AN EXPLORATION OF THE EFFECTS OF BEVACIZUMAB ON THE CORNEAL EPITHELIUM AND ENDOTHELIUM: AN IN VITRO AND CLINICAL STUDY

by

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B.Sc., University of British Columbia, 2017

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2021

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ABSTRACT

The avascular nature of the cornea is disrupted when corneal neovascularization (NV) causes the formation of new blood vessels onto the ocular surface resulting in opacification and subsequent blindness. The third most common cause of blindness worldwide is corneal disease and corneal NV is present in almost every case. Approximately 4.14% of patients who require eye care present with corneal NV and the major causes are infectious diseases, extended wear of contact lenses and vascular response to corneal transplantation.

Vascular endothelial growth factor (VEGF), a pro-angiogenic factor, plays the most critical role in the development of several retinal neovascular diseases, as well as in corneal NV. Its inhibition by anti-VEGF agents has proven to be successful in the management of these conditions. The most widely used therapeutic agent is bevacizumab (Avastin®), a full-length recombinant humanized monoclonal antibody against VEGF, due to its cost-effectiveness. Despite the widespread use of bevacizumab in ophthalmology, its use is considered off-label. Therefore, ongoing studies into the safety of this drug for the eye is important. Given that bevacizumab was initially used to treat retinal neovascular diseases, its safety for retinal cells is more robustly established. More recently, it has been studied as an off-label topical treatment for corneal NV. The safety and biocompatibility of bevacizumab on the cornea must be ensured for off-label use, particularly as there has been evidence for potential corneal cytotoxicity resulting in delayed epithelial wound healing. It is speculated that topical bevacizumab possibly interferes with the adhesion between the corneal epithelium and the basement membrane, leading to increased risk of corneal epithelial defects.
In this thesis, the primary objective is to investigate the effects of bevacizumab on the corneal epithelium and endothelium, focusing on two aims: 1) examine the cytotoxic effects of bevacizumab on the viability and metabolism of human corneal epithelial cells and endothelial cells, and 2) evaluate the adverse effects of intravitreal bevacizumab on the cornea in patients and report on the risk factors involved in developing corneal epithelial defects.
LAY SUMMARY

The healthy cornea is clear and transparent, and it is the outermost tissue layer of the eye. Many eye diseases can cause blood vessels to grow into the cornea which can in turn lead to vision loss from corneal scarring. Vascular endothelial growth factor (VEGF) is the key chemical factor involved in the development of many eye diseases in which abnormal blood vessels grow. Drugs that stop the action of VEGF are a treatment option for the management of these conditions. Bevacizumab (Avastin®) is an example of a drug that binds and inactivates VEGF.

The objective of this thesis is to investigate the effects of bevacizumab on the corneal epithelium and endothelium. There are two aims: 1) examine the toxic effects of bevacizumab on human corneal epithelial cells and endothelial cells, and 2) evaluate the adverse effects of intravitreal bevacizumab on the cornea in patients seen in the clinic.
PREFACE

The work contained within the main body of this thesis (Chapter Two and Chapter Three) was performed at the Eye Care Centre – Vancouver General Hospital in the Department of Ophthalmology and Visual Sciences at the University of British Columbia. All projects were approved by the University of British Columbia’s Research Ethics Board (B18-0192, B18-0123 and H19-00286).

At the current time, Chapter Two has been submitted for publication. I (Shayan Shokoohi) am the primary author and was responsible for performing laboratory experimental procedures, data analysis and manuscript composition. Dr. Sonia N. Yeung and Dr. Alfonso Iovieno were responsible for experimental design, reviewing the data analysis, revising the manuscript, obtaining funding and supervising the process.
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LIST OF ABBREVIATIONS

aFGF – acidic fibroblast growth factor
AMD – age-related macular degeneration
ARPE-19 – human retinal pigment epithelial cells
ATP – adenosine triphosphate
bFGF – basic fibroblast growth factor
CED – corneal epithelial defect
CME – cystoid macular edema
CNVM – choroidal neovascular membranes
DAMPs – damage-associated molecular patterns
DED – dry eye disease
DME – diabetic macular edema
DMEM – Dulbecco’s Modified Eagle Medium
DPBS – Dulbecco’s phosphate-buffered saline
ECM – extracellular matrix
EGF – epidermal growth factor
ERK – extracellular signal-regulated protein kinase
FBS – fetal bovine serum
FDA – Food and Drug Administration
FGF – fibroblast growth factor
GSDMD – gasdermin D
HBSS – Hank’s Balanced Salt Solution
HCEnCs – human corneal endothelial cells
HCEpCs – human corneal epithelial cells
HGF – hepatocyte growth factor
HIF-1 – hypoxia-inducible factor-1
IGF – insulin-like growth factor
IgG1 – immunoglobulin G1
IL – interleukin
IOP – intraocular pressure
IVB – intravitreal bevacizumab
KGF – keratocyte growth factor
MAPK – mitogen-activated protein kinase
MLKL - mixed lineage kinase domain-like pseudokinase
MMP – matrix metalloproteinase
NV – neovascularization
OSD – ocular surface disease
PDGF – platelet-derived growth factor
PDGFR – platelet-derived growth factor receptor
PDR – proliferative diabetic retinopathy
PGF – placental growth factor
PROSE – Prosthetic Replacement of Ocular Surface Ecosystem
ROP – retinopathy of prematurity
RVO – retinal vein occlusion
SFM – serum-free medium
TBUT – tear break-up time
TGF – transforming growth factor
TNF – tumour necrosis factor
VEGF – vascular endothelial growth factor
VEGFR – vascular endothelial growth factor receptor
ACKNOWLEDGEMENTS

My sincerest gratitude to my supervisor, Dr. Sonia N. Yeung, for her mentorship, support and patience over these last few years. I have learned immensely from you both professionally and personally and I am thankful for providing the space and freedom to grow on my own as a scientist. I am forever grateful for all the opportunities that have changed me for the better.

To members of my supervisory committee, Dr. Joanne A. Matsubara and Dr. Mahyar Etminan, I am truly appreciative of your continuous guidance and feedback that have made this experience an ease and very enjoyable.

I would like to extend a special thanks to Dr. Jing Cui for teaching me a multitude of techniques that will come very handy in my future. Your compassion and humour is endearing and I will never forget our light talks. To other members of the Matsubara Lab, Eleanor To, Erika, Siqi, Chris and Gideon, I thank you all very much for familiarizing me with a lab environment.

I must express a distinct thanks to Howard Meadows for first introducing me to the eye. Your enthusiasm and lightheartedness is infectious and I look up to you greatly.

Lastly, I would like to thank my family, Mom, Dad and my brothers, Aria and Sassan, for their incessant love and support. I could not have accomplished this without you. I am forever indebted to you.
DEDICATION

To Mom and Dad,

Thank you for your everlasting affection and support throughout my recurring incidents of doubt.
CHAPTER ONE: INTRODUCTION

1.1 Background Information

1.1.1 Angiogenesis and ocular neovascularization

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels and is the root cause of neovascularization within the eye. Angiogenesis is responsible for a wide variety of biological processes including wound healing, embryological development, tumour growth and metastasis and ocular diseases, and collectively, they provide us with an understanding of the mechanisms involved in the development of new blood vessels in the body [1].

