

Full Length Research Paper

Effects of preserved and unpreserved timolol maleate on the surface of the corneal epithelium of albino rabbits

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Accepted 18 July, 2011

Timolol maleate, ophthalmic solution, is frequently prescribed for glaucoma, although the use of this drop is likely to interfere with wound healing in the immediate corneal surgeries. The purpose of this study is to evaluate the toxicity of commercially prepared topical timolol maleate 0.005 and 0.05% and its preservative benzalkonium chloride (BAC) 0.0004 and 0.001%. And have also shown its preservative free effect on the corneal epithelia and changes in protein pattern. Rabbit (albino) corneal epithelial organ cultures were prepared as migrating (wounded) and non-migrating (unwounded) corneal epithelia. Corneas (n = 95) were removed and 7 mm epithelia were abraded mechanically. The abraded corneal epithelia (migrating) and non-abraded corneal epithelia (non-migrating) were incubated in modified supplemental hormonal epithelial media (SHEM) containing a) timolol maleate drop 0.005% concentration and 0.05% concentration supplied with BAC+ (0.001%) and b) BAC- (preservative free) timolol maleate (purified form) 0.005 and 0.05% and c) along with BAC+ 0.001 and 0.0004% concentration, d) only BAC with 0.001 and 0.0004% concentration. The media containing timolol maleate and BAC was replaced every 24 h. Phase contrast light microscopy of the ocular surface was carried out after haematoxylin-eosin staining and photographic documentation was performed at different time points 0, 24, 48, 72, and 96 h. 2-DE analysis demonstrated the changes in the proteins of migrating, non-migrating and treated corneal epithelia. Morphological studies confirmed the deleterious effect of timolol maleate 0.05% with BAC 0.001% and revealed enhanced corneal toxicity when compared to timolol 0.005% with the same concentration of BAC. Preservative BAC at 0.0004% showed no significant difference at protein level and morphological changes were also not observed on corneal epithelia. Both concentrations of timolol maleate with BAC 0.001% show down regulation of protein expressions. Corneal organ culture model has confirmed that topically applied timolol maleate 0.005 and 0.05% and its preservative BAC 0.001% showed deficiency in term of protein expressions, although the appropriate randomized clinical assessment are essential to certify these reports.

Key words: Timolol maleate, BAC, toxicity.

INTRODUCTION

In ophthalmic solutions, preservative is used to prevent contamination during the treatment period. Controversy exists in literature about the effects of β -adrenergic receptor (β -ARs) antagonist and their vehicles on corneal

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healing (Weissman et al., 1990; Hirano et al., 2004; Tan et al., 2002 and Baudouin 1996). Preservatives for ophthalmic solutions include benzalkonium chloride (BAC), chlorobutanol, parahydroxybenzoates and poly-sorbate.

Timolol maleate and its preservative BAC is used successfully worldwide as topical intraocular pressure lowering drug. However, side effects like dry eyes, 3532 Sci. Res. Essays

burning and corneal anesthesia has been reported during the use of timolol maleate BAC⁺ (Herreras, 1992; Costagliola, 2002; Ohtsuki, 2001, Thygesen, 2000). One of the most common preservative BAC for eye drops, has antibacterial effects (Friedlaender et al., 2006; Kowalski, 2006) and induces cytotoxic damage in conjunctival cells (de Saint Jean, 2000), with the thinning of cornea as well as stromal damage (Pisella, 2000). One clinical investigation has shown that the administration of timolol eye drops with its preservative BAC induces greater disruption of blood–aqueous barrier than the BAC alone (Miyake, 2001). It is therefore suggested that the addition of BAC to the eye drops or in beta blockers contributes to these adverse affect.

On account of the disparity in reported literature, a series of experiments have been designed for a better understanding of the extent of contribution of the commercially available β -blocker eye drop, timolol maleate and its preservative, BAC, and thereby, abet in the development of a safer therapeutic composition of the drug.

Hamard (2003) and his group observed that preserved beta blockers showed a pro-apoptotic effect on human trabecular cells *in vitro* whereas BAC alone is highly cytotoxic. A prominent observation in this study was that preserved antiglaucoma drugs showed stronger proapoptotic effects compared to unpreserved drugs. However, a number of ocular side effects like uveitis, have been reported due to use of beta blockers, example, timolol, betaxolol treatment for glaucoma (van Beek et al., 2000).

