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Morphological Alterations of the Palpebral Conjunctival Epithelium in a Dry Eye Model

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Abstract

Purpose—To investigate the normal palpebral conjunctival histology in C57BL/6 mice, and the structural changes that occur in a dry eye model.

Methods—24 male and female C57BL/6 mice, 8 untreated (UT) and 16 exposed to experimental ocular surface desiccating stress (DS). Ocular dryness was induced by administration of scopolamine hydrobromide (0.5 mg/0.2 ml) QID for 5 (DS5) or 10 (DS10) days. Counts and measurements were obtained using anatomical reference points and goblet cell density was investigated with a variety of stains.

Results—Near the junction between the lid margin and the normal palpebral conjunctiva, the epithelium had an average thickness of $45.6 \pm 10.5 \mu\text{m}$, 8.8 ± 2.0 cell layers, versus $37.7 \pm 5.6 \mu\text{m}$, 7.4 ± 1.3 layers in DS10 ($P < 0.05$). In the goblet cell populated palpebral region the normal epithelium was thicker ($P < 0.05$) than in DS5 and DS10. In the control, 43% of the goblet cells were covered by squamous epithelium, compared to 58% (DS5) and 63% (DS10) ($P < 0.05$). A decreased number of Periodic Acid Schiff (PAS) and Alcian blue stained goblet cells was observed in the dry eye. Not all goblet cells stained with PAS and Alcian blue.

Conclusions—The mouse palpebral conjunctival epithelium was structurally similar to the human. After DS the palpebral conjunctival epithelium decreased in thickness and goblet cell access to the surface appeared to be inhibited by surrounding epithelial cells, potentially slowing down their migration to the surface. Differential staining with PAS and Alcian blue suggests there may be different subtypes of conjunctival goblet cells.

Keywords

mouse; conjunctival morphology; desiccating stress; goblet cells

Introduction

‘Dry eye is defined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and

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inflammation of the ocular surface.¹ Almost 5 million Americans, 50 years or older - 3.23 million women and 1.68 million men (Miljanovic B, IOVS, 2007:48:E-Abstract 4293-B729) – have dry eyes.² However, the Dry Eye Workshop (DEWS) report suggests tens of millions more have less severe symptoms, which may become manifest only during stressful conditions for the ocular surface e.g., during contact lens wear, airline travel and drafty environmental conditions.³

Evaluating the pathological changes in the conjunctival epithelium in human dry eye patients requires biopsying one or more sites and is not feasible due to ethical considerations. As a consequence, studies investigating the pathology of the dry eye are facilitated by use of an animal model. The dry eye mouse has proved useful in assessing the effects of dryness on the ocular surface epithelial and immune/inflammatory cells.⁴ Although, the mouse is widely used for ocular surface studies, the literature has, surprisingly, offered only limited information regarding the structural and histological characteristics of the normal conjunctiva in both humans and mice.^{5, 6}

Morphological and dimensional alterations occurring in the palpebral conjunctival epithelium of dry eyes are yet to be investigated. Therefore, ultrastructural studies evaluating the normal and the dry eye palpebral conjunctival epithelium are needed and will enhance our understanding of the dry eye. An increased understanding of the dry eye palpebral conjunctival epithelium may help direct clinicians toward an improved diagnosis and management of dry eye sufferers and can potentially also be beneficial in the future when designing pharmacological agents intended to cure or relieve patient's symptoms and ocular surface disease.

The purpose of the present study was to utilize a histologic and morphometric approach to provide needed normative data on the normal palpebral conjunctiva and goblet cells in C57BL/6 mice, while also investigating structural changes occurring in an established dry eye mouse model.

