RESEARCH PAPER

Assessment of size and nucleo-cytoplasmic characteristics of the squamous cells of the corneal epithelium

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Purpose: The aim was to objectively assess size, nucleus and nucleo-cytoplasmic ratio features of squamous cells from the corneal epithelium

Methods: The corneas of recent post-mortem sheep eyes were either glutaraldehyde-fixed for transmission electron microscopy or impression cytology samples taken, glutaraldehyde-fixed and stained with Giemsa. From the specimens for impression cytology, a representative region was photographed from 12 different samples taken from the central region and 16 different samples taken from mid-peripheral regions of the corneal epithelium. Images were subjected to morphometry after overlays were generated.

Results: Electron microscopy revealed a very distinctive stratified corneal epithelium with several superficial layers, confirming the squamous phenotype. Impression cytology from such superficial layers revealed a cell size of 60.1 ± 4.8 μm, nucleus dimension of 12.3 ± 1.5 μm, cell area of 2,419 ± 416 μm² and nucleus area of 131 ± 31 μm². A nucleo-cytoplasmic ratio based on nucleus-to-cell length had a mean of 0.207 ± 0.022, while a cytoplasm-to-nucleus length ratio was 3.975 ± 0.474. Estimates of the nucleo-cytoplasmic ratio based on areas had a mean value of 0.059 ± 0.011. Very similar results were found for mid-peripheral corneal epithelium.

Conclusions: The results strongly indicate that the squamous phenotype of the superficial corneal epithelial cells is characterised by a large size, large nucleus and low nucleo-cytoplasmic ratio. These morphological characteristics show a notable resemblance to data obtained from impression cytological studies on human conjunctival epithelial cells showing severe squamous metaplasia.

The ocular surface is a specialised mucous epithelium divided into two main zones (namely, the corneal epithelial surface and the bulbar / palpebral conjunctival surface) with two even more specialized zones (namely, the limbal epithelial surface separating the cornea and bulbar conjunctiva and the marginal zone of the palpebral conjunctiva).[1] The bulbar and palpebral conjunctiva, as well as the fornix conjunctiva, contain numerous mucin-secreting cells (the goblet cells).[1,2] These goblet cells have openings (orifices) to the surface[3–5] and at least for bulbar conjunctiva, these goblet cell orifices can be shown to have a very distinct spatial organisation.[5] The marginal zone of the palpebral conjunctiva, also known as the oculo-mucocutaneous junction (OMCJ), should not include goblet cells but these are expected to be found in regions immediately adjacent to the mucocutaneous junction as part of the normal palpebral conjunctival epithelium.[6,7]

Based on numerous histology-based studies on human and animal corneas, it has long been established that the superficial corneal epithelial cells of the mammalian cornea exhibit a true squamous phenotype in that the cells are flattened and can desquamate (or exfoliate) from the corneal surface.[1,8–12] The same squamous phenotype with markedly flattened superficial cells, perhaps with added keratinisation of the cells, can also be expected for cells across the marginal zone of the conjunctiva.[6,7]

The cells at the surface of the normal healthy conjunctiva do not exhibit a squamous phenotype but rather are composed of a mixture of non-flattened 'cuboidal' or 'columnar' cells interspersed with the goblet cells.[1] Under conditions of stress, the non-squamous conjunctival epithelial cells can undergo a transformation in phenotype. This change in the cells is commonly referred to as 'squamous metaplasia'.[13] Associated with this transformation, lower numbers of goblet cells can be expected.[2,14]

