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Safety of sodium hyaluronate eye drop with C12-benzalkonium chloride

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ABSTRACT

Purpose: In this study, we investigated the effects of commercially available multi-dose sodium hyaluronate 0.1% (Hyalein®; Santen, Osaka, Japan) containing 0.003% C12-benzalkonium chloride (BAC) on the Corneal epithelium and its degree of safety.

Methods: Japanese white male rabbits were divided into four groups. The corneas of each group exposed to one of the following solutions: sodium hyaluronate 0.1%, C12-BAC, C12, 14, 16-BAC Mixture, and Hank's Balanced Salt Solution (HBSS) (as control), respectively. Corneal transepithelial electrical resistance (TER) changes after 60 s of exposure to the above solutions were measured in living rabbits. TER reflects the barrier function of the epithelium. In addition, scanning electron microscopy was used to examine the acute effects of the above solutions on the integrity of the corneal epithelium of four groups.

Results: There was no significant decrease in the corneal TER after exposure of the cornea to Hyalein® eye drops as compared to HBSS control eyes. Also, BAC mixture solution and C12-BAC did not produce any significant decrease in the corneal TER as compared to HBSS control eyes. All the corneal epithelium exposed to Hyalein®, 0.003% C12-BAC and 0.003% BAC mixture exhibited a regular appearance of the superficial cells with a high density of microvilli.

Conclusion: This study confirms that Hyalein® has no acute hazardous effect on corneal epithelium.

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Hyalein; sodium hyaluronate; benzalkonium chloride; alkyl chain length; cornea; transepithelial electrical resistance

Introduction

Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface[1]. Dry eye is one of the most common ophthalmologic problems, and it is estimated that up to one-third of the population worldwide may be affected. The effect on quality of life is substantial because of symptoms such as pain and irritation, which have a negative effect on ocular health, general health, and well-being and often disrupt daily activities[2].

Aqueous, mucin and lipid layers constitute the tear film and are essential for maintaining homeostasis of the ocular surface. In particular, mucin contributes to aqueous coverage and stability of the ocular surface by making the corneal and conjunctival epithelial surface hydrophilic[3]. Dry eye is presumably caused by abnormalities in tear film composition or tear volume. Quantitative and qualitative abnormalities in tear fluid cause tear film destabilization and keratoconjunctival epithelial disorders, including damage to the mucin layer in the keratoconjunctival epithelium[4]. This in turn leads to subjective symptoms, such as eye discomfort, foreign body sensation, or visual disturbance, eventually lowering patients' quality of life (QOL)[2].

Artificial tears are often effective in relieving symptoms in mild and moderate dry eye by replenishing deficient tear volume. Unfortunately, the low ocular residency time of watery formulations necessitates frequent instillation, whilst more viscous artificial tears blur vision and interfere with blinking[5].

Hyaluronic acid (sodium hyaluronate) is a glycosaminoglycan distributed throughout both the vitreous humour and synovial fluid. Sodium hyaluronate (SH) is capable of holding large quantities of water, and ophthalmic solutions containing SH are the currently preferred tear-film substitute[5,6]. SH consists of repeating disaccharide units of N-acetyl-D-glucosamine and sodium-D-glucuronate. Several authors have reported the use of SH in artificial tears. Between blinks its relatively high viscosity improves tear film stability and reduces washout from the ocular surface, but reduced viscosity under shear stress permits uninterrupted blinking. Additionally, SH effectively binds water and resists dehydration, and promotes epithelial wound healing[5,6].

Formulations containing SH are commercially available in Japan in single-dose containers without preservatives, and in multi-dose containers with preservatives. The multi-dose 0.1% SH preserved with 0.003% benzalkonium chloride (BAC) (Hyalein® 0.1%; Santen, Osaka, Japan)[7].

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The most commonly used antimicrobial preservative in topical eye drops is the quaternary ammonium cationic surfactant, BAC, which is a homologous mixture of N-alkyldimethylbenzyl ammonium chlorides with N-alkyl chain lengths ranging from C8 to C18[8]. In ophthalmology, commonly administered pharmacotherapeutic agents contain commercially produced BAC, which consists of three homologues that have different N-alkyl chain lengths (C12, C14, and C16). On the other hand, numerous studies have revealed that there are deleterious corneal effects associated with BAC that include destabilization of the tear film[8], death of corneal and conjunctival epithelial cells[9,10], morphological changes in the corneal epithelial cells[9,11], and the reduction of the corneal epithelial barrier function[12]. Previous reports seem to suggest that BAC homologues can result in different degrees of ocular toxicity. However, presently there are few reports that have examined the BAC homologue-induced ocular surface impairment.

