International Journal of
Ophthalmology and Clinical Research

Sia et al. Int J Ophthalmol Clin Res 2016, 3:048
Volume 3 | Issue 1
ISSN: 2378-346X

Case Report: Open Access

Non-stimulated Tear Sample Collection Using Polyvinyl Alcohol (PVA) Foam and Polyester Wick

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Abstract

Background: Tear fluid analysis contributes to the greater understanding of various ocular and systemic diseases. Obtaining adequate samples for tear analysis requires an effective collection method. The direct aspiration method using capillary micropipette may be challenging to the operator and patients especially when collecting non stimulated tear samples. The purpose of this study is to assess efficacy of polyvinyl alcohol (PVA) foam and polyester wick (PW) for non-stimulated tear sample collection.

Methods: Thirty subjects participated in the first part of the study to quantify tear volume in seconds using PVA foam (Microstaar foam tip plunger, Staar Surgical) and PW (Transorb Wick, Filtrona). Tear volume absorbed (VA), volume recovered (VR) and recovery ratio (RR) were determined. Tear protein analysis included major protein profile and total protein concentration (TPC). Twelve subjects participated in the second part of the study to quantify lysozyme, lactoferrin, IgA and serum albumin by enzyme-linked immunosorbent assay (ELISA) technique from tears collected from PVA and PW for 3 minutes.

Results: Ninety samples were collected (PVA = 45, PW = 45) for first part of the study. VA by PVA (6.80 ± 1.29 µL) and PW (6.44 ± 1.36 µL) were comparable (p = 0.267) whereas VR from PVA (3.89 ± 1.12 µL) was less compared to PW (5.66 ± 1.35 µL, p = 0.051). RR from PVA was significantly less than in PW (29% vs. 67%, p < 0.001). Mean TPC analyzed from 12 PVA samples (16.90 ± 2.72 mg/mL) and 21 PW samples (16.60 ± 2.02 mg/mL) were comparable (p = 0.674). Major tear proteins profiles (79 kDa, 27 kDa, and 18-14 kDa) were identical regardless of collection material used. Lysozyme, lactoferrin, IgA and serum albumin detected by ELISA were from 6 PVA samples (0.26 ± 0.03mg/mL, 9.80 ± 3.13mg/mL, 0.88 ± 0.15 mg/mL and 0.88 ± 0.33, 113 respectively) and 9 PW samples (0.30 ± 0.01 mg/mL, 11.62 ± 2.07 mg/mL, 1.10 ± 0.73 mg/mL and 0.88 ± 0.25 mg/mL, respectively) were comparable.

Conclusion: Both PVA and PW showed similar ability to absorb tear fluid but PVA was less efficient in tear fluid recovery.

Keywords

Tear collection, Polyvinyl alcohol, Polyester wick, Tear protein

Background

Evaluation of the tear film is often done through clinical assessment of its production, stability and evaporation rate [1,2]. Over the years, several studies have focused on the contribution of tear film biochemical composition in specific disorders of the eye [3-5]. Valid conclusions from the analysis of tear biochemical composition necessitate effective tear fluid collection. Various methods and materials for tear fluid collection have been described in the literature (Figure 1) [6-9]. The 'standard' and most common method is through direct aspiration using a glass capillary micropipette. This, however, is slow, inconsistent, poorly tolerated by patients, and is not suitable for use in children. In our experience, both the operator and patient seem to have increased dissatisfaction with this method especially when collecting non-stimulated tears as it uses a delicate material which could easily break; and it requires a longer collection time. Other investigators reported that the collection time by this method was affected by the relative viscosity of the tears and that air bubbles entering the lumen slowed down tear collection [9]. Because of its drawbacks, glass capillary micropipettes are not highly regarded in the clinical setting and remain a tool primarily in research.

