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Effect of tear fluid sampling and processing on total protein quantity and electrophoretic pattern

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Abstract:

Human tears contain more than 1500 proteins that could be diagnostically relevant. To date, numerous candidates on a biomarker of protein origin were identified for ocular and systemic diseases. However, the suitable sampling method is still the subject of discussion. To address the need for a description of sampling methods properties for possible clinical analyses, we studied a total protein concentration and electrophoretic pattern of tear fluid collected by capillary tubes, Schirmer strips, cellulose microsponges, and flushing. The total protein concentration was 4.339 μg/μL ± 1.905 μg/μL, 0.967 μg/μL ± 0.117 μg/μL, 0.022 μg/μL ± 0.016 μg/μL, and 0.008 μg/μL ± 0.006 μg/μ for the capillary tubes, Schirmer strips, flushing, and cellulose microsponges, respectively. Sodium dodecyl sulfate polyacrylamide electrophoresis showed the different patterns of tear proteins obtained by the above-mentioned sampling methods. These differences could originate from the use of a bigger amount of extraction reagent that was not used in the case of capillary tubes, and retention of the proteins by strips and sponges. Taken together, capillary tubes, Schirmer strips, cellulose microsponges, and flushing represent sensitive and convenient sampling methods for tear fluid collection. For the isolation of proteins from strips and sponges, and for the flushing, less than 100 μL of a reagent should be used to ensure the sufficient concentration of the biomarkers in a trace amount.

Keywords:

Biomarkers, polyacrylamide electrophoresis, tear fluid, tear proteins

Introduction

The great efforts toward personalized medicine set new challenges in clinical diagnostics. One of the consequences is the rapid growth of the studies dealing with the use of nonstandard sampling materials (tears, saliva).[1,2] From these biological fluids, tear fluid shows immense potential to diagnose ocular and systemic diseases[1] and to monitor response to the treatment on the molecular level.[3] Its advantages lie in availability, noninvasive collection, and lower variability of the components compared to blood serum or plasma. Despite intensive research, tear fluid is not used in practice yet.

Although tear fluid potential was shown in numerous studies, it is still not clear how the sampling methods, use of anesthetics, sample processing, and handling before an analysis affect the final results. Tear fluid could be collected using microcapillary tubes, Schirmer strips, various rods, tips, and sponges from highly absorptive material, and the flushing method. Microcapillary tubes are small diameter thin-walled tubes that exhibit capillary action. The tear fluid sampling is conducted by gently placing a capillary to the lower eyelid with attention not to touch a cornea if basal tears are acquired. Schirmer strips are strips of sterile filter paper primarily used for the assessment of tear fluid production. It is placed inside the lower eyelid with or without anesthetics to obtain basal or reflex tears, respectively. Cellulose microsponges

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are multipurpose sponges with a high absorption rate. Depending on their shape, they can be inserted into an inferior cul-de-sac of the eye, or gently attached to an eye surface in the area of the inferior lower lid. The flushing method is a special procedure for the acquirement of the content of the eye surface. A flush itself is done using a normal saline solution that is gently pipetted into an inferior cul-de-sac of the eye and subsequently withdrawn.

In search of the most suitable method, several studies compared their properties. Flushing and cellulose rods were shown to provide qualitatively the same results of major proteins as microcapillaries, although the total protein concentration was lower.[4,5] Schirmer strips, too, gave similar results when compared to capillaries; however, the changes in the minor proteins were detected.[6,7] On the other hand, Li *et al*.[8] found differences in the major proteins between Schirmer strips and capillary tubes, but the extraction protocols varied between the studies. These inconsistencies imply the need for more studies and the well-defined sampling conditions, processing, and storage of the tears.

For this purpose, we analyzed the total protein content and electrophoretic pattern of four tear fluid collection methods: glass microcapillaries, Schirmer strips, cellulose microsponges, and flushing method under defined conditions.

Methods

Subjects

Ten adult volunteers (5 males, 5 females) aged 24–43 years had been recruited randomly in the cross-sectional study. The inclusion criteria were the absence of chronic systemic and ocular diseases. All volunteers underwent a general ophthalmologic examination before the tear fluid collection. The volunteers were excluded from the study if the presence of dry eye disease, seasonal allergy, or undiagnosed condition affecting tearing occurred. One volunteer was excluded due to failure to produce neither basal nor reflex tears.

