Quantitative evaluation of the effects of artificial tears on the corneal epithelium

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Abstract

Two drops of a chlorobutanol --or benzalkonium chloride-- preserved artificial teardrops were instilled in to the right eye of six female grey rabbits (2 kg) at 21.00 and 09.00. A group of six control animals received no eyedrops. The animals were euthanized at 15.00 h and the central region of the corneal epithelium essessed by scanning electron microscopy using a digitizer pad/computer system. After recovery from the exposure to artificial teardrops, there were up to 2% exfoliating cells evident at the ocular surface but with no difference between the two products; controls had no cell exfoliation. After recovery from the chlorobutanol-preserved artificial tears, the distribution of surface areas of the squamous cells (n = 500 cells evaluated) was shifted to slightly larger values compared to controls but the number of epithelial cell craters /cell was unchanged from controls. Following recovery from benzalkonium chloride-preserved artificial tears, the cell areas were shifted o significantly smaller values than controls and there were fewer epithelial cell craters/cell. The methods and assessment protocol are presented as a basis for objectively comparing different types of eyedrops on the corneal surface where there is no overt cytotoxicity.

Introduction

The use of artificial tear pharmaceuticals in patients with irritated or dry eyes is commonplace[1]. Those pharmaceutical products that are intented to be used many times by a patient (e.g. 5 mL multiuse bottles of eyedrops) will usually contain preservative agents. These preservative agents are included so as to reduce the risk of substantial multiplication of most microorganisms should the container or the actual solution become accidentally contamined with microorganisms such as bacteria or fungi. A large number of these chemical preservative agents have been tried over the last 40 years[2-5]. Several of these agents have become widely used and have an acceptable record of effi-

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cacy in maintaining the sterility of eyedrops[6-8]. Significant microbial growth can occur however, presumably if the level of pathogen contamination is high or there is repeated exposure of the eyedrop container to the pathogen(s).[6-8] As a consequence, it is necessary that the concentrations of chemical preservative agents included in the pharmaceuticals be sufficiently high so as to be able to effect as rapid inactivation or destruction of the microorganisms as possible. However, in deciding on the concentration of the preservative agent that is to be included in the eyedrops, consideration must be given to the potential cytotoxic effects of the preservative agent on the tissues of the eye, especially the corneal and conjunctival epithelia. As a result of these considerations, it can be stated that the overall toxicity risk, with the most commonly used concentrations of these preservative agents, is very low. That this statement is a valid reflection of the use of preserved eyedrops comes from the overall scarcity of reports on complications (that could be atributed to cytotoxic effects or even microbial contamination) arising from the use of such eyedrops. This overall situation needs to be carefully distinguished from the occasional occurrence of allergic or hypersensitivity reactions to preservatives (e.g. thimerosal [9]) or any other ingredients of the eyedrops. [10] Despite this repeated use of multiple-use eyedrop products without consequence, preservative agents continue to receive adverse publicity. Part of the reason for this situation is that there is only limited information on the effects of these preservatives on the ocular surface of the living eye.

Scanning electron microscopy has been used for many years to evaluate the effects of numerous chemicals (including preservative agents), drugs and pharmaceuticals on the surface of the corneal epithelium. [11-13] For the most part, the technique has been used to provide high magnification images of the actual cells at the epithelial surface.

The images have been only subjectively assessed in most cases. Furthermore, the technique has been largely used to only document the cellular damage or cell exfoliation that can occur within an hour of application of the test substance to the ocular surface. Scanning electron microscopy can however also be used to provide images of the mosaic of cells at the epithelial surface that can then be subjected to a quantitative analysis by planimetry. Discrete cell changes and low levels of cell exfoliation associated with artificial tear use have been quantified in this way [13, 14].

Overall however, despite the large number of published papers reporting the effects of preservative agents on the corneal epithelium (as assessed by scanning electron microscopy [12]), few of the studies compare preservative agents under conditions resembling clinical use of eyedrops and none of these comparative studies has been quantitative.

