Dry eye is a multifactorial disease of the tears and the ocular surface resulting in symptoms of discomfort, visual disturbance, and tear film instability [1]. It is estimated that more than 5 million Americans aged 50 years old or older have symptomatic dry eye and that approximately 20 million or more are affected by dry eye of any severity [2]. Although dry eye is broadly classified into either evaporative or aqueous deficient or a mixture of both, the multifactorial nature of the disease suggests the underlying mechanisms and contributing factors are more complex [1].

One important factor in maintaining a healthy ocular surface is the composition of the tear film, which consists of lipids, aqueous, soluble proteins, and mucins. The lipids of the tear film slow the evaporation of the aqueous tears, in addition to providing a barrier from tear film spillage over the lid margins. The aqueous plays a role in hydration of the ocular surface to provide the optimal refractive surface, as well as for physiologic processes involved in homeostasis. For instance, the aqueous contains essential cytokines, proteins, and immune factors to clear away debris, protect the eye from pathogens, and keep the eye moisture rich [3].

Ocular mucins are highly O-glycosylated proteins with large molecular weights and are present in two main forms: a secreted form in the tears and a membrane-associated form in the glyocalyx, which is a protective barrier on the cornea and the conjunctiva [4,5]. The primary secreted mucin, the gel forming mucin MUC5AC, has the major role of lubricating the ocular surface and is secreted by the goblet cells of the conjunctiva [6]. The secreted mucins also aid in clearing away pathogens and debris. Although three membrane-associated mucins (MAMs) have been identified on the ocular surface as a part of the glyocalyx (MUC1, MUC4, and MUC16), MUC16 has been shown to play the...
This research was conducted in accordance with the Declaration of Helsinki with Institutional Review Board approval at the University of Alabama at Birmingham and adhered to the ARVO statement on human subjects. Fifteen subjects without ocular surface disease were recruited. Informed consent was obtained from subjects after explanation of the purpose of the study and the procedures involved for collection of tear film samples. All subjects provided a thorough ocular and medical history and confirmed absence of dry eye using the ocular surface disease index (OSDI). Subjects underwent clinical examination, including visual acuity and ocular health assessment using a slit-lamp biomicroscope to confirm the absence of ocular surface disease. Subjects were excluded if artificial tears or ophthalmic medication had been used within 48 h before the study visit. Subjects were excluded if there were signs of meibomian gland dysfunction, blepharitis, papillary conjunctivitis, or greater than mild gradings of conjunctival redness. Subjects included in the study had no complaints of dry eye and no previous diagnosis of dry eye disease.

**Tear sample collection:** Subjects presented for tear film collection on three separate days for each of the three different tear collection methods selected for this study. The tear collection methods selected for this study include Schirmer’s strip, basal tear collection, and flush tear collection; the order of tear collection method was randomized for each subject and used on both eyes during the visit. For the first method, a Schirmer’s strip (TearFlow Diagnostic Test Strip; Rancho Cucamonga, CA) was placed at the temporal canthus of each eye for 5 min without anesthetic. The strips were then removed from the eyes, and the amount of wetting in millimeters was recorded by observing the location of the leading edge of moisture on the printed millimeter marks. Strips were placed in centrifuge tubes to be stored immediately at −80 °C. To collect basal tears, microcapillary tubes (Drummond Scientific, Broomall, PA) of various sizes (1, 2, and 5 µl) were carefully placed at the lower tear meniscus temporarily to allow for tears to be collected via capillary action. Collection stopped once a maximum of 15 µl was collected or 3 min had passed to prevent reflex stimulation of tears. For the third method, 60 µl of saline was pipetted on the eye followed by the subject closing the eye and moving the eye around to ensure mixing of the saline with the tears. The subject was instructed to tilt his or her head to the side and open the eye as the tears were collected using a 15 µl capillary tube (Drummond Scientific). For both methods, the volume of tears collected was calculated based on the length of the tears collected in the tubes given the diameter and then recorded.

METHODS

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Tear fluid was expelled into microcentrifuge tubes using a bulb dispenser followed by immediate freezing at −80 °C.

