The Effects of Increasing Ocular Surface Stimulation on Blinking and Tear Secretion

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PURPOSE. To investigate the effect of varying levels of ocular surface stimulation on the timing and amplitude of the blink and tear secretion.

METHODS. Following instillation of fluorescein dye, increasing levels of air flow were directed toward the central corneas of 10 healthy subjects. Interblink interval (IBI), tear meniscus height (TMH), and fluorescence intensity were measured simultaneously. Because blinking can obscure changes in TMH, we developed novel measures of tear secretion by calculating tear meniscus fluorescein concentration (TMFC) from intensity using a mathematical model. The change of TMH and TMFC over trials and the slope of the TMFC within each IBI (IBI-TTR) were further calculated.

RESULTS. The mean IBI was decreased by 8.08 ± 8.54 seconds from baseline to maximum air stimulation. The TMH increase was highly variable (0.41 ± 0.39 mm) among subjects, compared to the fluorescence tear turnover metrics: decrease in TMFC of 2.84 ± 0.98 natural logarithm or $\ln(\%)$ and IBI-TTR of $0.065 \pm 0.032 \ln(\%)/\text{sec}$. Ocular surface stimulation was highly correlated with the TMFC and IBI-TTR, but less so with TMH (Pearson's $r = 0.71, 0.69$, and $0.40, P < 0.01$, respectively). Blinking and tearing were significantly correlated with each other (Pearson's $r = 0.56, P < 0.01$), but tearing lagged behind by an average of 6.54 ± 4.07 seconds.

CONCLUSIONS. Blinking and tearing share a common origin with sensory stimulation at the ocular surface. Both showed a dose-response increase with surface stimulation and were correlated with each other. These methods can potentially be used to understand alterations in ocular surface sensory function and associated protective responses in dry eye and other disorders of the ocular surface.

Keywords: tear dynamics, tear secretion, blinking, ocular surface, dry eye

Dry eye is a common disease that affects millions in the United States^{1,2} and worldwide.^{3,4} It is considered a multifactorial condition driven by stress to the ocular surface due to tear film instability and hyperosmolarity.⁵ Under normal conditions, the ocular surface is protected from stress by two reflex responses, blinking^{6–8} and tearing.^{9,10} During a full blink, the upper lid wipes the ocular surface, spreading the newly secreted tears over the surface,^{11,12} and reflex tearing occurs in the presence of adverse stimuli.^{9,13} Thus, blinking and tearing both contribute to tear film wetting of the ocular surface,¹² which is required for ocular surface maintenance and lubrication,¹⁴ and to create a smooth optical surface for good vision.^{15,16} However, despite their importance in promoting a healthy ocular surface, the dynamic responses of blinking and tearing to ocular surface stimulation and their connection to each other remain unclear.

A number of studies have demonstrated that the ocular surface sensory input plays an important role in blinking.^{6,7,17–19} Lubricated eye drops^{7,17} or anesthetics,^{7,18,20} which are expected to reduce sensory input, are known to significantly decrease the blink rate (BR). Conversely, laboratory experiments involving air blowing onto the surface of the eye^{6,7,17,19} or clinical studies of dry eye patients^{19,21,22} found

significant increases in BR, presumably due to elevated ocular surface irritation. Recently, we have shown a linear relationship between pneumatic ocular surface stimulation and blinking when the pneumatic stimulus was gradually increased.²³ These results suggest that ocular surface neurons are able to both detect and quantify a stimulus, and then modulate the blink response appropriately.

Ocular surface stimulation is also thought to be involved in so-called basic or unstimulated tear secretion, most likely through detection of evaporation by cold receptors,^{24,25} as well as reflex tearing in response to adverse stimuli,^{9,13} where it serves to wash out foreign objects and wet the ocular surface. Tear volume is often measured clinically by the Schirmer tear test,^{26,27} but the method can lack repeatability^{28,29} and cannot easily be used to measure the dynamics of the tearing response.¹⁰ Tear meniscus height (TMH) has also been commonly used to measure tear volume or secretion, using either a slit-lamp biomicroscope^{30,31} or optical coherence tomography (OCT),^{11,32–34} but blinking can alter TMH,¹¹ rendering it potentially problematic for investigating dynamic changes in the reflex tearing response over time.

