

# INHIBITORY EFFECTS OF TIMOLOL MALEATE AND ITS PRESERVATIVE BENZALKONIUM CHLORIDE ON CORNEAL EPITHELIAL MIGRATION IN A RABBIT ORGAN CULTURE SYSTEM

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## ABSTRACT

**Purpose:** Timolol maleate, the ophthalmic solutions, is frequently prescribed for glaucoma, although the use of this drop is likely to interfere with wound healing in corneal surgeries. A possible factor that may influence rates of corneal wound healing is how the solutions are preserved. The purpose of this study to evaluate the effect of commercially prepared topical timolol maleate and its preservative benzalkonium chloride (BAC-) 0.0004% and 0.001% or its preservative free effect on the rate of corneal epithelial wound healing. **Methods:** In this study, New Zealand white rabbits corneas (n= 80) were removed and 7mm epithelia were abraded mechanically. The abraded corneal epithelia were incubated in modified supplemental hormonal epithelial media (SHEM) containing a) timolol maleate drop 0.005% and 0.05% 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 hours. Photographic documentation of Richardson stained, non-healed areas, was performed at time points 0, 24, 48, 72, 96 and 120 hours of post wounding. **Results:** The timolol maleate (0.05%) BAC accelerated wound healing and wound closure significantly ( $p < 0.001$ ), compared to controls. Delayed wound healing and wound closure at 120 hours was demonstrated only by timolol maleate drop at both concentrations (0.005% and 0.05%). Corneas treated only with BAC (0.001%) demonstrated delayed healing and wound closure was observed at 120 hours of post wounding. However, timolol maleate with relatively low concentration of BAC 0.0004% did not show any significant effect on wound healing ( $p < 0.05$ ). **Conclusion:** Corneal organ culture model has confirmed that topically applied timolol maleate inhibited corneal wound healing with its preservative BAC, although the appropriate randomized clinical assessment are essential to certify these reports.

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### KEY WORDS

Timolol maleate; benzalkonium chloride, cornea; wound healing

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## [I] INTRODUCTION

Beta blockers eye drops contain preservative to prevent contamination during the treatment period of the patient. Preservatives for ophthalmic solutions include benzalkonium chloride (BAC), chlorobutanol, parahydroxybenzoates and polysorbate.

Timolol maleate has been the drug of choice in the treatment of ocular hypertension and associated glaucoma for over two decades [1-3]. Drug related complications including chronic systemic and ocular side effects as a result of prolonged usage are a major concern [4-8]. Among the ocular side effects, corneal re-epithelialization remains a debatable issue. While

some studies have reported  $\beta$ -blockers to significantly delay corneal epithelial wound healing [9-11], others strongly suggest non-deleterious effects on this phenomenon [12, 13].

One of the leading factors attributed to impaired wound healing following  $\beta$ -blocker administration is the presence of benzalkonium chloride (BAC), a preservative used in almost all  $\beta$ -blocker eye drop preparations. Numerous reports describe the preservative cytotoxicity which has been investigated extensively [16-18] and have revealed BAC to cause a delay in wound closure [14-16]. However it has been documented that

BAC alone does not appear to exert any effect on corneal epithelial wound healing [12].

The first evidence to a biological function for beta blocker in wound healing came from an early study indicating that beta blocker delayed skin wound healing in newt limbs [19]. However subsequent studies in other epithelia yielded conflicting results. For example, it has been reported that beta blocker either delay [10, 20] or enhance [12] corneal epithelial wound healing.

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  $\beta$ -blocker eye drop, timolol maleate and its preservative, BAC, and thereby, abet in the development of a safer therapeutic composition of the drug. Thus, our work uncovers the effects of timolol maleate, a commonly prescribed ophthalmic preparation, on corneal epithelial wound healing following buffering of the effects of BAC. Clearly, further investigation will improve our understanding of the corneal wound healing and hopefully escort to the development of new therapies to enhance wound healing.

## [II] MATERIALS AND METHODS

### 2.1. Animals

New Zealand white rabbits (n= 40) weighing 2-3 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 a University slaughter house within 30 minutes following decapitation.