The cell morphological characteristics have been noted for cell samples obtained by conjunctival impression cytology (CIC) rather than by conventional histology. As visualised by CIC, normal human conjunctival surface cells[15,16] and those from sheep eyes[17] are small with a prominent nucleus occupying much of the cell, that is, having a 'high' nucleo-cytoplasmic ratio based on assessments of the relative size (area) of the nucleus in relation to the surrounding cytoplasm or overall cell size. Transformed conjunctival cells appear to be substantially enlarged,[13,15,18–20] with a notable change in nucleo-cytoplasmic ratio,[19,21] which becomes
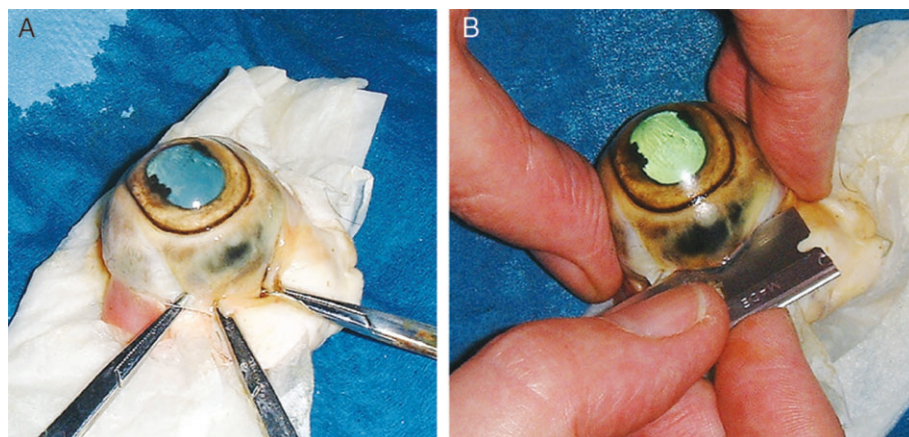
Figure 1. Photographs to illustrate set up of sheep eyeball for surface irrigation and drop-wise fixation (A) and the outcome of the fixation process, where the cornea retains its normal morphology as the eyeball is cut into with a razor (B).

reduced, that is, the nucleus occupies less of the cell compared to the cytoplasm. This is a feature included in a number of similar grading schemes for CIC-procured samples.[13,18,19,22,23] The grading of the nucleo-cytoplasmic ratio is usually noted as being 1:1 or 1:2 and to change to 1:6 or greater, although the basis of these numbers has never been clearly defined (so warranting further objective analyses).

From a perspective that the squamous metaplasia reflects a morphological transformation of conjunctival cells to a more squamous phenotype, a logical question is to ask how closely the transformed cells of the conjunctiva resemble the normal superficial epithelial cells of the cornea, both in terms of cell size and their nucleo-cytoplasmic ratio. Surface cells have been removed from both animal and human corneas with a collodion solution[24] and have been reported to contain large flattened cells with prominent nuclei surrounded by large amounts of cytoplasm. This appearance is consistent with a squamous phenotype and that which would be expected, if the same cells were collected by corneal impression cytology (cornea IC);[12] however, results from various human corneal impression cytology studies have been inconsistent and provided ambiguous evidence for squamous cells being present.[25–31]

Overall, obtaining suitable impression cytological samples from normal human corneas has been considered a challenge.[12] As an alternative approach, therefore, corneal impression cytology was undertaken on post-mortem animal eyes, with added verification (by transmission electron microscopy) to assess the size and nucleo-cytoplasmic characteristics of the squamous cells of the corneal epithelium.

METHODS

Tissue selection

The material for the present studies was from recent post-mortem sheep eyes obtained from a local slaughterhouse by special arrangements.[17,32] The eyes were earlier washed in cold tap water and, over a five-year period, small batches of eyes (usually 10 to 15) in closed containers were received at the laboratory within two hours post-mortem. The eyes were inspected for overall quality (that is, no indications of gross corneal damage or disease) and larger eyes (from outbred eyes) were then selected based on the globe mass and horizontal corneal diameter. The whole globe weights were assessed to within 0.1 g after trimming off all excess fat, tissue and the optic nerve. The eyeball was occasionally wetted with one per cent saline (at room temperature) during these preparatory steps to reduce the chance of desiccation-related changes in the eyes. Horizontal corneal diameter (HCD) was measured to within 0.5 mm with a calliper-based rule.