Neovascularization (NV) results from a complex interaction between pro-angiogenic factors and anti-angiogenic factors that stimulate or inhibit endothelial cell differentiation, proliferation, migration and maturation, respectively (Fig. 1.1) [1]. When a tissue experiences hypoxia, i.e. a lack of oxygen, it results in increased levels of hypoxia-inducible factor-1 (HIF-1) which stimulates the expression of pro-angiogenic factors, leading to NV [2]. These pro-angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PGF) and platelet-derived growth factor (PDGF) [2]. More specifically, the VEGF and FGF families have been shown to play a pivotal role in the development of several retinal neovascular diseases, such as neovascular age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), retinal vein occlusion (RVO) and retinopathy of prematurity (ROP) [2].
Figure 1.1 Pro-angiogenic factors and anti-angiogenic factors involved in neovascularization (Image source: Feizi et al., 2017 [3] with permission under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)).

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor [3]. The most vital member of the VEGF family is VEGF-A which is secreted by a wide range of heterogeneous cells, such as macrophages, T-cells, fibroblasts, pericytes, astrocytes, retinal pigment epithelial cells and corneal cells (epithelium, keratocytes and endothelium) [3]. VEGF-A takes its effect by binding to and activating two tyrosine kinase receptors VEGFR1 and VEGFR2 leading to the promotion of angiogenesis, vascular permeability, cell migration and gene expression [3].
The FGF family includes acidic FGF (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) [1]. Both aFGF and bFGF are produced by a variety of cell types including vascular endothelial cells, fibroblasts, smooth muscle cells, astrocytes, granulosa cells, adrenocortical cells, retinal pigment epithelial cells and many malignant cell types [1]. In vivo, bFGF has angiogenic activity and is highly expressed by endothelial cells during tumor angiogenesis and in vitro, bFGF promotes endothelial cell proliferation, protease production and chemotaxis [1].

Current findings suggest that there is no single growth factor that acts alone to cause ocular neovascularization. It has been demonstrated that bFGF promotes angiogenesis through its effect on VEGF-A, VEGF-C and VEGF-D production, thus signifying a role for bFGF in regulating VEGF levels. In vitro, VEGF-induced angiogenesis was blocked by the addition of bFGF antibodies. This indicates a complex relationship between bFGF and VEGF in promoting ocular neovascularization [1-3].

1.1.2 Corneal neovascularization

The cornea is the clear, dome-shaped anterior part of the eye which covers the iris, pupil and anterior chamber. A healthy cornea is transparent and avascular in order to provide a proper anterior refractive surface. New blood vessels that irregularly grow from pre-existing vascular structures surrounding the cornea lead to corneal NV [3]. Corneal NV is not a diagnosis, but a non-specific response to various clinical insults [3]. It occurs in a wide range of corneal pathologies, such as contact lens-related hypoxia, chemical burns, allergy, trauma, infectious keratitis, limbal stem cell deficiency, corneal graft rejection, inflammatory disorders, autoimmune diseases and
congenital diseases [3]. It can cause a decrease in visual acuity as a result of edema, persistent inflammation, scarring and intrastromal protein and lipid deposition [3].

In corneal NV specifically, VEGF and bFGF are not the only biological factors that play a role in promoting angiogenesis. It has been shown that PDGF is also heavily involved with cell growth and division, tissue remodeling and angiogenesis [3]. The binding of two ligands, PDGF-A and PDGF-B, with their appropriate receptors, PDGFR-α and PDGFR-β, has been associated with corneal NV [3]. Other angiogenic factors that have been linked to corneal NV include matrix metalloproteinase (MMP) and interleukin-1 (IL-1) [3]. One member of the MMP family, MMP14, has been shown to interact with receptor VEGFR1 causing an enzymatic reaction that is crucial for VEGF-induced angiogenesis [3]. Additionally, IL-1 is a proinflammatory molecule produced by fibroblasts, macrophages and neutrophils, and it stimulates the expression of adhesion molecules, chemokines and growth factors that result in NV [3].

1.1.3 Anti-VEGF agents

Although angiogenesis is a multifaceted process induced by many factors, it has been determined by a large number of experimental studies that VEGF-A plays the most critical role and it is the main target in recent anti-angiogenic therapies for the treatment of several ocular diseases characterized by NV. The most commonly used anti-VEGF agents for treating retinal neovascular diseases are bevacizumab, ranibizumab and aflibercept.

Bevacizumab (Avastin®) is a full-length, humanized monoclonal antibody which inhibits all biologically active isoforms of VEGF-A. It is a recombinant IgG1 antibody with a molecular
weight of 149 kDa and it initially received FDA approval for the treatment of metastatic colon cancer [3]. Its use in ophthalmology is off-label, however it is most widely used as an intravitreal injection for the treatment of numerous retinal neovascular diseases [3]. More recently, it has also been studied as topical or subconjunctival application for the management of corneal NV [3].

Ranibizumab (Lucentis®) is a humanized, monovalent monoclonal antibody which inhibits all biologically active VEGF-A isoforms, similar to bevacizumab. It is a recombinant IgG1 antibody fragment with a molecular weight of 48 kDa and it has received FDA approval for the treatment of neovascular AMD. Due to its smaller size compared to bevacizumab, it has a greater affinity for VEGF-A and is able to penetrate retinal vessels more efficiently. It is only intended for intravitreal injection as it has an enhanced VEGF-A binding potential [3].

Afibercept (Eylea®) is a recombinant fusion protein which not only inhibits VEGF-A and VEGF-B isoforms, but also interacts with PDGF and PGF, and counteracts the ligand-induced activation of receptors. It contains an IgG backbone fused to extracellular VEGF receptor sequences of the human VEGFR1 and VEGFR2, thus serving as a soluble decoy receptor. It has received FDA approval as an intravitreal injection for the treatment of neovascular AMD. Considering its inhibition of VEGF-A, VEGF-B, PDGF and PGF, it is believed to have a higher efficacy than bevacizumab and ranibizumab [3].

1.1.4 Anti-VEGF therapy for corneal neovascularization

Over the years, anti-VEGF agents have demonstrated great success in the treatment of several retinal neovascular diseases, thus their potential for the management of corneal NV has
been evaluated as well. Studies assessing the safety and efficacy of anti-VEGF agents against corneal NV are less abundant, but more investigations have been done in recent years. Nevertheless, these agents have limited efficacy and each pose their own challenges and undesirable side effects.

Topical application of bevacizumab has been shown to partially reduce corneal NV in both animal and human studies [4]. In an in vivo study, topical bevacizumab was delivered to 30 eyes of 27 patients and a 61% decrease in mean vascularized area and a 24% decrease in vessel diameter was observed [5]. In another study, 10 eyes of 10 patients received topical bevacizumab and a 47.1% reduction in mean neovascular area and a 54.1% reduction in vessel caliber was reported [6]. Subconjunctival administration of bevacizumab has also shown positive results in treating corneal NV. In a study consisting of 18 patients with lipid keratopathy secondary to corneal NV, the extent, centricity and percentage of involved corneal surface of the NV were measured and all parameters of corneal NV improved significantly with subconjunctival bevacizumab [7]. Another study that included 10 patients with major and minor corneal NV received 2.5 mg of subconjunctival bevacizumab and determined that it resulted in a reduction of the total area and extent of NV for up to 3 months after injection [8]. It remains unclear, however, whether subconjunctival administration is more effective than topical application and more studies comparing the two techniques must be carried out before a conclusion can be reached.

