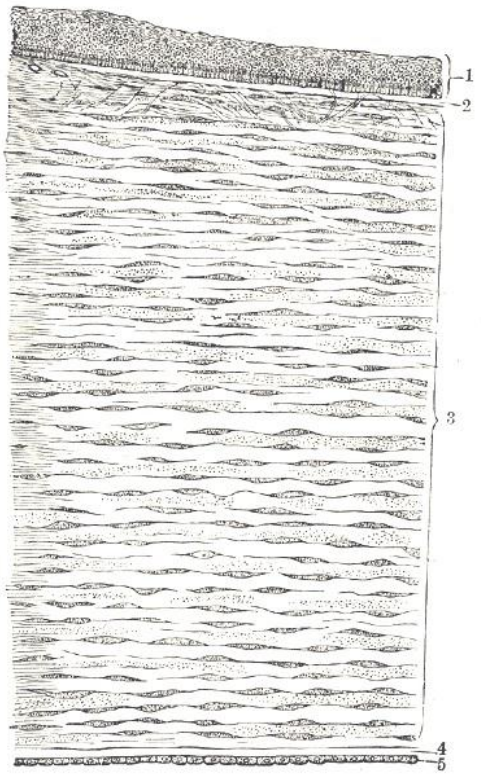

Figure 1: Vertical section of human cornea from near the margin. (Waldeyer.) Magnified.

1. **Epithelium**
2. **Bowman's layer**
3. **Stroma**
4. **Descemet's membrane**
5. **Endothelium**

(Gray's Anatomy of the Human Body, ***originally published in 1918 and therefore lapsed into the public domain)***

The cornea is well protected from pathogens and the external environment by tight junctions and its constant epithelial-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.[5,11,12] 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.[5] After divisions, mature cells 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.[5] The epithelial cell turnover rate has been found to be approximately 7 days in normal corneas.[13] The rapid epithelial renewal rate and continuous shedding of superficial epithelial surface cells reduces the time and opportunity for mechanical stresses and potentially adherent and infectious organisms to contact the corneal surface.[14]

The presence of tight junctions (Figure 2) in the corneal epithelial layers 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.[10,15] Tight junctions are formed from two integral transmembrane proteins, occludin and claudins, and several membranes associated proteins, ZO-1, ZO-2 and ZO-3. ZO-1 is localized in the apical region of superficial epithelial cell-cell junctions.[16]

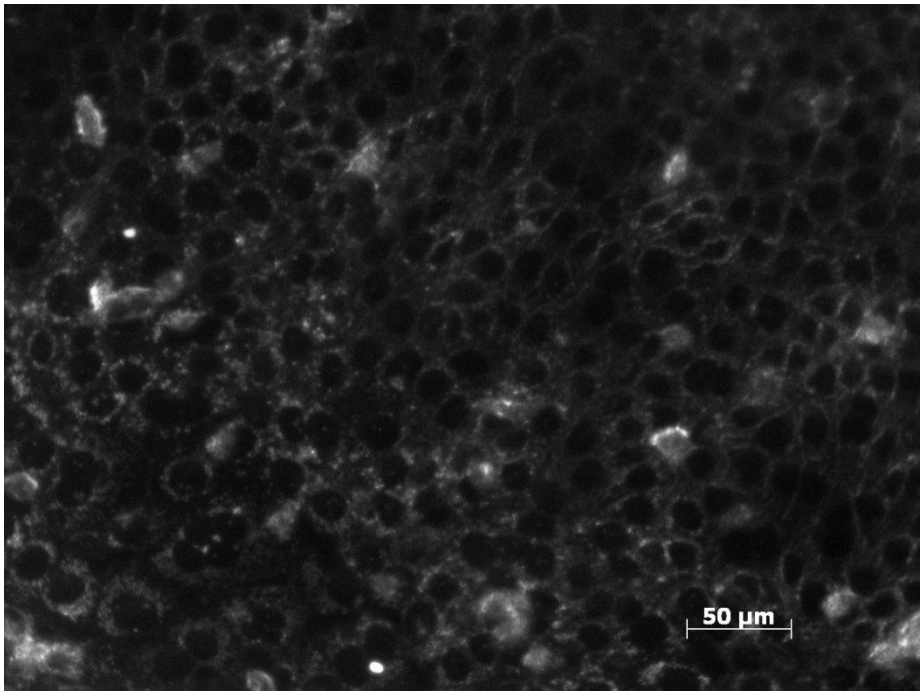

Figure 2: ZO-1 stained immortalized HCEC (Fluorescent Microscopy)

The conjunctiva is the thin, transparent mucous membrane overlying the sclera. It has three parts; palpebral, forniceal and bulbar and histologically consists of epithelium and stroma. There are numerous other cell types' resident within the epithelium besides epithelial cells, such as goblet cells, melanocytes, Langerhans' cells and lymphocytes. Goblet cells are responsible for the secretion of the majority of conjunctival mucins. The accessory lacrimal glands of Krause and Wolfring are located in the conjuncrival stroma and are thought to be accountable for the baseline tear production.[17]

The cornea is a vital tissue which must retain its integrity to preserve vision and protect against harmful bacterial infections and other insults. A compromised corneal epithelial could decrease the defense systems of the ocular surface, and increase the risk of serious infection.

Contact lenses are worn by over 125 million of people worldwide and the complications associated with wearing them are well recognized. Contact lens wear causes changes in the cornea in terms of structure, cell turnover, tear production and oxygen levels. These changes can produce problems and may also worsen pre-existing conditions. To achieve a better understanding of the effect of various factors such as inflammation, infection, contact lenses, therapeutics and environment on the human cornea, investigations sampling the cornea need to be undertaken.

1.2 METHODS FOR SAMPLING THE CORNEA

1.2.1 Introduction

Our current abilities to study changes to human corneal epithelial cells (HCEC) are restricted by the availability of non-invasive techniques. There are a number of techniques for collecting cells from the ocular surface. These techniques include impression cytology, irrigation and contact lens cytology.

1.2.2 Impression Cytology

Impression cytology can collect several layers of cells from a localized area of the conjunctiva.[18] It is carried out by pressing a small piece of special filter paper against the conjunctiva for a few seconds, after which it is removed (Figure 3). This can be repeated on the same area two or three times on the conjuctiva. The filter paper is then fixed to a glass slide, stained and examined under a microscope. Although not recommended due to the

potential of irreversible damage and increased risks of infection following the procedure, this method has also been used on the cornea in two recent studies.[19,20] The process also requires the use of an anesthetic which may affect results; lidocaine has been shown to induce corneal cytotoxicity.[21] A major drawback with this invasive sampling technique is the potential of tissue damage from the anesthetic, the pressure exerted on the cells and the mechanics of peeling off the filter which can cause an unpredictable influence on the morphological appearance of collected cells.

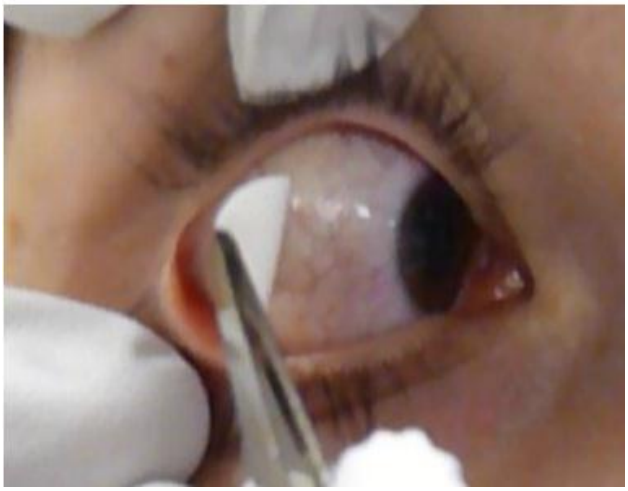

Figure 3: Impression Cytology (Courtesy of CCLR 2010)

1.2.3 Irrigation

The noncontact corneal irrigation chamber (NC-CIC) is non-invasive eyewash that collects cells from the precorneal tear film.[3] The subject is seated with forehead resting comfortably against the head-rest and the NC-CIC irrigating tip is position below the centre of the subject's cornea. Both lids are retracted slightly and the NC-CIC is raised vertically until the irrigating tip is 2 mm below the corneal apex. Each irrigation involves the delivery

of 10 ml of sterile saline solution to the central cornea over a period of 30 seconds. The cells collected are stained and examined under a microscope. This technique has been used extensively by others groups to study corneal epithelial cells interactions with bacteria.[22,23] However, low cell yields (less than 100 cells) are obtained and thus this technique is not suitable for routine examination of HCEC.

1.2.4 Contact Lens Cytology

Contact lens cytology involves placing a disposable soft contact lens which has been rinsed thoroughly in sterile basic tear solution and is placed directly on the cornea of a subject. The contact lens is allowed to stabilize on the eye for a period of 2 minutes and is then removed directly from the cornea. This is accomplished without moving the lens onto the conjunctiva to minimize contamination with conjunctival cells. The soft contact lens is then draped over the end of a 20 mm diameter glass test with the front surface of the contact lens against the convex surface of the glass. The exposed back surface of the contact lens is rinsed vigorously into a 50 ml beaker with approximately 10 ml of sterile basic tear solution directed on the lens with a 30 ml syringe with a 23-gauge blunt needle. The cells are stained and the cell suspension is filtered through a 13 mm diameter polycarbonate filter and transferred to a glass slide and examined with a microscope.[24] While higher cell yields are obtained with contact lens cytology, this method introduces additional factors such as reduced shear forces from the lids, hypoxia and chemical components (from the multipurpose solution or packaging solution).[25]

1.2.5 Ocular Surface Cell Collection Apparatus

Due to poor cell yields, the information gained from these techniques has been limited and thus has not enabled reliable studies of HCEC. While *in vitro* models using animal or cultured tissues may offer an alternative to human investigations, their predictions cannot yet entirely mimic the responses of the human eye.[26] *In vitro* HCEC monolayer models have been criticized for being overly sensitive and multilayer models are more difficult to culture.[27] At the Centre for Contact Lens Research (CCLR), a new ocular surface cell collection apparatus (OSCCA) has been developed to collect HCECs (Figure 4). Similar to the NC-CIC, with the OSCCA, the participant places his/her forehead onto a head rest with his/her gaze towards the work-bench. The OSCCA funnel is aligned directly under the ocular surface of interest (in this case the cornea) and its height is adjusted until the edge of the funnel is either restricted by the nose or the fountain rests, two cm from the eye. Ten ml of warmed sterile phosphate buffered saline is then delivered to the desired area (the cornea) over approximately 30 seconds. The cell suspension is concentrated by centrifugation and stained, then examined under a microscope. The OSCCA allows safe and efficacious collection of human corneal epithelial cells.[2] The collected cells may provide the ability to examine cytological changes to the human cornea over a wide variety of situations.

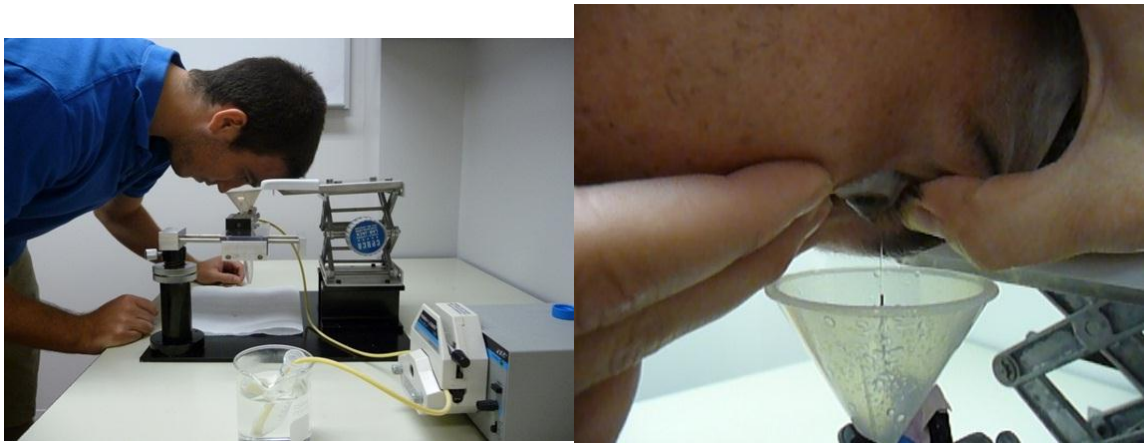

Figure 4: The Ocular Surface Cell Collection Apparatus Device and close up of saline delivery.

1.3 CORNEAL STAINING

1.3.1 Introduction

The use of sodium fluorescein to assess the ocular surface is a well-known technique. It involves the insertion of the fluorescent dye onto the surface of the eye which is then examined under blue light. In healthy eyes, sodium fluorescein does not stain corneal epithelial cells. The fluorescein is believed to highlights area of epithelial cell disruption on the surface of the cornea, "staining" it.[28] The extent and intensity of the dye is said to represent the level of human corneal cell disruption.[28] Despite the widespread use of fluorescein, its effects on cells and mechanisms involved in staining remain unclear.

Sodium fluorescein is sometimes classified as a vital dye. There is some deliberation as to whether this is the correct description as there are some discrepancies over the definition of the term vital dye. A vital dye usually infers that the agent enters living cells which are not damaged.[29] However, some suggest that a vital dye means it is an agent that only enters living cells which are damaged.[30] In the ophthalmology world, sodium

fluorescein is traditionally viewed as an agent that only enters damaged cells at the ocular surface and not healthy cells. The exact mechanisms which give rise to ocular surface staining are not well understood. And until this subject can be clarified, any discussions on whether or not sodium fluorescein is a vital dye are irrelevant.

Sodium fluorescein is optimally excited by 495 nm light which is within the range typically emitted from a standard white bulb in a slit lamp biomicroscope. It is problematic viewing the fluorescence of the dye (510 – 520 nm) under white light conditions because this narrow wavelength band of fluorescence has low contrast against the wide wavelength white light band making up the general background illumination.[28] There are two methods employed to overcome this problem and enhance the appearance of the ocular surface staining. The first method is to limit the spectral radiance of the illuminating source to match the ideal excitatory spectrum for sodium fluorescein. This is achieved by introducing a blue filter into the illumination system of the biomicroscope in order that only radiation between 400 and 500 nm reaches the ocular surface, providing a much better contrast for the clinician.[28] This can be improved further by using a barrier filter as part of the view system. This filter is able to absorb the reflected blue light and transmit the long wavelength of the ocular surface fluorescence. This provides bright green areas of fluorescence on a dark background. Usually, a yellow photographic Kodak Wratten 12 filter has been used for this purpose as its spectral transmission properties decrease the overlap between the excitation and emission spectra of fluorescein.

Corneal staining is a valuable clinical tool for assessing corneal epithelial integrity with the slit lamp. Corneal staining grading scales were developed to help clinicians monitor changes of the cornea and choose a course of action. Corneal staining can take on a number

of clinical appearances which can all categorized in terms of intensity (punctate, diffuse, coalescent), shape (arc, linear or patch) and location (central, superior, inferior, nasal or temporal corneal areas).[28]

Superficial punctate fluorescence is found in normal subjects, contact lens wearers, and dry eyes. Although the examination of corneal staining is undertaken every day all over the world by eye practitioners, it is shocking that the underlying cellular mechanisms of fluorescein staining are so poorly understood. There are many hypotheses on how fluorescein reacts with corneal epithelial cells: surface pooling, uptake by cells, ingress around cells and multipurpose contact lens solution interactions.