### 2.2. Wound Model

Rabbit corneal epithelial organ cultures were prepared as described previously [21, 22]. The Albino rabbit eyes were used to prepare migrating (n=60) and nonmigrating (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. Following three washes in saline, 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 1-3 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 5-7 minutes 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 CO<sub>2</sub> incubator; media was replaced every 24 hours. The media containing beta blocker (timolol maleate)/or and BAC was also similarly processed.

### 2.3. Treatment Groups

The rabbit eyes were randomly assigned to one of four treatment group; each group consisted of twenty eyes. One group served as SHEM

treated control. Timolol maleate and its trade names and manufacturers, vehicle, Benzalkonium chloride (BAC) are given in Table- 1.

### 2.4. 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 desired concentrations.

Table: 1. Timolol Maleate and BAC Treatment

Group	Treatment	Con. (%)	Trade Name
1	SHEM (Control)	-	Sigma Aldrich
2	Timolol Maleate (D)	0.005	Remington Pharmaceutical
	Timolol Maleate (P) BAC-	0.005	Sigma Aldrich
	Timolol Maleate (P) BAC+ Timolol Maleate (P) BAC+	0.005+0.0004% 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+ Timolol Maleate (P) BAC+	0.05+0.0004% 0.05+0.001	Sigma Aldrich
4	BAC	0.0004% 0.001%	Sigma Aldrich

### 2.5. Wound analysis

At 0, 24, 48, 72, 96 and 120 hours every cornea in each group was Richardson stained and its size with remaining epithelium defect was measured and photographed. For the statistical analysis, a one way ANOVA was applied. The results were presented as mean  $\pm$  SD and a value of  $p < 0.05$  is considered to be statistically significant.

## [III] RESULTS

Corneas treated with the beta blocker timolol maleate in its drop and preservative free form along with BAC 0.001% showed delayed healing whereas timolol maleate with 0.0004% BAC did not show any significant delay. However, in corneas treated with SHEM (control) wound closure occurred at 72 hour of post wounding. A comparison of data obtained on the corneal epithelial wound healing (48 hours, which is the active phase of migration) rates of all organ culture preparations is summarized in Table-2. At 24 hours there was no difference in the wound healing rates among all the treated corneas.

Table. 2: Rate of Corneal Epithelial Wound Healing at 48 Hours of Post Wounding.

Treatment	Con (%)	Number of eyes	Rate of Healing at 48 hours (mm <sup>2</sup> /hr±SEM)	P-value compared with SHEM treated control	Time for wound closure (hours)	
SHEM	-	6	0.61 ± 0.98	-	72	
Timolol Maleate (D)	0.005	6 each	0.53 ± 1.88	P<0.001	120	
Timolol maleate (P) BAC-	0.005		0.58 ± 1.24		72	
Timolol Maleate (P) BAC+	0.0004		0.56 ± 1.46		72	
Timolol Maleate (P) BAC+	0.001		0.55 ± 1.57		96	
Timolol Maleate (D)	0.05		0.50 ± 0.52		120	
Timolol Maleate (P) BAC-	0.05	6 each	0.58 ± 0.10	P<0.001	72	
Timolol Maleate (P) BAC+	0.0004		0.57 ± 1.47		72	
Timolol Maleate (P) BAC+	0.001		0.52 ± 1.47		96	
BAC only	0.0004		0.59 ± 0.99		P<0.05	96
	0.001		0.48 ± 1.77			120

The corneas treated with beta blocker timolol maleate drop (Remington) 0.005% which contained BAC (0.001%) showed wound closure at 120 hours. Whereas at the same concentration of timolol maleate preservative free BAC- group of corneas showed wound closure at 72 hour. We observed a delay in wound healing and subsequent wound closure at 96 hours (p<0.001) in corneas treated with a combination of 0.001% BAC with 0.005% timolol maleate, whereas 0.0004% BAC with 0.005% timolol maleate did not show any significant difference [Figure- 1].

Corneas treated with timolol maleate drop 0.05% [Figure- 2] and its preservative free form showed wound closure at 120 and 72 hours respectively whereas 0.0004% BAC with 0.05% timolol maleate revealed no significant effect on the rate of healing. Timolol maleate with 0.001% BAC took longer time to heal and the wound closure occurred at 96 hours of post wounding (p<0.001). Similarly only BAC 0.001% treated corneas showed delayed wound closure compared to the control and it also profoundly impedes wound closure at 120 hours [Figure- 3]. However, we observed facilitated wound healing in corneas treated with 0.0004% BAC alone (p<0.05) following wound closure at 96 hours [Figure- 4 A, B, C].

