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Article title: Clinical and biochemical analysis of the ageing tear film

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Synopsis

Biochemical and clinical tear film distinctions, between two populations differing in age by over 30 years, were observed - exemplified by a decrease in lacrimal gland protein concentration paralleled by a reduction in tear secretion and stability.

Abstract

Background Tear film stability is important for healthy visual function, yet little is known of the ageing mechanisms. The aim of this study was to investigate parallels between biochemical changes and clinical physical parameters, which occur in the tear film of two subject populations differing in age by over 30 years.

Methods Two distinct age groups were chosen: eleven 'younger' (23.7±2.1 years) and nineteen 'older' (63.0±4.0 years) subjects. A series of clinical tests were performed to access tear volume, tear film stability and general ocular health. Tear protein analyses from extracted Schirmer strips were conducted with the Agilent 2100 Bioanalyzer.

Results Clinical investigations highlighted significant differences between the age groups. For example: McMonnies scores ($p = 0.009$) and bulbar redness ($p = 0.038$) were higher for the older group whereas tear meniscus height was larger ($p = 0.018$) in the younger group. Similarly, relative plasma-derived albumin levels were higher (17.1±12.4%) in the tears of the older, compared to the younger (5.0±9.6%) group. A protein peak at ~23 kDa was observed in 53% of the older group samples but in only 36% of the samples of the younger subjects ($p = 0.122$).

Conclusions Distinct differences in tear film composition between the two age groups were observed. Parallels in terms of clinical symptoms which reflected a biochemical response (and vice versa) were found, but specific correlations between clinical measurements and biomarkers for individual subjects were not observed.

Keywords: tear protein, tear film stability, corneal haemostasis, ageing biomarkers

Introduction

Inevitably, the eye, like any other part of the body, succumbs to age in very specific ways. These can be divided into those which act directly on the eye (e.g. glaucoma and cataracts) and systemic disorders which affect the eye indirectly (e.g. diabetes and cardiovascular disease). Sight deterioration has a huge impact on quality of life and is arguably the most significant consequence of ocular ageing. Ocular surface ageing can present itself in many different manifestations including a reduction in palpebral aperture, a decrease in lid tension, the onset of pingueculae and changes to tear film structure and function.

This study focuses on the tear film which plays a vital role in maintaining healthy visual function and protecting the corneal surface. The functions of the tear film include lubrication and maintenance of a smooth refracting surface in addition to supporting the innate and acquired immune ocular defence. Normal tear film function is reliant upon the combined involvement of the corneal surface, adjacent glands, eyelids and cellular and muscular processes. These foremost anterior ocular functional structures, collectively known as the lacrimal functional unit[1] work harmoniously to supply, regulate and control the tear film. A comprehensive review of the ocular surface and lacrimal functional unit in terms of ageing (and the potential link with dry eye) was published recently.[2] Age-related changes in these ocular components can impinge on normal tear film composition and function. The overall picture of ocular surface ageing seems to be dominated by the impairment of aqueous function - principally by evaporation and inadequate secretion. However, there is, as yet, no single identifiable mechanism that explains the reduction in tear stability and the umbrella term “dry eye syndrome” often used to describe the multifactorial origins of the condition.[3]

The effects of the ageing on the tear film can be considered from both clinical and biochemical viewpoints. Clinical events can be measured directly using a wide range of point-of-care methodologies, on the other hand biochemical measurements usually require complex laboratory techniques. In general, clinical measurements have been given more attention and consequently information regarding the biochemical response of the ageing tear film is somewhat lacking. The dynamic and interactive mucin, aqueous and lipid layers of the tear film cover the ocular surfaces. They comprise many components including mucins, glycoproteins, proteins, peptides, lipids and electrolytes. Changes to just one constituent of tears could have a detrimental effect on tear film function.

Tear proteins play a range of important roles in ocular immunity and homeostasis and comprise both indigenous proteins,[4,5,6] and those that result from vascular leakage.[7,8] Although tear protein analysis is a key and well-studied technique[4,9] and the role of individual proteins is well known, little is understood about their role in tear film ageing. In this study, the principal indigenous tear proteins (lysozyme, lipocalin, secretory IgA and lactoferrin) which make up around 80% (~6 mg/ml) of total tear protein[4] were investigated. Lysozyme is an antibacterial enzyme, while lactoferrin is an iron binding protein which functions to inhibit bacterial growth. Lipocalin binds small hydrophobic molecules for transport and is a lipid scavenger at corneal surface. Secretory IgA (sIgA) is the primary antibody in tears and critical for host ocular immunity. Serum albumin in tears was also measured and used as a marker of vascular permeability. The aim of this pilot study was to investigate and compare individual tear protein profiles in two subject populations, differing in age by over 30 years - in conjunction with a range of routine point-of-care standard clinical measurements.

