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# Identification of 9-*O*-acetyl-*N*-acetylneuraminic acid in normal canine pre-ocular tear film secreted mucins and its depletion in *Keratoconjunctivitis sicca*

A.P. Corfield[1], S.R. Donapaty[2], S.D. Carrington[2*], S.J. Hicks[2†], R. Schauer[3] and G. Kohla[3]

[1] *Mucin Research Group, Clinical Sciences at South Bristol, Level 7, Bristol Royal Infirmary, Bristol, BS2 8HW, UK,* [2] *Department of Anatomy, School of Veterinary Sciences, University of Bristol, Southwell Street, Bristol BS2 8EJ, UK,* [3] *Biochemisches Institut, Universität Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany*

***O*-Acetylated sialic acids have been reported in many sialoglycoproteins where they mediate a variety of immune and other biological events. We have previously demonstrated that the protective mucus barrier on the surface of the canine eye contains sialoglycoproteins. We have also investigated the occurrence of *O*-Acetylated sialic acids in these ocular mucins. Mucus aspirated from the surface of normal dog eyes and those with keratoconjunctivitis sicca (KCS) was fractionated into three pools by density gradient centrifugation. Sialic acids comprised 0.6–0.9% of the dry weight of the mucins isolated. The sialic acid profile in these pools was examined using HPLC. *O*-Acetylated sialic acids, mainly Neu5,9Ac$_2$, were detected in normal animals and made up 10–30% of the total sialic acids detected. A doubling of the sialic acid content was found in KCS mucins, but the level of 9-*O*-Acetylated sialic acid was reduced below 4% of total.**

**Histological analysis of conjunctival tissue from normal and KCS dogs showed the presence of sialic acids, detected with the $\alpha$(2–6) sialic acid-specific lectin *Sambucus nigra*, in the goblet cells and corresponding to the staining pattern for MUC5AC, the major ocular-secreted mucin gene product. In KCS animals a disruption of the normal pattern of conjunctival goblet cells was seen with preservation of the pattern of lectin binding observed in normal animals.**

**Thus the data demonstrate the presence of mono-*O*-Acetylated sialic acids in normal canine ocular mucins and a loss of this population of sialic acids in dry eye disease in spite of a significant increase in total sialic acids in KCS mucin.**
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## Introduction

Mucus, a complex protective secretion, is synthesised by surface and glandular epithelial cells including specialised secretory 'goblet' cells. It occurs at mucosal surfaces as a viscoelastic, water insoluble glycoprotein gel [1,2]. In order for higher organisms to thrive and survive, they must continually maintain the mucus gels that protect their mucosal surfaces. Individual characteristics of mucus composition depend on anatomical location and are achieved by organ-specific differences in the expression of all mucus components. These include the mucins, responsible for the viscoelastic properties of mucus and which show organ-specific patterns of MUC peptide and glycosylation [1–3]. The glycosylation of mucins has attracted wide attention and many studies have focused on the oligosaccharide components of mucins in normal and pathological situations [1,4–6].

The acidic sugar sialic acid is a common feature of many mucins and is responsible for a variety of functions in these molecules including viscoelasticity and receptors for bacteria [7–9]. The sialic acids comprise a family of related monosaccharides, occurring as peripheral residues in oligosaccharide chains. The most common forms found in mammals are the *N*-acetyl and *N*-glycolyl forms of neuraminic acid and their corresponding mono- and oligo-*O*-acetyl esters [7–9].