1.3.2 Surface Pooling

The simplest form of corneal staining is when there is indentation at the corneal surface. Surface irregularities or defects left by an absence of cells cause fluorescein to pool in punctate areas. A common clinical instance of corneal staining is the due to the formation mucin balls. [31] Mucin balls are tiny translucent spheres of mucin (proteins) formed from the gradual movement of the contact lens across the tear film. Mucin balls get entrapped beneath a contact lens and often leave a temporary imprint in the epithelium.[32] The fluorescein, when added to the ocular surface, pools in these indented areas caused by the interaction of the contact lens and mucin balls. It has been shown that although there may be some transient pooling of fluorescein in a mucin ball-related corneal impression, there is no tendency for increased punctate staining in patients with mucin ball formation.[31] In another study designed to examine the role of corneal surface irregularities in dry eye, no strong correlation

between corneal surface regularity and corneal staining was observed.[33] Corneal staining due to these irregularities cause relatively large indentations in the cornea. There are also smaller events where there is corneal staining due to the accumulation of fluorescein in gaps left by shed epithelial cells.[34] It has been demonstrated experimentally that the irrigation of damaged rabbit corneas does not easily remove the fluorescent stain. These studies all tend to suggest that pooling over surface irregularities is unlikely responsible for this observation.[34]

1.3.3 Ingress Around Cells

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.[10,15] An intact epithelium plays an important role in the physiology of the cornea. Intercellular gaps created by the loss of these tight junctions allow deep penetration and trapping of fluorescein between cells. It has been suggested that low levels of fluorescein are able to enter a healthy epithelium through the tight junctions,[30] however experimental evidence has yet to support this hypothesis. Another study showed that after a period of 30 minutes in an *in vitro* model of a stratified human corneal epithelium prevented penetration of 96% of the fluorescein applied to the apical surface.[35]

1.3.4 Uptake by Cells

Studies in rabbits and humans suggest that both living and dead cells take in fluorescein, although not all cells with fluorescein uptake are visible under the slit lamp microscope.[30,36] This was clearly demonstrated in single rabbit corneal epithelial cells that were stained with fluorescein after exposure to chemical preservatives known to cause staining in humans as well as mechanical trauma, creating slit lamp views similar to those seen in clinical practice in cases of contact lens solution sensitivity or corneal abrasion. After slit lamp verification of the fluorescein staining pattern, rabbits were euthanized and the corneas excised for viewing with high magnification on a fluorescent laboratory microscope.[34] No evidence of accumulation of fluorescein on the corneal surface or in intercellular spaces that resulted in corneal staining was found.[34] Recently, it was shown using impression cytology that punctate fluorescent spots, which disappeared after impression cytology, correlated with sodium fluorescent stained cells on the membrane.[20] Using a rabbit model, fluorescence on the corneal surface was also associated with the uptake of fluorescein by individual cells. Bandamwar *et al* found that healthy cells stained with fluorescein but not at a level to cause hyperfluorescence. However, apoptotic cells took up high levels of fluorescein and were hyperfluorescent and could be visualized with the slit lamp as micropunctate staining. Dead cells took up minimal fluorescein and were not visible with the slit lamp.[37] Superficial punctate fluorescein staining of the corneal epithelium visualized with the slit lamp thus appears to correspond to the presence of damaged epithelial cells.[37]

1.3.5 Solution Toxicity?

Solution induced corneal staining (SICS) (Figure 5) has been observed with certain combinations of contact lens solutions and silicone hydrogel lenses.[38,39] It is also referred to as solution sensitivity and has been hypothesized to be a result of a toxic reaction.[3840] The appearance of SICS is typically fine punctuate spots and usually most prominent in the peripheral cornea with only marginal central involvement.[38] SICS has also been reported to be asymptomatic.[38,40] The presence of SICS is most evident during the first 2-4 hours of contact lens wear with residual SICS after 6 hours of contact lens wear.[38,40,41] Significantly more asymptomatic SICS was observed when PureVision lenses (Bausch & Lomb) were used in combination with the preservative found in Polyhexamethylene Biguanide (PHMB) based solution ReNu Multiplus (Bausch & Lomb) than with the polyquiad-based solution OptiFree Express (Alcon).[38] The mechanism behind SICS is still unknown. It is suggested that certain components within the contact lens solution, mainly the preservative, can be adsorbed onto the lens surface and then released after lens insertion, causing a toxic reaction.[38] SICS may thus be a direct result of the contact lens solution causing premature apoptosis of the superficial corneal epithelial cells, cells which would then uptake high amounts of fluorescein and result in the observed corneal staining.

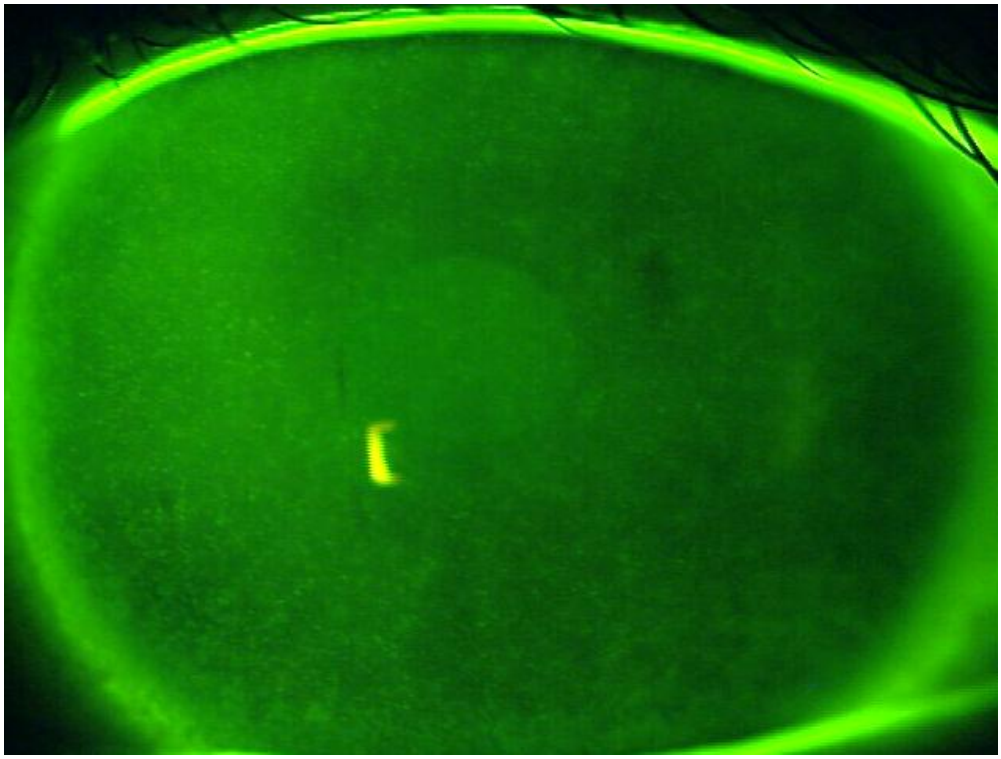

Figure 5: Solution Induced Corneal Staining (Courtesy of CCLR 2011)

Recent research suggests that the transient hyperfluorescence observed in multipurpose solution (MPS) users is a nonthreatening phenomenon that is etiologically different from corneal staining observed in pathological and physiological situations. This new understanding supports the beliefs that the corneal hyperfluorescence in MPS users, which has also recently been termed preservative-associated transient hyperfluorescence (PATH), is an artifact with no known complications.[42] It is believed that PATH does not signify a pathological process. All preservatives found in MPS are taken up by all soft contact lenses during the soaking period.[43] The amount absorbed and rate of absorption depends on the lens material and the preservative. After the lens is inserted in the eye the lens then releases the preservative into the tear film and the rate of release depends on the preservative in the MPS and contact lens material combination.[43,44] In the presences of

PHMB a preservative found in some MPS, the modeled cell membrane is not affected.[42] The preservative PHMB has an extremely strong affinity for fluorescein and is 50-times greater than that for polyquatemium-1/POLYQUAD, another MPS preservative. Thus the other current hypothesis related to SICS is that the fluorescein adheres to the PHMB on the eye, which was released from the contact lens.[42] The superficial punctate of the visual signal may results from many PHMB molecules aggregating and binding with fluorescein.[45] This explanation of corneal staining is limited and does not explain the corneal staining found with other contact lens/MPS combinations.

1.3.6 Conclusion

Sodium fluorescein remains the main clinical diagnostic tool used to assess the integrity of the cornea, although the mechanisms involved in the interaction of the superficial oculars cells and the dye itself are not yet well understood. Although PATH provides a logical explanation, it does not answer why corneal staining is present without the interaction of PHMB. PATH also fails to address why the combination of Pure Vision lens soaked in ReNu (a PHMB containing disinfecting solution) leads to significant corneal staining while soaking a different lens in ReNu or the same lens in a different PHMB containing disinfecting solution results in less staining. With recent research, it is becoming more evident that individual damaged superficial corneal epithelial cells are stained with fluorescein and SICS may be evidence of that damage. Continuing research on this topic will provide the complete answer to this phenomenon that has been debated for decades.

1.4 PURPOSE OF THE INVESTIGATION AND SCIENTIFIC QUESTIONS

Corneal infiltrates and microbial keratitis are commonly observed during adverse reactions associated with contact lens wear.[46,47] Our current abilities to study changes to HCEC are restricted by the need for non-invasive techniques. There are a number of techniques for collecting cells from the ocular surface, namely impression cytology, contact lens cytology. However due to poor cell yields, the information gained from these techniques has been limited and thus has not enabled reliable studies of HCEC. While in vitro models using animal or cultured tissues may offer an alternative to human investigations, their predictions cannot yet entirely mimic the responses of the human eye. The OSCCA allows safe and efficacious collection of human corneal epithelial cells[2] and may provide the ability to examine cytological changes to the human cornea during lens wear. The overall objective of this project was to demonstrate the efficacy and reliability of the OSCCA as a tool to collect human corneal epithelial cells and examine cytological changes to the human cornea. This will be achieved by characterizing the phenotype and viability status of cells collected from the ocular surface using the OSCCA and by comparing the obtained results with samples collected using other non-invasive techniques.

Primary questions

(1) Is the OSCCA an efficient and reliable tool to collect human corneal epithelial cells?

(2) Can we measure the viability status of cells collected from the ocular surface using the OSCCA?

By comparing different viability stains, we can attempt to determine how sodium fluorescein interacts with the cells collected from the ocular surface, which leads to the ***secondary question:***

(3) What does sodium fluorescein actually stain?

2 CHARACTERIZATION OF COLLECTED CELL PHENOTYPE

2.1 INTRODUCTION

The corneal and conjunctiva epithelia have unique cytokeratins (CKs) patterns. Cytokeratins are a complex group within the intermediate filament family and are present in almost all invertebrates' epithelial cells. Keratins exist in a 1:1 ratio of type I (acidic, including CK9 to CK20) and type II (basic, including CK1 to CK8). The keratins of the corneal epithelium have been shown to be composed of a major keratin pair, formed by the acidic keratin, CK12 and the basic keratin, CK3, together with a minor keratin pair acidic keratin, CK14 and basic keratin CK5. The types of keratins synthesized are specific to the development of the stage and the phenotype of the cells.[48] Cytokeratins characteristic of nonkeratinized, stratified (CK4 and CK13), simple (CK8 and CK19), and glandular epithelia (CK7) are present in the superficial layer of normal human conjunctival epithelium.[49]

To determine if the OSCCA is an efficient and reliable tool to collect human corneal epithelial cells, we must be able to differentiate the corneal epithelial cells from the neighbouring conjunctival epithelial cells. The expression of epithelial cell specific markers, cytokeratins (CK3 and 19), was investigated for *ex vivo* cells collected with the OSCCA using immunohistochemistry. CK3 and CK19 have been demonstrated to discriminate between corneal and conjunctival epithelia.[50] AE5 is a monoclonal antibody against CK3 keratin, which stains all layers of the normal human corneal epithelium but does not stain the conjunctival cells, whereas CK19 stains the conjunctiva but not the corneal epithelium.

As part of the cell collection, the viability of the cells collected with the OSCCA was determined with fluorescent microscopy and the cytological dyes, Hoechst and propidium

iodide (PI). Hoechst is a nuclear stain which is readily taken up by live and early apoptotic cells in suspension and stains DNA by binding to A-T pairs preferentially. PI is a nuclear stain which may permeate into cells following late-stage apoptosis or death.

2.2 MATERIALS AND METHODS

2.2.1 OSCCA Cell Collection

The OSCCA funnel was sterilized with alcohol (either ethanol spray or wipes) and left to dry. It was then rinsed to remove any residual alcohol or fibers. A test tube was secured into position under the funnel and sterile PharMed tubing was attached to the external needle tail. One ml of sterile phosphate buffered saline (PBS) which had been warmed to 35 ºCelsius (using a water bath) was pumped through the tube until expelled from the needle tip. The participant then placed their forehead onto the head-rest with their gaze towards the work-bench. The OSCCA funnel was aligned directly under the ocular surface of interest (in this case the cornea) and its height adjusted until the edge of the funnel was either restricted by the nose or the fountain rests 2-cm from the eye. Ten ml of warmed PBS was then delivered to the desired area over approximately 30 seconds.

Cells were collected using the OSCCA. The OSCCA was used bilaterally on the eyes of five participants. Samples were pooled together after centrifugation and divided into two equal groups. One group was used to measure cell viability while the other group was used for the immunostaining process. Cells were examined as soon after collection as possible (0-15 minutes), to minimize any potential damage or distortion. To concentrate the cells, the individual suspensions were centrifuged (at 1800 rpm for 10 minutes at 25ºCelsius), 9 ml of

solution was aspirated, and the remaining 1 ml was pooled in 15ml conical tube and was concentrated again to 2 ml. 1 ml was then transferred to two separate wells in a 24-well plate. This protocol was repeated three times, with a wash-out period of 3 days minimum.

2.2.2 Immunohistochemistry

Prior to cell staining, a solution of 1 mg/ml poly-D-lysine (average MW 400,000) in distilled water was prepared. This solution was then used to coat the wells of a 24-well tissue culture plate. This was done by incubating the 24-well plate with 250 µl per well of the poly-D-lysine solution for 10 minutes. The wells were then wash multiple times with distilled water and allowed to air dry. The 1 ml cell suspension was added to the well and incubated for 10 minutes at room temperature. The cells were then fixed using 250µl of -20°C methanol solution, which was removed after two minutes. The surfaces were left to air dry. Cells were permeabilized with 500 µl of 0.2% Triton-X solution for five minutes, and well surfaces were blocked with 200µl of a 1:50 fetal bovine serum (FBS) solution for 20 minutes. The samples were then incubated for one hour at room temperature with 200 µl of a 1:50 dilution of primary mouse antibody anti-epithelial keratin AE5 (Millipore, USA) per well. After washing twice with 500 µl of 0.2% Triton-X solution, the cells were incubated in the dark with 200 µl of the secondary donkey-anti-mouse FITC antibody solution (1:50 dilution in PBS containing 1% FBS) for one hour at room temperature. Primary human corneal and conjunctival (HConjEC) epithelial cells (ScienCell, Carlsbad, CA) and an impression cytology sample of the conjunctiva stained with both CK3 and CK19 were used as positive and negative controls. They were immunostained following the same procedures

as described, except that negative controls were incubated without primary antibodies. Two SV40-immortalized HCEC lines were also compared. One line was obtained from Dr. M. Griffith (Ottawa Eye Research Institute, Ottawa, Canada) and the other was obtained from RIKEN BioResource Center, Tsukuba, Japan (#RCB 2280). The *ex vivo* cells collected with the OSCCA were observed using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA).