In the present study, an objective comparison was made of the effects of an exposure of the corneal epithelium *in vivo* to two different preservative agents included in the same type of polyvinyl alcohol-based eyedrop. Particular attention was given to the extent of cell exfoliation, [14] the surface areas of the residual squamous cells [13] and a cell surface feature that is often called the epithelial hole or crater. [15-18]

Materials and methods

Animals and treatment protocols

Female grey (Dulch Belt) rabbits were housed individually in Canadian Council for Animal Care (CCAC) -approved cages and quarters and provided with unrestricted access to food and water. All procedures were reviewed by and approved by the local, CCAC -approved animal care committee. The animals were firts acclimatized to the University facilities for 7 to 9 days after receipt from a local supplier. An artificial light: dark cycle of 14:10 h was imposed with the light cycle starting at 06.00. After aclimatization, the animals (2.0 to 2.2 kg) were checked by slit-lamp biomicroscopy

ınd assigned to control or test groups. The test ınimals received two drops of an artificial tear ıolution in the right eye only at 21.00 local time ınd the eyedrop instillation was repeated at 09.00 he following morning. Control animals received ıo eyedrops. At 15.00 h (i.e. 6 h after the instillaion of the second set of eyesdrops for the test aninals), the rabbits were euthanized with an overlose of T-61 euthanasia solution (0.5 mL/kg) adninistered via a peripheral ear vein. The right eye vas used for the electron microscopy studies.

Preparation of corneas for scanning electron nicroscopy

Immediately after euthanasia, all neck blood essels were severed and the animal drained over sink about 30 s. With the animals then placed on heir left side, two drops of a glutaraldehyde fixaıve solution were carefully applied to the surface he right eye. The lids were then resected to the rbital rim, two more drops of fixative applied and ıe eyeball carefully enucleated. The corneas was ıen prepared for scanning electron microscopy s previously detailed [12]. In brief, the technique ıvolves the occasional application of drops of the xative solution to the surface of the eyeball over period of 70-80 min at room temperature be)re the aqueous humor of the eye was replaced 'ith fixative solution and the cornea excised on a :leral rim. The fixative was a freshly prepared soıtion of 2% w/v glutaraldehyde in 80 mM sodium ıcodylate buffer and was warmed to 35 to 36°C ıst before use. The pH was adjusted to 7.2 to 7.4 'ith a few drops of dilute hydrochloric acid. The nal solution osmolarity was 330-340 mOsm/kg ıd ultraviolet absorbance spectroscopy was rouıely used to check that the polymer content of ıe fixative was less than 5% [19].

Scanning electron microscopy and image ıalysis

Six corneas were successfully processed for each ' the three experimental groups (controls, ılorobutanol-preserved eyedrops and benzalkonium chloride-preserved eyedrops). From three of the corneas in each group, the inferior-nasal quadrant was taken for analysis and the superior-temporal quadrant was taken from the other three corneas in each group. Scanning electron microscopy was performed using a Hitachi S570 microscopy operating at 15kV. From each quadrant, a series of micrographs were taken at a position close to the apex of the quadrant, i.e. within 1.5 mm of the true apex of the corneal surface. The micrographs were all taken at a working distance of 8 mm and with the epithelial surface normal to the electron beam (to within ±5°). All micrographs were identified only by a number code at the time of the assessments being made. Micrographs were taken at 200 X at-stage magnifi-cation and printed at 10 x 8 inches to allow assessments of approximately 0.35 mm^2 portions of the corneal surface. From these micrographs, assessments were made of any regions of the ocular surface where any from of cell damage or abnormality was evident. These regions of approximatel 0.06 mm^2 were quantitatively assessed by mannual planimetry [13] using a commercial digitizer pad and computer software (Bioqant IV, R & M Biometrics. Nashville, TN). Micrographs were also taken at 500 X at-stage magnification (and also printed at 10 x 8 in) from the same portion of the corneal surface. These micrographs were used to measure the surface area of a total of 500 to 550 squamous cells from each experimental group (i.e. approximately 90 cells/corneal quadrant) by use of the digitizer pad system [13]. All surface area values were core- ceted for fixation and processing related tissue shrinkage as previously detailed [13].and only groups of fully tesselated cells were used in these morphometric analyses. Finally, from micrographs taken at 500 X magnification, a count was made of the number of crater-like surface structures on the surface of the 500 to 550 cells used for surface area measures. Only those crater-like structures that had a conspicuous colar or rim [16,17] were included in the counts.