Alternatives to direct aspiration are absorption and recovery techniques using different materials such as Schirmer strips, polyurethane minisponges, polyester fiber rods, and cellulose acetate rods [7-9]. Jones et al. [9] reported that direct aspiration by glass capillary micropipette and absorption recovery techniques by polyester wick are equally efficient. Polyester wick also has greater clinical utility and can facilitate routine analysis of the tear film [9] Seifert et al. [10] were able to develop an immunoassay for the tear

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Citation: Sia RK, Ryan DS, Howard RS, Haymes S, Zhou Y, et al. (2016) Non-stimulated Tear Sample Collection Using Polyvinyl Alcohol (PVA) Foam and Polyester Wick. Int J Ophthalmol Clin Res 3:048

Received: January 08, 2016: **Accepted:** February 20, 2016: **Published:** February 23, 2016

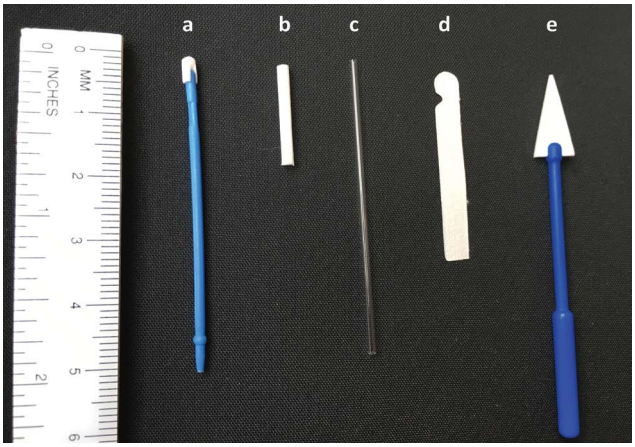

Figure 1: Different types of tear collection methods: **a**) polyvinyl alcohol foam tip plunger, **b**) polyester fiber rod wick, **c**) glass capillary micropipette, **d**) filter paper strip and e) polyvinyl alcohol microsponge.

glycoprotein lacritin using tears collected with the polyester fiber rod wick. This method was also employed in a study that determined tear nerve growth factor levels in patients with diabetic retinopathy [11].

Polyvinyl alcohol (PVA) foam is widely used commercially and also in ophthalmology. It is highly absorbent, nontoxic, and less friable, making it an ideal microsurgical sponge used in laser-assisted in situ keratomileusis (LASIK) and the preferred delivery device for anti-proliferative agents in glaucoma surgery [12]. Inic- Kanada, et al. [13] have tested three ophthalmic PVA sponges (Merocel, Pro-Ophtha and Weck-Cel) to quantify cytokines and they found varying efficacy among the sponges, attributing the differences to the structure or sponge matrix. The PVA material in Merocel has 100% open pores in its structure and has no dead-end pockets that may hold residues which make it highly absorbent and fast-wicking. The PVA material in Pro- Ophtha appears to have a comparable structure as the quantification results were similar, albeit less effective to Merocel. The material in Weck-Cel is made of natural cellulose which is also highly absorbent but its sponge matrix is more pronounced and may have micropockets that can trap residues [13].

A PVA foam tip plunger device developed by Staar Surgical (Monrovia, California) is intended for use in the Microstaar delivery system for collamer intraocular lens (IOL) implantation [14]. In surgery, it is assembled with a spring-loaded injector device to deliver the IOL into the eye. The unique design of the foam tip plunger device makes it suitable for tear collection. Experienced personnel can easily collect tears by holding on to the plastic mini-hand piece while the PVA foam tip safely absorbs tear fluid from the patient's eye.

In this study, we compared the PVA foam to the polyester wick to determine which method demonstrated greater efficacy in tear absorption and release, as well as, post-collection protein concentration and protein composition.

Methods

The research protocol was reviewed and approved by the Walter Reed National Military Medical Center Institutional Review Board (Reference numbers 352844 and 365836) prior to implementation. Adult volunteers with no history, signs and symptoms of ocular surface disease were enrolled in the study. Informed consent for participation in the study was obtained from each subject after counseling on the risks and benefits of their participation and the study was carried out in accordance with the tenets of the Declaration of Helsinki.

The study was conducted in two parts. The first part investigated efficacy of the test materials by determining tear volume, major tear protein profile and total protein concentration. To further assess efficacy, the second part of the study quantified specific proteins: lysozyme, lactoferrin, IgA and serum albumin. Serum albumin was used as a marker for ocular irritation and stress.

