

Tear Proteomics in Young Malays with Dry Eyes

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ABSTRACT

Introduction: Changes in tear protein concentrations may reflect ocular surface health. This study analyzes changes in tear protein concentrations of young Malays with dry eye (DE) and determines its association with the clinical findings. **Methods:** Subjects were screened using McMonnies questionnaire (MDEQ) and fluorescein tear break up time (TBUT). Total tear protein concentration (TTPC) was determined using Bradford's technique and specific tear protein (slgA, lysozyme, lactoferrin and human serum albumin (HSA)) concentrations were determined using SDS-PAGE. Parametric and nonparametric tests were used to compare means between groups. Spearman correlation was used to determine the association between variables measured. **Results:** A total of 42 subjects (21 DE and 21 NDE) were included. Mean MDEQ score for DE was 16.00 ± 1.48 and NDE was 8.47 ± 3.47 . Mean TBUT for DE was 3.47 ± 0.47 s and NDE was 4.98 ± 0.43 s. Mean TTPC for DE and NDE was 9.84 ± 2.40 mg/ml and 8.96 ± 1.84 mg/ml respectively. Mean slgA, lysozyme, lactoferrin and HSA for DE was 0.54 ± 0.10 mg/ml, 1.68 ± 0.17 mg/ml, 1.47 ± 0.25 mg/ml, 0.06 ± 0.03 mg/ml and for NDE was 0.57 ± 0.09 mg/ml, 2.04 ± 0.19 mg/ml, 1.75 ± 0.23 mg/ml, 0.06 ± 0.03 mg/ml accordingly. Significant differences were noted in MDEQ score ($p=0.01$), TBUT ($p=0.01$), lactoferrin ($p=0.01$) and lysozyme ($p=0.01$) but not in TTPC ($p=0.19$), HSA ($p=0.74$) and slgA ($p=0.24$) between groups. Significant correlations were noted between TBUT with lactoferrin ($r=0.02$, $p=0.02$) and lysozyme ($r=0.63$, $p=0.01$) and between MDEQ score with lactoferrin ($r=-0.34$, $p=0.02$) and lysozyme ($r=-0.64$, $p=0.01$). **Conclusions:** There are changes in specific tear protein in dry eye patients, which correlate well with clinical results. Tear protein analysis may play an important role in the diagnosis of the dry eye.

KEYWORDS: Tears, protein analysis, electrophoresis, dry eye

INTRODUCTION

The tear film is a highly specialized and carefully structured moist film that covers the cornea and conjunctiva. It contains mucins, proteins, lipids, lipoproteins, glycolipids and metabolites that lubricate, protect and provide nutrition to the cornea.¹ Normal tear volume is around 6-7 μ L μ L, and the production rate is around 1 to 1.2 μ L/min for non stimulated (basal) tears and greater than 5 μ L/min for stimulated (reflex) tears.^{2,3} Qualitatively and quantitatively; the tear composition must be maintained to ensure healthy and functional visual system. Any abnormalities affecting the constituents or the volume may disrupt the stability of the tear film and result in dry eye.^{4,5}

Dry eye is characterized by tear film instability and damaged exposed surface epithelium, which causes chronic irritation of the ocular surface. Fluorescein tear break up time (TBUT) is the common clinical procedure used to assess tear stability. Even though this technique has been questioned for its repeatability and high variability that exist between within and in between subjects^{6,7} and between instruments,⁸ eye care practitioners have used it widely. Ethnic differences have been quoted as one of the factors that affects TBUT values. In the western population, mean TBUT value is around 15 seconds (s).^{9,10} Eye with value of less than 10 s is considered abnormal. Cho and Brown¹¹ found that 90% of Hong Kong Chinese has TBUT values of less than 10 s. Significant difference in TBUT values was found between different ethnic groups in Scotland, with Caucasians having the highest value.¹² In Malaysia, the mean TBUT values for normal young Malays was 7.8 ± 1.89 s with around 88% of the subjects having TBUT of equal or less than 10 s.¹³

Another common variable measured to be related to ocular surface dryness is the tear's protein concentration. Versura *et al.* analyzed tear protein variations in patients with evaporative dry eye disease (TBUT \leq 10 seconds) and compared them to tears of healthy subjects (TBUT \geq 10 seconds).¹⁴ Their

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results showed a significant decrease in levels of lactoferrin, lipocalin-1 and lipophilinA-C in patients with evaporative dry eye disease. Yanwei et al. compared lactoferrin levels between 40 dry eye (Schirmer < 5 mm, TBUT < 10 seconds) and 35 normal patients (Schirmer > 5 mm, TBUT > 10 seconds).¹⁵ Their results showed a significant decrease in lactoferrin levels in dry eye patients compared to healthy subjects. Vitali *et al.* found variable lactoferrin results, which were not concordant with other more diagnostic tests such as rose bengal staining, Schirmer's test and other ocular symptoms.⁶ Ng *et al.* analyzed tear protein of young normal Hong Kong Chinese using Bradford and modified Lowry methods.¹⁶ Their results showed similar protein patterns with those reported values from Caucasian subjects, but the concentrations of the major proteins were not in concordance with previous reports. The authors attributed this to the large variability in the method used.