Fluorophotometry has classically been used to assess tear volume and flow,^{35,36} but it requires specialized equipment that

measures a relatively small area of the tear film with relatively low temporal resolution. The fluorescein tear clearance test can be considered a clinical adaptation of fluorophotometry, in that the decline of fluorescence is monitored at various time points over a 15- to 30-minute time period.^{35,37,38} Often the fluorescein tear clearance test involves repeated instillation of fluorescein strips³⁹ and the use of visual scales to grade fluorescence,⁴⁰ with the goal of differentiating dry eye from normal subjects.^{39–42} Few studies have investigated the dynamic, rapid changes in tear secretion over time that would be expected to occur in response to increasing stimulation of the ocular surface.

Ocular surface stimulation is known to lead to an increased tear secretion, especially if the stimulus is presented to the central corneal area.^{9,13} Both the blinking and tearing pathways begin with stimulation of corneal neurons that project to the sensory complex in the brainstem through trigeminal afferent fibers.⁴³ Then, blink pathways project to the facial motor nucleus and orbicularis oculi, while tearing pathways project to the superior salivatory nucleus and finally to the lacrimal gland.⁴³ However, the relationship between tearing and blinking, the main protective responses of the ocular surface, has not been fully explored despite their common origins.

Despite the importance of these ocular surface protective responses, we were not aware of any human studies investigating these two responses simultaneously. Therefore, in this pilot study, we developed a human-based laboratory model to investigate the effect of varying levels of ocular surface stimulation on the timing and amplitude of the blink and tear secretion responses over time. We developed a novel method, based on fluorophotometry^{35,36} but using images of the inferior tear meniscus obtained from a slit-lamp biomicroscope for measuring tear secretion with high spatial and temporal resolution. Only young and healthy subjects were recruited to determine these responses within a normal physiological range to avoid any potential effects from corneal neurons altered in disease.^{44–46}

METHODS

Subjects

The study was conducted at the Borish Center for Ophthalmic Research at the Indiana University School of Optometry, Bloomington, Indiana. It adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at Indiana University. Informed consent was obtained from each subject prior to beginning the study.

Ten young, healthy subjects were recruited for the study. The number of subjects was chosen based on an earlier study of blinking in response to surface stimulation.²³ Subjects reporting ophthalmic disorders including dry eye or ocular allergies, contact lens wear, or any systemic disease or oral medications were excluded. At the beginning of the study, subjects were told that the reason for the study was to examine the tear film while they were engaged a computer task. They were not informed that the study purpose was to monitor blinking and tear secretion until the study was completed in order to avoid voluntary or other altered blinking⁴⁷ or possible changes in tear secretion due to subject expectations.

Procedures

Subjects reported to our laboratory for two visits in this investigation. The purpose of the first visit was to estimate the level of pneumatic stimulus that triggered an increased BR to

set the air stimulus levels for each individual in the second visit. The experimental data were collected during the second visit using these air stimulus levels. All subject visits were scheduled between 10 AM and 3 PM.⁴⁸

Visit 1: Estimation of BR Threshold. During the first visit, subjects filled out the Dry Eye Questionnaire⁴⁹ to assess habitual symptoms of ocular irritation and dry eye. They were seated behind a slit-lamp biomicroscope ($\times 12$ magnification, Zeiss 20SL; Carl Zeiss, Oberkochen, Germany) fitted with two cameras for simultaneous recording of the inferior tear meniscus (7.5 Hz, GRAS-20S4C-C; Point Grey, Ludwigsburg, Germany) and upper lid movement during blinking (30 Hz, Basler piA640-210gm; Basler AG, Ahrensburg, Germany). Only the right eye was tested in order to ensure the same angle between the esthesiometer stimulus tube and the cornea in our experimental setup. The other eye was manually held shut by the subject.

A self-adhesive 2-mm-diameter reflective white dot (3M Company, St. Paul, MN, USA) was gently positioned on the margin of the upper lid of the right eye to record eyelid movement during the blink, as described previously.²³ To visualize tear turnover, 1 μ m 2% fluorescein was instilled into the temporal aspect of the inferior tear meniscus. During testing, subjects looked down slightly (approximately 5°) to place the inferior meniscus over the cornea (not limbus or conjunctiva) where visualization of the meniscus was best for image analysis purposes. The inferior tear meniscus was illuminated using the slit-lamp biomicroscope light source with a cobalt blue filter positioned at an angle of 30° temporally and a Wratten number 8 filter (Eastman Kodak Company, Rochester, NY, USA) over the observation port. The area illuminated by the slit-lamp biomicroscope beam was positioned below the pupil to avoid initiation of reflex tearing secondary to the bright light. As described in our previous studies, subjects played a computer game viewed through a beam splitter throughout the measurements to keep the effect of concentration on blinking as constant as possible.^{6,23}