#### [IV] DISCUSSION

Corneal epithelial wound healing following exposure to varying concentrations of the tested commercial ophthalmic solutions timolol maleate was prompted in the absence of preservative (BAC).

Numerous reports described the toxic effect of beta blockers along with their preservative, BAC, disparity in peer-reviewed literature demand more extensive studies to better understand the contribution of each in corneal toxicity [2, 3, 19].

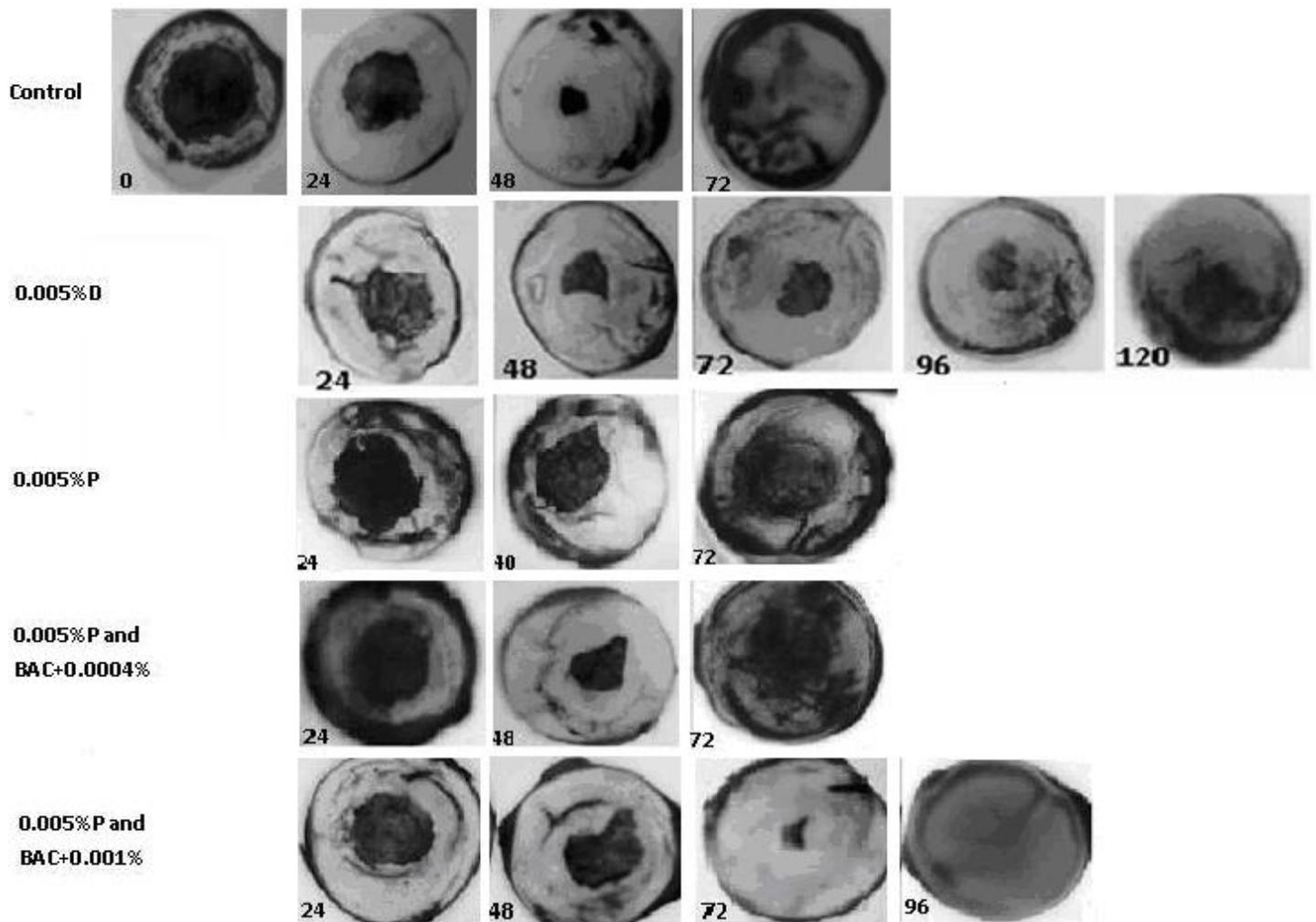
The present study was conducted to investigate the extent of inhibitory effect on corneal epithelial wound healing induced by the commercially available β-blocker, Timolol maleate, following buffering of the effects of its preservative, BAC. These effects on ocular surface tissue may be caused in part by preservatives usually applied with the therapeutic agent [25].