The downside to anti-VEGF agents is that they also inhibit VEGF functions that are essential for cell growth and proliferation, leading to complications and side effects. These functions include wound healing, corneal nerve regeneration, control of vascular tone and
formation of collateral vessels [3]. It is speculated that topical, but not subconjunctival, bevacizumab possibly interferes with the adhesion between the corneal epithelium and the basement membrane leading to delayed wound healing and stromal thinning [3]. This also explains the increased risk of corneal epithelial defects associated with topical bevacizumab [4]. Corneal epithelial defects normally heal within 7-10 days after a corneal injury, however if rapid re-epithelialization does not occur within two weeks, a persistent corneal epithelial defect (CED) develops (Fig. 1.2), making the eye prone to infection, stromal ulceration, perforation, scarring and significant vision loss [9]. This side effect is troublesome in the treatment of corneal NV for patients who present with corneal wounds or injuries [4]. Some studies suggest that shorter treatment durations and lower dosage may improve the safety profile of bevacizumab, however further randomized, controlled clinical trials must be carried out to confirm this [4].

![Figure 1.2](Image source: Vaidyanathan et al., 2019 [9] with permission under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/)).

Ranibizumab has been derived from its parent antibody bevacizumab and is approximately one-third the size, therefore it may have better corneal penetration and diffusion. The binding epitope of ranibizumab was further refined resulting in a higher affinity antigen-binding fragment [10]. Additionally, it has been shown that topical ranibizumab effectively treats corneal NV with a significant decrease in vessel diameter and neovascular area, but no effect on the length of blood
vessels [11]. This suggests that ranibizumab mainly promotes the narrowing of corneal NV more than a reduction in their length [3]. Comparisons of the anti-angiogenic effect between ranibizumab and bevacizumab varies greatly in the literature. Some studies state no significant differences between the two agents, while other studies demonstrated fewer new vessels, shorter blood vessels, lower corneal haziness and less inflammation in patients treated with bevacizumab [3]. However, it was found that topical ranibizumab was superior to bevacizumab in terms of its onset of action and degree of efficacy [12].

Afibercept has also shown favorable results in treating corneal NV in mouse models. Cursiefen et al. demonstrated that area of vascularization decreased from 49% in control mice to 2.3% in mice treated with afibercept in a suture-induced corneal NV model [13]. Similarly, Oliveira et al. presented that the area of corneal NV was 1.05 mm² in untreated controls at day 4 and was significantly reduced to 0.11 mm² in mice treated with afibercept at day 4 in a bFGF-induced corneal NV model [14]. Additionally, some evidence suggests that afibercept may increase corneal graft survival more than bevacizumab and ranibizumab in mice [4]. In a recent in vitro study, Kang et al. compared the cytotoxic effects of bevacizumab, ranibizumab and afibercept on corneal epithelial cells and found that ranibizumab and afibercept cause less damage to the corneal epithelium in cases of patients with pre-existing epithelial defects [15]. Since afibercept binds all isoforms of VEGF-A and VEGF-B with greater affinity as well as PDGF and PGF, it was demonstrated that it has a longer duration of effect in the eye between 10 and 12 weeks after injection, thus it may prove to be more effective than bevacizumab and ranibizumab for the treatment of corneal NV [16].
1.1.5 Wound healing process of the corneal epithelium

The corneal epithelial wound healing process is highly regulated and very complex, and it involves many growth factors, matrix and degradation proteins, and receptors. The epithelial basement membrane lies between the basal epithelial cells and the stroma [9]. The periphery of the cornea, known as the limbus, contains epithelial stem cells which proliferate into the basal epithelium and basal cells [9]. Epidermal growth factor (EGF) assists the proliferation and migration process by promoting synthesis of nucleic acids in epithelial cells and stromal fibroblasts, and also stimulating the production of extracellular matrix proteins and fibronectin by epithelial cells [9]. Upon injury to the epithelium, inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), are released and keratocytes respond to IL-1 and produce growth factors, such as hepatocyte growth factor (HGF) and keratocyte growth factor (KGF), which influence the migration and proliferation of epithelial cells [9]. Insulin-like growth factor (IGF) and transforming growth factor beta (TGF-β) control differentiation and growth of stromal keratocytes and epithelial cells, and PDGFs control migration and proliferation of keratocytes, and thymosin-β4 promotes re-epithelialization and epithelial migration [9]. After successful re-epithelialization, the stroma can adhere to the regenerated epithelial layer via hemidesmosomes anchoring to fibrils [9]. If the epithelium fails to attach to the stroma, persistent, non-healing CEDs develop.

1.1.6 Cost-effectiveness of bevacizumab

Despite evidence that ranibizumab and aflibercept may be more effective for the treatment of corneal NV with fewer side effects, bevacizumab remains the most widely used and researched anti-VEGF agent due to its cost-effectiveness. For the treatment of diabetic macular edema (DME),
bevacizumab costs $60 per dose, while ranibizumab costs $1,170 per dose and aflibercept costs $1,850 per dose [17]. These agents may be given 9 to 11 times in the first year of treatment and on average, 17 times over 5 years, therefore total costs can be substantial [17]. Over the course of a year, total mean costs per patient for bevacizumab, ranibizumab and aflibercept are $4,100, $18,600 and $26,100, respectively [17]. Hence, ranibizumab and aflibercept are not cost-effective in comparison to bevacizumab for the treatment of corneal NV. These concerns pose a challenge for clinicians, patients and policymakers when safety and efficacy of drugs are at odds with their cost-effectiveness.

**1.2 Significance**

The third most common cause of blindness worldwide is corneal disease and corneal NV is present in almost every case [18]. Approximately 4.14% of patients who require eye care, equal to around 1.4 million people per year, present with corneal NV and the major causes are infectious diseases, extended wear of contact lenses and vascular response to corneal transplantation [18]. Infectious keratitis, which can be either bacterial, viral, fungal or parasitic, is highly prevalent worldwide with 6 million people having chlamydial infections and 270,000 people having onchocerciasis infections, and herpetic keratitis affecting 500,000 cases in the USA [19]. Furthermore, in a study that evaluated human corneal buttons excised during keratoplasty, it was determined that 19.9% displayed angiogenesis [20]. Additionally, 1.3% of 9 million contact lens wearers, particularly users of soft hydrogel lenses, presented with new corneal vessels [21]. The widespread presence of these corneal pathologies paints a picture of the prevalence of corneal NV that exists worldwide and anti-angiogenic therapy, especially with the most cost-effective agent bevacizumab (Avastin®), allows for the successful treatment of this condition. This study will
provide safety data on the concentrations of bevacizumab used clinically. Specifically, this study will provide more data on the toxicity of bevacizumab injected intravitreally on retinal pigment epithelial cells, and investigate the safety of bevacizumab on corneal epithelial and endothelial cells *in vitro*, as well as examine clinical data for toxicity of intravitreal bevacizumab on the cornea of patients treated with this drug.

1.3 Research Objectives and Hypotheses

The main objective of this thesis is to investigate the effects of bevacizumab on the corneal epithelium and endothelium, focusing on two aims:

- **Aim 1:** Examine the cytotoxic effects of bevacizumab on the viability and metabolism of human corneal epithelial cells and endothelial cells.
  - **Hypothesis 1:** Bevacizumab significantly reduces the viability and metabolism of human corneal epithelial cells and endothelial cells at concentration of 5.00 mg/ml, the dose used for the topical treatment of corneal neovascularization.