Chemicals and pharmaceuticals

All chemicals used for the electron microscopy were of the highest purity grade available and were

obtained from J.B.E.M. Inc, Larval, Quebec. All solutions were prepared in double-distilled water. The artifical tear products were kindly provided by Allergan Inc (Canada). The LIQUIFILM TEARS™ products contain 1.4% polyvinyl alcohol and were preserved with either chlorobutanol or benzalkonium chloride.

Results

Scanning electron microscopy evaluation of the corneal surface at 1000 X at-stage magnification

Illustrated in (Figure 1) are representative micrographs showing the appearance of the normal corneal epithelial surface at 1000 X and 5000 X at-stage magnification. These magnifications were chosen since the allow resolution not only of the cell-cell borders and epithelial craters but also the actual surface features of the cells. The epithelial surface can be seen to be composed of a continuous mosaic of cells that appear to be in very close contact or apposition to one another. The cell surfaces are decorated both with a uniform mosaic of microplicae (with only occasional solitary microvilli) and the epithelial craters. These micrographs are presented to show that the epithelial surface is qualitatively similar when compari controls with eyedrop-treated epithelia. The n crographs are representative not only of the s samples studied for each group but also of the cc neal surface at mid-peripheral and peripheral sit as well.

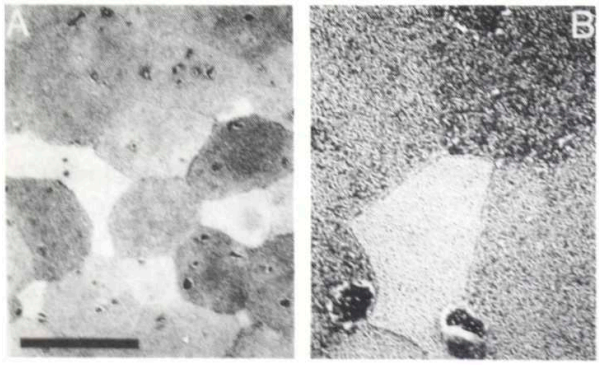

Figure 1 Representative scanning electron micrographs taken from the close to the apex of the normal rabbit corneal epithelial surface in the superior-temporal quadrant. (A) 1000 X, (B) 5000 X, bar indicates 4.2 and 8.4 μm respetively in A and B (corrected for tissue shrinkage)

Quantitative evaluation of cellular exfoliatio or other alterations of the epithelial surfa evaluated at 200 X and 500 X magnification.

Micrographs taken at 200 X magnification wer used to assess the presence of any gross alteratior in the epithelial surface. The results are presente in (Table 1). The analyses show that the corne surface appeared to be largely uncompromised b the exposure to the preservative agent-containin eyedrops. The incidence of exfolialting or othe wise abnormal cells averged only 3.5% and did nc exceed 5%. No difference was seen between th two treatments. From micrographs taken at 500 magnification, occasional exfoliating cells wer evident in addition to the occasional cell tha showed evidence of partial surface disruption

TABLE 1
ASSESSMENTS OF CELL EXFOLIATION AND OTHER SURFASE DAMAGE

	DAMAGED REGIONS (Percentage of epithelial surfase analyzed)	NUMBERS OF EXFOLIATING CELLS (Percentage ofcells analyzed)
Controls	0.26 ± 0.11	0.42 ± 0.07
Chlorobutanol	2.36 ± 0.95	1.70 ± 0.26
Benzalkonium	1.07 ± 0.72	2.11 ± 0.34

6 micrographs were evaluated for each set of data (means ±SD)

nucleus changes or had uplifted edges (suggestive of the initial phases of exfoliation). Three examples are illustrated in (Figure 2) and the numbers of such cells detailed in (Table 1). For control corneas, only 1 of 510 cells analyzed was designated as being abnormal. Both of the treatments with

