

Sialic Acid in Normal Human Tear Fluid

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Purpose: We measured the concentration of sialic acid, the terminal component of mucin, in normal diluted human tears.

Methods: Twenty-microliter tear samples were collected from 31 healthy volunteers (average age = 50.7 years) using micropipette after 50 μ L instillation of saline. We investigated the correlation of concentration between glycoprotein and sialic acid and the difference between the right and the left eyes, as well as the reproducibility of the sampling procedure.

Results: There was significant correlation of the concentration between glycoprotein and sialic acid (right eye: $r = 0.952$, $P < .0001$; left eye: $r = 0.976$, $P < .001$). There was no significant difference in concentration between the right and the left eyes. Also, the reproducibility was considered acceptable in three measurements of sialic acid using the present procedure. The sialic acid concentration in normal diluted tears was 37.1 μ g/mL on average.

Conclusion: Our data indicates that sialic acid concentration is an indicator for the concentration of glycoprotein and that this method of measurement is applicable to the analysis of mucin-deficient disorders. **Jpn J Ophthalmol 2001;45:327-331** © 2001 Japanese Ophthalmological Society

Key Words: Glycoprotein, mucin, PAS method, sialic acid, tear fluid.

Introduction

The tear film covers the ocular surface epithelium and is considered to have three layers: lipid layer, aqueous layer, and mucus layer.¹ The mucus layer is composed of mucins, γ -globulins, and other proteins.² Mucin is one of the particularly important components in considering dry eye condition, because it is the main component of the mucus layer, and is thought to contribute to tear stability and viscosity.³ Mucin has a high molecular weight, over 250 kDa, and contains many oligosaccharide side chains (>50%),⁴ which contain sialic acid and lactose. However, the exact composition of mucin has never been determined.²

N-acetyl neuraminic acid, one of the sialic acids, is considered to accelerate mucus viscosity and to contribute to tear stability.⁵ The measurement of mucin is difficult because of its high molecular weight. It is reported that mucin is measured using the periodic acid-Schiff (PAS) method in stomach and small intestine, because mucin is composed of many glycoproteins.⁶ In another previous report, sialic acid levels were measured as a parameter of mucin because salivary mucin has many sialic acids.⁷ In addition, sialic acid was detected in rabbit lacrimal gland fluid.⁸ Moreover, sialic acid has also been detected in human tear fluids,^{9,10} being derived from soluble glycoproteins in tears, exclusive of major tear proteins, such as secretory immunoglobulin A (IgA), lactoferrin, and lysozyme. In our study, we further investigated the correlation between glycoprotein concentration and sialic acid concentration in normal human diluted tears, and investigated whether sialic acid concentration can be considered a quantitative indication of tear mucin.

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Materials and Methods

Subjects

Subjects comprised 31 normal healthy volunteers (17 men, 14 women, aged 50.7 ± 16.0 years, mean \pm SD) with no dry eye symptoms. The criteria for normal was no ocular past history, no abnormalities of cornea or conjunctiva as assessed by slit-lamp examination, no staining of fluorescein and rose bengal, and normal cotton thread values (>10 mm), fluorescein breakup time (BUT) (>5 seconds), and Schirmer I test (>5 mm). Informed consent was obtained after a full explanation of the procedures and all examinations that would be performed.

Collection of Tear Samples

With the subjects in a supine position, 50 μ L saline warmed to 35°C was instilled in the nasal side of 1 eye by an Eppendorf micropipette, while pulling down the punctum without anesthesia and with as little stimulation as possible. After three forced blinks, a 20- μ L diluted tear sample was collected by another micropipette from the temporal side of the palpebral fissure. Tear samples were stored at -80°C until assay. For the analysis of the correlation between the PAS method and the sialic acid concentration method, a 50- μ L sample was collected from each eye, after the instillation of 100 μ L of saline. A 20- μ L diluted tear sample was used for the PAS method, and another 20- μ L tear sample was used for the sialic acid measurement method.

