Decrease in Corneal Damage due to Benzalkonium Chloride by the Addition of Sericin into Timolol Maleate Eye Drops

Noriaki Nagai¹, Yoshimasa Ito¹,²*, Norio Okamoto³ and Yoshikazu Shimomura³

¹ School of Pharmacy and ²Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka, 577-8502, Japan.
³ Department of Ophthalmology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511, Japan.

Abstract: We investigated the protective effects of sericin on corneal damage due to benzalkonium chloride (BAC) used as a preservative in commercially available timolol maleate eye drops using rat debrided corneal epithelium and a human cornea epithelial cell line (HCE-T). Corneal wounds were monitored using a fundus camera TRC-50X equipped with a digital camera; eye drops were instilled into the rat eyes five times a day after corneal epithelial abrasion. The viability of HCE-T cells was calculated by TetraColor One; and Escherichia coli (ATCC 8739) were used to measure antimicrobial activity. The reducing effects on transcorneal penetration and intraocular pressure (IOP) of the eye drops were determined using rabbits. The corneal wound healing rate and rate constants (kₜ) as well as cell viability were higher following treatment with 0.005% BAC solution containing 0.1% sericin than in the case of treatment with BAC solution alone; the antimicrobial activity was approximately the same for BAC solutions with and without sericin. In addition, the kₜ for rat eyes instilled with commercially available timolol maleate eye drops containing 0.1% sericin was significantly higher than that of eyes instilled with timolol maleate eye drops without sericin, and the addition of sericin did not affect the corneal penetration or IOP reducing effect of commercially available timolol maleate eye drops. A preservative system comprising BAC and sericin may provide effective therapy for glaucoma patients requiring long-term anti-glaucoma agents.

Key words: Sericin, benzalkonium chloride, cornea, timolol maleate, preservative

1 INTRODUCTION

The most common preservative used in ophthalmic preparations used to treat glaucoma and ocular surface diseases is benzalkonium chloride (BAC), most often used at a concentration of 0.01% (range, 0.005-0.02%) in topical multi-dose solutions¹. BAC is known to have a strong preservative effect, and its surface-active effects increase the corneal penetration of the main component. Therefore, BAC has been seen as an effective preservative and indispensable in the preparation of eye drops. However, BAC has been shown to be highly toxic both in vitro and in vivo due to a stimulatory effect on epithelial cell death²–¹⁰. The BAC is a quaternary ammonium compound that has been shown to hasten the drying of the tear film¹¹–¹⁵, worsen preexisting dry eye,¹⁶ and affect both the cornea and conjunctiva¹⁷. In addition, BAC is a pro-inflammatory or pro-apoptotic mediator because it induces oxidative stress³–¹¹ or significantly alters precorneal mucus⁻⁻. Clinically, these iatrogenic effects are found most frequently for eye drops used to treat long-term pathologies such as glaucoma. The side effects of BAC seem to be both dose- and time-dependent, increasing with larger amounts used for longer periods. Recently, new preservative system without BAC has been in development. Travatan Z® (Alcon, TX, U.S.A.) is an anti-glaucoma eye drop formulation to be preserved with a non-BAC system (sofzia) patented by Alcon. The sofzia preservative system of Travatan Z® consists of boric acid and zinc chloride, which is less damaging than BAC to the ocular surface of glaucoma patients receiving long-term eye drop therapy. However, for reasons of versatility, this potent preservative system has not yet been introduced because the sofzia preservative system is not applicable to other eye drops (only Travatan Z®). Taken together, improvements to the BAC preservative system that do not
cause corneal epithelial cell damage remain a high priority.

As the proteins fibroin and sericin are the main constituents of silk, with fibroin contributing 70 to 80% and sericin 20 to 30% of the total cocoon weight\(^{13,15}\). When cocoons or raw silk are used for textiles, the sericin is mostly removed from the cocoon and disposed of unused. However, sericin has recently been investigated for its activities in biotechnological fields. It has been reported that sericin enhances the attachment and growth of mouse and human fibroblasts\(^{14,15}\). Terada et al. found growth promotion in several human cell lines and mouse hybridomas when sericin was added to the culture media\(^{16}\). We also reported that isotonic solutions of sericin are stable, and that sericin instillation has a potent effect in promoting wound healing and wound-size reduction in rats\(^{17}\). In addition, sericin has protective effects against freezing stress in *Escherichia coli*\(^{18}\), and acts as an antioxidant to inhibit tyrosinase and lipid peroxidation\(^{13}\). In this study, we investigated the protective effects of sericin on BAC-induced corneal damage using rat debrided corneal epithelium and a human cornea epithelial cell line (HCE-T). In addition, we demonstrate the usefulness of a new preservative system for timolol maleate (TM) eye drops consisting of BAC and sericin.

