Effect of Diabetes Mellitus on Ocular Surface Tight Junctions and Glycocalyx

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Effect of Diabetes Mellitus on Ocular Surface Tight Junctions and Glycocalyx

A Thesis by

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Irvine, CA
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Submitted in partial fulfillment of the requirements for the degree of
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Effect of Diabetes Mellitus on Ocular Surface Tight Junctions and Glycocalyx

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ABSTRACT

Effect of Diabetes Mellitus on Ocular Surface Tight Junctions and Glycocalyx

by Saleh Mohammed G Alfuraih

Diabetes mellitus is the disease of the century that affects many body organs. In the eye, diabetes mellitus causes retinopathy, cataract, glaucoma, papillopathy, and ocular surface disease. Ocular surface abnormalities in patients of diabetes mellitus include impairment of corneal epithelial barrier function, conjunctival defects, increased incidence of corneal and conjunctival infections and higher prevalence of dry eye disease. Impairment of tight junctions and glycocalyx, which are critical for ocular surface barrier function, may underlie these diabetes-associated ocular surface defects. Therefore, the present study was designed to investigate the effect of high glucose exposure and type I diabetes mellitus on the conjunctival tight junction proteins, tear secretion and corneal glycocalyx.

Cultured human conjunctival epithelial cells were exposed to high glucose (15 mM and 30 mM) concentrations for 24 and 72 hours. Trans-epithelial electrical resistance and scratch assay were performed to quantify barrier functions and cell migration. Gene and protein expression of tight junction proteins: claudin-1, claudin-2, claudin-3, ZO-1, ZO-2, ZO-3, and occludin was quantified using real time PCR and western blotting. Type I diabetes was induced in mice by streptozotocin injection. Phenol red thread test, fluorescein staining and wheat-germ agglutinin corneal staining and confocal microscopy of whole mount corneas were performed to quantify tears, keratopathy and corneal glycocalyx area.
Our data demonstrates that high glucose causes a significant decrease in trans-epithelial electrical resistance of cultured human conjunctival epithelial cells. However, high glucose did not modulate the cellular migration or protein expression of claudin-1, ZO-1,-2,-3 or occludin. Interestingly, an increase in gene expression of all the tight junction proteins was observed at 72-hour exposure with 15 mM glucose. This effect on gene expression is likely due to the cellular osmotic stress caused by glucose since mannitol also caused the similar increase after 72 hours exposure. Type I diabetes caused a significant decreased in the tear film volume which was accompanied by corneal keratopathy and a decrease in the area of corneal glycocalyx. In summary, our data demonstrates that high glucose impairs the conjunctival epithelial cell barrier functions, but the alterations in cellular migration and tight junction proteins are not the likely cause of conjunctival epithelial barrier dysfunction. Type I diabetes mellitus causes tear film abnormalities and corneal keratopathy which is accompanied by a concurrent decrease in corneal glycocalyx.
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CHAPTER I

INTRODUCTION
Diabetes mellitus is an endocrine disease characterized by persistently high levels of blood glucose that can cause many serious health issues [Brownlee, 2001; Forbes and Cooper, 2013; Sayin et al., 2015]. The incidence of diabetes mellitus is alarmingly rising all over the world and it will be of epidemic proportions in the near future [Whiting et al., 2011]. Approximately 240 million people worldwide are affected by diabetic mellitus, and this number is going to increase nearly to 370 million by 2030 [IDF Diabetes Atlas, 2019]. The countries experiencing the highest incidence of diabetes are China, India and United States. In the United States, more than 30 million people are suffering from diabetes mellitus and surprisingly a large percentage of population is prediabetic [American Diabetes Association statistics, 2019].

Diabetes mellitus causes significant morbidity especially due to microvascular complications resulting in neuropathy, nephropathy and retinopathy [Brownlee, 2001; Forbes and Cooper, 2013]. Besides retinopathy, diabetes mellitus also can cause many other ocular complications such as ocular surface diseases [Yoon et al., 2004], glaucoma [Zhao and Chen., 2017], cataract [Pollreisz and Schmidt-Erfurth, 2010] and diabetic papillopathy [Regillo et al., 1995; Bayraktar et al., 2002]. Incidence of corneal keratopathy and persistent corneal epithelial defects is disproportionally high in patients of diabetes mellitus [Ljubimov, 2017]. Abnormalities in epithelial cell density or alterations in the corneal epithelial maturation have also been detected in the cornea of diabetes mellitus patients [Ljubimov, 2017; Barsegian et al., 2018]. Clinical studies using anterior fluororometry-assisted tracking of corneal fluorescein penetration have consistently demonstrated an impairment of corneal epithelial barrier function in diabetic patients [Gekka et al., 2004]. These studies have also shown that the frequency and severity of
corneal epithelium barrier impairment correlated with the serum levels of HbA1c [Gekka et al., 2004]. In addition to these corneal barrier function abnormalities, patients of diabetes mellitus have been reported to suffer from conjunctival defects as well. Abnormalities in conjunctival capillaries, including dilatation, increased tortuosity and leakage, have been reported in patients suffering from diabetes mellitus [Cheung et al., 2001; Owen et al., 2008]. Patients with diabetes mellitus suffer from significantly higher incidence of corneal and conjunctival infections [Ljubimov, 2017]. The corneal and the conjunctival epithelial cells express high level of tight junction proteins that serve as a strong barrier against any microbial invasion [Ban et al., 2003; Yoshida et al., 2009]. There are more than 40 protein in tight junctions which help to regulates the movement of macromolecules, ions, and water [Yoshida et al., 2009]. These proteins also play an important role in maintaining the cell polarity by regulating the lipids distributions within the membrane [Campbell et al., 2017]. Diabetes mellitus has been shown to alter the expression of tight junctions in many tissues include retina, cardiac, neuronal, renal cells [Barber et al., 2000; Dias and Griffiths, 2014; Mongelli-Sabino et al., 2017].

Glycocalyx is a layer that covers the plasma membrane of many cells. It is made up of glycoproteins, proteoglycans and glycosaminoglycans. It functions as a barrier between the cells and their surroundings [Egberts et al., 1984; Devuyst et al., 2014; Alphonsus and Rodseth, 2014]. The ocular surface glycocalyx is made up of a network of corneal epithelial membrane bound glycosylated mucins MUC1, MUC4, MUC16 and galactin-3 [Mantelli et al., 2013]. This glycocalyx serves as a protective barrier in addition to the tight junction proteins expressed by ocular surface epithelial cells [Mantelli et al., 2013]. The glycocalyx also serves an important function of retaining water and keeping
the ocular surface moist [Prydal et al., 1992; Davidson and Kuonen, 2004]. Our lab and other research groups have demonstrated that systemic diseases can cause an alteration in corneal glycocalyx [Uchino, 2018; Shamloo et al., 2019]. Our lab has also demonstrated that exposure of cultured corneal and conjunctival epithelial cells to high glucose causes alterations in the gene expression of MUC1, MUC 4 and MUC16 [Unpublished data].

Dry eye syndrome or keratoconjunctivitis sicca is an ocular surface disease that can cause feeling of dryness, redness and foreign body sensation in the eye. If left untreated it can cause damage to the cornea and conjunctiva resulting in significant visual morbidity. The incidence of dry eye is significantly higher in diabetic patients [Yoon et al., 2004; Sayin et al., 2015; Misra et al., 2016]. Tear film is made up of muco-aqueous layer covered by a lipid layer. Tear film is an important part of the eye and plays a role to protect the ocular surface from infections, mechanical damage and keeps it hydrated and lubricated. Studies have shown that diabetic patients have decreased tear production, an abnormal ocular surface sensitivity and higher incidence of dry eye [Rosenberg et al., 2000; Yoon et al., 2004; Alves et al., 2008; Misra et al., 2016]. Moreover, increased osmolarity of tears and inflammation of ocular surface has also been reported in patients suffering from diabetes mellitus [Fuerst et al., 2014; Sağdıç et al., 2013].

The impact of diabetes mellitus-associated hyperglycemia on tight junction proteins and ocular surface glycocalyx has not been investigated. If diabetes mellitus adversely affects the tight junction proteins and the integrity of ocular surface glycocalyx, such damage may account for the loss of ocular surface barrier integrity and the higher
incidence of corneal/conjunctival infections and dry eye observed in patients of diabetes mellitus.