2.2.3 Cell Viability

Viability of the HCECs was also assessed. One µl of the fluorescent cytological dyes Hoechst 33342 (H) and propidium iodide (PI) (Vybrant® Apoptosis Assay Kit #5, Molecular probes, OR, USA) were added to each sample collected. The fluorescent cytological dyes were incubated in the dark for 20 minutes prior examination. Epithelial cells were manually counted using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA).

2.3 RESULTS

As described in the previous section, corneal epithelial cells differ from others by their expression of CK3 keratin. The cell marker AE5 binds to cells expressing CK3, which allows them to be differentiated. The proportion of collected cells stained by AE5 was 58±17% (Mean±SD) (Figure 6) with a range of 32% to 74% of cells staining for AE5. Viability count showed that 525±139 cells stained with PI and 543±87 stained with Hoechst (n=3). The conjunctival impression cytology samples stained positively with CK19 and negatively with AE5 (Figure 7). However contrary to the expected, both primary and

immortalized corneal and conjunctival *in vitro* cells stained positively with CK19 and AE5 (Figure 8).

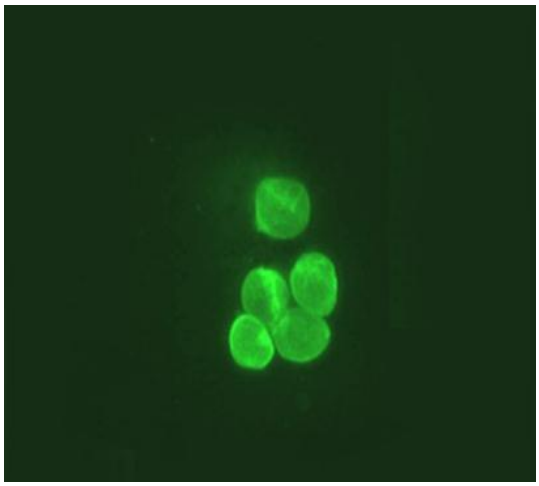

Figure 6: Aggregate of CK3+ stained cells

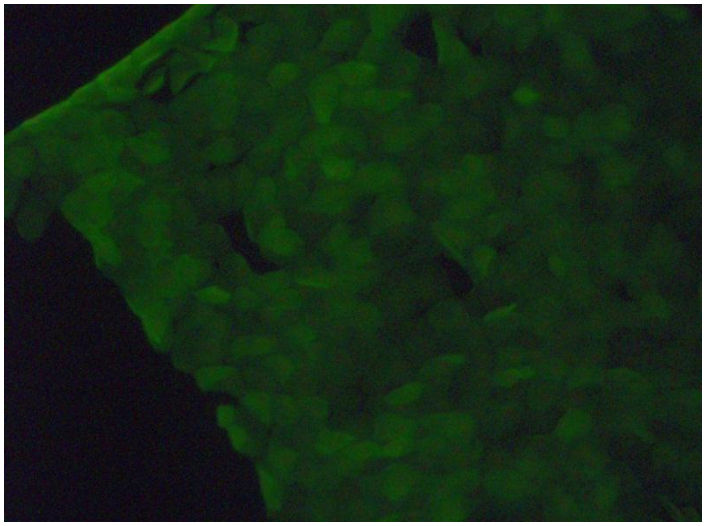

Figure 7: Conjunctival impression cytology sample stained positively with CK19.

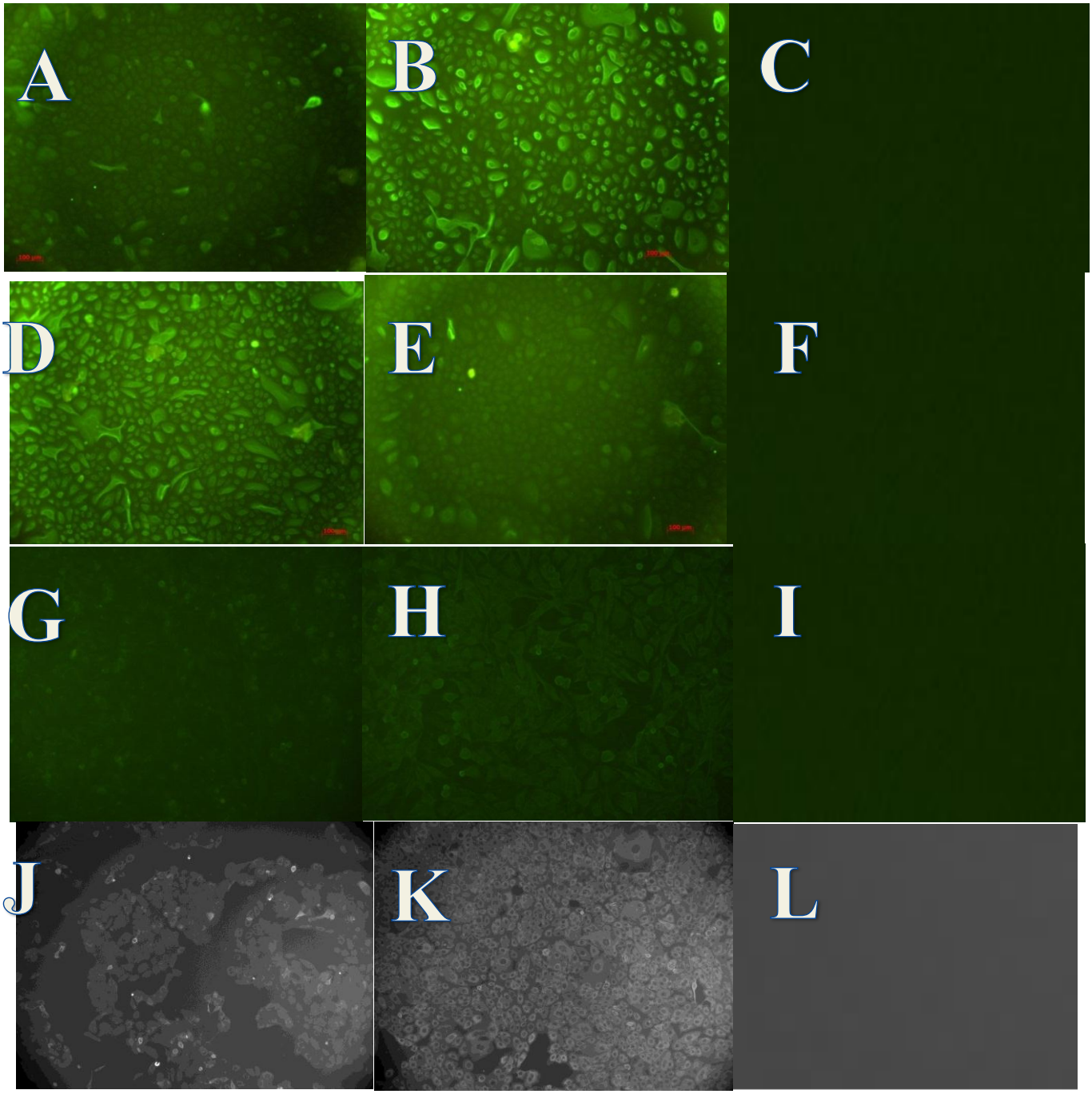

Figure 8: A: Primary HCEC CK3+, B: Primary HCEC CK19+, C: Primary HCEC (no primary antibody) D: Primary HConjEC CK3+, E: Primary HConjEC CK19+, F: Primary HConjEC (no primary antibody), G: Immortalized HCEC (Ottawa) CK3+, H: Immortalized HCEC (Ottawa) CK19+, I: Immortalized HCEC (Ottawa) (no primary antibody), J: Immortalized HCEC (Japan) CK3+, K: Immortalized HCEC (Japan) CK19+, Immortalized HCEC (Japan) (no primary antibody)

2.4 DISCUSSION

Assessing viability of collected cells was challenging. We attempted to quantify viability by calculating the number of live/apoptotic cells collected represented by Hoechst positive cells and dead/necrotic cells collected represented by PI. However, there was an unanticipated double staining of cells whereby the dyes were not mutually exclusive and cells were found to stain both for PI and Hoechst (see Figure 9). This meant that these cells were counted twice once as live and once as dead, which prevented us to reliably report data on the live and dead population in the OSCCA collected cells. This problem is also discussed in the recently published paper by Peterson *et* al.[2] Moreover, there was also an overlap of the fluorescence from PI+ nucleated cells into the blue spectrum. This was also confirm in a control experiment where only PI was added to collected cells and was viewed under the blue filter of the fluorescent microscope (Figure 11). As can be seen some faint red cells as well as some gray/blue nucleus are apparent, the latter cells may potentially be counted as Hoechst positive stain cells during routine counts with PI and Hoechst.

The lack of exclusivity with the dyes is surprising as Hoechst is mostly reported as being a live and early apoptotis stain while PI only stains for dead cells. Hoechst also stained the cell cytoplasm and not just the nucleus of the HCEC, which may be an issue with using too high of a concentration. Others have also referred to Hoechst as staining live, apoptotic and necrotic cells, which would thus explain the significant overlap between PI and Hoechst.[69] It is also possible that the nature of these shed cells may lead to unexpected results with viability certain combination of viability dyes, as has been reported previously with exfoliated mucosal cells.[70]

Nonspecific Hoechst staining of debris was also observed. This fact compounded with the lack of exclusivity between nuclear dye fluorescence made it difficult to reliably count HCEC and meant that no true differentiation between viable and non-viable cells could be made without time consuming comparisons between microscope views. To investigate further the proportion of and changes in viability of non-invasive collected cells, an alternative set of cytological dyes need to be employed and is investigated in chapter 4.

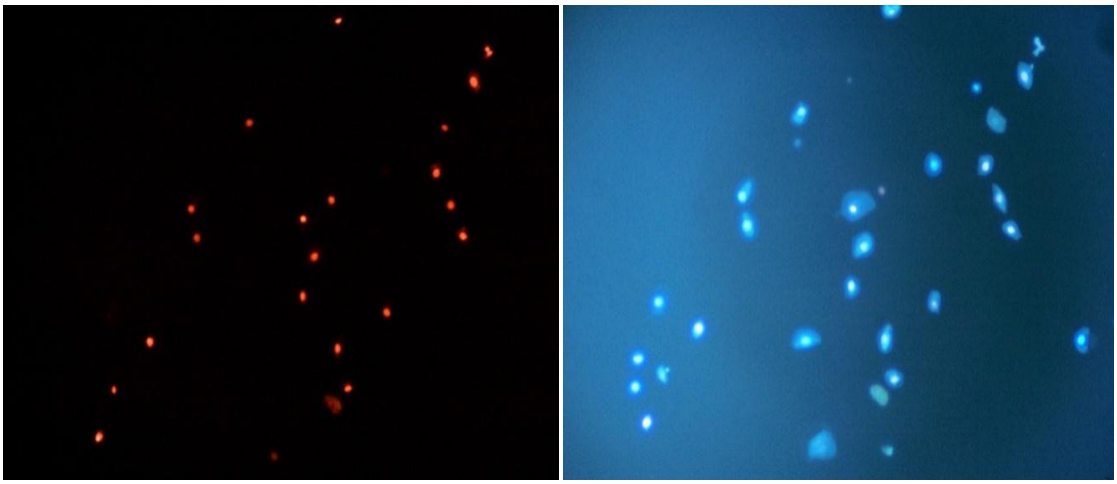

Figure 9: Image of collected cells indicating the same cells highlighted with both Hoechst and PI visible with red and blue excitation filters.

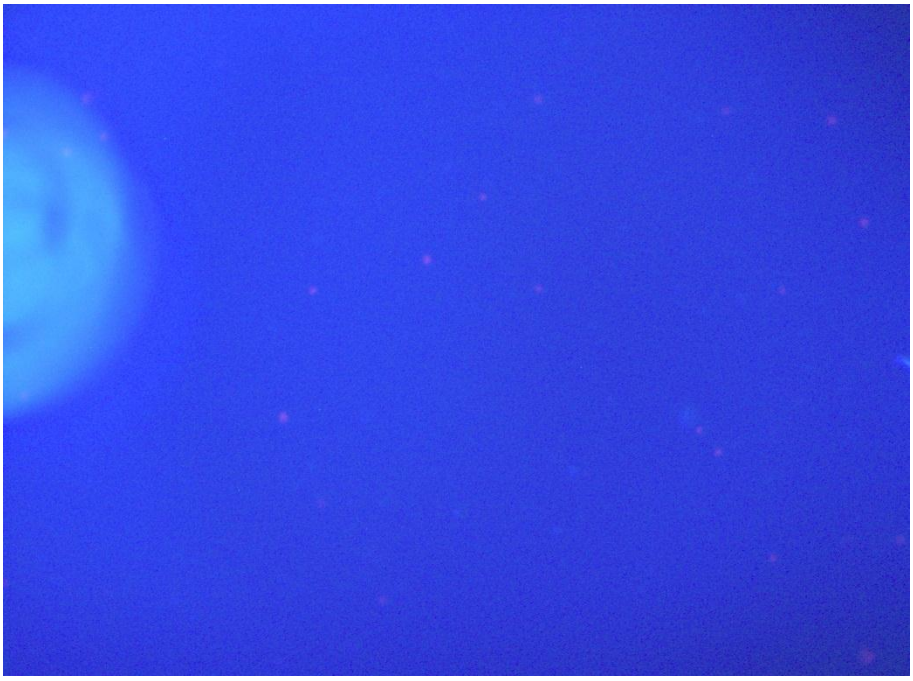

Figure 10: Image of collected cells stained with only PI viewed under the blue excitation filter.

