Water-Soluble Antioxidants in Human Tears: Effect of the Collection Method

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Purpose. To resolve differences in published data on tear antioxidant levels by comparing the concentration of water-soluble antioxidants in human reflex tears collected by capillary tube and by the Schirmer strip collection method and in basal and reflex tears collected using the Schirmer strip method.

Methods. Yawn-induced reflex tears (collected simultaneously by capillary tubes and by Schirmer strips) and basal tears (by Schirmer strips and using local anesthetic) were collected from 12 healthy subjects. Tear cysteine, ascorbate, glutathione, urate, and tyrosine were measured by high-performance liquid chromatography within a few minutes of collection.

Results. Cysteine, ascorbate, glutathione, and tyrosine were 5 to 10 times higher ($P < 0.01$) in both reflex and basal tears collected by Schirmer strip compared with reflex tears collected by capillary tube from the same subject. Urate levels were slightly but nonsignificantly higher in Schirmer strip samples ($P > 0.05$).

Conclusions. The conflict in published data on tear antioxidants is caused by differences in collection methods. With the exception of urate, antioxidants accumulate to very high levels in corneal cells. Spuriously high antioxidant levels in tears collected using Schirmer strips, therefore, are most probably caused by contamination with intracellular constituents. The capillary tube collection method is proposed as the method of choice for reflex tear collection for biochemical studies. This less-invasive method facilitates the evaluation of tear antioxidant levels as a biomonitoring tool for corneal health. Although moderately increased antioxidant levels may be beneficial, the authors hypothesize that marked increases may indicate damage to the ocular surface. (*Invest Ophthalmol Vis Sci.* 2001;42: 3130–3134)

Tear fluid protects the external surface of the eye. Tears can be described as of two types: reflex tears, which are induced by a stimulus such as yawning, irritation, or bright light, and basal tears, which are the nonstimulated secretion of the tear glands. The major function of tears is to maintain corneal health by diluting, flushing out, or neutralizing foreign bodies and chemicals and reactive oxygen species (ROS).[1–4] Owing to its exposed nature, the corneal surface is at particular risk of oxidative damage by photo-induced and environmental ROS.[5–8] Antioxidants in tears act to oppose such damage.[3,4] Several low-molecular-weight antioxidants have been found in human tears. These include endogenous compounds, such as cysteine, glutathione, urate, and tyrosine and dietary ascorbate.[3,4]

There is considerable interest in the possible role of tears in corneal health, but to date there are few publications on tear antioxidants.[1,3,4,9–13] Most previously published data were obtained on tears collected using Schirmer strips.[1,3,10] This method of tear collection is well established for measuring tear volume; however, the volume of tears collected by Schirmer strip is very small, and obtaining an accurate composition analysis is difficult.[14] Evaporation of water from the small tear sample captured on the strip may significantly increase the apparent concentration of solutes, including antioxidants. Schirmer strips are also invasive, and damage to ocular surface cells by these strips could occur. It has been reported that the use of Schirmer strips is associated with elevated plasmin concentrations in the tear samples, indicating that cells on the conjunctival surface are damaged.[14] Vascular fragility caused by strip-induced irritation in the lower cul-de-sac of the eye (where Schirmer strips are usually placed during tear collection), and injuries to the conjunctival surface may change the composition of the tears collected.[14] Because many antioxidants are found in blood plasma and are highly concentrated within cells,[15–22] transudation of vascular fluid and/or leakage from damaged cells at the site of collection onto the Schirmer strip could lead to a significant increase in antioxidant concentrations of the tear fluid collected. This could help account for the lower levels of ascorbate and urate recently reported, in which capillary tubes, rather than Schirmer strips, were used for tear collection.[4,9,11,13] The capillary tube collection method is much less invasive than the Schirmer strip technique. A small, disposable glass capillary tube is placed just above the lower tear meniscus and with care, minimal contact between the tip of the capillary tube and the globe can be achieved.[23] Antioxidant concentrations in tears collected by capillary tubes, therefore, may give a more accurate indication of tear composition.

The main purpose of this study was to compare the concentration of water-soluble antioxidants in human reflex tears collected by Schirmer strips and by capillary tubes. A secondary purpose was to investigate the difference between basal and reflex tears collected using the Schirmer strip method. The tear components of interest were cysteine, ascorbate, glutathione, urate, and tyrosine.