Cornea preparation for transmission electron microscopy

The ocular surface of three selected eyeballs, processed many months apart, was fairly for-cibly irrigated with 1 per cent saline at room temperature[33] to ensure removal of any adherent material. Individual eyeballs were placed on absorbent tissue with the cornea facing upwards and three to five haemostat clamps applied to the edges of the cut conjunctiva to help ensure that the conjunctiva, limbus and corneal surface were stretched during fixation (Figure 1A). A few drops of freshly-prepared fixative solution were applied to the ocular surface every few minutes over a period of 90 minutes. The fixative was at room temperature and composed of 2 per cent glutaraldehyde in 80 mmol/l sodium cacodylate buffer, pH 7.2 to 7.4, 320 to 340 mOsm/kg.[32,33] The anterior chamber fluid was then exchanged for fixative using a syringe and further drops of fixative applied to the surface over five to 10 minutes. The eyeball was then cut using a single-edged razor blade several millimetres from the limbus (Figure 1B). The excised cornea, with its scleral rim, was placed in a vial of fixative in a 4°C refrigerator for later processing. Following previously detailed protocols,[34] a narrow strip of tissue across the horizontal meridian of the cornea was cut out and two blocks of tissue (approximately 2.0 by 1.0 mm) were obtained from the central corneal region and processed and examined essentially as previously detailed.[32] Both blocks were processed for light microscopy (with thick sections being stained with toluidine blue), and then thin sections prepared from one block from each cornea with multiple sections (10 to 20) from each corneal block being examined. Thin sections were stained with lead citrate and uranyl acetate.

Impression cytology

A total of 20 selected eyes were processed for impression cytology at intervals over a five-year period. After thorough washing with 1 per cent saline, individual eyes were set up as shown in Figure 1A and then several drops of balanced salts solution (BSS) were applied to the cornea. The eye was then left for five minutes to air dry at room temperature prior to impression cytology. For 12 corneas, the impression cytology was from the central region of the cornea, while for eight others, separate samples were taken from both the nasal and temporal aspects of the cornea.

Corneal impression cytology was then undertaken using a 10 mm diameter Biopore® filter as available in a Millicell®-CM unit 0.4 PICM 012550

Morphometric parameter	Abbreviation	Detail of calculation
Cell shape	L : S ratio	LONG / SHORT.
Nucleus-to-cell length ratio	LNLONG ratio	NUCLONG / LONG.
Cytoplasm-to-nucleus length ratio	LONGLN ratio	(LONG-NUCLONG) / NUCLONG.
Nucleus-to-cytoplasm area ratio	NU/CYT area ratio	AREANUC / (AREAC-AREANUC).
Cytoplasm-to-nucleus area ratio	CYT/NUC area ratio	(AREAC-AREANUC) / AREANUC.

Table 1. Secondary morphometric measures from impression cytology

(Millipore, Co. Cork, Ireland) essentially following previous protocols developed for conjunctiva.[16,17,32] One side of the filter unit was marked with a vertical line to facilitate placement and serve as a reference for the horizontal meridian, the filter orientation and location with respect to the limbus, so to avoid any chance of peripheral epithelium being assessed. The eyeball was firmly held resting on the bench top, the filter unit fairly firmly placed on the selected region of the corneal surface, and held there for two or three seconds before being removed. Specimens were either taken from the central cornea or mid-peripheral regions. For the latter, with the horizontal corneal diameters all being over 20 mm, it was relatively easy to locate the two impression cytology filters side-by-side to each other across the corneal surface. With just the middle regions of the filters assessed, the two mid-peripheral samples should be from a location approximately five to six millimetres from the corneal central region. All specimens for impression cytology were fixed with the same glutaraldehyde solution used for the transmission electron microscopy and then stained with Giemsa.

From each specimen filter, a single representative image was selected for objective analysis, where a monolayer of cells predominated. Only the middle portion of the filters was examined, so as to get cells from as close to the central cornea as possible (n = 12) or as close to the mid-periphery as possible (n = 16). The cells were photographed at 200 times magnification, a scale bar of 100 µm (including the sample ID number) affixed to the print, a JPEG image file generated and projected at a final magnification of approximately 1,000 times. On the projected image, a paper overlay was generated,[16,17] to show the positions of the cell and nuclear borders (Figure 3). On the overlay, measurements were taken of the longest dimension of the cell (LONG), the shortest dimension (SHORT), the longest dimension of the cell nucleus (NUCLONG), the area of the cells (AREAC) and the area of the nucleus (AREANUC).