Measurement of corneal transepithelial electrical resistance (TER) is a suitable method for evaluating corneal permeability and irritancy quantitatively and continuously. TER reflects the barrier function of the epithelium. Lower corneal TER means more electrical current penetrates through the damaged superficial cells and tight junctions between them. In addition, it is reported to be a very sensitive test for measuring electrical properties of the cornea[12]. We developed a new method of measuring the TER of live rabbit cornea. In this method, the cornea is not damaged by the experimental procedure and the TER is stable before drug administration. To measure corneal TER, we used a volt-ohm meter which generates ±20uA AC square wave current at 12.5 Hz. Therefore, it was able to measure TER every 0.08 s. In addition, TER was monitored with a recorder, which shows TER changes continuously[13].

In previous studies, after developing a new *in vivo* method of measuring the TER of rabbit corneas, we demonstrated that BAC concentrations between 0.005% and 0.02% immediately caused acute corneal barrier dysfunction[14,15]. Also, from our previous study[16], we conclude that the BAC homologue induced acute corneal epithelial toxicity is dependent upon the alkyl chain length. Among the BAC homologues, C12-BAC exhibited the lowest corneal impairment, whereas C14-BAC induced the most severe impairment.

In this study, we investigated the corneal epithelial cell damage of commercially available SH 0.1% (with 0.003% C12-BAC) and compare its effect with same concentration (0.003%) of C12-BAC and C12, 14, 16-BAC mixture solutions using our novel technique.

Materials and methods

Chemicals

Commercially available SH 0.1% (Hyalein®; Santen, Osaka, Japan) (with 0.003% C12-BAC) was used in this study. Benzyldimethyldodecylammonium chloride (C12-BAC) was purchased from Sigma-Aldrich, Inc, (St Louis, MO, USA). BAC 10% solution (mixed BAC) was obtained from Wako Pure Chemical, Co, (Osaka, Japan) and was composed of 3 BAC homologues that included approximately 67% C12-BAC, 28% C14-BAC, and 6% C16-BAC.

Hank balanced salt Ca2+ and Mg2+ free solution (HBSS) was obtained from Invitrogen, Corp, (Carlsbad, CA). BAC homologue test solutions were prepared in HBSS. The concentrations for the C12-BAC homologue and C12, 14, 16-BAC mixture were set at concentration of 0.003%.

Experimental animals

Japanese white male rabbits (KBT Oriental, Tosu, Japan) weighing 2.5–3.0 kg were individually housed in cages under a controlled temperature (21 °C) and humidity (50% ± 5%) and a 12:12-h light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Initiation of the study occurred once the rabbits had reached weights of 3.0–4.0 kg, which is the point where corneal diameters were of a suitable size for experimentation. The rabbits had free access to food and water. All experiments in the present study confirmed to the guiding principles in the care and use of animals (DHEW Publication, National Institutes of Health 80–23), the Association for Research in Vision and Ophthalmology Resolution for the use of animals in ophthalmic research, and the Declaration of Helsinki.

Corneal TER measurements in vivo

The rabbits were anaesthetized with an intramuscular injection of 30-mg/kg ketamine (Ketalar, Sankyo, Tokyo, Japan) and 5-mg/kg xylazine (Celactal; Bayer Health Care, Osaka, Japan). The experimental procedure was started within 10 min of the induction of anaesthesia. After a slit-lamp examination of the eyes to confirm that the cornea was intact, adhesive tape was applied so that one eye was kept open, whereas the other was kept closed. After a small incision was made with an 18-gauge sharp needle (Terumo, Tokyo, Japan) in the peripheral cornea, a 1.0-mm diameter custom-made Ag/AgCl electrode (Physiotech, Tokyo, Japan) was inserted into the anterior chamber. A 6.0-mm internal diameter (0.28-cm^2 inner area) nitrile rubber O-ring (Union Packing; SAN-EI, Osaka, Japan) was fixed on the cornea using biomedical adhesive (Alon-Alpha A; Sankyo, Tokyo, Japan). Subsequently, 80 μL of HBSS was placed inside the ring, with the second electrode then placed in HBSS on the cornea. This initial procedure was carefully conducted to avoid damaging the center of the cornea.