The purpose of this study was to determine the tear protein concentration of young Malays using the Bradford and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) methods. Both techniques have been widely used by previous investigators to determine the total and specific protein concentrations in tears.^{17,18} Some researchers suggested the use of SDS-PAGE followed by Coomassie blue staining and densitometric scanning as diagnostic tools for dry eye syndrome.¹⁷ This study investigates the total and specific protein concentrations of young Malays with dry eye symptoms and compares them with tear proteins in normal subjects. The association of tear protein concentrations with clinical findings (TBUT and McMonnies score) will also be determined. The outcomes of this study may improve our understanding and management of dry eye patients, particularly within the Asian region.

MATERIALS AND METHODS

Subjects were recruited through advertisements on bulletin boards of Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur campus. Informed consent was obtained from all subjects prior to data collection. This study was approved by the Medical Ethics Committee of UKM and followed the tenets of the declaration of Helsinki. The inclusion criteria were young Malays, who were non-contact lens wearers, refractive error of less than $\pm 3.00D$ and no history of anterior segment disease or surgery. Clinical evaluation was conducted at the Optometry Clinic and tear protein analysis was done at the Biochemistry Lab, Faculty of Health Sciences, Universiti Kebangsaan Malaysia Kuala Lumpur Campus.

All subjects were screened using the McMonnies dry eye questionnaire (MDEQ) to ascertain the number, type and frequency of dryness symptoms at the beginning of the study.¹⁹ Given the popularity of this survey among Optometrists in Asia; it is reasonable for us to use it in this study. The MDEQ was invented

in 1986, and it consists of 12 questions that focus on clinical risk factors for dry eye.¹⁹ The questions employ response options that vary in number and type and provide a score from 0 to 45. Respondents were required to answer all the 12 questions and the score of each question (which has a weighted scoring scale) was calculated. Scores above 14.5 are consistent with a dry eye. Tear stability of every subject were measured using the fluorescein tear break up time (TBUT). The technique was described in an earlier report.²⁰ The subject's upper bulbar conjunctiva was swiped with a saline wetted fluorescein strip (Haag-Streit International, Switzerland). Subjects were told to close their eyes for approximately 10 seconds and then to open their eyes, blink twice and to keep the eye naturally open and look straight ahead (in primary position) with chin firmly on the chinrest of a slit lamp biomicroscope. Cobalt blue light with diffuse illumination and 10X magnification was used to determine the appearance of dark spots or streaks at the five different regions of the cornea (nasal, temporal, inferior, superior and nasal). Time from the last blink to the first appearance of random dark spots or streaks was taken as TBUT. Three consecutive readings were taken from each eye using a stopwatch, and the mean was recorded. Corneal staining was evaluated using fluorescein and slit lamp biomicroscope and graded following the Efron grading scale.²¹ Room temperature was set between 23 to 25°C and room humidity level was between 45 to 50% at the time of investigation.

Tears were collected in the clinic using a sterilized glass micro-capillary tube. Around 50µl of tears was collected from each subject. The time required for collection varied between 25 and 50 minutes. After collection, the tears were stored at -80°C until all subjects had completed clinical examination. The total tear protein concentration (TTPC) was determined using the Bradford Method. It is a rapid and sensitive colorimetric protein assay based on absorbance shift of the blue dye for quantitation of protein in a solution. This technique was carried out using Bio-Rad Protein assays. The assay reagent was prepared by diluting one part of reagent concentrate (Bio-Rad Laboratories, USA) with four parts of distilled water. Then, 10µl of sample (optimized with 50X dilution after a repeatability test) and 10µl of standard following serial dilution was mixed with 200µl of diluted assay reagent in a 69 micro-well plate. The absorbance of the mixture was measured at 595nm with a micro-plate reader after a least 5 minute incubation time at room temperature (Bio-Rad Technical Bulletin 1069). The obtained mean values from triplicate samples were then calculated to determine the TTPC for each tear samples.