Materials and Methods

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 24 mice of both genders, 6–8 weeks of age were utilized for this experiment. Eight untreated (UT) C57BL/6 mice (control) were used for morphometric assessment and description of the normal palpebral conjunctiva. The data obtained from the untreated mice were compared to data obtained from the palpebral conjunctiva of 16 mice, 8 per group who were exposed to experimental ocular surface desiccating stress (DS) for 5 or 10 days (DS5 and DS10). DS was created by injection of scopolamine hydrobromide (0.5mg/0.2ml) QID, alternating between the left and right flanks.⁷ Mice (up to 5 per cage) were exposed to a constant air draft for 16hr/day from a fan placed 6" away from a side of their cage. Room humidity was maintained at 35–40% and temperature at 80°F. Aqueous tear production/volume was assessed using cotton thread (Quick Zone Thread; Oasis; Glendora, CA, USA).⁸ Following euthanization, eyes with surrounding eyelids were dissected, the right eyes processed for histology and the left eyes for immunohistochemistry.

Histochemistry

The eyes with surrounding eyelids were fixed in 10% buffered formalin over night and embedded in paraffin. 5 µm thick serial sections from each sample were cut with a microtome (Microm HM 340E). The sections were deparaffinized and stained with 0.5% periodic acid-schiff (PAS) stain, Alcian blue (pH 2.5) or with MUC5AC antibody (SC-20118 Santa Cruz, Santa Cruz biotechnology, Inc; Santa Cruz, CA, USA) for

identification of goblet cells. Digital images of four sections, (eight eye lids), from 3 animals per group were captured (DXM, 1200; Nikon; Melville; NY, USA) and evaluated by image analysis software (NIS Elements, Nikon; Melville, NY, USA). The number of positively stained goblet cells was counted and the length of the basement membrane between the first and last goblet cell measured. In addition, the MUC5AC positive area was also measured. The data are presented as the average number of goblet cells and total goblet cells/mm per eye lid.

MUC5AC Immunohistochemical staining

After deparaffinization, heat induced antigen retrieval was performed for 20 minutes in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0). Subsequently, the sections were treated with 0.3% H₂O₂ in PBS to quench the endogenous peroxidase activity and then incubated with an avidin-biotin block (Vector Laboratories; Burlingame; CA, USA) for 10 minutes each. After blocking with 20% normal goat serum in PBS for 30 minutes, the rabbit polyclonal primary antibody, SC-20118 (Santa Cruz; Santa Cruz; CA, USA) (1:200) diluted in 5% goat serum was applied and incubated for 60 minutes at room temperature. Next, the secondary antibody (goat anti-rabbit) (BD Pharmingen CN 550338; San Jose, CA, USA) (1:50) in 5% goat serum was applied for 30 minutes also at room temperature. The tissue was then incubated in an ABC solution (Vectastain Elite ABC Kit, Vector Laboratories Inc; Burlingame, CA, USA), and stained with a diaminobenzidine (DAB) solution (NovaRed substrate kit, Vector, CN-4800, red stain; Vector Laboratories Inc; Burlingame, CA, USA) for 2 minutes. The reaction was terminated in distilled water, the sections counterstained with Mayer's Hematoxylin, rinsed in tap water, dehydrated, cleared and mounted with 1–2 drops of Permount (SP15-100, Fisher Chemicals; Pittsburgh, PA, USA). Normal rabbit serum concentration (1:200) was substituted for the primary antibody as an irrelevant control.

Light (LM) and Transmission Electron Microscopy (TEM)