The TER was measured in real time using a volt-ohm meter (EVOMX; World Precision Instruments, Sarasota, FL, USA) that generated a ± 20 μA AC square wave current at 12.5 Hz. Data were recorded using a thermal array recorder (WR300-8; Graphtec, Tokyo, Japan). In a period of just a few seconds, 1 mL of the test solutions were gently poured into the ring, with overflow aspirated, followed by exposure for 60 s. The results were then calculated as a percentage of the pre-exposure TER value (100%). This specific methodology and photographs of the *in vivo* corneal TER measurement system have been previously published[13–16].

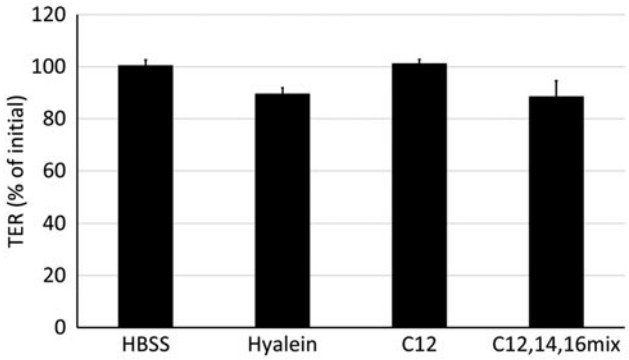

Figure 1. Corneal TER changes after exposure to HBSS, C12,14,16-BAC, C12-BAC, and Hyalein for 60 s. Data represent the percentage compared to the pre-exposure value.

SEM observation

The rabbits were divided into four groups. The corneas of each group exposed to one of the following solutions: SH 0.1%, C12- BAC, C12, 14, 16-BAC mixture, and HBSS (as control) respectively. The rabbits were anaesthetized with an intramuscular injection of 30-mg/kg ketamine and 5-mg/kg xylazine. Corneas were evenly soaked in the testing solutions for 60 s. After the corneal washing, the rabbits were immediately killed using a lethal dose of intravenous sodium pentobarbital (Nembutal; Dainippon Pharmaceutical, Japan). The corneas were carefully excised, fixed in 4% glutaraldehyde in 0.05 M of cacodylate buffer for 1 h, and then postfixed in 1% osmium tetroxide in veronal acetate buffer containing 0.22 M of sucrose. The fixed materials were dehydrated through a series of ethanol washes. Corneas were placed in t-butyl alcohol, treated in a freeze-drying apparatus (EIKO ID-2; EIKO, Japan), and spatter coated with gold using an auto fine coater (JEOL JFC-1600; JEOL, Japan). After processing, the surface of the corneal epithelium was observed by a scanning electron microscope (SEM) (Hitachi S2360; Hitachi, Japan).

Statistical analysis

All results were expressed as the mean ± standard error of three experiments. Statistical comparisons were performed using an analysis of variance followed by a Tukey Kramer test for the TER measurements. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

TER changes

The mean corneal TER for the live rabbits used in this study was 2133.4 ± 344.1 $\Omega.cm^2$. Figure 1 shows the TER changes that occurred after corneal exposure to HBSS, SH, C12-BAC, and C12, 14, 16-BAC mixture solutions. The relative TER value was 106 ± 2.0%. There was no significant decrease in the corneal TER after exposure of the cornea to SH eye drops (relative TER value =89.7 ± 1.5%) as compared to HBSS control eyes. Also, BAC mixture solution (relative TER value =88.6 ± 4.5%) and C12-BAC (relative TER value =101.3 ± 6.0%) did not produce any significant decrease in the corneal TER as compared to HBSS control eyes.

SEM observation

The superficial cells of the cornea of control eyes exposed to HBSS solution were normal in appearance with normal microvilli revealed with SEM (Figure 2(A)).

All the corneal epithelium exposed to SH, 0.003% C12-BAC, and 0.003% BAC mixture exhibited a regular appearance of the superficial cells with a high density of microvilli (Figure 2(B–D), respectively).