Statistics

All data were entered into a computer software package (Systat v. 11, Systat, Evanston, Illinois, USA) for generation of global statistics for the primary data (mean ± SD et cetera) and graphics. Software spreadsheets were used to calculate a series of secondary morphometric data for the nucleo-cytoplasmic ratios (Table 1). All distributions were assessed for normality using the default Shapiro-Wilk test, as installed in Systat. Inter-sample comparisons (that is, between different impression cytology filters from central or peripheral regions) were usually carried out using non-parametric (Friedman rank order) tests, both because of borderline normality and the slight differences in sample numbers, but where appropriate, some intra-corneal comparisons (that is, of the two specimens taken from the same cornea) were made using a Student's paired t-test (with Bonferroni correction applied) because the data sets were normal and had equal numbers of samples with statistical significance set at p < 0.05 in all cases.

RESULTS

Eye samples

The measured weights of the 23 carefully selected eyeballs were between 16.2 and 21.7 g for an overall mean (± SD) of 19.6 ± 1.2 g. The horizontal corneal diameters were between 21.5 and 23.5 mm for a group mean of 23.2 ± 1.2 mm. The central corneal thickness values were between 0.691 and 0.797 mm for a group average of 0.702 ± 0.083 mm.
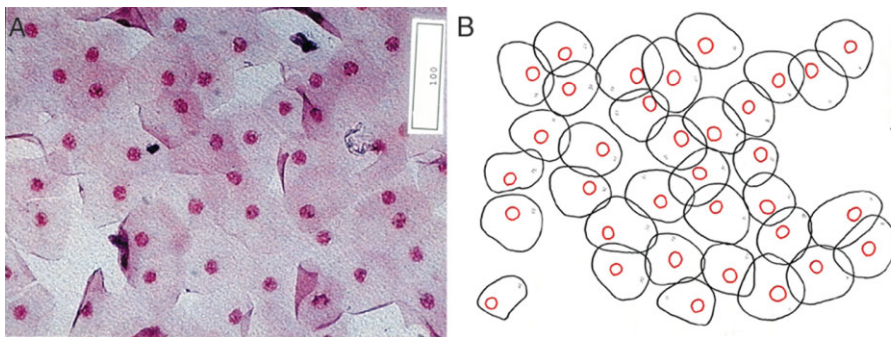
Fine structure of the ovine corneal epithelium as assessed by transmission electron microscopy

These assessments were undertaken to verify that the corneas did have superficial squamous cells. The initial washing provided clean corneal and conjunctival surfaces and the light reflex from the corneal surface is visible in Figure 1A. The use of the haemostat clamps to both weigh and stretch the external ocular tissue is done to obtain a cornea (and conjunctiva) that is free of obvious gross structural artefacts. The worst of these is that the cornea collapses slightly, distorts during fixation and / or shows numerous wrinkles or even some obvious folds. As shown in Figure 1B, as the eyeball was cut after fixation, the cornea did not sag and can be removed with close to its natural curvature being preserved. The image also shows that the cornea and conjunctiva change colour as a result of the fixation (becoming somewhat-to-substantially yellowed) and the surface reflex was routinely dulled (Figure 1B).