- **Aim 2:** Evaluate the adverse effects of intravitreal bevacizumab on the cornea in patients and report on the risk factors involved in developing corneal epithelial defects.
  - **Hypothesis 2:** Repeated intravitreal injections of bevacizumab may result in delayed or non-healing corneal epithelial defects in a subset of patients.
2.1 Introduction

The biocompatibility of intravitreal bevacizumab has been established over the past two decades for the safe and effective treatment of retinal neovascular diseases. The topical application of bevacizumab, however, for the treatment of corneal NV raises concerns due to its potential corneal cytotoxicity. It is speculated that topical bevacizumab possibly interferes with the adhesion between the corneal epithelium and the basement membrane, resulting in delayed wound healing and stromal thinning, which can in turn lead to corneal epithelial defects [3]. Also, previous reports show conflicting evidence of the concentration of bevacizumab that is toxic to human corneal cells, ranging from 1.0 mg/ml up to 5.0 mg/ml when applied topically to the cornea [15,22,23].

In this in vitro, experimental study, we assess the cytotoxic effects of bevacizumab (Avastin®) on the viability and metabolism of two corneal cell lines: human corneal epithelial cells (HCEpCs) and human corneal endothelial cells (HCEnCs) across clinically relevant concentrations (0.313-5.00 mg/ml) after a 24-hr. treatment period [15,22,23]. Given that published cytotoxicity of bevacizumab on human retinal pigment epithelial cells (ARPE-19) range from 2.50-5.00 mg/ml [24,25], we also studied ARPE-19 for comparison and as a positive control.
2.2 Methods

2.2.1 Cell culture

Human Corneal Epithelial Cells (HCEpCs)

An immortalized human corneal epithelial cell line was purchased from ATCC (Manassas, USA) and cultured on T25 flasks (Corning) containing corneal epithelial cell basal medium (ATCC) supplemented with corneal epithelial cell growth kit (ATCC) consisting of 0.2% corneal epithelial growth factor, 5 µg/ml apo-transferrin, 1.0 µM epinephrine, 0.4% extract P, 100 ng/ml hydrocortisone hemisuccinate, 6 mM L-glutamine and 5 µg/ml rh insulin. The cells were cultured in a 37°C, 5% CO₂ incubator, medium was changed every 2-3 days until 90% confluence, then were passaged using 0.25% trypsin-EDTA (Gibco). Enzyme activity was quenched by trypsin neutralizing solution (ATCC) containing 10% fetal bovine serum (FBS) in Dulbecco’s phosphate-buffered saline (DPBS). Cells at passage 4 were used for experiments.

Human Corneal Endothelial Cells (HCEnCs)

An immortalized human corneal endothelial cell line (B4G12) was purchased from DSMZ (Braunschweig, Germany) and cultured on T75 flasks (Corning) coated with 10 µg/ml laminin (Sigma) and 10 mg/ml chondroitin sulfate (Sigma). The medium consisted of Human Endothelial-SFM (Gibco) supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Gibco). The cells were cultured in a 37°C, 5% CO₂ incubator, medium was changed every 2-3 days until 90% confluence, then were passaged using 0.25% trypsin-EDTA (Gibco). Enzyme activity was quenched by protease inhibitor cocktail (Sigma) in 500-fold dilution with Hanks’ balanced salt solution (HBSS; Gibco). Cells at passage 7 were used for experiments.
Human Retinal Pigment Epithelial Cells (ARPE-19)

An immortalized human retinal pigment epithelial cell line was purchased from ATCC (Manassas, USA) and cultured on T75 flasks (Corning) containing Dulbecco’s Modified Eagle Medium and Ham’s F-12 1:1 mixture (DMEM/F12; HyClone) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were cultured in a 37°C, 5% CO₂ incubator, medium was changed every 2-3 days until 90% confluence, then were passaged using 0.25% trypsin-EDTA (Gibco). Enzyme activity was quenched by complete growth medium containing 10% FBS. Cells at passage 13 were used for experiments.

2.2.2 ApoTox-Glo Triplex Assay

The ApoTox-Glo Triplex Assay was purchased from Promega (Madison, USA) to determine HCEpC, HCEnC and ARPE-19 cell viability, cytotoxicity and apoptosis following manufacturer’s instructions. Two substrates GF-AFC and bis-AAF-R110 were added and produced fluorescent signals proportional to viability and cytotoxicity, respectively. Substrate GF-AFC is able to enter cells and is only cleaved by live-cell proteases and becomes inactive once cell membrane activity is lost. Conversely, substrate bis-AAF-R110 is not able to enter cells and is only cleaved by dead-cell proteases leaked from cells which lack membrane integrity. The cleaved substrates produce two products, AFC and R110, which have different excitation and emission spectra, allowing them to be measured simultaneously at two wavelength sets: 400nm_{Ex}/505nm_{Em} (viability) and 485nm_{Ex}/520nm_{Em} (cytotoxicity). Subsequently, Caspase-3/7 substrate was added and produced a luminescent signal proportional to the amount of caspase activity present. Substrate Caspase-3/7 results in cell lysis, followed by caspase cleavage of the substrate by apoptotic cells, generating a “glow-type” luminescent signal produced by luciferase. Fluorescence and
luminescence were measured with Synergy H1 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA).

2.2.3 Mitochondrial ToxGlo Assay

The Mitochondrial ToxGlo Assay was purchased from Promega (Madison, USA) to determine HCEpC, HCEnC and ARPE-19 membrane integrity and adenosine triphosphate (ATP) levels following manufacturer’s instructions. Cells were treated in serum-free RPMI 1640 medium supplemented with 2.0 g/L sodium bicarbonate and 1.8 g/L galactose, as opposed to glucose which would serve as a non-mitochondrial ATP source, in order to be more responsive to mitochondrial insults and primarily rely on mitochondrial oxidative phosphorylation to produce ATP. First, cell membrane integrity was assessed by measuring a distinct protease activity associated with necrosis using a fluorogenic peptide substrate bis-AAF-R110 to detect dead cell protease activity. A loss in membrane integrity is associated with an increase in cytotoxicity. Next, ATP Detection Reagent was added, causing cell lysis and producing a luminescent signal proportional to the amount of ATP present. Fluorescence and luminescence were measured with Synergy H1 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA).

2.2.4 Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Results were analyzed by one-way ANOVA with Dunnett’s post hoc test for multiple comparisons to the untreated control using GraphPad Prism Version 8 (GraphPad Software Inc., San Diego, USA). All data are presented as percentage of the control with the control normalized to 100%. P < 0.05 was considered statistically significant.
2.3 Results

2.3.1 Effect of bevacizumab on cell viability, cytotoxicity and apoptosis

The ApoTox-Glo Triplex Assay was used to assess cell viability, cytotoxicity and apoptosis of the three cell types in response to clinically relevant concentrations of bevacizumab, ranging from 0.313 mg/ml to 5.00 mg/ml, after a 24-hr. treatment period. Cell viability of HCEpCs was unaffected up to concentration of 2.50 mg/ml, however a significant decrease \((p=0.0015)\) was observed at concentration of 5.00 mg/ml (Fig. 2.1A). A significant increase \((p=0.0032)\) in cytotoxicity was also seen at concentration of 5.00 mg/ml (Fig. 2.1B). Apoptosis, detected by caspase-3/7 activity, remained unchanged across all concentrations (Fig. 2.1C).

![Figure 2.1](image)

**Figure 2.1** Effect of bevacizumab (Avastin®) on HCEpCs viability (A), cytotoxicity (B) and apoptosis (C) detected by ApoTox-Glo Triplex Assay following a 24-hr. treatment period. Data presented as mean ± SEM \((n=8)\) with significant differences between the treated group and untreated control designated as **\(P<0.01\)**.

