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Acute Corneal Toxicity of Diquas

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Key Words
Diquafosol · Benzalkonium chloride · Alkyl chain length · Cornea · Transepithelial electrical resistance

Abstract
Aim: This study aimed to investigate acute corneal toxicity of commercially available diquafosol 3% ophthalmic solution (Diquas®), which contains C12 benzalkonium chloride (BAC) as a preservative. ***Methods:*** Corneal transepithelial electrical resistance (TER) changes after a 60-second exposure to Diquas® (diquafosol 3% preserved with 0.0075% C12 BAC); 0.0075% C12 BAC and 0.0075% C12, C14, C16 BAC mixture were measured in living rabbits. Corneal damage was also examined by scanning electron microscopy (SEM). Hank's balanced salt solution (HBSS) was used as a control. ***Results:*** Diquas® and 0.0075% C12 BAC did not produce any significant decrease in the corneal TER as compared to the HBSS control eyes. There was a significant decrease in the corneal TER after exposure of the cornea to the 0.0075% C12, C14, C16 BAC mixture ($p < 0.01$). SEM revealed that the superficial cells of the corneas exposed to the 0.0075% BAC mixture were damaged and exhibited degenerated microvilli. Conversely, the superficial cells of corneas exposed to Diquas® or 0.0075% C12 BAC appeared normal and had normal microvilli under SEM examinations. ***Conclusion:*** The acute corneal toxicity of Diquas® is less than that of the 0.0075% BAC mixture. Diquas® preserved with 0.0075% C12 BAC did not show acute corneal toxicity.

Introduction

A significant amount of information on the pathophysiology and therapy for dry eye has been published [1, 2]. According to these studies, the essential factors in dry eye are the tear film and the corneal and conjunctival epithelium, as these 2 factors complement each other. Damage to either of these factors results in a cycle that induces dry eye. Based on these findings for the pathophysiology, the treatment of dry eye has now shifted from prescribing artificial tears for hydration or lubrication of the ocular surface to administering agents that stimulate the epithelium to secrete aqueous tears or mucin [1, 2].

Commercial available 3% diquafosol ophthalmic solution (Diquas®; Santen Pharmaceutical Co., Ltd., Osaka, Japan) was introduced exclusively in Japan in December 2010. It contains diquafosol, which is a dinucleotide derivative that exhibits P2Y2 receptor agonist activity, as the active ingredient. The P2Y2 receptor is distributed widely in the body, and its endogenous agonists are adenosine triphosphate (ATP) or uridine triphosphate (UTP). At the ocular surface, the receptors are expressed in the cornea, conjunctiva including goblet cells and meibomian gland, and it has been reported that ATP and UTP promote fluid and mucin secretion in the conjunctival tissue [3]. In the conjunctival epithelium, diquafosol accelerates fluid transport from the serosal to mucosal (tear) side via chloride channel activation after intracellular calcium ion concentration elevation [3, 4]. Moreover, diquafosol reportedly facilitates the secretion of soluble mucin from

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conjunctival goblet cells and stimulates the expression of membrane-associated mucin on the corneal epithelium [4, 5]. Therefore, the actions of diquafosol stabilize the tear film on the ocular surface and provide therapeutic relief for dry eye. Several clinical studies have demonstrated the superior effect of diquafosol for the treatment of dry eye [6–8]. However, water and mucin secretion facilitated by diquafosol has only been confirmed by in vitro models and in the in vivo rabbit [9], cat [10] and rat models [11]. As a result of these previous studies [3–11], diquafosol is confirmed to be safe for the ocular surface and for the treatment of dry eye.

Diquas® contains sodium diquafosol (30 mg/1 ml), dibasic sodium phosphate, sodium chloride, potassium chloride, sodium edetate hydrate, a pH adjuster and benzalkonium chloride (BAC; 0.0075% C12-BAC). The most commonly used antimicrobial preservative in topical eye drops is the quaternary ammonium cationic surfactant, BAC, which is a homologous mixture of N-alkyl dimethyl benzyl ammonium chlorides that have different N-alkyl chain lengths (C12, C14 and C16) [6]. On the other hand, numerous studies have revealed that there are deleterious corneal effects associated with BAC that include destabilization of the tear film [10], death of the corneal and conjunctival epithelial cells [13, 14], morphological changes in the corneal epithelial cells [15] and the reduction of the corneal epithelial barrier function [16].

