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EFFECTS OF TOPICAL BENZALKONIUM CHLORIDE ON CORNEAL EPITHELIUM

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The present study was designed to investigate the long and short term topical effects of Benzalkonium chloride (BAC) on the corneal epithelium with low and high concentrations used in commercial ophthalmic preparations. Forty eight guinea pigs (ninety six eyes) of Dunkin Hartley strains were procured from NIH Islamabad, and randomly divided into four long term (A) and four short term treatment (B) groups. The analysis of the results showed significant decrease ($p<0.05$) in the thickness and number of epithelial cell layers. The incidence of epithelial desquamation, erosions, and ulceration was more in those experimental groups which received higher concentration of BAC more frequently than those receiving lower concentration and instilled less frequently. It is, therefore, suggested that a better and safe substitute for BAC or preservative free eye drops should be formulated to prevent the hazards of this toxic substance.

Nearly all of ophthalmic products, from artificial tears to contact lens solutions, contains presservatives.[1] Benzalkonium chloride (BAC) is being commonly used as a preservative in commercially available eye preparations since 1930. Studies from late 1970s have shown that this quaternary ammonium compound denatures proteins and causes lysis of cytoplasmic membranes.[1,2] Mammalian cells are unable to neutralize BAC, and corneal epithelium is damaged by its entrance through liposomes or other intracellular vacuoles; this induces cytotoxic damage to conjunctival cells and corneal stroma, resulting in thinning of the cornea.[3] BAC induces two different patterns of cell death; apoptosis and necrosis in a dose dependant manner. Both patterns of cell death can be induced by different concentrations of BAC.[4,5] *In vitro* studies, using cultureed human conjunctival cells, have demonstrated that BAC induced cell damage ranging from arrest of cell growth at very low (0.0001%), apoptosis at medium (0.001%), and necrosis at high (0.05 to 0.1%) concentrations.[6] In a study to determine the effects of anti-inflammatory drugs alone and those containing preservatives, Hendrix[7] reported that BAC caused rounding and shrinkage of cells with varying concentrations; superficial epithelial cells were loosened or lost and the deeper cells were shrunken. There was disruption of intercellular attachments and loss of the plicate appearance at a concentration of 0.01% in rabbit corneas. Baudouin and Lunardo[8] reported that BAC, through its surfactant effect, altered tear film stability and reduced its break up time at a concentration of 0.005%, resulting in corneal epithelial cell wrinkling and peeling. Pisella *et al.*[9] conducted a study on 5288 patients, examined by 250 ophthalmologists in France to evaluate the prevalence of ocular toxicity caused by topical anti-glaucoma preparations containing BAC as a preservative. The incidence of conjunctival hyperaemia (38%), conjunctival follicles (20%), and superficial punctate keratitis (18%) was recorded in these patients. This survey showed that ocular surface impairment was not a marginal phenolmenon in these patients but rather occurred in a large number of patients and, therefore, constituted a real healthcare concern.

There is a poor awareness of the potential toxicity of BAC on ocular tissues on account of approval of its use by WHO vide CAS Registry No. 8001-54-5 (WHO INN).[10] Moreover, Food and Drug Administration (FDA) of USA has also listed it as a safe preservative.[11] The ophthalmologists, therefore, do not take into account the effects of BAC on cornea while prescribing ophthalmic preparations containing this preservative. Thus, the complications caused by BAC usually pass unnoticed. Patients with pre-existing conjunctival and corneal diseases, such as dry eye syndrome, are especially more prone to its toxic effects since eyes with decreased tear production may not be able to wash away the preservative as effectively as in normal eyes or when the lachrymal outflow passages are partially or totally obstructed[12] increasing the contact time of the drug to the corneal surface.

MATERIALS AND METHODS

Forty eight guinea pigs of Dunkin Hartley strain, weighing between 350-600 grams and 8-9 weeks old were used in the study. These animals were procured from the National Institute of Health, Islamabad. The animals were kept in the experi-

mental research laboratory of the University of Health Sciences, Lahore (Pakistan), at a temperature of 24-27 °C under 12:12 hours light/dark (LD) cycle. The humidity was maintained between 45-65%. The animals were fed ad libitum on fresh lettuce, cabbage, carrot top, grass, maize and fruits. All experimental protocols were observed in compliance with the Ethical committee of the University of Health Sciences Lahore.

