Comparison of In Vivo Efficacy of Different Ocular Lubricants in Dry Eye Animal Models

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PURPOSE. To compare the efficacy of three types of ocular lubricants in protecting corneal epithelial cells in dry eye animal models.

METHODS. Ocular lubricants containing 0.1% or 0.3% sodium hyaluronate (SH), carboxymethylcellulose (CMC), or hydroxypropyl methylcellulose (HPMC) were tested. First, ocular lubricant containing 0.002% fluorescein was dropped onto the rabbit cornea. The fluorescein intensity as an index of retention was measured. Second, a rabbit dry eye model was made by holding the eye open with a speculum, and 50 μL of each ocular lubricant was dropped onto the cornea. After 3 hours, the corneas were stained with 1% methylene blue (MB), and the absorbance of MB was measured. Third, a rat dry eye model was treated with the ocular lubricants for 4 weeks, and the corneal fluorescein staining was scored. Eyes treated with physiological saline were used as controls. Finally, immunohistochemistry was used to analyze occludin, an epithelial barrier protein, in cultured human corneal epithelial cells pretreated with ocular lubricants and desiccated for 20 or 60 minutes.

RESULTS. Our results showed that 0.3% SH had a significantly longer retention time than the other lubricants (all P < 0.01). The absorbance of MB was significantly lower in the 0.3% SH group. The corneas of rats exposed to 0.3% SH had significantly lower fluorescein staining scores. A significantly higher number of occludin-positive cells were found after exposure to 0.3% SH than other lubricants.

CONCLUSIONS. Ocular lubricant containing 0.3% SH would be preferable to treat patients with dry eye syndrome.

Keywords: ocular lubricant, animal model, cornea, dry eye syndrome

Dry eye disease is a relatively common condition affecting approximately 10% to 20% of the adult population. It 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. Use of ocular lubricants is the primary treatment for dry eye disease. Although there are many effective ocular lubricant formulations, sodium hyaluronate (SH), carboxymethylcellulose (CMC), and hydroxypropyl methylcellulose (HPMC) are the main components of ocular lubricants most commonly prescribed.

Investigations have been done on the effectiveness of ocular lubricants in protecting the cornea from dehydration in a porcine dry eye model, to ameliorate the tear film instability in a rabbit model, and to treat mild to moderate dry eyes in patients. We have investigated the in vitro efficacy of the three ocular lubricants mentioned for their ability to retain water and to protect human corneal epithelial cells (HCECs) from dehydration. However, there have been no reports of in vivo comparisons of these three types of ocular lubricants for their abilities to protect the corneal epithelial cells against desiccation and to treat dry eye disease in animal models.

Thus, the purpose of this study was to compare the protective and therapeutic efficacies of three representative commercial ocular lubricants in vivo. To accomplish this, we used a rabbit model representing the evaporative type of dry eye and a rat model representing the aqueous-deficient type of dry eye. We compared three types of commercial ocular lubricants that contain the ingredients most often used to treat dry eye—0.1% or 0.3% sodium hyaluronate (SH), carboxymethylcellulose (CMC), or hydroxypropyl methylcellulose (HPMC)—for their efficacy in protecting the corneal epithelial cells in a rabbit dry eye model and their therapeutic efficacy in a rat dry eye model. In addition, the retention of the three types of ocular lubricants on rabbit corneas was evaluated.

MATERIALS AND METHODS

Ocular Lubricants

The commercial ocular lubricants tested were 0.1% and 0.3% SH (Hyalein Mini; Santen Pharmaceutical Co., Ltd., Osaka, Japan), CMC (Refresh Plus; Allergan, Inc., Irvine, CA, USA), and HPMC (Tears Naturale Free; Alcon, Fort Worth, TX, USA). Physiological saline solution (PSS) was used as the control (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan).

Animals

Male Japanese white rabbits (Kitayama Labs Co., Ltd., Nagano, Japan) weighing 1.7 to 2.5 kg and male 4-week-old Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) were purchased and acclimated for at least 1 week prior to the experiments. All animals were kept under standard pathogen-free conditions at 22°C ± 3°C, 50% ± 20% humidity, and 12 hours of light and 12 hours of darkness.
hours of darkness. The rabbits were fed approximately 130 g food per day, and both were given access to water ad libitum. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ability of Retention of Ocular Lubricants on the Corneal Surface