Tear volume determination

A total of thirty subjects participated in the first portion of the study. Three tear samples were collected sequentially from the left eye of each subject. Tear collection was completed using both a polyvinyl alcohol foam (Microstaar foam tip plunger, Staar Surgicals Monrovia, CA) and a 2.0 mm × 15.0 mm polyester wick (Transorb Wick, Filtrona, Richmond, VA) (Figure 1a and 1b, respectively). To control for order effect, subjects were randomized to one of two groups. In group 1, the sequence of tear collection was: PVA foam, polyester wick, PVA foam. The order of collection material for group 2 was: polyester wick, PVA foam, polyester wick. Subjects wearing contact lenses were asked to remove their lenses and wait for five minutes before proceeding with the tear collection procedure. After instilling one drop of 0.5% proparacaine on the cornea and conjunctiva, excess fluid around the eye was blotted dry. Subjects were asked to wait for a period of two minutes. Tears were collected by gently and intermittently placing the test material on to the inferior cul-de-sac of the subject's eye for 60 seconds. Subjects were given a five minute interval between collections. At the end of the tear collection, subjects were asked for their preferred test material based on comfort.

To determine the tear volume absorbed, the test material was placed in a 1.5 ml Eppendorf tube/pipette tip basket prior to tear collection and weighed using an analytical balance. The test material was subsequently re-weighed after tear collection. Tear volumes were determined assuming tear fluid density equaled 1g/mL [7]. Absorbed tear fluid was recovered from the test material by batch centrifugation for each subject at 4,400 rpm for seven minutes using an Eppendorf Centrifuge (Westbury, NY).

Tear volume recovered was equal to the difference of the pre- and post-centrifugation weight of the 1.5 ml Eppendorf tube. Recovery ratio or ratio between volume recovered and volume absorbed was also determined and expressed as a percentage.

Tear protein quantification

The tear total protein concentration (TPC) was determined using an established protocol [7] with minor modifications. Briefly, a 3 µL aliquot of each tear fluid sample as well as 3 µL aliquots from a serial dilution of bovine serum albumin (BSA) standard solution were spotted on a filter paper (Whatman 1MM). Filters were air-dried at room temperature for ten minutes, fixed for five minutes in 5% trichloroacetic acid, rinsed for five minutes in 80% ethanol, and stained for 20 minutes in 0.5% Coomassie blue dissolved in 45% isopropanol/10% acetic acid. The filters were washed thoroughly in 7% acetic acid until the background was almost white and then dried using a hair dryer. Blue-stained areas corresponding to each sample or standard solution were cut out of the filter and eluted using 1.0 mL of a mixture comprising 66% (vol/vol) methanol and 0.5% ammonia. Concentrations of eluted proteins were determined using a spectrophotometer by measuring absorbance at 630 nm. Tear protein concentrations were expressed in reference to BSA.

Polyacrylamide gel electrophoresis

Tear samples (5 µL) were mixed with 5 µL NuPAGE Lithium Dodecyl Sulfate (LDS) sample buffer and 2 µL NuPAGE antioxidant (Invitrogen, Carlsbad, CA), heated at 95°C for 10 minutes and subjected to SDS-PAGE analysis using the XCell Mini-Cell electrophoresis unit (Invitrogen). Samples were loaded onto 4-12% NuPAGE Novex Bis-Tris gels together with standards (SeeBlue Plus2, Invitrogen) and separated under reducing conditions in MOPS-SDS running buffer. Electrophoresis was run at a constant voltage of 200 V for 50 minutes. Gels were stained with Colloidal Blue Staining Kit (Invitrogen).

Specific tear protein quantification

Twelve healthy volunteers participated in this portion of the study. Tear samples were collected using PVA foam and polyester wick. Tear collection from the left eye was done under topical

anesthesia for three minutes. A five minute interval was given between each tear collection. The concentrations of four specific tear proteins, lactoferrin, lysozyme, albumin, and immunoglobulin A (IgA), in the collected tear samples were determined by ELISA assays following manufacturers' (Abnova Corporation, Bethyl Laboratories, Alpha Diagnostic International) instructions. The tear samples were diluted 1:200,000 for lactoferrin, 1:50,000 for lysozyme, 1:20,000 for albumin and 1:10,000 for immunoglobulin A.

Data analysis

Data are presented as the mean ± standard error of the mean (SEM). Since the primary outcomes volume absorbed, volume recovered, recovery ratio and TPC did not satisfy assumptions of normality based on the Shapiro-Wilk test, a nonparametric approach to the analysis of crossover designs was used to test for period and sequence effects [15]. In the absence of such effects, the difference between treatments was examined using the Wilcoxon signed rank test. The Spearman's rho correlation was used to examine the relationship of the volume absorbed to the recovery ratio. Tear protein concentrations were averaged for subjects with two samples per tear collection method (e.g. two PVA samples or two polyester wick samples). Specific tear protein concentrations were compared using the Wilcoxon rank sum test.