In the present report we provide evidence that timolol maleate ophthalmic solution ingredients are cytotoxic to corneal epithelium at certain concentration following buffering effects of its preservative BAC. Clearly, further investigation will improve our understanding of the corneal epithelium and hopefully escort to the development of new therapies to minimizing its toxicity.

MATERIALS AND METHODS

Animals

New Zealand white rabbits (n= 30) weighing 2 to 3 kg used in this study was in conformity to the Declaration of Helsinki on the "Guiding Principle in Care and Use of Animals". Fresh eyes were obtained from the University slaughter house within 30 min following decapitation.

Organ culture model

Rabbit corneal epithelial organ cultures were prepared as described previously (Mushtaq et al., 2007, 2011). The Albino rabbit eyes were used to prepare migrating (n=60) and non-migrating (n=20) corneal epithelia in organ culture. Four sets of experiment were

performed in triplicates. The integrity of the corneas was checked with fluorescein. Rabbits with intact corneas were decapitated and their eyes processed immediately on ice. For migrating corneal epithelia, the corneas were demarcated with a 7 mm trephine, and

the epithelium within this region was subsequently removed with a scalpel blade (#10) under a dissecting microscope (Olympus M081). The corneas were then excised along 1 to 3 mm scleral rim. For non-migrating epithelium, excised similarly without scraping the epithelium and rinsed in Hanks Balanced Salt Solution (HBSS) (Sigma, St.Louis, MO) and disinfected for 5 to 7 min in antibiotic-antimycotic solution. The corneas were again rinsed in HBSS and transferred to modified Supplemental Hormonal Epithelial Media (SHEM) [24] containing fetal calf serum (5%). The cultures were then incubated at 37°C in a CO₂ incubator. The media was replaced after 24 h. The media containing beta blocker (timolol maleate) was replaced every 24 h. The media containing timolol maleate and BAC was also similarly processed.

Treatment groups

The rabbit eyes were randomly assigned to one of four treatment group; each group consisted of six eyes. One group served as control and only treated with SHEM. Timolol maleate and its trade names and manufacturers, vehicle, Benzalkonium chloride (BAC) are given in Table 1. In timolol maleate (D) a preservative, benzalkonium chloride (BAC⁺), is major constituents where as purified (P) form of timolol maleate BAC⁻, formerly is identical to timolol maleate BAC⁺ except that it lacks a preservative BAC.

Drugs

Commercially available timolol maleate eye drop (0.5% Remington) was used with 0.05% and 0.005% concentrations. Preservative BAC free (BAC⁻) timolol maleate (purified form, Sigma-Aldrich) was also prepared with concentrations, similar to eye drop, 0.05 and 0.005% and (BAC⁻) (0.001 and 0.0004%). Timolol maleate and BAC were dissolved directly in SHEM medium to give the desire concentrations.

Light microscopy

Non-migrating, migrating and treated rabbit corneas that had been healing *in vitro* for 0, 24, 48 and 72 h were fixed overnight in 10% formalin. The samples were first hydrated with gradual increased concentration of ethanol ((70, 80, 95% × 2) and then dehydrated with three changes of absolute ethanol. The ethanol was cleared twice with xylene that was subsequently in filtered with paraffin. All the treatments were carried out for one hr each. The tissues were then embedded in paraffin blocks for half an hour in an oven at 60°C. Sections (5 μ m thick) were cut with a microtome (Shandon AS325). Deparaffinization was then allowed for another half an hr with xylene. The sections were then placed on glass slides and air-dried at room temperature. The slides were deparaffinized in xylene with two treatments of xylene for five minutes each. Further washing was carried out in crude ethanol and 95% ethanol again for five minutes each. The sections were then rinsed with distilled water.