Thick sections of such corneas routinely showed undistorted and wrinkle-free corneas with well-defined parallel arrays of stromal lamellae. The corneal epithelial layer was of uniform thickness as was the posterior limiting lamina and endothelial cell layer. Ovine corneas, at least those of the breed studied (Scottish Black face), lack a notable anterior limiting lamina but have a distinct epithelial basement membrane (Figure 2A). Figure 2A was typical of all blocks processed and sections examined showing a very notably stratified epithelium with distinctly large basal cells, smaller intermediate (wing) cells and several layers of flattened cells more superficially. As illustrated, it was routine to find portions of these surface cells with densely staining cytoplasm and appearing intact. There could also be immediately adjacent cells that were only lightly stained with toluidine blue (not shown) or uranyl acetate /lead citrate (Figure 2A). In some sections, as illustrated, a few cells at two or three layers deeper in the epithelium also showed this sparse staining. In Figure 2B is shown a selected region at higher magnification to illustrate a very small region of the surface where such sparse staining was evident (at lower magnification). This image shows these most superficial cells to have obvious signs of degeneration with substantial reduction in cytoplasmic density. Other adjacent cells, including those in the next layer(s), are
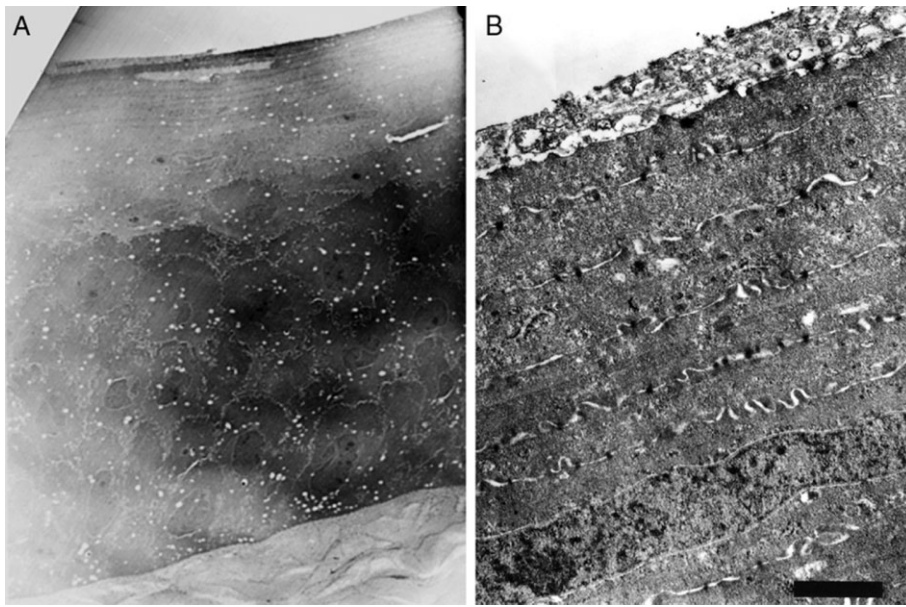
Figure 2. Representative low magnification transmission electron microscopic (TEM) image of sheep corneal epithelium (A) and a higher magnification image (B) to illustrate the distinct stratification of the more superficial squamous cells that can be overlain with necrotic cells. Scale bars represent 15 μm (A) and 1.2 μm (B).

Figure 3. Representative corneal epithelial impression cytology (IC) image from central region of cornea (A) and the resultant overlay drawn from the image (B). The scale bars are indicated by the vertical white box in (A) and as the vertical line in (B) and are equal to 100 μm.

Impression cytology of central corneal epithelium

Impression cytological specimens from the central corneal region of 12 different eyes showed a filter surface completely covered with cells, that is, around 75 mm² of cells. As examined at lower final magnification (40 or 100 times), almost all the cells appeared to be uniform in staining, which was generally light, indicative of thin (attenuated, flattened) cells. Examination at moderate magnification (200 times, Figure 3A) routinely indicated that a high proportion of the cells were in a monolayer or just slightly multi-layered.

In Figure 3A is shown a typical example of a specimen of impression cytology, with the same or very similar appearance found in many microscopic fields across the middle regions of the filters, both taken from the central and mid-peripheral regions of the corneal surface. The overlay generated from the image is shown in Figure 3B to illustrate the process and where any minor artefacts were ignored prior to the morphometry being undertaken. In some instances, there were a few spaces between the cells, usually of similar size to individual cells. In some locations across the filter surface, these spaces were somewhat larger and perhaps occupying the space of three or four cells. Some of the spaces (gaps) between cells could be locations where necrotic cells were originally present but with inadequate material retained on the impression cytology filter to show staining.