**Protein extraction, precipitation, and quantification:** For protein extraction from the Schirmer strips, 100 mM ammonium bicarbonate was added to the strips and allowed to solubilize for 1 h at room temperature. Following centrifugation to pellet the strip, the supernatant was transferred to a new microcentrifuge tube. Acetone was added at an 8:1 ratio and incubated at −20 °C overnight to precipitate the protein. Protein was pelleted using centrifugation and subsequently washed once with fresh acetone. After aspiration of acetone with care to avoid disturbing the protein pellet, the pellet was resuspended in a 1:100 ratio of protease inhibitor and PBS (1X; BioWhittaker w/o Ca++, Mg++, pH 7.4; Lonza, Basel, Switzerland) solution. To maximize the tear protein and mucin yield, acetone was used as a precipitation agent in the basal and flush tear samples. A similar protocol was followed for the tear samples, including the addition of prechilled acetone, overnight incubation, centrifugation, and resuspension in a protease inhibitor solution. The standard microplate protocol for the Bradford protein assay using standard curves was followed for determining the total protein concentration in all of the samples [20,23,24].

**Mucin analyses:** All samples from the right eyes were analyzed for MUC5AC whereas all samples from the left eyes were analyzed for MUC16. Ten micrograms of total protein from each individual sample were denatured in 4X Laemmli buffer. Each individual sample was then loaded in a lane into a 1% (w/v) agarose gel and run in electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3, 0.1% sodium dodecyl sulfate) for 2 h at room temperature for protein separation. The HiMark prestained protein ladder was used to mark molecular weights from 30 to 460 kDa (Thermo Fisher Scientific; Waltham, MA). Capillary transfer overnight was used to transfer the proteins from the gel to the nitrocellulose membrane via diffusion. The transfer stack consisted of the gel, membrane, and two pieces of thick, blotting filter paper (Bio-Rad Laboratories; Hercules, CA). The stack was placed horizontally over a reservoir filled with transfer buffer (0.6 M NaCl, 60 mM sodium citrate), over a “wicking” piece of filter paper, whose ends were in contact with the reservoir buffer. The topmost layers of the filter paper in the transfer stack are initially dry; thus, diffusion pulls the buffer through the wick, through the layers of the transfer stack, and toward the dry layers on the top of the stack. This process is stimulated by placing a stack of paper towels and a weight on top of the whole stack, which ensures that all the layers are in close contact and that the buffer will diffuse quickly throughout.
all the layers. Confirmation of complete transfer was made the following morning through visualization of the total transfer of the stained ladder from the gel to the nitrocellulose membrane. After transfer, membranes were blocked with bovine serum albumin (BSA) in PBS and incubated with either MUC5AC antibody CLH2 (1:200) or MUC16 antibody OC125 (1:20) for western blot analysis. Blots were developed with enhanced chemiluminescence (ECL; Thermo Scientific Pierce), and the chemiluminescent signals were captured with the Odyssey Fc Imaging System (LI-COR Biosciences; Lincoln, NE).

Statistical analysis: The relative amounts of MUC5AC and MUC16 were quantified with densitometry using Image Studio Lite v5.2 (Biosciences, LI-COR Inc.) and compared for statistically significant differences between the tear collection methods using the non-parametric Kruskal–Wallis test in SPSS 22 (IBM; Armonk, New York) and GraphPad Prism 7.02 (La Jolla, CA). The Wilcoxon matched-pairs signed-rank test was used to compare the tear volumes, tear protein, and protein concentrations collected between the right and left eyes for each method. Dunn’s multiple comparisons test was used for pairwise post-hoc comparisons. Statistical analysis: p < 0.05 was considered statistically significant.

RESULTS

Subjects in this study included three men and 12 women with an average age of 25.8±3.0 years (median, 24.5 years; range, 23–33 years). Tear film samples containing less than 10 μg of total protein were not used for analysis; the samples not analyzed included one basal tear sample, one Schirmer sample, and 15 flush tear samples. This left 38 right eye (OD) and 36 left eye (OS) samples out of 45 total samples collected per eye. The median tear volume collected using the flush method was 17 µl (the interquartile range [IQR] = 15.25–17.00 µl) for right eyes and 17 µl (IQR = 16.70–17.00 µl) for left eyes with no difference between eyes (Z = 7, p = 0.44; Figure 1A). Collection of basal tears with a microcapillary tube yielded a median tear volume of 15 µl (IQR = 9.75–15.00 µl) for right eyes and 15 µl (IQR = 10.10–15.50 µl) for left eyes with no difference between eyes (Z = –3, p = 0.81; Figure 1A). The median amount of wetting on the Schirmer’s strip was 33.0 mm (IQR = 23.75–35.00 mm) for right eyes and 30.5