Hemotoxylin-eosin staining

Hemotoxylin-eosin staining was carried out to visualize the corneal epithelial layers under light microscopy. Briefly, the sections were incubated in hemotoxylin for one minute, dehydrated in acid ethanol (1%) for 5 min, washed with distilled water, dehydrated again in

ethanol (70 and 95%) for 5 min each and incubated in eosin (1%) for 1 min. The excess stain was removed by incubation in absolute

ethanol, carboxylene (20%) and pure xylene for 5 min each. The samples were immediately mounted onto glass slides. The glass
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Table 1. Timolol maleate and BAC treatment.

| Group | Treatment | Concentration (%) | Trade name |
|-------|-------------------------------------|-------------------|--------------------------|
| 1 | SHEM (control) | - | - |
| 2 | Timolol maleate (D) | 0.005 | Remington Pharmaceutical |
| | Timolol maleate (P)BAC ⁻ | 0.005 | Sigma Aldrich |
| | Timolol maleate (P) BAC+ | 0.005+0.0004 | Sigma Aldrich |
| | Timolol maleate (P) BAC+ | 0.005+0.001 | Sigma Aldrich |
| 3 | Timolol maleate (D) | 0.05 | Remington Pharmaceutical |
| | Timolol maleate (P)BAC ⁻ | 0.05 | Sigma Aldrich |
| | Timolol maleate (P) BAC+ | 0.05+0.0004 | Sigma Aldrich |
| | Timolol maleate (P) BAC | 0.05+0.001 | Sigma Aldrich |
| 4 | BAC | 0.0004,0.001 | Sigma Aldrich |

Timolol maleate (D): Timolol maleate drop already contained BAC. Timolol maleate (P) BAC+: Timolol maleate purified form contained BAC. Timolol maleate (P) BAC⁻: Timolol maleate purified form without BAC.

slide containing sections were then placed on cover slips pre-poured with mounting medium DPX (Di-N-Butyle Phthalate in Xylene) solution. The corneal epithelia were viewed and photographed using a microscope (Olympus).

Extraction of protein

After 48 h, the migrating, treated with (timolol maleate, BAC) and non-migrating epithelia from the corneas were harvested and collected in 1 ml 0.5% CHAPS extraction buffer containing benzamidine 5 mM, N-ethylmaleimide 5 mM, 6-aminohexanoic acid 0.1 M and 5 µg/ml PMSF. Harvested epithelium was dispersed by sonication and centrifuged at 12000×g for 15 min, the process was repeated three times, supernatants were pooled and dialyzed to remove salts and detergents (Panjwani, 1995). Proteins were estimated by BCA assay (Pierce Chemical Co., Rockford, IL), using BSA as a standard.

2-D Electrophoresis

Iso-electric focusing (IEF) was carried out with immobiline dry strips kit (GE Healthcare Life Science, NJ, USA) using Multiphor II electrophoresis system according to the manufacturer's instructions. Briefly IEF strips (11 cm, pH 3 to 10) were allowed to rehydrate over night in the rehydration buffer containing 8 M urea, 2% CHAPS, 0.1 M DTT, and 0.5% IPG (immobiline PH gradient) buffer (pH 3 to 10), the strips were overlaid with mineral oil.

Epithelial proteins from both migrating (48 h), non-migrating (control) and treated corneas were solubilized in sample solution containing 9 M urea, 50 mM DTT, 2% pharmalyte (3 to 10) and 0.5% Triton X-100 and loaded onto caps at the cathodic end of the rehydrated IPG strips, and covered with low-viscosity paraffin oil. IEF was run at 150 V for an hour. The voltage was progressively increased from 300 to 600 V during first three hours, followed by 3500 V for two hours. Before the second dimension run, the IPG strips were equilibrated for 15 min with a solution of 0.05 M Tris-HCl, 6 M urea, 30% glycerol and 1% SDS. In the second dimension proteins were separated on 10% SDS-PAGE gels. The gels were

silver stained and image analysis performed with software (Bionumeric BioSystematica, Tavistock, United Kingdom) to determine the percent that each spot contributed to the total protein on the gel.

RESULTS

Histochemical analysis

To examine the cellular reorganization of the migration corneal epithelium (controls) with time, 1 µm thick sections were examined with eosin-hemotoxylin (Figure 1a, b, c, d, e and f) staining at 0, 24, 48, 72 and 96 h following wounding. Figure 1c shows a progressive thinning of the epithelium at 24 h. The leading edge of the epithelium was composed of a single cell layer (Figure 1d, e) after 48 and 72 h. At 96 h, the epithelium was composed of 2 to 3 cell layers (Figure 1f), however, the normal thickness as compared to non-migrating corneal epithelium (Figure 1a) was not restored even after 96 hrs of wounding (Figure 1f). Non-migrating corneal epithelium showed 5 layers of cells (Figure 1a) no significant effect was seen when 0.0004% BAC were used either alone or with both concentration of timolol maleate (data not shown).