Statistical analysis was performed using SPSS for Windows version 16.0. A p value of < 0.05 was considered statistically significant. The exact p-values are presented.

Results

Thirty (30) participants, with mean age of 31.4 ± 8.7 years comprised the first part of the study whereas 12 participants with mean age of 27.8 ± 7.3 years comprised the second part of the study. A majority of the participants were male in both the first study (24 of 30) and in the second study (9 of 12) as the study population was comprised of active duty military service members.

Tear volume determination

The order of collection had no effect on tear volume absorbed, volume recovered, or recovery ratio (Figure 2): there was no period effect (i.e. differences due to 1st period vs 2nd period testing), for volume absorbed ($p = 0.430$), volume recovered ($p = 0.163$), or recovery ratio ($p = 0.250$). There was no effect of sequence (i.e. testing for a carryover effect) for volume absorbed ($p = 0.959$), volume recovered ($p = 0.830$) or recovery ratio ($p = 0.683$). The first two collection periods from 30 subjects (PVA foam = 30 and polyester wick = 30) represented the outcomes. The tear volume absorbed by the PVA foam at 6.80 ± 1.29 µL (range 0.50 to 21.90 µL) was not significantly different from the polyester wick at 6.44 ± 1.36 µL (range 0.40 to 29.30 µL, $p = 0.267$). The tear volume recovered from PVA foam at 3.89 ± 1.12 µL (range 0.00 to 17.40 µL) was lower than the polyester wick at 5.66 ± 1.35 µL (range 0.00 to 28.80 µL, $p = 0.051$). The recovery ratio of the PVA foam was significantly lower at 29 ± 6% (range 0 to 100%) versus the polyester wick at 67 ± 5% (range 0 to 98%, $p < 0.001$). Figure 3 shows that PVA foam had a consistently lower recovery ratio for all volume absorbed quantities. It also demonstrates the relationship between the recovery ratio as a function of volume absorbed for both PVA foam and polyester wick. It shows that the recovery ratios from both PVA foam and polyester wick increased as the tear volume absorbed increased. Spearman's rho correlation analysis was conducted and a positive, significant correlation was found between the volume absorbed and recovery ratio for both PVA foam ($r_s = 0.728$, $p < 0.001$) and polyester wick ($r_s = 0.909$, $p < 0.001$).

There was insignificant or minimal discomfort reported from either collection method however 83% of the subjects preferred PVA foam over the polyester wick. No adverse effects were observed during the entire study with either collection method.

Total tear protein quantification

From the 90 collected tear samples, 33 (36.7%) contained adequate volume (≥ 3 µL) of tear fluid for protein analysis, of which 12 samples were from PVA and 21 from the polyester wick. Results showed that the mean TPC from the PVA foam was 16.90 ± 2.72 mg/mL while the mean TPC from the polyester wick was 16.60 ± 2.02 mg/mL ($p = 0.674$). Based on the molecular weights as reported in another study, [7] the major tear proteins were represented and were most likely lactoferrin (79 kDa), sIgA-light chain (27 kDa), tear-specific pre albumin (TSPA, 18 kDa) and lysozyme (14 kDa). The profiles were similar to those collected using PVA foam and polyester wick. As tears were collected, regardless of collection order, the level of minor 66 kDa protein, serum albumin was observed to be similar (Figure 4). However, serum albumin appeared to vary between subjects.

