THE USE OF FLUORESCENT QUENCHING IN STUDYING THE CONTRIBUTION OF EVAPORATION TO TEAR THINNING

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Vision Sciences in the Graduate School of The Ohio State University

By

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ABSTRACT

It is hypothesized that evaporation is the major mechanism of tear film thinning between blinks. This can be demonstrated through the ‘self-quenching’ property of fluorescein. At low concentrations, fluorescent efficiency is independent of concentration, while at high concentrations, fluorescent efficiency falls rapidly. If tear film break up is due to evaporation, then at high concentrations, the measured fluorescein intensity will decay as the fluorescein becomes more concentrated within the tear film. The purpose of this study is to determine if tear film thinning corresponds with changes in the fluorescent intensity of fluorescein in the tears.

Thirty subjects were recruited for this study (34.3 ± 13.1 years of age, 57% female). At baseline, 1µL of 2% fluorescein was placed in the right eye of each subject. Two concurrent 20 second open-eye spectral interferometer recordings, capable of measuring tear film thickness and fluorescein intensity, were obtained. Five minutes after the initial 2% drop instillation, 1 µL of 10% fluorescein was placed in the right eye of each subject. Two concurrent interferometry measurements were obtained. The fluorescein decay rate was compared to the tear film decay rate using nonparametric statistical analyses.
The mean fluorescent decay rate for the first trial of 10% fluorescein was 3.09% per second, while the second trial of 2% fluorescein decay rate was 0.50% per second. The difference between these two decay rates was significant (p < 0.0005, Wilcoxon Sign Rank). The difference in mean thinning rates for the first trial of the 10% fluorescein (1.83% per second) and second trial of the 2% fluorescein (1.58% per second) was not significant (p = 0.76, Wilcoxon Sign Rank). The significant difference in fluorescein decay observed between the 2% and 10% fluorescein trials provides further evidence that evaporation is the major mechanism contributing to tear film thinning between blinks.
DEDICATION

To My Parents:

For helping me achieve my dreams, and supporting me every step of the way.
ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance of my advisor, Dr. Jason Nichols. His expertise, patience, and encouragement were invaluable throughout the entire thesis process. He has helped me grow into a better researcher and clinician. I could not have asked for a more dedicated mentor.

I am deeply grateful for the kind support and dedication of Dr. Ewen King-Smith. His brilliance and passion for research on a daily basis is truly inspirational. Thank you for helping me achieve my goals, and for giving me a ‘shot at glory’.

I would like to thank Dr. Kelly Nichols for serving on my thesis committee. Her clinical insight was instrumental in editing and refining this thesis.
VITA

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CHAPTER 1: INTRODUCTION

Dry Eye Disease

Definition

In 2007 the International Dry Eye Workshop (DEWS), comprised of a team of international experts, was created to provide an evidence-based review of the epidemiology, classification, mechanism, diagnosis, and management of dry eye disease [1-4]. Within this review the DEWS committee re-defined dry eye to encompass new knowledge about its pathology and effect on visual function [4]. The new definition recognizes dry eye as a multifactorial disease which has the potential to damage the ocular surface through tear film instability leading to increased osmolarity and surface inflammation [4]. Dry eye disease can encompass symptoms of ocular discomfort and can cause visual disturbances which may greatly impact an individual’s ability to perform daily activities [5-6]. Severe cases of dry eye disease can lead to increased risk of infection or visual impairment [3].

Epidemiology

Dry eye is believed to be one of the most common ocular problems in industrialized countries [5, 7]. There are significant challenges in studying the prevalence of dry eye including the variability in reported patient symptoms and their lack of correlation to clinical tests and ocular signs [3]. Worldwide prevalence of dry eye is
estimated to be about 8 to 34 percent [3, 5, 7-8]. Within the United States, an estimated 10 million people suffer from moderate-to-severe dry eye symptoms, which are among the leading causes of patient visits to eye care professionals [5, 9].

The prevalence of dry eye is known to increase with age. An estimated 10 to 15 percent of the older adult population having definitive dry eye [10]. The prevalence of dry eye is also greater among women [11-12]. Nearly half of women between the ages of 35 and 60 complain of occasional mild dry eye symptoms [10]. In addition, Nichols et al. reported that more than 50 percent of contact lens patients suffer from dry eye symptoms [13].