Sections of corneas incubated with timolol maleate (D) drops, (P) purified (0.005, 0.05%) and BAC (0.001%) was observed only at 48 h of incubation. Timolol maleate (D) (0.005%) treated corneal epithelium showed a significant thinning of the corneal epithelium (Figure 1g) where desquamations were seen in same concentration (P) with BAC 0.001% (Figure 1h). Timolol 0.05% (D) and (P) with BAC 0.001% (Figure 1i and j treated samples did not show thinning, however, detachment of the corneal

epithelial layer was seen at 48 h. Only in BAC (0.001%) treated a complete detachment and extreme
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desquamation of corneal epithelium was evident (Figure 1k).

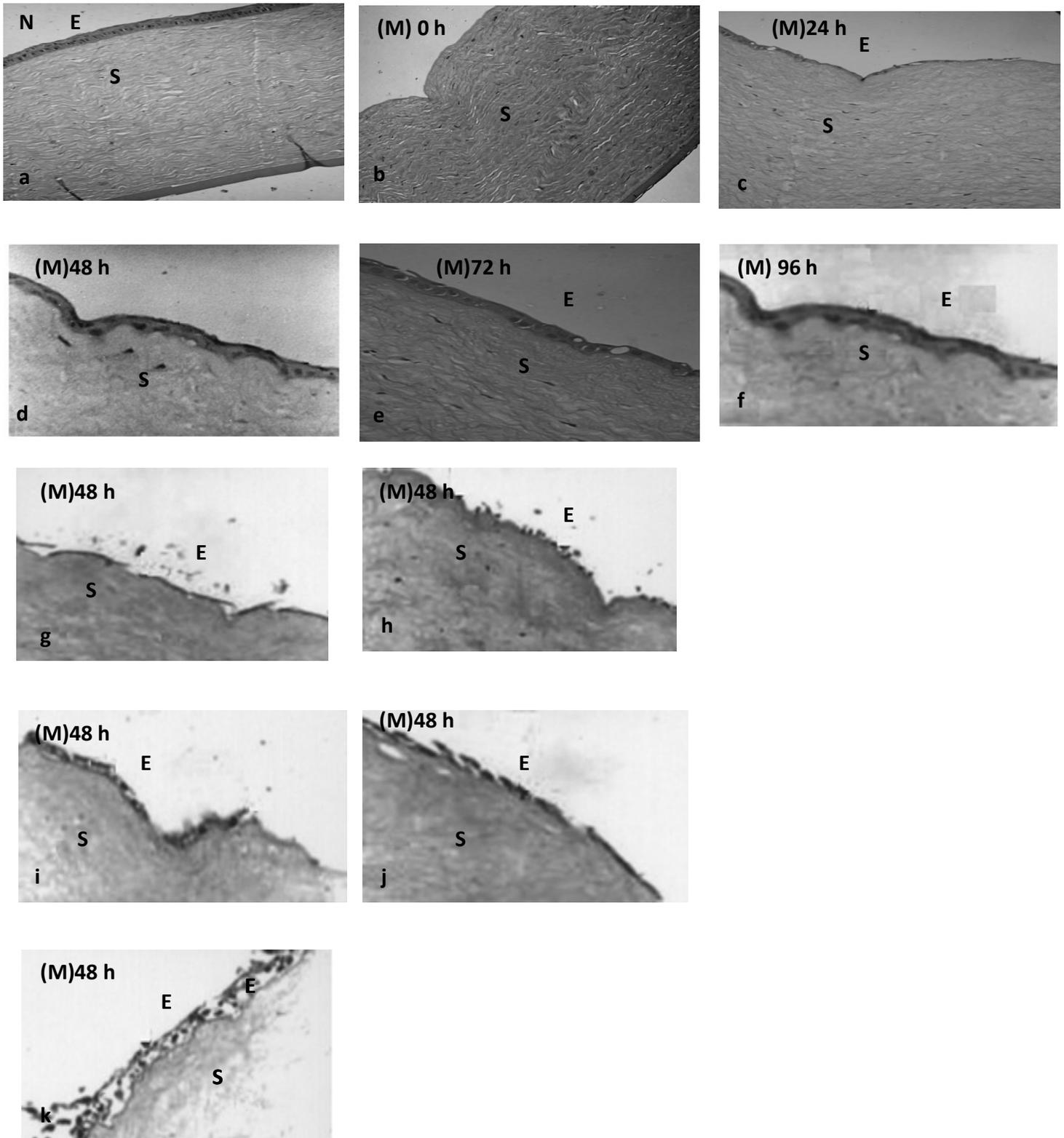