To whom correspondence should be addressed: A.P. Corfield, Mucin Research Group, Clinical Sciences at South Bristol, Jenner Yard, Bristol Royal Infirmary, Bristol, BS2 8HW, UK.
**Present address*: Room 231, Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University College Dublin, Belfield, Dublin 4. Ireland. E-mail: Stephen.Carrington@ucd.ie
*†Present address*: Senior Research Fellow, School of Applied Sciences, University of Wales Institute Cardiff, Western Avenue, Cardiff CF5 2YB, South Wales, UK. E-mail: shicks@uwic.ac.uk

The *O*-acetylated sialic acids are frequently ignored or remain undetected due to their chemical lability. Nevertheless, the mono-*O*-acetylated forms are widespread [7,8,10]. The majority of 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9$Ac_2$) identified has been reported in *N*-linked oligosaccharides [7,8,10], although it is also present in human colonic mucins on *O*-linked glycans together with oligo-*O*-acetylated sialic acids [11]. A particular focus has been haematopoietic cells where Neu5,9$Ac_2$ has been found in mouse erythrocyte membranes and functions in the alternative complement pathway [12]. It is also present in human *T*-lymphocyte ganglioside antigens and is implicated in *T*-cell differentiation [13,14]. Neu5,9$Ac_2$ blocks the binding of the sialic acid-dependent B cell adhesion molecule CD22 [15,16] and increased levels have recently been implicated in the pathology of visceral leishmaniasis [17] and childhood acute lymphoblastic leukemia [18], where cell surface sialoglycoproteins in the peripheral blood mononuclear cells carry Neu5,9$Ac_2$ determinants. In addition, the same *O*-acetylated sialic acids serve as recognition sites for the binding of influenza *C* viruses [19] and are involved in blocking the binding of malarial parasites to erythrocytes [20].

The surface of the eye is a specially adapted mucosal surface, part of which (the cornea) is the major refractive surface of the eye. The pre-ocular tear film surmounts the cornea, and the surrounding conjunctiva essentially represents a highly regular and transparent mucus gel [21]. Keratoconjunctivitis sicca (KCS, dry eye syndrome) is a condition in which a variety of abnormalities of the lacrimal apparatus (which includes the lacrimal gland, ocular surface, eyelids and nasolacrimal drainage system) may lead to abnormal instability of the pre-ocular tear film, with varying degrees of ocular surface dessiccation and changes in the character of the ocular surface mucus secretions. A common manifestation of this condition results from autoimmune damage to the lacrimal gland and a reduction in the flow of tears. In such cases, there is an immune-mediated destruction of lacrimal tissue [22], a decrease in conjunctival goblet cell density [23,24] and accumulation of copious quantities of abnormal viscous mucus [25].

The normal, secreted ocular mucins of the dog [26] and man [27,28] are known to be sialoglycoproteins. Furthermore, examination of ocular mucins in a spontaneous canine model of KCS [26] has shown an increase in sialic acids [29]. This indicates that part of the mucosal response to this condition involves a biosynthetic modulation of sialic acid metabolism.

The current study was designed to examine the sialic acid content of canine sialomucin in the normal and KCS eye. The results show that mono-*O*-acetylated sialic acid is a feature of normal ocular mucin and that this ester is greatly diminished or lost in canine KCS.

## Materials and methods

### Materials

The following chemicals were obtained from the Sigma Chemical Company, Poole, UK: caesium chloride, propionic acid, guanidine hydrochloride, orcinol**,** acylneuraminate lyase, nitrocellulose membrane. Dowex AG 50W-X8, 20-50 mesh, and AG 2-X8, 200–400 mesh, ion exchange resins were from Bio-Rad Laboratories, GmbH, D-80901 Munich, Germany. Dojindo Laboratories Inc., Kumamoto, Japan, supplied 1,2-diamino-4,5-methylenedioxybenzene. HPLC solvents methanol and acetonitrile of HPLC-grade as well as Lichrosphere 100 RP18 column, (4 mm diameter, 250 mm length, and 5 $\mu$m particle size) were from Merck, Darmstadt, Germany. UF-0.5 microconcentrator with 3 kDa cut off were supplied by Schleicher & Schuell, Dassel, Germany. The fluoresceine isothiocyanate (FITC)-conjugated lectins *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA) and wheat germ agglutinin (WGA), SNA-biotin and MAA-biotin lectin conjugates, WGA-horse radish peroxidase (HRP) conjugate and streptavidin-HRP conjugate were products of Medac GmbH, Hamburg, Germany. The anti-sialyl-Tn antibody TKH2 and rabbit anti-mouse immunoglobulin-horse radish peroxidase conjugate was purchased from Dako, Peterborough, UK. Anti-MUC5AC antibody 21M1 was the kind gift of Dr J. Bara, Centre de Recherche Paris Saint Antoine, INSERM U55, France. Bovine submandibular gland mucin (BSM) was prepared as described elsewhere [30].