The image, relative to the 100 μm scale marker white box at the top right of the image, reveals the cells to be fairly uniform in area with the overall size being large. The nuclei were also prominent and large. There is also a single cell (not included in the overlay) at the middle-left edge of the image shown, where a non-staining circular region in the middle of the cell indicates where a nucleus once was but this was uncommon.

For the example shown, the cell longest dimension (LONG) averaged 63.0 μm, the shorter dimension (SHORT) averaged 50.8 μm and the average longest dimension of the nuclei (NUCLONG) was 13.4 μm. The average cell area (AREAC) was 2,663 μm² and the average nucleus area (AREANUC) was 151 μm². Similar numbers were found for the other 12 samples (Table 2). For any particular sample, the variance in any of the values (calculated as the coefficient of variation, COV) was

clearly intact and show normal cytoplasmic density.

All three corneas (with multiple sections examined) showed several superficial cell layers (Figure 2A). These cells were obviously flattened, with their thickness being around 1.0 μm (range 0.8 to 1.3 μm) and only rarely was a noticeable part of a nucleus seen in these superficial cells. Very obviously nucleated cells were routinely seen a few layers deeper down into the epithelial layers with a large proportion of the slightly thicker cells being occupied by a normal appearing nucleus. Overall, the lack of obvious nuclei in sections through the most superficial cells should not be taken to indicate that these cells are a-nucleate. For these superficial cells, desmosomes to adjacent cell layers appear to be generally intact and the intercellular spaces, while clearly defined, do not show unusual dilation (indicative of oedema).

Location	LONG	SHORT	NUCLONG	AREAC	AREANUC
Central	60.1 ± 4.8	49.9 ± 4.5	12.3 ± 1.5	2,419 ± 416	131 ± 31
Nasal-mid periphery	64.4 ± 6.6	50.5 ± 5.2	12.7 ± 1.7	2,738 ± 529	125 ± 21
Temporal-mid periphery	61.4 ± 72	51.3 ± 5.4	13.1 ± 2.2	2,601 ± 577	129 ± 27

All data as mean ± SD, with the LONG, SHORT and NUCLONG dimensions being in microns (μm) and the AREA and AREANUC values being in square microns (μm²).

Table 2. Primary morphometry measures from impression cytology

Location	L : S ratio	LNLONG ratio	LONGLN ratio	NU/CYT area ratio	CYT/NUC area ratio
Central	1.213 ± 0.048	0.207 ± 0.022	3.975 ± 0.474	0.059 ± 0.011	17.89 ± 3.13
Nasal-mid periphery	1.304 ± 0.034	0.196 ± 0.017	4.202 ± 0.581	0.052 ± 0.019	18.79 ± 3.04
Temporal-mid periphery	1.221 ± 0.067	0.224 ± 0.031	3.788 ± 0.402	0.065 ± 0.022	18.02 ± 3.84

All data as mean ± SD and are in relative units.

Table 3. Secondary morphometry measures from impression cytology

between nine to 14 per cent for linear values and 15 to 22 per cent for area values.

Overall, the cells were not round but had a slightly greater length (LONG) to width (SHORT) giving a mean L : S ratio of 1.213 (Table 3, top line). The nucleo-cytoplasmic ratio, as assessed by the nuclear length compared to the longest dimension of the cells (that is, LNLONG ratio) had a mean 0.207, a value which could also be considered as an integer ratio close to 1:5. An alternative measure of the size of the surrounding cytoplasm to the size of the nucleus is that of the LONGLN ratio, which had a mean value of 3.975, a value that could be considered as being close to 1:4. For completeness, similar nucleo-cytoplasmic ratios based on area measures were also calculated with the nucleo-cytoplasmic area ratio having a mean of 0.059 and the CYT/NUC area ratio being 17.89.

Impression cytology of mid-peripheral corneal epithelium

For a second set of eight eyes, impression cytology was undertaken from the mid-peripheral region of the cornea, either side of the central region. Overall, the quality of cell sampling from the more peripheral regions was usually less with only some 75 per cent of the filter surface being covered with cells.