Rabbits were anesthetized by intramuscular injections of a mixture of 2% xylazine (Selactar, Bayer Healthcare, Leverkusen, Germany) and 50 mg/mL ketamine hydrogen chloride (Ketalar; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan). Their eyes were held open with a speculum, and 50 μL each of the three types of ocular lubricants containing 0.002% fluorescein sodium (Sigma-Aldrich Co., St. Louis, MO, USA) was dropped onto the corneas. One lubricant was tested per cornea. The retention of the ocular lubricant on the corneal surface was determined by measuring the fluorescein intensity on the corneal surface with a commercial slit-lamp fluorometer (Anterior Fluorometer FL-500; Kowa Co., Ltd., Tokyo, Japan). The fluorescein intensity was measured in a 2-mm-diameter circle centered on the apex of the cornea. The measurements were made at 2, 5, 10, 20, and 30 minutes after the instillation of the lubricants. Physiological saline solution with the same concentration of fluorescein was used as the control. The right eyes of a total of six rabbits were studied for each ocular lubricant group.

Protective Effectiveness of Ocular Lubricants in Rabbit Evaporative-Type Dry Eye Model

Rabbits were anesthetized with urethane (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and their eyes were held open with a speculum to test the effectiveness of the ocular lubricants in protecting the corneal epithelial cells in an evaporative-type dry eye model.6,7 The room temperature was 22°C to 25°C, and the relative humidity was 45% to 55%. Immediately after opening the eye, 50 μL of each of the three types of ocular lubricants was dropped onto the eye, and the eye was kept open for 3 hours. After the exposure, the corneas were stained with 50 μL of 1% methylene blue (MB) solution, a vital stain that is taken up by dead or damaged cells but not by living cells. The excess MB was then rinsed off with PSS. The rabbits were killed by an overdose of sodium pentobarbital and cells were fixed in 95% ethanol at 4°C for 30 minutes and in 37°C in an atmosphere of 5% CO₂ in air.

Human corneal epithelial cells were seeded in 4-well culture plates (LAB-TEK II Chamber slide; Thermo Fisher Scientific KK, Yokohama, Japan) at a concentration of 1 × 10⁵ cells/well and cultivated for 5 days. The supernatant was removed and replaced with different lubricants of 0.1% SH, 0.5% SH, 0.5% CMC, or 0.3% HPMC for 1 hour. Then, the supernatants were removed, and the cells were exposed to room air (room temperature, 23.8°C–24.0°C; humidity, 41%) for 20 or 60 minutes for desiccation. Plates were re-filled with culture medium at 37°C for 15 minutes, and cells were fixed in 95% ethanol at 4°C for 30 minutes and in acetone at room temperature for 1 minute. After exposure to 1% skimmed milk in PBS, a blocking buffer, at room temperature for 30 minutes, the anti-occludin antibody (×50, goat IgG; Santa Cruz Biotechnology, Dallas, Texas, USA) was added, and cells were left overnight at 4°C. Cells were rinsed with PBS, and a second antibody (×2000, donkey anti-goat IgG AlexaFluor 488; Invitrogen, Carlsbad, CA, USA) with propidium iodide (PI) solution (×400; Invitrogen) was applied for 1 hour. Cells were rinsed with PBS again and examined under fluorescence microscope (BIOREVO BZ-9000; KEYENCE Corp., Osaka, Japan). Six fields in each group were photographed. The total number of cells stained with PI and the number of cells showing occludin-positive borders were counted in each field. The occludin-positive cell rate was calculated as follows: occludin positive cell rate = (number of occludin-positive cells/total cell number) × 100%.

Therapeutic Effectiveness of Ocular Lubricants in Rat Aqueous-Deficient–Type Dry Eye Model

Rats were anesthetized with sodium pentobarbital, and the lateral exorbital lacrimal gland was excised from both eyes. The wound was sutured using Michel suture clips (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and treated with antibiotic ointment (Tarivid; Santen Pharmaceutical Co.). As shown, these rats have a significantly decreased volume of tear secretion to almost one-half the volume of that of normal rat eyes.8,9 The instillation of the ocular lubricants was started 8 weeks after the surgery. The three types of ocular lubricants were instilled six times per day for 4 weeks. Slit-lamp examinations were performed at 1, 2, and 4 weeks after the beginning of treatment. For this part of the study, 1% fluorescein solution was used to stain the cornea, and the degree of superficial punctuate keratitis (SPK) was scored by the area and density of the fluorescein staining. The cornea was divided into equal upper, middle, and lower areas, and the score was based on the density of staining, which ranged from 0 for none, 1 for mild, 2 for moderate, and 3 for severe. For each section, a minimum score was 0.5, and the scores were summed to obtain the final score for a maximum of 9.9 The scorer was masked to the type of treatment received by the rats.