An instrument similar to a pneumatic esthesiometer^{50,51} was used to stimulate the cornea with air flow, as in our previous study.²³ The air intensity from the stimulus tip was recorded by a customized LabVIEW 5.1 program (National Instruments, Austin, TX, USA), and time stamps were used to match the air stimulus with blinking and tear meniscus data. The air stimulus was aimed at the central cornea at a distance of 15 mm from a slightly temporal (12°) and slightly inferior (5°) direction, so that it did not block the slit-lamp view of the inferior meniscus or the subject's vision while playing the computer game. Its position was constantly monitored by a calibrated side-mounted camera (LifeCam VX-5000, Microsoft, Redmond, WA, USA).

To obtain the baseline response, each subject's BR was first recorded with no air stimulus for 2 minutes. This time was divided into four equal sections to obtain the mean and standard deviation of the baseline blink for each subject. The air stimulus was systematically increased in a step size of 50 mL/min every 30 seconds until the observer noted an obvious increase in BR; then the trial was stopped. The threshold for increased blinking was calculated as the level of air stimulus that elevated the BR by 3 standard deviations higher than the mean BR obtained during baseline. This threshold level was used to set the six levels of pneumatic stimuli to be tested at the next study visit by multiplying it by 0 (baseline: no air stimulus), 0.25, 0.5, 0.75, 1.00, and 1.25, thereby estimating a range of sub- to suprathreshold stimuli producing an increased BR for each subject.

Visit 2: Experiment. During the second study visit, baseline and the effect of the five levels of pneumatic stimuli on the blinking and tear secretion were determined. As in the

first visit, a small white dot was placed on the upper eyelid to monitor upper lid movement to measure the blinks. A small black dot was drawn in the center of the lower eyelid using a cosmetic eye liner (Maybelline Eyestudio Master Precise, black; Maybelline, New York City, NY, USA) to enable registration of the lower meniscus images. One microliter 2% fluorescein was instilled into the right eye before each trial. Each stimulus level trial began with 30 seconds of no stimulus, followed by 1 minute of the stimulus, followed by 30 seconds without stimulation. Stimulus levels were presented in a random order, including the baseline measurement, which involved monitoring subjects for 2 minutes without stimulation. There were 5-minute breaks between trials.

Analysis

Blink Rate (BR) and Interblink Interval (IBI). As in our previous study,²³ a customized MATLAB (The Mathworks, Natick, MA, USA) program was used to track the white dot on the upper eyelid to determine the occurrence of each blink that extended to or reached beyond the middle of the preblink palpebral aperture. The BR and IBI were calculated for each level of air stimulation. This method was similar to that of many previous studies on blinking.^{23,52–54}

In order to measure the time point at which BR was significantly increased after air stimulation, a spike density analysis (Gaussian kernel: 0.3 seconds) was used to transform the raw blink data.⁵⁵ The time at which the spike density exceeded 3x standard deviations above the prestimulus level was used to define the blink increase time.

Tear Secretion and Turnover. One of the purposes of this study was to develop a new method for examining the dynamics of tear secretion and tear turnover from the inferior tear meniscus that quantified both the height of the inferior tear meniscus and its fluorescence intensity from each image. To control eye movements, all frames in each video were registered by the black marker on the lower lid (After Effects; Adobe, San Jose, CA, USA). A customized MATLAB program was used to create a rectangular region of interest (ROI) centered on the lower meniscus (Fig. 1A) or moved slightly nasally to avoid any bright reflections, if present. A pixel intensity curve was computed for each vertical column across the ROI. In order to use the information from all the columns in each image and remove the tilt of the meniscus across the ROI, the intensity curve of each column was cross-correlated and vertically aligned with the first column, and then all columns were averaged. This allowed us to summarize the information from a two-dimensional image into a one-dimensional matrix (Fig. 1B). Use of all columns across the ROI of an image increased the signal strength (detection of the TMH) and decreased the image noise, such as fluorescein spots spilled on the lower lid (Fig. 1B, arrow). Fluorescence intensity was converted into a false color scale where blue represents low pixel intensity and red represents high pixel intensity (Fig. 1C). This same analysis was performed for every image in the trial and a color map was generated, allowing visualization of the TMH and fluorescein intensity over time (Fig. 1D).