Overall, very similar results were obtained for the mid-periphery as compared to the central corneal region (compare top line with lines 2 and 3 in Tables 2 and 3) and the nasal and temporal aspects also provided very similar results (compare lines 2 and 3 in the Tables). The squamous cells were large, had large nuclei and low nucleo-cytoplasmic ratios. Inter-sample comparisons revealed no statistical differences (Friedman) in the primary morphometric data between central and mid-peripheral samples (p ≥ 0.1). Similarly, intra-sample comparisons also failed to reveal any predictable differences between nasal and temporal aspects of the mid-peripheral corneal epithelium (Student's t-tests, p ≥ 0.2).

DISCUSSION

Overall, the broad intent of the present study was to try to define better the morphology of the squamous cells of the ocular surface, specifically that of the corneal epithelium. With such information, the extent (using objective measures) of the squamous transformation of the bulbar conjunctiva (especially in humans) could be defined better and understood. An animal model system was used as a source of corneal cells, namely the post-mortem ovine eye. As verified by transmission electron microscopy,

the more superficial cells of the ovine corneal epithelium (as expected) are clearly squamous in nature, being flattened and arranged in several layers, a result considered to be in agreement with conventional histological studies.[8–11] Surface cells removed from human corneas with a collodion solution have been reported to be large, flattened and with prominent nuclei surrounded by large amounts of cytoplasm.[24] Impression cytological samples of this surface in ovine eyes revealed the morphology of these cells to also show distinct squamous characteristics with the cells being large, having large nuclei and a 'low' nucleo-cytoplasmic ratio. For ovine inter-palpebral bulbar conjunctiva, the cells are small, have smaller nuclei and a 'high' nucleo-cytoplasmic ratio.[17] Based on numerous assessments, the ocular surface of the ovine eyes used for the present studies does not routinely stain with either rose Bengal or lissamine green,[17] indicating a normal healthy ocular surface.

The results obtained for these post-mortem ovine eyes appears to contrast rather substantially to several previous reports on the cytological (morphological) appearance of human corneal epithelial cells obtained by impression cytology, with it also being unclear in some cases whether samples were from normal or diseased eyes.[25–29] These reports have generally been descriptive, mainly being based on low magnification images of specimens of impression cytology that have allowed for qualitative comparisons between the corneal epithelium and the adjacent epithelia of the limbal and even bulbar conjunctival regions. From such images, peripheral corneal cells appear rather larger than adjacent conjunctival cells (albeit also elongated)[25] or only slightly larger[26,29] but with a 'low' nucleo-cytoplasmic ratio.[29] Normal corneal epithelial surface cells have also been considered to be very similar to surrounding conjunctival cells being relatively small and having a high nucleo-cytoplasmic ratio.[28,30] Both small and also larger cells were noted in another study with both high and low nucleo-cytoplasmic ratios, perhaps arising from a 'risk of conjunctival cell contamination or misinterpreting the peripheral rim of conjunctival cells'.[27] In another study, it is commented that cells from the inferior cornea showed 'mild epithelial squamous metaplasia'.[31]

From the present analyses of ovine corneal epithelial cells, some preliminary estimates can be generated to allow for

definition of what the expected values for an ocular surface squamous cell could (or should) be. As an example, in part based on other proposals for these metrics,[16,19] the nucleo-cytoplasmic area ratio in any particular set of corneal epithelial cells should likely be less than 0.1. Using the group mean standard deviation, this could be defined better by a 95 per cent confidence interval (based on 1.96 SD). For central corneal epithelium, this could be between 0.038 and 0.080 for the nucleo-cytoplasmic area ratio.

Overall, the present morphometric analyses indicate that squamous cells of the ovine corneal epithelium are large, have large nuclei and a 'low' nucleo-cytoplasmic ratio. With it being so well established that the superficial cells of the mammalian corneal epithelium have a squamous phenotype, it would be rather surprising if normal human corneal epithelial cells (as assessed by impression cytology) did have a non-squamous phenotype (with small size and a 'high' nucleo-cytoplasmic ratio). The reason for this discrepancy can be resolved only with further study and it would be extremely useful for more quantitative investigations to be undertaken on normal human corneas.

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