A few drops of 2% glutaraldehyde in 80mM sodium cacodylate buffered, 330mOsm/kg fixative⁹ was immediately applied to the right cornea and eyelids after euthanasia with CO₂ inhalation. Subsequently, the eyes with attached lids were carefully removed and immersed in fixative for 4–6 hours to ensure proper cross linking and preservation of the tissue. Subsequently the tissue was processed according to an established protocol.¹⁰ After fixation, transverse pieces (1×1.5 mm) were cut from both the superior and inferior eyelids. Next, the tissue pieces were washed three times in sodium cacodylate buffer (pH 7.4) at room temperature for 10 minutes per wash. The samples were then immersed in a freshly prepared 1% solution of osmium tetroxide in 100mM sodium cacodylate buffer for 1 hour under dim light. After osmification the samples were washed several times in sodium cacodylate buffer at 10 minutes per wash. A Leica EM TP tissue processor (Leica Microsystems; Buffalo Grove, IL, USA) was used for dehydration, transition, infiltration and embedding. The tissue samples were dehydrated through a graded alcohol series (30% –100% in 6 steps) at room temperature and infiltrated with propylene oxide. Embedding with agitation was achieved through an initial 2:1 mixture of propylene oxide and Araldite resin for 3 hours followed by overnight immersion in a 1:1 mixture of propylene oxide and Araldite resin. Thereafter, the tissue samples were immersed in a propylene oxide and Araldite resin (SPI) 1:3 mixture for 4–8 hours before being transferred to 100% Araldite resin overnight. The tissue samples were then oriented in embedding molds and left 12 hours for polymerization in an oven at 60 °C. An ultramicrotome (MT-7000) (Research Manufacturing Co. Inc; Tucson, AZ, USA), was used to cut thick (0.5 –1 µm) and ultrathin transverse sections (60–100 nm). The thick sections were stained with 1% toluidine blue for examination with an Olympus BX51 (Olympus America; Center Valley, PA, USA) light microscope (LM). Slightly overlapping digital images were captured at 100X, montages assembled using PanaVue ImageAssembler

3 (Sharelt Inc; Quebec, Canada) and printed (Roland FJ-52, Roland DGA; Irvine, CA). First, the thickness of the conjunctival palpebral epithelium was measured in triplicate using NIH Image J Software and the number of cell layers counted in the marginal, central and forniceal palpebral conjunctiva. Second, the number of goblet cells was counted on toluidine blue stained sections based on histological criteria. Cells meeting one or more of the following criteria, a triangular shaped nucleus, cytoplasm packed with secretory granules or a bloated or ballooning cell body, were classified and counted as goblet cells. Their location within the epithelium (basal or superficial) was also determined.

For morphological analysis ultrathin sections were mounted on oval slot (2×1 mm) formvar/carbon coated copper grids (FCF2010-Cu, Electron Microscopy Sciences; Hatfield, PA, USA). These sections were double stained, first, in 3.5% uranyl acetate for 20 minutes, followed by Reynold's lead citrate for 10 minutes at room temperature. The stained grids were examined in a Tecnai G2 Bio Twin Spirit (FEI Company; Hillsboro, OR, USA) Transmission Electron Microscope (TEM). TEM micrographs of goblet cells were captured digitally at a magnification of 890X.

Morphometry

Stereology is a widely used technique for obtaining three dimensional data from sampling two dimensional data (e.g from tissue sections).¹¹ An empirical determination was made to establish that 10 goblet cells from each sample were sufficient for accurate morphometric analysis. A systematic uniform sampling (SURS) strategy was employed for unbiased selection of 10 goblet cells from each sample to include in the data analysis.

To determine goblet cell surface density (S_v) and the volume fraction (V_v) of granules per goblet cell, the point counting method was used, and this involved randomly placing an oriented cycloid grid over the goblet cell of interest.^{12, 13} Surface density (S_v) was calculated by the formula, $S_v = (2 \cdot \Sigma I) / (l/p \cdot \Sigma P)$ where I = the number cycloid intersects with the goblet cell plasma membrane or granule plasma membrane, P = points falling on the entire goblet cell or on granules and l/p equals a constant with correction for linear magnification.^{11, 14} The volume fraction (V_v) was calculated by counting all dots falling on granules and dividing by all dots falling on the goblet cell. To investigate the volume fraction (V_v) of goblet cells in the entire palpebral conjunctival epithelial goblet cell region a point counting grid was used. (V_v) was computed by counting all dots falling on goblet cells and dividing by the total number of dots falling on the palpebral conjunctival epithelium. The average area per goblet cell in each of the three conditions was also measured.