Rat eyes with excised exorbital lacrimal gland were treated with PSS and used as positive controls. Rats without removal of the exorbital lacrimal gland served as negative controls. The right eyes of four rats were tested for each ocular lubricant group.

Epithelial Barrier Function Protein Assay After Desiccation

Sodium hyaluronate (Kewpie, Tokyo, Japan), CMC (Daiichi Kogyo Seiyaku Co., Ltd., Tokyo, Japan), and HPMC (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) were purchased. The lubricants were dissolved in Dulbecco’s modified Eagle’s medium (DMEM)/F12 culture medium (Nacalai Tesque, Kyoto, Japan) for epithelial barrier function protein assay. The SV-40 immortalized HCEC line was purchased from RIKEN BioResource Center (Ibaragi, Japan) for the in vitro studies. Cells were incubated in DMEM/F12 culture medium with 15% fetal bovine serum, 5 μg/mL insulin, 10 ng/mL human epidermal growth factor, and 40 μg/mL gentamicin in cell culture flasks at 37°C in an atmosphere of 5% CO₂ in air.

Human corneal epithelial cells were seeded in 4-well culture plates (LAB-TEK II Chamber slide; Thermo Fisher Scientific KK, Yokohama, Japan) at a concentration of 1 × 10⁵ cells/well and cultivated for 5 days. The supernatant was removed and replaced with different lubricants of 0.1% SH, 0.5% SH, 0.5% CMC, or 0.3% HPMC for 1 hour. Then, the supernatants were removed, and the cells were exposed to room air (room temperature, 23.8°C–24.0°C; humidity, 41%) for 20 or 60 minutes for desiccation. Plates were re-filled with culture medium at 37°C for 15 minutes, and cells were fixed in 95% ethanol at 4°C for 30 minutes and in acetone at room temperature for 1 minute. After exposure to 1% skimmed milk in PBS, a blocking buffer, at room temperature for 30 minutes, the anti-occludin antibody (×50, goat IgG; Santa Cruz Biotechnology, Dallas, Texas, USA) was added, and cells were left overnight at 4°C. Cells were rinsed with PBS, and a second antibody (×2000, donkey anti-goat IgG AlexaFluor 488; Invitrogen, Carlsbad, CA, USA) with propidium iodide (PI) solution (×400; Invitrogen) was applied for 1 hour. Cells were rinsed with PBS again and examined under fluorescence microscope (BIOREVO BZ-9000; KEYENCE Corp., Osaka, Japan). Six fields in each group were photographed. The total number of cells stained with PI and the number of cells showing occludin-positive borders were counted in each field. The occludin-positive cell rate was calculated as follows: occludin positive cell rate = (number of occludin-positive cells/total cell number) × 100%.

Statistical Analyses

All data are expressed as the mean ± SEM. Tukey variant tests were used to determine the significance of the differences of
the three ocular lubricants in the fluorescein intensity on the rabbit corneal surface, the MB absorbance of the dissected rabbit corneas, the fluorescein staining scores on the rat corneas, and the positive cell rates for staining occludin. Comparisons between positive and negative control groups and between no desiccation and desiccation groups were tested by Student’s t-tests. A $P < 0.05$ was taken to be significant.

**RESULTS**

**Retention of Different Ocular Lubricants on Corneal Surface**

The amount of ocular lubricant containing fluorescein remaining on the corneal surface was determined by measuring the fluorescein intensity. All lubricants had higher values than controls at all times. Among all lubricants tested, 0.3% SH had significantly higher fluorescein intensities at all times ($P < 0.01$; Tukey test). The fluorescein intensity of HPMC was significantly higher than that of 0.1% SH at 2 minutes (all $P < 0.05$). At 5, 10, 20, and 30 minutes, the differences in the fluorescein intensity of eyes with 0.1% SH, CMC, and HPMC were not significant (all $P > 0.05$). At 60 minutes of desiccation, but 0.3% SH treatment was effective after 20 minutes (all $P < 0.05$; Tukey test). Also, the fluorescein staining scores of eyes treated with 0.3% SH were significantly lower than those of eyes treated with HPMC 4 weeks after treatment ($P < 0.05$, Tukey test; Fig. 3A). Representative slit-lamp microscopic photographs are shown in Figure 3B.