Classification

As the definition and knowledge of dry eye disease has evolved over the past decade, so has the classification of its disease process. Recently, the International Dry Eye Workshop developed an etiopathogenic classification scheme to reflect current understanding that deficiencies in tear film quantity or quality can be categorized by either low tear production or by excessive tear evaporation. The DEWS report defines two main classes of dry eye disease.

The first class is a result of excessive tear evaporation, termed evaporative dry eye (EDE). EDE is due to excessive water loss from the ocular surface in the presence of normal lacrimal gland function. Most commonly, this involves intrinsic etiologies like meibomian gland dysfunction. Less common are extrinsic etiologies, such as contact lens wear or ocular allergies [4, 14-15].
The second class is a result of low tear production, termed aqueous-deficient dry eye (ADDE). ADDE is due to a failure of lacrimal tear secretions. This can be a result of Sjogren’s syndrome or non-Sjogren’s lacrimal gland deficiencies or obstructions [4]. It should be noted however that the DEWS report acknowledges that a clear clinical separation between ADDE and EDE may be difficult, and both classifications types may be present simultaneously [4]. Khanal describes dry eye as a continuum disease with most patients demonstrating significant overlap between the two dry eye subtypes. Furthermore, Khanal results show that tear evaporation plays a significant role in both EDE and ADDE [16].

**Causative Mechanisms**

The DEWS report proposes that the two etiopathogenic classes of dry eye disease act through two causative mechanisms, tear hyperosmolarity and tear film instability [4]. The first mechanism, tear film hyperosmolarity is a sensitive indicator of dry eye disease [17-18]. Osmolarity is the measure of solute concentration, defined as the number of osmoles of solute per liter of solution. Tear film osmolarity depends on the ratio of evaporation rate to tear secretion rate [19]. Thus, tear film hyperosmolarity can occur in instances of increased evaporation, as in EDE, or decreased aqueous production, as in ADDE. Both of these scenarios lead to increased osmolarity of the tear film. Increased osmolarity stimulates an inflammatory cascade of inflammatory cytokines and matrix metalloproteinases (MMPs) on the ocular surface which leads to apoptotic death of surface epithelial cells and goblet cells [20-22]. A reduction in mucus secretion, produced
by goblet cells, contributes even further instability of the tear film, which can trigger additional inflammatory events.

Tear film instability, the second mechanism of dry eye is commonly observed by reduced tear breakup time. Tear film instability can be the initiating event of dry eye, in instances of ocular allergy, xerophthalmia, and contact lens wear [4]. In addition, instability and breakup can occur as a result of inflammation from a prior hyperosmotic tear film [4].

The Human Pre-Corneal Tear Film

To understand the classification, etiologies, mechanisms, and treatment of dry eye disease, a thorough understanding of human tear film structure and function is needed.

Structure and Function

The classical description of the human pre-corneal tear film was first provided by Wolff in 1946. He described a three layered structure comprised of an anterior lipid layer, a middle aqueous layer, and a deep mucin layer [23]. While this model is generally accepted, there has been much controversy concerning contribution each of these layers has to the overall make-up of the tear film and their relative thickness [24-28]. Reported values of the pre-corneal tear film (PCTF) thickness, measured using a variety of invasive and noninvasive methods range from 3µm to 46µm [29]. Recent studies using non-invasive spectral interferometry by King-Smith et al. have measured an average tear film thickness of 2.7µm [30].
The tear film is the primary interface between the ocular system and the visual world. It is essential that the tear film be of adequate thickness, transparency, and composition for maintaining proper ocular surface function and clear vision [4, 31-32]. Korb has described four essential functions of the human pre-corneal tear film: creating a smooth refracting surface for light, lubricating and washing debris from the ocular surface, providing antimicrobial protection, and supplying nutrients to the surface cells [33].