Methods

Study population

Two age groups were enrolled; one 'younger' ≤27 years (n=11; female, n=7) and one 'older' ≥58 years (n=19; female, n=8). While no 'overall-health' questionnaires were taken, the participants were questioned about their ocular and general health. None of the participants were previously diagnosed with any eye-related problems or had previously undergone surgery on their eyes. None of the participants suffered from allergies or were taking medications. Contact lens wearers (n=4, two subjects in each group) were not excluded but participants were asked not to wear lenses on the day of the study.

Study protocol

Measurements were performed in the order listed below with at least five-minute intervals between each test. The same examiner carried out each tests on participants within a 6-hour window of time (10:00 - 16:00) in an attempt to minimise diurnal variation. All tests were carried out in temperature-controlled, air-conditioned optometry clinic.

Questionnaires

Subjects completed a McMonnies[10] and a Ocular Surface Disease Index (OSDI)[11] questionnaires.[12] Expected McMonnies scores are 11.7±6.0 for <25 year olds and 17.6±6.0 for >45 year olds.[13] OSDI scores of <12 indicates a normal result.[14]

Anterior ocular health

A Takagi SM-70 (Takagi Seiko Co., Ltd., Nagano-ken, Japan) slit lamp (x16 and x40 magnification) was used to assess ocular surface health of both eyes. Bulbar, limbal and tarsal redness and corneal neovascularisation were subjectively graded from 0 to 4 on the Efron scale.[15] A grade of 1 to 2 is normal for bulbar redness, but younger people are more often found to have a grade of 0. A grade of up to 2 is normal for limbal and tarsal redness. One grade was recorded and reported for each area assessed.

Tear meniscus height

A non-invasive assessment of the tear margin using the Takagi SM-70 slit lamp (x16 and x40 magnification) was conducted.[16] Three measurements of central tear meniscus height of the right eye were taken, to the nearest 0.1 millimetre (normal value 0.1 - 0.4 mm) using the scale on the eyepiece. An average of these values was reported.

Tear film break-up time (TBUT)

TBUT was measured using a non-invasive placido-based topography system using a Topcon D300 (Topcon Corporation, Japan). The placido rings were projected onto the cornea of the left eye using white light. Each subject was asked to blink three times and then to keep their eye open for as long as possible after the third blink. A video recording was taken and TBUT was noted manually as the first appearance of break-up of the placido rings. An average of three measurements was reported and there was a five-minute interval between each test. A TBUT >10 s is generally considered normal and <5 s is indicative of dry eye.[17]

Tear osmolarity

Tear osmolarity was recorded using the TearLab Osmolarity System (TearLab Corporation, CA., USA). The osmolarity test pen, with a freshly placed new chip test card, gently extracts 50 nl of tears from the tear meniscus at the lateral canthus. The card was then inserted into the instrument where osmolarity is measured in mOsm/L. Both eyes were tested and an average of the two osmolarities was reported. A value of 302±8 mOsm/L is considered

normal, with values of 315±11 and 336±22 mOsm/L indicating mild/moderate and severe dry eye respectively.[18] The instrument was calibrated at the start of each day using the high and low osmolarity standards provided with the kit.

Tear flow - Schirmer strip (SS) test

The SS test was used to measure tear flow rate.[19] An absorbent SS (TearFlo®, HUB Pharmaceuticals LLC, US) was inserted into the medial lower conjunctival sac (no anaesthesia was used), the subject was allowed to blink as normal but kept their eyes open throughout. After 5 minutes the strip was removed and the wetting length of the SS was recorded (in millimetres), or the time was recorded after 35 mm was absorbed. Mean wetting length values ≥10 mm are typical for normal healthy eyes but values ≤10 mm can indicate dry eye.[20,21]

Tear film collection

There are many ways to collect tears, each of which can influence the results. Presently, there is no one recommended method, so the SS collection method was selected allowing the concurrent measurement of tear flow and tear fluid collection. This is in line with the current DEWS report which states "Schirmer strips are preferred by patients for comfort and perceived safety."[3] The left eye was used for SS collection. To extract the tear fluid from the strip, using a piggyback system (a small microcentrifuge tube with a small hole in the bottom placed in a larger microcentrifuge tube), the SS was centrifuged at 10,000 rpm for 5 mins and the eluted tear film collected. Extracted samples were stored (< 72 hours) at 4˚C.