Statistical Analysis

One way analysis of variance (ANOVA) with Dunnett post hoc testing was performed to compare morphometry, palpebral conjunctival epithelial thickness measurements and counts between the three conditions (UT, DS5 and DS10). Statistical significance was set to ($P < 0.05$). Data are presented as mean $\pm$ SD. Prism 4.0 software (GraphPad Software Inc; La Jolla, CA, USA) was utilized for the statistical analysis.

Results

The first part of this study utilized light microscopy and investigated whether morphological differences existed between the normal superior and inferior palpebral conjunctival epithelium. The only difference noticed between the two lids was at the lid margin, where the superior conjunctival epithelium was approximately 14 μ m and 3 cell layers thicker than

the inferior. Since no other differences were found between the two lids, the data are reported as the combined averages $\pm$ SD from the superior and inferior lids.

At the junction of the lid margin and palpebral conjunctiva, the epithelium can be described as a stratified multi-layered epithelium devoid of goblet cells. In this region the epithelium was composed of 8.8 ± 2.0 cell layers with average thickness of $45.6 \pm 10.5 \mu\text{m}$. Going in the direction towards the fornix over the next $372.1 \pm 79.3 \mu\text{m}$ the epithelium tapered down to 3–4 layers of cells but lacked goblet cells. However, goblet cells were present over the next $1356.3 \pm 154.9 \mu\text{m}$ where the epithelium maintained an approximately uniform thickness (Figure 1).

In the goblet cell-rich region the thickness of the normal conjunctival epithelium averaged $23.5 \pm 4.0 \mu\text{m}$ and was thicker ($P < 0.05$) than the DS5 ($18.2 \pm 2.8 \mu\text{m}$) and the DS10 ($19.0 \pm 3.1 \mu\text{m}$). When comparing regional epithelial thickness variations all three palpebral conjunctival regions (marginal, central and forniceal) showed a statistically significant difference in thickness between the DS5 and DS10 compared to the control (UT) (Figure 1, Table 1A). A decreased thickness and a loss of epithelial cells were also noticed at the junctional lid margin in the DS10 compared to the normal (Table 1A). There was no statistically significant difference ($P > 0.05$) in the number of goblet cells between the superior and inferior lids or between the different treatment groups (Figure 2A). However, in normal eyes, 43% of the goblet cells were covered by epithelial cells, compared to 58% for the DS5 and 63% for the DS10 ($P < 0.05$) (Figure 2B and Figure 3).

Since no difference in number of goblet cells was found between the normal and the two dry eye groups in toluidine blue plastic embedded sections, morphological analysis was utilized to investigate whether the volume fraction of goblet cells per area of epithelium changed in the dry eye. After taking into account the loss of epithelial volume (reduction of epithelial thickness), following DS no statistical differences were found between groups when evaluating volume fractions of goblet cells, volume fractions of granules per goblet cell or the surface densities of goblet cell and granule membranes (Table 1B).

Finally, this study investigated whether experimental dryness would decrease the number of goblet cells stained with mucous specific stains. This experiment showed a statistically significant decrease of PAS and Alcian blue positive goblet cells in DS5 and DS10 compared to the UT (Table 2). Certain goblet cells were noted to stain with only one, but not both of mucin specific stains (arrows in Figure 4).

Discussion

This study represents the first attempt to utilize a systematic, histologic approach to morphometrically and dimensionally assess the normal palpebral conjunctival epithelium, while also investigating structural changes occurring in the dry eye. To ensure proper cross-linking and preservation of the tissue, a fixation protocol that approximates physiological osmolality and pH values and, as a result, minimizes tissue shrinkage and distortion was employed. The histological approach chosen allowed the observer to conduct accurate intra-conjunctival measurements by utilizing well defined specific reference points. In addition to counts and measurements, a stereological approach was used to conduct morphometric analysis.¹⁴ Design-based stereology became the method of choice since it allows the observer to sample in two dimensions (e.g. from microscope slide), and relate the interpretations to three dimensional space.