Figure 1. Histological analysis of migrating and non-migrating corneal epithelia at different time intervals 24, 48, 72 and 96 h of post wounding, using haematoxylin-eosin staining a) (N) non-migrating (control) and (M) migrating corneal epithelia b) at 0 h c) 24 h, d) 48 hrs, e) 72 h and f) 96 h, incubation in timolol maleate drop at 48 h g) with 0.005% concentration, h) timolol maleate (P) with BAC 0.001%, i) with timolol maleate drop

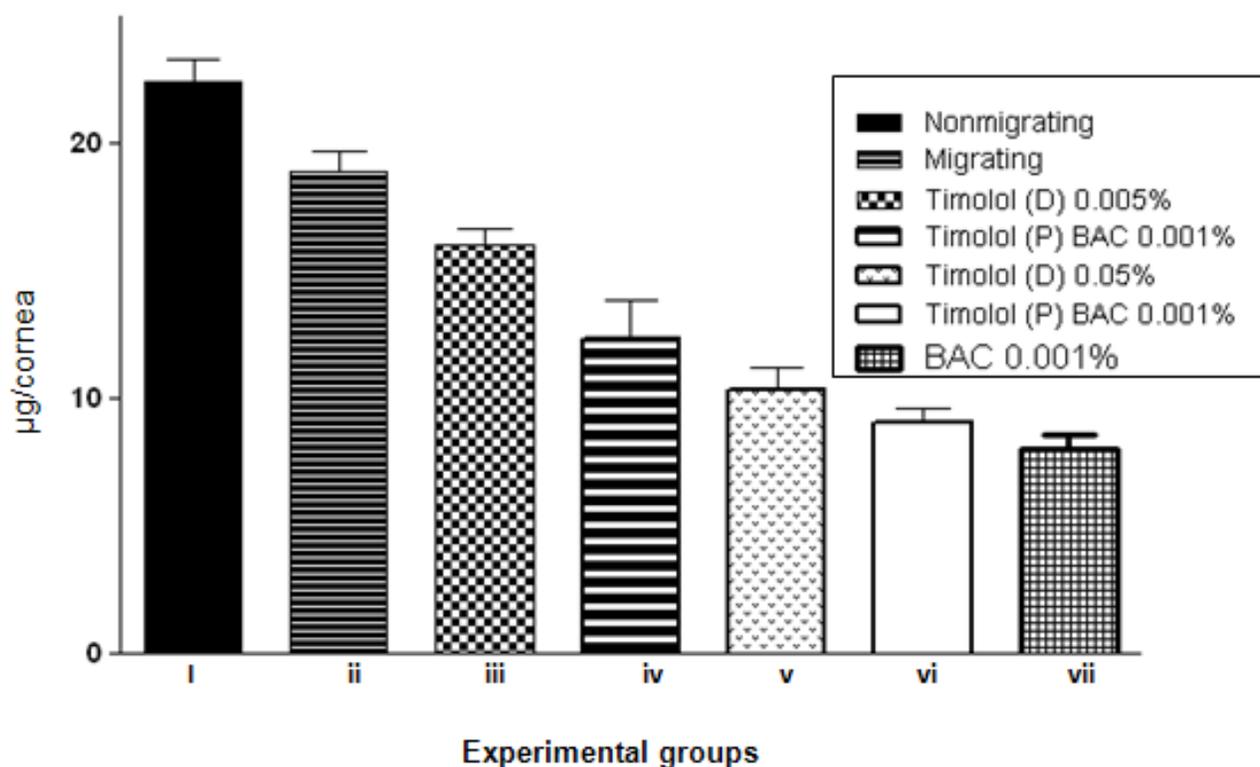


Figure 2. Comparisons of the total µgs of proteins extracted at 48 hrs in i) migrating corneal epithelia ii) migrating iii) timolol maleate (D) treated with 0.005% and iv) (P) with BAC 0.001% v) timolol maleate (D) 0.05%, vi) (P) with BAC 0.001% and vii) only BAC 0.001%. Error bars indicate the standard error of mean. $P > 0.001$. n = number of corneas (n = 10 each). D: drop; P:purified form.