### Animals

Conjunctival tissue, and mucus aspirates were obtained from normal dogs, and dogs with KCS. The study included 11 normal female and 16 normal male Greyhound dogs, from which materials were collected after euthanasia by barbiturate overdose. Samples were also collected, from three female and 5 male dogs with Keratoconjunctivitis Sicca (KCS/dry eye) after routine topical analgesia with Ophthaine (Bristol, Myers, Squibb, New York, USA). All these animals had recorded Schirmer-I tear test levels of $<10$ mm of wetting/min on three separate occasions. They were of various breeds, and aged between 2 and 5 years. Samples were collected as part of a routine veterinary clinical workup, and the informed consent of the dogs' owners was obtained before they were taken. As samples were collected from animals at a clinical center it was not possible to collect appropriate breed matched control samples within a reasonable timescale. The control greyhound group is large, includes both genders and represents a large breed with relatively low inbreeding.

### Histochemical analysis

Tissue from the ventral conjunctival fornix of 6 (4 male, 2 female) normal dogs and 3 dogs (2 male, 1 female) with KCS was fixed in 95% ethanol for 18 h at room temperature before routine processing into paraffin wax. Sections of 5 $\mu$m were cut. Histochemistry was performed as described before for Alcian Blue/PAS [31], high-iron diamine (HID)/Alcian Blue [32] and mild PAS [33,44].

Sections were stained with the FITC-conjugated lectins wheat germ agglutinin (WGA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) at 10 $\mu$g/ml as described before [35]. Negative control incubations included lectin mixed with 200–500 mM Neu5Ac in each case. Lectin binding for biotinylated SNA and MAA was detected by incubation with streptavidin-HRP conjugate 1/3000 and visualised with diaminobenzidine. Sections were also incubated under standard conditions with the mouse monoclonal antibodies 21M1 and TKH2 at a dilution of 1/200, followed by rabbit anti-mouse immunoglobulin-horse radish peroxidase conjugate at 1/3000. Visualisation was with diaminobenzidine.

### Mucus collection and purification

Mucus samples were collected and purified as described before [26]. Briefly, this included gentle aspiration from the ocular surface of animals using low level suction from a water powered pump, and immediate mixing with 3–6 ml of 4 M guanidine hydrochloride in phosphate buffered saline containing a cocktail of protease inhibitors. Samples from normal male and female dogs, and dogs with KCS were pooled in order to obtain a suitable quantity of mucin for analysis. Purification was carried out by density gradient centrifugation, the dispersed mucus samples were adjusted to 1.40 g/ml with solid CsCl and centrifuged at 150,000 $\times$ g for 24 h. Floating membranous material was separated, and fractions of 0.5 ml were collected. The gradients were divided into three pools, Pool 1 $<$ 1.30 g/ml, Pool 2 1.3 $<$ 1.36 g/ml and Pool 3 1.36 $<$ 1.47 g/ml. High molecular weight material was obtained by gel filtration on Sepharose CL 2B and desalted using Sephadex G10.

The purity of the samples was checked by agarose gel electrophoresis and vacuum blotting onto PVDF membrane as detailed before [26].

### Agarose-gel electrophoresis and vacuum blotting

Sample concentration was determined prior to electrophoresis using dot blotting with WGA detection. Samples of 50 $\mu$l were suspended in loading buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0, containing 0.1% SDS, 1% bromophenol blue and 10% glycerol), incubated at 80°C for 3 min and loaded onto horizontal agarose gels (40 mM Tris-acetate, 1 mM EDTA, 0.1% SDS, 1% agarose, pH 8.0). Gels were run at 20 V for 18 h at room temperature, and blotted onto PVDF membrane using a 2016 vacuum blotting unit (LKB) in 20 xSSC (3 M NaCl, 3.3 M sodium citrate pH 7.0) for 2 h at 40 mbar. Glycoconjugates were detected using HRP-conjugated wheat germ agglutinin.