Our central hypothesis is that high glucose due to diabetes mellitus causes impairment of ocular surface barrier functions by decreasing the expression of tight junction proteins and causing redaction in glycocalyx. Therefore, the present study is designed to investigate:

**Aim 1:** the effect of acute (24 hours) and chronic (72 hours) high glucose exposure on the tight junction proteins using cultured human conjunctival epithelial cells.

**Aim 2:** the effect of type I diabetes mellitus on tear secretion and ocular surface glycocalyx using a mouse model of type I diabetes.
CHAPTER II

LITERATURE REVIEW
Ocular Surface, Glycocalyx, Lacrimal Functional Unit and Tear film

The ocular surface is made up of cornea and conjunctiva. It is covered by a thin layer of fluid called tear film.

i. Conjunctiva

The term “conjunctiva” comes from the Latin word meaning "to bind together." The conjunctiva is a semi-transparent thin membrane that covers the eyeball above the sclera and the inner surface of the eyelids. The conjunctiva covering the sclera is referred to as bulbar conjunctiva and the conjunctiva covering under the eyelids is termed as palpebral conjunctiva (Figure 1A). The palpebral conjunctiva is further divided into marginal, tarsal and orbital conjunctiva [Chigbu, 2013].

![Figure 1: Anatomy (A) and histology (B) of conjunctiva.](https://www.sciencephoto.com/media/703697/view/conjunctiva-illustration-illustration;A) (https://www.slideshare.net/kmrhssl/anatomy-of-conjunctiva B)

Histologically, conjunctiva consist of two layers (Figure 1B): the epithelium and the substania propria [Shields and Shields, 2019]. The conjunctival epithelium consists of two to seven layers of nonkeratinized, stratified columnar epithelial cells joined by tight junction and desmosomes to provide barrier against the environmental insults. The conjunctival epithelium has also abundant goblet cells; which secrete mucins into the tear film [Kim et al., 2011; Uchino et al., 2013].
ii. Limbus

Limbus is anatomically indistinguishable but histologically distinct circular area that forms the junction between conjunctiva and cornea (Figure 2). In humans, it is about 1.5 mm wide. Limbus contains stem cells and its primary function is to continually regenerate conjunctival and corneal epithelial cells [Van Buskirk, 1989; Chigbu, 2013; Sridhar, 2018]. The epithelial cells generated by limbus migrate to cover the ocular surface. The conjunctival side of limbus has between eight to ten layers of epithelial cells. Limbal conjunctival epithelium is continuous with the epithelium of the bulbar conjunctiva [Van Buskirk, 1989; Sridhar, 2018].

![Figure 2: Anatomical location of limbus.](https://example.com/limbus.jpg) (Riordan-Eva et al., 2018)

iii. Cornea

The cornea is a thin transparent tissue with an average thickness of 550 μm and an average diameter of about 11.7 mm horizontally and 10.6 mm vertically in adult humans. Histologically, the cornea has five different layers, including epithelium, bowman's layer, stroma, Descemet's membrane, and endothelium. The corneal epithelium has approximately five or six layers of cells that are in continuation with the epithelium of the limbus and bulbar conjunctiva. Cornea has three different types of epithelial cells which include squamous, wing and basal cells. The corneal epithelium
provides a substantial barrier to protect the eye from any external materials such as dust, microorganism, and allergens [Chigbu and Coyne, 2015; Sridhar, 2018].

iv. Glycocalyx

The glycocalyx is thin “fuzzy” layers made up of glycoproteins, proteoglycans and glycosaminoglycans covering the external part of the plasma membrane of cells. In humans, the thickness of glycocalyx varies from organ to organ approximately being 0.5 to 5.0 μm [Uchimido et al., 2019]. In ocular surface, glycocalyx is about 0.5 μm thick [Uchino, 2018]. Glycocalyx serves as barrier between the cells and the surrounding environment [Alphonsus and Rodseth, 2014; Devuyst et al., 2014]. It protects the cell membrane from any stress or physical actions that affect its integrity [Monne et al., 2013]. Glycocalyx can be altered in many diseases and also influences the properties of cell membranes [Uchino, 2018, Shamloo et al., 2019]. The ocular surface corneal and conjunctival epithelial cells produce O-glycosylated transmembrane mucins including MUC1, MUC4 and MUC16. These membrane-bound mucins and galactin-3 together form the glycocalyx at the ocular surface epithelia (Figure 3) working to protect the cells from any chemical or mechanical damages [Mantelli et al., 2013]. In addition, it plays an important function of keeping the ocular surface lubricated and moist [Davidson and Kuonen, 2004].
v. Lacrimal function unit

It consists of meibomian gland, lacrimal gland, lids, conjunctiva, and cornea and the sensory nerves innervating these structures (Figure 4). Together, lacrimal functional unit is responsible for secretion and maintenance of the tear film [Stern et al., 2004].

vi. Tear Film

The tear film in humans consists of two layers, the muco-aqueous layer primarily secreted by conjunctiva, cornea and the lacrimal gland and the lipid layer secreted the meibomian gland [Dartt and Willcox, 2013]. The tear film serves the important functions of maintaining humid environment to avoid the ocular surface from becoming dry, lubricating it for smooth eyelids movement, inhibiting the growth of microorganisms by
flushing, and providing the cornea with oxygen and nutrients. Normally the tear volume in each eye varies roughly from 2 to 7 µl. The tear film contains albumin, immunoglobulins IgA, IgG and IgE, lysozymes, potassium, sodium, chloride, glucose and urea [McDermott, 2013]. The deficiency of tear film can lead to dry eye disease and abnormalities in the ocular surface epithelium. The patients who suffer from dry eyes have many symptoms such scratchy sensation, itching, increase in mucus secretion, redness, burning, difficulty of eyelids movement and pain [Ljubimov, 2017]. The dry eye syndrome can lead to decreased vision, and if left untreated can cause corneal ulceration, corneal scarring, corneal thinning and infections [Matossian et al., 2019].

**Ocular Surface Barrier Function**

The ocular surface is perpetually exposed to the external environment, which makes it vulnerable to microbes, allergens, and chemicals. Corneal and conjunctival epithelial cells form the outermost layer of the ocular surface and provide a substantial barrier against these environmental insults. The tight junctions are highly expressed in the ocular surface epithelial cells and serve to create a strong barrier to protects the eye from outside environments [Ban et al., 2003; Yoshida et al., 2009; Mescher, 2018]. The tightness of tight junction in corneal epithelium is greater as compared to conjunctival epithelium [Yoshida et al., 2009].

**Biology of Inter-cellular Junctions**

The intercellular junctions are made up of i) tight junctions, ii) adherens junctions, and iii) desmosomes (Figure 5). Both corneal and conjunctival epithelial cells express these intercellular junctions [Yoshida et al., 2009].
i) Tight junctions

Tight junctions are localized on the apical surface of epithelial cells. One of the significant functions attributed to the tight junctions is to restrict the paracellular movement of large molecules, ions and pathogens, thus significantly contributing to the barrier function of epithelial cells [Anderson and Van Itallie, 2009; Leong and Tong, 2015; Lee et al., 2018]. These junctions contain an assembly of integral transmembrane proteins that are further connected to the peripheral membrane proteins present in the cytosol (Figure 7). Four transmembrane proteins present in the tight junctions include claudins, occludin, junctional adhesion molecules, and tricellulin [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. The peripheral proteins are zonula occludens such as ZO-1, ZO-2, ZO-3 and serve to connect the transmembrane proteins to the cytoskeletal proteins [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018].

The claudins protein family consists of 27 members who share a common feature of four transmembrane spanning region, forming two extracellular loops and cytosolic C and N terminals [Krause et al., 2008; Tsukita et al., 2019]. Claudins demonstrate a tissue-specific distribution with various combinations of the 27 subtypes being expressed in the
epithelial cells of different organs [Krause et al., 2008; Tsukita et al., 2019]. Claudin-1,-2,-4,-7,-9, and Claudin -14 expressions have been detected in both human corneal and conjunctival epithelial cells. However, claudin- 3 was detected only in corneal epithelial cells and claudin-10 being selectively expressed in the conjunctival epithelial cells [Yoshida et al., 2009].

