Corneal Alternations Induced by Topical Application of Benzalkonium Chloride in Rabbit

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Abstract

Benzalkonium chloride (BAC) is the most common preservative in ophthalmic preparations. Here, we investigated the corneal alternations in rabbits following exposure to BAC. Twenty-four adult male New Zealand albino rabbits were randomly divided into three groups. BAC at 0.01%, 0.05%, or 0.1% was applied twice daily to one eye each of rabbits for 4 days. The contralateral untreated eyes were used as control. Aqueous tear production and fluorescein staining scores of BAC-treated eyes were compared with those of controls. The structure of the central cornea was examined by in vivo confocal microscopy. Expression of mucin-5 subtype AC (MUC5AC) in conjunctiva was detected by immunostainig on cryosections. Corneal barrier function was assessed in terms of permeability to carboxy fluorescein (CF). The distribution and expression of ZO-1, a known marker of tight junction, and reorganization of the perijunctional actomyosin ring (PAMR) were examined by immunofluorescence analysis. Although there were no significant differences between control and BAC-treated eyes in Schirmer scores, corneal fluorescein scores and the number of conjunctival MUC5AC staining cells, in vivo confocal microscopy revealed significant epithelial and stromal defects in all BAC-treated corneas. Moreover, BAC at 0.1% resulted in significant increases in central corneal thickness and endothelial CF permeability, compared with those in control eyes, and endothelial cell damage with dislocation of ZO-1 and disruption of PAMR. Topical application of BAC can quickly impair the whole cornea without occurrence of dry eye. A high concentration of BAC breaks down the barrier integrity of corneal endothelium, concomitant with the disruption of PAMR and remodeling of apical junctional complex in vivo.

Introduction

Preservatives are a major component of the ophthalmic preparations to provide antimicrobial activity and prevent decomposition of the active drug in multi-dose bottles. Benzalkonium chloride (BAC) is the most common preservative in ophthalmic preparations for its apparently good safety/efficacy profile [1]. Furthermore, as a soluble antimicrobial agent and cationic surfactant, BAC was found to have the ability to enhance penetration of active compounds [2,3]. However, a large number of experimental and clinical studies have shown that long-term use of topical drugs with BAC may induce ocular surface changes, causing ocular discomfort, tear film instability, loss of goblet cells, inflammation, conjunctival squamous metaplasia, epithelial apoptosis, subconjunctival fibrosis and the potential risk of failure for further glaucoma surgery [1].

BAC is most often used at a concentration of 0.01% (ranging from 0.004% to 0.02%) in ophthalmic preparations [4]. As long-term clinical testing with low concentration of BAC is difficult to conduct on animals [5], many researchers have investigated acute toxic effects of high concentrations of BAC (approximately 1-50 times the commercial concentrations) on the ocular surface. In 1944, Swan found that BAC causes punctate disturbances to corneal epithelium at concentration of 0.04%, and edema and cellular desquamation with corneal lesions at a concentration of 0.1% [6]. Since then, much clinical and experimental evidence has been obtained to support the notion that the toxic effect of BAC on the ocular surface is primarily related to its concentration. Recently, Liang et al. showed that following short- and repeated exposure to 0.02% BAC, a large number of inflammatory cells were present in rabbit corneal epithelial basal layer and stroma [7]. Although a few studies have proven that topical application of high concentration of BAC (0.25% or 0.5%) induced corneal endothelial cell edema, even disappearance [5,8], in vivo toxic effect of BAC on the barrier function of corneal endothelium remains unclear.

Aqueous humor movements into the cornea are required as a source of nutrition for cells residing within the corneal stroma. The corneal stroma has a tendency to swell due to the presence of non-diffusible, negatively charged molecules such as hydrophilic glycosaminoglycans. The corneal endothelium is thought to be solely responsible for the maintenance of stromal deturgescence, which is prerequisite for corneal transparency [9,10]. In healthy cornea, the endothelium forms a semi-permeable barrier that regulates fluid and solute exchange between the nutrient-rich aqueous humor and avascular corneal stroma, and tight junctions are an integral component of this barrier [10–12]. The tight junctions of the corneal endothelium are focally present around endothelial cells and serve to prevent the movement of aqueous humor from anterior chamber into corneal stroma. The tight
Aqueous Tear Production

