Density and Distribution of Canine Conjunctival Goblet Cells

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Conjunctival goblet cells (GCs) were quantitated to establish baseline values for density and distribution of these cells in healthy canine eyes. From each of 18 sites, tissue was collected, sectioned at 2 μm, and stained with periodic acid Schiff stain. Within each sampling site, 500 epithelial cells (GCs, squamous, polygonal, and basal epithelial cells) were counted and the ratio of GCs to total epithelial cells was computed as an index of goblet cell density or goblet cell index (GCI). A heterogeneous distribution of canine conjunctival goblet cells was demonstrated. Lower nasal fornix (LNf) and adjacent sites, lower middle fornix (LMf) and lower nasal tarsal (LNT), had the highest mean densities of goblet cells. In contrast, GCs were essentially absent from the upper and lower bulbar areas. Remaining sites had intermediate GCIs. Sex differences in GCIs were noted for LNF and LNT sites. Mean tear film breakup times (BUTs) were determined, and, for normal beagle dogs, were 19.38 (±4.80 sec) OS and 19.96 (±5.01 sec) OD. The similarities between canine and human conjunctival goblet cell distributions support the use of the dog for studying the conjunctival mucous system.


Conjunctival epithelial goblet cells are the source of an undissolved gel-like mucin which forms the deepest of three tear film layers.1-3 Goblet cell mucin (gel mucin) covers the glycocalyces of conjunctival and corneal epithelial cells and forms a multipurpose "mucociliary blanket" over the ocular surface.4,5 Gel mucin serves as a corneal wetting agent by forming a hydrophilic interface between the aqueous tear layer and the hydrophobic corneal epithelium and fills in surface irregularities of the epithelial cells thereby providing an optically smooth corneal surface.4-6 Goblet cell mucin protects the ocular surface by trapping particulate debris and bacteria, and contributes to local immunity by providing a medium for the adherence of immunoglobulins (IgA) and microbical lysozymes.4-7

Ocular diseases associated with quantitative and qualitative deficiencies of the tear gel mucin layer are characterized by instability of the tear film with subsequent drying of the ocular surface.8,9 Conjunctival inflammation, squamous metaplasia, ocular pain, corneal ulceration, scarring and opacification, and blindness have been associated with these diseases. Spontaneously occurring GC deficiency with associated mucin insufficiency has recently been reported in dogs.10

Tear film breakup time (BUT), tear mucin measurement, and GC quantitation are methods used to assess presence and functional capacity of preocular gel mucin.11-13 Lack of standardization and variables related to performance of the procedure have limited the usefulness of tear BUT as a diagnostic and research tool.14,15 Mucin has been quantified by measuring hexosamine and O-linked oligosaccharides.12,16 Because specimens collected for biochemical studies are aqueous tear samples, the value of these measurements has been questioned.17,18 Morphologic study of the conjunctiva and GC counts provide an indirect measure of mucous production. Recent findings indicate that determination of GC density is a more sensitive indicator of ocular surface health than methods which measure aqueous tear mucin content directly.17,18 Accurate interpretation of GC quantities using impression cytology depends upon prior knowledge of GC frequencies at accessible sampling sites.19,21

In domestic and laboratory animals considerable species variation has been noted in the location and

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Materials and Methods

Animals

Twenty-four healthy young adult laboratory beagle dogs (10 males and 14 females) between 9-18 months of age were studied and eyes of each animal were screened for normality using a hand-held slit-lamp biomicroscope and a binocular indirect ophthalmoscope. Schirmer tear tests and tear film breakup tests were performed 5 days prior to tissue collection and euthanasia. All animals used in this study were cared for and treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Schirmer Tear Test

A Schirmer tear test (STT) strip was inserted into the inferior conjunctival culdesac of each eye of each dog and the tear levels were recorded after 60 seconds.

Tear Film Breakup Test

The dogs were sedated with xylazine and ketamine to minimize ocular movements. Tear film breakup time (BUT) was recorded following instillation of two drops of fluorescein dye into the conjunctival sac of each eye. After allowing two to three blinks to distribute the fluorescein, the eyelids were held open manually. A 2 mm slit beam light with cobalt blue filter and X16 magnification on a stationary slit-lamp biomicroscope were used for the tear BUT tests. Time was measured from the last blink to the appearance of the first spot of fluorescein disruption.

Tissue Collection, Processing and Sampling

After 5 days, each animal was anesthetized, the eyelids were sutured together with 4-0 silk, the periorcular area was aseptically prepared and the orbits were exenerated. The orbital contents, extraocular muscles, and periorcular skin were excised and discarded leaving the closed conjunctival sac attached to the globe. Subconjunctival connective tissues were separated from the episclera with a round-tipped surgical blade. The conjunctiva was reflected to the limbus for the entire 360° limbal circumference.