Ethical approval

After informing the volunteers about the objectives and risks of the study, informed consent was obtained based on the Declaration of Helsinki and approved by Pavol Jozef Šafárik University in Košice Ethical Committee with the number 7N/2018a.

Tear fluid collection

Tear fluid samples were collected between 9.00 and 12.00 a. m. to eliminate possible variability caused by diurnal rhythm.[9] Tears were collected from the lower eyelid for all patients in the same order: with capillary tube (total volume 20 μL, Sigma-Aldrich, Steinheim, Germany) from the left eye [Figure 1a], Schirmer strip (Madhu Instruments Pvt. Ltd., New Delhi, India) from the right eye [Figure 1b], cellulose microsponge (Sugi® Eyespear, Kettenbach, Eschenburg, Germany) from the left eye [Figure 1c], and flushing with 100 μL saline (Fresenius Kabi, Verona, Italy) from the right eye without an anesthetic [Figure 1d]. The Schirmer strip was left in the eye until the tears reached 10 mm. The cellulose microsponge was attached to the tear film until it was visibly swollen (about 5 s). Flushing was performed with 100 μL of normal saline solution that was pipetted into an inferior cul-de-sac of the eye and subsequently withdrawn. Between samplings from the same eye, 5-min break was ensured. The samples were put into the microcentrifuge tubes, kept, and transferred on ice. Tear fluid from the capillary tube was recovered immediately after the collection by the air pressure applied to the capillary using an automatic pipette. Tear fluid from the cellulose microsponges was recovered by adding 100 μL of a normal saline solution on the microsponge placed in the tube, the tube was pierced at the bottom, put into the larger tube, centrifugated at 21 000×g for 5 min at 20°C, and supernatants were used for the analyses. The samples from the capillary tubes, cellulose microsponges, and flushing method were aliquoted, and all samples were stored at −80°C until analysis. Tear fluid from the Schirmer strip was recovered directly before relevant analysis by adding 100 μL of PBS +1% Triton X-100 to the tubes. Tubes were incubated overnight at 4°C, pierced at the bottom, put into the larger tubes, and centrifuged at 21 000 x g for 3 min at 4°C.

Protein quantification

Total tear fluid protein quantification was determined according to the Bradford method. Briefly, absorption of the samples with Bradford Reagent (Sigma-Aldrich, Steinheim, Germany) was measured spectrophotometrically on Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Friedrichsthal, Germany) at a wavelength of 595 nm. All samples were analyzed in duplicate. Statistical analysis was performed using the Kolmogorov–Smirnov test and Shapiro–Wilk test to test the normality of our data. Paired Student's *t*-test was carried out to assess the differences between groups, and $P < 0.05$ was considered statistically significant.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

7.5 μL of tear fluid sample was mixed with loading buffer and loaded on the 15% sodium dodecyl sulfate (SDS) polyacrylamide minigels under nonreducing conditions. Electrophoresis was run at a constant current of 20 mA until the color dye reached the end of the gel. After electrophoresis, the gels were stained with 0.5%

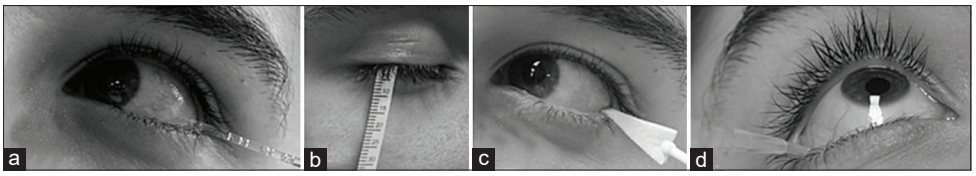

Figure 1: Tear fluid sampling using (a) capillary tube, (b) Schirmer strip, (c) cellulose microsponge, (d) flushing

Coomassie Brilliant Blue R-250 (SERVA, Heidelberg, Germany) in 40% isopropanol (Sigma-Aldrich, Steinheim, Germany) and 10% acetic acid (ITES Vranov, Vranov nad Topľou, Slovakia) for 20 min at room temperature and destained for 4 h in 40% methanol (Sigma-Aldrich, Steinheim, Germany) and 10% acetic acid. Subsequently, the gels were scanned and analyzed using ImageJ software (public domain).