Functional Layers

The lipid layer is primarily secreted by meibomian glands located along the upper and lower lid margin and are regulated by autonomic an androgen influences. It is a bilayer chiefly comprised of polar lipids (phospholipids, fatty acids, and free cholesterol) which interact with the aqueous layer, and relatively non-polar lipids (cholesterol esters) which interact with the atmosphere [34-35]. The lipid layer has been considered a vital structure in retarding the evaporation of the underlying aqueous layer [36]. Tear breakup time is inversely correlated with the rate of evaporation, and is positively correlated with lipid layer thickness [36]. Traditionally values of lipid layer thickness range from 40nm to 100 nm [29, 36-37]. King-Smith et al. using spectral interferometry measured a mean lipid layer thickness of 42 nm [38].

The aqueous layer is generally regarded as the thickest layer of the tear film. It is primarily produced by the main lacrimal glands with help from the accessory lacrimal glands of Krause and Wolfring. Control and composition of lacrimal gland secretions is regulated by autonomic innervations and androgen influences; this is one reason why
lower rates of secretion are observed in postmenopausal women [12, 39]. The aqueous layer contains oxygen, glucose, and electrolytes which provide nourishment to the avascular anterior cornea. A failure of the cornea to receive enough oxygen can result in corneal edema and inflammation, causing a disruption in optical quality [40-41]. The tear film also contains a number of specialized proteins which are mostly antibacterial, antiviral, or otherwise involved in the protection of the eye [42].

The mucus layer is comprised mainly of hydrophilic glycoporetins called mucins. Mucins are mainly produced by goblet cells within the conjunctiva and specialized epithelial cells located on the cornea. Certain types of mucins form the glycocalyx which is found on the apical side of the corneal epithelium. This structure stabilizes the thick aqueous layer of the tear film on an otherwise hydrophobic corneal surface [43].

**Controversy in Tear Film Thinning**

The tear film is in a constant state of flux, continuously thinning when the eye is open and refreshing with every blink [10]. In both ADDE and EDE, the tear film thinning rates are higher than normal, leading to decreased tear film break-up times between blinks. Tear breakup occurs when the tear thickness is reduced to zero and the superficial lipid layer comes into contact with the glycocalyx [44].

There are two possible mechanisms in which tear film thinning can occur from the ocular surface: evaporation (outward flow) and tangential flow (parallel flow) [45]. Both mechanisms are illustrated in figure 1 below. It has been also suggested that absorption (inward flow) of the tears into the cornea may play a minor contribution to
It has been a subject of controversy as to which plays the most important role in tear film thinning.

**Possible mechanisms of breakup**

A. Tangential flow. B. Evaporation.

Figure 1: Proposed Mechanisms of Tear Film Thinning and Break-Up (King-Smith).

**Inward Flow**

The contribution of absorption to tear film thinning seems insignificant [44]. Nichols et al. were unable to measure any thickening of the corneal epithelium during tear film thinning [45]. Furthermore, several studies have demonstrated that it seems more likely for water to flow in an outward direction, from the cornea into the tear film [46]. In addition, any contribution of evaporation to the thinning would increase the osmolarity of the tear film, setting up as osmotic gradient which would cause osmotic flow into the tear film from the corneal epithelium [19].

**Tangential Flow**

Tangential flow is the parallel flow of the tear film across the ocular surface. This causes redistribution of tear thickness creating areas of tear film thinning and other areas
of tear film thickening [45]. Thus, despite local change in tear volume and osmolarity, the actual total volume of aqueous remains unchanged. Thickness changes in tangential flow can be driven by both pressure gradients and surface tension gradients (Marangoni flow) [45]. These forces have been shown to have significant contributions to special instances of tear film break-up including meniscus thinning, in the presence of corneal elevations, in small areas of thickened lipid layer, during partial blinks, and immediately following complete blinks [47-51]. Some studies suggest that tangential flow plays a more pivotal role than evaporation in rapid tear film thinning leading to tear break-up [45]. More recently however, the same authors demonstrated that tangential flow is not quick enough to explain the observed thinning rate of the tear film [52].

**Evaporation**

Evaporation is the outward flow of water from the tear film into the surrounding air. This results in tear film thinning across the entire corneal surface, as concluded by the observation of interference fringes of the pre-corneal tear film [44]. Unlike tangential flow, evaporation results in a decrease of aqueous volume and potential increase in the osmolarity of the tear film [18].