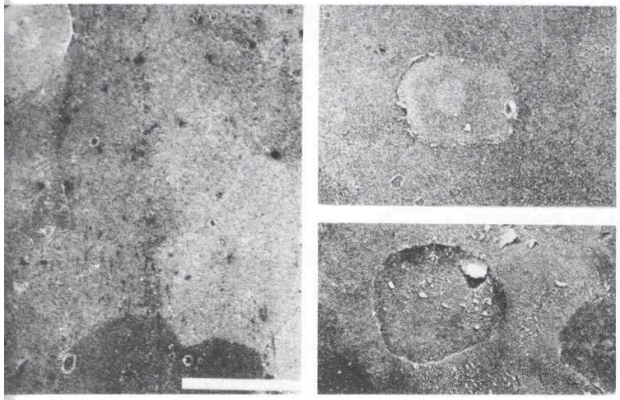

Figure 2 Scanning electron microscope images of the corneal surface to illustrate the appearance of isolated exfoliating cells observed after treatment with either of the eyedro- ps. For quantification, see Table 1. Bar indicates 42 μm (corrected for tissue shrinkage).

artifical tears resulted in the appearance of small numbers of exfoliating or abnormal cells. The relative incidence of these cells was however very low when assessed as a percentage of the approximately 500 cells that were analyzed from the 6 micrographs of each test group, i. e., 1,7% and 2.1% respectively for the chorobutanol and benzalkonium preserved artificial tears respectively

Qualitative evaluation of the appearance of the epithelial surface after exposure to artifical teardrops.

In Figure 3 and 4 are illustrated representative scanning electron micrographs of the corneal epithelial cells obtained 6 h after the exposure to artifical tears containing chlorobutanol (Figure 3) or benzalkonium chloride (Figure 4). For the chlorobutanol - exposed epithelia, the electron microscope image was routinely of relatively low contrast compared to controls (compare Figure 1A with Figure 3A) but the cell - cell borders were still evident and there was little evidence of cell damage etc. The main reason for the low contrast image is that the surface of many of the cells was routinely found to be covered with small to large quantities of an amoprhous material. However, that

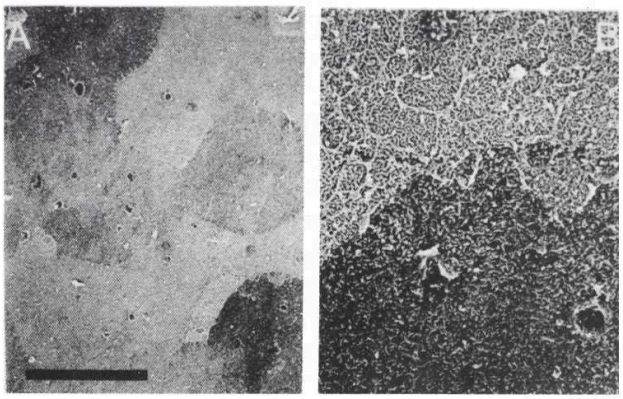

Figure 3 Representative scanning electron micrographs taken from close to the apex of corneas 6 hours after exposure to two sets of eyedrops preserved with chlorobutanol. Other details as Figure 1.

this is just a superficial coverage (perhaps of degraded mucins) is evident from the higher magnification images (Figure 3B) which cleary show the presence of normal - appearance microplicae on the cell surface. Following recovery from the benzalkonium chloride treatment, the electron images all showed less contrast than controls (compare Figure 1A with figure 4A) but the image quality was generally superior to that seen following the chlorobutanol exposure. The cell - cell borders were well resolved and the cell surface features such as the microplicae largely unchanged (Figure 4B)

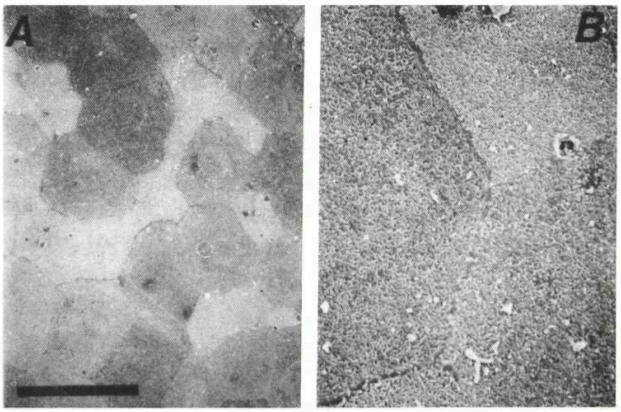

Figure 4 Representative scanning electron micrographs taken from close to the apex of corneas 6 hours after exposure to two sets of eyedrops preserved with benzalkonium chloride. Other details as Figure 1.