2 EXPERIMENTAL

2.1 Animals and regents

Male Wistar rats and rabbits were housed under standard conditions (12 h/d fluorescent light (07:00-19:00), 25°C room temperature), and allowed free access to a commercial diet (CE-2 or CR-3, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the Kinki University School of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Pure Sericin\(^{TM}\) (30 kDa) was kindly donated by Seiren Co., Ltd. (Fukui, Japan). Commercially available 0.5% TM eye drops (Timoptol\(^{®}\)) and 0.4% Benoxil were obtained from Santen Pharmaceutical Co., Ltd. (Osaka, Japan), and BAC was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). *Escherichia coli* (E. coli, ATCC 8739) was provided by the National Institute of Technology and Evaluation (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

2.2 Preparation of BAC solution and commercially available TM eye drops with or without sericin

Sericin and BAC were each dissolved in saline and used to prepare BAC solutions with or without sericin, which were then filtered through a Minisart CE (pore size of 0.20 μm, Costar, MA, USA). No adsorption of sericin or BAC to the filter was observed. The BAC eye drop with or without sericin were adjusted to pH 7.0 by the addition of 1N NaOH solution. For the preparation of commercially available TM eye drops with or without 0.1% sericin, 0.04 ml of filtrated saline or 5% sericin solution was added to 1.96 ml of commercially available TM eye drops. BAC concentrations used were determined according to the generally used range in many ophthalmic solution products; 0.01%-0.1% sericin solutions were used in this study.

2.3 Instillation of eye drops in rats

Ten microliters of saline, BAC or commercially available TM eye drops were instilled into the eyes of rats subjected to corneal abrasion (next section) five times per day (9:00, 12:00, 15:00, 18:00 and 21:00). The eyes were kept open for approximately 1 min following instillation to prevent the eye drops from washing out.

2.4 Image analysis of corneal wound healing in rats

Rats, 7 weeks of age, were anesthetized with pentobarbital (30 mg/kg, i.p.), and a patch of corneal epithelium was removed with a BD Micro-Sharp\(^{TM}\) (blade 3.5 mm, 30°, Becton Dickinson, Fukushima, Japan) as described previously\(^{17,19}\). The areas of debrided corneal epithelium were as follows: saline, 10.14 ± 0.52 mm\(^2\); Sericin, 10.30 ± 0.71 mm\(^2\); 0.005% BAC with or without sericin, 10.86 ± 0.88 mm\(^2\); 0.01% BAC with or without sericin, 10.75 ± 0.87 mm\(^2\); 0.02% BAC with or without sericin, 11.19 ± 1.07 mm\(^2\); 11.01 ± 1.13 mm\(^2\); commercially available TM eye drops with or without sericin, 10.75 ± 0.79 mm\(^2\); 10.96 ± 0.81 mm\(^2\) (mean ± S.E. for 4-11 independent rat corneas). The debrided corneal epithelium was dyed by instilling a solution containing 1% fluorescein (Alcon Japan, Tokyo, Japan) and 0.4% Benoxil (Santen Pharmaceutical Co., Ltd., Osaka, Japan). Changes in the corneal wounds were monitored under a TRC-50X fundus camera (Topcon, Tokyo, Japan) equipped with a digital camera (EOS Kiss Digital N, Canon Inc., Tokyo, Japan)\(^{17,19}\), and the images obtained were analyzed with Image J\(^{20}\). The amounts of corneal wound healing (%) were calculated according to equation 1:

\[
\text{Corneal wound healing (\%)} = \frac{(\text{wound area}_{0h} - \text{wound area}_{12-36h})}{\text{wound area}_{0h}} \times 100 \tag{1}
\]