Expression of occludin has also been detected in the corneal and conjunctival epithelial cells [Yoshida et al., 2009]. Like claudins, occludin also has four transmembrane domains and two extracellular loops [Krause et al., 2008; Cummins, 2012]. However, the function of occludin in tight junctions is less clear as compared to claudins. Occludin is proposed to regulate the movement of large macromolecule across the tight junction barrier. Occludin is phosphorylated at threonine and serine residues and upon phosphorylation it is mainly localized in the plasma membrane. Dephosphorylation of occludin leads to its migration to cytoplasm [Cummins, 2012]. The phosphorylation of occludin seems to be essential in interacting with other tight junction proteins like ZO-1 [Cummins, 2012; Bhat et al., 2019] Consequently, the phosphorylation of occludin may maintain the permeability and stability of tight junctions [Bhat et al., 2019] whereas its dephosphorylation may promote leakiness [Bhat et al., 2019].

The junctional adhesion molecules are also the important component of tight junctions [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. These molecules structurally belong to immunoglobulin like single span family of proteins and have an extracellular domain, a single transmembrane domain and a cytoplasmic tail [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. Junctional adhesion molecules interact with adjacent cell’s tight junction by the extracellular domain. The
cytoplasmic tail is involved in cytosolic protein interactions. Besides, playing a role in tight junction formation, junctional adhesion molecules also play a role in cell proliferation and migration [Itoh and Bissell, 2003].

**Figure 6: Various protein components of tight junctions and adherence junctions.** (Singh et al., 2010)

Zonula occludens, ZO-1, ZO-2, ZO-3, connect the junctional proteins claudin and occludin to actin cytoskeleton. These proteins have also been shown to be present in the tight junctions of corneal and conjunctival epithelial cells [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018; Bhat et al., 2019]. The roles of ZOs proteins in tight junctions is still under investigation. Studies show that cells deficient in ZO-1 could still preserve the tight junction structure and function and demonstrate the normal permeability barrier but have a delay in the expression of occludin and claudins [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018; Bhat et al., 2019] On the other hand, epithelial cells deficient in ZO-2 and ZO-3 did not have any notable effect on their tight junctions. [Lee et al., 2018].
ii) Adherens junctions

Adherens junctions or zonula adherens are localized below the tight junctions in the polarized epithelial cells at the apical region of the intercellular cleft and act as a zipper-like seal between adjacent cells [Niessen, 2007; Campbell et al., 2017; Shigetomi et al., 2018]. The adherens junctions maintain the cell-cell adhesion, regulate the organization of the actin cytoskeleton and also play a role in the gene transcription [Niessen, 2007; Campbell et al., 2017; Shigetomi et al., 2018]. Structurally, adherens junctions contains E-cadherin, p120 catenin, beta-catenin, and alpha-catenin. Along with cadherins, catenins are also essential for formation, function, and maintenance of adherens junctions [Niessen, 2007; Campbell et al., 2017; Shigetomi et al., 2018].

iii) Desmosomes

Desmosomes, also known as macula adherens, are located at the basal side of the epithelia. The desmosomes have unique structures like disc shapes with two identical structures that match the adjacent cell surface. The desmosomes consist of proteins from cadherins family, which include desmocollin and desmoglein [Garcia et al., 2018]. Desmosomes also contain Armadillo proteins family proteins, plakoglobin and plakophilin [Garcia et al., 2018]. Desmosomes play an essential role in providing strength to the cell conjunction and cytoskeleton. Desmosomes may also work as a strong barrier between the cells.

Diabetes Mellitus: Etiology and Epidemiology

Diabetes mellitus has the origin of its name in Greek word diabetes meaning "to siphon" and the Latin word mellitus meaning "sweet" [Cowap and Parry, 2015], denoting the key disease symptom of excessive passing of sweet-tasting urine. Diabetes mellitus
is a serious metabolic disorder that has the phenotype of hyperglycemia. Diabetes mellitus has several subtypes that are caused by an interaction between environmental factors and genetics. The etiology of diabetes mellitus is multi-factorial. The diabetes mellitus is classified based on the pathogenic process leading to hyperglycemia. There are two main categories of diabetes mellitus, Type 1 and Type 2. Type 1 diabetes mellitus is caused by an autoimmune response resulting in destruction of beta cells of pancreas. This ultimately leads to almost complete insulin deficiency [Powers et al., 2018]. The type 2 diabetes mellitus is a heterogeneous disorder characterized by a combination of insulin resistance, decreased insulin secretion and increased hepatic glucose production. The other types of diabetes mellitus include those caused by genetic defects in insulin secretion, action, and metabolic abnormalities that decrease insulin secretion, abnormality of mitochondria and decreased glucose tolerance [Powers et al., 2018]. Also, the mutation in insulin can cause rare disorders characterized by severe insulin resistance. In some cases, the diabetes mellitus may be developed due to chronic pancreatitis or cystic fibrosis, when the islets become damaged. [Powers et al., 2018].

According to the International Diabetes Federation publication “The Diabetes Atlas”, the prevalence of diabetes mellitus has risen dangerously over the past few decades. In 1985, there were approximately 30 million cases rising to a whopping 415 million cases in 2017. This number is further projected to rise to 642 million by 2040. In 2015, the countries with largest number of individuals suffering from diabetes mellitus include China (109 million) followed India (73 million), then United States (30 million), Brazil (14 million), and the Russian Federation (9 Million). As reported by the Center for Disease Control and Prevention, the united states have 9.4% of the population diagnosed
with diabetes. Additionally, there are about 34% more people who have prediabetes [Xu et al., 2013; Cho et al., 2018; Powers et al., 2018].

While the number of patients of both type 1 and type 2 diabetes mellitus is increasing around the world, the prevalence of type 2 diabetes mellitus is more rapidly increasing compared to type 1. Life-style changes such as reduced physical activity level, increased obesity, and the aging population has been proposed to contribute to the increased incidence of diabetes mellitus. The rate of type 1 diabetes is also increasing by roughly 5% per year around the world and patients are being diagnosed at younger ages. The high incidence of diabetes mellitus will lead to tremendous health burden worldwide [Powers et al., 2018].

**High glucose-mediated Cellular and Pathological Alterations**

Diabetes mellitus causes damage to many organs in the human body. The vascular damage is considered to be a major underlying cause of many diabetes-associated complications. The diabetic complications based on vascular damage are divided into two major categories: microvascular and macrovascular. The microvascular complications are specifically thought to be responsible for diabetes-associated neuropathy, nephropathy and retinopathy while the macrovascular complications account for diabetes-associated coronary and peripheral artery diseases [Funk, 2019].

Strict regulation of duration and degree of hyperglycemia has been shown to decrease diabetes-associated microvascular complications suggesting that high glucose plays a critical role in diabetes-associated complications. Hyperglycemia has been proposed to cause damage by four pathways (Figure 7): activation of polyol pathway,
increased formation of advanced glycation end product (AGE), increased hexosamine formation, and activation of the protein kinase C (PKC) [Funk, 2019].

**Figure 7: Hyperglycemia-mediated cellular changes that trigger diabetes-associated complications.** (Hammer and McPhee, 2018)

The polyol pathway has been proposed to constitute one-third of intracellular glucose flux. Most of the cells have aldose reductase which is an enzyme reducing toxic aldehyde to alcohol. When the concentration of glucose becomes high in the cells, the polyol pathway converts the glucose to sorbitol then to fructose by oxidation. The pathway utilizes nicotinamide adenine dinucleotide phosphate (NADPH) which is mandatory to regenerate the glutathione and thiol. The pathway causes both the osmotic and oxidative stress on the cells. However, recent evidence suggests the greater damage is because of oxidative stress due to NADPH depletion rather than osmotic stress [Lorenzi, 2007].

The advanced glycosylation end-product (AGE) are formed by an initial non-enzymatic Millard reaction between the free amino groups of the proteins and carbonyl group of glucose (Figure 9). AGE formation is subdivided into three stages. In early stage, glucose reacts with free amino group of proteins to form unstable Schiff base intermediate that then undergoes the Amadori rearrangement to be form more stable intermediate. In
second stage, Amadori products degrade to dicarbonyl compounds such as deoxyglucosones and glyoxal by oxidation and dehydration (Figure 8). In the last stage, advanced glycosylation end-products (AGE) are formed by cyclization, oxidation and dehydration reactions. Further increase in glycation leads to the protein cross linking causing structural changes and accumulation of AGEs and crosslinked proteins [Singh et al., 2014; Funk, 2019].