Evaluation of surface areas of squamous cells after exposure to artificial tears

From each set of 6 micrographs of part of the central region of the corneal epithelial surface, a total of 500 to 550 cells were digitized. Examples of tracing overlays of the cell borders are given in (Figure 5) to illustrate the overall effect observed. The squamous cells appear to have a similar range of sizes when the control micrograph (Figure 5A) is compared to the micrograph from a chlorobutanol-treated epithelium (Figure 5B). The squamous cell of the corneal epithelium treated with benzalkonium chloride however were generally smaller (Figure 5C).

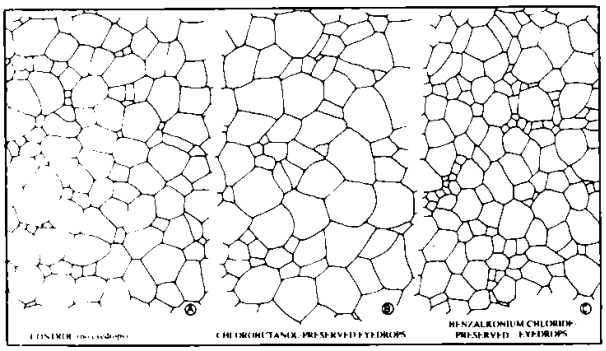

Figure 5. Representative tracing overlays made from scanning electron micrographs to illustrate differences in cell sizes between controls (A; from Figure 1A), Chlorobutanol -exposed epithelia (B; from Figure 2A) and benzalkonium chloride-exposed epithelia (C; from Figure 4A).

Morphometric measures and analyses provide an unambiguous documentation of the change in surface areas of the squamous cells. These results are presented in (Figure 6). For each group of corneas, the cell area data was pooled. A histogram of the cell surface areas from the control group (Figure 6A) revels a wide range of cell sizes from <50 μm^2 to 2127 μm^2. The distribution is clearly skewed to larger cell areas and is clearly non-Gausian. The average cell area (n = 510) was 525 μm^2 and the median cell area was 388 μm^2. Following recovery from the two sets of exposure to the chlorobutanol-preserved artificial teardrops, a similar range of cell sizes was observed, i.e. <50

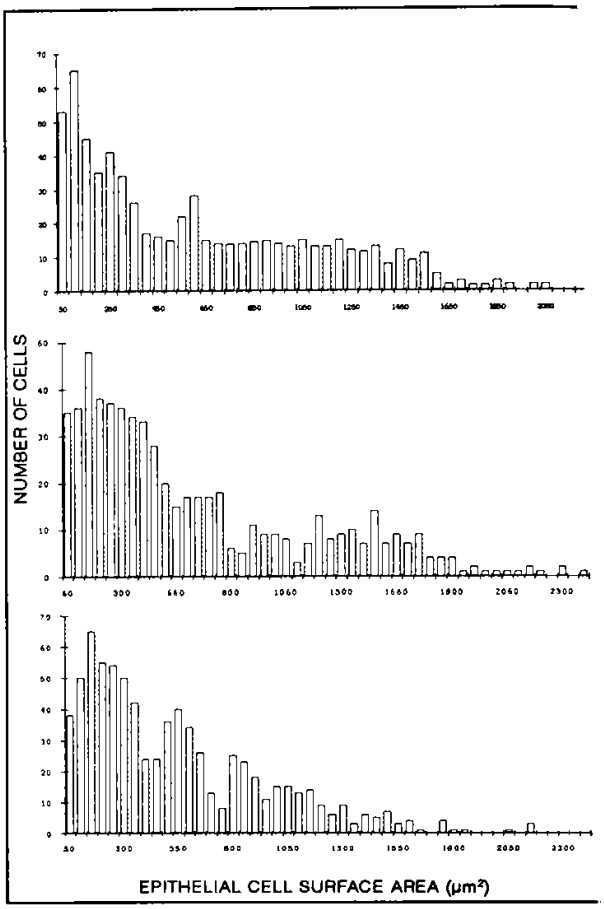

Figure 6. Histograms to illustrate distributions of squamous cell sizes at the corneal epithelial surface. The data was pooled from 6 corneas for each group and the cell surfase area values are corrected for tissue shrinkage. (A) control corneas, (B) chlorobutanol - exposed corneas , (C) benzalkonium chloride - exposed corneas. Other detials as Figure 1