There is also a level of uncertainty whether or not the cells were corneal. The primary HCEC and HConjEC did not behave as expected. It is expected that the primary HCEC would stain positively with CK3 and negativity for CK19 and vice versa for the HConjEC. This was not the case. It has been shown before that primary cells lines do not always behave as expected, in the IOBA-NHC normal human conjunctiva epithelial cell line (IOBA-University of Valladolid, Valladolid, Spain), CK19 was not detected in either young or passaged IOBA-NHC cells.[51] There was also a large range (42%) between the percentages of AE5+ stained cells between the samples. This was within the range of Peterson *et al.* (2011) who reported 75±14% K3+ stained cells[2] and Zhou *et al.* (2000) who reported collecting 61% corneal cells with contact lens cytology.[5] Although the OSCCA jet was aimed the cornea, there was a large potential that the OSCCA was also washing out the tear film, which could include many different cell types, also the physics of saline hitting the cornea can cause the saline to spread beyond the

cornea and onto the conjunctiva. Also since almost the entire population of cells collected was stained with PI and hence were presumably dead, this may have contributed to non-specific antibody binding creating a false positive, as dead cells are notorious for nonspecifically binding antibodies.[52] Dead cells may also not express any CK3, thus contributing to a false negative. It is also possible that the cells collected may be terminally differentiated corneal epithelial cells which may have different expression of these keratins. The literature on keratins expression in corneal epithelial cells remains contradictory.[48-51,71] Initial results were also obtained in rabbit corneal epithelial cells which may show different expression compared to human.[71] Finally, there is also the possibility that false positives may occur from the secondary antibody containing free FITC molecules which may bind to corneal epithelial cells (since we have shown, see next section, that corneal epithelial cells have the ability to stain with sodium fluorescein alone). However this was controlled for in experiments with washing but also by using negative controls whereby incubation were performed with just the primary antibody or just the secondary antibody. This verified that auto-fluorescence was not at play and that nonspecific binding of fluorescein did not occur with our cells. One cannot exclude the fact that the secondary antibody may not have bound specifically to its primary antibody. This would be difficult to test for and such a mechanism would also potentially apply to both CK19 and CK3 results and thus cannot help in explaining the contradictory results that were obtained.

Although there are uncertainties in the type of cells being collected and the viability status using Hoechst and PI with further research and experimentation with different stains this apparatus could provide more confident results.

3 INVESTIGATIONS OF DIAGNOSTIC DYES SODIUM FLUORESCEIN AND LISSAMINE GREEN ON HUMAN CORNEAL EPITHELIAL CELLS

3.1 INTRODUCTION

Previous reports have suggested that sodium fluorescein (NaFl) and other diagnostic dyes may actually cause or exacerbate ocular surface changes such as corneal staining.[53] Lissamine green (LG) is another vital dye for evaluating the ocular surface. It is commonly used to access the conjunctiva.[54] LG which contains 2 aminophenol groups has not been reported to be toxic, teratogenic, or carcinogenic when used in concentrations typical of ophthalmic preparations.[55] LG has been reported to share similar staining characteristics with rose bengal. LG has been found to be a viable substitute for rose bengal when assessing ocular surface disease, particularly as it relates to patient tolerance as patients have been shown to tolerate the instillation of LG better than rose bengal because of decreased discomfort and shorter duration of symptoms.[56] LG has been reported to stain mucus and dead or degenerating cells.[55] The objective of this study section was to investigate the potential cytotoxic effect of sodium fluorescein and lissamine green on the cells of the human corneal epithelium.

3.2 MATERIAL AND METHODS

3.2.1 *Ex vivo*

Cells were collected with the OSCCA following the OSCCA Cell Collection protocol in section 2.2.1.

This investigation was conducted in accordance with the tenets of the Declaration of Helsinki. The University of Waterloo ethics committee approval was acquired before the

investigation began and written informed consent was obtained from participants following explanation of the study procedures. Five healthy participants were recruited, excluding those using systemic or topical medications, or with any ocular pathology. Prior to each study visit the participants were required to undertake a two day wash-out where swimming and contact lens wear was prohibited. All 5 participants attended the clinic on each of 6 study visit days. Visits were scheduled between 09:30-13:00. Each visit day was separated by at least 48 hours.

A prewash was performed on each participant before each instillation treatment to attempt to control for previous environmental conditions and remove any external debris. The OSCCA was used to deliver 10ml of warmed PBS to each eye. After the prewash, for visits 1-3, 0, 1, 6 instillations of 5µl of 1% sodium fluorescein was delivered to the lower conjunctival fornix via pipette. There was a 3 minute gap between each sequential instillation. This protocol was repeated for visits 4-6 with 15µl of 0.5% lissamine green. After 2 hours, the cells were collected with the OSCCA to be analyzed.

After each cell collection, eyes were examined using slit-lamp biomicroscopy and corneal staining and conjunctival staining was graded. Cells were concentrated following the protocol in section 2.2.1. One µl of the fluorescent cytological dyes Hoechst 33342 (H) and propidium iodide (PI) (Vybrant® Apoptosis Assay Kit #5, Molecular probes, OR, USA) were added to each sample collected. The fluorescent cytological dyes were incubated in the dark for 20 minutes prior examination. The cells were examined using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA). Epithelial cells were manually counted and categorized as Hoechst nucleated, Hoechst ghost, PI, sodium fluorescein and lissamine green stained cells.

3.2.2 In vitro

Immortalized human corneal epithelial cells (HCEC) were cultured in a keratinocyte serum-free medium (KSFM) supplemented with bovine pituitary extract, epidermal growth factor and pen-strep. Once confluent in the culture flask, cells were washed with 5ml of PBS and then trypsinized with 3ml of TryplExpress (Invitrogen, Burlington, ON, Canada) and allowed to incubate for 15 minutes at 37^0C. 10ml of F12/Serum (Invitrogen, Burlington, ON, Canada) was added to the culture flask and the contents of the culture flask were transfer into a 15ml conical tube and were centrifuged for 7 minutes at 230g's at room temperature. The supernatant was aspirated and the cells were resuspended in 5ml of KSFM. A 20µl sample of cells was mixed with 20ul trypan blue (Invitrogen, Burlington, ON, Canada.) 10µl of this mixture was transferred to a hemocytometer and counted. 8 x 10^4 cells were seeded in a 48-well tissue culture treated polystyrene plate and grown until confluent. Dilutions of NaFl (0.004%, 0.02%, 0.67% and 1%) and LG (0.002, 0.004, and 0.01%) in KSFM were placed on top of the adherent monolayer HCEC and incubated for 2 hours at 37^0C. The experiment was run in triplicate and repeated on a separate day. To measure cytotoxicity of the products released from the diagnostic dyes, the MTT cellular viability assay was performed. After two gentle rinses 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 24 hours at 37^0C, cells were lysed with dimethyl sulfoxide and transfer to a 96 well plate and absorbance read at 595nm (Thermo MultiSkan Spectrum Photometer, Fischer Scientific, Ottawa, ON, Canada). An AlamarBlue assay (Invitrogen, Burlington, ON, Canada) was also performed to measure cytotoxicity. Cells were incubated with 10% alamarBlue in phenol-red free RPMI medium at 37ºC for 2 hours. Fluorescence was read with an excitation of 530 nm and emission was

recorded at 590 nm (Thermo MultiSkan Spectrum Photometer, Fischer Scientific, Ottawa, ON, Canada). All results are expressed as relative viability compared to cells grown in the absence of the diagnostic dyes. All results are reported as means ± standard deviation. To evaluate the significances of the difference in cell viability and counts, 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.

3.3 RESULTS

3.3.1 Ex vivo

Figure 11 illustrates results of cell staining with the different dyes and categorized as Hoechst nucleated, Hoechst ghost, PI, sodium fluorescein and lissamine green stained cells.

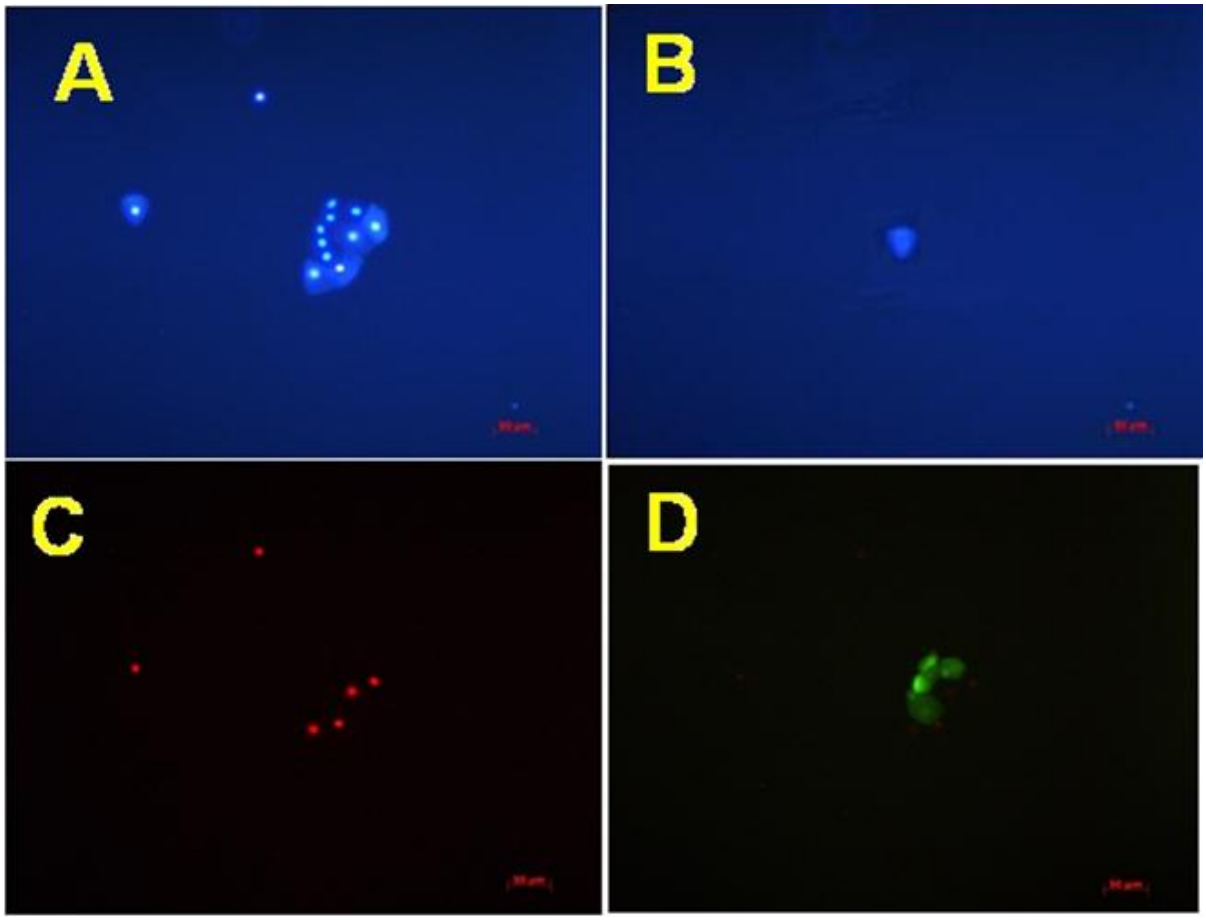

Figure 11: Stained cells. A: Hoechst-nucleated; B: Hoechst-ghost; C: propidium iodide; D: sodium fluorescein.

For sodium fluorescein, 0, 1, and 6 instillations, 421±220, 517±342 and 386±262 Hoechst-stained epithelial cells were counted (Table 1). Hoechst-ghost stained epithelial cells PI-stained epithelial cells were counted (table 1). No trends between the number of instillations and number of cells collected were found (R^2 values lower than 0.02). There also was no significant difference ($p>0.05$) between the number of cells collected per instillation treatment (Table 1). Similar results were obtained with lissamine green. For 0, 1, and 6 instillations, 440±226, 646±252 and 608±283 Hoechst-stained epithelial cells were counted; hoechst-ghost stained epithelial cells and PI-stained epithelial can also be found in Table 1. Again no trends

(R^2 values lower than 0.03) were found and there was no significant difference ($p>0.05$) between the number of cells collected per instillation treatment (Table 1). When comparing the number of epithelial cells collected with fluorescein versus with lissamine green, no significant difference was found ($p>0.05$).

Instillation / Dye	0	1	6	p-value
NaFl				
H-nucleated	421±220	517±342	386±262	P > 0.58
H-ghost	58±23	68±44	53±22	P > 0.60
PI	383±192	463±327	334±219	P > 0.53
LG				
H-nucleated	440±226	646±252	608±283	P > 0.22
H-ghost	55±25	72±41	71±40	P > 0.56
PI	423±249	596±228	540±226	P > 0.28

Table 1: Counts of ex vivo cells with 0,1, or 6 instillations of sodium fluorescein and lissamine green collected with the OSCCA.

It was also observed that following the single fluorescein instillation, 36±39 collected cells stained brightly with both fluorescein and Hoechst and after the six sequential instillations, 38±29 collected cells stained brightly with both fluorescein and Hoechst. The fluorescein-stained cells excluded PI as shown in Figure 12. There was no significant difference ($p>0.05$) between the number of instillations and number of fluorescein-stained cells collected. In our cell collections following lissamine green instillations, none of the cells stained with lissamine green.

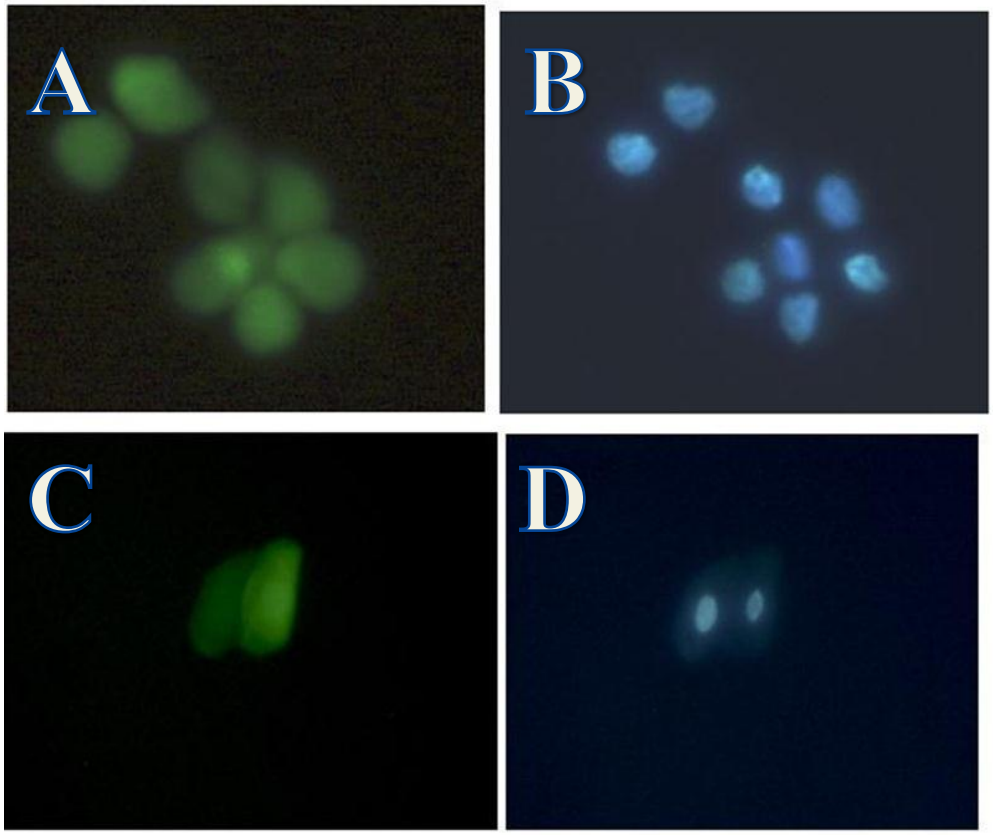

Figure 12: Sodium fluorescein stained cells(A&C) also stained with Hoechst(B&D) but not propidum iodide

3.3.2 In vitro

At 2 and 24 hours, the MTT assay showed no differences in viability between all the NaFl concentrations tested (up to 1%). However at high concentrations of NaFl, a significant difference in viability was observed with the AB assay between 2 and 24 hours ($p<0.05$) (Figure 13). At 2 and 24 hours, with LG, regardless of concentration and the viability assay used no significant difference in viability was observed (Figure 14).