Methods

Aqueous Tear Production

The use of this microscope has been described previously [17]. A drop of carboxer gel (Alcon Laboratories, Fort Worth, TX) was applied as coupling medium between the applanating lens and the cornea. A diode laser is used as a light source with a wavelength of 670-nm. The objective of the microscope is immersion lens (Olympus, Hamburg, Germany), magnification × 60, which is covered by a polymethyl methacrylate cap. Images consist of 384 × 384 pixels, allowing a scanning area of 400 µm² with lateral and vertical resolutions of both 1 µm and a magnification up to 800 times. The center of the cap was applanated onto the central cornea by adjusting the controller, and in vivo digital images of the cornea were visualized directly on the computer screen. The central cornea was examined and more than 10 images were taken for each of the following structure: superficial and basal epithelium, stroma, and endothelium. The mean central corneal thickness was calculated based on the depth difference between the most superficial epithelium and the endothelium and was recorded as the average of a minimum of three individual acquisitions. All measurements were performed by a single investigator masked to the specific experimental conditions. At the end, the epithelial cell size, epithelial basal cell and endothelial cell density were automatically calculated using the tomography-associated software. Cell density was recorded as cells per square millimeter. Based on 10 images, the means and standard deviations were calculated for each parameter.

Measurement of Permeability to Carboxy fluorescein (CF)

Corneal epithelial barrier function was evaluated on the basis of measurement of corneal permeability to CF (0.3%, Cohasset, MA). The fluorophotometric methods were modified for rabbits from a previous report in rat [18]. In brief, 10 minutes after 40 µL of CF was applied to each ocular surface, the animals (n = 3 per group) were euthanized with overdose of pentobarbital sodium and cornea were excised. Each cornea was washed 3 times in 1mL of balanced salt solution (BBS; Alcon Laboratories, Fort Worth, TX) for 5 minutes per wash, and places in a tube with a tube with 1mL BBS. Each tube was wrapped in aluminum foil to protect the solution from light and placed on an orbital shaker for 90 minutes. The concentration of CF (nmol/µL) was measured by using a Gilford Fluoro IV fluorometer (Corning, Oberlin, OH).

Under a surgical microscopy, aqueous humor (0.1ml) was removed from the anterior chamber with 33G NanoFil Injector System (World Precision Instruments, Sarasota, FL, USA) through the lateral corneal limbus. Twenty minutes after 0.1 µL of CF was injected into the anterior chamber, the animals (n = 3 per group) were euthanized. The corneas were excised, and endothelial permeability to CF was measured as described above.

Immunofluorescein Staining

After in vivo examinations, rabbits (n = 5 per group) were euthanized after intravenous injection of a lethal dose of pentobarbital sodium. The eyes (including bulbar conjunctiva) were enucleated, and the nasal and temporal bulbar conjunctivias were snap-frozen in liquid nitrogen and cut into 8-µm-thick sections. Sections were fixed in acetone for 10 minutes at 4°C. After washing in phosphate-buffer saline (PBS), the sections were incubated with 1% bovine serum albumin (BSA) to block nonspecific binding, and then overlaid with a 1:50 dilution of mouse anti-rabbit MUC5AC antibody (Abscam, Cambridge, UK) overnight at 4°C. The second day, the sections were washed with PBS, incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cell Signaling Technology, Inc., Danvers, MA) for 1 hour at 4°C, followed by three washes, with three
washed in PBS and nuclei counterstaining with 1:100 dilution of Hoechst 33342 dye (Sigma).