Methods

Experiments were performed to determine the water-soluble antioxidant components in tear fluid from healthy young adults and to compare the antioxidant levels in reflex tears collected by capillary tubes and by Schirmer strips (without use of local anesthetic) and in basal tears collected by Schirmer strips (with use of local anesthetic). Twelve Chinese subjects (seven men, five women), aged from 22 to 29 years, were recruited with their informed consent. All subjects had apparently normal general and ocular health. None were smokers or users of vitamin supplements, and none wore contact lenses. Subjects in this self-controlled study attended our clinic on one occasion only between the hours of 9 AM and 3 PM. Reflex tears were collected simulta-

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TABLE 1. Comparison of Water-Soluble Antioxidant Concentrations in Human Tears Collected by Capillary Tubes and by Schirmer Strips (Current Study)

	Reflex Tears Collected by Capillary Tubes	Reflex Tears Collected by Schirmer Strips without Local Anesthetic	Basal Tears Collected by Schirmer Strips with Local Anesthetic	Plasma* (μM)	Intracellular* (approximate μM in noncataractous human crystalline lens)
Cysteine	0.7 ± 0.2 (0.4–1.0)†	6.0 ± 1.7 (3.6–9.8)	12.5 ± 8.9 (4.8–37.9)‡	<12	150
Ascorbate	24.4 ± 11.0 (11.0–53.6)†	120.4 ± 108.0 (29.3–258.6)	393.3 ± 412.8 (138.4–1453.1)‡	25–100	2000
Glutathione	3.7 ± 2.5 (1.6–10.6)†	25.1 ± 16.8 (10.0–65.2)	75.8 ± 66.8 (12.6–183.3)‡	<4	3000
Urate	98.1 ± 41.9 (54.8–194.4)	116.5 ± 74.2 (50.3–308.1)	166.0 ± 83.5 (73.7–322.0)‡	150–450	1000
Tyrosine	2.7 ± 1.3 (1.5–5.5)†	10.8 ± 4.8 (5.6–21.1)	29.8 ± 18.7 (10.7–76.9)‡	50–150	400

* Representative plasma and intracellular concentrations (from published reports[15,18–20,22]) are given for reference. Collection data are expressed as mean micromolar ± SD, with ranges in parentheses.

† Significantly lower than the level in reflex tears collected using Schirmer strips ($P < 0.01$).

‡ Significantly higher than the level in corresponding reflex tears collected by Schirmer strips ($P < 0.05$); however, when corrected for volume, there were no significant differences ($P > 0.05$).

neously from both eyes of each subject (one by capillary tube and the other by Schirmer strip). Basal tears were collected, from the eye previously used for reflex tear collection by capillary tube, 30 minutes after reflex tear collection. This study was approved by the ethics subcommittee of the university, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 2000.

A preweighed Schirmer strip (weighed in a clean, sealed Eppendorf tube; Eppendorf, Fremont, CA) was placed onto the outer canthus of one eye, randomly selected, of each subject, and reflex tears were collected for 5 minutes. Simultaneously, a disposable capillary tube (Drummond Scientific Co., Broomall, PA) was used to collect tears from the other eye, according to the procedure of Callender and Morrison.[23] Within 30 minutes of reflex tear collection, basal tears were collected as follows: One drop of local anesthetic (0.5% benoxinate HCl, Alcon Ltd., London, UK) was instilled into the eye from which tears had been collected by capillary tube. After 10 seconds, a preweighed Schirmer strip was placed on the outer canthus, and basal tears collected for 5 minutes. After tear collection, the wet Schirmer strip was immediately put into the same tube as before, the tube was sealed to avoid evaporation, and the strip was reweighed to determine the weight of tears collected. This was translated into volume by using the tear density, which was calculated by weighing a known volume of the reflex tears collected by capillary tube from the same subject. Tears were eluted from strips using 50 μl phosphate buffer (HPLC mobile phase), and approximately 30 μl eluate was transferred into the sample cup for measurement. Approximately 30 μl of reflex tears collected by capillary tube were transferred directly into a sample cup. The sample cups were immediately placed in the autosampler compartment of an HPLC system (Millennium; Waters Alliance, Milford, MA), and the antioxidants of interest (cysteine, ascorbate, glutathione, urate, and tyrosine) were measured concurrently, according to the HPLC method of Gogia et al.[3]