Corneal epithelial healing rates in response to an abrasion in organ cultures were carried out, which is a well established experimental means of evaluating corneal toxicity [26]. In the present organ culture model we considered it appropriate to determine the optimum concentration of BAC at which the rate of healing was similar to that of the untreated controls. This

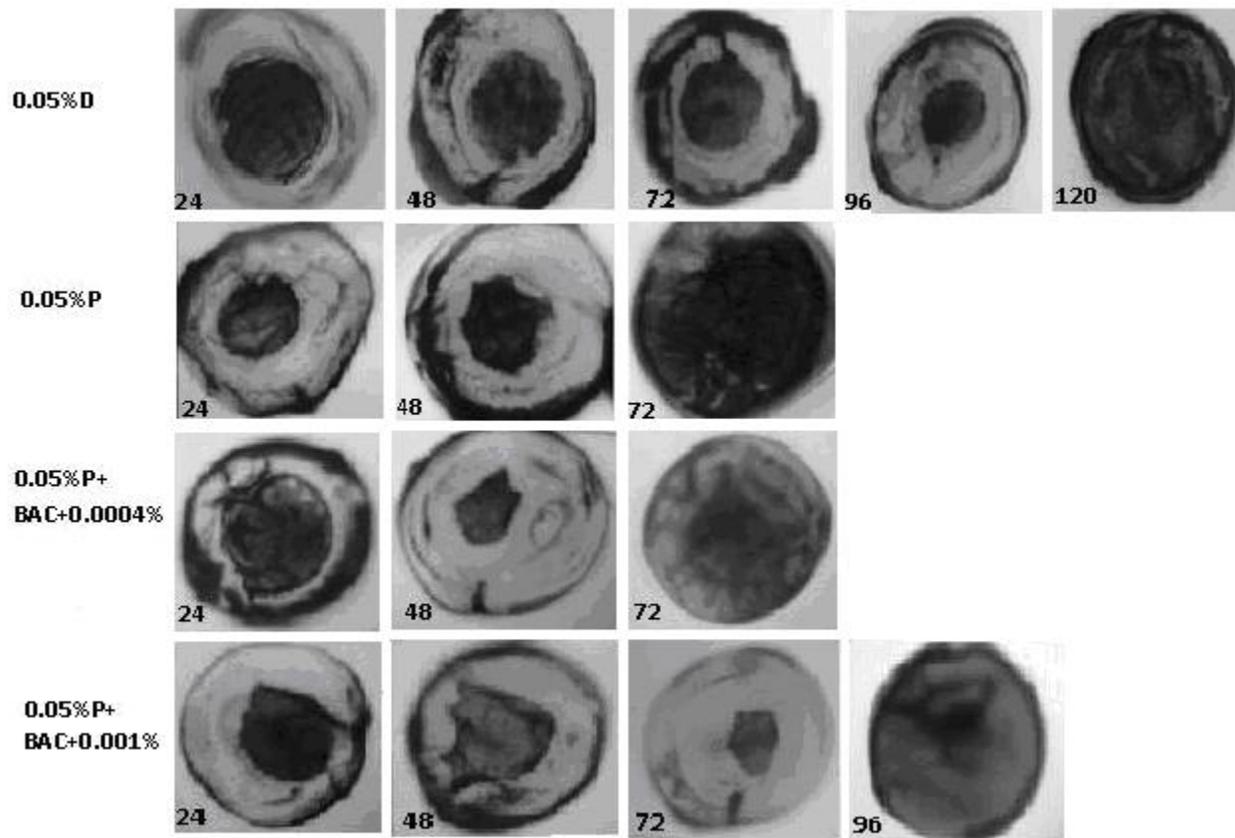
buffering effect was found to occur at a concentration of 0.001% of BAC. On the contrary the same concentration of preservative (0.001%) in the presence of two different concentrations of Timolol maleate, (0.005% and 0.05%) induced a delay in the wound healing rate. Our observations are consistent with a number of studies that have reported  $\beta$ -blockers to inhibit corneal re-epithelialization [10, 11] and BAC to significantly retard wound healing upon prolonged use [10]. Trope et al [9] showed Betagan treated corneas to induce mild superficial epithelial changes with loss of microvilli or slight