Under a dissecting microscope (Model SZ40; Olympus, Tokyo, Japan), the retina, lens, and iris were discarded and four incisions were made in each cornea. Each cornea was fixed in situ in PBS with 4% paraformaldehyde for 5 minutes, and then was permeabilized with acetone for 3 minutes at -20°C. Subsequently, the corneas were washed in PBS with 1% Triton X-100 and 1% dimethyl sulfoxide (DMSO; TD buffer) as described previously [19,20]. To block nonspecific staining, tissue blocks were incubated in 1% BSA diluted in TD buffer for 1 hour. Then, the tissues were placed in 1:100 of mouse anti-rabbit ZO-1 antibody (Zymed, Carlsbad, CA) for 8 hours with agitation at 4°C. Next, the tissues were washed with TD buffer, placed in Alexa Fluor 488-conjugated secondary antibody (1:500 dilution; Molecular Probes; Eugene, OR) for 1 hour at 4°C. After distilled water wash, the whole-mount cornea tissues were mounted endothelial side up on a slide and stained with a nuclear fluorescence dye, 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame CA).

For PAMR staining, the whole mount corneas were incubated in 1:1000 of Texas-red conjugated phalloidin (Molecular Probes, Eugene, OR) overnight with agitation for 4 hours at 4°C.

**Statistical Analysis**
Quantitative data are presented as mean ± SD and were analyzed by Dunnett multiple comparison test. P < 0.05 was considered statistically significant.

**Results**

**Aqueous Tear Production and Fluorescence Staining**
There was no statistically difference between the BAC-treated and control eyes in aqueous tear production (P > 0.1) (Fig. 1A). In addition, no substantial fluorescein staining was detected in the eyes treated with BAC, even at the highest concentration (Fig. 1F).

**In Vivo Confocal Microscopy Analysis**
The corneal superficial epithelial cells of control rabbits had a polygonal mosaic appearance with brightly reflective nuclei.
endothelial change, with presence of hyporeflective spots at many
0.1% BAC treated rabbits exhibited significant abnormal
damage on the endothelial cells (Fig. 5B, 5C). However,
ance, with some larger hexagonal cells also apparent (Fig. 5A).
structures in both groups were decreased compared with 0.01%
of normal cell borders. The numbers of membrane bridge-like
objects against a dark background. Hyperreflective membrane
bridge-like structures (<0.5 μm diameter) were occasionally
and some of these structures formed distinct intercellular
bridge between two or more cells (Fig 4A, arrow). These highly
distinctive intercellular membrane bridge-like structures were
generally short and straight and largely resemble membrane
nanotubes observed in the mouse cornea.21 The abundance
of these structures was significantly increased from 10.1±1.1 per
square millimeter in the control to 160±15.8 per square
millimeter in the cornes treated with 0.01% BAC, suggesting
that they play an important role in vivo in cell-cell communication
during the BAC-induced inflammation (Fig. 4B, 4E). The 0.05% and
0.1% BAC induced inflammatory cell infiltration in the basal
epithelium. The inflammatory cells were counted, giving densities
of 560.3±28.3 and 368.2±16.5 cells/mm² in the eyes treated with
0.05% BAC and 0.1% BAC, respectively. At both of the
concentrations, the number of basal cells was significantly higher
than that of control (Fig. 3C-E).

In control anterior stroma, keratocyte nuclei were seen as bright
objects against a dark background. Hyperreflective membrane
bridge-like structures (<0.5 μm diameter) were occasionally
observed, and some of these structures formed distinct intercellular
bridge between two or more cells (Fig 4A, arrow). These highly
distinctive intercellular membrane bridge-like structures were
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nanotubes observed in the mouse cornea.21 The abundance
of these structures was significantly increased from 10.1±1.1 per
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millimeter in the cornes treated with 0.01% BAC, suggesting
that they play an important role in vivo in cell-cell communication
during the BAC-induced inflammation (Fig. 4B, 4E). The 0.05% and
0.1% BAC induced whole stromal disorganization, with loss
of normal cell borders. The numbers of membrane bridge-like
structures in both groups were decreased compared with 0.01%
BAC treated rabbits, and yet still significantly higher than in the
control (Fig. 4C-E).

The control endothelial cells had a polygonal mosaic appearance,
with some larger hexagonal cells also apparent (Fig. 3A). Application of 0.01% and 0.05% BAC seemed not to inflict
significant damage on the endothelial cells (Fig. 3B, 3C). However,
0.1% BAC treated rabbits exhibited significant abnormal
endothelial change, with presence of hyporeflective spots at many
cell-cell borders (Fig. 5D, arrow). Cell density in the endothelium
was not significantly affected by BAC treatment (Fig. 5E).