The TMH was determined when fluorescence intensity exceeded a criterion level within each image. This criterion was chosen as the minimal pixel intensity that provided a stable estimate of the tear meniscus edges during the prestimulus period (edge variability was ≤ 2 pixels for upper edge and ≤ 4 pixels for the lower edge). The lower edge criterion value was higher due to the frequent spillover of tears onto the lower lid, rendering those data more noisy than for the upper edge of the meniscus that abutted the cornea (Fig. 1B, arrow). The TMH increase was measured within each trial (Fig. 2A). We also measured the time when the TMH began to

FIGURE 1. An example of image analysis of the lower meniscus. (A) Raw color image. The white box shows the region of interest (ROI). (B) The intensity curve within the ROI across the vertical position. The blue curves represent the intensity curves from each column, overlapped after cross-correlation, with the red curve representing the average intensity. The arrow indicates the noise in the raw image, due to the fluorescein on the eyelid. (C) A false color scale converted from the intensity curve. Red represents high intensity and blue represents low intensity. (D) A color map generated from a trial, indicating both tear meniscus height (TMH) and fluorescein intensity over time.

increase after air stimulus was initiated (defined as an increase of 1/10 of the maximum TMH increment for that trial, Fig. 2A, black star).

During each trial, fluorescein concentration declined as newly secreted tears entered the meniscus and drained out through the canaliculi. However, tracking fluorescence intensity to monitor new tear secretion can potentially be complicated by fluorescence quenching and the nonmonotonic relationship between concentration and intensity (see Fig. 3B).^{36,56,57} For that reason, we calculated the tear meniscus fluorescein concentration (TMFC) from intensity based on a previously established mathematical model^{56,57}:

$$I = k(1/(1 + (f/f_0)^2))(1 - e^{-abf/w^{*10}}) \quad (1)$$

where I is the fluorescein intensity, which was standardized by the maximum intensity within each subject; k is a constant equal to 1; f is fluorescein concentration (%); f_0 is the critical fluorescein concentration (0.2%), a is a molar extinction coefficient ($7.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$), b is the film thickness (cm), and w is molecular weight of fluorescein (376 g/M). The highest fluorescein intensity value within each image should occur at the location of greatest film thickness (meniscus depth), and thus was used in this calculation. The tear meniscus depth (b) was not measured directly, but was estimated from the TMH using an average ratio (1.56) based on previously published TMH and depth data.^{33,58–60}

Equation 1 shows that a given fluorescein intensity yields two fluorescein concentrations: one in the dilute regimen and the other in the quenching regimen (Fig. 3B). Because the fluorescein concentrations should decrease over time only as new tears are secreted and tears drain, the initial fluorescein concentration value was determined from the initial fluorescein intensity and the direction of intensity change over time. As Figure 2B shows, we calculated the TMFC decrease over the trial and the time when TMFC started to decline (decrease to 1/10 of its maximum change within the trial, Fig. 2B, black star).

FIGURE 2. Examples of calculated metrics. (A) The maximum increase of the TMH within a trial (TMH increase, *vertical arrows*). The TMH increase time is defined when the TMH reaches 1/10 of its maximum difference within a trial (*black star*). (B) The maximum decrease in TMFC within a trial (TMFC decrease, *vertical arrows*). The TMFC decrease time is defined when the TMFC reaches 1/10 of the maximum difference within a trial (*black star*). (C) The slope of the fluorescein concentration was measured between blinks. (D) The linear slope was calculated from each segment (*green markers*, IBI-TTR).

Images with tear film fluorescence too low to analyze were excluded from this analysis.

To minimize the effect of blinking on measurements of tear secretion, we developed a novel metric (Figs. 2C–D) based on calculating the slope of the fluorescein concentration change within each IBI. This metric was adapted from fluorophotometry,^{35,38} in which the changing slope of the fluorescein concentration over time indicates the tear turnover rate (TTR).^{35,37} Theoretically, the TTR will be heavily influenced by the blink rate, presumably due to increased tear drainage.⁶¹ Therefore, in this study, TTR was measured during each interblink interval (IBI-TTR), thus enabling comparison of tear secretion rates among subjects who blinked at different rates.

In order to validate the TMFC calculations⁶² and to check the calibration of our slit-lamp biomicroscope imaging system, we constructed an eye model to mimic the lower tear meniscus. The eye meniscus model curvature of the upper sphere (mimicking the cornea) was 7.8 mm and of the lower sphere (mimicking the lower lid) was 12 mm, creating an angle

of 76.5° .³⁴ Two microliters of 48 fluorescein concentrations (from 0.001 to 1%) were pipetted into the eye model meniscus and imaged under the same experimental conditions as for the human subjects in this study.