Fluorescein staining

A minim was used to apply a ~2 µl drop of 1% w/v fluorescein sodium solution (Bausch and Lomb) to the tear meniscus of both eyes. The drop was allowed to spread over the ocular surface. Staining was visualised using a Takagi SM-70 slit lamp (x16 and x40 magnification) fitted with a Wratten 12 filter and cobalt blue illumination. Staining of both eyes were visually subjectively scored (0 – 4) on the Efron grading scale.[15] A normal ocular surface (zero to mild staining) has a grade of 0 to 2.[14]

Biochemical laboratory-based protein analysis

Tear samples were analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., CA., USA) in combination with Protein 230 Plus LabChip kits (sensitivity 0.005 mg/ml).

Overall protein profiles are calculated with each analyte presented as a function of the total protein detected.[4,9]

Statistical analysis

One-way ANOVA tests (p <0.05 indicates a statistically significant difference) were used to analyse the data. Z-tests were used to determine the statistical difference between groups on a single characteristic, i.e. number of subjects (%). Correlations between biochemical and age group data and clinical and age data are shown as p values. Linear correlation calculation was used to identify any links between the biochemical and the clinical data, where a value of R-squared (R^2) close to 1 provides an indication of a good correlation.

Ethics

All the studies described herein had received prior ethics approval by the appropriate committee at Aston University and conformed to the tenets of the Declaration of Helsinki. Written informed consent from each subject was obtained once the requirements of the study had been explained.

Results

Clinical observations and measurements

A total of 30 participants were enrolled. Average ages of the "young" and "old" groups were 23.7±2.1 and 63.0±4.0 respectively. Overall summaries of mean (±SD) data for all parameters are shown in Table 1. Statistical analysis indicated that the McMonnies dry eye questionnaire was significantly (p = 0.009) higher for the older group, (although the older cohort's higher scores do not put these subjects into the dry eye category) whereas the OSDI was not significantly different (p = 0.983). The mean results of the anterior eye grading scale assessment revealed no significant differences between the age groups for limbal and tarsal redness, corneal neovascularisation, or corneal staining. The bulbar redness was significantly (p = 0.040) lower, and tear meniscus height (p = 0.001) significantly higher in the younger age group. Statistical analysis indicated that the TBUT was significantly lower in the older age group (p = 0.045), but that there was no significant difference between the age groups in tear osmolarity (p = 0.688). The SS wetting length and collection rate (wetting length divided by collection time) were significantly higher in the younger age group (p = 0.003 and p = 0.001 respectively) and in all cases the time taken to collect the tear film from the older group

was five minutes. Although contact lens wear has been known to influence tear film quality and composition, the results for the contact lens wearers did not deviate significantly from the mean in this study.

Table 1. Comparison of clinical and biochemical measurements for the two (≤27 and ≥58) age groups. Data are expressed as the mean (±SD) with *p* value correlations from one-way ANOVA analysis. A Z-test was used to determine the statistical significance between subject percentages.

Details	**Age group**	**Scale/Range**	**≤27**	**≥58**	***p***
Subject	Age	n/a	23.7±2.1	63.0±4.0	NA
	Cohort (#)	n/a	11	19	NA
Questionnaires	McMonnies	0-45	5.0±3.2	10.7±5.6	0.009
	Ocular Surface Disease Index	0-100	4.7±5.0	4.6±3.7	0.983
Anterior health	Bulbar redness	0 to 4	1.3±5.5	1.7±0.4	0.038
	Limbal redness	0 to 4	1.3±0.6	1.4±0.4	0.550
	Tarsal redness	0 to 4	1.1±0.6	1.2±0.7	0.675
	Corneal neovascularisation	0 to 4	0.7±0.6	0.7±0.5	0.279
	Corneal staining	0 to 4	0.8±0.8	1.3±1.0	0.116
Tear Parameters	Tear osmolarity (mOsm/L)	302±8 is normal[18]	297.1±4.6	298.5±8.2	0.688
	Tear meniscus height (mm)	0.1 to 0.4 is normal	0.18±0.05	0.14±0.05	0.018
	Collection rate (mm/min)	35mm/5min max	9.5±5.0	3.9±1.7	0.001
	TBUT (s)	<10s in abnormal	11±5.5	6.8±4.4	0.045
	Schirmer strip wetting length (mm)	0 to 35	31.3±6.1	21.4±8.3	0.003
Protein Analysis	No of Peaks Detected	n/a	12.9±2.1	14.9±2.7	0.031
	Lysozyme (%)[a]	n/a	34.4±11.9	24.8±8.0	0.016
	Lipocalin (%)[a]	n/a	17.4±5.4	13.5±6.7	0.119
	sIgA (%)[a]	n/a	2.2±2.2	5.2±2.9	0.010
	Lactoferrin (%)[a]	n/a	23.6±7.8	21.8±9.3	0.618
	Albumin (%)[a]	n/a	5.0±9.6	17.1±12.4	0.011
	~88 kDa protein (%)[a]	n/a	0.16±0.39	0.76±0.82	0.432
	~23 kDa protein (%)[a]	n/a	0.09±0.18	0.49±0.62	0.122
	albumin positive subjects (%)	n/a	82	100	0.080
	88 kDa positive subjects (%)	n/a	18	59	0.390
	23 kDa positive subjects (%)	n/a	36	53	0.122