We also measured central corneal thickness in the BAC-treated
and control eyes. In control eyes, the mean central cornea
thickness was 355±6 μm. Central corneal thicknesses was
significantly increased by 57.2% in eyes that received 0.1% BAC, but BAC at lower concentration did not induce significant
change in mean corneal thickness (Fig. 6A). These data thus
suggested that higher BAC concentration leads to corneal edema
as a result of a loss of corneal endothelial barrier function.

Permeability Measurement
As shown in Figure 6B, treatment with BAC significantly
increases epithelial permeability, compared with untreated
control. Endothelial permeabilities measured for control versus
0.1% BAC treated cornes equaled 16 ± 5.2 mmol/μL and 125 ±
14.7 mmol/μL. These values were significantly different
(P<0.001). In contrast, there was no significant difference of
dermal CF uptake between control and lower BAC concen-
tration treated cornes (Fig. 6C).

Immunofluorescence Staining Analysis
Immunofluorescence staining of conjunctival tissues with
MUC5AC antibody revealed that MUC5AC positive cells were
located in the nasal and temporal bulbar conjunctivas of BAC-
treated and control eyes (Fig. 1C, 1D). Statistical analysis showed
no significant difference between the BAC-treated and control
eyes in the density of conjunctival MUC5AC positive cells
(Fig. 1B).

We next examined the effect of BAC on the expression of ZO-1
in the corneal epithelium. In the control cornes, ZO-1 is localized
contiguously at the superficial cell-cell boundaries and accordingly
stained uniformly at the cell borders (Fig. 7A). In contrast, ZO-1
staining was patchy and discontinuous in eyes treated with BAC
(Fig. 7B-D). These observations thus suggested that BAC induced
disruption of the localization of ZO-1 and loss of superficial cells in
many areas of the epithelium.

To further corroborate presence of significant damage on
the endothelial cell-cell borders, as revealed by in vivo confocal
microscopy, we next examined the organization of the AJC. As
shown by confocal images in Figure 8, a continuous linear pattern
of ZO-1 staining at the boundaries of adjacent endothelial cells
was found in the control eyes (Fig 8). In both the 0.01% and
0.05% BAC-treated eyes, the pattern of ZO-1 distribution is

![Figure 2. Toxic effect of BAC on the morphology of rabbit corneal epithelial superficial cells.](image-url)

Representative in vivo confocal images of the corneal epithelium in different groups. (A) Untreated control. (B) 0.01% BAC. (C) 0.05% BAC. (D) 0.1% BAC. Mean cell size at the epithelial surface was shown in (E). Note that the size of surface cells in the corneal epithelium of eyes treated with 0.1% BAC was significantly larger than that of control eyes. Data are mean ± SD of values from eight eyes per group. ** P<0.01 (Dunnett test).

doi:10.1371/journal.pone.0026103.g002
similar to that of control (data not shown). A contiguous distribution of ZO-1 at intercellular border was found to be retracted and dislocation from the cell-cell border in eyes treated by 0.1% BAC, as evidenced by discontinuities in ZO-1 localization (Fig. 8B, arrow). These observations thus suggested that high BAC concentration could disrupt the TJs in the rabbit corneal endothelium in vivo.

We also investigated remodeling of the PAMR in response to BAC in vivo. PAMR is formed by the actin cytoskeleton at the AJC, and is structurally and functionally coupled to cytoplasmic domains of TJs and AJs. In control and BAC at low concentration treated eyes; F-actin formed a characteristic dense bend of PAMR (Fig 8C). In contrast, PAMR compacted into a contractile ring in 0.1% BAC treated eyes (Figure 8D).

Discussion

BAC is the most frequently used preservative in multi-dose eyes drops for its apparently good safety/efficacy profile. However, the generally good bactericidal outcomes have tended to overshadow the significant number of patients who undergo ocular discomfort after long-term use. Therefore, prophylaxis and treatment of toxic reactions associated with BAC have become important clinical issues. In the present study, we utilized an animal model to examine the toxic effect of BAC on the whole cornea. Our choice of different concentrations of BAC in this experiment was based on clinical practice and previous experimental studies. BAC is most often used in ophthalmic preparations at a concentration of 0.01% [4]. BAC at 0.1% was found to cause corneal edema [6] and was used to develop a rabbit dry eye model [21]. The 0.05% BAC concentration was added in this set of experiments, so as to better characterize the changes in the cornea found between the 0.01% and the 0.1% BAC concentrations.

