THE ROLE OF DAMAGE ASSOCIATED MOLECULAR PATTERNS IN DRY EYE INFLAMMATION

By

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THESIS

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Abstract

Purpose: It has been shown that inflammation is an important part of dry eye disease (DED), which may lead to degradation of visual quality and the ocular surface. Toll-like receptors (TLR) have been shown to stimulate the production of dry eye associated inflammatory cytokines and matrix metalloproteinase (MMP). The purpose of this thesis was to determine if (1a) damage associated molecular patterns (DAMPs) which are thought to activate TLRs are increased on the ocular surface in dry eye subjects and to assess whether a low humidity environment (LHE) can modulate (1b) DAMP ocular surface levels (n=9 DED subjects) and (2) higher order aberrations (n=33 normal subjects).

Methods: Ocular surface disease index (OSDI), corneal and conjunctival staining, phenol red thread test (PRTT), osmolarity, sodium fluorescein tear break up time (TBUT), conjunctival erythemia, and/or meibomian gland expression were analyzed in each subject. The tear film (10-20µl) was collected and analyzed for HMGB-1 (ELISA) and HSP 27, 60, 70, 90 (Luminex Assay). Conjunctival impression cytology (CIC) samples were analyzed for cytokine and MMP mRNA or DAMP protein analysis. These data were collected under normal building temperature and humidity conditions (1) or following 2hr LHE (2a). To examine changes in visual performance, high and low contrast Log MAR visual acuity was measured and corneal topographies of the subject's right eye were measured using the Nidek OPD Scan III and the Topcon KR-1W before and after 1hr LHE (2b).

Results: (1a) Thirty normal (n=15) or DED (n=15) subjects qualified for the study. In the DED subjects, there was a statistically significant increase in OSDI, TBUT, corneal staining and conjunctival staining ($p \leq 0.05$). HMGB-1 was found to have significantly higher levels in the tear film of DED subjects compared to normals ($p \leq 0.05$) while there no difference in HSP-27, HSP-60, HSP-70, HSP-90α. In the CIC samples, there was no difference in HMGB-1, HSP-27, HSP-60 and HSP-90α, while HSP-70 levels were significantly decreased in DED subjects (1b). Following 2hr LHE, corneal staining was significantly increased and MMP-9 mRNA significantly upregulated in the conjunctiva by 2.4 ± 1.0 fold. There was no significant change in HMGB-1 or HSPs tear levels. There was no significant difference between visual acuity or third, fourth, and the third-sixth higher order aberrations using the KR-1W or the OPD-Scan III following LHE (2).

Conclusions: Dry eye subjects had higher levels of HMGB-1 in their tear film (1a) and LHE (2hrs) did not increase HMGB-1/HSPs levels despite additional corneal damage and increased MMP-9 mRNA expression in DED patients. (1b). LHE (1hr) did not increase HOA or alter visual acuity which may be a result of the short LHE duration (1hr vs 2hr) (2). These data suggest the DAMPs maybe involved in DED, but their ability to stimulate inflammation remains to be determined.

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List of Abbreviations

DED	Dry Eye Disease
HOS	Hyperosmolar Stress
TLR	Toll-Like Receptor
MMP	Matrix Metalloproteinase
IL	Interleukin
TNF	Tumor-Necrosis Factor
PAMPs	Pathogen Associated Molecular Patterns
DAMPs	Damage Associated Molecular Patterns
HMGB1	High Mobility Group Box-1
HSP	Heat Shock Proteins
LHE	Low Humidity Environment
CL	Contact Lens
RH	Relative Humidity
PRR	Pattern Recognition Receptors
TBUT	Tear Break Up Time
PRTT	Phenol-Red Thread Test
SS/ non-SS	Sjögren's syndrome/ non-Sjögren's syndrome
HOA	Higher Order Aberrations
TIR	Toll-IL1 Receptor

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Chapter 1: Introduction

Dry Eye Disease (DED) is a chronic, multifactorial ocular surface condition that affects 1.68 million men and 3.23 million women over the age of 50 in the United States with prevalence expected to increase as the population ages(1),(2),(3). Affecting one in four people who present to ophthalmic clinics in the US, DED is believed to be one of the most prevalent ocular surface conditions (4). Symptoms vary from mild to severe discomfort with one study finding severe dry eye to be as impactful to quality of life as severe angina or dialysis (5). Sufferers may experience burning, tearing, epiphora, or foreign body sensation, dryness, scratchiness and soreness. Yet, an estimated 43% of patients present with no symptoms at all, making diagnosis and clinical management of DED difficult (6)(7). Risk factors for DED include age, gender, contact lens (CL) wear, medication use, and irritation on waking, previous treatment for dry eye (8)(7). Oral medications play a significant role in DED. Hypertension medication, diuretics, antihistamines, oral steroids and antidepressants are all correlated with DED symptoms (8)(7)(2)(9). However not all medications lead to DED. For example, the risk for DED is decreased in patients using angiotensin converting enzyme inhibitors (8)(10). One risk factor for dry eye found was previous treatment for dry eye, which shows how ineffective treatments are for helping those with DED (7). The burden of DED is physical and economical costing our health care an estimated $3.84 billion annually and $55.4 billion to the US society overall making it a public health issue that cannot be ignored.(11).

Dry Eye Disease can be broken into two main categories: aqueous deficient dry eye and evaporative dry eye. Aqueous deficient dry eye can be divided in to Sjögren's syndrome dry eye and non-Sjögren's syndrome dry eye (12). Non-Sjögren's Syndrome

dry eye (Non-SS) stems from a problem with the lacrimal gland or signaling to the lacrimal gland without the presence of an autoimmune disease. This includes lacrimal gland deficiency, lacrimal gland block, reflex block, or a systemic medication induced decrease in tear production (13). The most severe form of aqueous deficient DED is due to Sjögren's syndrome (SS) which consists of autoimmune inflammation of the lacrimal gland (12). Sjögren's is an autoimmune disease more commonly found in females that presents with dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia). Since it is a slow progressing and chronic disease with symptoms that overlap many of the other autoimmune diseases, diagnosis of SS is often difficult and often requires collaborating with a rheumatologist. It is important to determine whether SS is primary SS or secondary SS since it is common for multiple autoimmune diseases to be present at once. If SS is present, other systemic complications may be presents including fatigue (70%), respiratory complications (10%), interstitial nephritis, cutaneous involvement, arthralgias, gastrointestinal manifestations that stem from dryness of the pharynx and esophagus, thyroid diseases (20%), and other neurological manifestations (2-60%) (12). SS sufferers are at an increased risk for lymphoma therefore careful monitoring is important (12). In order to differentiate aqueous deficient dry eye from evaporative the following tests should be performed: sodium fluorescein tear break-up time (TBUT), ocular surface staining, and Schirmer's test (12).

Evaporative dry eye can be caused by extrinsic or intrinsic factors (12). Intrinsic factors include abnormalities or disease states of the eyelid including meibomian gland dysfunction (MGD), decreased or incomplete blinking, or drug induced abnormalities of the eyelid (12). Extrinsic factors include contact lens wear, preservative interactions, or

ocular surface conditions such as allergies (12). Meibomian glands are holocrine sebaceous glands, meaning the entire cell and its contents are secreted, and are located along the margin of the upper and lower lid (14). They are responsible for excreting the lipid layer of the tear film which aides in reducing evaporation. Different environments effect the gland's production. When meibomian glands are exposed to desiccating stress, there is an increase in cell proliferation as well as an increase in lipid production but also consequently an increase in protein which has an effect on the quality of meibom expression (14). This buildup of protein may be what begins the MG disease state (14). Up to 47% of patients at an eye care professional's office are reported to have some form of MGD (15). In MGD, an anterior movement of the junction between keratinized skin and non-keratinized conjunctiva, or the mucocutaneous junction is seen (Marx line) (14). In normal eyes, Marx line is located posterior to the meibomian gland orifices towards the conjunctiva, however in MGD this line is found either on top of the gland or anterior to the gland (16). This shift in the position of the mucocutaneous junction, which is visible in humans by vital dye staining (NaFl and Lissamine Greeen) of Marx line, is strongly correlated to MGD (16).

1.1 Dry Eye Inflammation

The cornea and conjunctiva are covered by a pre-corneal tear film that contains proteins and nutrients to help nourish and protect the cornea. The tear film is described as a three-component structure; a thin layer of low polarity lipids, an aqueous layer, which composes about 80% of the tear volume and a mucin layer that helps to stabilize the tears on the eye. In a healthy subject, the tear film is constantly being produced then evaporates, is absorbed, or drains via the upper or lower punctum. This creates a fragile

dynamic that is integral in maintaining ocular health and its disruption leads to inflammation in three different ways. First, the decreased in production of tears means that there is a decrease in the innate anti-inflammatory factors, such as lactoferrin, found in tears (17). Secondly, there is an increase in many different types of pro-inflammatory cytokines such as interleukin-1 (IL)-1 along with inflammatory cells that invade the epithelium (17). Finally, there is an increase in latent pro-inflammatory cytokines that are present as a first defense for invading pathogens (17). Inflammation was not traditionally considered a component of dry eye until the US Food and Drug Administration approved the use of cyclosporine emulsion for the treatment of DED in 2002. With this approval, there was a shift in research that aimed at looking at the inflammatory pathway and its role in DED.

It has been shown that part of the cycle of inflammation comes from hyperosmolar stress (HOS). In DED, a decrease in tear production, or increase in tear film evaporation, leads to a tear film with a decreased aqueous component, leading to an increased concentration of salts and creating an environment of HOS for the corneal epithelium (18). This increase in osmolarity is shown in measurements of tear meniscus osmolarity. Typical osmolarity for a normal subject is around 302 mOsM/kg on average, however, the osmolarity in dry eye individuals is above 316 mOsM/kg and is reported to fluctuate substantially between blinks (19),(20),(18). This fluctuation in osmolarity between blinks has been suggested to be the predominate cause of the burning sensation often reported by dry eye suffers (18). One study asked subjects to keep their eye open as long as possible and rate discomfort(18). They then asked subjects to report discomfort felt after instillation of hyperosmolar drops ranging from 300-1000 mOsM/kg.

Sensations of discomfort were first felt at 450-460 mOsM/kg but were not reported to be as uncomfortable as holding their eye open until 800-900 mOsM/kg (18). This suggests that tear break up creates transient spikes of hyperosmolar tear film which may be what drives discomfort (18). Levels of HOS this high can be damaging to the corneal epithelium and have shown to be correlated with increased levels of pro-inflammatory cytokines and matrix metalloproteinase (MMPs) in both mice and human corneal epithelial cells (21),(22),(23).

Cytokines are a group of proteins that are used in intracellular communication (24). Although capable of eliciting a variety of responses, cytokines are most commonly known for their role in innate immunity (24). Interleukins are a subset of cytokines that are produced and interact with many different cell types (25). Currently, 15 different types of interleukins have been identified and many, such as IL-1, IL-6, and IL-8, are responsible for stimulating inflammation (24)(25). Early cytokines such as IL-1, IL-2, IL-4 and TNF- α typically are responsible for activating cytokines, such as IL-6, IL-8 and tumor necrosis factor (TNF)- α, further down the inflammatory pathway (26)(27). IL-1, -6, and -8 and TNF-α are known to be elevated in subjects with DED and are responsible for B cell antibody production, T-cell differentiation, and many other functions (27)(26). Furthermore, elevated levels interleukins are associated with clinical symptoms seen with DED including a direct correlation between corneal fluorescein staining with IL-1 and IL-8 (28).