The animals were randomly divided into groups (Tables 1 and 2). Each group contained six animals. BAC (Fluka, Germany) in different concentrations was used topically as eye drops which were prepared in normal saline and instilled in the right eye while the left eye of each animal served as a control and was treated only with normal saline solution.[13,14] Both short and long term effects were studied by using low and high concentrations of BAC comparable to those used in commercial ophthalmic preparations in concentrations of 0.0075% and 0.02%.[1]

Table 1: *Dosage protocol of short term study (Group A).*

Group	Frequency of administration[13]	Concentration of BAC
A - 1	Six times/day for 48 hours.	0.0075%
A - 2	Six times/day for 48 hours.	0.02%
A - 3	Six times/ day for one week.	0.0075%
A - 4	Six times/day for one week.	0.02%

Table 2: *Dosage protocol of long term study (Group B).*

Group	Frequency of administration[15]	Concentration of BAC
B-1	Twice daily for four weeks.	0.0075%
B-2	Twice daily for four weeks.	0.02%
B-3	Twice daily for eight weeks.	0.0075%
B-4	Twice daily for eight weeks.	0.02%

Preparation of BAC Solution:

Two different strengths of solutions containing BAC were prepared. The required amount of BAC was dissolved in distilled water, sodium chloride was added to make the final solution isotonic, similar to the tonicity of tear film.[17]According to Fialho and Cunha[17] the ideal pH for maximum comfort to the eye is 7.2 ± 0.2 for an ophthalmic preparation; hence, the pH of the solutions was adjusted to 7.2 with a pH meter using 0.1N HCL and 0.1N NaOH. The final volume of each solution was made up to 100 ml with distilled water.[18] The normal saline (0.9%) solution was similarly prepared for the control eyes.[8] The solutions were freshly prepared every week and stored in appropriately labelled glass bottles. One drop of topical solution containing BAC and normal saline was instilled, according to the schedule mentioned in Tables 1 and 2.

The animals were killed with an overdose of Pentothal Sodium injection given intraperitonially.[19] the right and left eyes of the animals were enucleated after the experimental period. The delicate nature of the cornea required special treatment; therefore, the schedule described by Bancroft and Gamble[20] was used for tissue processing. Automated microtome was used for sectioning and 6 µm thick sections were obtained and affixed to precleaned albuminised glass slides. These were stained with H&E techniques in a usual way. Micrroscopic study on the corneal epithetlium was carried out under a light microscope and observations were recorded on the following aspects:

a. Desquamation of cells.

b. Any erosion or ulceration.

c. Thickness calculated using method of Culling.[21]

RESULTS

The left eye (control) of the animals was examined daily for any gross change before its enucleation. The corneas appeared transparent, smooth and shiny and no gross change was observed in the control eyes (Fig. 1-A). The right (treated) eye, however, in group A-4 and B-4 showed ulceration of the treated eye. The ulcer finally resolved as a dense white opacity after the withdrawal of drug at the end of experimental period in two animals (Fig. 1-B).

Control Corneas (Fig 2):

Epithelium in the cornea of the control eyes consisted of 5-6 layers of cells of squamous stratified non-keratinized variety, comprising basal, middle and superficial layers. The low columnar cells formed a single basal layer adjacent to the basement membrane. The oval nuclei of these cells were aligned vertically along the long axis of the cells. The middle layer comprised 1-2 strata of polygonal cells with darkly stained round nuclei located in

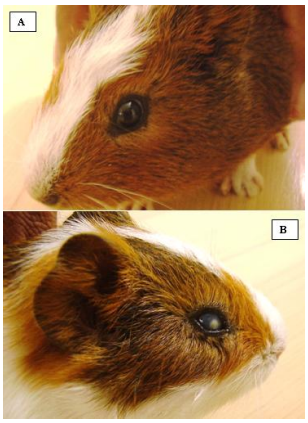

Fig. 1: Photograph of a guinea pig (Gp. A-4) showing transparent cornea of control eye (A) and corneal opacity in the treated eye (B) of the same animal.