desquamation.

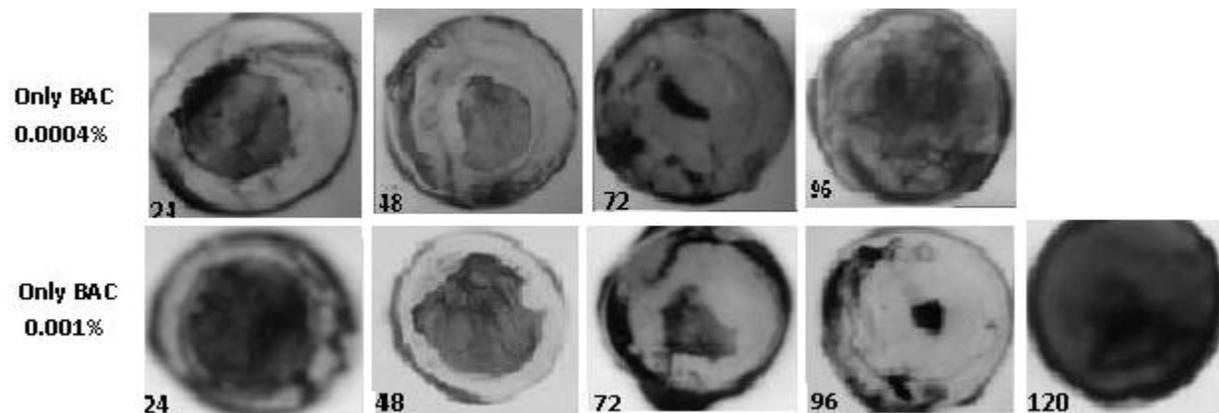
It is noteworthy that levobunolol, the  $\beta$ -blocker by itself has shown little effect on the corneal epithelial surface [13] and additionally enhanced the rate of wound healing [12]. Interestingly, BAC, which was found to disrupt the integrity of the outermost corneal epithelial layers, enhanced the transcorneal levobunolol flux and reduced its extent of metabolism [26]. This may provide an explanation for the combined toxic effect of BAC and levobunolol.