Matrix metalloproteinase (MMPs) are zinc-dependent extracellular endoproteinases that play a central role in tissue remodeling during inflammation, wound healing, and angiogenesis (29). On the ocular surface, MMP-9 is known to cleave

epithelial basement membrane and tight junction proteins which are responsible for creating the ocular surface barrier for invading pathogens (30) (31). In a non-diseased state, MMP-9 is responsible for corneal epithelium turnover which is supported by vitamin A deficient mice having lower levels of MMP-9 and lower levels of epithelial exfoliation (32)(33). In contrast, excess levels of MMPs have been shown to promote inflammation by damaging connective tissue in diseases such as arthritis and tissue ulcerations (34). MMP-9 is elevated in DED associated diseases such as MGD and Sjögren's syndrome and levels of MMP-9 correlate with the severity of the disease state (30)(27). When mice were subjected to a topical application of balanced salt solution four times per day, there was a significant increase in MMP-9 by the corneal epithelium as well as significant increase in the tear fluid (35). If MMP-9 knockout mice are subjected to HOS, they have less corneal surface damage then their wild type counterparts, an effect that is negated after MMP-9 is applied to the cornea (30). MMPs not only increase inflammation by remodeling, they are known activators of pro-inflammatory factors such as IL-1, TNF-α and latent pro-MMPs (23). When mice are subjected to experimental dry eye there is an increase in MMP-9 as well as increased expression of IL-1α, IL-1β and TNF-α (35)(36). Both corticosteroids and doxycycline suppress this expression showing how DED is relieved with their use (35). Activation of the pro-inflammatory cytokines by MMP-9 lead to a further increase in MMPs. One study showed that MMP-9 levels increase in response to TNF-α in salivary gland acinar cells suggesting TNF-α is responsible for the activation of MMP-9 (37). Furthermore, other pro-inflammatory cytokines such as IL-1, IL-6, are known to further activate MMPs, leading to further tissue degradation, and creating the chronic cycle of inflammation seen in DED

sufferers(34). The role of MMP-9 and IL-1 in DED has been suggested due to highest levels of MMP-9 and IL-1 in severe DED sufferers(27). Yet, the mechanism by which pro-inflammatory cytokines and MMPs are up-regulated in DED has not been established.

One proposed mechanism of pro-inflammatory cytokine and MMP up-regulation is via toll-like-receptor (TLR) activation. TLRs are transmembrane proteins found in most multicellular organisms that are an important component of innate immunity (38). They are located on the cell surface (TLR-1, -2, -4-6) or within the cell on intracellular vesicles (TLR-3, -7-9). There are currently ten known human TLRs and thirteen murine TLRs, which are capable of mediating activation of dendritic cells, macrophages, B cells, T cells and other antigen-presenting cells (39). TLRs act as a type of pattern recognition receptor (PRR) and, when activated, up-regulate expression of inflammatory cytokines such as IL-1, IL-6, and IL-8 and aide in the development of antigen-specific adaptive immunity (38) (40). TLR-2, -4, -5 and -9 have all been shown to be present in human corneal epithelial cells and are associated with corneal inflammation and ocular surface disorders (41)(42)(43)(44). All of the above TLRs, and all known TLRs except TLR-3, are at least partially dependent on the adaptor protein myeloid differentiation factor 88 (MyD88), with TLR-4 having both a MyD88 dependent and MyD88 independent pathway which involves Toll-IL1 Receptor (TIR) (42). Many different cells express TLRs on the human and mouse cornea and conjunctiva and their expression is modulated by hyperosmolar stress or experimental dry eye conditions (40)(45)(46)(43)(47)(48). TLR4 seems to play a major role in DED inflammation. TLR4 was one of the first PRR discovered and was originally known for its recognition of bacterial lipopolysaccharide,

which can cause septic shock (49). When primary human corneal epithelial cells were grown under HOS, there was an 8.18 fold increase in TLR4 protein expression which was supported by a 9.70 fold increase in mRNA expression (43). Further, when TLR4 was inhibited, there was a significant decrease in corneal staining and expression of TNFα and IL-1ß in a mouse model of dry eye (50). TLR4 as well as TLR5 have been shown to be up-regulated in a Sjögren's syndrome mouse model as well as many inflammatory cytokines (51). When TLRs are activated, there is an increase in both cytokines and MMP-9 mRNA and protein which may lead to the chronic cycle of inflammation so often seen in DED (47).

TLRs are activated by a diverse array of pathogen associated molecular patterns (PAMPs) found on bacteria, viruses, parasites and fungi, and also by endogenous damage associated molecular patterns (DAMPs) (46)(52)(53)(48). PAMPs that activate TLRs include lipids, lipoproteins, proteins and nucleic acids but PAMPs are not necessarily present in DED (54). This is where DAMPs may play a role. DAMPs are typically expressed to signal stress. DAMPs are found in the plasma membranes, nucleus, endoplasmic reticulum, cytosol and mitochondria of cells but are not recognized by the human immune system due to their protection by the cellular membrane (55) (56). However after necrosis, apoptosis, or active expression the cellular membrane deteriorates and/or the intracellular material is released (55). The DAMPs then go on to stimulate PRR including many of the same ones that are stimulated by PAMPs, showing the similarity between the two proteins (56). DAMPs can stimulate or modulate the immune system by affecting antigen presenting cells, eosinophils, mast cells, and neutrophils (56). This stimulation of the innate immune system leads to a more robust

activation of the adaptive immune system (57). Examples of DAMPs include high-mobility group box-1 (HMGB1), S100A proteins, and heat shock proteins (HSPs) (46)(56)(58). These sub-groups are classified based on localization or release (56). HMGB1 is traditionally thought to be released or secreted extracellularly while HSPs are enriched on the plasma membrane (56). Both HMGB1 and S100 have been shown to be released extracellularly after trauma leading to sterile inflammation while HSPs are found in conditions with low grade inflammation such as atherosclerosis (56)(59)(60)(61).

1.2 DAMPs in DED

S100 proteins consist of small calcium binding dimeric proteins that have a wide range of physiological functions including wound healing, oxidative stress, apoptosis, phagocyte recruitment and neutrophil migration and degranulation (58). The functions of the S100A family on the ocular surface can be categorized into three main groups; structural proteins, transcription factors, and immune modulators (58). Many of the S100A proteins (S100A1-A12) are expressed in human corneal and limbal epithelial cells, only S100A8 and A9 were found only at the surface of the cells suggesting a role in ocular inflammation (58). However, studies have shown that S100A8, A9, and A12 are pro-inflammatory proteins (62). This led researchers to investigate S100A family of proteins in relation to dry eye. To date, the only DAMPs increased with DED are the S100A family (63). Of this group, S100A8, A9, A4 and A11 were shown to be significantly upregulated in DED(63). Further analysis showed S100A8 and A9 levels correlated with severity of DED and inflammation with a 96% accuracy, 91% sensitivity and 90% specificity for detecting the disease when used in a 4-protein biomarker panel (63). There are multiple possibilities of how the S100A family is involved in the

pathogenesis of DED. When looking at S100A4, S100A8 and A9, S100A4 is capable of stimulating MMPs, while S100A8 and A9 are capable of activating stress-signaling pathways in DED (58). Since S100A8 and A9 are increased in Sjögren's Syndrome, we aimed to look if other DAMPs (HMGB-1 and HSPs) were increased in DED (64).

1.3 Treating DED Inflammation

Corticosteroids were the first option of treating dry eye inflammation. Their mechanism of action was two fold; interference with the glucocorticoid receptor mediated pathways as well as decreasing expression of proinflammatory cytokine and chemokine production and other proinflammatory genes (17). Corticosteroid use is a rapid and effective treatment for even severe keratoconjunctivitis sicca, yet they are more efficacious in short term use (2-4 weeks) since long term use has many potential side effects including toxicity, increased intraocular pressure, and cataracts (17).

Cyclosporine A (CsA) was the second approved topical drug for the treatment of DED inflammation. A safer drug for long term use, it's mechanism of action includes preventing activation of transcription factors for T-cell activation, cytokine production, and apoptosis inhibition by blocking the mitochondrial permeability transition pore (17). Treatment effects take longer than corticosteroids (about 1 month with increase until 6-month period) and no side effects were noted except burning after installation in 17% of subjects (17).

Other treatments aimed at targeting DED inflammation include, but are not limited to, the following: autologous serum which uses innate anti-inflammatory factors in order to quell the inflammatory pathway in DED and tetracyclines such as doxycycline

where a decrease in production of IL-1 and MMPs are observed, especially in those suffering from ocular rosacea (17).

1.4 Low-Humidity Environment and DED

It is well known that DED sufferers are more symptomatic under low humidity environments due to the decrease in relative humidity (RH) causing a significant increase in tear evaporation rate and tear production, and decrease in noninvasive tear break-up time (65). Furthermore sufferers are less symptomatic when exposed to an increase in RH. When 30 DED subjects were instructed to wear moisture chamber goggles for 90 minutes, there was steady improvement in comfort, increase in the tear meniscus, noninvasive TBUT and lipid layer until the 60 min mark (66). In fact, only a 10% change in RH causes a statistically significant increase in evaporation rates in aqueous deficient dry eye subjects (67). This effect of RH on ocular surface can be significant especially since it is common to spend most of a life in artificially created environments such as office buildings, airplanes, and automobiles.

Eye irritation such as redness, burning, and itching are commonly reported by people in visually demanding jobs as well as across the United States (68). Yet, effects of irritation extend past comfort and can affect the quality of life as well as overall ocular health (68). It has been noted that dry eye sufferers experience a decrease in visual acuity caused by changes to the precorneal tear film. Even minimally symptomatic subjects that are exposed to a 19% RH, versus the typical 30-40% found inside homes, for two hours will have negative impacts on the ocular surface (69)(70). It is believed that this decrease in visual acuity is due to an increase in higher order aberrations (HOA) as the tear film begins to thin (71). This increase in HOAs was shown when normal and DED subjects'

HOAs were serially measured 10 seconds post-blink (71). DED subjects not only had a significant increase in HOAs but the degree of change directly correlated with symptoms measured by the OSDI (71). This suggests that corneal topography may be an objective way to measure severity of DED (71).

An increase in HOAs and decrease in VA is not the only negative effect seen on the ocular surface after exposure to desiccating stress. One study looked at the inflammatory response in Sjögren Syndrome subjects before and after exposure to 5% RH and air flow for 2 hours (72). They found a significant increase in tear osmolarity, as well as a significant increase in IL-6, IL-8 and MMP-9 (72). These changes happened in normal and non-Sjögren Syndrome mild to moderate DED subjects as well. When subjected to 5% RH for two hours both groups had a significant increase in MMP-9 and the asymptomatic group showed a significant increase in IL-6 (73). This shows how quickly the ocular surface can be negatively impacted and how it can deteriorate when subjected to a RH that may be similar to those experienced in a building or hospital situation (74)(75).

1.5 General Hypothesis

For this thesis the general hypothesis is that DED and dry eye stress increases DAMPs and HOAs.