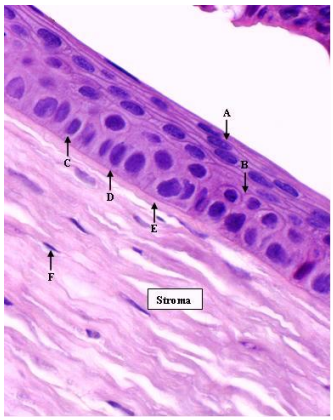

Fig. 2: Photomicrograph (Gp. A-2) of guinea pig cornea (control) showing squamous stratified epithelium with distinct cell boundaries. The superficial layer of flat squamous cells (A), middle layer of polygonal wing cells (B), and basal layer of low columnar cells (C) resting on a thin basement membrane-(D) are visible. Bowman's membrane (E) and underlying stroma containing fibroblasts (F) can also be seen. H and E stain. X 400.

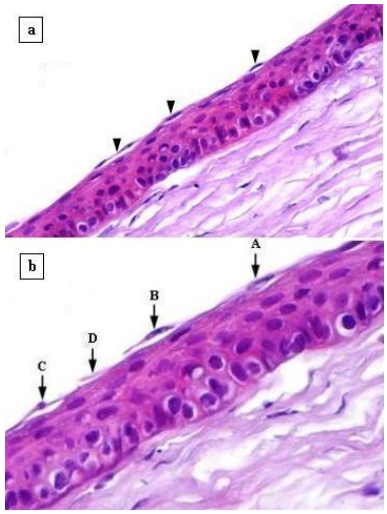

Fig. 3: (a) Photomicrograph of guinea pig cornea (Gp. A-3) showing early changes of desquamation (arrowheads) in the treated eye. H & E stain. X 200. (b) Photomicrograph showing late changes in the treated eye. Lifting of the superficial epithelial cells (A), detachment of cell in progress (B), scanty cytoplasm and shrunken nucleus (C) and a flake-like desquamating cell (D) can be appreciated. H and E stain. X 400.

the center of the cells. The superficial layer comprised 2-3 strata of flat squamous spindle shaped cells containing flattened nuclei in the central part of the cells. Distinct boundaries of the epithelial cells were discernible in sections (Fig. 2).

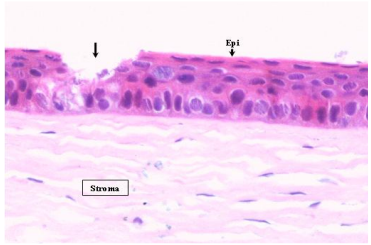

Fig. 4: Photomicrograph of guinea pig cornea (Gp. A-4) showing epithelium (Epi) and superficial punctuate keratitis (SPK) in the form of corneal-erosion (arrow). H and E stain. X 400.

Treated Corneas:

Desqamation of superficial epithelial cells was seen at places in groups A-2 and A-3 showing early (Fig. 3-a) and late stages of desquamation process (Fig. 3-b).

Superficial Punctate keratitis in the form of corneal erosions and ulceration of epithelium was seen in group A-4 (Fig. 4 and 5).

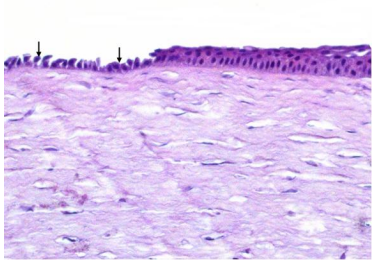

Fig. 5: Photomicrograph of guinea pig cornea (Gp. A-4) showing ulceration of epithelial cells (arrows), leaving the basal cells on H and E stain. X 200.

STATISTICAL ANALYSIS

Thickness in μ of epithelium in the treated groups A and B

The statistical analysis of groups A and B using independent sample t-test showed that the thickness of epithelium was significantly decreased $p < 0.05$) in the treated groups A-4 and B-4; A-4 control $\mu = 33.36$, S.E. = 0.799; A-4 treated $\mu = 16.46$, S.E. = 2.900, B4 control $\mu = 35.10$, S.E. = 1.463; B-4 treated $\mu = 26.00$, S.E. = 2.013 (Table. 3, Fig. 6).