As previously stated, the lipid layer plays an essential role in retarding the evaporation of the underlying aqueous layer [36]. This has been demonstrated in several studies in which removal of the lipid layer has resulted in a significant increase in tear film evaporation rates [24, 53]. In addition, some studies provide evidence that evaporation rates are increased in patients with dry eye and meibomian gland dysfunction due to alternations in lipid layer thickness and composition [14, 54-59].
Despite this evidence for the significance of evaporation in special pathological circumstances, controversy still exists as to the contribution evaporation makes to tear film thinning under normal conditions. A number of authors have concluded that evaporation plays only a small role if the superficial lipid layer remains intact [36, 54]. Furthermore, some studies suggest evaporation is not quick enough to make a significant contribution to tear film thinning rates under normal conditions [60-61]. A review paper by Mathers in 2003 summarized the findings of eighteen studies of the pre-corneal tear film evaporation rate [10]. King-Smith et al. found that these averaged values could only account for 20 to 25 percent of their own observed thinning rates using spectral interferometry in free-air conditions [44]. This discrepancy, which will be discussed in detail later, may be explained by evaporation measurements in free-air conditions being much more rapid than traditional measurements using closed-air preocular chambers.

**Spectral Interferometry**

Spectral interferometry is a noninvasive method employed to measure physical characteristics of the human tear film. The optical system is located in a dark room with only minimal ambient light present. A point source located behind a filter emits light above 600nm, which then passes through an aperture stop which is focused on the front of the cornea at normal incidence. Light is reflected from the ocular surfaces passes back through the optical system and is captured by a spectrophotometer as shown in figure 1 below.
The light that is captured in the spectrometer is the result of reflections from a number of different surfaces, including the air-tear interface and the tear-cornea interface. The intensity of reflected light is dependent mainly on the interference pattern of these two reflections. When these two reflections are completely out of phase, destructive interference occurs and the resultant intensity of reflected light is at a minimum. Conversely, when these two reflections are completely in phase, constructive interference occurs and the resultant intensity of reflected light is at a maximum.

The interference pattern of the reflected light will also vary depending on the wavelength of light. In other words, the intensity of the resultant reflected beam is dependent on the wavelength of light. This interference pattern at different wavelengths is termed “spectral oscillations”. The thickness of the tear film is proportional to the frequency of these oscillations, and tear film thinning rate can be determined if the measurement is taken over a period of time. Simultaneously a cobalt blue light is projected onto the cornea. The emitted intensity of fluorescein can be determined by
measuring the intensity of light at 521nm. The fluorescein decay rate can be determined if the measurement is done over a period of time.

**Fluorescein**

*Tear Break-Up Time with Fluorescein*

Fluorescein is an orange dye which fluoresces green when excited by blue light. It is used for many medical and ophthalmic applications. It is used in a number of dry eye diagnostic tests including fluorescein staining of the dead corneal epithelial cells, observation of the tear meniscus, and measurement of tear break up time[62].

The break-up time (TBUT) is the time that elapses in seconds from when the patient is instructed to blink following the instillation of fluorescein to the appearance of the first dark spot formation. This spot has traditionally been identified as an area of tear break-up [62-63]. A good quality and stable tear film will show a high TBUT, while and instable tear film will yield a low TBUT, therefore tear break-up time is considered to be reduced in all forms of dry eye [62].

A TBUT with fluorescein is one of the most common clinical tools used to evaluate tear film stability. It is used by 19 percent to 43 percent of clinicians [64-65]. Most commonly it is applied to the eye with a one percent fluorescein sodium impregnated strip which is wetted with a sterile drop of saline and applied to the conjunctiva. This method results in an extremely variable fluorescein concentration between individuals. Usually a TBUT value less than 10 seconds is used to identify dry eye patients [4]. The instillation of fluorescein has been shown to shorten the normal TBUT [66]. A noninvasive test of tear stability is one that does not involve the
installation of fluorescein, such as spectral interferometry. One study measured a mean TBUT value in normal subjects of 7.6 ±10.4 seconds, and a mean non-invasive TBUT of 11.2 ±6.8 seconds [67].

*Fluorescein Self-Quenching*

Fluorescein possesses a ‘self-quenching’ (extinction) property in that if the dye concentration is high enough, the linear relationship between concentration and fluorescent light output breaks down [68]. In other words at low concentrations, fluorescent efficiency is independent of concentration, while at high concentrations, fluorescent efficiency falls rapidly. The concentration at which quenching results in fluorescence decay is referred to as the critical concentration.