Similarly, cell viability of HCEnCs was unaffected up to concentration of 2.50 mg/ml, however a significant decrease \((p=0.0034)\) was observed at concentration of 5.00 mg/ml (Fig. 2.2A). A significant increase \((p=0.0035)\) in cytotoxicity was also seen at concentration of 5.00 mg/ml (Fig. 2.2B). Apoptotic activity remained unchanged across all concentrations (Fig. 2.2C).
Figure 2.2 Effect of bevacizumab (Avastin®) on HCEncs viability (A), cytotoxicity (B) and apoptosis (C) detected by ApoTox-Glo Triplex Assay following a 24-hr. treatment period. Data presented as mean ± SEM (n = 8) with significant differences between the treated group and untreated control designated as **P<0.01.

Lastly, cell viability of ARPE-19 was unaffected up to concentration of 2.50 mg/ml, however a significant decrease (p<0.0001) was observed at concentration of 5.00 mg/ml (Fig. 2.3A). A significant increase (p=0.0108) in cytotoxicity was also seen at concentration of 5.00 mg/ml (Fig. 2.3B). Apoptotic activity remained unchanged across all concentrations (Fig. 2.3C).

Figure 2.3 Effect of bevacizumab (Avastin®) on ARPE-19 viability (A), cytotoxicity (B) and apoptosis (C) detected by ApoTox-Glo Triplex Assay following a 24-hr. treatment period. Data presented as mean ± SEM (n = 8) with significant differences between the treated group and untreated control designated as *P<0.05 and ****P<0.0001.
2.3.2 Effect of bevacizumab on cell membrane integrity and ATP levels

The Mitochondrial ToxGlo Assay was used to evaluate cell membrane integrity and ATP levels of the three cell types in response to clinically relevant concentrations of bevacizumab, ranging from 0.313 mg/ml to 5.00 mg/ml, after a 24-hr. treatment period. Membrane integrity of HCEpCs diminished as seen with a significant increase (p=0.0032) in cytotoxicity at concentration of 5.00 mg/ml (Fig. 2.4). No significant changes in ATP levels were observed with increasing concentrations of bevacizumab up to 5.00 mg/ml (Fig. 2.4). These results suggest that bevacizumab does not induce mitochondrial toxicity in HCEpCs at 5.00 mg/ml as cellular ATP production is not impaired. Therefore, HCEpCs are able to maintain normal metabolism when exposed to bevacizumab up to concentration of 5.00 mg/ml for 24 hours.

![Figure 2.4](image)

**Figure 2.4** Effect of bevacizumab (Avastin®) on HCEpCs cytotoxicity (solid line) and ATP levels (dashed line) detected by Mitochondrial ToxGlo Assay following a 24-hr. treatment period. Data presented as mean ± SEM (n = 8) with significant differences between the treated group and untreated control designated as **P<0.01.**
Similarly, membrane integrity of HCEnCs diminished as seen with a significant increase (p=0.0035) in cytotoxicity at concentration of 5.00 mg/ml (Fig. 2.5). ATP levels, however, were not affected by increasing concentrations of bevacizumab up to 5.00 mg/ml (Fig. 2.5). These results suggest that bevacizumab does not induce mitochondrial toxicity in HCEnCs at 5.00 mg/ml as cellular ATP production is not impaired. Therefore, HCEnCs are able to maintain normal metabolism when exposed to bevacizumab up to concentration of 5.00 mg/ml for 24 hours.

![Figure 2.5](image-url) Effect of bevacizumab (Avastin®) on HCEnCs cytotoxicity (solid line) and ATP levels (dashed line) detected by Mitochondrial ToxGlo Assay following a 24-hr. treatment period. Data presented as mean ± SEM (n = 8) with significant differences between the treated group and untreated control designated as **P<0.01.

Lastly, membrane integrity of ARPE-19 diminished as seen with a significant increase (p=0.0108) in cytotoxicity at concentration of 5.00 mg/ml (Fig. 2.6). ATP levels remain unchanged with increasing concentrations of bevacizumab up to 5.00 mg/ml (Fig. 2.6). These results suggest that bevacizumab does not induce mitochondrial toxicity in ARPE-19 at 5.00 mg/ml as cellular ATP production is not impaired. Therefore, ARPE-19 are able to maintain normal metabolism when exposed to bevacizumab up to concentration of 5.00 mg/ml for 24 hours.


Figure 2.6 Effect of bevacizumab (Avastin®) on ARPE-19 cytotoxicity (solid line) and ATP levels (dashed line) detected by Mitochondrial ToxGlo Assay following a 24-hr. treatment period. Data presented as mean ± SEM (n = 8) with significant differences between the treated group and untreated control designated as *P<0.05.

2.4 Discussion

In this study, we evaluated the cytotoxic effects of bevacizumab on human corneal epithelial cells, endothelial cells, and retinal pigment epithelial cells across various concentrations for a 24-hr. treatment period. For all the experiments, we took the highest concentration of bevacizumab (5.00 mg/ml) which is typically used for the treatment of corneal NV, down to the lowest concentration (0.313 mg/ml) which is injected intravitreally (50 µl of 25 mg/ml stock; diluted in 4 ml of vitreous). Across all three cell types, we observed similar results of a decrease in cell viability at 5.00 mg/ml with a concomitant increase in cytotoxicity at 5.00 mg/ml, while apoptotic activity remained unchanged. After a 24-hr. treatment period, caspase-3/7 activity is similar to the untreated control, a profile consistent with cells undergoing primary necrosis caused by the toxicity of bevacizumab at concentration of 5.00 mg/ml, leading to non-apoptotic,
accidental, unprogrammed cell death by a foreign toxin, thus resulting in no increase in caspase-3/7 activity, which is a hallmark of apoptosis. Additionally, cell membrane integrity was compromised at 5.00 mg/ml, while no changes in ATP levels were observed. It is possible that we may be seeing a compensatory effect regarding similar levels of ATP production to the untreated control as the cells could be producing more ATP to account for the increased toxicity caused by high concentrations of bevacizumab. As the final step of the assay, the reagent lyses all the cells and determines the total amount of ATP available both intracellularly and extracellularly, therefore it is possible that ATP levels have not diminished, yet the cells have lost their membrane integrity. Nonetheless, we observe no interference with mitochondrial oxidative phosphorylation in ATP production and the cells appear to be capable of maintaining normal metabolic levels and mitochondrial function across the concentrations tested.

It is also possible that cell death may be occurring through pyroptosis or necroptosis. Pyroptosis is a form of regulated, programmed cell death instigated by intracellular or extracellular perturbations of homeostasis related to innate immunity [26]. When cells die, they release molecules, known as damage-associated molecular patterns (DAMPs), that alert the entire colony of a potential threat, resulting in elimination of dangerous cells [26]. It involves cellular swelling leading up to plasma membrane permeabilization, however, unlike apoptosis, it is dependent on inflammatory caspase-1 activation, followed by IL-1β and IL-18 secretion and subsequent proteolytic cleavage of gasdermin D (GSDMD) [26]. Active caspase-1 can also cleave GSDMD, indicating that DAMPs stimulating canonical inflammasome signaling can initiate pyroptosis [26]. Necroptosis is also a form of regulated, programmed cell death triggered by perturbations of the intracellular or extracellular microenvironment detected by specific death receptors including,
not limited to, FAS and TNFR1 [26]. It is crucially dependent on sequential activation of RIPK3 and mixed lineage kinase domain-like pseudokinase (MLKL) [26]. Once necroptosis is initiated by TNFR1, RIPK3 is activated by RIPK1 through an interaction between their respective RIP homotypic interaction motif domains and RIPK1 catalytic activity [26]. Conversely, RIPK3 can be activated by various DAMPs at the plasma membrane, leading to phosphorylation of MLKL and formation of MLKL oligomers that translocate to the plasma membrane and trigger plasma membrane permeabilization [26]. Nevertheless, in order to ascertain whether the cells are potentially dying by pyroptosis or necroptosis, we would have to look at markers specific to each cell death pathway.