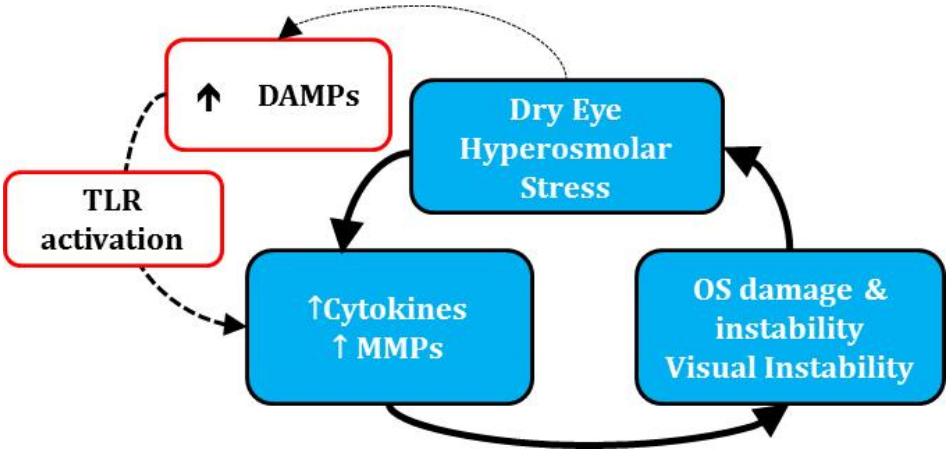


Figure 1.1. DED and dry eye stress increases DAMPs which lead to OS damage and instability causing visual instability and therefore an increase in HOAs.

1.6 Specific Aims

There are three specific aims for this thesis:

1) To quantitate the level of heat shock proteins (HSP) and high mobility group box-1 (HMGB-1) on the ocular surface in dry eye and normal subjects (1A) and in dry eye subjects before and after a 2 hour exposure to a low humidity environment (1B)

2) To quantitate higher order aberrations and visual acuity before and after a 1 hour exposure to a low humidity environment using the Topcon KR-1W and Nidek OPD Scan III

Chapter 2: The Role of Damage Associated Molecular Patterns in Dry Eye Inflammation under Normal and Low Humidity Exposure

2.1 Introduction

A three-component tear film, rich with a variety of proteins, covers the cornea and conjunctiva, provides nourishment, wards off infection, and creates a smooth refractive

surface for the eye. In DED a decrease in tear production, or increase in tear film evaporation, disrupts the ocular surface causing damage and symptoms of discomfort in sufferers. In 2007, The International Dry Eye Workshop (DEWS) defined dry eye as "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface"(76). The debilitating nature of symptoms are a public health issue that cannot be ignored and effects an estimated 14.4% of the general US population and 25% of patients at ophthalmic clinics, with numbers expected to rise in our aging population (9)(4). Although traditionally DED was diagnosed based on signs and symptoms, studies have shown that 43% subjects with objective evidence of DED are asymptomatic, leaving many undetected and untreated (6). This puts clinicians at a disadvantage since considerable variability in presentation and lack of consensus in objective testing make diagnosis and treatment difficult.

Inflammation is a known component of dry eye, yet the mechanism that drives the inflammatory response remains unclear. It is known that the tear film of a DED subject has higher osmolarity than that of a normal subject, which is believed to be due to the decrease in aqueous component from either increased evaporation or decreased tear production (21). This creates an environment of HOS for the corneal epithelium. When evaluating the effect of HOS to the epithelium, research correlated HOS to increased levels of pro-inflammatory cytokines and MMPs in both mice and humans (77)(23)(78). Pro-inflammatory cytokines such as IL-1 are known to activate MMPs that have been

shown to promote inflammation by destroying connective tissue (29). Yet how HOS stimulates the increase in cytokines and MMPs is not understood.

One proposed mechanism is that HOS activates TLRs. The latter have been found on human corneal and conjunctival cells and when cultured under HOS, primary human corneal epithelial cells have been shown to modulate TLR expression (43). TLRs are activated by PAMPs as well as endogenous DAMPs such as S100, HSPs, and HMGB-1 (52)(46)(79). A recent study which looked at the tear film biomarkers in subjects with DED found that there was a statistically significant increase in S100A8, A9, A4, and A11 when compared to normal subjects and a correlation between S100A8 and S100A9 to the severity of the disease (80). Since S100A proteins were found to be elevated in DED and S100A8/9, HMGB-1, and HSPs are commonly elevated in autoimmune diseases that are associated with DED such as rheumatoid arthritis and lupus, our study set out to determine if there were elevated levels of HSP and HMGB-1 in the tear film. Specifically this study examined if there was an increase in HSP and HMGB-1 in the conjunctiva and tear film of DED subjects compared to age/gender-matched normal subjects (Part 1A) as well as to evaluate whether there is an increase in DAMPs in dry eye subjects before and after a two hour exposure to a low humidity environment (Part 1B).

2.2 Methods

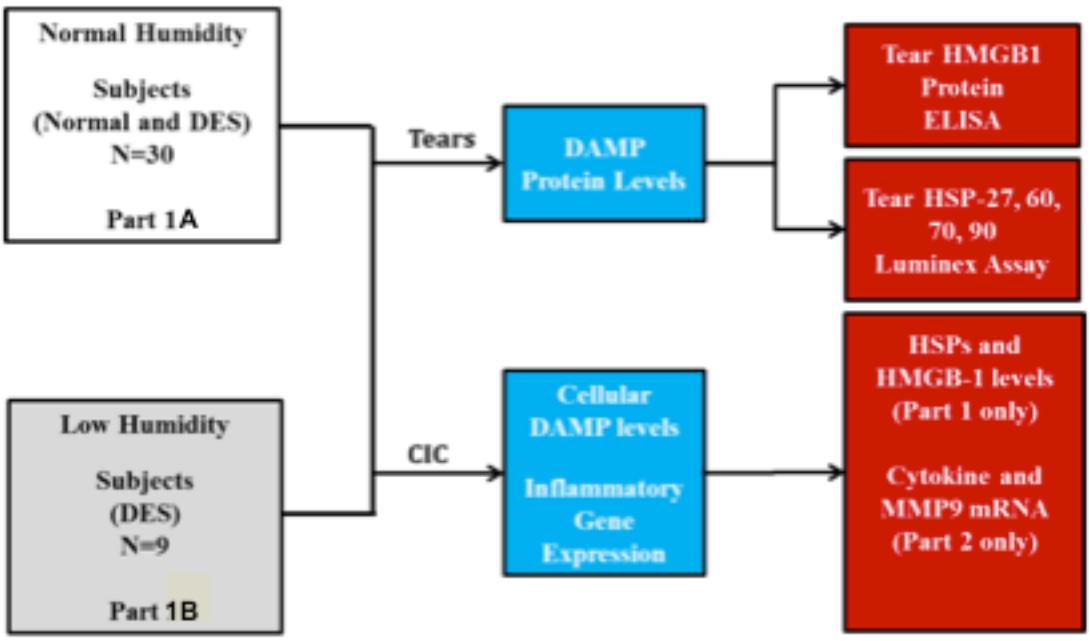


Figure 2.1: A flow diagram of the sample collection for examining DAMPs (HSP and HMGB-1) levels and inflammatory gene expression during normal humidity and following two-hour low humidity exposure.

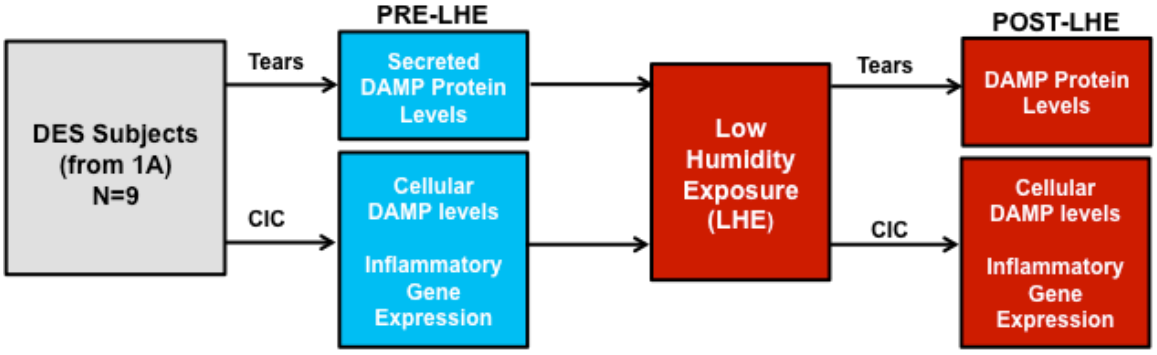


Figure 2.2: A flow diagram explaining the process of study 1B.

Subjects

All procedures were in accordance with the Tenets of the Declaration of Helsinki and were approved by the University of Houston's Institutional Review Board. Informed consent was thoroughly reviewed with each subject and written consent was obtained prior to enrollment into the study. All subject data was collected at the University of Houston. Forty-five normal and dry eye subjects were enrolled in the study. A clinical examination was taken under normal building humidity (78^{o}F and 40-45% humidity) (1A). Nine of the dry eye subjects were recruited for a smaller pilot study for clinical examination under normal building humidity and after dry arid humidity (72^{o}F and <10% humidity) (1B). All subjects recruited were over the age of 18 and were currently not using any topical medications other than rewetting drops. Exclusion criteria for both groups included anyone pregnant or nursing, contact lens wear, any ocular surgeries within the previous six months, any active eye diseases including ocular allergies but excluding dry eye disease, anyone currently taking doxycycline, Restasis, or steroids, and individuals with known allergy or sensitivity to fluorescein, lissamine green and topical anesthetics. All subjects were instructed not to instill artificial tears within the 2 hours prior to the appointment.

Normal and Dry Eye Classification

Subjects must have reported a symptom score greater or equal to 12 on the OSDI questionnaire and have one eye exceed the normal thresholds of three of the four following objective grading measures to be considered dry eye; tear production, corneal staining, conjunctival staining, and tear film stability. This classification scheme was derived based on a modified version of the classification schemes presented in papers by

Sambursky et al. and Sullivan et al. (81)(6) Overall, seven objective signs were analyzed for each subject.

a. Tear film osmolarity (Osm >308 mOsm/L=DED, TearLab Osmolarity System; TearLab Corporation, San Diego, CA) (82).
b. Tear production (phenol red thread test (PRTT≤10mm = DED)
c. Corneal and conjunctival epithelial staining (graded 0-3 in each of the 5 part grids as per CCLRU (83) with fluorescein and lissamine green, respectively; Cornea and Contact Lens Research Unit. Staining ≥4 in the combined 5 quadrants was considered DED, MGD Workshop Report),
d. Conjunctival erythemia (injection ≥ mild = DED; Dry Eye Workshop (DEWS).
e. Tear film debris (debris ≥ mild = DED; DEWS)
f. Meibomian glands (MG)/lids health (MG or lid variability present= DED; DEWS)
g. Tear film stability (fluorescein tear break up time, Dry Eye Test; Akorn, Chicago, IL DES= <10 secs).