**Purpose**

It is hypothesized that evaporation and tangential flow are the major mechanisms of tear film thinning under normal conditions. The contribution of these two possible mechanisms can be studied using the ‘self-quenching’ property of fluorescein. If tear film breakup is due to evaporation, the increase in fluorescein above the critical concentration will cause fluorescence decay as a result of quenching. If tear film breakup is due to tangential flow, fluorescence decay will be independent of concentration.
Chapter 2: Methods

Study Subjects and Design

Inclusion Criteria

Recruitment of subjects and the research protocol for this project was approved by The Ohio State University Institutional Review Board in accordance with the Declaration of Helsinki. Participants were required to be at least 18 years of age, and an informed consent was obtained from each subject.

Exclusion Criteria

Hormonal changes are thought to affect eyelid gland secretion and tear chemistry. Therefore, subjects who were currently pregnant or breastfeeding, as determined through self report, were excluded from participation. Current contact lens wearers were also excluded from participation in the study, as contact lens wear has been suggested to disrupt normal tear composition, production, and thickness. This alteration away from the tear films normal physiological state could change tear film thickness and thinning rates, which may in turn influence outcome measures.
**Study Design**

A double un-blinded, cross sectional study of The Ohio State University faculty and student population was conducted. Calculations of sample size for paired data analyses suggested that a minimum of 30 subjects would be needed to show a clinically meaningful effect. Each subject participated in one study visit lasting approximately 30 minutes. Subjects were asked to complete a demographic form, Ocular Surface Disease Index survey, and spectral interferometry measurement of their right eye.

**Data Collection**

Data collection was performed with the previously mentioned spectral interferometer which was modified to measure the tear film thickness, tear film thinning rate, and fluorescent intensity simultaneously. Two different concentrations of fluorescein were utilized in this study: a low concentration mixture composed of 2% concentrated fluorescein, and a high concentration mixture composed of 10% concentrated fluorescein. A total of four spectral interferometry measurements were obtained from each subject; two recordings using the low concentrated fluorescein and, two recordings using the high concentrated fluorescein.

For the first trial, 1µL of 2% fluorescein was placed in the right eye of each subject. A micro-pipette with a special nonstick tip was used. If necessary, any residual fluorescein that remained on the tip was wiped on the lower palpebral conjunctiva. The subject was then instructed to close his or her eye and roll it in a clockwise fashion to ensure that the fluorescein had been distributed across the ocular surface. These procedures helped ensure that a precise and consistent amount of fluorescein was placed into each eye. Once the subject was aligned in the interferometer, the first 20 second
recording was obtained. About one second after the start of the recording, the subject was instructed to blink, and then hold the eye open and steady for the remaining 19 seconds. After a rest period of about three minutes, a second recording was obtained; however, no fluorescein was added to the eye. For the second trial, 1µL of 10% fluorescein was placed in the right eye of each subject. The procedure described above was repeated, resulting in two concurrent interferometry readings obtained over a period of three minutes.

**Interferometric Data Processing**

Data from each reflection spectrum were analyzed starting two seconds after the subject was instructed to blink. This was to avoid the transient upward drift of the lipid and aqueous layers immediately following the blink. A computer program was employed to translate the raw data supplied by the spectrometer into the tear film and fluorescent outcome measures. The calculations used by this program have previously been described in detail [30].

**Ocular Surface Disease Index**

The diagnosis and classification of dry eye is controversial [69]. Several studies, as well as anecdotal clinical observation have found a poor correlation between clinical signs and patient reported dry eye symptoms [69-70]. Furthermore some investigators suggest patient symptoms are a more reliable and accurate indicator of dry eye disease [71-73]. The 1995 National Eye Institute Industry Workshop on Clinical Trials in Dry Eye concluded that all clinical trials concerning dry eye should include an assessment of subjective symptoms and functional lifestyle [74].
The Ocular Surface Disease Index (OSDI) is a standardized questionnaire consisting of 12 items used to determine the presence and severity of symptoms consistent with dry eye disease, and their impact on vision related functioning. The questionnaire was developed by the Outcomes Research Group at Allergan Inc. (Irvine, CA), and is considered a reliable instrument for measuring the severity of dry eye [75]. All OSDI scores were computed using the published OSDI scoring guidelines. Subjects scoring above or equal to 22 were classified as having at least mild dry eye. The OSDI was used to distinguish those subjects with dry eye disease from normal subjects.