Other studies have reported the cytotoxic effects of bevacizumab on human corneal epithelial cells and endothelial cells, although with varying results. Yoeruek et al. demonstrated that bevacizumab did not cause a significant decrease in cell viability or any morphological changes in HCEnCs up to a concentration of 5.0 mg/ml after 24-hr exposure [22]. Chalam et al. showed that bevacizumab was non-toxic to HCEpCs up to a concentration of 2.0 mg/ml after 24-hr exposure [23]. Conversely, Kang et al. determined that bevacizumab significantly decreased the cell viability of HCEpCs at concentrations of 1.0 mg/ml and 2.0 mg/ml after 24-hr exposure, as well as a significant reduction in HCEpC wound healing due to decreased expression levels of phosphorylated p38 mitogen-activated protein kinase (MAPK), a marker for cell migration [15]. In a clinical setting, reports of delayed corneal epithelial healing due to intravitreal bevacizumab has been published as well. Colombres et al. performed retrospective chart review on 850 eyes of 850 patients receiving intravitreal bevacizumab for various retinal neovascular diseases, and observed that 7 patients who had pre-existing corneal edema developed corneal epithelial defects
Importantly, none of the patients without corneal edema developed CEDs [27]. Thus, bevacizumab may not be ideal for treating patients with a pre-existing ocular surface disease.

One method that has been proposed for the treatment of corneal NV for patients who present with ocular surface disease (OSD) is the use of Prosthetic Replacement of the Ocular Surface Ecosystem (PROSE) device as a drug delivery system for bevacizumab. In a recent in vivo study, Yin and Jacobs evaluated the long-term outcome of using PROSE device in 13 patients with significant pre-existing OSD and reported that 12 patients had significant corneal NV regression and 10 patients had significant improvement in visual acuity over the 5-year average follow-up period with no ophthalmic or systemic complications [28]. They speculate that the PROSE device prevented any ophthalmic adverse events because (1) the ocular surface remains nourished and protected underneath the device, (2) a preservative-free, pH-balanced microenvironment is provided by the device to deliver the bevacizumab, (3) bevacizumab, a large molecule, is retained in the reservoir of the device for a longer duration possibly increasing its bioavailability to the cornea, and (4) the device lowers the cornea’s susceptibility to the many factors that may cause corneal NV, thus ending the vicious cycle between corneal NV and heightened inflammation [28]. Therefore, the PROSE device may prove to be an effective drug delivery system for the treatment of corneal NV for patients who present with severe OSD.

Despite evidence that high concentrations of bevacizumab (~5.00 mg/ml) may be toxic to the corneal epithelium, very low concentrations of topical bevacizumab are being explored as an effective modality against OSD. In a recent randomized, controlled clinical trial, Kasetsuwan et al. assessed the safety and efficacy of bevacizumab 0.05% (0.5 mg/ml) eye drops as a novel
treatment for Dry Eye Disease (DED) in 31 participants and concluded that all participants had significant improvements in tear break-up time (TBUT), an important diagnostic sign in DED, over the 12-week follow-up period with no adverse events [29]. Thus, this may be considered as an alternative treatment for DED to decrease inflammation as opposed to the use of steroids and cyclosporin-A which can have adverse events, such as elevated intraocular pressure, cataract, burning or stinging symptoms [30].

However, our study has several limitations. First, the responses of human corneal cells to bevacizumab in vivo may be different from cell lines tested in vitro, as in vivo conditions introduce other environmental factors and results may not be directly transferrable. Second, treatment period was limited to 24 hours as cells were treated in starving conditions to study molecular mechanisms and thus, longer incubation periods were not possible. Third, we were not able to investigate the effect of repeated topical applications of bevacizumab on corneal cells under in vitro conditions due to limitations of the assay. It is possible that there could be a depot effect or accumulative effect of the drug under in vivo conditions that could contribute to toxicity over time and repeated eye drops. Therefore, in vivo animal studies are necessary to assess how frequency and longevity of topical bevacizumab may affect the safety and efficacy of this treatment for corneal NV.

In conclusion, this in vitro study demonstrates that HCEpCs, HCEnCs and ARPE-19 experience a decrease in viability when exposed to bevacizumab at concentration of 5.00 mg/ml, however they are able to maintain normal metabolism and mitochondrial function at high concentrations typically used for the treatment of corneal neovascularization.
CHAPTER THREE: EFFECT OF INTRAVITREAL BEVACIZUMAB ON THE CORNEA IN PATIENTS

3.1 Introduction

Intravitreal bevacizumab (IVB) has safely and effectively been used for the treatment of various ocular conditions and its injection into the eye has been shown to reduce retinal neovascularization in clinical practice. Nevertheless, adverse drug events have been associated with IVB, including endophthalmitis, uveitis, retinal detachment, vitreous hemorrhage, among others [31]. Additionally, the repeated and long-term injections of bevacizumab can lead to increased risk of ocular and systemic complications.

The effect of IVB on the cornea has been minimally explored and it is speculated that anti-VEGF agents, such as bevacizumab, hinder the corneal epithelial wound healing process and lead to the development of corneal epithelial defects (CEDs) [27]. Many growth factors have been shown to be involved in the corneal epithelial healing process. Cytokines, such as tumour necrosis factor alpha (TNF-\(\alpha\)) and interleukin-1 (IL-1), are released as a result of injury to the corneal epithelium, initiating a cascade of events where dormant keratocytes become activated and produce hepatocyte growth factor (HGF) and keratocyte growth factor (KGF), which stimulate the migration and proliferation of epithelial cells [32]. Furthermore, matrix metalloproteinases (MMPs) are regulated by growth factors and play a vital role in healing and tissue remodeling by modifying adhesion interactions and the extracellular matrix [32]. It has also been demonstrated that the corneal epithelium expresses VEGF and its receptors, VEGFR1 and VEGFR2 [33]. These findings indicate a role for VEGF not only with its involvement in corneal neovascularization, but also in the corneal epithelial healing process.
In this retrospective clinical study, we investigate the adverse effects of IVB on the cornea in patients and report on the risk factors involved in developing CEDs in a subset of patients seen by the Cornea Service (UBC Department of Ophthalmology and Visual Sciences) who were receiving IVB for a retinal neovascular disorder between 2012 and 2020.

### 3.2 Methods

A retrospective chart review was performed on 127 eyes of 127 patients who had received IVB from January 1, 2012 to January 1, 2020 for several retinal conditions, including age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), diabetic macular edema (DME), choroidal neovascular membranes (CNVM) and cystoid macular edema (CME). The patients had received 1.25 mg of IVB in either one eye or both eyes every 4-6 weeks until resolution or stability of disease.

