Sodium Hyaluronate Decreases Ocular Surface Toxicity Induced by Benzalkonium Chloride–Preserved Latanoprost: An In Vivo Study

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PURPOSE. To investigate the effects of sodium hyaluronate (SH) on ocular surface toxicity induced by benzalkonium chloride (BAC)-preserved latanoprost.

METHODS. Twenty-one white rabbits (42 eyes) were randomly divided into three groups. The control group was untreated. The two experimental groups were treated with 0.02% BAC-containing latanoprost once a day combined with unpreserved 0.3% SH or PBS three times daily for 60 days. Schirmer test, fluorescein and rose bengal staining, and conjunctival impression cytology were performed on days 0, 31, and 61. Apoptosis of conjunctival epithelium was detected by TUNEL assay on day 61. Conjunctival inflammation was evaluated with light microscopy. Cornea and conjunctiva ultrastructure were observed by electron microscopy.

RESULTS. Compared with the control group, the PBS-treated latanoprost group showed increases in fluorescein and rose bengal scores, decreases in Schirmer scores, and goblet cell density (GCD) on days 31 and 61. Increases in inflammatory and apoptotic cells in conjunctiva, and ultrastructural disorders of the cornea and conjunctiva were also observed on day 61. Compared with the PBS-treated latanoprost group, the SH-treated latanoprost group showed decreases in fluorescein and rose bengal scores, and increases in Schirmer scores and GCD on days 31 and 61. Decreases in inflammatory and apoptotic cells in conjunctiva and amelioration of ultrastructural disorders were also observed.

CONCLUSIONS. Topical application of SH significantly decreased the ocular surface toxicity induced by BAC-preserved latanoprost. As a vehicle or neutralizing material, SH could be proposed to reduce ocular toxicity and protect the ocular surface in long-term BAK-preserved antiglaucoma medication treatment.

Keywords: sodium hyaluronate, ocular surface toxicity, benzalkonium chloride, latanoprost
Materials and Methods

Animals and Treatments

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the animal ethics committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (approval ID: 2010-028). Twenty-one female white New Zealand rabbits (2.5–3.0 months old, 2.0–2.5 kg, purchased from Guangdong Medical Laboratory Animal Center, Guangdong, China) were used. The rabbits were placed in cages (one rabbit per cage) and kept in standard conditions (room temperature 23°C ± 2°C, relative humidity 60% ± 10%, 12-hour/12-hour light/dark cycle) and fed on the standard laboratory diet with free access to water throughout the study. Before all experiments, the ocular surface integrity was examined by slit-lamp microscope (Topcon, Tokyo, Japan).

In this study, we used one type of commercially available unpreserved 0.3% SH eye drops (Hialid; Santen, Osaka, Japan) and one type of commercially available 0.005% latanoprost eye drops (Xalatan; Pfizer, New York, NY) containing the highest concentration of BAC (0.02%).

Twenty-one rabbits (42 eyes) were randomly divided into three groups. The control group was untreated for baseline comparison. The PBS-treated latanoprost group was administered with latanoprost eye drops once daily (10 PM) and 50 µL PBS (pH 7.4) three times daily (8 AM, 2 PM, 8 PM). The SH-treated latanoprost group was administered with latanoprost eye drops once daily (10 PM) and 50 µL 0.3% SH three times daily (8 AM, 2 PM, 8 PM).

In all groups, fluorescein staining, rose bengal staining, Schirmer test, and conjunctiva impression cytology (CIC) were performed sequentially following the methods described below on days 0, 31, and 61. At day 61, all rabbits were killed with an overdose of general anesthetics. The conjunctival apoptosis was detected by in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Conjunctival inflammation was evaluated with light microscopy. Histomorphological changes of cornea and conjunctiva were observed by light microscopy or scanning electron microscopy (SEM) or transmission electron microscopy (TEM).