This method was developed and used for in toto collection of conjunctiva and cornea from each eye and is illustrated in Figures 1-3. This procedure allowed consistent division of the conjunctivae of all eyes into dorsal and ventral halves. Following tissue collection the flat mount preparation was immersed in 10% buffered formalin and stored until tissues were processed histologically. For each half of each
conjunctival preparation, three horizontal zones (bulbar, fornix, and tarsal) were identified anatomically and each zone represented approximately equal areas. Vertical zones were established by dividing the tissue into three approximately equal areas identified as nasal, middle, and temporal zones. By combining horizontal and vertical designations, nine sites were defined for upper and lower conjunctiva and, therefore, a total of 18 sampling sites were identified per eye. Designations and notations for the 18 sites are given in Figure 4.

One eye was used from each of five randomly selected males and five randomly selected females. One animal from each sex group was randomly selected to contribute both eyes to the study. This resulted in the initial analysis of 12 eyes (six males-three left, three right; six females-three left, three right). For each of the 18 sites per eye a 6 mm diameter core sample was collected with a disposable cutaneous biopsy punch.

Samples were cut in half perpendicular to the conjunctival surface with randomly oriented planes of incision. The hemisections were placed side-by-side, cut face down, embedded in glycol methacrylate, and sectioned at 2 µm thickness with a LKB Histofax microtome (LKB Industries, Inc., Gaithersburg,
Goblet Cell Index

Goblet cell indices were determined by counting GC profiles versus all epithelial cell profiles, ie, GCs plus squamous, polygonal, and basal cells. Goblet cells were identified by the presence of PAS-positive intracellular material. Crescent-shaped nuclei associated with PAS-positive material were considered nuclei of GCs and were not included in the remaining epithelial cell counts. The ratio of GC profiles to total epithelial cell profiles for each site was computed as an index of GC density or goblet cell index (GCI).

A single linear section was selected from each site for epithelial cell quantitation. This selection was based on absence of technical artifacts. For each site, 500 total epithelial cells were counted using the ×40 objective and a standard light microscope. Five foci of 100 cells/focus were selected along the length of each linear section in the following manner: Cells in two focused high power fields, one at each end of the linear specimen, were counted just inside left and right tissue margins. Cells in a third high power field were counted at the center of the specimen and the fourth and fifth foci were located midway between the center and each end of the section. Counting was initiated at the left-hand side of a high power field and proceeded from left to right until a total of 100 epithelial cell profiles, including GCs, had been counted. To validate this sampling scheme, the entire population of cells was counted for some specimens (in addition to counting the five designated foci) and similar proportions were determined.

Because three sites (lower nasal fornix (LNf), lower middle fornix (LMf) and lower nasal tarsal (LNT) of the initial 12 eyes studied showed a difference between male and female eyes, expanded studies were performed on these sites. Additional samples (LNf, n = 11; LMf, n = 12; and LNT, n = 19) were processed and GCIs were determined for these three sites. Some samples were not usable because of artifacts and were not included in the study. Therefore, numbers were unequal for the three sites in the expanded study.

Statistical Analysis

Schirmer tear test (STT) and tear film breakup time (BUT) data were analyzed statistically for differences between left and right eyes using a two-tailed paired t-test. Sex differences for STT and tear BUT were tested with an independent two-sample t-test after averaging measurements across left and right eyes. Sex differences in GCI for each site were tested with one way analysis of variance (ANOVA). Multiple comparisons of mean GCIs for sites were made using ANOVA least squares differences (LSD). The assumption of equal variance for the GCI values across sites was tested with Levene’s test.

Results

Schirmer Tear Tests and Tear Film Breakup Times

The mean Schirmer tear test (STT) value was 23.42 mm/min (SD 6.25 mm/min) for left eyes (OS) and 24.29 mm/min (SD 5.58 mm/min) for right eyes (OD). Tear film breakup times (BUTs) were 19.38 (SD 4.80 seconds) OS and 19.96 (SD 5.01 seconds) OD. No significant differences were found between right and left eyes for STT (0.5 > P > 0.4) or BUTs (P > 0.5). Differences were not significant between eyes of males and females for STT (P > 0.5) or for tear BUTs (P > 0.5).

Goblet Cell Index

Summary data for goblet cell indices (GCIs) for all 18 sites from the initial 12 eyes studied (six males and six females) are recorded in Table 1. Marked differences in GCIs were noted among sites. Highest GCIs were found in two adjacent sites, LNF and LMf, in the lower fornix region of the conjunctiva (Table 1, Figures 5 and 6). The GCI for LNT, a site on the nasal area of the lower eyelid adjacent to LNF, was highest of the ten intermediate sites. Considering combined male and female data for the 12 sites with nonnegligible numbers of GCs, the two sites with the highest mean GCIs, LNF (0.299) and LMf (0.296), had the least variability, ie, smallest standard deviations (LNF 0.043; LMf 0.044).