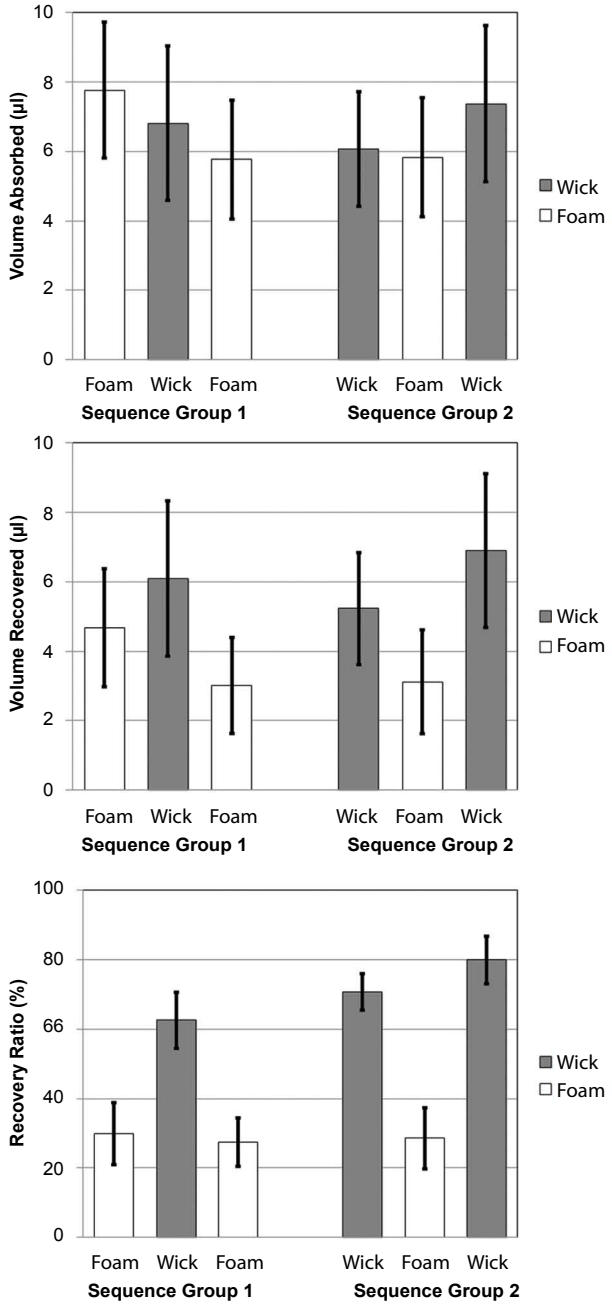

Figure 2: Test for order and sequence effect: 2A.) volume absorbed, 2B.) volume recovered and 2C.) recovery ratio by testing sequence group: PVA foam -polyester wick-PVA foam (sequence group 1); and polyester wick-PVA foam -polyester wick (sequence group 2). Data are presented as mean and standard error of the mean.

Specific tear protein quantification

From 24 tear samples collected, 15 samples (6 PVA foam and 9 polyester wick) had sufficient volume (≥ 1 µL) to perform ELISA for each of the four specific proteins. The major proteins, lysozyme, lactoferrin and IgA as well as serum albumin, were detected by ELISA technique in tears collected using either PVA foam or polyester

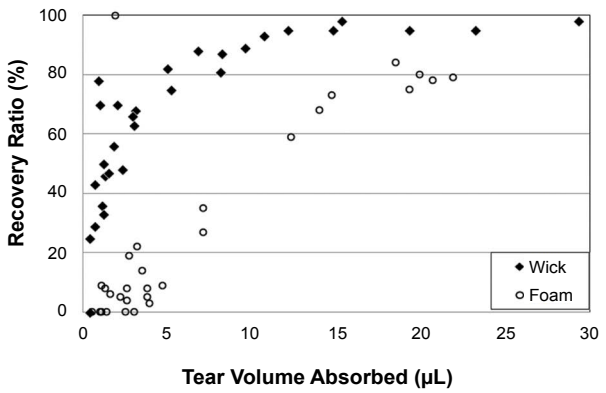

Figure 3: Relationship between tear volume absorbed and recovery ratio of PVA foam and polyester wick. The first two collection periods from 30 subjects (PVA foam = 30 and polyester wick = 30) were presented.