Low-humidity exposure (LHE) in Part 1B

Nine DED subjects from study 1A were recruited and exposed to low humidity conditions for 2 hours within the environmental chamber inside The Ocular Surface Institute located at the University of Houston, College of Optometry. Temperature ranged from 74.3-74.8^{o}F (mean 74.5 ^{o}F) while humidity ranged from 4.2-4.3%. Temperature and humidity were measured and recorded every 15 minutes. Additionally airflow was

increased using an individual fan mounted on the wall. Subjects were seated so that the fan was not directly pointed at their face but rather parallel to where they sat. During this time subjects were instructed to not use any lubricating eye drops or sleep. Drinkable fluids were allowed yet the amount was monitored since the subjects were not allowed to leave during the two hours of exposure to use the bathroom. During that time participants watched Netflix on a light-emitting diode television monitor. Subjects were evaluated based on the following parameters pre- and post-LHE.

Clinical Examination

Study 1A

A slit lamp was used to measure the conjunctival bulbar hyperemia and tear film debris based on the DEWS report and the Cornea and Contact Lens Research Unit (CCLRU) grading schemes. Meibomian gland inspissation and expression were graded in each eye based on the International Workshop on Meibomian Gland Dysfuntion. Lisamine green (GreenGlo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, California, USA) and sodium fluorescein strips (BioGlo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, California, USA) were wetted with saline and placed in the inferior fornix of each eye. Corneal and conjunctiva examination was video recorded and staining grade was confirmed by a masked observer. Corneal staining was graded using a cobalt-blue filter and a yellow Wratten no. 12 filter (Eastman Kodak, Rochester, New York, USA). The cornea was divided into central, superior, nasal, inferior, and temporal areas, as per the NEI workshop, and the corneal staining in each section was graded 0-4 based on the CCLRU scale (13). Similarly, the bulbar conjunctiva was divided to temporal-central,

temporal-superior, and temporal-inferior as well as nasal-central, nasal-superior, and nasal-inferior and conjunctival staining was graded 0-3 based on the Efron scale (84). For invasive tear break-up time, the subject was instructed to blink three times and then to refrain from blinking. Time was measured from the end of the last blink until the appearance of the first dry spot. This procedure was repeated three times and then an average was taken.

Study 1B

All subjects were examined before and after low humidity exposure. The second examination was performed while the subject remained in the low humidity chamber. The following clinical signs were graded as previously described: conjunctival bulbar hyperemia, tear film debris, meibomian gland inspissation and expression. A lissamine green and sodium fluorescein strip was individually placed in 200 µl of non-preserved saline and was allowed to soak for 1 minute. After the minute, the strip was immediately removed and the solution was capped to prevent any evaporation. Then 15µl of each solution was micropipetted into the inferior fornix of each eye. Corneal staining and conjunctival staining and invasive tear break up time was graded as previously mentioned. The subject's right and left eye were randomized using a random number generator and the results determined which eye the clinician first measured PRTT and which eye conjunctival impression cytology was performed before and after the chamber.

Conjunctival Impression Cytology

A single drop of 0.5% proparacaine hydrochloride anesthetic was instilled onto each eye. A sterile polyether sulfone membrane was cut into 8 equal pieces. (Supor® Membrane

Disc Filters, 0.2um pore size, 25mm, plain, PALL Life Sciences) Two of the eight pieces were used and samples were collected from the medial or lateral bulbar conjunctiva and were stored in either RLT lysis buffer (Qiagen) for RNA analysis or in PBS with 0.2% Tween with protease inhibitor (Complete Mini, Roche Diagnostics) for protein analysis using luminex or ELISA.

Quantitative RT-PCR

Total RNA from CIC membrane samples was extracted using the RNeasy Mini Kit (Qiagen). RT-PCR was used to quantitate relative mRNA expression of IL-6, IL-8, MMP-9. RP2 was used as housekeeping gene. cDNA was generated using iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD) using equal amounts of RNA. Samples containing no reverse transcriptase or water in place of RNA (no template control) served as negative controls. Ten microliters of cDNA reactions were analyzed using a CFX96™ Real-Time System (BIO-RAD). Real-time PCR amplification of cDNA was performed with SsoAdvanced™SYBR® Green Supermix (BIO-RAD, Hercules, CA) using PrimePCR™SYBR® Green specific primers for human IL-6, IL-8, MMP-9 and RP2. For each gene, samples were processed in triplicate and amplified gene products were normalized to RP2. The mean relative quantity of IL-6, IL-8 and MMP-9 was calculated then by use of the CFX Manager™ software

HMGB-1 ELISA and HSP Luminex Assays

Total tear and CIC protein was quantitated and processed using multibead Luminex assay for HSP assay (HSP27, HSP27pS78, HSP60, HSP70, HSP90α) and an ELISA to quantitated HMGB-1 (IBL International) as per manufactures protocol directions.

Statistical Analysis

Data analyses were conducted with STATA (StataCorp; College Station, TX). Comparison of normal and dry eye subject's clinical findings were compared using a Paired Student T-Test. Both intracellular and extracellular (tear) levels of DAMP's were compared using Mann-Whitney nonparametric test.

2.3 Results

Normal and DED Subjects Recruited

Of the forty-five subjects, fourteen were males and thirty-one were females with an average overall age of 57.24 ± 9.43. When categorizing the subjects, 15 qualified as normal and 15 qualified as DED subjects. The other 15 subjects showed either signs or symptoms but not both and therefore were not classified based on ambiguity. The subjects had a mean age ± SD for the normal and DED groups of 58.4 ± 9.6 and 56.3 ± 10.4 respectively. Of the normal subject group 60% were female while 66.7% were female in the DED group. The mean age ± SD of the nine dry eye subjects exposed to LWH study was 60.56 ± 12.86 years (Part 1B). Of the nine subjects 66.7% were female.

Normal and DED Subject Clinical Objective Measurements: Part 1A

When comparing clinical scores for normal and DED groups, there was a statistically significant difference between normal and DED subjects for OSDI, TBUT, corneal staining and conjunctival staining (Table 2.1). Compared to normal age-matched subjects, the DED subjects had significantly higher OSDI score (27.0 ± 14.3 vs. 14.3 ± 8.47), more corneal staining (5.13 ± 4.75 vs. 1.83 ± 2.37), and more conjunctival staining (7.57 ± 3.83 vs. 3.60 ± 2.65). There was no significant difference in PRTT for the normal and DED group (25.8 ± 5.12 vs 23.8 ± 7.98 respectively) These data show that there was a significant difference in clinical objective measurements between the normal and DED subjects.

DED Subject Clinical Objective Measurements pre- and post-LHE: Part 1B

When comparing the clinical signs pre- and post-LHE, only corneal staining was significantly ($p \leq 0.005$) increased (Table 2.2). Following LHE (2hrs) the corneal staining almost doubled and increased from 2.22 ± 1.82 (pre-LHE) to 4.06 ±2.10 (post-LHE). There was no significant difference found in PRTT, 20.9 ± 8.22mm (pre-LHE) to 22.9 ± 8.22mm (post-LHE), tear break-up time 4.04 ± 2.60s (pre-LHE) to 3.44 ± 1.37s (post-LHE), tear film osmolarity 309.6 ± 5.42 (pre-LHE) to 306.9 ± 12.57 mOsM/Kg (post-LHE) or conjunctival staining 3.22 ± 3.27 (pre-LHE) to 4.72 ± 4.30 (post-LHE). These data suggest that 2hrs of LHE can results in corneal damage while subjects paradoxically maintain their tear film quality.

Normal and DED Subject Tear Film DAMPs Levels: Part 1A

In order to determine if DAMPs were increased in the tear film of DED subjects compared to normal, the levels of HMGB-1 and HSP were analyzed using the ELISA and Luminex assay respectively. HMGB-1 was found to have significantly (p-value=0.007) higher levels in the tear film of DED subjects (18.31 ± 35.11 ng/ml) compared to a normal subjects (1.55 ± 3.96 ng/ml) while no difference was found between DED and normal subjects for HSP-27, HSP-60, HSP-70, or HSP-90α (Table 2.3 and Figure 2.2). These data suggest that HMGB-1 may play a role in the inflammatory pathway of DED, potentially by stimulating TLRs on the ocular surface.

Normal and DED Subject Conjunctival DAMPs Levels: Part 1A

To determine if there was an up-regulation of DAMP production within the cell, impression cytology samples were taken from the nasal and temporal bulbar conjunctiva of each eye. There was no difference in HMGB-1, HSP-27, HSP-60, or HSP-90α (p-value= 0.105 to 0.711) (Table 2.4) levels between the normal and DED subjects. There was a modest, but significant, decrease in HSP-70 (p-value =0.038) in DED patients (804.8 ± 602.8 MFI) compared to normal controls (1315.4 ± 909.58 MFI). These data suggests that HMGB-1 is secreted into the tears after being produced by the cell or maybe a result of passive release following cell death from ocular surface damage.

DED Subjects Tear Film DAMPs Levels Following LHE: Study 1B

Since a decrease in relative humidity is known to affect the tear film as well as signs and symptoms of dryness is DED, the levels of HMGB-1, HSP-27, HSP-60, HSP-70, HSP-

90α in the tear film were analyzed using the ELISA and Luminex assay respectively before and after LHE.

There was no significant increase in either HMGB-1 (p-value=0.4801) or the HSPs (p-value=0.2236 to 0.4404) after being subjected to the LHE for 2 hours (Table 2.5). These data suggest that either 1) DAMPs take longer than two hours to be produced or 2) DAMPs in DED subjects are already up-regulated before exposure to a LHE are not affected by short term changes to relative humidity 3) there is an increase in DAMPs levels but the overall levels are still below our threshold of detection (Figure 2.3).

mRNA Expression of IL-6, IL-8 and MMP-9 in the Conjunctiva Following LHE: Study1B

In order to determine whether there was an increase in intracellular levels of inflammatory cytokines and MMP-9 in the DED subjects before and after exposure to the LHE for 2 hours, CIC samples of the bulbar conjunctiva were taken from nasal and temporal locations (1 sample on each side) from either eye (determined at random) before exposure and then from the fellow eye after exposure to LHE significantly ($p \leq 0.05$) upregulated MMP-9 mRNA expression by 2.4 ± 1.0 fold. No significant increase in IL-6 or IL-8 was found. These data suggest that there was a significant increase in ocular surface inflammation following the LHE and correlates with the increase in corneal staining seen before and after exposure (Figure 2.4)

DED Subjects Tear Film HMGB-1 between study 1A and 1B

Since study 1B recruited previously seen DED subjects from 1A, the levels of HMGB-1 were compared from the first study to the second. There was a statistically significant

decrease (p-value: 0.0047) in HMGB-1 between study 1A and study 1B. This shows how large the variation of DAMPs are even within the same subjects.

2.4 Discussion

DED has long been one of the leading causes for a person to seek an eye care professional but often times patient symptoms do not match clinical signs. Although new technologies are coming to market every day, many of the treatments are palliative and few are capable of being used for the long term. Therefore, understanding the inflammatory pathway is vital so that more treatment options can be created and better targeted for DED. Studies have shown that DED leads to HOS that causes an increase in MMPs and inflammatory cytokines, but the pathogenesis behind this process is still unknown. Our proposed mechanism is that HOS leads to an increase in DAMPs which then are capable of activating TLRs. DAMPs are found in inflammatory conditions such as rheumatoid arthritis and transplant rejection, many of which are associated with dry eye (85)(86). Yet, few studies have looked at DAMPs in the tear film. Studies looking at biomarkers for DED have found a significant increase in the S100A family of proteins in DED subjects compared to normal subjects and Zhou et al.(63) found a positive correlation in DED severity and S100A8 and A9 (80)(87) but other DAMPs have not been found to be increased. Since the S100A family A8 and S100 A9 are found to be elevated in autoimmune diseases that are closely correlated to DED, this study aimed to determine if there was an increase in HSPs and HMGB-1, two other DAMPs found in the same autoimmune diseases as the S100A family.