Assays

Glycoprotein assay by PAS method.⁶ Each 20- μ L tear sample was placed in a 96-well plate, 80 μ L of distilled water was added, and the samples were incubated for 2 hours at 37°C after the addition of 10 μ L of 0.05% periodic acid containing 7% acetic acid. After periodate oxidation, 10 μ L of the Schiff solution (E. Merck, Darmstadt, Germany) was added to the sample solution. After leaving the solution for 30 minutes at room temperature to allow color development, the resulting solution absorbance was measured at a wavelength of 550 nm. Bovine submaxillary gland mucin (BSM; Sigma Chemical, St. Louis, MO, USA) was used for the standard curve, and provided a calculation of PAS-positive glycoprotein concentration in BSM equivalent.

Sialic acid assay.^{11,12} A 20- μ L tear sample, placed in a screw-capped 7-mL vial (Iwaki Glass, Osaka) was mixed with 1 mL of sulfuric acid (25 mM) (Nacalai

tesque, Kyoto). The vial was tightly closed and heated at 95°C for 1 hour to hydrolyze the sample. After the sample was cooled, 1 mL of 7 mM 1,2-diamino-4,5-methylenedioxybenzene (DMB; Dojin Chemical Laboratory, Kumamoto) dissolved in 1 M β -mercaptoethanol (Wako Pure Chemical, Osaka) and 18 mM sodium hydrosulfite (Nacalai tesque) was added. The mixture was heated at 60°C for 2.5 hours in the dark to develop the fluorescence. The reaction mixture was cooled in ice water to stop the reaction. A 20-mL aliquot of the resulting solution was injected into a high-performance liquid chromatograph (HPLC). The HPLC system (LC-10AD; Shimadzu, Kyoto) equipped with a fluorescence spectromonitor RF-10A_{XL} (Shimadzu) was used in reverse-phase mode. Excitation wavelength was 373 nm and emission wavelength was monitored at 448 nm. The stationary phase used was TSK gel ODS-80T_M column (Tosoh, Tokyo). A mixture of acetonitrile and 0.04 M potassium phosphate (10:90, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. Standard sialic acid (Nacalai tesque) was used to make the calibration line (relation between sialic acid concentration and peak area integration in HPLC), based upon which the measured sialic acid concentration was determined.

Examinations

Correlation in concentration between glycoprotein and sialic acid. Subjects were 10 normal healthy volunteers. We investigated the correlation between glycoprotein concentration and sialic acid concentration, and the difference in concentration between the right and left eyes of each subject. To avoid the influence of reflex tear, the tear collection of the left eye was performed 10 minutes after tear collection in the right eye. To evaluate the correlation in concentration between glycoprotein and sialic acid, the Spearman's correlation coefficient was used; also, to evaluate the difference in concentration between right eye and left eye, the Wilcoxon signed rank test was used. In both analyses, $P < .05$ was considered significant.

Reproducibility of sialic acid concentration measurement. Subjects were 7 normal volunteers (left eye only). We measured sialic acid concentrations of three tear samples collected by the same ophthalmologist (YN) on different days. We used the Levene test to analyze homogeneity of sialic acid concentration variance in each subject. $P < .05$ was considered significant.

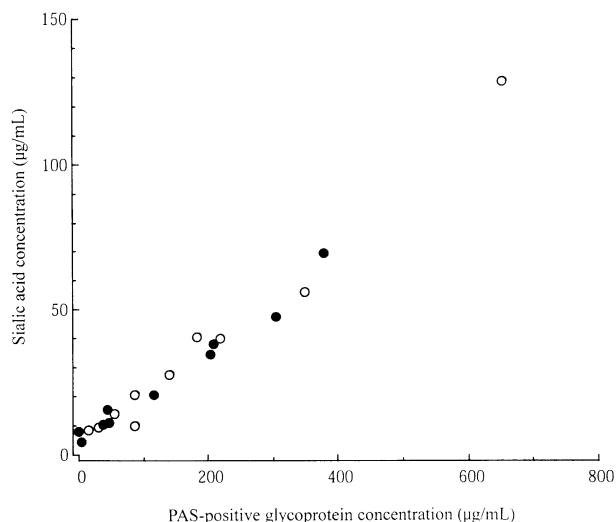


Figure 1. Correlation of concentration between glycoprotein and sialic acid in normal tear samples. There was significant linear correlation in both eyes (right eye: $r = 0.952$, $P < .0001$; left eye: $r = 0.976$, $P < .001$, $n = 10$, Spearman correlation coefficient). Concentration of periodic acid-Schiff (PAS)-positive glycoprotein in diluted tears was calculated in bovine submaxillary gland mucin. ○: right eye, ●: left eye.