Figure 8: Chemical reaction between glucose and proteins leading to advanced glycosylation end-products (AGE) formation. (Hammer and McPhee, 2018)

Excessive intracellular glucose causes increased de novo synthesis of diacylglycerol (DAG) which activates the protein kinase C (PKC). Activation of PKC can cause changes in gene expression which can cause excessive synthesis of extracellular matrix, altered the blood flow and impaired the endothelial permeability [Koya and King, 1998].

Lastly, the hexosamine pathway also play an important role in diabetes-associated microvascular diseases. The amino sugars synthesized by hexosamine pathway are converted to fructose-6-phosphate, then to glucosamine-6-phosphate. Fructose-6-phosphate-amidotransferase, the enzyme that catalyzes this reaction, uses glutamine as donor. The glucosamine-6-phosphate is then converted and activated to uridine-5-
diphosphate-N-acetylglucosamine which is the major precursor necessary for the biosynthesis of glycoproteins, glycosaminoglycans, proteoglycans and glycolipids [Schleicher and Weigert, 2000]. The overactivation of hexosamine pathway contributes to the insulin resistance, stimulates the protein expression of transforming growth factor-β, plasminogen activator inhibitor and excessive synthesis of extracellular matrix [Buse, 2006].

**Impact of Diabetes Mellitus on Ocular Surface Barrier and Tear Film**

In the eyes, diabetes can cause diabetic retinopathy, dry eye syndrome, glaucoma, and cataract [Bayraktar et al., 2002; Yoon et al., 2004; Pollreisz and Schmidt-Erfurth, 2010; Zhao and Chen., 2017]. Recent evidence suggests that diabetes can also have major detrimental effect on the ocular surface [Rosenberg et al., 2000; Yoon et al., 2004; Alves et al., 2008; Misra et al., 2016]. Patients of diabetes mellitus suffer from corneal keratopathy and persistent corneal epithelial defects [Ljubimov, 2017]. The density of corneal epithelial cells has been shown to be decreased in patients of diabetes mellitus and the corneal epithelial cell maturation is delayed [Barsegian et al., 2018]. Anterior fluorometry-assisted tracking of corneal fluorescein penetration has shown that patients of diabetes mellitus have an impairment of corneal epithelial barrier function in diabetic patients [Gekka et al., 2004]. These studies have also shown that the frequency and severity of corneal epithelium barrier impairment correlated with the serum levels of HbA1c [Gekka et al., 2004; Misra et al., 2016]. Patients of diabetes mellitus also show abnormalities in conjunctival capillaries, such as dilatation, increased tortuosity and leakage [Cheung et al., 2001; Owen et al., 2008]. Patients with diabetes mellitus also suffer from significantly higher incidence of corneal and conjunctival infections [Ljubimov,
Studies using type I diabetic rats have shown decreased tear secretion, altered wound healing and impaired corneal innervation [Yin et al., 2011].

Dry eye syndrome is reported more frequently in patients of diabetes mellitus. There is a correlation between the incidence of dry eye and the level of glycated hemoglobin, with a higher level of glycated hemoglobin, leading to a higher level of incidence of dry eye [Seifart and Strempel, 1994]. Studies have shown about 45% prevalence of both symptomatic and asymptomatic dry eye syndrome in patients with diabetes [Hom and De Land 2006]. Another study reported higher incidence with approximately 53% of patients with diabetes showing clinical signs of dry eyes [Manaviat et al., 2008]. These findings were further corroborated by another study also reporting 54% of patients with diabetes having dry eye syndrome, and there was a strong correlation between the duration of diabetes and dry eye syndrome. In older patients suffering from diabetes mellitus, the incidence of symptomatic dry eye disease is high, and it increases further with the advancing age. Furthermore, women suffering from diabetes mellitus are 50% more likely to get dry eye compared to male patients of diabetes mellitus [Rosenberg et al., 2000].
CHAPTER III

METHODOLOGY
Human Conjunctival Epithelial Cell Culture

Telomerase-immortalized human conjunctival epithelial cells were plated at a density of 1X10^6 cells onto a T75 flask (CytoOne® USA Scientific, Inc, Ocala, FL, USA). The cells were cultured in the supplemented keratinocyte growth medium (Table 1) at 37°C with 5% carbon dioxide and 95% humidity in a cell culture incubator (Forma series II water jacket, Thermo Fisher Scientific, Marietta, OH, USA). The media was replaced every two to three days. Once the cells reached 80% confluence, they were trypsinized and plated [Gipson et al., 2003].

Table 1: Composition of keratinocyte growth medium.

<table>
<thead>
<tr>
<th>No</th>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Keratinocyte Serum-Free Medium</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Human Epidermal Growth Factor</td>
<td>0.23ng/ml</td>
</tr>
<tr>
<td>3</td>
<td>Bovine Pituitary Extract</td>
<td>25µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>Calcium Chloride</td>
<td>0.4mM</td>
</tr>
<tr>
<td>5</td>
<td>Penicillin/Streptomycin</td>
<td>100U/ml</td>
</tr>
</tbody>
</table>

Treatment Protocol

The cells were exposed to either keratinocyte growth medium (control) or keratinocyte growth medium containing glucose; mannitol (15 mM; 30 mM) for 24h; 72h (Table 2). All the experiment mentioned below (TEER, Scratch, Gene Expression and Western blot) were performed in triplicate for each treatment group.

Table 2: Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>High Glucose</th>
<th>High Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>72 h</td>
<td>24h</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>15 mM</td>
<td>15 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 mM</td>
<td>30 mM</td>
</tr>
<tr>
<td></td>
<td>15 mM</td>
<td>30 mM</td>
<td>15 mM</td>
</tr>
<tr>
<td></td>
<td>15 mM</td>
<td>30 mM</td>
<td>15 mM</td>
</tr>
</tbody>
</table>
**Trans-epithelial Electrical Resistance (TEER)**

The cells were seeded onto collagen-coated 3.0 μm polytetrafluoroethylene (PTFE) trans-well membranes inserts (Costar® Corning incorporated, Kennebunk, ME, USA) placed onto 12-well plates. The cells were allowed to grow to a 100% monolayer. The trans-epithelial electrical resistance was measured using chopstick electrode method (Axon® Instruments) [Srinivasan et al., 2015].

**Cell Migration Assay**

The cells were plated in 12-well plates and allowed to reach 100% confluence. The scratch was performed on the cells in two perpendicular straight lines using a 1000 μl pipette tip [Hou., 2013]. The cells were imaged at the same position just above where the two perpendicular scratch lines intersected at time points immediately after scratch (0), and then at 6, 12, 24, 48 and 72 hours using a 4X objective phase contrast lens on a bright-field microscope (Keyence BZ-X 710, Itasca, IL, USA). The re-epithelization of the scratch area was quantified by measuring the distance between the two edges of the scratch lines using Image J software [NIH].

**Isolation of mRNA and Preparation of cDNA**

The cells were plated in a 12-well plate. A commercially available kit (QIAGEN’s RNeasy Mini Kit) was used to isolate the mRNA from cultured human conjunctival epithelial cells. Briefly, the media was aspirated from each well and 350 μl of RNase inhibitor containing RLT buffer was added to each well to cause cell lysis. The lysate was then transferred to the QIA shredder spin column and centrifuged to further facilitate shredding.
The flow-through was collected and an equal volume of 70% molecular biology grade ethanol was added to the lysate to precipitate the DNA. The lysate was transferred to the RNeasy spin column which binds RNA. The column was then centrifuged at 10,000 rpm for 30 sec. The column was washed with RW1 buffer and RPE buffer to remove any residual protein contamination. Lastly, 30 μl of RNase-free water was added to the column membrane and incubated for five minutes at room temperature followed by centrifugation for 1 minute to elute the RNA. The isolated RNA was immediately used for cDNA preparations.