When comparing levels of HMGB-1 and HSP-27, HSP-60, HSP-70 and HSP-90α, only HMGB-1 had a significant increase in DED versus normal subjects. This increase

was found only with extracellular/secreted proteins located in the tears and was not present intracellularly in the CIC samples. Although the exact source or mechanism for release is not well understood, the presence of HMGB-1 and other DAMPs in the tear film may set up a chronic cycle of inflammation by the production of cytokines and MMPs via TLR activation.

Among the seven objective signs evaluated, only corneal or conjunctival staining showed a slight trend when compared to HMGB-1. However, correlation of HMGB-1 to corneal and conjunctival staining was minimal (R^2= 0.0477 and 0.1835 respectively (Figure 2.5 and 2.6). None of the HSPs showed a trend for any of the objective signs showing DED inflammation may not be properly quantified based on signs alone.

When looking at changes in DED signs and symptoms after exposure to a low humidity environment, one study found that there was an increase in MMP-9 and IL-6 after a two hour exposure to 5% relative humidity (73). Since there is an increase in cytokines in DED subjects after a two hour exposure to LHE, it was speculated that there may be an increase in DAMPs in DED subjects after a two hour exposure to a LHE (73).

One limitation of this study was the DED classification scheme. A difficulty that clinicians frequently face is that DED signs and symptoms are often in disagreement with one another making diagnosis and treatment very difficult. After a review of the literature, there was a plethora of classification schemes, however many focused on either signs or symptoms alone which leaves room for debate on whether subjects are really DED or not. This study wanted to minimize the possibility that subjects were misclassified, which was complicated by the fact that most subjects were mild to moderate DED making classification even more difficult. It was determined that a subject

needed to have both symptoms and multiple signs or neither signs and symptoms in order to be classified as DED or normal respectively, which resulted in the loss of 15 subjects from the study. This was an unfortunate result, however room for debate on whether subjects were properly classified was minimized. For the first study, sample collection of tears was capped around 10 μL for some of the early subjects which limited the amount of tears for analysis. This error was corrected for the second study and a minimum of 15 μL was collected with most samples at the 20 μL level. Although it was attempted to minimize reflex tearing while collecting samples, there was inevitably tearing that could not be controlled for which may have diluted the samples. Finally, if CIC collection were repeated, an attempt would be made to more accurately visualize the amount of sample collected with each membrane in order to estimate the amount of RNA collected. While some samples had adequate RNA for analysis, the RNA tended to have low yield and therefore made analysis difficult.

In conclusion, our study has now shown that there is an increase in HMGB-1 in normal vs DED subjects. This study furthers supports previous studies where S100A was found to be elevated in DED subjects (87). Although HSP was not found to be elevated in this study, tears are constantly being replenished and drained making the capture of a true tear profile difficult. Since HSP have been shown to be associated with HMGB-1 and S100A, more research is needed to investigate whether they are elevated under other conditions such as longer desiccating stress. Since DAMPs are increased in DED, they may be what activate TLRs and lead to the increase in cytokines and MMP-9. If so this is a major breakthrough in the pathogenesis of inflammation in DED and may allow researchers to target and treat DED inflammation.

	OSDI Score	PRTT (mm)	TBUT (sec)	Corneal Staining	Conjunctival Staining
Normal	14.33 ± 8.47	25.80 ± 5.12	6.29 ± 3.64	1.83 ± 2.37	3.60 ± 2.65
DES	26.93 ± 13.81	23.83 ± 7.98	2.62 ± 2.17	5.13 ± 4.75	7.57 ± 3.83
p-value	0.005	0.429	0.002	0.023	0.003

Table 2.1: Comparison of normal and dry eye subjects in Study 1A using a Student's T-test. There was a statistically significant difference between normal and DED subjects for Ocular Surface Disease Index (OSDI), tear break-up time (TBUT), corneal staining and conjunctival staining. Values represent mean ± standard deviation

	PRTT (mm)	TBUT (sec)	Corneal Staining	Conjunctival Staining	Osmolarity
Before LHE	20.89 ± 8.22	4.04 ± 2.60	2.22 ± 1.82	3.22 ± 3.27	309.56 ± 5.42
After LHE	22.94 ± 6.78	3.44 ± 1.37	4.06 ± 2.10	4.72 ± 4.30	306.94 ± 12.57
P-Value	0.4117	0.4039	0.0046	0.2290	0.4647

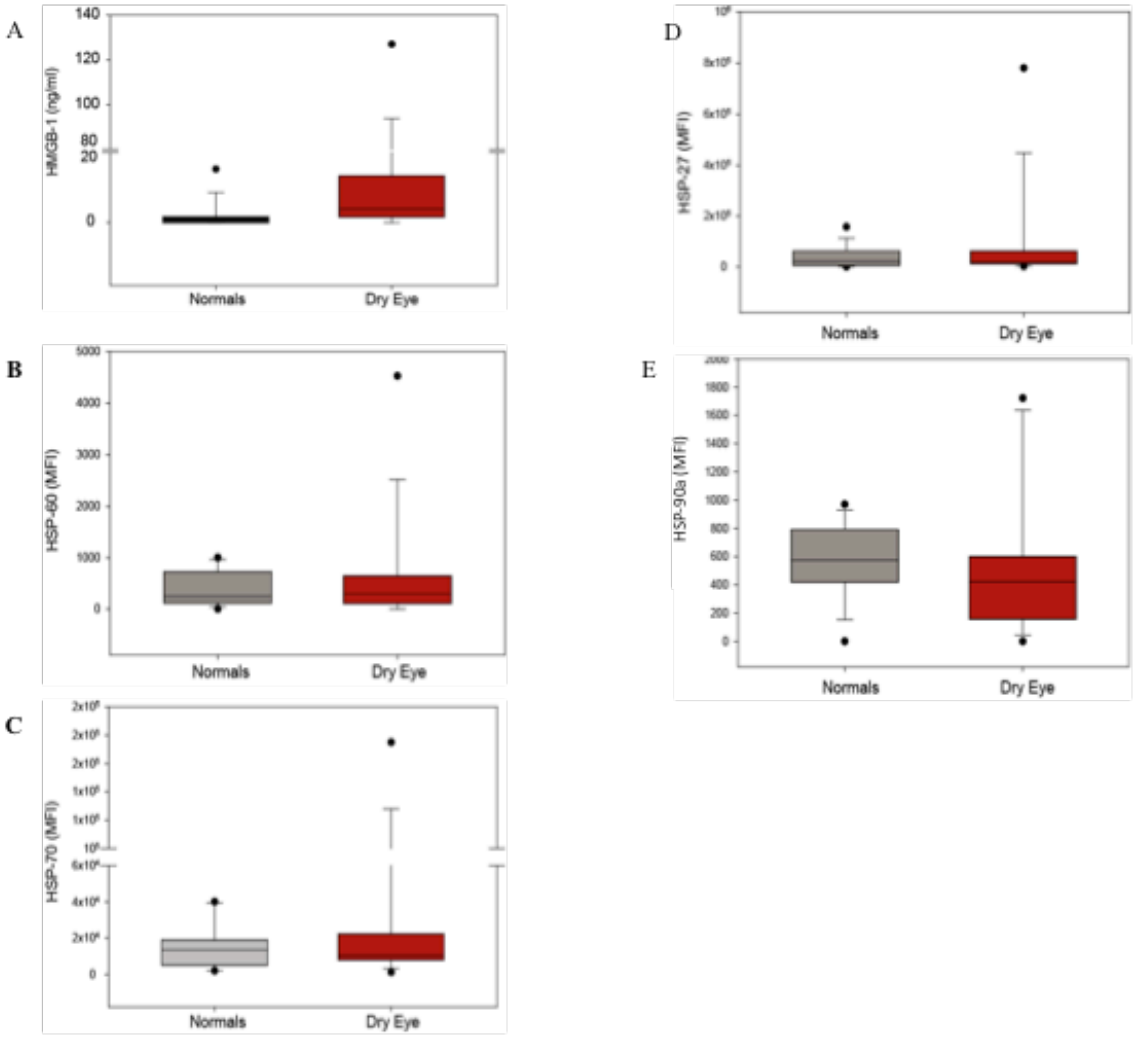
Table 2.2: Comparison of dry eye subjects in Study 1B before and after a two-hour low humidity exposure (LHE). All data was checked for normality using the Shapiro-Wilk Normality Test. The paired T-test was used for each set of data except conjunctival staining for which a One Sample Sign Test was performed. There was no significant difference found in PRTT, TBUT, tear film osmolarity (mOsm/Kg) or conjunctival staining. Values represent mean ± standard deviation.

	HMGB-1	HSP 27	HSP 60	HSP 70	HSP 90
Normal	1.55 ± 3.96	36674.3 ± 43325.0	386.3 ± 329.2	561.4 ± 253.3	13892.3 ± 12051.8
DES	18.31 ± 35.11	94697.8 ± 198914.5	667.4 ± 1131.0	567.6 ± 541.9	28295.6 ± 46778.2
p-value	0.007	0.645	0.833	0.984	0.682

Values represent the mean ± standard deviation

Table 2.3: Tear Film Levels DAMPs in Normal and DED Subjects in Study 1A

Normality was tested using the Shapiro-Wilk Normality test. A t-test was performed on HSP-27 and HSP-90α while a Mann-Whitney test was performed on the other data. Comparison of the DAMPs (high mobility group box-1 (HMGB-1, ng/ml) and heat shock proteins (HSP) mean fluorescent intensity (MFI) found in the tears of dry eye vs normal subjects under normal humidity conditions. Values represent mean ± standard deviation.



Figures 2.3: Boxplots of the levels of DAMPs found in Tears in Study 1A. Levels of HMGB-1 (A) and the mean fluorescent intensity (MFI) of HSP-27 (B), HSP-70 (C), HSP-60, HSP-90α (E) were compared in the tears of normal vs DED subjects. The middle line in the box represents the median, the lower and upper box represents the first and third quartile, whiskers reflect the 95% confidence interval of the mean and filled circles are outliers from 95% confidence interval. HMGB-1 was found to be significantly increased in DED subjects compared to normal ($p \leq 0.05$) while no significant difference was found in the HSPs.