Sialic acid concentration in diluted normal tear fluid. Subjects were 14 normal women (age: 61.1 ± 9.0 years, mean \pm SD). We measured the sialic acid concentrations in diluted tear samples.

Results

Correlation of Concentration Between Glycoprotein and Sialic Acid

Sialic acid concentration in right eyes and left eyes was 35.6 ± 36.5 $\mu\text{g/mL}$ (mean \pm SD), and $26.0 \pm$

21.6 , respectively. Also, glycoprotein concentration in right eyes and left eyes was 181.9 ± 193.3 $\mu\text{g/mL}$ BSM equivalent (mean \pm SD), and 134.5 ± 132.6 , respectively.

There was a significant linear correlation of concentration between glycoprotein and sialic acid concentration in each eye, although it varied among individuals (right eye: $r = 0.952$, $P < .0001$; left eye: $r = 0.976$, $P < .001$) (Figure 1). In addition, there was no significant difference in glycoprotein and sialic acid concentrations between right eyes and left eyes ($P = .223$, $.432$, respectively, Wilcoxon signed rank test) (Figure 2).

Reproducibility of Sialic Acid Concentration Measurement

Sialic acid concentration in all subjects was 57.3 ± 28.9 $\mu\text{g/mL}$ (mean \pm SD).

The first, second, and third sialic acid concentration measurements were 55.5 ± 31.0 $\mu\text{g/mL}$ (mean \pm SD), 56.3 ± 30.6 , and 60.1 ± 29.8 , respectively. The homogeneity of variance in test results the third time showed that reproducibility was excellent (Figure 3).

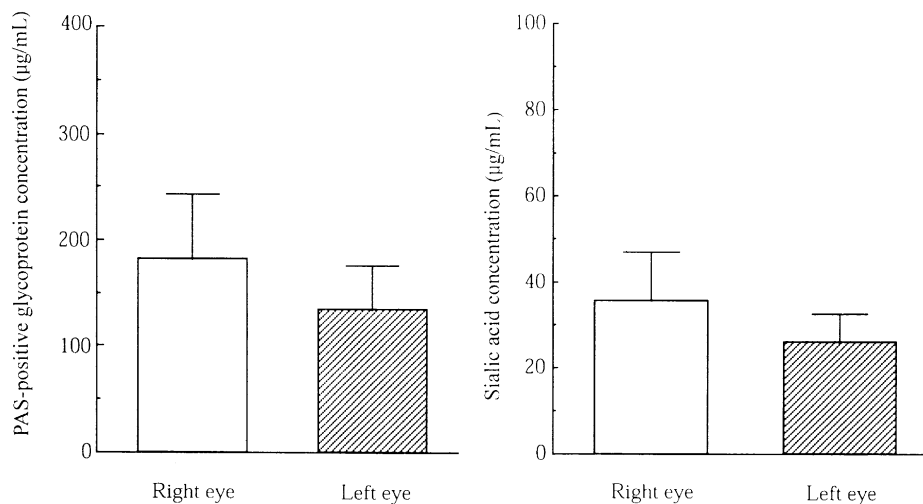
Sialic Acid Concentration in Diluted Normal Tear Fluid

Sialic acid concentration in diluted tear fluid of normal subjects was 37.1 ± 28.3 $\mu\text{g/mL}$ (mean \pm SD).

Discussion

Mucin is thought to play a key role in tear stability;³ therefore, a method for evaluating mucin is expected to be useful in investigating the pathology of

Figure 2. Difference in glycoprotein and sialic acid concentrations between right and left eyes. There was no significant difference in periodic acid-Schiff (PAS)-positive glycoprotein and sialic acid concentrations between right and left eyes ($P = .223$, $P = .432$, $n = 10$, Wilcoxon signed rank test). Concentration of PAS-positive glycoprotein in diluted tears was calculated in bovine submaxillary gland mucin.