Fluorescein and Rose Bengal Staining

One microliter of 1% fluorescein sodium was instilled in the conjunctival sac and then rinsed with PBS 1 minute later. The eyes were examined and photographed under a slit-lamp microscope with a cobalt blue filter and a digital camera (Topcon, Tokyo, Japan). Five corneal areas were considered, the area of positive corneal staining was scored from 0 (absent) to 3 (diffuse loss of epithelium).\(^{16}\)

Thirty minutes later, the disappearance of staining was observed under a slit-lamp microscope. One microliter of 1% rose bengal was instilled into the conjunctival sac. Fifteen seconds later, the scores were graded according to the van Bijsterveld grading system under a slit-lamp microscope (Topcon).\(^{17}\)

Measurement of Aqueous Tear Production

After intramuscular injection of a mixture of 50 mg ketamine and 25 mg chlorpromazine and topical application of proparacaine (Alcaine), two 3.5 × 3.5-mm round nitrocellulose filter papers (Pall Corporation, New York, NY) were applied separately on the nasal and temporal bulbar conjunctiva and pressed for 10 seconds with constant pressure. The specimens were fixed with 95% ethanol and stained with periodic acid Schiff (PAS) and hematoxylin reagents. The number of goblet cells was counted under a light microscope (Olympus, Tokyo, Japan) in a masked fashion. Three nonadjacent high-power fields (HPFs, ×400) of each specimen were selected randomly for counting, and an average was calculated. The morphology of the conjunctival epithelium was graded according to the Nelson method.\(^{18}\)

TUNEL Assay

End-stage apoptosis was detected by in situ TUNEL labeling on paraffin sections of the superior bulbar conjunctiva. Formalin-fixed and paraffin-embedded 5-µm-thick tissue sections were dewaxed and rehydrated in a graded ethanol series (100%, 95%, 85%, 70%), rinsed with PBS, permeabilized by 0.1% Triton X-100 and 0.1% sodium citrate, incubated with terminal deoxynucleotidyl transferase solution and converter-peroxidase according to the manufacturer’s instructions (in situ cell death detection kit-POD; Hoffmann-La Roche Co. Ltd., Basel, Switzerland), immersed in 3,3’-diaminobenzidine (DAB), and counterstained with hematoxylin. The samples were observed and photographed under a light microscope. TUNEL-positive apoptotic cells in the conjunctival epithelia were distinguished by brown immunoreactivity.

Light Microscopy

The superior bulbar conjunctiva was fixed in 10% formalin. After dehydration in a graded ethanol series, the specimens were embedded in paraffin and cross-sectioned. The histological changes were observed by hematoxylin-eosin staining. The number of inflammatory cells in the conjunctival epithelium and stroma were counted in a masked manner. Three nonadjacent HPFs (×400) of each specimen were selected randomly for counting, and an average was calculated.

Scanning Electron Microscopy

Corneas were fixed with 2.5% glutaraldehyde, postfixed with osmium tetroxide, dehydrated in a graded ethanol series, dried with hexamethyldisilazane, sputter-coated with gold, and examined under SEM (Quanta 200; Philips, Amsterdam, The Netherlands).

Transmission Electron Microscopy

Cornea and conjunctiva were fixed with 2.5% glutaraldehyde, postfixed with osmium tetroxide, embedded in resin, cut in sections with 60-nm thickness, then examined under TEM (Tecnai G2; Philips).

Statistical Analysis

Statistical analyses were performed using the SPSS software version 13.0 (SPSS Inc., Chicago, IL). Repeated-measures ANOVA...
Bonferroni posttest) was applied to make comparisons of aqueous tear production and conjunctival goblet cell density among the groups over the study period. One-way ANOVA (Bonferroni posttest) was applied to make comparisons of conjunctival inflammation among the groups on day 61. Kruskal-Wallis test (Bonferroni posttest) was applied to make comparisons of fluorescein and rose bengal staining scores. P values less than 0.05 were considered statistically significant.

RESULTS

Fluorescein and Rose Bengal Staining

There were significant differences in fluorescein (Kruskal-Wallis test, $H = 31.078$, $P < 0.001$) and rose bengal (Kruskal-Wallis test, $H = 28.470$, $P < 0.001$) staining scores among the groups on day 61. Compared with the control group, fluorescein (Bonferroni, $P < 0.001$, Fig. 1A) and rose bengal (Bonferroni, $P < 0.001$, Fig. 1B) staining scores increased in the PBS-treated latanoprost group on day 61. There was no significant difference of the scores between the SH-treated latanoprost group and the control group. In the SH-treated group, fluorescein (Bonferroni, $P < 0.001$, Fig. 1A) and rose bengal (Bonferroni, $P < 0.001$, Fig. 1B) staining scores were significantly lower than the PBS-treated group.