	HMGB-1	HSP 27	HSP60	HSP70	HSP90α
Normal	384.64 ± 227.08	6823.80 ± 1980.49	1380.08 ± 504.06	1315.35 ± 909.58	1573.42 ± 659.54
DES	406.55 ± 257.94	5509.52 ± 2420.44	1157.71 ± 555.45	804.82 ± 602.77	1151.47 ± 611.75
p-value	0.711	0.115	0.261	0.023	0.080

Table 2.4: Intracellular Protein levels of DAMPs in Normal and DED Patients in Study 1A

Normality was tested using the Shapiro-Wilk test. Normality was checked using the Shapiro-Wilk Test. HMGB-1 was analyzed using the Wilcoxon Signed-Rank Test while the others were analyzed using a Student T-Test. Comparing intracellular levels of HMGB-1 (ng/ml) and the mean fluorescent intensity (MFI) of HSP-27, HSP-60, HSP-70, and HSP-90α in DED subjects compared to normal subjects. Values represent mean ± standard deviation

	HMGB-1	HSP27	HSP 60	HSP70	HSP90α
Before LHE	101.1 ± 137.2	4610 ± 7240	271 ± 610	494 ± 872	1190 ± 1410
After LHE	61.5 ± 70.6	2260 ± 2250	907 ± 2580	969 ± 2400	1210 ± 1560
P-Value	>0.05	0.4404	0.2546	0.2236	0.4404

Table 2.5: Tear Film Protein levels of DAMPs in DED Patients Before and After LHE Exposure in Study 1B

All statistics were performed using the Wilcoxon Signed-Rank Test. Comparing tear film levels of HMGB-1 (ng/ml) and the mean fluorescent intensity (MFI) of HSP-27, HSP-60, HSP-70, and HSP-90α in DED subjects before and after a two-hour low humidity environment exposure. P-value calculated using Wilcoxon Signed-Ranked test. Values represent mean ± standard deviation

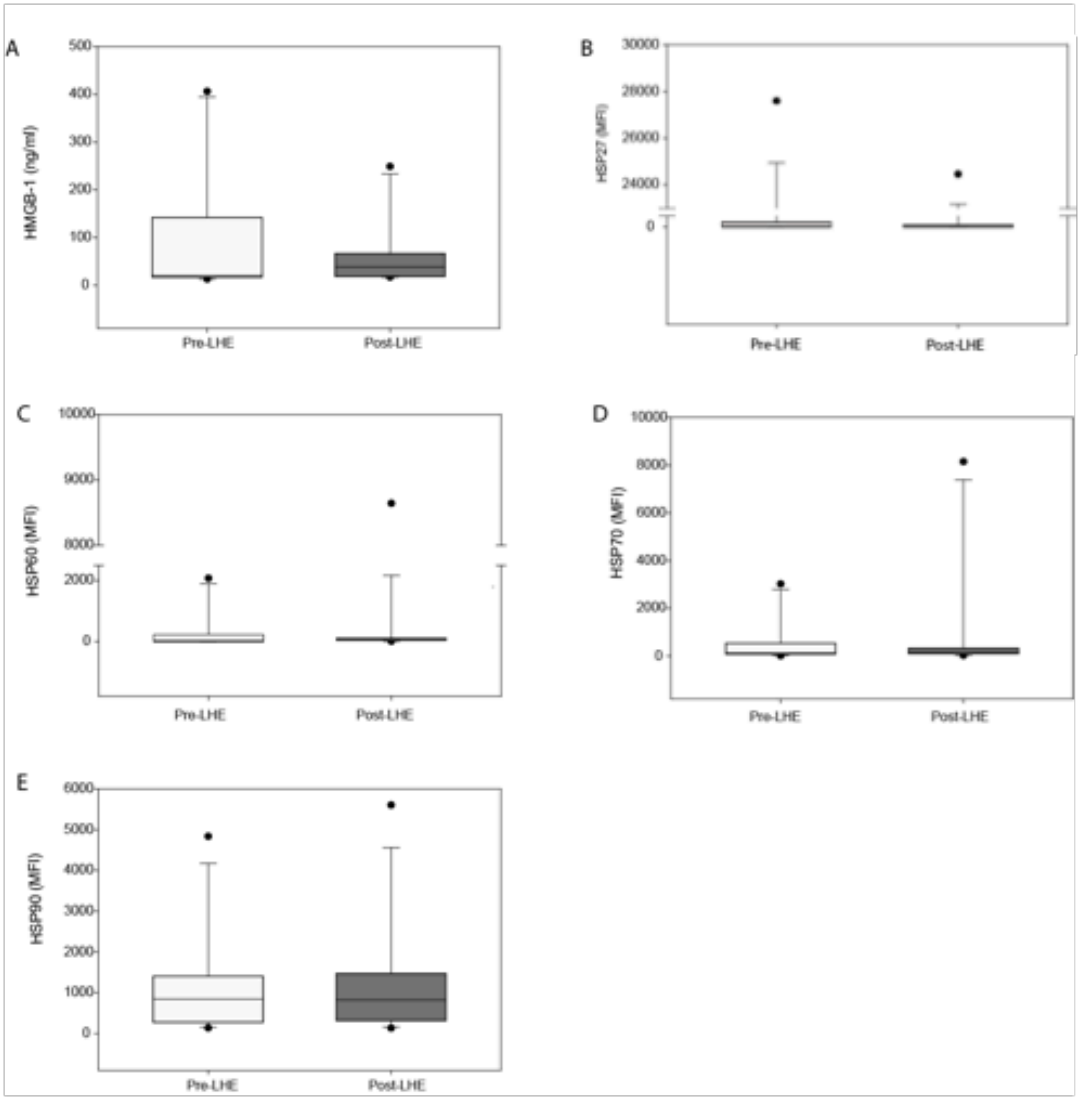

A
HMGB-1 (ng/ml)
500
400
300
200
100
0
Pre-LHE
Post-LHE
B
HSP27 (MFI)
30000
28000
26000
24000
0
Pre-LHE
Post-LHE
C
HSP60 (MFI)
10000
9000
8000
2000
0
Pre-LHE
Post-LHE
D
HSP70 (MFI)
10000
8000
6000
4000
2000
0
Pre-LHE
Post-LHE
E
HSP90 (MFI)
6000
5000
4000
3000
2000
1000
0
Pre-LHE
Post-LHE

Figure 2.4: Boxplots of the levels of DAMPs in dry eye subjects pre- and post-LHE in Study 1B. Levels of HMGB-1 (A) and the mean fluorescent intensity (MFI) of HSP-27 (B), HSP-60 (C), HSP-70, HSP-90 (E) were compared pre- and post-LHE. The middle line in the box represents the median, the lower and upper box represents the first and third quartile, whiskers the 95% confidence interval of the mean and filled circles are outliers from 95% confidence interval. No significant increase in HMGB-1 or the HSPs were found before and after two hour LHE.

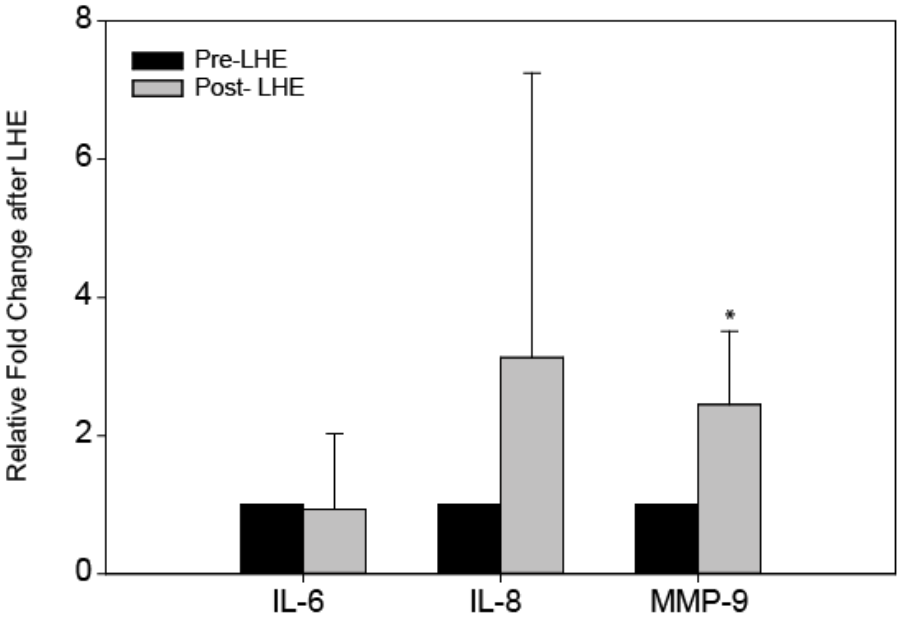


Figure 2.5: mRNA Expression of IL-6, IL-8 and MMP-9 in the CIC Samples Following LHE in Study 1B. Mean fluorescent intensity (MFI) of IL-6, IL-8 and MMP-9 were compared before and after a two-hour exposure to a LHE. There was no significant difference found in IL-6 and IL-8 but MMP-9 was found to have a 2.4 fold increase after exposure.

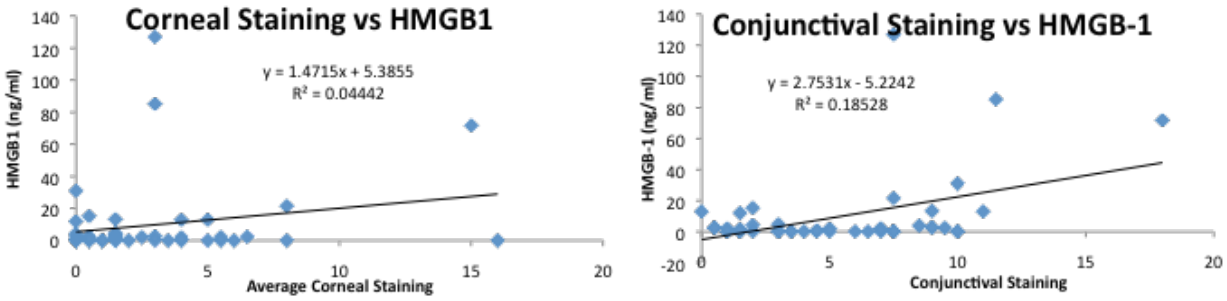


Figure 2.6: Corneal (A) and Conjunctival (B) staining scores compared to levels of HMGB-1 in Study 1A. Values of HMGB-1 were compared to the clinical signs of corneal staining with sodium fluorescein and conjunctival staining with lissamine green.

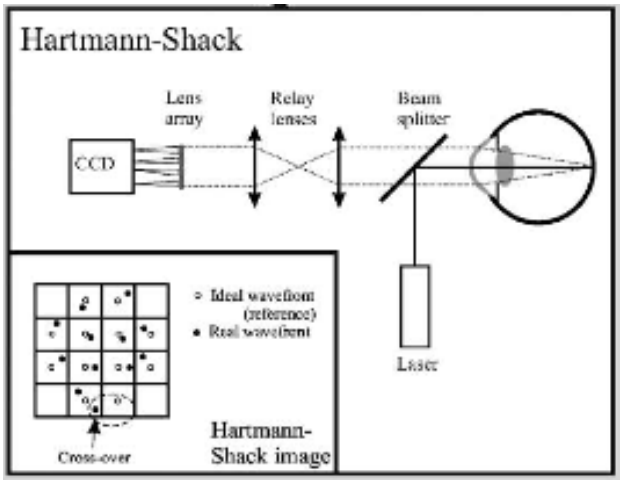
Chapter 3: The Effect of Low Humidity Exposure on Visual Performance

3.1 Introduction

It has been noted that dry eye sufferers experience a decrease in visual acuity, yet there was no known hypothesis as to why until G. Rieger's work was published in The British Journal of Ophthalmology in 1992 (70). In his paper, Rieger examined visual acuity in thirty subjects with DED and noticed an improvement in visual acuity when instilling an artificial tear in one eye compared to the subject's other control eye. Reiger deduced from the data that tear film degradation was responsible for degradation of vision, however it was difficult to objectively determine that the visual changes were directly influenced by tear film. This was validated with a study by Denoyer that showed that there was an increase in HOA measurements taken 10 seconds post-blink in dry eye subjects yet, no significant change in HOAs in normal subjects (71). Furthermore, there was a strong correlation between severity of DED reported on OSDI scores and the amount that HOAs changed post blink. This led the authors to suggest that serial corneal topography can be used as an objective measurement for determining the severity of DED a person has. Since it is known that DED subjects report an increase in symptoms in lower humidity environments, McCulley et al. examined just how much of a change in RH was needed to affect one's tear film. He showed that as low as a 10% change in RH can cause a statistically significant increase in evaporation rates in aqueous deficient dry eye subjects (67). In fact, further studies have shown that even minimally symptomatic subjects who are exposed to a 19% RH environment for two hours show negative impacts on the ocular surface (69).