Statistical Analysis. Both TMH and TMFC were smoothed using the Loess method with a span of 5% to reduce noise.⁶³ Since the TMFC decays exponentially over time, TMFC was transformed by natural logarithm (ln).^{35,37} The Pearson's correlation coefficient was used to determine the association between the air intensity, IBI, and tear meniscus metrics.

RESULTS

Subjects

The average ($\pm$ SD) age of study subjects was 23.9 ± 1.7 years (range, 22–27 years). Five of the 10 subjects were female. The median Dry Eye Questionnaire (DEQ-5) score⁶⁴ was 3 (range, 0–7) and none of the subjects reported a previous dry eye diagnosis on the DEQ or thought they had dry eye.⁶⁵ The average ($\pm$ SD) Schirmer I tear test and TBUT were 16.1 ± 13.2 mm/5 min (range, 1–35 mm) and 7.2 ± 5.9 seconds (range, 2–22 seconds), respectively. None of the subjects showed corneal or conjunctival staining. The average ($\pm$ SD) of the temperature and humidity in exam room were $24.2 \pm 1.1^\circ\text{C}$ and $44.0 \pm 1.6\%$, respectively.

Calibration of TMFC Using an Eye Model

Fluorophotometry is performed using an instrument designed to measure changes in tear film fluorescence over time, whereas a slit-lamp biomicroscope, even with the appropriate filters, is not designed for purpose. Thus, we calibrated our system⁶² using an eye model and inferior meniscus closely mimicking that of the human eye. Figure 3A shows some of the images collected from the eye model with known fluorescein concentrations, ranging from low to high fluorescein concentrations. Fluorescence intensity reached its maximum at the 0.08% concentration and then rapidly decreased due to concentration self-quenching.^{36,56} As Figure 3B shows, the raw data from the eye model (blue dots) fit well with the calculated mathematical model (blue line, $R^2 = 0.9807$), establishing that our slit-lamp system was able to detect different fluorescein concentrations and we could use the mathematical model to calculate the TMFC from its intensity.

FIGURE 3. (A) Eye model images with known fluorescein concentrations. (B) The calculated TMFC from the eye model, with the raw data points (*blue*) and best fitted line from the mathematical model. The *red lines* show the calculated TMFC from all the subjects and trials.

FIGURE 4. Blinking and tear secretion responses (subject 6) to six levels of air stimulation from 0 to 148 mL/min (A–F). Outlined in red (A) baseline response) are a meniscus color map at the top, plots of the TMFC (blue) and TMH (red) in the middle, the IBI-TTR (green) and timing of each blink (vertical bars) at the bottom. The dashed lines indicate the timing when the air stimulus was turned on and off. (B–F) These metrics with increasing air stimulation, as noted in the upper right.

The red lines in Figure 3B are the data from subjects in this study, which align well with the eye model data.

Tear Secretion and Turnover

Figures 4 and 5 show data from two individuals with the order of trials derandomized for presentation. The vertical streaks

within the color maps of the menisci were due to eye movements or blinks that altered the ROI in individual frames. It is clear from Figure 4 (subject 6) that there was very little change in TMH and TMFC until air stimulation was ≥ 87 mL/min (Figs. 4D–F), when the TMH increased and the TMFC decreased. At 148 mL/min, these changes were more marked

FIGURE 5. Blinking and tear secretion responses for subject 9. Formatting is identical to that in Figure 4. Note the lack of change in TMH in (F) when rapid blinking occurred.

FIGURE 6. Tear dynamics as a function of air intensity (A–C). A regression line was fitted for all subjects (Pearson's $P < 0.01$).

and blinking also increased rapidly at the higher levels of air stimulation. The average IBI-TTR was -0.0041 , -0.0026 , -0.0020 , -0.0299 , -0.0531 , and -0.1326 $\ln(\%)/\text{sec}$ from baseline to maximum air stimulation, respectively. The average ($\pm$ SD) time of TMH increase, TMFC decrease, and blink increase was 5.61 ± 1.29 , 4.56 ± 0.10 , and 0.54 ± 0.53 seconds, respectively, for the last three trials.