**Protective Efficacy in Dry Eye Rabbit Model**

To compare the ability of the three types of ocular lubricants to protect corneal epithelial cells from desiccation, the rabbit evaporative-type dry eye model was used. The level of MB extracted from the corneas in closed eyes (i.e., negative controls) was $0.064 \pm 0.008$, indicating that there was only minimal damage to the epithelial cells. In contrast, the absorbance of corneas exposed to PSS (i.e., positive controls) was $0.161 \pm 0.023$ ($P = 0.001$, Student’s t-test). Among the corneas exposed to the three types of ocular lubricants, only the corneas exposed to 0.3% SH had a significantly lower level of absorbance of MB than that of the positive control ($P = 0.021$, Tukey test). The absorbance of 0.3% SH–instilled corneas was also significantly lower than that of HPMC ($P = 0.011$, Tukey test; Fig. 2). Although the corneas treated with 0.1% SH and CMC had a lower absorbance of MB than the positive controls, the differences were not significant ($P = 0.216$ and $P = 0.664$, respectively; Tukey test).

**Efficacy in Dry Eye Rat Model**

To determine the effect of the ocular lubricants in a dry eye rat model, the eye drops were instilled daily beginning 8 weeks after the removal of the exorbital lacrimal glands. The eyes treated with PSS (positive control) had a significantly higher corneal fluorescein staining score than that of rats whose lacrimal glands were intact (negative control) at all times (all $P < 0.01$; Student’s t-tests; Fig. 3A). Compared with positive controls, all eyes treated with the ocular lubricants had a lower fluorescein staining score, but only eyes treated with 0.1% or 0.3% SH were significantly lower (all $P < 0.05$; Tukey test). Also, the fluorescein staining scores of eyes treated with 0.3% SH were significantly lower than those of eyes treated with HPMC 4 weeks after treatment ($P < 0.05$, Tukey test; Fig. 3A). Representative slit-lamp microscopic photographs are shown in Figure 3B.

**Protective Effects of Lubricants on Occludin, a Barrier Function Protein of HCECs**

To study the underlying mechanism of the protective effects of the three lubricants, the presence of occludin, an epithelial tight junction protein in HCECs was determined immunohistochemically. The HCECs were pretreated with each of the lubricants and then placed under desiccation conditions. Our results showed that occludin was not present around the cell borders after the desiccation (Figs. 4A, 4B). All lubricants had some protective effect on the expression of occludin after 20 minutes of desiccation, but 0.3% SH treatment was effective over a wider area than treatment with 0.1% SH, 0.5% CMC, and 0.3% HPMC. The effect of 0.3% SH was effective even after 60 minutes of desiccation. The 0.3% SH had the highest rate of occludin-positive cells of all the lubricants at 20 minutes (all $P < 0.01$, Tukey test; Fig. 4C). At 60 minutes, the positive rate of 0.3% SH was also significantly higher than for 0.1% SH, 0.5% CMC, and 0.3% HPMC (all $P < 0.05$, Tukey test; Fig. 4D).
molecules as evidenced by our finding that high intensity of the ocular surface. In addition, SH may not only contribute to the stability of the tear film, and the wettability of SH is attributed to the sponge-like structure of its flexible, open-coil conformation. The water retentive property of SH is measured by the retentive time on the cornea. Hydroxypropyl methylcellulose is less viscous than CMC but is known to be a superior cohesive and has emollient properties.

Although the effectiveness of SH, CMC, and HPMC has been examined in in vitro and in vivo studies, there has not been a single study that compared these three types of ocular lubricants for their ability to be retained by the cornea and their effectiveness in protecting corneal epithelial cells against desiccation. Our findings indicated that SH, CMC, and HPMC had significantly higher ocular surface retention times than PSS as the control, and of these, 0.3% SH had the highest ability to protect the corneal epithelial cells from desiccation. This effect was concentration dependent.

Sodium hyaluronate is a high molecular weight, naturally occurring glycosaminoglycan. It is a long molecule with a flexible, open-coil conformation. The water retentive property of SH is attributed to the sponge-like structure of its polysaccharide chains. This property is also believed to contribute to the stability of the tear film, and the wettability of the ocular surface. In addition, SH may not only "hold" water but also act as a reservoir of slowly releasing water molecules as evidenced by our finding that high intensity of fluorescein was detected on the corneal surface for 0.3% SH at all times of the experiment. Our data are in agreement with our earlier report and observations by Nakamura et al. who showed that SH could retard water loss from filter paper or when placed atop an agar gel in a dose-dependent manner. In addition, our findings support the data reported by Snibson et al. that the ocular surface residence time of SH was significantly longer than HPMC.

