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Goblet cell density estimate differences in impression cytology samples varies with different magnification of images

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ABSTRACT

Purpose: To assess the impact of using different microscope magnifications for the goblet cell density (GCD) estimates from conjunctival impression cytology (CIC) samples from healthy individuals

Methods: In a prospective study, CIC specimens were collected from the superior bulbar conjunctiva (12 o'clock, 5 mm from limbus) of 20 adult subjects (average age 22 years) onto Millicell-CM membranes and Giemsa stained. A region from each CIC filter containing reasonably high numbers of goblet cells was imaged by light microscopy at a final magnification of 400X and then the same region assessed at 200X and then 100X. The images were enlarged, the goblet cells marked and counted and GCD values/sq mm calculated.

Results: The mean GCD estimates at 400X magnification, 200X and 100X were 644 ± 180, 405 ± 72 and 365 ± 81 cells/sq mm respectively, and these values were statistically different ($p < 0.001$).

Conclusions: As a result of non-uniform distribution, a strategy to select a 400X high power microscope field (HPF) that appears to include a moderate number of goblet cells will have a probability (by at least 20:1) that the GCD estimates will likely be higher compared to those at 200X or 100X, and the probability for higher GCD values is at least 15:1 comparing assessments made at 200X to 100X. Investigators should use only one magnification, with that of a medium power field (200X final magnification) likely being the most useful.

1. Introduction

Conjunctival impression cytology (CIC) is a technique that can be used to collect cells from the conjunctiva of the living eye. The basic principle of the method is generally credited to Egbert and colleagues [1] who noted that following the application of a cellulose acetate (Millipore) filter paper onto the bulbar conjunctiva an impression was formed of the locations of the mucus secretions from the goblet cells usually present in the conjunctiva. These were visualized on the filter after staining it with periodic acid-Schiff (PAS). Egbert and colleagues also reported that non-goblet conjunctival cells as well as goblet cells could be removed onto the CIC filter. It is this outcome of CIC that has been routinely obtained by numerous investigators since the introduction of the technique. While Egbert and colleagues only noted that the density of goblet cells was 'roughly equivalent to the density of the impressions', the contribution of goblet cells to the CIC samples has been both subjectively assessed and counts also made their actual numbers [2]. The assessment of both goblet cells and non-goblet (epithelial) cells by CIC can be considered as a largely non-invasive method to evaluate ocular surface physiology and inflammation (including in contact lens wearers) [3–6], Notwithstanding, analysis of the published literature indicates substantial variability in the outcome of CIC-based assessments of conjunctival goblet cell density, with in vivo confocal microscopy of the conjunctiva to assess goblet cells also yielding rather variable outcomes [2].

For goblet cell density (GCD), average values between 24 and 2226/mm^2 have been reported from nominally normal eyes [2]. This outcome might be considered as that expected because of various published comments made on the variability in GCD assessments. For example, in one early study reporting on goblet cell counts in CIC samples, it was commented that "marked variations in goblet cell densities do occur…" [7], while in another is a note that 'while investigating 300 normal persons, we found GC counts varying by more than 100% (even with imprints from the same patients)' [8]. In later studies, such comments have been repeated, in that there can be unevenly distributed and/or clumped goblet cells [9], that 'variability in superficial conjunctival histology across a given sample occurs'[10] or that in some regions (of the CIC) filter) the GCD was found to be 'more condensed' than in other regions [11]. These characteristics could, in part, be forwarded as a reason for the substantial differences in normal GCD values, but does not explain why there should be such notable differences in average GCD values between studies.

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An aspect of goblet cell assessments in CIC samples that has not received notable attention is the actual magnification used to make the counts, and thus estimate a density value/unit area. In a previous retrospective pilot study [12], five CIC samples that had been taken from the nasal aspect of the inter-palpebral (normally exposed) conjunctiva were analysed at 3 different microscope magnifications to assess how even small differences in goblet cell counts could result in very large changes in the GCD estimates when a high magnification (40X objective, 400X final magnification) field was used. A secondary result from this analysis was that the GCD estimates appeared to be predictably greater the higher the magnification used. A larger scale prospective study was thus undertaken to further evaluate this possible variability in goblet cell numbers that can be visualized across CIC samples taken from the superior (covered) bulbar conjunctiva of normal healthy young adults, especially in relation to the magnification chosen for the image assessment.

2. Methods

2.1. Subjects and impression cytology method

Following approval by the Life Sciences ethics committee of the university, conjunctival impression cytology (CIC) was carried out on nominally healthy young adult (student) subjects essentially as previously detailed [12], except that the sample site was the superior bulbar conjunctiva (12 o'clock). Contact lens wearers were excluded, as were any subjects who had refractive surgery. The subjects were informed of the procedure and a consent form signed, with all procedures involving these human subjects being in accordance with the tenants of Helsinki. All subjects first completed the Glasgow-Caledonian Ocular Comfort Questionnaire [13], that includes a visual analogue scale for the subjects to report their ocular comfort. For the current version, the scale is an unmarked horizontal line bounded by UNCOMFORTABLE at one end to COMFORTABLE at the other, with subjects being asked to draw a vertical line for their comfort for each eye separately.