### Analysis of sialic acids

#### *Preparation of total sialic acids*

The total sialic acid content of mucin samples from each of the density gradient pools from normal and KCS animals was determined by the Orcinol/$Fe^{3+}$ test [36] using 100 $\mu$g of each pooled mucin sample. Samples of 150 $\mu$g from each pooled sample of normal and KCS mucins, were hydrolysed at a final concentration of 2 M propionic acid for 4 h at 80°C [37], and passed through a UF-0.5 microconcentrator (3 kDa cut off). They were then washed twice with 500 $\mu$l of distilled water. The combined effluent was divided into three equal parts, purified as described below, and lyophilised. One sample was taken directly for HPLC analysis, the second was treated with 0.1 M NaOH for 30 min at room temperature to saponify *O*-acyl groups before analysis by HPLC, and the third sample was used to identify sialic acids by the acylneuraminate lyase assay after saponification [36].

The total sialic acid released from each sample was purified by ion-exchange as previously described [36]. This purification optimises the preservation of *O*-acetyl esters on sialic acids. Briefly, samples dissolved in distilled water were passed through a 2 ml column of Dowex AG 50W-X8, $H^+$-form, and washed with 10 ml water. The combined effluent was passed over a column of 2 ml Dowex AG 2X8, formate form, washed with 10 ml of distilled water, and the sialic acids were eluted with 5 ml 0.6 M formic acid. The eluates were freeze-dried and analysed as indicated above. Authentic standards of sialic acids were prepared from bovine submandibular gland mucin as before [36].

#### *Identification of sialic acids by HPLC*

Sialic acids were analysed fluorimetrically by HPLC using the method of Hara et al. [38]. Sialic acids (20 $\mu$g) were mixed with 20 $\mu$l of 1,2-diamino-4,5-methylene dioxybenzene solution and incubated for 1 h at 56°C in the dark. The derivatised sialic acid mixture (5 $\mu$l) was injected onto an RP18 cartridge in acetonitrile-methanol-water (9:7:84, v:v:v) at a flow rate of 0.5 ml/min. Elution was monitored with a 980-fluorescence detector (Applied Biosystems, Weiterstadt, Germany) with an excitation wavelength of 343 nm and a cut-off emission filter of 389 nm. The retention times of the sialic acids were compared with authentic sialic acids from bovine submandibular gland mucin [36].

## Results

### Histological analysis of canine conjunctival tissue

Confirmation of the major secreted mucin MUC5AC in the majority of conjunctival goblet cells was seen with the 21M1 antibody, showing superficial mucosal distribution and glandular structures (Figure 1a). Similar patterns were observed histochemically using the periodic acid-Schiff/Alcian Blue stain (Figure 1b). Most goblet cell vesicles stained purple (approx 75%), with some showing pink. Some membranes also stained positively (purple). The mild-PAS technique, which is more specific for sialic acids, stained the majority of goblet cells and surface membranes (Figure 1c). Saponification resulted in little difference to the PAS/AB stain while mPAS gave weaker staining (data not shown). Use of reagents specific