Discussion

BAC is a major preservative component in eye drops used to prevent bacterial contamination in multi-dose bottles during the treatment period of eye diseases. Such bactericidal agents are necessary for patient safety because the multi-use containers for eye drops often lead to improper use. However, concerns were raised about the cytotoxicity of BAC, which is a known irritant and could potentially damage the delicate ocular surface in the millions of patients who use eye drops routinely over many years[17]. BAC also disrupts the precorneal tear film and may cause adverse effects including dry eye, tearing, burning, and foreign body sensations[8]. However, the toxic preservative BAC is still an important excipient found in the vast majority of eye drop formulations. BAC is a mixture of alkylbenzyldimethylammonium chlorides used for the bactericidal and microbicidal activity of its C12 and C14 alkyl derivatives[10].

The corneal epithelium, which in direct contact with topically instilled drugs, is recognized as the primary source of the corneal barrier function. Therefore, evaluations of corneal epithelial barrier function have been performed to assess the corneal toxicity of ophthalmic agents, including BAC[12–14]. Tear flow also plays an important role in the protective mechanism that eliminates instilled drugs from the precorneal area. The turnover of tears rapidly dilutes the instilled BAC to 26% of its original concentration in 1 min and to 9% in 5 min[18]. This fact led us to realize that corneal toxicity assays performed over long periods of time do not sufficiently reflect actual clinical conditions, whereas evaluations of BAC-induced corneal toxicity over short periods of time could provide valuable data. In the current study, we used a corneal exposure period of 60 s to evaluate the acute corneal epithelial effect induced by the tested solutions.

Many methods have been used to evaluate corneal irritation and permeability induced by ophthalmic drugs. Ocular irritability is conventionally tested according to modified procedure of Draize by scoring the degree of damage to rabbit eyes[19]. Alternative methods include evaluation of toxicity in cultured ocular cells[20], direct confocal microscopic analysis[21], and various other approaches using isolated animal corneas[22,23]. Corneal drug permeability has been evaluated by diffusion experiment *in vitro*[24]. The epithelial barrier function in humans has been examined by measuring the permeability of fluorescence[25].