Wavefront aberrations are measured using a wave-front sensor to compare the shape of a standard light wave that passes through the eye with a hypothetical perfect refracting surface. It then measures the difference between expected and measured data and calculates all of the points where the cornea deviates from the ideal model. The two aberrometers in this study utilize different methods; the Topcon KR-1W (KR-1W) utilizes Hartman Shack aberrometery (Figure 3.1A), while the Nidek OPD-Scan II (OPD) uses slit retinoscopy (Figure 3.1B).

A KR-1W



B OPD Scan III

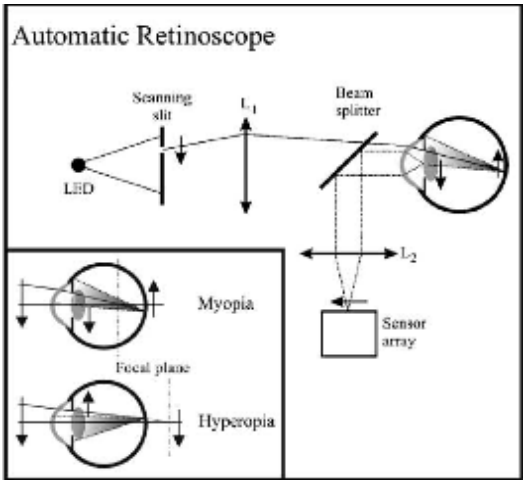


Figure 3.1: The different measurement methods utilized by the Hartmann-Shack method, KR-1W (A) and slit-retinoscopy method OPD Scan III (B) . The KR-1W utilizes the Hartmann-Shack method, uses a beam of light along the eye's line of sight, which is reflected off the retina and exits the eye. The beam is then directed through a series of lenses which creates multiple beams of lights. The position of the new beam is compared to the ideal wavefront and differences are measured. The Automatic Retinoscope uses a light placed behind a fast moving slit. The slit is projected on the retina. Depending on the refractive error, the beam moves at a certain speed and direction. The sensor records these values and the machine moves to a different meridian to produce a wavefront map.

Knowing that disruptions in the tear film lead to an increase in corneal aberrations in dry eye subjects, it was wondered if there was an increase in aberrations in normal subjects who were subjected to low humidity conditions. If there is a change, are current topographers repeatable and a reliable way to measure HOAs in varying levels of humidity? These questions are vital, especially with the increased popularity of wavefront guided LASIK and cataract surgeries and differences in humidity between cities or indoor and outdoor environments that subjects are subjected to before surgery.

The purpose of this study was two-fold- 1) To determine whether an exposure time of one hour in a low humidity environment significantly changes measured corneal aberrations and 2) To study the repeatability of HOA measurements as assessed with the Topcon KR-1W and the Nidek OPD-Scan III in response to changes in relative humidity.

3.2 Methods

The study was conducted in the environmental chamber at The Ocular Surface Institute located at the University of Houston College of Optometry. All procedures were in accordance with the Tenets of the Declaration of Helsinki Scotland amendment, 2000, and were approved by the University of Houston's Institutional Review Board. Informed consent was thoroughly reviewed with each subject and written consent was obtained prior to enrollment into the study.

Subjects

Forty-six normal male and female subjects between the ages of 21 and 58 were recruited. Subjects could be soft contact lens wearers, however no rigid gas permeable lenses were allowed. Subjects could not have any history of previous ocular surgeries. The subjects were told to wear their glasses to the appointment and were first screened

using a very specific history questionnaire asking about medical and ocular history as well as current medications and artificial tears. If anything was revealed that affected the ocular surface, then the subject was disqualified. The subjects were administered the OSDI survey and were disqualified if scores were out of the normal range. After the subjects were screened, their visual acuities were measured using the high and low contrast Log MAR charts. The subjects then took a Dry Eye Questionnaire (DEQ10) that inquired about any symptoms they may be experiencing related to dry eyes. The subjects then entered the low humidity room for exactly an hour where they were instructed not to sleep and where food, water, artificial tears, etc were not allowed. A camera was installed in the low humidity chamber to make sure subjects were following instructions. The relative humidity was monitored throughout the time the subject was in the low humidity chamber. After an hour, the examiner measured high and low contrast Log MAR visual acuities and the subjects took a post-chamber survey asking details about dry eye symptoms felt after the hour.

Measurements

Higher order aberrations were measured on the subject's right eye with the KR-1W and OPD Scan-III before and after entering the low humidity environment. The investigator then recorded the before and after third through 6^{th} order higher order aberrations for a 4mm pupil. This accounted for the majority of aberrations that are not the subjects' spherical or cylindrical refractive error.

Data Analysis

The Mann-Whitney test or paired t-test was used to compare the 3^{rd}, 4^{th} and 3^{rd}-6^{th} corneal HOA's before and after exposure to the LHE. Furthermore, the data was plotted

using Bland-Altman plots to see if there were any trends in the difference vs means. The intraclass correlation coefficient was also assessed when comparing the reliability of the measurements between the Nidek OPD-Scan III and the Topcon KR-1W. Overall, these statistics compared corneal 3^{rd} order, 4^{th} order, and total 3^{rd}-6^{th} order aberrations before and after low humidity exposure as well as make an analysis between the two aberrometers.

3.3 Results

Patient Data

The average age ± SD was 26.06 ± 6.99 years (median age = 24). Of the 47 original subjects, 13 were excluded due to OSDI's outside of the normal range, one subject was excluded due to the inability to get topography readings on their eye. Of the remaining 24 were female and 10 were male.

Low Humidity Environment

The RH was recorded as 18.12 ± 3.23% with an average temperature of 80.92 ± 2.66 °F when the subjects entered the low humidity environment and 16.24 ± 3.18% with a temperature of 79.82 ± 14.08 °F when the subjects exited the chamber. RH never exceeded 24%.

Visual Acuity

Before entering the low humidity environment, average high contrast visual acuity ±SD was -0.02 ± 0.12 logMAR while low contrast visual acuity was 0.09 ± 0.15 logMAR, while high and low contrast after the hour exposure were -0.02 ± 0.14 and 0.11 ± 0.16 respectively. There was no statistically significant difference between pre and post

low humidity visual acuity readings for either high or low contrast (p-value = 0.7414, 0.5419 respectively).

Higher Order Aberration Readings

The average third, fourth, and the third-sixth higher corneal aberration values for the Topcon OPD pre-exposure were 0.095 µm, 0.058 µm, and 0.114µm, respectively, while the average post-exposure values for third, fourth, and total higher corneal aberrations were 0.098 µm, 0.069 µm, and 0.121 µm, respectively. The average third, fourth, and the third-sixth higher corneal aberration values for the Nidek pre-exposure were 0.317 µm, 0.223 µm, and 0.416 µm, respectively, while the average post-exposure values for third, fourth, and total higher corneal aberrations were 0.134 µm, 0.098 µm, and 0.177 µm, respectively. There were no statistically significant differences in the third, fourth, and the third-sixth higher order corneal aberrations between pre- and post-exposure to the low humidity environment (see Table 3.1).

There was no difference in repeatability of the corneal HOA measurements using the OPD or KR-1W. However, the OPD had a greater number of outliers (21 vs 10 when using the Iglewicz and Hoaglin's outlier test with a Z score ≥ 3.5) as well as a greater standard deviation.

3.4 Discussion

For the purpose of this study, a change in humidity from around 41% to around 17% was considered relatively similar to a change in humidity one may experience in Houston summers when going from outdoors to indoors. Since it has been shown that as little as a 10% change in humidity lead to changes in tear evaporation (67), it was hoped

that a 20% decrease would induce measurable optical changes even in a person with a robust tear film. Since no change in aberrations was seen, it suggests that a normal, non-symptomatic subject's tear film was stable enough to withstand the low humidity stress that it was submitted to for the hour. However, more research is needed to see if a longer time in that environment or a greater change in relative humidity would lead to increases in HOAs that the topographer would detect.

Although there was no statistical difference between the repeatability of the Topcon KR-1W and the Nidek OPD SCAN III, the OPD SCAN III had a greater amount of outliers and a greater standard deviation than the KR-1W suggesting there may be a difference with an increased number of subjects.

Dry eye disease is a public health concern with up to one-third of adults in the United States reporting symptoms(76). In fact, dry eye symptoms are one of the leading causes for people within the United States to seek their eye care professional. With numbers expected to rise, clinicians are faced with a dilemma since even mild dry eye can cause contact lens intolerance and drop out. Unable to wear contact lenses and unhappy with glasses, many of these subjects seek other forms of visual correction including wavefront guided LASIK. Studies have shown that up to 75% of subjects undergoing LASIK have preoperative dry eye (88), and preoperative dryness puts the subject at risk for severe postoperative dryness and can potentially adversely affect postoperative visual outcome and satisfaction (89).

Since the first femtosecond laser was approved by the US food and Drug Administration in 2001 for laser-assisted in situ keratomileusis (LASIK), technology has evolved and the lasers have become more complex and accurate. Laser technology has

evolved from correcting 1st order refractive errors to create a customized refractive procedure led by wavefront guided technology (90). With the increase in precision and efficacy, the market for laser-assisted surgeries has grown and now lasers are often used in a myriad of surgeries ranging from LASIK to femtosecond laser-assisted cataract surgery to keratoplasty. Accurate aberrometry readings are critical in order to achieve the best visual outcome, but the aberrometry reading changes depending on multiple external influences including blink rate and clinical or subclinical DED. Studies have shown that HOAs significantly change post-blink in dry eye subjects and that there is no significant change in their normal counterparts (71), and that exposure to a LHE can induce dry eye changes (67) yet little work has been done to investigate how exposure to a LHE can effect aberrometer readings. In order to continue to improve laser-assisted surgeries, more research is needed to determine optimal surgical conditions, which possibly need to include humidity monitors.