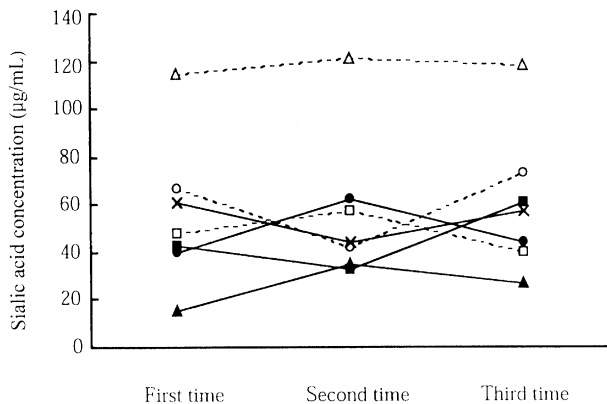


Figure 3. Reproducibility of sialic acid concentration measurement. Homogeneity of variance test results the third time showed excellent reproducibility ($F = 1.37$, $P = .293$, Levene test). Each symbol represents 1 subject ($n = 7$).

ocular surface disorders, such as dry eye. A number of methods for evaluating mucin have been reported, such as evaluation by impression cytology on conjunctival goblet cells,^{13,14} evaluation by rose bengal staining on the epithelium of mucus membrane,¹⁵ evaluation of mucin decrease by BUT of tearfilm.³ In addition, the ferning test reportedly evaluates the quantitative change of mucin.¹⁶ This method is thought to estimate the mucin quantity relative to the mucin/protein ratio in tear fluid.^{17,18} Various methods for quantifying the mucin in vivo have been reported as mentioned above; however, it is difficult to say that any methods is sufficiently accurate.

We investigated whether the sialic acid concentration in tear fluid serves as a quantitative indicator for tear mucin. First, we measured glycoprotein as an indicator for mucin in tear fluid by the PAS method. Periodic acid-Schiff staining is a staining of glucose, and measured PAS-positive glycoprotein is applicable as an index of mucin in stomach,^{6,19} small intestine,⁶ and conjunctiva.²⁰ Also, it is reported that there are few glycoproteins other than mucin; therefore, PAS-positive glycoprotein is considered to be almost all mucin.² Our results showed a significant correlation of concentration between sialic acid and PAS-positive glycoprotein. This demonstrates that the tear fluid sialic acid concentration measurement is useful in estimating mucin.

The pipetting method used in our study differed from the capillary method used in previous reports.^{9,10} To investigate the validity of the present method, we compared sialic acid concentrations between tears collected by the capillary method without stimulation and tears collected by the present method, from the same persons ($n = 5$). Our results

showed that sialic acid concentrations assessed by the capillary method and by the present method were 85.2 ± 45.5 and 60.8 ± 44.9 $\mu\text{g/mL}$, respectively, when we converted sialic acid concentration taking into consideration that tear fluid samples were diluted about eight times, if the average tear volume is considered to be 7 mL.²¹ This result indicates that the present method is quite compatible with the capillary method in collecting tear fluid. Because the absolute amount of sialic acid in tear fluid is reported as 1.14 mmol/L by Cebezas et al,⁹ and 0.9–1.8 mmol/L by Kuizenga et al,¹⁰ the result in our study was low, at 0.12 mmol/L. We speculate that the reason for this may be the differences in stimulating intensity and collecting method between our study and these previous reports. Therefore, we consider it difficult to make a comparison.

Kuizenga et al reported that the sialic acid in tear fluid is derived from glycoprotein, such as mucin, because by their methods using neuraminidase, sialic acid was not detected simultaneously with lactoferrin or secreted IgA, the main proteins in tear fluid.¹⁰ As our method using DMB is of high sensitivity, in the present study the origin of the measured sialic acid may differ from that in their report. However, we consider that measured sialic acid concentration in tear samples reflects glycoprotein concentration, because there was excellent correlation between PAS-positive glycoprotein concentration and sialic acid concentration. Also, the sialic acid concentration in each person's tear fluid was almost unchanged in the sampling in our study method. Therefore, our method can be considered to be reproducible because of the good reproducibility of the three measurements. Moreover, this method may make it possible to care for dry-eye patients whose sialic acid concentrations are low, because the DMB methods used in this study have high sensitivity, making our sampling method applicable to a tear-deficient subject. In our preliminary examination, our measurement method for sialic acid was 10 times more sensitive than the PAS method. This method can therefore be used in severe dry-eye patients whose tear volume is extremely low, as it enables us to measure low concentrations of glycoproteins, unlike the PAS method. Next, we will use this method to investigate the association between mucin and the pathophysiology of aqueous-deficient dry eye.

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