[a] as a function of total protein detected
n/a = not applicable

Protein analysis

Figure 1 shows individual protein composition data points for all subjects. Inspection reveals no obvious discrimination between male and female protein composition for either age group, whereas significant differences in overall average protein levels were observed between age groups. The number of protein peaks detected were statistically significantly lower in the younger age group ($p = 0.031$) (Table 1). Relative plasma-derived albumin levels were higher (17.1±12.4%) in the tears of the older, compared to the younger (5.0±9.6%) age group ($p = 0.011$), likewise relative mean sIgA levels increased from 2.2±2.2% in the younger group to 5.2±2.9% in the older group ($p = 0.010$). On the other hand lysozyme was significantly higher in the younger age group ($p = 0.016$). In general, an age-related reduction in lysozyme, lipocalin and to a lesser extent lactoferrin, relative concentration, was observed. Conversely, sIgA, albumin and the lower concentration 23/88 kDa protein peaks showed an average increase in relative concentration with age. The main (indigenous) tear proteins were omnipresent at ~0.1 – 2 mg/ml concentrations, whereas albumin, and the 23/88 kDa proteins were not detected (by the technique used) in all samples. The percentage of subjects presenting positive for the 88 kDa protein peak in the older cohort at 59% was much higher than the 18% positive samples in younger age group ($p = 0.390$). The same was true for the 23 kDa protein peak (53% vs 36%) ($p = 0.122$). Interestingly, all of the older cohort presented with albumin positive tear samples compared with the lower 82% of the younger cohort ($p = 0.080$). A positive correlation between albumin levels and the relative concentrations of both the 23 kDa ($R^2 = 0.521$) and the 88 kDa ($R^2 = 0.843$) proteins was found.

Discussion

The observed clinical measurements largely follow the trends presented in the literature. Average TBUT values, for example, reduced with age as did those reported by Tiffany and Gouveia, although their recorded decrease was larger.[22] SS tear wetting lengths measured here showed a significant decrease from younger to older age group ($p = 0.003$) consistent with the general consensus that tear flow rate and stability decline with age.[23,24] No significant difference in osmolarity was found between the two age groups and while an increase in osmolarity is observed with dry eye patients[25,26] there is disagreement in the literature as to whether osmolarity increases specifically with age in the healthy eye.[27,28]

Differences in tear film protein composition between the two age cohorts were clearly observed; the significant reductions in lysozyme, lipocalin and, to a lesser extent, lactoferrin concentration are in line with age-related decreases quoted in the literature.[22,23,29] The decline in these lacrimal gland proteins parallels the clinical observation that an overall slowdown in tear secretion and stability occurs with age. This reduction in bio-availability may adversely affect the antimicrobial and lipid binding capability of the tear film. Interestingly, in contrast with lysozyme, lipocalin and lactoferrin, a small yet significant increase in relative concentration of sIgA with age was observed. This reflects the fact that the nervous stimuli, secretion pathway and movement of sIgA from the lacrimal gland into the tear film differs from that of lysozyme, lipocalin and lactoferrin, which are produced by the acinar cells of the main lacrimal gland and directly secreted into the tear film. sIgA, being produced by plasma cells residing in the lacrimal gland, relies on specific polymeric IgA receptors which transports it across the acinar cell membrane into the tear film.[30] Lower overall active lacrimal aqueous secretion rates are consistent with increased relative sIgA concentrations, because decreases in lysozyme, lipocalin and lactoferrin mean that sIgA increases proportionally in relative concentration.