**Statistical Analysis**

The statistical analysis was performed using Microsoft Excel (Seattle, WA) and IBM’s SPSS software (Chicago, IL). It cannot be assumed that tear film thickness and thinning rates follow a normal probability distribution; therefore, nonparametric statistical analyses were used to compare the fluorescein decay rate to the tear film decay rate.

Within the analysis, the second trial of the low concentration (2%) fluorescein was used along with the first trial of the high concentration (10%) fluorescein. This allowed for a Wilcoxon Sign Rank test to compare the fluorescein decay rate as well as the tear film decay rate between the least concentrated and most concentrated trials. A p-value of less than 0.05 was considered to be significant.
CHAPTER 3: RESULTS

Demographics and Baseline Data

Thirty individuals participated in the study, and data was analyzed from all 30 subjects. The mean age of this population was 34.3 ± 13.1 years, and 57% were female. The mean OSDI score was 9.86 ± 9.09. Five subjects scored over 22 on the OSDI, indicating possible dry eye. All subjects were included in the statistical analysis as their exclusion did not produce statistically different results. The mean temperature within the room was 22.1 degrees Celsius, and the mean humidity was 48%.

Interferometry Illustrated Fluorescent Quenching

Figure 3 is an example of spectral interferometry data representing tear film thickness and fluorescence over twenty seconds. As previously noted the analysis began with data collected 2 seconds after the initial blink in order to avoid any transient movement and thickness changes of the tear film. Data collected after any unintentional subsequent blink was not analyzed. The graph on the right shows the low concentration fluorescein trial, while the graph on the left shows the high concentration fluorescein trial. While the subjects thinning rate between both trials is relatively similar, the fluorescence decay rate is very different.
Tear Film Thinning Rate

Table 1 is a summary of the mean study data for tear film thickness and thinning rates. The mean tear film thinning rate was 3.09 µm/min for the low concentration trial and 4.34 µm/min for the high concentration trial. The mean initial thickness was 3.82 µm and 3.59 µm, respectively. The thinning rate divided by the starting thickness of the tear film yields a percent thinning rate per second. The mean percent thinning rate was 1.58% per second for the low concentration trial and 1.83% per second for the high concentrated trial. The difference between these percent thinning rates was not significant (p= 0.76, Wilcoxon Sign Rank).

Figure 3: Study data illustrating low fluorescein and high fluorescein concentration trials.
Table 1: Mean Tear Film Thickness and Thinning Rates

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<th>Low Concentrated Fluorescein</th>
<th>High Concentrated Fluorescein</th>
<th>P-Value</th>
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</thead>
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<tr>
<td>Mean Initial Thickness (µm)</td>
<td>3.82 ± 0.86</td>
<td>4.40 ± 1.68</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean Thinning Rate (µm/sec)</td>
<td>0.05 ± 0.08</td>
<td>0.07 ± 0.13</td>
<td>0.91</td>
</tr>
<tr>
<td>Mean Thinning Rate (µm/min)</td>
<td>3.09 ± 4.69</td>
<td>4.34 ± 7.82</td>
<td>0.91</td>
</tr>
<tr>
<td>Mean Percent Thinning Rate per Second</td>
<td>1.58 ± 2.54 %</td>
<td>1.83% ± 3.04</td>
<td>0.758</td>
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Fluorescein Decay Rate

Table 2 is a summary of the mean study date for fluorescein decay rate. The mean fluorescein decay rate was 4.11 units per second for the low concentration trial and 16.57 units per second for the high concentration trial. The mean percent fluorescein decay rate for the low concentration trial was 0.50% per second, while it was 3.09% per second for the high concentrated trial. The difference between these two decay rates was significant (p < 0.0005, Wilcoxon Sign Rank).