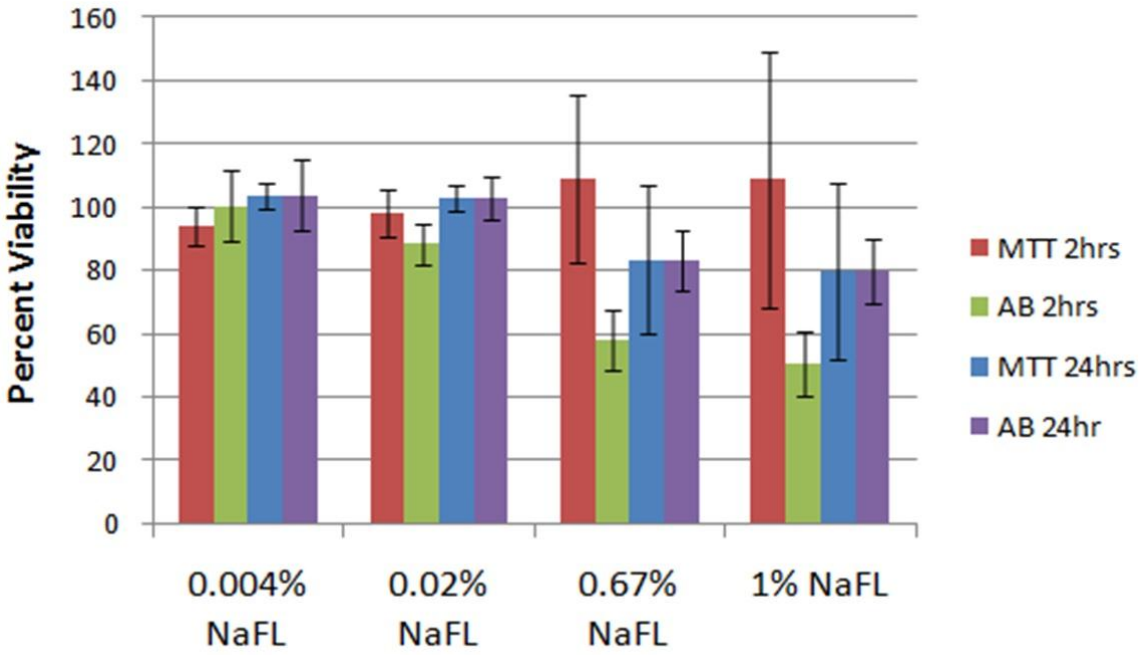

Figure 13: MTT and AB assay of percent viability of *in vitro* HCECs incubated with sodium fluorescein (NaFl) at 0.004%, 0.02%, 0.67%, and 1% dilutions (v/v)

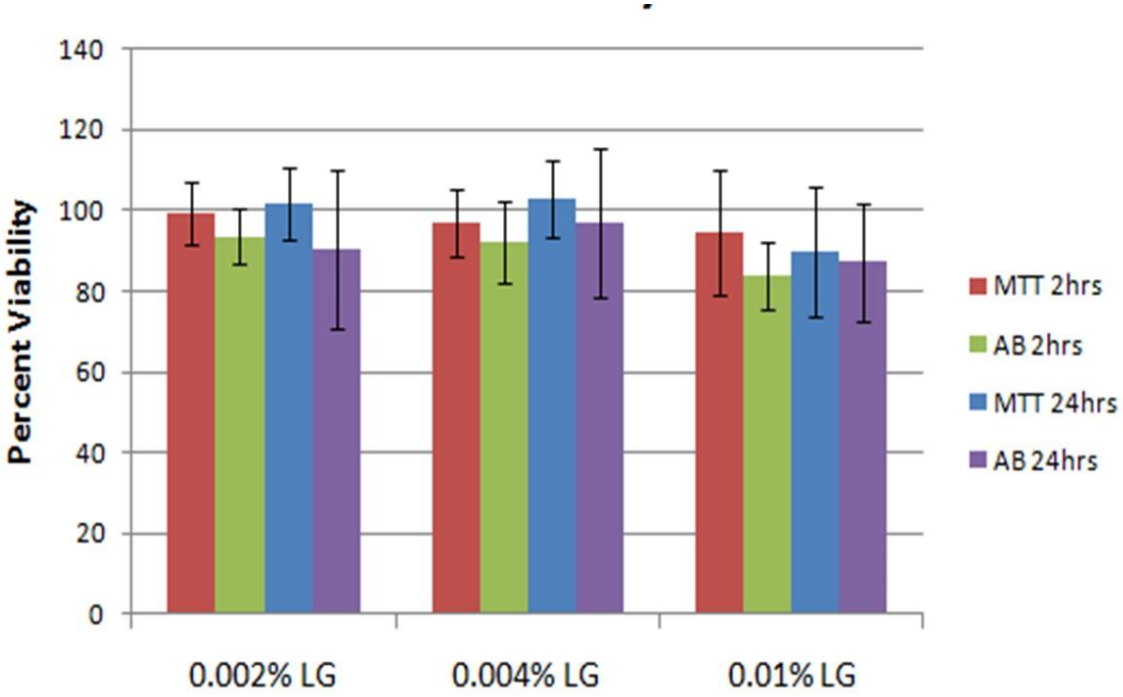

Figure 14: MTT and AB assay of percent viability of *in vitro* HCECs incubated with lissamine green (LG) at 0.002%, 0.004%, and 0.01% dilutions (v/v)

Light microscopy observations revealed that cells exposed to 1% NaFl showed a rounder appearance compared to control cells (no dye). Cells exposed to LG appeared to be normal (Figure 15).

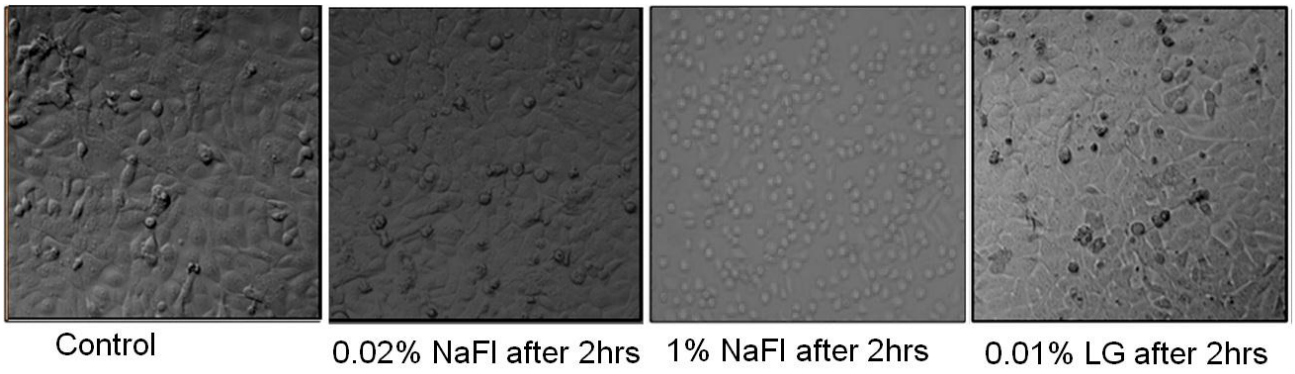

Figure 15: SV-40 immortalized HCECs after 2 hour exposure to sodium fluorescein (NaFl) and lissamine green (LG).

3.4 DISCUSSION

In the ex vivo cell collection experiments, neither sodium fluorescein nor lissamine green instillations appeared to significantly increase cell shedding after 2 hours. This is an indication that these concentration and amount of time left on the eye of these ophthalmic dyes are safe to use and are not cytotoxic to the patient. There was a high variability between same treatments on different days. This could be due to many incontrollable environmental factors ie. weather, dust, allergies, sleep, etc that the participant maybe be exposed to. Debris such as make-up also made it difficult to count Hoechst and PI cells. An interesting finding was the ability to collect

fluorescein-stained cells. One could postulate that sodium fluorescein stains damaged or apoptotic epithelial cells but not necrotic or dead cells. This agrees with the recent paper by Mokhatarzadeh *et al.* that sodium fluorescein is staining individual human superficial corneal epithelial cells.[20] It also agrees with the recent poster by Bandamwar *et al.* whose rabbit model had shown that fluorescence on the corneal surface is due to the uptake of fluorescein by individual cells. The same group also reported that in their *in vivo* rabbit model, superficial punctate fluorescein staining of the corneal epithelium visualized with the slit lamp corresponds to the presence of damaged epithelial cells.[37]

The ability to collect fluorescein stained cells *ex vivo* using the OSCCA provides new means to study contact lens solution based corneal staining and may help understand and identify the mechanisms involved in solution induced corneal staining. The results of the viability MTT assay on the *in vitro* cells showed that the dilutions of sodium fluorescein was not cytotoxic, however light microscopy showed that the HCEC were beginning to change phenotype. The HCEC incubated with sodium fluorescein were smaller and rounder compared to the controls (see Figure 16). These results tend to suggest that *in vitro* exposure to sodium fluorescein could be cytotoxic to HCEC and that sodium fluorescein can induce apoptosis in HCEC. The same concentration of sodium fluorescein was used in the *ex vivo* and *in vitro* study, however it is likely the concentration of sodium fluorescein in the eye is much lower on the HCEC after 2 hours: NaFl will be diluted with tear turnover and blinking may further remove NaFl from the corneal surface. In the *in vitro* experiment as there was no medium exchange, and thus the NaFl concentration on the HCEC remained constant. The *in vitro* HCEC model used in this experiment was a monolayer compared to in the *in vivo* situation where the cornea is composed of multiple layers of cornea epithelial cells. With that into consideration, a monolayer may be

more sensitive compared to a multilayer when testing these diagnostic dyes. Future work on multilayer models should be considered.

In conclusion neither sodium fluorescein nor lissamine green instillations appear to significantly increase cell shedding *in vivo* or cell death in vitro after 2 hours. These findings suggest that the OSCCA and HCEC model are both sufficiently sensitive enough methods to study corneal staining.

4 DEVELOPMENT OF VIABILITY STAINING PROTOCOL

4.1 INTRODUCTION

The previous chapters outlined a few issues that were encountered when determining viability of OSCCA collected cells. The significant overlap with the cytological dyes, Hoechst (supposed to stain only live cells) and PI (dead cell stain). To further investigate the proportion and changes in viability of collected cells, an alternative set of cytological dyes and methods needed to be identified. Hoechst33342 (H3570) , Live/Dead Cell vitality Assay Kit (C12 resazurin/SYTOX® Green), calcein and ethidium homodimer, caspase assay, and a luminescence based ATP assay were identified as being potential stains that could be used to assess cell viability in ex vivo collected cells. Table 2 lists the characteristics of the proposed assays to determine collected cell viability.

Name	Description	Equipment	Pros	Cons
Hoechst 33342 and PI	Fluorescence detection of the compacted state of the chromatin in apoptotic cells	Flow Cytometry Fluorescent Microscopy Fluorescent spectrophotometry	Used in previous *ex vivo* studies.[2]	Stains debris Colour bleeds through channels
Live/Dead Cell vitality Assay Kit (C12 resazurin/SYTOX® Green)	a two-color fluorescence assay that distinguishes metabolically active cells from injured cells and dead cells.	Flow Cytometry Fluorescent Microscopy Fluorescent spectrophotometry	Resazurin is nontoxic	Dead Cells Fluoresce green which would interfere with Sodium fluorescein
Calcein and ethidium homodimer assay	The Calcein AM/EthD-1 assay can simultaneously determine live and dead cells with two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity.	Flow Cytometry Fluorescent Microscopy Fluorescent spectrophotometry	Used in previous *in vitro* and *ex vivo* studies.[57,58] Viable and dead cells can be counted in the same field	Healthy Cells Fluoresce green which would interfere with Sodium fluorescein
CaspACE™ FITC-VAD-FMK	Fluorescent apoptosis marker Allows delivery of the inhibitor into the cell where it irreversibly binds to activated caspases	Flow Cytometry Fluorescent Microscopy	Used in previous *in vitro* study.[59] Single Reagent Can be combined with PI	Uses FITC, which would interfere with Sodium fluorescein
ATP assay	ATP assay system is based on the production of light caused by the reaction of ATP with added Luciferase and D-Luciferin	Luminometer	Very sensitive <20 cells	Unable to visualize with a microscope

Table 2: Characteristics of the proposed assays to determine collected cell viability

This chapter will present the investigation the Live/Dead® Cell vitality Assay Kit (C12 resazurin/SYTOX® Green) (Invitrogen, Burlington, ON, Canada) and the Live/Dead® Viability/Cytoxicity kit for mammalian cells (calcein AM/ethidium homdimer-1) (Invitrogen, Burlington, ON, Canada) as well as looking at some other methods using a cytospin and a fluorescent plate reader.

Live/Dead® Cell vitality Assay Kit (C12 resazurin/SYTOX® Green) provides a simple, two-color fluorescence assay that distinguishes metabolically active cells from injured cells and dead cells. The assay is based on the reduction of C12-resazurin to red-fluorescent C12-resorufin in metabolically active cells and on the uptake of the cell-impermeant, green-fluorescent nucleic acid stain, SYTOX Green dye, in cells with compromised plasma membranes (usually late apoptotic and necrotic cells). In this assay, dead cells emit mostly green fluorescence and healthy, metabolically active cells emit mostly red fluorescence; injured cells exhibit reduced red and green fluorescence.[60]

The LIVE/DEAD® Viability/Cytotoxicity Kit (calcein AM/ethidium homodimer-1) quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity by binding to live cells which have intracellular esterases that convert nonfluorescent, cell-permeable calcein acetoxymethyl to the intensely fluorescent calcein. The red-fluorescent ethidium homodimer-1 enters dead cells which have damaged membranes; the ethidium homodimer-1 enters damaged cells and is fluorescent when bot to nucleic acids. It is adaptable to most eukaryotic cells where cytotoxic conditions produce these cellular effects.[61] Calcein Blue, AM (Invitrogen, Burlington, ON, Canada) was also purchased as an alternative to the regular calcein-AM. This was implemented

since the *ex vivo* studies aimed at looking at the effects of sodium fluorescein which also emits a green colour.

4.2 METHODS

4.2.1 *In vitro* model

Immortalized HCEC were cultured in a KSFM supplemented medium with bovine pituitary extract, epidermal growth factor and pen-strep. Once confluent in the culture flask, cells were washed with 5ml of PBS and then trypsinized with 3ml of TryplExpress (Invitrogen, Burlington, ON, Canada) for 15 minutes at 37ºC. 10ml of DMEM/FBS was added to the culture flask and the contents of the culture flask were transferred into a 15ml conical tube and were centrifuged for 7 minutes at 230g's at room temperature. The supernatant was aspirated and the cells were resuspended in 5ml of KSFM. A 20µl sample of cells was mixed with 20ul trypan blue (Invitrogen, Burlington, ON, Canada.) 10µl of this mixture was transferred to a hemocytometer and counted. 8 x 10^4 cells were seeded in a 48-well tissue culture treated polystyrene plate and grown for 24 hours. Cells were washed three times with PBS and treated with KM/PBS (100 µl KM + 100 µl PBS) or a KM/BAK (100 µl + 100 µl Bausch&Lomb Collyrium for Fresh Eyes) with a final 0.005% concentration of BAK solution for 1 hour at 37ºC. Cells were washed three times with PBS and 200 µl of PBS was added to the wells. Following the manufacturer's protocols, 2 µL of 50 µM C12-resazurin and 2 µL of the 1 µM SYTOX green dye or 0.5 µl of 50 µM calcein AM and 1 µL of the 2 mM homodimer-1 was added to the cells and incubated at 37ºC for 20 minutes. The cells were examined using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA).