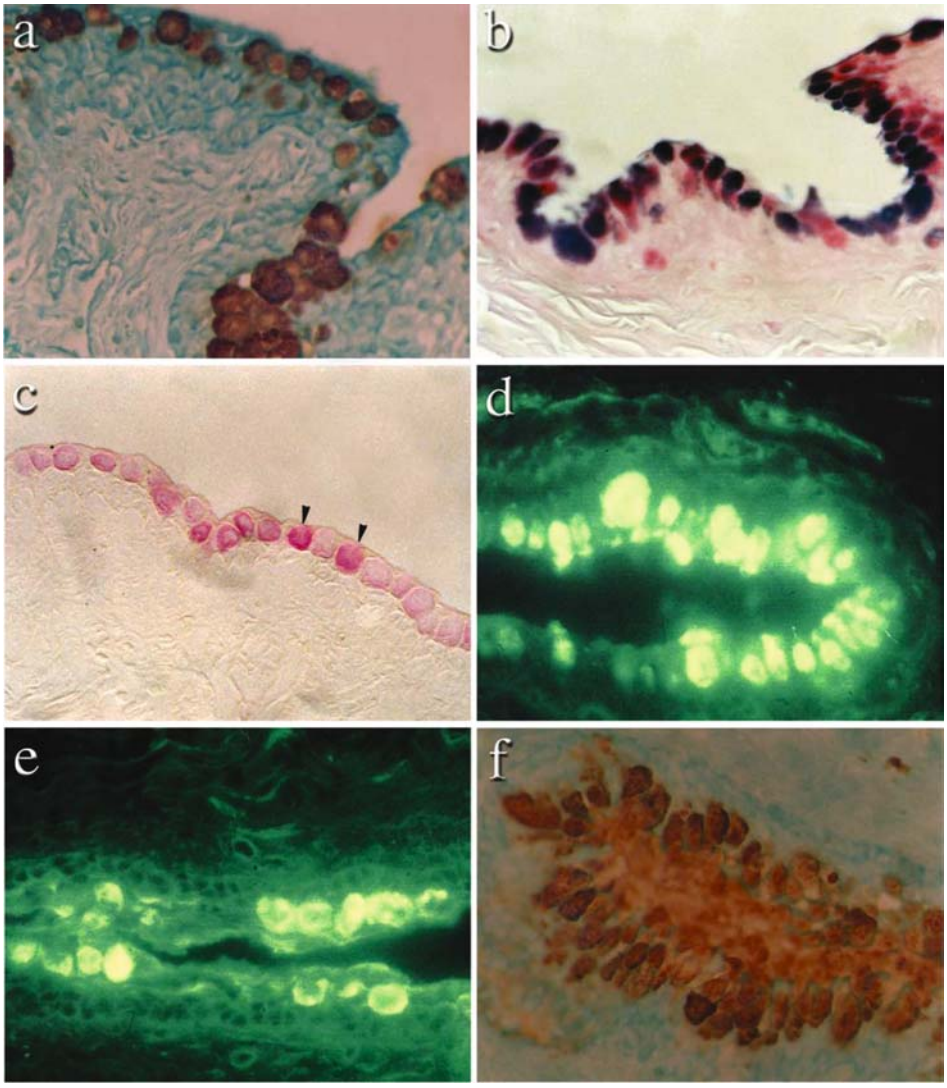


**Figure 1.** Histology of normal canine conjunctival tissues. Tissue sections from normal canine conjunctiva were stained with the anti-MUC5AC antibody 21M1 (a), Alcian Blue/PAS (b), mild-PAS (c), SNA for $\alpha$(2–6)-linked sialic acids (d), WGA (e) and antibody TKH2 against sialyl-Tn (sialyl-$\alpha$(2-6)GalNAc) (f). Details of the methods are given in the text. Magnification is $\times$40 in all cases.