Table 2: Mean Fluorescein Intensity and Decay Rates

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<th>High Concentrated Fluorescein</th>
<th>P-Value</th>
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<tr>
<td>Mean Initial Fluorescein Intensity</td>
<td>637.47 ± 381.47</td>
<td>672.09 ± 649.72</td>
<td>0.5530</td>
</tr>
<tr>
<td>Mean Fluorescein Decay Rate (per sec)</td>
<td>4.11 ± 6.78</td>
<td>16.57 ± 29.34</td>
<td>0.0027</td>
</tr>
<tr>
<td>Mean Percent Fluorescein Decay Rate per Second</td>
<td>0.50 ± 1.46 %</td>
<td>3.09 ± 3.55%</td>
<td>0.0005</td>
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Percent Fluorescein Decay Rate vs. Percent Tear Film Thinning Rate

Figure 4 is a comparison of the mean thinning and fluorescein decay rate shown in percent change per second.
CHAPTER 4: DISCUSSION

Fluorescent Quenching within the Tear Film

Quenching occurs when the concentration of fluorescein reaches a ‘critical concentration’ and is defined as the point where the linear relationship between concentration and fluorescent intensity breaks down [68]. In 1967, Maurice found that the critical concentration at which quenching becomes significant within the tear film is approximately 2 mg ml⁻¹ [76]. In other words, for low concentrations of fluorescein in the tear film, much less that the critical concentration of 0.2%, fluorescent efficiency is independent of concentration. At concentrations much above the critical concentration of 0.2%, fluorescent efficiency falls rapidly with increasing concentration.

This is illustrated in figure 5 below. Quenching occurs in the tear film when the actual concentration of fluorescein equals the critical concentration of fluorescein, as indicated by the vertical red line. The left side of the graph illustrates when fluorescein is below the critical concentration. In this instance fluorescein efficiency is independent of concentration. The right side of the graph illustrates when fluorescein is above the critical concentration. In this instance fluorescein efficiency falls rapidly with increasing concentration, and fluorescent intensity output is reduced.
There are two possible mechanisms by which tear film thinning and subsequent breakup can occur on the ocular surface: tangential flow (parallel flow) and evaporation (outward flow). In this study the relative contribution of these two mechanisms were examined using the phenomenon of fluorescein quenching (extinction).

In tangential flow, fluorescein will flow tangentially with the tear film during breakup. Fluorescein concentration will remain unchanged and thus there is no potential for quenching to occur, regardless of concentration. In evaporation, fluorescein will become concentration within the tear film as the aqueous evaporates. Fluorescein concentration is increasing. Quenching will occur at high concentrations, that is concentrations above the critical concentration.

It has been shown that fluorescein concentration decreases in the tears at a rate of $11.85 \pm 3.31\%$ per minute in normal individuals [77]. The tear volume has been measured to be approximately $7 \pm 2 \mu l$ [78]. The addition of $20 \text{ mg ml}^{-1}$ (2%) fluorescein
during our low concentration trial would yield an approximate tear film concentration of 2.5 mg ml\(^{-1}\) (0.025\%). This concentration would decrease to below the critical concentration 4-5 minutes after initial drop instillation.

**Fluorescent Quenching and Evaporation**

As seen in figure 2 there is a significant difference in the fluorescence decay rate between the low concentration and high concentration trial. This indicates quenching is occurring in the high concentration trial due to the increasing concentration of fluorescein as a result of evaporation. Quenching would not be observed in tangential flow, where fluorescein concentration would remain unchanged between blinks in both the low and high concentrated trials.

In addition, figure 2 illustrates that the difference in mean percent thinning rates between the two trials were not significant. This indicates that the significant difference in fluorescence decay rate between the two trials is not related to a difference in tear film thinning rates.

**Evaporation and the Lipid Layer**

The lipid layer has been considered essential in preventing evaporation of the underlying aqueous layer [36]. Mishima, Maurice, and later Iwata were among the first to show a significant increase in tear evaporation rate in rabbits when the lipid layer was removed [24, 53]. Conversely, in vitro work found that lipids did not prevent the evaporation of underlying saline [79]. Since then many authors have demonstrated increase evaporation rates in humans with compromised lipid layers or in those with
meibomian gland dysfunction [14, 54, 57]. In 1995, work by Craig demonstrated a relationship between lipid layer structure and tear film stability [80].