The rates of corneal wound healing, represented by the corneal wound healing rate constant \(k_{Ht} (\text{h}^{-1})\), over the period 0-36 h after corneal epithelial abrasion were calculated according to equation 2:

\[
H_t = H_w \cdot (1 - e^{-k_{Ht} \cdot t}) \tag{2}
\]

where \(t\) is time (0-36 h) after corneal abrasion, and \(H_w\) and \(H_t\) are the percentages of corneal wound healing (%) at time \(\infty\) and \(t\), respectively.
2.5 Cell culture and treatment

The immortalized human corneal epithelial cell line (HCE-T) developed by Araki-Sasaki et al.\(^{21}\) was used in this study. HCE-T cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 (GIBCO, Tokyo, Japan) containing 5% (v/v) heat-inactivated fetal bovine serum and 0.1 mg/mL streptomycin and 1000 IU/mL penicillin (GIBCO, Tokyo, Japan). In the experiment, HCE-T cells (1 × 10^⁴ cells) were seeded in 96-well microplates (IWAKI, Chiba, Japan). Saline or 0.02% BAC solution with or without 0.01%-0.1% sericin was added to the cell cultures one day after seeding, and the cells were stimulated for 0-120 sec. Following stimulation, culture medium containing Tetra-Color One (SEIKAGAKU Co. Tokyo, Japan) was added, and the absorbance (Abs) at 490 nm was measured, and cell viability was calculated according to the manufacturer’s instructions as represented by equation 3:

\[
\text{Cell viability (％)} = \frac{\text{Abs}_{\text{treatment}}}{\text{Abs}_{\text{non-treatment}}} \times 100
\]  

(3)

2.6 Antimicrobial activity of BAC with or without sericin

BAC solutions with or without 0.1% sericin were tested for antimicrobial activity against *E. coli* (ATCC 8739). The organism was selected based on Japanese Pharmacopoeia (JP) test protocols\(^{22}\). According to the standard methodology, the bulk dilution was split into 10 mL aliquots, which were inoculated with between 10^⁵ and 10^⁷ colony-forming units (CFU)/mL of *E. coli* (ATCC 8739) (1 organism per aliquot) and incubated in the presence of saline, sericin or BAC solution with or without 0.1% sericin at 20°C to 25°C. Sampling and enumeration of the inoculated samples were done at 2, 7, 14 and 28 days. One mL aliquots were serially diluted in phosphate buffer, plated in duplicate on soybean-casein digest agar (Casein soya bean digest agar for JP general test, Wako, Osaka, Japan), and incubated at 31°C for 3 days. Raw data counts were converted to log (CFU) values. Since the samples were diluted at least 1:10 at the time of testing, 10 CFU reduction is the lowest sensitivity allowed by the test.

2.7 In vitro transcorneal penetration of TM from commercially available TM eye drops with or without sericin

The *in vitro* transcorneal penetration of commercially available TM eye drops with or without 0.1% sericin was examined using the method of Iwata et al.\(^{23}\) Adult Japanese albino rabbits weighing 2.5 to 3.0 kg were killed by injecting a lethal dose of pentobarbital into the marginal ear vein. The eyes were removed and the corneas were carefully separated from other ocular tissues. The individual corneas were placed on a methacrylate cell designed for transcorneal penetration studies. The side of the chamber (donor chamber) exposed to the exterior surface of the cornea was filled with commercially available TM eye drops with or without 0.1% sericin. The other side of the chamber (reservoir chamber) was filled with 10 mM HEPES buffer (pH 7.4) containing 136.2 mM NaCl, 5.3 mM KCl, 1.0 mM KH2PO4, 1.7 mM CaCl₂, and 5.5 mM glucose. The experiments were performed at 35°C for 6 h. Fifty microliters of sample solution was withdrawn from the reservoir chamber at the indicated time intervals and replaced with the same volume of buffer. TM concentration in the samples was determined by the following HPLC method. Fifty microliters of filtrate was added to 50 µL methanol containing 10 µg propyl p-hydroxybenzoate (internal standard), and the mixture solution were filtered through a Chromatodisk 4A (pore size 0.45 µm, Kurabo Industries Ltd., Osaka, Japan). The solution (10 µL) was injected onto a Mightysil RP-18 (3 µm, column size: 2.0 mm × 50 mm) column (Kanto Chemical Co., Inc., Tokyo, Japan) using a Shimadzu LC-10AD system equipped with a column oven CTO-6A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of 25 mM phosphate buffer (pH 7) containing 30% methanol and 10% acetonitrile at a flow rate of 0.2 mL/min, the column temperature was 35°C, and the wavelength for detection was 294 nm. Corneal viability was monitored by measuring thickness (0.0625 cm, average for 5 rabbits, no significant changes in thickness were observed over the 6 h period). The obtained data were analyzed by following equations:

\[
J_t = \frac{K_n \cdot D \cdot C_{TM}}{\delta} = K_n \cdot C_{TM}
\]  