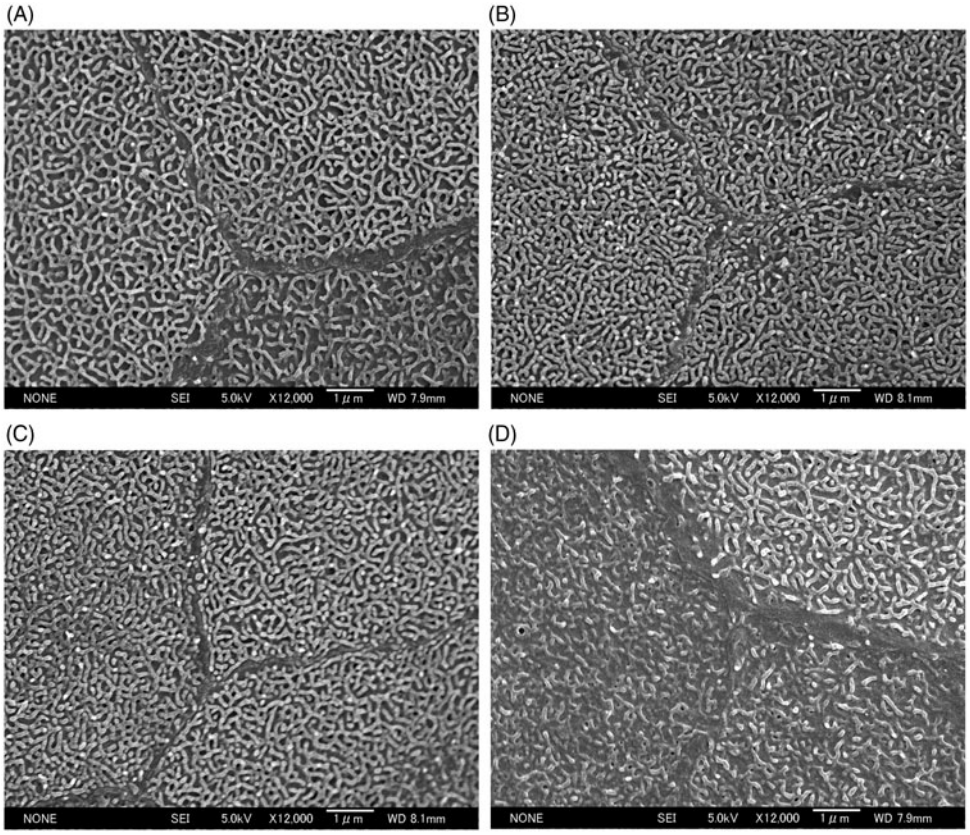

Figure 2. Scanning electron microscopy images of the corneal epithelium after 60 s of exposure to HBSS (A), Hyalein (B), C12-BAC (C), and C12,14,16-BAC Mix (D), (12,000× magnification). Images A, B, C, and D show that the corneal epithelial structures remain almost intact.

In general, TER reflects the barrier function of epithelium, with lower corneal TER values indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and tight junctions existing in the epithelium. Thus, TER is a sensitive, reliable, and versatile test of corneal epithelial barrier function and useful indicator of corneal toxicity[13–16]. Our developed novel corneal TER measurement system *in vivo* using custom-designed thin stick electrodes and a volt-ohm meter can measure the barrier function of the intact cornea in rabbits. This design more accurately reflects the clinical instillation of ophthalmic drugs and gives us a relevant data about the acute corneal toxicity of some eye drops[13–16].

In this study, there was no statistical difference between all SH eye drop, 0.003%BAC mixture, and to 0.003%C12-BAC solutions as compared to HBSS control solution. In this study, we were able to confirm that all tested solutions including SH did not cause any corneal impairment by performing a histological analysis using SEM. The SEM-based histological analyses of the corneas treated with 0.003% BAC mixture, 0.003% C12-BAC, and SH indicated that the appearance of the superficial layer was almost intact. In our previous studies, we assessed changes in the TER induced by instillation with 0.02–0.005% BAC and found that these concentrations rapidly caused acute dysfunction of the corneal barrier and significant decline in TER was detected. On the other hand, low concentrations of BAC (0.001% and 0.002%) did not induce a decline in the TER. Also, we confirmed significant decreased cell viability using normal rabbit corneal epithelial cell line (NRCE) cells after exposure to each 0.02–0.005% concentration of BAC. On the other hand, low concentrations of BAC (0.001% and 0.002) did not induce a decline in the cell viability[13–14]. This study confirms our previous study that assumed that acute corneal barrier dysfunction occurs if the concentrations of BAC ranged between 0.005% and 0.02%[13–14]. Because all tested solutions including SH contain 0.003% BAC, there are no significant changes as regard TER and SEM examinations between tested solutions and HBSS control group. Although the BAC concentration (0.003%) is lower in SH than other ophthalmic eye drops but all manufactured multi-dose ophthalmic products are required to resist contamination by passing antimicrobial efficacy testing (AET) in the USA, Japan, and the European Union before its commercial use.

SH is a linear polymer built from repeating disaccharide units containing N-acetylglucosamine and glucuronic acid. The polymer is stabilized by hydrogen bonds parallel with the chain axis and behaves in solution as an expanded random coil. The coil can be regarded as a highly hydrated sphere containing approximately 1000-fold more water than polymer. Native hyaluronate is an important structural element of the extracellular matrix and widely exists in vertebrate tissues, notably in the vitreous body of the eye ball. A minority of studies showed recently that SH significantly reduces BAC induced cytotoxic effects *in vitro*[26].

Clinical research has shown that SH could improve grading scores of conjunctival impression cytology for treating severe dry eye in Sjogren's syndrome patients[27].

Yu et al.[28] 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[28].

SH has 0.003%C12-BAC as a preservative and at this concentration it does not affect the corneal permeability and has no deleterious effect on corneal epithelium as revealed with SEM examination. Patients with dry eyes require long-term management and unless patients can use eye drops with comfort, safety, and convenience, adherence to treatment would not be guaranteed and treatment efficacy is severely reduced. This study confirms that SH has no acute hazardous effect on corneal epithelium, but the probability to be used for long term without any deleterious effect on corneal epithelium must be studied in later researches.

From this study and previous study, we emphasize that use of C12-BAC instead of the commercially available BAC and at lower concentration might provide greater security and safety for patients during ophthalmological pharmacotherapy.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing, we confirm that we have followed the regulations of our institutions concerning intellectual property.

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