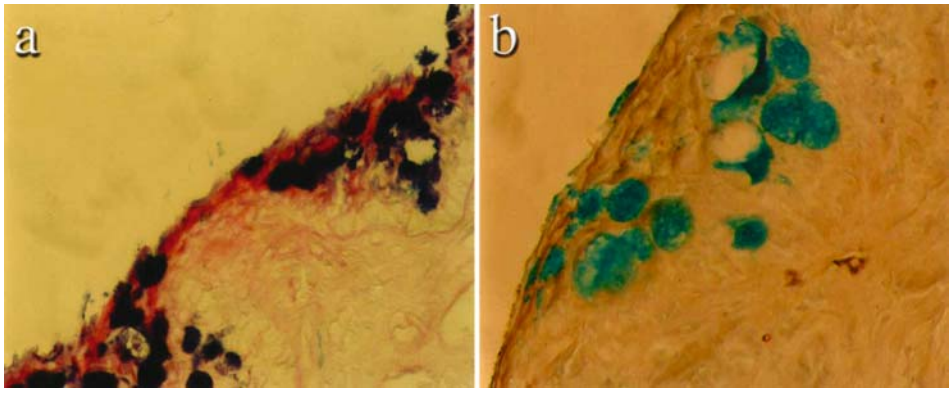


**Figure 2.** Histology of KCS canine conjunctival tissues. Tissue sections from animals with KCS stained with periodic acid-Schiff/Alcian Blue (a) and high iron diamine/Alcian Blue (b). Magnification is $\times$40 in both cases.

for sialic acid-containing epitopes, but reflecting the type of glycosidic linkage, confirmed a relative abundance of $\alpha$(2–6)-sialoglycoconjugates in most conjunctival goblet cells. The two sialic acid specific lectins SNA (specific for $\alpha$(2–6)-linked sialic acids) and MAA (specific for $\alpha$(2–3)-linked sialic acids) gave contrasting results. SNA-staining was strong in goblet cell vesicles (Figure 1d) while no detectable binding was found for the $\alpha$(2–3)-specific lectin MAA (not shown). WGA gave also strong staining of goblet cell vesicles (Figure 1e) and most cellular membranes, in keeping with its wider specificity for both *N*-acetylhexosamine and sialic acids. Staining with the anti-sialyl-Tn antibody TKH2 showed strong reaction with the goblet cell vesicles and luminal mucus (Figure 1f) Goblet cell vesicles stained with the HID/Alcian Blue method revealed a preponderance of blue, sialomucin reactivity while approx. 30% were positive for sulfomucin (data not shown).

Analysis of conjunctival tissue from KCS animals (Figure 2a and b) showed disruption of the normal pattern of goblet cells (see Figure 1b), and a loss of the continuous surface expression with almost exclusive blue staining for sialomucins and no significant brown sulphomucin reactivity. Staining with lectins showed the same binding seen in normal pattern with strong SNA and WGA reactivity but a negative MAA result (data not shown).

## Purification of mucin

The purified mucins showed a characteristic buoyant density of approx. 1.41 g/ml on CsCl density centrifugation for both normal and KCS animals. Pools 2 and 3 contained the majority of the mucins. The material eluting in the $V_0$ fraction after gel filtration on Sepharose CL 2B, was used for further experiments. Agarose gel electrophoresis and vacuum blotting showed typical polydisperse bands for mucins in all three pools. Pool 1 was found to contain some lower molecular weight glycoproteins, in keeping with its light buoyant density and in agreement with previous analyses of normal and KCS canine ocular mucus [26,39].

## Analysis of sialic acids

### *Total sialic acid content*

The sialic acid content of normal dog ocular mucin (Table 1). ranged from 0.55–0.85% of mucin dry weight with highest levels in Pool 3 (1.36 < 1.47 g/ml). In KCS animals, approximately twice this amount was found, with a range of 0.80–1.36% of mucin dry weight. The distribution between the pools was similar to the normal animals.

### *Identification of O-Acetylated sialic acids by HPLC*

The distribution of sialic acids in normal and KCS dogs is shown in Table 1 and a typical HPLC elution profile for a normal pooled mucin sample is shown in Figure 3. HPLC analysis confirmed the presence of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) in mucins from both normal and KCS dogs. The individual sialic acids were identified on the basis of their relative retention times compared with standard sialic acids prepared from BSM.