Protein yield

Proteins were extracted with CHAPS extraction buffer after 48 h. The protein yield of the BAC and timol maleate (D) and (P) treated migrating corneal epithelium was expressed as µg of protein/cornea total of 60 corneas (Figure 2). A decrease concentration was seen in the BAC 0.001% treated samples when compared with timolol maleate 0.005% and 0.05% D and P form.

Protein components

CHAPS-extractable proteins (100 µg) were subjected to 10% SDS-PAGE. The proteins were visualized by silver staining (SS). Figure 3 shows the 2D electrophoretic patterns of the treated and control (saline treated) corneal epithelial proteins. The total number of spot (S) obtained after silver staining were approximately 20-22 spots but the differences were seen among 12 proteins spots (Table 2).

Timolol maleate (0.005 and 0.05%) D and P with and without BAC (0.001%) treated samples showed

approximately 20 and 16 bands in the silver stained gels. The molecular weight of the protein components ranged from approximately 20 to 120kd. Table 2 presents the apparent molecular weight and the relative quantity (%) applied in each lane (%) of the components (P1-P12). The 80 kd protein component (P1) with pI 7.8 only present low intensity 0.24% in only BAC treated (0.001%) samples while high intensity was observed in all preparations. However 74 kd (P2) with pI 5.8 absent in BAC treated (0.001%) samples as compared to other timolol maleate BAC treated samples. The 78 kd (P3) with pI 3.5 component was absent in non-migrating samples while observed in migrating as 0.64% and timolol maleate both drops and purified form with BAC treated (0.001%) samples. The 58 kd (P5) component with pI 7.7 only present in migrating and non migrating samples and absent in all other treated preparations, however 60 kd (P9) with pI 3.2 absent in only BAC treated 0.001% samples. The 45 kd (P11) with pI 9.2 was seen only in migrating and non-migrating while 42 kd (P12) with pI 3.1 present only 0.005% timolol maleate drops treated samples.