Measurement of corneal transepithelial electrical resistance (TER) is a suitable method for evaluating corneal permeability and irritancy both quantitatively and continuously. In addition, it also been shown to be a very sensitive test for measuring the electrical properties of the cornea [16]. As reported in a previous study, we developed a new method for measuring the TER of live rabbit cornea that does not damage the cornea during the experimental procedure, thereby ensuring that the TER is stable before drug administration [17]. With this method, we were able to measure the TER every 0.08 s and monitored the TER with a recorder that continuously showed the TER changes.

In this previous study and in a further study, we also demonstrated that BAC concentrations between 0.005 and 0.02% immediately caused acute corneal barrier dysfunction [17, 18]. In a subsequent study [19], we investigated the BAC homolog-induced acute corneal epithelial toxicity and concluded that it was dependent upon the alkyl chain length and its concentration. Among the BAC homologs examined, 0.0025% C12-BAC exhibited the lowest corneal impairment, whereas 0.01% C14-BAC induced the most severe impairment. However, the first commercial solution of diquafosol in Japan (Diquas®) is proposed in a preserved formulation (0.0075% C12-BAC) in higher concentration than previously examined, which raises a number of issues.

Because of the importance and widespread use of Diquas® in Japan, we used our technique to investigate the acute corneal toxicity caused by this eye drop, which contains 0.0075% C12-BAC.

Materials and Methods

Chemicals

Commercially available diquafosol 3% ophthalmic solution (Diquas®; Santen Pharmaceutical, Osaka, Japan) containing 0.0075% C12-BAC was used in this study. Benzyl dimethyl dodecyl ammonium chloride (C12-BAC) was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., USA). The 10% BAC solution (BAC mixture) was obtained from Wako Pure Chemical, Co. (Osaka, Japan) and consisted of 3 BAC homologs that included approximately 67% C12-BAC, 28% C14-BAC and 6% C16-BAC. Ca2+ and Mg2+ free Hank's balanced salt solution (HBSS) was obtained from Invitrogen Corp. (Carlsbad, Calif., USA). BAC homolog test solutions were prepared in HBSS. The C12-BAC homolog and the C12, 14, 16-BAC mixture concentrations were set at 0.0075%.

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-hour light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Rabbits had access to food and water at all times and were raised until they reached weights of 3.0–4.0 kg, which is the point where the corneal diameters were of a suitable size for experimentation. The study was initiated once the rabbits reached the correct weight. All experiments in the present study conformed to the guiding principles of 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 and approved by the animal Ethics Committee of Nagasaki University School of Medicine.

Corneal TER Measurements in vivo

The rabbits were anesthetized with 30 mg/kg intramuscular injection of 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 anesthesia. 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 using an 18-gauge sharp needle (Terumo, Tokyo, Japan) to make a small incision in the peripheral cornea, a 1.0 mm diameter custom-made Ag/AgCl electrode (Physio-Tech Co., Ltd., 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). After placing 80 μl of HBSS inside the ring, a second electrode was then placed in the HBSS on the cornea. This initial procedure was conducted with extreme care in order 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) 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). Over a period of just a few seconds, 1 ml of each of the test solutions was gently poured into the ring, with the overflow aspirated. For each solution, there was an exposure time of 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 [17–19].

SEM Examination

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg of ketamine and 5 mg/kg of 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; Sumitomo Dainippon Pharmaceutical, Osaka, Japan). The corneas were carefully excised, fixed in 4% glutaraldehyde in 0.05 mol/l cacodylate buffer for 1 h, and then post-fixed in 1% osmium tetroxide in veronal acetate buffer containing 0.22 mol/l 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 sputter 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 (Hitachi S2360; Hitachi, Tokyo, Japan).

Statistical Analysis

All results were expressed as the mean ± SE of 3 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 2,484.9 ± 406.9 Ω·cm^2. Figure 1 shows the TER changes that occurred after corneal exposure to HBSS, Diquas®, C12-BAC and the C12, 14, 16-BAC mixture solution. There was no change in the corneal TER after exposure to the HBSS solution (relative TER value 100.6 ± 2.0%). There was a significant decrease in the corneal TER after exposure of the cornea to the BAC mixture solution (relative TER value 51.7 ± 9.9%; $p < 0.01$).