**Table 1.** Sialic acids in normal and KCS ocular mucins. Individual sialic acids were identified by HPLC relative to standards prepared from BSM and are expressed as a percentage of all sialic acids detected. The identity was confirmed using acylneuraminate lyase digestion after saponification

| | Percentage (%) of total sialic acids | | | | | |
|---|---|---|---|---|---|---|
| | Normal | | | KCS | | |
| *Sialic acid* | *Pool 1* | *Pool 2* | *Pool 3* | *Pool 1* | *Pool 2* | *Pool 3* |
| Neu5Ac | 78.1 | 78.8 | 60.4 | 71.8 | 89.1 | 95.6 |
| Neu5Gc | 8.3 | 7.6 | 6.4 | 9.1 | 7.7 | 3.2 |
| Neu5Gc9Ac | 1.4 | 0 | 0 | 0 | 0 | 0 |
| Neu5,9Ac$_2$ | 12.2 | 13.6 | 33.2 | 19.1 | 3.2 | 1.2 |
| Total* | 0.58 | 0.55 | 0.85 | 0.80 | 1.02 | 1.36 |

*Total sialic acids in ocular mucin pools were measured using the orcinol/$Fe3^+$ assay, and are expressed as a percentage of the dry weight of mucin.

Low amounts of *N*-glycolyl-9-*O*-acetylneuraminic acid (Neu5Gc9Ac) were observed only in Pool 1 from normal animals. Mucins from normal dogs contained between 60% and 80% of unsubstituted sialic acid as Neu5Ac. Mono-*O*-acetyl sialic acid Neu5,9Ac$_2$ made up greater than 10% in Pools 1 and 2 and over 30% in Pool 3. Low amounts of Neu5Gc, approx. 8%, were found in all pools.

The mucins from KCS animals contained the same types of sialic acids but Neu5,9Ac$_2$ constituted below 4% of all sialic acids except for Pool 1 which contained 19%. Similar levels of Neu5Gc were found to the normal mucins. The reduction in

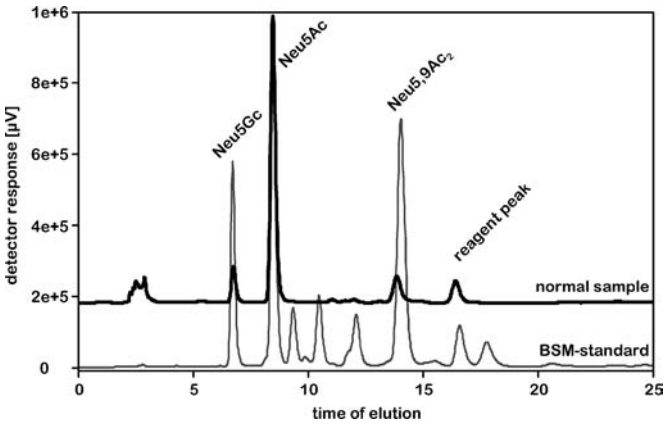


**Figure 3.** Detection of sialic acids in normal canine ocular mucins by HPLC. Profile for sialic acids from normal canine mucin pool 3 is shown relative to standard sialic acids isolated from BSM.

the proportion of *O*-acetylated sialic acids in KCS mucins was accompanied by an increase in Neu5Ac.

## Discussion

Previous studies from our laboratories have demonstrated that the secreted ocular mucins in the dog are sialylated glycoproteins corresponding to Muc5AC [26,29,39]. This paper extends these findings to show that *O*-acetylated forms of sialic acids exist in the normal mucus glycoproteins. Furthermore, an increase of total sialic acid is associated with mucins from dogs with dry eye syndrome, together with an overall reduction in the proportion of *O*-acetylated forms.

Identification of sialic acids in canine conjunctival cells producing ocular mucins is demonstrated using immunohistochemistry with a variety of reagents showing varied specificity for sialic acids (Figure 1) and similarity with the corresponding human tissue [40]. Mucin is readily detected in the goblet cells using periodic acid-Schiff (PAS)/Alcian Blue and mild-PAS techniques. The anti-MUC5AC antibody staining confirms this localisation with respect to the mucins. The location of sialic acid containing epitopes in mucins and cellular membranes is demonstrated by lectin staining. These include WGA, with a broader specificity for glycan detection, and SNA with a specificity for $\alpha$(2–6)-linked sialic acid residues. Staining for $\alpha$(2–3)-linked sialic acid with MAA lectin was absent. Further support for the presence of $\alpha$(2–6) linkages comes from the positive reaction of conjunctival goblet cells with the anti-sialyl-Tn (sialyl $\alpha$(2–6)-GalNAc) antibody TKH2. Confirmation of these oligosaccharide structures at the chemical level is, however, still outstanding.