Our data showed that similar to the human, the mouse palpebral conjunctival epithelium can be described as stratified, multi-layered, rich in goblet cells with surface cells that feature a glycocalyx. The superior human palpebral conjunctiva is stated to have only two layers of

cells with a third layer appearing close to the fornix, while the inferior palpebral conjunctiva has on average 3–4 layers of cells.⁶ Both the superior and inferior C57BL/6 mouse palpebral conjunctiva had an average of 3 cell layers. This finding agrees with what has been documented for the human⁶, rabbit^{15, 16}, gerbil¹⁷ and guinea pig¹⁸ but contrasts with the 5–8 layers of cells reported for the Sprague-Dawley rat^{19, 20}.

The present study is unique in providing epithelial thickness values for the different regions, and, in addition, morphometrically defining the portion of the lid containing goblet cells in the normal and dry eye palpebral conjunctiva. The latter is particularly applicable to the mouse conjunctiva where the goblet cells may only be visualized in a limited region. In addition, our study shows for the first time that dry eye is associated with a decrease in epithelial thickness and cell numbers. This reduction may be due to increased friction from desiccation of the tissue, and/or increased frequency of blinking. The lid wiper is defined as the portion of the marginal conjunctiva of the upper lid that spreads the tear film over the ocular surface.²¹ Clinically, this area has shown positive dye (rose bengal, lissamine green and/or fluorescein) staining in patients with dry eye symptoms.^{21, 22} The conjunctival epithelial thinning and reduction of epithelial cells at the junction of the lid margin and the conjunctiva noted in the current study may be a potential explanation behind the staining of the lid wiper in the dry eye.

In the mouse palpebral conjunctiva the goblet cells were present as either isolated cells or arranged in clusters. This configuration is in agreement with what has been reported for the mongolian gerbil, human and rabbit¹⁵ but contrary to the guinea pig where goblet cells have been reported to be arranged singly¹⁸ and to the Sprague-Dawley rat, where goblet cells are found only in cluster¹⁹.

Goblet cells, their granules and secretion have been investigated ultrastructurally in other organs e.g. lung and airway epithelium^{23, 24} and intestines^{25, 26}. A few studies are available providing ultrastructural information on conjunctival goblet cells in different species e.g. the human^{27–29}, rabbit^{15, 16}, rat^{17, 20, 29–31} and guinea pig^{18, 32, 33}. The goblet cells were reported to be on average 11–15 μm (rabbit)^{15, 16} and 15–20 μm (human)³⁴ in height and rich in secretory granules. These membrane bound granules were spheroid or ovoid in shape and between 0.3–1 μm in diameter.^{15, 27–29} They had heterogeneous or homogenous electron density and their shades varied from light gray to very dark.^{15, 16, 27, 28}

Only two studies were found that incorporated ultrastructural analysis of the normal mouse conjunctiva. While these studies provided valuable information on goblet cells and their granules, they do not address conjunctival epithelial and goblet cell morphology in the dry eye.^{35, 36} The study by Aargona *et al.*³⁵ used the superior bulbar conjunctiva and investigated the effects of tear substitutes on the conjunctival epithelium in 3 month old CD1 mice. Paz *et al.*³⁶ studied the role of calcium in mucin packaging within goblet cells, in C57/129/sv hybrids; but did not identify the conjunctival region examined nor were the ages of the mice reported. Furthermore none of these two studies provided thickness measurements of the conjunctival epithelium or studied the same region of the conjunctiva (palpebral) as reported in the current study.

In the present study, ultrastructurally, the goblet cell granules were found to be relatively homogenous with different shades of gray to black. A variety of granule shades were observed both within and between goblet cells. There was no difference in granule appearance between the unstressed and dry conditions and thus, all shades were seen in all conditions. Aargona *et al.*³⁵ reported a homogenous light gray granule appearance while Paz *et al.*³⁶ reported goblet cells with mostly dark granules in normal mice and a mixture of electron-dense granules in a group treated with sodium hyaluronate. Because all these

varieties of granules were seen in our samples, this study agrees with both of these previous reports.