4.2.2 *Ex vivo* collection

Cells were collected with the OSCCA following the OSCCA Cell Collection protocol in section 2.2.1. To concentrate the cells, the individual suspensions were centrifuged (at 1800 rpm for 10 minutes at 25ºCelsius) with 1ml of FBS, 10.5ml of solution was aspirated and the remaining 0.5ml was transferred into a 48-well plate. One µl of the fluorescent cytological dyes C12 resazurin and SYTOX® Green were added to a series of samples and 0.25 µl of calcein AM (blue and green) and 1 µl of ethidium homodimer-1 were added to a second series of samples. Following the kit instructions, cells were incubated for 20 minutes at 37ºC. Cells were then immediately observed using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA)

4.2.3 Fluorescent Microplate Reader

Similar to the *in vitro* model from above, HCEC were seeded into a 96-well tissue culture treated polystyrene plate and grown for 24 hours. Cells were washed three times with PBS and treated with varying concentrations of BAK in KM (0.0025%, 0.0050% and 0.0100%) in triplicate for 15 minutes at 37ºC. Samples were washed three times and PBS and 100 µl of PBS and 100 µl of PBS solution at 1 µM calcein AM and 2 µM ethidium homodimer-1 were added to the samples and incubated for 30 minutes and read with the Spectramax M5e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) using the LiveDead setting with Wavelength excitations are ~494nm and ~528nm and emissions are ~525nm and ~620nm respectively.

A similar experiment was attempted on collected cells from the OSCCA. Cells were concentrated to 200 µl and transferred to a 96-well plate. 0.25 µl of both calcein AM and ethidium homodimer-1 were added to the samples and allowed to incubate for 30 minutes. The

samples were than read with the Spectramax M5e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

4.2.4 Cyto-spin

An alternate method was experimented with the collected *ex vivo* cells (n=3). After cells were concentrated via the method above, 200 µl was transferred to a disposable 3-well Cell Concentrator (Iris, MA, USA). The cell concentrator was spun in a StatSpin® Cytofuge (Iris, MA, USA) for 4 minutes at 1000rpm. The slides were stained with 0.25 µl of Hoechst and 0.25 µl of PI and were incubated for 30 minutes in the dark at room temperature. They were cover-slipped and observed using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA).

4.3 RESULTS

4.3.1 Live/Dead® Cell vitality Assay Kit (C12 resazurin/SYTOX® Green)

The *in vitro* experiments showed that the C12 resazurin stained the metabolically active cells red and the cells compromised plasma membranes green. This was evident in the control wells, where cells were exposed to their normal growth medium. In the control well the majority of the HCECs fluoresced red (live), while a few cells fluoresced green (compromised/dead) (Figure 16). The *in vitro* cells treated with the solution containing the BAK resulted in the majority of the cells fluorescing green, indicating a compromised plasma membrane. Although, many of those cells also fluoresced red, indicating some metabolic activity. In the presence of BAK, the cell morphology was also different compared to the control: the cells were rounder compared to the elongated live cells (Figure 17).

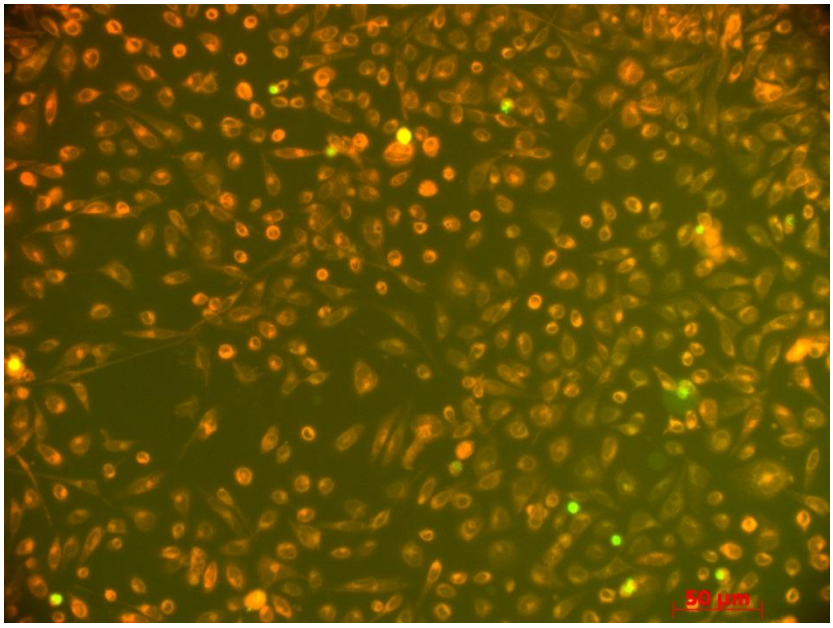

Figure 16: Control *in vitro* HCECs stained with C12 resazurin(red-live)/SYTOX® Green (dead/compromised)

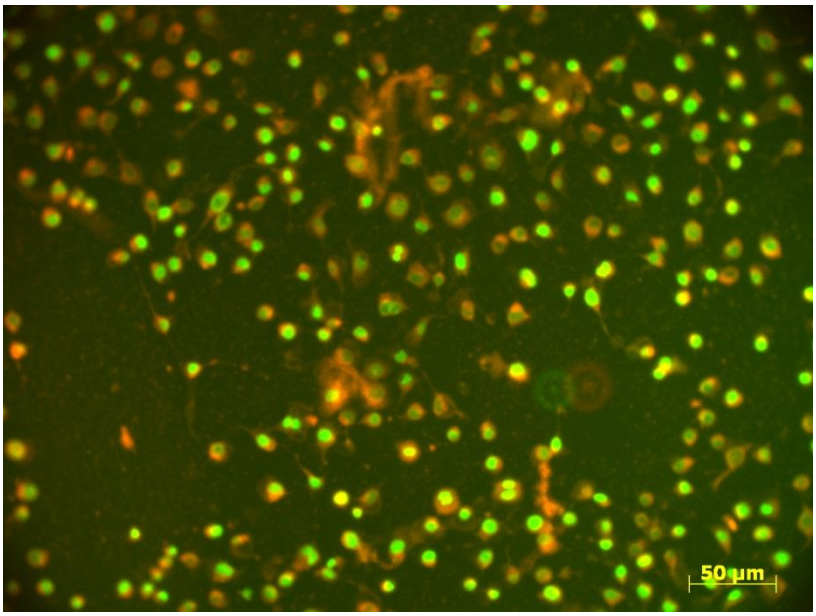

Figure 17: BAK treated (1 hour) *in vitro* HCECs stained with C12 resazurin(red-live)/SYTOX® Green (dead/compromised)

The OSCCA collected cells had similar results to the Hoechst and PI of the previous experiments. There was an overlap of the stains, the same cells stained with both the C12 resazurin and SYTOX® green (Figure 18).

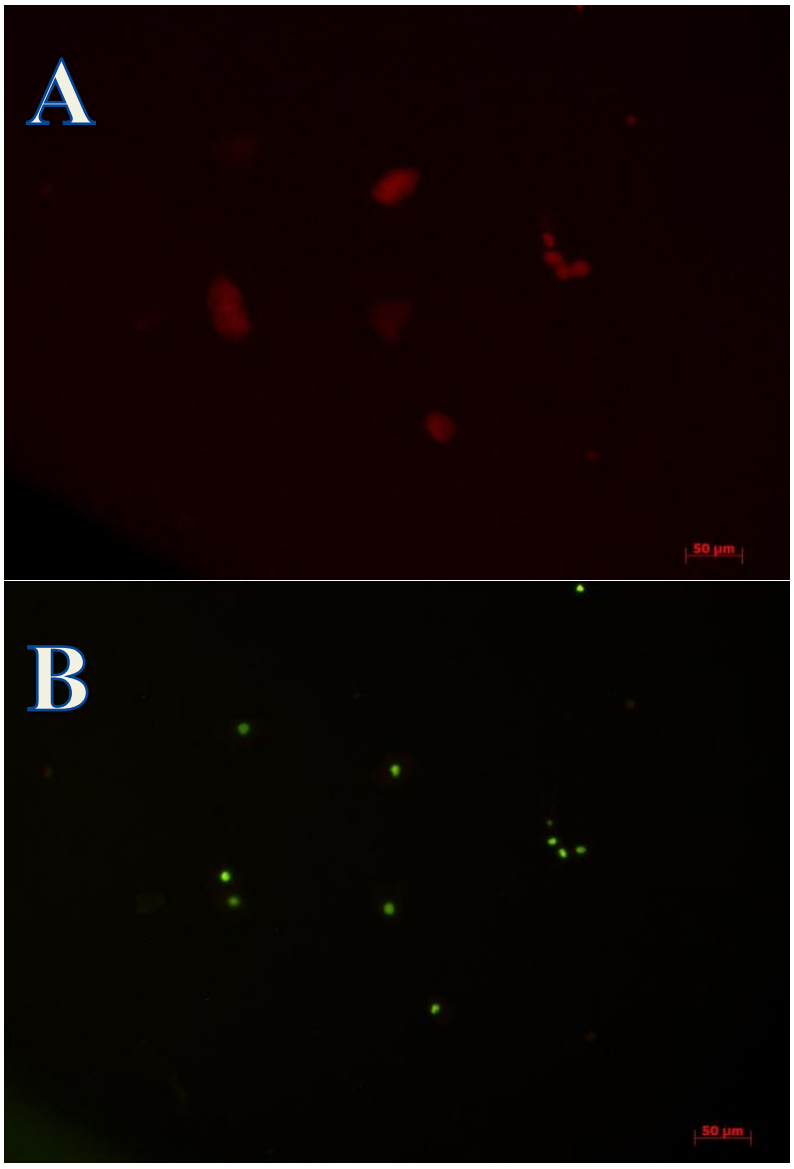

Figure 18: Image of collected cells indicating the same cells highlighted with both C12 resazurin (live) (A) and SYTOX® green (dead/compromised) (B)

4.3.2 The LIVE⁄DEAD® Viability⁄Cytotoxicity Kit (calcein AM/ethidium homodimer-1)

Similar *in vitro* experiments were performed with calcein AM and ethidium homdimer-1 stains. Calcein Blue AM was added to the LIVE⁄DEAD® Viability⁄Cytotoxicity Kit (calcein AM/ethidium homodimer-1) to show that the calcein blue AM stains the exact same cells as the

regular calcein AM (which stains cells green.) Both calcein AMs stained the majority of the live *in vitro* HCECs blue and green indicating the intracellular esterase activity. The HCECs with loss of plasma membrane integrity fluoresced red with the ethidium homodimer-1 (Figure 19). When treated with BAK all the *in vitro* cells stained with just the ethidium homodimer-1 (Figure 19).

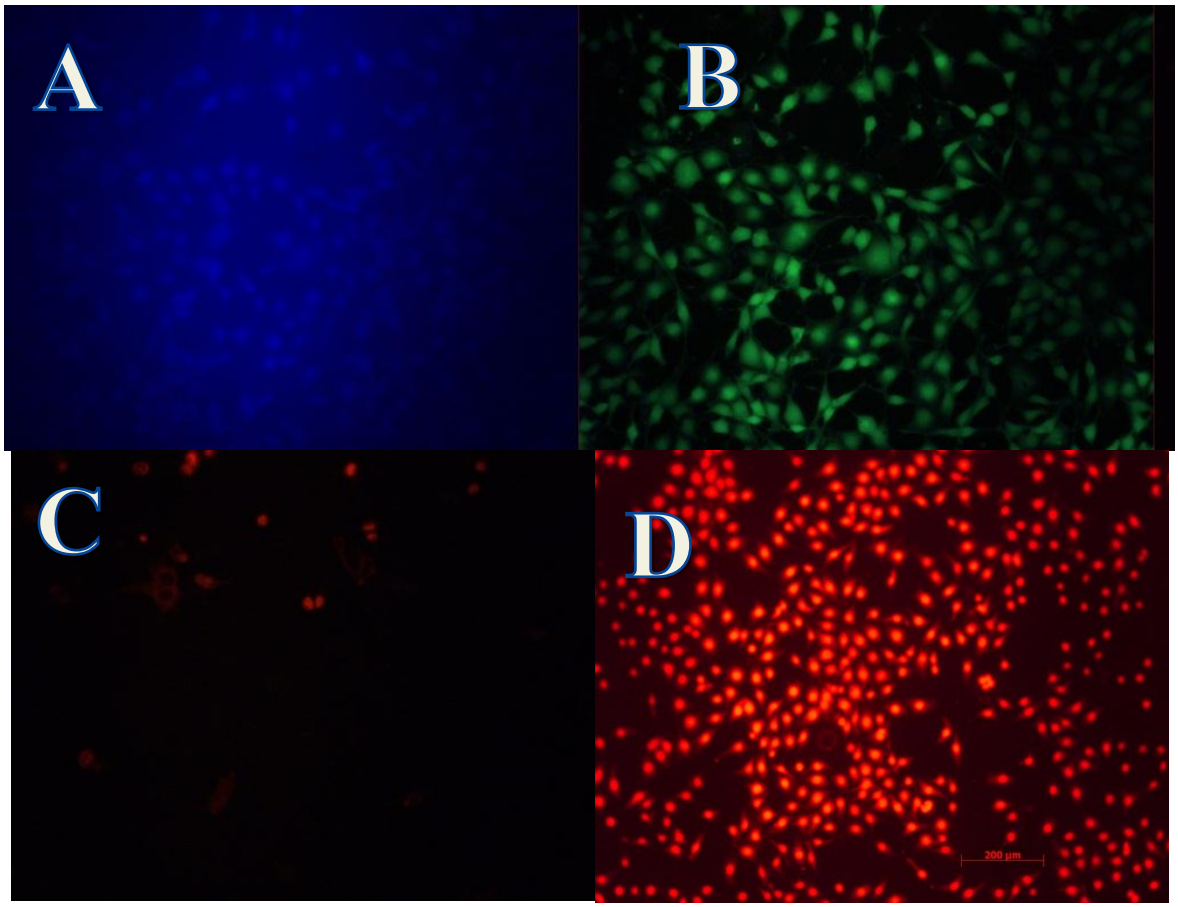

Figure 19: *In vitro* HCECs stained with (A) calcein blue AM (live stain), (B) calcein (green) AM (live stain) and (C) ethidium homodimer-1 (red) (dead stain), (D) BAK treated HCEC stained with ethidium homodimer-1

The OSCCA collected cells differed with the cytological dyes, calcein AM/ethidium homodimer-1 compared with C12 resazurin/SYTOX® green and the Hoechst/PI. There was

very little overlapping between the calcein AM and the ethidium homodimer-1. Similar to the *in vitro* experiment, it also showed that the calcein blue AM and the calcein (green) AM were staining the identical cells (Figure 20).