to 2410 μm^2 (Figure 6B). However, the distribution was now not only skewed to larger areas but showed clear indications of bimodality with peaks at both 150 μm^2 and around 1500 μm^2. The number of cells with surface areas of 199 μm^2 or smaller was clearly less than that seen in the control group. The mean cell area (n = 536) was slightly larger than controls at 537 μm^2 but the median cell area was 353 μm^2. A rather different histogram was obtained for the squamous cell surface areas after recovery from two exposures to the benzalkonium

chloride-preserved artificial teardrop (Figure 6C). While essentially the same range of cell sizes was observed (i.e. <50 to 2179 μm^2), there were fewer cells with areas over 1100 μm^2. This change was accompanied by the appearance of a higher number of cells with areas between 200 and 899 μm^2. As an overall result, the average cell surface area was smaller than that for the control corneas (i.e. 482 compared to 525 μm^2). A small decrease in the median cell area resulted from the change in the distribution (i.e. 372 compared to 388 μm^2 in the controls).

Quantitative assessment of the epithelial cell surface craters.

With the special preparative methods used in this study, crater-like structures with an encircling colar or ring were evident on all cell types in all three of the experimental groups of corneas (i.e. controls, chlorobutanol-and benzalkonium chloride - exposed corneas). It can be qualitatively noted that the epithelia exposed to benzalkonium chloride (Figure 4A) clearly had fewer craters than the chlorobutanol - exposed or control corneas. In order to quantitatively assess the occurrence of these features on the cells, a count was simply made of the number of craters on each of the cells that was digitized for the cell surface area measures. The results of these counts are presented in histogram form in (Figure 7). For control corneas, 140 of 510 cells (i.e. 27.5%) did not have any obvious crater-like structure on their surface. Similarly, for the chlorobutanol-exposed epithelia, 159 of 536 cells (29.1%) did not have obvious craters. In contrast, following recovery from the exposure to the benzalkonium chloride-preserved artificial teardrops, 42.9% (219 of 511) of the cells did not have obvious crater-like structures on them. For all of the rest of cells, one or more crater-like structures were evident. The distributions of crater numbers was very similar for control cells and those exposed to chlorobutanol-containing eyedrops 6 hours previously (compare Figures 7A and 7B), e.g. 144 cells had 1 crater in both these groups while

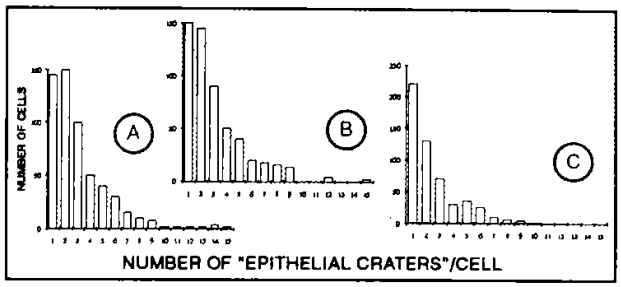

Figure 7. Histograms to show incidence of cells with different numbers of epithelial craters (holes). The cells analyzed with the same as those in Figure 4. (A) control corneas, (B) chlorobutanol - exposed corneas, (C) benzalkonium chloride - exposed corneas. See text for criteria for counting of epithelial craters.

94 and 90 cells respectively had 2 craters. However, these numbers were rather lower for those cells that had been exposed to the benzalkonium chloride, e.g. 137 cells with 1 crater and only 79 cells with 2 craters etc. (Figure 7C). The cells observed after benzalkonium chloride exposure thus have a lower incidence of crater-like structures on their exposed surfaces.