### 3.3 Results

Nine patients (5 male/4 female) of 127 total patients at the Cornea Service of University of British Columbia with an age range of 69 to 98 who had received IVB monthly developed CEDs (7.1%). Of these, 3 patients had AMD, 3 had PDR, 2 had CNVM and 1 had CME (Table 3.1). Notably, all patients had a pre-existing corneal complication, including corneal ulcer, stromal edema, neurotrophic corneal decompensation, herpetic keratitis, corneal thinning, stromal swelling and limbal stem cell deficiency (Table 3.1). All 9 eyes developed CEDs after uneventful IVB injection despite following proper management steps. Number of monthly IVB injections before CED ranged from 5-11. Importantly, none of the patients without a corneal complication developed CEDs.
### Table 3.1 Clinical data of nine patients who developed CEDs after IVB

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age / Sex</th>
<th>Previous Diagnosis</th>
<th>Frequency of IVB</th>
<th>Corneal Complication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98/F</td>
<td>AMD</td>
<td>Monthly</td>
<td>Corneal ulcer</td>
</tr>
<tr>
<td>2</td>
<td>72/M</td>
<td>PDR</td>
<td>Monthly</td>
<td>Stromal edema</td>
</tr>
<tr>
<td>3</td>
<td>87/F</td>
<td>AMD</td>
<td>Monthly</td>
<td>Neurotrophic corneal decompensation</td>
</tr>
<tr>
<td>4</td>
<td>69/M</td>
<td>PDR (DME)</td>
<td>Monthly</td>
<td>Herpetic keratitis</td>
</tr>
<tr>
<td>5</td>
<td>85/F</td>
<td>CNVM</td>
<td>Monthly</td>
<td>Corneal thinning</td>
</tr>
<tr>
<td>6</td>
<td>88/M</td>
<td>CNVM</td>
<td>Monthly</td>
<td>Stromal swelling</td>
</tr>
<tr>
<td>7</td>
<td>80/M</td>
<td>CME</td>
<td>Monthly</td>
<td>Neurotrophic corneal decompensation</td>
</tr>
<tr>
<td>8</td>
<td>69/M</td>
<td>PDR</td>
<td>Monthly</td>
<td>Stromal edema</td>
</tr>
<tr>
<td>9</td>
<td>88/F</td>
<td>AMD</td>
<td>Monthly</td>
<td>Limbal stem cell deficiency</td>
</tr>
</tbody>
</table>

AMD, age-related macular degeneration; PDR, proliferative diabetic retinopathy; DME, diabetic macular edema; CNVM, choroidal neovascular membranes; CME, cystoid macular edema

After the development of CEDs, patients were initially treated with aggressive lubrication using non-preservative artificial tears or hydrogel, topical antibiotic coverage for infection prophylaxis, followed by bandage contact lenses to protect the corneal surface against any mechanical trauma, as well as offer high oxygen permeability to assist with the healing process. Additionally, some cases were treated with topical steroids if there was an inflammatory component noted to the ocular surface or anterior chamber.
3.4 Discussion

In this study, we assessed the effect of intravitreal bevacizumab on the cornea in patients and the risk factors involved in developing corneal epithelial defects in a subset of patients who were receiving IVB between 2012 and 2020. Out of 127 patients, we found that 9 patients (7.1%) developed CEDs after uneventful IVB injections. All 9 eyes showed delayed epithelial healing despite following appropriate post-injection treatment plan in all cases. Common risk factors among all patients were pre-existing corneal complications, such as corneal ulcer, stromal edema, neurotrophic corneal decompensation, herpetic keratitis, corneal thinning, stromal swelling and limbal stem cell deficiency. It is of utmost importance to note that none of the eyes without a corneal complication developed CEDs. Therefore, bevacizumab may be involved in delaying the wound healing process of an injured or unstable corneal epithelium.

Few clinical studies have reported the development of CEDs due to IVB. In a retrospective study, Wong et al. analyzed medical records of 186 patients (203 eyes) who had received a total of 578 injections of 1.25 mg IVB, and reported the occurrence of corneal adverse events in three eyes (1.5%) following IVB injection [34]. In another study, Colombres et al. examined the charts of 850 eyes of 850 patients receiving IVB, and noticed 7 patients (0.8%) who had pre-existing corneal edema developed CEDs [27]. This possibly designates a role for bevacizumab in delaying the healing process of a compromised ocular surface. The prevalence of CEDs following IVB in our study is higher (7.1%) than these 2 studies, likely as our series was derived from the Cornea Service of a busy tertiary retinal center. Therefore, the Cornea Service would have received referrals from the region for any complications related to IVB.
One explanation for seeing this effect of IVB on the cornea is that although bevacizumab is injected into the posterior segment, there is reflux onto the ocular surface following injection and also migration of the drug intraocularly from the posterior segment to the anterior chamber. This explains the observed effect of IVB on delaying corneal epithelial healing and forming persistent CEDs. Patients who present with high intraocular pressure (IOP) may be at increased risk for reflux of drug post-injection as this has been designated as one of the several factors that encourages the reflux of IVB onto the ocular surface [35]. Furthermore, stromal and epithelial edema may cause a rupture in Bowman’s layer and the epithelial basement membrane, leading to poor adhesion of the epithelium and the formation of subepithelial bullae, which in turn may lead to persistent CEDs [36]. This vicious cycle between corneal epithelial breakdown and wound healing yields a state of persistent inflammation, and the inhibition of VEGF with bevacizumab possibly disturbs the corneal physiology and cultivates an inflammatory state that hinders wound healing.

The effect of bevacizumab on delayed corneal epithelial healing has been investigated in animal models. Kim et al. demonstrated that topical bevacizumab eye drops delayed corneal epithelial healing in rabbit eyes, and more importantly decreased the expression of integrins [37]. Integrins are adhesion molecules that play a crucial role in corneal wound healing by facilitating interactions between contiguous cells and the extracellular matrix (ECM) and thus, control cellular properties such as proliferation, migration, differentiation and apoptosis [38]. Alpha5beta1 integrin is the classic receptor for fibronectin and assists cellular attachment to the ECM [39]. VEGFR1, one of the receptors for VEGF, is present within the ECM and directly binds alpha5beta1 integrin to enhance migration [40]. Therefore, it is likely that inhibition of VEGF by
bevacizumab impedes the function of fibronectin and restricts the attachment of the corneal epithelium to Bowman’s layer, resulting in slow re-epithelialization of the cornea.

Another possibility for delay in corneal epithelial healing could be related to VEGF and its receptor, VEGFR2. They become active following epithelial injury and are highly expressed in proliferating corneal epithelium, keratocytes and corneal endothelium [41]. Gan et al. demonstrated that VEGF expression was inhibited in the rabbit cornea and completely diminished in the epithelium peripheral to the leading edge, while epithelial cells at the wound edge highly expressed both VEGF and VEGFR2 [41]. It is possible that the application of topical bevacizumab on the injured rabbit cornea inhibited the function of VEGF in proliferation of the corneal epithelium, leading to delayed healing.

Nonetheless, this study has limitations. First, the small sample size of 127 patients derived solely from the Cornea Service provides limited data on the true prevalence of corneal adverse events associated with IVB. To address this, review of all charts of the Retina Service for the same region would be able to more accurately demonstrate how common this serious complication is. However, in this context, it is possible that any corneal complication would not have presented back to the Retina Clinic, but rather would have been seen in an Emergency Department, Cornea Clinic, or General Ophthalmologist’s office. Another limitation is that the Retina Service may have treated minor corneal complications themselves without involving the Cornea Service. This would have been captured if all charts of the Retina Service for the same region were reviewed. Finally, given that many patients treated at our tertiary center travel from other regions for care, it is possible that corneal complications could have arisen in their home regions and therefore treated
by their own ophthalmologists closer to home. This data would not have been captured in the charts of the Retina Service.