To check whether there was any antioxidant or contamination of the tear sample due to contact with the capillary tubes, Schirmer strips, or local anesthetic, three blank samples were prepared by collecting mobile phase into a capillary tube, absorbing mobile phase onto a Schirmer strip, eluting as described earlier, and adding 10 μl of local anesthetic to 55 μl mobile phase. In addition, recovery of added antioxidants was determined by measuring freshly prepared combined standard (containing all five antioxidants of interest), with the same procedures used to prepare the three blank samples. Combined standard (100 μM) was prepared freshly on each testing occasion by mixing equal parts of 500 μM standards of each antioxidant of interest (cysteine, ascorbate, glutathione, urate, and tyrosine). The 500 μM standards were prepared fresh from stock solutions as follows: cysteine (20 mM) was prepared from solid (BDH Chemicals Ltd., Poole, UK) in extra pure water, and dissolving was facilitated by the addition of a few drops of 0.2 M HCl; glutathione (10 mM) was prepared from solids (Sigma Chemical Co., St. Louis, MO) in extra pure water; urate (2 mM) and tyrosine (20 mM) were prepared from solids (BDH Chemicals Ltd.) in extra pure water to which a few drops of 1 M NaOH had been added to facilitate dissolving. The stock solutions of cysteine, glutathione, urate, and tyrosine were aliquoted (separately), stored at −70°C, and thawed just before use. Ascorbate (10 mM, from D-L extra pure crystals; Merck, Darmstadt, Germany) was prepared in extra pure water just before use.

The HPLC system consisted of a solvent pump and injector with sample cooling function (Waters Alliance), a C18 precolumn (5 μm, 3.9 × 20 mm; Guard-Pak Waters Sentry), and a reversed-phase C18 analytical column (5 μm, 5 × 250 mm; Resolve, Isco Inc.). The detector (996 PDA; Waters), UV detector, and electrochemical detector were connected in series. The output was calculated on computer (Millennium software, version 3.05.07; Waters Alliance) and the peak areas were recorded. The mobile phase was 0.2 M KH_2PO_4-H_3PO_4 (Merck, Darmstadt, Germany) at pH 2.7, delivered at 1 ml/min. The injection volume was 20 μl, and the retention times for cysteine, ascorbate, glutathione, urate, and tyrosine were, respectively, 3.0, 4.2, 5.0, 9.4, and 10.7 minutes, indicating good separation and clear identification of these antioxidants. The injection interval used was 12 minutes. Calibration and precision were performed on each occasion using 2.5, 5.0, 7.5, and 10.0 μl injection volumes of the freshly prepared combined standard.

The distributions of the data were not significantly different from normal (one-sample Kolmogorov-Smirnov D tests, $P > 0.05$), and parametric tests were therefore used. Results were statistically analyzed using the paired *t*-test to detect differences between tears collected by the two methods and differences between reflex and basal tears collected using Schirmer strips. $P < 0.05$ was regarded as statistically significant.

RESULTS

Precision was acceptable for all antioxidants: within-run and between-run coefficients of variation were, respectively, less than 3.0% ($n = 10$) and 10.0% or less ($n = 5$) in each case. No absorption peaks were obtained in three sets of blank sample, indicating no antioxidant contamination (results not shown). Results showed 100% recovery of all antioxidant components in antioxidant standard from samples collected into capillary tubes or mixed with local anesthetic. Antioxidant standards collected onto Schirmer strips and eluted, as described earlier, showed more than 90% recovery for ascorbate, glutathione, and tyrosine and approximately 60% recovery for both cysteine and urate. This relatively low recovery could indicate poor elution due to binding of cysteine and urate onto the strip. However, cysteine is unstable, and it is likely that some was degraded during collection and elution from the strip.

TABLE 2. Previously Published Data and Findings of the Current Study on Antioxidants in Human Tears

	Herzig and Hurwitz[9]	Howard et al.[10]	Kuizenga et al.[11]	Paterson and O'Rourke[1]	Gogia et al.[3]	Mendelsohn et al.[13]	Choy et al.[4]	Current Study
Ascorbate	Not done	65 ± 34†	Not detectable	220–1310 (Range, no mean given)‡	116 ± 16† 665 ± 49‡	Not done	23 ± 9.6*	24.4 ± 11.0* 120.4 ± 108.0† 393.3 ± 412.8‡
Urate	53.6–89.3 (Range, no mean given)*	Not done	Not done	Not done	131 ± 24† 328 ± 48‡	99.4 (no SD given)*	68 ± 46*	98.1 ± 41.9* 116.5 ± 74.2† 166.0 ± 83.5‡
Cysteine	Not done	Not done	Not done	Not done	12.9 ± 4.5† 48.5 ± 14.6‡	Not done	Not done	0.7 ± 0.2* 6.0 ± 1.7† 12.5 ± 8.9‡
Glutathione	Not done	Not done	Not done	Not done	16.3 ± 4.1† 107.1 ± 22.8‡	Not done	Not done	3.7 ± 2.5* 25.1 ± 16.8† 75.8 ± 66.8‡
Tyrosine	Not done	Not done	Not done	Not done	20.8 ± 4.2† 45.6 ± 3.2‡	Not done	Not done	2.7 ± 1.3* 10.8 ± 4.8† 29.8 ± 18.7‡