Evidence for the participation of $\alpha$(2–6)-linked sialic acids in ocular surface defence has come from studies of bacterial binding. *Pseudomonas aeruginosa* interacts with scarified or immature epithelial surfaces through sialic acid residues on the cornea [41]. Pili from the bacteria bind to $\alpha$(2–6)-linked sialic acid in a corneal membrane glycoprotein [42]. Ocular mucus rich in these linkages may provide a defence against the attachment of *Pseudomonas aeruginosa* to the corneal surface.

Comparison of the histology of canine conjunctiva from normal with KCS-animals (Figures 1 and 2) demonstrates morphological aberrations of the mucin secreting goblet cell population consistent with those in human forms of KCS [43,44].

It also confirms that the conjunctiva of affected dogs are still capable of producing goblet-cell mucins. Our earlier biochemical studies suggest that the nature of the mucus secretion in canine dry eye syndrome is abnormal with respect to mucin electrophoretic behaviour, loss of subunit structure and altered glycosylation [26]. This work also showed that the content of sialic acid in purified ocular mucins determined is in the range of 0.5–0.8% of dry weight and is increased in canine KCS [29]. This is confirmed in the current study (Table 1).

Mono-*O*-acetylated sialic acid is found in the two mucin-rich fractions from the density gradients (Pools 2 and 3) rising to over 25% of total sialic acids in Pool 3. The importance of Neu5,9$Ac_2$ is further underlined in the mucins from KCS animals where this form is greatly reduced with concomitant increase of the non-substituted Neu5Ac (Table 1). This result identifies a molecular defect in KCS and suggests that *O*-acetylated sialic acids play a role in the normal protective function attributed to ocular mucins. It represents a further example of pathological loss of sialic acid *O*-acetylation. Previous studies in the gastrointestinal disease have demonstrated *O*-acetylation defects [11].

The occurrence and importance of mono-*O*-acetylated sialic acids is well known [7–9]. The formation of Neu5,9$Ac_2$ may arise from the action of an O-acetyltransferase directly at position 9, or at position 7 with subsequent migration to position 9 [7–9]. In addition migration of the acetyl group from position 7 to 9 may also occur under the conditions used to prepare the sialic acids for analysis [7–9]. An examination of potential binding partners for *O*-acetylated-mucins at the ocular surface is now required. Candidates may originate from the white cells present in the eye, in particular during sleep when the eyes are closed [46] or from bacteria, where a range of sialic acid binding adhesins and lectins have been identified [41,42,47]. Another potential function of *O*-acetylated sialic acids may be an anti-adhesive role as is known from the influenza A and B hemagglutinin and the siglecs, the family of eukaryote sialic acid specific lectins [48]. The more hydrophobic *O*-Acetylated sialic acids may also facilitate the attachment of surface lipid film to mucins within the layer.

In conclusion, the mucins present at the ocular surface of the normal dog contain a significant proportion of *O*-acetylated sialic acids. This has not previously been described. This work adds to known examples from other cells and tissues where functions have been ascribed to these sialic acid variants [45]. The observation that this form of sialic acid is greatly depleted in mucins from animals with keratoconjunctivitis sicca is also novel: as is the finding that the total sialic acid content of ocular mucins is doubled in KCS. An increase in sialic acid has also been reported in human dry eye tears [49]. The pathology of this ocular disease in dogs is linked with defective sialic acid metabolism. It is possible that a reduction in the expression of acetyl-CoA: sialate 7(9)-*O*-acetyltransferase (EC 2.3.1.45) occurs during this process or that endogenous or bacterial sialate *O*-acetylesterase activity causes the loss. The level at which this loss occurs and its relation to drying and inflammation at the ocular surface during disease remains to be determined.

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