For the 12 original eyes, sex differences in GCIs for each site were tested statistically using one-way ANOVA for which no significant differences were found for 15 of the 18 sites. Significant differences were discovered for the three highest GCI sites, LNF, LMf, and LNT (LNF P < 0.001; LMf P = 0.050; LNT P = 0.007). Goblet cell indices were similarly low or negligible for all six bulbar sites for both males and females.

To investigate further possible sex differences in GCIs for the three highest sites, additional male and female samples were processed (see Materials and Methods), for LNF (n = 11), LMf (n = 12), and LNT (n = 19) sites. Of the additional tissues studied, some animals were represented by two eyes while other animals were represented by one eye. After increasing the sample size, the mean GCIs for male and female
samples at the LMF site (Table 2) were not different statistically ($P = 0.930$). Two sites, LNF and LNT, remained significantly different (LNF $P = 0.001$; LNT $P = 0.040$).

Considering all data, mean GCIs for each of the 18 sites were compared using an ANOVA least-significant-difference (LSD) method of multiple comparisons. Results of the LSD multiple comparisons ($P < 0.05$) with combined male and female data are given in Table 3. If an animal had both right and left eyes represented, each eye was considered independently. Those sites included in the same group were statistically indistinguishable. Sites with the highest GCIs, LNF (0.300) and LMF (0.290), were statistically the same and were designated as group A. The six bulbar regions had few or essentially no GCs (GCI < 0.035) and formed a distinct group designated F. The remaining 10 sites comprised four intermediate groups (B, C, D, and E) with GCIs ranging from LNT (0.141) to LNT (0.242). For the 10 sites in the intermediate groups, considerable overlapping of mean GCIs was observed (Table 3). Because generally similar patterns were noted for males and females analyzed separately, pooling of data across sexes was justified despite differences between sexes noted for two sites.

Because the bulbar sites had negligible numbers of GCs, they were excluded from the analysis and the ANOVA LSD multiple comparisons were recomputed for the remaining 12 sites. Although some minor changes in the overlapping of groups were observed, results were similar to those shown in Table 3 in that the highest GCI sites (LNF and LMF) remained statistically indistinguishable. The third highest GCI site (LNT) was no longer significantly different from the second highest GCI site (LMF).

### Discussion

In canine and human conjunctiva GCs are absent, or present in extremely low numbers, on the perilimbal bulbar surface and are present in highest numbers in the lower nasal fornix, lower middle fornix, and lower nasal palpebral sites. Although little is known about factors that influence normal conjunctival GC density and distribution, the degree of conjunctival hydration has been proposed as a significant exogenous factor.26 We concur that surface hydration is an important determinant in GC location and density. However, contrary to Kessing's idea of surface hy-

### Table 1. Goblet cell indices (GCIs) for 18 sites of initial 12 eyes studied

<table>
<thead>
<tr>
<th>Site</th>
<th>Combined male and female $(n = 12)$</th>
<th>Female $(n = 6)$</th>
<th>Male $(n = 6)$</th>
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<tbody>
<tr>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
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<tr>
<td>LNF</td>
<td>0.299</td>
<td>0.043</td>
<td>0.335</td>
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<td>LMF</td>
<td>0.290</td>
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<td>0.320</td>
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<td>LNT</td>
<td>0.238</td>
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<td>LTF</td>
<td>0.248</td>
<td>0.070</td>
<td>0.236</td>
</tr>
<tr>
<td>UFF</td>
<td>0.228</td>
<td>0.062</td>
<td>0.242</td>
</tr>
<tr>
<td>UTf</td>
<td>0.223</td>
<td>0.065</td>
<td>0.237</td>
</tr>
<tr>
<td>UMf</td>
<td>0.214</td>
<td>0.060</td>
<td>0.202</td>
</tr>
<tr>
<td>UNf</td>
<td>0.214</td>
<td>0.081</td>
<td>0.244</td>
</tr>
<tr>
<td>LNF</td>
<td>0.208</td>
<td>0.082</td>
<td>0.215</td>
</tr>
<tr>
<td>LMT</td>
<td>0.190</td>
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<td>0.212</td>
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<tr>
<td>UTt</td>
<td>0.178</td>
<td>0.072</td>
<td>0.201</td>
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<tr>
<td>LMt</td>
<td>0.141</td>
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<tr>
<td>LAt</td>
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<td>0.021</td>
<td>0.011</td>
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<tr>
<td>LAt</td>
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<td>0.011</td>
<td>0.000</td>
</tr>
<tr>
<td>LTB</td>
<td>0.001</td>
<td>0.002</td>
<td>0.000</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Location of high, intermediate and low density GC areas (left eye). Note that the two sites with the highest goblet cell indices (GCIs) are adjacent in the lower conjunctival fornix (LNF and LMF sites). Goblet cells are essentially absent from all six bulbar sites. The remaining ten tarsal and fornix sites have intermediate GC densities.
Means and Standard Errors for GCIs