In contrast, the Diquas® (relative TER value 89.4 ± 4.5%) and the C12-BAC (relative TER value 87.7 ± 1.3%) did not produce any significant decrease in the corneal TER as compared to the HBSS control eyes. There was a significant difference between the BAC mixture and both the Diquas® and the C12-BAC with regard to the TER changes ($p < 0.01$).

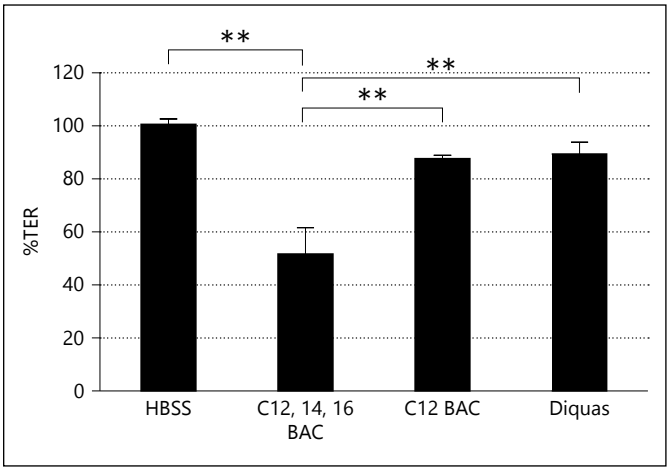

Fig. 1. Corneal TER changes after exposure to HBSS, 0.0075% C12, 14, 16 BAC mixture, 0.0075% C12 BAC and diquafosol for 60 s. Data represent the percentage compared to the pre-exposure value. Each value is the mean ± SE (n = 3). ** $p < 0.01$ as compared with the 0.0075% C12, 14, 16 BAC mixture (Tukey–Kramer test).

SEM Observation

Scanning electron microscopy (SEM) showed that the superficial cells of the cornea of the control eyes exposed to the HBSS solution were normal in appearance with normal microvilli (fig. 2).

Although the corneal epithelium exposed to 0.0075% C12-BAC and Diquas® exhibited a regular appearance of the superficial cells with a high density of microvilli (fig. 3, 4), detached superficial cells and degenerated microvilli were observed in the corneal epithelium after exposure to the 0.0075% BAC mixture (fig. 5).

Discussion

BAC is a major preservative component in eye drops used to prevent bacterial contamination in multi-dose bottles during the treatment period for various eye diseases. These bactericidal agents are necessary for patient safety, as the multi-use containers for the eye drops can be improperly used [20]. However, concerns have been raised about the cytotoxicity of BAC, which is a known irritant and which can potentially damage the delicate ocular surface in the millions of patients who routinely use these types of eye drops over a period of many years [20].

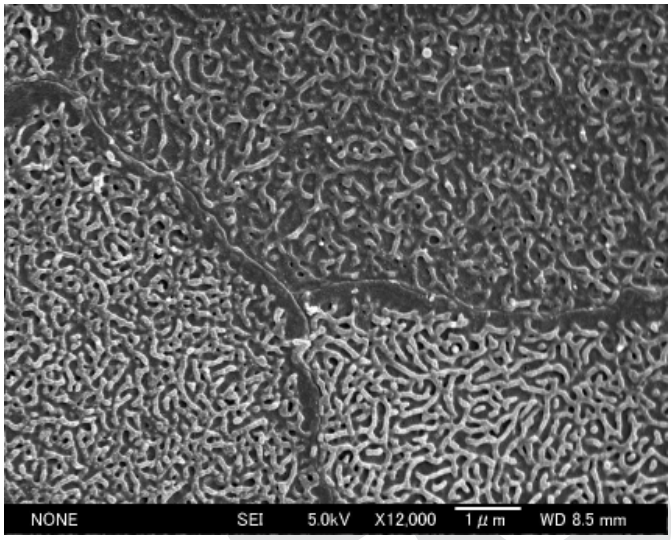

Fig. 2. SEM image of the corneal epithelium after 60 s of exposure to HBSS (magnification, ×12,000). The image shows that the corneal epithelial structures remain almost intact.