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Figure 2. Bradford results for the total protein and protein concentrations showing individual amounts, as well as the median and the interquartile range, for each method and eye. A: The median total protein amount obtained using Schirmer’s strip was 34.56 µg/µl for the right eyes and 47.93 µg/µl for the left eyes. For basal tears, the median total protein amount was 32.08 µg/µl for the right eyes and 65.58 µg/µl for the left eyes. For flush tears, the median total protein amount was 39.27 µg/µl for the right eyes and 46.93 µg/µl for the left eyes. B: The median protein concentration using Schirmer’s strip was calculated to be 0.69 µg/µl for the right eyes and 1.03 µg/µl for the left eyes. For basal tears, the median protein concentration was 0.64 µg/µl for the right eyes and 1.31 µg/µl for the left eyes. For flush tears, the median protein concentration was 0.79 µg/µl for the right eyes and 0.96 µg/µl for the left eyes. * p<0.05 between the eyes for Schirmer tears and *** p<0.001 between the eyes for basal tears.
mm (IQR = 16.25–35.00 mm) for left eyes with no difference between the eyes (Z = –19, p = 0.43; Figure 1B).

From the Bradford assays, the median right eye total protein was 34.56 µg (IQR = 25.48–40.24 µg) using Schirmer's strip, 39.27 µg (IQR = 23.60–69.43 µg) for flush tears, and 32.08 µg (IQR = 24.33–40.52 µg) for basal tears (Figure 2A). The median left eye total protein was 51.28 µg (IQR = 41.29–73.04 µg) using Schirmer’s strip, 47.93 µg (IQR = 36.02–61.97 µg) for flush tears, and 65.58 µg (IQR = 40.70–76.17 µg) for basal tears (Figure 2A). While a statistically significantly higher amount of tear protein was obtained for left eye Schirmer’s strips (Z = 71, p = 0.02) and for left eye basal tears (Z = 97, p = 0.0009) when compared to the amount obtained in the right eyes for each method, there was no difference in tear protein between eyes for flush tears (Z = 2, p = 0.94).

The protein concentration for each eye and method was calculated as the total protein in 1 µl of tear fluid. The median right eye protein concentration was 0.69 µg/µl (IQR = 0.51–0.80 µg/µl) using Schirmer’s strip, 0.64 µg/µl (IQR = 0.49–0.81 µg/µl) for basal tears, and 0.79 µg/µl (IQR = 0.47–1.39 µg/µl) for flush tears (Figure 2B). The median left eye protein concentration was 1.03 µg/µl (IQR = 0.83–1.46 µg/µl) using Schirmer’s strip, 1.31 µg/µl (IQR = 0.81–1.52 µg/µl) for basal tears, and 0.96 µg/µl (IQR = 0.72–1.24 µg/µl) for flush tears (Figure 2B). When the right and left eyes were compared, a higher protein concentration was found for left eye Schirmer’s tears (p = 0.03) and left eye basal tears (p = 0.002), but there was no statistically significant difference for flush tears (p = 0.87). Levene’s test for variance between techniques did not show a statistically significant difference (p = 0.48). There was also no statistically significant

Figure 3. Western blot results for detection of MUC5AC in the tear fluid collected using the three tear collection methods. Only samples that yielded greater than 10 µg of protein were probed using antibody CLH2 on individual samples of tears collected from the right eyes of human subjects. A: Results after probing tear samples collected using the Schirmer’s strip (n = 15). B: Results after probing flush tear samples (n = 9). C: Results after probing basal tear samples (n = 14). The ladder marker for 250 kDa is displayed for all blots.
Figure 4. Western blot results for detection of MUC16 in the tear fluid collected using the three tear collection methods. Only samples that yielded greater than 10 µg of protein were probed using antibody OC125 on individual samples of tears collected from the right eyes of human subjects. A: Results after probing tear samples collected using the Schirmer’s strip (n = 14). B: Results after probing flush tear samples (n = 7). C: Results after probing basal tear samples collected (n = 15). The ladder marker for 250 kDa is displayed for all blots.
difference in protein concentration when the tear collection methods were compared (H = 0.441, p = 0.80).