Questionnaire of the subject's comfort

The volunteers rated the subjective comfort or discomfort of each collection method at the scale of 0–5 (0 – very uncomfortable, 1 – uncomfortable, 2 – slightly uncomfortable, 3 – slightly comfortable, 4 – comfortable, and 5 – very comfortable) at the end of procedures. The sum for each method was calculated and the highest points indicated the most comfortable way of sampling. Statistical analysis was performed using Kolmogorov–Smirnov test and Shapiro–Wilk test to test the normality of our data. Statistical significance of the results was tested using Wilcoxon sign-ranked test, and $P < 0.05$ was considered statistically significant.

Results

Protein quantification

To specify the total protein content obtained by each collection method, we performed a spectrophotometric measurement according to Bradford. The highest protein levels were detected in the tear fluid collected by the capillary tubes (mean 4.339 μg/μL ± 1.905 μg/μL) followed by the Schirmer strips (0.967 μg/μL ± 0.117 μg/μL), flushing (0.022 μg/μL ± 0.016 μg/μL), and the lowest was identified in cellulose microsponges (mean 0.008 μg/μL ± 0.006 μg/μL) [Figure 2].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

For the assessment of qualitative protein changes, SDS polyacrylamide gel electrophoresis was performed. The electrophoretic pattern differed among the sampling methods with the widest bands and the highest number of bands in the capillary tubes [Figure 3] which is consistent with the total protein quantity measurement. All samples provided bands at about 80, 18, 14, and 13 kDa. The additional bands were seen at above 245 kDa, between 58 and 80 kDa in capillary tubes and Schirmer strips, and between 46 and 58 kDa and below 46 and 17 kDa in capillary tubes.

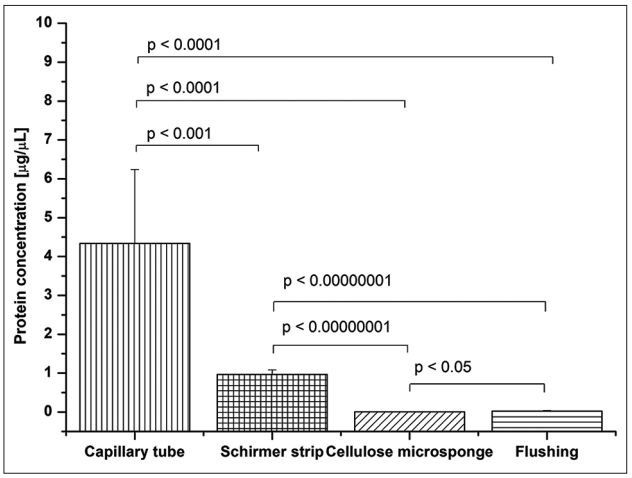

Figure 2: Total protein content obtained by capillary tubes, Schirmer strips, cellulose microsponges, and flushing. There were statistically significant differences in total protein content among the sampling methods (expressed as *P* values)

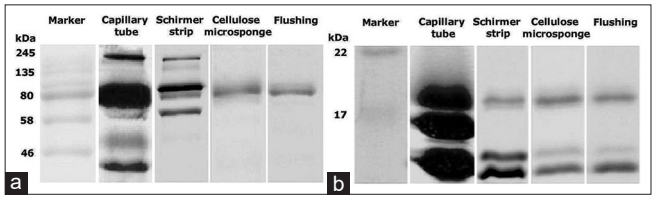

Figure 3: Representative sodium dodecyl sulfate polyacrylamide gel electrophoregrams of tear fluid proteins (V = 7.5 μL/well) with (a) higher and (b) lower molecular weight collected by the capillary tubes (4.339 μg/μL ± 1.905 μg/μL) Schirmer strip (0.967 μg/μL ± 0.117 μg/μL), cellulose microsponge (0.008 μg/μL ± 0.006 μg/μL), and flushing (0.022 μg/μL ± 0.016 μg/μL)

Subject's comfort

Due to the existence of several sampling methods, we also decided to assess the comfort of patients during sampling. For this purpose, the questionnaire was given to the volunteers to rate each collection method after sampling. The highest rating achieved the flushing method (37 points/45 points), the lowest capillary tubes (18 points/45 points) [Table 1]. Flushing and cellulose microsponges were significantly more comfortable when compared to capillary tubes ($P < 0.05$).