(4)

\[
\tau = \frac{\delta^2}{6D}
\]  

(5)

\[
Q_t = J_t \cdot A \cdot (t - \tau)
\]  

(6)

where \(J_t\) is the TM penetration rate, \(K_n\) is the cornea/preparation partition coefficient, \(D\) is the diffusion constant within the cornea, \(C_{TM}\) is the TM content in the ophthalmic preparation, \(\tau\) is the lag time, \(\delta\) is thickness of the cornea, \(\delta\) is the total amount of TM appearing in the reservoir solution at time \(t\), and \(A\) is the effective area of the cornea (0.78 cm²). \(J_t\) and \(\tau\) were estimated by fitting each penetration profile to Eq. 6. The penetration coefficient through the cornea, \(K_n\), is given by \(J_t/C_{TM}\). A nonlinear least-squares computer program was employed for the calculation\(^{24}\).

2.8 Measurement of intraocular pressure in rabbits

Intraocular pressure (IOP) enhancement in rabbits was induced by keeping them in a darkroom for 5 h (11:00 – 16:00)\(^{25}\). A calibrated tonometer TonoPen XL (Medtronic SOLAN, FL, USA) was used to monitor the IOP after surface anesthesia (0.4% Benoxil ophthalmic solution) instilled into the rabbit eyes. The IOPs were monitored at intervals of 5-15 min for 90 min after the instillation of 40 µL of commercially available TM eye drops with or without sericin. Differences in the IOPs (ΔIOP, mmHg) among rabbits receiving different preparations of drugs and saline were measured. The area under the curve for ΔIOP (AUCΔIOP, mmHg·min) was calculated according to equation
sericin. The cell viabilities of HCE-T cells treated with BAC solutions co ntaining
while those instilled with 0.02 BAC solutions without sericin.

2.9 Statistical analysis
All values are presented as mean ± standard error of the mean (S.E.). Unpaired Student’s t-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way analysis of variance followed by Dunnett’s multiple comparison. P values less than 0.05 were considered significant.

3 RESULTS
3.1 Preventive effects of sericin on the stimulation of BAC in the cornea
Figure 1 shows corneal wound healing levels following the instillation of BAC solutions with or without sericin. Table 1 shows the rate constants ($k_H$) for corneal wound healing of rat eyes treated by the instillation of BAC solutions with or without sericin. The levels of corneal wound healing of rat eyes instilled with saline were almost entirely healed 36 h after corneal epithelial abrasion. The corneal wounds of rat eyes instilled with saline were almost entirely healed 36 h after corneal epithelial abrasion (99.6%). The corneal wounds of rat eyes instilled with 0.02% BAC solutions showed 6.5% healing while those instilled with 0.02% BAC solutions containing 0.1% sericin showed 34.8% healing 12 h after corneal epithelial abrasion. The $k_H$ for rat eyes instilled with BAC with sericin was significantly higher than that of eyes instilled with BAC solutions without sericin (Table 1). Figure 2 shows the changes in cell viability of HCE-T cells following treatment with BAC solutions containing 0.01%–0.1% sericin. The cell viabilities of HCE-T cells treated with BAC solution for 10 or 20 sec were approximately 26.9% or 4.1%, respectively. On the other hand, the cell viability of HCE-T cells treated with BAC solutions containing sericin were higher than in the absence of sericin, and the cell viability of HCE-T cells increased with increasing sericin concentration. Figure 3 shows the antimicrobial activity of 0.005% BAC solution with or without 0.1% sericin. No antimicrobial activity was observed by the addition of saline and 0.1% sericin. The 0.005% BAC solution with 0.1% sericin showed high antimicrobial activity approximately equal to that of BAC solution without sericin.