The majority of previously reported studies investigating goblet cell density light microscopically, counted either PAS or Alcian blue positive goblet cells. The present study detected a decreased number of goblet cells in the dry eye when either of these stains was used, and this finding is in agreement with previous reports.^{7, 37, 38} A different staining pattern was observed in MUC5AC immunohistochemical stained sections than those stained with PAS or Alcian blue. Donut or apical immunostaining was noted in many cells (Figure 4A). In contrast to sections stained with PAS and Alcian Blue where individual goblet cells could be easily identified, boundaries between individual cells often could not be visualized in MUC5AC stained sections, therefore, we were unable to determine the effects of experimental dry eye on the density of MUC5AC positive cells. Comparison of the location of cells stained with PAS or Alcian blue in serial sections shows a differential staining pattern for these two stains suggesting that there is more than one type of goblet cell (Figure 4B, C). A change in histochemical characteristics of the goblet cells has previously been reported as a consequence of treatments with different tear substitutes.³⁵

The number of goblet cells counted in toluidine blue histological sections was not affected by DS. While the number of goblet cells counted in these sections was less than that counted in Alcian Blue or PAS stained paraffin sections, this is explained by the difference in section thickness (0.5 μ m thick for toluidine blue sections versus 5 μ m thick for paraffin sections). The thinner toluidine blue plastic sections provided increased resolution making it possible to identify and count goblet cells based on specific histologic criteria independent of mucin content (acidic or neutral) within the cell. By light microscopy, DS is associated with distinct epithelial shrinkage (~25%) and, when this shrinkage is taken into account,³⁹ it is apparent that the volume fraction of goblet cells within the epithelium is not altered by DS. Hence, while DS has no effect on goblet cell numbers, it induces a generalized epithelial shrinkage with no net change in the goblet cell volume fraction. The same surface density and volume fraction of granules could be explained either by the granules having the same size or by the granules being smaller and more numerous in the dry eye.

We have previously reported that aqueous tear production is markedly reduced in this mouse model that uses systemic scopolamine administration and exposure to a drafty low humidity environment to induce an aqueous deficient dry eye.⁷

Earlier studies have proposed that parasympathetic innervation regulates goblet cell secretion.⁴⁰ In the current study, scopolamine injections was used to inhibit glandular secretions by pharmacological blockade of cholinergic receptors. If the administered scopolamine injections had an effect on goblet cell secretion, a larger volume fraction of goblet cells and an increased area of mucin in the dry eye epithelium would be expected as a result of the blocked secretion. However, this was not seen and therefore, we would like to conclude that the results shown in this study were not a side effect of the administered injections. Interestingly tarsal (meibomian) glands are reported to receive a facial nerve parasympathetic innervation like the lacrimal gland^{41, 42} and, consequently the administration of the scopolamine could have a similar inhibitory effect on the tarsal glands. Such an effect would potentially mimic the meibomian gland dysfunction which is often an important factor in dry eye disease. The present study has not demonstrated the involvement of the tarsal glands in this model but this could potentially be the case.

In conclusion, this study discovered a decreased epithelial thickness accompanied with a loss of cell layers at the junctional lid margin, in the dry eye. The decrease in number of PAS and Alcian blue stained goblet cells in experimental dry eye indicates that cells

producing neutral or acidic mucin are susceptible to the dry eye environment. Due to a greater epithelial coverage, migration of the goblet cells towards the surface appeared to be decelerated by surrounding epithelial cells in the dry eye.

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Figure 1.

Normal palpebral conjunctiva illustrating its three regions in toluidine blue plastic embedded sections. M=Marginal, C=Central and F=Forniceal. Close to the lid margin (arrow) is the stratified multi-layered epithelium thickest and devoid of goblet cells. The region where the goblet cells are present is indicated between the two white arrow heads (triangles). (Magnification 100X).