Aqueous Tear Production

There were significant differences in aqueous tear production (repeated-measures ANOVA, $F = 5.72$, $P < 0.01$) among the groups over the study period. Compared with the control group, aqueous tear production (Bonferroni, both $P < 0.001$) decreased in the PBS-treated latanoprost group on days 31 and 61. There was no significant difference of aqueous tear production between the SH-treated latanoprost group and the control group. In the SH-treated group, aqueous tear production (Bonferroni, both $P < 0.01$) was more significantly increased than in the PBS-treated group on days 31 and 61 (Fig. 2A).

Changes of Goblet Cell Density and Conjunctival Epithelium Morphology

There were significant differences in goblet cell density (GCD) (repeated-measures ANOVA, $F = 17.38$, $P < 0.001$) among the groups over the study period. Compared with the control group, GCD decreased in both PBS-treated (Bonferroni, both $P < 0.001$) and SH-treated (Bonferroni, both $P < 0.05$) latanoprost groups on days 31 and 61. In the SH-treated group, GCD (Bonferroni, $P < 0.01$, $P < 0.001$, respectively) was more significantly increased than in the PBS-treated group on days 31 and 61 (Fig. 2B). Grading scores increased from 0 to 2 on day 61 in the PBS-treated latanoprost group. Squamous metaplasia developed with time shown as the flattening of conjunctival epithelial cells and increase of nucleocytoplasmic ratio. However, grading scores were only 0 to approximately 1 in the SH-treated latanoprost group (Fig. 3).

Inflammatory Cell Infiltration in Conjunctiva

There was a significant difference in the number of inflammatory cells (one-way ANOVA, $F = 74.62$, $P < 0.001$) among the groups on day 61. Compared with the control group, the number of inflammatory cells in the conjunctival epithelium and stroma increased in both the PBS-treated (Bonferroni, $P < 0.001$) and SH-treated (Bonferroni, $P < 0.05$) latanoprost groups on day 61. In the SH-treated group, conjunctival inflammatory cell infiltration (Bonferroni, $P < 0.001$) was significantly less than in the PBS-treated group on day 61 (Fig. 4).

Apoptosis in Conjunctival Epithelia

TUNEL staining revealed marked apoptosis in the conjunctival epithelium in the PBS-treated latanoprost group. However, apoptosis was visibly relieved in the SH-treated latanoprost group (Fig. 5).

Ultrastructural Changes of Cornea and Conjunctiva

Under the SEM, the cornea showed many bright cells covered with high-density microvilli in the control group. In the PBS-treated latanoprost group, the cornea showed a large number of dark cells covered with seriously destroyed microvilli, diffused epithelia exfoliation and deformation, and cytoplasm wrinkling. However, there were many bright cells covered with high-density microvilli and minimal cytoplasm wrinkling in the SH-treated latanoprost group (Fig. 6).
Under TEM, sharp and high-density microvilli could be seen on the outer surfaces of corneal epithelial cells in the control eyes and the SH-treated eyes. Intracellular structures such as mitochondria, Golgi apparatus, and rough endoplasmic reticulum were also clearly presented. In corneal epithelial cells of the PBS-treated latanoprost group, flat and low-density microvilli, granular clumps in the nuclear chromatin, widening of the perinuclear cisterna (PN), and swelling of intracellular structures were observed (Fig. 7).

Similar changes were observed in conjunctival epithelial cells under TEM. Abundant microvilli on the epithelial cells and secretory granules in the goblet cells could be seen in the control group. In the PBS-treated latanoprost group, decreased microvilli, nuclear chromatin condensation of the epithelial cells, and reduction of secretory granules in the goblet cells were observed. These damages were greatly mitigated in the SH-treated latanoprost group (Fig. 8).

**DISCUSSION**

Hyaluronate is not only an important component of extracellular matrix but also interacts with cells during several cellular pathways, such as embryonic morphogenesis, inflammation, and wound repair, as well as chemoresistance. It regulates cell movement and transport of extracellular components. It could inhibit nitric oxide–induced apoptosis and dedifferentiation of articular chondrocytes, and upgrade VEGF expression in rabbit Achilles tendon. At present, SH is widely used as a kind of substitute for tears. Clinical research has shown that SH could improve grading scores of conjunctival impression cytology for treating severe dry eye in Sjögren’s syndrome patients. In this study, we demonstrated that SH could decrease ocular surface toxicity induced by benzalkonium chloride–preserved latanoprost. To our best knowledge, this has been the first in vivo study to demonstrate the function of SH to decrease the toxicity induced by BAC-preserved antiglaucoma drops.