3.2 Effects of sericin on stimulation, transcorneal penetration and IOP of TM eye drops
Table 2 summarizes the pharmacokinetic parameters calculated from the data for corneal healing (Fig. 4), in

\[
AUC_{\Delta IOP} = \int_0^t \Delta IOP \, dt
\]  

Briefly, $AUC$ was determined according to the trapezoidal rule up to the last IOP measurement point.

\[Fig. 1\] Corneal Wound Healing of Rat Eyes Treated by the Instillation of Saline, 0.02% BAC solution, and 0.02% BAC solution containing 0.1% Sericin. The degree of corneal wound healing (%) was calculated according to equation 1 in EXPERIMENTAL. 0.02% BAC solutions with or without 0.1% sericin were instilled into rat eyes five times per day. The data are presented as means ± S.E. of 5-10 independent rat corneas. *P < 0.05, vs. BAC-instilled rat eyes.

Table 1 Effect of BAC Solutions with or without 0.1% Sericin on the Rate of Corneal Wound Healing in Rats.

<table>
<thead>
<tr>
<th>BAC Solution</th>
<th>Corneal wound healing rate constant ($k_H \times 10^{-2}$/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5.41 ± 1.15</td>
</tr>
<tr>
<td>0.1% Sericin</td>
<td>5.48 ± 1.07</td>
</tr>
<tr>
<td>0.005% BAC</td>
<td>without sericin 3.49 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>with sericin 5.26 ± 1.34</td>
</tr>
<tr>
<td>0.01% BAC</td>
<td>without sericin 1.88 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>with sericin 4.15 ± 0.86*</td>
</tr>
<tr>
<td>0.02% BAC</td>
<td>without sericin 0.40 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>with sericin 2.81 ± 0.73*</td>
</tr>
</tbody>
</table>

The rate of corneal wound healing was calculated according to equation 2 (see EXPERIMENTAL). Solutions were instilled into the eyes of rats five times a day following corneal abrasion. The data are presented as means ± S.E. of 4-10 independent rat corneas. *P < 0.05, vs. BAC solution without sericin for each category.

\[vitro\] transcorneal penetration and change of IOP. Figure 4 shows corneal wound healing levels following the instillation of commercially available TM eye drops with or without sericin. The $k_H$ for rat eyes instilled with TM eye drops containing sericin was significantly higher than that of eyes instilled with TM eye drops without sericin (Table 2), and the levels of corneal wound healing of rat eyes in-
Improvement of BAC Preservative System using Sericin

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Fig. 2 Effect of BAC Solutions with or without Sericin on the Viability of HCE-T Cells. HCE-T cells in 96-well microplates were treated with saline, 0.02% BAC solution, or 0.02% BAC solutions containing 0.01 - 0.1% sericin for 0 - 120 sec. Cell viability was calculated using TetraColor One according to equation 3 in EXPERIMENTAL. The data are presented as means ± S.E. of 8-15 experiments. *P < 0.05, vs. 0.02% BAC-treated HCE-T cells.

Fig. 3 Effect of 0.1% Sericin on the Antimicrobial Activity of 0.005% BAC Solution. BAC solutions with or without 0.1% sericin were tested for antimicrobial activity against E. coli (ATCC 8739). Raw data counts were converted to log_{10} values, and are presented as means ± S.E. of 5 independent experiments.

stilled with TM eye drops with and without sericin were approximately 79.3% and 65.8%, respectively, 24 h after corneal epithelial abrasion. In the in vitro transcorneal penetration experiments, the amount of penetrated TM increased linearly for 6 h after the addition of TM eye drops with or without 0.1% sericin into the donor chambers, and there were no significant differences in the amount of penetration between the two eye drop formulations (with or without 0.1% sericin, Table 2). On the other hand, the IOP enhancement in rabbits was induced by keeping them in a darkroom; after 5 h in the dark, the IOP of rabbits rose by 7.1-9.6 mmHg as compared with untreated rabbits (16.5 mmHg). TM eye drops containing 0.1% sericin reduced the enhanced IOP, and the IOP reducing effects of TM eye drops both with and without sericin were similar (Table 2).