Thickness of epithelium in the treated group A

Analysis of variance (ANOVA) showed that there was a significant decrease in the thickness of epithelium among the treated groups A1, A2, A3 and A4.

Post-Hoc test, using the Tukey (HSD) showed that this difference was also significant: p-value < 0.05 (Table 4). There was a significant decrease in the thickness of epithelium in the treated group A4 as compared to groups A1, A2 and A3 respectively (Fig. 7).

Table 3: *Independent sample t-test.*

Group compared	df	Significance (2-tailed)	Mean Difference	SE Difference
A1	10	0.360	1.30000	1.35655
A2	10	0.242	2.93333	2.35665
A3	10	0.126	2.06667	1.23792
A4	10	<0.0001*	16.90000	3.00847
B1	10	0.882	0.43333	2.85474
B2	10	0.714	-0.56667	1.50059
B3	10	0.234	3.90000	3.08246
B4	10	0.004*	9.10000	2.48931

*Significant

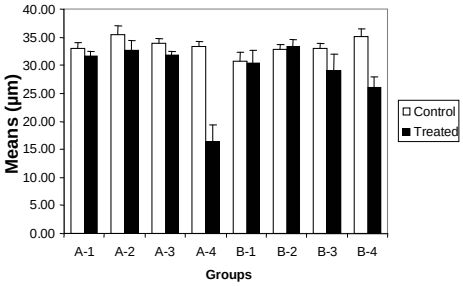

Fig. 6: Mean thickness of epithelium in the control and treatment groups.

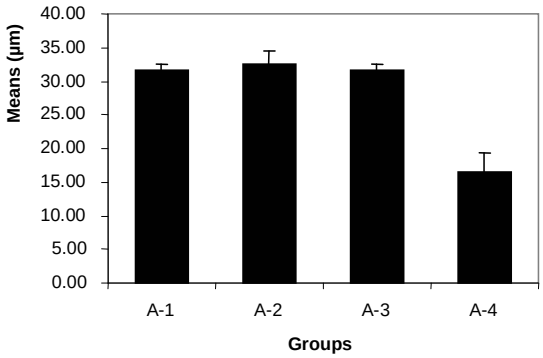

Fig. 7: Mean thickness of epithelium in treated group A

Thickness of epithelium in the treated group B

Analysis of variance (ANOVA) showed that there was no significant effect of Benzalkonim chloride on the thickness of epithelium among the treated groups B1, B2, B3 and B4. Post-Hoc test, using the Tukey (HSD) showed that this difference was also not significant: $p > 0.05$ (Table. 5, Fig. 8).

DISCUSSION

BAC interferes with the growth, multiplication, and metabolism of microbial organisms. It has similar effects on eukaryotic cells, which account for its cytotoxicity.[15] In the current study, changes were observed in corneal epithelium treated with BAC, especially in the region of central cornea, because this part of cornea is directly exposed to the instilled eye drops.[2] did not observe any gross damage to corneal epithelium when they used lower concentrations of BAC (0.001%) for up to 07 days. Although this observation was consistent with our study with low concentration of BAC (0.0075) higher concentrations (0.02) produced the gross changes in the cornea of experimental groups A-4 and B-4. In our study desquamation of epithelial cells was observed in 16.66% corneas of short term treated groups (A-2, A-3, and A-4) and 8.33% in long term treated groups B (B-4). The incidence of corneal erosions was 12.50% in group A and 0.00% in group B. Corneal ulceration was seen in 20.83%

Table 4: *Multiple comparisons of thickness of epithelium in treated eyes (Group A)*