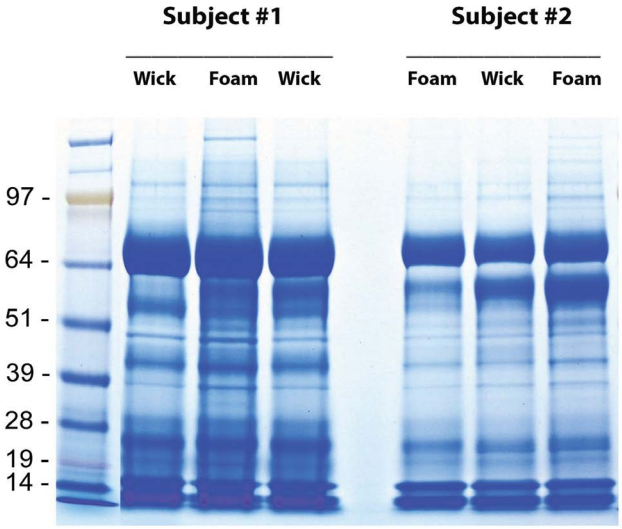

Figure 4: Representative electrophoretic profile of human tear collected from the same eye of each subject using a polyester wick (wick) and polyvinyl alcohol foam (foam). The profiles of major tear proteins lactoferrin (79 kDa), sIgA-light chain (27 kDa), and tear specific pre-albumin (18-14 kDa) and minor protein serum albumin (66 kDa) are present in both subjects and in all tear samples regardless of the collection materials used.

Table 1: Specific protein quantification in non-stimulated tears collected using PVA foam and polyester wick.

Tear protein	PVA foam (n = 6)	Polyester wick (n = 9)	P-value
Lysozyme (mg/mL)	0.26 ± 0.03	0.30 ± 0.01	0.955
Lactoferrin (mg/mL)	9.80 ± 3.13	11.62 ± 2.07	0.456
IgA (mg/mL)	0.88 ± 0.15	1.10 ± 0.24	0.776
Serum albumin (mg/mL)	0.88 ± 0.33	0.88 ± 0.25	0.955

*Data are presented as mean ± standard error of the mean. Wilcoxon rank sum test, $p < 0.05$, statistically significant.

wick. Table 1 shows the specific protein concentrations from the test materials.

Discussion

In this study, we evaluated the efficacy of PVA foam and polyester wick for non-stimulated tear collection. Based on our results, both materials showed the ability to absorb and release tear fluid. Both were able to absorb volumes near the range of estimated normal non-stimulated tear fluid volume of 6.2 ± 2.0 µL as reported by Mishima et al. [16]. Although utmost care was given to prevent tear stimulation during collection, subject response caused rapid fluctuations in tear flow resulting in the variable volumes absorbed. The outcomes also appeared to be affected by the type of material used. The PVA, a hydrophilic material, [17] was excellent in absorbing tear fluids. However, we believe because of its hydrophilic property, the PVA foam yielded lesser mean volume recovered than the polyester wick. The recovery ratio of the PVA foam was significantly lower compared to that of the polyester wick. In contrast to the PVA foam, the polyester wick is hydrophobic [18] and it readily released tear fluid as reflected by the recovery ratio. As shown in figure 3, the recovery ratio from both the PVA foam and the polyester wick increased as the volume absorbed increased. Figure 3 also shows that the PVA foam seemed to require absorption of at least 5 µL before effective tear fluid recovery. Moreover, the recovery ratio from PVA foam tip appeared to reach a plateau when the volume absorbed reached 20 µL as opposed to that of the polyester wick at about 10 µL. This means that the required volumes for getting the best recovery ratios were different between the two collection methods. For the PVA foam, 20 µL was needed for maximal recovery versus 10 µL for the polyester wick.

Collecting sufficient volume of tears for the purpose of protein analysis can be difficult [19,20]. Carefully collecting small volumes or allotting long collection time would be necessary to obtain adequate amount of samples without stimulating reflex tearing. The present study primarily focused on determining the efficacy of PVA foam and the polyester wick based on tear volume thus the tear collection was time-limited. For 60 seconds of sample collection, adding the capillary micropipette as a control arm in the first part of the study may seem impractical and may add unnecessary burden to the subjects and the operator. Theoretically, the micropipette method will yield 100% recovery if the tear fluid is adequately collected.

Another drawback of imposing a time limit is the possibility of inadequate tear volume collected for protein quantification. When the collection time was set at 60 seconds in the first part of the study, 33 out of 90 (36.7%) collected samples provided adequate volume for tear protein quantification; when collection time was set at three minutes in the second part of the study, 15 out of 36 (41.7%) samples had sufficient volume for the protein assays. These findings may have implications in the use of the test materials to collect non-stimulated or basal tear samples in subjects with dry eye.

Previous studies showed that non-stimulated tears had a total protein concentration of approximately 20 mg/mL while stimulated tears had a much lower concentration (3-7 mg/mL), reflecting the diluting effect of lacrimal gland fluid on tear protein concentration levels [19,21]. Topical anesthesia was given to lessen stimulation and further dilution expected with reflex tearing because our study was for test materials to come in contact with the surface of the eye. Measures were taken to lessen the diluting effect of topical anesthesia such as wiping off excess anesthetic fluid around the eye and allowing a two-minute interval between the instillation of the anesthesia and the tear collection. The inferior cul-de-sac was not blotted dry to minimize disruption of the ocular surface.