The specific protein levels (sIgA, lysozyme, lactoferrin and HSA) were determined using the Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method. The technique was conducted on an electrophoresis system (Bio-Rad Mini-Protean Tetra Cell, Bio-Rad Laboratories, USA)

using the Laemmli (Bio-Rad Laboratories, USA) buffer system. All non-reduced tear samples were diluted with the buffer solution in the optimized ratio of 1:4. Each 20 µl of the diluted samples were then loaded into the wells on top of the stacking gel (Bio-Rad Mini Protean Pre-Cast Gels, Bio-Rad Laboratories, USA). The protein markers were also prepared by similar methods and loaded into each well. Chosen commercially available protein markers included the 66.5kDa albumin (A3782), 82.4kDa lactoferrin (L0520), 14.3kDa lysozyme (L4919) and 400-420kDa IgA (I1010). The protein markers were all obtained from Sigma Chemical Company, USA. Molecular weight markers (Precision Plus Protein Kaleidoscope, Bio-Rad Laboratories, USA) with 10 multicolor recombinant proteins (10-250kDa) were run alongside the diluted tear samples and protein markers. Electrophoresis was executed at 100 V for 10 minutes and subsequently, 120 V for 60 minutes to ensure progressive protein mobility. The generated gels were then stained with Coomassie brilliant blue dye (Nacalai Tesque CBB Stain One, Japan) for an hour. After that, the destaining process was continued overnight and dried. The final processed gels were scanned (V313, Dell, USA) using the highest resolution dots per inch (dpi) and converted into digital image. The specific protein bands were identified and quantified using image analysis. The specific protein bands from the samples were identified via the molecular weights and protein markers. Image Meter Version 1.1 for Windows (Adobe Air 3.2, USA) was used to estimate the specific protein concentrations on the gels. The areas (mm²) of the specific protein bands were selected and compared to the protein marker's pixel by pixel.

The local background of the image was subtracted out from the analysis. The areas (mm²) were then calculated based on the protein marker's concentrations to obtain the specific protein concentrations.

Statistical Analysis

Statistical analysis was performed using SPSS (Version 20.0, SPSS Inc, Chicago, IL, USA). The clinical and threshold statistical significance was taken as $P < 0.05$. Parametric (Independent Samples T-Test) and nonparametric tests (Mann-Whitney U Test) were used to compare means between groups. Spearman test of correlation was used to find any association between variables measured.

RESULTS

A total of 42 Malay subjects (21 dry eye (DE) and 21 non dry eye (NDE)) aged between 19 to 31 years old participated in this study. The mean age for all subjects was 21.36 ± 2.36 years. Mean age for DE was 21.95 ± 2.92 and for NDE was 20.76 ± 1.44 . Mean spherical refraction for DE was -0.75 ± 1.09 DS and for NDE was -1.50 ± 2.11 DS. Mean MDEQ score for DE was 16.00 ± 1.48 and for NDE was 8.47 ± 3.47 . Tear stability was evaluated using the fluorescein TBUT. Mean TBUT for DE and NDE was 3.47 ± 0.47 s and 4.98 ± 0.43 s respectively. Evaluation of corneal health showed no significant staining in both groups. Statistical analysis indicates no significant difference ($p > 0.05$) between groups was noted in all parameters measured except for the mean MDEQ score and TBUT ($P = 0.001$; $P = 0.001$ respectively). Summary of results are shown in Table 1.

Table 1. Subjects demographic and clinical data

	Dry eye subjects	Non dry eye subjects	Significance level (p)
Number of subjects (n)	21	21	
Age (years)	21.95 ± 2.92	20.76 ± 1.44	0.372
Refractive error (DS)	-0.75 ± 1.09	-1.50 ± 2.11	0.441
Mean MDEQ score	16.00 ± 1.48	8.47 ± 3.47	0.001*
Mean TBUT (seconds)	3.47 ± 0.47	4.98 ± 0.43	0.001*

* $p < 0.05$ is considered significant

The Bradford method was used to determine the total tear protein concentration (TTPC). Mean TTPC for a DE subject was 9.84 ± 2.40 mg/ml and for NDE was 8.96 ± 1.84 mg/ml. No significant difference was detected between both groups ($P = 0.190$). The specific protein concentrations (slgA, lysozyme, lactoferrin and HSA) were determined using SDS-PAGE technique. Mean HSA concentration for DE was 0.060 ± 0.03 mg/ml and for NDE was 0.061 ± 0.028 mg/ml. Mean lactoferrin concentrations for DE and NDE subjects were 1.469 ± 0.248 mg/ml and 1.745 ± 0.224 mg/

ml respectively. Mean lysozyme concentration for DE subjects was 1.682 ± 0.166 mg/ml and for NDE subjects was 2.041 ± 0.186 mg/ml. Mean slgA concentrations for DE and NDE subjects were 0.543 ± 0.102 mg/ml and 0.569 ± 0.089 mg/ml respectively. Statistical analysis indicates significant difference in lactoferrin ($P = 0.001$) and lysozyme ($P = 0.001$) concentrations between both groups. Results are summarized in Table 2.