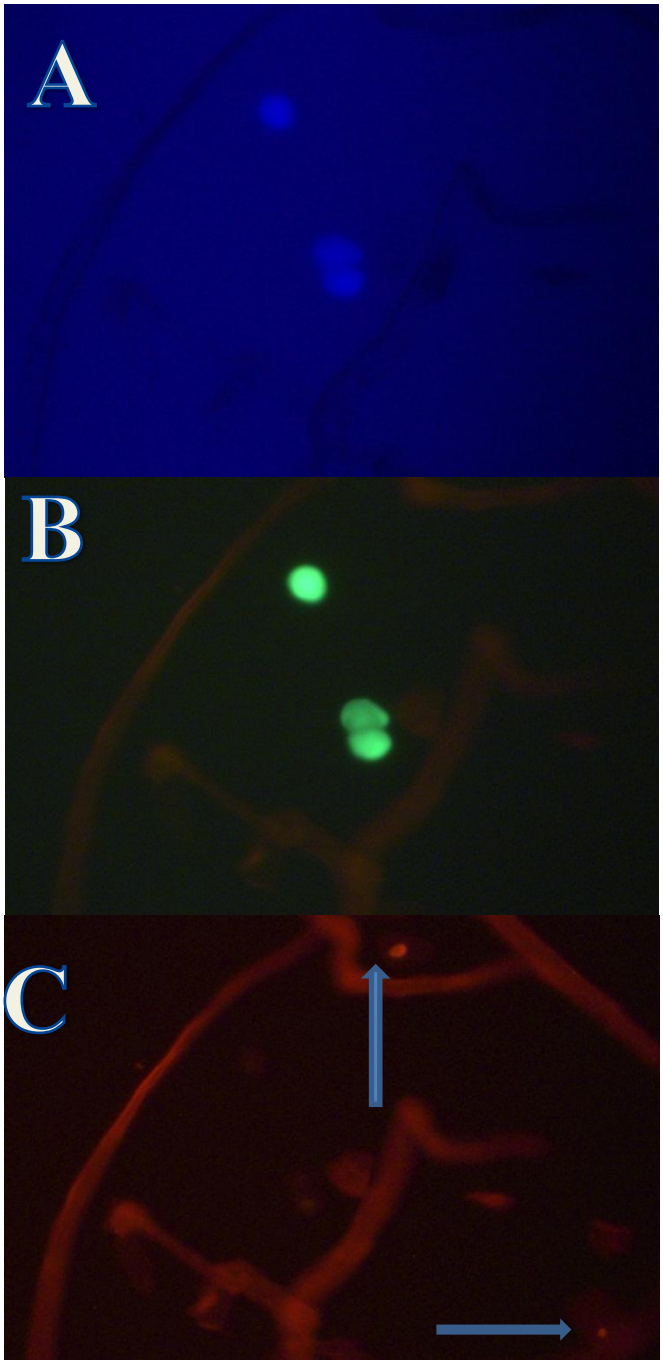

Figure 20: Image of collected cells indicating that (A) calcein blue AM and the (B) calcein (green) AM was staining identical live cells and there was no overlap with the (C) ethidium homodimer-1 which was staining cells with a comprised plasma membrane.

4.3.3 Fluorescent Microplate Reader

The Fluorescent Microplate reader clearly showed that cells exposed to the high concentrations of BAK (0.01% and 0.05%) were significantly different from the PBS control ($P<0.05$) (Figure 21). After just 15 minutes exposure, the higher concentrations of BAK 0.01% and 0.05%, had calculated viability of 28% and 40% respectively (100% viability being based on cells exposed to PBS).

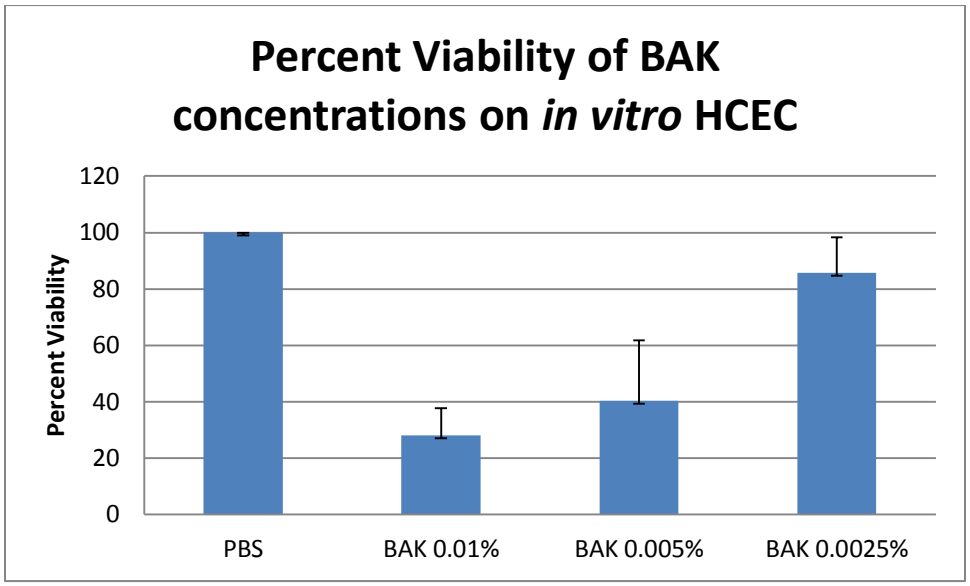

Figure 21: Percent Viability of BAK concentrations on *in vitro* HCEC

The fluorescent microplate reader experiment on the OSCCA collected was unsuccessful and no readings were obtained. This was likely due to the low cell numbers.

4.3.4 Cyto-Spin Technique

The cyto-spin technique showed some interesting results with Hoechst and PI. The same issue persisted with the overlapping of Hoechst and PI. However with Hoechst, with "cyto-spin" cells, only the cell nucleus fluoresced blue compared to our previous experiments where cells left

to settle in the well had Hoechst, stained not only the nucleus but the cell cytoplasm as well (Figure 22). In all cyto-spin experiments, no ghost cells were found.

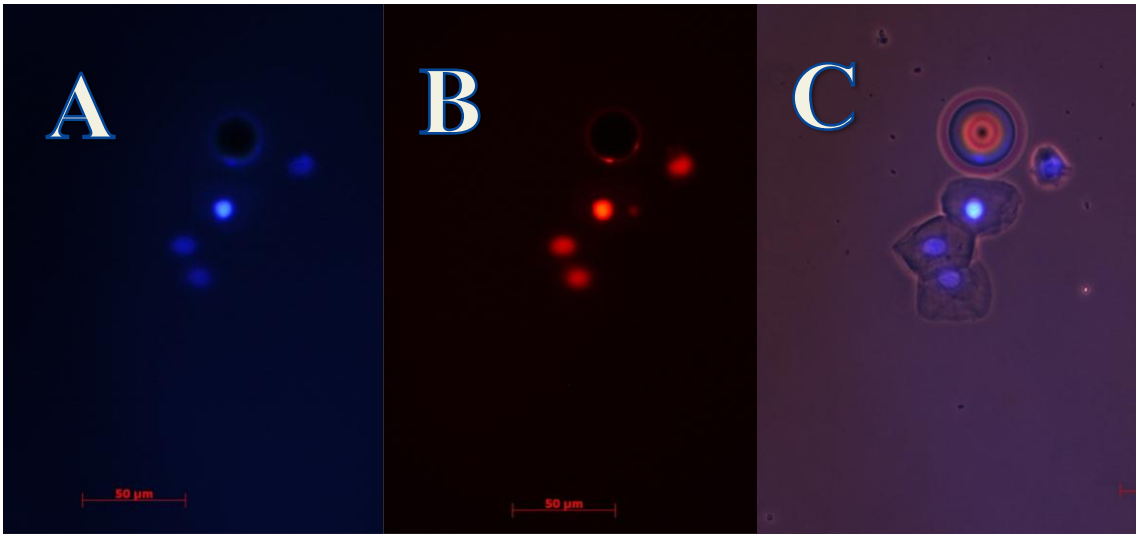

Figure 22: Image of collected cells indicating the same cells highlighted with both (A) Hoechst and (B) PI visible with red and blue excitation filters. Image (C) shows that only the cell nucleus was stained.

4.4 DISCUSSION

The investigation of different cytology dyes showed that calcein AM and ethidium homodimer-1 are the most suitable dyes to use with collected cells from the OSCCA. Due to the lack of overlap, calcein AM/ethidium homodimer-1 gives the ability to distinguish between live and dead cells in the cells collected from the OSCCA. Although the calcein blue AM signal is not as strong and as long lasting as calcein (green) AM it provides the ability to interchange the calcein (green) AM with the calcein blue AM to allow sodium fluorescein to be investigated without any interference of green fluorescence coming from the calcein (green) AM. Increasing the concentration of calcein blue AM may help in increasing the signal intensity. Based on the results, a visual representation of the cytological dyes is shown in Figure 23.

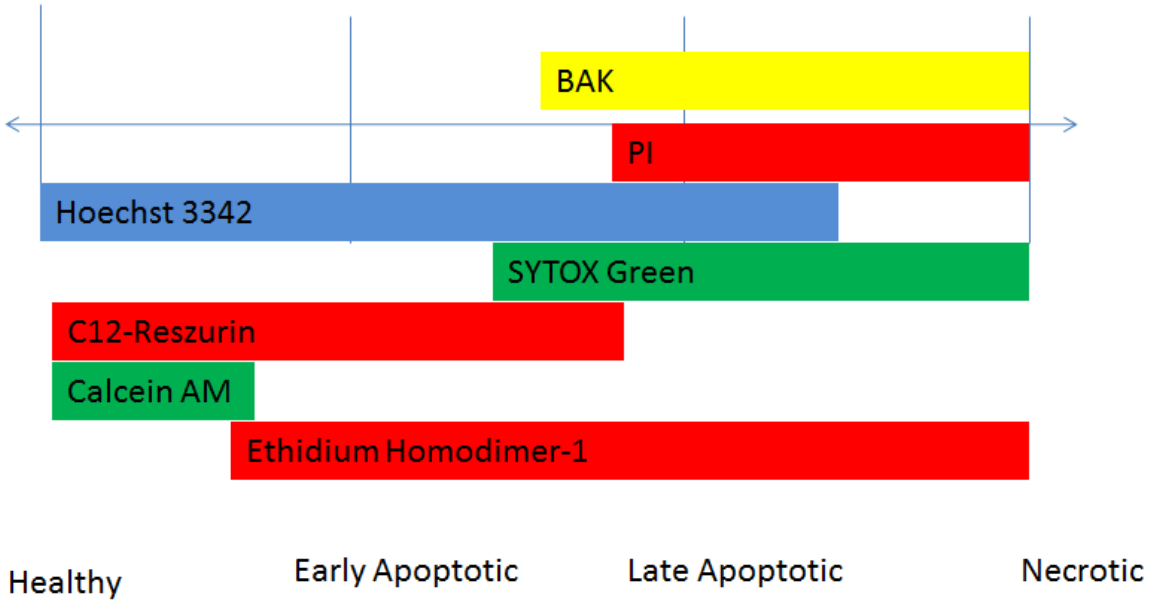

Figure 23: Visual representation of the estimated overlap of different cytological dyes

The cyto-spin technique provided some interesting results. No ghost cells were found using the cyto-spin technique, which makes sense since Hoechst is only supposed to only stain the nucleus and not the cell membrane or cytoplasm. This is also beneficial when manually counting cells since only the nucleus is being stained; there is less confusion whether or not a cell or debris is being counted. The cyto-spin also provides another method to attach cells to a surface different from the poly-D-lysine used in chapter 2.

The fluorescent microplate reader technique was a quick and simple method to show viability on the *in vitro* model. It confirmed results from many studies that BAK even at low concentrations is toxic to HCEC.[62-64] However, when used to detect changes in the collected cells from the OSCCA, results were not favorable and likely related to the low cell numbers. Pooling a large number of samples may remedy this problem and provide a quick method to determine

viability instead of the timely counting method; however this would prevent looking at individual changes in the cornea and may thus not be appropriate for clinical studies.

5 INVESTIGATIONS OF BENZALKONIUM CHLORIDE AND SODIUM FLUORESCEIN ON HUMAN CORNEAL EPITHELIAL CELLS

5.1 INTRODUCTION

Benzalkonium Chloride (BAK) is a common preservative found in many commercially available ophthalmic formulations from comfort drops to anti-glaucoma preparations. Studies have shown that mammalian cells are unable to neutralize BAK, and the corneal epithelium is damaged by its entrance through liposomes or other intracellular vacuoles which induces cytotoxic damage.[65] BAK induces two different patterns of cell death; apoptosis and necrosis in a dose dependent manner.[62 66] *In vitro* studies have also shown that BAK induced cell damage at concentrations as low as 0.0001%[63] and high levels of toxicity have also been observed in corneal and conjunctival cells.[67] In the previous chapter it was made evident that a solution containing 0.01% BAK was quite damaging to the HCECs (Figure 17). Despite the reported *in vitro* evidence of the cytotoxicity potential of BAK, the Food and Drug Administration (FDA) has listed it as a safe preservative.[64]

This chapter will look at the impact of BAK and the association of sodium fluorescein on ocular cells collected non-invasively using the OSCCA and the new cytotoxic stains, calcein blue AM and ethidium homodimer-1 from the previous chapter.

5.2 MATERIALS AND METHODS

Cells were collected with the OSCCA following the OSCCA Cell Collection protocol in section 2.2.1.

This investigation was conducted in accordance with the tenets of the Declaration of Helsinki. The University of Waterloo ethics committee approval was acquired before the

investigation began and written informed consent was obtained from participants following explanation of the study procedures. Five healthy participants were recruited, excluding those using systemic or topical medications, or with any ocular pathology. Prior to each study visit the participants were required to undertake a two day wash-out where swimming and contact lens wear was prohibited. All 5 participants attended the clinic on each of 3 study visit days. Each visit day was separated by at least 48 hours.

A prewash was performed on each participant before each instillation treatment to attempt to control for previous environmental conditions and remove any external debris. The OSCCA was used to deliver 10ml of warmed PBS to each eye. After the prewash each participant's eyes were examined using slit-lamp biomicroscopy. Two µl of sodium fluorescein was instilled in each eye with a capillary tube. Corneal staining and conjunctival staining was graded and corneal global staining scores (GSS) were calculated. After the biomicroscopy each participant underwent a contra-lateral randomized instillation of 50 µl of 0.01% BAK solution (Bausch&Lomb Moisture Eyes) in one eye and 50µl of sterile in the opposite eye. Fifteen minutes post instillation, the participants eyes were reassessed using slit lamp biomicroscopy and the GSS was recalculated and cells were collected with the OSCCA. After each cell collection, eyes were again examined using slit-lamp biomicroscopy and the GSS was calculated. Cells were examined as soon after collection as possible to minimize any potential damage or distortion. To concentrate the cells, 0.5 ml of FBS was added and the individual suspensions were centrifuged (at 1800 rpm for 10 minutes at 25ºCelsius), the solution was aspirated to 200µl and was transferred into a 96-well plate. 0.25 µl of calcein blue AM (Invitrogen, Burlington, ON, Canada) and 0.50 µl of ethidium homodimer-1 (Invitrogen, Burlington, ON, Canada) were added to wells and incubated for 45 minutes in the dark at room temperature. The cells were

examined using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss, ON, CA). Live and dead cell counts were established and sodium fluorescein stained cells were also counted. Mutuality of sodium fluorescein stained cells were compared to calcein blue AM and ethidium homodimer-1. All results are reported as means ± standard deviation. To evaluate the significances of the difference in cell viability and counts, a t-test was carried out. A p-value of less than 0.05 was required for statistical significance.