A loss of the integrity of the rabbit corneal epithelial cells has been documented by blocking the blinking of rabbit eyes with a speculum. This model has been used to study the aqueous-deficient type dry eye syndrome. Compared with the positive control receiving PSS drops, SH had a better protective effect at both the 0.1% and 0.3% concentrations. This efficacy was found as early as 1 week following the beginning of application. In contrast, no significant effectiveness was noted for CMC and HPMC in reducing the corneal staining score at all times, which is consistent with the results of our rabbit dry eye models.

The rheologic properties of SH solutions have given it a place in ophthalmologic surgery. Dilute solutions of SH have high viscosities with low shear rates, but at high shear rates (e.g., during blinking) they undergo a marked reduction in viscosity. This pseudoplasticity allows its topical use. Our findings support the reports that SH treatment is better in healing corneal lesions, in reducing the degree of keratitis, and in increasing corneal surface regularity than CMC.

The underlying mechanisms of protection from desiccation by SH could be explained, at least in part, by our immunohistochemical study of the epithelial barrier function protein assay. Human corneal epithelial cells pretreated with 0.3% SH had significantly more intact epithelial-cell tight junctions than the other lubricants. This finding indicated that SH may benefit
the cells by retaining or preserving more water and, hence, preserve more functional tight junctions during cell desiccation. For in vivo studies, another possibility may be that SH could play a role in reducing the inflammatory response caused by the desiccation. Sodium hyaluronate has been shown to reduce the level of the inflammatory marker CD44, a hyaluronate receptor, that is known to be overexpressed in patients with dry eye disease. Sodium hyaluronate has also been shown to be beneficial for corneal epithelial cell migration and elongation in vitro. All of these effects would favor the protection of corneal epithelial cells from desiccation and contribute to the healing of damaged corneal epithelial cells.

Some other factors such as product viscosity should also be taken into consideration when interpreting our data. The retention of a fluid on the eye of a nonblinking animal, such as rabbit, will be determined mostly by viscosity. Our earlier study showed that the viscosity was 0.93 mPa·S, 3.83 mPa·S, 27.81 mPa·S, 3.04 mPa·S, and 6.74 mPa·S for PSS, 0.1% SH, 0.3% SH, CMC, and HPMC, respectively. The highest viscosity of 0.3% SH could account for the significantly higher ocular surface retention and protective effects in the rabbit models.

**Figure 3.** Therapeutic effectiveness of three types of ocular lubricants in an exorbital lacrimal gland-extirpated rat dry eye model. (A) Corneal fluorescein staining scores indicative of loss of the epithelial integrity are shown. Data represent the mean ± SEM of four eyes of four rats. Negative controls were rats with intact lacrimal gland, and positive controls were dry eye model rats receiving PSS. The statistical significances are shown as **P < 0.01 versus negative control (Student’s t-test); #P < 0.05 and ##P < 0.01 versus positive control (Tukey variant test); and ¶P < 0.05 versus HPMC (Tukey variant test). (B) Representative slit-lamp microscopic photographs showing corneal fluorescein staining at 4 weeks after ocular lubricant exposure.
Figure 4. Epithelial cell barrier function protein assay against desiccation. Human corneal epithelial cells were pretreated with different lubricants and desiccated for 20 minutes (A, C) and 60 minutes (B, D). Cells were stained with anti-occludin antibody with propidium iodide (PI) solution and examined under fluorescence microscope (original magnification ×40). The occludin-positive cell rate was calculated. Data represent the mean ± SEM of six fields in each group. The statistical significances are shown as ##P < 0.01 versus no desiccation (Student’s t-test); *P < 0.05 and **P < 0.01 versus desiccation (Tukey variant test); ¶¶P < 0.01 versus 0.1% SH (Tukey variant test); $$$P < 0.01 versus CMC (Tukey variant test); ††P < 0.01 versus HPMC (Tukey variant test).
There are several limitations of our study. We studied only three types of ocular lubricants, and other types of commercially available eye drops such as glycerin, polyvinyl alcohol, and hydroxyethylcellulose were not tested. In addition, the ocular lubricants evaluated were all preservative free, and because ocular lubricants containing different types of preservatives are sometimes prescribed, they should also be evaluated in dry eye animal models. It should be remembered that not all dry eyes can be cured by ocular lubricants alone, and other factors such as ocular surface inflammation, corneal epithelial cell abnormality, and Meibomian gland dysfunction should also be considered when initiating appropriate therapy for any given case.19,20 The combined effectiveness of ocular lubricants with other types of eye drops, such as anti-inflammation drops, should also be evaluated in future studies.

In conclusion, our results indicate that ocular lubricants containing SH are superior to those containing CMC and HPMC in inflammation drops, should also be evaluated in future studies.

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