Discussion

To contribute to previous studies that analyzed differences between the sampling methods, we

compared the total protein content and electrophoretic pattern among glass microcapillaries, Schirmer strips, cellulose microsponges, and the flushing method. The volumes of 100 μL of reagents that we used for the extraction of proteins from the Schirmer strips and cellulose microsponges provided a lower protein concentration in comparison with capillary tubes. Similarly, Kenny *et al*.[10] isolated tear fluid proteins from Schirmer strip with 500 μL phosphate buffer with a cocktail of peptidase inhibitors and gained the range of 0.05–0.3 μg proteins/μL. On the other hand, Posa *et al*.[6] used only the centrifugal forces and isolated 4.4–4.7 μg of proteins/μL. However, Markoulli *et al*.[11] who used the same isolation procedure achieved a wider range of 0.6–6.6 μg of proteins/μL.

Considering microsponges, Lee *et al*.[12] obtained statistically insignificant differences of protein amount when compared to Schirmer strips or capillaries (4.1 ± 0.31 μg/μL or 5.0 ± 0.76 μg/μL, respectively), except microsponges from polyvinyl acetal, and they also used reagent-free centrifugation.

Similarly, the flushing with 100 μL of the saline solution gave a low protein concentration in this study what was manifested also on the electrophoregram. When 20 and 60 μL were used, 3.8 and 3.3 μg of proteins/μL were obtained, respectively.[11] A summary of previous literature is in Table 2.

Table 1: Rating of the sampling methods by volunteers

Sampling method	Volunteers' rating (points)
Capillary tube	18/45
Schirmer strip	25/45
Cellulose microsponge	36/45
Flushing	37/45

The highest score means the more comfortable method

Although based on these studies and our results, it might seem that isolation of tear fluid proteins from strips and sponges without a reagent, using the centrifugal forces, yields quantitatively better results [Table 2], the retention of proteins in the strips and sponges should be considered.[13] This could be also the reason for differences manifested on electrophoregram. We showed an electrophoretic pattern that differed among the methods. Similarly, several studies demonstrated qualitative differences in electrophoregrams of tear fluid collected by capillary tubes and Schirmer strips.[8,14] On the other hand, Posa *et al*.[6] showed only minor electrophoretic changes in protein patterns between capillaries and Schirmer strips. However, they also proved that an addition of 15 μL of phosphate buffer after centrifugation of a strip provides further protein extraction. Considering flushing, it remains questionable if we are capable to flush an eye obtaining the same tear fluid content as to collect by the capillaries. Taken together, capillary tubes provided the highest protein content with the most numerous different types of proteins represented by the bands on the electrophoregram. Together with the results of the above-mentioned studies, capillary tubes represent a standard for comparison of other methods.

Our work further demonstrated that the most comfortable sampling methods were cellulose microsponges and flushing. We assume that the major reason is the time efficiency that is a nonnegligible advantage when the prevalence of ophthalmological diseases is still rising.

Conclusion

To sum it up, the total tear fluid proteins and their electrophoretic pattern differed among capillary tubes, Schirmer strip, cellulose microsponges, and flushing sampling method. For a specific analysis, the properties

Table 2: Summary of protein concentration and volume used for elution in sampling methods and the advantages and disadvantages of the methods

Sampling method	Elution volume (μL)	Protein concentration (μg/μL)	Reference	Advantages	Disadvantages
Capillary tube	-	7.14±2.22	[4]	No elution needed	Unsuitable for dry eye
	-	4.7-4.9	[6]		Risk of reflex tearing
	-	5.0±0.76	[12]		Uncomfortable
Schirmer strip	500	0.05-0.3	[10]	Available at clinics	Unsuitable for dry eye
	-	4.4-4.7	[6]		Retention of proteins
	-	0.6-6.6	[11]		More demanding sampling processing
	-	4.1±0.31	[12]		Uncomfortable
Cellulose microsponge	-	5.2±0.95	[12]	Comfortable	Unsuitable for dry eye
	-	4.7±0.6	[12]	Time-saving	Retention of proteins
					More demanding sampling processing
Flushing	20	3.8	[11]	Comfortable	Questionable reproducibility
	60	3.3	[11]	Suitable for dry eye	Diluted sample
	60	3.79±1.51	[4]	Time-saving	

of a sampling method should be considered. Whereas the processing of a sample in the clinical laboratories depends on the routinely performed specific analysis, it is, therefore, not necessary to establish the only general tear fluid sampling method for all kinds of analyses.

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Conflicts of interest

The authors declare that there are no conflicts of interests of this paper.

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