Although quite a few studies suggested that the active component latanoprost did not induce overexpression of various inflammatory cytokines, and was even responsible for protective effects against BAC toxicity by its antioxidative properties, evidence showed that BAC-preserved latanoprost induced toxicity on conjunctival epithelial cells in vitro. We demonstrated significant time-dependent ocular surface toxicities in 0.02% of the BAC-latanoprost treated group in this study, including decreases in basal tear secretion and goblet...
cell density, increases in inflammatory cell infiltration, and apoptosis rates. It seemed that the protective effects of latanoprost were not enough to neutralize the toxicities of BAC.

BAC induces damage in the superficial structure and integrity. Fluorescein staining means the disruption of corneal epithelial cell-cell junctions and damaged corneal epithelial cells. Rose bengal staining means deficiency in tear film (especially mucin layer), which covers corneal and conjunctival epithelial cells. The Schirmer test is the classic method for measuring the production of the aqueous component in tears. Microvilli on the surfaces of corneal and conjunctival epithelial cells play a key role in the adherence of tear film mucin layer to epithelial cells. Under SEM and TEM, ultrastructural disorders of the epithelial cells and microvilli could be observed clearly. The results of our study showed that SH could decrease fluorescein and rose bengal staining induced by application of BAC-preserved latanoprost and could increase aqueous tear production and protect ultrastructures, such as microvilli on the epithelial cells. A rational explanation may be that SH, a viscous biopolymer with negative charges, can neutralize the toxic cationic charge of the remaining BAC quaternary ammonium.

Moreover, viscoelastic properties and long ocular surface residence time of SH have rendered it effectively increase tear film stability and corneal wettability, and reduce the tear evaporation rate. It was recently reported that a hyaluronate receptor, CD44, is expressed in human and rabbit corneas. SH promoted human cornea epithelial cell migration by the adhesion between CD44 receptor.

Dogan et al. found that the apoptosis rates of epithelial cells were higher in glaucoma patients; however, no difference was found between different antiglaucoma medications. In a reconstructed three-dimensional model of human corneal epithelial cells (3D-HCEs), BAC induced more positive TUNEL staining (representing late stage of apoptosis and cell death) in the superficial cell layers and more activation of caspase-3 (representing early stage of apoptosis) in the deeper layers. We also demonstrated that BAC-preserved latanoprost increased TUNEL staining especially in the superficial layer of conjunctival epithelium; nevertheless, this phenomenon was significant suppressed by topical application of SH. Because protection against ocular surface disorders could be achieved by suppression of apoptosis, we consider that SH could effectively protect the ocular surface during topical antiglaucomatous therapy.

**Figure 4.** Representative images of conjunctival paraffin sections with hematoxylin-eosin staining on day 61 in each group (HP, ×400). (A) The control group showed normal rabbit conjunctiva with few lymphocyte infiltration in the epithelium and stroma. (B) The PBS-treated latanoprost group showed remarkable inflammatory cell infiltration. (C) The SH-treated latanoprost group showed less inflammatory cells infiltration in the conjunctiva. (D) Alterations of the inflammatory cells count in the conjunctival epithelium and stroma in each group on day 61. Compared with the control group, both PBS-treated (P < 0.001) and SH-treated (P < 0.05) latanoprost groups showed a trend of increased inflammatory cell infiltration. In the SH-treated group, conjunctival inflammatory cell infiltration was significantly less than the PBS-treated group (P < 0.001).
There are three possible mechanisms through which SH decreases the apoptosis rate. First, BAC can induce reactive oxygen species overproduction, which is associated with alterations of the mitochondrial respiratory chain and results in stretching of mitochondrial crests and formation of megamitochondria. The mitochondrial dysfunction induces the activation of apoptogenic proteases with secondary endonuclease activation and consequent apoptosis. SH is rich in hydroxyl functions, which may potentially absorb reactive oxygen species induced by BAC. Second, several recent studies indicated that BAC-induced P2X7 receptor activation could cause cytolytic pore formation and lead to apoptosis. SH physically coated cell membrane via strong links with CD44 receptors. At the same time, it masked the P2X7 receptors, and inhibited the P2X7 receptors to be activated. Finally, toxicological studies showed in conjunctival cell lines, the prostaglandin analogue seemed to present cytoprotective and antioxidative effects against BAC-induced toxicity.