Figure 5 shows data from another individual (subject 9), who showed an even more marked increase in blinking with higher levels of air stimulation (Fig. 5F). However, in this case, the TMH increased only slightly, even under maximum stimulation, whereas fluorescence clearly decreased rapidly. Thus, at 290 mL/min , there appears to be very high tear secretion in the absence of a corresponding increase in TMH, presumably due to increased tear drainage caused by the extremely rapid blinking. Therefore, in this case, TMFC appeared to provide a more reliable measure of tear secretion overall, compared to TMH. The average IBI-TTR (absolute value) was -0.0016 , -0.0079 , -0.0160 , -0.0162 , -0.0546 , and -0.0685 $\ln(\%)/\text{sec}$ from baseline to maximum air stimulation, respectively. The average ($\pm$ SD) time of TMH increase and TMFC decrease was 5.84 ± 4.34 and 7.57 ± 1.16 seconds, respectively (excluding the last trial due to low fluorescence). The average blink increase time was very close to 0 seconds.

The data for all 10 subjects are plotted as a function of air intensity in Figure 6. Figure 6A shows the increase of TMH with air stimulation. There was a high individual variation in the TMH, which 0.41 ± 0.39 mm for all subjects. Five subjects showed only minimal changes of TMH during all the trials, presumably due to very high blink rates that acted to rapidly pump tears out of the lower meniscus, thus failing to increase TMH (see subject 9, Fig. 5). The TMH of the other five subjects increased to various levels, and then most decreased at the highest air stimulation, probably also due to very high rates of blinking. In contrast with a previous study,⁹ we found an overall poor correlation between air intensity and the TMH increase (Pearson's $r = 0.40$, $P < 0.01$), perhaps due to the sustained stimulus used in this study.

The decrease in the TMFC (Fig. 6B) shows a much less variable response between subjects than the TMH, probably because both blinks and tear secretion will decrease the TMFC. Most subjects showed a decline in the TMFC with surface stimulation, especially at the higher air flow rates. The average TMFC decrease was 2.84 ± 0.98 $\ln(\%)$ for all subjects. However, two subjects (subjects 3 and 5) showed no apparent response to air stimulation except at the highest level. This was most likely due to setting the levels of air stimulation too low for those subjects. Six out of the remaining eight subjects showed a significant linear correlation between air intensity and TMFC (Pearson's $r > 0.79$, $P < 0.05$) with an average slope 0.011 ± 0.007 . We also found an overall high correlation between air intensity and TMFC (Pearson's $r = 0.71$, $P < 0.01$).

Figure 6C shows the relationship between increasing air stimulation and the average IBI-TTR (across the stimulus). Similarly to TMFC findings, six subjects showed a significant correlation between air intensity and IBI-TTR (Pearson's $r > 0.74$, $P < 0.05$) with an average slope of $3.883 \pm 2.374 \times 10^{-4}$. There was a significant linear correlation between air intensity and IBI-TTR (Pearson's $r = 0.69$, $P < 0.01$).

Blink Response

At baseline, the IBI was highly variable among subjects, with an average IBI of 9.47 ± 8.79 seconds. The data were similar to those in our previous study²³ and thus are not shown here. For most subjects, the IBI decreased with increasing air stimulation, and the average decline was 8.08 ± 8.54 seconds for all subjects. At maximum air stimulation the average IBI was markedly lower than baseline (1.39 ± 1.11 seconds). There was a significant linear correlation between stimulus air intensity and IBI after log transformation ($r = -0.471$, $P < 0.001$).

Tear Secretion and Blinking

Our data in Figures 4 through 6 reveal that increasing corneal stimulation increases both the blink rate and tear secretion. For Figure 7, data were combined from all subjects in order to compare the relative timing of the increased blinking (BR) and tear secretion (IBI-TTR) to increased surface stimulation. In order to standardize the number of data points from each subject, each trial was segmented into sixty 2-second windows and the average IBI-TTR and BR were calculated within each window for each stimulus air intensity level. The horizontal dashed lines represent 3 standard deviations from the mean value observed during the prestimulus period. There was not much change in either BR or IBI-TTR in the first three levels of stimulus air intensity (0, 0.25, and 0.5 of the threshold intensity identified in visit 1). However, further increase in stimulus intensity led to increase in both blinking and tear secretion. From baseline to maximum levels of air stimulation, the peak BRs were 21, 27, 30, 78, 87, and 114 blinks/min, and the peak IBI-TTR were -0.0346 , -0.0718 , -0.0968 , -0.1585 , -0.3391 , and -0.4777 $\ln(\%)/\text{sec}$, respectively. Interestingly, both the BR and IBI-TTR responses spiked transiently and then declined during the 1-minute stimulus period (Figs. 7D–F), even though the stimulus was maintained.