We have now shown that topical application of BAC, even at a low concentration, breaks down rabbit corneal epithelial barrier function, as revealed by measurement of permeability to CF. ZO-1, which is a marker of tight junction of corneal epithelium, is contiguous at superficial cell-cell border [22,23], and its dislocation...
is an index of the loss of tight junction integrity [24,25]. In this study, we found that in control eyes, ZO-1 distribution was contiguous at the cell-cell borders at the surface of the rabbit corneal epithelium. In contrast, in BAC treated corneas, ZO-1 immunoreactivity was patchy and discontinuous in the superficial epithelium. Recent studies have proven that exposure to BAC induce a continuous decline in corneal transepithelial electric resistance in rabbit [26,27]. Our study extends these finding and confirms that topical application of BAC disrupt the tight junctions between superficial cells in the rabbit corneal epithelium in vivo. We found that the highest BAC concentration used here (0.1%) not only resulted in a significant increase in the size of cells at the surface of the corneal epithelium, but also induced maximum corneal surface barrier damage, as shown by the severe CF uptake. These findings suggest that BAC at high concentration causes corneal surface epithelial cell edema and more adversely affect the barrier integrity of the corneal epithelium.

Our results showed a dose-response relationship for BAC-treated corneal epithelial basal layer, with low (0.01%) BAC concentration inducing basal cell edema, whereas the high (0.05% and 0.1%) BAC concentrations caused inflammation and increase in cell density. Liang et al reported that topical repeated application of BAC induced important inflammatory cell infiltration in rabbit corneal epithelial basal layer and stroma [7]. In 3D-reconstituted corneal epithelium, Pauly et al found that the number of proliferative cells increased after BAC treatment [28]. Our results were consistent with these studies, showing that topical application of BAC quickly impairs the corneal epithelium.

Membrane nanotubes that connect two or more cells have been found in vitro in a number of cells types including rat pheochromocytoma cell lines, normal rat kidney cells [29], and primary cultures of dendritic cells (DCs), macrophages, human peripheral blood NK cells, and B cells [30]. Recently, long and fine (<0.8 μm in diameter) membrane nanotube-like structures on bone marrow-derived major histocompatibility (MHC) class II+ DCs have been documented in the corneal stroma of normal mouse. The frequency of these nanotubes was significantly increased in corneas subjected to trauma and LPS, suggesting that these structures play an important role in vivo in cell-cell communication between widely spaced dendritic cells during inflammation [31]. Using in vivo confocal microscopy, we found that a few of long and hyporeflective membrane bridge-like structures (<0.5 μm in diameter) were distributed in normal corneal anterior stroma. Four days after application of 0.01% BAC, the number of membrane bridge-like structures in anterior central stroma increased from 10.1±1.1 per square millimeter to 160±15.8 per square millimeter. Such a rapid and significant modulation of membrane bridge-like structures density suggests that they serve as critical structures in BAC at low concentration inducing corneal pathological changes. We found that the density of

![Figure 5. Toxic effects of BAC on the morphology of the rabbit corneal endothelium.](image)

![Figure 6. Toxic effects of BAC on central corneal thickness and CF uptake in the rabbit.](image)
ZO-1 in the corneal endothelium.