The RNA was reverse transcribed to cDNA using a commercially available kit (SuperScript® III First-Stand, Invitrogen, CA, USA). A 7 μl sample of isolated RNA was combined with 1 μl of 50 μM oligo(dT)20 primer, 1 μl of 10mM dNTP mix and 2 μl of DEPC-treated water. The mixture was incubated at 65°C for 5 minutes using MiniAmp Thermocycler (Applied Biosystems, Thermo Fisher Scientific, Singapore) followed by incubation on ice for 1 minute. A 10 μl of cDNA Synthesis mix (Table 3) was then added to the above reaction mixture.

The mixture was incubated at 50°C for 50 minutes and then, at 85°C for 5 minutes. Lastly, 1 μl RNase H was added to the reaction and incubated at 37°C for 20 minutes.

**Table 3: Composition of cDNA synthesis mixture.**

<table>
<thead>
<tr>
<th>No</th>
<th>Ingredient</th>
<th>Quantity/Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 X RT buffer</td>
<td>2 μL</td>
</tr>
<tr>
<td>2</td>
<td>25 mM MgCl2</td>
<td>4 μL</td>
</tr>
<tr>
<td>3</td>
<td>0.1 M DTT</td>
<td>2 μL</td>
</tr>
<tr>
<td>4</td>
<td>RNaseOUT™ (40 U/ μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>5</td>
<td>Superscript® III RT (200 U/ μL)</td>
<td>1 μL</td>
</tr>
</tbody>
</table>
Real-Time PCR

The gene expression of the tight junction proteins claudin-1,-2,-3, ZO-1,-2,-3 and occludin was quantified using Real-Time PCR. The PCR master mix was prepared as shown in the table 4 and using the primers shown in table 5. A total of 20 µl was used per reaction (1µl cDNA+19 µl of master mix).

Table 4: Composition of Real-Time PCR master mix.

<table>
<thead>
<tr>
<th>No</th>
<th>Ingredient</th>
<th>Quantity/PCR Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bullsey EvaGreen qPCR Master Mix</td>
<td>10µl</td>
</tr>
<tr>
<td>2</td>
<td>Reverse Primer of Target Gene</td>
<td>2µl</td>
</tr>
<tr>
<td>3</td>
<td>Forward Primer of Target Gene</td>
<td>2µl</td>
</tr>
<tr>
<td>4</td>
<td>cDNA Sample</td>
<td>1µl</td>
</tr>
<tr>
<td>5</td>
<td>DEPC water</td>
<td>5µl</td>
</tr>
</tbody>
</table>

QuantStudio™ 3 Real-Time thermocycler (Applied Biosystems, Thermo Fisher Scientific, Singapore) thermocycler was used to run the PCR reactions.

Table 5: Nucleotide sequences of forward and reverse primers for mRNA of human tight junction proteins and β-actin.

<table>
<thead>
<tr>
<th>No</th>
<th>Protein</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZO-1</td>
<td>5'-GCA GCC ACA ACC AAT TCA TAG-3'</td>
<td>5'-GAA AGG TAG TAA GGG ACT GGA GAT G-3'</td>
<td>NM_001355015.1</td>
</tr>
<tr>
<td>2</td>
<td>ZO-2</td>
<td>5'-AGG ATG CCG TTC TCT ACC T-3'</td>
<td>5'-CAC AAG CCA GGA TGT CTC TAT AC-3'</td>
<td>NM_004817.4</td>
</tr>
<tr>
<td>3</td>
<td>ZO-3</td>
<td>5'-GGG GGG AAA GTT CAG TAG ATT-3'</td>
<td>5'-GGC ACT CTG TAG ATG TCA TAG C-3'</td>
<td>NM_001267561.1</td>
</tr>
<tr>
<td>4</td>
<td>CLA-1</td>
<td>5'-CCA GTT AGA AGA GGT AGT GTG AAT-3'</td>
<td>5'-CAG CCA GCT GAG CAA ATA AAG-3'</td>
<td>NM_021101.4</td>
</tr>
<tr>
<td>5</td>
<td>CLA-2</td>
<td>5'-CCT CCA TCC CAC TCT TGT TAT G-3'</td>
<td>5'-CAT CCT GCA TCC TGC TTT CT-3'</td>
<td>NM_020384.3</td>
</tr>
<tr>
<td>6</td>
<td>CLA-3</td>
<td>5'-CCA AGG CCA AGA TCA CCA T-3'</td>
<td>5'-GGT TGT AGA AGT CCC GGA TAA TG-3'</td>
<td>NM_001306.3</td>
</tr>
<tr>
<td>7</td>
<td>Occludin</td>
<td>5'-GGA AGG TTC TGG TGT GAA CTA A-3'</td>
<td>5'-CTG AAA GGT GGT TGA GAG GAT TA-3'</td>
<td>NM_002538.4</td>
</tr>
<tr>
<td>8</td>
<td>β-actin</td>
<td>5'-GGA CCT GAC TGA CTA CCT CAT-3'</td>
<td>5'-CGT AGC ACA GCT TCT CCT TAA T-3'</td>
<td>NM_001101.3</td>
</tr>
</tbody>
</table>

The thermocycler settings for PCR were: Step 1 (Activation): 50°C for 2 minutes then 95°C for 2 minutes for thermal activation of the modified inactive form of Taq
polymerase. **Step 2 (PCR):** 40 cycles of: 95°C for 15 seconds to cause cDNA denaturation and 60°C for 1 minute for annealing and extension. **Step 3 (Melt Curve):** Continuous: 95°C for 15 seconds then 60°C for 1 minute then 95°C for 15 seconds.

**Western Blotting**

The protein extracts were prepared by adding radioimmunoprecipitation assay (RIPA) buffer containing Halt™ protease inhibitor (Thermo Scientific, Rockford, IL, USA) to the cells. The cells were incubated in RIPA buffer at 4°C for 15 min on a shaker followed by removal of any adherent cells using a cell scraper. The cells were further lysed by passing through a 21-gauge needle and incubated on ice for 60 min. The lysate was then centrifuged at 14,000g for 15 min at 4°C. The supernatant was collected as total cell lysate. The protein concentration was determined by BCA method using a commercially available kit (Pierce™ BCA protein Assay Kit., Thermo Scientific, Rockford, IL, USA). The protein extracts were suspended in loading buffer containing β-mercaptoethanol (Bio-Rad) and heated at 95°C for 5 min. Protein samples were resolved by loading on 10% SDS-PAGE gels (Mini-PROTEAN® TGX™, Bio-Rad Laboratories Inc, Carlsbad, CA, USA) and running at 100V for about 85 minutes using 1X TRIS-glycine-SDS buffer. The resolved proteins were transferred from gel onto the polyvinylidene difluoride membranes using wet transfer at 100V for 60 minutes. The membranes were blocked in 5% blocking buffer (5% bovine serum albumin in distilled water) for 1 hour. The membranes were then incubated with primary antibodies overnight at the concentrations shown in the table 6. The membranes were washed three times with 0.1% TBST (Tween 20 in Tris buffered saline), followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hour and then three washings with TBST. Glyceraldehyde-3-phosphate
dehydrogenase (GAPDH), was used as house-keeping gene. The blot was developed using enhanced chemiluminescence reagent (Lumigen Inc., Southfield, Michigan, USA) and imaged using a chemiluminescence gel imager (Bio-Rad Laboratories Inc, Carlsbad, CA, USA). The digital quantification of western blots was performed using Image J software. The quantification intensity was normalized against GAPDH bands.

Table 6: Polyclonal and monoclonal antibodies used for Western Blot.

<table>
<thead>
<tr>
<th>No</th>
<th>Primary Antibody</th>
<th>Concentration/per mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZO-1</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>ZO-2</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>3</td>
<td>ZO-3</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>CLA-1</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Occludin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>6</td>
<td>Anti GAPDH</td>
<td>1:10000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No</th>
<th>Secondary Antibody</th>
<th>Concentration/per mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Donkey Anti-Rabbit IgG HRP</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Mouse Model of Type I Diabetes

The animal protocol was approved by Institutional Animal Care and Use Committee of Chapman University. Female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) 6 weeks old and weighing from 16-18 were used in this study. To induce type I diabetes, the mice were injected with a single intraperitoneal dose of 200 mg/kg streptozotocin dissolved in citrate buffer, pH 4.5 [Deeds et al., 2011].