Table 2 Effect of Commercially Available TM Eye Drops with or without 0.1% Sericin on Corneal Wound Healing, Transcorneal Penetration, and IOP.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>without sericin</th>
<th>with sericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{H} (× 10^{-2}/h)</td>
<td>2.37 ± 0.49</td>
<td>3.86 ± 0.42*</td>
</tr>
<tr>
<td>J_{L} (× 10^{2} nmol/cm^{2}/h)</td>
<td>4.20 ± 0.73</td>
<td>4.12 ± 0.76</td>
</tr>
<tr>
<td>k_{P} (× 10^{-4} cm^{2}/h)</td>
<td>2.42 ± 0.60</td>
<td>2.45 ± 0.59</td>
</tr>
<tr>
<td>k_{m} (× 10^{-4})</td>
<td>1.51 ± 0.37</td>
<td>1.49 ± 0.36</td>
</tr>
<tr>
<td>τ (× 10^{-1} h)</td>
<td>4.06 ± 0.01</td>
<td>4.09 ± 0.02</td>
</tr>
<tr>
<td>D (× 10^{-5} cm^{2}/h)</td>
<td>1.61 ± 0.01</td>
<td>1.57 ± 0.02</td>
</tr>
<tr>
<td>AUC (mmHg·h)</td>
<td>4.44 ± 0.49</td>
<td>4.30 ± 0.45</td>
</tr>
</tbody>
</table>

Parameters were calculated according to equations 2, 4-6, and 7 (see EXPERIMENTAL). The data are presented as means ± S.E. of 5-6 independent rats or rabbits. *P < 0.05, vs. commercially available TM eye drops without sericin for each category.

Fig. 4 Corneal Wound Healing of Rat Eyes Treated by the Instillation of Commercially Available TM Eye Drops with or without 0.1% Sericin. The degree of corneal wound healing (%) was calculated according to equation 1 in EXPERIMENTAL. Commercially available TM eye drops with or without 0.1% sericin were instilled into rat eyes five times per day. The data are presented as means ± S.E. of 5-6 independent rat corneas. *P < 0.05, vs. commercially available TM eye drop-instilled rat eyes.
4 DISCUSSION

Damage to the cornea can result in scarring or opacification, causing visual defects that compromise transparency, and that can even lead to a complete loss of vision. Clinically, a delay in corneal wound healing caused by anti-glaucoma drugs is a serious problem for glaucoma patients. It has been reported that the main toxic effects of anti-glaucoma agents are related to the doses of the preservatives commonly used in eye-drop formulations\(^1\),\(^2\),\(^10\),\(^26\), the most common of which in ophthalmic preparations is BAC. In this study, we investigated the preventive effects of sericin on corneal damage by BAC using rat debrided corneal epithelium and HCE-T cells. In addition, we determined the usefulness of BAC containing sericin for the preparation of anti-glaucoma eye drops.

In studies to evaluate the effects of BAC on corneal wound healing, the selection of the experimental animal is very important. The rat debrided corneal epithelium model has been used in studies aimed at the development of corneal healing drugs\(^27\),\(^28\), and the mechanism of corneal wound healing in this model is similar to that in humans. Therefore, we used the rat debrided corneal epithelium model to compare corneal damage levels in eyes treated by the instillation of BAC solutions with or without sericin.

The corneal wounds of rats eyes instilled with saline showed approximately 51.4\% healing at 12 h and 86.2\% healing at 24 h after corneal epithelial abrasion, and the wounds were almost completely healed by 36 h after abrasion. We previously reported that the instillation of 1-10\% sericin has a potent effect in promoting corneal wound healing in rats\(^17\). However, the instillation of 0.1\% sericin, a very low concentration, was not observed to increase the corneal wound healing rate. The corneal wounds of rat eyes instilled with 0.02\% BAC solution containing sericin were 34.8\% healed 12 h after corneal epithelial abrasion, but only 6.5\% healed in rat eyes instilled with 0.02\% BAC solution in the absence of sericin (Fig. 1). In addition, we evaluated the effects of sericin on corneal damage and the antimicrobial activity by BAC using HCE-T cells and E. coli (Fig. 2 and 3). The viability of HCE-T cells treated with BAC solutions containing sericin was higher than that of cells treated with BAC solutions without sericin, and the cell viability increased with increasing sericin concentrations. A 0.005\% BAC solution containing 0.1\% sericin shows high antimicrobial activity and antimicrobial activity approximately equal to that of TM eye drops with or without sericin. Therefore, these data suggest that the sericin diminishes some of the side effects of BAC. In addition, we investigate that the effect of 0.005\% BAC solution containing 0.1\% sericin on hemolysis rate using red blood cell (RBC) of rabbit, which is evaluation model of cell injury. The RBC membrane is broken by the treatment with 0.005\% BAC for 20 min, and the hemolysis rate is 98.9 ± 1.6\% (n = 3). However, the hemolysis rate by the treatment with 0.005\% BAC containing 0.1\% sericin for 20 min is 21.5 ± 1.4\% (n = 3). From these results, the sericin may prevent the membrane disruption by BAC.