Figure 5. Representative images of the conjunctival TUNEL staining on day 61 (HP ×400). (A) The control group showed few apoptotic cells in the epithelium. (B) The PBS-treated latanoprost group showed large amounts of apoptotic cells (brown) particularly among the epithelial and the goblet cells. (C) The SH-treated latanoprost group showed a significant drop in the number of apoptotic cells.

Figure 6. Representative images of SEM for the corneal epithelium on day 61. (A) the control group showed many bright cells with hexagonal shape ([A-1], SEM ×3000), high-density microvilli ([A-2], SEM ×24,000). (B) The PBS-treated latanoprost group showed diffuse cell peeling, loss of hexagonal shape, and plasma cell wrinkling ([B-1], SEM ×3000), diffuse loss of microvilli ([B-2], SEM ×24,000). (C) The SH-treated latanoprost group showed mild cell peeling and slight cell wrinkling ([C-1], SEM ×3000), and high density of microvilli ([C-2], SEM ×24,000).
protective effects of latanoprost became possibly apparent after BAC neutralization by SH. That is to say, we can hypothesize that SH and latanoprost play a synergic role in reducing BAC-induced toxicity.

A more severe side effect of BAC-preserved antiglaucoma drops is chronic ocular surface inflammation. Broadway and colleagues found that subclinical inflammation induced by the use of topical preserved antiglaucoma drugs was associated with a significantly lower trabeculectomy success rate in comparison with patients undergoing initial trabeculectomy. Baudouin et al. showed that glaucoma patients treated over 1 year, even though clinically asymptomatic, exhibited significant expression of HLA-DR, ICAM-1, IL-6, IL-8, and IL-10 in the conjunctival epithelium. BAC-preserved drugs may cause apoptosis in epithelial cells, increased renewal of the conjunctival epithelium, release of inflammatory cytokines, and further activation of inflammatory cells. Our study demonstrated that SH could effectively decrease inflammatory cell infiltration induced by BAC in the conjunctival epithelium and stroma. Ex vivo and in vivo studies showed that BAC-preserved latanoprost induced fewer positive cells for inflammatory marker expression compared with 0.02% BAC-preserved timolol or 0.02% BAC alone. In this work, the latanoprost anti-inflammatory effect observed may be promoted after BAC neutralization by SH. Besides the antioxidative nature and possible cooperation with latanoprost, SH may decrease expression of apoptosis-related inflammatory markers, Fas, APO2.7, HLA-DR, and CD40 through CD44 mediation, just like in treatment of moderate dry eye syndrome and superficial keratitis. A limitation of this study is lack of detecting the expression of inflammatory cytokines. Our further understanding of the mechanism was limited due to the poor availability of antibodies against rabbit proteins. Further studies are required.

GCD is a key parameter that reflects the overall health of the ocular surface. Conjunctival impression cytology is the most effective noninvasive technique for counting goblet cells. Current studies showed that BAC might trigger a direct toxicity on goblet cells and inhibit mucin production in goblet cells. Pisella et al. found that in patients treated with BAC-preserved antiglaucoma drugs, the lowest levels of MUC5AC-positive cells were related to the highest HLA-DR and ICAM-1 levels, thus emphasizing that inflammation in conjunctiva was associated with a highly significant decrease in goblet cells. Inflammatory immune environment caused apoptosis of goblet cells and decreased production of secretory granules, resulting in an unstable tear film, thereby aggravated ocular surface damage and stimulated the inflammation cascade of the epithelial cells. Thus, the eye entered a vicious cycle. In our study, SH significantly improved GCD and conjunctival squamous metaplasia. Moreover, TEM also showed more glycogen inside goblet cells in the SH-treated latanoprost than in the PBS-treated group. SH possibly maintains goblet cell density and stimulates mucin synthesis by neutralizing BAC and
also through its antioxidative and anti-inflammatory properties, which might help the ocular epithelium break out of the vicious cycle of apoptosis and inflammation, and goblet cell loss.

The model consisting of regular applications that mimic the clinical setting in rabbit eyes does not reflect the real ocular surface reactions in patients, even rabbit corneal tissue appears to be more sensitive to the exposure of antiglaucoma medications than human tissues. But it may emphasize the actions of SH and the histomorphological changes that are difficult to obtain from patients.

In conclusion, our in vivo study displayed that SH significantly reduced the ocular surface toxicity induced by one commonly used antiglaucoma medication, preserved latanoprost. As a vehicle or neutralizing material, SH could be proposed to reduce ocular toxicity, and protect the ocular surface in long-term BAC-preserved latanoprost treatment.

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