Craig and Tomlison studied the effect of lipid layer structure and tear film evaporation rates. The evaporation was derived from the vapor pressure as measured by the Servomed Evaporimeter while at a different point in time the lipid layer structure was observed with interference fringes using the Keeler Tearscope [36]. They found that where the lipid layer is absent, or not confluent, the tear film is unstable and tear evaporation is significantly increased. Conversely, where the tear film is stable, and the lipid layer is intact, regardless of lipid thickness, tear evaporation is retarded.

Most recently King-Smith et al. applied a novel method of spectral interferometry capable of simultaneously measuring lipid layer thickness and tear film thinning rates [38]. They found a highly correlated relationship when tear film thinning rate was analyzed using a slow/rapid dichotomy. The study provides evidence that slow thinning rates may correspond with a good evaporation barrier, whereas rapid thinning may correspond with a relatively poor evaporation barrier. This poor barrier of evaporation may due to inadequate thickness or insufficient composition. These results further support our results that the main contribution to thinning rate comes from evaporation.

**Contributions from Tangential Flow**

The contribution of tangential flow to tear film thinning has previously been studied by observing the movement of the lipid layer and hence the underlying aqueous layer as a result of viscous drag [46, 49]. The movement of the lipid layer was observed to be too slow to explain the thinning rate of the tear film between blinks, implying that
evaporation and not tangential flow is the main cause of tear film thinning and break-up. The author also consider tangential flow of the aqueous layer independent of lipid layer movement, but through theoretical analysis conclude that this movement only makes a minor contribution to observed tear film thinning.

**Previous Measures of Evaporation**

Previous investigators have claimed tear film evaporation is too slow to explain tear film break up. Holly states that “approximately 5 to 10 minutes of continuous evaporation would be required to eliminate the tear film under normal conditions. [60]” A review of the evaporation rates reported in the literature, as conducted by Mathers in 2004, found that the mean reported evaporation rate was 0.75 µl per minute. Even when considering the most conservative measurement of tear film thickness, evaporation would result in tear film breakup in 3 to 4 minutes. This is much longer than the observed average tear break up time of normal subject (8.5 ± 6.4 seconds) [63].

As mentioned, the results of this study show that the decay of fluorescence between blinks is consistent with tear thinning from evaporation rather than tangential flow. This discrepancy may be explained by the use of pre-ocular chambers used in most previous experimental studies. It is believed that these chambers may block normal air currents over the cornea. As shown in Figure 6, a thick layer of humid air can build up in front of the cornea, creating resistance to evaporation. In free air conditions, normal air currents prevent the build up of human air.
This theory was supported by Kimbal who found significantly different tear film thinning rates under each of these conditions using spectral interferometry. His results of increased tear thinning under free air conditions suggest that evaporation is quick enough under normal conditions to be primary means by which the tear film thins. Recently, experimental measurements of tear film evaporation rates using ventilated chambers have been similar to tear film thinning rates obtained using spectral interferometry [41, 74]. Ventilated chambers allows air currents to circulate over the cornea similar to natural free air conditions and negates the development of a thick humid layer which may cause a barrier to evaporation.

Clinical Implications

If tear film thinning is due primarily to evaporation than the osmolarity of the thinned tear film should be greatly increased. This has considerable implications for
understanding the pathophysiology, diagnosis, and treatment of dry eye disease. For instance, this challenges the traditional interpretation of fluorescein tear breakup time. The formation of black spots on the cornea is probably not due to an absence of the tear fluorescein, indicating an exposed corneal surface. Rather it may be the result of fluorescent quenching indicating a highly concentrated area of fluorescein in a thin tear film layer.
CHAPTER 5: CONCLUSION

The decay of fluorescence between blinks may be attributed to fluorescent quenching. Quenching is a result of changing fluorescent concentration between blinks, indicating that evaporation and not tangential flow is the main contributor to tear film thinning and break-up. This conclusion supports previous interferometry studies which measured tear film thinning rates in free air conditions. Additional questions still remain about the relationship between tear film thinning, tear film breakup, and dry eye disease. Furthermore, it is still unclear as to how fluorescent quenching applies to clinical observations of fluorescein tear film break up times.