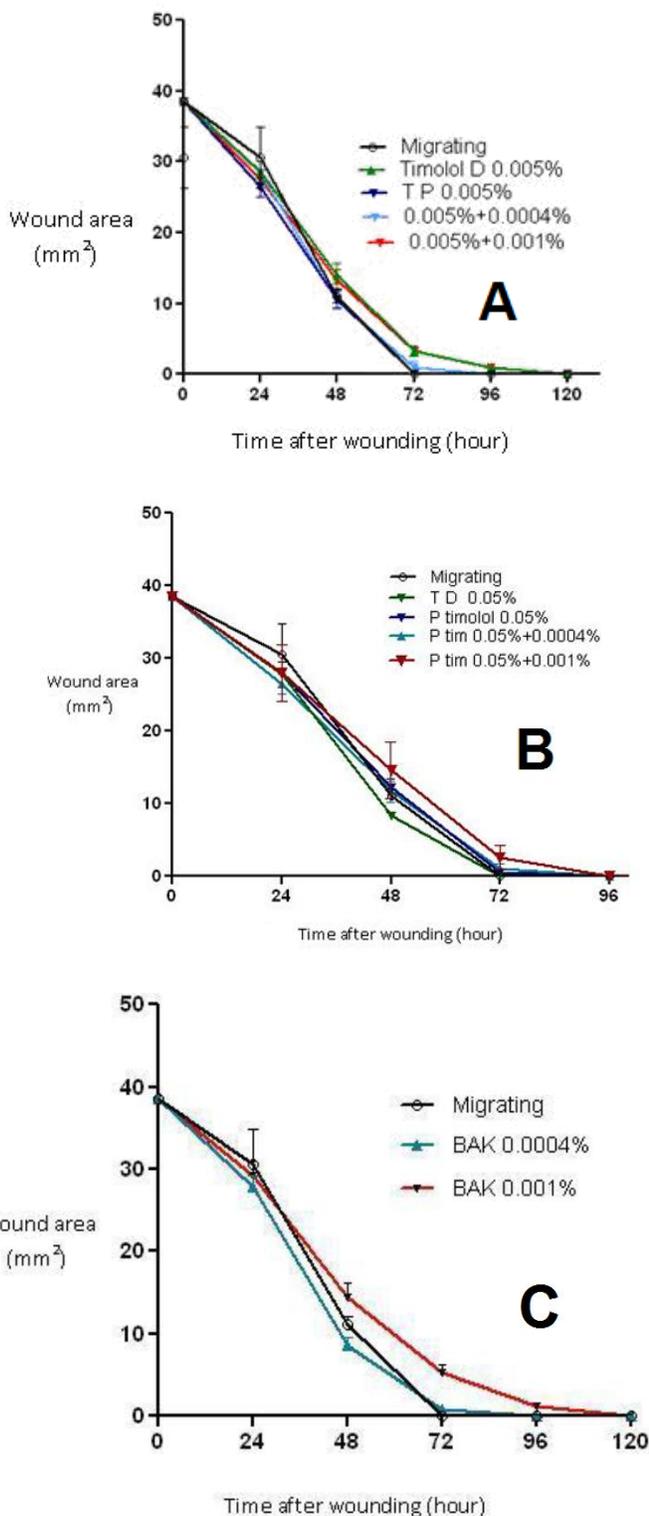
**Fig. 1:** Corneal epithelia treated with timolol maleate (Drop 0.005% and BAC- 0.005% with combination of BAC+ 0.0004% and 0.001% concentration). Wound healing was observed at different time interval represents 24, 48, 72, 96 and 120 hours. Scraped cornea after harvesting of epithelium 0 hour, arrows indicates 7 mm scraped region. At 72 hours, complete wound closure occurred at this time point in control. Corneas visualized with Richardson stain show the migrating epithelial cell from the wound margin to the center of wound.



**Fig. 2:** Corneal epithelia treated with timolol maleate (Drop 0.05% and BAC- 0.05% with combination of BAC+ 0.0004% and 0.001% concentration). Wound healing was observed at different time interval represents 24, 48, 72, 96 and 120 hours. At 72 hours, complete wound closure occurred at this time point in control. Corneas visualized with Richardson stain show the migrating epithelial cell from the wound margin to the center of wound.



**Fig. 3:** Corneal epithelia treated with only BAC 0.0004% and 0.001% were analyzed for wound healing at different time interval represents 24, 48, 72, 96 and 120 hours of post wounding. Corneas visualized with Richardson stain show the migrating epithelial cell from the wound margin to the center of wound.



**Fig. 4:** In vitro wound healing of timolol maleate treated corneal epithelia with (A) drop 0.005%, BAC- and BAC+ 0.0004% and

0.001%. (B) Timolol maleate 0.05% BAC- and BAC+ 0.0004% and 0.001% concentrations (C) only BAC 0.0004% and 0.001% with control (without treatment) normal migrating corneal epithelia in organ culture. This shows average epithelial wound area at 24, 48 and 72 hours after wounding. The figure shows the phases of wound healing 0-24, 24-48, 48-72, 72-96 and 96-120 hours. Time to complete wound closure is seen at 72 hours in control group (without treatment). Each value represents six corneas and error bar indicates SEM.

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 inhibition in the rate of healing of the corneas [Figure- 4 A, B, C]. In the light of our results it is doubtful whether the concentrations to which the corneal epithelium is exposed to beta blockers (containing preservative) after surgeries in the common clinical therapeutics doses should be continued. Further investigations of individual components of beta blockers are necessary to fully understand ophthalmic solution cytotoxicity

#### FINANCIAL DISCLOSURE

This work is not supported by any grant.

#### CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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