DISCUSSION

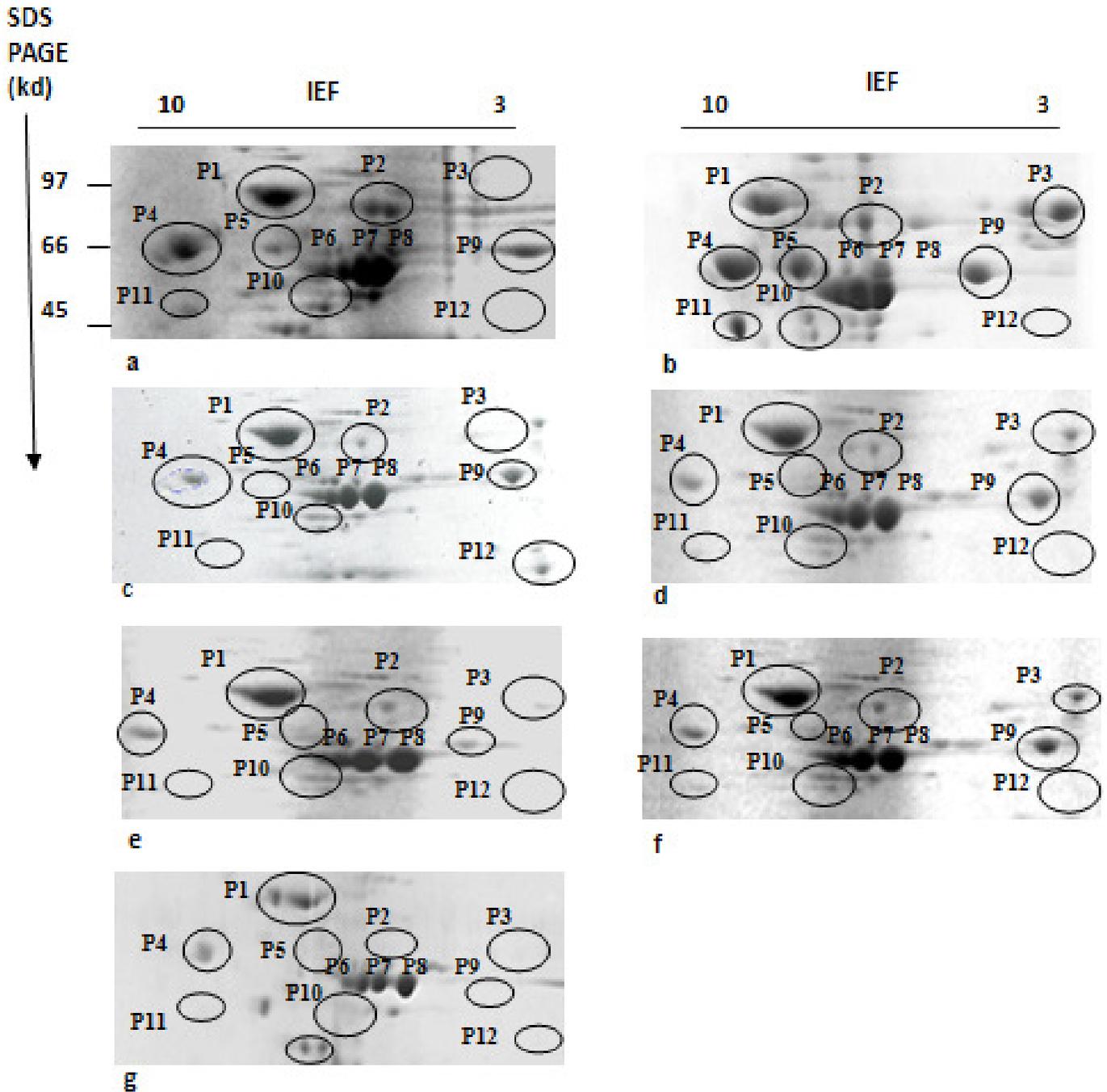


Figure 3. 2-D representative map of CHAPS-extractable proteins of a) non migrating, b) migrating epithelia extracted at 48 hrs of post wounding using pH 3-10 strips after incubation with c) timolol maleate (D 0.005%) only d) timolol maleate (P 0.005%) in combination with BAC (0.001%) e) timolol maleate (D 0.05%) f) timolol maleate with BAC (0.001%), g) only BAC 0.001%.For second dimension 10% polyacrylamide gels were used and silver stained used to visualize the proteins spots.

its purified form on the proteins and corneal epithelial surface of the migrating corneal epithelium have been

studied in corneal organ culture model. Timolol maleate is chosen for two reasons (i) frequent use in the

treatment of glaucoma, a major eye disorder in both developed and developing countries and (ii) debatable conclusions in literature of their effects on corneal epithelial migration. Previous studies that have attempted to analyze the effect of BAC alone on corneal epithelial wound healing have used several different approaches,

Table 2. Protein profile of migrating corneal epithelia treated with timolol maleate with and without BAC analyzed on 2-D gels using Bionumeric software programme.