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Combined Male and Female</th>
<th>Female</th>
<th>Male</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
<td>S.D.</td>
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<td>LNf</td>
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<td>LMf</td>
<td>24</td>
<td>0.290</td>
<td>0.047</td>
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<tr>
<td>LTn</td>
<td>31</td>
<td>0.242</td>
<td>0.074</td>
</tr>
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</table>

Although a previous topographical study has provided clinically useful information, the method used was not directly applicable to the clinical study of spontaneous conjunctival disease. We have developed a new method, which is less disruptive to the conjunctival mucosa, for harvesting and preparing the conjunctiva in toto. This method allows collection of samples from multiple sites for microscopical studies of conjunctiva. Because 18 different sampling sites may be consistently identified using this new method, predictable sampling of multiple sites from one eye or sampling of comparable sites from different eyes is possible.

Because of the elasticity of conjunctiva and the nonlinearity of processed tissue, we believe proportion indexing methods provide a more consistent means of quantitating GC profiles histologically than linear counting methods. Because of the inherent variability in the number of epithelial layers present at various sites within the conjunctiva, we further believe that expressing the ratio of GC profiles/100 total epithelial cell profiles more accurately reflects
actual volumetric relationships than alternative indexing methods, such as GCs/100 basal epithelial cells.

Since, in the present study, the two areas with highest GCIs (LNf and LMf) were also characterized by the least variability in GC numbers, these are recommended as the most appropriate biopsy sites for quantitating canine conjunctival GCs in experimentally-induced or spontaneously-occurring ocular surface diseases. In the live animal, the lower conjunctival fornix is easily exposed and readily accessible for sampling. Furthermore, the elasticity of fornical conjunctiva allows the tissue to be easily grasped and elevated for incisional biopsy.

Because this study has established that extremely low GCIs occur at all bulbar areas in normal canine eyes, cellulose acetate (CA) impression methods applied to bulbar conjunctiva would seem to be inappropriate for quantitating GCs in diseased canine eyes. Furthermore, since it is technically difficult to insert CA strips onto fornix or palpebral conjunctiva in unanesthetized animals, the CA method would appear to be a less advisable sampling procedure for GC quantitation in the dog than histological examination of conjunctiva.

Tear film breakup time (BUT), another noninvasive technique, has been regarded as a clinically useful procedure for assessing the stability of the tear film in man. In the present study tear BUTs are reported for the first time in normal dogs. In this study, a combination of tranquilizer and dissociative anesthetic agent was administered prior to performing BUTs. Anesthetic and preanesthetic drugs have been shown to reduce aqueous tear secretions and could, thereby, influence BUT. In addition, this protocol may be undesirable in animals with spontaneous disease. Although tear BUT may be a useful screening procedure, morphological study of conjunctival biopsy specimens with GC quantitation appears to be a more definitive method for evaluating production of precocular gel mucin in the dog.

Although the influence of sex on GC numbers is not totally clear from this study, a significant sex effect was noted at two sites. Based on recent reports of endocrine influences on lacrimal tissues and conjunctiva, it is not surprising that differences in GCIs between sexes might be seen. Since the sites which showed sex differences were among sites with highest GC densities, it is possible that greater variability within intermediate GC sites accounted for their lack of significant sex differences. Additional studies are necessary to further clarify the influence of sex on conjunctival GC densities.

The present study has demonstrated quantitatively a heterogeneous distribution of conjunctival GCs in young adult beagle dogs. The highest densities of GC were noted in the lower nasal fornix and adjacent sites (lower nasal tarsal and lower middle fornix) and, with respect to relative densities of GCs, canine conjunctiva closely resembles human conjunctiva. In the dog, intermediate densities of conjunctival GCs were found in the remaining fornix and tarsal areas and the prelimbal bulbar conjunctiva contained essentially no GCs. The spontaneous occurrence of canine tear deficient disease and the similarities which exist between canine and human conjunctivae support the use of the dog as a model species for studying the conjunctival mucous system.

Key words: canine conjunctival GC, goblet cell index, precocular mucin, conjunctival biopsy sites, goblet cell density and distribution

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