Both eyes were then anesthetized with a single drop of preservative-free anaesthetic (either Minims® Oxybuprocaine 0.4% or Minims® Lidocaine 4% with fluorescein 0.25% (Bausch and Lomb Pharmaceuticals, Kingston-up-Thames, UK), and the subjects asked to close their eyes for 15–30 s after each instillation. The 10 subjects treated with oxybuprocaine also had fluorescein applied to their temporal conjunctiva from a saline pre-wetted fluorescein strip (Bio-Glo or BioFluoro). All subjects were then asked to take a seat behind a slit lamp while the ocular surface was assessed, as well as observations being made (by the author) of the fluorescein-highlighted tear meniscus over a few minutes.

After approximately 6 to 7 min since anaesthetic instillation, a single CIC sample was taken off the superior bulbar conjunctiva of the left eye using a 10 mm diameter Biopore membrane (Millicell™–CM units, Millipore Corp., Cork, Ireland) (Fig. 1), with the center of the filter being aimed for a location approximately 5 mm from the limbus. A vertical line was drawn (with an indelible marker) on the edge of the plastic filter support ring so that the filter could be oriented to the 12 o'clock position. A few minutes later, further fluorescein was re-applied and the exposed bulbar conjunctiva examined to verify the location of the impression [14].

2.2. Staining, image acquisition and objective analysis of impression cytology samples

The CIC samples were first allowed to briefly air dry at room temperature (RT) and then, within a few minutes, a single drop of 2% glutaraldehyde in 80 mm sodium cacodylate buffer (pH 7.2–7.4, 320–330 mOs/L) was applied to the filter surface and left at RT for 15 min. Excess fixative was shaken off and the filter unit returned to its packaging for storage in a cool place. At a later date, individual filters were retrieved, the surface rinsed with saline and then the filter unit immersed in 99% methanol for 2 min at RT, then immersed in distilled water for 1 min and then a commercial Giemsa stain solution (Sigma, Kingston-up-Thames, UK; product number G3032) for 2 min. The stained filter was briefly washed with tap water then examined. Firstly, an image was taken of the stained filter surface using a scanner (Fig. 1A) so that the size of the stained area could be measured with a digitizer pad.

Using an Olympus Vannox light microscope, the surface of each filter was briefly viewed at very low magnification (4X objective lens) to assess consistency of the cellular material. Next, the entire surface of the filter was examined using both 10X and 20X objective lenses to locate regions where there was essentially a monolayer of cells, and a representative image captured at 200X final magnification (Fig. 1B). Such images were used to grade the monolayer portions of the specimens according to a 4 point scale derived, in part, from original proposals made by Nelson and modified by the author [15] (Fig. 2). Lastly, for each filter surface, a region that appeared to include reasonably high numbers of goblet cells was selected and then imaged at high magnification (40X objective, total magnification 400X). Then, and essentially without any substantial repositioning of the specimen on the microscope stage, the same region of the filter was then re-examined using a medium power magnification (20X objective, 200X total magnification) and then at low power (10X objective, 100X total magnification). Three images, specifically for the assessment of goblet cells were thus taken from each CIC sample (see Fig. 3), and these different images extended over areas of 0.036 mm^2, 0.143 mm^2 and 0.57 mm^2 respectively [12].

The images were acquired through a JVC TK-1280E video camera attached to the microscope and printed in colour onto 11.5 × 8.5 cm photographic grade paper. To the images, a numerical code ID that included an appropriate scale bar was attached and these were then

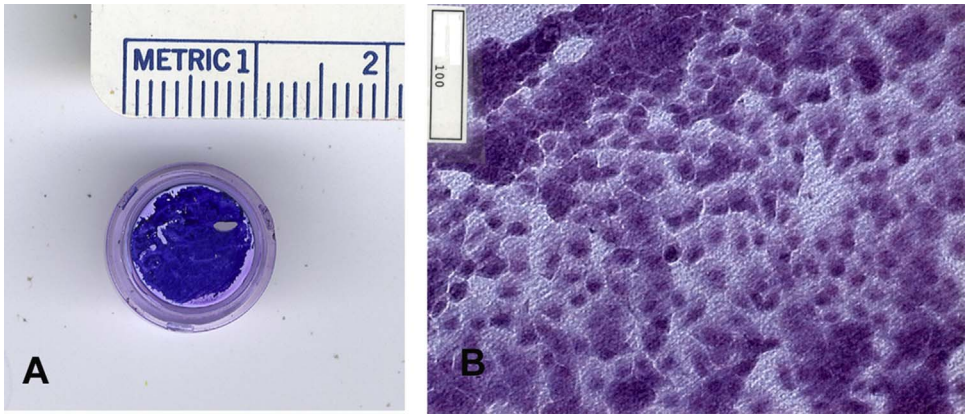

Fig. 1. (A) Representative image of a Giemsa-stained filter, along with a mm rule, from which the total area occupied by the collected material could be measured, (B) representative example of a medium power (200X final magnification) field showing essentially a monolayer of cells which were assigned grade 0. The height of the rectangular white box (where the image ID was originally placed) represents 100 μm.