Figure 7. Toxic effects of BAC on localization of ZO-1 in the rabbit corneal epithelium. Corneal tissue blocks prepared from a control eye (A) or from eyes treated with 0.01% (B), 0.05% (C) or 0.1% BAC (D). ZO-1 staining was observed as a continuous linear pattern along with superficial cell-cell borders in normal rabbit corneal epithelial cells. In contrast, ZO-1 staining was patchy and discontinuous in eyes treated with BAC (B, C, and D). Note that BAC induced disappearance of ZO-1 in a concentration-dependent manner. doi:10.1371/journal.pone.0026103.g007

Figure 8. Toxic effects of BAC on the PAMR and distribution of ZO-1 in the corneal endothelium. (A, C) Untreated control eyes. (B, D) 0.1% BAC treated eyes. In untreated eyes, ZO-1 distribution is contiguous at the endothelial cell-cell border (A) and the characteristic organization of cortical actin with intact PAMP is observed (C). In contrast, BAC at 0.1% induced the dispersion of ZO-1(B), as evidence by discontinuities in ZO-1 distribution (C, arrow) and the disruption of PAMR (D). doi:10.1371/journal.pone.0026103.g008

These structures were less increased in the eyes treated with 0.05% and 0.1% BAC, probably because high concentration primarily caused deep tissue damage. To our knowledge, we are the first group to report the existence of the long and hyporeflective structures in the central corneal stroma of the group to report the existence of the long and hyporeflective caused deep tissue damage. To our knowledge, we are the first group to report the existence of the long and hyporeflective structures in the central corneal stroma of the rabbit eye. Further studies are needed to determine whether the toxicity of BAC could induce significant corneal stromal alterations. Our results, taken together, indicate that the rabbit cornea is very sensitive to the toxicity of BAC. Therefore, the direct toxic effect on the whole cornea should be taken into account if BAC is used to establish an animal dry eye model.

Paul et al. performed a series of toxicological assessments in the rat eye after instillations of BAC [8]. They found that BAC at low concentrations (0.01% and 0.1%) induced damage restricted to the the epithelium, whereas the highest concentrations (0.25% and 0.5%) caused epithelial denudation as well as major stromal and endothelial damage. In our study, in vivo confocal microscopy revealed destructuring of the corneal stroma with presence of a lot of membrane bridge-like structures in eyes treated by lower (0.01% and 0.05%) concentration of BAC. Moreover, 0.1% BAC induced significant corneal endothelial cell defects. The reason for these discrepancies is not immediately clear; one possible explanation is due to species differences.

An important function of the corneal endothelium is to provide a barrier to the aqueous humor movements. The tight junction plays an important role in the establishment and maintenance of the barrier function of the endothelium [32,33]. The tight junctions of corneal endothelium can be disrupted as a result of aging, inflammatory reaction, iatrogenic injury, and genetic disorders such as Fuch’s dystrophy [34]. Our data suggest that topical application of high BAC concentration can induce loss of barrier integrity in the rabbit corneal endothelium in vivo. There are several lines of evidence to support this notion: First, application of 0.1% BAC, but not that of lower BAC concentrations, resulted in significant increases in central corneal thickness and endothelial permeability, compared with those in control eyes (P<0.01). This finding means that high BAC concentration can lead to significant corneal edema as a result of a loss of corneal barrier function. Second, in vivo confocal microscopy revealed presence of hyporeflective spots at many endothelial cell-cell borders in 0.1% BAC treated eyes, indicating that high BAC concentration inflict significant damage on the endothelial cell junctions. Third, in control and low concentrations of BAC-treated eyes, ZO-1 expression exhibited a continuous linear pattern of at cell-cell boundaries in the endothelium, whereas in eyes treat with 0.1% BAC, ZO-1 immunoreactivity was discontinuous, indicating the disruption of tight junctions. Moreover, 0.1% BAC induced the compaction of PAMR into a contractile ring. Taken together, the present study clearly shows that topical application of high concentration of BAC can not only damage the corneal epithelium and stroma, but also break down the barrier integrity in the rabbit corneal endothelium in vivo.

In summary, our study demonstrated that topical application of BAC can quickly impair the whole cornea without occurrence of dry eye. BAC at high concentration induces disruption of the barrier integrity in the corneal endothelium. These findings may...
provide additional information for our understanding of the toxic effect of BAC on the cornea.

Acknowledgments

The authors thank Hui He for her technical support.

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Author Contributions

Conceived and designed the experiments: WC Z.Li. Performed the experiments: WC Z.Li JH ZZ LC YC. Analyzed the data: WC Z.Li. Contributed reagents/materials/analysis tools: WC Z.Li. Wrote the paper: WC.