In conclusion, this clinical study demonstrates that the development of CEDs or corneal adverse events are more likely with IVB injections for patients who present with pre-existing corneal complications. Physicians administering IVB should be aware of this possible effect and instruct patients to seek urgent care should they experience any redness, pain, or blurring of vision.
CHAPTER FOUR: CONCLUSIONS

4.1 Thesis Summary

4.1.1 Chapter Two

The topical application of bevacizumab, an anti-VEGF agent, is one of the more effective treatment options for corneal neovascularization today. Nevertheless, the repeated and long-term use of these topical eye drops has been associated with potential corneal cytotoxicity. In Chapter Two, we assessed the effect of bevacizumab on the viability and metabolism of human corneal epithelial cells (HCEpCs), human corneal endothelial cells (HCEnCs), and human retinal pigment epithelial cells (ARPE-19) across clinically relevant concentrations (0.313-5.00 mg/ml) after a 24-hr. treatment period. We found that across all three cell types, there was a decrease in viability at concentration of 5.00 mg/ml and an increase in cytotoxicity at concentration of 5.00 mg/ml, while no changes in apoptotic activity were observed. This suggests that the cells undergo primary necrosis, non-apoptotic, unprogrammed cell death caused by a foreign toxin, when exposed to bevacizumab at high concentrations. Additionally, cell membrane integrity was lost at concentration of 5.00 mg/ml, while no changes in ATP levels were observed. Therefore, no interference with mitochondrial oxidative phosphorylation in production of ATP was seen and the cells appear to maintain normal metabolism and mitochondrial function at high concentration of bevacizumab used for the treatment of corneal neovascularization.

4.1.2 Chapter Three

Intravitreal bevacizumab (IVB) has effectively been used for the treatment of various retinal neovascular diseases. However, repeated and long-term use have led to increased risk of
ocular and systemic complications. In animal studies, there have been reports of delayed corneal epithelial healing due to IVB. In Chapter Three, we examined the adverse effects of IVB on the cornea in patients, and reported the development of non-healing corneal epithelial defects (CEDs). We found that 9 eyes out of 127 eyes developed CEDs after uneventful IVB injections and all 9 eyes showed delayed epithelial healing despite following appropriate post-injection treatment plan in all cases. All 9 patients had presented with pre-existing corneal complications, such as corneal ulcer, stromal edema, neurotrophic corneal decompensation, herpetic keratitis, corneal thinning, stromal swelling and limbal stem cell deficiency, which led to the development of non-healing CEDs upon receipt of IVB. Importantly, none of the patients without corneal complications developed CEDs. It is possible that IVB had an effect on the ocular surface due to reflux post-injection, thus causing an effect on the corneal epithelium and resulting in delayed corneal epithelial healing and the formation of persistent CEDs. Therefore, if a patient presents with an unstable or injured ocular surface or a history of corneal disease, physicians should be aware of this possible effect of IVB on the cornea and instruct patients to seek urgent care should they experience any redness, pain or blurry vision.

4.2 Future Directions

4.2.1 Chapter Two

The experimental work in this chapter can be supplemented with exploring the cytotoxicity of other anti-VEGF agents, such as ranibizumab, aflibercept and pegaptanib, on human corneal cells. More recent anti-VEGF agents may offer improved safety profiles on corneal epithelial and endothelial cells, and lessen the toxicity that has been associated with bevacizumab. Furthermore, the constant development and testing of new anti-VEGF agents with increased efficacy and greater
affinity for all VEGF isoforms would be beneficial to ensure fewer side effects and adverse drug events in the treatment of corneal neovascularization. Currently, research is focused on the inhibition of other steps of the VEGF signaling pathway, such as targeting the downstream tyrosine kinase pathway initiated by VEGF receptors, VEGFR1 and VEGFR2. However, they are mainly being developed for the treatment of retinal neovascular diseases and are aiming for enhanced retinal penetration, which may also prove to be effective for corneal neovascularization. If these new drugs exhibit better efficacy for corneal neovascularization, it could obviate the need for corneal transplantation in cases where vision loss had occurred due to corneal scarring.

Additionally, blocking other growth factors of the angiogenic pathway could prove to be more advantageous than only VEGF inhibition. Sunitinib, an anti-VEGF/anti-PDGF agent, was approved in 2006 for the treatment of renal cell carcinoma and gastrointestinal stromal tumor [42]. It would be noteworthy to investigate whether this drug can effectively inhibit corneal NV in animal studies by using a suture-induced, bFGF-pellet or chemical burn model, and measuring outcomes such as neovascular area, vessel caliber, mean diameter of corneal neovessels and invasion area [43-45].

Furthermore, it would be critical to examine the effect of these anti-angiogenic agents on protein levels that are highly involved in cell migration, proliferation and attachment of the corneal epithelium. P38 MAPK plays a vital role in cell migration and thus, studying the effect of these drugs on its activation through Western blot analysis would provide valuable information about the pathways disturbed [46]. Also, extracellular signal-regulated protein kinase (ERK) is heavily involved in cell proliferation and tracking its phosphorylation by Western blot analysis could
possibly allow for better understanding of how these agents disrupt the healing process of the corneal epithelium [47]. The role of alpha5beta1 integrin should be further explored regarding its involvement in the attachment of the corneal epithelium to the basement membrane, which would further clarify the role of fibronectin and the ECM in facilitating epithelial wound healing [40].

Lastly, use of primary cells and animal models of corneal neovascularization as aforementioned would be the next step to further investigate the toxicity in vivo of repeated topical bevacizumab applications on the health of the cornea, as this would most closely replicate the clinical scenario. These animal models would also be instructive in determining the most effective concentration of anti-VEGF agent necessary to effectively reduce or eliminate corneal neovascularization. As well, the other modalities used clinically for the treatment of corneal neovascularization could be directly compared, such as topical steroid drops and laser treatments to the vessels themselves.

4.2.2 Chapter Three

The clinical study in this chapter would benefit from exploring the risk of intravitreal injections of other anti-VEGF agents, such as ranibizumab and aflibercept, on developing CEDs. It is currently unknown whether a small molecule, such as ranibizumab, has similar toxic effects on the cornea at a cellular level. Similarly, aflibercept which inhibits not only VEGFR1 and VEGFR2, but also PDGF and PGF, has not yet been studied for its adverse effects on the cornea and thus the formation of non-healing CEDs in patients receiving this drug is not known. A large database study would determine the incidence of developing CEDs in relation to the usage of different anti-VEGF agents. As adverse events are reportable to the Food and Drug Administration
(FDA), a comparative evaluation of corneal adverse events through the reporting website may further allow us to determine more accurate risk of corneal complications with the use of intravitreal anti-VEGF agents.

It would also be favorable to examine whether a threshold number of injections beyond which corneal complications are more likely, or whether certain pre-existing corneal conditions place patients at an even higher risk of corneal adverse events post-injection of anti-VEGF agents. This could be examined through a large scale regional or province-wide review of all patients receiving anti-VEGF agents with subgroup analysis for risk stratification.
REFERENCES