| Protein spots (P) | Observed Mr/pl | % of spot volume of N | % of spot volume of M | % of spot volume of Timolol maleate (D) 0.005% | % of spot volume of Timolol maleate (P) 0.005% BAC 0.001% | % of spot volume of Timolol maleate (D) 0.05% | % of spot volume of Timolol maleate (P) 0.05% BAC 0.001% | % of spot volume of BAC 0.001% |
|-------------------|----------------|-----------------------|-----------------------|--|---|---|--|--------------------------------|
| P1 | 80/7.8 | 0.76 | 0.64 | 0.78 | 0.69 | 0.75 | 0.77 | 0.24 |
| P2 | 74/5.8 | 0.43 | 0.22 | 0.11 | 0.13 | 0.15 | 0.16 | - |
| P3 | 78/3.5 | - | 0.64 | - | 0.25 | - | 0.34 | - |
| P4 | 67/9.3 | 0.45 | 0.58 | 0.25 | 0.18 | 0.20 | 0.21 | 0.28 |
| P5 | 58/7.7 | 0.12 | 0.54 | - | - | - | - | - |
| P6 | 58/6.6 | 0.43 | 0.53 | 0.41 | 0.44 | 0.46 | 0.45 | 0.21 |
| P7 | 60/6.2 | 0.45 | 0.46 | 0.44 | 0.47 | 0.48 | 0.49 | 0.21 |
| P8 | 62/6.0 | 0.65 | 0.62 | 0.50 | 0.64 | 0.12 | 0.46 | 0.35 |
| P9 | 60/3.2 | 0.15 | 0.52 | 0.35 | 0.45 | 0.10 | 0.25 | - |
| P10 | 48/7.2 | 0.21 | 0.24 | 0.13 | 0.10 | 0.32 | 0.22 | - |
| P11 | 45/9.2 | 0.10 | 0.48 | - | - | - | - | - |
| P12 | 42/3.1 | - | - | 0.24 | - | - | - | - |

Mr: Molecular mass (kd).N: Non-migrating.M: Migrating.pl: isoelectric point.

and are therefore difficult to compare. However, experiments on the long-term effects of BAC have shown strong evidence of toxicity to the ocular surface membrane (Martone et al., 2009; Sosne et al., 2006). This is consistent with the light microscopic observations that show severe thinning of the epithelial layer in samples treated with 0.001% BAC following 48 h of incubation in BAC supplemented media. Exposure of primary cultures of human corneal epithelial cells to a single dose of BAC (0.01%) caused cell retraction, cessation of normal cytokinesis, cell movement, mitotic activity and degeneration of epithelial cells (Tripathi et al., 1992). Since this study was conducted *in vitro*, it was essential to determine the concentration at which the rate of healing of BAC treated samples were similar to the rate of healing of normal migrating corneal epithelium obtained in the organ culture model. It was apparent that at 0.0004% BAC treated samples

(data not shown), no significant change was observed with respect to normal migrating corneal epithelium, when analyzed by light microscopy.

Our previous studies (Mushtaq et al., 2007, 2011) supported that albumin precursor (69 kd) and Hsp70 involved in cell matrix adhesion and play role in cell-cell interaction. In this study we have also demonstrated that preserved and unpreserved timolol maleate inhibited hsp70 and albumin precursor (P2 and P4) while Hsp70 protein was absent in 0.001% BAC treated samples.

The other proteins profile shows the 78, 60 and 42 kd acid protein to be of significance. The two-dimensional patterns of corneas incubated with timolol maleate eye drops and purified (0.005 and 0.05%) show an absence of a 78 kd (P3) and 58 kd (P5) protein with a pl of 3.5 and 7.7, respectively. These protein changes may be related to severe epithelial thinning evident after

48 h of treatment. Unlike the BAC 0.001% treated samples, desquamation is observed at 48 h of incubation. This indicated that timolol maleate exert toxic effects even with and without the preservative. Polat et al (1999) using transmission electron microscopy have shown that levobunolol caused loss of microvilli, increase in glycogen particles, nuclear indentation, widening of the intercellular spaces and cytoplasmic vacuolization in epithelium. The major difference is the absence of a 58kd protein with a pl of 7.7 in all treated samples, however down-regulation of a 80, 58, 62 kd spots at pl 7.8, 6.6 and 6.2 respectively in BAC 0.001% as compared to the timolol treated samples. This suggests that the down-regulation of the specific proteins results in the inhibition of the normal healing rate.

This is consistent with the findings of Baudouin et al. (1998) that showed preservative free group of beta blocker has more potential to surface of

cornea compared to preservative beta blocker.

Conclusion

This study convincingly indicates that following buffering of the adverse effects of BAC, timolol maleate remains a potential hazard for the corneal wound healing. The BAC, which is used as a preservative in almost all beta-blockers, has shown toxicity to the corneal surface. Whether or not BAC is the main factor responsible for ocular surface toxicity remains to be determined. However, these results need to be confirmed *in vivo* for a conclusive study of the consequences of long term use of preservatives and timolol maleate on ocular surface.

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