The obvious correlation between tear secretion (IBI-TTR) and blinking (BR) shown in Figure 7 for data averaged across all subjects was examined quantitatively (Fig. 8) via a scatter plot of IBI-TTR and IBI for each subject and each stimulus intensity level. The IBI and the IBI-TTR were significantly correlated after log transformation as shown in Figure 8 (Pearson's $r = -0.556$, $P < 0.001$).

From Figures 7D through 7F we can see that the spike in IBI-TTR was delayed slightly relative to the spike in BR. In

FIGURE 7. Aggregate data for the BR and IBI-TTR with six air levels (baseline to maximum stimulation) for all subjects (A-F). The blue markers are the blink rate (BR), and the red markers are the IBI-TTR. The dashed lines represent 3 standard deviations from the mean value (prestimulus in the first 30 seconds of each trial). The black vertical lines indicate the timing when the air stimulus was turned on and off.

Figure 9, we show histograms of the times at which blinking and tearing (TMH and TMFC) started to change after stimulus onset (response latency) for all subjects. Blinking increased quickly after the air stimulus began, with an average blink onset time of 0.60 ± 1.12 seconds. The tearing response, as measured by TMH and TMFC in this study, occurred later, with the average onset time of 6.30 ± 4.80 and 7.29 ± 3.95 seconds, respectively. The average time difference between blinking and tearing onset was 6.54 ± 4.07 seconds for all subjects in all trials.

DISCUSSION

This study is the first, to our knowledge, to measure the dynamics of blinking and tear secretion simultaneously in response to ocular surface stimulation. In order to measure tear secretion along with the blink response, we developed new methods of image analysis, adapted from fluorophotometry,^{35,37} that can be employed without specialized equipment. Our results support the hypothesis that ocular surface stimulation increases both blink rate²³ and tear secretion,⁹

and demonstrate that these two protective responses are significantly correlated with each other. The establishment of a dose-response relationship between ocular surface stimulation, blinking,²³ and tearing⁹ in young, healthy subjects has implications for the dry eye condition, where the ocular surface sensory response,^{44,45,66} blinking,^{19,21} and tearing^{66,67} are all known to be altered.

In this study, we developed novel methods for quantifying tear secretion to add to our previous apparatus for measuring changes in blink rate,²³ so that both could be simultaneously measured in response to known levels of ocular surface stimulation. Traditionally, TMH has been used to estimate tear volume^{30,68,69} or secretion.⁷⁰ However, we found that high blink rates apparently obscured tear secretion in some cases, as measured by TMH (Fig. 6A), presumably due to the increased drainage of tears with rapid blinking.⁶¹ This caveat potentially limits TMH as a measure of tear secretion and could lead to

FIGURE 8. Correlation between the IBI and IBI-TTR with a regression line.

FIGURE 9. Histograms of the response times for the blink and tear secretion into the meniscus for all subjects and all trials.

marked underestimates of reflex tearing when accompanied by increased blinking, even using direct volumetric measurement such as OCT.^{33,34}

For these reasons, we developed a method, adapted from fluorophotometry,^{35,38,67} calculating changes in fluorescein concentration from fluorescein intensity. The tear secretion rates were indirectly quantified from the decline of fluorescein during the interblink. This method is based on the idea that tear secretion into the inferior tear meniscus should lead to decreased fluorescein concentration. Thus, unlike TMH, the effect on TMFC will be measurable even in the presence of very high blink rates, until the concentration of dye is too low to be detectable. In this study, we instilled a very small amount (1 μ L) of fluorescein dye at the beginning of testing with minimal changes in TMH or TMFC during the prestimulus period (Figs. 4, 5), so this was unlikely to affect our results. The difficulty with this method lies in interpreting changes in tear meniscus fluorescence over time and in properly converting fluorescence intensity to concentration. To address this issue, we developed an in vitro method to verify known fluorescein dye concentrations with our slit-lamp biomicroscope and digital camera system.⁶² Figure 3 demonstrates that our in vitro eye model data and calculated TMFC data from experiments (in vivo) both closely fitted in a previously determined mathematical model.^{56,57}

Another potential issue with the calculation of TMFC from pixel intensity was that the inferior meniscus depth (or film thickness) was needed but was unknown. In this study, we estimated the meniscus depth from the TMH at baseline, using the ratio of height to depth values published in previous studies.^{33,58–60} This ratio agrees well among investigations with very different study populations and thus appears to provide a good surrogate for measured depth. In addition, we found that changes in this measure have very little effect on calculated concentrations when the film thickness exceeds 0.15 mm. In this study, the average estimated meniscus depth was 0.16 ± 0.04 mm during baseline and became much greater during tear secretion (0.21 ± 0.09 mm). Thus, potential errors in calculating the TMFC produced by estimating film thickness (meniscus depth) in this study should have been minimal.