Collection data are expressed as mean micromolar ± SD.
* Reflex tears collected by capillary tubes.
† Reflex tears collected by Schirmer strips.
‡ Basal tears collected by Schirmer strips.

The antioxidant levels in tears collected by capillary tubes and Schirmer strips are presented in Table 1, along with representative plasma and intracellular concentrations of the relevant antioxidants for reference. Because there are no complete data sets for antioxidants in human cornea or conjunctiva, the published intracellular concentrations given are for human, normal crystalline lens.[15,18–20,22] Results showed that the levels of cysteine, ascorbate, glutathione, and tyrosine were significantly ($P < 0.01$) higher in reflex tear samples collected by Schirmer strips without local anesthetic compared with tears collected concurrently by capillary tubes from the other eye of the same subject. Urate levels were slightly but nonsignificantly higher in reflex tears collected by the Schirmer strip method ($P = 0.1573$). These results indicate release of intracellular antioxidants into the reflex tears during the period of contact between the Schirmer strip and the palpebral conjunctiva. In basal tears collected by Schirmer strips, antioxidant levels were apparently higher than in the reflex tears collected onto Schirmer strips; however, when concentrations were corrected for volume (which was lower in the case of basal tears) a basal-reflex concentration ratio of approximately 1.0 was found for each antioxidant. This indicates that the traumatic effect of the Schirmer strip was the same, whether or not anesthetic was used, and that the different molar concentrations found in basal and reflect tears collected by Schirmer strips was due to the greater dilution of released intracellular antioxidants in reflex tears.

DISCUSSION

Within the eye, production of ROS is likely to be high, owing to photo-oxidation processes, and this induces oxidative stress.[5–8] Oxidative stress plays a role in both the causes and consequences of ocular disorders, including keratitis, glaucoma, cataract, and age-related maculopathy.[24–30] The importance of maintaining an optimal antioxidant defense to maintain an optimal state of health is becoming widely recognized and explored.[31,32] The antioxidant status of tear fluid is of interest, because tears constitute the first barrier protecting the cornea against oxidative damage from radiation, atmospheric oxygen, and pollutants.[1–4,12] Published data on tear antioxidants are scarce. However, there is one published report of cysteine, glutathione, and tyrosine levels in tears. The few reports of tear ascorbate and urate show conflicting results (Table 2).[1,3,4,9–11,13]

The variation in published results for tear ascorbate and urate could be due to differences in methodology for sample collection and measurement, or to differences in the subjects studied. The ascorbate method used by Kuizenga et al.[11] has a limit of detection of 60 μM, and therefore their inability to detect ascorbate in reflex tears cannot be taken to mean that no ascorbate was present. Most other investigators used an HPLC method to measure ascorbate and urate[1,3,10]; however, Choy et al.[4] used specific enzyme-linked colorimetric methods. These methods have been reported with good precision and linearity.[4,10,33] Methodological differences in measurement, therefore, cannot account for the differences in tear ascorbate and urate seen in Table 2. Most previous studies have involved Caucasian subjects.[1,3,9–11] Our subjects, however, are Chinese.[4] It is possible that Caucasian and Chinese eyes are somewhat different in terms of antioxidant composition, and this deserves investigation. It is likely, however, that the Schirmer strip method of sample collection affects tear composition and is responsible for the differences seen. Direct conjunctival contact with a Schirmer strip during tear collection may induce some degree of vascular transudation or cell damage, with leakage of plasma and intracellular constituents.[14] Alternatively, some cells may adhere to the strip, and their contents are then eluted from the strip, along with the tears. As can be seen in Table 1, the intracellular levels of cysteine, ascorbate, glutathione, and tyrosine are high.[15–17,19,21] Even a small amount of cellular leakage or transfer would cause a marked increase in tear antioxidant levels. Urate, a purine degradation product, does not show such a large intra- or extracellular differential in concentration as do other antioxidants, and thus cell damage or leakage per se would not be expected to cause a marked increase in the tear urate level.