A **B**

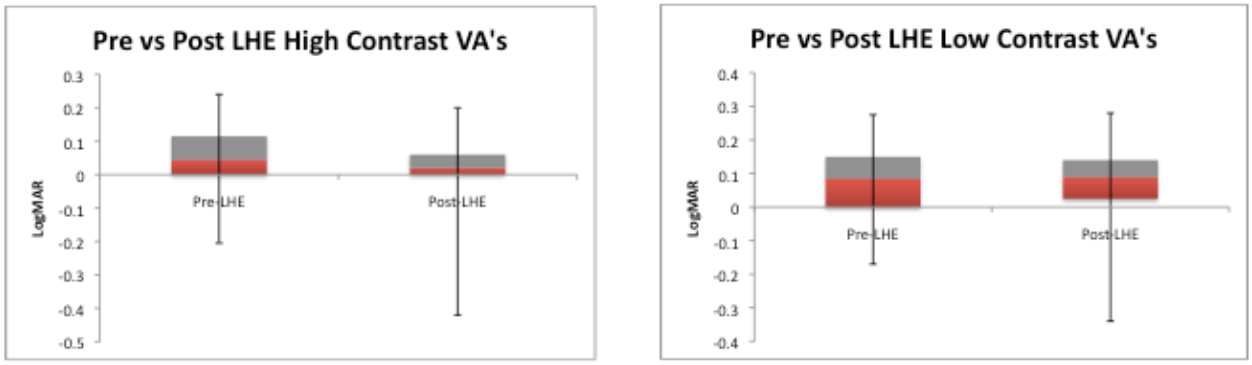


Figure 3.2 High (A) and low contrast (B) visual acuity pre and post 1 hour low humidity exposure (LHE) in logMAR scale. The center line represents the mean and the box represents the first to third quartile. The bars represent the 95 percentile. No significant difference was found between pre and post exposure visual acuities for either high or low contrast acuity charts**.**

	Topcon			Nidek		
	3rd	4th	3rd-6th	3rd	4th	3rd-6th
Pre-Chamber	0.095	0.058	0.114	0.098	0.069	0.121
Post-Chamber	0.098	0.069	0.121	0.317	0.223	0.416
P-Value	0.946	0.142	0.527	0.857	0.960	0.711

Table 3.1. Average RMS values (μm) for the 3rd, 4th and 3rd-6th higher order aberrations (HOA). Taken with the Topcon KR-1W and Nidek OPD Scan III. There was no significant difference between pre and post chamber readings for either the 3^{rd}, 4^{th}, or 3^{rd}-6^{th} higher order aberrations.

Chapter 4: Overall Summary, Conclusions and Future Directions

This study examined the levels of DAMPs on the ocular surface of normal and dry eye subjects to further understand the mechanisms of dry eye inflammation under normal and low humidity exposure. (1) HMGB-1 was shown to be elevated in the tear film of DED subjects when compared to normal subjects. Although HSPs were not shown to be elevated in this study, the higher variance in the DED group suggests a difference may be found with more subjects and therefore further research should be done. Furthermore, when DED subjects underwent two hours of desiccating stress (2), there was a significant increase in MMP-9 mRNA (p-value < 0.05) and corneal staining (p value <0.05) but no change seen in IL-6 or IL-8. These ocular surface changes might result in changes in HOA, but unfortunately this was not examined in these patients. When looking at DAMPs in the tear film and conjunctiva in DED subjects before and after LHE, there was no significant increase in HMGB-1 or any of the HSPs. Together this shows that a LHE increases damage seen on the ocular surface in DED, however a two hour exposure may not be enough time to upregulate intracellular or extracellular levels of DAMPs in DED subjects. Overall, these data suggests that DAMPs may play a role in TLR activation, which triggers a self-perpetuating inflammatory cascade seen in chronic dry eyes.

An increase in higher order aberrations post blink is one of the causes of subjective blur commonly reported in DED subjects. The final section of the thesis examined how a normal tear film can withstand variations in humidity people commonly experience when going from outdoor settings to indoor. It has been shown that LHE can affect the stability of the tear film, yet when a normal tear film is subjected to a low humidity environment for an hour, there is no significant increase in HOAs measured by

either the Topcon KR-1W or the Nidek OPD Scan III. However, this study did not look at the ocular surface or the tear film and so results can only be attributed to the detection level of the aberrometers to the HOAs. Further investigation should compare measurements of the Topcon KR-1W to non-invasive TBUT and corneal NaFl staining to see if the LHE exposure causes any objective changes to the tear film and if these changes are captured by the aberrometers.

The main findings of this thesis are as follows:

- HMGB-1 was increased, while HSP70 was decreased in the tear film of DED subjects when compared to normal subjects
- HSP27, HSP 27pS78, HSP60 and HSP90α were not increased in the tear film of DED subjects.
- There was no significant intracellular increase in either HSPs or HMGB-1 in DED subjects
- MMP-9 mRNA upregulated after a 2 hour exposure to 4.2-4.3% humidity which may be a potential source of corneal damage.
- There was no significant increase in measureable HOAs before and after 1hr LHE to a <24% relative humidity. Changes in HOA are thought to occur as a result of ocular surface damage. Unfortunately, this portion of the study did not address if 1hr LHE resulted in ocular surface damage, but it is possible that only 1hr exposure to LHE is not long enough to create sufficient ocular surface damage to increase HOA or modulate visual acuity in normal subjects.

Further investigation of DAMPs in DED should include the following:

- **Repeat of intracellular and tear film HSPs with a greater sample size and/or more severe DED subjects since most of the study's subjects were mild to moderate DED**. The trend of the data suggests that there would be a significant difference between the amount of HSP in the tear film and intracellularly with a greater sample size. If there is an increase in HSPs then it will further strengthen the theory that DAMPs are involved in DED pathogenesis. It also remains to be determined if DAMPs can activate human ocular surface cells and result in additional inflammation.
- **A study determining if there is a difference in DAMPs between normal and DED subject's pre- and post- LHE (2hr) exposure.** Although we found an increase in HMGB-1 in DED subjects compared to normal subjects and that there was not a significant increase in DAMPs seen pre-and post- LHE exposure in DED subjects, it would be interesting to see if there is a change in the difference between normal and DED levels of DAMPs pre- and post-LHE.

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Appendix

1) OSDI:

Ocular Surface Disease Index© (OSDI©)[2]

Ask your patients the following 12 questions, and circle the number in the box that best represents each answer. Then, fill in boxes A, B, C, D, and E according to the instructions beside each.

Have you experienced any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light? . .	4	3	2	1	0
2. Eyes that feel gritty?	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	4	3	2	1	0
5. Poor vision?	4	3	2	1	0

Subtotal score for answers 1 to 5 (A)

Have problems with your eyes limited you in performing any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading?.	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?.	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A

Subtotal score for answers 6 to 9 (B)

Have your eyes felt uncomfortable in any of the following situations *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions?.	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned?. . .	4	3	2	1	0	N/A

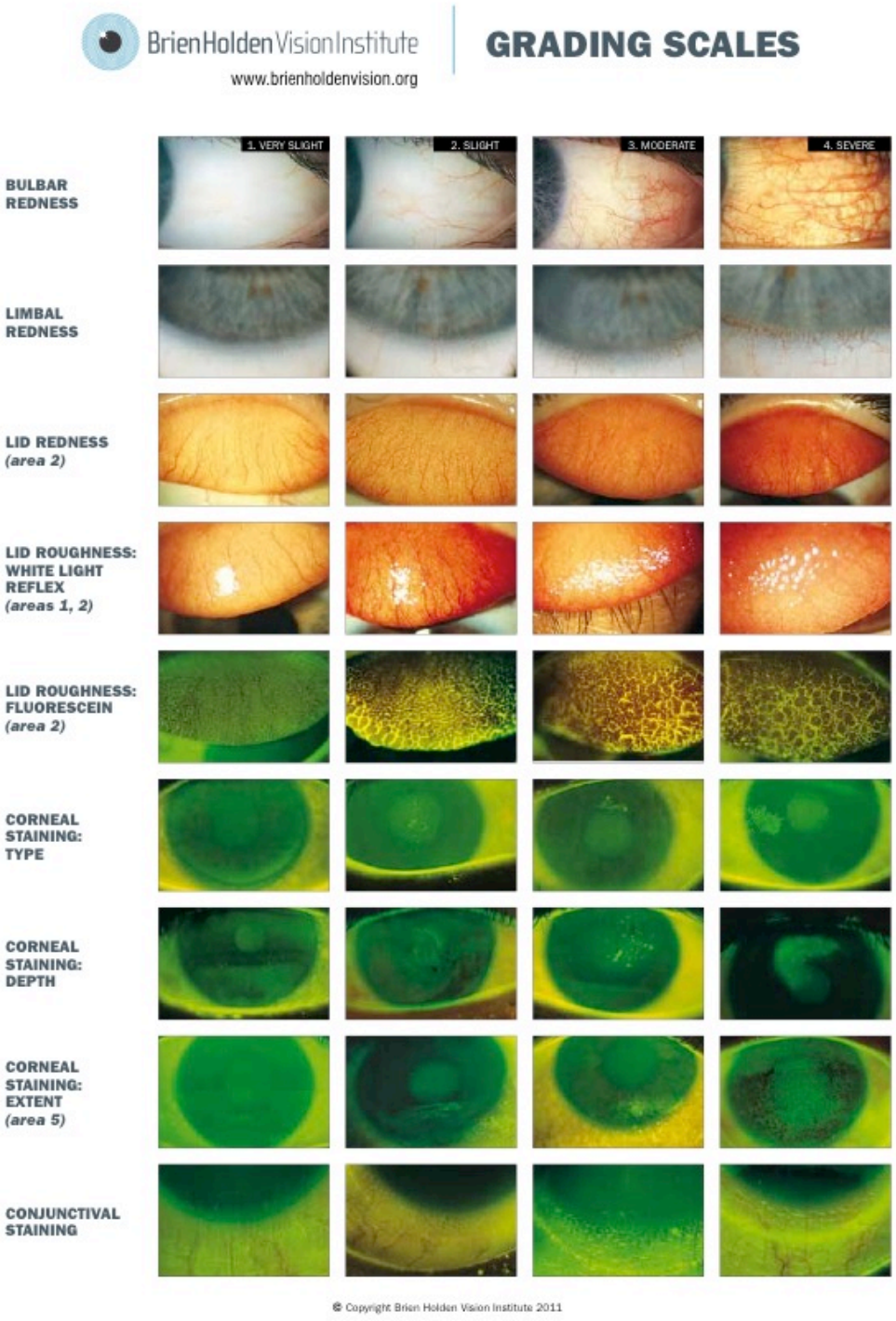
Subtotal score for answers 10 to 12 (C)

Add subtotals A, B, and C to obtain D
(D = sum of scores for all questions answered) (D)

Total number of questions answered
(do not include questions answered N/A) (E)

Please turn over the questionnaire to calculate the patient's final OSDI© score.

2) CCLRU:


BrienHolden Vision Institute
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GRADING SCALES
1. VERY SLIGHT
2. SLIGHT
3. MODERATE
4. SEVERE
BULBAR REDNESS
LIMBAL REDNESS
LID REDNESS (area 2)
LID ROUGHNESS: WHITE LIGHT REFLEX (areas 1, 2)
LID ROUGHNESS: FLUORESCEIN (area 2)
CORNEAL STAINING: TYPE
CORNEAL STAINING: DEPTH
CORNEAL STAINING: EXTENT (area 5)
CONJUNCTIVAL STAINING
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