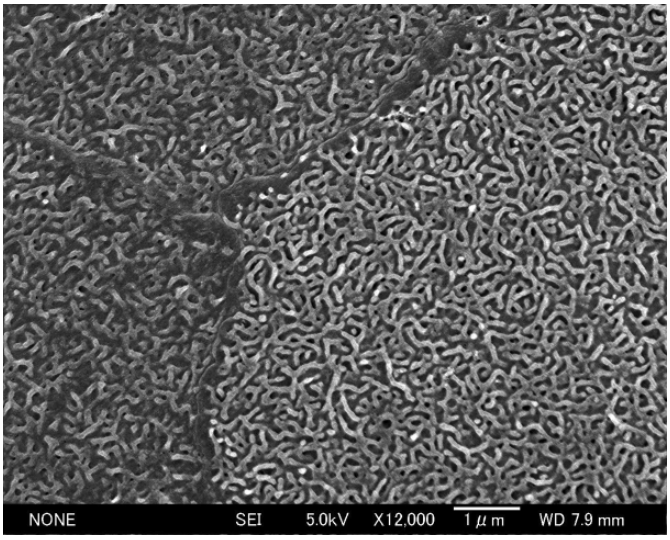

Fig. 4. SEM image of the corneal epithelium after 60 s of exposure to diquafosol (magnification, ×12,000). The image shows that the corneal epithelial structures remain almost intact.

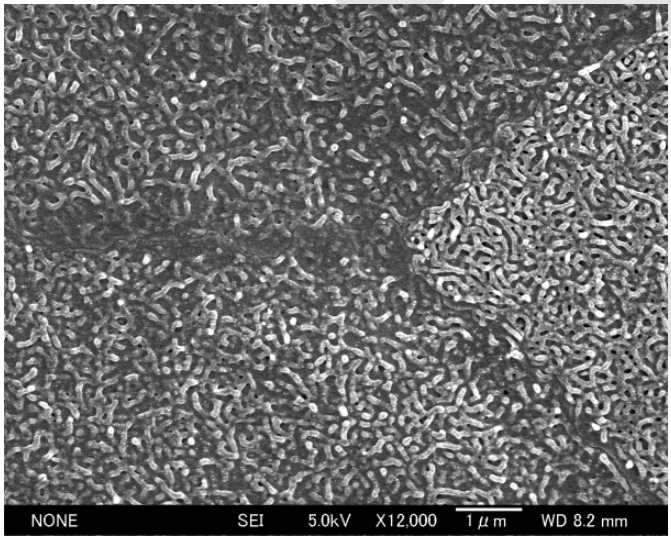

Fig. 3. SEM image of the corneal epithelium after 60 s of exposure to 0.0075% C12 BAC (magnification, ×12,000). The image shows that the corneal epithelial structures remain almost intact.

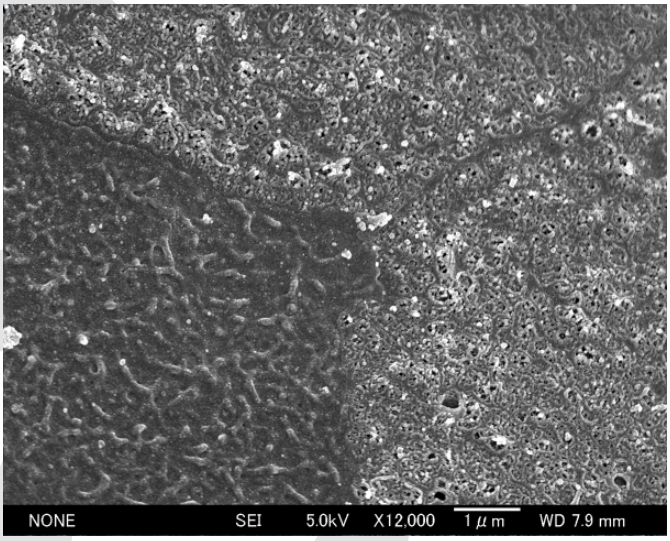

Fig. 5. SEM image of the corneal epithelium after 60 s of exposure to 0.0075% C12, 14, 16 BAC mixture (magnification, ×12,000). The images show injured corneal epithelial structures including degenerated microvilli.