In this self-controlled study, results are consistent with this rationale, and the effect of Schirmer strips on tear antioxidant composition is shown very clearly. Tears collected by capillary tubes, with minimal conjunctival contact, contained cysteine, ascorbate, glutathione, and tyrosine at levels 5 to 10 times lower than those found in reflex tears collected by Schirmer strips. Urate was slightly, but nonsignificantly higher in Schirmer strip tears. It should be noted here that the recovery of cysteine and urate from Schirmer strips was only 60%.

Results presented were not corrected for this relatively low recovery, and the true levels of cysteine and urate in Schirmer strip tears may have been approximately 40% higher than those presented in Table 1. Nonetheless, our conclusion that tears collected by Schirmer strips have spuriously high levels of water-soluble antioxidants owing to contamination by intracellular contents, still stands. The results obtained on basal tears collected by Schirmer strips were higher than those on reflex tears obtained using the same collection method and from the same subject. However, this apparent difference was caused by a greater dilution of intracellular constituents in the higher volume reflex tears, and antioxidant concentrations appeared no different when corrected for volume.

Ocular tissues and fluids are rich in various antioxidants, and this is likely to reflect high demand.[3,6,15–17,19,21] The eye is uniquely exposed to light and to the external environment. As the first physical barrier to oxidative damage, tear fluid is believed to be an important site of ocular antioxidant defense.[1–3,6,13] Our data showed that the reflex tear levels of the antioxidants, cysteine, ascorbate, urate, glutathione, and tyrosine, were quite low. This could be a reflection of high antioxidant utilization in situ, or rapid cellular uptake by the cornea. Currently, the source of tear antioxidants is not clear, however; and it is possible that corneal cell turnover and leakage are responsible. If utilization or cellular uptake of antioxidants were high, however, fresh lacrimal gland secretion would be expected to contain higher amounts of antioxidants than tears in contact with the cornea and exposed to light and air. Alternatively, if some or all the antioxidants in tears come from corneal cell turnover and leakage, then fresh lacrimal gland secretion would be expected to have lower levels of antioxidants than tears that had been in contact with the cornea. This has important implications, because in one scenario high antioxidant levels in tears could mean enhanced corneal supply and defense, whereas in the other it could indicate corneal damage.

Results presented here show also that tears collected using Schirmer strips contained significant amounts of intracellular constituents. We suggest, therefore, that this method of tear collection be limited to studies of tear volume. Furthermore, previously published biochemical data on tears collected using Schirmer strips should perhaps be re-examined in light of the data presented here.

In this present study, we measured the antioxidants cysteine, ascorbate, glutathione, urate, and tyrosine in tears. We have reported previously that the total antioxidant capacity of reflex tears is approximately 400 μM and that ascorbate and urate account for approximately half of this.[4] The small amounts of cysteine, glutathione, and tyrosine detected in tears in this present study, however, cannot account for the remaining 50%, of the total antioxidant capacity. There are clearly as yet unidentified antioxidant(s) in tears; thus, further work is needed in this area.

In summary, vascular fragility and cell injury or transfer caused by the Schirmer strip affect tear composition and result in spuriously high antioxidant levels in tears. The use of a capillary tube for tear collection is much less invasive, and is suggested as the method of choice for reflex tear collection for biochemical studies. There is currently no suitable method available for collection of basal tears for biochemical analysis. Indeed, it has been suggested that even when basal tears are collected using the standard protocol of Schirmer strips and an anesthetized cornea, some degree of reflex tearing still occurs.[34] Tears contains several antioxidants, including cysteine, ascorbate, glutathione, urate, and tyrosine, in addition to as yet unidentified antioxidant(s). The source of tear antioxidants remains to be established. Depending on the source (corneal cell leakage or lacrimal gland secretion), measurement of tear antioxidant levels may be useful to assess defense status, or may reflect corneal damage. In the first scenario, increased levels may be beneficial and decreased levels would indicate increased oxidative stress, whereas in the second scenario, increased levels would act as a biomarker of damage to the ocular surface. Further work is needed to clarify this. In either case, however, measurement of tear antioxidants may be a useful tool to monitor corneal health.

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