An increase in albumin, which accounts for ca 50% of total serum protein, is indicative of plasma leakage into tears and serves as a biomarker for vascular permeability and inflammation.[8] A significant increase was observed in relative albumin concentration and detection rate in the older cohort; albumin was present in all tear samples of the older cohort compared with 82% for the younger group. There is some logic in expecting that the changes observed here point to a progressive shift in tear film composition to a plasma protein-rich biological fluid which will ultimately influence tear film dynamics. Similarly, there may be pointers to mechanisms and links between biomarkers and clinical response, which are undoubtedly more difficult to establish. Limbal and bulbar hyperaemia, for example, are a function of increased blood flow at the anterior ocular surface, but from the clinical observations recorded in Table 1, it is only bulbar hyperaemia that gives a significant clinical correlation with albumin influx. It may well be that a greater age differential would produce stronger correlations.

Increases in the number of protein peaks detected and the occurrence of peaks at ~23 and 88 kDa, in the older tear film, are interesting findings in terms of the ageing tear film. A possible identity of the 23 kDa protein peak is lacrimal proline-rich protein-4 (LPRR4) which is

similar to proline rich proteins in saliva - known for their antimicrobial and lubrication roles.[31] Interestingly, LPRR4 down-regulation has been suggested as a marker of dry eye.[32] Other potential 23 kDa candidates include prolactin, of the cytokine superfamily involved in haemostasis and secretion, and lacritin, which promotes basal tear secretion.[33-35] It is interesting that in this study there was generally a higher proportional concentration of this protein peak and increase in incidence with age, whereas in the literature all three of these potential 23 kDa protein candidates have been observed to occur at a lower concentration or be down-regulated in dry eye subjects.[32,34,35]

The apparent paradox - that age is a risk factor for dry eye, yet here we see this protein peak up-regulated in the older cohort - is intriguing. Correlations of these results with dry eye studies must be made with caution, however, the older cohort here did not show a propensity for dry eye symptomology, as borne out by the osmolarity results and questionnaires. The positive correlation between albumin levels and the relative concentrations of the 23/88 kDa proteins supports a common vascular origin. Although the 23/88 kDa proteins are always associated with the presence of albumin, the reverse is not true. Whether their influx into tears is a cause or a consequence of an acute ocular response, and their role in protection from the onset of dry eye, remains to be determined.

Conclusion

In general, a correlation between the signs and symptoms of ocular disorders and tear film properties remains elusive. Although clinical and biochemical tear compositional changes were apparent with age, no one clinical parameter correlated directly with a specific biochemical change for individual subjects, even though general trends were observed. Table 2 highlights the key findings in this study and summarises the age-related clinical and biochemical changes. It is clear that clinical indicators are reflected in a biochemical response. An example of this is the higher incidence of corneal staining and bulbar redness, which are concurrent with a greater influx of albumin - a key biomarker of vascular permeability and ocular redness. Similarly, a reduction with age in lysozyme, lipocalin and lactoferrin - the main aqueous secretory tear proteins - is mirrored in a reduction in tear flow rate, TBUT and overall tear secretion. It is important to distinguish the effects of the ageing process in the compromised and in the uncompromised eye – not least to clarify the need for medical intervention. This work has identified changes and unresolved questions which could

prove to be important in understanding the progressive ageing of the tear film. These centre, in particular, on the identity and role of the ~23 and 88 kDa proteins.

Table 2. A summary of the key clinical and biochemical changes with an increase in age.

Clinical Measurement	Biochemical Assay Measurement	Change with Increasing Age
McMonnies score Corneal staining Bulbar redness	Albumin sIgA 23 kDa protein 88 kDa protein Number of protein peaks detected	**Increase**
OSDI Tear osmolarity Tarsal/limbal redness Corneal neovascularisation		**No change**
SS wetting length TBUT Tear flow rate Tear meniscus height	Lysozyme Lipocalin Lactoferrin	**Decrease**

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Figure Caption:

Figure 1. Individual subject data points for the two age cohorts, split into male versus female, with the individual protein peak percentages shown as a function of total protein detected for lysozyme, lipocalin, lactoferrin, sIgA, albumin, 23 kDa protein and 88 kDa protein (shown in order). The *p* values show one-way ANOVA correlations.