Assay of mucins in tears collected with three different methods: MUC5AC and MUC16 were detected in all samples as all lanes displayed identifiable bands at or greater than 460 kDa [10]. The MUC5AC median relative signal intensities from Schirmer’s strip, basal tears, and flush tears were 2.86 (n = 15, IQR = 2.54 – 3.21), 1.65 (n = 14, IQR = 1.34 – 3.10), and 1.67 (n = 9, IQR = 1.42 – 1.72), respectively, (H = 9.5, p = 0.009; Figure 3). The MUC16 median relative signal intensities from Schirmer’s strip, basal tears, and flush tears were 1.88 (n = 14, IQR = 1.43 – 2.61), 5.24 (n = 15, IQR = 4.16 – 6.21), and 2.45 (n = 7, IQR = 1.85 – 2.48), respectively (H = 18.1, p = 0.001; Figure 4). Post-hoc pairwise comparisons for MUC5AC showed a significant difference between Schirmer’s strip and flush tears (p = 0.01; Figure 5A). Post-hoc pairwise comparisons for MUC16 showed significant differences between basal tears and Schirmer’s strip (p = 0.0003) and between basal tears and flush tears (p = 0.006; Figure 5B).

DISCUSSION

The results of this study confirm the presence of gel-forming MUC5AC and shed membrane-associated MUC16 in human tear fluid, as well as demonstrating differences in the amount of mucin obtained when various tear collection methods are used. Although all three tear collection methods described here are easily performed and tolerated, the results show the importance of selecting a method based on the mucin of interest when investigating these glycoproteins in the tear film. The microcapillary collection method for basal tears yielded the highest relative concentration of MUC16 based on densitometry, which was statistically significantly different from the amount obtained with either Schirmer’s strip or in flush tears. However, tear collection using Schirmer’s strip yielded the highest MUC5AC concentration although it was statistically significantly different only from the flush tears. Although it was hypothesized that the Schirmer’s strip method would yield a higher concentration of MUC16 as the strip pulls conjunctival epithelial cells when it is removed from the eye, reflex tearing induced by irritation from the strip, as well as the capture of goblet cells, may have contributed to a greater yield of MUC5AC secretion from goblet cells.
with this method [25]. By this reasoning, the MUC16 concentration in the tears may be unaffected by reflex tearing and may explain why Schirmer’s strip did not yield the highest amount of MUC16.

The tear protein concentration averaged around 1 µg/µl across all three methods which was lower than what has been reported by Posa et al. at 4.8 µg/µl for basal tears versus 4.6 µg/µl from Schirmer’s strip. However, Powell et al. reported an average protein concentration of 2.82 µg/µl when using the acetone precipitation method used in this study [24]. Although in this study, Schirmer’s strip was performed at the same time with the right eye strip inserted first, on average, the left eye Schirmer’s strip protein concentration was higher than that for the right eye (OS M = 1.03 µg/µl versus OD M = 0.69 µg/µl). This similar trend was seen also for basal tear collection (OS M = 1.31 µg/µl versus OD M = 0.64 µg/µl), which may be explained by the subject’s apprehension with the collection starting on the right eye and less apprehension when the collection moved to the left eye. Previous studies have shown differences in proteins and tear film composition in basal versus reflex tears which may aid in explaining these differences although care was taken to avoid reflex collection of tears during the collection of basal tears [18,26].

Previous studies have shown the presence of MUC5AC and MUC16 protein in human tear fluid [27,28]. Most studies used the flush tear method or Schirmer’s strip to collect tears. A study by Spurr-Michaud used the flush tear method to demonstrate shed MUC16 in the tears for the first time [10]. As mentioned, soluble forms of membrane-associated mucins exist in the tears as extracellular domains. Therefore, collection of tears to investigate these mucins may not be representative of membrane-associated mucins in the glyocalyx. Usage of techniques such as impression cytology may need to be employed to investigate membrane-associated mucins as they emanate from conjunctival epithelial cells.

In conclusion, the microcapillary method for basal tears can be used in studies investigating MUC16 in human tear fluid. Although the Schirmer’s strip method may obtain more MUC5AC, the strip as it is applied may be collecting a small amount of MUC5AC from the palpebral conjunctiva which may differ in molecular weight and structure from MUC5AC secreted in the tears. Moreover, the reflex effect of the Schirmer’s strip may induce release of MUC5AC from goblet cells and thus may not be representative of the basal levels of MUC5AC in the tear film. Finally, although flush tears may seem an optimal method to use especially in severe dry eye patients, dilution of the tears with saline yields less than desirable amounts of MUC5AC and MUC16 for subsequent analysis.

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