5.3 RESULTS

There was no clinically significant corneal staining found post instillation of either 0.01% BAK or sterile saline after 15 minutes of exposure. On average, there were 428 cells collected on eyes treated with BAK compared to 434 cells collected from the control eyes (sterile saline instillation). On average the BAK group had counts of 25±14, 403±156, 17±13 and the sterile saline control group had counts of 17±13, 417±241, 14±13 for calcein blue AM, ethidium homodimer-1 and sodium fluorescein. No significant difference ($P>0.05$) was found (Figure 24). The BAK group had a viability of 6.6±4.7% compared to the sterile saline control group of 5.3±4.7%. Again no significant difference ($P>0.05$) was found. Cells that stained with sodium fluorescein stained exclusively with calcein blue AM and not ethidium homodimer-1. (Figure 25)

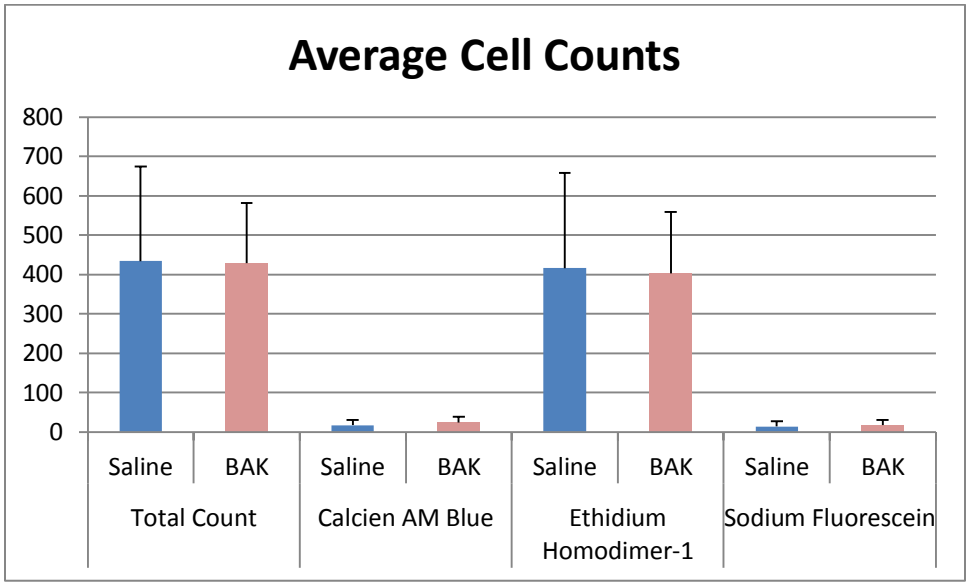

Figure 24: Average cell counts between sterile saline control and BAK

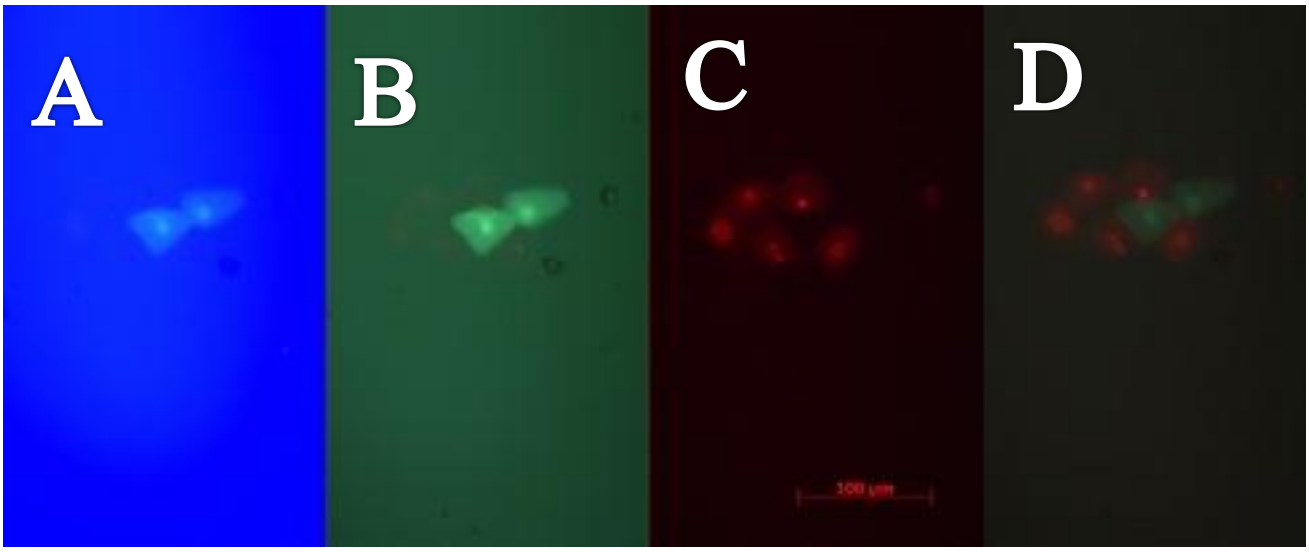

Figure 25: Splitview image of one view field containing OSCCA-collected cells staining with (a) Calcein AM Blue (live stain), (b) sodium fluorescein and (c) ethidium homodimer-1 (dead stain). (d) shows the overlay of sodium fluorescein and ethidium homodimer-1 staining indicating the absence of dead stain in the sodium fluorescein stained cells.

5.4 DISCUSSION

The calcein AM and ethidium homodimer-1 allowed to appropriately determine the percentage of the collected cell population that was alive, which had not been possible previously due to the significant overlap in staining with Hoechst and PI. Also compared to Hoechst and PI, calcein AM and eithidium provided a much cleaner sample with less debris being stained. Similar overall counts were made between this experiment and the one in chapter 3. The PI counts were also very similar to the ethidium homodimer-1 counts which make sense, since they both stain cells that are damaged or dead. The low calcein AM counts seem very logical, it can be assumed that the majority of cells shed from the eye are damaged or dead. The number of sodium fluorescein stained cells shed from the eye was slightly higher in the previous experiment, but still within the same range. This slight increase could be attributed to the higher amount of sodium fluorescein used in the previous experiment. It also provides more evidence that the BAK in this experiment had no effect on corneal staining in this experiment.

It was expected that the BAK drop would have negative effects on the corneal epithelium because the extreme toxic effects found in *in vitro* and *in vivo* animal models.[62,63,67] However these studies involved either a static model where there was nothing to simulate blinking (*in vitro* models) or multi doses of BAK over a longer people of time. It is very likely there was no difference between the sterile saline group and the BAK group because the solution was quickly diluted in the tear film and potentially blinked away and the BAK did not get the chance to interact with the superficial cells on the ocular surface in the healthy normal study participants. Timing may have impacted the effect of BAK on the corneal epithelial cells. In this experiment examinations were done after 15 minutes of exposure to BAK, which may not have been a sufficient amount of time to observe cellular changes and increase cell shedding. However prior

to the experiment a pilot study investigated a 30 minute exposure time to BAK and no cellular changes were observed. Since there were no differences in cellular change, the 15 minute exposure time was used. Participants with the BAK treated eye did not exhibit any signs of corneal staining. It was also found in experiments performed in parallel in the Gorbet lab, that sodium fluorescein failed to show any "corneal staining" on *in vitro* HCEC after two hours. The results contradicted to a study showing increased corneal staining with a 15-20 minute exposure to an anesthetic containing BAK.[72] However the other ingredients in the anesthetic may have contributed to the corneal staining. Although there was no significant reaction precaution still should be taken when using ophthalmic solutions containing BAK. Patients with pre-existing conjunctival and corneal diseases, such as dry eye syndrome, are especially more prone to the toxic effects of BAK.[68] Their eyes, which often have decreased tear production may not be able to wash away the preservative as effectively as normal eyes. These patients may also experience the lachrymal outflow passages that are partially or totally obstructed, which would increase the contact time of the BAK solution on the ocular surface.[68]

Interestingly, similar results were found in the experiment in chapter 3, cells that stained with sodium fluorescein stained exclusively with calcein blue AM and not ethidium homodimer-1. Cells that had intracellular esterase activity were stain with calcein blue AM and sodium fluorescein. Cells with a loss of plasma membrane integrity, presumably necrotic and dead cells only stained with the ethidium homodimer-1. Again, one could postulate that sodium fluorescein stains damaged or apoptotic epithelial cells but not necrotic or dead cells.

6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

In conclusion, the answers to the scientific questions raised in this thesis are as follows:

(1) Is the OSCCA an efficient and reliable tool to collect human corneal epithelial cells?

Determining the ocular cell types collected with the OSCCA was a challenge. There was a high level of uncertainty whether or not the cells collected were in fact corneal or conjunctival epithelial cells. It was found that 58% of cells collected with the OSCCA stained with the corneal epithelial cell marker. Similarly, in a study by Zhou *et al.* they determined they only 61% of cells collected using contact lens cytology were corneal epithelial cells.[5] Chapter 2 discussed that there is a large potential that the OSCCA is also washing out the tear film, which could include many different cell types. Also the physics of saline hitting the cornea can cause the saline to spread beyond the cornea and onto the conjunctiva, potentially collecting further cells that are not on the cornea. The majority of the cells that the OSCCA collected were dead. The results from chapter 5 showed that approximately 95% of the cells collected from the OSCCA were non-viable, which is similar to the 89% non-viable cells collected by Wilson *et al.*[3] It has been shown that dead cells are notorious at contributing to non-specific antibody binding which may explain our difficulties in clearly identifying collected cells as corneal epithelial versus conjunctival cells. In order to confidently distinguish the cell types, future work must be done. Using flow cytometry and having a much larger sample negating all the dead cells may potentially enable the characterization of living cells, the only possibility to obtain a clear representation of the types of cells collected with the OSCCA.

One may also envision installing a video-camera and a screen to actually visualize the flow of saline hitting the cornea and its spread. Ideally, adding a biocompatible dye to the saline

solution during the video would not provide further means to study the spread of the saline jet. While such a setup would not provide a clear answer on cell type that are being collected as the saline will still mix the tear film, it would allow us to better visualize the target of the saline jet and gain a better understanding (and control via feedback) of the ocular surface that is being washed.

(2) Can we measure the viability status of cells collected from the ocular surface using the OSCCA?

Chapter 2 and 3 showed the Hoechst and PI were not optimal stains to measure the viability status of cells collected with the OSCCA because there was an unanticipated overlap of the fluorescence from PI+ nucleated cells into the blue spectrum and the Hoechst stained both live and dead cells. Previous work by Wilson *et al.*[3] had used acridine orange as a live stain and thus it is difficult to know if the nature of the shed cells or the Hoechst/PI combination itself resulted in the poor outcome of the first investigated viability protocol. Chapter 4 looked at other cytological stains and concluded that the LIVE/DEAD® Viability/Cytotoxicity Kit (calcein AM/ethidium homodimer-1) was the most appropriate stain to use with the OSCCA collect cells due to the lack of overlap between stains. Calcein AM/ethidium homodimer-1 was also used on cells collect by contact lens in an experiment by Zhou *et al.*[5] If sodium fluorescein was a part of the experiment, the calcein (green) AM could be easily replace with calcein blue AM. The calcein AM/ethidium homodimer-1 was able to show a clear difference between live and dead cells. Although the fluorescent microplate reader was unable to detect enough fluorescence, pooling a large number of samples may remedy this problem and provide a quick method to determine viability instead of the timely counting method. The cyto-spin is also beneficial in certain situations. The cyto-spin allows cells to be attached to a surface of a glass side, which

has better optical properties for photographic benefits when using confocal microscopy. The cyto-spin also obtained cleaner where less debris was stained compared to the multiwall plate method.

An issue that still needs to be remedied with measuring the viability of the collected cells is time. Manually counting cells is very time consuming process and limits the number of samples that can be processed without jeopardizing other samples that are in the queue. Automatic methods need to be investigated.

(3) What does sodium fluorescein actually stain?

Chapter 3 showed that cells that stained with sodium fluorescein stained with only Hoechst and not PI. Since Hoechst stains live and early apoptotic cells and PI stains cells that are late stage apoptotic, necrotic and dead cells, we can conclude that sodium fluorescein stains live and early apoptotic cells. Similarly in chapter 5 it was found that cells that stained with sodium fluorescein stained exclusively with calcein blue AM, suggesting cells possessed intracellular esterase activity and did not lose their plasma membrane integrity as they did not stain with ethidium homodimer-1. The results agree with the recent paper by Mokhatarzadeh *et al.* that sodium fluorescein is staining individual human superficial corneal epithelial cells using impression cytology in conjunction with DAPI staining with confocal fluorescent microscopy.[20] It also agrees with the recent poster by Bandamwar *et al.* whose rabbit model had shown that fluorescence on the corneal surface is due to the uptake of fluorescein by individual cells and superficial punctate fluorescein staining of the corneal epithelium visualized with the slit lamp corresponds to the presence of damaged epithelial cells.[37] In conclusion, this thesis is one of the

first reports to identify clearly in shed human corneal cells that sodium fluorescein stains live, damaged or apoptotic epithelial cells but not necrotic or dead cells. The fact that sodium fluorescein stains live cells has significant implications on the way staining is currently viewed in the field of vision science.[28,73] While it may highlight area of corneal disruption, one cannot say anymore that fluorescein stains dead cells in the cornea. Potentially in SICS, the superficial layer of corneal cells may not stain with sodium fluorescein at all and solution toxicity may compromise the superficial epithelial layer allowing sodium fluorescein to stain the live corneal cells beneath. Our findings also would not support the PATH theory of corneal staining that sodium fluorescein just merely adheres to PHMB molecules.

Overall, the OSCCA enables to sample the cornea and collect 434±241 shed cells in a wash. Under normal conditions, as examined mainly in this thesis, this represents a small amount of shed cells and potentially less than 0.1% of cells from the superficial layer of the cornea. However, conditions such as lens wear, exposure to lenses that have been soaked in different cleaning solutions or dry-eye are likely to result in changes to cell shedding, either in the number of cells being collected or in their phenotype. The methods developed in this thesis enable to characterize OSCCA collected cells and will contribute to advance knowledge on the mechanisms involved in cell shedding at the ocular surface. As for corneal staining, there are still many questions that need to be answered to determine the actual mechanisms involved in corneal staining. For example does SICS stains cells the same way that alcaine stains cells? What is the sodium fluorescein attached to on the individual cells? Is it inside the cell or just bound to the membrane? How do superficial cells compare to the cells one layer beneath them? It is expected that using the OSCCA and the methods developed herein will continue to contribute to gain a better understanding of the complex puzzle that corneal staining represents, so that

optometrists and ophthalmologists around the world may one day know what fluorescein actually stains.

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