Discussion

In this study, the effect of preservative agent-containing artificial teardrops on the surface cells of the corneal rabbit epithelium has been objectively assessed. Measures of cell cytotoxic effects (assessed by estimates of the number of exfoliating cells), non-toxic cell changes (assessed by cell surface area measures) and surface membrane-related phenomena (assessed by counts of cell craters) are all presented as methods of quantitative evaluation of the effects of artificial teardrops on the scanning electron microscope image of the ocular surface. Such methods are time-consuming but provide an objective means of comparing chemicals, drugs or pharmaceuticals. Such quantification is clearly neccessary since even control epithelia (where such micrographs have been presented) have often been different both between studies from the same laboratories and between laboratories. [12] The most likely reason for these

differences is a lack of standardization of fixation protocol, even in studies from the same group of researchers in some cases. [12] Subjective grading schemes have been proposed [20,21] and used by some investigators [20-22] and may well be sufficient to distinguish between effects at the gross level, e.g. cell exfoliation or conspicuous cell damage. The assessments however appear to require that the electron micrographs be taken at very high magnifications (e.g. 2000 X to 3000 X magnification) so that the microplicae (or microvilli) on the cell surfaces can be seen. As a result, a large number of separate micrographs need to be taken to obtain a representative sample [21].

The study was designed only to assess the effects of occasional use of an artificial teardrop on the corneal epithelial surface in a healthy eye. It was not intended as a cytotoxicity evaluation neither was the interest in documenting the acute cellular or ultrastructural changes that clearly can occur when the ocular surface is exposed to single or multiple drops of preservative agent-containing ophthalmic solutions. Numerous other studies have provided documentation of the acute effects (i.e. within 30 min) of the effects of higher concentrations of benzalkonium chloride-containing solutions on the ocular surface; gross cell exfoliation has been reported in several scanning electron microscope studies. [11,22-27] Similar results have been obtained from light microscope [24, 28, 29] or transmission electron microscope studies. [30] While the concentrations of benzalkonium chloride used in some of the studies have been close to those commonly used in commercially-available eyedrops, the exposure has either been intense (i.e. 10-15 drops administered over several minutes to an hour) or continuous (i.e. the continuous application over many minutes or even immersion of the globe for a few minutes in preservative agent-containing solution) [22-28, 30]. The present studies provide information on the state of the epithelial surface well after the eyedrops were instilled and were designed to show that the exfoliation that can be seen with benzalkonium chloride (or even chlorobutanol [14]) is not progressive when just a couple of drops of the eyedrops are instilled.

The reason for the observed effects is not entirely clear at this time. Several tentative conclusions can however be drawn to provide a basis for further investigations. The overall lack of cell exfoliation seen after recovery from exposure to the chlorobutanol-preserved eyedrops reflects the proable fact that the peak exfoliation rate has not been realized. In a previous study, [14] it was observed that, at the same period of time after exposure of the epithelial surface to these eyedrops, the percentage exfoliating cells was maximal at some time between 3 and 5 days of twice daily use. The shift to slightly larger squamous cells presumably reflects a reduced rate of exfoliation of squamous cells, at least in the short term. For the benzalkonium chloride-exposed epithelia, while little cell exfoliation was evident at 6 hrs after the last set of eyedrops, the occurrence of higher numbers of smaller cells suggests that significant cellular exfoliation had occurred during the period after the eyedrop instillation; the surface being viewed by scanning electron microscopy would thus reflect a newly uncovered layer of cells. [31] The kinetics of these changes, over the 6 h period, thus need to be studied in more detail both with the dosage used in these studies and with more frequent instillation of the eyedrops. The significantly reduced numbers of well-formed epithelial craters (epithelial holes) after recovery from benzalkonium chloride exposure can be taken both as an indication that the most superficial layer of cells has been lost after the treatment [21] and that the nature of the tear film and epithelial surface intercation is immature. [18] It remains to be established if these craters reflects exocytosis (of intracellular vesicles [17]) or an ongoing process of endocytosis and exocytosis of surface mucin. [18]

ACKNOWLEDGEMENTS

This study was made possible by a research operating grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada.

Allegan Inc is thanked for their generous provision of samples of the commercially available eyedrop products but the study was not financially supported by the company. The author has no proprietary interest in these or any other artifical tear formulations.

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