Blood Glucose Monitoring

The blood glucose was monitored by nicking the tail to collect a blood drop in a glucose strip. The blood glucose was quantified using a glucometer (OneTouch Verio IQ® Life Scan Europe, China).

Tear Quantification

The tear secretion was quantified in both eyes using the commercially available phenol red thread (Zone Quik, FCI Ophthalmic, Japan). Briefly, the thread was placed in
the lower eyelid of the mice for 60 seconds. The phenol red thread is pH sensitive and when wetted by tears changes from yellow to red. After 1 minutes, the thread was removed and the length of the red color was measured in millimeters [Choi et al., 2015; Choi, 2016].

**Fluorescein Staining and Keratopathy Scoring**

The mice were anesthetized using ketamine (80 mg/kg) and xylazine (5 mg/kg). A 2 µl sterile solution of 0.1% of sodium fluorescein was applied to both eyes followed by washing with 1X PBS. After 10 minutes, the mice were imaged under cobalt blue filter using a slit-lamp (Kowa SL-17, Japan) equipped with a digital camera. The captured corneal images were divided into four hypothetical quadrants for scoring the keratopathy using a previously published method [Zhang et al., 2014]. Each quadrant was scored as follows: no staining = 0; slightly punctate staining less than 30 spots = 1; punctate staining more than 30 spots, but not diffuse = 2; diffuse staining but no positive plaque = 3; positive fluorescein plaque = 4. The scores of each quadrant were added to arrive at a final grade (total maximum possible score = 16). Corneal keratopathy scoring was not performed in blinded manner.

**Tissue Harvesting and Cornea Isolation**

The mice were euthanized with CO2 using AVMA compliant equipment. The right eyelids along with the eyeballs was harvested and fixed in 4% paraformaldehyde overnight followed by gradient dehydration by immersing in with 15% sucrose for 24 hours and 30% of sucrose for another 24 hours. The corneas were isolated under a dissecting microscope.
**Glycocalyx staining, Confocal imaging and Quantification**

The corneas were placed in a 12-well plate and washed with 1X PBS on a shaker for 5 minutes followed by blocking with 5% bovine serum albumin for 20 minutes. The corneas were then stained with Alexa 488 conjugated wheat germ agglutinin (Thermo Fisher Scientific, Hanover Park, IL, USA) using a dilution of 1.5 µl of stock solution in 1 mL of 1X PBS for 20 minutes at room temperature [Kataoka et al., 2016]. The corneas were then washed with 1X PBS for 10 minutes twice. The stained corneas were then placed on a slide followed by DAPI containing mounting media. The corneas were imaged using confocal microscope. The quantification of glycocalyx stained area was performed using Image J software (provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) but was not performed in a blinded manner.

**Statistical Analysis**

All the experiments were performed in triplicate for each treatment group. The data is presented as mean ± Standard error of mean. Statistical analysis was performed using GraphPad Prism software (Version 8, San Diego, CA, USA). The cell culture data was analyzed using Two-Way ANOVA followed by Tukey’s post-hoc test for multiple comparisons. The statistical analysis for animal studies was done using One-Way ANOVA followed by Dunnett’s post-hoc test. A p value of <0.05 was considered statistically significant.
CHAPTER IV

RESULTS
Effect of High Glucose on Trans-Epithelial Electrical Resistance (TEER)

To test whether high glucose causes an impairment of conjunctival epithelial cell barrier functions, we cultured a monolayer of human conjunctival epithelial cells on a trans-well membrane. Typically, monolayer of conjunctival epithelial cells with intact barrier functions shows high electrical resistance across the apical to basal surface making TEER a reliable test [Srinivasan et al., 2015]. Chopstick electrodes placed on the top and bottom of the monolayer were used to measure TEER. As is shown in Figure 9A, treatment of human conjunctival epithelial cells with 15 and 30mM glucose caused an average of 14% and 5% decrease in TEER after 72 hours of exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). No significant decrease in TEER was observed at 24 hours suggesting that a persistent exposure to high glucose may be required to cause an impairment of barrier functions.

It should be noted that mannitol only at 30 mM concentration caused a 4% decrease in TEER after 72 hours of exposure whereas 15 mM of mannitol did not cause any notable decrease in TEER either after 24 or 72 hours of exposure (Figure 9B).

Effect of High Glucose on Cell Migration

To test the effect of high glucose exposure on cellular migration of conjunctival epithelial cells, we used the scratch assay. Figure 10 shows the representative phase contrast microscopy images of conjunctival epithelial cells exposed to high glucose and mannitol respectively at various tested time points after preforming the scratch wounding.

The quantification of the scratch length as a percentage change to the baseline length showed that high glucose (Figure 11A) and mannitol (Figure 11B) had no effect on the time dependent decrease in the scratch length. The data suggests that neither high
glucose nor mannitol had any significant effect on the migration of human conjunctival epithelial cells.

**Effect of High Glucose on Claudin Family Gene and Protein Expression**

Tight junctions play an important role in maintaining the cellular barrier functions [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. Claudin family of proteins are integral part of tight junctions [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. Therefore, we next tested the effect of high glucose exposure on the gene and protein expression of claudin-1,2 and 3. Figure 12A shows that glucose at 15 mM concentration caused an average of 4.81-fold increase in claudin-1 gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). On the other hand, glucose at 30 mM concentration did not modulate claudin-1 gene expression either with 24- or 72-hours exposure. Figure 12B shows that mannitol also caused a significant 3.4- and 2.2-fold increase in claudin-1 at 15mM and 30mM after 72 hours of exposure (* p< 0.05 compared to control cells exposed to media containing no mannitol).

Figure 13A shows that glucose 15mM concentration caused a 2.31-fold increase in claudin-3 gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). As can be seen in Figure 13B, mannitol also caused a significant 2.74- and 1.74- fold increase in claudin-3 at both 15mM and 30mM after 72 hours of exposure (* p< 0.05 compared to control cells exposed to media containing no mannitol). In contrast to claudin-1 and claudin-3, high glucose and high mannitol did not cause any significant change in claudin-2 gene expression (Figure 13).
We further tested the effect of high glucose exposure on protein expression of claudin family of proteins. High glucose or high mannitol did not cause any significant change in claudin-1 protein expression either at 24 or 72 hours of exposure (Figure 14). Furthermore, claudin 2 or claudin 3 protein expression could not detected in conjunctival epithelial cell extracts using western blot.

**Effect of High Glucose on Zonula Occludens Family Gene and Protein Expression**

Zonula occludens (ZO) family of proteins tether the cytoplasmic chain of claudin proteins to the cytoskeletal actin filaments [Itoh and Bissell, 2003; Ban et al., 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. Therefore, we next tested the effect of high glucose exposure on zonula occludens family of proteins. Figure 15A shows that 15 mM glucose caused an average of 1.81-fold increase in ZO-1 gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). Figure 15B shows that mannitol also caused a significant 1.52-fold increase in ZO-1 at 15mM after 72 hours of exposure (* p< 0.05 compared to control cells exposed to media containing no mannitol). It should be noted that no change in ZO-1 gene expression was observed with glucose or mannitol exposure after 24 hours.

In contrast to the noted increase in ZO-1 gene expression, high glucose or high mannitol did not cause any significant change in ZO-1 protein expression either at 24 or 72 hours of exposure (Figure 16).

Figure 17A shows that 15 mM glucose caused a 3.71-fold increase in ZO-2 gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). Figure 17B shows that mannitol also caused
a 2.69- and 1.85-fold increase in ZO-2 at 15mM and 30mM after 72 hours of exposure (*p< 0.05 compared to control cells exposed to media containing no mannitol).

However, high glucose or high mannitol exposure did not cause any significant change in ZO-2 protein expression either at 24 or 72 hours of exposure (Figure 18).

Finally, we also tested the effect of high glucose exposure on the gene and protein expression of ZO-3. Figure 19 shows that 15 mM glucose caused a significant 4.75-fold increase in ZO-3 gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). Similar to glucose, mannitol also caused a significant 3- and 1.71-fold increase in ZO-3 at 15mM and 30mM after 72 hours of exposure (*p< 0.05 compared to control cells exposed to media containing no mannitol).

As was the case with claudin-1, ZO-1 and ZO-2, high glucose or high mannitol did not cause any significant change in ZO-3 protein expression either at 24 or 72 hours of exposure (Figure 20).