Table 2. Summary of mean tear protein concentrations dry eye and non-dry eye subjects

	Dry eye subjects	Non dry eye subjects	Significance level (p)
Total tear protein concentration (TTPC)	9.84 ± 2.40mg/ml	8.96 ± 1.84mg/ml	0.190
slgA	0.543 ± 0.102mg/ml	0.569 ± 0.089mg/ml	0.237
Lysozyme	1.682 ± 0.166mg/ml	2.041± 0.186mg/ml	0.001*
Lactoferrin	1.469 ± 0.248mg/ml	1.745 ± 0.224mg/ml	0.001*
HSA	0.060 ± 0.03mg/ml	0.061 ± 0.028mg/ml	0.734

*p<0.05 is considered significant

Correlation between MDEQ scores and TBUT values with tear protein concentrations were analyzed using Spearman's test of association. Significant correlations were noted between MDEQ scores with TTPC (P=0.014), lactoferrin (P=0.015) and lysozyme (P=0.001) concentrations. Similar correlations were also found between TBUT with lactoferrin (P=0.022) and lysozyme concentrations (P=0.001).

DISCUSSION

In this study, tear protein characteristics of young Malays with dry eye symptoms were compared to normal subjects, and their associations to TBUT and MDEQ scores were determined. The McMonnies dry eye questionnaire (MDEQ) was used to elucidate the dry eye symptoms of subjects in this study. The survey has been shown to have high sensitivity and specificity for dry eye diagnosis, where a referent value of 14.5 or more denotes dry eye.²¹ In the present study, the scores of MDEQ between both groups were significantly different with values ranged from 14.5 to 18 for DE subjects and 5 to 13 for NDE subjects. Tear stability was determined using fluorescein TBUT technique. Mean TBUT of DE subjects was significantly lower than NDE subjects with values ranged from 3 to 5s and 5 to 7s respectively. Result from the present study support earlier findings of TBUT in Asian population.^{11,13} In reports with Caucasian subjects, TBUT value of less than 10s is considered as abnormal.^{9,10} The discrepancy was probably due to the differences in tears characteristics of Asian population.¹⁵

Detailed analysis of tear protein concentrations were conducted in this study using the Bradford and SDS-PAGE techniques, and the outcomes showed significant correlations with TBUT and MDEQ scores. However, the results showed higher TTPC than found in previous investigations.^{14,16} The inconsistency was probably due to the method used to collect tears.^{17,18} Earlier works have shown that methods of tear collection can critically affect the tear protein concentrations.²² Ng et al. postulated that stimulated tear reflex might increase the normal rate of tear secretion, thus diluting the normal tear protein concentration, rendering it to a lower value.¹⁶ However, other factors such as concentration of the

chemical assay used, age and race of subjects may also influence the results.^{17,18} Results of the present study also demonstrated insignificant differences between TTPC in DE and NDE subjects. These results were in agreement with Caffery *et al.* who demonstrated no significant difference in TTPC between patients with keratoconjunctivitis sicca and normals in their study.²³ However, their results showed significant difference in TTPC between normal and patients with Sjögren's syndrome.

The specific proteins that were analyzed in this study include HSA, lactoferrin, lysozyme and slgA. However, significant differences were only noted in lactoferrin and lysozyme concentrations between both groups. Concentrations of both proteins were found to be higher in DE than NDE groups. These findings were in accordance with several earlier investigations.^{14,24,25} Janssen and van Bijsterveld noted significant reduction in lactoferrin and lysozyme concentrations in the tear samples of dry eye subjects using SDS-PAGE technique.²⁵ Versura et al. demonstrated significant decrease of lactoferrin levels in patients with evaporative dry eyes.¹⁴ Similar results were also found in patients with keratoconjunctivitis (KCS).²³ It is possible that the reduction in lactoferrin and lysozyme concentrations were associated with a deficiency in the aqueous production. Around 20-40% of total protein in the tears aqueous layer is made up of lysozyme. Deficiency in the aqueous layer will result in reduction of the lysozyme concentration. According to Danjo *et al.*, the lacrimal gland produces lactoferrin. Therefore, the function of the lacrimal gland can be evaluated by determination of level of tear lactoferrin regardless of differences in pathogenesis of underlying diseases.²⁶

CONCLUSION

This study provides the first data on tear protein concentrations of young Malays. There are changes in specific tear protein in dry eye patients, which correlate well with clinical results. The results indicate the importance of tear protein analysis in the diagnosis of dry eye and may be used as future reference.

CONFLICTS OF INTEREST

None declared.

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