It is important to confirm the usefulness of BAC containing sericin in the preparation of anti-glaucoma eye drops. Glaucoma is characterized by nerve degeneration that results in the disappearance of retinal ganglion cells, visual field loss, excavation of the optic disk, and ophthalmopapillitis\(^27\),\(^28\). The major risk factor for glaucoma is elevated IOP, which leads to apoptosis and a loss of retinal ganglion cells\(^29\). In treating glaucoma, the focus is on the reduction of IOP and the prevention of retinal and optic nerve damage. Anti-glaucoma eye drops are frequently used in clinical treatment, and treatment with anti-glaucoma eye drops must be continued in glaucoma patients even if they cause corneal damage. Recently, anti-glaucoma combination eye drops, such as Xalacom® (lotanoprost/TM combination eye drops), Duotrav® (travoprost/TM combination eye drops) and Cosopt® (dorzolamide hydrochloride/TM combination eye drops), have been developed. Eye drops containing TM are currently the most prescribed glaucoma medications, and the preservative used in eye drops containing TM is BAC. Therefore, we investigated the corneal wound healing rate following the instillation of commercially available TM eye drops containing saline or sericin using the rat debrided corneal epithelium model. The rate constants for corneal wound healing (k\(_h\)) for rat eyes instilled with TM eye drops containing sericin were significantly higher than those of eyes instilled with TM eye drops without sericin (Table 2), and the levels of corneal wound healing of rat eyes instilled with TM eye drops with and without sericin were approximately 79.3\% and 65.8\% 12 h after corneal epithelial abrasion, respectively. These results indicate that TM eye drops without sericin are much more toxic than TM eye drops with sericin. It is known that the surface-active effects of BAC increase the corneal penetration of the main component. Therefore, BAC solutions with or without sericin were examined for their ability to penetrate the cornea and reduce IOP using rabbits. In an in vitro transcorneal penetration experiment, the amount of TM penetrating the cornea increased linearly up to 6 h after the addition of TM eye drops with or without 0.1\% sericin into the donor chambers with no significant differences observed between the presence or absence of sericin (Table 2). The instillation of TM eye drops containing 0.1\% sericin reduced the enhanced IOP in rabbits in kept in a darkroom, and the IOP reducing effect of TM eye drops containing sericin is similar to that of TM eye drops. These results indicate that the addition of sericin may provide effective therapy for patients requiring long-term anti-glaucoma eye drops. Further studies are needed to elucidate the usefulness of BAC containing sericin for anti-glaucoma eye drop preparations. Therefore, we are now investigating the versatility and antimicrobial

\( \text{Table 2} \)

<table>
<thead>
<tr>
<th>BAC solution</th>
<th>k(_h) (1/min)</th>
<th>IOP reduction (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>( \text{&lt;0.1} )</td>
<td>( \text{-} )</td>
</tr>
<tr>
<td>TM + 0.005% BAC</td>
<td>( \text{&gt;0.1} )</td>
<td>( \text{&gt;0.1} )</td>
</tr>
<tr>
<td>TM + 0.1% sericin</td>
<td>( \text{&gt;0.1} )</td>
<td>( \text{&gt;0.1} )</td>
</tr>
</tbody>
</table>

\( k\(_h\): \text{rate constant for corneal wound healing} \)

\( \text{IOP}: \text{intraocular pressure} \)
activity of sericin using other anti-glaucoma eye drops and organisms.

5 CONCLUSION

The present study demonstrates that BAC solutions containing sericin are tolerated better on the rat ocular surface than the classic BAC only preservative system. In addition, sericin does not affect the antimicrobial activity of BAC against E. coli or the corneal penetration of TM from commercially available TM eye drops. A BAC plus sericin preservative system may provide effective therapy for glaucoma patients requiring long-term anti-glaucoma agents.

References

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