**Effect of High Glucose on Occludin Gene and Protein Expression**

Figure 21 shows that 15 mM glucose caused a significant 2.66-fold increase in occludin gene expression after 72 hours exposure (* p< 0.05 compared to control cells exposed to media containing normal concentration of glucose). Similar to glucose, mannitol also caused a significant 1.89-fold increase in Occludin at 15mM after 72 hours of exposure (*p< 0.05 compared to control cells exposed to media containing no mannitol).
Just like claudin-1 and ZOs, high glucose or high mannitol did not cause any significant change in occludin protein expression either at 24 or 72 hours of exposure (Figure 22).

**Induction of Type I diabetes**

Figure 23 shows the random postprandial blood glucose levels in mice injected with a single intraperitoneal dose of streptozotocin (200 mg/kg). A mean blood glucose levels of 500 and 530 mg/dl were noted at week 1 and week 2 respectively after streptozotocin injection which were significantly (p<0.05) high compared to the average baseline blood glucose levels in these mice. The high blood glucose values after streptozotocin administration confirmed the onset of type I diabetes in these mice.

**Effect of Type I diabetes mellitus on Tear Secretion**

Figure 23 shows a mean baseline phenol red thread value of 3.3 mm in mice. At 1 week after the onset of hyperglycemia due to diabetes mellitus, a significant (p<0.05) drop in the phenol red thread test values was noted suggesting a decrease in the tear film. A partial recovery in this noted decrease in tear film was observed at week 2 after the induction of diabetes suggesting the possibility of a compensatory response by lacrimal functional unit.

**Effect of Type I diabetes mellitus on Corneal Keratopathy**

The top panel in figure 24 shows representative fluorescein images of mouse corneas before and at week 1 and week 2 after the onset of diabetes-associated hyperglycemia. As can be seen in the images, corneas of diabetic mice show corneal punctate and plaque staining. Quantification of fluorescein stained images showed a mean score of 5 and 9 at week 1 and week 2 respectively after the onset of diabetes
mellitus suggesting the presence of corneal keratopathy in these mice. The decrease in tear film typically precedes the onset of keratopathy which explain the higher score of corneal keratopathy observed at week 2 as compared to week 1.

**Effect of Type I diabetes mellitus on Corneal Glycocalyx**

Corneal epithelial cells express membrane-tethered mucins MUC1, MUC4 and MUC 16 on their apical surface [Mantelli et al., 2013]. Galectin 3 forms a continuous network with these membrane-tethered mucins to form glycocalyx [Mantelli et al., 2013]. Wheat germ agglutinin lectin binds to the sialic acid residues present on these mucins and has been previously used to stain the corneal glycocalyx [Kataoka et al., 2016; Shamloo et al., 2019]. The top panel in figure 25 shows the Z-stack confocal images of the mouse corneas stained for glycocalyx using Alexa 488-conjugated wheat germ agglutinin. The top view of confocal z stack images shows a dense and uniformly distributed glycocalyx staining in the corneas of control mice. On the other hand, corneal glycocalyx is sparse and patchy in the corneas obtained from mice at week 1 and week 2 after the onset of diabetes mellitus. The glycocalyx-stained area was quantified as a percentage of the total area using binary image analysis. As can be seen from the quantification data presented in figure 25, a significant (p<0.05) decrease of 20% and 12% in glycocalyx-stained area was observed in the cornea of mice at week 1 and week 2 after the onset of diabetes mellitus.
Figure 9: Effect of high glucose (A) and mannitol (B) on trans-epithelial electrical resistance (TEER) in cultured human conjunctival epithelial cells.

*p<0.05 compared to control
Figure 10: Representative phase contrast microscopy images showing the effect of glucose (Left) and mannitol (Right) on migration of human conjunctival epithelial cells measured using scratch assay.
Figure 11: Time-dependent effect of high glucose (A) and mannitol (B) on migration of human conjunctival epithelial cells measured over 72 hours as a percent change in the length of scratch compared to the length at the start (Baseline).
Figure 12: Effect of high glucose (A) and mannitol (B) on claudin-1 gene expression in human conjunctival epithelial cells quantified using real-time PCR.

* p<0.05 compared to control
Figure 13: Effect of high glucose (A) and mannitol (B) on claudin-2 (Left) and claudin-3 (Right) gene expression in human conjunctival epithelial cells quantified using real-time PCR.

* p<0.05 compared to control
Figure 14: Effect of high glucose (A) and mannitol (B) on claudin-1 protein expression in human conjunctival epithelial cells quantified using western blotting and densitometric image analysis.
Figure 15: Effect of high glucose (A) and mannitol (B) on ZO-1 gene expression in human conjunctival epithelial cells quantified using real-time PCR.

* p<0.05 compared to control
Figure 16: Effect of high glucose (A) and mannitol (B) on ZO-1 protein expression in cultured human conjunctival epithelial cells quantified using western blotting and densitometric image analysis.
Figure 17: Effect of high glucose (A) and mannitol (B) on ZO-2 gene expression in human conjunctival epithelial cells quantified using real-time PCR.

* p<0.05 compared to control
Figure 18: Effect of high glucose (A) and mannitol (B) on ZO-2 protein expression in cultured human conjunctival epithelial cells quantified using western blotting and densitometric image analysis.
Figure 19: Effect of high glucose (A) and mannitol (B) on ZO-3 gene expression in human conjunctival epithelial cells quantified using real-time PCR.
* p<0.05 compared to control
Figure 20: Effect of high glucose (A) and mannitol (B) on ZO-3 protein expression in cultured human conjunctival epithelial cells quantified using western blotting and densitometric image analysis.
Figure 21: Effect of high glucose (A) and mannitol (B) on occludin gene expression in human conjunctival epithelial cells quantified using real-time PCR.

* p<0.05 compared to control
Figure 22: Effect of high glucose (A) and mannitol (B) on occludin protein expression in cultured human conjunctival epithelial cells quantified using western blotting and densitometric image analysis.
Figure 23: Random post-prandial blood glucose levels (Top Panel) and Tear volume (Bottom Panel) in mice prior to (Before Diabetes) and at week 1 and week 2 (After Diabetes) after a single intraperitoneal injection of streptozotocin
* p< 0.05 compared to before diabetes
Figure 24: Corneal keratopathy score in mice prior to (Before Diabetes) and at week 1 and week 2 (After Diabetes) after a single intraperitoneal injection of streptozotocin measured using images obtained from slit-lamp after fluorescein staining.

* p< 0.05 compared to before diabetes
Figure 25: Quantification of glycocalyx in corneas obtained from nondiabetic mice and mice at week 1 and week 2 (Diabetic) after a single intraperitoneal injection of streptozotocin measured using alexa-488 conjugated wheat germ agglutinin staining and confocal microscopy.

* p< 0.05 compared to Non-diabetic
CHAPTER V
DISCUSSION
Diabetes mellitus has been shown to cause loss of barrier function in many organs. Induction of diabetes mellitus in animals or exposure of cultured cells to high glucose has been shown to cause an impairment in the transport and integrity of blood brain barrier microvessels, changes in intestinal permeability and a compromise of selective glomerular barrier filtration [Hawkins et al., 2007; Prasad et al., 2014; Mongelli-Sabino et al. 2017; Thaiss et al., 2018; Shen et al., 2019; Eftekhari et al., 2020]. In the ocular tissue, high glucose exposure of cultured retinal epithelial cells has been shown to compromise the barrier functions [Villarroel et al., 2009; Xia et al., 2017]. The present study for the first time demonstrated that high glucose exposure can also compromise the barrier function of cultured human conjunctival epithelial cells as indicated by a decrease in TEER. It is worthwhile to note that a significant decrease in TEER was observed only at 72 hours suggesting that a persistent exposure to high glucose is likely to cause a decrease in conjunctival epithelial cell barrier function whereas conjunctival cells may be able to tolerate transient high glucose exposure with any notable detrimental effect. Our lab has also observed a similar trend in high glucose-mediated decrease in TEER in corneal epithelial cells as well.