Figure 2.

(A) Average number of goblet cells (GC) in toluidine blue plastic embedded sections. No statistical significance ($P > 0.05$) was found between the number of goblet cells between the superior and inferior lids or between the three conditions. (B) Percent covered goblet cells (GC). Data showed a statistically significant difference in percent covered goblet cells between the 5 and 10 day dry eye. Data are presented as average $\pm$ SD. Asterisk = ($P < 0.05$).

Figure 3. Micrographs illustrating goblet cells at the surface (A) or covered by epithelial cells (B) and (C). Bar = 5μm

Figure 4.

The same region of the conjunctiva stained with three different stains. Images illustrates that same goblet cell is not stained with all three stains. A = MUC5AC, B= Alcian blue and C= Periodic Acid Schiff (PAS). Arrows indicate two apparent examples. Black arrows = Example 1 and White arrows = Example 2. (Magnification 400X).

(A) Regional conjunctival epithelial thickness variations. Junctional region is where the palpebral conjunctival epithelium meets the epithelium of the lid margin. (B) Volume fraction (Vv) and surface density (Sv) of granules or goblet cells.

Table 1

Table 1A

	Normal (UT)		5D		10D	
	Thickness (μm)	Cell layers	Thickness (μm)	Cell layers	Thickness (μm)	Cell layers
Marginal	22.8 ± 2.5	3.5 ± 0.6	18.0 ± 3.3*	3.2 ± 0.8	18.4 ± 2.8*	2.8 ± 0.7
Central	26.3 ± 3.0	3.3 ± 0.6	18.5 ± 3.1*	3.4 ± 0.5	19.7 ± 4.4*	2.6 ± 0.6
Forniceal	21.4 ± 2.1	3.3 ± 0.4	18.1 ± 2.6*	3.0 ± 0.5	18.8 ± 2.1*	2.7 ± 0.6
Junctional lid margin	45.6 ± 10.5	8.8 ± 2.0	41.8 ± 6.5	8.0 ± 0.9	37.7 ± 5.5*	7.4 ± 1.3*

Table 1B

	Normal (UT)		5D		10D	
	(Vv) of goblet cells in the histologically defined goblet cell region (%);		(Vv) of goblet cells in the histologically defined goblet cell region (%);		(Vv) of goblet cells in the histologically defined goblet cell region (%);	
(Vv) of goblet cells in the histologically defined goblet cell region (%);	12.5 ± 3.7		15.5 ± 3.2		14.0 ± 5.0	
(Vv) of granules per goblet cell (%);	43.6 ± 6.7		42.6 ± 3.1		43.2 ± 2.9	
(Sv) of goblet cells (μm ⁻¹);	1.3 ± 0.2		1.2 ± 0.1		1.3 ± 0.1	
(Sv) of granules (μm ⁻¹);	11.7 ± 0.6		11.3 ± 0.9		10.2 ± 0.2	

Data are presented as mean ± SD. Asterisk = (P < 0.05).

Table 2

Counts of goblet cells stained with mucin specific stains. The data show the average number of goblet cells or the number of goblet cells/mm for Alcian blue and Periodic Acid Schiff (PAS).

	Normal (UT)	5D	10D
Alcian blue			
Number of goblet cells	68.9 ± 13.3	50.2 ± 11.6*	55.0 ± 9.1*
Goblet cells/mm	46.0 ± 10.9	38.8 ± 7.4*	37.1 ± 11.7*
PAS			
Number of goblet cells	73.8 ± 16.5	55.3 ± 13.1*	52.6 ± 9.8*
Goblet cells/mm	54.4 ± 7.8	48.0 ± 8.3*	41.3 ± 9.5*

UT = Untreated. Data are presented as average ± SD. Asterisk = (P < 0.05).