In order to better separate the effects of blinking from tear secretion, we developed a novel metric, the IBI-TTR. Tear drainage depends on the pressure gradient between the meniscus and canaliculi, which is created as the eyelid begins to move upward, uncovering the puncta during the opening phase of the blink; but then the pressure declines rapidly.^{71–73} In this study, we measured the slope of the TMFC during the interblink period while the eye was fully open, thus minimizing the effect of tear drainage on the TMFC. Therefore, the TMFC decrease in the interblink period should be primarily due to the effect of newly secreted tears, and thus provides a measure sensitive to the high secretion rates during reflex tearing.

Both of these newly developed metrics for tear flow and secretion, the TMFC and IBI-TTR, showed a dose-response relationship with increasing ocular surface stimulation. This effect was much less marked with TMH, most likely for the reasons discussed above. Previous studies suggest that basal tear secretion is mainly due to the activation of cold receptors,^{24,74} whereas reflex tearing is mainly triggered by stimulation of polymodal nociceptors in response to noxious stimulation.^{9,13} In this study, tear secretion was relatively similar in the first three trials and markedly increased with air level 4, suggesting that the noxious threshold for stimulation of polymodal nociceptors was breached at that level.¹³ Increasing surface stimulation beyond level 4 led to an even greater response, which is consistent with corneal electrophysiology studies demonstrating that polymodal nociceptors encode the

intensity of the stimulus.^{75–77} Noxious stimulation of polymodal nociceptors is also the most likely explanation for the dose-response relationship between ocular surface stimulation and blinking, especially for strong surface stimulation.^{9,13} Thus, the correlation between the blinking and tearing response is not surprising, given that both arise from stimulation at the ocular surface.⁴³

The blinking and tear responses showed other similarities, aside from their correlation to each other. In air stimulation levels 4 to 6, both showed an initial spiked response and then diminished, even while the stimulus was maintained (Fig. 7). This may be due to the neural characteristics of corneal receptors, which, when stimulated, fire at a high rate initially, followed by a gradual decline, even though the stimulus is maintained at the same level.^{74,78,79} This response may have clinical implications for the dry eye condition, in which chronic or sustained stress to the corneal surface by an inadequate tear film may not elicit the appropriate protective responses (blinking and tear secretion), resulting in potential damage on the ocular surface.⁸

Although the blinking and tearing responses were correlated and showed similar patterns, the initial timing of the responses differed. The blink response to ocular surface stimulation was very rapid (onset time < 1 second), whereas increased tearing was delayed by approximately 6 seconds. The reason for this difference in timing is unclear, but it is most likely due to later aspects of their pathways since they share the initial portion.⁴³ Blinking involves innervation of the orbicular muscles, while tearing requires stimulation of the lacrimal gland to secrete tears, which then must move into the lower meniscus.⁸⁰ Thus, the delay in tear fluorescein in the lower meniscus may represent the time needed for tears to flow into the menisci following secretion. When viewed from the standpoint of protecting the eye from a foreign body, the difference in timing of the two responses makes sense. Immediate, rapid blinking would help to quickly displace a foreign object away from the central cornea, which then could be washed out of the eye by reflex tearing.

This study examined the timing and extent of the two main reflexive, protective mechanisms available to the ocular surface in response to adverse stimulation.^{5,14} We developed novel techniques, adapted from fluorophotometry^{35,37} and calculating fluorescein concentration from intensity. We also developed a novel metric, using the rate of fluorescence decline between blinks (IBI-TTR), to measure tear secretion in response to ocular surface stimulation. Both the blinking and tearing response originate with ocular surface stimulation and thus depend on initiation by ocular surface sensory nerves.⁴³ While this study tested young, healthy subjects to establish normal protective response of the ocular surface, these methods can be used to further understand potential deficiencies in these responses in ocular surface conditions such as dry eye.^{19,44,45,66}

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