Comparison among groups		Mean difference (I-J)	(SE)	Level of significance p-value
group (I)	group compared (J)			
A1	A2	-0.96667	2.56249	0.981
	A3	-0.10000	2.56249	1.000
	A4	15.16667	2.56249	<0.0001*
A2	A1	0.96667	2.56249	0.981
	A3	0.86667	2.56249	0.986
	A4	16.13333	2.56249	<0.0001*
A3	A1	0.10000	2.56249	1.000
	A2	-0.86667	2.56249	0.986
	A4	15.26667	2.56249	<0.0001*
A4	A1	-15.16667	2.56249	<0.0001*
	A2	-16.13333	2.56249	<0.0001*
	A3	-15.26667	2.56249	<0.0001*

*Significant

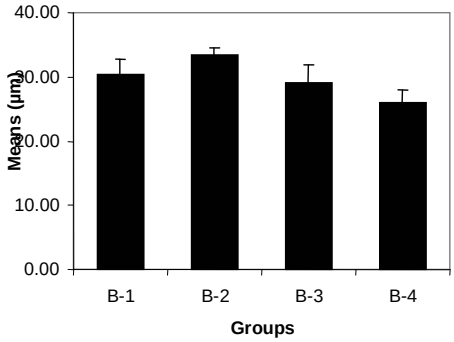

Fig. 8: Mean Thickness of Epithelium in Treated Group B

Table 5: *Multiple Comparisons of Thickness of Epithelium in Treated eyes (Group B)*

Comparison among groups		Mean Difference (I-J)	(SE)	Level of Significance P-value
group (I)	group compared (J)			
B1	B2	-3.03333	3.16660	0.774
	B3	1.30000	3.16660	0.976
	B4	4.33333	3.16660	0.532
B2	B1	3.03333	3.16660	0.774
	B3	4.33333	3.16660	0.532
	B4	7.36667	3.16660	0.125
B3	B1	-1.30000	3.16660	0.976
	B2	-4.33333	3.16660	0.532
	B4	3.03333	3.16660	0.774
B4	B1	-4.33333	3.16660	0.532
	B2	-7.36667	3.16660	0.125
	B3	-3.03333	3.16660	0.774

in Group A (A-4) and 4.16% in Group B-4. Lemp and Zimmerman[22] reported comparable lesions in their study i.e., numerous superficial corneal erosions and ulceration with eye drops containing BAC. Ichijima *et al.*[23] observed superficial epithelial cells desquamation at 0.005% BAC which proportionately increased with concentration gradient. Doughty[24] observed 5% exfoliation of the epithelial cells at the ocular surface of rabbit after treatment with artificial tears containing BAC. In our study mean thickness of epithelium showed significant decrease ($p<0.05$) in treated groups A-4 and B-4 (Fig. 6, 7, and 11) as a result of corneal ulceration probably due to the use of high concentration (0.02%) of BAC for relatively long duration to the other groups.

The main aim of the BAC is to reduce the eventual bacterial growth in the ophthalmic drops. This result, however, is not always achieved. Adair *et al.*[25] and Loughlin *et al.*[26] mentioned the cross-resistance of BAC to pseudomonas aeruginosa. Schein and colleagues reported 29% growth of Gram +ve and Gram -ve strains in the eye drops. These studies arouse doubts about the real capacity of BAC to prevent the risk of contamination. In fact, despite its limited antiseptic activity, BAC is not able to assure a sterile content inside the bottle. Rolando[12] also warned those who use contact lenses that the preservative absorbed by the lens prolongs its time of contact, thereby producing cumulative effects of BAC on the corneal epithelium. Recently, Brandt[27] has shown concern over the increased incidence of cataract in patients using BAC containing eye drops for long period. This is an alarming situation which demands for the search of a new non-toxic preservative or formulation of preservative free eye drops. International agencies and regulatory authorities like FDA also need to take up this problem, so that the hazards caused by BAC may be eliminated.

It is **Conclused** that the analysis of the results showed significant decrease ($p< 0.05$) in the thickness and number of epithelial cell layers. The incidence of epithelial desquamation, erosions and ulceration was more in those experimental groups which received higher concentration of BAC more frequently than those receiving lower concentration and instilled less frequently. It is, therefore, suggested that a better and safe substitute for BAC or preservative free eye drops should be formulated to prevent the hazards of this toxic substance.

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