Under normal physiological conditions, conjunctival epithelial cells in the eye undergo a quick turnover of about 72 hours [Van Buskirk, 1989; Chigbu, 2013; Sridhar, 2018]. Newly formed conjunctival epithelial cells generated at the limbus migrate to the sclera and eyelids to form a normal protective barrier [Van Buskirk, 1989; Chigbu, 2013; Sridhar, 2018]. Thus, an impairment of conjunctival epithelial cellular migration can significantly compromise the barrier functions. However, our data demonstrates that high glucose exposure does not affect the migration of human conjunctival epithelial cells, thus
suggesting that impairment of cellular migration of human conjunctival epithelial cells may not contribute to the high glucose-associated impairment of barrier functions.

Intercellular adhesion junctions are critical for maintaining the normal barrier functions of epithelial and endothelial cells [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018]. Three such class of cell to cell adhesion junctions include tight junctions, adherens junctions, and desmosome. Among these junctions, tight junctions provide the most critical barrier function regulating the intercellular passage of molecules and ions based on their size and composition [Itoh and Bissell, 2003; Anderson and Van Itallie, 2009; Lee et al., 2018; Bhat et al., 2019]. Tight junctions also serve the ‘fence function’ by restricting the movement of molecules in the plasma membrane from apical to basolateral side. Adherens junctions, on the other hand, join the actin filaments of adjacent cells together thus providing the structural adherence function [Niessen, 2007; Campbell et al., 2017; Shigetomi et al., 2018]. Desmosomes provide even stronger adherence by linking the intermediate filaments between the adjacent cells [Garcia et al., 2018]. Since tight junctions provide the most critical function of providing the barrier, therefore we next tested the effect of high glucose exposure on the protein and gene expression of various components of tight junctions to delineate their role in the noted decrease in barrier function of conjunctival epithelial cells after high glucose exposure.

Claudins are the backbone of tight junctions and play an essential in their barrier function [Krause et al., 2008; Tsukita et al., 2019]. Claudins can be classified as closed or selectively permeable [Bhat et al., 2019]. Claudin-1 and claudin-3 belong to the closed group while claudin 2 is selectively permeable to anions and water [Bhat et al., 2019]. Conjunctival epithelial cells have been shown to express high levels of claudin-1 [Yoshida
et al., 2009]. In agreement with the previously published report [Yoshida et al., 2009], our data also demonstrated high mRNA and protein expression of claudin-1 in the conjunctival epithelial cells. However, exposure of human conjunctival epithelial cells to high glucose did not modulate the protein expression of claudin 1. Interestingly, high glucose at 15 mM caused an increase in the gene expression of claudin-1 after 72 hours of exposure. Our data also demonstrated the detection of claudin-2 and claudin-3 mRNA transcripts but we were unable to detect the protein levels claudin 2 and claudin 3 suggesting that these two claudins may not be physiologically relevant for contributing to the barrier function of human conjunctival epithelial cells.

Claudins have a conserved PDZ domain through which they interact with the intracellular ZO molecules to provide an anchoring function [Krause et al., 2008; Campbell et al., 2017; Tsukita et al., 2019]. Since high glucose did not affect the protein expression of claudin-1, we next tested the possibility that high glucose may compromise the anchoring of claudin-1 by decreasing the levels of ZO proteins. Our data demonstrates the presence of mRNA transcripts and protein levels of all the three members of ZO family (i.e ZO1, 2 and 3) in the human conjunctival epithelial cells. Our data is in agreement with previous reports showing the presence of ZOs in human conjunctival epithelial cells [Yoshida et al., 2009]. However, exposure of human conjunctival epithelial cells to high glucose did not modulate the proteins levels of any of the three ZOs. On the other hand, high glucose at 15 mM, exactly like its effect on claudin-1, caused an increase in the gene expression of ZO1, 2, and 3 after 72 hours exposure.

Lastly, we tested the effect of high glucose exposure on occludin. Occludin is the protein present in the tight junctions that acts as a zipper to hold the adjacent cells [Krause
et al., 2008; Cummins, 2012]. Our data shows the presence of mRNA transcript and protein levels of occludin in human conjunctival epithelial cells. However, exposure of human conjunctival epithelial cells to high glucose had no impact on its protein levels. In a surprisingly consistent manner along with the claudin-1, ZO 1, 2 and 3, high glucose at 15 mM caused an increase in the occludin gene expression as well after 72-hour exposure.

Overall, our data suggests that a decrease in the protein expression of claudin-1, ZOs family and occludin is not the likely underlying cause to the observed high glucose exposure-mediated impairment of human conjunctival epithelial cells barrier function. Besides claudin-1, human conjunctival epithelial cells have also been reported to express other claudins such as claudin-4 and claudin-10 [Yoshida et al., 2009]. Therefore, it is possible that high glucose-mediated decrease in claudins-4 or 10 may contribute to the noted decrease in barrier function and may be addressed by the future studies. It is also possible that tight junction protein components, other than claudins, ZOs and occludin, may be impacted by high glucose and may be contributing to the compromised barrier function. Lastly, our study did not test the impact of high glucose of the phosphorylation status or confirmation changes of intercellular junction proteins. Such alteration can also contribute to high glucose-mediated compromise of barrier function with causing any decrease in the protein levels. An observed increase in the gene expression of the tight junction proteins as observed in the present study may be a compensatory cellular response to preserve the barrier functions and to partial circumvent the damaging effect of high glucose exposure. This hypothesis is especially supported by the fact the observed increase was only observed at 15 mM glucose and was a delayed response.
This effect on gene expression is likely due to the cellular osmotic stress caused by glucose since mannitol at the same concentration also caused the similar increase after 72 hours exposure. On the other hand, detrimental effect of 30 mM glucose or mannitol may be too high to overwhelm any compensatory cellular response.

Membrane-tethered mucins along with galectin form ocular surface glycocalyx which together with tight junction provide additional barrier functions and keeps the eye surface hydrated and lubricated [Mantelli et al., 2013]. Previous studies in our lab have demonstrated that high glucose can adversely affect the expression of membrane-tethered mucins in human corneal and conjunctival epithelial cells [Unpublished data]. Therefore, we further tested the effect of diabetes mellitus on glycocalyx using a mouse model of type I diabetes mellitus. In agreement with our in vitro data, we notice that type I diabetes mellitus caused a significant decrease in the area of corneal glycocalyx. This decrease in glycocalyx was accompanied by a concomitant reduction in the volume of tear film suggesting the onset of dry eye. It is interesting to notice that a partial recovery in glycocalyx area and tear film volume was observed at week 2 after the onset of diabetes as compared to week one. This partial recovery suggests that increased tear secretion from other lacrimal functional units such as meibomian gland or lacrimal gland may try to compensate for the diabetes-mediated damage to the glycocalyx. It is likely that the persistent hyperglycemia may continue to cause further damage to the glycocalyx and can also be detrimental to other parts of lacrimal functional units, thus eventually causing more severe dry eye. To answer these questions, we need to perform longer duration studies for 4-8 weeks after the induction of diabetes and such studies are currently underway in our lab.
CHAPTER VI
CONCLUSION
The results of our data demonstrate that exposure of conjunctival epithelial cells to high glucose causes a decrease in trans-epithelial electrical resistance, suggesting that high glucose causes impairment of conjunctival epithelial barrier function. However, exposure of conjunctival epithelial cells to high glucose did not impact cellular migration at various time points. Further, our data also demonstrated that exposure of conjunctival epithelial cells to high glucose did not cause any change in protein expression of claudin-1, ZO-1, ZO-2, ZO-3, and occludin suggesting that a decrease in tight junction proteins may not account for the impairment of barrier function. On the other hand, exposure of conjunctival epithelial cells to high glucose caused an increase in gene expression of claudin-1, ZO-1, ZO-2, ZO-3, and occludin at 72 hours suggesting a likely compensatory physiological response to counter the detrimental effects of high glucose on barrier functions. Animal studies using type I mouse model of diabetes mellitus showed a significant decrease in the tear film and presence of corneal epithelial defects, suggesting that diabetes mellitus cause tear film dysfunction which may underlie diabetes mellitus-associated corneal keratopathy. The tear film dysfunction and corneal keratopathy were accompanied by a concomitant decrease in the area of corneal glycocalyx.
## APPENDIX

<table>
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<th>MATERIALS</th>
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<td>Alexa 488 conjugated wheat germ agglutinin</td>
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BIBLIOGRAPHY