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 the corneal epithelial barrier function have been performed as a way to assess the corneal toxicity of ophthalmic agents, including BAC [16–18]. Tear flow also plays an important role in the protective mechanism that eliminates instilled drugs from the pre-corneal area. The turnover of tears has been shown to rapidly dilute the instilled BAC to 26% of its original concentration at 1 min and to 9% at 5 min [21]. This finding led us to realize that the corneal toxicity assays performed over long periods of time may not sufficiently reflect the 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 the modified procedure of Draize et al. [22] that scores the degree of damage that occurs in rabbit eyes. Alternative methods include evaluation of toxicity in cultured ocular cells [23], direct confocal microscopic analysis [24] and various other approaches that use isolated animal corneas [25]. Corneal drug permeability has been evaluated in vitro by the use of a diffusion experiment [26]. The epithelial barrier function in humans has been examined by measuring the permeability of fluorescence [27].

In general, TER reflects the barrier function of the epithelium, with lower corneal TER values indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and the tight junctions that exist in the epithelium. Thus, TER is a sensitive, reliable and versatile test of the corneal epithelial barrier function and a useful indicator of corneal toxicity [17–19]. We have previously developed a novel in vivo corneal TER measurement system that uses custom-designed thin stick electrodes and a volt-ohm meter to measure the barrier function of the intact cornea in rabbits. This design more accurately reflects the clinical instillation of ophthalmic drugs and provides relevant data on the acute corneal toxicity of some eye drops [17–19].

In our current study, we determined that the TER of the cornea of normal live rabbits did not significantly change after exposure to Diquas® or to C12-BAC solutions. There was also no statistical difference between the Diquas® and the C12-BAC solutions when compared to the HBSS control solution. In contrast, there was marked reduction of the corneal TER measurement after exposure to the BAC mixture solution that contained the same concentration (0.0075%) as the ophthalmic solution. Furthermore, there was also a significant statistical difference between the BAC mixture and the other tested solutions with regard to the TER measurement ($p < 0.01$).

In this study, our SEM histological analysis was also able to confirm that the BAC mixture caused corneal impairment. The SEM-based histological analyses of the corneas treated with 0.0075% C12-BAC and Diquas® showed there was an almost intact superficial layer.

Our current findings also confirmed the results of our previous study that examined the BAC homologs and showed that lower concentrations of C12-BAC did not cause any changes in the TER and that C14-BAC induced the most severe impairment [19]. Overall, our results suggest that acute corneal barrier dysfunction is the greatest for the BAC mixture. The decrease in the TER that was caused by the BAC mixture can be attributed to the synergic activity of the BAC homologs that were of various concentrations within the solution. Our previous study suggested that this toxicity may primarily be attributable to C14-BAC, which makes up approximately 28% of this solution. A previous US clinical trial study evaluated the clinical efficacy of diquafosol as a treatment for dry eye [7]. Diquafosol was approved for treating dry eye in Japan in late 2010. Since cyclosporine A has not been approved as a treatment for dry eye in Japan, sodium hyaluronate ophthalmic solutions have been widely used to treat the syndrome for many years along with preservative-free artificial ophthalmic solutions. More recently, a Japanese phase 2 clinical placebo-controlled trial of diquafosol for dry eye reported that the diquafosol was at least equal to or more effective than the sodium hyaluronate for treating dry eye in terms of reducing ocular surface staining scores and symptomatology [8].

While Diquas® uses C12-BAC (0.0075%) as the preservative, SEM examinations have shown that this concentration does not affect the corneal permeability and has no deleterious effect on the corneal epithelium. In addition, we confirmed in a previous study that equivalent minimum inhibitory concentrations against Escherichia coli for all BAC homologs were noted at 25 mg/ml (0.0025%) [19]. So, C12 BAC has the same antimicrobial effect as BAC mixture. Patients with dry eye syndrome require long-term management and unless patients can comfortably, safely and conveniently use these types of eye drops, adherence to the treatment cannot be guaranteed and thus, treatment efficacy will be severely reduced. Although the findings of our current study showed that Diquas® had no acute hazardous effect on the corneal epithelium, definitive proof that long-term use has no deleterious effect on the corneal epithelium will need to be confirmed in further investigations.

In conclusion, Diquas® preserved with 0.0075% C12 BAC did not show acute corneal toxicity. Based on our current and previous studies, it should be noted that our findings do demonstrate that the use of C12-BAC instead of the commercially available BAC might provide greater security and safety for patients during ophthalmological pharmacotherapy.

Disclosure Statement

The authors have no competing conflicts of interest to report.

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