Quantification showed that the total protein concentration (TPC) from the polyester wick and PVA foam were comparable. The mean TPC from the polyester wick (16.60 ± 2.02 mg/mL) and the PVA foam (16.90 ± 2.72 mg/mL) were higher than the TPC obtained from a study with similar tear quantification methodology and standard. In the first part of our study, both PVA foam and the polyester wick were held in contact with the surface of the anesthetized eye for 60 seconds which may have facilitated atraumatic and non-stimulated tear collection, as indicated by higher tear protein concentrations.

The TPC in both PVA foam and polyester wick could also reflect cellular proteins since tears were collected by bringing the test materials in contact with the inferior cul de-sac. But even with the conventional capillary micropipette method, it could be difficult to obtain tear fluid that is not mixed with at least some cellular proteins. Green-Church et al. [22] were able to identify and compare cellular and serum proteins collected by direct aspiration through capillary micropipette and by absorption recovery techniques by Schirmer strips.

Analysis of major polypeptide profiles from tear fluid from the PVA foam and polyester wick were found to be similar (Figure 4). Based on

previous works, it is thought these molecular weights represent lactoferrin (79 kDa), sIgA-light chain (27 kDa), tear-specific pre-albumin (TSPA, 18 kDa) and lysozyme (14 kDa) [23-25]. Variations in level of the 66 kDa protein, which is thought to represent serum albumin, obtained in our study were also reported by Ng et al. [26] Levels of the blood-derived protein serum albumin in the tear film depended on the permeability of the blood capillaries of the conjunctiva which could have been sensitive to ocular irritation or stress [26].

We attempted to quantify proteins from non-stimulated tears collected. The collection time for this portion of the study was increased to three minutes in an effort to increase the volume of samples to analyze four proteins by ELISA technique. We attempted to collect samples by micropipette to serve as control however, this method proved to be challenging if done without evoking stimulation. Using ELISA technique, we were able to detect and quantify major tear proteins lysozyme, lactoferrin and IgA from PVA foam tip and polyester wick. Serum albumin was relatively low supporting our assumption that the sample collection using either PVA foam or polyester wick were done with minimal trauma to the ocular surface.

In evaluating major tear proteins, tear samples can be collected without the risk and the discomfort of the capillary micropipette method when PVA foam or polyester wick are utilized. Because this study focused on obtaining non-stimulated tear samples, future research could also be directed in determining the impact of stimulated tear collection or reflex tearing on total and specific tear protein concentrations. Population assessment for variability of polypeptide profiles should also be investigated as this was beyond the scope of this study. When recovery ratio is low, the possibility of tear proteins retained within the collection material matrix should also be considered. Rinsing the collection material with buffer solutions such as phosphate buffered saline (PBS) prior to centrifugation process may be helpful however dilution may impede detection of proteins. Chemical extraction process may also be an option to remove protein from the matrices [27].

Conclusion

Each tear collection method has its own advantages and disadvantages. It is therefore up to the clinician's or the investigator's discretion as to which method to use, depending on objectives of the study. Different tear collection procedures may produce varying tearing response and varying concentration of tear proteins recovered. PVA foam and polyester wick appear to be good alternatives to standard capillary micropipette in collecting non-stimulated tear fluid for protein analysis. Both techniques are well-tolerated, producing minimal discomfort. Comparing PVA foam and polyester wick, the polyester wick seems to be the more efficient in terms of tear volume. On the other hand, PVA foam was easy to use and was preferred for comfort which may be due to its rounded tip compared to the blunt tip of a polyester wick. PVA foam tip is commercially available as an individually sterilized and packed item, needing no pre collection preparation and sterilization. Considering the low recovery ratio of the PVA, it may not be suitable for tear collection in patients with aqueous deficient dry eyes.

Acknowledgements

The authors thank Bader Fileta for carrying out the tear analysis and Lamarr Peppers for administrative support.

Financial Support

There was no financial support, public or private, used to fund this study.

Proprietary Interest

The authors have no financial interest in any product, drug, instrument, or equipment discussed in this manuscript.

Disclaimer

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